Ex Vivo Activity of Methotrexate versus Novel Antifolate Inhibitors of Dihydrofolate Reductase and Thymidylate Synthase against Childhood Leukemia Cells

Robert Mauritz, Marcel W. Bekkenk, Marianne G. Rots, Rob Pieters, Enrico Mini, Christina H. van Zantwijk, Anjo J. P. Veerman, Godfried J. Peters, and Gerrit Jansen

Departments of Medical Oncology [R. M., M. G. R., G. J. P., G. J.] and Pediatric Hematology/Oncology [M. W. B., M. G. R., R. P., C. H. V. Z., A. J. P. V.], University Hospital Vrije Universiteit, 1081 HV Amsterdam, the Netherlands, and Department of Preclinical and Clinical Pharmacology, University of Florence, 50134 Florence, Italy [E. M.]

ABSTRACT

Leukemic cells of 27 children [14 patients with initial acute lymphoblastic leukemia (iALL), 8 patients with relapsed ALL (rALL), and 5 patients with acute nonlymphoblastic leukemia (ANLL)] were evaluated for their sensitivity to methotrexate (MTX) and five novel antifolate drugs, which have the potential to circumvent MTX resistance. The novel antifolates include a polyglutamatable [edatrexate (EDX)] and a lipophilic (trimetrexate) inhibitor of dihydrofolate reductase and two polyglutamatable inhibitors (ZD1694 and GW1843U89) and one lipophilic inhibitor (AG337) of thymidylate synthase (TS).

Drug activity was assessed via the determination of in situ inhibition of TS activity after exposing leukemic cells to antifolate drugs for: (a) 3 h, followed by a 15-h drug-free period; and (b) 18 h of continuous exposure. For human CEM leukemia cell lines with well-defined mechanisms of resistance to MTX, in situ TS inhibition correlated with the growth-inhibitory effects of MTX and the novel antifolates (r = 0.86–0.93; P < 0.01). Although a wide interpatient variability in MTX sensitivity was observed within the three leukemia groups, the median drug concentration required to inhibit TS activity to 50% of untreated controls (T50) for a 3-h exposure to MTX was similar for iALL and rALL cells but was up to 9-fold higher in ANLL cells. After a 3-h exposure, EDX, ZD1694, and GW1843U89 displayed a markedly (10–150-fold) increased potency over MTX in all leukemia groups with comparable T50 values for ANLL and iALL cells. Compared with a 3-h MTX exposure, continuous exposure resulted in lower T50 values for iALL (14-fold), rALL (14-fold), and ANLL cells (85-fold). In comparison to MTX, the T50 values in these groups were also lower for EDX (1.6–3.5-fold), ZD1694 (2.1–4.3-fold), and GW1843U89 (15–35-fold). On short-term exposure, the lipophilic drugs trimetrexate and AG337 displayed markedly less potency as compared with that of long-term exposure.

In conclusion, the efficacy of novel antifolates against childhood leukemia cells can be tested with the in situ TS inhibition assay. These novel antifolates displayed a greater efficacy than MTX against childhood leukemia cells and may have potential for the circumvention of MTX resistance in ANLL cells.

INTRODUCTION

The folate antagonist MTX3 is an essential drug in treatment regimens for children with ALL (1, 2). Nowadays, over 95% of children newly diagnosed with ALL will reach complete remission with combination chemotherapy. About 70% of these children can anticipate prolonged disease-free survival and are likely to be cured (3). However, this implies that in a considerable number of children with ALL, the leukemia relapses, despite aggressive therapy.

A small number of children with leukemia are diagnosed with ANLL. The prognosis of children with ANLL is much worse than that of those with ALL; only 30–40% of patients with childhood ANLL can anticipate event-free survival (3). ANLL cells are considered intrinsically resistant to MTX, due to a molecular mechanism that involves a decreased polyglutamylation of MTX via the enzyme FPGS and an increased breakdown of MTX polyglutamates via FPGH (4, 5).

It is generally accepted that resistance to chemotherapeutic drugs is a major reason for treatment failure. Several studies in our laboratory have demonstrated that in vitro drug resistance is correlated with a poor clinical outcome in childhood leukemia (6, 7). Unfortunately, the sensitivity of leukemic cells to MTX could not be evaluated by cell culture assays, because cell death during the incubation results in the presence of sufficient rescuing factors such as thymidine, hypoxanthine, and folates in the medium to protect the remaining cells from the cytotoxic action of MTX (5, 8–10).

3 The abbreviations used are: MTX, methotrexate; TMQ, trimetrexate; EDX, edatrexate; ALL, acute lymphoblastic leukemia; rALL, relapsed ALL; iALL, initial ALL; ANLL, acute nonlymphoblastic leukemia; DHFR, dihydrofolate reductase; TS, thymidylate synthase; FPGS, folylpolyglutamate synthetase; FPGH, folylpolyglutamate hydrolase; T50, drug concentration required to inhibit TS activity to 50% of untreated controls; RFC, reduced folate carrier; dUrd, 2′-deoxyuridine; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.
Extensive research in the last decades has elicited several mechanisms by which neoplastic cells acquire resistance to MTX \textit{in vitro} (11, 12). Four mechanisms of resistance to MTX have been characterized in detail: (a) impaired membrane transport of MTX into the cell via the RFC (13–15); (b) decreased retention of MTX due to a decrease in intracellular polyglutamylation (16, 17); (c) increased expression (18) of DHFR, the target enzyme of MTX; or (d) kinetic alterations of DHFR (19).

