Biochemical Effects of 2'-Fluoro-5-methyl-1-β-d-arabinofuranosyluracil and 2'-Fluoro-5-iodo-1-β-d-arabinofuranosylcytosine in Mouse Leukemic Cells Sensitive and Resistant to 1-β-d-Arabinofuranosylcytosine

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ABSTRACT

2'-Fluoro-5-methyl-1-β-d-arabinofuranosyluracil (FMAU), like 2'-fluoro-5-iodo-1-β-d-arabinofuranosylcytosine (FIAC), has potent antiviral activity, unlike FIAC, it also has antileukemic effects. The two agents and 1-β-d-arabinofuranosylcytosine (ara-C) are compared herein. Concentrations inhibiting thymidine (dThd) incorporation into DNA by 50% are for FMAU, FIAC, and ara-C, respectively, in L1210/0, 32, 353, and 0.2 μM and in L1210/ara-C, 17, >10,000, and 3,900 μM. Other FIAM analogs, 2'-fluoro-5-iodo-1-β-d-arabinofuranosyluracil and 2'-fluoro-5-ethyl-1-β-d-arabinofuranosyluracil, inhibit dThd incorporation equally in ara-C-sensitive and -resistant cells; however, their potencies are weaker than that of FIAC. Similar results are obtained when [3H]deoxyadenosine is used as a precursor of incorporation. In L1210/0 cells, incorporation of [2-14C]FMAU radioactivity into DNA is competitively inhibited by dThd and deoxycytidine (dCyd), whereas the incorporation of [2-14C]FIAC radioactivity is competitively inhibited by dCyd but not appreciably by dThd. In L1210/ara-C cells, incorporation of [2-14C]FMAU is competitively inhibited by dCyd but not by dThd. In L1210/0 cells, FMAU has little inhibitory effect on the tritium release from [5-3H]deoxyuridine but markedly inhibits the incorporation of [2-14C]deoxyuridine into DNA. FIAC, by contrast, predominately inhibits the release of tritium from [5-3H]deoxyuridine but has little effect on subsequent incorporation into DNA. These results suggest that (a) FIAC, but not FMAU, is cross-resistant to ara-C; (b) FMAU is particularly effective against L1210/ara-C cells; (c) FIAC behaves metabolically like dCyd, and FMAU like dThd and dCyd; (d) dCyd and dThd may be used as chemotherapeutic modulators; and (e) FIAC predominately inhibits dThd kinase and/or thymidine monophosphate synthetase, whereas FMAU predominately inhibits DNA polymerase and/or nucleotide kinases. Similar conclusions were obtained when P815/0 and P815/ara-C cells were used. The relative potencies of FIAC and FMAU in inhibiting dThd incorporation into DNA in leukemic sublines correlate with cytotoxicity in vitro and chemotherapeutic effects in vivo.

INTRODUCTION

ara-C remains one of the most widely used agents against acute leukemias despite such shortcomings as rapid metabolic inactivation and development of resistance by the host toward the drug (12, 18, 21). The search for compounds better than ara-C for cancer chemotherapy has been a major effort in these laboratories and elsewhere (1, 11, 17). The present paper reports several 2'-fluoro-arabinofuranosynucleosides that have biochemical effects which are substantially different from that of ara-C, especially in cell lines that are resistant to ara-C. FMAU and its related 2'-fluoro-arabinofuranosynucleosides, FIAC, FIAU, and FEAU, synthesized by Reichman et al. (27) and Watanabe et al. (32), have been shown previously to be potent antithrombotic agents with varying degrees of cytotoxicity (3, 7, 9, 16, 23, 32). FMAU also inhibits leukemia in mice (2). The present results indicate that, in murine leukemic cells, FMAU, unlike many of its analogs, stands out as an inhibitor which blocks incorporation of nucleoside precursors into DNA in cell lines both sensitive and resistant to ara-C. Thus, it becomes of interest to compare biochemical effects of FMAU and related analogs in leukemic cells in vitro to explore the nature of their selective actions. The present results also indicate that the relative potency of FIAC and FMAU in inhibiting precursor incorporation into DNA correlates to their cytotoxicity in vitro and in vivo (2).

