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Unique Inhibitory Effect of 1-(2′-Deoxy-2′-fluoro-β-L-arabinofuranosyl)-5-methyluracil 5′-Triphosphate on Epstein-Barr Virus and Human DNA Polymerases

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ABSTRACT. 1-(2′-Deoxy-2′-fluoro-β-L-arabinofuranosyl)-5-methyluracil (L-FMAU) was shown to have potent antiviral activity against Epstein-Barr virus (EBV) without any cellular toxicity at concentrations up to 200 μM (Yao *et al.*, *Biochem Pharmacol* **51:** 941–947, 1996). The 5′-triphosphate of L-FMAU was not a substrate for EBV or cellular DNA polymerases, but could inhibit the elongation reaction, 3′-to-5′ exonuclease activity, and nucleotide turnover catalyzed by EBV DNA polymerase. DNA synthesis catalyzed by human DNA polymerases was inhibited to a lesser extent. The inhibition pattern of EBV DNA polymerase by L-FMAU-5′-triphosphate (L-FMAU-TP) was consistent with an uncompetitive mechanism when dNTP or template-primer were used as the variable substrates. The K_i values were 38 ± 10 μM for the elongation reaction, and about 50 ± 10 μM for both nucleotide exchange and 3′-to-5′ exonuclease reactions, values that were 10–20 times less than that for GMP. L-FMAU-TP is the first nucleoside 5′-triphosphate shown to have such unique behavior toward DNA polymerases. EBV DNA polymerase could be one of the targets for the inhibitory effect of L-FMAU-TP on EBV replication. BIOCHEM PHARMACOL **55**;8:1181–1187, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. L-nucleoside; L-FMAU-TP; EBV; DNA polymerases

EBV§ is a major pathogen associated with infectious diseases and malignancies in humans [1–3]. Several nucleoside analogs such as acyclovir, gancyclovir, FIAU, and D-FMAU have been shown to have different degrees of activity against EBV replication in cell culture [4–6]. The mechanism of their action was thought to be the incorporation of the 5′-monophosphates of the nucleoside analogs into viral DNA as a result of a preferential interaction of EBV DNA polymerase with their 5′-triphosphate metabolites. Recently, L-FMAU, an L-thymidine analog, was shown by us to have potent activity against EBV and HBV [7, 8]. L-FMAU could be phosphorylated stepwise to its 5′-triphosphate form, L-FMAU-TP, in human cell cultures. Preferential phosphorylation of L-FMAU in EBV replicating cells was noted, which could be due to the fact that L-FMAU is a substrate of EBV thymidine kinase (unpublished data). L-FMAU-TP, unlike the triphosphate metabolites of other anti-EBV agents such as acyclovir, gancyclovir, FIAU, and D-FMAU, which are in the D-configuration of nucleosides, is not a substrate for human or EBV DNA polymerases [7].

In this paper, we present data demonstrating that L-FMAU-TP inhibits DNA elongation, 3′-to-5′exonuclease activity, and nucleotide turnover catalyzed by EBV DNA polymerase, and, to a lesser extent, DNA elongation catalyzed by human DNA polymerases. These results demonstrated that an L-dNTP analog could serve as a DNA polymerase inhibitor without being a substrate for the enzyme. EBV DNA polymerase could be one of the targets that results in the inhibitory effect of L-FMAU on EBV replication without significant toxicity to host cells.

MATERIALS AND METHODS

Chemicals

[γ-^{32}P]ATP (6000 Ci/mmol) and T4 polynucleotide kinase were purchased from the Amersham Corp.; dNTPs were obtained from Boehringer. L-FMAU (Fig. 1) was a gift of Dr. C. K. Chu, University of Georgia. L-FMAU-TP (ammonium salt) was synthesized from L-FMAU according to a procedure described previously [9]. The purity of L-FMAU-TP was 95–98% as confirmed by HPLC analysis. Kieselgel 60 F_{254} cellulose thin-layer chromatographic plates were purchased from Merck Inc. The oligonucleotides were synthesized on an Applied Biosystems 380A DNA synthesizer at the Yale Oligonucleotide Synthesis

