Mammalian DNA Polymerases α, β, γ, δ, and ϵ Incorporate Fialuridine (FIAU) Monophosphate into DNA and Are Inhibited Competitively by FIAU Triphosphate†

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ABSTRACT: Fialuridine [FIAU, 1-(2′-deoxy-2′-fluoro-β-D-arabinofuranosyl)-5-iodouridine] was used in clinical trials for chronic hepatitis B virus infection and was extremely toxic. Evidence suggested targets of FIAU toxicity included mitochondria, but toxic mechanisms were unclear. Since FIAU is a thymidine analog, we reasoned that triphosphorylated FIAU (FIAUTP) could be incorporated into mitochondrial DNA by DNA pol-γ and into genomic DNA by DNA polymerases α, β, δ, and ϵ. All five purified mammalian DNA polymerases incorporated FIAUMP into the nascent DNA chain during *in vitro* DNA synthesis. When FIAUTP was substituted for dTTP, oligonucleotide products were generated efficiently by DNA pol-γ and were similar to those generated in the presence of the four normal dNTPs. In contrast, oligonucleotide products generated by the four nuclear DNA polymerases in the presence of FIAUTP were significantly reduced in length relative to those generated in the presence of dTTP. In parallel kinetic assays, FIAUTP competitively inhibited the accumulation of radiolabeled dTTP into DNA by DNA pol-γ. The K_i with DNA pol-γ was 0.04 μM, the lowest K_i among the mammalian DNA polymerases. Competition between FIAUTP and dTTP and the relative ease of accumulation of FIAUMP in mitochondrial DNA by DNA pol-γ *in vitro* together may relate to clinical FIAU toxicity.

Chronic hepatitis from hepatitis B virus (HBV)[1] infection afflicts approximately one million Americans and up to 5% of the world's population (Hoofnagle & DiBisceglie, 1989). Major goals of therapy in chronic HBV infection include reducing or eliminating viral replication and halting the progress of irreversible hepatocellular disease (Hoofnagle & Alter, 1984). A cell line derived by cotransfection of HBV genomes into human hepatoblastoma cells continuously produced mature virions, replicative DNA intermediates, and large amounts of viral polypeptides (Lampertico et al., 1991). The nucleoside analogs 1-(2′-deoxy-2′-fluoro-β-D-arabinofuranosyl)-5-iodouracil (fialuridine, FIAU) and 1-(2′-deoxy-2′-fluoro-β-D-arabinofuranosyl)-5-iodocytosine (FIAC) each reduced intracellular and extracellular HBV DNA abundance in those cells (Korba & Gerin, 1992). The anti-HBV activity of FIAU was selective and dependent, in part, upon its intracellular phosphorylation (Staschke et al., 1994). FIAU was used to treat chronic hepatitis in clinical trials at the NIH where unexpected toxicity included death of some patients (Touchette, 1993).

In viral illnesses, therapy may include treatment with antiviral nucleoside analogs (ANAs). Many of the ANAs for treatment of HBV, human immunodeficiency virus-1 (HIV-1) and other viruses share structural similarities. Both FIAU and zidovudine (AZT) are thymidine ANAs. AZT also is triphosphorylated intracellularly (DeClercq, 1991). AZTTP interferes with the HIV-1 reverse transcriptase (Yarchoan & Broder, 1987), effectively inhibits mitochondrial DNA pol-γ *in vitro* compared to nuclear DNA pols (Huang et al., 1990; Keilbaugh et al., 1990; Izuta et al., 1991; Chen et al., 1991; Lewis et al., 1994), and causes structural changes in mitochondria *in vivo* (Lewis et al., 1991, 1992). On the basis of clinical features and pharmacologic data, we reasoned that FIAU toxicity may relate to inhibition of DNA pol-γ by FIAUTP and to accumulation of FIAUMP in mitochondrial DNA (mtDNA) by DNA pol-γ. To test these possibilities, we compared the polymerization of FIAUMP into DNA *in vitro* by mitochondrial DNA pol-γ to its polymerization by the nuclear DNA pol-α, DNA pol-β, DNA pol-δ, and DNA pol-ϵ. In parallel, we defined K_is of FIAUTP with each mammalian DNA polymerase. All five DNA polymerases incorporated FIAUMP into DNA, but FIAUMP accumulated in DNA by DNA pol-γ most efficiently. Furthermore, the K_i of FIAUTP with DNA pol-γ (0.04 μM FIAUTP) was the lowest among the DNA polymerases examined.

