Proc. Natl. Acad. Sci. USA
Vol. 93, pp. 3592–3597, April 1996
Medical Sciences

Fialuridine and its metabolites inhibit DNA polymerase γ at sites of multiple adjacent analog incorporation, decrease mtDNA abundance, and cause mitochondrial structural defects in cultured hepatoblasts

(antiviral agents/hepatitis B/nucleoside analogs/mitochondrial myopathy)

WILLIAM LEWIS*†, ERIC S. LEVINE*, BRONE GRINIUVIENE*, KEVIN O. TANKERSLEY*, JOSEPH M. COLACINO‡, JEAN-PIERRE SOMMADOSSI§, KYOICHI A. WATANABE¶, AND FRED W. PERRINO‖

*Department of Pathology and Laboratory Medicine, College of Medicine, University of Cincinnati, 231 Bethesda Avenue, Cincinnati, OH 45267-0529; ‡Lilly Research Laboratories, Indianapolis, IN 46285; §Department of Pharmacology, University of Alabama School of Medicine, Birmingham, AL 35294; ¶Department of Pharmacology, Sloan-Kettering Institute for Cancer Research, New York, NY 10021; and ‖Department of Biochemistry, Wake Forest University Medical Center, Winston-Salem, NC 27157

Communicated by Douglas C. Wallace, Emory University School of Medicine, Atlanta, GA, December 13, 1995 (received for review October 3, 1995)

ABSTRACT **The thymidine analog fialuridine [1-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)-5-iodouracil (FIAU)] was toxic in trials for chronic hepatitis B infection. One mechanism postulated that defective mtDNA replication was mediated through inhibition of DNA polymerase-γ (DNA pol-γ) by FIAU triphosphate (FIAUTP) or by triphosphates of FIAU metabolites. Inhibition kinetics and primer-extension analyses determined biochemical mechanisms of FIAU, 1-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)-5-methyluracil (FAU), and 1-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)uracil triphosphate (TP) inhibition of DNA pol-γ. dTMP incorporation by DNA pol-γ was inhibited competitively by FIAUTP, FMAUTP, and FAUTP (K_i = 0.015, 0.03, and 1.0 μM, respectively). By using oligonucleotide template–primers, DNA pol-γ incorporated each analog into DNA opposite a single adenosine efficiently without effects on DNA chain elongation. Incorporation of multiple adjacent analogs at positions of consecutive adenosines dramatically impaired chain elongation by DNA pol-γ. Effects of FIAU, FMAU, and FAU on HepG2 cell mtDNA abundance and ultrastructure were determined. After 14 days, mtDNA decreased by 30% with 20 μM FIAU or 20 μM FMAU and decreased less than 10% with 100 μM FAU. FIAU and FMAU disrupted mitochondria and caused accumulation of intracytoplasmic lipid droplets. Biochemical and cell biological findings suggest that FIAU and its metabolites inhibit mtDNA replication, most likely at positions of adenosine tracts, leading to decreased mtDNA and mitochondrial ultrastructural defects.**

Hepatitis B virus (HBV) infection is a common disease that afflicts over 250 million people worldwide (1). Principal goals in chronic HBV infection include reduction or elimination of viral replication and halting progress of irreversible hepatocellular damage (2). 1-(2-Deoxy-2-fluoro-β-D-arabinofuranosyl)-5-iodocytosine (FIAC) demonstrated efficacy in herpesvirus infections (3). Metabolites of FIAC include 1-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)-5-iodouracil (FIAU), 1-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)-5-methyluracil (FMAU) and 1-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)uracil (FAU) (4, 5). FIAU and FMAU were efficacious against HBV, and each reduced the abundance of HBV DNA in the HepG2 cell line (6, 7). FIAU was used in clinical trials to treat chronic HBV. Serious side effects caused premature termination of the trials and led to vigorous investigations of toxic mechanisms (8–10).

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Although the mechanism of FIAU toxicity was not well understood, possibilities included conversion of FIAU and its metabolites into triphosphates and subsequent inhibition of cellular DNA polymerases. Findings from both the clinical trials and preclinical data implicated defective mtDNA replication in FIAU toxicity (10). FMAU and FIAU caused mitochondrial toxicity to cultured HepG2 hepatoblastoma cells, and the drugs accumulated intracellularly (11). Although intramitochondrial concentrations were not determined, toxicity features included increased lactate, formation of intracytoplasmic lipid droplets, drug incorporation into mtDNA, and altered mitochondrial morphology.

In this work, we explored biochemical and cell biologic effects of each FIAU analog on DNA polymerase-γ (pol-γ) activity, on incorporation into DNA *in vitro*, on mtDNA replication, and on mitochondrial ultrastructure. FIAUTP and FMAUTP (TP, triphosphate) both have very low inhibition constants compared to FAUTP. All three analogs are effective alternative substrates for incorporation into DNA *in vitro* by DNA pol-γ. Template–primer function was dramatically impaired upon incorporation of these analogs at two or three consecutive positions in the nascent DNA chain. FIAU and FMAU at similar doses caused accumulation of intracellular lipid droplets and disruption of mitochondrial cristae. FAU did not cause such changes. HepG2 mtDNA decreased with both FIAU and FMAU treatment but remained at control levels even with a 5-fold higher dose of FAU. These results suggest inhibition of DNA pol-γ and defective mtDNA replication may be central to toxic processes.

MATERIALS AND METHODS

Materials. [^{3}H]dTTP was from ICN. [^{32}P]ATP was from Amersham. FIAU was provided by Oclassen Pharmaceuticals (San Rafael, CA). FIAUTP and FAUTP were synthesized by T. E. Mabry and C. D. Jones at Lilly Research Laboratories (Indianapolis). The purity of each was >99% and was confirmed by HPLC. FMAUTP was synthesized by Moravek Biochemicals (La Brea, CA). For each compound, the structures were verified by proton NMR, mass spectrometry, and UV spectroscopy. FIAU was from Lilly Research Laboratories, and

Abbreviations: DNA pol-γ, DNA polymerase-γ; FAU, 1-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)uracil; FIAC, 1-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)-5-iodocytosine; FIAU, fialuridine or 1-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)-5-iodouracil; FMAU, 1-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)-5-methyluracil; HBV, hepatitis B virus; MP, monophosphate; TP, triphosphate.

