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# Toxicity, Metabolism, DNA Incorporation with Lack of Repair, and Lactate Production for 1-(2′-Fluoro-2′-deoxy-β-D-arabinofuranosyl)-5-iodouracil in U-937 and MOLT-4 Cells

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## SUMMARY

Two cell lines, U-937 and MOLT-4, were used to investigate the toxicity, DNA incorporation, and effect on mitochondria of 1-(2′-fluoro-2′-deoxy-β-D-arabinofuranosyl)-5-iodouracil (FIAU) and its putative metabolite 1-(2′-fluoro-2′-deoxy-β-D-arabinofuranosyl)-uracil (FAU). After 72-hr incubation, the $IC_{50}$ values for FIAU were 6.4 μM for U-937 cells and 26 μM for MOLT-4 cells. $IC_{50}$ values for FAU were 10-fold higher in both cell lines. Incubation for 24 hr with 10 μM [2-$^{14}$C]FIAU led to 2.1% and 0.93% replacement of thymidine in DNA of U-937 and MOLT-4 cells, respectively. The predominant radioactive species measurable in DNA was FIAU. A similar incubation with [2-$^{14}$C]FAU resulted in 4-fold lower DNA incorporation of a single radioactive species that coeluted with 1-(2′-fluoro-2′-deoxy-β-D-arabinofuranosyl)-5-methyluracil (FMAU). There was no evidence of a selective repair process after DNA incorporation of FIAU or FAU (FMAU). Increased intracellular concentrations of FIAU triphosphate and incorporation into DNA were associated with an increase in cellular toxicity. Continuous exposure to a clinically achievable concentration of FIAU, 0.44 μM, produced a constant DNA incorporation of 0.80% and 0.11% for U-937 and MOLT-4 cells, respectively. FIAU was not readily metabolized to FAU or iodouracil by human liver *in vitro*. Compared with 2′,3′-dideoxycytidine as a positive control, after 12 days of continuous exposure of U-937 and MOLT-4 cells to FIAU there was no evidence of increased lactate production. These data negate several possible mechanisms (DNA chain termination, DNA polymerase inhibition, one form of selective mitochondrial poisoning, and FAU-mediated toxicity) and provide clues for possible mechanisms (FIAU triphosphate concentration and DNA incorporation). Further work is needed to develop a complete explanation for the delayed hepatic toxicity observed in the investigational clinical trials of FIAU.

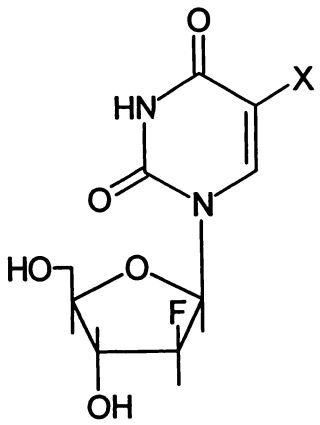
Clinical trials of FIAU (Fig. 1) as an agent against hepatitis B virus ended prematurely with severe delayed toxicities. The most prominent effect was hepatotoxicity, producing death in five of the 15 patients and the requirement for liver transplantation in two other subjects (1, 2). Lactic acidosis was an end-stage observation in this group.

Because a large number of nucleoside analogs have been investigated as antiviral or anticancer drugs, several potential mechanisms for the delayed toxicity can be hypothesized. Among the possibilities are inhibition of host cellular enzymes (including DNA polymerase) by nucleotides derived from FIAU, chain termination of DNA synthesis, and impaired transcription secondary to incorporation of FIAU into host cellular DNA. These DNA-linked events might occur in either mitochondrial or nuclear portions of the cell.

Although there are no published pharmacology studies of FIAU administration in humans or animals, preclinical and clinical studies of the related pyrimidine FIAC were published >10 years ago. After dosing with FIAC, substantial quantities of FIAU are formed in humans (3) and rodents (4). Other metabolites of FIAC included FAC, FAU, and FMAU. FAC, FIAU, and FMAU were found in DNA extracted from murine intestines (5). Because of the study design, it was not possible to determine whether FAU was derived from FIAU or FAC. Aside from antiviral effects, there are two reports of direct biochemical studies of FIAU in cell culture. When Vero cells were incubated with FIAU, only FIAU was found in DNA, with no evidence of FMAU (6), and HepG2/G3 cells incubated with [$^{14}$C]FIAU produced large amounts of [$^{14}$C]FIAU phosphorylation, whereas no radioactivity greater than background was found in the perchloric acid-insoluble fraction (7).

