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Gene expression profiling of leukemia T-cells resistant to methotrexate and 7-hydroxymethotrexate reveals alterations that preserve intracellular levels of folate and nucleotide biosynthesis☆

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ABSTRACT

In vitro treatment of human T-cell leukemia cells with 7-hydroxymethotrexate, the major metabolite of methotrexate resulted in acquired resistance as a result of the complete loss of folypolyglutamate synthetase (FPGS) activity. This was in contradistinction to the major modality of antifolate resistance of impaired drug transport in leukemia cells exposed to methotrexate. To identify the genes associated with methotrexate and 7-hydroxymethotrexate resistance, we herein explored the patterns of genome-wide expression profiles in these antifolte-resistant leukemia sublines. mRNA levels of the reduced folate carrier, the primary influx transporter of folates and antifolates, were down-regulated more than two-fold in methotrexate-resistant cells. The dramatic loss of FPGS activity in 7-hydroxymethotrexate-resistant cells was associated with alterations in the expression of various genes aimed at preserving reduced folates and/or enhancing purine nucleotide biosynthesis, e.g. methylene tetrahydrofolate reductase, glycinamide ribonucleotide formyltransferase, adenosine deaminase, cystathionine β synthase, as well as the ATP-dependent folate exporters *BCRP/ABCG2* and *MRP1/ABCC1*. The observed changes in gene expression were generally not paralleled by acquired DNA copy numbers alterations, suggesting transcriptional regulatory mechanisms. Interestingly, gene expression of DNA/RNA metabolism and transport genes were more profoundly altered in methotrexate-resistant subline, whereas in 7-hydroxymethotrexate-resistant cells, the most profoundly affected groups of genes were those encoding for proteins involved in metabolism and cellular proliferation. Thus, the present investigation provides evidence that 7-hydroxymethotrexate induces gene expression alterations and an antifolate resistance modality that are distinct from its parent drug methotrexate.

1. Introduction

During the past six decades, the rate of success in curing childhood acute lymphoblastic leukemia (ALL) has increased from less than 10% to more than 80%. This dramatic improvement is largely due to optimization of the use of existing chemotherapeutic agents. Methotrexate (MTX) is the major anti-metabolite (Fig. 1) employed in combination chemotherapeutic regimens for childhood ALL. Treatment failure is mainly related to acquired drug resistance or selection of pre-existing molecular alterations facilitating antifolate resistance.

The mechanisms of action and resistance to MTX and its major metabolite 7-hydroxymethotrexate (7-OHMTX) have been characterized in some detail [1,2]. Decreased cellular uptake via the reduced folate carrier (RFC/SLC19A1) [3] or increased active efflux

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Abbreviations: MTX, methotrexate; 7-OHMTX, 7-hydroxymethotrexate; IC_{50}, the drug concentration inhibiting cell growth by 50%; DHFR, dihydrofolate reductase; TS, thymidylate synthase; RFC, reduced folate carrier; FPGS, folylpolyglutamate synthetase; FPGH, folylpolyglutamate hydrolase; GARFT, glycinamide ribonucleotide formyltransferase; MRP, multidrug resistance-associated protein.

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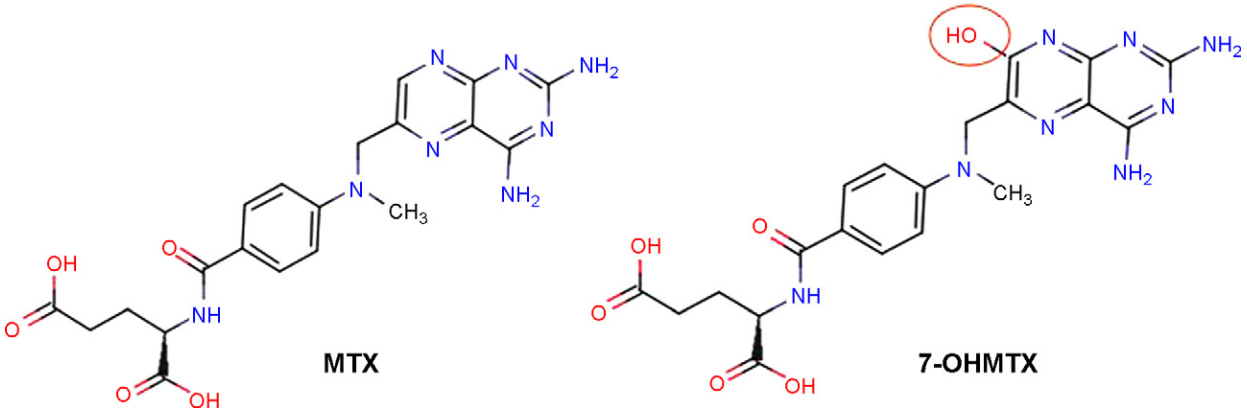

