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Review article

Mechanisms for the anti-hepatitis B virus activity and mitochondrial toxicity of fialuridine (FIAU)[1]

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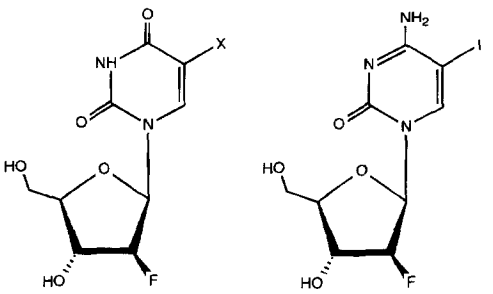
Abstract

Fialuridine (FIAU) is a thymidine nucleoside analog with activity against various herpesviruses and hepatitis B virus (HBV) in vitro and in vivo. In a clinical evaluation for its use as a treatment for chronic HBV infection, long term oral administration of FIAU resulted in severe multi-organ toxicity characterized by a delayed onset and refractory lactic acidosis. These clinical manifestations led to the hypothesis that the toxicity of FIAU was mediated through mitochondrial dysfunction, possibly as a result of the inhibition of mitochondrial DNA polymerase γ and/or incorporation of FIAU into mitochondrial DNA. In addition to describing the anti-HBV activity of FIAU, this review discusses results from in vitro experiments carried out by various laboratories in an effort to evaluate and understand more fully the mitochondrial toxicity of FIAU.

Keywords: FIAU; Fialuridine; Mitochondrial toxicity; Hepatitis B

1. The 2'-fluoroarabinosylpyrimidine nucleoside analogs, FIAU and FIAC

The structures for FIAU (fialuridine; 1-(2-deoxy-2-fluoro-1-β-D-arabinofuranosyl-5-iodo)uracil) and FIAC (1-(2-deoxy-2-fluoro-1-β-D-arabinofuranosyl-5-iodo)cytosine) are shown in Fig. 1. These compounds were first developed for their activity against herpes viruses (Lopez et al., 1980; Colacino and Lopez, 1983; Lin et al., 1983; Schat



FIAU: X = I
FMAU: X = CH_3
FAU: X = H

FIAC

Fig. 1. Chemical structures for 1-(2-deoxy-2-fluoro-1-β-D-arabinofuranosyl)pyrimidine nucleoside analogs.

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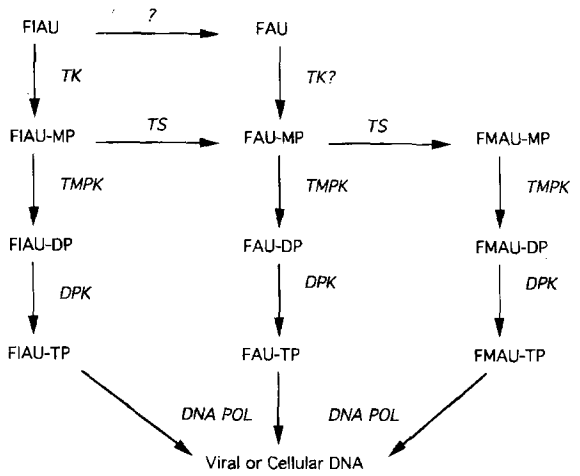

Fig. 2. Metabolic pathways for FIAU. FIAU is phosphorylated by thymidine kinase to the 5'-monophosphate. The 5'-triphosphate of FIAU can serve as an alternate substrate in place of TTP for the support of DNA synthesis. MP, DP and TP indicate 5'-mono-, 5'-di- and 5'-triphosphates, respectively. TK, thymidine kinase; TS, thymidylate synthase; TMPK, thymidylate kinase; DPK, nucleoside diphosphate kinase; DNA pol, DNA polymerase. (The figure is adapted from Schinazi et al., 1986 and Klecker et al., 1994).

et al., 1984; Schinazi et al., 1986) and more recently were found to have potent activity against hepatitis B viruses in vitro (Korba and Gerin, 1992; Staschke et al., 1994) and in the duck (Fourel et al., 1992) and woodchuck (Fourel et al., 1990; B. C. Tennant, personal communication) models of hepatitis B virus infection.

The 2'-fluoroarabinofuranosyl pyrimidine nucleoside analogs are phosphorylated preferentially by thymidine kinase encoded by herpes simplex viruses (Kreis et al., 1982; Colacino and Lopez, 1983; Chou et al., 1987) but can be phosphorylated by pyrimidine nucleoside kinases of cellular origin (Cheng et al., 1981; Schat et al., 1984; Staschke et al., 1994; Klecker et al., 1994; Cui et al., 1995). In the metabolic pathways shown in Fig. 2 (adapted from Schinazi et al., 1986 and Klecker et al., 1994), FIAU is phosphorylated by thymidine kinase. Earlier work demonstrated that thymidylate synthase can catalyze the deiodination and methylation of 5-iodo-deoxyuridylate (Garrett et al., 1979). Similarly, in cell free experiments, thymidylate synthase catalyzed the de-iodination of FIAU-MP to FAU-MP and the methylation of FAU-MP to FMAU-MP (Braun et al., 1982). Consistent with these earlier results, recently FAU was found to incorporate into the DNA of whole cells not as FAU but, rather, as FMAU (Klecker et al., 1994). The monophosphates of FIAU, FMAU and FAU are further phosphorylated to di- and tri-phosphates. The triphosphates of FIAU and FMAU, and possibly to a slight extent, FAU, are recognized by cellular and/or viral DNA polymerases and can be incorporated, to a greater or lesser extent, into cellular and/or viral DNA. Although FIAU can be chemically deiodinated to form FAU, it is not a substrate for deiodination by thymidylate synthase (Braun et al., 1982) and it is not known which enzyme, if any, is responsible for the bioconversion of FIAU to FAU (Schinazi et al., 1986). Interestingly, FAU itself has only marginal anti-HBV activity (Staschke et al., 1994) and is relatively non-cytotoxic in HepG2 cells (Cui et al., 1995).