Studies of individual parameters of MTX resistance in leukemia cells have pointed to: (a) relatively small levels of DHFR gene amplification (2–4-fold) or 3–10-fold relative elevations in DHFR content in subpopulations of ALL cells (20, 21); (b) a heterogeneous picture of MTX transport capacities in ALL cells (20, 22–24); and (c) a proliferative and lineage-dependent activity of FPGS and FPGH (5, 25–27).

In recent years, the improved knowledge of the critical determinants of MTX sensitivity and resistance enabled and stimulated the development of novel antifolate drugs with the aim of circumventing clinical resistance to MTX (1). In addition to DHFR, other crucial enzymes in folate metabolism, such as TS, have been exploited as targets for the development of novel antifolates (28, 29). Novel antifolate-based TS inhibitors, which are currently being evaluated in clinical trials, include ZD1694 (Tomudex), GW1843U89, and AG337 (Thymitaq) (30–32). Like the DHFR inhibitor EDX (33), ZD1694 (30) and GW1843U89 (31) are more efficiently transported via the RFC and are better substrates for FPGS than MTX. AG337 (32) and the DHFR inhibitor TMQ (1) are lipophilic drugs designed to display activity against tumor cells, regardless of their RFC or FPGS status. Clinical studies indicate that some of the novel antifolates have promising activity in solid tumors (29).

In the present study, we investigated whether these new antifolate drugs displayed enhanced efficacy against leukemia, so that they may be applied to circumvent MTX resistance. Because the MTX sensitivity of leukemic blast cells could not be evaluated with cell culture assays (34, 35), we used the \textit{in situ} TS inhibition assay to determine the activity of MTX versus EDX, TMQ, ZD1694, GW1843U89, and AG337 (Fig. 1) against a panel of sensitive and MTX-resistant cultured human CCRF-CEM lymphoblasts and subsequently against clinical specimens of 27 children with ALL or ANLL.

**MATERIALS AND METHODS**

**Chemicals.** RPMI 1640 and FCS were obtained from Life Technologies, Inc. (Grand Island, NY). [5-\textsuperscript{3}H]dUrd (22 Ci/mmol) was purchased from Moravek Biochemicals, Inc. (Brea, CA). Ficoll Isopaque (Lymphoprep, 1.077 g/ml) was purchased from Nyegaard (Oslo, Norway). Earle’s balanced salt solution was obtained from NPBI International (Emmer-Compascuum, the Netherlands). All other chemicals were of the highest purity available.

**Drugs.** MTX was a gift from Pharmachemie (Haarlem, the Netherlands). EDX was a gift from Ciba-Geigy (Basel, Switzerland). ZD1694 (Tomudex; Raltitrexed) was a gift of Dr. J. J. McGuire (Grace Cancer Drug Center, Buffalo, NY). TMQ was obtained from Warner-Lambert/Parke Davis (Ann Arbor, MI).

**Cell Lines.** Human leukemia CCRF-CEM cells and MTX-resistant sublines were maintained in RPMI 1640 containing 2.2 \textmu M folic acid and supplemented with 10% FCS, 2 mm glutamine, 100 units/ml penicillin, and 100 \mu g/ml streptomycin at 37°C in a 5% CO\textsubscript{2} humidified atmosphere. The MTX-resistant sublines used in this study include: (a) CCRF-CEM/T, a RFC transport-defective cell line (36, 37); (b) CCRF-CEM/E, characterized by 18-fold DHFR gene amplification (38); and (c) CCRF-CEM/R30dm, a cell line that has 1% of parental FPGS activity (17), generously provided by Dr. J. J. McGuire (Grace Cancer Drug Center, Buffalo, NY). CCRF-CEM/T and CCRF-CEM/E were cultured in the presence of 1 and 0.1 \textmu M MTX, respectively. Before the experiments, the cells were cultured without MTX for at least three passages.

**Preparation of Patient Samples.** Leukemic cells were obtained for routine diagnostic procedures from bone marrow and peripheral blood samples of 27 children with leukemia.
Fig. 2  Outline of the in situ TS inhibition assay. 1, transport via the RFC; 2, cell entry of lipophilic compounds (passive/facilitated diffusion); 3, polyglutamylation by FPGS and breakdown by FPGH; 4, DHFR catalyzes the formation of reduced folates, which serve as cofactors in the conversion of dUMP to dTMP by TS; 5, when cells are incubated with [5-3H]dUrd, tritium will be released as [3H]H2O; 6, inhibitory effect on DHFR; 7, inhibitory effect on TS.

