Proc. Natl. Acad. Sci. USA
Vol. 91, pp. 12003–12007, December 1994
Pharmacology

Fialuridine accumulates in DNA of dogs, monkeys, and rats following long-term oral administration

(DNA incorporation/nucleosides/antiviral/hepatitis)

FRANK C. RICHARDSON*, JEFFERY A. ENGELHARDT*, AND RONALD R. BOWSHER†

*Toxicology Research Laboratories, Lilly Research Laboratories, Eli Lilly and Company, Greenfield, IN 46140; and †Drug Disposition and Bioanalytical Research, Lilly Research Laboratories, Eli Lilly and Company, Indianapolis, IN 46202

Communicated by Louis Sokoloff, August 5, 1994

ABSTRACT **Accumulation of the antiviral nucleoside analogue fialuridine (FIAU; 1-(2′-deoxy-2′-fluoro-β-D-arabinofuranosyl-5-iodouracil) in genomic DNA was examined with a modified version of a recently developed RIA for FIAU. DNA was obtained from tissues of dogs administered FIAU at 0, 1, 2, or 3 mg/kg of body weight per day for 90 days, monkeys administered FIAU at 0 or 25 mg/kg per day for 30 days, and rats administered FIAU at 0, 255, or 510 mg/kg per day for 70 days. FIAU incorporation was observed in all species. In the rat, FIAU was incorporated into DNA of all tissues examined, with highest concentrations in the liver followed by jejunum, spleen, and heart. FIAU was also incorporated into sperm DNA. Incorporation rates were as high as 11,000 pmol of FIAU per μmol of thymidine or 1 FIAU molecule per 90 thymidine molecules. In dogs and rats, the extent of incorporation was dose-dependent. Across species, FIAU concentrations in DNA were not singly dependent on the total dose administered but also may have been dependent on the duration of exposure. These studies show that FIAU accumulates to high concentrations in genomic DNA of liver as well as other tissues during chronic oral administration and suggest that net accumulation of FIAU in DNA may be a critical step in FIAU-induced toxicity.**

Fialuridine [1-(2′-deoxy-2′-fluoro-β-D-arabinofuranosyl)-5-iodouracil; FIAU] (Fig. 1) is a nucleoside analogue shown to be an effective drug against hepatitis B virus (HBV) infection (1). The ability of 1-(2′-deoxy-2′-fluoro-β-D-arabinofuranosyl)-5-iodocytosine (FIAC) triphosphate to inhibit HBV DNA polymerase activity (2, 3) and the inhibition of viral DNA replication by FIAU (4, 5) suggests that the antiviral mechanism of FIAU involves direct inhibition of viral DNA polymerase. FIAU reduced serum HBV DNA levels in individuals treated for up to 30 days and showed promising results in longer exposures until severe lactic acidosis accompanied in some cases by irreversible liver failure and pancreatitis caused termination of the clinical trials (6).

In part because of the lactic acidosis observed in the clinical trials, it has been proposed that FIAU may selectively affect mitochondrial function. Depletion of muscle mitochondrial DNA (mtDNA) has been observed with dideoxycytidine (ddC) (6) and 3′-azido-3′-deoxythymidine (AZT) (7) and has been suggested as a possible mechanism for the delayed toxicity of FIAU. Unlike AZT and ddC, however, FIAU has a free 3′-OH, which in theory permits internal incorporation into full-length DNA. Acute studies have shown that FIAU is incorporated into the DNA of several tissues in mice after a single dose of FIAC. FIAC is converted to FIAU *in vivo* (9). Moreover, FIAU-containing DNA (FIAU-DNA) is present in the small intestine of mice 24 hr after a single dose of FIAU at 20 mg/kg of body weight

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.

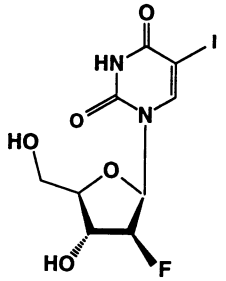

FIG. 1. Structure of fialuridine.