2. The in vitro anti-hepatitis B virus activity of FIAU

It has been observed that the extent of phosphorylation and the potency of anti-herpesvirus activity of 2'-fluoroarabinosyl nucleoside analogs is, in part, cell-specific (Schat et al., 1984). Similarly, the in vitro anti-HBV activity of FIAU is determined, at least in part, by the host cell, and is correlated to the extent of its intracellular phosphorylation over time (Staschke et al., 1994). In cell culture systems, phosphorylated FIAU accumulated in human hepatoblastoma cells but not in chicken liver cells incubated in radiolabeled FIAU over a 72-h period. Using Southern blot analysis of intracellular HBV replicative intermediates, FIAU displayed relatively little antiviral activity ($EC_{50} = 156\ \mu M$) in chicken liver cells that constitutively replicate duck hepatitis B virus. In contrast, FIAU was quite potent ($EC_{50} = 0.075\ \mu M$) in human hepatoblastoma cells that replicate the same virus (Staschke et al., 1994). Along these lines, the requirement for the phosphorylation of the nucleoside analog, 3'-fluoro-2',3'-

dideoxythymidine (FLT), for anti-duck hepatitis B virus activity in vivo has been described (Löfgren et al., 1990).

The 5'-triphosphate of FIAU is an inhibitor directly of DHBV endogenous DNA polymerase with an IC_{50} of 0.038 μM in the presence of 0.2 μM TTP (Schilke et al., 1994). Similarly, Hantz et al. (1984) showed that the 5'-triphosphate of FIAC inhibited human HBV DNA polymerase with an IC_{50} of 0.05 μM. Of interest, in terms of FIAU metabolism, is our recent observation that the 5'-triphosphate of FAU, at a concentration of up to 100 μM in the presence of 0.2 μM TTP, does not inhibit DHBV endogenous DNA polymerase (A. J. Baxter and J. M. Colacino, unpublished observations).

Hepatitis B viruses use a unique mechanism for reverse transcription of the viral RNA pregenome in which the reverse transcriptase acts as a protein primer for viral DNA synthesis (Wang and Seeger, 1992; Wang and Seeger, 1993). DHBV reverse transcriptase which is transcribed and translated in vitro becomes covalently linked to a primer containing four nucleotides with the wildtype sequence GTAA. In a reaction in which the concentration of TTP was 10 μM, FIAU-TP inhibited the incorporation of TMP into the protein primer in a dose-dependent fashion with an IC_{50} of approximately 0.66 μM but did not inhibit the covalent attachment of dGMP to the reverse transcriptase (Staschke and Colacino, 1994). Using a construct that is transcribed and translated in vitro to yield the DHBV reverse transcriptase and a primer for DHBV DNA synthesis that has the sequence GTAC rather than GTAA, 100 μM FIAU-TP did not interfere with the covalent attachment of dGMP to the reverse transcriptase or with the incorporation of dAMP into the DNA primer. It did abrogate the incorporation of TMP and markedly decreased the incorporation of dCMP, now taking the place of the second A in the wildtype sequence. These data are consistent with the incorporation of FIAU into the DHBV DNA primer as an analog of thymidine. The incorporation of the following nucleotide is allowed since FIAU has an intact 3'-OH. However, the incorporation of the subsequent nucleotide is inhibited or slowed down markedly. In this way, at least for DHBV DNA priming, FIAU acts as a 'de facto' chain terminator similar to what has been described for DHPG (9-(1,3-dihydroxy-2-propoxymethyl)guanine), an acyclo analog of guanosine that also contains a 3'-OH group (Reardon, 1989).

3. The in vivo anti-hepatitis B virus activity of FIAU

As did other 2'-fluoropyrimidine nucleoside analogs (Fourel et al., 1990), FIAU displayed potent antiviral activity in the woodchuck model of HBV infection (B. C. Tennant, personal communication). In one experiment designed to evaluate the antiviral efficacy of FIAU, 18 woodchucks were infected at 3 days of age with woodchuck hepatitis virus (WHV) and all of the animals became chronic WHV carriers. Treatment, consisting of once per day intraperitoneal injections, was initiated when the animals were 9 months of age. Six animals received placebo, 6 received 0.3 mg FIAU per kg per day for 28 days, and 6 received 1.5 mg FIAU per kg per day for 28 days. At 1.5 mg per kg per day, FIAU displayed a potent anti-WHV effect, as demonstrated by a nearly 100% reduction in plasma WHV DNA levels. Within this 28 day study and at these dosages, there was no evidence of toxicity. However, in a recent experiment designed to evaluate further the in vivo toxicity of FIAU, toxicities similar to those observed in a clinical trial (see below) were evident in woodchucks treated orally for 11–12 weeks with FIAU at a dosage of 1.5 mg per kg per day (B. C. Tennant, personal communication).

4. Mitochondrial dysfunction hypothesis

FIAU was evaluated clinically for its potential as an anti-HBV drug. Oral administration of FIAU resulted in a dramatic decrease in the level of HBV DNA in patients with chronic hepatitis B virus infection (Fried et al., 1992). However, long term oral administration of FIAU was accompanied by severe toxicity characterized by multi-or-

gan involvement including muscle, nerve, the liver and the pancreas. Additionally, microvesicular fat infiltration of the liver, and severe and refractory lactic acidosis were evident. Importantly, there was a delayed onset of these toxicities and, tragically, five people died (Stevenson et al., 1995; McKenzie et al., 1995). These clinical events combined with the proposed mechanism of AZT-induced myopathy (Gertner et al., 1989; Dalakas et al., 1990; Arnaudo et al., 1991; Lamperth et al., 1991; Lewis et al., 1991; Lewis et al., 1992; Lewis et al., 1994a; Mhiri et al., 1991) and 2',3'-dideoxycytidine (ddC)-induced peripheral neuropathy (Dubinsky et al., 1989) led to the working hypothesis that the multi-organ toxicity of FIAU was mediated by mitochondrial dysfunction, possibly through inhibition of DNA polymerase γ, the polymerase which is responsible for the replication of mitochondrial DNA.

