Antiviral Activity and Toxicity of Fialuridine in the Woodchuck Model of Hepatitis B Virus Infection

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Woodchucks were used to study the antiviral activity and toxicity of fialuridine (FIAU; 1′-2′-deoxy-2′fluoro-1-β-D-arabinofuranosyl-5-iodo-uracil). In an initial experiment, groups of six chronic woodchuck hepatitis virus (WHV) carrier woodchucks received daily doses of FIAU by intraperitoneal injection for 4 weeks. At 0.3 mg/kg/d, the antiviral effect was equivocal, but at 1.5 mg/kg/d, FIAU had significant antiviral activity. No evidence of drug toxicity was observed during the 4-week period of treatment or during posttreatment follow-up. In a second experiment, groups of nine WHV carriers or uninfected woodchucks were given 1.5 mg/kg/d of FIAU orally for 12 weeks, and the results compared with placebo-treated controls. After 4 weeks, the serum WHV-DNA concentration in the FIAU-treated carrier group was two to three logs lower than that in the placebo-treated group. After 12 weeks of FIAU treatment, serum WHV DNA was not detectable by conventional dot-blot analysis, hepatic WHV-DNA replicative intermediates (RI) had decreased 100-fold, and hepatic expression of WHV core antigen was remarkably decreased. No evidence of toxicity was observed after 4 weeks, but, after 6 to 7 weeks, food intake decreased and, after 8 weeks, the mean body weights of woodchucks treated with FIAU were significantly lower than controls. Anorexia, weight loss, muscle wasting, and lethargy became progressively severe, and all FIAU-treated woodchucks died or were euthanized 78 to 111 days after treatment began. Hepatic insufficiency (hyperbilirubinemia, decreased serum fibrinogen, elevated prothrombin time), lactic acidosis, and hepatic steatosis were characteristic findings in the final stages of FIAU toxicity in woodchucks. The syndrome of delayed toxicity in woodchucks was similar to that observed previously in humans treated with FIAU, suggesting that the woodchuck should be valuable in future investigations of the molecular mechanisms of FIAU toxicity in vivo and for preclinical toxicological evaluation of other nucleoside analogs before use in patients. (HEPATOLOGY 1998;28:179-191.)

Fialuridine (FIAU; 1′-2′-deoxy-2′fluoro-1-β-D-arabinofuranosyl-5-iodo-uracil) is a pyrimidine nucleoside analog with antiviral activity against herpes viruses1,2 and hepatitis B virus (HBV).3,4 During the summer of 1993, patients with chronic HBV infection that had been treated with FIAU for 9.5 to 13 weeks developed signs of severe and, in some cases, fatal hepatotoxicity associated with lactic acidosis.5,6 The observed toxicity had not been anticipated on the basis of earlier 2- and 4-week clinical trials of FIAU in human patients or on the basis of preclinical toxicological studies using conventional laboratory animals.5

The toxicological studies that preceded the FIAU clinical trials7,8 and the studies that followed9 used healthy animals with normal hepatic morphology and function. FIAU toxicity, however, had been observed in patients with chronic HBV infection, and it became necessary to know if HBV-induced liver disease had been an essential factor in the pathogenesis of the observed human drug toxicity.10 To investigate this question, we used woodchucks chronically infected with the woodchuck hepatitis virus (WHV) as a model of HBV infection. WHV belongs to the genus Orthohepadnavirus (family: Hepadnaviridae), of which HBV is the prototype member.11 Woodchucks with naturally acquired12,13 or experimentally14,15 chronic WHV infection develop progressively severe chronic hepatitis and hepatocellular carcinoma (HCC), similar to the diseases observed in humans with chronic HBV infection.11-15

The experiments reported here were designed to assess the antiviral activity of FIAU against WHV, and to determine the possible influence of hepadnavirus infection on toxicity by comparing the effects of FIAU in normal woodchucks and in chronic WHV carriers.
MATERIALS AND METHODS

Experimental Animals. The woodchucks used in these studies were the offspring of WHV-negative females from a breeding colony maintained at Cornell University. WHV-infected woodchucks were inoculated subcutaneously at 3 days of age with 103.3 ID50 of virus in 0.1 ml. The woodchucks were inoculated with varying doses of WHV. Infectivity of the standardized infectious serum pool (WHVTP) had been established previously by titration in adult woodchucks. Approximately 70% of woodchucks inoculated as neonates in this manner with the WHVTP pool became chronic WHV carriers. Pups from WHV-infected litters and uninfected control litters were weaned at 6 to 8 weeks of age. The diet consisted of laboratory animal chow formulated for rabbits (Agway Big Red Rabbit Food, Syracuse, NY), but specially pelleted in blocks for woodchucks. Diet and water were provided ad libitum.

Experimental Procedures. FIAU was provided as a dry powder (Lilly Research Laboratories, Indianapolis, IN). Two separate experiments that involved the administration of FIAU to woodchucks were conducted. The objective of Experiment 1 was to determine the relationship of dose to antiviral activity in a 4-week treatment protocol. Woodchucks in Experiment 1 were 10 months of age when the study was begun, and approximately equal numbers of males and females were used in each of three experimental groups. One group of six chronic WHV carriers received FIAU at a dosage of 0.3 mg/kg/d by intraperitoneal injection, a second group of six WHV carriers received FIAU at a dosage of 1.5 mg/kg/d intraperitoneally, and a group of six chronic WHV carriers received the isotonic saline vehicle intraperitoneally as placebo. All groups were treated for 4 weeks. FIAU treatment in Experiment 1 was initiated December 9, 1992, and a 12-week posttreatment follow-up period was completed March 30, 1993. After learning during the final week of June, 1993, of the toxicity caused by FIAU treatment in HBV patients, the woodchucks of Experiment 1 were re-examined on July 2, 1993, and subsequently were monitored for evidence of drug-related toxicity for 38 additional weeks, when the study was terminated.