Mononuclear cells were isolated by centrifugation with Ficoll Isopaque (500 × g at interphase, 25 min) as described previously (34). The isolated leukemic cells were washed twice in Earle’s balanced salt solution containing 0.1% BSA and suspended in RPMI 1640 supplemented with 15% FCS, 100 IU/ml penicillin, 100 µg/ml streptomycin, 200 µg/ml gentamycin, 0.125 µg/ml fungizone, 2 mm glutamine, 5 µg/ml insulin, 5 µg/ml transferrin, and 5 ng/ml sodium selenite.

Patient Characteristics. Fourteen patients were diagnosed with iALL, eight patients were diagnosed with rALL, and five patients had ANLL. The iALL group consisted of 10 male and 4 female patients whose ages varied between 9 months and 17 years (median age, 3 years); the immunophenotypes were common ALL (n = 8), preB (n = 3), and T-ALL (n = 3), and the WBC count ranged between 2.8 and 283 × 10⁶ cells/liter (median, 14.7 × 10⁷ cells/liter). The rALL group included 7 male patients and 1 female patient whose ages ranged from 6-17 years (median age, 9 years); the immunophenotypes were common ALL (n = 5), preB (n = 1), proB (n = 1), and T-ALL (n = 1). The ANLL group consisted of three male and two female patients whose ages ranged from 3 to 14 years (median age, 11 years), and the French-American-British classifications were M1 (n = 1), M2 (n = 2), M4 (n = 1), and M5 (n = 1); the WBC count ranged from 17.4-300 × 10⁶ cells/liter (median, 31 × 10⁷ cells/liter).

TS Inhibition Assay. The in situ TS inhibition assay provides a rapid assessment of the dynamic processes of transport, polyglutamylation, and inhibition of in situ TS activity by antifolate drugs (39-41). Determination of TS in situ activity by antifolate drugs with the \(^{3}H\)H2O release assay is in essence similar to the use of the \(^{3}H\)H2O release assay for the catalytic TS assay in cell extracts, which is a routine assay in our laboratory (41, 42). Cells were incubated with [5-3H]dUrd that is intracellularly phosphorylated to [5-3H]dUMP, the substrate for TS. This enzyme catalyzes the conversion of [5-3H]dUMP to dTMP, during which reaction [3H]H2O is formed and released in the medium. Thus, the amount of [3H]H2O released correlates with the activity of TS, whereas the extent of inhibition of the TS activity reflects the potency of antifolate drugs (Fig. 2).

We initially incubated the leukemic cells with [5-3H]dUrd for 3 h according to the protocol of Rodenhuis et al. (40). However, we noted that 3-h incubations with [5-3H]dUrd produced a [3H]H2O release signal that was insufficient to obtain a reliable antifolate inhibition profile in 40% of the samples of childhood leukemia cells (data not shown). Therefore, as an adaptation to the protocol of Rodenhuis et al. (40), we extended the incubations with [5-3H]dUrd from 3 h to 14 h to obtain an adequate signal of [3H]H2O release in 90% of the childhood leukemia samples, thereby preventing selection bias.

In situ TS activity was determined after two different drug incubation conditions. The first condition refers to exposure of the cells to six different concentrations of an antifolate drug for 3 h, after which the cells were washed twice and suspended in drug-free medium for another 15 h. This condition was designated “short-term exposure.” The second condition was a parallel experiment in which the blast cells were continuously incubated with six different concentrations of antifolate drugs for 18 h. This condition was designated “continuous exposure.” The short-term exposure condition was included to determine the duration of inhibition of TS activity after removal of the extracellular drug, which mainly reflects the drug retention within the cells as a result of polyglutamylation.

Cells were washed and suspended in cell culture medium at a density of 2 × 10⁶ cells/ml (CCRF-CEM cells) or 4 × 10⁶ cells/ml (leukemic cells). Incubations were carried out in 3-ml capped Falcon round-bottomed tubes containing 150-µl aliquots of cell suspension. Controls were incubated without drugs, and blanks were incubated without both drugs and cells. All incubations were performed in a shaking water bath at 37°C. In clinical specimens, the in situ TS activity was measured by the
addition of [5-3H]dUrd to the medium at a final concentration of 1.0 μM (specific activity, 2.7 μCi/nmol) 4 h after the start of the incubation (total dUrd incubation time, 14 h).

When the cell lines were studied, [5-3H]dUrd was added 1 h before the end of the incubation (final concentration, 1.0 μM; specific activity, 0.7 μCi/nmol). The reaction was stopped by adding 150 μl of ice-cold 35% trichloroacetic acid solution, and after the addition of 750 μl of 10% activated charcoal solution, the suspensions were mixed and centrifuged at 800 g for 30 min at 4°C (42). A 450-μl sample of the supernatant was counted for radioactivity. The inhibition of the in situ activity of TS was expressed as a TS150 value. TS150 values were calculated assuming a linear dose-response curve between the two flanking concentration points using Microsoft Excel software.

