Circumvention of Methotrexate Resistance in Childhood Leukemia Subtypes by Rationally Designed Antifolates


Resistance to methotrexate (MTX) may contribute to treatment failure as observed in one fifth of children diagnosed with common/preB-acute lymphoblastic leukemia (c/preB-ALL). The risk for relapse is partly associated with diagnosis with common/preB-acute lymphoblastic leukemia (c/preB-ALL), T-lineage ALL (T-ALL), and acute myeloid leukemia (AML). The ex vivo potency of several antifolates (MTX, trimetrexate [TMQ], GW1843U89, multitargeted antifolate [MTA], Raltitrexed, and ZD9331) was studied via in situ inhibition of thymidylate synthase (TS). After short-term exposure, relapsed c/preB-ALL (rALL, n = 21), T-ALL (n = 22), and AML (n = 22) were 3-fold, 10-fold, and 6-fold less sensitive to MTX (P ≤ .001) compared with initial c/preB-ALL (n = 43). This difference in resistance was not observed for TMQ. Also for GW1843U89 and MTA, no resistance was observed in rALL and AML compared with c/preB-ALL. T-ALL compared with c/preB-ALL tended to be less resistant to GW1843U89 (3-fold) and MTA (6-fold) than to MTX (10-fold) (P = .06). Raltitrexed was more active against c/preB-ALL compared with the other subtypes. After 21 hours continuous incubation, T-ALL and AML samples were equally sensitive as c/preB-ALL to MTX, but rALL was 3-fold resistant to MTX compared with initial c/preB-ALL (P = .003). The resistance of ALL was circumvented by TMQ (1-fold; P = .03) and GW1843U89 (1.4-fold; P = .004). Novel antifolates except MTA, displayed a more potent TS inhibition than MTX during continuous exposure. These results suggest that MTX resistance in AML and ALL can be circumvented by continuous exposure, and that novel antifolates should be explored further for MTX-resistant T-ALL, rALL, and AML cells.

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in one third of the AML samples, whereas also high levels of (altered) DHFR have been reported for AML blasts. We recently described that cellular resistance to MTX and the potency of new antifolates against blast cells from leukemia patients can be evaluated using an in situ thymidylate synthase (TS) inhibition assay. In the present study, we investigated the potency of novel antifolates designed to circumvent MTX resistance by (1) bypassing transport deficiency (trimetrexate) and (2) not being dependent on polyglutamylation (TMQ) and ZD9331, (3) having an improved affinity towards FPGS and RFC (GW1843U89), Raltitrexed, (4) multигtargeted antifolate (MTA), or by (5) targeting other enzymes than DHFR (GW1843U89, Raltitrexed, ZD9331, and MTA). We show that continuous long-term MTX exposure can cause MTX resistance in T-ALL and AML and that novel antifolates show potency to circumvent MTX resistance after short-term exposure in relapsed ALL, T-ALL, and AML.

MATERIALS AND METHODS

Patient Specimens
Bone marrow and peripheral blood samples were obtained after informed consent from 49 children with newly diagnosed common/pre-B-ALL, 22 with B-precursor ALL at relapse (rALL) previously treated with MTX, 24 with newly diagnosed T-lineage ALL, and from 26 AML patients (French-American-British [FAB] classifications: M0; M1; M2; M3; M4; M5; M6; M7; and 2 unknown). None of the patients have been treated with 1 of the novel antifolates. The number of antifolates screened per sample was dependent on the number of cells available. MTX sensitivity data for 75% of the untreated ALL samples, presented as reference values in the present report, have been reported previously. Immunophenotype of the ALL patients was determined by immunocytochemistry: c/preB-ALL was defined as HLA-DR⁺/CD19⁺ and terminal deoxynucleotidyl transferase (TdT) positive, excluding the proB-ALL phenotype (CD10⁺, cytoplasmic μ-chain negative). Samples were scored T-ALL when positive for TdT, CD3, and CD7. Newly diagnosed ALL patients were classified as high-risk if 1 or more of the following features were present: T-lineage phenotype, white blood cell counts ≥25x10⁶/L and younger than 1 or older than 10 years of age. Eighty percent of the newly diagnosed c/preB-ALL samples were derived from high-risk patients.