Fig. 1. The chemical structures of methotrexate (MTX) and its major catabolite 7-hydroxymethotrexate (7-OHMTX).

mediated by ABC transporters such as the multidrug resistance-associated proteins 1–5 (MRP1–5), the breast cancer resistance protein (BCRP/ABCG2) [4,5] and P-glycoprotein (MDR or ABCB1) [6] can lead to resistance to hydrophilic and lipophilic antifolates. The ability of cells to accumulate long chain MTX polyglutamates (MTX-PGs) is highly dependent on the activities of folylpolyglutamate synthetase (FPGS) and folylpolyglutamate hydrolase (FPGH). Furthermore, the susceptibility of cells to the cytotoxic effect elicited by MTX may also be influenced by mutations in the dihydrofolate reductase gene (*DHFR*) that result in a lower affinity for MTX, by increased activity of thymidylate synthase (TS), as well as by altered activities of other folate- and one-carbon metabolism enzymes.

The metabolite 7-OHMTX (Fig. 1) results from oxidation of MTX primarily by hepatic aldehyde oxidase. Intracellular conversion of MTX to 7-OHMTX has also been demonstrated in leukemia cells [7]. Although the affinity of DHFR for 7-OHMTX is markedly lower (>100-fold) than for MTX [8], the polyglutamated forms of this metabolite are more potent inhibitors of this enzyme [9,10]. Polyglutamated forms of MTX and polyglutamated conjugates of 7-OHMTX are also capable of inhibiting a number of other folate-dependent enzymes involved in *de novo* purine biosynthesis such as TS, glycinamide ribonucleotide formyltransferase (GARFT) and aminoimidazole carboxamide ribonucleotide formyltransferase (AICART).

We have recently reported that prolonged exposure of human leukemic T-cells to 7-OHMTX leads to antifolate resistance that attenuates the efficacy of MTX [11]. While the primary mechanism of resistance to MTX was a marked reduction in RFC-mediated drug uptake, resistance to 7-OHMTX was due to a dramatic decrease (>98%) in FPGS activity, which enhanced resistance to short-term (4 h) exposure to MTX more than 100-fold. To further characterize the molecular mechanisms associated with acquired resistance to MTX and 7-OHMTX in human leukemia cells, we characterized gene expression profiles and alterations in gene copy numbers using high density microarrays. The results revealed disparate cellular responses to MTX and 7-OHMTX that may facilitate the identification of novel cellular targets and modalities of resistance to 7-OHMTX as well as possibly suggesting novel approaches to overcome antifolate resistance and design individualized antifolate-based drug therapy of human leukemia.

2. Materials and methods

2.1. Materials

The HG-U133A GeneChip oligonucleotide microarray and all reagents required for microarray analysis were purchased from Affymetrix (Affymetrix Inc., Santa Clara, CA, USA). TaqMan reagents and gene expression assays were obtained from Applied Biosystems (Stockholm, Sweden) including: folylpolyglutamate synthetase (*FPGS*, No.: Hs00191956 m1), folylpolyglutamate hydrolase (*FPGH*/*GGH*, No.: Hs00608257_m1), the reduced folate carrier (*RFC*/*SLC19A1*, No.: Hs00161870_m1), glycinamide ribonucleotide transformylase (*GART*, No.: Hs00531926_m1), 5-aminoimidazole-4-carboxamide ribonucleotide formyltransferase (*AICART*, No.: Hs00269671_m1), 5-methyltetrahydrofolate-homocysteine methyltransferase (*MTR*, No.: Hs00165188_m1), 5,10-methylenetetrahydrofolate reductase (*MTHFR*, No.: Hs00195560_m1), multidrug resistance-associated proteins 4 (*MRP4*, No.: Hs00195260), and 5 (*MRP5*, No.: Hs00194701_m1), and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*, No.: Hs99999905_m1).