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§ *Abbreviations:* EBV, Epstein-Barr virus; HSV, herpes simplex virus; HBV, hepatitis B virus; L-FMAU, L-FMAU-MP, and L-FMAU-TP, 1-(2′-deoxy-2′-fluoro-β-L-arabinofuranosyl)-5-methyluracil and its 5′-mono- and triphosphates, respectively; D-FMAU, 1-(2′-deoxy-2′-fluoro-β-D-arabinofuranosyl)-5-methyluracil; FIAU, 1-(2′-deoxy-2′-iodo-β-D-arabinofuranosyluracil-5-methyluracil; DTT, dithiothreitol; TPA, 12-O-tetradecanoylphorbol-13-acetate; and PMSF, phenylmethylsulfonyl fluoride.

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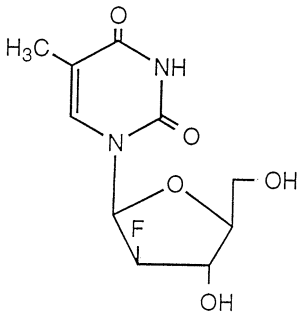

FIG. 1. Structure of L-FMAU.

Facility and were purified by electrophoresis through an 8 M urea–15% polyacrylamide gel. The sequences of the oligonucleotides used in this study are presented below:

3′-GGCAGTTAAGGAAATTCAAAATTGGAA

CGCCGGCA-5′ (35-mer)

5′-CCGTCAATTCCTTTA-3′ (15-mer)

The primer 15-mer oligonucleotide was labeled at the 5′-position with T4 polynucleotide kinase and [γ-^{32}P]ATP and was subsequently used as a substrate for 3′-to-5′ exonuclease assays. For a study of the elongation reactions, the [5′-^{32}P]-15-mer oligonucleotide was annealed to the 35-mer oligonucleotide as described [10]. The complex was purified through a Sephadex G-25 column and was used as a substrate for DNA elongation.

Cells

Raji cells were grown at 37° in RPMI 1640 medium supplemented with 10% fetal bovine serum and 100 μg/mL of kanamycin. To induce EBV DNA polymerase, cells were treated with 30 ng/mL of TPA and 4 mM of *n*-butyrate for 48–60 hr as described previously [11].

Purification of DNA Polymerases

EBV DNA polymerase was purified through successive chromatographic steps (DEAE cellulose, phosphocellulose, heparin agarose, and single-stranded DNA cellulose) as described previously [12] with some modifications. Only those fractions containing DNA polymerase activity that could be stimulated by 150 mM of $(NH_4)_2SO_4$ and were sensitive to phosphonoformic acid were pooled for further purification. Purification of human DNA polymerases was described previously [13], as was that of HSV DNA polymerase [14]. All buffer solutions contained 10% glycerol and protease inhibitors (benzamidine and PMSF, 1 mM each; leupeptin and pepstatin, 1 μg/mL each).

Enzyme Assays

EBV DNA polymerase was routinely assayed as described [7]. Each reaction mixture (8 μL) contained 50 mM of Tris–HCl buffer, pH 8.0, 4 mM of $MgCl_2$, 1 mM of DTT, 150 mM of KCl, 10% glycerol, 5 nM of 5′-[^{32}P]primer–template complex, and 100 μg/mL of BSA. The reaction was started by the addition of the enzyme fraction, and the reaction mixture was incubated for 30–60 min at 37°. Concentrations of dNTPs and inhibitors are shown in the legends to the figures. Reaction assays for human DNA polymerases have been described [13].

The 3′-to-5′ exonuclease activity was assayed using 5′-[^{32}P]-15-mer oligonucleotide. The reaction was run under the same conditions as the elongation reaction but without dNTPs. Products of primer extension or 3′-to-5′ exonuclease reactions were separated by 15% polyacrylamide–8 M urea gel electrophoresis. After electrophoresis, the gels were exposed directly to Kodak x-ray film at −80° overnight.