Mononuclear cells were isolated within 48 hours after sampling by centrifugation (500 g, 25 minutes) with Ficoll Isopaque and washed to a concentration 1x10⁶ cells/mL) with Ficol Isoquaque and washed twice in RPMI containing 1% fetal calf serum (FCS) with 10% to 20% of patients centrifugation at 300g and suspended at 1x10⁶ cells/mL in culture medium (RPMI 1640 containing 20% heat inactivated FCS, 100 IU/mL penicillin, 100 µg/mL streptomycin, 0.125 µg/mL fungizone, 200 µg/mL gentamycin, 2 mmol/L L-glutamine, 5 µg/mL insulin, 5 µg/mL transferrin, and 5 ng/mL sodium selenite). If necessary, contaminating normal cells were eliminated using monoclonal antibodies linked to magnetic beads, as previously described. At the time of assay, the percentage of blasts was greater than 80% as morphologically determined by May-Grünwald-Giemsa staining of the cytopsins. Samples, which had been cryopreserved in 10% dimethyl sulfoxide and 20% FCS, were assayed shortly after cells were thawed, washed, and resuspended in prewarmed culture medium.

Chemicals
MTX was a gift from Pharmachemie (Haarlem, The Netherlands). Trimetrexate (5-methyl-6-(3,4,5-trimethoxyphenyl) amino) methyl] 2,4-quinoxalinediamido (TMQ), GW1843U89 (TS)-2-(5-(1,2-dihydro-3-methyl-1-oxo-benzo(F)quinazolin-9-yl)methyl)-amino)-1-oxo-2-isoin- dolinyl)glutaric acid) and MTA (N-[4-[2-(2-amino-3,4-di-hydro-4-oxo-7H-pyrrolo[2,3-d]pyrimidin-5-ylmethyl]-benzoyl]-L-glutamic acid, LY231541) were provided by Warner-Lambert/Parke Davis (Ann Arbor, MI), Glaxo Wellcome (Research Triangle Park, NC), and Eli Lilly Research Laboratories (Indianapolis, IN), respectively. Raltitrexed (Tomudex, ZD1694) and ZD9331 (gamma tetrazole analogue of 2-desamino-2,7-dimethyl-N⁴⁰ propargyl-2'-fluoro-5,8-dideazafolic acid) were obtained from Zeneca Pharmaceuticals (Macclesfield, UK).

FCS, penicillin, streptomycin, fungizone, gentamycin, and L-glutamine were obtained from Flow Laboratories (Irvine, UK), bovine serum albumin (BSA) from Organon Technika (Oss, The Netherlands), and Ficol Isoquaque (Lymphoprep 1.077 g/mL) was purchased from Sigma (Zwijndrecht, The Netherlands) and [5-3H]-2'-deoxyctydylitate (25 Ci/mmol) from Moravek Biochemicals (Brea, CA). RNAzol was purchased from Campro Scientific (Weenendaal, The Netherlands). Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV-RT) from Promega (Madison, WI) and Taq Polymerase from Pharmacia Biotech (Roosendaal, The Netherlands).

