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Effect of Fialuridine on Replication of Mitochondrial DNA in CEM Cells and in Human Hepatoblastoma Cells in Culture

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Fialuridine (FIAU) is a nucleoside analog with potent activity against hepatitis B virus in vitro and in vivo. In this report, the effect of FIAU on mitochondrial DNA (mtDNA) replication in vitro was investigated. CEM cells, a cell line derived from human T cells, were incubated for 6 days in up to 20 μM FIAU. Total cellular DNA was isolated, normalized for the number of cells, and slot hybridized to a probe specific for mtDNA sequences. Treatment of CEM cells with FIAU did not result in a dose-dependent decrease in the amount of mtDNA. In contrast, dideoxycytidine (ddC) inhibited mtDNA replication by 50% at a concentration of approximately 0.1 μM. After 6 days of incubation, both compounds displayed a 50% toxic dose at a concentration of approximately 2 μM in CEM cells and approximately 34 μM in human hepatoblastoma cells (HepG2). In further experiments, CEM cells were incubated for 15 days in up to 2.5 μM FIAU, and again, no inhibition of mtDNA was observed. Over a 6-day incubation, FIAU, at concentrations of up to 200 μM, also failed to inhibit mtDNA replication in either HepG2 or HepG2 cells which constitutively replicate duck hepatitis B virus. In contrast, ddC inhibited mtDNA replication in these cells with a 50% inhibitory concentration of approximately 0.2 μM over a 6-day incubation. Treatment of cells with either FIAU or ddC resulted in a dose-dependent increase in lactate levels in the cell medium, indicating that any effect of FIAU on mitochondrial function may not be related to inhibition of mtDNA replication on the basis of the in vitro data. Alternative explanations for mitochondrial toxicity are considered.

Fialuridine [FIAU; 1-(2′-deoxy-2′-fluoro-β-D-arabinofuranosyl)-5-iodouracil] is a nucleoside analog with activity against hepatitis B virus (HBV) in vitro (12, 22) and against woodchuck hepatitis virus in chronically infected woodchucks (22a). Recently, in clinical studies, severe toxicity was associated with the oral administration of FIAU. The toxicity profile included (i) muscle, nerve, pancreas, and liver involvement; (ii) delayed onset of symptoms; (iii) microvesicular fat infiltration of the liver; and (iv) severe, refractory lactic acidosis (11a). Considering this toxicity profile and the possible mechanism of zidovudine-induced myopathy (7, 9, 13–16, 18) and 2′,3′-dideoxycytidine (ddC)-induced peripheral neuropathy (8), the hypothesis that the multiorgan toxicity of FIAU is mediated by mitochondrial dysfunction, possibly through inhibition of DNA polymerase gamma has been suggested. Along these lines, the 5′-triphosphate of FIAU was found to be a potent inhibitor of DNA polymerase gamma (14a). In this report, we investigate the ability of FIAU to inhibit mitochondrial DNA (mtDNA) replication in CEM cells and in human hepatoblastoma (HepG2) cells in vitro.

MATERIALS AND METHODS

Cells and reagents. CEM cells ($T_{CCRF-CEM}$), a cell line derived from human T cells, and human hepatoblastoma cells (HepG2) were purchased from the American Type Culture Collection, Rockville, Md. Human hepatoblastoma cells which constitutively produce duck HBV (HepG2/G3 [10]), were obtained from William S. Mason (Fox Chase Cancer Center, Philadelphia, Pa.). CEM cells were cultured in suspension in RPMI 1640 medium with L-glutamine supplemented with 10% fetal bovine serum and gentamicin. Human hepatoblastoma cells were cultured as monolayers in minimum essential medium with Earle's salts and L-glutamine supplemented with 10% fetal bovine serum, 1 mM sodium pyruvate, penicillin, and streptomycin and, for duck or human HBV-producing cells, 400 μg of geneticin per ml (22). Cell culture media, fetal bovine serum, and antibiotics were purchased from GIBCO BRL Life Technologies, Inc. (Gaithersburg, Md.). Geneticin was purchased from Sigma Chemical Co. (St. Louis, Mo.). FIAU was obtained from Oclassen Pharmaceuticals, Inc. (San Raphael, Calif.). ddC was purchased from Sigma Chemical Co.