2.2. Comparison of plasma MTX and 7-OHMTX levels in MTX treated ALL patients

Plasma levels of MTX and 7-OHMTX previously determined in 49 childhood ALL patients by HPLC [12]. The conditions for sample collections and procedures and HPLC assay applied are according to previously published information [13]. In short, plasma samples were obtained after intravenous infusion of MTX (5–8 g/m^2) during 24 h. Blood samples were collected 20–23 h after beginning of MTX infusion (steady-state concentration), at 36 h and once every 6 h afterward until plasma-MTX concentration fell below <0.2 μM. Samples were generally drawn from a central venous catheter, while the steady-state samples were obtained from a peripheral vein.

2.3. Antifolate-resistant cells and tissue culturing

MOLT-4 cells derived from a human T-cell ALL were purchased from American Type Culture Collection (Rockville, MD, USA). The generation and functional characterization of drug resistant derivatives have been previously reported [11]. In short, this involved sequential exposure (12–18 steps each of 4 days) of parental MOLT-4 cells to gradually increasing concentrations of MTX (1–300 nM) or 7-OHMTX (50–30,000 nM). This resulted in sub-lines with greater than 50-fold increased resistance to MTX or 7-OHMTX relative to parental cells. Cells were cultured in RPMI-1640 medium (supplemented with 10% fetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine) at 37 °C under a humidified air containing 5% CO_2. For experimental studies cells were cultured in drug-free-medium for at least three passages, and harvested during the logarithmic phase growth at a density (0.8–1.5) × 10^6/ml as determined by cell counting using a Coulter Multi-sizer (Coulter Electronics, Luton, United Kingdom).

2.4. Isolation and quality verification of RNA

Triplicate inoculates of parental and resistant cells were thawed, washed and cultured in a standard medium (see above) for four passages, following which 10^7 cells of each cell type were collected by centrifugation. High quality RNA was extracted using the RNeasy Midi kit in accordance with the manufacturer's instructions (RNeasy Midi Handbook; Qiagen, KEBO Lab, Spånga, Sweden). A NanoDrop ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies, Wilmington, DE) was used to determine RNA concentrations, and to verify its quality and purity, whereby RNA with an OD ratio of 1.99–2.0 at 260/280 was found acceptable.

2.5. Experimental conditions and analysis of expression arrays

The experimental procedures and analyses were in accordance with the instructions of the manufacturer (Technical manual of Affymetrix GeneChip products). In short, complementary DNA (cDNA) synthesized from 11 μg of total RNA, was used for synthesis and isolation of biotin-labelled complementary RNA (cRNA), and fragmented to a mean size of ~50–100 nucleotides. Samples were subsequently analyzed in triplicate on Affymetrix U133A GeneChips containing 22,283 sets of probes for approximately 17,000 different species of mRNA. After hybridization for 16 h at 45 °C, washing and staining with streptavidin-R-phycoerythrin, the arrays were scanned in an Agilent Gene Array Scanner (Affymetrix). The arrays were first analyzed using GeneChip Operating Software (GCOS 1.4; Affymetrix), and data files were subsequently analyzed utilizing the GeneSpring 7.2 software. Normalization was performed with the same software employing default normalization parameters as follows: for each sample the raw data were divided by the 50th percentile of all measurements, and for each gene the raw data were divided by the median level of expression of the specific samples in question. Data from sets of probes designed to detect the expression of genes that failed to fulfil the criteria for detection (labelled "absent" or "marginal" in all microarrays) and an intensity value less than 50 (to reduce background signals) were eliminated. For statistical calculations, analysis of variance (ANOVA) were used ($p < 0.05$), and Benjamin and Hochberg false discovery rate were used for multiple testing correction. After the filtration steps, 863 probe sets with significantly altered expression levels between parental and/or MTX resistant and/or 7-OHMTX resistant cells remained for further analysis (Fig. 3). All microarray data were submitted to http://www.ncbi.nlm.nih.gov/geo/.

