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THE INTRACELLULAR PHOSPHORYLATION OF (−)-2′-DEOXY-3′-THIACYTIDINE (3TC) AND THE INCORPORATION OF 3TC 5′-MONOPHOSPHATE INTO DNA BY HIV-1 REVERSE TRANSCRIPTASE AND HUMAN DNA POLYMERASE γ

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Abstract—(−)-2′-deoxy-3′-thiacytidine (3TC)† has been shown to be a potent, selective inhibitor of HIV replication *in vitro*, which requires phosphorylation to its 5′-triphosphate for antiviral activity. The intracellular concentration of 3TC 5′-triphosphate in phytohaemagglutinin (PHA)-stimulated peripheral blood lymphocytes (PBL) shows a linear dependence on the extracellular concentration of 3TC up to an extracellular 3TC concentration of 10 μM. At this extracellular concentration of 3TC, the resulting intracellular concentration of 3TC 5′-triphosphate is 5 μM. This value is similar to the inhibition constant (K_i) values for the competitive inhibition of human immunodeficiency virus type 1 (HIV-1) reverse transcriptase and human DNA polymerases (10–16 μM) by 3TC 5′-triphosphate. Since the concentration of 3TC producing 90% inhibition (IC_{90}) of HIV replication in PBLs has been reported to be 76 nM, the antiviral activity of 3TC requires intracellular concentrations of 3TC 5′-triphosphate, which would result in very little inhibition of reverse transcriptase if its sole mode of action was competitive inhibition. This apparent discrepency may be explained by the ability of 3TC 5′-triphosphate to act as a substrate for reverse transcriptase. Primer extension assays have shown that 3TC 5′-triphosphate is a substrate for HIV-1 reverse transcriptase and DNA polymerase γ, resulting in the incorporation of 3TC 5′-monophosphate into DNA. In the case of DNA polymerase γ, the product of this reaction (i.e. double-stranded DNA with 3TC 5′-monophosphate incorporated at the 3′-terminus of the primer strand) is also a substrate for the 3′-5′ exonuclease activity of this enzyme. This may explain the low levels of mitochondrial toxicity observed with 3TC.

Key words: 3TC; 3TC 5′-triphosphate; intracellular phosphorylation; HIV-1 reverse transcriptase; chain termination; DNA polymerase γ

Human immunodeficiency virus (HIV), the etiological agent of AIDS,‡ encodes the enzyme reverse transcriptase (EC 2.7.7.49) within the viral gene *pol*. Reverse transcriptase is a multi-functional enzyme with RNA-dependent DNA polymerase (RNAdepDNApol), DNA-dependent DNA polymerase (DNAdepDNApol), and ribonuclease H activities [1]. This enzyme has been shown to be essential for the viral replicative cycle. It represents a viable antiviral drug target, as it posseses biochemical properties quite distinct from those of human DNA polymerases [2].

3TC ((−)4-amino-1-[-2-(hydroxymethyl)-1,3,-oxathiolan-5-yl]-(1H)-2-pyrimidinone) has been identified as a potent, selective inhibitor of HIV replication, in a number of human T-lymphoblastoid and monocyte/macrophage cell lines *in vitro* [3]. 3TC has also been demonstrated to have extremely low cytotoxicity [4] and to be phosphorylated to the 5′-triphosphate in peripheral blood lymphocytes (PBL) [5]. 3TC 5′-triphosphate has an inhibition constant (K_i) value of approximately 10 μM for the inhibition of both the RNAdepDNApol and DNAdepDNApol activities HIV-1 reverse transcriptase, and is a competitive inhibitor with respect to 2′-deoxycytidine 5′-triphosphate (dCTP) [6]. In the same study [6], the K_i value for mitochondrial DNA polymerase γ was found to be 16 μM. This apparent lack of selectivity in the inhibition of DNA polymerases by 3TC 5′-triphosphate is in marked contrast to the very low cytotoxicity of 3TC *in vitro*. Furthermore, data on the intracellular phosphorylation [5] have shown that 3TC at an extracellular concentration of 10 μM gave rise to an intracellular concentration of 3TC 5′-triphosphate of approximately 10 pmoles/10^6 cells within phytohaemagglutinin

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† 3TC is a trademark of Glaxo Group Ltd., Glaxo House, Greenford Road, Greenford, Middlesex UB6 OHE, U.K.

‡ Abbreviations: AIDS, acquired immunodeficiency syndrome; AZT, 3′-azido-3′-deoxythymidine; dAMP, 2′-deoxyadenosine 5′-monophosphate 3,4-DCI, (3,4-dichloroisocoumarin); dCMP, 2′-deoxycytidine 5′-monophosphate; ddCMP, 2′,3′-dideoxycytidine 5′-monophosphate; ddCTP, 2′,3′-dideoxycytidine 5′-triphosphate; dCTP, 2′-deoxycytidine 5′-triphosphate; DTT, dithiothreitol; E64, (L-trans-epoxysuccinyl-leucylamide-(4-guanidino)-butane); 3TC, (−)-2′-deoxy-3′-thiacytidine; DNAdepDNApol, DNA-dependent DNA polymerase; HIV-1, human immunodeficiency virus type 1; IC_{90}, concentration producing 90% inhibition; k_{cat}, catalytic constant; K_i, inhibition constant; PHA, phytohaemagglutinin; PBL, peripheral blood lymphocyte; PMSF, phenylmethylsulphonyl fluoride; RNAdepDNApol, RNA-dependent DNA polymerase; k_{cat}/K_m, specificity constant; TBE, tris-borate-EDTA; dTMP, 2′-deoxythymidine 5′-monophosphate.

