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

By Marianne G. Rots, Rob Pieters, Gert J an L. Kaspers, Christina H. van Zantwijk, Paul Noordhuis, Rob Maurit, Anjo J. P. Veerman, Gerrit Jansen, and Godefridus J. Peters

Methotrexate (MTX) is not cytotoxic 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 \(^3\)H-dUMP to dTMP and \(^3\)H\(_2\)O, 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, \(\text{TSI}_{50}\) 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. \(\text{TSI}_{50}\) 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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From 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. This treatment failure may be explained by unfavorable clinical pharmacokinetics, regrowth potential of the residual leukemic cells, and by cellular drug resistance. 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. 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 assay or by other 4-day culture assays, such as the differential staining cytotoxicity (DiSC) assay. 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. 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. 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 lymphocytes due to degradation of DNA and RNA. Because on average 35% of ALL cells derived from patients spontaneously die during 4 days of culture, thereby releasing Hx and TdR,


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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.5 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 x 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 gentamycin, 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 x 10^6 cells/mL, U937 and CEM cells at 0.1 x 10^6 cells/mL for the MTT assay and at 2 x 10^5 cells/mL for the TSA.

Chemicals. MTX was obtained as a gift from Pharmachemie (Haarlem, The Netherlands). Fluoro-MTX (F-MTX), a nonglutamylated analogue of MTX, was obtained from Pharmachemie.22 Agouron Pharmaceuticals (La Jolla, CA), Ciba-Geigy (Basel, Switzerland), Glaxo Wellcome (Research Triangle Park, NC), Wernher-Lambert/Parke Davis (Ann Arbor, MI), and Zeneca (Macles Field, UK), respectively, supplied FCS, penicillin, streptomycin, fungizone, gentamycin, and L-glutamine. Solutions of MTX were prepared from Pharmachemie reagent-grade MTX (three MTX-resistant sublines were provided by Dr. H. van Besien, K.U. Leuven, Belgium). Tri-octylamine/1,1,2-tri-chloro-tri-fluoro-ethane (1/4; vol/vol) was used as a substrate.

Measurements of Hx and TdR. After 4 days of culture, microculture plates were kept at −20°C, at which temperature Hx and TdR are stabilized, 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-ethylamine/l,1,2-tri-chloro-tri-fluoro-ethane (1/4; vol/vol). This mixture was centrifuged for 1 minute at 12,000g. The separation of the nucleosides was quanod in the supernatant was performed on a reverse-phase microsphere C18 column (Chrompack; 100 x 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 tetrabutylammonium hydrogensulphate (TBAP), at pH 6 (0.5% sodium Tween 80 and 50% 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 TSA. Inhibition of TS was determined in whole cells based on a previously described assay 23,29 by measuring the TS-catalyzed conversion of 3H-dUMP to dTMP and 3H2O. A total of 135 µL cell suspensions (1 x 10^5 cells/mL; 4 x 10^5 cells/mL when deoxyuridine was used as a substrate) were incubated at 37°C with either 15 µL RPMI as controls or with 15 µL MTX 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 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/µmol) 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 of 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 TSA. Inhibition of TS was determined in whole cells based on a previously described assay 23,29 by measuring the TS-catalyzed conversion of 3H-dUMP to dTMP and 3H2O. A total of 135 µL cell suspensions (1 x 10^5 cells/mL; 4 x 10^5 cells/mL when deoxyuridine was used as a substrate) were incubated at 37°C with either 15 µL RPMI as controls or with 15 µL MTX 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 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/µmol) 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 of 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 TSA.
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 (IC_{50} 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^{-7} mol/L (Fig 1A and B). To investigate whether Hx and TdR can protect cells from growth inhibition by high MTX concentrations (>10^{-7} mol/L), but IC_{50} values increased fourfold and fivefold for HL60 and U937, respectively. TdR (10 μmol/L) by itself increased the IC_{50} 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 IC_{50} could be calculated (IC_{50} > 10^{-3} 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. IC_{50} 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. LC_{50} 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 nondividing 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.