In our study, two human-derived cell lines, U-937 and MOLT-4, were used to investigate the cellular pharmacology of FIAU. Acute cellular toxicity (growth inhibition), intracellular nucleotide production, and incorporation of FIAU into DNA were investigated for 1–3-day periods. Cells were also

**ABBREVIATIONS:** FIAU, 1-(2′-fluoro-2′-deoxy-β-D-arabinofuranosyl)-5-iodouracil; FIAUTP, 1-(2′-fluoro-2′-deoxy-β-D-arabinofuranosyl)-5-iodouracil triphosphate; FAU, 1-(2′-fluoro-2′-deoxy-β-D-arabinofuranosyl)uracil; FMAU, 1-(2′-fluoro-2′-deoxy-β-D-arabinofuranosyl)-5-methyluracil; FIAC, 1-(2′-fluoro-2′-deoxy-β-D-arabinofuranosyl)-5-iodocytidine; FAC, 1-(2′-fluoro-2′-deoxy-β-D-arabinofuranosyl)cytidine; IdUrd, 5-iodo-2′-deoxyuridine; EdUrd, 5-ethyl-2′-deoxyuridine; dThd, thymidine; DDC, 2′,3′-dideoxycytidine; HPLC, high performance liquid chromatography.



FIAU → FIAUMP → FIAUDP → FIAUTP → DNA
↓ TS
FAU → FAUMP
↓ TS
FMAU → FMAUMP → FMAUDP → FMAUTP → DNA

**Fig. 1.** Structures of FIAU (X = I), FAU (X = H), and FMAU (X = $CH_3$) and possible metabolic pathways for FIAU and FAU. Thymidylate synthase (*TS*) has the potential to de-iodinate FIAU and to methylate FAU, resulting in FMAU monophosphate (*FMAUMP*). After additional phosphorylation, incorporation into nuclear DNA is through DNA polymerase $\alpha$ and $\beta$, whereas incorporation into mitochondrial DNA occurs through DNA polymerase $\gamma$. *FIAUMP*, FIAU monophosphate; *FIAUDP*, FIAU diphosphate; *FAUMP*, FAU monophosphate; *FMAUDP*, FMAU diphosphate; *FMAUTP*, FMAU triphosphate.

exposed to FIAU for 12 days to determine whether there was delayed toxicity associated with lactate production, as shown for dideoxynucleosides, particularly DDC (8, 9, 10).

To evaluate the potential role of FAU in the expression of toxicity from FIAU, cells were also incubated directly with FAU. Acute toxicity, nucleotide formation, and DNA incorporation were determined for FAU. Fresh slices of human liver and subcellular hepatic fractions were used to investigate the formation of FAU from FIAU.

## Materials and Methods

**Enzymes and chemicals.** DNase I (type II) from bovine pancreas, phosphodiesterase I (type VII) from *Crotalus atrox*, lactate enzymatic determination kit, Krebs-Henseleit bicarbonate buffer, IdUrd, EdUrd, and dThd were obtained from Sigma Chemical Co. (St. Louis, MO). FIAU was synthesized and kindly provided by K. A. Watanabe, Memorial Sloan Kettering Cancer Center (New York, NY) (11). Unlabeled FAU, radiolabeled [2-$^{14}$C]FIAU (56 mCi/mmol), [2-$^{14}$C]FAU (56 mCi/mmol), [*methyl*-$^{3}$H]FMAU (330 mCi/mmol), [6-$^{3}$H]IdUrd (20 Ci/mmol), and [*ethyl*-$^{3}$H]EdUrd (10 Ci/mmol) were obtained from Moravek Biochemicals (Brea, CA).

**Cellular incubation.** The suspension cell lines U-937 (human histiocytic lymphoma) and MOLT-4 (human peripheral blood acute lymphoblastic leukemia) were obtained from the American Type Culture Collection (Rockville, MD) and grown at 37° in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, in humidified 5% $CO_2$. The U-937 cell line was chosen for its ability to de-iodinate 5-IdUrd (12), and MOLT-4 was the cell line used in previous studies of mitochodrial inhibition (9, 10). Cells were in logarithmic growth for 24 hr before drug incubations. Cells were split to the desired cell density and drug was added to begin the experiment. Cell counts were obtained with an Elzone 180 particle counter (Particle Data, Elmhurst, IL).

**Biochemical determinations after 24-hr incubations.** Cells were split to 300,000–400,000 cells/ml in fresh medium containing 10 $\mu$M [$^{14}$C]FIAU, [$^{14}$C]FAU, or [$^{3}$H]FMAU. Cells were harvested at 24 hr for DNA incorporation and nucleotide measurement. For the repair study, cells were harvested, washed twice in medium at 4°, and suspended in drug-free medium at 37°. Aliquots of each replicate were used for further splitting and measurement of nucleotides and DNA incorporation. Repair studies were performed by splitting the cells to 300,000, 150,000, and 75,000 cells/ml for harvest at 24, 48, and 72 hr, respectively. The DNA incorporation was determined at each harvest time and compared with the expected value obtained by dilution of label due to cell growth. DNA incorporation was determined using the following equation: percentage incorporation = 100 × ([drug]/([dThd] + [drug])).

**Cytotoxicity after 72-hr incubations.** Cells were split to 20,000 cells/ml in fresh medium containing up to 1000 $\mu$M FIAU or FAU. At 72 hr, the cells were counted and the cell growth was reported as a percentage of control growth. The drug concentration resulting in 50% growth inhibition ($IC_{50}$) was determined from a fit of the sigmoidal $E_{max}$ equation, $E = E_{max} - (E_{max} \times C^n)/(IC_{50}^n + C^n)$, where $E$ is effect, $E_{max}$ is maximum growth, $C$ is concentration, and $n$ is the sigmoidicity factor.