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[1] Abbreviations: HBV, hepatitis B virus; FIAU, 1-(2′-deoxy-2′-fluoro-β-D-arabinofuranosyl)-5-iodouracil; FIAUMP, 1-(2′-deoxy-2′-fluoro-β-D-arabinofuranosyl)-5-iodouracil monophosphate; FIAUTP, 1-(2′-deoxy-2′-fluoro-β-D-arabinofuranosyl)-5-iodouracil triphosphate; FIAC, 1-(2′-deoxy-2′-fluoro-β-D-arabinofuranosyl)-5-iodocytosine; ANAs, antiviral nucleoside analogs; HIV-1, human immunodeficiency virus-1; AZT, 3′-azido-3′-deoxythymidine; AZTTP, 3′-azido-3′-deoxythymidine triphosphate; DNA pol, DNA polymerase; dNTP, deoxynucleoside triphosphate; BSA, bovine serum albumin; mtDNA, mitochondrial DNA; 2-ME, 2-mercaptoethanol.

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MATERIALS AND METHODS

Materials. Reagents were analytical grade I. [^{32}P]dTTP and [^{3}H]dTTP were from Amersham. FIAU was provided by Oclassen Pharmaceuticals, San Rafael, CA. FIAUTP was synthesized by T. E. Mabry and C. D. Jones, Lilly Research Laboratories, Indianapolis, IN. FIAUTP purity was >99% and was confirmed by HPLC. The structure was verified by proton NMR, mass spectrometry, and UV spectroscopy. Synthetic ribo- and deoxyribonucleotide polymers were obtained from Pharmacia. They were annealed in 10 mM Tris-HCl, pH 7.5, and 1 mM EDTA at 65 °C for 15 min and slowly cooled to room temperature. Poly[d(A-T)]·poly-[d(A-T)] was prepared according to established methods (Schachman et al., 1960). The phagemid DNA template was derived from pBluescript II KS$^+$ (Perrino & Mekosh, 1992). The 17 base oligonucleotide primer is complementary to nucleotide position 627−643 of the phagemid DNA. The dNTPs were obtained from Sigma.

DNA polymerases. The five mammalian DNA polymerases were purified in our laboratories by published methods. Each enzyme represented an extensively purified or homogeneous preparation. The specific activities of the DNA polymerases ranged from 1200 to 50 000 units/mg. A unit of polymerase activity catalyzed the incorporation of 1 nmol of total nucleotide per hour at 37 °C.

With each DNA polymerase, the enzyme was purified from more than one tissue to verify that tissue source did not impact on the results. Kinetics of the enzymes and K_ms for dTTP with each DNA polymerase were essentially identical irrespective of tissue source of a given enzyme. The tissue sources of the respective DNA polymerases and the respective methods of purification were as follows. DNA pol-α was prepared from calf thymus or from human myeloblasts as the four-subunit DNA pol-α−primase complex (Perrino & Mekosh, 1992; Perrino & Loeb, 1989). For each preparation, the amount of DNA pol-α protein (140−180 kDa) was estimated from SDS silver-stained gels by comparing staining intensities with β-galactosidase (Sigma, Grade VIII). Specific activity was estimated to be between 10 000 and 15 000 units/mg for the calf thymus and human DNA pol-α, respectively. DNA pol-β (specific activity of 50 000 units/mg) was obtained from the Novikoff hepatoma (Stalker et al., 1976) or from *Escherichira coli* containing the cloned rat DNA pol-β gene (Date et al., 1988). DNA pol-γ (specific activity of 3300 units/mg) was prepared from bovine liver or heart [described in Lewis et al., (1994) and modified from Meyer and Simpson (1968, 1970) and Mosbaugh (1988)]. DNA pol-δ was purified from calf thymus through step 6 of the methods of Lee et al., 1984) with a specific activity of 9500 units/mg. DNA pol-δ and DNA pol-ε from human myeloblasts were purified using methods of Syväoja et al., (1990) through step 5. DNA pol-δ and DNA pol-ε specific activities from those preparations were 3500 and 1200 units/mg, respectively. DNA pol-δ was separated from proliferating cell nuclear antigen during chromatography using DEAE-Sepharose to generate the low processivity form of the enzyme.

