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Effect of 1-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)-5-ethyluracil on mitochondrial functions in HepG2 cells

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Abstract

The effects of 1-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)-5-ethyluracil (D-FEAU) on mitochondrial functions were examined in HepG2 cells. D-FEAU between 0.1 and 10 μM had no apparent inhibitory effect on cell proliferation for 2-week period; however, D-FEAU caused a decrease in mitochondrial DNA (mtDNA) content in a dose-dependent manner with an IC_{50} value of 2.7 μM. A 20.9% of increase in lactic acid production was observed after the cells were incubated with 10 μM of D-FEAU for 4 days without substantial effect being detected at 0.1 and 1 μM. In addition, no significant changes on mitochondrial morphology were observed in the cells treated with 10 μM of D-FEAU for 14 days under the electron microscope.

Keywords: D-FEAU; Mitochondrial function; Hepatitis B; Hepatotoxicity

1. Introduction

D-FEAU, along with a series of fluoroarabinofuranosyl 5-substituted pyrimidine nucleosides such as 1-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)-5-iodocytosine (D-FIAC), 1-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)-5-iodouracil (D-FIAU) and 1-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)-5-methyluracil (D-FMAU), was shown to be highly active against several herpes group viruses both in vitro and in vivo (Mansuri et al., 1987; Soike et al., 1990; Trousdale et al., 1992). The greatest advantage for D-FEAU, however, comes to its much lower toxicity toward host cells compared with the other fluorinated nucleosides, which is partly due to its most selective inhibitory effect on viral DNA synthesis while allowing cellular DNA synthesis to continue (Chou et al., 1987; Kong et al., 1988). More importantly, recent studies have demonstrated that D-FEAU could induce an immediate, and sustained inhibition on woodchuck hepatitis virus replication in a woodchuck animal model without apparent toxic effects (Fourel et al., 1990), a fact that warrants further development of D-FEAU as a possible anti-HBV agent.

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The fatal liver toxicity associated with long term administration of D-FIAU (Touchette, 1993; Dusheiko, 1994) has further stressed the necessity for preclinically evaluating the effect of D-FEAU, which is a close analogue of D-FIAU, on host cell mitochondrial functions as mitochondrial damage was well indicated in D-FIAU-induced toxicity (Parker and Cheng, 1994). Our previous investigations using a human hepatoblastoma HepG2 cell line have demonstrated that D-FIAU and its in vivo metabolite D-FMAU incorporate into mtDNA of cells and lead to a marked mitochondrial dysfunction as evidenced by disturbance in cellular energy metabolism and detection of micro- and macrovesicular steatosis (Cui et al., 1995). The HepG2 cell line retains many characteristics of hepatocytes and is an adequate in vitro model to examine the drug's interaction with liver functions (Darlington et al., 1987; Sassa et al., 1987). Therefore, in present study, the same cell line was used to examine the effect of D-FEAU on mitochondrial functions.

2. Materials and methods

2.1. Materials

The HepG2 cell line was purchased from the American Type Culture Collection (Rockville, MD). D-FEAU was obtained from Dr R.F. Schinazi (Emory University, Decatur, GA). MEM with non-essential amino acids, sodium pyruvate, dialyzed fetal bovine serum and $10\times$ trypsin-EDTA were purchased from GIBCO BRL (Grand Island, NY). Lactic acid assay kit was purchased from Boehringer Mannheim Corp.(Mannheim, Germany).

2.2. Cell cultures

The HepG2 cells were grown in 75 cm^2 tissue culture flasks in MEM with non-essential amino acids supplemented with 10% heat-inactivated dialyzed fetal bovine serum, 1% sodium pyruvate, and 1% penicillin/streptomycin. The medium was changed every three days and cells were subcultured once a week.

2.3. Effect of D-FEAU on cell growth

The HepG2 cells from stock culture were diluted and plated in 12-well cell culture clusters with 2.5×10^4 cells/ml in each well. D-FEAU with concentrations between 0.1 and 10 μM or no compound (control) were added into medium of each well. After 4 days of incubation, fresh medium with the tested compound was changed every other day until termination of the experiment at 14 days. Every other day the cells were counted as previously described (Cui et al., 1995).

2.4. Lactic acid determination

HepG2 cells (2.5×10^4 cells/ml) were plated into 12-well cell culture clusters and treated with various concentrations of D-FEAU under similar conditions described above. After 4 days of incubation, cell number in each well was determined with a hemocytometer and lactic acid content in the medium was measured by using a Boehringer lactic acid assay kit, following the supplier's instructions.