Cytotoxicity assays. CEM cells were plated into 96-well microtiter plates at a concentration of 10^4 cells per well in a total volume of 180 μl. Stock solutions of drugs (10 mM) were diluted in media, sonicated, and filter sterilized. Drugs (20 μl of the appropriate dilution) were added immediately following plating of the cells, after which the cells were incubated at 37°C in 5% CO_2 for 2, 4, and 6 days. Viability of CEM cells was determined by the soluble formazan assay as described previously (23). Optical densities were determined, and inhibition curves were generated by using a Vmax Kinetic Microplate Reader (Molecular Devices, Menlo Park, Calif.). All assays were done in triplicate, and the standard deviation was 8.5% of the sample mean or less in all cases.

HepG2 cells which constitutively produce human HBV (22) were seeded into 25-cm^2 flasks at an initial density of 10^6 cells per flask and incubated in the absence or presence of increasing concentrations of FIAU or ddC. After 6 days, the cell monolayers were washed with phosphate-buffered saline (PBS) and trypsinized. The trypsin was neutralized with medium containing 10% fetal bovine serum, and the cells were collected by centrifugation, washed with PBS, and resuspended in PBS. The number of cells at each concentration of FIAU or ddC was determined in duplicate by direct cell counting with the aid of a Coulter counter, and the percent reduction at each drug concentration was calculated. The concentration of drug

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required to inhibit cell replication by 50% was determined by linear regression analysis.

Slot and Southern blot analyses. CEM cells were incubated in the absence or presence of increasing concentrations of ddC or FIAU. To insure cell replication over the course of the experiment, the medium was changed every 3 days, at which time the cells were diluted to the initial cell density. At 3, 6, and 9 days, total cell DNA was prepared according to the method of Chen and Cheng (3), and 10^5 cell equivalents of DNA were applied to a nylon filter by using a slot blotting manifold and hybridized to a mtDNA-specific probe. The probe was prepared from a clone of mtDNA obtained from P. May (Lilly Research Laboratories, Indianapolis, Ind.). Briefly, mitochondrial DNA, spanning nucleotides 6937 to 7516 of the mitochondrial genome, was cloned into the *Sma*I site of pBluescribe M13 (+/−) (17). Plasmid DNA containing the mtDNA sequences was digested with *Bam*HI and used for run-off transcription (Promega, Madison, Wis.) to generate a riboprobe 603 nucleotides in length which is labeled with [^{32}P]CTP and is specific for mtDNA sequences encoding cytochrome oxidase C and Ser tRNA. Hybridization of the riboprobe to slot blots of total cellular DNA was carried out at 55°C in the presence of 50% deionized formamide, and the blots were washed twice with 1× SSC (1× SSC = 0.15 M NaCl, 0.015 M sodium citrate)–0.1% sodium dodecyl sulfate (SDS) at room temperature and twice with 0.1× SSC–0.1% SDS at 65°C.

Hybridizations to control for total cellular DNA content were carried out with a probe specific for Alu DNA sequences (Oncor, Gaithersburg, Md.) which was labeled with ^{32}P by random priming (Prime-It II kit; Stratagene, La Jolla, Calif.). Hybridizations for the detection of Alu sequences were carried out at 42°C in the presence of 50% deionized formamide. A Betascope 603 BlotAnalyzer (Betagen, Mountain View, Calif.) was used to analyze the hybridization signals in order to quantify the effect on the levels of mitochondrial DNA.