Experiment 2 was designed specifically to assess the possible influence of chronic hepadnavirus infection on FIAU toxicity. Thirty-six 24- to 26-month-old adult woodchucks of both sexes were used. One group of 9 woodchucks, seronegative for markers of WHV infection, received FIAU at a dosage of 1.5 mg/kg/d orally for 12 weeks. A second group of 9 WHV-negative woodchucks received water vehicle daily for 12 weeks as placebo. A third group of 9 chronic WHV carrier woodchucks received FIAU at a dosage of 1.5 mg/kg/d orally for 12 weeks, and a fourth group of 9 chronic WHV carrier woodchucks received water vehicle as placebo for 12 weeks. A dose syringe was used to administer aqueous FIAU solution or placebo into the cheek, and this was followed by administration of 4 to 5 ml of semipurified liquid diet (Liquid Woodchuck Control Diet, Dyets, Inc., Bethlehem, PA) to ensure consumption of drug or placebo. A posttreatment follow-up period of 12 weeks was originally planned. Because of FIAU toxicity, however, the longest posttreatment survival was 27 days. Experiment 2 was initiated February 8, 1994.

Woodchucks in Experiments 1 and 2 were observed daily at the time they received food and water, at the time drug or placebo was administered, and each time they were anesthetized to obtain blood samples or liver biopsies. Abnormalities in appearance or behavior, including alterations in food and water intake, were recorded. When experimental woodchucks became cachectic or moribund, they were euthanized (ketamine/xylazine anesthesia, followed by pentobarbital overdose). In Experiment 2, each time an FIAU-treated woodchuck died or was euthanized, a placebo-treated woodchuck matched by sex and WHV status also was euthanized. Experiments were conducted in accordance with the Guide for the Care and Use for Laboratory Animals (National Academy Press, revised 1996) and were reviewed and approved by the Cornell University Institutional Animal Care and Use Committee.

Hematology and Clinical Biochemistry. Blood samples were obtained with woodchucks under ketamine (50 mg/kg) and xylazine (5 mg/kg) anesthesia before initiation of treatment, during the period of FIAU treatment, and during posttreatment follow-up. Blood samples were obtained routinely from the femoral vein or artery. Venous blood samples used to measure blood pH and Pco2 (bicarbonate) were obtained from the external jugular vein. Complete blood counts were performed using an electronic cell-counting system (Model 5+ IV, Coulter Corporation, Hialeah, FL), except for differential leukocyte counts that were performed manually. An automatic chemical analyzer was used for the measurement of aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (AP), γ-glutamyl transpeptidase (GGT), bilirubin, sodium, potassium, chloride, calcium, inorganic phosphorus, glucose, blood urea nitrogen (BUN), creatinine, creatine kinase, lipase, and amylase (DACOS, Coulter Corporation). Automated procedures also were used for measurement of prothrombin time and fibrinogen (BioQuest FibroSystem, Becton-Dickinson and Co., Cockeysville, MD). Sorbitol dehydrogenase (SDH) was determined using fructose as substrate, and the conversion of NADH to NAD quantitated spectrophotometrically (Sigma Diagnostics, St. Louis, MO). Blood lactate was determined enzymatically by the conversion of lactate to pyruvate by lactate dehydrogenase and by measuring the conversion of NAD to NADH spectrophotometrically (Sigma Diagnostics).

In Experiment 2, urine specimens were collected after 8, 10, and 12 weeks of treatment from anesthetized woodchucks, and analyses of urine were performed (Multistix, Bayer Corp., Diagnostics Division, Elkhart, IN).

Serological Tests for WHV Markers and Nucleic Acid Analyses of Liver. Tests for WHV surface antigen (WHsAg), antibody to WHV core antigen (anti-WHc), and antibody to WHV surface antigen (anti-WHs) were performed using WHV-specific enzyme-linked immunosorbent assays. Test serum was diluted 1:100 so that WHsAg, anti-WHc, and anti-WHs were determined under saturating conditions. Sample-negative ratios greater than 3.1 were considered positive.

The influence of FIAU on WHV replication was determined by comparing the levels of serum WHV DNA and the hepatic WHV nucleic acids of FIAU-treated chronic WHV carriers with those of placebo-treated controls before, during, and following treatment. The WHV-DNA levels of serum were determined by dot-blot hybridization analysis. Serum samples were centrifuged at 14,000g for 2 minutes. Aliquots (10 µL or 100 µL) were denatured with an equal volume of 2.0 mol/L NaOH/20 × SSC for 30 minutes at room temperature and immediately applied to nitrocellulose (presoaked in sterile 20 × SSC) under vacuum. After entry into the membrane, samples were washed with 0.4 mL sterile 1.0 mol/L Tris-HCl (pH 7.4)/2 mol/L NaOH, and then with 0.4 mL 20 × SSC. The membrane was then rinsed with sterile 2 × SSC (2 minutes), air-dried (5 minutes), and baked for 30 minutes at 80°C under vacuum. The membranes were then hybridized with a full-length (3.2-kb), gel-purified WHV-DNA probe as previously described. The sensitivity cut-off of this assay is approximately 1.0 pg WHV DNA (3.3 × 10^6 WHV genome equivalents) per milliliter of serum. WHV nucleic acids (replicative intermediates [RI], monomeric WHV DNA, WHV RNA) were measured by Southern and Northern blot analysis using liver specimens obtained at biopsy or at postmortem, immediately following euthanasia.