In Situ TS Inhibition Assay
Because antifolate sensitivity of patient-derived leukemia samples cannot be determined using conventional cytotoxicity assays, we determined TS inhibition in intact cells based on a previously described assay, measuring the TS-catalyzed conversion of 3H-deoxy-uridylitate (3H-dUMP) to deoxythymidylate (dTMP) and 3H 2 O. In cell lines, we previously demonstrated a good correlation between antifolate sensitivity determined by conventional cytotoxicity assays and the in situ TS inhibition assay for MTX and for novel rationally designed antifolates. In short, 135-µL cell suspensions (1 x 10⁶ cells/mL) were incubated at 37°C with either 15 µL RPMI as controls or with 15 µL drug solution. Blanks in triplicate were included containing 135 µL culture medium and 15 µL RPMI. Two conditions were tested: (1) continuous incubation in which cells were incubated with drugs for 21 hours and (2) short-term exposure in which the drug was washed away after 3 hours followed by an 18-hour drug-free period. Five concentrations of each antifolate were tested in duplicate for each condition as demonstrated in Table 1. Controls without drug were included in triplicate for both conditions. [5-3H]-2'-deoxyctydylitate (final concentration 1 µmol/L, 2.5 Ci/mmol) was added 4 hours after the start of the experiment as precursor for [5-3H]-dUMP, the substrate for TS.

After a total incubation time of 21 hours, cells were put on ice and 150 µL 35% ice-cold trichloroacetic acid (TCA) was added together with 750 µL 10% activated charcoal solution (10 g washed charcoal, 0.5 g Dextran, and 2.5 g BSA in 100 mL demineralized water). After vortexing, samples were left on ice for 30 minutes and centrifuged (800g, 30 minutes, 4°C); 450 µL of the aqueous phase, containing 3H O 2, was transferred to a scintillation vial and counted for radioactivity. After subtraction of the mean blank counts, the data were evaluated by

<table>
<thead>
<tr>
<th>Antifolate</th>
<th>Short Incubation (µmol/L)</th>
<th>Continuous Incubation (µmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MTX</td>
<td>0.156-40</td>
<td>0.0039-1</td>
</tr>
<tr>
<td>TMQ</td>
<td>0.08-50</td>
<td>0.0016-1</td>
</tr>
<tr>
<td>GW1843U89</td>
<td>0.001-0.63</td>
<td>0.00025-0.064</td>
</tr>
<tr>
<td>MTA</td>
<td>0.039-10</td>
<td>0.039-10</td>
</tr>
<tr>
<td>Raltitrexed</td>
<td>0.0013-0.32</td>
<td>0.00063-0.16</td>
</tr>
<tr>
<td>ZD9331</td>
<td>0.04-25</td>
<td>0.0016-0.4</td>
</tr>
</tbody>
</table>
calculation of the concentration of drug needed to inhibit 50% of the control TS activity, assuming a linear dose-response curve between the 2 flanking concentration points. Data were expressed as TSI<sub>50,short</sub> referring to the continuous exposure condition and as TSI<sub>50,cont</sub> for the short exposure condition. To evaluate the potency of novel antifolates to circumvent MTX resistance within individual samples, normalized TSI<sub>50</sub> ratios were calculated by dividing the TSI<sub>50</sub> values for each antifolate with the median TSI<sub>50</sub> value obtained for that antifolate for the group of “MTX-sensitive” c/preB-ALL samples.

Competitive Template Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

RNA was extracted from five million cells by the RNAzol method and reversed transcribed by Moloney Murine Leukemia Virus reverse transcriptase, as described by the manufacturer with minimal modifications. Competitive templates (CTs) were designed for RFC, DHFR, TS, FPGS, and FPGH and dissolved in standardized mixtures to avoid irreproducibilities as described elsewhere (manuscript submitted). PCRs were performed by adding different primer pairs to different aliquots of the same mastermix containing patients’ cDNA and CT mixture. PCR products (cDNA, CT, and heterodimers) were separated on agarose gel and measured by densitometry.

Statistics

The Mann-Whitney U test was applied to evaluate differences in antifolate sensitivity between the group of c/preB-ALL versus the group of T-ALL, relapsed ALL, or AML cells. The Wilcoxon signed ranks test was conducted to analyze the potency of a novel antifolate to circumvent MTX resistance within individual samples by comparing the normalized TSI<sub>50</sub> ratios for MTX with those for a novel antifolate. To analyze MTX resistance within individual samples by comparing the normalized TSI<sub>50,short</sub> values for MTX with those for a novel antifolate. To analyze MTX resistance within individual samples by comparing the normalized TSI<sub>50</sub> values for MTX with those for a novel antifolate.