5. Effect of FIAU on mitochondrial DNA replication and function in cultured cells

To address the hypothesis that FIAU clinical toxicity was mediated through mitochondrial dysfunction, the effect of FIAU on mitochondrial DNA replication and function in cell culture was studied (Colacino et al., 1994; Cui et al., 1995). Initially, we looked at the cytotoxicity of FIAU in CEM cells that are derived from human T cells. After 6 days of incubation, the IC_{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 that constitutively produce HBV, after 6 days of incubation, FIAU displayed a IC_{50} of 34.9 $\pm$ 4.9 μM while ddC displayed a IC_{50} of 34.3 $\pm$ 4.0 μM. Therefore, FIAU and ddC were equivalently toxic to these cells also. By comparison, in HepG2 cells after 6 and 14 days of incubation, FIAU displayed considerably lower IC_{50}'s of 2.1 μM and 1.4 μM, respectively, as reported by others (Cui et al., 1995).

The effect of cytotoxic concentrations of ddC and FIAU on the replication of mitochondrial DNA (mtDNA) in CEM cells was determined using slot blot analysis to quantify DNA that hybridizes to a riboprobe specific for mtDNA sequences (Colacino et al., 1994). Since ddC is a selective inhibitor of mtDNA replication in cultured cells (Chen and Cheng, 1989; Chen and Cheng, 1992), the effect of this nucleoside analog on mtDNA replication was evaluated for comparative purposes. The inhibitory effect of ddC on mtDNA replication could be detected after 3 days of incubation and became more pronounced after 6 and 9 days of incubation. The concentration of ddC required to inhibit mtDNA replication by 50% was approximately 0.1 μM. In contrast, no inhibitory effect of FIAU (up to 20 μM) on the replication of mtDNA in CEM cells was detected under these conditions. Also, FIAU did not cause a concentration-dependent decrease in the amount of mtDNA as determined by Southern blot analysis of DNA from CEM cells incubated in up to 2.5 μM FIAU for 15 days (Colacino et al., 1994). When DNA from these cells was digested with *Bam*H1, a restriction endonuclease that cuts once within the 16.5 kbp mitochondrial DNA genome, a single 16.5-kbp band that hybridized to the mtDNA specific probe was readily observed by Southern blot analysis. No major 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. FIAU had no effect on the abundance of mtDNA in human hepatoblastoma cells at concentrations well above the recently published IC_{50}'s for this compound in these cells (Colacino et al., 1994; Cui et al., 1995). Additionally, as the case for CEM cells, no major deletions of mtDNA were detected by Southern blot analysis of DNA digested with *Bam*HI (Colacino et al., 1994). In contrast, ddC inhibited mtDNA replication in these cells with an IC_{50} of approximately 0.2 μM (Colacino et al., 1994).

The inability of FIAU to inhibit mtDNA replication did not necessarily preclude an inhibitory effect on mitochondrial function that would result in an adaptive cellular response characterized by the stimulation of anaerobic glycolysis leading to an increase in lactic acid production (Stryer, 1988). Therefore lactic acid levels in cell supernatant media were determined in an effort to evaluate mitochondrial function in cells incubated in FIAU. In HepG2 cells that replicate human HBV,

FIAU increased lactic acid levels in cell culture supernatant in a concentration-dependent manner. After 6 days of incubation, levels of lactate were increased by 100%, 250% and 500% with 10 μM, 100 μM and 200 μM FIAU, respectively (Fig. 3). Similar results were obtained using HepG2/G3 cells which constitutively replicate duck hepatitis B virus, HepG2 cells, or CEM cells (Colacino et al., 1994). Similarly, Cui et al. (1995) have demonstrated that lactic acid production was increased by approximately 100% in HepG2 cells incubated for 4 days in 10 μM FIAU. Under these conditions, there was no appreciable change in the abundance of mitochondrial DNA. In contrast, treatment of U-937 cells with up to 5 μM FIAU or MOLT-4 cells with up to 20 μM FIAU for 12 days did not result in the increased production of lactic acid, whereas treatment with ddC did, leading to the conclusion that lactate production in this system would not have predicted the clinical outcome for FIAU (Klecker et al., 1994).

6. Incorporation of FIAU into total cellular DNA

The toxicity profile of a particular nucleoside analog is determined by the metabolism of that

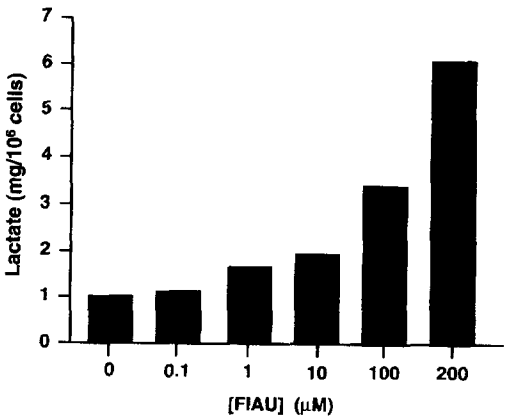

Fig. 3. Effect of FIAU on lactate production by human hepatoblastoma cells which replicate human hepatitis B virus. Cells were incubated for 6 days in medium containing the indicated concentration of FIAU. The medium was changed daily and on day 6, aliquots were clarified of cells by centrifugation and the amount of lactate in the supernatant was determined.