(10). While the biological consequences of FIAU incorporation into DNA remain unclear, *in vitro* studies with 9-D-arabinofuranosyl-2-fluoroadenine (F-ara-A) (11) and 1-D-arabinofuranosylcytosine (araC) (12) have correlated toxicity with incorporation into genomic DNA, suggesting that incorporation of FIAU into genomic DNA could also lead to cytotoxicity. Since clinical deterioration only occurred after long durations of exposure, it could be hypothesized that not simply incorporation but net accumulation of FIAU in DNA could be critical to the delayed toxicity of FIAU. Therefore, studies were conducted to measure FIAU accumulation in DNA of rats, monkeys, and dogs following long-term oral administration.

In the past, measuring FIAU incorporation into DNA was done by using standard radiolabeling techniques and only in acute pulse–dose studies (9, 10). These techniques are not amenable to assessing FIAU accumulation in DNA following long-term dosing regimens (90 days) and in larger species (dogs and monkeys) because of the large amounts of radioactivity required and the safety hazards associated with administering large quantities of radioactivity to animals over extended periods of time. Recently, however, the development of a sensitive radioimmunoassay (RIA) for use on biological fluids (13) has made it feasible to measure concentrations of FIAU-DNA under such conditions.

MATERIALS AND METHODS

All DNA samples were obtained from animals used in studies conducted in the Toxicology Division of Eli Lilly and Company. In this manuscript, full descriptions of all toxicology studies have not been provided; complete reports will be published elsewhere. Only information necessary to the interpretation of FIAU-DNA incorporation has been provided. Since toxicological evaluation was the primary purpose of these studies, the doses for each species vary and are

Abbreviations: FIAU, 1-(2′-deoxy-2′-fluoro-β-D-arabinofuranosyl)-5-iodouracil (fialuridine); FIAU-DNA, FIAU-containing DNA; HBV, hepatitis B virus; FIAC, 1-(2′-deoxy-2′-fluoro)-β-D-arabinofuranosyl)-5-iodocytosine; AZT, 3′-azido-3′-deoxythymidine; ddC, dideoxycytidine; mtDNA, mitochondrial DNA.

based on the maximum tolerated dose determined in previous toxicology studies.

Rats. Male and female F-344 rats 5–7 weeks old (Taconic Farms) were divided into groups of five and given a total oral dose of FIAU of 0, 255, and 510 mg/kg per day suspended in 10% acacia for 70 days. The total daily dose was divided and administered each day in three equivalent doses over a 12-hr period. Animals were housed individually in ventilated racks located in rooms with a 12-hr light/dark cycle and a climate-controlled environment (20.5–23°C and 30–70% humidity). Purina Rodent Chow 5002 and water were provided *ad libitum* to animals in all groups. Animals were killed by CO_2 asphyxiation 24 hr after the last dose of FIAU. Tissues collected for DNA isolation included: epididymal sperm, liver, heart, small intestine, and spleen. All tissues, except sperm, were collected and kept frozen at −70°C until DNA isolation could be performed; sperm was stored at −20°C. DNA was isolated 4–5 days after tissue collection. Hepatic nuclei were isolated by low-speed differential centrifugation. Liver was homogenized (Dounce homogenizer, Wheaton Scientific) in sucrose buffer [4 mM Hepes (Sigma)/0.25 M sucrose (Fisher Scientific)/4 mM K_2HPO_4/1.2 mM EDTA (Sigma), pH 7.3] and centrifuged at 600 × *g*. The nuclear pellet was then washed in sucrose buffer, recentrifuged at 600 × *g*, and stored at −70°C until DNA was isolated.

Dogs. Male and female beagle dogs, 8–13 kg (Marshall Farms, North Rose, NY), were given oral doses of FIAU of 0, 1, 2, or 3 mg/kg per day suspended in 10% acacia once a day continuously for 90 days. Selected high-dose and control dogs were maintained off treatment for a period of 5 weeks to examine the reversibility of the treatment effects. Dogs were euthanized using Succumb (Anthony Products, Arcadia, CA). Dogs were housed individually in rooms with a 12-hr light/dark cycle and a climate-controlled environment (20.5–23°C and 30–70% humidity). Purina Canine Diet 5007 and water were provided *ad libitum*. In all cases, tissues for DNA analysis were obtained 24 hr after the last dose of FIAU or at the end of the reversibility phase and stored at −70°C until DNA isolation was performed. Hepatic nuclei were isolated and stored as described for rats.