RESULTS

Potency of MTX to Inhibit In Situ TS Activity in Childhood Leukemia Subtypes

To determine whether relative clinical chemoresistance of relapsed c/preB-ALL, T-lineage ALL and AML compared with c/preB-ALL was reflected by ex vivo testing of MTX sensitivity, we used the in situ TS inhibition assay as described previously. Ranges of TSI<sub>50</sub> values are presented in Table 2. Compared with c/preB-ALL, the median TSI<sub>50,short</sub> values for MTX were higher for relapsed c/preB-ALL, T-lineage ALL and AML (Table 3 and Fig 1A through D [closed squares]).

Because a continuous exposure to MTX can bypass some potential mechanisms of MTX resistance, cells were continuously exposed to MTX. TSI<sub>50,cont</sub> values for MTX were higher for relapsed c/preB-ALL cells compared with initial c/preB-ALL cells. In contrast, TSI<sub>50,cont</sub> values for MTX in T-ALL and AML samples were comparable to those in c/preB-ALL (Table 4 and Fig 1A through D [open squares]).

Potency of Novel Antifolates to Inhibit In Situ TS Activity in c/preB-ALL

Of the novel antifolates, only the polyglutamatable TS-inhibitors (GW1843U89 and Raltitrexed) displayed lower TSI<sub>50,short</sub> values than MTX (Table 3 and Fig 1A). MTA, an inhibitor of both DHFR and TS displayed similar TSI<sub>50,short</sub> values. Both nonpolyglutamatable antifolates, TMQ, and ZD9331 showed a lower activity than MTX; the median TSI<sub>50,short</sub> for the

Table 3. Median TSI<sub>50,short</sub> Values (µmol/L) for MTX and Novel Antifolates and Normalized TSI<sub>50,short</sub> Ratios in Childhood Leukemia Subtypes

<table>
<thead>
<tr>
<th>Initial c/preB-ALL</th>
<th>Relapsed c/preB-ALL (ALL)</th>
<th>T-ALL</th>
<th>AML</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TSI&lt;sub&gt;50,short&lt;/sub&gt; (µmol/L)</td>
<td>TSI&lt;sub&gt;50,short&lt;/sub&gt; (µmol/L)</td>
<td>Normalized TSI&lt;sub&gt;50,short&lt;/sub&gt; Ratio</td>
</tr>
<tr>
<td>MTX</td>
<td>0.38</td>
<td>1.1*</td>
<td>2.9</td>
</tr>
<tr>
<td>TMQ</td>
<td>1.5</td>
<td>1.3</td>
<td>0.85‡</td>
</tr>
<tr>
<td>GW1843U89</td>
<td>0.0067</td>
<td>0.0037</td>
<td>0.55*</td>
</tr>
<tr>
<td>MTA</td>
<td>0.21</td>
<td>0.33</td>
<td>1.6§</td>
</tr>
<tr>
<td>Raltitrexed</td>
<td>0.0096</td>
<td>0.047‡</td>
<td>4.9</td>
</tr>
</tbody>
</table>

TSI<sub>50,short</sub> values were measured by the in situ TS inhibition assay after a 3-hour drug exposure followed by an 18-hour drug-free period. Mann-Whitney U test was applied to analyze differences between untreated c/preB-ALL and relapsed c/preB-ALL (ALL), T-ALL or AML. Normalized TSI<sub>50,short</sub> was calculated as (TSI<sub>50,short</sub> for individual sample)/(median TSI<sub>50,short</sub> for group of c/preB-ALL). Wilcoxon signed ranks test was applied to analyze differences between normalized TSI<sub>50,short</sub> ratios for a novel antifolate and for MTX. No results are presented for ZD9331 because the majority of the samples required more than the highest concentration tested in this study to inhibit 50% of the TS activity.