MATERIALS AND METHODS

Chemicals and Radiochemicals. FIAC, FIAU, FMAU, and FMAC were synthesized by Reichman et al. (27) and Watanabe et al. (32). ara-C and ara-U were obtained from The Upjohn Co., Kalamazoo, Mich. [3H-methyl]dThd (2.0 Ci/mmol), [3H]cytidine (28.6 Ci/mmol), and [5-3H]dCyd (38.1 Ci/mmol) were purchased from New England Nuclear, Boston, Mass. [2-14C]FIAC was synthesized as described previously (7). [2-14C]dUrd (48.5 mCi/mmol) was purchased from Schwarz/Mann, Orangeburg, N. Y., and [5-3H]dAdo (13.5 Ci/mmol) and [5-3H]dUrd (23 Ci/mmol) were purchased from ICN Pharmaceuticals, Inc., Irvine, Calif. [2-14C]dUrd (59 mCi/mmol) was purchased from Moravek Biochemicals, Inc., Brea, Calif. All nonlabeled natural nucleosides were obtained from Sigma Chemical Co., St. Louis, Mo. 3,4,5,6-Tetrahydrodouridine was supplied by the Drug Research and Development Branch, National Cancer Institute, Bethesda, Md. Radioactivity was determined with a Packard Tri-Carb Model 3775 liquid scintillation spectrometer using Liquisint scintillation fluid. Liquisint was purchased from National Diagnostics, Inc., Somerville, N. J.

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The abbreviations used are: ara-C, 1-β-d-arabinofuranosycytosine; FMAU, 2'-fluoro-5-methyl-1-β-d-arabinofuranosyluracil; FIAC, 2'-fluoro-5-iodo-1-β-d-arabinofuranosyluracil; FEAU, 2'-fluoro-5-ethyl-1-β-d-arabinofuranosyluracil; FMAC, 2'-fluoro-5-methyl-1-β-d-arabinofuranosylcytosine; ara-U, 1-β-d-arabinofuranosyluracil; dThd, thymidine; dCyd, deoxycytidine; dUrd, 5-iodo-2'-deoxyuridine; dAdo, deoxyadenosine; dUrd, deoxyuridine; SFU, 5-fluorouracil.

T-L. Su, K. A. Watanabe, and J. J. Fox, unpublished results.
Cell Lines. L1210/0 and P815/0 cells and their sublines resistant to ara-C, L1210/ara-C and P815/ara-C, respectively, were maintained by passage in C57BL/6 x DBA/2 (hereafter called BD2F1) mice. Approximately 1 x 10^6 cells were inoculated i.p. every 6 days for each passage. Day 5 ascites cells were collected and used for experiments. Cells beyond Day 5 were frequently contaminated by erythrocytes and were not used. The resistant cell lines were derived by treating BD2F1, mice bearing L1210/0 or P815/0 leukemia with ara-C, 60 mg/kg/day, i.p. for 6 days for six treatment cycles. The cell line resistant to ara-C was maintained by giving the same doses of ara-C on Days 1 and 2. The ara-C in vivo treatment was discontinued at least 1 week prior to the use of L1210/ara-C and P815/ara-C cells. The BD2F1, mice bearing L1210/0 or P815/0 leukemia died on Day 7 or Day 8 after i.p. inoculation of 10^6 cells, ara-C, 60 mg/kg/day, i.p. for 6 days increased by 85 to 90% the life span of these animals. Similar treatment to mice bearing L1210/ara-C or P815/ara-C did not show any increase in life span. L1210/ara-C cells have been previously shown (30) to contain less than 2% of the dCyd kinase of L1210/0 cells. The regimen of 5FU, 28 mg/kg/day, i.p. for 6 days also increases by 85 to 90% the life span of mice bearing L1210/0 leukemia. Similar treatment with 5FU did not increase the life span of mice bearing L1210/5FU leukemia.

Assays. For studying precursor incorporation into cellular DNA, an incubation mixture was made in Eagle’s base medium (1 ml), containing 10% fetal calf serum, 2 mM L-glutamine, 1 mM 3,4,5,6-tetrahydrodrolidine, a cytosine nucleoside deaminase inhibitor (6), and 10 to 48 x 10^5 leukemic cells. Except for the [2-14C]FIAC and [2-14C]FMAU experiments, the mixtures were preincubated with various concentrations of 2'-fluoro-arabinofuranosylcytosine for 37° for 10 min, followed by the addition of a radioactive pulse of 1 µCi, 0.5 mmol of [3H]dThd or [3H]dAdo for 30 min. The pulse was terminated by adding 3 ml of cold 10% perchloric acid. The radioactivity in the acid-insoluble DNA fraction was measured by the method described previously (8).

