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A possible role for methotrexate in the treatment of childhood acute myeloid leukaemia, in particular for acute monocytic leukaemia

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Abstract

Acute myeloid leukaemia (AML) is thought to be methotrexate (MTX)-resistant. However, a small study suggested that acute monocytic leukemia (AML-M5) is sensitive to MTX. We measured MTX accumulation/polyglutamylation in 20 AML-nonM5, 37 AML-M5 and 83 common/preB-acute lymphoblastic leukaemia (c/preB-ALL) samples. Membrane transport was determined in 11 childhood AMLs (including 3 AML-M5) and in 25 c/preB-ALL samples. MTX sensitivity was determined in 23 AML-nonM5, 15 AML-M5 and 63 common/preB-ALL samples using the thymidylate synthase (TS) inhibition assay. MTX transport was higher in AML samples compared with c/preB-ALL precluding a transport defect in AML. Accumulation of long-chain polyglutamates MTX-Glu_{4-6} was 3-fold lower for AML-nonM5 compared with c/preB-ALL cells (median 268 versus 889 pmol MTX-Glu_{4-6}/10^9 cells; $P \leqslant 0.001$); for AML-M5 samples, median accumulation of MTX-Glu_{4-6} was 0 pmol/10^9 cells ($P \leqslant 0.001$). After short-term MTX exposure, AML-nonM5 was 6-fold more resistant to MTX compared with c/preB-ALL cells (2.16 versus 0.39 μM; $P < 0.001$), while AML-M5 was 2-fold more resistant ($P = 0.02$). In both AML-nonM5 and AML-M5 cells, MTX resistance was circumvented by continuous MTX exposure (median TSI_{50} values: 0.052 and 0.041 μM, respectively) compared with a c/preB-ALL value of 0.066 μM. In conclusion, AML-M5 is relatively sensitive to MTX compared with other AML-subtypes even though polyglutamylation of MTX is poor. Using continuous exposure, AML-nonM5 and AML-M5 cells were at least as sensitive to MTX as c/preB-ALL cells. This report suggests that MTX might be an overlooked drug in the treatment of childhood AML.

Keywords: Acute monocytic leukaemia; Childhood acute myeloid leukaemia; Methotrexate; Polyglutamylation; Reduced folate carrier

1. Introduction

Childhood acute myeloid leukaemia (AML) has an event-free survival (EFS) of 25–50% [1–3], which is markedly lower than the reported EFS of 80% for precursor B-acute lymphoblastic leukaemia (ALL) patients [4]. Although aggressive induction therapy results in remission rates of up to 90% [3,5], many children with AML relapse. At relapse, the prognosis for patients with AML is generally poor, which might be associated with the presence of primary or secondary cellular drug resistance [6,7]. Therefore inclusion of different agents in the (re-)induction therapy may be beneficial.

The antifolate methotrexate (MTX) is generally not included in the treatment of AML, since early clinical trials failed to demonstrate convincing responses to MTX in AML patients. The use of MTX was not explored further, possibly also as a result of the success of the use of anthracyclines and cytosine arabinoside [9]. MTX resistance in AML cells may be related to defects in membrane transport [10] or in polyglutamylation [11–14]. The inefficient polyglutamylation was associated

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with a low activity of the enzyme catalysing the polyglutamylation, folylpolyglutamate synthetase (FPGS) [14,15] and with a high activity of the enzyme responsible for degradation of the glutamate side chain, folylpolyglutamate hydrolase (FPGH) [14]. Additional factors such as high levels of dihydrofolate reductase (DHFR), the main target enzyme of MTX, might also negatively affect the response of AML cells to MTX [16,17].

Recently, two small studies described that MTX polyglutamylation patterns in eight samples of acute monocytic leukaemia patients (AML-M5) [18] and two of acute megakaryocytic leukaemia patients (AML-M7) [19] were comparable to those obtained for paediatric precursor B-ALL samples, a phenotype considered to be highly MTX-sensitive. Consequently, it was suggested that the prognosis of these AML subtypes might be improved by treatment with MTX [18,19]. AML-M5 represents 13–22% of the AML cases and frequently occurs in infants. Secondary leukemia after treatment with epipodophyllotoxins also often presents as AML-M5 [20].

In the present report, we studied MTX resistance and its circumvention in AML-M5 versus AML-nonM5 relative to common/preB-ALL. This was done by analysis of MTX membrane transport, MTX accumulation, of subsequent polyglutamylation to the pharmacologically important metabolites MTX-Glu_{4-6} and of *in situ* inhibition of thymidylate synthase (TS).