DNA Polymerase Assays. For the primer extension assays, the 17 base oligonucleotide primer was labeled with ^{32}P at the 5′ position and hybridized to the phagemid DNA template at a 1:1 molar ratio (Perrino & Mekosh, 1992). Reaction mixtures (20 μL) were prepared for each of the five DNA polymerases containing the 17-mer-primed DNA template (0.5 pmol) and the following: for DNA pol-α, 20 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 2 mM dithiothreitol (DTT), 2-ME and 100 μg/mL BSA (Perrino & Mekosh, 1992); for DNA pol-β, 25 mM Tris-HCl, pH 8.4, 5 mM 2-mercaptoethanol (2-ME), 1 mM $MnCl_2$, and 15% glycerol (Stalker et al., 1976; Mosbaugh & Meyer, 1980); for DNA pol-γ, 25 mM Tris-HCl, pH 8.0, 100 mM NaCl, 5 mM 2-ME, and 1 mM $MnCl_2$ (Meyer & Simpson, 1968; Mosbaugh, 1988); for DNA pol-δ, 40 mM HEPES, pH 6.5, 1 mM $MgCl_2$, 10 mM KCl, 2 mM DTT, 0.03% Triton X-100, 2% glycerol, and 80 μg/mL BSA (Lee et al., 1984; Syväoja et al., 1990); for DNA pol-ε, 50 mM HEPES, pH 7.5, 15 mM $MgCl_2$, 10 mM DTT, 0.03% Triton X-100, 20% glycerol, and 200 μg/mL BSA (Syväoja et al., 1990). The concentration of each dNTP was 20 μM as indicated in the legend to Figure 1. Extension reactions were performed at 37 °C for 30−60 min with 0.04−0.12 units of DNA polymerase. Reaction products were processed as described (Perrino & Loeb, 1989) and analyzed by electrophoresis through 15% polyacrylamide sequencing gels. Gels were fixed in 10% methanol/10% acetic acid, vacuum-dried, and exposed to Kodak XAR-5 film.

For the kinetic assays, each of the DNA polymerases was measured using the above reaction conditions with the optimal synthetic polynucleotide template and dNTP concentration defined in the literature (Mosbaugh & Meyer, 1980; Fry & Loeb, 1986; Burgers, 1989; Rein et al., 1990; Wang, 1991). To determine K_m for dTTP, velocity vs substrate concentration experiments were performed for each DNA polymerase using a range of dTTP concentrations from 0.2 to 100 μM. The K_ms for dTTP were calculated from reciprocal plots using 6−10 different concentrations that generated evenly spaced reciprocals in the region flanking the K_m. Four dTTP concentrations were selected for the K_i analysis using four different inhibitor concentrations. For DNA pol-α, the template was 100 μM poly[d(A-T)]·poly-[d(A-T)] and 10−25 μM dNTPs. For DNA pol-β, the template was 100 μM poly(rA)·oligo(dT_{12-18}) (5:1 molar ratio) and 20−40 μM dNTPs. For DNA pol-γ, the template was 50 μM poly(rA)·oligo(dT_{12-18}) (5:1 molar ratio) and 0.6−4.0 μM dNTPs. For DNA pol-δ, the template was 100 μM poly[d(A-T)]·poly[d(A-T)], and nucleotides were 40 μM dATP and 10−25 μM dNTP. For DNA pol-ε, the template was 100 μM poly(dA)·oligo(dT_{12-18}) (5:1 molar ratio) and 5−20 μM dNTPs. Assays were carried out in 30−62.5 μL volumes at 30 or 37 °C for 30−60 min using [^{32}P]dTTP or [^{3}H]dTTP. For each enzyme, the assay conditions gave linear kinetics with respect to time and enzyme concentration. In every experiment, the reported dTTP concentration represented the total of radiolabeled and unlabeled dTTP. Assays were terminated by placing reaction mixtures in ice. After addition of 10 μg of carrier DNA to each tube, the DNA product was precipitated by adding 0.5 mL of cold 10% trichloroacetic acid containing 20 mM sodium pyrophosphate. Precipitates were collected on Whatman GF/C filters, washed three times with 0.1 M HCl containing 20 mM sodium pyrophosphate, and washed once with 95% ethanol. The radioactivity was determined by liquid scintillation spectrometry. All enzyme−inhibitor assays were replicated 2−4 times. Each assay point was performed in triplicate within each run. Calculated arithmetic means from

