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Short communication

Fialuridine is phosphorylated and inhibits DNA synthesis in isolated rat hepatic mitochondria

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Abstract

Fialuridine (FIAU) is a thymidine analog effective against hepatitis B virus. Toxicity associated with FIAU treatment included clinical signs consistent with mitochondrial dysfunction, including severe lactic acidosis. To understand further the mechanism of FIAU toxicity, we examined the effect of FIAU on DNA synthesis in mitochondria. Mitochondria isolated from livers of naive rats were treated in vitro with concentrations of FIAU or FIAU triphosphate (FIAU-TP) ranging from 0.1 to 200 μM. A 14 or 32% decrease in mitochondrial DNA synthesis compared to controls was observed when isolated mitochondria were treated with 25 μM FIAU or FIAU-TP, respectively. Since it is thought that nucleosides must be phosphorylated to inhibit DNA polymerase, studies were conducted to determine whether isolated rat mitochondria could phosphorylate FIAU. Results using lanthanum chloride precipitation and HPLC analysis showed that enzymes present in a mitochondrial lysate were capable of phosphorylating FIAU to form FIAU monophosphate. © 1997 Elsevier Science B.V.

Keywords: FIAU; Phosphorylation; Mitochondria; DNA synthesis

Fialuridine [FIAU; 1-(2′-deoxy-2′-fluoro-1-β-D-arabinofuranosyl)-5-iodouracil] is a thymidine analog evaluated in clinical trials for use in the treatment of chronic hepatitis B infection. Clinical trials were halted due to severe toxicity which included clinical signs consistent with mitochondrial dysfunction, including severe lactic acidosis (McKenzie et al., 1995). It has been hypothesized that FIAU toxicity was due to FIAU incorpora-

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tion into mitochondrial DNA (mtDNA) (Parker and Cheng, 1994; Lewis et al., 1994). In addition, incorporation of FIAU into mtDNA of HepG2 cells has been demonstrated (Cui et al., 1995; Colacino et al., 1996).

In order to further understand the mechanism of FIAU toxicity, hepatic mitochondria were studied in vitro to determine whether FIAU could inhibit mtDNA synthesis. Briefly, naive F344 rats (Taconic Farms; Germantown, NY) were euthanized by decapitation following an overnight fast, and mitochondria were isolated using differential centrifugation as previously described (Johnson and Lardy, 1967; Hoke et al., 1989). DNA synthesis in isolated mitochondria was determined by measuring the incorporation of [^{3}H]thymidine or [^{3}H]deoxyadenosine triphosphate into TCA precipitable material after a 90 min incubation following the addition of all components necessary for mtDNA synthesis, including mitochondrial energy requirements and DNA synthesis components as described (Banki and Anders, 1989). Controls were isolated from the same animal and received identical treatment as treated mitochondria but were not exposed to FIAU or FIAU-TP.

Initial studies in isolated mitochondria revealed a marked inhibition in mtDNA synthesis by FIAU and FIAU-TP (FIAU-TP) when [^{3}H]thymidine was used as the indicator of DNA synthesis (Fig. 1). Further studies by this laboratory showed that this decrease was due largely to competition between [^{3}H]thymidine and FIAU or FIAU-TP for uptake into the mitochondria (data not shown). This competition has been observed previously with other thymidine analogs such as AZT (Simpson et al., 1989). In order to avoid competition issues, studies were modified to use [^{3}H]dATP in place of [^{3}H]thymidine as the indicator of DNA synthesis as previously described (Simpson et al., 1989).

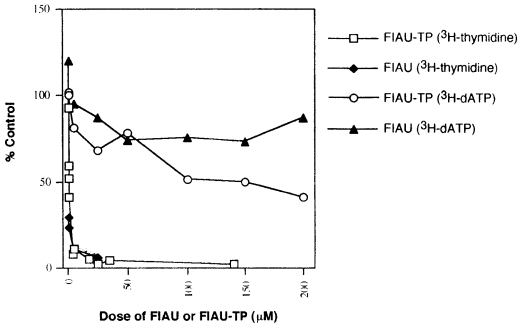

Fig. 1. DNA synthesis in mitochondria isolated from naive rats using [^{3}H]thymidine or [^{3}H]dATP incorporation as the indicator of synthesis.