2. Patients and methods

2.1. Patient samples

Leukaemic blast cells were obtained by density gradient centrifugation (Lymphoprep, 1.077 g/ml, Nycomed Pharma, Oslo, Norway) from 59 children with AML, classified according to the French–American–British (FAB) classification system [21] as M0 ($n=1$), M1 ($n=6$), M2 ($n=9$), M3 ($n=2$), M4 ($n=9$), M5 ($n=31$) and M7 ($n=1$). From all patients and/or their parents we received informed consent to perform scientific research with these samples. Polyglutamylation data [14] and MTX sensitivity data [22] have been reported previously for 23 and 12 AML samples, respectively. In order to increase the number of AML-M5 samples, eight adult AML-M5 samples were also analysed for MTX polyglutamylation efficiency. Since the MTX resistance parameters did not differ between paediatric AML-M5 cells and those obtained from adult AML-M5 patients, the data were pooled for statistical evaluations. The MTX resistance parameters were compared with those obtained within a group of 111 children older than 1 year, newly diagnosed with common- or preB-ALL (c/preB-ALL) as determined by positive immunocytochemical staining for French–American–British (HLA-DR) human leucocyte antigen-DR, terminal deoxynucleotidyl, cluster of differentiation CD10 and CD19, together with negative staining for surface immunoglobulin.

All samples contained more than 80% of leukaemic cells; for some samples contaminating non-malignant cells were removed by monoclonal antibodies linked to magnetic beads, as previously described [23]. For all assays, cells were suspended in Roswell Park Memorial Institute (RPMI) 1640 Dutch Modification (Gibco BRL, Breda, The Netherlands) containing 20% fetal calf serum (FCS) (Gibco BRL), 2 mM L-glutamine (ICN, Costa Mesa, CA, USA), 5 μg/ml insulin, 5 μg/ml transferrin, 5 ng/ml sodium selenite (Sigma, St Louis, MO, USA) and antibiotics [24].

2.2. Initial rate of MTX membrane transport

Leukaemia cells (5×10^6) were washed with RPMI containing 2% FCS and resuspended in 1 ml Magnesium HEPES Sucrose (MHS) buffer (20 mM HEPES, 225 mM Sucrose, pH 7.4 set with MgO). This anion-free buffer has been designed to measure maximal reduced folate carrier (RFC)-mediated MTX membrane transport due to the lack of competition of anions and abolishment of MTX efflux [25]. Cells were incubated with [^{3}H]-MTX (final concentration 2 μM, 2 Ci/mmol) for 15 min, washed three times with ice-cold phosphate-buffered solution (PBS) and resuspended in 350 μl PBS. Cell counting and viability determination by trypan blue exclusion was performed on 20 μl of cell suspension; 300 μl were used for ß-scintillation counting. A control for membrane transport specificity consisted of cells which were incubated at 37°C in the presence of [^{3}H]-MTX with an excess of unlabelled MTX (final concentration 1 mM, 4 μCi/mmol). Data for the initial rate of membrane MTX transport were evaluable when over 85% of the cells were viable after incubation in MHS buffer and are expressed as pmol MTX transport/min/10^6 viable cells.

2.3. Polyglutamylation of MTX

Accumulation and polyglutamylation of MTX were assayed as previously described [14]. In short, leukaemic cells (10×10^6/5 ml) were incubated at 37°C for 24 h with 1 μM [^{3}H]-MTX (Moravek, Brea, CA, USA; final specific activity: 2 Ci/mmol), washed and counted for cell number and for radioactivity. Polyglutamylated metabolites were separated by high performance liquid chromatography (HPLC).

2.4. In situ TS inhibition assay

Depending on the number of cells available per sample, MTX sensitivity was determined by measurements of the MTX-induced inhibition of the TS-catalysed