Histopathological and Ultrastructural Studies of Liver. Liver biopsy specimens were obtained while animals were under general anesthesia (ketamine/xylazine) using 14-gauge disposable biopsy needles (Biopsy-Cut, C.R. Bard, Inc., Covington, GA) directed by ultrasound imaging. The needle was inserted at a site near the ventral midline just caudal to the xiphoid cartilage and directed dorsolaterally and somewhat cranially into the margin of the left lateral lobe of the liver. One specimen was placed immediately in liquid nitrogen and stored at −70°C before nucleic acid analysis. A second specimen was fixed in phosphate-buffered formalin, embedded in paraffin, sectioned, and stained with hematoxylin-eosin for conventional light
microscopic examination. Sections of paraffin-embedded tissue also were stained immunohistochemically for WHsAg and WHcAg using a peroxidase method and polyclonal rabbit antibodies raised against the respective WHV antigens. A third specimen obtained from woodchucks of Experiment 2 was chopped into 1-mm cubes, fixed in diluted Karnovsky’s solution, embedded in plastic, thin-sectioned, and examined by electron microscopy. A fourth specimen was placed in OCT medium (Tissue-Tek, Sakura Finetek USA, Inc., Torrance, CA) and snap-frozen in a pentane/solid CO2 slurry. Frozen sections (some of which were fixed in formalin but not otherwise processed) were stained for fat with Oil Red O.

Hematoxylin-eosin-stained liver biopsy sections were examined under code without reference to treatment. The severity of portal hepatitis, parenchymal hepatitis, bile duct proliferation, and fatty change was scored using a scale of 0 to 4. Steatosis also was classified qualitatively as microvesicular, macrovesicular, or mixed. Oil Red O–stained sections were used to confirm the presence and severity of steatosis. Histological specimens stained for WHsAg or WHcAg were scored (scale of 0 to 4) for intensity of staining and the percentage of hepatocytes stained, and the primary intracellular localization of the antigen was recorded.

Postmortem examinations were performed on all woodchucks that died or were euthanized. In Experiment 2, the postmortem procedures for placebo-treated woodchucks were identical to those used for woodchucks treated with FIAU. Immediately following euthanasia, five separate specimens of liver were obtained and processed and examined in a manner similar to that used for liver biopsies.

Analyses of FIAU in Serum and Liver. In Experiment 2, serum samples were collected for FIAU analysis during the treatment phase 14.5 to 18 hours following FIAU administration and in surviving woodchucks during posttreatment follow-up. The serum concentration of FIAU was determined using a competitive binding radioimmunoassay based on a rabbit antibody raised against FIAU (5'-O-hemisuccinate conjugated to keyhole limpet hemocyanin). The sensitivity (0.2 ng/ml), specificity (negligible interference from known FIAU metabolites and endogenous nucleosides), and interassay variability (5.0% to 19.7%) of this procedure have been described.

For determination of hepatic FIAU incorporated into cellular DNA, specimens of liver were obtained immediately following euthanasia and processed by a previously described procedure. Briefly, DNA was isolated from whole liver homogenate using the ASAP Genomic DNA Isolation Kit (Boehringer Mannheim, Corp., Indianapolis, IN). DNA extracted from liver homogenate was denatured by heating to 95°C for 5 minutes, and the solution placed on ice. DNA was hydrolyzed with snake venom phosphodiesterase and bacterial AP FIAU measured by radioimmunoassay, and the results expressed as picomoles of FIAU per micromoles of thymidine determined by high-pressure liquid chromatography.

Statistical Methods. The Student’s t test was used for comparisons of continuous data (solute concentrations, enzyme activity, FIAU content of hepatic DNA) between WHV-infected and uninfected woodchucks, and between FIAU-treated and placebo-treated groups. Each test included only samples taken at the same time point. Mean values were expressed ± SD. For comparisons of ordinal data between groups (histopathological scores), Wilcoxon’s rank sum test was used. All these tests used α = 0.05 and were one-tailed. Preliminary tests between WHV-negative and WHV-positive groups treated either with FIAU or placebo were performed using two-tailed tests, and when P ≥ .50, results of WHV-positive and negative groups were combined for further analysis.

RESULTS

Antiviral Activity of FIAU. In Experiment 1, intraperitoneal treatment with FIAU at 1.5 mg/kg/d was associated with a significant 10-fold decrease in serum WHV DNA after 1 week of treatment (P = .01). After 4 weeks of treatment, the average decrease was 100-fold versus placebo-treated controls (P = .001). Serum WHV DNA gradually increased after drug withdrawal, but at all time points examined throughout the 12-week posttreatment period, the values were significantly lower than those of the controls (P < .001). In woodchucks treated with FIAU at 0.3 mg/kg/d, mean serum WHV-DNA levels were not significantly different from those in the placebo-treated controls, although some treated woodchucks temporarily had lower WHV-DNA levels during drug treatment.

Hepatic WHV-DNA RI were measured in Experiment 1 before treatment, at the end of the 4-week treatment period, and at 4 and 12 weeks following drug withdrawal. In woodchucks treated with FIAU at 1.5 mg/kg, mean hepatic RI at the end of the 4-week treatment period were threefold less than those in placebo-treated controls, and the difference was highly significant (P = .002). Levels of hepatic WHV RI increased in all individuals within 4 weeks after completion of treatment at the 1.5-mg/kg/d FIAU dosage, but remained significantly below control values (P = .02). Twelve weeks after treatment ended, the mean levels of hepatic WHV RI were less than those in the control group, but the difference was not significant (P = .06). In woodchucks treated with FIAU at 0.3 mg/kg/d, levels of hepatic WHV RI were not altered. No significant differences were observed between control woodchucks and woodchucks treated with FIAU at either 0.3 or 1.5 mg/kg/d in hepatic episomal (3.2-kb) WHV-DNA genomes or hepatic WHV RNA. There was no serological evidence of resolution of WHV infection, and all FIAU- and placebo-treated woodchucks remained test-positive for WHsAg and anti-WHc, and test-negative for anti-WHs at the end of the study.