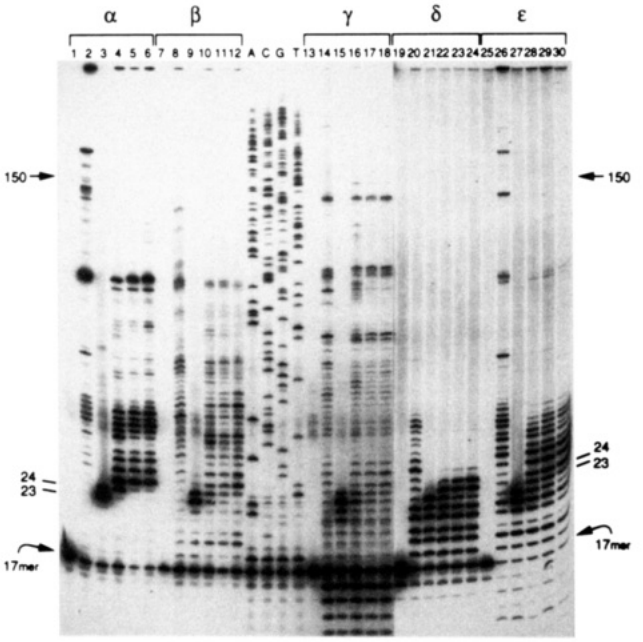

FIGURE 1: 1. Accumulation of FIAUMP in DNA catalyzed by mammalian DNA polymerases. The 5′ ^{32}P-labeled 17-mer oligonucleotide was hybridized to the phagemid DNA template and extended with the DNA pol-α (lanes 1−6), DNA pol-β (lanes 7−12), DNA pol-γ (lanes 13−18), DNA pol-δ (lanes 19−24), and DNA pol-ϵ (lanes 25−30) in reaction mixtures prepared as described under Materials and Methods. The nucleotides present in reactions were as follows: 20 μM dATP, dCTP, dGTP, and dTTP (lanes 2, 8, 14, 20, and 26), 20 μM dATP, dCTP, and dGTP (lanes 3, 9, 15, 21, and 27), 20 μM dATP, dCTP, dGTP, and 1 μM FIAUTP (lanes 4, 10, 16, 22, and 28), 20 μM dATP, dCTP, dGTP, and 5 μM FIAUTP (lanes 5, 11, 17, 23, and 29), and 20 μM dATP, dCTP, dGTP, and 20 μM FIAUTP (lanes 6, 12, 18, 24, and 30). Extension reactions were at 37 °C for 30 min (DNA pol-α and DNA pol-β) or 60 min (DNA pol-γ, DNA pol-δ, and DNA pol-ϵ) with 0.12 units of DNA pol-α, 0.10 units of DNA pol-β, 0.04 units of DNA pol-γ, 0.08 units of DNA pol-δ, and 0.04 units of DNA pol-ϵ. Control reactions contain no DNA polymerase (lanes 1, 7, 13, 19, and 25). Dideoxy sequencing reactions using the same DNA template-primer are indicated as A, C, G, and T for the dideoxynucleotide used in the reaction. The positions of the starting 17-mer primer and oligonucleotide products are indicated.

the triplicate assays were utilized to plot kinetic data using the least−squares method.

RESULTS

Accumulation of FIAUMP into DNA by Mammalian DNA Polymerases. The ability of DNA pol- α, DNA pol-β, DNA pol-γ, DNA pol-δ, and DNA pol-ϵ to catalyze the accumulation of FIAUMP into a nascent DNA chain was determined by analyzing the products of *in vitro* DNA synthesis reactions (Figure 1). Using an oligonucleotide-primed natural DNA template, in the presence of all four natural dNTPs each of the DNA polymerases extended the 17-mer primer to generate oligonucleotide products that range from 18 to >150 nucleotides in length (Figure 1, lanes 2, 8, 14, 20, and 26). When dTTP was omitted from reactions, the major elongated oligonucleotide products generated by the DNA pol-α and DNA pol-β corresponded to a 23-mer and 24-mer (Figure 1, lanes 3 and 9). For DNA pol-γ, DNA pol-δ, and DNA pol-ϵ the products were distributed between 18 and 24 nucleotides in length (Figure 1, lanes 15, 21, and 27). The products up to 23 nucleotides in length resulted from extension of the 17-mer primer by addition of correct

