Differential Methotrexate Resistance in Childhood T- Versus Common/PreB-Acute Lymphoblastic Leukemia Can Be Measured by an In Situ Thymidylate Synthase Inhibition Assay, But Not by the MTT Assay

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Methotrexate (MTX) is not cytototoxic to patient-derived acute lymphoblastic leukemia (ALL) cells in total-cell-kill assays, such as the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, putatively due to the rescue effects of hypoxanthine and thymidine released from dying cells. This was mimicked by a diminished methotrexate (MTX) cytotoxicity for the cell lines HL60 and U937 in the presence of hypoxanthine, thymidine, or lysed ALL cells. However, enzymatic depletion or inhibition of nucleoside membrane transport did not result in MTX dose-dependent cytotoxicity in patient samples. Alternatively, a thymidylate synthase inhibition assay (TSIA), based on inhibition of the TS-catalyzed conversion of Hx-dUMP to dTMP and H2O2, correlated with the MTT assay for antifolate sensitivity in four human leukemia cell lines with different modes of MTX resistance. For 86 ALL patient samples, TS50 values after 21 hours exposure to MTX were not different between T- and c/preB-ALL \((P = .46)\). After 3 hours incubation with MTX followed by an 18-hour drug-free period, T-ALL samples were 3.4-fold more resistant to MTX compared with c/preB-ALL samples \((P = .001)\) reflecting the clinical differences in MTX sensitivity. TS50 values correlated with MTX accumulation \((r = - .58, P < .001)\). In conclusion, the TSIA, but not the MTT assay, can measure dose-response curves for MTX in patient-derived ALL cells and showed relative MTX resistance in T-ALL compared with c/preB-ALL.

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Acute lymphoblastic leukemia (ALL) is the most frequently occurring type of cancer in children. Today, more than 95% of the children with newly diagnosed ALL will reach complete remission by combination chemotherapy. However, the leukemia will relapse in one third of the patients.1 This treatment failure may be explained by unfavorable clinical pharmacokinetics,2 regrowth potential of the residual leukemic cells, and by cellular drug resistance.3 Testing of in vitro drug resistance, eg, by using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, has provided good correlations with clinical outcome.4,5 Unfortunately, the cytotoxicity of methotrexate (MTX), a cornerstone in the treatment of ALL, cannot be evaluated on primary patient-derived leukemic cells by the MTT assay6-12 or by other 4-day culture assays, such as the differential staining cytotoxicity (DiSC) assay.13 A test system, which allows the determination of MTX resistance of primary ALL cells, might provide additional prognostic information, thereby facilitating the identification of low- and high-risk patients. In addition, an in vitro assay may be used to determine the relation between clinical and cell biological features and MTX resistance.

In contrast to the nonproliferating patient-derived ALL cells, MTX-induced growth inhibition can easily be observed in cell lines using the MTT assay.14 The mechanism of action of MTX is through the inhibition of folate requiring enzymes in the pyrimidine and purine de novo synthesis pathways, ultimately resulting in an inhibition of the synthesis of DNA, RNA, and protein.15 However, cells can be rescued from this cytotoxic action by salvage pathways in which, eg, hypoxanthine (Hx) is converted to inosine monophosphate (IMP) and subsequently to adenosine monophosphate (AMP) and guanosine monophosphate (GMP). Inhibition of the de novo synthesis of thymidylate catalyzed by thymidylate synthase (TS) can be bypassed by the consumption of exogenous thymidine (TdR), which will be converted by thymidine kinases to dTMP. Hx and TdR have been reported to be released by spontaneously dying lymphocytes16 due to degradation of DNA and RNA. Because on average 35% of ALL cells derived from patients spontaneously die during 4 days of culture,10 thereby releasing Hx and TdR, this may explain the absence of a dose-response curve of MTX for ALL cells. Additional evidence for this hypothesis is provided by a report in which enzymatic depletion of Hx and TdR resulted in MTX cytotoxicity on GKT, a leukemia xenograft, which is unable to grow ex vivo, similar to ALL cells.17 This approach, however, was not applied to samples directly derived from patients, and so far it is unknown whether total-cell-kill assays can be adapted for the screening of cells derived from pediatric ALL patients for MTX sensitivity.