nucleoside analog and the extent to which it is incorporated into nuclear DNA, mtDNA, or both (reviewed in Flint, 1994). Specifically, the cytotoxicity of 9-β-D-arabinofuranosyl-2-fluoroadenine (Huang et al., 1990) or 1-β-D-arabinofuranosylcytosine (Kufe et al., 1980) was correlated directly with the incorporation of these nucleoside analogs into DNA. Previous studies (Grant et al., 1982; Chen et al., 1986; Richardson et al., 1994; Klecker et al., 1994, Cui et al., 1995) have demonstrated that FIAU is able to incorporate into total cellular DNA. Furthermore, a linear relationship between the amount of FIAU incorporated into U-937 or MOLT-4 cellular DNA at 24 h and cytotoxicity at 72 h was established (Klecker et al., 1994). DNA from the same HepG2 cells used in the Southern blot experiments discussed above was examined for the presence of FIAU using a sensitive and specific radioimmunoassay developed recently (Bowsher et al., 1994). Total cellular DNA from cells incubated in FIAU was hydrolyzed to completion with DNase and the hydrolysate was treated with phosphatase as previously described (Richardson et al., 1994). Released FIAU was detected by radioimmunoassay using a specific antiserum raised in rabbits immunized with a hemisuccinate analog of FIAU coupled to KLH (Bowsher et al., 1994). HepG2 cells, HepG2/G3 cells and HepG2 cells that replicate human HBV were treated for 6 days in up to 200 μM FIAU and total cellular DNA was isolated and prepared for radioimmunoassay of FIAU content. In each case, the amount of FIAU was normalized to total thymidine content. FIAU was incorporated into DNA in a dose-related fashion between 0.1 and 100 μM with a drop off at 200 μM, probably due to toxicity (Fig. 4). For HepG2 cells, the incorporation of FIAU at 100 μM was equivalent to approximately 1 FIAU moiety for every 25 thymidines. In these experiments, FIAU was concluded to be the predominant metabolite since the specificity of the radioimmunoassay is such that FAU and FMAU are cross-reactive at a level of only 0.15% and 0.12%, respectively, with antiserum raised against FIAU (Bowsher et al., 1994).

In U-937 and MOLT-4 cells incubated for 24 h in 10 μM radiolabeled ^{14}C-FIAU, incorporation

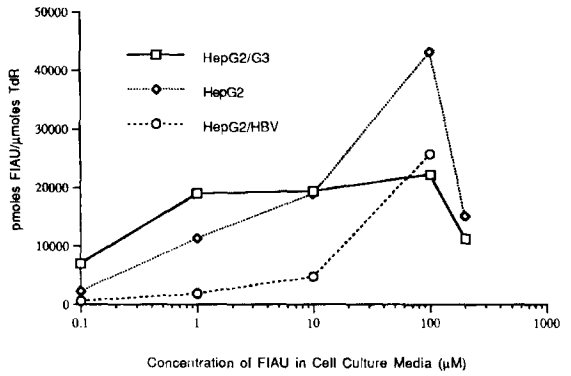

Fig. 4. Incorporation of FIAU into DNA in human hepatoblastoma cells. Human hepatoblastoma cells (HepG2), HepG2 cells which replicate duck hepatitis B virus (HepG2/G3), and HepG2 cells which replicate human hepatitis B virus (HepG2/HBV) were incubated for 6 days in medium containing the indicated concentration of FIAU. The cells were harvested by trypsinization, total cellular DNA was extracted and purified, and FIAU content was quantified using radioimmunoassay as previously described (Bowsher et al., 1994). The amount of FIAU was normalized to the total amount of thymidine in each sample.

of FIAU into total cellular DNA was 2.1% and 0.9% the level of thymidine, respectively, and the majority of the radioactivity in DNA was identified by HPLC as FIAU while 0.4% of the radioactivity was identified as FMAU (Klecker et al., 1994). Importantly, there was no evidence for the selective repair of DNA after incorporation of FIAU (Klecker et al., 1994). Additionally, results from in vivo studies demonstrated that FIAU was not rapidly removed from DNA isolated from treated dogs indicating that removal is not mediated by an active repair mechanism in this system (Richardson et al., 1994).

7. Mode of incorporation of FIAC into DNA

Using the method of nearest neighbor base sequence analysis (Jossee et al., 1961; Müller et al., 1975), the mode of incorporation of FIAC, the aminated metabolic precursor of FIAU (Chou et al., 1981), into cellular DNA was studied (Chou et al., 1983). Briefly, when DNA is digested with micrococcal nuclease and spleen phosphodiesterase, internal nucleotides are released as 3'-monophosphates while terminal residues are released as free nucleosides. The products of the enzymatic digest can be separated by polyethyleneimine (PEI) thin layer chromatography (TLC). In a control experiment, Vero cells (African green monkey kidney cells) infected with herpes simplex virus type 1 were incubated with radiolabeled thymidine. Digestion with both enzymes followed by PEI-TLC showed that most of the radioactivity was associated with TMP while a significantly lesser amount was associated with free thymidine, as expected since thymidine is not a chain terminator. When DNA from cells incubated in radiolabeled FIAC was digested with micrococcal nuclease and spleen phosphodiesterase followed by PEI-TLC, the results were similar to those obtained with radiolabeled thymidine (Fig. 5). This indicates that FIAC (and presumably FIAU) does not act as a chain terminator. It was similarly concluded that FIAU is not a chain terminator based on the high levels of dThd replacement by FIAU in DNA (Klecker et al., 1994). Also, when DNA containing FIAU was isolated from treated rats and subjected to hydrolysis from the 3'-end there was a continuous release of FIAU until digestion was complete suggesting that FIAU was located at internal sites in the DNA (Richardson et al., 1994). Further experiments are necessary to determine the number of nucleotides that can be incorporated after the insertion of FIAU. It has not been determined rigorously whether FIAU can act as a 'de facto' chain terminator of DNA in whole cells, as was shown for the cell-free priming of duck hepatitis B virus DNA synthesis (Staschke and Colacino, 1994).