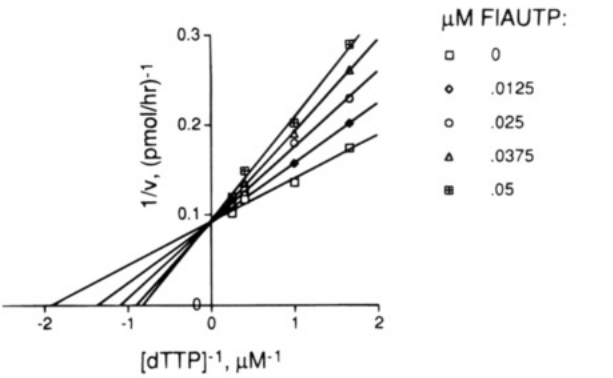

FIGURE 2: 2. Lineweaver−Burk plot of FIAUTP inhibition of DNA pol-γ. By varying the concentration of dTTP substrate (0.6−4 μM dTTP) and relating the FIAUTP concentration (0−0.05 μM FIAUTP) to the reciprocal of the enzyme velocity, a family of lines was generated. Inhibition of dTTP incorporation by FIAUTP was competitive. Assays were performed in 50 μL volumes in buffer containing 50 μM poly(rA)•oligo(dT$_{12}$−18) (5:1 molar ratio), 10 μCi/mL [^{3}H]dTTP (final concentration: 2 μM), and 0.01−0.04 units of enzyme. Reactions were for 30 min at 30 °C.

nucleotides. The 24-mer resulted from subsequent addition of an incorrect nucleotide opposite the first dAMP in the DNA template. When dTTP was omitted, no oligonucleotide products were detected beyond the 24-mer position for any DNA polymerase reaction. These control reactions demonstrated that all of the DNA polymerases effectively utilize the natural DNA template. In the absence of dTTP no template-directed DNA synthesis was detected past sites that corresponded to template dAMP.

When the FIAUTP was added in reactions containing dATP, dCTP, and dGTP, each of the DNA polymerases generated oligonucleotide products that were 24 nucleotides in length or greater. This indicated that all five DNA polymerases insert FIAUMP opposite dAMP in the DNA template (Figure 1, lanes 4−6, 10−12 16−18, 22−24, and 28−30) and that FIAUTP served as an alternate substrate for the DNA polymerases in these reactions. Furthermore, oligonucleotide products greater than 25 nucleotides in length were detected for all concentrations of FIAUTP and for all DNA polymerases tested. These latter results demonstrated that all five of the mammalian DNA polymerases inserted FIAUMP and extended from the FIAUMP 3′ terminus to incorporate the analog into duplex DNA.

Mitochondrial DNA pol-γ efficiently catalyzed the accumulation of FIAUMP into DNA. Oligonucleotide products generated by DNA pol-γ in the presence of 20 μM FIAUTP were essentially indistinguishable from those generated in the presence of 20 μM dTTP (compare Figure 1, lane 18 with lane 14). On this basis, FIAUTP served as an effective substitute for dTTP with respect to template-directed polymerization by DNA pol-γ. In the presence of 20 μM FIAUTP, oligonucleotide products formed by each nuclear DNA polymerase were reduced in length compared to those generated by the respective polymerase in the presence of 20 μM dTTP (for DNA pol-α, compare lane 6 with lane 2; for DNA pol-β, compare lane 12 with lane 8; for DNA pol-δ, compare lane 24 with lane 20; for DNA pol-ϵ, compare lane 30 with lane 26).

Inhibition of Mammalian DNA Polymerases by FIAUTP. Pharmacologic inhibition of the mammalian DNA polymerases by FIAUTP was determined in kinetic assays using preferred synthetic template/primers. No differences in