(PHA)-stimulated PBLs. Since the volume of a eukaryotic cell is about 1 pL [7], this indicates that an intracellular concentration of 3TC 5′-triphosphate equivalent to the K_i value is attained only when the extracellular concentration of 3TC is two orders of magnitude greater than the antiviral concentration producing 90% inhibition (IC_{90}). This apparent discrepancy between potent and selective antiviral effect and weak enzyme inhibition may be explained by the ability of 3TC 5′-triphosphate to act as a substrate for reverse transcriptase. As 3TC does not contain a hydroxyl group at any position analogous to the 3′-hydroxyl group of the natural 2′-deoxynucleosides, the use of 3TC 5′-triphosphate as a substrate by HIV-1 reverse transcriptase effectively terminates chain extension beyond its incorporation into the HIV DNA genome. Other nucleoside analogues have been shown to act as chain terminators after intracellular phosphorylation to the corresponding 5′-triphosphates [8–10].

We have investigated the dependency of the intracellular concentration of 3TC 5′-triphosphate in PBLs on the extracellular 3TC concentration to determine intracellular 3TC 5′-triphosphate concentrations at a concentration of 3TC that gives rise to an antiviral effect. The ability of HIV-1 reverse transcriptase to utilize 3TC 5′-triphosphate as a substrate for the incorporation of 3TC 5′-monophosphate into DNA was assessed using an incorporation assay. Kinetic constants for HIV-1 reverse transcriptase (RNAdepDNApol and DNAdepDNApol activities) were determined for 3TC 5′-triphosphate and 3′-azido-3′-deoxythymidine (AZT) 5′-triphosphate acting as substrates. The interaction of 3TC 5′-triphosphate with DNA polymerase γ was also investigated to assess the selectivity of 3TC 5′-triphosphate. DNA polymerase γ is one of the key enzymes required for mitochondrial DNA synthesis [11], and inhibition of mitochondrial DNA synthesis by the 5′-triphosphates of nucleoside analogues has been proposed to be a major cause of the toxicity of other nucleoside analogues [12, 13]. In addition to its DNAdepDNApol activity, it also possesses a 3′-5′ exonuclease activity [14], unlike reverse transcriptase, which represents one of the few known mechanisms of DNA repair within the mitochondrion. Selective inhibition of reverse transcription by 3TC 5′-triphosphate might result from 3TC 5′-triphosphate being a very poor substrate for the DNAdepDNApol activity of DNA polymerase γ, or from the chain-terminated product of the reaction (i.e. double-strandeded DNA with 3TC 5′-monophosphate at the 3′-terminus of the primer strand) being a substrate for the 3′-5′ exonuclease activity. The ability of 3TC 5′-triphosphate to act as a substrate for DNA polymerase γ and the ability of the 3′-5′ exonuclease activity of DNA polymerase γ to excise any incorporated 3TC 5′-monophosphate was assessed.

MATERIALS AND METHODS

Materials

The (−) enantiomer of 3TC 5′-triphosphate and AZT 5′-triphosphate were synthesized at Glaxo Research and Development Ltd. dCTP and 2′,3′-dideoxycytidine 5′-triphosphate (ddCTP) were purchased from the Sigma Chemical Co. (Poole, U.K.). All other reagents were of the highest grade available.

Intracellular phosphorylation of [^{3}H] 3TC in uninfected, PHA-stimulated peripheral blood lymphocytes

The materials and methods used are those described [5] with the following differences. Peripheral blood lymphocytes were incubated at 37° for 4 hr with a mixture of [^{3}H]-3TC and 3TC to a final concentration of 0.1 μM to 500 μM. The limit of detection of [^{3}H]-3TC 5′-triphosphate is below 100 nM.

Enzyme purification

HIV-1 reverse transcriptase (from strain BH10) was cloned, expressed, and purified as described [15]. DNA polymerase γ was purified from HeLa (Ohio) cells using the procedures described [6] with the following alterations: The proteinase inhibitors L-transepoxysuccinyl-leucylamide-(4-guanidino)-butane (E-64), at a reaction concentration of 10 μM, and 3,4-dichloroisocoumarin (3,4-DCI), at 10 μM, replaced phenylmethylsulphonyl fluoride (PMSF) and soya bean trypsin inhibitor in the protocol. The initial anion-exchange chromatography step, using Q-Sepharose Fast Flow (Pharmacia, U.K.), was changed to Resource Q (Pharmacia, U.K.), using a 6-ml column at a flow rate of 10 mL/min with a 2-column volume post-load wash, followed by a 10-column volume gradient. The fractions containing the DNA polymerase γ were diluted four-fold in 20 mM potassium phosphate, pH 7.5, prior to loading on a double-stranded DNA cellulose column, replacing an overnight dialysis. The addition of a step was added to remove contaminating nucleases. This involved using a 16-mm × 100-cm column packed with Superdex 200 prep grade (Pharmacia, U.K.), running at 1 mL/min in 20 mM potassium phosphate buffer, pH 7.5, containing 1 mM dithiothreitol (DTT) and 20% glycerol (v/v). The DNAdepDNApol and 3′-5′ exonuclease activities of DNA polymerase γ co-purified; their migration on gel permeation chromatography was consistent with a molecular mass of approximately 190 kDa. The purity of the enzyme was assessed on a 7.5% SDS-PAGE silver stained gel, where a single band of approximately 140 kDa was observed (data not shown). The molecular mass as determined by both gel permeation chromatography and SDS-PAGE was identical to human DNA polymerase γ, as observed by Wong and Gray [11]. The sensitivity of DNA polymerase γ, DNAdepDNApol, and 3′-5′ exonuclease activities to a range of inhibitors was identical to that of Wang and Gray [11] (data not shown).