2.5. Effects of D-FEAU on mtDNA content

After 14 days of incubation, cells (5×10^4 per sample) incubated with D-FEAU under various concentrations and no compound (control) were boiled under alkaline condition and the DNA was immobilized on a Zeta-Probe membrane (Bio-Rad, Richmond, CA) by using a slot-blot apparatus. The mtDNA on the membrane was quantitated with a specific human oligonucleotide mitochondrial probe, encompassing nucleotide positions 4212-4242 (Anderson et al., 1981). A gel-purified 625-base pair fragment of a human β-actin cDNA plasmid was used as a probe for standardizing the amount of total cellular DNA loaded on the membrane.

2.6. Morphological evaluation

HepG2 cells (2.5×10^4 cells/ml) were plated into 35×10 mm tissue culture dishes and 10μM of D-FEAU or no compound (control) were added to each dish. After 14-day incubation period, cells were fixed, post-fixed, dehydrated, em-

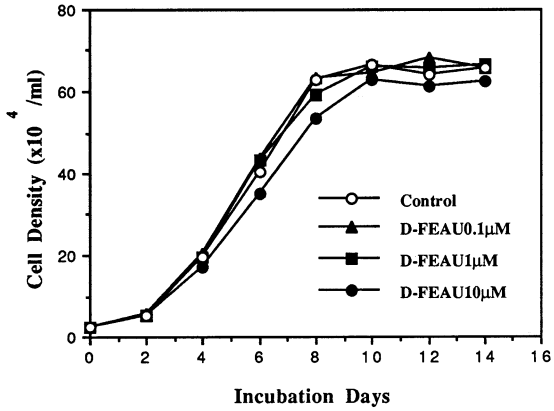

Fig. 1. Effect of various concentrations of D-FEAU on HepG2 cells growth.

bedded and sectioned as previously described (Cui et al., 1995). Finally, cells were examined with a Hitachi 7000 electron microscope.

3. Results

3.1. Effect of D-FEAU on HepG2 cell proliferation

HepG2 cell growth was counted in the presence of 0.1–10 μM of D-FEAU. As shown in Fig. 1, D-FEAU between 0.1 and 10 μM had no apparent effect on cell proliferation over a 14-day period.

3.2. Effect of D-FEAU on mitochondrial functions in HepG2 cells

As summarized in Table 1, D-FEAU had no substantial effect on lactic acid production of HepG2 cells with only 20.9% increase at concentration of 10 μM. However, a dose-dependent reduction of mtDNA content was indicated in HepG2 cells after 14-day exposure to D-FEAU between 0.1 and 10 μM with an IC_{50} value of 2.7 μM.

3.3. Morphological evaluation

In our electronmicroscopy study, D-FEAU-treated HepG2 cells manifested a slight increase in lipid droplet formation as compared with control cells, while no obvious morphological change of mitochondria was detected (Fig. 2 and Fig. 3).

4. Discussion

The clinical use of antiviral nucleoside analogues is limited by their seriously adverse effects, which have been attributed to a delayed mitochondrial toxicity over the past few years (Parker and Cheng, 1994). This hypothesis was confirmed in many previous studies on ddC-induced peripheral neuropathy (Chen and Cheng, 1989, 1992) and AZT-induced myopathy (Dalakas et al., 1990; Lewis et al., 1992), in which a preferential depletion of mtDNA content was observed. On the other hand, our results on D-FIAU-induced liver toxicity demonstrated that both D-FIAU and its in vivo metabolite D-FMAU were incorporated into mtDNA of HepG2 cells and led to marked mitochondrial dysfunction, despite the lack of inhibition on mtDNA synthesis (Cui et al., 1995). These data suggested that different mecha-

Table 1
Effect of D-FEAU on mitochondrial functions in HepG2 cells.Values are means ± SD of three different experiments

Compound	Concentration (μM)	Lactic acid production (mg/10^6cells)	Ratio to control of mtDNA synthesis (%)
Control	0	2.34 ± 0.07	100
D-FEAU	0.1	2.33 ± 0.03 (0%)[a]	88.4 ± 44.6
	1	2.54 ± 0.08 (8.5%)	68.6 ± 22.5
	10	2.83 ± 0.08 (20.9%)	30.9 ± 6.6

[a] The number in the parentheses is the percentage of increase in lactic acid production compared to control.