*P < .001; †P < .01; §P < .05; ¶P < .1.
DHFR inhibitor TMQ was 4-fold higher than for MTX, but for ZD9331, TSI_{50,short} values were generally higher than the highest concentration used in the assay (25 µmol/L).

Prolonged exposure to the antifolates showed a completely different pattern. For all drugs, except for MTA, median TSI_{50,cont.} values were lower than for MTX (Table 4 and Fig 1A).

**Potency of Novel Antifolates to Circumvent MTX Resistance in Relapsed c/preB-ALL (rALL)**

Relapsed c/preB-ALL compared with initial c/preB-ALL. Because several of the novel antifolates displayed a differential better activity than MTX in c/preB-ALL, we also tested these compounds in the MTX-resistant subtypes. No significant differences in TSI_{50,short} values were observed between rALL and initial c/preB-ALL for TMQ, GW1843U89, MTA, or ZD9331 (Table 3 and Fig 1B). When exposed to Raltitrexed, the median TSI_{50,short} was higher for rALL compared with initial c/preB-ALL.

Relapsed c/preB-ALL is the only subgroup in this study that remained more resistant to MTX during continuous incubation compared with initial c/preB-ALL (Table 4 and Fig 1B). In contrast, no significant differences in TSI_{50,cont.} values between rALL and initial c/preB-ALL cells were observed for TMQ, GW1843U89, MTA, or ZD9331. For Raltitrexed, however, the median TSI_{50,cont.} was higher for rALL compared with c/preB-ALL.

Novel antifolates compared with MTX (relapsed c/preB-ALL). To evaluate the relative potency of each antifolate compared with MTX within individual samples, normalized TSI_{50} ratios were calculated by dividing the TSI_{50} values obtained for each individual rALL sample by the median TSI_{50} value obtained for the group of initial c/preB-ALL samples. The median normalized TSI_{50,short} ratio for rALL was 3 for MTX (Table 3). The median normalized TSI_{50,short} ratios were lower for TMQ and GW1843U89 than for MTX (Table 4). No significant decrease
Potency of Novel Antifolates to Circumvent MTX Resistance in T-ALL

T-ALL compared with initial c/preB-ALL. TSI50,cont. values for GW1843U89 and Raltitrexed were higher for T-ALL samples compared with c/preB-ALL samples, while no significant differences between T-ALL and c/preB-ALL were observed for TMQ and for MTA (Table 3 and Fig 1C). As for MTX, no differences in TSI50,cont. values were observed between the group of T-ALL samples and the group of c/preB-ALL samples for TMQ, GW1843U89, and ZD9331 (Table 4). T-ALL samples, however, were more resistant to MTA and to Raltitrexed compared with c/preB-ALL after continuous exposure.

Novel antifolates compared with MTX (T-ALL). The median normalized TSI50,short was 10 for MTX in the group of T-ALL samples (Table 3). The median normalized TSI50,short ratio was significantly lower for TMQ and tended to be lower for GW1843U89 and MTA. For Raltitrexed, normalized TSI50,short ratios were not significantly different from those obtained for MTX. The median normalized TSI50,cont. was 1.4 for MTX in the group of T-ALL samples (Table 4). Normalized TSI50,cont. ratios were lower for TMQ, equal for GW1843U89, but higher for MTA, Raltitrexed, and ZD9331 compared with MTX.

Potency of Novel Antifolates to Circumvent MTX Resistance in AML

AML compared with c/preB-ALL. The median TSI50,short value was lower for TMQ and higher for Raltitrexed in the group of AML compared with c/preB-ALL samples (Table 3 and Fig 1D). TSI50,cont. values were also lower for TMQ and for Raltitrexed for AML compared with c/preB-ALL (Table 4). No significant differences between AML and c/preB-ALL were observed for GW1843U89 and MTA after short exposure or during continuous exposure.