Monkeys. Six female rhesus monkeys, 2.5 kg, were administered FIAU at either 0 or 25 mg/kg per day by the nasogastric route once a day for 30 days. Monkeys were housed individually in rooms with a 12-hr light/dark cycle and a climate-controlled (20.5–23°C and 30–70% humidity) environment and were fed Purina Primate Chow 5048. Animals were euthanized using Succumb, and tissues were immediately frozen and stored at −70°C until DNA isolation was performed.

DNA Isolation and Hydrolysis. DNA was isolated from either liver nuclei or whole-liver tissue by using the A.S.A.P. Genomic DNA Isolation Kit (Boehringer Mannheim). DNA was dissolved in 200 μl of TE (10 mM Tris/1 mM EDTA) and quantitated by UV absorbance at 260 nm. In addition, A_{260}/A_{280} ratios were used to evaluate DNA purity.

Because the FIAU polyclonal antibody did not react with FIAU in intact DNA (Table 1), it was necessary to hydrolyze the DNA enzymatically prior to RIA analysis. One hundred microliters of sample (4–40 μg of DNA) was enzymatically hydrolyzed with 10 units of DNase I from bovine pancreas (Sigma) in 10 mM Tris/10 mM $MgCl_2$ for 2 hr at 37°C. Subsequently, 10 μl of 1 M Tris·HCl, 3 units of bacterial alkaline phosphatase (Sigma), and 0.5 unit of snake venom phosphodiesterase (Worthington) were added, and hydrolysis was allowed to proceed at 37°C overnight. After hydrolysis, 20–30 μl of hydrolysate was removed and saved for evaluation of nucleoside content. The remaining hydrolysate was used for FIAU quantification by competitive RIA.

3′-Hydrolysis of FIAU-DNA. To determine if FIAU was incorporated primarily at the 3′ end of DNA, FIAU-DNA was sequentially digested from the 3′ terminus by using snake venom phosphodiesterase. Specifically, a solution of FIAU containing DNA (200 μg/ml) from low-dose rats (255 mg/kg per day) was denatured by heating to 95°C for 5 min and immediately placed on ice. To the solution was added 14.3 units of bacterial alkaline phosphatase (Sigma), 10 units of snake venom phosphodiesterase (Worthington), 100 μl of 1 M Tris (pH 8.3), and 100 μl of 10 mM Tris/10 mM $MgCl_2$. The sample was incubated at 37°C, and aliquots were removed at 0 time, 5 min, 10 min, 20 min, 40 min, 2 hr, 3 hr, and 6 hr. The aliquots were analyzed by HPLC (described below) as measured by the thymidine concentration and by the release of FIAU. (As shown in Table 1, FIAU is not recognized by the RIA polyclonal antibody when incorporated into intact DNA.)

Table 1. Comparison of FIAU RIA results from enzymatically hydrolyzed and nonhydrolyzed DNA

Sample	FIAU in DNA,* pmol	
	Nonhydrolyzed DNA	Hydrolyzed DNA
Dog DNA (50 μg)†	0	80.8
Rat DNA (4 μg)‡	0	92.9

*Since thymidine concentrations could not be determined for nonhydrolyzed DNA, the amount of FIAU is the amount present in the listed μg of DNA.
†Dog DNA was obtained from a dog dosed orally with 3 mg of FIAU per kg per day for 90 days.
‡Rat DNA was obtained from a rat dosed orally with 510 mg of FIAU per kg per day for 70 days.

HPLC Analysis. To standardize the amount of FIAU in DNA and the extent of enzymatic hydrolysis, the concentration of thymidine was quantified by using a 1090M Hewlett–Packard HPLC system equipped with a diode array detector. HPLC conditions were as follows: an RP-18-S reversed-phase HPLC column (5-μm particle size; 4 mm × 25 cm; Supelco), an isocratic gradient buffer of 50 mM potassium phosphate, pH 6.5/10% (vol/vol) MeOH; a flow rate of 1.5 ml/min and a UV monitor at 260 nm. A standard curve for quantification of thymidine (elution time of ≈6 min) was constructed by using the linear regression program in the JMP software (SAS Institute, Cary, NC).