For measuring tritium release from [5-3H]dUrd, the method of Roberts (28), as modified by Kalman and Yalowich (22), was used.

Dose-Effect Analysis. Dose-effect relationships for inhibiting precursor incorporation into DNA were analyzed by the median effect plot (4, 10) where the concentration of inhibitor (I) is related to the effect in terms of fractional inhibition (I) based on the median effect equation of the mass action law:

\[
\log([f]^{-1} - 1)^{-1} = \log(I) - m \log(I_{so})
\]

(A)

where I_{so} is the concentration that is required to produce 50% inhibition and m is a Hill-type coefficient (4) that represents apparent kinetic order. A plot of \( y = \log([f]^{-1} - 1)^{-1} \) versus \( x = \log(I) \) gives a slope m and the intercept of the plot at \( y = 0 \) gives a log(I_{so}) value and thus the I_{so} (or ED_{so}) value. By transforming the median effect equation, the median effect concentration (ED_{so}) can also be calculated by:

\[
I_{so} = \text{antilog}(-y\text{ intercept}/m)
\]

(B)

After m and I_{so} in Equation A have been determined by linear regression analysis, the concentration of inhibitor (I) that is required to produce any degree of inhibition [e.g., ED_{so} (i.e., I = 0.9), ED_{so} (I = 0.95), etc.] can be readily calculated from Equation A.

RESULTS

Relative Potency of Inhibition. Studies of dose effects of the different arabinofuranosylnucleoside analogs in inhibiting the natural nucleoside incorporation into DNA of P815 cells provided data that fitted linear median effect plots (Chart 1). L1210 cells gave similar results. The slopes of the plots ranged from 0.8 to 1.2, except FEAU in L1210/ara-C cells which gave a shallow slope. The linearity of the plots and unity of slopes (m = 1) indicate that the dose-effect relationships can be approximated by the first-order mass action law (i.e., Michaelis-Menten-type relationships). In P815/0 cells, the relative potency for inhibiting [3H]dThd incorporation into DNA decreased in the following order: ara-C » FMAU > FIAU > FIAC > FEAU > ara-U. For P815/ara-C cells, the order is: FMAU > FIAU > FEAU > ara-C > ara-U > FIAC (see Table 1). FMAU was the most potent inhibitor in ara-C-resistant cells, whereas ara-C and FIAC showed close cross-resistance. FIAU or FIAC inhibited [3H]dThd incorporation in both ara-C-sensitive and -resistant cell lines to a similar extent, but the potencies of FIAC and FEAU were much weaker than that of FMAU. ara-U was a weak inhibitor in both ara-C-sensitive and -resistant cells. L1210/0 cells were inhibited in a pattern similar to that for P815/0, whereas L1210/ara-C cells were inhibited in a manner similar to P815/ara-C cells (Table 1). Similar patterns of inhibition were observed when [3H]dAdo was used instead of [3H]dThd (Table 1).

Inhibition Pattern of Analogs. In order to explore the mode of action of FIAC and FMAU at the cellular level, competition studies were carried out with labeled natural nucleoside versus unlabeled FIAC or FMAU, and also, to be shown later, with labeled [2-14C]FIAC or [2-14C]FMAU versus unlabeled natural nucleosides. The relative rates for [2-14C]FIAC and [2-14C]FMAU incorporation into nucleic acid were also compared. As shown in Table 2 and Chart 2 for P815/0 cells, [3H]dCyd incorporation (K_{m} = 0.79 µM) was competitively inhibited by FIAC (K_{i} = 20.8 µM) or by ara-C (K_{i} = 0.08 µM); [3H]dThd incorporation (K_{m} = 1.2 µM) was noncompetitively inhibited by FIAC (K_{i} = 11.1 µM) or by ara-C (K_{i} = 0.02 µM). For P815/ara-C cells, [3H]dCyd incorporation (K_{m} = 1.2 µM) was 10-fold less than for P815/0 cells and the incorporation was also competitively inhibited by FIAC (K_{i} = 19.8 µM), whereas [3H]dThd incorporation (K_{m} = 0.66 µM) was relatively inert to FIAC (K_{i} = 1334 µM).

