METHOTREXATE (MTX), a key drug in the curative regimen of children with acute lymphocytic leukemia (ALL), is less effective in the treatment of acute myeloid leukemia (AML). An important correlate of this difference in sensitivity to MTX was found to be the high levels of MTX accumulation in pre-B ALL cells as compared with AML cells, due to polyglutamylation of this drug. However, as has been observed previously, there are some myeloid cell lines such as THP-1 that show adequate accumulation of intracellular MTX polyglutamates and as a result are sensitive to MTX, as are approximately 10% of AML patients. Like folates, intracellular MTX is extensively metabolized to polyglutamate derivatives, which are retained within the cell in the absence of extracellular drug, whereas, under the same conditions, the parental compound rapidly effluxes from the cell. The ability to remain within the cell is influenced by the chain length so that the long chain MTX polyglutamates (three to six glutamates) are retained much longer than MTX or MTX diglutamate. MTX polyglutamates result in a prolonged dihydrofolate reductase (DHFR) inhibition, and thymidylate synthetase (TS), glycaminide ribonucleotide (GAR), and aminoimidazole carboxamidine (AICAR) transformylases are inhibited, as well.

The amount of long chain polyglutamates in cells represents a result of the balance between the activity of two different enzymes, folylpolyglutamate synthetase (FPGS), which adds up to 4 glutamyl groups in a polyglutamate form, and folylpolyglutamate synthetase. K_m values did not change with partial purification of this enzyme, indicating that interfering substances in crude lysates were not responsible for this difference. We postulate that there may be differences in the properties of this enzyme related to tissue specific expression. In contrast to MTX, both Tomudex (Zeneca Pharmaceuticals, Wilmington, DE) and 1843U89, potent inhibitors of thymidylate synthetase, have low K_m values for folylpolyglutamate synthetase, and polyglutamate forms of these inhibitors are accumulated to the same degree in both myeloid and lymphoid acute leukemia cells, paralleling the equivalent cytotoxicity found between myeloid and lymphoid leukemia cell lines. Based on these results, we believe a clinical trial of Tomudex in patients with acute myeloid leukemia is warranted.

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intracellular levels of MTX, Tomudex, and 1843U89 and their polyglutamates were measured by exposing cell lines to 1 μmol/L radiolabeled drug for 24 hours. The cells were then washed twice with PBS at 4°C and the pellets were suspended in 0.5 mL of boiling 50 mmol/L sodium phosphate (pH 5.5), boiled for an additional 5 minutes and centrifuged at 15,000 g for 10 minutes at 4°C. Supernatants were stored at −20°C until high performance liquid chromatography (HPLC) analysis was performed. HPLC separations were done on an absorbosphere C18 column (5-μm particle size, 4.6 × 250 mm) equipped with a 1 cm guard cartridge column of the same material from Alltech Associates (Deerfield, IL) using a Spectra Physics 8800 HPLC gradient pump from Thermo Separation (Fullerton, CA).^{18}

**FPGS assay.** FPGS activity in cell extracts was determined as previously described.^{10,20} Crude extracts were prepared from cell pellets by suspension in 5 vol of extraction buffer (50 mmol/L Tris-HCl, 20 mmol/L KCl, 10 mmol/L MgCl₂, 5 mmol/L dihydrotestosterone, pH 7.6) at 4°C, sonication (5 seconds × 3 at intervals of 30 seconds) and centrifugation (4°C) at 20,000 g for 20 minutes. The FPGS assay mix contained, in a final volume of 250 μL and at pH 8.4, 100 mmol/L Tris-HCl (pH 8.85), 10 mmol/L adenosine triphosphate (ATP), 20 mmol/L MgCl₂, 20 mmol/L KCl, 10 mmol/L dihydrotestosterone (DTT), 4 mmol/L L-[3H]glutamate, enzyme and substrate at different concentrations (either MTX, Tomudex, or 1843U89). The reactions were stopped after 2 hours by the addition of 1 mL of 5 mmol/L L-glutamate (pH 7.5) at 4°C. The unreacted [3H]glutamate was separated from the polyglutamates by a Sep-Pack C₈ cartridge reversed phase chromatography from Waters (Milford, MA). Activity of FPGS was expressed in pmol [3H]glutamate incorporated/mg protein/hour at 37°C.