Histological examination of hepatic biopsies from the FIAU- and placebo-treated woodchucks before beginning the study demonstrated mild to moderate degrees of portal and parenchymal hepatitis. Biopsies of liver obtained at the end of FIAU treatment and at 4 and 12 weeks after drug treatment ended were examined, and neither the severity of hepatic inflammation nor the expression of either WHsAg or WHcAg were influenced by treatment.

In Experiment 2, treatment with FIAU at an oral dose of 1.5 mg/kg/d had a prompt and marked effect on viral replication. Within 2 weeks, the mean serum WHV-DNA level in FIAU-treated woodchucks was 10-fold less than that in the placebo-treated woodchucks (P < .001) (Fig. 1). Within 4 weeks, the mean WHV-DNA level in the FIAU-treated group was two to three logs less than that in the placebo group (P < .001) and, after 10 weeks, was below the range detectable by quantitative blot hybridization analysis. The serum WHV-DNA levels of carriers receiving placebo were unchanged during the study (Fig. 1).

In Experiment 2, two- to threefold reductions of hepatic WHV-DNA RI occurred after 4 weeks of FIAU treatment and, after 12 weeks, WHV-DNA RI had decreased 10-fold (both P < .001 vs. placebo) (Fig. 2). Reductions of episomal WHV-DNA genomes and WHV RNA were not observed after 4 weeks of treatment, but had declined an average of twofold after 12 weeks of treatment (data not shown).

At the end of the 12-week treatment, percutaneous liver biopsies were obtained from six surviving chronic WHV carriers that had received FIAU and from six that received placebo. The median scores for WHcAg expression before initiation of therapy were 3/4 in the placebo group and 3.5/4
in the FIAU-treated group. After 4 weeks of treatment, the median scores for WHcAg expression were 3/4 in both the placebo- and FIAU-treated groups. At 12 weeks, the median WHcAg expression score was 3/4 in the placebo group and 1/4 in the FIAU-treated group (P < .001). The median scores for expression of WHsAg in hepatocyte plasma membranes were 2/4 and 1/4 in the placebo-treated and FIAU-treated groups, respectively, after 12 weeks of treatment (P = .12). The median scores for cytoplasmic expression of WHsAg in single hepatocytes were 1/4 in both the placebo-treated and FIAU-treated groups.

**Toxicity of FIAU.** In Experiment 1, there were no physical signs of FIAU toxicity in woodchucks treated at either 0.3 mg/kg/d or 1.5 mg/kg/d for 4 weeks. No significant differences between the mean body weights of FIAU-treated woodchucks and placebo-treated controls were observed during treatment or the follow-up period. There were no FIAU-related hematological or clinical biochemical changes during treatment or during posttreatment follow-up. In biopsies of liver obtained at the end of treatment and at 4 and 12 weeks after treatment ended, there were no histological differences between FIAU- and placebo-treated woodchucks in severity of hepatitis, steatosis, or in the expression of WHc and WHsAg.

At the end of the original 12-week posttreatment period (March 30, 1993), the mean body weight of the FIAU high-dose group (3.4 ± 0.3 kg) at this time was significantly lower, however, than that of the placebo-treated controls (3.8 ± 0.3 kg; P = .03), and for the remainder of the posttreatment study (9 months), mean body weights of the FIAU high-dose group were lower than those of the controls (data not shown).

From initiation of FIAU treatment on December 9, 1992, until termination of follow-up studies on March 1, 1994, no FIAU treatment-related alterations were observed in serum tests for hepatic injury or hepatic function (GGT, SDH, ALT, AST, AP, bilirubin) or renal function (BUN and creatinine). Serum WHV DNA of the FIAU high-dose group had returned to pretreatment levels by the time posttreatment follow-up was initiated 25 weeks after drug administration ended.

Between September 30 and December 2, 1993, two placebo-treated controls and two low-dose, FIAU-treated woodchucks were euthanized because of the development of HCC (neoplasms considered to be a direct result of infection with WHV). Between February 16 and March 1, 1994, the final surviving woodchucks from the study were euthanized. Postmortem examinations were performed on five of six of the original FIAU high-dose group, three of six of the FIAU low-dose group, and four of six of the placebo-treated controls. In woodchucks from all three groups, varying degrees of chronic hepatitis were found. The severity was considered to be that expected for chronic WHV carriers that were approximately 2 years of age. Similar degrees of hepatic neoplasia were observed in FIAU-treated and control groups. There were no gross or histopathological lesions attributable to FIAU treatment.

**FIG. 1.** Serum WHV DNA (pg/mL) of chronic WHV carrier woodchucks treated orally for 12 weeks with FIAU (1.5 mg/kg/d; n = 9) or with placebo (n = 9). After 4 weeks, there was a 100- to 1,000-fold reduction of serum WHV DNA in the FIAU-treated group compared with controls (P < .001), and the difference was sustained during the remainder of the study.