For Southern blot analysis (21), cells were treated for the indicated time in the absence or presence of increasing concentrations of drugs and collected by centrifugation (CEM cells in suspension) or by trypsinization and centrifugation (HepG2 cell monolayers). The cell pellets were resuspended in medium, counted with the aid of a Coulter counter, pelleted, and resuspended in lysis solution (10 mM Tris-HCl [pH 7.3], 0.5% Sarkosyl, 20 mM EDTA [pH 8.0]). RNase (final concentration, 50 μg/ml) was added, and the lysate was incubated for several hours at 37°C, after which 100 μg of proteinase K (final concentration, 100 μg/ml) was added and the lysate was incubated overnight at 37°C. The DNA was extracted with phenol-chloroform and then chloroform and precipitated with sodium acetate and ethanol. The DNA pellets were washed with 80% ethanol and resuspended in sterile distilled water. DNA was quantified by spectrophotometry at 260 nm. The indicated amounts of DNA were mock digested or digested with *Bam*HI according to the manufacturer's instruction (Bethesda Research Laboratories, Inc., Gaithersburg, Md.) and electrophoresed through agarose. DNA in the gels was stained with ethidium bromide and visualized under UV illumination before denaturation and transfer to nitrocellulose or Nytran membranes (Schleicher & Schuell, Keene, N.H.). Southern blot analysis was conducted by standard procedures (20). The size of the band which hybridized to the mtDNA-specific probe was estimated by comparison to *Hin*dIII fragments of lambda DNA which were electrophoresed in the same gel.

HepG2/G3 cells, a human hepatoblastoma cell line which constitutively replicates duck HBV (10), were seeded into 25-cm^2 flasks (10^6 cells per flask) and incubated for 6 days in the absence or presence of increasing concentrations of ddC (up to 100 μM) or FIAU (up to 200 μM). For slot blot analysis, total cell DNA was prepared according to the method of Chen and Cheng (3), and 10^5 cell equivalents of DNA were applied to nylon filters and hybridized to a ^{32}P-labeled probe specific for human Alu sequences to control for the DNA amount or to a ^{32}P-labeled mtDNA-specific probe. Southern blot analysis of HepG2/G3 DNA was performed as for CEM DNA.

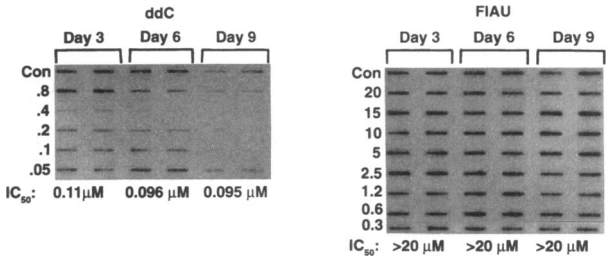

FIG. 1. Effect of ddC or FIAU on mtDNA replication in CEM cells. Cells were incubated in the absence or presence of the indicated concentrations (con) of ddC or FIAU as described in Materials and Methods. Cell medium was changed every 3 days, at which time the cells were diluted to the initial cell density. Each slot represents 10^5 cell equivalents of DNA. The blots were hybridized to an mtDNA-specific probe, and the signals were quantified with a Betascope 603. Hybridizations were normalized to DNA levels in corresponding blots hybridized to an Alu-specific probe. IC_{50}s were calculated by linear regression analysis. For ddC, the IC_{50}s were determined without the 0.8 μM concentration.

Determination of L-lactic acid in cell culture media. CEM cells and hepatoblastoma cells were incubated for 6 days in the absence or presence of increasing concentrations of ddC or FIAU. The cell culture medium was changed every 3 days for CEM suspension cells and every day for hepatoblastoma cells in monolayers. On day 6, a 5-μl aliquot of clarified cell culture medium was evaluated for lactate content by using an L-Lactic Acid UV-Method Kit (Boehringer-Mannheim, Indianapolis, Ind.) according to the manufacturer's instructions. Each assay was performed at least twice, and interassay results were reproducible.

RESULTS

Cytotoxicity of ddC and FIAU in CEM and hepatoblastoma cells. After 2 days, the concentration of ddC required to reduce CEM cell viability by 50% (50% toxic concentration [TC_{50}]) was 10 μM while the TC_{50} of FIAU was >10 μM. After 4 days of incubation, ddC was slightly more toxic than FIAU, having a TC_{50} of 4 μM compared with 10 μM for FIAU. After 6 days of incubation, the TC_{50} of ddC was 1.5 μM while that of FIAU was 1.9 μM; i.e., ddC and FIAU were equivalently cytotoxic in CEM cells. In HepG2 cells which constitutively produce HBV, after 6 days of incubation, FIAU displayed a TC_{50} of 34.9 ± 4.9 μM while ddC displayed a TC_{50} of 34.3 ± 4.0 μM. Therefore, FIAU and ddC were equivalently toxic to these cells. Similar results were obtained for HepG2 and HepG2/G3 cells (data not shown).