Table 1: K_m for dTTP and K_i for FIAUTP for Mammalian DNA Polymerases Using Synthetic Polynucleotide Template/Primers[a]

enzyme	tissue source	template	K_m dTTP	K_i FIAUTP
DNA pol-α	calf thymus	poly[dA-T]•poly[d(A-T)]	4.8 ± 0.3	0.31 ± 0.06
DNA pol-β	Novikoff hepatoma	poly(rA)•oligo(dT)	18 ± 5.5	0.88 ± 0.13
DNA poly-γ	bovine liver	poly(rA)•oligo(dT)	0.5 ± 0.02	0.04 ± 0.004
DNA pol-δ	calf thymus	poly[d(A-T)]•poly[d(A-T)]	14 ± 9.0	1.1 ± 1.2
DNA pol-ε	human myeloblast	poly(dA)•oligo(dT)	9.1 ± 5.9	0.13 ± 0.07

[a] Each enzyme was assayed under optimal conditions with preferred templates. All results are expressed as μM.

enzyme kinetics were detected for a given DNA polymerase extracted from different tissue sources. The Lineweaver–Burk plot (Dixon & Webb, 1979) from assays with FIAUTP and DNA pol-γ demonstrated competitive inhibition kinetics (Figure 2). The K_i of 0.04 μM FIAUTP was determined from the replot of the slopes (data not shown).

The remaining mammalian DNA polymerases were examined kinetically with FIAUTP as an inhibitor in similar systems with their preferred template/primers. Kinetic data obtained are summarized in Table 1. FIAUTP effectively inhibited the incorporation of radiolabeled dTTP using the purified DNA pol-γ in the *in vitro* assay system that used poly(rA)•oligo(dT). The K_i of FIAUTP with DNA pol-γ was the lowest among the mammalian DNA polymerases (Table 1). For DNA pol-α, the preferred template/primer was poly-[d(A-T)]•poly[d(A-T)], and the K_m for dTTP was 4.8 μM. The K_i for FIAUTP with DNA pol-α was 0.31 μM. Using purified DNA pol-β, and poly(rA)•oligo(dT) as the template/primer, the K_m for dTTP was 18 μM and the K_i for DNA pol-β was 0.88 μM FIAUTP. With DNA pol-δ, the preferred template/primer was poly[d(A-T)]•poly[d(A-T)]. The K_m for dTTP was 14 μM and the K_i was 1.1 μM FIAUTP. For DNA pol-ε, the template/primer was poly(dA)•oligo(dT), and the K_m was 9.1 μM dTTP. The K_i of FIAUTP was determined to be 0.13 μM.

Incorporation of radiolabeled dTTP into the synthetic polynucleotide template/primers was inhibited by FIAUTP with all of the cellular DNA polymerases irrespective of the tissue source. The K_i for FIAUTP was determined to be approximately 10-fold lower than the corresponding K_m for dTTP with each DNA polymerase. As expected, unphosphorylated FIAU (the pharmaceutical compound) did not inhibit DNA pol-γ substantially *in vitro*; at 1000 μM FIAU, polymerization by DNA pol-γ was 85% of that detected in the absence of FIAU (data not shown).

DISCUSSION

FIAU toxicity was manifested clinically by hepatic steatosis, lactic acidosis, pancreatitis, and myopathy (Touchette, 1993). The pathologic and clinical findings suggested that one target organelle of FIAU toxicity might be mitochondria of liver or muscle. This was based in part on shared clinical features of FIAU toxicity and some heritable mitochondrial illnesses [reviewed in Wallace, 1992)].

Previously, other investigators and members of our group showed AZTTP inhibited DNA pol-γ *in vitro* (Keilbaugh et al., 1990; Izuta et al., 1991; Chen et al., 1991; Lewis et al., 1994). Data from our study (Lewis et al., 1994) indicated that such inhibition yielded mixed kinetics with competitive and noncompetitive inhibition. This inhibition could be important pharmacologically in clinical AZT toxic myopathy of skeletal muscle and a recently described AZT-induced hepatic toxicity (Freiman et al., 1993; Dalakas et al., 1990). The K_i and K_i' determined for AZTTP with DNA pol-γ each were higher than the K_m for dTTP with DNA pol-γ, but both K_i and K_i' were in pharmacologic range.

We based our present studies of FIAUMP accumulation into DNA by mammalian DNA polymerases on the pharmacologic mechanism of FIAUTP, clinical and pathologic features of FIAU toxicity, and the mechanisms of toxicity of well-studied nucleoside analogs that demonstrate mitochondrial toxicity, such as AZT. A key structural distinction between FIAUTP and AZTTP is the 3′-OH present on FIAUTP and not on AZTTP. The 3′-OH is necessary for extension from the incorporated analog by the DNA polymerases during chain elongation.