**FIG. 2.** Hepatic WHV-DNA RI of chronic WHV carrier woodchucks treated orally for 12 weeks with FIAU (1.5 mg/kg/d) or with placebo. There was a 10-fold reduction in hepatic WHV-DNA RI associated with FIAU treatment (P < .001).
In Experiment 2, there was no clinical, hematological, or biochemical evidence of FIAU toxicity during the first 4 weeks of oral drug treatment. Histological examination of hepatic biopsies obtained after 4 weeks demonstrated no differences between groups of uninfected and WHV carrier woodchucks treated with FIAU and corresponding groups of placebo-treated controls. These initial observations were similar to those of Experiment 1 in which there was no conclusive evidence of toxicity after intraperitoneal administration of FIAU for 4 weeks.

In Experiment 2, the first evidence of FIAU toxicity was observed after 6 to 7 weeks. Both uninfected and WHV carrier woodchucks treated with FIAU had moderate decreases in food intake, and their contact bedding (wood chips) was less soiled than that of placebo-treated woodchucks. After 8 weeks, the mean body weights of both WHV carrier and uninfected woodchucks receiving FIAU were significantly lower than those of the placebo groups (P = .05) (Fig. 3). Thereafter, decreased food intake, progressive weight loss, muscle wasting, weakness, and lethargy were conspicuous clinical signs in FIAU recipients.

The first death in the study was in a placebo-treated WHV carrier that was euthanized after 75 days because of signs of hepatic failure associated with the development of HCC. Thereafter, all woodchucks that died or that were euthanized following drug withdrawal. In FIAU-treated woodchucks not infected with WHV, mean survival, median survival, and the survival range were similar to FIAU-treated WHV carriers (85 ± 5, 84, and 79-99 days, respectively). Six of the uninfected, FIAU-treated group died during the final week of treatment, and three died during the first 15 days after treatment ended. With the exception noted, woodchucks treated with placebo all were clinically normal when euthanized as controls for FIAU-treated woodchucks. At the end of the 12-week period of FIAU treatment, on average, placebo recipients weighed more than twice as much as woodchucks treated with FIAU (Fig. 3).

**Hematology and Clinical Biochemistry.** During the 12-week course of FIAU treatment, no statistically significant differences were observed between FIAU- and placebo-treated groups in mean erythrocyte count, hemoglobin, hematocrit, or leukocyte count. FIAU-treated groups had mean thrombocyte counts that were half the corresponding values of placebo-treated groups after 12 weeks, suggesting moderate FIAU-induced bone marrow suppression.

No statistically significant treatment-related alterations in mean serum sodium, potassium, calcium, or chloride were observed. No effect of treatment on serum inorganic phosphorus was observed until the end of the 12-week treatment period when hyperphosphatemia developed in the FIAU-treated groups. Increases in serum inorganic phosphorus were associated in most cases with elevated BUN, indicating that the cause was renal insufficiency. Remarkably, serum creatinine was not similarly increased.

The plasma bicarbonate concentrations of both FIAU-treated groups were less than those of placebo-treated woodchucks beginning at 8 weeks (Fig. 4) and remained significantly lower (P < .001). The blood pH of FIAU recipients, however, did not change until the twelfth week of treatment when there was an abrupt decrease. Mean base excess was correspondingly decreased in FIAU-treated groups compared with controls at the end of drug treatment (P < .001) (Fig. 5). Serum lactate was measured after 8, 10, and 12 weeks of treatment. Only during the final week of drug administration were increases in serum lactate of 3- to 10-fold observed in FIAU-treated individuals (Fig. 6).

In chronic WHV carriers that received placebo, progressive increases were observed in the serum activities of ALT (data not shown), AST, and SDH (Fig. 7). Corresponding increases in the activity of these enzymes were not seen in uninfected, placebo-treated woodchucks, indicating that the increases were related to the chronic hepatitis associated with WHV infection. Remarkably, increased SDH, ALT, and AST were not observed in FIAU-treated chronic WHV carriers except in final samples. Similarly, the mean activities of serum GGT and AP were lower in WHV carriers treated with FIAU than in placebo-treated carriers. During the final week of treatment and following treatment, elevations in serum GGT activity of 10- to 100-fold were observed in four WHV carriers and four uninfected woodchucks treated with FIAU. These terminal elevations in GGT and similar changes in AP were attributed directly to FIAU-induced liver injury. Total serum bilirubin remained within normal limits until the tenth week of FIAU treatment when elevations associated with clinical icterus were observed in both WHV-positive and -negative, FIAU-treated woodchucks (Fig. 8).
During week 12 of treatment, mean serum fibrinogen levels in FIAU-treated woodchucks were less than those in placebo-treated controls among both the WHV-positive (P < .04) and WHV-negative (P < .001) woodchucks (Fig. 9). Correspondingly, during week 12, prothrombin times in FIAU-treated woodchucks were greater than those in placebo-treated controls (P < .001) (Fig. 9).

Serum amylase and serum lipase activities were measured as possible indicators of pancreatic injury. In three placebo-treated WHV carrier woodchucks, progressive increases in serum amylase were observed during the course of treatment, but similar elevations in serum amylase were not observed in placebo-treated, uninfected woodchucks, suggesting that the elevations in WHV carriers receiving placebo were part of the natural history of WHV infection. The three placebo-treated WHV carriers with elevated serum amylase had corresponding increases in serum GGT activity (a marker of HCC development in woodchucks). The congruent elevations in GGT and amylase were associated with development of HCC, and the elevations in serum amylase may have been of hepatic origin.

Elevations of four- to fivefold in serum lipase activity were observed terminally in three WHV carriers and in three uninfected woodchucks treated with FIAU, suggesting possible drug-induced pancreatic injury. There was, however, no gross evidence of acute hemorrhagic pancreatitis at necropsy. The only histological change in the pancreas associated with FIAU treatment was acinar atrophy. Woodchucks that had elevations in serum lipase also had elevations in BUN, suggesting that terminal elevations in lipase were related to diminished glomerular filtration.