†To whom reprint requests should be addressed.

FMAU and FAU were synthesized as described (12). Synthetic ribo- and deoxyribonucleotide polymers were obtained from Pharmacia. The 20-mer (5′-TAATACGACTCACTATAGGG-3′), 35-mer (5′-TGGAGCTCCAATTAGCCCTATAGTGAG-TCGTATTA-3′), 23-mer (5′-TTGATATTAAAAGAGACA-AATTT-3′), and 50-mer (5′-GCGAAGCTTAGGAGGT-AAAAAAAAATGAAATTTGTCTCTTTTAATATCAA-3′) oligonucleotides were synthesized in the Cancer Center of Wake Forest University. dNTPs were from Sigma. DNA pol-γ (specific activity, 3300 units/mg) was prepared from bovine liver (13, 14). A unit of polymerase activity catalyzed incorporation of 1 nmol of total nucleotide per hour at 37°C.

DNA Polymerase Assays. For inhibition kinetics (see Fig. 1), reaction mixtures (50 μl) contained 50 mM Tris·HCl (pH 8.0), 100 mM NaCl, 5 mM 2-mercaptoethanol, 1 mM $MnCl_2$, 50 μM poly(rA)·oligo(dT_{12-18}) (5:1 molar ratio) prepared as described (13), 0.01 unit of DNA pol-γ and [^{3}H]dTTP as indicated in the figure legends. The K_m value for dTTP was determined in velocity versus substrate concentration experiments using a range of dTTP concentrations from 0.2 to 100 μM. Assays were carried out at 37°C for 30 min and terminated on ice, and the amount of radioactivity in acid-insoluble material was determined on Whatman GF/C filters by liquid scintillation spectrometry (13). Assay conditions gave linear kinetics with respect to time and enzyme concentration. All enzyme–inhibitor assays were replicated 2–4 times. Each assay point was performed in triplicate within each experiment. Calculated arithmetic means from the triplicate assays were used to plot kinetic data using the least squares method.

For primer extension assays shown in Figs. 2 and 3, the 20-mer was labeled with ^{32}P at the 5′ position and hybridized to the 35-mer template at a 1:1 molar ratio (15). For assays in Fig. 4, the 23-mer was radiolabeled and hybridized to the 50-mer. Reaction mixtures (10 μl) contained 50 mM Tris·HCl (pH 8.0), 100 mM NaCl, 5 mM 2-mercaptoethanol, 1 mM $MnCl_2$, 100 μg of bovine serum albumin per ml, 0.25 pmol of DNA template primer, 0.01 unit of DNA pol-γ, and nucleotides as indicated in the figure legends. Reactions were stopped in 70% ethanol, dried, and resuspended in 95% formamide. Products were separated by electrophoresis through 15% urea polyacrylamide gels and quantified by phosphorimagery (Molecular Dynamics). To ensure steady-state kinetic conditions (see Fig. 2), reactions were performed so that <10% of the template primers were elongated (16).

Tissue Culture of Liver Cells. HepG2 cells (human hepatoblastoma) from American Type Tissue Collection (HB 8065) were cultured in minimal essential medium with Glutamax I (L-alanyl-L-glutamine; GIBCO/BRL), 10% fetal bovine serum, 100 units of penicillin per ml, and 100 μg of streptomycin per ml. Cells were treated with FIAU (1–50 μM), FMAU (1–20 μM), or FAU (10–100 μM), and each compound was replenished every 2–3 days. Controls were treated with an equivalent concentration of dimethyl sulfoxide vehicle (0.1%).

Southern Hybridization. Total cellular DNA was extracted according to standard methods (17, 18). DNA (10 μg per sample) was cut using the restriction enzyme *Bam*HI (10 units/μg of mtDNA). The mixture was incubated at 37°C for 2.5 h whereupon 0.1 volume of 10× stop solution (10% glycerol/10 mM EDTA, pH 8.0/0.2% bromophenol blue) was added. The mixture was loaded onto a 1% agarose gel in 1× TAE buffer (0.04 M Tris-acetate/0.001 M EDTA, pH 8.0) and electrophoresed overnight at 1 V/cm. After electrophoresis, DNA was transferred onto Hybond-N membranes (Amersham) as described (19). pMM26 (a cDNA probe that includes the 12 S and 16 S mitochondrial ribosomal subunits, cytochrome *b*, and 4 subunits of the respiratory chain NADH dehydrogenase) was used to detect mtDNA (20, 21). β-actin cDNA served as a probe for HepG2 nuclear DNA (18, 22). Labeling of cDNA was performed using random primer methods (Amersham) and [^{32}P]dCTP (specific activity, 3000 Ci/mmol; 1 Ci = 37 GBq; New England Nuclear) according to manufacturer's directions. Filters were incubated in 1 ml per 15-cm^2 filter area of prehybridization buffer (Denhardt's solution/1% SDS/1 M NaCl) for a minimum of 3 h at 65°C. Then 1×10^6–10^7 cpm/ml of the appropriately radiolabeled cDNA was added. Hybridization proceeded overnight at 65°C. Filters were washed at room temperature twice in 2× SSC/0.1% SDS for 15 min, twice in 0.5× SSC/0.1% SDS, and twice in 0.1× SSC/0.1% SDS. Radioactive signals on blots were quantitated using a Betagen (Waltham, MA) Betascope model 603 blot analyzer. After autoradiography, blots were stripped by incubating twice for 10 min in 0.05× SSC/10 mM EDTA, pH 8.0/0.1% SDS heated to 100°C. The number of mitochondrial genomes per sample was determined on blots as a ratio of mitochondrial pMM26 to β-actin radioactive signals. This ratio was independent of DNA load.