Our results showed that all of the mammalian DNA polymerases incorporated FIAUMP into duplex DNA. However, our comparative analysis of the mammalian DNA polymerases supports the hypothesis that inhibition of DNA pol-γ by FIAUTP and accumulation of FIAUMP into mtDNA might be central to toxic mechanisms of FIAU. When FIAUTP is substituted for dTTP in reaction mixtures, DNA pol-γ is capable of catalyzing DNA synthesis on a natural DNA template generating nascent DNA products like those observed in the presence of the four natural dNTPs. This result indicates that FIAUTP is an excellent analog for dTTP in the DNA pol-γ catalyzed reaction and is a less effective analog with the other DNA polymerases [for reviews, see Cozzarelli (1977) and Wright and Brown (1990)].

The direct competition between FIAUTP and dTTP was measured kinetically. In support of the findings of FIAUMP accumulation into DNA by DNA pol-γ, FIAUTP was a competitive inhibitor of DNA pol-γ for incorporation of radiolabeled dTTP. The K_i of 0.04 μM for FIAUTP with hepatic DNA pol-γ was the lowest for all the mammalian DNA polymerases.

Purified DNA pol-γ used in this study was not from human tissue. However, DNA polymerases exhibit considerable conservation of primary sequences from many eukaryotic sources (Fry & Loeb, 1986; Burgers, 1989; Wang, 1991). The amino acid sequence of mammalian DNA pol-γ has not been established, but DNA pol-γ activity from rat liver, porcine liver, and bovine heart revealed kinetics similar to those of bovine hepatic DNA pol-γ used in this work (Lewis et al., 1994; Meyer & Simpson, 1968, 1070; Mosbaugh, 1988). It is reasonable to expect that human hepatic DNA pol-γ would be enzymologically similar to DNA pol-γ from other mammalian sources. On the basis of our data, FIAUMP is likely to accumulate in mtDNA *in vivo*. This accumulation of FIAUMP in mtDNA may have further biologic implications by affecting transcription of mitochondrial genes or subsequent rounds of mtDNA replication.

DNA polymerases γ, δ, and ε contain 3′ → 5′ exonuclease activities as integral parts of the enzymes, whereas DNA

polymerases α and β do not (Wang, 1991). Results here suggest that the presence or absence of 3′ → 5′ exonuclease activity can not be used to predict the relative sensitivity of the enzyme to inhibition by FIAUTP. The exonucleases associated with DNA polymerases are proofreading exonucleases that preferentially remove mispaired nucleotides. The base-pairing properties of FIAUMP-dAMP are not known, but the accumulation of FIAUMP into DNA opposite dAMP by all five DNA polymerases tested suggests that the geometric requirements necessary for accurate DNA synthesis are relatively well preserved in the FIAUMP-dAMP structure (Echols & Goodman, 1991). Thus, FIAUMP-dAMP might be recognized poorly by the proofreading exonucleases. It is also possible that differences in sensitivity to FIAU reflect the relative abilities of the DNA polymerase to distinguish FIAUTP from dTTP at the active site of the DNA polymerase during the insertion step or the subsequent elongation step. Thus, the relative sensitivity of the DNA polymerases might reflect the kinetic contribution provided by the enzyme at the DNA polymerase active site and not the presence or absence of a 3′ → 5′ exonuclease activity.

FIAUMP was incorporated into DNA by all of the mammalian DNA polymerases. Using a natural DNA template and synthetic polynucleotide templates *in vitro*, mitochondrial DNA pol-γ incorporated FIAUMP into DNA more efficiently than did the nuclear DNA polymerases. These results might relate to clinical and pathologic findings that point to mitochondria in liver and skeletal muscle tissues as possible target organelles in FIAU toxicity. Our findings do not unequivocally prove the mechanism of clinical FIAU toxicity. It remains to be established that FIAU is phosphorylated within the mitochondrion, transported into the mitochondrion, or that intramitochondrial concentrations of FIAUTP are sufficiently high to compete with dTTP. In addition, FIAU (or metabolites of FIAU) may interfere with mitochondrial metabolism directly or indirectly. Since FIAUMP is incorporated in DNA synthesized by the nuclear DNA polymerases, it is possible that FIAUMP incorporated into genomic DNA affects transcription of nuclear genes or genomic DNA replication or repair.

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