Effect of ddC and FIAU on the replication of mtDNA. As shown in Fig. 1, the inhibitory effect of ddC on mtDNA replication could be detected after 3 days of incubation. At this time, the concentration of ddC required to inhibit mtDNA replication by 50% (IC_{50}) was approximately 0.11 μM. By day 6, the IC_{50} was 0.096 μM, and by day 9 the IC_{50} was 0.095 μM. In contrast, no inhibitory effect of FIAU (up to 20 μM) on the

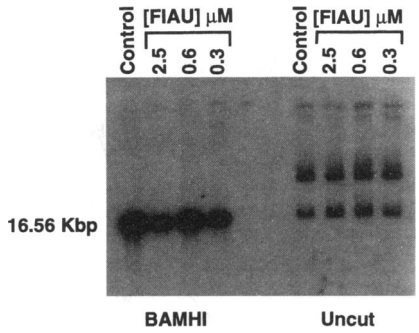

FIG. 2. Southern blot analysis of CEM cells exposed to FIAU for 15 days. CEM cells were incubated in the absence or presence of FIAU as described in the legend to Fig. 1. Cells were harvested, and total cellular DNA was obtained as described in Materials and Methods. DNA (10 μg per lane) was electrophoresed through 0.8% agarose, denatured, and transferred to nitrocellulose, and the immobilized DNA was hybridized to an mtDNA-specific riboprobe. The size of the *Bam*HI mtDNA band was determined by comparison to lambda DNA digested with *Hin*dIII and electrophoresed in the same gel. Equal amounts of DNA were present in each lane as determined by ethidium bromide-UV visualization.

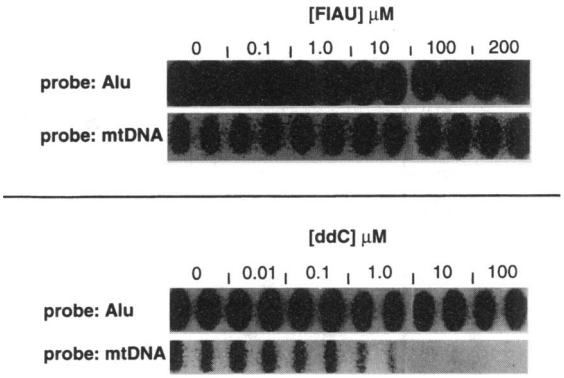

FIG. 3. Effect of ddC or FIAU on mtDNA replication in HepG2/G3 cells. Cells were plated at a density of 10^6 per 25-cm^2 flask and incubated for 6 days in the absence or presence of increasing concentrations of ddC or FIAU, with daily changes of the medium. Total cellular DNA was processed as described in Materials and Methods, and 10^5 cell equivalents of DNA were applied to each slot. Parallel blots were hybridized to a probe specific for Alu sequences or mtDNA sequences. The image is a digital printout from the Betascope 603.

replication of mtDNA in CEM cells was observed under these same conditions (Fig. 1).

No FIAU concentration-dependent decrease in the amount of DNA which hybridized to the mtDNA specific probe could be detected by Southern blot analysis of uncut DNA (Fig. 2). As expected, a single 16.56-kbp band which hybridized to the mtDNA-specific probe was readily observed by Southern blot analysis of DNA digested with *Bam*HI. Also, in this limited Southern blot analysis, no gross deletions of mtDNA were detected since the size of the mtDNA-specific band was the same in the DNA from control cells as in DNA from FIAU-treated cells. However, it should be noted that multiple digestions of mtDNA with a panel of various restriction enzymes might reveal deletions induced by FIAU which were not apparent in this study.

In HepG2/G3 cells, FIAU had no effect on the replication of mtDNA, while ddC inhibited mtDNA replication with an IC_{50} of approximately 0.2 μM (Fig. 3) as determined by slot blot hybridization analysis. Also, FIAU had no effect on the replication of mtDNA in HepG2 cells (data not shown).