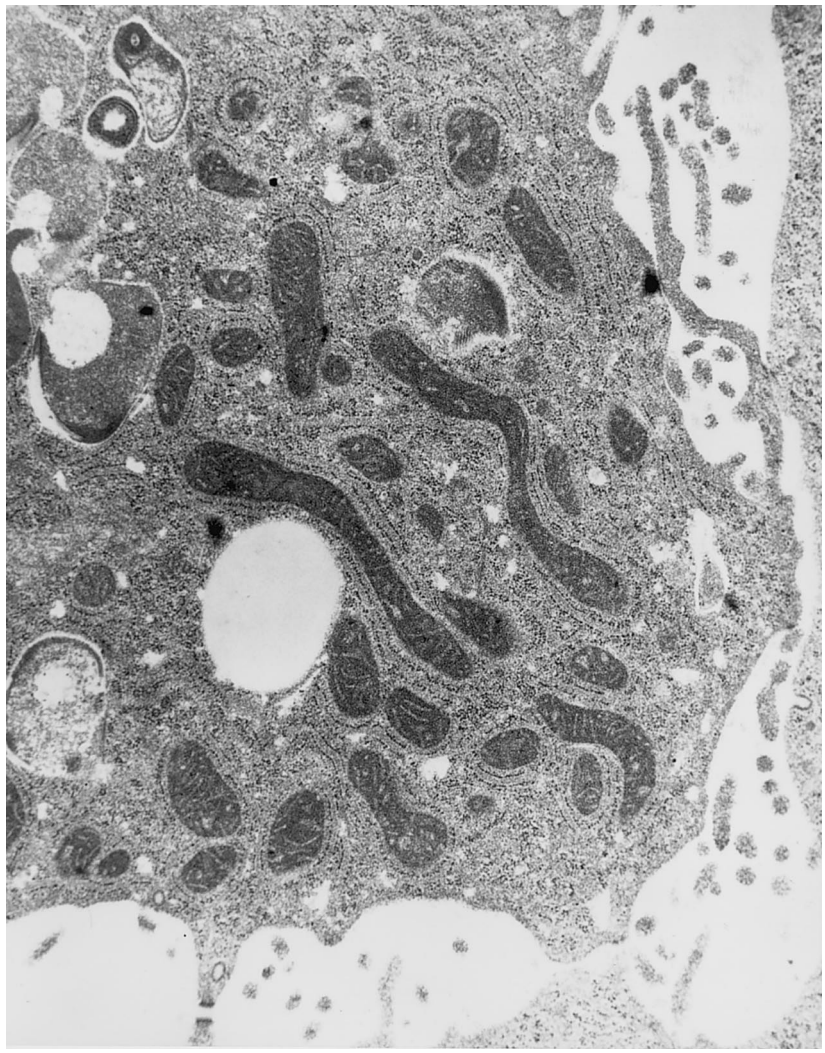

Fig. 2. Electron micrograph of control HepG2 cells after 14 days of incubation; magnification 30 000 × .

nisms may be underlying mitochondrial damage caused by different nucleoside analogues, and emphasized the need to screen newly developed antiviral nucleoside analogues for their possible mitochondrial toxicity in preclinical study.

In comparison to other fluorinated nucleoside analogues such as D-FIAC, D-FIAU, and D-FMAU, D-FEAU has shown strong activity against HSV and woodchuck hepatitis virus replication with much less toxicity against host cells (Chou et al., 1987; Kong et al., 1988). In our laboratory, an enzyme study also demonstrated that D-FEAU 5′-triphosphate (D-FEAU-TP) possesses a potent EC_{50} of 0.07 μM in regard to the inhibition of woodchuck hepatitis virus DNA polymerase activity (unpublished data). In addition, the pharmacokinetics study on D-FEAU in mice and rats has confirmed that D-FEAU has good bioavailability, distribution, and adequate half life (Kong et al., 1992), which makes it a more worthy antiviral candidate. However, no data have been reported about the effect of D-FEAU on mitochondrial functions, which may play a key role in the toxic side effects associated

with the use of antiviral nucleoside analogues. Our previous studies on D-FIAU-induced hepatotoxicity (Cui et al., 1995) and a group of potential anti-HBV nucleoside analogues (Cui et al., 1996) have indicated that human hepatoblastoma HepG2 cell line is a relevant model to evaluate the possible mitochondrial toxicity of newly developed drugs in liver cells.

In our current study, D-FEAU under pharmacologically relevant concentrations had no effect on HepG2 cell proliferation, which is consistent with its low host cell toxicity reported by the others (Chou et al., 1987; Kong et al., 1988). Interestingly, the effect of D-FEAU on cell growth is quite different from that of D-FIAU and D-FMAU in our earlier study, even though these nucleoside analogues have very similar chemical structure. Our earlier study had shown that both D-FIAU and D-FMAU inhibited HepG2 cell growth in a dose-dependent manner with IC_{50} of both compounds being less than 5 μM. As for their effects on mitochondrial functions, D-FEAU also manifested a distinctive profile compared with D-FIAU and D-FMAU.