Some samples of leukemia cells were used to find out which concentrations of the different drugs had to be included. The following ranges of six drug concentrations were used (continuous and short-term exposure, respectively): (a) MTX, 0.01–0.5 and 0.2–100 μM; (b) TMQ, 0.005–1 and 0.05–2.5 μM; (c) EDX, 0.025–0.1 and 0.025–100 μM; (d) ZD1694, 0.01–0.25 and 0.01–50 μM; (e) GW1843U89, 0.001–0.1 and 0.005–50 μM; (f) AG337, 0.1–10 and 1–50 μM. Due to the limited number of fresh leukemic cells available, we were not able to test the complete panel of antifolate drugs for each patient. Comparisons of TS150 values from fresh and cryopreserved samples from leukemia patients showed a good correlation, which indicates that the in situ TS inhibition data are reproducible.

Statistical Methods. The Spearman ranking correlation coefficient was calculated for the relationship between in situ TS inhibition and growth inhibition. Differences in TS inhibition between the three leukemia subsets were determined with the Mann-Whitney U test.

Cell Growth Inhibition Studies. The inhibitory effects of the folate antagonists on the growth of parental and MTX-resistant CCRF-CEM cell lines were determined as described previously by Westerhof et al. (36). Briefly, exponentially growing cells were prepared at a density of 1 × 10^6 cells/ml, distributed in 24-well plates, and exposed to various concentrations of drugs. The cells were incubated for 72 h at 37°C in a 5% CO2 humidified atmosphere. Cell counts and viability were determined in a hemocytometer by trypan blue exclusion. IC50 values were defined as the drug concentrations at which cell growth is inhibited by 50% compared to untreated controls.

For three of the four CEM cell lines, drug sensitivity profiles were also assessed with the MTT assay (34). A good correlation (r = 0.93; P < 0.01) was found between the IC50 values obtained with the MTT assay (data not shown) and the data obtained with the dye exclusion method.

RESULTS

Growth Inhibition of MTX-sensitive and -resistant CCRF-CEM Cell Lines. The growth-inhibitory effects of MTX and the novel antifolates EDX, TMQ, ZD1694, GW1843U89, and AG337 against the parental CCRF-CEM cell line (CCRF-CEM/S) and the MTX-resistant sublines CCRF-CEM/T (transport defective), CCRF-CEM/E (elevated DHFR), and CCRF-CEM/R30dm (low FPGS) are listed in Table 1. Compared to the parental cells, CCRF-CEM/T and CCRF-CEM/E cells were markedly resistant to MTX (240- and 49-fold, respectively), whereas the IC50 value for MTX in CCRF-CEM/R30dm cells was only 2-fold higher than that in parental cells. CCRF-CEM/T and CCRF-CEM/R30dm cells were sensitive to the lipophilic antifolate TMQ, whereas CCRF-CEM/E cells exhibited a 12-fold cross-resistance to TMQ. EDX, ZD1694, and GW1843U89 were more potent growth inhibitors of CCRF-CEM/S cells than was MTX. Compared to the parental cells, CCRF-CEM/T cells were more than 100-fold resistant to EDX, ZD1694, and GW1843U89. CCRF-CEM/E cells were cross-resistant to EDX but were sensitive to ZD1694 and GW1843U89. CCRF-CEM/R30dm showed the most predominant cross-resistance to ZD1694. AG337 was more than 200-fold less potent than MTX against CCRF-CEM/S cells. No significant differences in the IC50 values for AG337 were observed between the parental cell line and the MTX-resistant sublines.

In Situ TS Inhibition in MTX-sensitive and -resistant CCRF-CEM Cell Lines. The in vitro inhibitory action of MTX, TMQ, EDX, ZD1694, GW1843U89, and AG337 on TS activity in the parental cell line CCRF-CEM/S and its MTX-resistant sublines was measured with the TS inhibition assay.
Table 2  Inhibitory effects of antifolates on TS activity of the CCRF-CEM leukemia cell line and MTX-resistant sublines

Cells were incubated with drugs at various concentrations for 3 h followed by a 15-h drug-free period or continuously for 18 h. The in situ TS activity was determined by incubating cells with [5-3H]dUrd and measuring release of [3H]H2O. TSI50 values were determined in at least three different experiments.

<table>
<thead>
<tr>
<th>Drug</th>
<th>TSIC50 (nm)</th>
<th>CCRF-CEM/S a</th>
<th>CCRF-CEM/T b</th>
<th>CCRF-CEM/E c</th>
<th>CCRF-CEM/R30dm d</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3 h</td>
<td>18 h</td>
<td>3 h</td>
<td>18 h</td>
<td>3 h</td>
</tr>
<tr>
<td>MTX</td>
<td>3.270</td>
<td>22</td>
<td>&gt;100,000</td>
<td>2,930</td>
<td>&gt;100,000</td>
</tr>
<tr>
<td>TMQ</td>
<td>410</td>
<td>8.6</td>
<td>340</td>
<td>11</td>
<td>995</td>
</tr>
<tr>
<td>EDX</td>
<td>335</td>
<td>5.0</td>
<td>31,700</td>
<td>465</td>
<td>64,470</td>
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<tr>
<td>ZD1694</td>
<td>49</td>
<td>11</td>
<td>18,400</td>
<td>885</td>
<td>95</td>
</tr>
<tr>
<td>GW1843U89</td>
<td>320</td>
<td>6.2</td>
<td>25,600</td>
<td>315</td>
<td>190</td>
</tr>
<tr>
<td>AG337</td>
<td>&gt;100,000</td>
<td>500</td>
<td>&gt;100,000</td>
<td>320</td>
<td>44,400</td>
</tr>
</tbody>
</table>

a CCRF-CEM/S, parental cell line.
b CCRF-CEM/T, transport defect.
c CCRF-CEM/E, elevated DHFR.
d CCRF-CEM/R30dm, low FPGS.