Preparation of HepG2 Cells for Transmission Electron Microscopy. Methods (with minor modifications) resembled those used with cultured myotubes and myocardial cells (18, 20, 21). After 14 days exposure to the analog at given doses, the cell samples on glass were fixed in 12-well Falcon dishes in 2% glutaraldehyde/0.1 M cacodylate (pH 7.2) for 1 h at 4°C. The cells were rinsed and postfixed in 1% OsO_4 (Sigma) in 0.05 M cacodylate (pH 7.2) for 1 h. Following osmication and rinses, the HepG2 cells were dehydrated with graded ethanols and embedded in "Spurr" (23). Each sample was sectioned (100 nm), stained with lead citrate and uranyl acetate, examined, and photographed on a Phillips model 300 electron microscope (Phillips Electronic Instruments, Mahwah, NJ).

RESULTS

Inhibition Kinetics of DNA pol-γ. The nucleotide analogs examined all inhibit dTMP incorporation into DNA by DNA pol-γ. The ability of each analog to compete with dTTP and effectively reduce the amount of radiolabeled dTMP incorporated into DNA was determined by measuring K_i values (Fig. 1). Lineweaver–Burk plots from reactions containing FIAUTP (Fig. 1*A*), FMAUTP (Fig. 1*B*), and FAUTP (Fig. 1*C*) demonstrated that these analogs were competitive inhibitors of dTTP in the DNA pol-γ catalyzed reaction. The K_i values were 0.015 μM, 0.030 μM, and 1.0 μM for FIAUTP, FMAUTP, and FAUTP, respectively. The K_m value for dTTP was 0.75 μM.

Primer Extension Analysis. Products generated by DNA pol-γ in the presence of FIAUTP, FMAUTP, and FAUTP were examined using primer extension analysis to determine if these thymidine analogs served as alternative substrates or direct inhibitors of DNA pol-γ. Incorporation of each of the analogs was first examined opposite a single adenosine (target A) in the template (Figs. 2 and 3). The primer extension analysis showed that DNA pol-γ incorporates FIAUMP, FMAUMP, and FAUMP opposite adenosine in DNA as efficiently as dTMP. We used a steady-state kinetic assay (24) to quantify the insertion step of these three thymidine analogs into DNA by DNA pol-γ. A 20-mer primer was hybridized to a 35-mer DNA template so that the 3′ terminus was positioned 2 nt from the target A in the template (Fig. 2). In the presence of dCTP, the 20-mer was elongated to a 21-mer. Insertion of dTMP, FIAUMP, FMAUMP, and FAUMP at the target site was detected as the appearance of 22-mer oligonucleotides. The amounts of 22-mer detected in these reactions were dependent on the concentrations of dTTP, FIAUTP, FMAUTP, and FAUTP. Quantitation of the oligomer products showed saturation kinetics for insertion of dTMP, FIAUMP, FMAUMP, and FAUMP with apparent K_m values of 0.085, 0.19, 0.18, and 0.065 μM and apparent V_{max} values $(22\text{-mer}/21\text{-mer})_{max}$ of 1.4, 2.7, 3.3, and 1.7, respectively. The V_{max}/K_m values for insertion of the analogs of dTMP differ by <2-fold, indicating that DNA pol-γ inserts all three of these thymidine analogs into DNA efficiently.