In the present study, we investigated several adaptations of the MTT assay for the screening of ALL cells from childhood leukemia patients for MTX sensitivity or resistance, but no dose-dependent cytotoxicity of MTX was observed. Alternatively, we showed that an in situ thymidylate synthase inhibition assay (TSIA)18,19 could be used to determine relative sensitivities of cell lines to MTX, obtaining similar data as for the MTT assay. Therefore, we optimized this indirect MTX resistance assay for the screening of patient-derived leukemic cells. The results show that the TSIA provides dose-response curves in patient-derived ALL cells with large interpatient variation and that this assay can detect differential MTX resistance in T- versus c/preB-ALL after short-term exposure. This is in accordance with the clinically observed relative chemoresistance of T-ALL compared with c/preB-ALL cells.20

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MATERIALS AND METHODS

Patient specimens. Bone marrow and peripheral blood samples were obtained from newly diagnosed pediatric ALL patients; infants (<12 months old) and patients with pro-B (CD10− precursor B-lineage) and mature B-ALL were excluded. Mononuclear cells were isolated by centrifugation (500g, 25 minutes) with Ficoll Isopaque, as described previously.9 After isolation, cells were washed twice in RPMI containing 1% fetal calf serum (FCS) with 10-minute periods of centrifugation at 300g and suspended at 2 × 10^6 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 gentamicin, 2 mmol/L L-glutamine, 5 µg/mL insulin, 5 µg/mL transferrin, and 5 ng/mL sodium selenite). Remaining cells were cryopreserved in RPMI containing 20% FCS and 10% dimethyl sulfoxide (DMSO) in liquid nitrogen.

Cell lines. HL60, a human promyelocytic leukemia cell line, and U937, derived from a patient with monoblastic leukemia, and four human T-lymphoblastic leukemia cell lines CCRF-CEM (the parental CEM/S and three MTX-resistant sublines with either defective MTX transport, increased dihydrofolate reductase [DHFR], or defective MTX-polyglutamylation)21 were grown as suspension cultures in RPMI medium 1640 supplemented with 10% heat-inactivated FCS and 1 mmol/L L-glutamine. Cultures were maintained in exponential growth at 37°C in a humidified atmosphere of 95% air/5% carbon dioxide. In the experimental set up, HL60 cells were suspended at 0.2 × 10^6 cells/mL, U937 and CEM cells at 0.1 × 10^6 cells/mL for the MTT assay and at 2 × 10^6 cells/mL for the TSIA.

Chemicals. MTX was obtained as a gift from Pharmachemie (Haarlem, The Netherlands). Fluoro-MTX (F-MTX), a nonglutamylated derivative, was obtained from newly diagnosed pediatric ALL patients; infants (<12 months old) and patients with pro-B (CD10− precursor B-lineage) and mature B-ALL were excluded. MTX was obtained from Wellcome (Northwich, UK), as well as thymidine, hypoxanthine, xanthine, thymidine phosphorylase (TP), and xanthine oxidase (XO). Sigma (Zwijndrecht, The Netherlands), as well as thymidine, hypoxanthine, xanthine, thymidine phosphorylase (TP), and xanthine oxidase (XO). Organon Technika (Oss, The Netherlands), and Ficoll Isopaque (Lymphoprep 1.077 g/mL) was provided by Nyegaard (Oslo, Norway). Insulin, transferrin, sodium selenite, and MTX were purchased from Sigma (Zwijndrecht, The Netherlands), as well as thymidine, hypoxanthine, thymidine phosphorolysis (TP), and xanthine oxidase (XO). 2-(aminocarboxyl)-N-(4-amino-2,6-dichlorophenyl)-4-[5,5-bis-(4-fluorophenyl)-1-phenyl-1,2-piperazineacetamide (R75231) was kindly provided by Dr. H. Van Belle (Janssen Pharmaceuticals, Beerse, Belgium), 5-[H]-2'-deoxyuridine (22 Ci/mmol) and 5-[H]-2'-deoxycytidine (25 Ci/mmol) were purchased from Moravek Biochemicals (Brea, CA).