Nucleotide Turnover

The assay that measures the conversion of dNTP to dNMP was performed essentially as described [15]. The reaction mixture in a final volume of 20 μL contained 50 mM of Tris–HCl buffer, pH 8.0, 6 mM of $MgCl_2$, 1 mM of DTT, 80 μg/mL of poly(dC)oligo$(dG)_{12-18}$, 8 μM of [α-^{32}P]dGTP (2500 cpm/pmol), 5 units of EBV DNA polymerase, and different amounts of L-FMAU-TP. One unit of EBV DNA polymerase activity is defined as the amount of enzyme catalyzing the incorporation of 1 nmol of dTMP into DNA per 1 hr under the conditions used. After incubation at 37° for 40 min, 2 μL of a mixture containing both 20 mM of dGMP and 20 mM of dGTP (as markers for TLC) and 500 mM of EDTA was added to the incubation mixture. The products in 10-μL reaction mixtures were analyzed by Kieselgel 60 F_{254} thin-layer chromatography in the following system: dioxane:25% NH_4OH:H_2O, 6:1:4. The R_f values for dGTP, dGDP, and dGMP were 0.2, 0.47, and 0.63, respectively. The assays were done in the presence of a control (identical assays performed but without the template). The TLC sheets were cut into 0.5-cm pieces, added to scintillation fluid, and counted. The remaining 10 μL was used to detect the incorporation of dGMP residues into DNA.

RESULTS

Inhibition of Primer Extension Reaction Catalyzed by EBV DNA Polymerase with L-FMAU-TP

Previously, we reported that L-FMAU-TP could not be utilized as a substrate by EBV DNA polymerases or human DNA polymerases α, β, γ, and δ [7]. Therefore, the incorporation of the L-FMAU-MP residues into viral DNA might not be the mechanism of antiviral activity. To determine whether L-FMAU-TP is an inhibitor of EBV or human DNA polymerases α, β, ε, and γ, the effect of this compound on primer extension was examined. The complex of a 5′-[^{32}P]-15-mer oligonucleotide annealed to a 35-mer oligonucleotide was used as a primer-template for