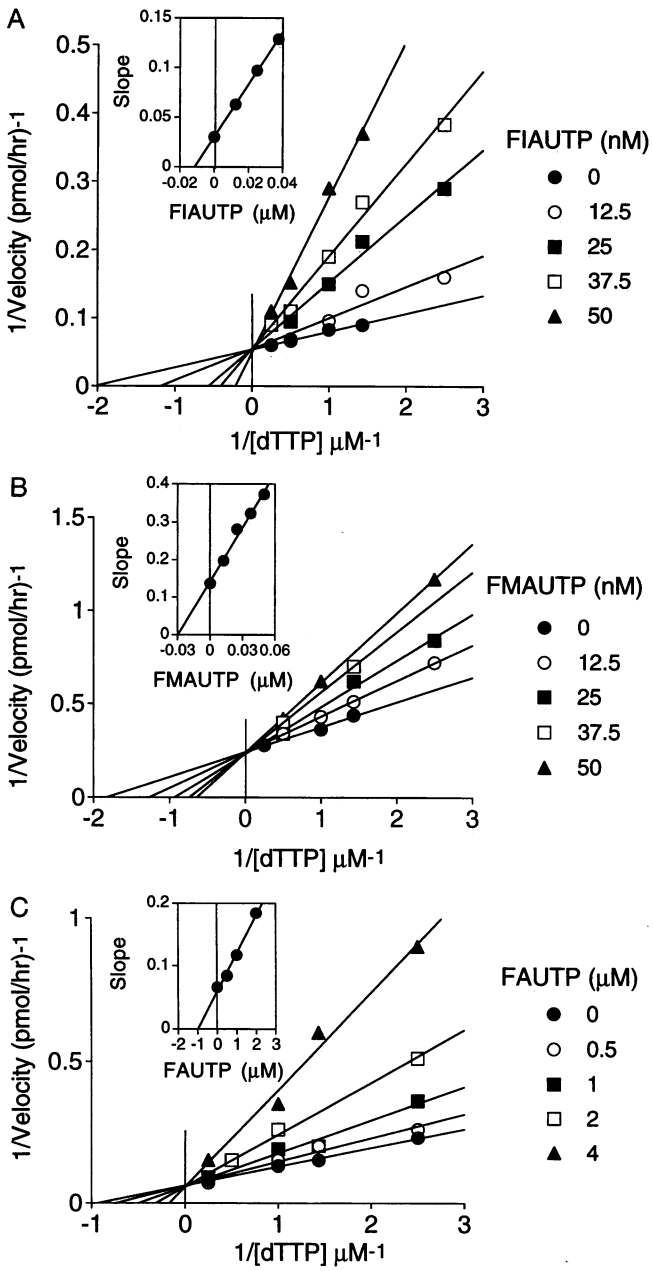

FIG. 1. Inhibition of DNA pol-γ by FIAUTP, FMAUTP, and FAUTP. Incorporation of [^{3}H]dTMP was measured in reactions prepared as described containing 0.4–4.0 μM [^{3}H]dTTP and the indicated concentrations of FIAUTP (*A*), FMAUTP (*B*), and FAUTP (*C*). Inhibition of dTMP incorporation was competitive with each. The K_i values from Dixon plots (*A–C Insets*) were K_i(FIAUTP) = 0.015 μM, K_i(FMAUTP) = 0.03 μM, and K_i(FAUTP) = 1.0 μM.

Incorporation into DNA required extension from the FIAUMP, FMAUMP, and FAUMP 3′ termini by DNA pol-γ. To determine the efficiencies of extension from the analogs relative to dTMP, four time course reactions were prepared using the 35-mer/20-mer DNA template (Fig. 3). These reactions contained dCTP, dATP, and either dTTP (lanes 3–5), FIAUTP (lanes 7–9), FMAUTP (lanes 11–13), or FAUTP (lanes 15–17). In each of these reactions oligomer products up to 26 nt in length were detected. The generation of 26-mer products required insertion and extension from the thymidine analogs. In control reactions, dATP was excluded and DNA pol-γ elongated the 20-mer to the 22-mer position (Fig. 3, lanes 2, 6, 10, 14). The amounts of the oligomer products obtained in these reactions indicated that the analogs were incorporated

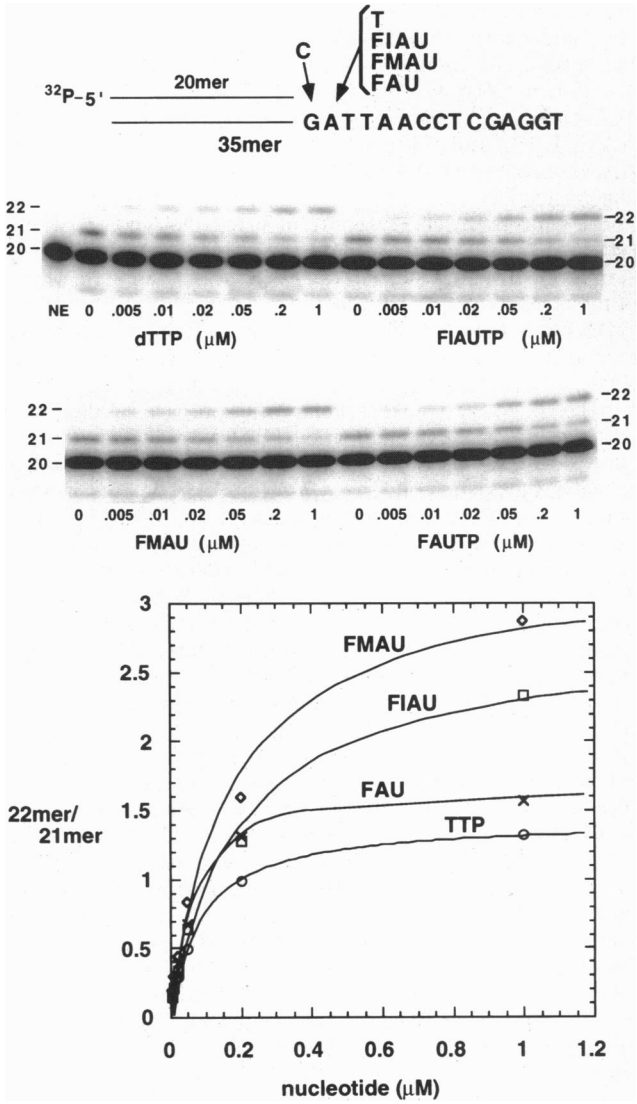

FIG. 2. Kinetics of FIAUMP, FMAUMP, and FAUMP (MP, monophosphate) insertion by DNA pol-γ. The radiolabeled 20-mer was hybridized to the 35-mer to produce the indicated template–primer. Primer extension reactions were prepared and contained 0.2 μM dCTP and the indicated concentrations of dTTP (○), FIAUTP (□), FMAUTP (◇), or FAUTP (×). Incubations were 10 min at 37°C. Elongated primes are shown, and quantitation was as described in *Materials and Methods.*

into the DNA product at positions opposite single adenosines by DNA pol-γ as efficiently as dTMP. The analog-containing oligonucleotide products migrated more rapidly (FIAUTP and FAUTP) or more slowly (FMAUTP) in the polyacrylamide gel than those generated in the presence of dTTP. These differences in electrophoretic migration provided evidence that the analog was incorporated into the nascent DNA. The insertion kinetics (Fig. 2) and extension analysis (Fig. 3) demonstrated that FIAUTP, FMAUTP, and FAUTP serve as effective alternative substrates during DNA polymerization by DNA pol-γ.