8. Interaction of the 5'-triphosphate of FIAU with mammalian DNA polymerases

The interaction of the 5'-triphosphate of FIAU with mammalian DNA polymerases was investigated (Lewis et al., 1994b). A 5'-^{32}P-labeled 17-mer oligonucleotide was hybridized to the phagemid DNA template and extended with DNA polymerase α, β, γ, δ, or ϵ. When FIAUTP was used in place of TTP for the support of in

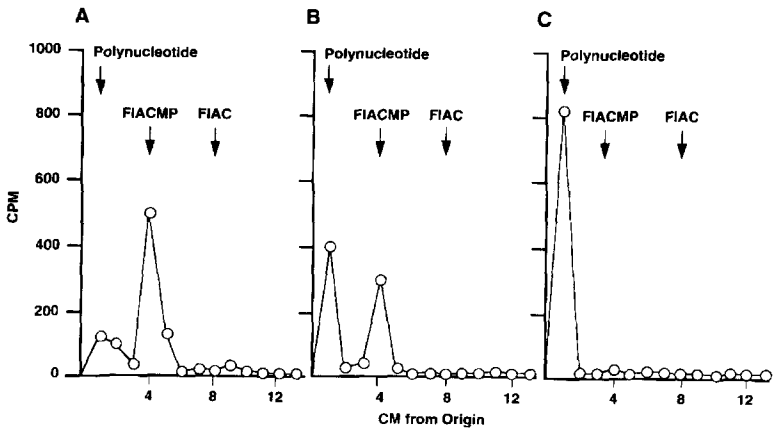

Fig. 5. Mode of incorporation of FIAC into total cellular DNA. DNA was extracted from Vero cells infected with HSV-1 and incubated in radiolabeled FIAC and digested with (A) micrococcal nuclease and spleen phosphodiesterase, (B) spleen phosphodiesterase, or (C) no enzyme (mock digest control). The enzymatic digests were subjected to polyethyleneimine thin layer chromatography to separate radiolabeled 3'-monophosphorylated from free nucleoside. Polynucleotide (calf thymus DNA), FIAC monophosphate and FIAC markers were visualized under ultraviolet illumination. CPM, counts per minute.

vitro DNA synthesis, all DNA polymerases generated products of 24 nucleotides or greater, indicating that FIAUTP can support in vitro DNA synthesis as an alternate substrate in place of TTP. Of the five DNA polymerases, mitochondrial DNA polymerase γ most efficiently catalyzed the accumulation of FIAUMP into DNA and the oligonucleotide products generated were indistinguishable from those generated in the presence of 20 μM TTP. In contrast, in the presence of 20 μM FIAUTP, oligonucleotide products formed by each of the other DNA polymerases were reduced in length compared to those generated by the respective DNA polymerase using 20 μM TTP.

As demonstrated by Lineweaver-Burke analysis, FIAUTP inhibited competitively the incorporation of radiolabeled TTP into DNA by DNA polymerase γ of bovine origin (Lewis et al., 1994b). The Ki of 0.04 μM for DNA polymerase γ was the lowest Ki among the mammalian DNA polymerases, although the KmTTP/KiFIAUTP ratio for DNA polymerase γ was lower than that for DNA polymerases α and ϵ, and approximately equal to that of DNA polymerases β and δ (Table 1). Although the mtDNA polymerase used in these studies was of bovine origin and results may be different with the human derived enzyme, these findings are instructive. The competition between FIAUTP and TTP along with the relative ease of incorporation of FIAUMP into a DNA template by DNA polymerase γ in vitro may relate to the potential of FIAU for mitochondrial toxicity. Recently, the inhibition of DNA polymerases β and γ by various nucleotide analogs was investigated (Cherrington et al., 1994). In this study (Table 2), FIAUTP had a Ki of 0.031 μM for DNA polymerase γ and 1.43 μM for DNA polymerase β. Similar to FIAUTP, ddCTP displayed Ki's of 1.32 μM and 0.034 μM for DNA polymerases β and γ, respectively. In contrast, the diphosphate of HPMPC, a nucleotide analog which is undergoing clinical evaluation for the treatment of CMV retinitis (Jaffe, 1994), had large Ki's for DNA polymerases β and γ, 520 μM and 299 μM, respectively.

The high Ki of HPMPCpp for DNA polymerase γ is encouraging for the predicted lack of mitochondrial toxicity of this compound whereas the relatively low Ki's of ddCTP and FIAUTP for DNA polymerase γ would anticipate toxicity mediated through mitochondrial dysfunction. However, the results from experiments in which interactions of nucleoside triphosphate analogs with isolated and purified DNA polymerases are examined must be interpreted cautiously since

Table 1
Enzyme kinetic analysis of inhibition of mammalian DNA polymerases by the 5'-triphosphate of FIAU[a]

Enzyme	Tissue source	Km for TTP (μM)	Ki for FIAUTP (μM)
DNA pol α	Calf thymus	4.5	0.23
DNA pol β	Novikoff hepatoma	8.0	0.75
DNA pol γ	Bovine liver	0.5	0.04
DNA pol δ	Calf thymus	27	2.9
DNA pol ϵ	Human myeloblast	4.4	0.24

[a]Adapted from Lewis et al. (1994b).

inhibition of mammalian mitochondrial DNA polymerase by nucleoside triphosphate analogs is not always directly correlated with toxicity in humans. As an example, the triphosphate of the anti-HIV and anti-HBV compound, (-)-2'-deoxy-3'-thiacytidine (3TC) inhibits mammalian DNA polymerase γ competitively with respect to dCTP and has a relatively low Ki for the enzyme (Hart et al., 1992). However, in a phase I/II study of 3TC given orally to HIV-positive patients, no dose limiting toxicity was observed, even after 1 year of therapy (van Leeuwen et al., 1995). Additionally, 3TC had no substantial effect on lactic acid production and did not alter mitochondrial morphology in HepG2 cells (Cui et al., 1995).

Recently, in one study of 16 nucleoside triphosphate analogs, no clear quantitative or qualitative correlation between inhibition of DNA polymerases (especially DNA polymerase γ) and the inhibition of mtDNA synthesis in cultured Molt-4 cells could be established (Martin et al., 1994). As an additional example, the 5'-triphosphate of 5-chloro-2',3'-dideoxy-3'-fluorouridine (935U83) was found to be a potent inhibitor of DNA polymerase γ even though 935U83 itself did not induce abnormalities in neurophysiology or neuropathology in monkeys dosed for 30 days or 6 months with up to 700 mg of compound per kg per day (Daluge et al., 1994). Also, 935U83 did not inhibit specifically mtDNA replication in CEM cells (Daluge et al., 1994). It would be of interest to determine the effect of 935U83 on lactate levels in cultured CEM or HepG2 cells since this was not addressed in the above study.