Analogs as substrate. Comparison was made for the relative rates of incorporation for [2-14C]FIAC with the same concentration of [3H]dCyd and [3H]dUrd in P815/0 cells (Table 3). The rates of incorporation were [3H]dCyd > [3H]dUrd > [2-14C]FIAC. It is of interest to note that [2-14C]FMAU incorporation in P815/0 cells was 0.95 ± 0.2 pmol/10^6 cells/15 min which was similar to that of [2-14C]FIAC (0.91 pmol/10^6 cells/15 min). [3H]dCyd, [2-14C]FIAC, and [3H]dUrd incorporation was more strongly inhibited by FMAU than that by FIAC, FIAU, or FIAC. Competition by Natural Nucleoside. Although [2-14C]FIAC and [2-14C]FMAU were incorporated to a similar extent into the DNA of P815/0 cells (Chart 3), the cells exhibited a marked difference in their susceptibility to the natural nucleosides, dCyd and dThd. The incorporation of radioactivity of [2-14C]FIAC was competitively inhibited by dCyd (K_{i} = 1.1 µM) but was rather insensitive toward dThd (K_{i} = 66.7 µM). By contrast, [2-14C]FMAU incorporation was potently inhibited by dThd (K_{i} = 0.9 µM) and by dCyd (K_{i} = 1.3 µM), both in a competitive manner. Incorporation of [2-14C]FMAU radioactivity into the DNA of L1210/ara-C cells was 50-fold higher than that of [2-14C]FIAC (Chart 4). Since ara-C cells are known to be deficient in dCyd kinase (14, 24, 30, 31) and since FIAC behaves metabolically like dCyd, the poor incorporation for [2-14C]FIAC is not unexpected. In L1210/ara-C cells, its incorporation was competitively inhibited by dCyd; by contrast, dThd was not a competitive inhibitor. For [2-14C]FMAU radioactivity incorporation, dThd served as a potent competitive inhibitor. Surprisingly,
Effects of FMAU and FIAC on ara-C-resistant Cells

A. P815/0  
B. P815/Ara-C

Chart 1. Median effect plot for dose-effect relationships of arabinofuranosylnucleosides for inhibiting [3H]dThd incorporation into DNA in P815 cells sensitive (A) and resistant (B) to ara-C.

Table 1

The median effect concentrations (ED₅₀) for inhibiting precursor incorporation into DNA of cells sensitive and resistant to ara-C by various arabinofuranosyl nucleosides

The incorporation of radioactive precursor into acid-insoluble DNA fraction is described in "Materials and Methods." In control experiments where no inhibitor was present, the incorporation of [3H]dThd and [3H]dAdo into DNA averaged 6.3 and 0.15 pmol/10⁸ cells/30 min, respectively. The dose-effect relationships were analyzed by the median effect plots (see Chart 1) and calculated by a rearranged median effect equation (Equation B):

\[ Dₐₒ = \frac{\text{antilog}(-y \text{ intercept/m})}{m} \]

where \( Dₐₒ \) is ED₅₀ and m is the regression slope of the plot.