RIA for FIAU. After enzymatic hydrolysis, FIAU was measured by a sensitive and specific RIA method with minor modifications (13). Briefly, each binding reaction consisted of 400 μl of radioiodinated FIAU (25 pg), 50 μl of hydrolysate sample, and 50 μl of rabbit anti-FIAU antiserum (diluted 1:225). The binding reaction mixture was mixed and incubated for 18–24 hr at 4°C. The bound and free forms of radiolabeled FIAU were separated by adding 100 μl of cold 1% (wt/vol) bovine gamma globulin, followed by 1 ml of cold 20% polyethylene glycol. After decanting the supernate, the radioactivity in the precipitate was measured in a γ counter. A VAX computer was used to analyze the RIA data by a weighted four-parameter logistic algorithm (13). The FIAU concentration in test samples was estimated from a standard curve for concentrations from 0.1 to 3500 ng/ml.

Since the RIA was developed for quantification of FIAU in serum and urine, it was necessary to validate the assay for use with hydrolysates of DNA. Therefore, 20 aliquots of 40 μg each of DNA were analyzed to determine the extent of nonspecific inhibition of binding to the antibody caused by the DNA hydrolysate. The average inhibition plus 2 SDs was then used to determine the lower limit of quantification for measuring FIAU in DNA. To assess effects of the DNA hydrolysate on the shape of the RIA standard curve, aliquots of enzymatically hydrolyzed calf thymus DNA (40 μg) were spiked with known quantities of FIAU ranging from 1.5 to 1100 pmol. The recorded FIAU concentrations from the RIA

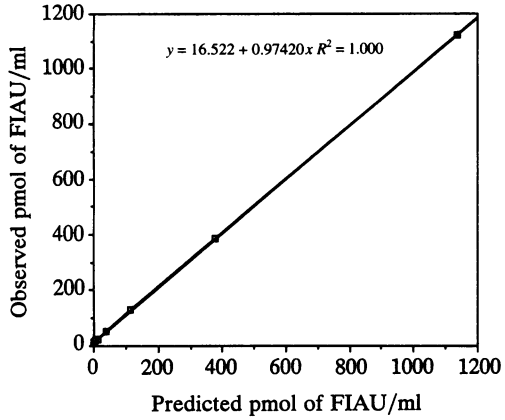

FIG. 2. Comparison between pmol of FIAU per ml added to 40 μg of enzymatically digested DNA and pmol of FIAU per ml observed in the FIAU RIA.

were then regressed against the actual amount of FIAU added in the assay by linear regression analysis with the JMP software. The slope and the intercept were used to determine the effects of the DNA hydrolysate on the performance of the RIA.

Final Values. The extent of FIAU incorporation was listed as pmol of FIAU per μmol of thymidine.

RESULTS

The RIA was originally developed for analysis of FIAU in serum and urine samples. Table 1 shows that binding of polyclonal antibody to the tracer was not inhibited by nonhydrolyzed DNA containing FIAU but was inhibited by FIAU-DNA following enzymatic hydrolysis. Since the DNA was isolated by the A.S.A.P. Genomic Isolation Kit, one can also conclude that the isolation technique effectively removed any FIAU not associated with DNA.