**Partial purification of polyglutamate synthetase.** FPGS was partially purified based on previously described methods.^{10,12} Cell extracts were prepared as follows: 9 × 10⁷ cells (CCRF-CEM) and 12 × 10⁷ cells (K562) were suspended in 40 mL and 65 mL extraction buffer, sonicated as described and centrifuged at 20,000 g for 20 minutes. Streptomycin sulfate at a final concentration of 1% was added to the supernatant solution and the mixture stirred for 1 hour. The precipitate was removed by centrifugation at 16,000g for 30 minutes and discarded. Solid ammonium sulfate was slowly added to the streptomycin sulfate supernatants with stirring at 4°C until 40% saturation was reached. The solution was stirred for 1 hour and then centrifuged for 30 minutes at 27,000 g. The pellets were redissolved in 12 mL and 16 mL extraction buffer, respectively. After overnight dialysis against one change of 5 L of Tris-HCl 50 mmol/L, DTT 5 mmol/L, and benzamidine 10 mmol/L pH 7.5 buffer, the dialyzed fractions were applied to P11 phosphocellulose (Whatman Ltd, Maidstone, UK) columns that had been equilibrated with the same buffer. The columns were eluted with a linear gradient of KCl (0 to 200 mmol/L) in the same buffer. Enzyme activity was detected in one peak in both preparations.

**Patient samples.** Leukemia cells were obtained from heparinized bone marrow aspirates or leukapheresis collections after obtaining written informed consent. The samples contained greater than 90% blasts cells. Mononuclear cells were isolated using a Ficoll Hypaque centrifugation gradient at 4°C and washed twice with PBS. The cells for the FPGS assay were pelleted and stored at −70°C until analysis. Cells were resuspended in RPMI 1640 with 10% dFBS and 1% glutamine, for the polyglutamylation assay.

**Subcellular fractionation.** For the subcellular fractionation of CCRF-CEM and K562 cells and the measurement of subcellular distribution of FPGS, we used a modification of the method described by Lin et al^{21} and the same procedures for the enzymatic determination of the mitochondrial, cytosolic, and lysosomal markers. Briefly, cells were pelleted by centrifugation at 300g for 5 minutes and resuspended in homogenization solution (HMS, 250 mmol/L sucrose, 1 mmol/L EDTA, pH 6.9). The cells were disrupted in a nitrogen cavitation device after exposing them to a pressure of 360 p.s.i. for 30 minutes and then centrifuged at 900g for 6 minutes to allow sedimentation of nuclei and unbroken cells. The supernatant was transferred to another tube and stored on ice, the pellet (nuclear fraction) was washed and resuspended in HMS. The second supernatant was combined with the first and then centrifuged. The supernatant (total postnuclear supernatant, PNS) was centrifuged at 10,000g for 15 minutes and the pellet was stored on ice. The supernatant was recentrifuged at 10,000g for 15 minutes to give a final supernatant (cytosolic fraction). The two last pellets were combined and resuspended in HMS to obtain the mitochondrial fraction.

**RESULTS**

Four cell lines, two lymphoid (CCRF-CEM and MOLT-3) and two myeloid (K562 and HL-60) were studied. CCRF-CEM, MOLT-3, and HL-60 were found to be more sensitive to 24-hour exposure of MTX, as compared with K562 cells, varying from 4- to 15-fold, as measured by IC₅₀ values (Table 1). In contrast, all four cell lines were equally and much more sensitive to Tomudex and to 1843U89 than MTX (Table 1). When polyglutamate levels were measured after incubation with radiolabeled MTX, Tomudex, and 1843U89, respectively, the lowest levels of MTX polyglutamates were found in K562 cells, while a high percentage of Tomudex and 1843U89 (80% or greater) were found as polyglutamates in all the cell lines. All four cell lines did not show any difference in MTX uptake as measured by the PT430 displacement assay.^{22} Differences in DHFR protein levels were not found by Western blot analysis (data not shown).