MTT and DiSC assay in cell lines. Individual wells of a 96-well microculture plate were filled with 50 µL cell suspension, 25 µL medium, and 25 µL MTX (final concentrations ranging from 10^-11 to 10^-7 mol/L). Plates were incubated in a humified incubator in 5% CO2 for 3 days at 37°C. Subsequently, 10 µL of MTT solution (final concentration, 0.45 mg/mL) was added and incubated for 3 hours at 37°C. Formazan crystals were dissolved with 100 µL of 0.04 N HCl-isopropyl alcohol (acid isopropanol). The concentration resulting in 50% inhibition of the cell growth (IC50) was determined by assigning 100% to the mean value for the OD of the control wells at day 3 after subtraction of the mean blank value. To distinguish between cell growth inhibition and cell kill, the OD after 3 days was corrected for the mean OD observed for the control wells at the day of drug addition.21 In some experiments, Hx, TdR, or lassed ALL cells were added to the medium and IC50 values were determined relative to control cells also incubated in the presence of these additives. Lyzed leukemic cells (final concentration, 1 × 10^5/mL) were obtained by snap-freezing and thawing three times.

Measurements of Hx and TdR. After 4 days of culture, microculture plates were kept at -20°C, at which temperature Hx and TdR are stable,26 until analyzed. To determine the concentration of Hx and TdR, 7 µL of 80% trichloroacetic acid (TCA) was added to 70 µL medium and put on ice for 30 minutes. After 2 minutes of centrifugation at 12,000g, the supernatant was neutralized by the addition of 140 µL of tri-ocetylamine/1,2-tri-chloro-tri-flouro-ethane (1:4, vol/vol). This mixture was centrifuged for 1 minute at 12,000g. The separation of the nucleosides wasoquo bases in the supernatant was performed on a reverse-phase microsphere C18 column (Chrompack; 100 × 4.6 mm, 3 µm) at a flow rate of 1 mL/min. Eluents were made of 60 mmol/L KH2PO4 in 5 mmol/L tetraethylammonium hydrogensulphate (TBA), pH 6 (solvent A) and 50 mmol/L acetonitril (solvent B). A gradient (2 minutes 2% B, followed by linear increase of 2% to 40% B over 10 minutes) separated the peaks, which were identified by their absorbance ratios and retention times by comparison with standards. Column output was monitored at 254 and 280 nm.

In situ TSIA. Inhibition of TS was determined in whole cells based on a previously described assay27,28 by measuring the TS-catalyzed conversion of 3-14C-dUMP to dTMP and 3-3H2O2. A total of 135 µL cell suspensions (1 × 10^6 cells/mL) containing 15 µL culture medium and 15 µL RPMI. Two conditions were tested: (1) continuous incubation in which cells were incubated with or without drugs for 21 hours (five final concentrations ranging from 0.0039 µmol/L to 1 µmol/L for MTX; from 0.039 µmol/L to 10 µmol/L for F-MTX) and (2) short exposure in which the drug was washed away after 3 hours followed by an 18-hour drug-free period (five final MTX concentrations ranging from 0.156 µmol/L to 40 µmol/L), based on pilot experiments reported elsewhere.27 Controls were included in triplicate for both conditions. [5-3H]-2'-deoxycytidine or [5-3H]-2'-deoxyuridine (final concentration, 1 µmol/L, 2.5 Ci/mmol) was added 4 hours after the start of the experiment as precursor for dUMP, the substrate for TS. After a total incubation time of 21 hours, cells were put on ice and 150 µL 35% ice-cold TCA was added together with 750 µL 10% activated charcoal solution (10 g washed charcoal, 0.5 g dextran, and 2.5 g bovine serum albumin [BSA] in 100 mL.)