Fig. 3  Efficacy of MTX against blast cells of patients with iALL, rALL, and ANLL. Drug efficacy was determined with the in situ TS inhibition assay after a continuous 18-h drug exposure (left half) as well as after a short-term (3-h) exposure followed by an additional 15-h incubation in drug-free medium (right half). Drug efficacy is depicted as TSI50 values. Each patient is represented by a ○, ■, median TSI50 values. In the first and fifth column, the median TSI50 values for the different drugs in a MTX-sensitive and three MTX-resistant human leukemia cell lines (CCRF-CEM/S (○), CCRF-CEM/T (■), CCRF-CEM/E (△), and CCRF-CEM/R30dm (●)) are depicted.

The results are shown in Table 2 and Figs. 3–8 (columns designated CEM) and serve as reference for the data of the childhood leukemic cells.

CCRF-CEM/T and CCRF-CEM/E cells were more than 30-fold less sensitive to MTX than were CCRF-CEM/S cells when in situ TS activity was measured after short-term MTX exposure followed by a drug-free period (Fig. 3). CCRF-CEM/R30dm cells were also markedly less sensitive to MTX than were CCRF-CEM/S cells. After 18 h of continuous exposure to MTX, TSI50 values of CCRF-CEM/T and CCRF-CEM/E cells were 130-and 12-fold higher than that of CCRF-CEM/S cells, but CCRF-CEM/R30dm cells displayed an almost wild-type sensitivity.

CCRF-CEM/T cells were 80–375-fold less sensitive to short-term exposure to EDX (Fig. 5), ZD1694 (Fig. 6), and GW1843U89 (Fig. 7) than were CCRF-CEM/S cells. In contrast, the activity of TMQ (Fig. 4) and AG337 (Fig. 8) against CCRF-CEM/T cells was similar to that of CCRF-CEM/S cells. CCRF-CEM/E cells showed a low sensitivity to EDX and TMQ after short-term exposure. However, the short-term exposure results of these cells for ZD1694, GW1843U89, and AG337 were comparable with those of the parental cells. When compared to CCRF-CEM/S cells, CCRF-CEM/R30dm cells were approximately 30-fold less sensitive to the short-term exposure of EDX and ZD1694 and 4-fold less sensitive to that of GW1843U89, whereas a sensitivity to TMQ and AG337 similar to that of CCRF-CEM/S cells was found. The sensitivity patterns of CCRF-CEM/T and CCRF-CEM/E cells after continuous exposure to the different antifolates were comparable with the short-term patterns of these cell lines. However, for CCRF-CEM/R30dm cells, we observed a different sensitivity pattern after continuous exposure as compared to short-term exposure. CCRF-CEM/R30dm cells were 8-fold resistant to continuous exposure to EDX and ZD1694 as compared with wild-type cells.
I.

Fig. 4  Efficacy of TMQ against blast cells of patients with iALL, rALL, and ANLL. Drug efficacy was determined with the in situ TS inhibition using the TS assay after a continuous 18-h drug exposure (left half) as well after a short-term (3-h) exposure followed by an additional 15-h incubation in drug-free medium (right half). Drug efficacy is depicted as TSI50 values. Each patient is represented by a ○, median TSI50 values. In the first and fifth column, the median TSI50 values for the different drugs in a MTX-sensitive and three MTX-resistant human leukemia cell lines [CCRF-CEM/S (▽), CCRF-CEM/T (●), CCRF-CEM/E (▲), and CCRF-CEM/R30dm (♦)] are depicted.

Fig. 5  Efficacy of EDX against blast cells of patients with iALL, rALL, and ANLL. Drug efficacy was determined with the in situ TS inhibition using the TS assay after a continuous 18-h drug exposure (left half) as well after a short-term (3-h) exposure followed by an additional 15-h incubation in drug-free medium (right half). Drug efficacy is depicted as TSI50 values. Each patient is represented by a ○, median TSI50 values. In the first and fifth column, the median TSI50 values for the different drugs in a MTX-sensitive and three MTX-resistant human leukemia cell lines [CCRF-CEM/S (▽), CCRF-CEM/T (●), CCRF-CEM/E (▲), and CCRF-CEM/R30dm (♦)] are depicted.

However, for MTX, TMQ, and AG337, a similar sensitivity was observed for CCRF-CEM/R30dm cells and CCRF-CEM/S cells. CCRF-CEM/R30dm cells were 7-fold more sensitive to continuous exposure to GW1843U89 than were CCRF-CEM/S cells.

Relation between in Situ TS Inhibition and Growth inhibition. The relationship between the IC50 values for the CCRF-CEM cell lines obtained with the 72-h growth inhibition assay and the TSI50 values obtained with the in situ TS inhibition assay is depicted in Fig. 9. A good correlation between the two assays was found. The correlation between growth inhibition and in situ TS was higher for the continuous exposure condition (r = 0.93; P < 0.01) than it was for short-term exposure condition (r = 0.86; P < 0.01).