In CCRF-CEM, MOLT-3, and HL-60 cells, the predominant polyglutamate form of MTX was MTX glu₄, and MTX glu₄, while the predominant form of MTX in K562 cells was MTX glu₁. Tomudex polyglutamate forms in all four cell types were glu₆, and glu₄, while 1843U89 diglutamates were found almost exclusively in the four leukemia cell types (data not shown). Of the three drugs, 1843U89 is clearly the most potent and correlated with high levels of the diglutamate of this drug achieved in all four cell lines. As cytotoxicity of K562 cells to MTX was much less than the other three cell types and was correlated to low levels of MTX polyglutamates formed, we then attempted to understand the basis for these results. The activity of FPGS in crude extracts of K562 cells was similar to that in CCRF-CEM and MOLT-3 cells, (1,220 ± 30 v 1,380 ± 180 and 1,140 ± 40 pmol/mg/h, respectively), but lower than that in HL-60 cells (3,330 ± 880 pmol/mg/h), indicating that the level of this enzyme activity was not responsible for the low level of polyglutamates formed. When γ-glutamyl hydrolase activity was measured in extracts of the four cell lines, K562 cells were found to have a lower level of this activity when compared with CCRF-CEM cells (data not shown), therefore, the lack of accumulation of MTX polyglutamates in K562 cells could not be attributed to increased MTX polyglutamate breakdown.

MTX is transported into leukemia cells via the reduced
Materials and Methods.

Limited by this transport process. As has been observed in ence in the \( K_m \) value between the two cell lines persisted, is important for conversion of folate antagonists to polygluta-

30-fold to 80-fold purification following chromatography on

centrifugation of the crude extract of the two cell lines (see

extracts were prepared as described in Materials and Methods. Substrates at different concentrations were used to measure enzyme activity

doing enzyme activity and to determine \( K_m \) and \( V_{\text{max}} \) values. Polyglutamates formation was measured after 24-hour exposure to radiolabeled MTX as described in Materials and Methods.


to determine \( K_m \) and \( V_{\text{max}} \) values. Polyglutamates formation was measured after a 24-hour exposure to radiolabeled drugs as also described in Materials and Methods. Cells extracts were prepared as described in Materials and Methods. Substrates at different concentrations were used to measure enzyme activity

between the two cell lines was also investigated. After differential centrifugation of the crude extract of the two cell lines (see Materials and Methods), we detected activity only in the cytosolic fraction, \( K_m \) values did not change in the two cell lines as

dmissible to the differences were due to inhibitors or stimulating substances present in the crude extracts (data not shown).

The possibility that differing proportions of cytosolic and mitochondrial FPGS enzyme with different properties could explain the different kinetic parameters of the enzyme in the two cell lines was also investigated. After differential centrifugation of the crude extract of the two cell lines (see Materials and Methods), we detected activity only in the cytosolic fraction and no activity was found in the mitochondrial or the nuclear fractions. When we measured the kinetic parameters of FPGS with MTX as a substrate in the cytosolic fraction, \( K_m \) values did not change in the two cell lines as compared with the values obtained with the crude extracts.

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>( K_m ) (( \mu \text{mol/L} ))</th>
<th>( V_{\text{max}} ) (pmol/mg/h)</th>
<th>Long Chain MTX Polyglutamates (pmol/10⁷ cells)</th>
<th>Long Chain MTX Polyglutamates as % of Total Polyglutamates</th>
</tr>
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<tbody>
<tr>
<td>ALL</td>
<td>48</td>
<td>14,450</td>
<td>9</td>
<td>90</td>
</tr>
<tr>
<td>ALL</td>
<td>23</td>
<td>1,065</td>
<td>12.7</td>
<td>66</td>
</tr>
<tr>
<td>ALL</td>
<td>47</td>
<td>1,832</td>
<td>16.3</td>
<td>86</td>
</tr>
<tr>
<td>Lymphoid BC/CML</td>
<td>45</td>
<td>6,600</td>
<td>30.8</td>
<td>88</td>
</tr>
<tr>
<td>AML</td>
<td>75</td>
<td>1,490</td>
<td>3.36</td>
<td>24</td>
</tr>
<tr>
<td>AML</td>
<td>83</td>
<td>2,030</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>AML</td>
<td>100</td>
<td>1,000</td>
<td>ND</td>
<td>ND</td>
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<th>Diagnosis</th>
<th>( K_m ) (( \mu \text{mol/L} ))</th>
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Cell lines were exposed to different concentrations of these drugs and cell viability measured as described in Materials and Methods. Polyglutamates formation was measured after 24-hour exposure to radiolabeled drugs as also described in Materials and Methods. Cells extracts were prepared as described in Materials and Methods. Substrates at different concentrations were used to measure enzyme activity

determine \( K_m \) and \( V_{\text{max}} \) values. Polyglutamates formation was measured after 24-hour exposure to radiolabeled MTX as described in Materials and Methods.