FIAU did not inhibit mtDNA replication nor induce deletions large enough to result in the altered mobility of the 16.56-kbp mtDNA band as determined by Southern blot analysis of *Bam*HI-digested DNA from HepG2/G3 cells (Fig. 4). In contrast, ddC effected a concentration-dependent decrease in the amount of 16.56-kbp DNA which hybridized to the mtDNA-specific riboprobe. Identical results were obtained with HepG2 cells and HepG2 cells which constitutively replicate human HBV (data not shown).

FIAU increases levels of lactic acid in cell culture media of CEM cells and human hepatoblastoma cells. The inability of FIAU to inhibit mtDNA replication does not preclude an inhibitory effect on mitochondrial function which would result in an adaptive cellular response characterized by the stimulation of anaerobic glycolysis leading to an increase in lactic acid production. Therefore, lactic acid levels in the cell supernatant medium were determined in an effort to evaluate mitochondrial function. As shown in Fig. 5, both ddC and FIAU increased lactic acid levels in the cell culture medium in a concentration-dependent manner. After 6 days of incubation, 1 μM ddC elevated lactic acid levels to 250% of the control level in T cells (Fig. 5A), while at a concentration of 100 μM, ddC elevated lactic acid levels to 200% of the control level in HepG2/G3 cells (Fig. 5B), to 138% in HepG2 cells (Fig. 5C) and to 175% in HepG2 cells which constitutively produce HBV (Fig. 5D, HepG2/HBV). Similarly after 6 days of incubation, 20 μM FIAU elevated lactic acid levels to 225% of the control level in T cells (Fig. 5A), while at a concentration of 200 μM, FIAU elevated lactic acid levels to 388% of the control level in HepG2/G3 cells (Fig. 5B), to 188% in HepG2 cells (Fig. 5C), and to 510% in HepG2/HBV cells (Fig. 5D).

DISCUSSION

We investigated whether FIAU, a pyrimidine nucleoside analog with anti-HBV activity in vitro and in vivo, is able to inhibit mtDNA replication in vitro. No selective inhibitory effect on mtDNA replication was observed in CEM or human hepatoblastoma cells. Furthermore, no inhibitory effect of FIAU (up to 2.5 μM) could be demonstrated in CEM cells which were incubated for 15 days in the presence of this

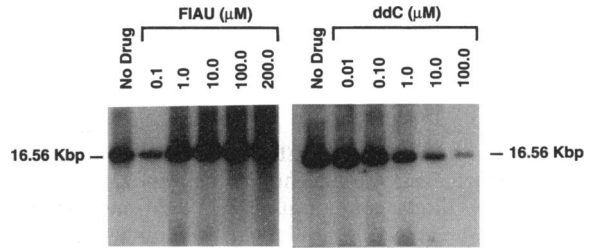

FIG. 4. Southern blot analysis of HepG2/G3 cells exposed to ddC or FIAU. The cells were incubated as described in the legend to Fig. 3, and total cell DNA was prepared for Southern blot analysis as described in Materials and Methods. DNA (2.5 μg) was digested with *Bam*HI, electrophoresed through 0.6% agarose, denatured, and transferred to a Nytran membrane. The immobilized DNA was hybridized to an mtDNA-specific riboprobe. There were equal amounts of DNA in each lane as determined by ethidium bromide-UV visualization, with the exception of the lane representing FIAU/0.1 μM, which was underloaded.

