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-Jan L. Kaspers, Christina H. van Zantwijk, Paul Noordhuis, Rob Mauritz, 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, 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. 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.

© 1999 by The American Society of Hematology.

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.4-6 Unfortunately, the cytotoxicity of methotrexate (MTX), a cornerstone in the treatment of 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

From the Departments of Pediatric Hematology/Oncology and Medical Oncology, University Hospital Vrije Universiteit, Amsterdam, The Netherlands.

Submitted July 29, 1998; accepted September 30, 1998.

Supported by Grant No. VU 94-679 from the Dutch Cancer Society.

Address reprint requests to Marianne G. Rots, Department of Pediatric Hematology/Oncology, University Hospital Vrije Universiteit, PO Box 7057, 1007 MB Amsterdam, The Netherlands; e-mail: marianne.rots@azu.nl.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. section 1734 solely to indicate this fact.

© 1999 by The American Society of Hematology.

0006-4971/99/9303-0001$3.00/0

From www.bloodjournal.org by guest on September 24, 2017. For personal use only.
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 x 10^6 cells/mL in culture medium (RPMI 1640 containing 20% heat-inactivated FCS, 100 U/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-polyglutamylatation)21 were grown as suspension cultures in RPMI 1640 containing 20% heat-inactivated FCS, 100 U/mL penicillin, 10 µg/mL streptomycin, 0.15 IU/mL) and XO (final concentration, 0.02 U/mL) at 37°C to 35°C in a humified 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 TSIA.

Chemicals. MTX was obtained as a gift from Pharmachemie (Haarlem, The Netherlands). Fluoro-MTX (F-MTX), a nonglutamylated MTX analogue,22 was kindly provided by Dr J.J. McGuire (Grace Cancer Center, Buffalo, NY). AG337, edatrexate, and ZD1694 were provided by Agouron Pharmaceuticals (La Jolla, CA), Werner-Lambert/Parke Davis (Ann Arbor, MI), and Zeneca (Maccles Field, UK), respectively. FCS, penicillin, streptomycin, fungizone, gentamycin, and L-glutamine were obtained from Flow Laboratories (Irvine, UK), bovine serum albumin from Organon Technika (Oss, The Netherlands), and Ficoll Isopaque (Lymphoprep 1.077 g/mL) was provided by Nyegaard (Oslo, Norway). Insulin, transferrin, sodium selenite, and MTT were purchased from Sigma (Zwijndrecht, The Netherlands), as well as thymidine, hypoxanthine, thymidine phosphorylase (TP), and xanthine oxidase (XO). 2-(aminocarbonyl)-N-(4-amino-2,6-dichlorophenyl)-4-[5,5-bis-(4-fluorophenyl)-1-piperazineacetamide (R75231) was kindly provided by Dr H. van Belle (Jansen Pharmaceuticals, Beerse, Belgium).

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,8 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 (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 casu quo bases 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 tetra-butylammonium hydrogensulphate (TBA), at 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 assay18,19 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 (four times 15 minutes with TP (final concentration, 0.15 µmol/L) and XO (final concentration, 0.02 U/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

MTT and DSC 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.20 In some experiments, Hx, TdR, or lysed ALL cells were added to the medium and IC50 values were determined relative to control cells also incubated in the presence of these additives. Lysed leukemic cells (final concentration, 1 x 10^6/mL) were obtained by snap-freezing and thawing three times.

From www.bloodjournal.org by guest on September 24, 2017. For personal use only.
was most obvious when Hx and TdR were added together.

The results are expressed as mean values of three experiments. In all cases, the amount of radioactivity and 10 µL for cell survival. The remaining 900 µL was applied to HL60 and U937. The results are expressed as mean values of three experiments. Data were expressed as TSI50,cont. referring to the continuous exposure condition and as TSI50,short for the short exposure condition. Experiments in which cell lines were assayed for antifolate sensitivity were performed by incubating 2 × 10^6 cells/mL applying the same time schedule. In this case, ^3^H-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 polyglutamylolation. Of samples for which a sufficient number of cells was available, 10^6^ cells were exposed for 24 hours to 1 µmol/L [^3^H]-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 TSI 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 polyglutamylation parameters. The Mann-Whitney U test was performed to determine differences in MTX sensitivity between T- and preB-ALL. All tests were performed by applying SPSS 7.5 for Windows (SPSS Benelux BV, Gorinchem, The Netherlands) software.