conversion of [^{3}H]-deoxyuridine monophosphate (dUMP) to dTMP and ^3H_2O [26]. Leukaemic cells ($\times 10^5$ cells/ml in 150 μl) were exposed to MTX for 21 h (continuous exposure; five final concentrations: 0.0039–1 μM in duplicate) or for 3 h after which the cells were washed and incubated for an additional 18 h in drug-free culture medium (short-term exposure; five final concentrations: 0.156–40 μM in duplicate), as previously described [27,28]. (5-[^{3}H]-2′-deoxycytidine (Moravek, Brea, CA, USA; final concentration of 1 μM, specific activity: 2.5 Ci/mmol) was added 4 h after the start of the experiment as a precursor for [^{3}H]-dUMP. Blanks (containing culture medium) and controls (cell suspensions without MTX) were included in triplicate. The TS-catalysed conversion of [^{3}H]-dUMP to dTMP and ^3H_2O was stopped by the addition of trichloroacetic acid and [^{3}H]-deoxycytidine monophosphate (dCMP), [^{3}H]-dUMP and unconverted [^{3}H]-deoxycytidine was absorbed by active charcoal. Samples were counted for radioactivity by ß-scintillation counting. Data were expressed as $TSI_{50,cont.}$ (concentration of MTX necessary to inhibit 50% of the control TS activity during long-term continuous exposure) and $TSI_{50,short}$ (referring to the short-term exposure).

2.5. Statistics

The non-parametric Mann–Whitney U test (two-tailed) was performed to analyse differences between AML-M5, AML-nonM5 and c/preB-ALL samples. A P value of <0.05 was considered to be significant.

3. Results

MTX membrane transport was higher for the 11 AML compared with the 25 c/preB-ALL samples (0.018 pmol MTX/min/10^6 cells versus 0.0054 pmol/MTX/min/10^6 cells; $P=0.01$) (Fig. 1). The three AML-M5 samples demonstrated an efficient MTX membrane transport.

Subsequently, accumulation and polyglutamylation were measured in AML-nonM5, AML-M5 and c/preB-ALL samples. MTX accumulation was not different between the 20 AML-nonM5 samples and 83 c/preB-ALL samples (1096 versus 1364 pmol MTX-$Glu_{1–6}$/10^9 cells; $P=0.14$), nor did we find a significant difference for the AML-M4 samples (1101 pmol MTX-$Glu_{4–6}$/10^9 cells) compared with either c/preB-ALL or AML-M5. MTX accumulation was lower in the 37 AML-M5 samples compared with c/preB-ALL (869 versus 1364 pmol MTX-$Glu_{1–6}$/10^9 cells; $P=0.001$) (Fig. 2). Polyglutamylation efficiency was lower in AML-nonM5 compared with c/preB-ALL samples and extremely poor for AML-M5 samples (Fig. 3). Of the total accumulated

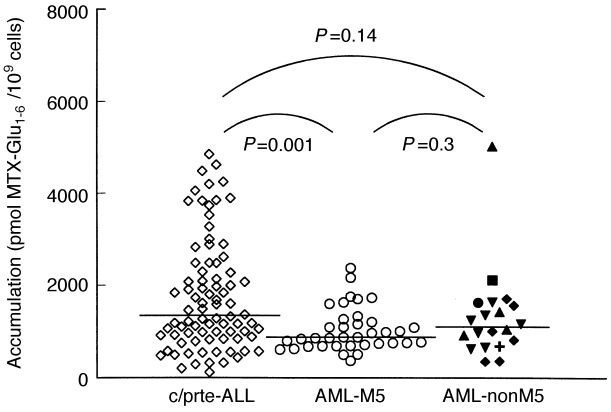

Fig. 2. Accumulation of MTX after 24 h exposure of childhood leukaemic cells to 1 μM [^{3}H]-MTX. Data are expressed as pmol MTX-$Glu_{1–6}$/10^9 cells. Symbols represent individual patient samples; the median value is represented by a horizontal line. AML-nonM5 is subdivided into (+) AML-M0, (▲) AML-M1, (▼) AML-M2, (■) AML-M3, (◆) AML-M4, (●) AML-M7. The subgroup AML-M4 was not significantly different from AML-M5.

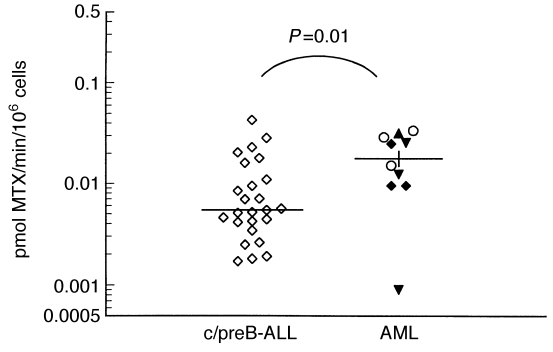

Fig. 1. Initial rates of methotrexate (MTX) membrane transport for 25 common- or preB-acute lymphoblastic leukaemia (c/preB-ALL) and 11 acute myeloid leukaemia (AML) samples determined after 15 min exposure to [^{3}H]-MTX in an anion-free buffer. Each symbol represents an individual patient sample; the median value is represented by a horizontal line. AML is subdivided into (+) AML-M0, (▲) AML-M1, (▼) AML-M2, (◆) AML-M4, (o) AML-M5.