Results of exposure of isolated rat mitochondria to FIAU and FIAU triphosphate for 90 min using [^{3}H]dATP showed a decrease in mtDNA synthesis compared to controls (Fig. 1). FIAU-TP significantly reduced mtDNA synthesis at 100, 150 and 200 μM, while FIAU significantly reduced mtDNA synthesis at 50, 100, and 150 μM. (Statistical analysis used Dunnett's test $P < 0.05$). The reason that a decrease at 200 μM FIAU was not observed was not clear. Since it has been suggested that nucleosides must be phosphorylated to inhibit DNA polymerases (Martin et al., 1994), our results, demonstrating an inhibition of DNA synthesis by FIAU, suggest that mitochondria can phosphorylate FIAU. Therefore, studies were conducted to determine whether, in fact, mitochondria isolated from rat liver could phosphorylate FIAU.

Following isolation of mitochondria as described above, the final mitochondrial pellet was resuspended in 300 μl dH_2O and lysed using 300 μl freshly prepared lyse buffer (2% Triton X-100, 10 mM NaF, 1.5 mM $MgCl_2$, 10 mM Tris, pH 7.6). Incubation buffer (50 mM Tris (pH 7.5), 5 mM ATP, 5 mM $MgCl_2$, 5 mM NaF, 1 mM 2-mercaptoethanol) was added to the lysate to give approximately 0.8 mg protein/ml (Wolcott and Colacino, 1989). Lysate was then dispensed into sterile tubes containing radiolabeled FIAU, thymidine, or dideoxycytidine (ddC). Thymidine was used as a positive control to test for the presence of thymidine kinase, while dideoxycytidine was used as a negative control to test for the presence of contaminating cytosolic kinases (Chen and Cheng, 1992; Eriksson et al., 1991). Following incubation at 37°C for 1 h, triplicate samplings of phosphorylation reactions were collected. Phosphorylated compounds were isolated by lanthanum chloride precipitation as previously described (Wolcott and Colacino, 1989).

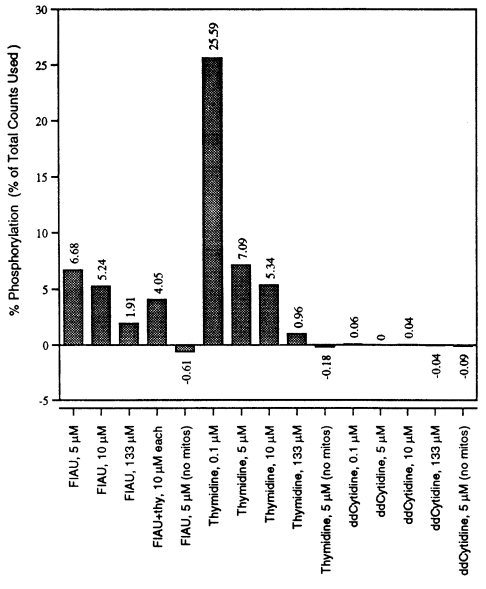

Fig. 2. $LaCl_3$ precipitation of phosphorylated products.

Identification of phosphorylated nucleotides was made by high performance liquid chromatography (HPLC) analysis and liquid scintillation counting after an overnight extraction in 60% methanol (final concentration) at $-20°C$, followed by centrifugation to remove pelleted protein as previously described (Cui et al., 1995). Internal standards contained FIAU triphosphate or thymidine triphosphate partially digested with calf intestinal phosphatase (Boehringer Mannheim, Indianapolis, IN). In addition, phosphorylation of the nucleosides was verified by enzymatic dephosphorylation prior to $LaCl_3$ precipitation.

Results showed that at identical concentrations, FIAU and thymidine were phosphorylated to nearly the same degree, while dideoxycytidine was not phosphorylated (Fig. 2). The addition of non-radioactive thymidine to the phosphorylation reaction tube containing [^{14}C]FIAU resulted in a net decrease in precipitable radioactive material, suggesting that there was competition between FIAU and thymidine for phosphorylation enzymes. The lack of ddC phosphorylation substantiated the absence of cytosolic kinases in the mitochondrial preparation. HPLC analysis showed that, over time, there was a shift in radioactivity from the FIAU peak to the FIAU monophosphate peak (Fig. 3). This shift amounted to 5% of the total reactivity, which agreed with results obtained from $LaCl_3$ precipitation experiments. There was no evidence of FIAU diphosphate or FIAU triphosphate (Fig. 3). Additional studies showed no evidence of thymidine diphosphate or thymidine triphosphate following 60 min of phosphorylation reaction time (data not shown). The reason for this is not clear, and may be due to reaction conditions which resulted in unstable di- and tri-phosphated nucleosides and/or inactive specific mitochondrial kinases. The disappearance of radioactivity precipitated by $LaCl_3$ following enzymatic dephosphorylation provided additional support that FIAU and thymidine had been phosphorylated (Fig. 4).