Chain termination assay

Chain termination studies were carried out using an oligodeoxynucleotide primer annealed to either MS2 RNA (Boehringer Mannheim, U.K.) as a template for RNAdepDNApol activity, or M13mp18(+) DNA (Pharmacia) as a template for RNAdepDNApol activity. For investigating the effects of 3TC 5′-triphosphate, the primers 5′-AGC-CAC-TCC-GAA-GTG-3′ (RNAdepDNApol) or 5′-ATG-CCA-CTA-CGA-AGG-3′ (DNAdepDNApol) were used, and 5′-CCA-CTC-CGA-AGT-GCG-3′ (RNAdepDNApol) or 5′-ACG-AAG-GCA-CCA-ACC-3′ (DNAdepDNApol) for AZT 5′-triphosphate studies. The oligodeoxynucleotide primers were synthesized by β-cyanoethyl chemistry, on solid-phase, using an Expidite 8909 oligonucleotide synthesiser (Millipore, Watford, Herts, U.K.). Purification of

the oligodeoxynucleotide primers was carried out by anion-exchange chromatography using a DfB hplc system (Pharmacia, U.K.) with a 4-mm × 25-cm Nucleopac PA-100 column (Dionex, U.K.) running a 40–60% gradient of 1.8 M ammonium acetate in 10% (v/v) acetonitrile, pH 6.0, at 1.5 mL/min with detection at 254 nm.

The 5′ end-labelling with [γ-^{32}P]ATP was carried out using T4 polynucleotide kinase, free of 3′-phosphatase activity (Boehringer Mannheim, U.K.), according to the manufacturer's instructions. The reaction was terminated by heating for 15 min at 95°. The 5′ end-labelled oligodeoxynucleotide was annealed to either MS2 RNA or M13mp18(+) DNA at a ratio of 2:1. The mixture was incubated at 60° for 15 min prior to rapid cooling on ice.

The HIV-1 reverse transcriptase assays were carried out under the conditions discussed [6], but with 1.9 pmoles of oligodeoxynucleotide primer annealed to 3.8 pmoles of RNA or DNA templates, 58.5 ng of HIV-1 reverse transcriptase per 50 µL assay, and a single nucleotide triphosphate (3TC 5′-triphosphate or AZT 5′-triphosphate) as substrate. Aliquots of 6 µL were removed at fixed times (0–9 min) and added to 3 µL of stop solution (95% (v/v) formamide, 20 mM EDTA, 0.05% (v/v) bromophenol blue, and 0.05% (v/v) xylene cyanol FF) to terminate the reaction.

The DNA polymerase γ DNAdepDNApol assay contained a final concentration of 50 mM Tris-HCl pH 8.0, 2.5 mM $MgCl_2$, 150 mM KCl, 5 mM DTT, 10 mM NaF, 2 mM Na_2VO_3, and 1.71 pmoles M13mp18(+) annealed to a 3.42 pmoles of end-labelled oligodeoxynucleotide primer with 3TC 5′-triphosphate or dCTP as a substrate in a final volume of 50 µL. The mixture was pre-incubated at 37° for 15 min prior to the addition of 5 µL DNA polymerase γ (0.35 µg/mL). Aliquots of 6 µL were removed at fixed times (0–9 min), and the reaction terminated as for HIV-1 reverse transcription. The experiments involving DNAdepDNApol and 3′-5′ exonuclease activity were performed over a longer period (0–240 min) to observe the products of the 3′-5′ exonuclease.

Polyacrylamide gel electrophoresis was performed under denaturing conditions with 20% polyacrylamide (v/v), 1 × Tris-borate-EDTA (TBE), 7M urea gel. The gel was pre-run for 1 hr at 2300 V. Samples were heated to 95° for 3 min and rapidly cooled on ice prior to loading on the gel. After electrophoresis the gel was autoradiographed for 1 hr with X-Omat film (Kodak, U.K.). The resulting substrate and product brands were excised from the gel and counted on a scintillation counter (Wallac, U.K.) using Optiphase Safe II scintillation fluid.

Synthesis of 16-mer oligodeoxynucleotide primers

The synthesis of 16-mer oligodeoxynucleotide primers with either 3TC 5′-monophosphate or 2′,3′-dideoxycytidine 5′-monophosphate (ddCMP) at their 3′ end was catalysed by deoxynucleotidyltransferase. The 3′ end incorporation was performed with oligodeoxynucleotide primer (10 pmoles) 5′-ATG-CCA-CTA-CGA-AGG-3′, 3TC 5′-triphosphate or ddCTP (20 pmoles) in 200 mM potassium cacodylate, 25 mM Tris-HCl, 2.5 mM $CoCl_2$, and 0.25 mg/mL BSA, pH 6.6 at 37°. The reaction was initiated with 50 units of deoxynucleotidylexotransferase (Boehringer Mannheim, U.K.) to give a final volume of 50 µL. The 16-mer product was separated from the 15-mer substrate using the hplc purification method described above.