<table>
<thead>
<tr>
<th>Cells</th>
<th>Labeled precursor</th>
<th>ara-C</th>
<th>FMAU</th>
<th>FIAC</th>
<th>FEAU</th>
<th>ara-U</th>
</tr>
</thead>
<tbody>
<tr>
<td>L1210/O</td>
<td>[3H]dThd</td>
<td>0.20 ± 0.15</td>
<td>32 ± 20</td>
<td>113 ± 68</td>
<td>363</td>
<td>560</td>
</tr>
<tr>
<td></td>
<td>[3H]dAdo</td>
<td>0.13 ± 0.03</td>
<td>25 ± 5</td>
<td>163 ± 39</td>
<td>4010 ± 2600</td>
<td></td>
</tr>
<tr>
<td>L1210/ara-C</td>
<td>[3H]dThd</td>
<td>3900 ± 1100</td>
<td>17</td>
<td>112</td>
<td>&gt;10,000</td>
<td>6300</td>
</tr>
<tr>
<td></td>
<td>[3H]dAdo</td>
<td>10,000</td>
<td>25</td>
<td>&gt;10,000</td>
<td>3200</td>
<td></td>
</tr>
<tr>
<td>P815/0</td>
<td>[3H]dThd</td>
<td>0.045 ± 0.044</td>
<td>12 ± 4</td>
<td>45</td>
<td>88 ± 25</td>
<td>630</td>
</tr>
<tr>
<td></td>
<td>[3H]dAdo</td>
<td>0.16 ± 0.13</td>
<td>22 ± 5</td>
<td>180 ± 12²</td>
<td>&gt;10,000²</td>
<td></td>
</tr>
<tr>
<td>P815/ara-C</td>
<td>[3H]dThd</td>
<td>1350</td>
<td>7</td>
<td>&gt;10,000</td>
<td>500</td>
<td>4,470</td>
</tr>
<tr>
<td></td>
<td>[3H]dAdo</td>
<td>324</td>
<td>19 ± 8</td>
<td>&gt;10,000</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*a Mean ± S.D. for 2 experiments; each experiment used 4 to 6 concentrations of inhibitor.

*b Mean ± S.D. of 3 experiments; each experiment used 4 to 6 concentrations of inhibitor.

Table 2

Inhibition of [3H]dCyd and [3H]dThd incorporation into DNA by FIAC

Vₘₐₓ is abbreviations for maximal rate, half-saturation concentration, and inhibition constant, respectively, for the cellular biochemical studies which are not necessarily identical to those determined in pure enzyme systems. Kᵢ is also referred to as inhibition index (5) which can be calculated from the double-reciprocal plot by:

\[ Vₘₐₓ = \frac{\text{concentration of inhibitor used}}{(\text{slope in presence of inhibitor}) / (\text{slope in absence of inhibitor}) - 1} \]

Vₘₐₓ, Kᵢ, and Kᵢ are abbreviations for maximal rate, half-saturation concentration, and inhibition constant, respectively, for the cellular biochemical studies which are not necessarily identical to those determined in pure enzyme systems. Kᵢ is also referred to as inhibition index (5) which can be calculated from the double-reciprocal plot by:

\[ \text{Vₘₐₓ} = \frac{\text{concentration of inhibitor used}}{(\text{slope in presence of inhibitor}) / (\text{slope in absence of inhibitor}) - 1} \]

¥ Mean ± S.D. of 2 experiments. Other are calculated results derived from a single experiment using 4 to 6 data points.

dCyd did not inhibit [2-¹⁴C]FMAU incorporation in L1210/ara-C cells but enhanced its incorporation (Chart 4B).

In order to reexamine this unexpected observation, incorporation of [2-¹⁴C]FMAU was further analyzed in L1210/0 (Chart 4C) and in L1210/ara-C cells (not shown). The maximal rate of incorporation of [2-¹⁴C]FMAU radioactivity at a saturating concentration of FMAU was nearly identical for both L1210/0 and L1210/ara-C cells (1.0 and 1.2 pmol/10⁸ cells/45 min, respectively). The former was competitively inhibited by both dCyd (Kᵢ = 0.34 μM) and dThd (Kᵢ = 0.37 μM) (Chart 4D). L1210/ara-C cells, by contrast, were competitively inhibited by dThd (Kᵢ = 2.8 μM) but not by dCyd. In fact, an enhancement of [2-¹⁴C]FMAU incorporation by dCyd was again observed.

Effects on dUrd Metabolism. Inhibition by FIAC and FMAU of tritium release from [5-³H]dUrd and inhibition of incorporation...
Chart 2. Double-reciprocal plots for inhibition of incorporation of [3H]dCyd and [3H]dThd into DNA in P815/0 and P815/ara-C cells. A, P815/0 cells, dCyd versus FIAC; B, P815/0 cells, dThd versus FIAC; C, P815/ara-C cells, dCyd versus FIAC; D, P815/ara-C cells, dThd versus FIAC; E, P815/0 cells, dCyd versus ara-C; F, P815/0 cells, dThd versus ara-C. FIAC concentration was 100 µM and ara-C was 0.1 µM. Note that [3H]dCyd incorporation into P815/ara-C was a particularly low amount. The kinetic parameters for inhibition are summarized in Table 2.