**Serum and Hepatic FIAU.** The mean serum concentrations of FIAU as measured by radioimmunoassay remained relatively constant during FIAU treatment (Fig. 10). Among FIAU-treated woodchucks that died or were euthanized during the final week of treatment, two WHV-negative and three WHV carrier woodchucks had marked elevations in serum FIAU concentration, and one of these individuals accounted for the sharp increase in mean serum FIAU concentration after 12 weeks in the uninfected group of drug-treated woodchucks (Fig. 10). In four of the five, the marked elevations in serum FIAU concentration were associated with significant increases in BUN, suggesting that the increases, at least in part, were related to terminally decreased renal drug clearance. Following drug withdrawal, serum FIAU decreased rapidly to below the detectable limit (Fig. 10).

Specimens of liver obtained immediately following euthanasia were analyzed for FIAU integrated into cellular DNA. Results are summarized in Fig. 11. In WHV carriers, the mean value of 2,303 ± 812 pmol FIAU per micromole of thymidine was significantly higher (P < .05) than the mean value of 1,678 ± 249 pmol FIAU per micromole of thymidine in uninfected woodchucks. The hepatic DNA of all FIAU-treated woodchucks contained integrated FIAU, whereas levels in all placebo-treated woodchucks were below the background of the assay.
Microscopic Examination of Hepatic Tissues. The median portal hepatitis scores for the initial hepatic biopsy specimens obtained before treatment in Experiment 2 were 1/4 for chronic WHV carriers and 0/4 for uninfected woodchucks. Similar median scores were recorded in biopsy specimens obtained 4 weeks in FIAU-treated and placebo-treated WHV carriers. In biopsy specimens of liver taken after 12 weeks of treatment, the median portal hepatitis score for the six surviving FIAU-treated WHV carriers of 0.5/4 was significantly less than the median score of 2/4 in six placebo-treated chronic carriers (P = .03). The median steatosis scores of hepatic biopsies from all groups of woodchucks before initiation of FIAU treatment were 0/4, and similar median scores were present after 4 weeks of treatment. The median score of the combined WHV-infected and uninfected, FIAU hepatic biopsies was 2/4 after 12 weeks, which was significantly higher than the corresponding combined score of the placebo-treated woodchucks of 0/4 (P < .01).

Postmortem examinations were performed on all woodchucks that died or were euthanized. Remarkable atrophy of skeletal muscle and atrophy of abdominal and subcutaneous fat stores were observed in both WHV-positive and WHV-negative FIAU-treated woodchucks. The livers of the FIAU-treated groups were characteristically very pale, and three floated in formalin solution, suggesting increased fat content. Significant differences in median portal hepatitis scores between placebo (1/4) and FIAU-treated (0/4) WHV carriers were observed in the postmortem specimens of liver (P <

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Fig. 6. Serum lactate of WHV carrier and uninfected woodchucks treated with FIAU (1.5 mg/kg/d). Lactate measurements were made on the blood samples obtained during the twelfth week of treatment. Serum lactates of placebo-treated woodchucks were significantly lower than the FIAU-treated woodchucks either uninfected with WHV (P = .001) or that were chronic WHV carriers (P = .02).

Fig. 7. Serum activities of AST (A) and SDH (B) in WHV carriers and uninfected woodchucks treated with FIAU (1.5 mg/kg/d) or with placebo for 12 weeks. Beginning at 4 weeks, placebo-treated, WHV carriers had significantly increased AST and SDH activities compared with the FIAU-treated, WHV carriers (P = .01 and P = .02, respectively). The difference was maintained until the twelfth week of treatment when AST values increased in both uninfected and WHV carrier woodchucks treated with FIAU increased. Both AST and SDH activities increased remarkably in surviving woodchucks of both FIAU-treated groups following drug withdrawal.
.001). Piecemeal necrosis also was more severe in WHV carriers treated with placebo than in those treated with FIAU (P = .007), but there appeared to be no effect of FIAU on the severity of either parenchymal hepatitis, which was not observed (0/4) in either FIAU- or placebo-treated, WHV carriers or bile duct proliferation (median scores of 1/4 in both WHV carrier groups). The most impressive histological change was severe hepatic steatosis in FIAU-treated woodchucks. The steatosis was primarily microvesicular in nature but, in the most severe cases, was classified as both microvesicular and macrovesicular. In specimens collected at necropsy, the hepatic steatosis scores were zero in 17 of 18

![Fig. 8. Total serum bilirubin of WHV carriers and uninfected woodchucks treated with FIAU (1.5 mg/kg/d) or with placebo for 12 weeks. Statistically significant increases in mean bilirubin values were observed in the combined FIAU-treated woodchucks compared with placebo-treated controls only after 12 weeks of treatment (P < .05) and following drug withdrawal.](image)

![Fig. 9. Fibrinogen (A) and prothrombin time (B) of WHV carrier and uninfected woodchucks after 12 weeks of treatment with FIAU (1.5 mg/kg/d) or with placebo. Mean serum fibrinogen levels were significantly lower in WHV carriers (P = .04) and uninfected woodchucks (P < .001) treated with FIAU than in the corresponding placebo-treated groups. Mean prothrombin time was significantly higher in FIAU-treated groups than in placebo-treated woodchucks (P < .001).](image)

![Fig. 10. Serum concentrations of FIAU in WHV carrier (A) and uninfected (B) woodchucks treated (1.5 mg/kg/d) for 12 weeks. Dark points connected by dark lines represent mean values. Light lines represent individual woodchuck values. Graphs on the right provide detail of the decreases in serum FIAU that occurred following drug withdrawal.](image)
placebo-treated woodchucks and 1/4 in the remaining placebo-treated woodchuck, which was a chronic WHV carrier. Among the FIAU-treated woodchucks, 17 of 18 had steatosis scores of 1/4 to 4/4, with a median score of 3/4 for both WHV-positive and -negative FIAU-treated groups. In the remaining FIAU-treated animal (which was a chronic WHV carrier), the steatosis score was zero. The difference in median hepatic steatosis scores between placebo and FIAU-treated groups was highly significant (P < .001) (Figs. 12 and 13).