9. Incorporation of FIAU into mitochondrial DNA

The abilities of FIAU to incorporate into total cell DNA and of FIAUTP to serve as an alternate substrate for in vitro DNA synthesis by DNA polymerase γ have been discussed. Therefore, it was of interest to determine whether FIAU is incorporated into mtDNA in cultured cells (Cui et al., 1995; J. M. Colacino, J. W. Horn, D. M. Horn, and F. C. Richardson, submitted for publication). In our studies, HepG2 cells were incubated for 6 days in the absence of FIAU or in 10 μM or 50 μM FIAU after which time they were harvested and divided into nuclear and mitochondrial fractions. Nuclear DNA was obtained using ASAP DNA column chromatography (Boehringer Mannheim Biochemicals, Indianapolis) and mtDNA was isolated and purified using alkaline lysis (Tamura and Aotsuka, 1988). The DNA in each fraction was hydrolyzed with DNAse and treated with phosphatase and the FIAU content was determined using RIA. When cells were incubated for 6 days in 10 μM FIAU, incorporation occurred at a level of 15 881 pmoles FIAU per μmole thymidine in nuclear DNA and 468 pmoles FIAU per μmole thymidine in mtDNA. At 50 μM FIAU, incorporation occurred at a level of 25 750 pmoles FIAU per μmole thymidine in nuclear DNA and 590 pmoles FIAU per μmole thymidine in mtDNA. Thus, in cultured cells over a 6 day incubation, FIAU was incorporated into mtDNA although incorporation was greater into nuclear DNA. Another group reported that in HepG2 cells incubated for 4 days in 2 μM radiolabeled ^{14}C-FIAU, incorporation into nuclear and mtDNA occurred at a

Table 2
Inhibition of human DNA polymerases β and γ: kinetic constants[a]

	Km (μM)		Ki (μM)			
DNA pol	dCTP	TTP	HPMPCpp	ddCTP	AZTTP	FIAUTP
Beta	4.3	4.7	520	1.32	1.4	1.43
Gamma	0.21	0.54	299	0.034	18.3	0.031

[a]Adapted from Cherrington et al. (1994).

level of 90 pmoles FIAU/μg DNA and 9.6 pmoles FIAU/μg DNA, respectively, and that 94% of the radioactivity in DNA co-eluted with the authentic FIAU standard as determined by reverse phase HPLC (Cui et al., 1995).

These experimental results support the following mechanism which may explain, in part, the mitochondrial toxicity of FIAU. FIAU, having an intact 3'-OH and a relatively low Ki for DNA polymerases (in particular DNA polymerase γ) is incorporated into nuclear DNA and, importantly, into mtDNA. It may not be recognized as an aberrant nucleotide and in the absence of a mitochondrial postreplication repair system for the excision of internally incorporated nucleotide analog (Lewis and Dalakas, 1995), replication of mtDNA may continue for a time without an apparent decrease in abundance. The presence of FIAU in mtDNA may lead to alterations in mitochondrial gene expression. In contrast, nucleotide analogs lacking an intact 3'-OH, such as ddCTP, but which have Ki's similar in magnitude to that of FIAU for DNA polymerase γ (Cherrington et al., 1994), act as obligate chain terminators when incorporated into mtDNA. In this case, the DNA is not elongated and there is a more immediate decrease in mtDNA abundance. This scenario has been discussed by others (Parker and Cheng, 1994; Medina et al., 1994; Lewis and Dalakas, 1995).

10. Implications of FIAU incorporation into DNA

The ability of FIAU to incorporate into cellular DNA prompted us to consider the implications of such incorporation. We used a model system in which oligonucleotides representing the TPA response element (TRE) that binds specifically to activator protein 1 (AP-1) (Angel et al., 1987; Lee et al., 1987) were synthesized with FIAU in place of thymidines at critical positions in the AP-1 binding sequence. Binding of AP-1 to normal oligonucleotides or to oligonucleotides containing FIAU was determined by gel-shift analysis (K. A. Staschke, A. J. Baxter, J. C. Scheuring, T. E. Mabry, and J. M. Colacino, manuscript in preparation). Briefly, the normal, non-substituted oligonucleotide was end-labeled with ^{32}P and incubated with nuclear extracts derived from HeLa cells and the extent of its binding to AP-1 was set to 100%. When thymidine at position -3, -1, 1 or 7 (relative to the center of dyad symmetry of the AP-1 binding sequence) was replaced with FIAU, binding to AP-1 was approximately 82%, 28%, 86% and 51%, respectively, of the binding of the non-substituted oligonucleotide to AP-1. When thymidine at position 3 or 5 (each directly adjacent to the center of dyad symmetry of the AP-1 binding sequence) was replaced with FIAU, binding to AP-1 was abrogated. Thus, when FIAU replaces thymidine at positions within the sequence critical for binding to AP-1, binding is dramatically reduced. These results indicate that incorporation of FIAU into DNA may induce local conformational changes in DNA, as shown for duplex DNA-containing monofluoronucleotides (Bergstrom and Swartling, 1988) or may disallow the contact of key amino acid residues of the AP-1 transcription factor with the methyl group of thymidine since it is replaced by iodine in FIAU. The importance of methyl groups in thymidine for binding of the TRE with AP-1 has been demonstrated by replacing crucial thymidines with deoxyuridine and showing that binding of TRE to Fos/Jun (components of AP-1) is

strongly reduced or even abolished (Risse et al., 1989). In any case, these results indicate the potential for FIAU to interfere with DNA replication and/or normal gene expression by inhibiting DNA-protein interactions which are important for gene expression although the relevance of the results of these cell free experiments to the effects of FIAU in whole cells will require further investigation.