Incorporation of each thymidine analog opposite multiple adjacent adenosines in the template severely impaired DNA chain elongation by DNA pol-γ. The inhibitory effect of consecutive analog incorporation was demonstrated using a 50-mer/23-mer DNA template containing nine consecutive adenosine residues (Fig. 4). In the presence of all four dNTPs, DNA pol-γ copied the oligomer template to the end, generating products ranging from 24 to 50 nt in length. When

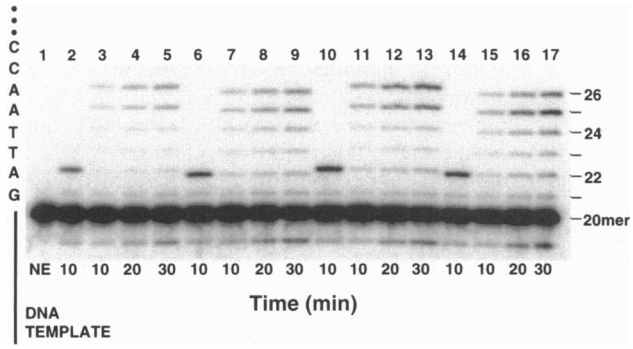

FIG. 3. Incorporation of FIAUMP, FMAUMP, and FAUMP by DNA pol-γ. The 35-mer/20-mer was incubated with DNA pol-γ at 37°C for the indicated times in primer extension reactions containing dCTP, dATP, and dTTP (lanes 3–5), FIAUTP (lanes 7–9), FMAUTP (lanes 11–13), or FAUTP (lanes 15–17) (each at 0.2 μM). Control reactions contained dCTP and dTTP (lane 2), FIAUTP (lane 6), FMAUTP (lane 10), or FAUTP (lane 14) (each at 0.2 μM) and no enzyme (lane 1). The sequence of the DNA template and the positions of the starting 20-mer primer and oligonucleotide products are indicated.

FIAUTP, FMAUTP, or FAUTP was substituted for dTTP, oligomer products were limited to 32 nt in length, and no DNA synthesis was detected past the nine consecutive adenosine residues. This demonstrates that incorporation of multiple adjacent analog residues can impair template–primer function and lead to inhibition of chain elongation by DNA pol-γ.

mtDNA Abundance in Treated HepG2 Cells. Cells were incubated with FIAU (1–50 μM), FMAU (1–20 μM), or FAU (10–100 μM) for up to 3 weeks. Total cellular DNA was extracted. The relative abundance of mtDNA was determined by comparing band intensities obtained using the pMM26 probe to those obtained using the nuclear-encoded β-actin probe (Fig. 5). After 2 weeks, FIAU-treated HepG2 cells revealed decreased steady-state mtDNA abundance at 20 μM or higher concentrations (Fig. 5*A*); 20 μM FIAU caused a 30% decrease and 50 μM FIAU caused a 90% decrease. After 3 weeks, 1 μM FIAU caused a 20% decrease in mtDNA and 20 μM caused a 50% decrease. FMAU treatment caused a similar decrease in mtDNA abundance. After 1 week, 20 μM FMAU decreased abundance of mtDNA by 40%. Similar effects were observed at 2 and 3 weeks, when mtDNA was 60% of controls

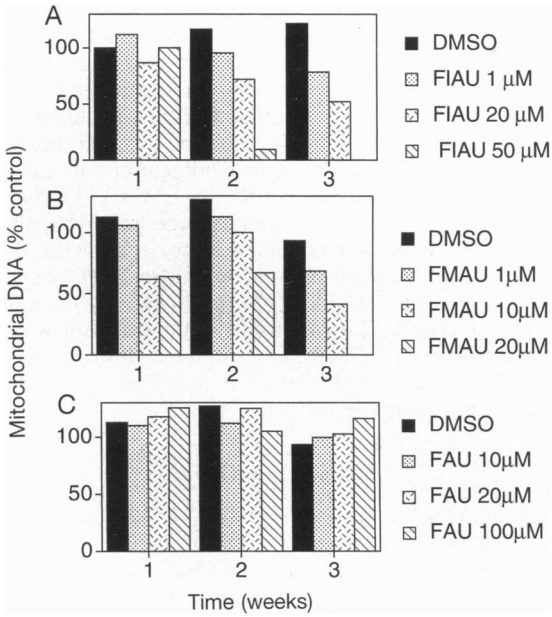

FIG. 5. Southern analysis of HepG2 cell mtDNA. HepG2 cells were treated with FIAU (1–50 μM), FMAU (1–20 μM), or FAU (10–100 μM). Extracted DNA underwent Southern transfer and blotting with pMM26. Radioactive signal was analyzed using a Betascope and was normalized with β-actin cDNA. FIAU causes essentially no change in steady-state mtDNA abundance at 1 week. At later times, a dose-dependent decrease in mtDNA occurred. FMAU (10 μM) appeared to be slightly more potent, decreasing mtDNA levels after 1 week. FAU appeared to be substantially less potent than FIAU. mtDNA levels remained near control values after 3 weeks of 100 μM FAU.

(Fig. 5*B*). In contrast, significantly higher doses of FAU produced no comparable decreases in HepG2 mtDNA. After 3 weeks of 100 μM FAU, mtDNA remained near control levels (Fig. 5*C*). These results showed that FIAU and FMAU reduced mtDNA in cells at lower doses and with shorter exposure than the FAU treatment.

Ultrastructural Changes of HepG2 Cell Mitochondria. Ultrastructural evaluation of mitochondria was undertaken in drug-treated HepG2 cells. The 2-week time point was selected because clear decreases in mtDNA abundance were demon-