Table 3
Effects of 2'-fluoro-arabinofuranosylpyrimidine on incorporation of labeled nucleosides into acid-insoluble fraction in P815/0 cells

<table>
<thead>
<tr>
<th>Substrate (10 µM)</th>
<th>Control</th>
<th>+0.9% NaCl solution</th>
<th>+FIAC (100 µM)</th>
<th>+FIAU (100 µM)</th>
<th>+FMAC (100 µM)</th>
<th>+FMAU (100 µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>[3H]dCyd pmol/10^6 cells/15 min</td>
<td>0.91</td>
<td>15.0</td>
<td>14.3</td>
<td>16.4</td>
<td>15.0</td>
<td>16.4</td>
</tr>
<tr>
<td>[3H]dUrd pmol/10^6 cells/15 min</td>
<td>4.3</td>
<td>4.1</td>
<td>2.9</td>
<td>4.1</td>
<td>4.1</td>
<td>4.1</td>
</tr>
</tbody>
</table>

Means of 3 measurements. The maximum range around any value was ±8%.

of [2-¹⁴C]dUrd into DNA were studied. The tritium release involves dThd kinase and TMP synthetase (19, 22, 28), whereas the incorporation into DNA, in addition to these 2 early metabolic steps, also involves nucleotide kinases and DNA polymerase. Thus, by analyzing overall inhibition (inhibition of incorporation into DNA) and the inhibition of early steps (dThd kinase and/or TMP synthetase), the inhibition to the later steps (nucleotide kinases and/or DNA polymerase) can be estimated

Chart 3. Double-reciprocal plot for inhibiting incorporation of radioactivity of [2-¹⁴C]FIAC and [2-¹⁴C]FMAU into DNA of P815/0 cells by dCyd or dThd. Note that incorporation of radioactivity of [2-¹⁴C]FIAC was strongly inhibited by dCyd, the inhibition index (5) (K_i = 1.1 µM) but not appreciably by dThd (K_i = 66.7 µM). In contrast, the incorporation of radioactivity of [2-¹⁴C]FMAU was strongly inhibited by both dThd (K_i = 0.9 µM) and dCyd (K_i = 1.3 µM).
Effects of FMAU and FIAC on ara-C-resistant Cells

L1210/Ara-C

![Graph A](image)

L1210/0

![Graph B](image)

C

![Graph C](image)

Chart 4. Double-reciprocal plots for incorporation of radioactivity of [2-14C]FIAC (A) and [2-14C]FMAU (B) into DNA of L1210/ara-C cells and [2-14C]FMAU into DNA of L1210/0 cells (C) and the effects of dCyd and dThd on their incorporation. The following parameters were obtained for [2-14C]FIAC in L1210/ara-C cells; $V_m = 0.02$ pmol/10^6 cells/45 min; $K_m = 11.4 \mu M$; $K_i$ for dThd = 12.9 $\mu M$ and for dCyd = 2.0 $\mu M$. For [2-14C]FMAU in L1210/ara-C; $V_m = 1.2$ pmol/10^6 cells/45 min; $K_m = 3.3 \mu M$; $K_i$ for dThd = 2.8 $\mu M$ and $K_i$ for dCyd activation = 3.9 $\mu M$. For [2-14C]FMAU in L1210/0; $V_m = 1$ pmol/10^6 cells/45 min; $K_m = 2.0 \mu M$; $K_i$ for dThd = 0.37 $\mu M$ and for dCyd = 0.34 $\mu M$. The activation index ($K_i$) was calculated by:

$$
\text{concentration of activator/slope in absence of activator/slope in presence of activator} - 1
$$

Other parameters are defined in the legend to Table 2.