**DNA incorporation after 72-hr incubations.** U-937 cells were split to 120,000 cells/ml in fresh medium containing 0, 0.3, 1, 2, 10, or 20 $\mu$M [$^{14}$C]FIAU and MOLT-4 cells were split to 120,000 cells/ml containing 0, 0.3, 8, 15, 30, or 60 $\mu$M [$^{14}$C]FIAU. At the end of 72 hr the cells were counted and harvested, and the DNA was extracted and analyzed as described below.

**Long term DNA incorporation after 12-day incubations.** Duplicate sets of cells were split to 20,000 cells/ml in fresh medium containing 0.44 $\mu$M [$^{14}$C]FIAU. At the end of 4 days, each cell replicate was counted and split to 20,000 cells/ml in fresh medium containing 0.44 $\mu$M [$^{14}$C]FIAU. This cycle was repeated once more to complete 12 days of continuous exposure to FIAU. DNA incorporation was measured on the last day of each cycle.

**Long term toxicity after 12-day incubations.** MOLT-4 cells, in triplicate, were split to an initial density of 200,000 cells/ml in fresh medium containing 0, 0.1, 0.2, or 0.4 $\mu$M DDC or 1.5, 4, 8, or 20 $\mu$M FIAU. U-937 cells were split to an initial density of 200,000 cells/ml in fresh medium containing 0, 0.1, 0.2, or 0.4 $\mu$M DDC or 1, 2, or 5 $\mu$M FIAU. Every 2 days, the cells were centrifuged at 1500 × $g$, medium was removed, and the cells were split to 200,000 cells/ml with fresh medium containing drug. This cycle was repeated several times to complete 12 days of continuous exposure. After 12 days of drug exposure, U-937 cells were grown for 6 more days (three cycles) in fresh medium without drug. The cell count and the lactate concentration in medium were determined before and after each cell splitting. The doubling time was estimated from the number of cell doublings at the end of each 2-day interval, and the lactate concentration was reported as the increase in lactate in medium divided by the cell number at the end of each 2-day interval.

**Nucleotide and DNA collection.** Soluble nucleotides and DNA incorporation were determined by previously reported methods. Typically, 2–6 × $10^6$ cells were washed once with phosphate-buffered saline and extracted with 0.3 ml of acetonitrile and 0.2 ml of water to obtain nucleotides (13). The DNA pellet was washed twice with 5% trichloroacetic acid, incubated for 90 min at 37° in 0.25 M sodium hydroxide, and precipitated and washed with 5% trichloroacetic acid twice, followed by two washes with phosphate-buffered saline (14, 15). The DNA pellet was digested for 90 min at 37° in 0.5 ml containing 50 units of DNase I, 0.024 units of phosphodiesterase, 2 mM magnesium chloride, and 25 mM potassium phosphate buffer, pH 7.4. Sufficient contaminating alkaline phosphatase was present in the crude enzyme preparations to complete the hydrolysis of the nucleotides to their respective nucleoside products (15).

**Measurement of drugs in media.** The drugs in the incubation media of cell cultures, liver slices, and S9 fractions were analyzed directly by HPLC or extracted by protein precipitation with acetonitrile (0.1 ml of sample plus 1 ml of acetonitrile) before analysis. Sample and

acetonitrile were mixed and centrifuged at 13,000 × g for 5 min, and the supernatant was transferred to a separate tube, dried under vacuum, and stored at −20° until analysis.

**HPLC.** Soluble nucleotides were separated by gradient, ion-pair, reverse phase elution at 1 ml/min on a Beckman Ultrasphere ODS column (5 μm, 4.6 × 250 mm). Solvent A consisted of 2.5 mM tetrabutylammonium hydrogen sulfate, 20 mM potassium phosphate, pH 6.6, with 2% (v/v) acetonitrile. Solvent B was a 50%/50% (v/v) mixture of solvent A and acetonitrile. During the analysis a linear gradient was run from 0% to 60% solvent B in 60 min, followed by an equilibration period of 30 min before the next injection. Detection was performed at 254 nm in tandem with an on-line radioactivity detector.

The enzymatic digests of DNA samples and the medium extracts were chromatographed using a Beckman Ultrasphere ODS column (5 μm, 4.6 × 250 mm). An isocratic mobile phase consisting of 10 mM sodium acetate, pH 5, with 6% (v/v) acetonitrile, at a flow of 1 ml/min, was used for the separation. Detection was performed at 254 nm in tandem with an on-line radioactivity detector.

**Human liver.** Human liver slices (approximately 8-mm diameter and 300-μm thick, 10 mg of blotted weight) were prepared by In Vitro Technologies (Baltimore, MD). These liver samples, which were medically unsuitable for transplantation, were obtained from the International Institute for the Advancement of Medicine (Exton, PA). Twenty slices were incubated for up to 2.5 hr in Krebs-Henseleit bicarbonate buffer, pH 7.5, containing 9 μM [$^{14}$C]FIAU, [$^{3}$H]IdUrd, or [$^{3}$H]EdUrd. The drug was measured in the incubation medium as described.