Transmission electron microscopic examination of representative hepatic, cardiac, and skeletal muscle specimens have been described elsewhere. There was remarkable damage of hepatic mitochondria in FIAU-treated woodchucks including conspicuous swelling of mitochondria, dissolution of cristae, and homogenization and decreased density of matrix. Significant ultrastructural damage of mitochondria also was found in heart, skeletal muscle, and diaphragm, and, in all of these tissues, fat droplet accumulation was present.

**DISCUSSION**

As reported previously in vitro and in patients with chronic HBV infection, FIAU treatment of woodchucks with chronic WHV infection resulted in prompt and highly significant inhibition of viral replication. In Experiment 1, the antiviral effect of FIAU was sustained following treatment, and serum WHV DNA remained below the levels of placebo-treated controls for more than 12 weeks after drug withdrawal. With most other polymerase inhibitors, rapid viral recrudescence follows drug withdrawal.

The syndrome of FIAU-induced toxicity observed in woodchucks was similar to that observed in clinical patients. In HBV patients, no evidence of toxicity was observed after 4 weeks of FIAU treatment, and few side-effects were observed during the first 8 weeks. Thereafter, fatigue, nausea, paresthesia, and abdominal pain resulted in premature discontinuation of treatment in 3 of 10 patients. Similarly, no signs of FIAU toxicity were observed in woodchucks after 4 weeks of treatment in either Experiment 1 or 2. Diminished food intake was first recognized in Experiment 2 after 6 to 7 weeks, and there was significant weight loss by 8 weeks. During the final 4 weeks of treatment and after treatment, progressively severe signs of anorexia, weight loss, muscle wasting, weakness, and lethargy were observed in all woodchucks that received FIAU.

In both humans and woodchucks, the most severe signs of FIAU toxicity including hepatic failure and lactic acidosis were observed after treatment for more than 9 weeks. Such signs were observed in 7 of 10 clinical patients and developed even after treatment was suspended. Similar signs developed in FIAU-treated woodchucks and deaths occurred either during the twelfth week of treatment or within 4 weeks following drug withdrawal.

In Experiment 2, progressive increases in serum AST, ALT and SDH activities were observed in placebo-treated, chronic WHV carriers but were not seen in uninfected placebo-treated woodchucks, indicating that the increased SDH and transaminase activities were attributable to chronic WHV infection. In contrast to placebo-treated WHV carriers, FIAU-treated WHV carriers did not have elevations in SDH and transaminase activities, suggesting that drug treatment was
responsible for suppressing the elevations in the activity of these enzymes. Only terminally, when there was evidence of multiorgan failure, were remarkable AST elevations observed in FIAU-treated woodchucks, and this was not a consistent finding. In human patients with FIAU-induced hepatic failure, serum transaminase activities were not characteristically elevated.5

In postmortem specimens of liver, moderate to severe microvesicular or mixed microvesicular and macrovesicular hepatic steatosis was found in all but one FIAU-treated woodchuck. This histological finding was essentially identical to that reported in human patients.5 The ultrastructural findings of mitochondrial swelling, dissolution of cristae, and decreased density of mitochondrial matrix in the liver of FIAU-treated woodchucks5,6 also were similar to those reported in human FIAU toxicity.5,6

In patients with signs of severe FIAU toxicity, there often was biochemical evidence of pancreatic injury, and in all five patients that died, there was postmortem evidence of pancreatitis.5 In FIAU-treated woodchucks, serum amylase activity was not elevated, but increases in serum lipase activity were observed terminally in six of these woodchucks. At postmor-
tem, there were no gross or microscopic lesions of hemorrhagic pancreatitis. The only morphological abnormality in the pancreas of FIAU-treated woodchucks was acinar cell atrophy. Whether this was a direct drug effect or was secondary to decreased food intake could not be determined. The BUN was consistently elevated in woodchucks with increased serum lipase activity, so the latter may have been related to decreased renal clearance. As noted in the Results section above, serum creatinine was inexplicably not elevated even in woodchucks with a 10-fold increase in BUN.

Some patients with FIAU toxicity had symptoms of neuropathy and/or myopathy. In woodchucks, even after anorexia and weight loss were obvious in FIAU-treated woodchucks, neurologic or muscular deficits were not observed. As described elsewhere, however, fatty metamorphosis and mitochondrial changes present in skeletal muscle, diaphragm, and heart suggested drug-induced myopathy. These results support the clinical and biochemical evidence that FIAU toxicity in woodchucks involved injury to multiple organs.

The observations made on patients suggested that mitochondrial dysfunction was important in the pathogenesis of FIAU toxicity. Hepatic steatosis, lactic acidosis, and ultrastructural abnormalities of mitochondria were similar to those of Reyès syndrome, in which mitochondrial injury is believed to be a critical factor in pathogenesis. Haptic steatosis and lactic acidosis have been observed in patients treated with other nucleoside analogs including ddC, ddI, AZT, and AZT, and multiorgan involvement similar to that associated with FIAU toxicity has been attributed to nucleoside-induced mitochondrial damage.