Analysis of 40 μg of calf thymus or control dog DNA showed that the DNA hydrolysate resulted in nonspecific inhibition equivalent to 14 ± 2 pmol/ml of FIAU. Because thymidine cross-reacts 0.02% as well with the antibody as does FIAU (13), the nonspecific inhibition of binding to the antibody produced by hydrolyzed DNA is probably due to thymidine. This is supported, first, by the fact that nonhydrolyzed DNA and enzymes plus buffers gave lower nonspecific inhibition by factors of 6–10, and, second, by previous studies showing that the ED_{50} for thymidine was 0.25 mM (13). This concentration is similar to the thymidine concentration generated by enzymatic digestion of 40 μg of DNA in 150 μl (0.22 mM). Fig. 2 plots the correlation between the amount of FIAU in the RIA based on the matrix previously developed (13) and the amount of FIAU actually placed in the DNA hydrolysate. The slope of ≈1 (0.97) as well as the goodness of the linear fit (R^2 = 1.0) showed that the DNA hydrolysate caused little or no alteration in the shape or slope of the RIA response. In addition, the intercept of 16.5 pmol of FIAU per ml was extremely close to the average of the 20 samples used to determine nonspecific inhibition. Therefore, a simple subtraction of 14 pmol of FIAU per ml was sufficient to correct for the effects of the DNA hydrolysate matrix. Samples with FIAU concentrations in the RIA of less than the background plus 2 standard deviations (a total of 18 pmol of FIAU per ml) were considered to be zero in the assay. Since the amount of DNA used in this assay was ≈40 μg/145 μl, or ≈0.22 μmol of thymidine per ml, the limit of detection for the assay was ≈82 pmol of FIAU/μmol of thymidine. Some treated rat livers contained FIAU-DNA concentrations that were off the linear scale for the RIA; therefore, a 1:10 dilution of the enzyme hydrolysate was performed. This dilution lowered the nonspecific binding, since the equivalent of 4 μg of DNA was analyzed. The nonspecific inhibition was calculated to be 1.1 ± 0.5 ng/ml and was subtracted from RIA results. The limit of quantification for 4 μg of DNA hydrolysate was 2.15 ng/ml.

In dogs, high levels of FIAU had accumulated in hepatic DNA during chronic exposure (Tables 2 and 3). The FIAU-DNA concentration was dose-dependent but not dose-proportional, with a 3-fold increase in dose resulting in an ≈2-fold increase in FIAU-DNA concentration. The FIAU-DNA concentration was also sex-dependent with concentrations in males ≈80% of that in females (Table 2). Removal of FIAU treatment for 35 days resulted in a diminution of FIAU-DNA concentrations of 41–61% (Table 2). The fact that this comparison was between whole-liver DNA and nuclear DNA is not a complicating factor, since additional studies in dogs (data not shown) and rats (see below) have shown little difference between FIAU-DNA concentrations in whole-liver DNA and nuclear DNA. FIAU was also present in DNA of quadriceps femoris (Table 3). Although only one measurement from each sex was made, the concentrations were comparable, and both were lower than in hepatic DNA by a factor of 5–6.

FIAU accumulated to an average level of 606 pmol/μmol of thymidine in hepatic DNA from two female monkeys exposed to 25 mg of FIAU per kg per day for 30 days, compared with three controls that showed zero. A separate analysis gave similar results, with a third monkey having a value of 468 pmol of FIAU per μmol of thymidine (data not shown).

In rats, FIAU was found in DNA obtained from all tissues examined (Table 4). Liver had the highest concentration of FIAU-DNA, with levels as high as 11,000 pmol of FIAU per μmol of thymidine or 1 FIAU molecule per 90 thymidine molecules. In contrast to dogs, FIAU-DNA concentrations in rat liver DNA were consistently 2-fold greater in males than in females. Concentrations in DNA from isolated hepatic nuclei were essentially the same as concentrations in DNA isolated from whole liver. After liver, the next highest FIAU-DNA concentrations were in jejunum and spleen followed by heart and sperm.

Table 2. FIAU-DNA concentrations in livers of dogs given FIAU orally for 90 days

	FIAU, pmol/μmol of thymidine (±SEM)			
Sex	0 mg/kg*	1 mg/kg*	2 mg/kg*	3 mg/kg*
F	0	1315 ± 171 (4)†	2261 ± 635 (3)†	2845 ± 230 (4)‡
F (rev)	0			1739 (1)†
M	0	1106 ± 184 (4)†	1511 ± 268 (3)†	2276 ± 80 (4)‡
M (rev)	0			941 (2)†

The number of dogs is shown in parentheses. F, female; M, male; F (rev) and M (rev), dogs removed from FIAU for 5 weeks prior to tissue collection.
*Daily dose of FIAU.
†DNA from whole liver.
‡DNA from nuclei.

Table 3. FIAU-DNA concentrations in skeletal muscle of dogs given FIAU orally for 90 days

Sex	FIAU, pmol/μmol of thymidine (±SEM)	
	0 mg/kg*	3 mg/kg*
M	0	550
F	0	487

One dog of each sex was used. M, male; F, female.
*Daily dose of FIAU.