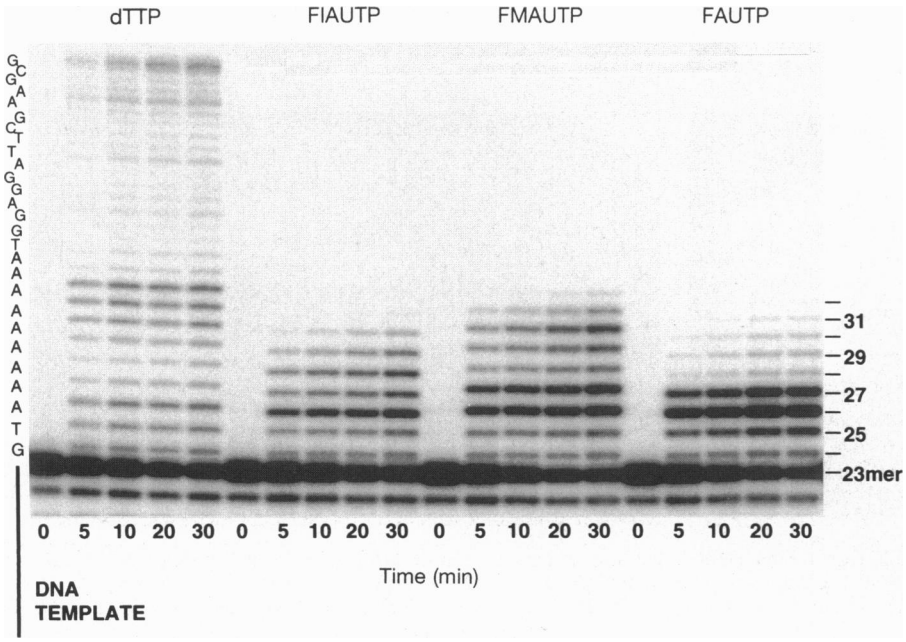

FIG. 4. Incorporation of multiple, adjacent FIAUMPs, FMAUMPs, and FAUMPs by DNA pol-γ. The 50-mer/23-mer was incubated with DNA pol-γ at 37°C for the indicated times in primer extension reactions containing dCTP, dATP, dGTP, and either dTTP, FIAUTP, FMAUTP, or FAUTP (each at 1.0 μM). The sequence of the DNA template and the positions of the starting 23-mer primer and oligonucleotide products are indicated.

strated with both FIAU and FMAU treatment (see Fig. 5). In control HepG2 cells, mitochondria were abundant, cristae were dense, and lipid droplets were rare (Fig. 6*A*). For each condition, 100 cells were evaluated by transmission electronic microscopy. Viability of all treatment groups was determined to be identical to controls by Trypan blue dye exclusion (data not shown). Treated cells revealed mitochondrial and cytoplasmic changes. Treatment with 20 μM FIAU revealed HepG2 ultrastructural changes that include irregularly shaped mitochondria, decreased cristae density, and disruption of cristae (Fig. 6*B*). Intracytoplasmic lipid droplets (L) were also abundant (Fig. 6*B*). FMAU (20 μM; Fig. 6*C*) caused a prominent increase in cytoplasmic lipid droplets which is similar to that seen with FIAU treatment. However, with 20 μM FAU, neither mitochondrial defects nor lipid droplets were apparent (compared Fig. 6 *A* with *D*).

DISCUSSION

In clinical trials for chronic hepatitis, FIAU demonstrated severe toxicity that required its discontinuation. Manifestations of toxicity included hepatic failure, lactic acidosis, neuropathy, pancreatitis, cardiomyopathy, and skeletal myopathy. Hepatic steatosis was prominent (8–10). The pathological and clinical findings suggested that one subcellular target of FIAU toxicity may be mitochondria of affected tissues such as liver, heart, or skeletal muscle. Neither the biochemical mechanism of the toxicity nor an etiologic agent was pinpointed.

Other investigators and our group studied mechanisms of toxicity of various thymidine nucleoside analogs (13, 14, 25–32). We showed that FIAUTP competitively inhibits incorporation of dTMP by all of the mammalian DNA polymerases and serves as an alternative substrate *in vitro*. The K_m/K_i ratios range from 13 to 70, indicating that FIAUTP is a potent inhibitor of mammalian DNA polymerases. The lowest K_i value was observed with mitochondrial DNA pol-γ, suggesting that very low concentrations of FIAUTP might inhibit this enzyme and reduce mtDNA synthesis (13).

Previous studies addressed the potential roles of FIAU and its metabolites in the serious side effects. IC_{50} values for FAU were 10-fold higher than those with FIAU. FMAU was the metabolite detected in DNA of FAU-treated cells (32). FIAU was found in DNA from liver, spleen, and heart tissues of various animal species (33). FIAU incorporates into DNA, and the relative abundance of FIAU in mtDNA is greater than that in nuclear DNA (11). We demonstrate that FIAUTP, FMAUTP, and FAUTP each competively inhibited the incorporation of dTMP by DNA pol-γ. The K_m(dTTP)/K_i(FIAUTP) = 50 and K_m(dTTP)/K_i(FMAUTP) = 25 values indicate that FIAUTP and FMAUTP are more potent inhibitors of DNA pol-γ than FAUTP with a K_m(dTTP)/K_i(FAUTP) = 0.75. This difference might be attributed to a very high affinity of FIAUTP and FMAUTP for the dTTP binding site relative to FAUTP.

The greater inhibitory effects of FIAU and FMAU might relate to interactions between DNA polymerase and DNA upon incorporation of the analogs. Analogs were incorporated efficiently into DNA at positions of single analog addition. Dramatic decreases in chain elongation were observed when multiple adjacent analogs were incorporated. It is possible that DNA pol-γ is trapped in a dead-end complex on the analog-containing DNA template(s). These *in vitro* results suggest that FIAU, FMAU, and FAU are likely to be incorporated into