The OD of wells containing MTX, but no cells, was subtracted from the OD of corresponding wells containing cells and MTX. Leukemic cell survival (LCS) was calculated by the equation: LCS = (ODday 4, treated wells/ODday 4, control wells) × 100%. In simultaneous experiments, the medium was precipitated for 15 minutes with TP (final concentration, 0.15 IU/mL) and XO (final concentration, 0.02 IU/mL) at 37°C to deplete TdR and Hx. Nucleoside transport was inhibited by the addition of R75231 (final concentration, 1.65 µmol/L). The DSC assay was performed as described elsewhere.24
was most obvious when Hx and TdR were added together. HL60 and (B) U937. The results are expressed as mean values of three experiments.

A dose-dependent growth inhibition was induced by incubating 2 × 10^6 cells/mL applying the same time schedule. In this case, ^3H-deoxyuridine was added as a substrate 1 hour before the end of the experiment (final concentration, 1 µmol/L, 0.7 Ci/mmol).

MTX accumulation and polyglutamylation. Of samples for which a sufficient number of cells was available, 10^6 cells were exposed for 24 hours to 1 µmol/L [3H]-MTX (2 Ci/mmol), washed three times in ice-cold phosphate-buffered saline (PBS) and resuspended in 1 mL ice-cold PBS. Total accumulation was determined by counting 90 µL for radioactivity and 10 µL for cell survival. The remaining 900 µL was centrifuged and analyzed for polyglutamate formation by high-performance liquid chromatography (HPLC) as described previously.

Statistics. To analyze correlations between parameters obtained with the TSIA and with the MTT assay, the Wilcoxon signed rank sum test was applied. The Spearman’s rank correlation coefficient was calculated for a relation between MTX sensitivity and polyglutamylating activity parameters.

RESULTS

Prevention of MTX-induced growth inhibition on leukemic cell lines. A dose-dependent growth inhibition was induced by MTX for the leukemic cell lines HL60 and U937, as determined with the MTT assay (IC50 values, 8.8 mmol/L ± 1.0 and 11.0 mmol/L ± 4.0, respectively). For both cell lines, cell growth was completely inhibited at MTX concentrations higher than 10^{-5} mol/L (Fig 1A and B). To investigate whether Hx and TdR can protect cells from MTX-induced growth inhibition, these metabolites were added to the medium. Hx (25 µmol/L) did not protect the cells from growth inhibition by high MTX concentrations (>10^{-7} mol/L), but IC50 values increased fourfold and fivefold for HL60 and U937, respectively. TdR (10 µmol/L) by itself increased the IC50 value of MTX by 2.7-fold and 3.8-fold, respectively. In contrast to the addition of Hx, TdR partly protected cells from high MTX concentrations (Fig 1A and B). When Hx and TdR were added simultaneously, complete prevention of the MTX effect was observed for U937 (Fig 1B). Also for HL60 cells, no IC50 could be calculated (IC50 > 10^{-7} mol/L) (Fig 1A). To test the hypothesis that compounds released by dying patient-derived ALL cells prevented MTX-induced cell kill in the MTT assay, lysed ALL cells were added to HL60 and U937 cultures. IC50 values increased fourfold for both cell lines (Fig 1A and B).

Lack of in vitro MTX cytotoxicity in patient-derived blast cells using the MTT assay. The effect of MTX was determined for blast cells of 83 leukemic patients by the MTT assay. LC50 values could, however, not be determined in 90% of the samples because the leukemic cell survival in the presence of MTX did not decrease below 50% of the control cell survival. This lack of MTX cytotoxicity was observed even at very high MTX concentrations up to 5.5 × 10^{-3} mol/L, as shown for two patients in Fig 2. By applying the DiSC assay, also no dose-response curves were obtained (data not shown).