The DNA polymerase γ 3′-5′ exonuclease assay was carried out under similar conditions to the DNAdep-DNApol assay, and contained a final concentration of 50 mM Tris-HCl pH 8.0, 2.5 mM $MgCl_2$, 50 mM KCl, 5 mM DTT, 10 mM NaF, 2 mM Na_2VO_3 with 17.1 pmoles M13mp18(+) annealed to 3.42 pmoles of the above end-labelled 16-mer oligodeoxynucleotide primer, and 3TC 5′-triphosphate or dCTP as a substrate in a final volume of 50 µL. Prior to the 3′-5′ exonuclease assay, the annealed mixture was applied to an S-400 microspin column (Pharmacia, U.K.) to remove any unannealed primer. The purified primer/template mixture was applied to a 20% non-denaturing PAGE gel containing 1 × TBE and run at 1000V for 2 hr, maintaining the temperature below 40°, to confirm the absence of free primer. The purified primer/template mixture was pre-incubated at 37° for 15 min prior to the addition of 5 µL DNA polymerase γ (0.35 µg/mL).

Data analysis

The initial rates from the time courses were converted to pmoles of nucleoside monophosphate incorporated/min/L: of the assay. The steady-state values of both the catalytic constant (k_{cat}), and the K_m were obtained using a nonlinear curve fit kinetics package (Grafit, Erithacus, Software, U.K.) for the Michaelis-Menten equation of initial velocity vs nucleoside triphosphate, at a fixed primer-template concentration. As it was not possible to obtain a saturating primer template concentration, all steady-state kinetic values must be regarded as apparent.

RESULTS

Intracellular phosphorylation of 3TC in PHA-stimulated PBLs

The formation of intracellular 3TC metabolites within PHA-stimulated PBLs (Fig. 1) was found to be dependent on the extracellular concentration of 3TC. The intracellular concentration is based on the assumption (stated in the introduction) that the volume of a eukaryotic cell is about 1 pL. In the range of extracellular 3TC concentrations between 0.1 µM and 10 µM, the level of intracellular 3TC 5′-triphosphate was linearly dependent on the extracellular 3TC concentration. The maximum intracellular 3TC 5′-triphosphate concentration detected was 17 µM, which was attained when the extracellular concentration of 3TC was 300 µM. The intracellular metabolism of 3TC in PBLs has been shown to be unaffected by infection with HIV-1 [5].

HIV-1 reverse transcriptase catalysed incorporation of 3TC 5′-monophosphate into DNA

DNA polymerase catalysed addition of 3TC 5′-monophosphate onto the 3′ end of a DNA primer strand results in termination of primer extension. To measure this, an oligodeoxynucleotide primer was end-labelled with ^{32}P and then annealed to an RNA or DNA template (Fig. 2A and B, respectively). The oligodeoxynucleotide primer sequence was chosen to be complementary to the template such that the first base incorporated in DNA polymerization would be deoxycytidine 5′-monophosphate (dCMP), or a cytidine analogue, 3TC 5′-monophosphate, or ddC 5′-monophosphate. The primers were chosen to anneal to overlapping regions of the templates in order to minimize possible effects of the secondary structure of the template nucleic acids. The reaction was initiated by

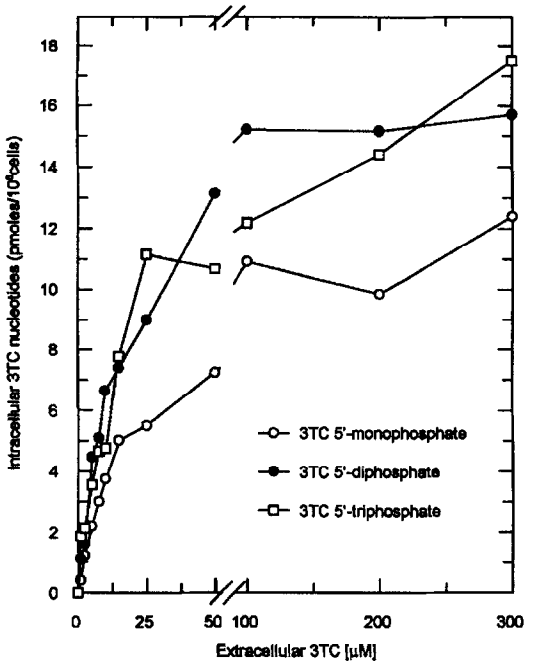

Fig. 1. Effect of extracellular 3TC concentration on intracellular 3TC nucleotide concentrations in uninfected PHA-stimulated PBLs, after 4 hr incubation.

the addition of HIV-1 reverse transcriptase, which utilizes 3TC 5′-triphosphate as a substrate incorporating 3TC 5′-monophosphate onto the 3′ end of the oligodeoxynucleotide primer. The reaction products were separated on a denaturing gel system. The oligodeoxynucleotide product containing 3TC 5′-monophosphate was excised from the gel and quantified.

The K_m for HIV-1 reverse transcriptase catalysed incorporation 3TC 5′-monophosphate onto an oligodeoxynucleotide primer annealed to MS2 RNA (Table 1) is 5.1 μM. By comparison, the K_m value of AZT 5′-monophosphate was found to be 0.45 μM. The specificity constant (k_{cat}/K_m) results show that HIV-1 reverse transcriptase would utilize AZT 5′-triphosphate approximately two orders of magnitude more efficiently as a substrate compared to 3TC 5′-triphosphate. The results for 3TC 5′-monophosphate incorporation into the oligodeoxynucleotide primer strand (Table 2), under DNAdepDNApol conditions, show a K_m of 205 μM. This K_m is 40 fold higher than that observed for RNAdepDNApol activity (Table 1), and well above the maximum observed intracellular concentration of 3TC 5′-triphosphate. The effect of AZT 5′-triphosphate on incorporation of its 5′-monophosphate into a DNA primer (Tables 1 and 2) show an 83-fold difference of RNAdepDNApol over DNAdepDNApol activity. This difference is also observed in the k_{cat}/K_m value, although to a lower degree.