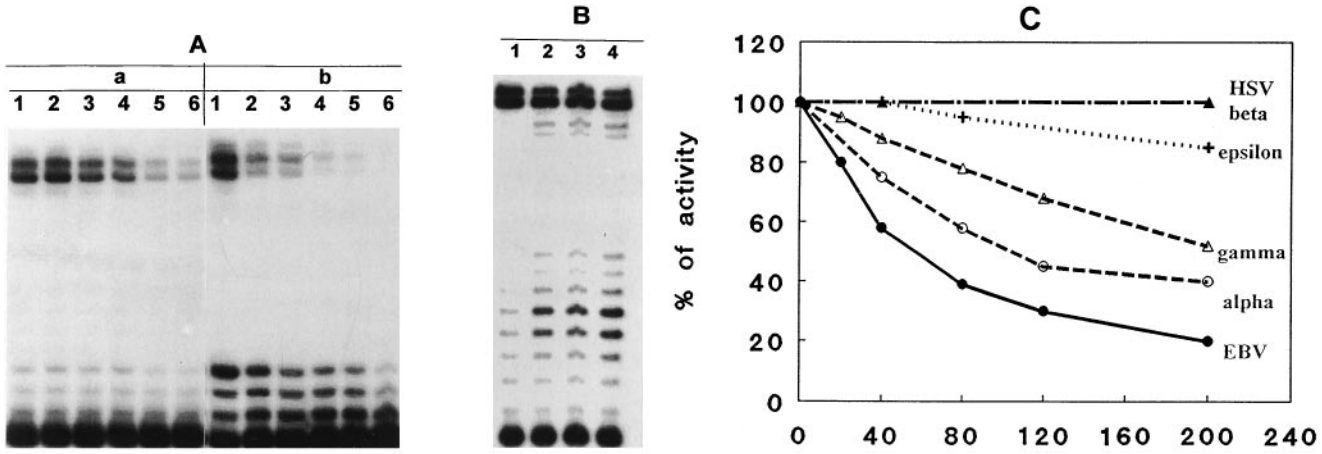

FIG. 2. Gel electrophoresis of DNA elongation products catalyzed by EBV (A) and HSV (B) DNA polymerases in the presence of L-FMAU-TP. Reaction mixtures contained: 20 μM of dATP, dGTP, dTTP each and 1 μM of dCTP (a); 20 μM of dATP, dGTP, dCTP each and 1 μM of dTTP (b); 5 μM all four dNTPs (B). Reaction conditions are described in Materials and Methods. Lanes 1 (A and B): reactions without L-FMAU-TP. Lanes 2–6 (A): in the presence of 10, 20, 50, 100, and 200 μM of L-FMAU-TP, respectively. Lanes 2–4 (B): in the presence of 100, 200, and 500 μM of L-FMAU-TP. (C) Concentration dependence of inhibition of DNA polymerases by L-FMAU-TP. Reaction mixtures contained 5 μM of dATP, dCTP, dGTP, and dTTP each. Reaction conditions were optimal for each enzyme [13] and are described in Materials and Methods. One hundred percent activity corresponds to the amount of products synthesized by DNA polymerases in the absence of L-FMAU-TP. The amount of products was measured by a computer densitometer as described in Materials and Methods. Values are the means of four independent experiments.

DNA elongation reaction. The reactions were conducted under conditions optimal for each enzyme and described in Materials and Methods.

Figure 2 shows the gel electrophoresis pattern of the elongation products catalyzed by EBV (panel A) and HSV (panel B) DNA polymerases in the presence of different amounts of L-FMAU-TP. The yield of the total products synthesized by EBV DNA polymerase decreased with increasing concentrations of L-FMAU-TP when either dCTP (Fig. 2A, panel a), or dTTP (Fig. 2A, panel b) was used at limiting concentrations (1 μM). The difference in the amount of products was no more than 50% when the dTTP concentration was changed 20 times (2b, 2a). Figure 2 also demonstrates that L-FMAU-TP could not compete with dCTP for its incorporation into DNA, although L-FMAU was a substrate for deoxycytidine kinase.* In contrast, HSV DNA polymerase, an enzyme similar in many ways to EBV DNA polymerase, was not inhibited by up to 500 μM of L-FMAU-TP. Inhibition patterns for different human DNA polymerases by L-FMAU-TP were also obtained (data not shown). The bands on the x-ray film, representing the total product synthesized by EBV, HSV, or human DNA polymerases α, ε, β, and γ in the presence of increasing concentrations of L-FMAU-TP, were quantitated with the aid of a densitometer (Molecular Dynamics), as described previously [16]. Figure 2C shows that the total amount of elongation products catalyzed by EBV, HSV, or human DNA polymerases decreased with increasing concentrations of L-FMAU-TP. Experimental measurements were carried out within the linear range between the yield of product formation and time. The concentrations of L-FMAU-TP that inhibited the yield of products by 50% were 45, 100, and greater than 150 μM for EBV, γ, and α DNA polymerases, respectively. DNA polymerases β, ε, and HSV were relatively resistant to L-FMAU-TP. We studied the primer extension reactions as a function of dNTP or primer–template concentrations to examine the type of inhibition exerted by L-FMAU-TP on EBV DNA polymerase (Fig. 3). The left lane (Fig. 3A) shows DNA synthesis catalyzed by EBV DNA polymerase in the absence of L-FMAU-TP when 5 μM of dNTP each were included in the reaction. The next lane shows that 50 μM of L-FMAU-TP inhibited the reaction by 50–60%, and that the values of inhibition were approximately the same while the concentrations of dATP, dTTP, dCTP, or dGTP were increased from 5 to 500 μM. The results indicated that L-FMAU-TP could not compete with any dNTPs for incorporation into DNA (Fig. 3A). When the inhibition of EBV DNA polymerase by L-FMAU-TP was examined as a function of the concentration of primer-template [poly(d-C)oligo$(dG)_{12-18}$], the results indicated that L-FMAU-TP inhibited the reaction in a linear, uncompetitive manner with respect to primer–template with an apparent K_i of 38 ± 10 μM (Fig. 3B).