Human liver, medically unsuitable for transplantation, was also obtained through the Washington Regional Transplant Consortium (Washington, DC). Liver tissue was homogenized in 10 volumes of buffer (150 mM KCl, 100 mM sodium phosphate, 1 mM EDTA, pH 7.4) using a blender (three or four 10-sec bursts). The homogenate was centrifuged at 13,500 × g for 20 min, and the resulting S9 supernatant was stored at −70° until it was used. The S9 fraction (100 μl) was incubated for 1 hr with 900 μl of buffer (100 mM sodium phosphate, 1 mM EDTA, 5 mM $MgCl_2$, pH 7.4) containing 9 μM drug. The drug was measured in the incubation medium as described.

## Results

Continuous incubation of cells with FIAU for 72 hr resulted in a steep concentration-response curve, with $IC_{50}$ values for FIAU of 6.4 μM for U-937 cells and 26 μM for MOLT-4 cells (Fig. 2). With 1 μM FIAU there was little effect on cell growth, whereas with 100 μM FIAU cell growth was almost completely inhibited. The $IC_{50}$ values for FAU after 72 hr in culture were 10-fold greater than the $IC_{50}$ values found for FIAU in both U-937 and MOLT-4 cells (data not shown).

Nucleotide standards were not available for FIAU, FAU, or FMAU. After 24-hr incubation with 10 μM FIAU, the HPLC-separated nucleotides were presumed to be FIAU monophosphate, FIAU diphosphate, and FIAUTP. The triphosphate formed after incubation with 10 μM FAU eluted with the same retention time on HPLC as did the triphosphate formed after incubation with FMAU. After 24-hr incubation with 10 μM FIAU or FAU, the concentrations of FIAU and FMAU triphosphates in both cell lines were between 1.9 and 4.8 pmol/$10^6$ cells (Table 1).

There was a linear relationship between the amount of FIAUTP in cells at 24 hr and the percentage of control growth at 72 hr in both cell lines (Fig. 3). The data from the two cell lines were identical. This relationship between FIAUTP concentration and toxicity did not hold for the 72-hr incubation (Fig. 3).

There was also a linear, but not identical, relationship between the amount of FIAU incorporated into DNA at 24 hr and toxicity at 72 hr (Fig. 4). The same relationship held when the 72-hr DNA incorporation was compared with toxicity at 72 hr (Fig. 4). The DNA incorporation in both cell lines increased 2-fold between 24 hr and 72 hr of incubation (~2.5 cell doublings).

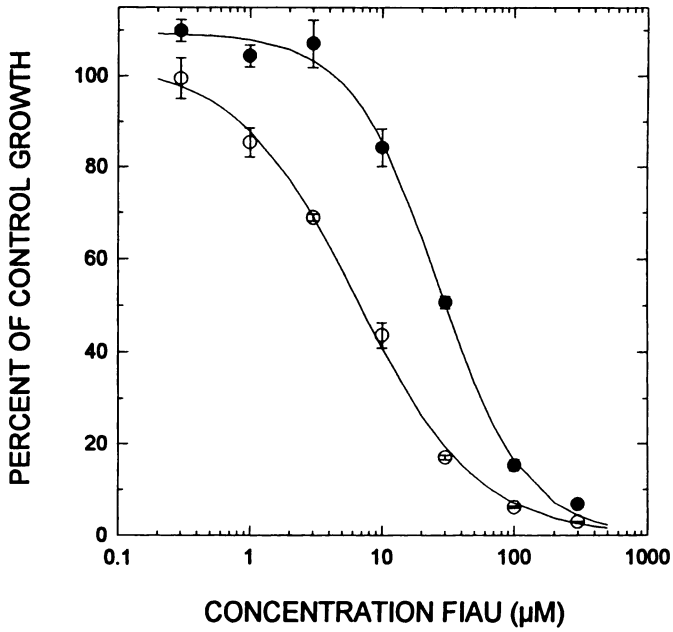



**Fig. 2.** Effect on cell growth of 72-hr continuous exposure to FIAU. U-937 cells (○) had an $IC_{50}$ value of 6.4 μM, whereas MOLT-4 cells (●) had an $IC_{50}$ value of 26 μM. Data points are the means of triplicate samples; *error bars*, standard deviations. *Lines*, fit of the data with the sigmoidal $E_{max}$ equation described in Materials and Methods.

TABLE 1

**DNA incorporation of FIAU and FAU**

U-937 and MOLT-4 cells were incubated with FIAU or FAU for 1, 4, 8, or 12 days. Data were from either duplicate (0.44 and 90 μM) or triplicate (10 μM) incubations. DNA incorporation was determined as percentage replacement of thymidine; nucleotides were measured as described in Materials and Methods.

| Drug | Time | Concentration | DNA incorporation | | Nucleotide triphosphates | |
|---|---|---|---|---|---|---|
| | | | U-937 | MOLT-4 | U-937 | MOLT-4 |
| | *day* | *μM* | *% of thymidine* | | *pmol/$10^6$ cells* | |
| FIAU | 1 | 10 | 2.13 | 0.93 | 4.8 | 3.3 |
| | 1 | 90 | 4.0 | 2.5 | | |
| | 4[a] | 0.44 | 0.82 | 0.11 | | |
| | 8[a] | 0.44 | 0.79 | 0.10 | | |
| | 12[a] | 0.44 | 0.78 | 0.11 | | |
| FAU | 1 | 10 | 0.50 | 0.19 | 1.9 | 1.9 |

[a] Day of harvest during continuous-exposure experiment.