Fig 1. Dose-response curves of MTX in absence or presence of 25 μmol/L Hx, 10 μmol/L TdR, or 1 × 10^6/mL lysed primary ALL cells for (A) HL60 and (B) U937. The results are expressed as mean values of three experiments ± SD. Protection from the MTX-induced growth inhibition was most obvious when Hx and TdR were added together.
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. 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 IC₅₀ values determined in the MTT assay and both the TSI₅₀,cont. (r = .89, P < .001, Fig 3) and the TSI₅₀,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 TSIA²⁹ required 2 × 10⁶ 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 [³H]₂O formed was increased fourfold when deoxycytidine was used as a substrate compared with deoxyuridine, reducing the amount of cells to 0.1 × 10⁶ cells per drug per concentration. No difference in TSI₅₀ 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 TSI₅₀ values were observed (data not shown).

TSIA in T- and c/preB-ALL. A large interpatient variation in MTX sensitivity was observed both in TSI₅₀,cont. ranging from 0.0067 to 0.76 µmol/L MTX and in TSI₅₀,short, ranging from <0.156 to >40 µmol/L MTX (Fig 5). The median TSI₅₀,cont was not significantly different between 29 T- and 51 c/preB-ALL samples (0.061 v. 0.104 µmol/L MTX; P = .46). The median TSI₅₀,short, however, was 3.4-fold higher in T-ALL compared with c/preB-ALL (1.66 v. 0.49 µmol/L; P = .001), but with a large overlap between both groups (Fig 5).

The role of MTX polyglutamates. F-MTX is a nonglutamatable analogue of MTX, with otherwise similar characteristics. Therefore, by measuring the sensitivity to both MTX and...
incubation period followed by an 18-hour drug-free period.

Patients after 21 hours of continuous MTX incubation and after a 3-hour
obtained in fresh samples and after cryopreservation from 10 pa-

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obtained by the use of \([3H]\)-dUrd and \([3H]\)-dCyd as substrates in the in

TSI 50, cont. F-MTX/TSI 50, cont. MTX was median 7 (range, 3 to
21), and the sensitivity for F-MTX was significantly related to
MTX \((r = .93; P < .001; \text{ Fig } 6)\). After incubation in drug-free medium, TS activity was fully recov-
nered 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 assay\(^9\)-\(^{12}\) and the DiSC assay\(^{13}\)
cannot be used for this purpose due to salvage by purines and
thymidine, which protect cells from the cytotoxic effects of
MTX.\(^{26,31-34}\) 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.\(^{16}\) In our experiments, the concentrations of metabo-
lites released by \(1 \times 10^6\) 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 lysate. 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 spontane-
ously during the 4 days of culture in the MTT assay,\(^9\) 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 concentra-
tion 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 concentra-
tion of 10,000 cells/well, cells were protected from MTX

cytotoxicity.\(^{16}\) 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.\(^{33}\) 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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<tr>
<td></td>
<td>r</td>
<td>P-value</td>
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<tr>
<td>TSI₅₀,cont. MTX</td>
<td>−.51</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>TSI₅₀,short MTX</td>
<td>−.58</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>TSI₅₀,cont. MTX/ TSI₅₀,short MTX</td>
<td>−.28</td>
<td>.14</td>
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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 polyglutamylation 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 deoxycytidine by deoxyuridine reduced the number of cells needed, as triptum is released faster from deoxycytidine than from deoxyuridine. This could be associated with the fact that conversion of deoxycytidine to dUMP is catalyzed by the cell cycle-dependent thymidine kinase I, 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 monophosphosphate 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 MTX than c/preB-ALL cells (p = 0.001). 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 polyglutamylation can be overcome during continuous MTX exposure, which has also been described for cell lines. 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 MTX than c/preB-ALL cells (p = 0.001). This is in accordance with the reported low accumulation of long chain polyglutamates in T-ALL, which are preferentially retained inside the cell.

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 TSI50,short/TSI50,cont. and the percentage MTX present as MTX-Glu4-6.

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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