The incorporation and excision of 3TC 5′-monophosphate from DNA catalysed by DNA polymerase γ

3TC 5′-triphosphate was a substrate for HeLa cell DNA polymerase γ in the chain termination assay (under different buffer conditions), catalysing incorporation of 3TC 5′-monophosphate at the 3′ end of the primer. Plots of product concentration against time were linear over the 10-minute time-course of each experiment. The K_m value of DNA polymerase γ for 3TC 5′-triphosphate (Table 3) is 3 μM, which is similar to that of the natural substrate, dCTP.

Prolonged incubation of 3TC 5′-triphosphate and primer-template in the presence of DNA polymerase γ results in the formation of products derived from both the polymerase activity and the 3′-5′ exonuclease activity of this protein, as shown in Fig. 3 and Table 4. As expected, no incorporation (DNAdepDNApol activity) occurs in the absence of 3TC 5′-triphosphate (lanes 2–7), but there is an increase in the rate of incorporation with increasing levels of 3TC 5′-triphosphate (lanes 20–25, 2.84 μM, and lanes 26–31, 284 μM). The rate of appearance of 14-mer and 13-mer exonuclease products (lanes 2–7, 20–25 and 26–31) shows that the exonuclease activity of DNA polymerase γ is independent of the 3TC 5′-triphosphate concentration over the range of concentrations tested. This also holds for dCTP at the concentration levels tested. The absence of a band running as a 17-mer (lanes 8–19, dCTP), which would be the result of misincorporation of dCMP for 2′-deoxyadenosine 5′-monophosphate (dAMP), is consistent with the high fidelity of DNA polymerase γ, in contrast to the low fidelity of reverse transcriptase. At 2.84 μM 3TC 5′-triphosphate, an attainable intracellular concentration, the rate of appearance of products resulting from the 3′-5′ exonuclease activity DNA polymerase γ is slightly greater than the rate of formation of the chain-terminated 16-mer (Table 4). However, at higher levels of 3TC 5′-triphosphate (284 μM) (Table 4), the rate of incorporation was greater than the rate of appearance of products of the exonuclease reaction. Similar behaviour was observed for the addition and excision of dCMP at 3′ end of the same primer.

To determine whether the 3′-5′ exonuclease activity of DNA polymerase γ was capable of excising 3TC 5′-monophosphate from the 3′ end of a primer, an oligodeoxynucleotide primer containing 3TC 5′-monophosphate at the 3′ end was synthesized (see Materials and Methods). An excess of template over primer was used to ensure that all the primer was present as a duplex. The use of gel filtration ensured that any primer that had failed to anneal was removed. Furthermore, the nondenaturing PAGE gel confirmed that there was no free primer (data not shown). The use of this substrate in exonuclease assays (Fig. 4, lanes 7–12 and Table 5) shows that DNA polymerase γ (3′-5′ exonuclease activity) is able to excise 3TC 5′-monophosphate from the 3′ end of a 16-mer primer annealed to M13mp18(+) DNA. The autoradiograph (Fig. 4) shows a typical exonuclease ladder, where there is a progression to shorter oligodeoxynucleotides with time. The (15mer-13mer) bands present on the enzyme blank (lanes 1–6) are due to contaminating 3′-5′ exonuclease activity present only in the end-labelling reaction, and show no time dependency in the 3′-5′ exonuclease assay. The rates for the excision of ddCMP and dCMP (Table 5) were similar to that of 3TC 5′-monophosphate.

DISCUSSION

The antiretroviral activity of a nucleoside analogue is dependent both upon intracellular phosphorylation to its 5′-triphosphate and the interaction of the 5′-triphosphate

Fig. 2. Primer/template complexes for the chain termination assay utilizing 3TC 5′-triphosphate as a substrate. (A) RNA-dependent DNA polymerization and (B) DNA-dependent DNA polymerization.

Table 1. Kinetic constants for HIV-1 reverse transcriptase (RNAdepDNApol)

Substrate	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m ($M^{-1} \times min^{-1}$)
3TC 5′-triphosphate	5.1 ± 1.0	0.0078 ± 0.0005	1.5×10^3
AZT 5′-triphosphate	0.45 ± 0.04	0.3085 ± 0.0055	6.9×10^5

Table 2. Kinetic constants for HIV-1 reverse transcriptase (DNAdepDNApol)

Substrate	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m ($M^{-1} \times min^{-1}$)
3TC 5′-triphosphate	205 ± 64	0.0326 ± 0.0059	1.6×10^2
AZT 5′-triphosphate	37.4 ± 3.3	0.180 ± 0.0059	4.8×10^3

Table 3. Kinetic constants for HeLa cell DNA polymerase γ (DNAdepDNApol)

Substrate	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m ($M^{-1} \times min^{-1}$)
3TC 5′-triphosphate	3.18 ± 0.63	0.0232 ± 0.0009	7.3×10^3
dCTP	1.24 ± 0.52	0.0074 ± 0.0007	6.0×10^3