Prevention of rescue from MTX cytotoxicity. The lack of MTX cytotoxicity on nondonivating patient cells might be associated with the presence of high concentrations of TdR and Hx. Therefore, we determined the concentrations of these compounds in the control wells of four patient samples after 4 days of incubation. The concentration of Hx varied between 36.4 and 58.7 µmol/L, which was not increased relative to the concentrations measured in the corresponding wells without cells (Table 1). The TdR concentrations ranged between <0.1 and 2.1 µmol/L, which is twice the concentration measured in the absence of cells (Table 1). Preincubation of the medium with TP and XO markedly reduced the TdR and Hx concentrations in four samples measured (Table 1). However, this reduction in the concentration of the metabolites did not result in dose-response curves for 11 patient samples, as shown for a representative sample in Fig 2. Cell membrane nucleoside transport was inhibited by coincubation with R75231 to prevent protection of cells by the presence of TdR in the medium. For 12 samples tested, R75231 did not increase the cytotoxicity of MTX, as shown for one sample in Fig 2.
Optimization and validation of the in situ TSIA. An alternative method to screen cells for antifolate resistance is based on the inhibition of TS in intact cells.\textsuperscript{18,19,30} To validate the TSIA, we determined antifolate sensitivity obtained with the MTT assay and with the TSIA using the MTX-sensitive T-lymphoblastic leukemia cell line CCRF-CEM and three sublines, which are resistant to MTX due to defective transport, impaired polyglutamylation, or elevated DHFR. In addition to MTX, sensitivity to five other antifolates, mentioned in the Materials and Methods section, was determined. These compounds differ from MTX at the level of transport, polyglutamylation, and/or target enzyme. A strong and significant correlation was found between the $IC_{50}$ values determined in the MTT assay and both the TSIA\textsubscript{50,cont.} ($r = .89, P < .001$, Fig 3) and the TSIA\textsubscript{50,short} ($r = .66, P = .001$) in the TSIA. The Wilcoxon signed rank sum test showed that antifolate sensitivity, as measured with the TSIA after continuous exposure, was not different from antifolate sensitivity as measured with the MTT assay ($P = .69$).

Because the original TSIA\textsuperscript{19} required $2 \times 10^6$ cells per drug per time point, we modified the assay by testing different concentrations of drugs instead of different time points. In addition, the substrate was changed from deoxyuridine to deoxycytidine, which is more efficiently converted to dUMP, the direct substrate for TS. The amount of $^3$H$^2$O formed was increased fourfold when deoxycytidine was used as a substrate instead of deoxyuridine, reducing the amount of cells to $0.1 \times 10^6$ cells to $0.1 \times 10^6$ cells per drug per concentration. No difference in TSIA\textsubscript{50} values was observed using these substrates as shown in Fig 4A.

Comparison of fresh and cryopreserved samples from 10 primary acute leukemia patients showed that cryopreservation did not influence MTX-induced TS inhibition during the continuous drug exposure or the short drug exposure condition of the TSIA (Fig 4B). In paired blast samples isolated from peripheral blood and from bone marrow derived from seven patients, no differences in TSIA\textsubscript{50} values were observed (data not shown).

**TSIA in T- and c/preB-ALL.** A large interpatient variation in MTX sensitivity was observed both in TSIA\textsubscript{50,cont.}, ranging from 0.0067 to 0.76 $\mu$mol/L MTX and in TSIA\textsubscript{50,short}, ranging from $<0.15$ to $>0.40$ $\mu$mol/L MTX (Fig 5). The median TSIA\textsubscript{50,cont.} was not significantly different between 29 T- and 51 c/preB-ALL samples ($0.061 \pm 0.104$ $\mu$mol/L MTX; $P = .46$). The median TSIA\textsubscript{50,short}, however, was 3.4-fold higher in T-ALL compared with c/preB-ALL (1.66 $\pm 0.49$ $\mu$mol/L; $P = .001$), but with a large overlap between both groups (Fig 5).

The role of MTX polyglutamates. F-MTX is a nonglutamylatable analogue of MTX, with otherwise similar characteristics.\textsuperscript{22} Therefore, by measuring the sensitivity to both MTX and

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### Table 1. Concentrations of HX and TdR in the Culture Medium of Leukemic Blast Samples

<table>
<thead>
<tr>
<th>Sample No.</th>
<th>HX ($\mu$mol/L)</th>
<th>TdR ($\mu$mol/L)</th>
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<tbody>
<tr>
<td></td>
<td>In the Absence of ALL Cells</td>
<td>In the Presence of ALL Cells</td>
</tr>
<tr>
<td></td>
<td>$-XO$</td>
<td>$+XO$</td>
</tr>
<tr>
<td>1</td>
<td>67.8</td>
<td>$&lt;0.4$</td>
</tr>
<tr>
<td>2</td>
<td>56.6</td>
<td>$&lt;0.4$</td>
</tr>
<tr>
<td>3</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>4</td>
<td>37.2</td>
<td>$&lt;0.4$</td>
</tr>
</tbody>
</table>