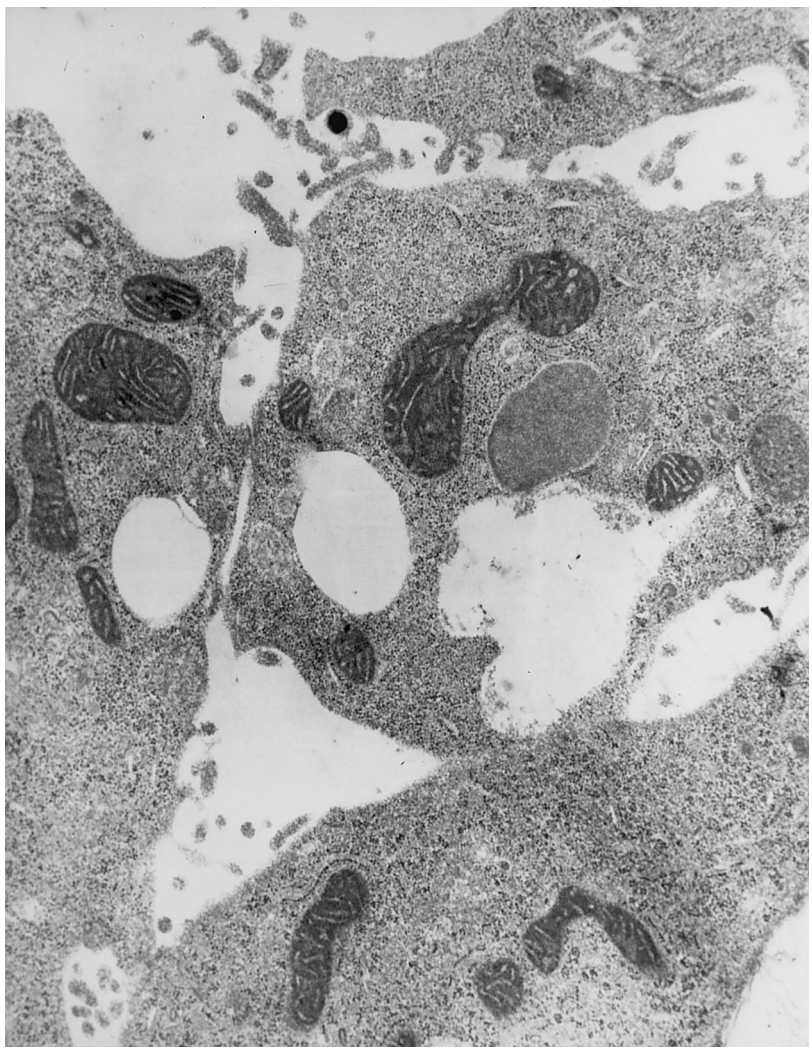

Fig. 3. Electron micrograph of HepG2 cells incubated over 14 days with 10 μM of D-FEAU; magnification 30 000 × .

Under the same tested concentrations, both D-FIAU and D-FMAU caused a dose-dependent increase in lactic acid production with over 100% enhancement at 10 μM after 4-day incubation, while they did not affect mtDNA synthesis (Cui et al., 1995). On the other hand, D-FEAU led to a dose-dependent reduction of mtDNA content with an IC_{50} value of 2.7 μM. Only a 20.9% increase in lactic acid production was detected at 10 μM of D-FEAU treatment. It has been known that most enzymes involved in oxidative phosphorylation are encoded by both mtDNA and nuclear DNA (Wallace, 1992). Our current results suggest that the increase in lactic acid production, a parameter specific to mitochondrial dysfunction, not only correlates with the extent of mtDNA inhibition (Tsai et al., 1994), but also is affected by nuclear DNA inhibition associated with different nucleoside analogues. Therefore, either genome damage may interfere with mitochondrial energy metabolism. As compared with the chemical structure of D-FIAU or D-FMAU, it seems that the 5-substituent group on the base ring is critical in determining the action of a nucleoside analogue on mtDNA synthesis. The depletion of mtDNA content is possibly due to the inhibition of D-FEAU-TP on DNA polymerase γ, an enzyme responsible for mtDNA synthesis.

In general, D-FEAU showed a much less toxic picture in HepG2 cells in terms of cell proliferation, lactic acid production as well as morphological changes. However, in our study, DFEAU did cause a reduction of mtDNA content, an important caution that should be taken into consideration in its further development. In fact, a recent study in the woodchuck model has shown a 12-week delayed toxicity with D-FEAU, a pattern different from that of D-FIAU and D-FMAU (Tennant, et al., 1998). Therefore, the possible development of D-FEAU as an antiviral agent may be hampered from a potential mitochondrial hepatotoxicity.

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