The principal radioactive species found in DNA after 24-hr incubation with FIAU eluted with FIAU (Fig. 5). When the conditions were optimized for concentration and specific activity, there was <0.4% incorporation of FMAU in DNA found in U-937 cells after 72-hr incubation (data not shown). Incubation with 10 μM FIAU resulted in DNA incorporation of 2.1 ± 0.3% and 0.93 ± 0.08% for U-937 and MOLT-4 cells, respectively (Table 1). DNA incorporation at 90 μM FIAU was 4.0% and 2.5% for U-937 and MOLT-4 cells, respectively. With constant exposure to 0.44 μM FIAU for 12 days (three 4-day cycles), the DNA incorporation was constant at 0.80 ± 0.02% and 0.11 ± 0.01% for U-937 and MOLT-4 cells, respectively.

FAU did not incorporate into DNA as FAU. The radioactive peak found in DNA digests after FAU incubation eluted with the same retention time as did radiolabeled FMAU (Fig. 5).

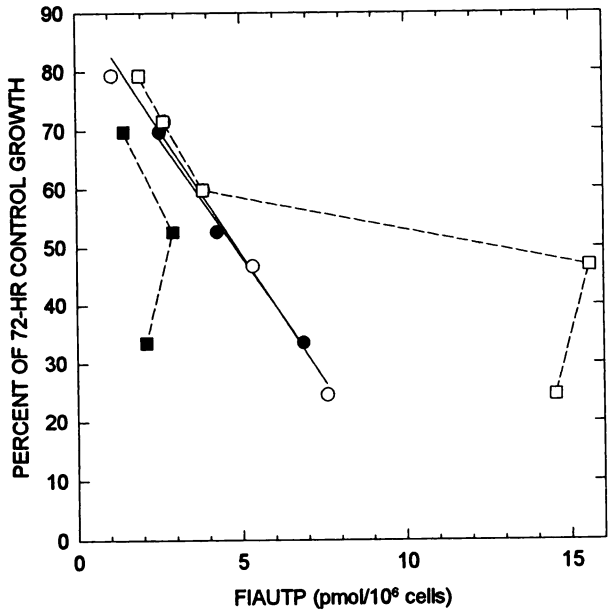



**Fig. 3.** Linear association of FIAUTP concentration with effects on cell growth after 24-hr (*circles*) but not 72-hr (*squares*) incubation with FIAU in U-937 (*open symbols*) and MOLT-4 (*solid symbols*) cells. The FIAUTP concentrations after 24 hr at the $IC_{50}$ were 4.9 and 4.8 pmol/$10^6$ cells for U-937 and MOLT-4 cells, respectively. The correlation coefficients were 0.992 and 0.987 for the linear regression fits for U-937 and MOLT-4 cells, respectively. Cell counts and FIAUTP concentrations were determined as described in Materials and Methods.

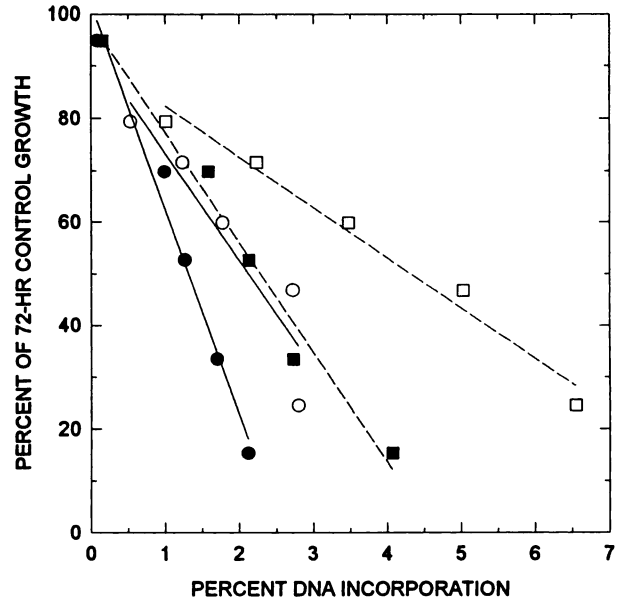



**Fig. 4.** Linear association of DNA incorporation with effect on cell growth after 24-hr (*circles*) and 72-hr (*squares*) incubation with FIAU. Percentage DNA incorporation at the $IC_{50}$ was 2-fold higher at 72 hr than at 24 hr for both U-937 (*open symbols*) and MOLT-4 (*solid symbols*) cells. Cell counts and DNA incorporation were determined as described in Materials and Methods.

The incorporation of FMAU in DNA after incubation with FAU was about 4-fold less than FIAU incorporation (Table 1).