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 ± 1.0 and 11.0 ± 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 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 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 coinubcation 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.](http://www.bloodjournal.org)
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 TSI(50)cont and TSI(50)short, measured by 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 required 2 × 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 deoxycytidine to deoxyuridine, which is more efficiently converted to dUMP, the direct substrate for TS. The amount of [H]2O formed was increased fourfold when deoxycytidine was used as a substrate instead of deoxyuridine, reducing the amount of cells to 0.1 × 10^6 cells/mL. 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 TSI(50)cont, ranging from 0.0067 to 0.76 μmol/L MTX and in TSI(50)short, ranging from <0.156 to >40 μmol/L MTX (Fig 5). The median TSI(50)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 TSI(50)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 its polyglutamates, a more accurate estimation of antifolate sensitivity could be obtained.

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

<table>
<thead>
<tr>
<th>Sample No.</th>
<th>Hx (μmol/L)</th>
<th>TdR (μmol/L)</th>
</tr>
</thead>
<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>Hx (μmol/L)</td>
<td>TdR (μmol/L)</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 × 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 TSI50,short) values 0.68). The ratio TSI50,short/TSI50,cont. 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.\(^{10}\) In our experiments, the concentrations of metabolites 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 spontaneously 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 concentration in well 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 on 10,000 cells/well, cells were protected from MTX-induced growth inhibition.\(^{16}\) 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>Total MTX Accumulation (pmol/10^9 cells)</th>
<th>Percentage MTX-Glu4-6</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>r</td>
</tr>
<tr>
<td>TSI_{50,cont.} MTX (µmol/L)</td>
<td>-.51</td>
</tr>
<tr>
<td>TSI_{50,short} MTX (µmol/L)</td>
<td>-.58</td>
</tr>
<tr>
<td>TSI_{50,cont.} MTX/TSI_{50,short} MTX</td>
<td>-.28</td>
</tr>
</tbody>
</table>

Spearman’s correlation coefficients (r) of TSI_{50} 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/10^9 cells (range, 314 to 1316 pmol/10^9 cells), median percentage MTX-Glu4-6 was 41% (range, 0% to 85%). For c/preB-ALL, median accumulation of MTX was 1364 pmol/10^9 cells (range, 521 to 3068 pmol/10^9 cells), median percentage MTX-Glu4-6 was 72% (range, 34% to 82%). TSI_{50} values were determined either after 21 hours continuous MTX exposure (TSI_{50,cont.}) or after 3 hours of MTX incubation followed by an 18-hour drug-free period (TSI_{50,short}).
to detect antifolate sensitivity.\textsuperscript{18,36,37} 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 tritium is released faster from deoxyuridine than from deoxycytidine.\textsuperscript{19} 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.\textsuperscript{38} Deoxycytidine is activated by deoxycytidine kinase, which has a higher activity in leukemic cells and is not cell cycle-dependent.\textsuperscript{38} The monophosphate dCMP is efficiently converted 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.\textsuperscript{29} This may be partly explained by resistance to MTX, as T-ALL displays a less efficient MTX polyglutamylation compared with c/preB-ALL.\textsuperscript{39,40} 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 compared with c/preB-ALL cells (\textit{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.\textsuperscript{41}

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 polyglutamylmatation can be overcome during continuous MTX exposure, which has also been described for cell lines.\textsuperscript{29,42,43} This is supported by the experiments with F-MTX, as the TS\textsubscript{50,cont.} values for MTX were strongly correlated with the TS\textsubscript{50,cont.} values obtained for the nonglutamylatable F-MTX.

Patient-derived leukemia samples, when continuously exposed to MTX for 21 hours, displayed TS\textsubscript{50} values varying almost 100-fold. High TS\textsubscript{50,cont.} values might reflect MTX resistance, as reviewed by Bertino\textsuperscript{44} 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.\textsuperscript{45} 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.\textsuperscript{46} 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 TS\textsubscript{50,short}/TS\textsubscript{50,cont.} and the percentage TSIA present as MTX-Glu\textsubscript{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.

REFERENCES

11. Hongo T, Fujiy Y, Igarashi Y: An in vitro chemosensitivity test for...


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

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