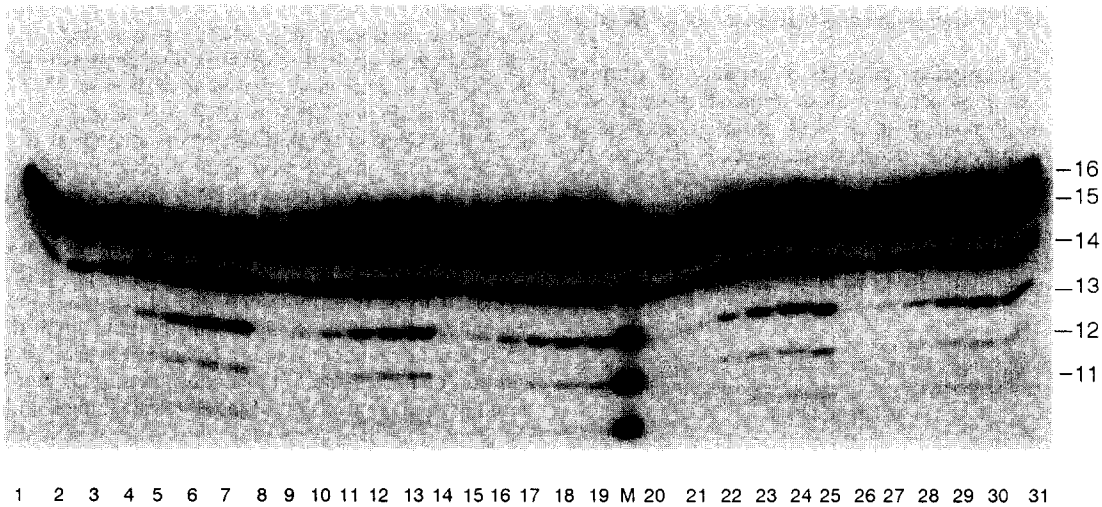

Fig. 3. Autoradiograph for HeLa cell DNA polymerase γ (DNAdepDNAApol and 3′-5′ exonuclease activity) in the presence or absence of 3TC 5′-triphosphate or dCTP. Lane 1, enzyme blank; lanes (2–7), DNA polymerase γ in the absence of triphosphate; lanes (8–13) and (14–19) 3 μM and 300 μM dCTP, respectively, in the presence of DNA polymerase γ; M (oligodeoxynucleotide markers, 11–16mer); lanes (20–25) and (26–31) 2.84 μM and 284 μM 3TC 5′-triphosphate, respectively, in the presence of DNA polymerase γ. The time courses (15, 30, 60, 120, 180, and 240 min) were carried out as described in Materials and Methods).

Table 4. HeLa cell DNA polymerase γ (DNAdepDNApol and 3′–5′ exonuclease activity) in the presence and absence of 3TC 5′-triphosphate or dCTP (1.00 ≡ 11.51×10^{-12} moles/min/L)

	Polymerase activity	3′–5′ exonuclease activity
No triphosphate	0.00	1.00
dCTP [3.00 μM]	0.63	1.00
dCTP [300 μM]	1.85	0.96
3TC 5′-triphosphate [2.84 μM]	0.76	0.97
3TC 5′-triphosphate [284 μM]	1.28	1.02

with the retroviral reverse transcriptase [16]. In earlier work it has been shown that at an extracellular concentration of 3TC of 10 μM, the intracellular concentration of 3TC 5′-triphosphate was 5 μM, which would be sufficient to inhibit measurably both reverse transcriptase and DNA polymerase γ [6]. We have extended these earlier data by examining the dependence of the intracellular 3TC 5′-triphosphate concentration on the extracellular 3TC concentration. The data (Fig. 1) show that an intracellular concentration of 3TC 5′-triphosphate that is similar in magnitude to the K_i value can be obtained only at high extracellular concentrations of 3TC. However, an extracellular concentration of 3TC equivalent to the IC_{90} concentration for inhibition of viral replication resulted in an intracellular concentration of 3TC 5′-triphosphate [4], which was below the detection limit of 100 nM. According to the Cheng-Prusoff equation [17], if 3TC 5′-triphosphate was acting purely as a competitive inhibitor of reverse transcriptase, the level of enzyme inhibition would be <1% at this low concentration of inhibitor. Furthermore, the high therapeutic indices for the inhibition of HIV replication by 3TC are inconsistent with the unselective competitive inhibition of DNA polymerases by 3TC 5′-triphosphate. Since competitive inhibition of reverse transcriptase activity does not appear to account for either the antiviral efficacy or the high selectivity of 3TC, we have further characterized intracellular phosphorylation of 3TC and the behavior of 3TC 5′-triphosphate as a substrate of DNA polymerases.

Once incorporated onto the 3′-terminus of a primer strand, the presence of a chain-terminating nucleoside analogue 5′-monophosphate halts further primer extension. As only one incorporation event is necessary to prevent the completion of reverse transcription [18], even a very low probability of incorporation at any one site can result in a potent inhibition of viral replication as a consequence of the large number of potential chain termination sites within the HIV genome. The k_{cat}/K_m values for 3TC 5′-triphosphate show that it is likely to be an order of magnitude more effective as a substrate for the RNAdepDNApol activity of HIV-1 reverse transcriptase than it is of this enzyme's DNAdepDNApol activity. Previously published studies of AZT 5′-triphosphate [19] acting as a substrate of HIV-1 reverse transcriptase, which used much shorter templates than those used in this study, show higher k_{cat} values, but exhibit similar differences between RNAdepDNApol and RNAdepDNApol activities for k_{cat} and K_m. The very much lower k_{cat} values determined in this study may result from the rate-limiting association between HIV-1 reverse transcriptase and its primer template complex, since the use of large heteropolymeric RNA or DNA templates with complex secondary structures could result in a large number of nonproductive interactions and, thus, a low k_{cat}.