Table 2 gives the DNA incorporation after 24-hr incubation with 9 μM FIAU or FAU. There was <20% growth inhibition at this concentration, compared with untreated controls (data not shown). No toxicity was observed for the remaining 72 hr of incubation in drug-free medium, indicating reversal of toxicity. The decrease in percentage incorporation on each successive day for each cell line was exactly as expected due to the dilution of DNA during normal growth (Table 2).

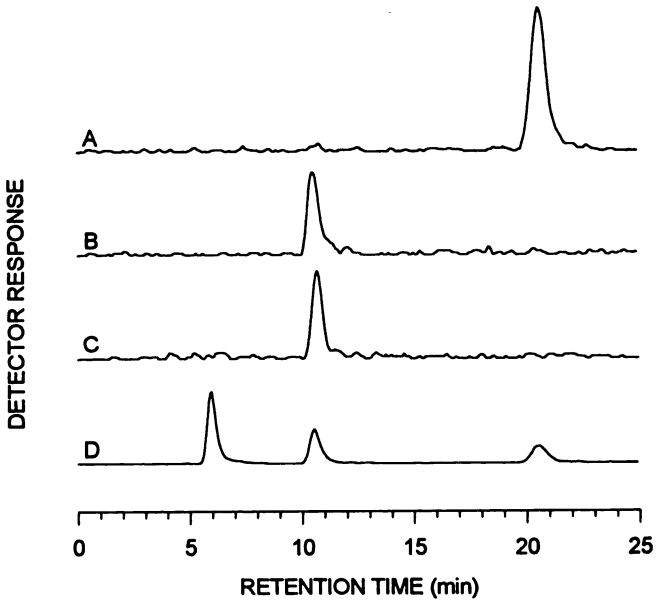



**Fig. 5.** Radiochromatograms of DNA digests. U-937 and MOLT-4 cells (~3 × $10^6$) were incubated for 24 hr with 10 μM drug and the DNA was extracted, digested, and analyzed by HPLC as described in Materials and Methods. The combined radiochromatograms represent the HPLC analysis of U-937 cell DNA digests after incubation with [$^{14}$C]FIAU (A), [$^3$H]FMAU (B), or [$^{14}$C]FAU (C). A standard solution (D) shows the relative retention times of [$^{14}$C]FAU (6 min), [$^3$H]FMAU (10.5 min), and [$^{14}$C]FIAU (20.5 min).

TABLE 2

**DNA incorporation and repair of FIAU and FAU**

U-937 and MOLT-4 cells were incubated for 24 hr with 9 μM FIAU or FAU. Day 0 began after the cells were washed free of drug, and DNA incorporation, as a percentage of thymidine, was determined on the following 3 days. Each value was the mean of duplicate samples. The expected incorporation was calculated as expected = $\text{found}_{\text{day 0}}/2^{\text{cell doublings}}$.

| | U-937 | | | MOLT-4 | | |
|---|---|---|---|---|---|---|
| | Cell doublings | DNA Incorporation | | Cell doublings | DNA Incorporation | |
| | | Expected | Found | | Expected | Found |
| | | % of thymidine | | | % of thymidine | |
| FIAU | | | | | | |
| Day 0 | | | 2.05 | | | 0.56 |
| Day 1 | 0.95 | 1.06 | 0.94 | 0.82 | 0.32 | 0.31 |
| Day 2 | 2.21 | 0.44 | 0.44 | 1.94 | 0.15 | 0.15 |
| Day 3 | 3.35 | 0.20 | 0.20 | 3.07 | 0.07 | 0.07 |
| FAU | | | | | | |
| Day 0 | | | 0.39 | | | 0.08 |
| Day 1 | 1.01 | 0.19 | 0.19 | 0.84 | 0.04 | 0.05 |
| Day 2 | 2.20 | 0.09 | 0.09 | 1.91 | 0.02 | 0.02 |
| Day 3 | 3.34 | 0.04 | 0.04 | 3.09 | 0.01 | 0.01 |

Both cell lines were cultured for 12 days with continuous exposure to DDC and FIAU. The lowest concentration of DDC (0.1 μM) had little effect on cell growth (Fig. 6A). Higher concentrations of DDC led to a concentration-dependent increase in doubling times in both U-937 and MOLT-4 cells (Fig. 6A). The doubling time in the 0.4 μM DDC incubation of U-937 cells continued to increase during the 6 days of incubation in drug-free medium (data not shown). After removal of DDC from the medium, the doubling time reverted to normal only in the cells incubated with the lowest concentration of DDC