Hydrolysis of FIAU-DNA from the 3′-end demonstrated continuous release of FIAU until all DNA had been digested (Table 5). In addition, preliminary analysis of DNA for fragmentation using electrophoretic separation (as would be expected if FIAU were located at the terminal ends) revealed no difference in DNA from FIAU-treated and untreated rats (data not shown). These results strongly suggest that FIAU is located in internal sites in the DNA.

DISCUSSION

Studies on the incorporation of FIAU into DNA *in vivo* have been limited to acute experiments in mice utilizing intraperitoneal injections of [^{14}C]FIAC (9) and [^{14}C]FIAU (10). Results presented in this manuscript also show that during long-term exposure, FIAU is incorporated into DNA and demonstrate that FIAU accumulates to high levels in DNA of rats, dogs, and monkeys. In rats, FIAU accumulated to the highest levels in the liver, the major organ of FIAU toxicity previously observed in the clinical trials. This was unexpected because high rates of nucleoside incorporation would be anticipated in tissues with high rates of DNA synthesis including spleen and jejunum as was indicated in studies with [^{14}C]FIAC in the mouse (9). The mechanism for the preferential accumulation in the liver remains unclear. Preliminary measurements in the spleen and liver of rats treated for 70 days with FIAU showed no difference in free FIAU concentrations between these two tissues (data not shown), suggesting that accumulation of the free drug is not a factor. Increases in this synthesis of DNA in liver compared with spleen, caused by chronic treatment or by differences in oral versus intraperitoneal administration or by both, could be responsible for the accumulation of FIAU-DNA in liver. Additional experiments designed to address this specific issue will be necessary to clarify the mechanism. Whatever the mechanism, these data support by example the position that nucleoside analogues may accumulate in DNA during long-term administration (14).

Table 4. FIAU-DNA concentrations in tissues from rats given FIAU orally for 70 days

Tissue	Sex	FIAU, pmol/μmol of thymidine (±SEM)		
		0 mg/kg*	255 mg/kg*	510 mg/kg*
Liver	M	0	5308 ± 336	11,138 ± 1073
	F	0	1680 ± 264	5,828 ± 476
Liver nuclei	M			10,101 (2)
	F			5,017 (2)
Jejunum	M	0	3943 ± 47	7,168 ± 197
Spleen	M	0	2235 ± 234	4,055 ± 323
Sperm	M	0	2398 ± 232	3,604 ± 172
Heart	M	0	1618 ± 67	2,311 ± 84

The average value ± SEM is shown when three animals were used. The number of rats other than three is shown in parentheses. F, female; M, male.
*This total daily dose of FIAU was divided into three doses to be administered each day.

Table 5. Enzymatic hydrolysis of FIAU-DNA

Time, min	FIAU,* pmol/μmol dT per ml of sample	Ratio pmol FIAU/μmol dT†
0	0/0.004	≈0
5	8.9/0.056	≈157
10	20.7/0.079	≈261
20	21/0.086	≈243
40	91.1/0.093	≈976
120	242/0.089	≈2719
180	303/0.098	≈3108
360	298/0.088	≈3397

*Final values of FIAU accounting for nonspecific inhibition of binding to RIA antibody caused by the DNA matrix (see Fig. 2).
†The ratio was computed from JMP (SAS Institute) prior to rounding of data in column 2.

In addition to demonstrating differences in tissue accumulation of FIAU-DNA, these studies also demonstrated species and gender differences in the accumulation of FIAU-DNA. Specifically, female dogs had FIAU-DNA concentrations in the liver that were 20% higher than in males. In contrast, FIAU-DNA concentrations in livers were 2-fold greater in male rats as compared with female rats (Table 4). The gender difference in dogs was most likely due to the ≈20% higher concentrations of FIAU in serum of female dogs (data not shown) as compared with male dogs. The underlying mechanism for the gender difference in rats is unknown.