All samples ($2 \times 10^6$ cells/mL) were incubated in 96-well plates for 4 days in the absence or presence of XO (final concentration, 0.02 IU/mL) and TP (final concentration, 0.15 IU/mL). The concentrations of TdR and HX were measured by HPLC as described in Materials and Methods. Abbreviation: ND, not determined.
F-MTX, more insight into the role of MTX polyglutamates would be provided. For F-MTX, no difference was observed for continuous exposure between T- and c/preB-ALL (median TSI 50, 9.21%, range, 4.5 to 21), and the sensitivity for F-MTX was significantly related to the sensitivity for MTX (r = .93; P < .001; Fig 6). After incubation in drug-free medium, TS activity was fully recovered in the majority of the samples incubated with F-MTX, in contrast to the results obtained with MTX.

Because the difference in retention of TS inhibition between F-MTX and MTX clearly indicated an important role for polyglutamylation of MTX, we determined MTX accumulation and polyglutamylation for 47 samples. TSI 50,cont. and TSI 50,short were both significantly correlated with MTX accumulation (Table 2). The ratio TSI 50,short/TSI 50,cont., excluding the two T- and the 16 B-lineage samples with TSI 50,short < 0.156 µmol/L, was not correlated with overall MTX accumulation, but rather with the percentage of MTX, which had been converted to the pharmacologically more important MTX-Glu4-6 (Table 2).

**DISCUSSION**

In this report, we showed that MTX resistance can be studied in primary ALL cells by an in situ TSIA. Conventional total cell kill assays such as the MTT assay⁹-¹² and the DiSC assay¹³ cannot be used for this purpose due to salvage by purines and thyridine, which protect cells from the cytotoxic effects of MTX. A test to determine MTX resistance is particularly important because MTX is an essential drug in the treatment of ALL, and evaluation of MTX resistance in primary ALL samples may improve the prognostic value of in vitro drug resistance testing. In addition, research on clinically relevant mechanisms of MTX resistance will be facilitated by the identification of resistant subgroups of patient samples.

In the present report, we showed that Hx, TdR, and lysed ALL cells can protect cell line cells against MTX-induced growth inhibition. Protection by lysed ALL cells can (at least partly) be explained by the release of metabolites such as Hx and TdR. In our experiments, the concentrations of metabolites released by 1 × 10⁶ lysed ALL cells/mL were high enough to increase TSI 50 values by fourfold. Higher concentrations of Hx (25 µmol/L) and TdR (10 µmol/L), however, were required for complete protection of U937 cells against MTX-induced growth inhibition. Also for HL60 cells, the simultaneous addition of Hx and TdR was more efficient in protecting cells compared with the ALL lystate. In the nonproliferating system of ALL lymphoblasts, however, the concentrations of Hx and TdR released by spontaneously dying patient cells may be sufficient to protect cells from MTX-induced death.

Because about 35% of the untreated ALL cells die spontaneously during the 4 days of culture in the MTT assay,¹⁰ the subsequent release of Hx and TdR into the medium partly explains the observed lack of MTX cytotoxicity in primary ALL cells obtained from patients. After 4 days of culture, we detected an increase in TdR concentration only and not in Hx concentration in wells containing cells versus wells without cells. This, however, might be a reflection of the consumption of Hx by the remaining cells to survive the 4 days of culture. The hypothesis that dying cells protect remaining cells from MTX cytotoxicity is supported by several observations. The dilution of plated ALL cells to less than 200 cells/well did result in a dose-dependent effect of MTX, whereas in experiments with an initial concentration of 10,000 cells/well, cells were protected from MTX by lysed ALL cells. In another study with a MTX nonresponsive xenograft, it was shown that incubation with XO and TP to degrade Hx and TdR, respectively, did result in MTX-induced growth inhibition.¹⁷