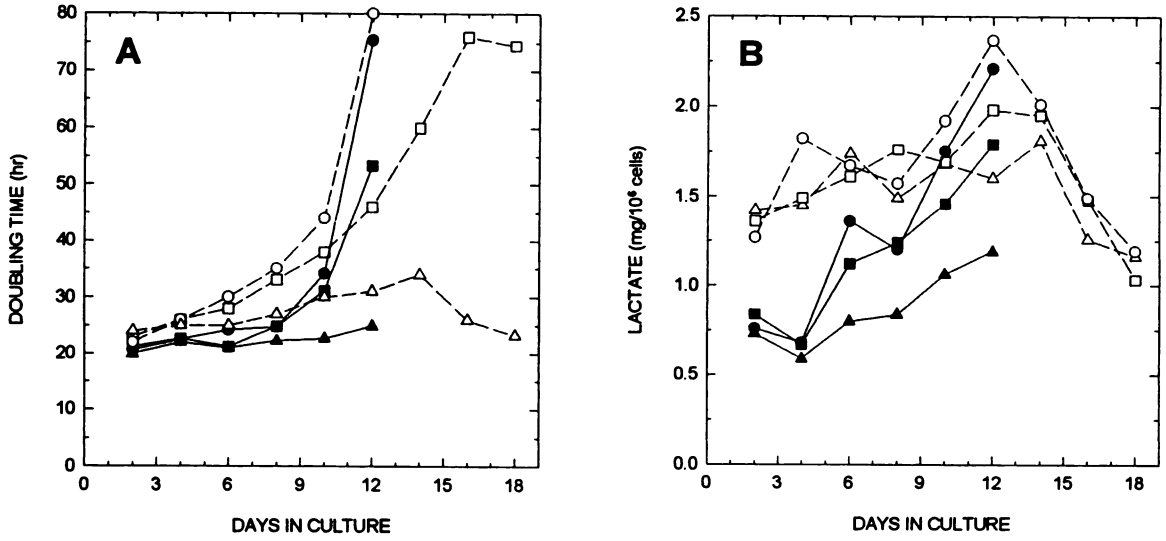



**Fig. 6.** Effect of DDC on cell doubling time (A) and lactate concentration (B) with continuous exposure in U-937 (*open symbols*) and MOLT-4 (*solid symbols*) cells. Cells were incubated with no drug, as control (not shown), or with DDC at 0.1 (*triangles*), 0.2 (*squares*), or 0.4 μM (*circles*) for 12 days of continuous exposure, followed by incubation in drug-free medium for an additional 6 days beginning on day 12. The doubling time was estimated from the number of cell doublings at the end of each 2-day interval, and the lactate concentration was determined by dividing the increased lactate in the medium by the cell number at the end of each 2-day interval. Control doubling times were 22 hr for both cell lines, and lactate concentrations were 1.2 and 0.7 mg/$10^6$ cells for U-937 and MOLT-4 cells, respectively.

(Fig. 6A). Lactate concentrations in both cell lines increased in proportion to the incubation concentration of DDC (Fig. 6B). There were lower initial lactate concentrations but larger increases in lactate concentration in the MOLT-4 cells, compared with U-937 cells. The lactate concentration returned to normal for all of the DDC incubations within 6 days after the drug was removed from the medium (Fig. 6B).

Although FIAU also had an effect on doubling time in both cell lines, the increase in doubling time began early in the time course and increased more gradually, compared with DDC-treated cells (Fig. 7A). The exception was with 5 μM FIAU in the U-937 cells between days 4 and 12. The doubling time could not be determined accurately due to the minimal growth at this concentration (data not shown). However, after removal of FIAU the doubling time returned to normal for all of the incubation concentrations with the U-937 cells (Fig. 7A). Lactate concentrations seemed to be higher than control values initially and to decrease to near or below control values near the end of 12 days of exposure (Fig. 7B). The effect was most pronounced at the highest concentration of FIAU in both cell lines. After removal of FIAU from the medium, lactate concentrations returned to normal for all of the FIAU concentrations (Fig. 7B).

Other observations of this study include the enzymatic stability of FIAU. The amount of FIAU measured in culture medium after 4 days of incubation with 0.44 μM FIAU, with a