Abbreviation: ND, not done (insufficient sample).
To investigate if this difference in FPGS $K_m$ values extended to fresh leukemia samples, we compared the $K_m$ and the $V_{max}$ of FPGS with MTX as a substrate in blasts from patients: three with AML, three with ALL, and from one patient with blastic crisis of chronic myeloid leukemia in which 90% of the blast cells expressed lymphoid markers (Table 2). Five of the seven patient samples were tested for polyglutamylation (Table 2). The PT430 competitive displacement flow cytometric assay performed on these blasts did not show impaired MTX transport (data not shown). PT430 accumulation has been used to measure DHFR levels, as well, and no evidence of increased DHFR binding was observed. The $K_m$ values obtained from extracts from blast cells from the four patients with lymphocytic leukemia were similar to those obtained for the CCRF-CEM cell line (40 ± 12 and 44 $\mu$mol/L, respectively), while the AML blast samples had values similar to those of K562 cell line (86 ± 12 and 92 ± 9 $\mu$mol/L, respectively) (Figs 1 and 2).

**DISCUSSION**

AML is considered intrinsically resistant to therapy with MTX.\(^1\)\(^-\)\(^3\)\(^,\)\(^7\) The reason for this resistance is believed to be the reduced formation of long chain MTX polyglutamates.\(^4\) When we studied MTX polyglutamylation in cell lines, we observed that the K562 cell line accumulated a lower amount of long chain polyglutamates than the other cell lines. The observation that FPGS activity was not low and the $\gamma$-glutamyl hydrolase activity in the K562 cells was not higher than the CCRF-CEM cell line prompted us to study the properties of FPGS in these cell lines and in fresh leukemia cells.

The consistent finding that emerged from our kinetic studies was the different $K_m$ values of FPGS with MTX as substrate between the AML and the ALL cell lines, which suggested that the enzyme may be different in the two forms of leukemia. The finding that the AML patient samples had a $K_m$ value similar to the AML cell line, whereas the ALL patients sample were similar to the ALL cell line, showed this did not occur solely in cell lines. Of interest is the lower $K_m$ also found from blast cells from the patient with blast crisis of chronic myelogenous leukemia, consistent with the diagnoses of a blast crisis of the lymphoid type.

The different $K_m$ values for FPGS between AML and ALL raised the possibility that there are two different enzymes or an alternative possibility examined was that an inhibitor of
FPGSs may be present in myelocytic leukemia. This latter hypothesis is made unlikely by the unchanged $K_m$ values we obtained after partial purification of the FPGS proteins from AML and ALL cells. It is also unlikely that differences in enzyme properties from mitochondria and the cytoplasm accounts for the results observed, as neither cell type was found to have appreciable mitochondrial enzyme activity.

Our findings of different FPGS activity in AML and ALL with different affinities for MTX may be explained by studies performed by Chen et al. in which alternative splice variants of the FPGS gene were identified. These additional splice variants may be responsible for the tissue-specific encoding of different isoforms of FPGS, which may explain the differences observed by us in leukemias of different cellular origin. We are currently in the process of examining AML and ALL cells for alterations in RNA expression level of these different isoforms.

The finding of a low $K_m$ for FPGS when using Tomudex or 1843U89 as a substrate in both leukemia cells and efficient formation of polyglutamates of tomudex and 1843U89 provides an explanation for the potency of these TS inhibitors in both leukemia subtypes, as compared with MTX. These new TS inhibitors may therefore be effective in the treatment of AML, as well as in ALL, in contrast to what is observed with MTX. Based on these findings, a clinical trial of Tomudex in the treatment of AML is planned at our institution.

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20. Jansen G, Schornagel JH, Kathmann I, Westerhof GR, Hor dijk GS, van der Laan BFAM: Measurement of folylpolyglutamate formation of polyglutamates of tomudex and 1843U89 provides an explanation for the potency of these TS inhibitors in both leukemia subtypes, as compared with MTX. These new TS inhibitors may therefore be effective in the treatment of AML, as well as in ALL, in contrast to what is observed with MTX. Based on these findings, a clinical trial of Tomudex in the treatment of AML is planned at our institution.
Disparate Affinities of Antifolates for Folylpolyglutamate Synthetase From Human Leukemia Cells

Giuseppe S.A. Longo, Richard Gorlick, William P. Tong, Emine Ercikan and Joseph R. Bertino