2.6. Quantitative real-time PCR (qRT-PCR)

qRT-PCR was performed on cDNA samples of parental and resistant cells in three independent experiments to quantify the expression of *FPGS*, *FPGH*/*GGH*, *RFC*/*SLC19A1*, *GART*, *AICART*, *MTR*, *MTHFR*, *MRP4*, and *MRP5*. *GAPDH* was analysed as a positive control as well as used for normalization.qRT-PCR reactions were carried out in 20 μl mixtures containing 9 μl cDNA template, 1 μl TaqMan® Gene Expression Assay and 10 μl TaqMan Fast Universal PCR Master Mix, No AmpErase UNG concentrated 2-fold. Amplifications involved 2 min at 50 °C (stage 1), and 10 min at 95 °C (stage 2), followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min (stage 3), and were carried out in 96-well optical PCR plates (N 801-0560, PerkinElmer) placed in an automated fluorometer (ABI PRISM 7700 Sequence Detection System, Applied Biosystems). The different mRNA species were normalized against *GAPDH* determined in the same samples. The values obtained for resistant cells were subsequently divided with the values for parental cells to allow comparisons. Further details are available in PerkinElmer Instruction Manual of 1997.

2.7. Array-based comparative genomic hybridization (array-CGH)

Genomic DNA was extracted from cultured cells using a commercial kit (Gen Elute, Sigma) and used for analyses of gene copy number alterations by array-CGH. The experimental procedures and data analyses were according to previously published information [14]. The tiling 33 k or 38 k BAC arrays used were produced at the SCIBLU Genomics Centre at Lund University, Sweden (www.lu.se/sciblu). Microarray slides were hybridized for 72 h at 37 °C with differentially labeled test and reference DNA (Cy5-dCTP and Cy3-dCTP) in the presence of Cot-1 DNA, washed under stringent conditions, and nitrogen blow-dried.

Arrays were subsequently scanned using a GenePix 4200A (Axon Instruments Inc., Union City, CA), and images were quantified with GenePix Pro 6.0 (Axon Instruments, Wheatherford, TX, USA) and uploaded in BioArray Software Environment, BASE (http://www.base.thep.lu.se/) [15]. The pin-based LOWESS algorithm [16] was used for data normalization. Relative copy numbers were determined from normalized $\log_2$ ratios, and altered regions were defined by CGH plotter [17] applying the thresholds >0.25 for gain, >1.0 for amplification, <−0.25 for loss and <−1.0 for homozygous loss. Mapping information and cytogenetic localization of clones were according to the UCSC genome browser (http://www.genome.ucsc.edu/; July 2004 freeze).

3. Results

As shown in Fig. 2, as high as 30–40 μM concentrations of 7-OHMTX, the primary catabolite of MTX, are readily attainable in the serum of childhood ALL patients treated with high-dose MTX [12]. Furthermore, while the parental drug MTX is efficiently cleared from the serum, relatively high levels (several μM) of the catabolite 7-OHMTX that exceed those of the parent drug by ~10-fold, persist for a long time in the serum. Accordingly, we explored the pattern of gene expression by leukemic cells that had acquired resistance to MTX or 7-OHMTX. Wild type MOLT-4 cells and their sublines selected for resistance to MTX or 7-OHMTX were characterized regarding gene expression profiles (Fig. 3; Table 1) and DNA copy number alterations (Table 2). Gene expression levels were compared to gene copy numbers (Table 3) and verified for selected genes by qRT-PCR (Table 4).