Novel antifolates compared with MTX (AML). The median normalized TSI50,short Ratio for MTX was 6 for AML samples (Table 3). Median normalized TSI50,short ratios were lower for TMQ, GW1843U89, and MTA than for MTX. The median normalized TSI50,cont. for MTX was 0.9 for AML samples (Table 4). Normalized TSI50,cont. ratios were lower for TMQ, equal for GW1843U89 and ZD9331, and higher for MTA and Raltitrexed compared with MTX.

Molecular Biological Correlates

To investigate whether antifolate sensitivity was correlated with mRNA levels of proteins involved in folate metabolism, we developed competitive templates to determine the mRNA levels of RFC, DHFR, TS, FPGS, and FPGH. No correlations were observed for antifolate sensitivity and mRNA levels of RFC, DHFR, or TS in a group of 16 samples for which these parameters were determined. However, for the enzymes involved in polyglutamylation, we observed a significant correlation between a high ratio of FPGH/FPGS mRNA levels and high TSI50,short values for MTX (r = .64, P = .007) and for MTA (r = .58, P = .030). For MTX, this could not be explained by a correlation with the mRNA levels of one of the enzymes independently. For MTA, however, high FPGH mRNA levels were correlated with high TSI50,short values (r = .58, P = .024), whereas low FPGS mRNA levels were correlated with high TSI50,cont. values (r = -.44, P = .051). Also for Raltitrexed and ZD9331, low FPGS mRNA levels were correlated with high TSI50,cont. values (r = -.77, P = .009 and r = -.49, P = .055, respectively). For GW1843U89, a trend was observed between low TSI50,short and high FPGS mRNA levels (r = -.45, P = .056).

DISCUSSION

In the present study, ex vivo experiments showed relative MTX resistance in relapsed c/preB-ALL, T-ALL, and AML samples compared with initial c/preB-ALL after short-term drug exposure. This MTX resistance could be circumvented by continuous drug exposure for T-ALL and AML. Thus, with respect to long-term continuous drug exposure, ALL at relapse was the only leukemia subgroup for which resistance to MTX was still observed. Five rationally designed folate antagonists (TMQ, GW1843U89, MTA, Raltitrexed, and ZD9331) displayed a marked potency to circumvent the observed MTX resistance in childhood leukemia cells.

We previously demonstrated that for cell lines, sensitivity to MTX and to novel antifolates, as measured via conventional cytototoxicity assays, correlated with sensitivity measured via the
in situ TS inhibition assay, both for short and continuous exposure conditions. As antifolate sensitivity of primary leukemia samples cannot be determined using conventional assays, as demonstrated and discussed previously, we determined the potency of novel antifolates versus MTX on the basis of in situ TS inhibition for all leukemia subgroups. The specific TS inhibitors GW1843U89, Raltitrexed, and ZD9331 showed markedly lower TSI0,cont. values than observed for MTX, which reflects their enhanced RFC-mediated membrane transport and more potent TS inhibition. Moreover, GW1843U89 and Raltitrexed were more efficiently retained during the drug-free period, as illustrated by a diminished difference between median values of TSI0,short and TSI0,cont. compared with this difference for MTX (Fig 1). This result is consistent with a superior polyglutamation of these compounds by FPGS as compared with MTX.  

To show whether MTX resistance can be circumvented by novel antifolates, we analyzed the differential potency of antifolates between the leukemia subtypes. rALL cells were resistant to MTX both after short-term exposure and after continuous exposure as compared with initial c/preB-ALL samples. Resistance to MTX after continuous drug exposure was not observed for T-ALL and AML, suggesting a different mechanism of MTX resistance in rALL compared with T-ALL and AML. Both after short-term and after continuous exposure, MTX resistance could be circumvented by TMQ and GW1843U89, but not by MTA and Raltitrexed in rALL. RFC transport defects, which have been reported to contribute to MTX resistance in rALL cells, can also reduce transport of Raltitrexed. On the other hand, residual RFC activity may still allow sufficient accumulation of GW1843U89 in rALL cells to confer drug sensitivity.