These studies demonstrated that mitochondria isolated from naive rat liver could phosphorylate FIAU and thymidine to form monophosphated nucleotides. These results are consistent with results using isolated TK1 and TK2 (Wang and Eriksson, 1996). However, these results are in contrast to studies where mitochondria isolated from HepG2 cells demonstrated no phosphorylation of FIAU (Cui et al., 1995). This difference

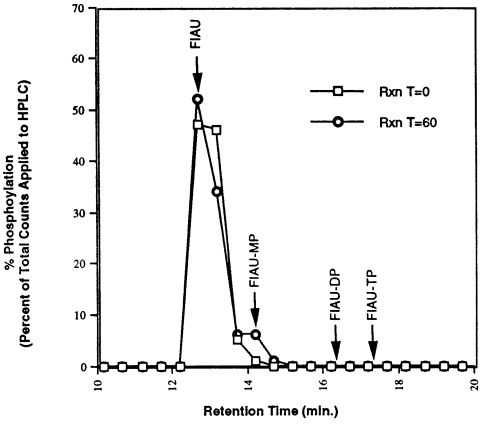

Fig. 3. HPLC analysis of 10 μM [^{14}C]FIAU phosphorylation reaction. The 1090M Hewlett-Packard HPLC system equipped with a diode array detector was set up to operate under the following conditions: a reversed-phase Supelco 5-8970, LC-18-T column (3 μm particle size; 15 cm $\times$ 4.6 mm); solvent A: H2O, solvent B: 0.1 M KPO_4/5 mM tetrabutylammoniun hydrogen sulfide, pH 6.0, (Sigma/Fluka, 39,684-2), solvent C1:70/30 ratio of solvent B/MeOH, pH 7.2; a flow rate of 1.5 ml/min and UV monitor at 260 nm.

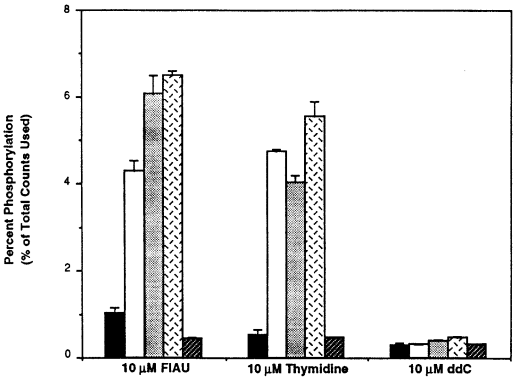

Fig. 4. Phosphorylation in mitochondrial preparation (time = 0–60 min), folllowed by enzymatic dephosphorylation (time = 60–120 min) Dephosphorylation was allowed to proceed at 37°C for 1 h, after which the samples were $LaCl_3$ precipitated. Dephosphorylation reaction mixture consisted of 10 mM Tris/10 mM $MgCl_2$, and 100 mM Tris–HCl (pH 8.3), 0.1 U of bacterial alkaline phosphatase (Sigma; St. Louis, MO), and 0.5 U of snake venom phosphodiesterase (Worthington; Freehold, NY). Filled square, phosphorylation reaction, time = 0 min; open square, phosphorylation reaction, time = 60 min; shaded square, phosphorylation reaction + dephosphorylation buffer, time = 60 min; open square with dots, phosphorylation reaction + dephosphorylation buffer, time = 120 min; closed square with dot, phosphorylation reaction followed by dephosphorylation reaction, time = 120 min.

may be due to the difference between species and/or cell lines or the fact that previous studies used intact mitochondria. The latter possibility seems unlikely since studies presented here showed FIAU inhibited mtDNA synthesis in intact mitochondria.

These in vitro studies demonstrating phosphorylation of FIAU by mitochondrial enzymes and inhibition of mitochondrial DNA synthesis by FIAU support further the theory that FIAU may exert its toxic effects through mitochondrial dysfunction, possibly through inhibition of polymerase gamma (Lewis et al., 1994) and/or incorporation of FIAU into mtDNA.

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