### Table 4

Inhibition of tritium release from [5-3H]dUrd and inhibition of incorporation of [2-14C]dUrd into DNA by FIAC and FMAU

<table>
<thead>
<tr>
<th>Cells</th>
<th>Experiment</th>
<th>Observed tritium release from [5-3H]dUrd</th>
<th>Observed incorporation of [2-14C]dUrd into DNA</th>
<th>Calculated % of inhibition of [2-14C]dUrd anabolism at DNA polymerase/nucleotide kinase steps a</th>
</tr>
</thead>
<tbody>
<tr>
<td>L1210/0</td>
<td>0.9% NaCl solution</td>
<td>1103 ± 47b</td>
<td>463 ± 18</td>
<td>16.8</td>
</tr>
<tr>
<td></td>
<td>+FIAC</td>
<td>29.1 ± 2.0</td>
<td>34.3 ± 4.8</td>
<td>59.9</td>
</tr>
<tr>
<td></td>
<td>+FMAU</td>
<td>19.0 ± 1.7</td>
<td>66.6 ± 1.4</td>
<td></td>
</tr>
<tr>
<td>L1210/5FU</td>
<td>0.9% NaCl solution</td>
<td>2263 ± 91</td>
<td>865 ± 2</td>
<td>18.0</td>
</tr>
<tr>
<td></td>
<td>+FIAC</td>
<td>33.2 ± 3.1</td>
<td>45.2 ± 1.1</td>
<td>70.9</td>
</tr>
<tr>
<td></td>
<td>+FMAU</td>
<td>9.4 ± 2.1</td>
<td>73.6 ± 0.4</td>
<td></td>
</tr>
<tr>
<td>L1210/ara-C</td>
<td>0.9% NaCl solution</td>
<td>1536 ± 90</td>
<td>713 ± 6</td>
<td>81.3</td>
</tr>
<tr>
<td></td>
<td>+FIAC</td>
<td>0.3 ± 0.3</td>
<td>0 ± 0.5</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>+FMAU</td>
<td>0 ± 0.6</td>
<td>81.3 ± 5.7</td>
<td></td>
</tr>
</tbody>
</table>

a Calculated by $(O_2 = 1 - [(1 - (\theta_a/\theta_d)](1 - (\theta_a/\theta_d))$, or $(1 - \theta_a, \theta_d) = (1 - (\theta_d/\theta_a))$, where $\theta$ is the fractional inhibition (see Ref. 10).

b Mean ± S.D.

### DISCUSSION

The present studies have shown that FMAU, unlike ara-C and FIAC, is an effective inhibitor of [3H]dThd incorporation into DNA in cell lines resistant to ara-C. Other nucleoside analogs tested were either less potent than FMAU or are cross-resistant to ara-C.

These results are in agreement with a preliminary paper (2) in which we have shown that FIAC is totally inactive in concentrations as high as 100 $\mu$g/ml against the growth of 2 lines resistant to ara-C, P815/ara-C and L5178Y/ara-C. On the other hand, FIAU and particularly FMAU are active against the growth of the L5178Y/ara-C and P815/ara-C. In addition, our preliminary results in vivo (2) also indicate that FIAC at 1000 mg/kg daily for 5 days has no significant effect in mouse leukemias P388, L1210, and P815. FMAU, on the other hand, is not only moderately effective against L1210 and P388 but is also particularly effective against P815/ara-C leukemia.

Based on the competitive inhibition pattern between FIAC and...
and dCyd given above (Charts, 2, A and C, and 3A) and based on the fact that L1210/ara-C cells are deficient in dCyd kinase (14, 30, 31), it can be concluded that dCyd kinase is a prerequisite for FIAC activity. L1210/ara-C data in Table 4 further support this proposition. Ruth and Cheng (29) reported that FMAU triphosphate and FIAC triphosphate inhibited DNA polymerase isolated from human HeLa cells to similar extents and that FMAU triphosphate has little ability to support DNA synthesis in the absence of competing substrate (dTTP or dCTP). These data suggest that HeLa cells are metabolically quite different from L1210 cells. It is also possible that data from the cell-free system cannot be extrapolated to the intact-cell system.