Table 5. HeLa cell DNA polymerase γ 3′–5′ exonuclease activity; activity relative to 3′–5′ exonuclease activity, in absence of triphosphate

Substrate	Initial velocity (moles/min/L)
5′ ATGCCACTACGAAGGC 3′	$11.58 \pm 0.06 \times 10^{-12}$
5′ ATGCCACTACGAAGG-dd*CMP* 3′	$6.7 \pm 0.76 \times 10^{-12}$
5′ ATGCCACTACGAAGG-*3TC5′-monphosphate* 3′	$7.69 \pm 0.13 \times 10^{-12}$

The interaction of the 5′-triphosphates of nucleoside analogues with cellular DNA polymerases has been proposed to be a cause of the toxicity associated with some members of this pharmacological class. We chose to investigate the interaction of 3TC 5′-triphosphate with DNA polymerase γ because inhibition of mitochondrial DNA synthesis by nucleoside analogue triphosphates has been implicated as a major cause of the toxicity of other nucleoside analogues, and because the results of a previous study indicated that 3TC 5′-triphosphate is a competitive inhibitor of DNA polymerase γ, with a K_i value determined to be 16 μM [6]. If 3TC 5′-triphosphate acted solely as a competitive inhibitor of DNA polymerases, the inhibition of these enzymes at therapeutically effective concentrations would be low (*vide supra*). However, as with HIV-1 reverse transcriptase, 3TC 5′-triphosphate may act as a substrate rather than an inhibitor of DNA polymerase γ.

Our data show that 3TC 5′-monophosphate is incorporated into DNA by DNA polymerase γ, a result consistent with the data of Chang *et al.* [20]. This indicates that 3TC 5′-triphosphate would be utilized as a substrate by DNA polymerase γ with an efficiency similar to that of the natural substrate, dCTP. (Unlike Chang *et al.*, we did not observe misincorporation during primer extension assays. The level of misincorporation observed by Chang *et al.* was surprising, as DNA polymerase γ from various sources normally exhibits a high degree of fidelity [21–23].) In principle, the incorporation of 3TC 5′-monophosphate into DNA by DNA polymerase γ might result in a significant inhibition of mitochondrial DNA synthesis if the enzyme were to be exposed to 3TC 5′-triphosphate *in vivo*. However, DNA polymerase γ, unlike reverse transcriptase, possesses a proof-reading (3′-5′) exonuclease activity, capable of excising nucleoside 5′-monophosphates from the 3′-termini of primer strands. This activity represents one of the few known mechanisms of DNA repair within the mitochondrion. DNA polymerization involves a balance between further addition of nucleoside 5′-monophosphates onto the 3′-terminus, and the excision of the 3′-terminal nucleotide. However, after the incorporation of a chain-terminating nucleoside 5′-monophosphate, further processive primer extension is impossible, which is likely to make the excision of the nucleoside analogue 5′-monophosphate highly probable. Since the data from the primer extension assay indicated that 3TC 5′-monophosphate could be incorporated onto the 3′-terminus of DNA by DNA polymerase γ, the ability of the 3′-5′ exonuclease activity

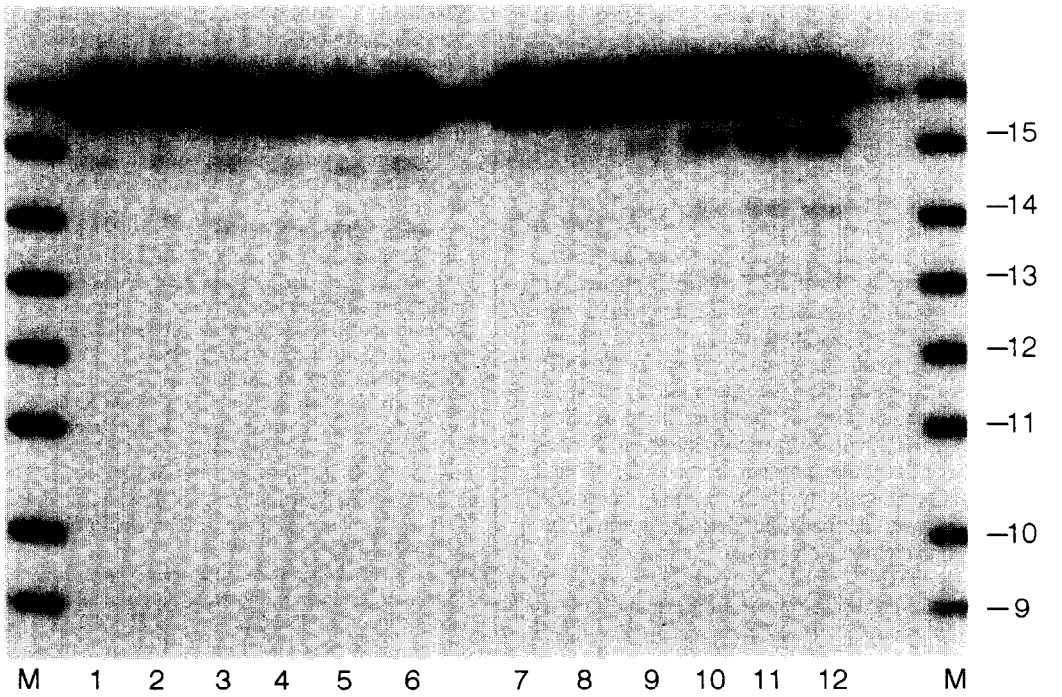

Fig. 4. Autoradiograph for the effect of HeLa cell DNA polymerase γ 3′-5′ exonuclease activity on (5′ ATGCCACTACGAAGG-*3TC 5′-monphosphate* 3′). M (oligodeoxynucleotide markers, 9–16mer); lanes (1–6), enzyme blank; lanes (7–12) in the presence of DNA polymerase γ and M (oligodeoxynucleotide markers, 9–16mer). The time courses (15, 30, 60, 120, 180, and 240 min) were carried out as described in Materials and Methods).

to excise incorporated 3TC 5′-monophosphate was investigated.