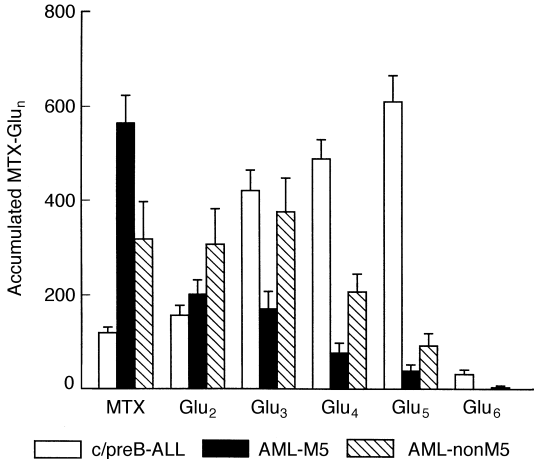

Fig. 3. Distribution of MTX polyglutamates in c/preB-ALL, AML-M5 and AML-nonM5 samples. Polyglutamates are presented as mean pmol/10^9 cells ± standard error.

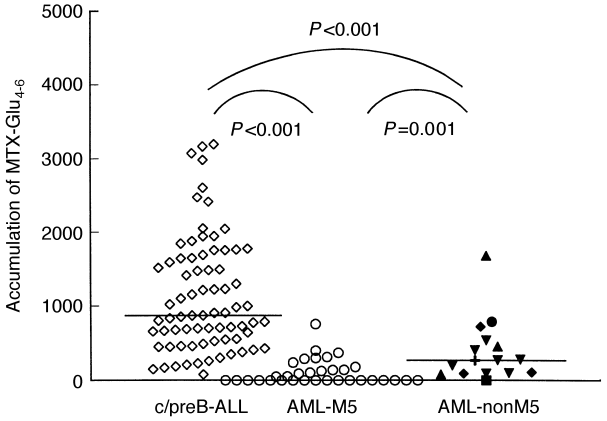

Fig. 4. Accumulation of MTX-$Glu_{4–6}$ after 24 h exposure of leukaemic cells to 1 μM [^{3}H]-MTX. Data are expressed as pmol MTX-$Glu_{4–6}$/10^9 cells. Symbols represent individual patient samples; the median value is represented by a horizontal line. An explanation of the symbols is provided in the legend of Fig. 2. The subgroup AML-M4 was not significantly different from AML-M5.

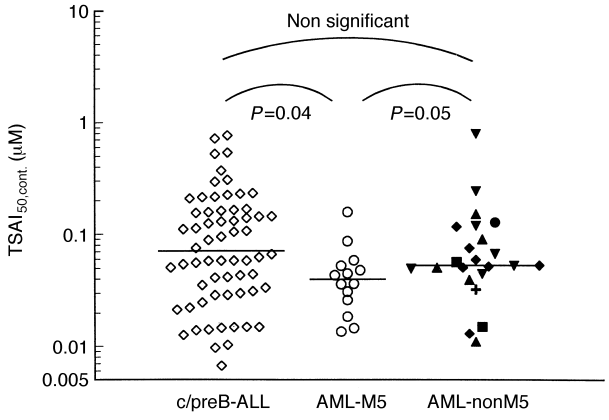

Fig. 6. Comparison of MTX sensitivity of c/preB-ALL, AML-M5 and AML-nonM5 as measured with the *in situ* TS inhibition assay. Results are expressed as the concentration of MTX resulting in 50% inhibition of the control *in situ* TS activity after 21 h continuous exposure to MTX ($TSI_{50,cont.}$). Symbols represent individual patient samples; the median value is represented by a horizontal line. An explanation of the symbols is provided in the legend of Fig. 2. The $TSI_{50,cont}$ was not significantly different in AML-M4 samples compared with AML-M5 samples.

MTX, a median of 29% was present as MTX-$Glu_{4–6}$ in the AML-nonM5 samples compared with 66% in the c/preB-ALL samples ($P < 0.001$), and 0% in the AML-M5 samples ($P < 0.001$). Consequently, the median accumulation of MTX-$Glu_{4–6}$ was 268 pmol MTX-$Glu_{4–6}$/10^9 AML-nonM5 cells compared with 889 pmol MTX-$Glu_{4–6}$/10^9 c/preB-ALL cells ($P < 0.001$) and 0 pmol/10^9 AML-M5 cells ($P = 0.001$) (Fig. 4).