Accumulation of FIAU in dog DNA was reversible as demonstrated by the 41–61% decrease in FIAU-DNA concentrations observed in dogs removed from treatment for 35 days. However, the results indicate that FIAU is not rapidly removed from genomic DNA, suggesting that removal does not occur via an active repair mechanism. FIAU-DNA concentrations could be reduced through hepatocyte turnover, which has been reported to range from 0.3–1.5% per day in rats and mice (15–17). Data on hepatocyte turnover in dogs is not available.

FIAU-DNA concentrations in dogs given a total dose of 90 mg of FIAU per kg over 90 days (1 mg/kg per day) were 2-fold higher than in monkeys given at total dose of 750 mg of FIAU per kg over 30 days (25 mg/kg per day). Differences in absorption or distribution did not appear to cause this difference, since maximum blood levels were 4-fold higher in monkeys than in dogs dosed 3 mg/kg per day (data not shown). The higher FIAU-DNA concentration in dogs compared with monkeys may be due in part to the longer exposure of dogs (90 days of treatment) compared with monkeys (30 days of treatment). Theoretically, duration of exposure can become a factor in determining the extent of accumulation of FIAU in DNA because FIAU incorporation is dependent on DNA synthesis, and the overall amount of DNA synthesis in a tissue is time dependent. Species variations in intracellular accumulation and/or phosphorylation may provide an additional explanation for these differences.

While it is clear that FIAU can accumulate to high concentrations in DNA, it is less clear what effect this incorporation has on DNA and cellular structure and function. Nucleoside analogue incorporation into DNA has been correlated with the cytotoxicity of 9-D-arabinofuranosyl-2-fluoroadenine (11) and 1-D-arabinofuranosylcytosine (12). Duplex structures containing monofluoronucleotides increase duplex stability and change conformation (18). Gemcitabine (a 2′-difluoropyrimidine nucleoside) can be incorporated into DNA (19–21). Incorporation of gemcitabine in the nascent strand and the template strand causes pausing in DNA synthesis (21, 22). Moreover DNA incorporation of gemcitabine correlates with cytotoxicity as determined by loss of clonogenicity in human lymphoblastoid T cells (22).

Chain terminators such as AZT and ddC are clastogenic (23) and, during continuous exposure, reduce mtDNA content—a possible mechanism by which they induce delayed myopathies (24, 25). FIAU is also a clastogen in genetic toxicology tests (J.A.E., M. L. Garriott, D. E. F. Kindig, and L. S. Schwier, unpublished data) and has been proposed to induce delayed toxicity that may be mediated through a direct effect on mtDNA. This issue remains to be resolved, as results in HepG2 cells have failed to reveal a reduction in mtDNA during FIAU treatment (26), while preliminary results from this laboratory indicate mtDNA depletion does occur *in vivo*.

Unlike AZT and ddC, which terminate DNA synthesis, the current investigation strongly suggests that FIAU accumulates into internal locations in the DNA. Whether FIAU accumulates in mtDNA remains to be determined; however, the high genomic content of FIAU reported here suggests that genomic DNA should be considered a target of FIAU toxicity. Despite these findings, it should be noted that the three species examined showed varying signs of systemic toxicity but no clinical signs of liver toxicity even at doses up to 1000× those used in the clinical trials (6). In addition, although increased apoptosis and dose-related nuclear atypia were observed in rat liver, no histologic changes were observed in livers of dogs and monkeys (J.A.E., unpublished data), despite relatively high concentrations of FIAU-DNA, suggesting that DNA incorporation may be a necessary step in FIAU toxicity but not a sufficient one.

An examination of the possible accumulation of nucleoside analogues following chronic exposure in animals had not been a part of the previous toxicological evaluation. This has been due in part to the lack of adequate technology to make such studies feasible. The development of an RIA for FIAU has allowed such a study to be conducted. These studies have shown that during long-term oral treatment, FIAU accumulates in DNA of treated animals and raises the possibility that other nucleoside analogues may do the same.

We thank Dave Oakes, John Scheuring, Janice Fogg, Jeffrey A. Kirkwood, and Debbie Horn for their expert technical assistance and Dr. Joseph Colacino for his critical review of this manuscript.