11. Effect of FIAU in dog hepatocytes

In toxicological studies, the treatment of beagle dogs with FIAU at 1, 2 or 4 mg per kg per day by capsule for 3 months resulted in the incorporation of FIAU into hepatocyte DNA, a decrease in mtDNA abundance, and a decrease in mitochondrial cytochrome C oxidase activity (J. A. Engelhardt, F. C. Richardson, P. Eacho, unpublished observations). In an effort to find an in vitro model to study the dog liver effects, the following experiment with FIAU was conducted (P. Eacho, P. Foxworthy, and J. Lawrence, personal communication). Dog hepatocytes were isolated from the dog liver by collagenase perfusion and the cells were plated on collagen-coated plastic and incubated in L-15 medium containing 5% fetal bovine serum and 0, 0.6, 6 or 60 μM FIAU for 3, 7 or 14 days. No evidence of FIAU cytotoxicity (at least as indicated by leakage of lactate dehydrogenase from hepatocytes) was observed. Furthermore, there were no changes attributable to FIAU in cytochrome C oxidase activity, a marker of mitochondrial function. (However, cytochrome C oxidase activity in control cells decreased considerably between days 3 and 14 of the experiment.) Likewise, there was no decrease attributable to FIAU in citrate synthase activity, a marker of nuclear gene function. Similar to the experiments using cultured HepG2 cells, FIAU was incorporated into dog hepatocyte DNA after 3, 7 and 14 days' exposure as determined by radioimmunoassay for the presence of FIAU (Fig. 6). Incorporation tended to increase as a function of time and concentration of FIAU, although the relationship was lost at 60 μM and 14 days.

Although FIAU was incorporated into DNA, the expected decrease in cytochrome C oxidase activity was not observed in cultured dog hepatocytes. Thus, under the conditions used above, the dog hepatocytes did not model predictively the effects of FIAU in dogs. The possibility remains that the duration of exposure in dog hepatocytes in vitro was not sufficiently long.

12. Effect of FIAU on cytochrome oxidase expression in cultured human hepatoblastoma cells

The effect of FIAU on cytochrome C oxidase activity in HepG2 cells was also examined. The cells (6 X 10^6 cells per 150 cm^2 flask) were incubated for 6 days in up to 200 μM FIAU. Northern blot analysis demonstrated that steady state levels of cytochrome C oxidase RNA from cells incubated in FIAU were not decreased as compared to controls (Fig. 7). In these cells within this time frame, treatment with FIAU did not result in a dose-dependent decrease in cytochrome C oxidase activity whereas at 200 μM FIAU, lactate levels were approximately 500% of the control levels. These results indicate that FIAU has an effect on mitochondrial function that is not corre-

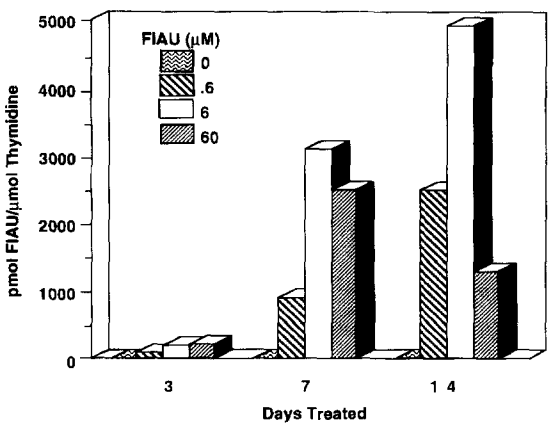

Fig. 6. Incorporation of FIAU into dog hepatocyte DNA. Dog hepatocytes were obtained by collagenase perfusion, plated onto collage coated plastic, and incubated for 3, 7 or 14 days in L15 medium containing the indicated concentration of FIAU. Total cellular DNA was obtained and FIAU content was quantified as described in the legend to Fig. 4.

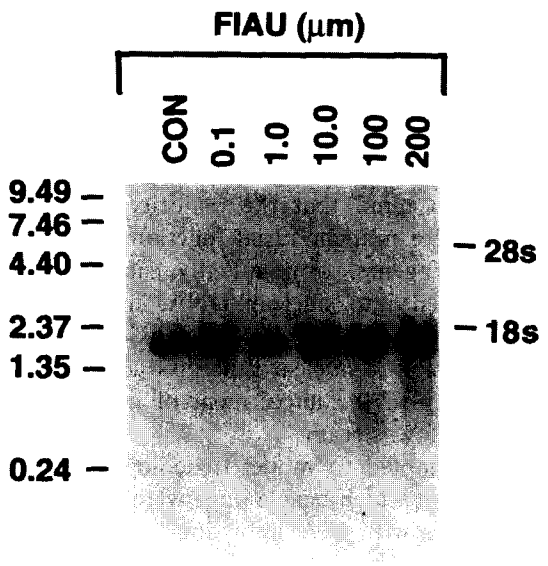

Fig. 7. Northern blot analysis of steady state levels of cytochrome oxidase RNA. HepG2 cells were incubated for 6 days in medium containing the indicated concentration of FIAU. Total RNA was prepared by the guanidinium isothiocyanate lysis procedure (Chirgwin et al., 1979) and 20 μg were electrophoresed through a formaldehyde-agarose gel, blotted to nitrocellulose and hybridized to a DNA probe specific for mitochondrial cytochrome oxidase sequences using standard procedures.

lated to mtDNA levels or to cytochrome C oxidase activity, at least in cell culture under these conditions.