Inhibition of 3′-to-5′ Exonuclease Activity of EBV DNA Polymerase with L-FMAU-TP

EBV DNA polymerase has an intrinsic 3′-to-5′ exonuclease activity that liberates dNMP residues from the primer terminus. This activity can be preferentially inhibited by purine nucleoside 5′-monophosphates, whereas at the same concentration pyrimidine nucleoside 5′-monophosphates are much weaker inhibitors [12, 17]. Among purine nucle-

* Liu S-H, Grove KL and Cheng Y-C, manuscript submitted for publication.

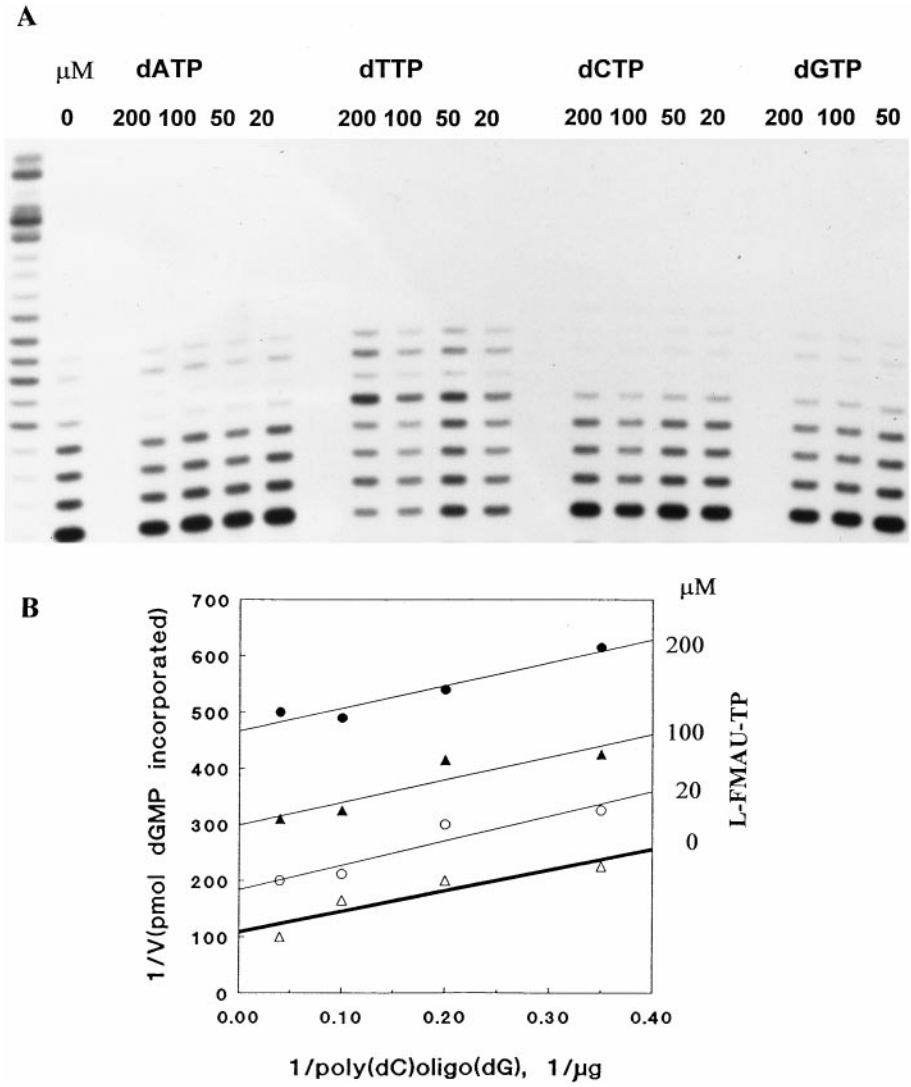

FIG. 3. (A) Effect of dNTPs on inhibition by L-FMAU-TP of DNA synthesis catalyzed by EBV DNA polymerase. Each reaction mixture contained 0.2 μM of complex [5′-[^{32}P]-15-mer to 35-mer oligonucleotide], 50 μM of L-FMAU-TP, 5 μM of dATP, dCTP, dTTP, dGTP each, and additional amounts of dATP, dTTP, dCTP, and dGTP, as shown. Left lane: synthesis without L-FMAU-TP. (B) Inhibition of EBV DNA polymerase by L-FMAU-TP as a function of poly(dC)oligo(dG)$_{12-18}$ concentration used as primer-template. dGMP incorporation was determined between 2 and 25 μg of poly(dC)oligo(dG)$_{12-18}$. Incubation time was 20 min. Reaction products were analyzed by filter assay [7]. Reaction conditions are described in Materials and Methods. Concentrations of L-FMAU-TP are shown near inhibition plots.