When an oligodeoxynucleotide containing 3TC 5′-monophosphate at the 3′-terminus was annealed to single-stranded DNA, the resulting duplex was indeed found to be a substrate for the 3′-5′ exonuclease activity of DNA polymerase γ. Overall, our results demonstrate that although 3TC 5′-triphosphate is a substrate for the DNAdepDNApol activity of DNA polymerase γ, the product of this reaction is a substrate for the 3′-5′ exonuclease activity. The ability of DNA polymerase γ to excise 3TC 5′-monophosphate from DNA may help to explain the lack of mitochondrial toxicity exhibited by 3TC. Other workers have attributed the lack of mitochondrial toxicity of 3TC to the inability of its 5′-triphosphate to be transported into mitochondria, and no information on the intramitochondrial concentration of 3TC 5′-triphosphate is available. Nevertheless, the majority of 3TC has been shown to cross the cell membranes through nonfacilitated passive diffusion *in vitro* ([20] and unpublished results), and to be phosphorylated by intracellular kinases [4, 20, 24], and enzymes potentially capable of phosphorylating 3TC have been identified within mitochondria [25]. Our experiments with DNA polymerase γ indicate that mechanisms of DNA repair may contribute to the high therapeutic index of this compound.

The dependence of the intracellular 3TC 5′-triphosphate concentration on the extracellular 3TC concentration also has implications for the development of viral resistance to nucleoside analogues by HIV. Mutations within the HIV genome conferring resistance to nucleoside analogues have all been shown to be associated with codon changes within the polymerase domain of the reverse transcriptase (e.g. M184V or M184I confers resistance to 3TC) [26]. For the phenotype of a virus isolate to be characterized as resistant, a significantly greater extracellular concentration of the nucleoside analogue must be used to achieve a given level of inhibition of viral replication than is required to attain the same level of inhibition of the wild-type virus. We have shown at therapeutically effective concentrations *in vitro* that there is a linear dependence of the intracellular concentration of 3TC 5′-triphosphate on the extracellular concentration of 3TC, which has a slope of approximately 0.5. Therefore, any mutation to reverse transcriptase must result in a large decrease in the magnitude of the parameter governing chain termination, k_{cat}/K_m, for the virus containing this mutation to be characterized as being resistant, because a large increase in the extracellular drug concentration results in a large increase in the intracellular concentration of the inhibitor of viral replication. A number of studies have shown that reverse transcriptases that contain mutations conferring resistance to 3TC have much reduced affinity for 3TC 5′-triphosphate [27]. This decrease in affinity will reduce the magnitude of k_{cat}/K_m for 3TC 5′-monophosphate incorporation, and therefore increase the probability of reverse transcription proceeding to completion. In contrast with the studies on 3TC-resistant reverse transcriptase, a number of studies of reverse transcriptases containing mutations associated with viral resistance to AZT have shown that these enzymes have affinities for AZT 5′-triphosphate that are little different from the wild-type enzyme. Recently, Carroll *et al.* [28] have shown that k_{cat}/K_m for AZT 5′-monophosphate incorporation was 25% lower for reverse transcriptase containing the AZT resistance mutations at codons 41, 67, 70, and 215 of reverse transcriptase. This change, though small, is sufficient to result in a two-fold increase in the number of

completed transcripts as calculated by the method of Goody *et al.*, [18]. Were AZT to be as well phosphorylated intracellularly as 3TC, it is unlikely that such a small change in the properties of reverse transcriptase would result in virus that could be characterized as being resistant. However, the available data show that the intracellular level of AZT 5′-triphosphate is very insensitive to the extracellular concentration of AZT as the result of the inefficient phosphorylation of AZT 5′-monophosphate. Since only a 3.5-fold increase in the intracellular concentration of AZT 5′-triphosphate is effected by a 1,000-fold increase in the extracellular concentration of AZT [29], a small change to the relative rates of 2′-deoxythymidine 5′-monophosphate (dTMP) and AZT 5′-monophosphate incorporation by HIV reverse transcriptase may be all that is required to allow the selection of HIV with a resistant phenotype. Further studies of the intracellular phosphorylation of AZT and other antiviral nucleosides will be necessary to establish the role that intracellular phosphorylation plays in determining the characteristics of resistant phenotypes.

In summary, 3TC 5′-triphosphate has been shown to act as a substrate for HIV-1 reverse transcriptase, resulting in incorporation of 3TC 5′-monophosphate at the 3′-terminus of oligodeoxynucleotide primers annealed to an RNA or DNA template. It was approximately 40-fold less effective as a substrate for DNAdepDNApol than RNAdepDNApol activities. The phosphorylation of 3TC to it's 5′-triphosphate in PBLs was linear up to 10 μM of extracellular 3TC, at which concentration the intracellular 3TC 5′-triphosphate concentration was estimated to be 5 μM. 3TC 5′-triphosphate is a substrate for DNA polymerase γ (DNAdepDNApol activity), but the product of this reaction (with 3TC 5′-monophosphate incorporated at the 3-terminus) is also a substrate for the 3′-5′ exonuclease activity of DNA polymerase γ. This may explain the low levels of mitochondrial toxicity observed with 3TC.

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