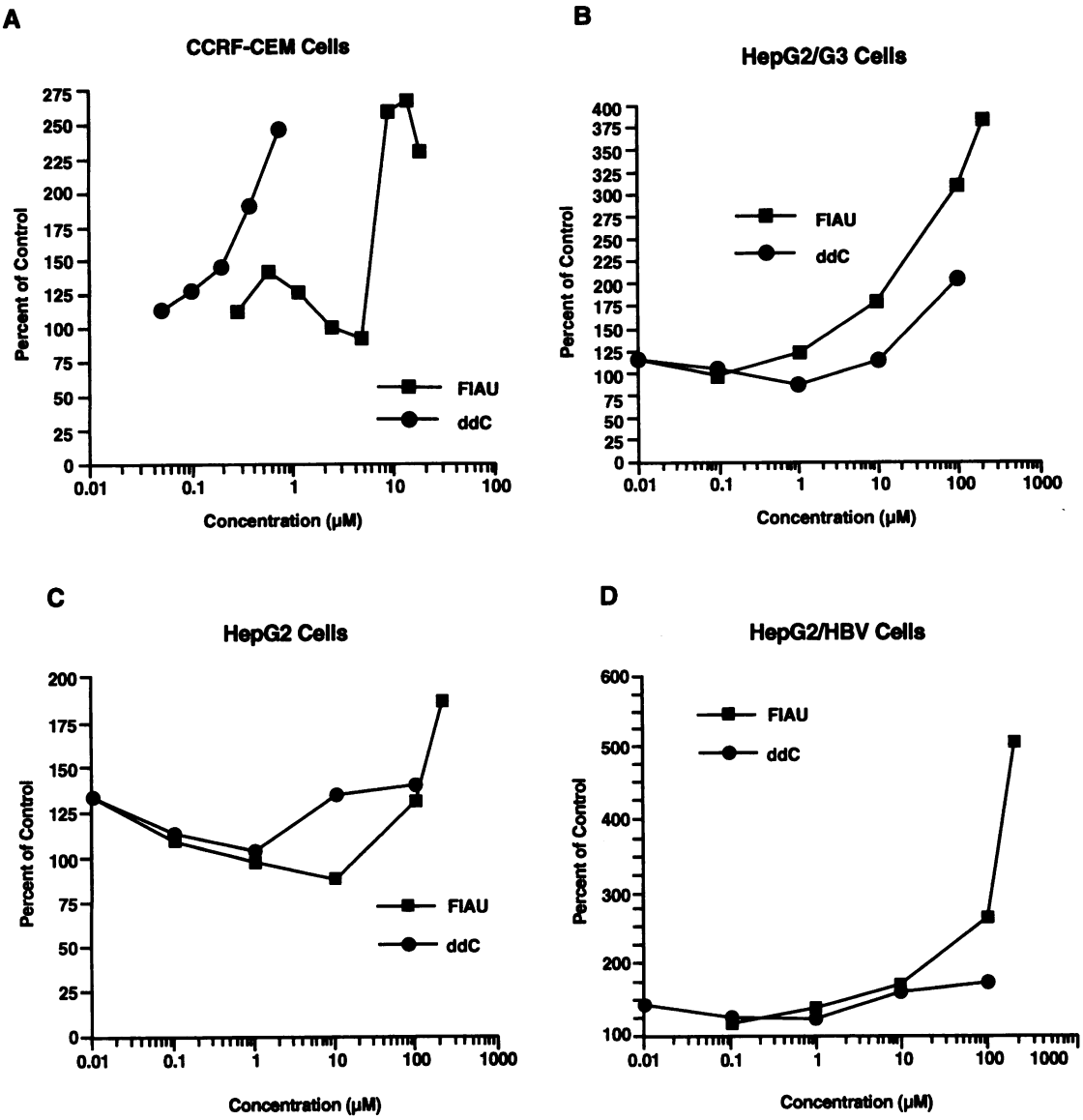

FIG. 5. Effect of ddC or FIAU on lactic acid levels in cell culture media. CEM cells were incubated for 6 days as described in the legend to Fig. 1. HepG2/G3, HepG2, and HepG2/HBV cells were incubated for 6 days as described in the legend to Fig. 3. On day 6, a 5-μl aliquot of clarified cell culture fluid was obtained for lactic acid determination as described in Materials and Methods. The total number of cells at each drug concentration was determined, and lactic acid levels (milligrams) were normalized to 10^6 cells. Lactic acid levels at each drug concentration were compared with those in cells incubated in drug-free medium, and the percentage of control levels was determined for each concentration.

nucleoside analog. Importantly, these concentrations of FIAU were shown to be cytotoxic to CEM cells as determined by the soluble formazan assay and to human hepatoblastoma cells as determined by direct cell counting and light-microscopic examination of the cells. In parallel experiments using the same conditions, ddC readily and selectively inhibited the replication of mtDNA with an IC_{50} of approximately 0.1 μM in CEM cells and approximately 0.2 μM in human hepatoblastoma cells. These results are consistent with the selective inhibitory effect of ddC on mtDNA replication (2, 3).