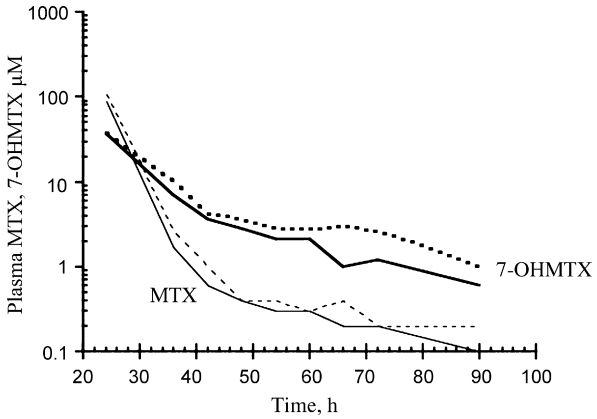

Fig. 2. Comparison of plasma levels for MTX and 7-OHMTX measured by HPLC in childhood ALL patients 24–90 h after administration of high dose MTX as part of their treatment [12]. The lines for MTX and 7-OHMTX (bold) represent the median values for all 49 cases studied, and dotted and dashed lines indicate the +2S.D. of plasma levels of MTX and 7-OHMTX, respectively.

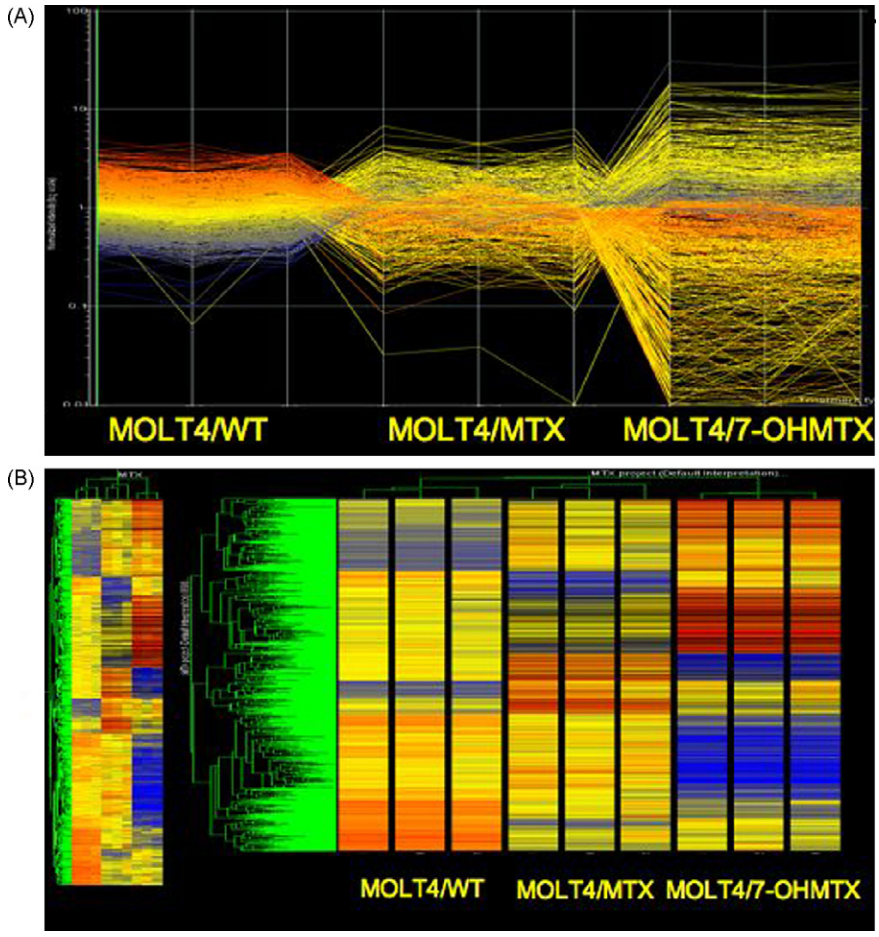

Fig. 3. (A) Gene expression patterns and (B) hierarchical clustering of parental cells (MOLT4/WT) and derivatives resistant to the MTX (MOLT4/MTX) or 7-OHMTX (MOLT4/7-OHMTX). Analyses based on three independent experiments were performed in GeneSpring 7.2, and the genes presented (n = 863) showed a more than two-fold difference in expression level. $P < 0.05$.