The incorporation of radioactivity of [2-14C]FMAU in P815/0 cells is competitively inhibited by both dThd (Ki = 0.9 μM) and dCyd (Ki = 1.3 μM), whereas the incorporation of radioactivity of [2-14C]FIAC is competitively inhibited by dCyd (Ki = 1.2 μM) but not appreciably by dThd (Chart 3). These low Ki values for inhibition by dCyd or dThd or both suggest that physiological levels of these substances may have a profound effect on [2-14C]FIAC and [2-14C]FMAU radioactivity incorporation in vivo. The dThd levels in serum vary in animal species (25). In mice, rats, monkeys, dogs, and humans, they are 0.68, 0.85, 0.23, 0.095, and 0.066 μM, respectively. The levels for dogs and humans are particularly low. If incorporation of FMAU into DNA is correlated to its cytotoxic effects, it can be expected that FMAU would be more toxic to dogs and humans than in other species. In another study (13), the levels of dCyd and dThd in rat plasma have been found to be 22 and 2 μM, respectively, which concentrations are considerably higher than or similar to the Ki values for dCyd and dThd given above. The low Ki values (see Charts 3 and 4) also suggest that exogenous dCyd or dThd may be used to modulate or rescue the effects of FIAC or FMAU in vivo.

The inhibition patterns of these studies have led to the conclusion that FIC behaves metabolically like dCyd in P815/0 (Charts 2A and 3A), P815/ara-C (Chart 2C), and L1210/ara-C (Chart 4A) cells. By contrast, FMAU behaves metabolically like dThd in P815/0 (Chart 3B), L1210/0 (Chart 4C), and L1210/ara-C (Chart 4B) cells. Interestingly, [3H]dThd incorporation was not inhibited by FIC in P815/ara-C cells (Chart 2D) and [2-14C]FMAU incorporation in L1210/0 cells (Chart 3A). Thus, dThd kinase or subsequent incorporation of TTP into DNA was not affected by FIC in these cells. In L1210/ara-C cells (Chart 4A), incorporation of [2-14C]FIC was very low, presumably due to deficiency of dCyd kinase (30, 31) which may also phosphorylate FIC. As shown in Chart 2, E and F, ara-C follows the same inhibitor pattern as FIAC in P815/0 cells. The incorporation of [2-14C]FIC in L1210/ara-C cells was low and only weakly and noncompetitively inhibited by dThd. The incorporation of [2-14C]FMAU was competitively inhibited by dCyd in P815/0 (Chart 3B) or L1210/0 (Chart 4C) cells, suggesting that FMAU, like dCyd, may be alternatively metabolized. [2-14C]FMAU incorporation was not inhibited by dCyd in L1210/ara-C cells (Chart 4B), and to some extent, potentiation of incorporation by dCyd kinase was observed (Chart 4B). The mechanism of this potentiation is not yet clear, but it may be related to the dual metabolic pathways for FMAU and to the deficiency in dCyd kinase in L1210/ara-C cells. These data indicate a basic and qualitative metabolic difference between ara-C-sensitive and -resistant cells. These observations also suggest that dCyd may be potentially useful to modulate and to increase therapeutic response to FMAU in ara-C-resistant tumors, while protecting host proliferative tissues which are known to be sensitive to ara-C.

It should be noted that the radioactivity of [2-14C]FIAC that was incorporated into the DNA of P815 cells was identified as FIAU by high-pressure liquid chromatography after purification of DNA and its enzymatic digestion to nucleosides (15, 20). A detectable amount of radioactivity of [2-14C]FIAC and [2-14C]FMAU appeared in the acid-insoluble 0.3 m KOH digestible fraction (e.g., the RNA fraction); this incorporation was not affected by 1 mM 3,4,5,6-tetrahydrodridine. The identity of the radioactivity of [2-14C]FMAU in DNA and RNA fractions remains to be determined.

Of particular interest is the finding that FMAU, FIAU, 2'-fluoro-1-β-o-arabinofuranosycytosine, and 2'-fluoro-1-β-d-arabinofuranosyluracil are metabolites of FIAC in vivo, which appeared in acid-soluble fractions of blood, small intestine, and liver in mice (6, 26). All of these metabolites are pharmacologically active agents (16, 32). In fact, the present study indicates that the metabolites of FIC, such as FIAU and FMAU, are more potent inhibitors of DNA synthesis than is FIAC (Charts 1 and 2). It appears, therefore, that FIAC may exert its effect in vivo because of its metabolites. The present in vitro studies which have incubation times of 15 or 45 min and the inclusion of a pyrimidine nucleoside inhibitor, 3,4,5,6-tetrahydridouridine, in the reaction mixture may have minimized the formation of FIC metabolites.

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Effects of FMAU and FIAC on ara-C-resistant Cells


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