To determine the influence of experimental parameters on the pattern of polyglutamylation, samples were also incubated in 10 μM MTX instead of 1 μM or in medium containing dialysed FCS instead of non-dialysed FCS for 2 AML-M5 patients. Although the total accumulation of MTX was increased by approximately 7-fold when samples were exposed to 10 μM MTX compared with 1 μM, the pattern of polyglutamylation did not change upon changing experimental conditions (data not shown).

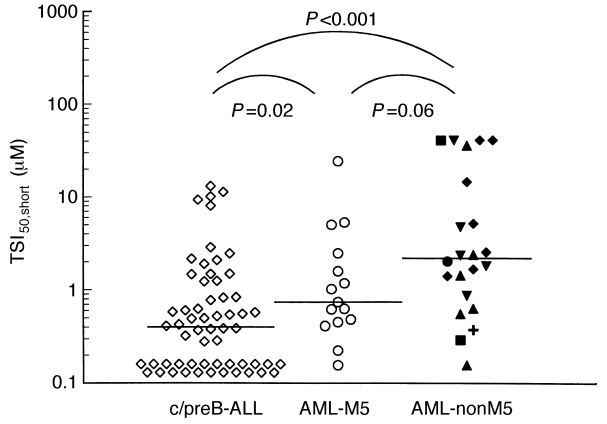

Fig. 5. Comparison of MTX sensitivity of c/preB-ALL, AML-M5 and AML-nonM5 as measured with the *in situ* TS inhibition assay. Results are expressed as the concentration of MTX resulting in 50% inhibition of the control *in situ* TS activity after 3 h MTX exposure followed by an 18 h drug-free period ($TSI_{50,short}$). Symbols represent individual patient samples; the median value is represented by a horizontal line. An explanation of the symbols is provided in the legend of Fig. 2. The $TSI_{50,short}$ in AML-M4 (median 5.06 μM) was significantly higher ($P = 0.002$) than in AML-M5.

The *in situ* TS inhibition assay was used to determine overall cellular MTX resistance for patient-derived leukaemic samples. Median $TSI_{50,short}$ was 6-fold higher for 22 AML-nonM5 samples compared with 59 c/preB-ALL samples (2.16 μM versus 0.39 μM; $P < 0.001$). For the 15 AML-M5 samples, median $TSI_{50,short}$ (0.73 μM) was 2-fold higher compared with the c/preB-ALL samples ($P = 0.02$), but tended to be 3-fold lower compared with AML-nonM5 samples ($P = 0.06$) and 7-fold lower than AML-M4 (median 5.06 μm) ($P = 0.002$) (Fig. 5). This relative MTX resistance in AML was completely circumvented by continuous MTX exposure; Median $TSI_{50,cont.}$ was not different between AML-nonM5 and c/preB-ALL. For AML-M5, the median $TSI_{50,cont.}$ (0.041 μM) was 1.6-fold lower compared with c/preB-ALL (0.066 μM; $P = 0.04$) and 1.3-fold lower compared with AML-nonM5 samples (0.052 μM; $P = 0.05$) (Fig. 6). There was no significant correlation between both TSI values and MTX accumulation.

4. Discussion

In this study, we demonstrate that MTX resistance in AML samples can be circumvented by continuous exposure and that AML-M5 samples were even more MTX sensitive compared with c/preB-ALL and compared with AML-nonM5. This is of particular interest since AML is generally thought to be intrinsically MTX

resistant [8,12,16], while c/preB-ALL is responsive to MTX-including regimens [4].

Previously, it has been shown that total MTX accumulation was not lower in AML compared with B-lineage ALL [11,12,14]. This suggests that membrane transport defects are not involved in causing MTX-resistance in AML. In line with this observation, no defects in MTX membrane transport were observed in the present study for the group of AML samples compared with c/preB-ALL. Interestingly, applying the fluorescent PT430 competition assay, Gorlick and colleagues reported that 35% of the AML samples displayed defective MTX transport as defined by incomplete (< 40%) displacement of PT430 from DHFR by MTX. The apparent discrepancy between the study by Gorlick and colleagues and the present study may be associated with different experimental set-ups and end-points. The PT430 displacement assay is an indirect measurement of MTX transport and assumes saturable, specific and competitive binding of PT430 to DHFR. Beyond this, since the competition assay requires at least 90 min of MTX incubation, factors other than RFC influx rates *per se* (e.g. MTX-polyglutamylation [14,29] and MTX efflux [30]) can determine the rate of PT430 displacement from DHFR. Under the conditions of our ^{3}H-MTX transport assay, efflux of MTX is minimal [25]. Thus, our assay reflects influx capacities rather than the results of a dynamic equilibrium of MTX influx and efflux, which is measured in the PT430 assay.