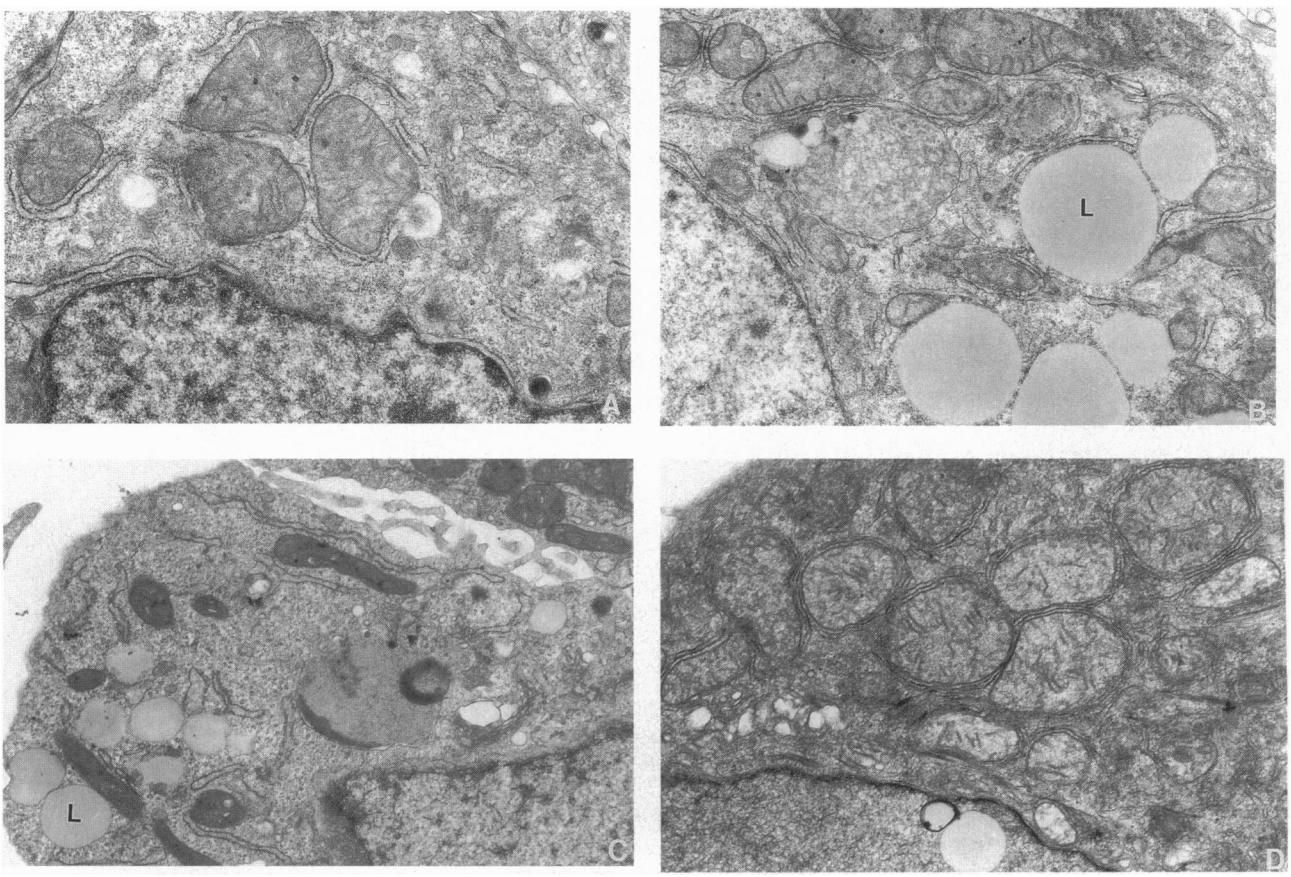

FIG. 6. Transmission electron photomicrographs from treated HepG2 cells after 14 days. (*A*) Control HepG2 cells without treatment. Abundant mitochondria are present with densely packed cristae. Lipid droplets are minimal. (*B–D*) HepG2 cells exposed to 20 μM FIAU (*B*), 20 μM FMAU (*C*), or 20 μM FAU (*D*). In *B* and *C*, lipid droplets (L) are present and decreased density of mitochondrial cristae is found. In *D*, little change is seen in the mitochondria. (×4300.)

mtDNA at single adenosines, but that inhibition of mtDNA synthesis is likely at sites of multiple adenosines.

The human mtDNA genome contains 1 run of eight, 6 runs of seven, 12 runs of six, and 24 runs of five consecutive adenosines (34). Primer extension analysis indicates that these "A tracts" may serve as replication barriers in the presence of FIAUTP, FMAUTP, and FAUTP. FIAU and FMAU decreased steady-state mtDNA abundance in treated HepG2 cells. Ultrastructural changes in mitochondria and increased intracellular lipid droplets were found with FIAU and FMAU treatment, but not with FAU.

Mitochondrial toxicity from nucleoside analogs receives increasing attention (30, 35). Awareness relates to identification of mitochondrial toxicity of nucleoside analogs (like zidovudine, fialuridine, didanosine, stavudine, and lamivudine) in selected tissues. Triphosphates of some nucleoside analogs are effective inhibitors of DNA polymerases, including DNA pol-γ (13, 14, 25–30, 36), and this inhibition could alter mtDNA replication in selected tissues *in vivo*. Biochemical and cell biologic studies here help delineate the roles of the parent compounds (e.g., FIAUTP) and metabolites (e.g., FMAUTP or FAUTP) in the pathogenesis of toxicity.

Biochemical studies here demonstrated the potential for each thymidine analog to inhibit DNA pol-γ, to be incorporated into DNA, and to affect greatly on DNA chain elongation. In the presence of FIAU and FMAU treatment, decreased abundance of steady-state mtDNA was found in parallel to loss of mitochondrial structural integrity. In contrast, FAU treatment caused little changes in either mtDNA abundance or in mitochondrial ultrastructure. It is possible that treatment with these analogs leads to decreased ability to utilize mitochondrial β-oxidation. This was supported by prominent intracytoplasmic lipid droplets with drug treatment. In other studies, lactate levels increased in FIAU- and FMAU-treated HepG2 cells (37, 38). Both biochemical and cell biologic data suggest that lower levels of FIAU and FMAU may exert mitochondrial toxicity in liver cells. Central to the cellular pathophysiology of FIAU toxicity are decreased mtDNA replication and inhibition of DNA pol-γ.

J. Morano is acknowledged for typing and J. Card for photography. Richard Montione performed the transmission electron microscopy. This research was supported by a University of Cincinnati Research Challenge Award (W.L.), by Grants FRA453 and DHP80B (F.W.P.), by a Faculty Research Award (J.-P.S.) from the American Cancer Society, by Grant 9510-IDG-1006 from North Carolina Biotech, and by Grants CA12197 and AI33239 from the Department of Health and Human Services.