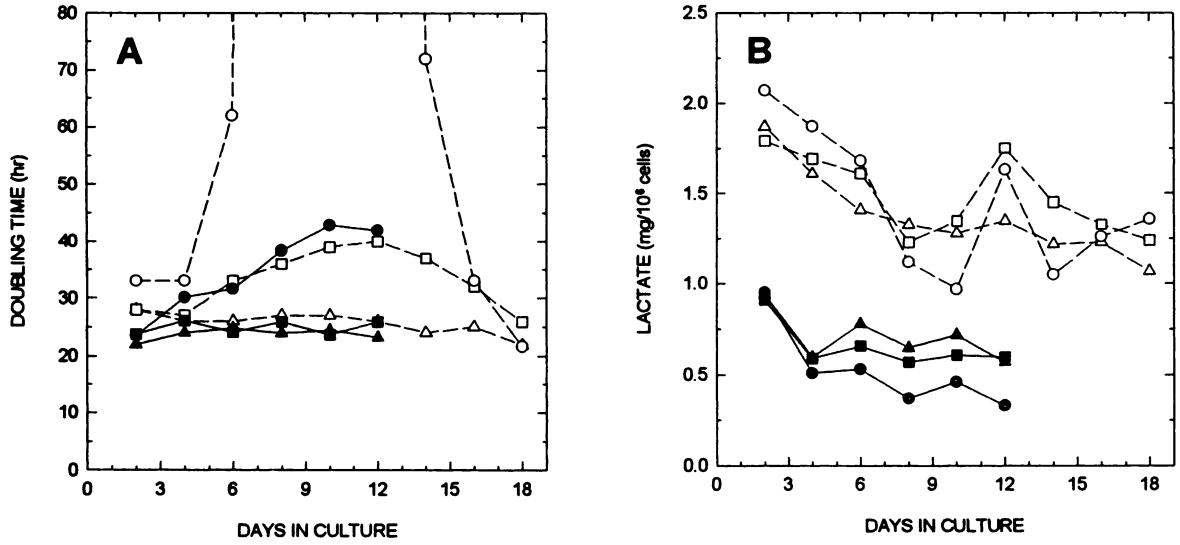



**Fig. 7.** Effect of FIAU on cell doubling time (A) and lactate concentration (B) with continuous exposure in U-937 (*open symbols*) and MOLT-4 (*solid symbols*) cells. Cells were incubated with no drug, as control (not shown), or with FIAU at 1 (△), 2 (□), or 5 μM (○) (U-937 cells) or 4 (▲), 8 (■), or 20 μM (●) (MOLT-4 cells) for 12 days of continuous exposure, followed by incubation in drug-free medium for an additional 6 days beginning on day 12. The doubling time was estimated from the number of cell doublings at the end of each 2-day interval, and the lactate concentration was determined by dividing the increased lactate in the medium by the cell number at the end of each 2-day interval. Control doubling times were 22 hr for both cell lines, and lactate concentrations were 1.2 and 0.7 mg/$10^6$ cells for U-937 and MOLT-4 cells, respectively.

final density of >0.5 × $10^6$ cells/ml, was within 4% of the FIAU measured in medium alone. There was no degradation of FIAU during incubations of 1 and 2.5 hr with S9 fractions and liver slices, respectively. In contrast, IdUrd and EdUrd were completely degraded to their respective bases within 1 hr of incubation with S9 fractions and liver slices.

## Discussion

Nucleotides were readily formed from FIAU and incorporated into growing strands of DNA. Based upon the similarity of FIAU to dideoxynucleosides and the report of small amounts of FIAU in DNA from murine tissues and mammalian cells in culture (6), chain termination of DNA synthesis might have been considered as a mechanism of action. Contrary to one report in HepG2/G3 cells, where FIAU nucleotides were produced in large quantities with lack of DNA incorporation (7), the high levels of dThd replacement by FIAU found in DNA in our experiments conclusively demonstrate that FIAU is not a chain terminator for DNA synthesis of mammalian cells.

The results for the U-937 and MOLT-4 cell lines suggest that toxicity of FIAU is associated with the intracellular concentration of FIAUTP and incorporation into DNA. For the same amount of toxicity, the DNA incorporation in U-937 cells was twice that found in MOLT-4 cells, but the incubation concentration was 4-fold less. Despite the observed difference in $IC_{50}$ values, the intracellular nucleoside triphosphate concentrations after 24 hr were similar at each level of toxicity for the two cell lines (Fig. 3). Nucleotide concentrations may serve as an early indicator of toxicity before the cells are affected by FIAU. A more diverse set of cell lines and exposure conditions would be required for a definitive conclusion. The association of toxicity with the increased FIAUTP levels, with a parallel increase in DNA incorporation, indicates that there was not a major inhibition of DNA polymerase by FIAU.

Based upon previous clinical (3) and preclinical studies (4) with FIAC, which functions as a prodrug for FIAU, it was not clear whether substantial amounts of FAU, which could contribute to the toxicity of FIAU, were formed. We found that FAU was phosphorylated, methylated to FMAU nucleotides, and incorporated into DNA. However, it is unlikely that FAU plays a role in the clinical toxicity of FIAU, because 1) no detectable FAU was formed from FIAU either in human liver incubates or in the cell lines and 2) FAU is 10-fold less toxic than FIAU.

Our studies of DNA incorporation do not differentiate between incorporation of FIAU into nuclear versus mitochondrial DNA. We attempted to independently assess mitochondrial toxicity by monitoring the doubling time and lactate production of cells cultured with FIAU. This model has been shown to be useful for studying the mitochondrial toxicity of DDC and several other dideoxynucleosides (8–10). Although we replicated the published results for DDC in MOLT-4 cells, the behavior of FIAU was qualitatively different, and lactate production in this system would not have predicted the clinical observations for FIAU. These experiments do not address all forms of mitochondrial toxicity. Indeed, the results demonstrate that multiple probes of mitochondrial function are necessary.

Subtle differences in nucleoside structure have a profound influence upon the catabolism, as well as the pharmacological effects, of the compounds. IdUrd was rapidly metabolized to its pyrimidine base, iodouracil. However, no catabolism was detected for incubations of FIAU, which differs from IdUrd only in the addition of a fluorine atom at the 2′-position and would be expected to generate the same base.

In summary, these data supply some helpful information regarding the toxicity of FIAU. Toxicity of FIAU in these cell lines was associated with its phosphorylation and incorporation into DNA. Although FAU is a potential metabolite of FIAU, it did not appear to contribute to the toxicity of FIAU, because it was not formed in cell culture and had lower potency. Production of lactate by MOLT-4 and U-937 cells exposed to FIAU does not predict the clinical observations. Further work is needed to develop a complete explanation for the clinical events and a prospective screen for avoiding future episodes of this nature.

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