We adapted the MTT assay conditions to block potential rescue effects by purine/pyrimidine salvage pathways. XO and TP indeed decreased the concentrations of Hx and TdR in the medium, but still no dose-response curves were observed for MTX on leukemic cell samples from 11 ALL patients in the MTT assay. This result could imply that residual levels of TdR and Hx were still sufficient to prevent the remaining patient cells from MTX cytotoxicity. Moreover, other purines have been described to rescue cells from MTX cytotoxicity such as guanosine and adenosine. These metabolites are also degraded...
by XO, but only after conversion to Hx. On the present chromatograms, these metabolites could not be evaluated. Another explanation may be found in an increase in extracellular folate pools due to the spontaneously dying cells. However, these folates would prevent MTX toxicity by competition for transport and polyglutamylation suggesting that higher concentrations of MTX should overcome this rescue.

Coincubation of R75231, described to inhibit nucleoside transport of Ehrlich ascites tumor cells, did not increase MTX cytotoxicity against ALL cells from patients. However, R75231 will only inhibit the transport of nucleosides, while bases such as Hx are still capable of entering the cell. In view of our cell line experiments and data provided by others, it is clear that Hx alone cannot fully rescue the remaining cells from MTX cytotoxicity. As already described, replacing FCS by dialyzed FCS to remove folates, nucleosides, and bases from the medium did not improve MTX cytotoxicity, but decreased leukemic cell survival in general. Altogether, it seems that different mechanisms of protection could be involved in patient-derived samples compared with proliferating cell lines.

Because no adaptations of the MTT assay resulted in dose-dependent MTX cytotoxicity curves for primary ALL cells, we investigated an alternative assay, described by others.

Table 2. Correlations Between the In Situ TSIA and MTX Accumulation or MTX Polyglutamylation

<table>
<thead>
<tr>
<th></th>
<th>Total MTX Accumulation (pmol/10⁹ cells)</th>
<th>Percentage MTX-Glu4-6</th>
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<tbody>
<tr>
<td></td>
<td>r</td>
<td>P</td>
</tr>
<tr>
<td>TSI₅₀,cont. MTX (µmol/L)</td>
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<td>&lt;.001</td>
</tr>
<tr>
<td>TSI₅₀,short MTX (µmol/L)</td>
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<td>TSI₅₀,cont. MTX/TSI₅₀,short MTX</td>
<td>-.28</td>
<td>.14</td>
</tr>
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</table>

Spearman’s correlation coefficients (r) of TSI₅₀ values for MTX of patient-derived leukemic blast cells obtained with the TSIA and MTX accumulation over 24 hours in 1 µmol/L [³H]-MTX or polyglutamylisation efficiency defined as percentage of total accumulated MTX present as MTX-Glu₄₋₆. For T-ALL, median accumulation of MTX was 630 pmol/10⁹ cells (range, 314 to 1316 pmol/10⁹ cells), median percentage MTX-Glu₄₋₆ was 41% (range, 0% to 85%). For c/preB-ALL, median accumulation of MTX was 1364 pmol/10⁹ cells (range, 521 to 3068 pmol/10⁹ cells), median percentage MTX-Glu₄₋₆ was 72% (range, 34% to 82%). TSI₅₀ values were determined either after 21 hours continuous MTX exposure (TSI₅₀,cont.) or after 3 hours of MTX incubation followed by an 18-hour drug-free period (TSI₅₀,short).
to detect antifolate sensitivity. In four cell lines and testing six antifolates, a highly significant correlation between the 18-hour TSIA and 3-day MTT assay suggested that the TSIA is a suitable antifolate sensitivity test for cell lines. For patient samples, replacing the substrate deoxouridine by deoxycytidine reduced the number of cells needed, as tritium is released faster from deoxycytidine than from deoxouridine. This could be associated with the fact that conversion of deoxouridine to dUMP is catalyzed by the cell cycle-dependent thymidylate kinase 1, which consequently has a low activity in resting cells. Deoxycytidine is activated by deoxycytidine kinase, which has a higher activity in leukemic cells and is not cell cycle-dependent. The monophosphate dCMP is efficiently deaminated to dUMP, the active substrate for TS. This modification, together with an increase in incubation times, resulted in an in situ MTX sensitivity assay, which can routinely be performed on cryopreserved and on fresh ALL patient cells and requires similar low numbers of cells as the conventional total cell-kill assays.