TS Inhibition in Leukemia Samples. The in situ TS inhibition assay was performed on fresh leukemic cells from 14 children with iALL, 8 children with rALL, and 5 children with ANLL using similar conditions as described for the CCRF-CEM cell lines. The results for MTX sensitivity are depicted in Fig. 3, and the results for for TMQ, EDX, ZD1694, GW1843U89, and AG337 sensitivity are depicted in Figs. 4–8, respectively. Table 3 summarizes the median TSI50 values for the three groups of patients as well as the ratios of the short-term exposure TSI50 values compared to the continuous exposure TSI50 values for all antifolates.

A wide interpatient variability in sensitivity to MTX was observed. After short-term exposure to MTX followed by an
incubation in drug-free medium, no difference in sensitivity between iALL and rALL cells was observed \((P = 0.53)\), whereas ANLL cells displayed a 9-fold lower \((P = 0.04)\) sensitivity to MTX (Fig. 3; Table 3). Cells from iALL, rALL, and ANLL patients were equally sensitive to MTX after continuous drug exposure for 18 h. Median TSI\(_{50}\) values for continuous exposure to MTX were up to 85-fold lower compared to short-term TSI\(_{50}\) values.

After short-term exposure conditions, the median TSI\(_{50}\) values for TMQ were similar in blast cells from all three patient groups (Fig. 4). These median TSI\(_{50}\) values for TMQ in iALL and rALL patients were similar to those of MTX (Table 3). ANLL cells seemed to be highly sensitive to continuous exposure to TMQ. For iALL and rALL cells, a wide variation was observed.

As for MTX and EDX (Fig. 5), the novel antifolates ZD1694 (Fig. 6) and GW1843U89 (Fig. 7) demonstrated an appreciable interpatient variability under the short-term exposure and the continuous exposure conditions. However, on the basis of median TSI\(_{50}\) values, EDX, ZD1694, and GW1843U89 were up to 150-fold more potent than MTX after short-term exposure. In particular, GW1843U89 appeared to be a very potent inhibitor of \textit{in situ} TS activity. In all patient groups, the ratios of short-term exposure TSI\(_{50}\) over continuous exposure TSI\(_{50}\) were lower for the novel antifolates EDX, ZD1694, and GW1843U89 as compared to that for MTX.
AG337

![Graph showing the inhibitory activity of AG337 against blast cells of patients with iALL, rALL, and ANLL. Drug efficacy was determined with the in situ TS inhibition using the TS assay after a continuous 18-h drug exposure (left half) as well as after a short-term (3-h) exposure followed by an additional 15-h incubation in drug-free medium (right half). Drug efficacy is depicted as TSI50 values. Each patient is represented by a closed circle, open square, or open triangle. Each median TSI50 value is depicted by a closed circle, open square, or open triangle. Arrows on markers indicate that the drug concentration required for TSI0 was higher than 50 μM.](image)

Fig. 8 Efficacy of AG337 against blast cells of patients with iALL, rALL, and ANLL. Drug efficacy was determined with the in situ TS inhibition using the TS assay after a continuous 18-h drug exposure (left half) as well as after a short-term (3-h) exposure followed by an additional 15-h incubation in drug-free medium (right half). Drug efficacy is depicted as TSI50 values. Each patient is represented by a closed circle, open square, or open triangle. Each median TSI50 value is depicted by a closed circle, open square, or open triangle. Arrows on markers indicate that the drug concentration required for TSI0 was higher than 50 μM.

### Table 3 Inhibitory effects of antifolates on the TS activity of cells from leukemia patients

<table>
<thead>
<tr>
<th>Cells</th>
<th>MTX</th>
<th>EDX</th>
<th>TMQ</th>
<th>ZD1694</th>
<th>GW1843U89</th>
<th>AG337</th>
</tr>
</thead>
<tbody>
<tr>
<td>iALL (n = 14)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.90</td>
<td>0.087</td>
<td>1.1</td>
<td>0.074</td>
<td>0.027</td>
<td>40</td>
</tr>
<tr>
<td>Short-term&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.065</td>
<td>0.041</td>
<td>0.030</td>
<td>0.024</td>
<td>0.0042</td>
<td>2.6</td>
</tr>
<tr>
<td>Continuous&lt;sup&gt;c&lt;/sup&gt;</td>
<td>35</td>
<td>4.6</td>
<td>61</td>
<td>4.4</td>
<td>5.7</td>
<td>13</td>
</tr>
<tr>
<td>Ratio&lt;sup&gt;d&lt;/sup&gt;</td>
<td>35</td>
<td>4.6</td>
<td>61</td>
<td>4.4</td>
<td>5.7</td>
<td>13</td>
</tr>
<tr>
<td>rALL (n = 8)&lt;sup&gt;e&lt;/sup&gt;</td>
<td>1.2</td>
<td>0.082</td>
<td>1.5</td>
<td>0.042</td>
<td>0.0079</td>
<td>&gt;50</td>
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<td>0.024</td>
<td>0.058</td>
<td>0.020</td>
<td>0.0024</td>
<td>1.6</td>
</tr>
<tr>
<td>Continuous&lt;sup&gt;c&lt;/sup&gt;</td>
<td>21</td>
<td>2.4</td>
<td>52</td>
<td>3.3</td>
<td>21</td>
<td>&gt;28</td>
</tr>
<tr>
<td>Ratio &lt;sup&gt;d&lt;/sup&gt;</td>
<td>21</td>
<td>2.4</td>
<td>52</td>
<td>3.3</td>
<td>21</td>
<td>&gt;28</td>
</tr>
<tr>
<td>ANLL (n = 5)&lt;sup&gt;f&lt;/sup&gt;</td>
<td>7.7</td>
<td>0.091</td>
<td>0.62</td>
<td>0.096</td>
<td>0.075</td>
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<tr>
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<td>0.043</td>
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<td>2.8</td>
<td>286</td>
<td>3.9</td>
<td>110</td>
<td>41</td>
</tr>
<tr>
<td>Ratio&lt;sup&gt;d&lt;/sup&gt;</td>
<td>151</td>
<td>2.8</td>
<td>286</td>
<td>3.9</td>
<td>110</td>
<td>41</td>
</tr>
</tbody>
</table>