Our results were initially surprising since a number of observations which are consistent with the ability of FIAU to inhibit mtDNA replication have been made: (i) the toxicity profile seen during the clinical evaluation of FIAU as a chemotherapeutic agent for the treatment of chronic HBV infection (11a), (ii) the ability of the triphosphate of FIAU to inhibit DNA polymerase gamma (14a), and (iii) the ability of zidovudine, an analog of thymidine, to inhibit mtDNA replication and to induce a deleterious effect on the mitochondrial structure (1, 4, 13–15). A number of possible explanations can be invoked to explain this apparent discrepancy. (i) As shown previously, FIAU was phosphorylated in hepatoblastoma cells (22) but was not a good substrate for mitochondrial thymidine kinase (6). Also, phosphorylation of radiolabeled [^{14}C]FIAU was not observed in isolated rat mitochondria (11b). Thus, the 5′-triphosphate of FIAU may have to be transported into the mitochondrial compartment in order to inhibit mtDNA replication. This transport may not occur efficiently in cell culture. (ii) The 5′-triphosphate of FIAU may be incorporated into mtDNA without causing a decrease in the abundance of

mtDNA. The incorporation of FIAU into mtDNA may lead to impaired gene function and ultimately mitochondrial dysfunction. Evidence for the incorporation of FIAU into mtDNA has been discussed previously (19). (iii) The incorporation of FIAU into nuclear DNA may alter nuclear gene expression such that one or more of the many proteins encoded by nuclear genes and necessary for mitochondrial function may be depleted. The incorporation of FIAU into cellular DNA has been documented previously (5, 11). (iv) In contrast to ddC which lacks a 3′-OH, FIAU contains a 3′-OH and may incorporate into mtDNA, thus necessitating a period of incubation longer than that used in the experiments reported here (>15 days) before depletion of mtDNA is noted. Preliminary data from experiments in which rats were given FIAU orally at a dosage of 500 mg/kg of body weight per day for 70 days demonstrate a decrease in the abundance of mtDNA in hepatocytes (8a). These results indicate that a chronic exposure to FIAU is necessary for depletion of mtDNA. (v) Treatment of cells with FIAU may lead to qualitative changes in mtDNA, such as small deletions (<100 bp) or a decrease in stability, which are not detectable by Southern blot analysis. These changes could lead to altered gene expression and, hence, mitochondrial dysfunction. (vi) Finally, the inhibition of mtDNA replication may be cell system dependent. Considering that the cells used in these studies (CEM and HepG2) are cell lines, it will be of interest to determine the effect of FIAU on the replication of mtDNA in vitro in peripheral blood mononuclear leukocytes or primary hepatocytes.

The results reported here, which demonstrate that FIAU increases lactic acid levels in a dose-dependent fashion in cell culture, are consistent with a drug-dependent impairment of mitochondrial function. Furthermore, since FIAU did not inhibit the replication of mtDNA in culture, our data demonstrate that the ability of certain nucleoside analogs to increase lactic acid production may not always be correlated with their ability to inhibit the replication of mtDNA. For example, 2′,3′-dideoxyinosine at 200 μM did not inhibit mtDNA replication in CEM cells but did increase lactic acid levels at that concentration (4). FIAU may have a direct effect on the respiratory chain and electron transfer. If so, mtDNA replication may proceed normally while mitochondrion function is impaired.

Although inhibition of mtDNA replication has been proposed to be the mechanism underlying the clinical toxicity of FIAU, it has not been demonstrated unequivocally. If the working hypothesis is correct, the results reported here demonstrate that screening nucleoside analogs by slot or Southern blot hybridization for their ability to inhibit mtDNA replication during a relatively short exposure of cells in culture is not sufficient to identify those compounds which may pose a real problem in the clinical setting.

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