1. Fried, M. W., DiBisceglie, A. M., Straus, S. E., Savarese, B., Beames, M. P., Hoofnagle, J. H. (1992) *Hepatology* **16**, 127A (abstr.).
2. Fourel, I., Hantz, L. C., Allaudeen, H. S. & Trepo, C. (1987) *Antiviral Res.* **8**, 189–199.
3. Hantz, O., Allaudeen, H. S., Ooka, T., DeClercq, E. & Trepo, C. (1984) *Antiviral Res.* **4**, 187–199.
4. Staschke, K. A., Colacino, J. M., Mabry, T. E. & Jones, C. D. (1994) *Antiviral Res.* **23**, 45–61.
5. Kroba, B. E. & Gerin, J. L. (1992) *Antiviral Res.* **19**, 55–70.
6. Macilwain, C. (1993) *Nature (London)* **364**, 275.
7. Lewis, L. D., Hamzeh, F. M. & Lietman, P. S. (1992) *Antimicrob. Agents Chemother.* **36**, 2061–2065.
8. Lewis, W., Gonzales, B., Chomyn, A. & Papoian, T. (1992) *J. Clin. Invest.* **89**, 1354–1360.
9. Grant, A. J., Feinberg, A., Chou, T.-C., Watanabe, K. A., Fox, J. J. & Philips, F. S. (1982) *Biochem. Pharmacol.* **31**, 1103–1108.
10. Chen, M. S., Van Nostrand, M. & Oshana, S. C. (1986) *Anal. Biochem.* **156**, 300–304.
11. Huang, P., Chubb, S. & Plunkett, W. (1990) *J. Biol. Chem.* **265**, 16617–16625.
12. Kufe, D. W., Major, P. P., Egan, E. & Beardsley, G. P. (1980) *J. Biol. Chem.* **255**, 8997–9000.
13. Bowsher, R. R., Compton, J. A., Kirkwood, J. A., Place, G. D., Jones, C. D., Mabry, T. E., Hyslop, D. L., Hatcher, B. L. & DeSante, K. A. (1994) *Antimicrob. Agents Chemother.* **38**, 2134–2142.
14. Oberg, B. & Johansson, N. G. (1984) *J. Antimicrob. Chemother.* **14**, Suppl. A, 5–26.
15. Eacho, P. I., Lanier, T. L. & Brodhecker, C. A. (1991) *Carcinogenesis* **12**, 1557–1561.
16. Richardson, F. C., Copple, D. M. & Eacho, P. I. (1992) *Carcinogenesis* **13**, 2453–2457.
17. Eldridge, S. R., Goldsworthy, T. L., Popp, J. A. & Butterworth, B. E. (1992) *Carcinogenesis* **13**, 409–415.
18. Bergstrom, D. E. & Swartling, D. J. (1988) in *Fluorine-Containing Molecules: Structure, Reactivity, Synthesis and Applications*, eds. Liebman, J. F., Greenberg, A. & Dolbier, W. R. (VCH, New York).
19. Ruiz van Haperen, V. W. T., Veerman, G., Vermorken, J. B. & Peters, G. J. (1993) *Biochem. Pharmacol.* **46**, 762–766.
20. Cuddy, D. P. & Ross, D. D. (1993) *Proc. Am. Assoc. Cancer Res.* **34**, 2490 (abstr.).
21. Schy, W. E., Hertel, L. W., Kroin, J. S., Bloom, L. B., Goodman, M. F. & Richardson, F. C. (1993) *Cancer Res.* **53**, 4582–4587.
22. Huang, P., Chubb, S., Hertel, L. W., Grindey, G. B. & Plunkett, W. (1991) *Cancer Res.* **51**, 6110–6117.
23. Phillips, M. D., Nascimbeni, B., Tice, R. R. & Shelby, M. D. (1991) *Environ. Mol. Mutagen.* **18**, 168–183.
24. Arnaudo, E., Dalakas, M., Shanske, S., Moraes, C. T., DiMauro, S. & Schon, E. A. (1991) *Lancet* **337**, 508–510.
25. Chen, C.-H. & Cheng, Y.-C. (1989) *J. Biol. Chem.* **264**, 11934–11937.
26. Colacino, J. M., Malcolm, S. K. & Jaskunas, S. R. (1994) *Antiviral Res.* **23**, Suppl. 1, 24 (abstr.).