oside 5′-monophosphates, GMP was the most potent inhibitor. GMP inhibited the exonuclease activity competitively with respect to the DNA substrate, whereas the chain elongation activity of EBV DNA polymerase is not inhibited by up to 5 mM of GMP [17]. The effects of L-FMAU-MP and L-FMAU-TP, in comparison to GMP, on 3′-to-5′ exonuclease activity of EBV DNA polymerase were studied. Figure 4 demonstrates that L-FMAU-MP, within a concentration range of 50–500 μM, had no effect on exonuclease activity. In contrast, L-FMAU-TP inhibited the activity by 50% at concentrations of less than 60 μM. L-FMAU-TP proved to be a 20-fold more potent inhibitor of 3′-to-5′ exonuclease activity than GMP, which inhibited 3′-to-5′ exonuclease activity by 50% at a concentration of 5 mM.

Inhibition of Nucleotide Exchange Reaction Catalyzed by EBV DNA Polymerase

To determine the effect of L-FMAU-TP on both the polymerase and the 3′-to-5′ exonuclease activities simulta-

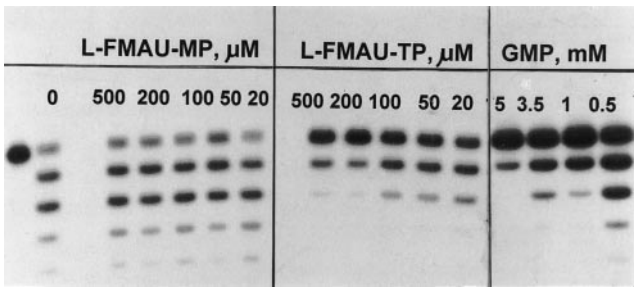

FIG. 4. Hydrolysis of [5′-^{32}P]-15-mer oligonucleotide by 3′-to-5′ exonuclease activity of EBV DNA polymerase in the presence of L-FMAU-MP, L-FMAU-TP, and GMP. Left lane: position of 15-mer oligonucleotide. Concentrations of compounds used are shown in the figure. The reaction mixture contained 5 nM of 5′-[^{32}P]-15-mer oligonucleotide and 2 units of EBV DNA polymerase. Samples were incubated for 30 min at 37°.