The mechanism responsible for FIAU-induced mitochondrial dysfunction and the role of mitochondrial dysfunction in FIAU toxicity are not yet fully understood. In tissue culture, the delayed toxicity of ddC has been comprehensively investigated and cytotoxicity and inhibition of cell growth correlated with mitochondrial injury. In vitro, ddC causes increased lactic acid production, ultrastructural changes in mitochondria, and accumulation of intracellular lipid vesicles. ddC also significantly decreased the abundance of mitochondrial DNA (mtDNA).

In vitro, FIAU also causes disturbances in mitochondrial function. In some, but not all reports, lactic acid production was increased by FIAU. In contrast to findings with ddC, which consistently produced significant decreases in steady-state abundance of mtDNA, variable results have been reported with FIAU from decrements that were slight or moderate to no effect on mtDNA abundance. ddC and FIAU both are competitive inhibitors of DNA polymerase γ with very low Kᵢ. Because ddC lacks the 3'-OH group necessary for DNA chain extension, incorporation of ddC into DNA results in obligatory chain termination and inhibition of mtDNA synthesis. Unlike dideoxynucleosides, FIAU has a functional 3'-OH group that allows chain extension and incorporation of FIAU into mitochondrial and nuclear DNA. It may follow that alterations of mitochondrial function and structure, at least in short-term in vitro studies, are the result of altering the genetic cascade within mitochondria.

While FIAU caused either no reduction or modest reduction in mtDNA in short-term tissue culture studies, substantial decrements in mtDNA abundance were found in the livers of clinical patients and in woodchucks with FIAU toxicity. Extended treatment of rats, monkeys, and dogs with FIAU also caused significant reduction of mtDNA in the liver and other tissues. When scaled on the basis of metabolic body size, the doses used in monkeys and rats were 10 to 100 times greater than those that caused toxicity in humans and woodchucks. The FIAU doses used in dogs, however, were similar to the dose that resulted in toxicity in woodchucks, and the reduction in hepatic mtDNA observed in dogs was comparable with that observed in woodchucks.

In Experiment 2, the toxicity of FIAU in woodchucks with chronic WHV infection appeared to be similar to that in uninfected woodchucks, suggesting that hepadnavirus infection did not contribute directly to FIAU toxicity. However, incorporation of FIAU into the cellular DNA of the liver was 37% greater in chronic WHV carriers than in uninfected woodchucks. WHV carriers had chronic hepatitis, which may have increased hepatocyte turnover, thereby facilitating incorporation of FIAU into cellular DNA. In mice, integration of FIAU into cellular DNA after single doses of radioactive drug was highest in tissues with the most rapidly dividing cells.

In studies of monkeys, rats, and dogs treated with FIAU, significant integration of FIAU was observed into cellular DNA in all three species. In rats, the liver appeared to be a major target for FIAU integration after 1 day, but after 77 days of FIAU treatment, the FIAU content of hepatic DNA was higher than the DNA of any other tissue. Rats treated with 255 or 510 mg/kg of FIAU for 70 to 110 days had levels of FIAU incorporated into hepatic DNA that were 10-fold higher than that observed in woodchucks in the present study. Dogs treated for 90 days with doses comparable with those used in woodchucks had levels of FIAU integrated into hepatic DNA that were similar to the woodchucks of this study.

FIAU toxicity in humans and woodchucks was associated with marked decreases in hepatic mtDNA. Comparable decreases in hepatic mtDNA were observed in monkeys, rats, and dogs treated with FIAU for 30, 70, and 90 days, respectively, but toxicity similar to that seen in humans and woodchucks was not observed. Incorporation of FIAU into cellular DNA of humans and the woodchucks described in the current study may have been important in determining FIAU toxicity, but no toxicity was reported in dogs with comparable levels of FIAU integrated into cellular DNA or in rats with levels of integrated FIAU that were 10 times higher than the levels formed in humans and woodchucks. Based on these observations, neither integration of FIAU into cellular DNA nor decreased abundance of mtDNA can be considered sufficient to explain the toxicity of FIAU. Perhaps other cellular targets of FIAU may be involved, e.g., the volume-activated chloride channel. Nucleoside analogs are known to block swelling-induced chloride current, and to diminish regulatory control of cell volume. This, in turn, could significantly alter regulation of intracellular pH, electrolyte content, and other metabolic functions, leading to mitochondrial damage and cell death.

In 3-month toxicological studies in mice and rats, no clinical signs of toxicity were observed even at doses of FIAU that were 100-fold greater than those that produced toxicity in woodchucks and humans. In rats, there was histological evidence of nuclear atypia, mitochondrial swelling, and increased apoptosis in the liver. In dogs that received doses of FIAU similar to those that produced toxicity in woodchucks and humans, no signs of toxicity or histological...
lesions in the liver were observed. Because toxicity of FIAU in woodchucks was not influenced by WHV infection, it was concluded that species-specific differences in the toxicity of FIAU exist between mice, rats, and dogs on one hand and humans and woodchucks on the other. FIAU, a prodrug of FIAU, and the closely related 5-methyl and 5-ethyl analogs all have been reported to have delayed toxicity in woodchucks similar to that observed with FIAU in this study. The similarity of the syndromes of FIAU toxicity in woodchucks and humans suggests that the woodchuck could be a valuable animal model for the recommended further investigation of the pathogenesis of FIAU toxicity and for the preclinical toxicological assessment of related nucleoside analogs undergoing development as potential human drugs.

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