<sup>a</sup>The complete panel of antifolate drugs could not be tested for each patient.

<sup>b</sup>TSI50 values after short-term exposure (3-h drug exposure followed by a 15-h drug-free period).

<sup>c</sup>TSI50 values after continuous (18-h) drug exposure.

<sup>d</sup>The mean of the ratios of the short-term exposure TSI50 value over the continuous exposure TSI50 value.

Cells were incubated with drugs at various concentrations. The activity of TS was determined by incubating the cells with [5-3H]dUrd and measuring the release of [3H]H2O. The results are presented as median TSI50 values, i.e. the concentration of drug required for 50% inhibition of TS activity.

After a short-term drug exposure to AG337 followed by a drug-free period, in situ inhibition of TS was only observed at drug concentrations higher than 50 μM (Fig. 8). Median TSI50 values for AG337 after continuous exposure were much higher than those for MTX or the other antifolate drugs; however, AG337 showed a comparable activity in leukemic cells from iALL, rALL, and ANLL patients.

### DISCUSSION

Unlike cell culture assays such as the MTT assay, the measurement of in situ TS inhibition was found to be a suitable assay for the initial screening of MTX sensitivity in childhood leukemia cells. Our results with the CCRF-CEM cell lines showed a good correlation (Fig. 9) between growth inhibition studies (Table 2) and the in situ TS inhibition assay (Table 3), which is in agreement with the findings of other studies with rat sarcoma cells, lung cancer cell lines, and a human HCT-8 colon carcinoma cell line (41, 43, 44).

The in situ TS inhibition assay has been applied for a tentative classification of the mechanisms of resistance to MTX in leukemic cells of a limited number of patients (40). In the present study, we used the panel of MTX-resistant CCRF-CEM cell lines primarily for validation of the in situ TS inhibition assay and to illustrate the effects on antifolate sensitivity for cell lines that display a single mechanism of resistance based on an extreme alteration in folate transport (1% residual MTX trans-
port in CCRF-CEM/T cells) or folate metabolism (18-fold elevated DHFR gene amplification in CCRF-CEM/E cells and 1% residual FPGS activity in CCRF-CEM/R30dm cells). With respect to clinical specimens, the in situ TS inhibition assay may not fully and quantitatively dissect the multifactorial mechanisms that underlie a biological variation in antifolate sensitivity or acquired resistance to MTX. Therefore, the in situ TS inhibition assay should be considered as a general screening assay for antifolate sensitivity in childhood leukemia cells, after which more specific assays should delineate the relative contribution of the different resistance mechanisms in the overall clinical sensitivity to MTX or other antifolates.

It can be anticipated that the TS inhibition assay, as used under the conditions described in this study (3-h drug exposure versus 18-h drug exposure), can elicit differences in MTX polyglutamylation efficiency but may not have sufficient fine-tuning to detect relatively small differences in MTX transport or in DHFR levels. It is of interest to note that we observed no difference in MTX sensitivity between the leukemic cells of patients at diagnosis and the leukemic cells of patients at relapse. This suggests that even when small reductions in MTX transport, MTX polyglutamylation or elevations in DHFR levels were acquired in rALL blasts, they did not influence the potency of in situ TS inhibition after 3 or 18 h of drug incubation. In contrast, a reduced sensitivity to MTX after a 3-h exposure was observed for ANLL cells as compared with iALL and rALL cells. This result is consistent with other studies that have shown that ANLL cells accumulate lower levels of long-chain MTX polyglutamates than ALL cells due to a decreased FPGS activity, an increased $K_m$ of FPGS for MTX, and an increased FPGH activity (4, 5, 45, 46). Although a constitutive decreased polyglutamylation can be a contributing mechanism of intrinsic resistance to short-term MTX exposure in ANLL cells, the results from Fig. 3 suggest that a prolonged exposure to MTX can confer comparable sensitivities to iALL and rALL cells. However, it should be taken into account that the number of samples tested in the present study is limited. Moreover, the results of the in situ TS inhibition assay might be influenced by differences in patient characteristics between the different leukemia subsets.