13. Ultrastructural studies

As elegantly shown by Medina et al. (1994), an important part of the evaluation of mitochondrial toxicity in vitro is an examination of mitochondrial morphology at the ultrastructural level. Therefore, the effect of FIAU, and for comparative purposes ddC, on mitochondrial structure using electron microscopy (EM) was investigated (Cui et al., 1995; J. M. Colacino, J. W. Horn, D. M. Horn, and F. C. Richardson, submitted for publication). In our studies, HepG2 cells were incubated for 6 days on Millicell-CM filters (Millipore Products Division, Bedford, MA) in media containing 0, 10, 100 or 200 μM FIAU or ddC. Cells incubated for 6 days in 10 μM, 100 μM or 200 μM ddC displayed altered mitochondrial structure including swelling of the mitochondrion, distortion of the inner cristae and matrix dissolution. The ddC-induced distortion of mitochondrial cristae was similar to that seen by others (Medina et al., 1994). When cells were incubated for 6 days in 10 μM FIAU, distortions of the cristae were apparent. These effects were more pronounced at 100 μM and 200 μM FIAU and included marked mitochondrial swelling with cristae loss and matrix dissolution. Recently, it has been shown by EM that mitochondria from HepG2 cells incubated for 4 days in 10 μM FIAU displayed a higher cristae density rather than matrix dissolution and that FIAU caused micro- and macrovesicular steatosis (Cui et al., 1995). The reasons for the differences between the two studies are not known. Recently, EM analyses of liver tissues from patients chronically infected with HBV and treated orally with FIAU revealed distortion of inner mitochondrial cristae and matrix dissolution rather than compaction of the inner cristae (Stevenson et al., 1995). Nonetheless, FIAU clearly induces alterations in mitochondrial morphology at the ultrastructural level which are apparent after 6 days of cell culture. These changes may be correlated directly to impairment of mitochondrial function.

14. Conclusion

FIAU showed promise as a treatment for chronic hepatitis B virus infection but the development of this compound was discontinued because of unanticipated and severe clinical toxicity which occurred following oral administration of 0.1 and 0.25 mg of FIAU per kg of body weight per day for more than 2 months (McKenzie et al., 1995). A newly developed radioimmunoassay for the sensitive and specific detection of FIAU has permitted a detailed assessment of the pharmacokinetics of this compound (Bowsher et al., 1994). When a single 5 mg dose of FIAU was given orally to 16 normal, healthy volunteers, a mean Cmax of 238 ng/ml (0.64 μM) was reached 0.5 h after administration and at 25 h, serum

concentrations of FIAU were approximately 6 ng/ml (0.02 μM). Because of the high sensitivity of the radioimmunoassay, FIAU displayed a prolonged elimination phase with a mean half-life ($t_{1/2}\beta$) of 29.3 hours. In this pharmacokinetic analysis, serum concentrations of FIAU were considerably lower than concentrations of FIAU (up to 200 μM) which induced cytotoxicity, increased lactic acid production, and altered mitochondrial morphology in cell culture. The clinical toxicity of FIAU, or any drug for that matter, is a function of its pharmacokinetics, i.e. absorption accumulation, half-life, elimination, compartmentalization into tissue and cellular components, and incorporation into nucleic acids (especially in the case of nucleoside analogs). These factors are difficult to model completely in cell culture experiments and thus it is difficult to extrapolate findings from in vitro experiments to the clinical situation. With these considerations in mind, the mechanism of the delayed toxicity of FIAU has been hypothesized to be mediated through mitochondrial dysfunction and in vitro experiments were conducted in an effort to understand more fully the mitochondrial toxicity of FIAU.

In various in vitro experiments carried out by independent groups, FIAU did not cause a concentration-dependent decrease in mtDNA abundance but did cause an increase in lactate levels in cultured cells (Colacino et al., 1994; Cui et al., 1995). This indicates that any deleterious effect of FIAU on the function of mitochondria in cultured cells may not be correlated directly to absolute levels of mtDNA. However, as the experiments discussed in this review demonstrate, the 5'-triphosphate of FIAU is used efficiently as an alternate substrate for TTP and is able to support in vitro DNA synthesis by DNA polymerase γ. Consistent with this is the observation discussed here and reported previously by others (Cui et al., 1995) that FIAU can become incorporated into mtDNA within whole cells. Since FIAU contains a 3'-OH, and in light of experimental data, the incorporation of FIAU into DNA may not lead to an obligatory decrease in mtDNA abundance but may affect nuclear and/or mitochondrial gene expression resulting in decreased mitochondrial function. Indeed, our experiments using an oligonucleotide substituted with FIAU, in place of thymidines important for binding to the transcription factor, AP-1, demonstrate the potential for the incorporation of FIAU leading to a disruption of normal protein-DNA interactions crucial for gene expression. It should be kept in mind that the majority of proteins necessary for mitochondrial function are encoded by nuclear genes. Consequently, since FIAU is incorporated into nuclear DNA, mitochondrial toxicity may be the result of cytotoxicity in which the expression of nuclear gene product(s) is inhibited leading to the impairment of mitochondrial function 'downstream.'

We attempted to model toxicological findings of studies in which beagle dogs were dosed orally for 90 days with FIAU. In dog hepatocytes obtained by collagenase perfusion, FIAU (up to 60 μM) did not cause a concentration dependent decrease in either cytochrome oxidase or citrate synthase function over a 14-day incubation period. Similarly, in HepG2 cells over a 6 day incubation, FIAU did not cause a decrease in steady state levels of cytochrome oxidase RNA or in cytochrome oxidase activity. With the exception of FIAU incorporation into hepatocyte DNA, these relatively short in vitro experiments did not model appropriately long term in vivo results and thus are not adequate for predicting mitochondrial toxicity induced by FIAU. Longer duration in vitro experiments as well as the functional evaluation of additional mitochondrial genes are necessary.

As discussed here and reported by others (Cui et al., 1995), electron microscopic analysis of HepG2 cells incubated for 6 days in FIAU demonstrated unambiguously that mitochondrial structure was altered providing a correlation with compromised mitochondrial function. These findings underscore the importance of an ultrastructural analysis for evaluating the effects of a candidate antiviral compound on mitochondrial structure. Such effects might not be considered especially in short term cell culture studies in which the abundance of mtDNA is unchanged or where mitochondrial function is not noticeably affected.

Experiments designed to study the effect of nucleoside and nucleotide analogs on mtDNA replication, lactate production in vitro, mitochondrial gene function, mitochondrial morphology at the ultrastructural level, and isolated and purified DNA polymerases α, β, δ, γ and ϵ, are important for a complete understanding of the toxicity of these agents. These studies combined with long term efficacy and safety studies, utilizing detection assays for nucleoside analogs which are highly sensitive and specific will help to minimize the risk involved in the clinical evaluation of nucleoside analogs as antiviral agents.

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