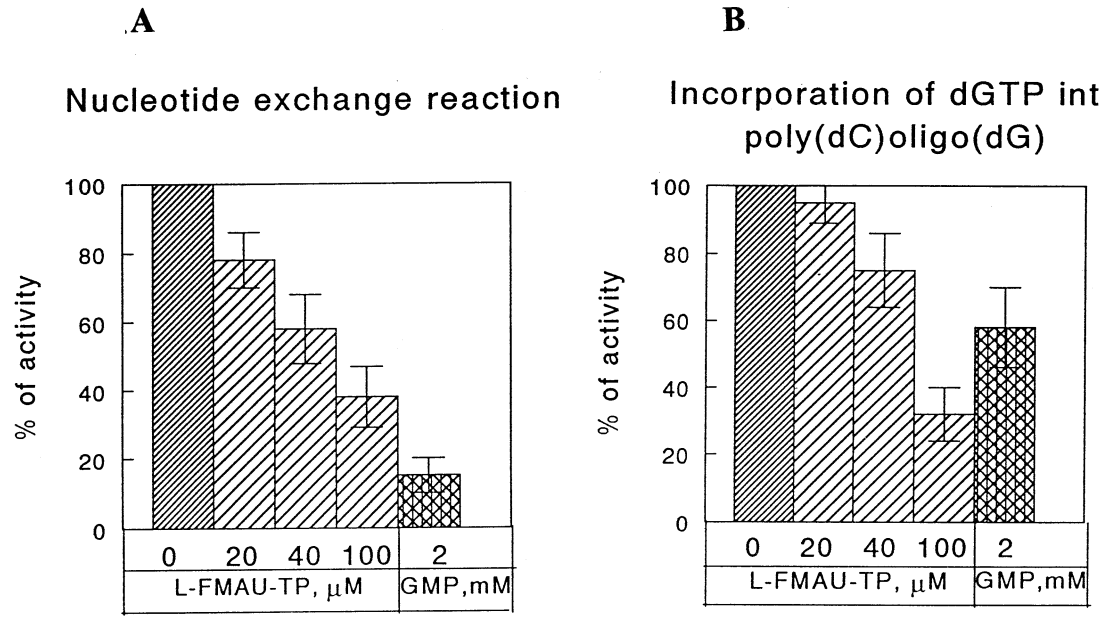

FIG. 5. (A) Nucleotide exchange reaction. (B) Incorporation of [α-^{32}P]dGMP into poly(dC)oligo(dG)$_{12-18}$ catalyzed by EBV DNA polymerase in the presence of L-FMAU-TP or GMP. Concentrations of L-FMAU-TP and GMP are shown in the figure. Background was defined as the measurement of radioactivity of the sample incubated without primer-template. The value was less than 10% when compared with the experimental mixture in the presence of template. Reaction mixtures and conditions are described in Materials and Methods. In panel A, the radioactivity of [^{32}P]dGMP obtained in the absence of inhibitors was taken as 100% and was equal to 11,200 cpm. In panel B, the radioactivity incorporated into the acid-insoluble material in the absence of inhibitors was taken as 100%. The 100% corresponds to 15,000 cpm. Values are the means (± SD) of three independent experiments.

neously, DNA-dependent nucleotide turnover, which is a characteristic of DNA polymerases possessing 3′-to-5′ exonucleases [18], was studied for EBV DNA polymerase. EBV DNA polymerase exhibited nucleotide turnover of dGTP to dGMP when either poly(dC)oligo(dG) or activated DNA was used as a template-primer. Figure 5 shows the effect of L-FMAU-TP on nucleotide turnover and on the extension reaction of oligo(dG) with [α-^{32}P]dGMP residues when [α-^{32}P]dGTP and poly(dC)oligo(dG) were used as substrate and primer-template. Both reactions were done in the presence of controls, which were identical assays but in the absence of the primer-template. Both reactions were inhibited by 50% at about the same concentration (40–60 μM). GMP could also inhibit both reactions as described previously [17].

DISCUSSION

Several nucleoside analogs in the D-configuration have been shown to be active as antiviral agents that can suppress or eliminate viral replication. Recently, several L-nucleoside analogs were discovered as a new class of compounds with potent inhibitory activity against HIV and HBV, as well as cancer. Among the L-nucleoside analogs, β-L-2′,3′-dideoxy-3′-thiacytidine (β-L-SddC, 3TC), β-L-5-fluoro-2′,3′-dideoxy-3′-thiacytidine (β-L-5FSddC), and β-L-5-fluoro-2′,3′-dideoxy-didehydrocytidine (β-L-5Fd4C) are in different stages of clinical development as antiviral compounds. All of these compounds are L-cytidine analogs, and none of them has potent anti-EBV activity. Recently, β-L-dioxalane-cytidine (β-L-OddC) has been reported to have potent anti-EBV activity [19]. However, β-L-OddC was shown to display high toxicity to the growth of a variety of cell lines, and now this compound is in phase I trials as an anticancer compound [20].