Our findings with the CCRF-CEM cell lines show that the in situ TS inhibition profiles for the second-generation folate antagonists TMQ, EDX, ZD1694, GW1843U89, and AG337 were comparable with the 72-h growth inhibition potency of these drugs. Furthermore, the absolute potency of in situ TS inhibition by EDX, ZD1694, and GW1843U89 in CCRF-CEM cell lines was substantially greater than that for MTX, which confirms that these compounds exhibit more efficient transport via the RFC and more efficient polyglutamylation via FPGS. An interesting finding was that CCRF-CEM/R30dm cells were 7-fold more sensitive to GW1843U89 than were CCRF-CEM/S cells (Table 2). A possible explanation for this might be that the low FPGS activity in CCRF-CEM/R30dm cells results in low pools of reduced folate cofactors, which leads to less competition for FPGS, enhancing the polyglutamylation of the drug.

The in situ TS inhibition profiles for childhood leukemia specimens showed a large interpatient variability that may reflect their antifolate drug sensitivity. As with the CCRF-CEM lines, the absolute potency of in situ TS inhibition by EDX, ZD1694, and GW1843U89 was markedly increased compared to that of MTX in the leukemia specimens, suggesting that these drugs were efficiently transported and polyglutamated. Further evidence for efficient polyglutamylation of EDX, ZD1694, and GW1843U89 in iALL, rALL, and ANLL cells was obtained from the fact that these drugs retained their marked activity after a 3-h incubation followed by a 15-h drug-free period. It was particularly noteworthy that EDX, ZD1694, and GW1843U89 showed a potent TS inhibitory potential against ANLL cells. This suggests that whereas an increased $K_m$ of FPGS for MTX resulted in decreased polyglutamylation and resistance to MTX, the unchanged high affinity of FPGS for EDX, ZD1694, and GW1843U89 still allowed optimal polyglutamylation and sensitivity for these drugs (46). In a clinical setting, high-dose MTX regimes are being used with the rationale to bypass possible...
MTX transport defects (47, 48). In fact, Masson et al. (25) and Synold et al. (49) showed that MTX accumulation and polyglutamylation in childhood leukemia cells were higher for the high-dose MTX regimen than they were for the low-dose MTX regimen. Data from this study suggest that a low-dose regimen may be effective for novel antifolates that are more effectively transported and polyglutamylated than MTX. Recently, Smith et al. (50) provided evidence for this concept by demonstrating that in childhood leukemia cells, enhanced transport and polyglutamylation of aminopterin conferred, at a low-dose regimen, a markedly improved accumulation and polyglutamylation of this drug compared to MTX. This observation has formed the basis for a currently ongoing Phase I clinical trial with ZD1694 for childhood leukemia (51). EDX and ZD1694 were shown to be active drugs for the treatment of some solid tumors in adults (33, 52, 53). Whether the more efficient transport and polyglutamylation characteristics of the novel antifolates will result in an improved therapeutic index in childhood leukemia needs to be evaluated in clinical trials. At this time, Phase I clinical trials with ZD1694 and AG337 for childhood leukemia are ongoing (54, 55). GW1843U89 may be another likely candidate for clinical evaluation in childhood leukemia, particularly in ANLL.

Lipophilic antifolate inhibitors of DHFR and TS were rationally designed to circumvent transport-related resistance to antifolates. In addition, it may be anticipated that lipophilic antifolates do not face the problem of delayed or chronic toxicities, which have been associated with the long-term cellular retention of polyglutamatable antifolates. After an 18-h exposure, TMQ was more active against ANLL cells than against iALL and rALL cells (Fig. 3). Collateral sensitivity to TMQ has been associated with RFC transport defects or with a low cellular folate status (56, 57). Because we could not observe significant differences in MTX transport capacities between ANLL and ALL cells, a diminished polyglutamylation of natural reduced folate cofactors could contribute to an increased sensitivity of ANLL cells for TMQ in the in situ TS inhibition assay. All leukemia specimens retained sensitivity for TMQ after a 3-h exposure and a 15-h drug-free period, which can possibly be explained by the fact that inhibition of DHFR by TMQ leads to elevated levels of dihydrofolate polyglutamates, which results in sustained inhibition of TS (58). Such an effect is not expected for the lipophilic TS inhibitor AG337. This drug requires continuous exposure to maintain TS inhibition; therefore, drug washout rapidly relieves TS. The same observations were made in a Phase I clinical trial for AG337 (59). Continuous infusions of AG337 resulted in TS inhibition, whereas discontinuation of AG337 infusions rapidly reactivated TS activity.

In conclusion, the in situ TS inhibition assay has proven to be a useful assay to assess the sensitivity or resistance to MTX in leukemia cell lines and cells from children with leukemia. Moreover, this assay can be applied for sensitivity screening of novel antifolates that may potentially circumvent MTX resistance due to more efficient RFC-(un)related transport or polyglutamylation.

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