Using the TSIA, samples from 86 children with ALL, both c/preB-and T-ALL, were screened for MTX resistance. Both subtypes are treated with MTX, but the outcome of T-ALL is worse in conventional therapy regimens compared with c/preB-ALL as reviewed by Uckun et al. This may be partly explained by resistance to MTX, as T-ALL displays a less efficient MTX polyglutamylation compared with c/preB-ALL. Although the TSIA might not always equate MTX-induced cytotoxicity, the relative in vitro MTX resistance for T- versus c/preB-ALL is confirmed by the TSIA measurements. When cells were allowed to efflux MTX during an 18-hour drug-free period after 3 hours of incubation, T-ALL samples were 3.4-fold more resistant to efflux MTX during an 18-hour drug-free period after 3 hours of incubation compared with c/preB-ALL cells ($P = .001$). This is in accordance with the reported low accumulation of long chain polyglutamates in T-ALL, which are preferentially retained inside the cell.

With the continuous 21-hour exposure condition, no difference in MTX sensitivity between T- and c/preB-ALL could be detected. This suggests that differences in polyglutamoylation can be overcome during continuous MTX exposure, which has also been described for cell lines. This is supported by the experiments with F-MTX, as the TSIA values for MTX were strongly correlated with the TSIA values obtained for the nonglutamylatable F-MTX.

Patient-derived leukemia samples, when continuously exposed to MTX for 21 hours, displayed TSIA values varying almost 100-fold. High TSIA values might reflect MTX resistance, as reviewed by Bertino due to (1) a defective transport leading to a lower intracellular concentration of MTX, (2) a mutation in DHFR, the main target enzyme, resulting in a low-affinity for MTX, or (3) a higher level of DHFR. The relative chemoresistance in T-ALL may also be related to the more frequent prevalence of subclones with elevated levels of DHFR in T-ALL compared with B-lineage ALL samples. In addition, a human T-cell leukemia cell line was reported to contain twofold higher DHFR protein and mRNA levels compared with a human B-lineage leukemia cell line. The TSIA, however, could not detect differences in MTX sensitivity between T- and c/preB-ALL samples in the continuous 21-hour exposure condition. This might be related to elevations in DHFR in subclones occurring in a range of 10% to 90% of the cells; an overall activity assay would not detect these differences. Moreover, the 21 hours of incubation in the TSIA may overcome small differences in DHFR content. In addition, several other factors involved in MTX cytotoxicity might obscure the influence of only one parameter when measured by the end point TSIA.

The overall accumulation of MTX, as measured after 24 hours, is one parameter of MTX resistance that is correlated with the MTX sensitivity as measured with the TSIA. The comparison of a short (3 hours) incubation followed by a drug-free period (18 hours) and a continuous (21 hours) exposure condition may help to investigate intracellular retention of MTX, possibly providing information on MTX polyglutamylation defects. This hypothesis is supported by the significant correlation found between the ratio of TSIA$_{21h}$/TSIA$_{0cont}$ and the percentage MTX present as MTX-Glu$_{4-6}$.

In conclusion, the total-cell-kill MTT assay could not be adapted to evaluate MTX cytotoxicity on patient-derived ALL samples. However, the indirect TSIA based on MTX-induced TS inhibition proved to be informative with respect to the extent of MTX sensitivity and resistance. Using this assay, T-ALL samples were more MTX-resistant compared with B-lineage ALL samples after a 3-hour MTX exposure, followed by an 18-hour drug-free period; on long-term MTX exposure, both phenotypes were equally sensitive.

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Differential Methotrexate Resistance in Childhood T- Versus Common/PreB-Acute Lymphoblastic Leukemia Can Be Measured by an In Situ Thymidylate Synthase Inhibition Assay, But Not by the MTT Assay

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