T-ALL was MTX resistant only after short-term exposure as compared with initial c/preB-ALL. This resistance could be circumvented by continuous MTX exposure, while the nonpolyglutamatable antifolate, TMQ, was equally potent in both subtypes. Because polyglutamation defects could be overcome by continuous exposure in cell line experiments, the circumvention of MTX resistance in these primary leukemia samples is in agreement with the observation that T-ALL samples are less capable of polyglutamation compared with initial c/preB-ALL samples. For TMQ, the circumvention of MTX resistance is of particular interest because increased DHFR levels have been reported as an additional mechanism of MTX resistance in T-ALL cells. Although DHFR is the target enzyme of TMQ, these small elevations of DHFR apparently do not result in cross-resistance to TMQ. The improved activity of TMQ over MTX might reflect its sufficient intracellular accumulation to levels that can sustain DHFR inhibition both in c/preB-ALL and T-ALL cells. T-ALL tended to be less resistant to GW1843U89 and MTA, but not to Raltitrexed, as compared with MTX. Despite the low number of samples tested for Raltitrexed sensitivity, this suggests that resistance due to a low FPGS activity, as reported for T-ALL cells previously, cannot be circumvented by the increased affinity of FPGS for Raltitrexed. This is consistent with studies on MTX-resistant human leukemia cell lines.  

MTX resistance only after short-term drug exposure, were sensitive to TMQ, GW1843U89, and MTA after short-term exposure. In contrast, Raltitrexed was not able to circumvent MTX resistance ex vivo in these AML samples. Whether this is solely due to the lower activity of FPGS along with altered kinetic properties, as reported for AML cells, or a multifactorial process involving decreased transport and increased FPGH activity, is presently unknown.

We and others previously described a good correlation between TSI0,short values, MTX polyglutamylation, and the ratio of FPGH/FPGS activities or FPGH/FPGS mRNA levels (present report). The present study shows no correlations for TSI0 values and mRNA expression of the target enzymes DHFR and TS, or of RFC, for any of the antifolates. This suggests that mutations or posttranslational modifications are of more importance in determining the overall response to these drugs. For GW1843U89 and MTA, correlations were observed between TSI0,short and mRNA expression levels of FPGH and FPGS, respectively. This might suggest that MTX resistance due to low or high mRNA expression of FPGH or FPGS, respectively, could not be circumvented by these antifolates. Despite these correlations, however, GW1843U89 and MTA demonstrated good potency to circumvent MTX resistance in childhood T-ALL and AML cells.

To date, only TMQ and Raltitrexed have been administered in pediatric clinical trials. Peak plasma levels obtained during these trials, together with plasma levels of GW1843U89 and MTA obtained in adults, are provided in Table 2. Although these values suggest that the presented TSI0 values for the novel antifolates are clinically achievable, some considerations have to be kept in mind. Binding of drugs to serum proteins (in vivo) or to plastic (in vitro) and technical parameters, such as incubation time, make it difficult to extrapolate in vitro data to in vivo values.

Although the poor prognosis of AML and ALL at relapse warrants novel treatment modalities, low numbers of pediatric patients are eligible for entry into phase I/II clinical trials. This is due to the low incidence of childhood cancer and the relatively good prognosis of the majority of the cases. Therefore, the agents to be chosen for further studies need to be prioritized. The availability of biochemical assays, such as the in situ TS inhibition assay, and molecular probes to analyze MTX-resistance parameters, may facilitate the initiation of phase I/II clinical trials in childhood leukemia with novel antifolates.

In conclusion, these ex vivo results suggest that novel antifolates could be promising candidates for further evaluation in clinical trials with T-ALL, rALL, and AML.

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