It has been demonstrated in this laboratory that a novel L-thymidine analog, L-FMAU, has potent anti-EBV [7] and anti-HBV [8] activity. L-FMAU can be phosphorylated stepwise to L-FMAU-TP by many human cell lines in culture. Both EBV and cellular thymidine kinases can utilize L-FMAU as a substrate [7]. Therefore, the concentration of phosphorylated metabolites in EBV replicating cells could be higher than that in cells not infected with EBV. In addition, deoxycytidine kinase could also utilize L-FMAU as a substrate (unpublished data). Interestingly, L-FMAU has little activity against HSV, but still can serve as a substrate for HSV thymidine kinase (unpublished results). The potent inhibitory activity of L-FMAU-TP toward EBV DNA polymerase but not toward HSV-1 DNA polymerase can explain, in part, the inhibitory effect of L-FMAU against EBV but not HSV, providing viral DNA polymerase as a target for the inhibitory effect. The mechanism of inhibition of EBV DNA polymerase by L-FMAU-TP differs from that of the triphosphate metabolites of antiviral agents such as acyclovir, D-FMAU, FIAU, gancyclovir, and pencyclovir, which are substrates for viral DNA polymerase and certain human DNA polymerases [21–23]. The behavior of L-FMAU-TP toward different

DNA polymerases is similar, to some degree, to β-L-TTP [24] and its analog, β-L-rTTP [25], which also have 3′-OH groups at ribose residues. β-L-rTTP was an inhibitor, but not a substrate for HIV reverse transcriptase [25]. DNA polymerases α and β were resistant to β-L-TTP up to 200 μM, and DNA polymerase γ was inhibited by 50% at about 50 μM of β-L-TTP under the conditions used [24]. No indication of the type of inhibition was reported. Others have shown that β-L-dTTP could not be incorporated into DNA by DNA polymerases β, δ, and γ, but that DNA polymerase α could incorporate two β-L-dTMP residues [26]. Recently, the substrate properties of β-L-dTTP and β-L-dCTP toward DNA polymerases α, β, and ε were examined, and it was shown that neither L-dTTP nor L-dCTP were substrates for the DNA polymerases tested [27]. The effect of β-L-dTTP on exonuclease activity associated with any DNA polymerases was not studied. Our results have shown that L-FMAU-TP is not a substrate for the DNA polymerases examined and is a relatively poor inhibitor of DNA elongation catalyzed by human DNA polymerases. However, L-FMAU-TP is an inhibitor of DNA elongation and 3′-to-5′ nucleotide excision catalyzed by EBV DNA polymerase with a K_i of 50 μM. None of the natural dNTPs prevented the inhibition of DNA synthesis catalyzed by EBV DNA polymerase by L-FMAU-TP. Uncompetitive inhibition of EBV DNA polymerase by L-FMAU-TP with respect to DNA template was observed. These results suggested that L-FMAU-TP bound to EBV DNA polymerase at a site different from the dNTP or template binding sites. Such an interaction resulted in the inhibition of both the DNA elongation catalyzed by EBV DNA polymerase and its associated 3′-to-5′ exonuclease activity.

In summary, L-FMAU-TP is not a substrate for EBV DNA polymerase but can inhibit both DNA chain elongation and exonuclease activity of EBV DNA polymerase with similar potency. To date, no other nucleoside 5′-triphosphate analogs have been described with such properties. The pattern of inhibition is uncompetitive toward substrates. L-FMAU-TP inhibition of human DNA polymerases is much weaker. The inhibition of EBV DNA polymerase by L-FMAU-TP may be one of the mechanisms responsible for the anti-EBV activity of L-FMAU.

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