1. Hoofnagle, J. H. & Alter, H. J. (1984) in *Viral Hepatitis and Liver Diseases*, eds. Dienstag, J. L. & Hoofnagle, J. H. (Grune & Stratton, New York), pp. 97–113.
2. Hoofnagle, J. H. & DiBisceglie, A. M. (1989) in *Antiviral Agents and Viral Diseases of Man*, eds. Galasso, G., Whitley, R. & Merigan, T. (Raven, New York), 3rd Ed, pp. 415–460.
3. Watanabe, K. A., Reichman, U., Hirota, K., Lopez, C. & Fox, J. J. (1979) *J. Med. Chem.* **22,** 21–24.
4. Grant, A. J., Feinberg, A., Chou, T.-C., Watanabe, K. A., Fox, J. J. & Philips, F. S. (1982) *Chem. Pharmacol.* **31,** 1103–1108.
5. King, D. H. (1991) *Transplant. Proc.* **23,** 168–170.
6. Lampertico, P., Malter, J. S. & Gerber, M. A. (1991) *Hepatology* **13,** 422–426.
7. Korba, B. E. & Gerin, J. L. (1992) *Antiviral Res.* **19,** 55–70.
8. Brahams, D. (1994) *Lancet* **343,** 1494–1495.
9. Marshall, E. (1994) *Science* **264,** 1530.
10. Manning, F. J. & Swartz, M., eds. (1995) *Institute of Medicine Review of the Fialuridine (FIAU) Clinical Trials* (Natl. Acad. Press, Washington, DC), pp. 98–104.
11. Cui, L., Yoon, S., Schinazi, R. F. & Sommadossi, J.-P. (1995) *J. Clin. Invest.* **95,** 555–563.
12. Chou, T. C., Feinberg, A., Grant, A. J., Vidal, P., Reichman, U., Watanabe, K. A., Fox, J. J. & Philips, F. S. (1981) *Cancer Res.* **41,** 3336–3342.
13. Lewis, W., Meyer, R. R., Simpson, J. F., Colacino, J. M. & Perrino, F. W. (1994) *Biochemistry* **33,** 14620–14624.
14. Lewis, W., Simpson, J. F. & Meyer, R. R. (1994) *Circ. Res.* **74,** 344–348.
15. Perrino, F. W. & Loeb, L. A. (1989) *J. Biol. Chem.* **264,** 2898–2905.
16. Perrino, F. W. & Mekosh, H. L. (1992) *J. Biol. Chem.* **267,** 23043–23051.
17. Blin, N. & Stafford, D. W. (1976) *Nucleic Acids Res.* **3,** 2303–2308.
18. d'Amati, G. & Lewis, W. (1994) *Lab. Invest.* **71,** 879–884.
19. Maniatis, T., Fritsch, E. F. & Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab. Press, Plainview, NY).
20. Lewis, W., Chomyn, A., Gonzalez, B. & Papoian, T. (1992) *J. Clin. Invest.* **89,** 1354–1360.
21. Lewis, W., Papoian, T., Gonzalez, B., Louie, H., Kelly, D. P., Payne, R. M. & Grody, W. W. (1991) *Lab. Invest.* **65,** 228–236.
22. Lewis, W. & Gonzalez, B. (1990) *Lab. Invest.* **62,** 69–76.
23. Spurr, A. R. (1969) *J. Ultrastruct. Res.* **36,** 31–43.
24. Boosalis, M. S., Petruska, J. & Goodman, M. F. (1987) *J. Biol. Chem.* **262,** 14689–14696.
25. Copeland, W. C., Chen, M. S. & Wang, T. S. F. (1992) *J. Biol. Chem.* **267,** 21459–21464.
26. Heidenreich, O., Kruhøffer, M., Grosse, F. & Eckstein, F. (1990) *Eur. J. Biochem.* **192,** 621–625.
27. Chen, C.-H. & Cheng, Y.-C. (1989) *J. Biol. Chem.* **264,** 11934–11937.
28. König, H., Behr, E., Lower, J. & Kurth, R. (1989) *Antimicrob. Agents Chemother.* **33,** 2109–2114.
29. Simpson, M. V., Chin, C. D., Keilbaugh, S. A., Lin, T. S. & Prusoff, W. H. (1989) *Biochem. Pharmacol.* **38,** 1033–1036.
30. Parker, W. B. & Cheng, Y.-C. (1994) *J. Natl. Inst. Health Res.* **6,** 57–61.
31. Staschke, K. A., Colacino, J. M., Mabry, T. E. & Jones, C. D. (1994) *Antiviral Res.* **23,** 45–61.
32. Klecker, R. W., Katki, A. G. & Collins, J. M. (1994) *Mol. Pharmacol.* **46,** 1204–1209.
33. Richardson, F. C., Engelhart, J. A. & Bowsher, R. R. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 12003–12007.
34. Anderson, S., Bankier, A. T., Barrell, B. G., de Bruijn, M. H. L., Coulson, A. R., Drouin, J., Eperon, I. C., Nierlich, D. P., Roe, B. A., Sanger, F., Schreier, P. H., Smith, A. J. H., Staden, R. & Young, I. G. (1981) *Nature (London)* **290,** 457–465.
35. Lewis, W. & Dalakas, M. C. (1995) *Nat. Med.* **1,** 417–422.
36. Izuta, S., Saneyoshi, M., Sakurai, T., Suzuki, M., Kojima, K. & Yoshida, S. (1991) *Biochem. Biophys. Res. Commun.* **179,** 776–783.
37. Levine, E. S. & Lewis, W. (1995) *FASEB J.* **9,** A424 (abstr.).
38. Colacino, J. M., Malcolm, S. K. & Jaskunas, S. R. (1994) *Antimicrob. Agents Chemother.* **38,** 1997–2002.