MTX resistance in AML is associated with defects in polyglutamylation as we and others have shown [11–14]. Polyglutamylation is necessary for the intracellular retention of MTX [31]. In the present study, we confirmed the relatively poor polyglutamylation profile of AML cells that was found in our previous report describing less samples. The poor polyglutamylation profile results in MTX resistance after short-term MTX exposure followed by a drug-free period (Fig. 5). Interestingly, the resistance can be overcome by 21 h of continuous exposure to MTX (Fig. 6). This result is consistent with observations in leukaemic cell lines [32–34] and patient-derived T-ALL cells [28] for which polyglutamylation defects were observed: long-term MTX exposure could overcome MTX resistance noted after a drug-free period. Likewise, in a variety of leukaemic samples with different polyglutamylation characteristics, the MTX-induced TS *in situ* inhibition was correlated with the potency of the nonpolyglutamatable F-MTX to induce TS inhibition after continuous exposure [28]. The observation of circumvention of MTX resistance in AML suggests the possible use of MTX administered as a continuous infusion in the treatment of AML.

Within the subtypes of AML, the AML-M5 samples were suggested to be relatively sensitive to MTX. This suggestion was based on the observation that AML-M5 samples were capable of accumulating similar amounts of the pharmacologically important long-chain MTX-$Glu_{4–6}$ as observed for c/preB-ALL samples [18]. Using the in situ TS inhibition assay, for which antifolate sensitivity data correlated with those derived from conventional cytotoxicity assays [27,28], we demonstrate in the present report that AML-M5 indeed is a relative MTX-sensitive AML-subtype. This relative MTX sensitivity, however, was not related to favourable polyglutamylation characteristics in our study: AML-M5 samples showed very poor MTX polyglutamylation. The apparent discrepancies between the present study and the study of Göker and colleagues [18] could not be explained by different experimental conditions (e.g. differences in MTX concentration and in culture media), as we determined for two AML-M5 samples. In this regard, we showed elsewhere that our polyglutamylation method revealed differences between c/preB-ALL, T-lineage ALL and AML [14] as also described by others [11–13,35,36]. Moreover, re-analysis of four samples by the group of Bertino (Memorial Sloan Kettering Institute, New York, NY, USA) showed the same polyglutamylation profiles as obtained by our laboratory (data not shown).

Therefore, the reason for the low level of MTX polyglutamylation of AML-M5 samples in the present study is not clear. Several explanations can be considered; FPGS might display a poor affinity for MTX in AML-M5 compared with other acute leukaemias as has been described for a AML-M3 and-M6 cell line and for 3 unclassified AML patient samples [37]. Moreover, high levels of DHFR, as described for AML [16,17] can bind MTX which may prevent MTX polyglutamylation [38]. To our knowledge, however, no data are available on DHFR levels in AML-M5 samples compared with AML-nonM5. Unfortunately, both functional DHFR and TS enzyme assays require large amounts of cells, precluding evaluation in our samples. Moreover, high levels of DHFR would be in contrast with the MTX sensitivity observed in this study after continuous MTX exposure in the in situ TS inhibition assay. Finally, high intracellular folate levels might abolish MTX polyglutamylation [39,40].

It is of interest to note that, despite the poor polyglutamylation of MTX, the TS inhibitory potency is retained in AML-M5 cells. This might be related to a relatively low efflux of MTX in AML-M5 or to a high efflux rate in AML-nonM5. Efflux of MTX can be mediated via multidrug resistance proteins (MRPs) [30,41] which are differentially expressed in AML cells [42,43]. Alternatively, this phenomenon might be explained by dihydrofolate-polyglutamates inhibiting TS [31], since levels of dihydrofolates increase upon DHFR inhibition by MTX. Because dihydrofolate is an

excellent substrate for FPGS [44], this can provide an additional inhibitory effect on TS.

In conclusion, long-term exposure can overcome MTX resistance in AML. Despite a poor MTX polyglutamylation, AML-M5 tended to be more MTX sensitive compared with AML-nonM5 samples. These results suggest that AML patients, particularly AML-M5, might benefit from continuous MTX exposure as a treatment.

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