3.1. Expression profiles of 7-OHMTX and MTX-resistant cells

Nine separate hybridizations were performed on the Human Genome U133A GeneChip representing triplicate inoculates of parental, 7-OHMTX- as well as MTX-resistant MOLT-4 cells. In total, 863 probe sets showed two-fold or more difference in expression levels between the three lines analysed (Fig. 3). Analysis of the gene expression patterns revealed 176 genes in MTX-resistant cells and 365 genes in 7-OHMTX-resistant cells, the expression of which was more than two-fold different from that in parental cells ($p < 0.05$) (Fig. 3). Forty-eight of these genes were common to both drug-resistant cell lines. Functional classification revealed frequent dysregulation of genes encoding signalling proteins in both resistant derivatives studied (Table 1). In the MTX-resistant subline, the expression of genes encoding proteins involved in DNA/RNA metabolism and membrane transport proteins were altered more profoundly, whereas in the 7-OHMTX-resistant cells, the most pronounced alterations were in transcripts coding for proteins involved in metabolism and cell proliferation (Table 1). The expression levels of certain genes with functions related to folate metabolism, purine biosynthetic pathway and transport of related substances are detailed in Table 3.

Table 1
Genes up- or down-regulated more than two-fold in resistant versus parental cells.

Functional category	Up-regulated genes		Down-regulated genes	
	MTX	7-OHMTX	MTX	7-OHMTX
RNA/DNA metabolism	10% (4)	3% (4)	16% (22)	10% (23)
Cell cycle	0% (0)	1% (1)	3% (4)	1% (2)
Development	5% (2)	4% (5)	4% (6)	10% (23)
Apoptosis	5% (2)	2% (3)	1% (1)	4% (9)
Transporter	10% (4)	4% (5)	4% (6)	5% (11)
Signaling	30% (12)	36% (49)	26% (35)	33% (75)
Stress response	5% (2)	4% (5)	0% (0)	1% (2)
Metabolism	10% (4)	22% (31)	17% (24)	14% (32)
Growth	15% (6)	11% (15)	20% (27)	15% (34)
Organization and proliferation	0% (0)	4% (5)	9% (13)	7% (16)
Other	10% (4)	9% (13)	0% (0)	0% (0)
Total	100% (n = 40)	100% (n = 136)	100% (n = 138)	100% (n = 227)

Table 2
Summary of copy number aberrations by array-CGH in parental and resistant cells.

Chr.	Parental	MTX resistant	7-OHMTX resistant
Copy number losses and homozygous deletions			
1	1q23.1–qter	1q23.3–qter	1p36.31–p36.32
2	2p24.1–p24.2	2p24.1–p24.2	2p24.1–p24.2
4	4q31.23–qter	4q31.23–qter	4q31.23–qter
5	5q13.2; q35.1–qter	5q35.1–qter	5q13.2; q35.1–qter
7	**7p21.3 hz**; **q34 hz**	**7q34 hz**	**7p21.3hz**; **q34hz**
9	9p21.1–pter/**p21.2–p21.3 hz**/**p23 hz**	9p21.1–pter/p21.2–p**21.3hz/p23hz**	9p21.1–pter/**p21.2–p21.3 hz**
10	–	10p12.1–pter	10p12.32–pter
11	**11p14.3–p15.1hz**; q25–qter	**11p14.3–p15.1hz**; q25–qter	**11p14.3–p15.1hz**; q25–qter
13	–	–	13q22.1–q34
14	14q11.1–11.2; **q11.2hz**	**14q11.2 hz**	**14q11.2 hz**
19	19q13.2	19q13.2	19q13.2
22	22q12.3	–	–
Copy number gains and amplifications			
1	1p32.3; 1p34.1–p36.31	1p32.2–36.31; 1p36.32–pter	1p32.3–p36.31
3	3p21.1–p21.31	–	–
4	4p16.3–pter	–	4p16.3–pter
5	–	5p15.33–pter	5p15.33–pter
6	–	6p21.1–p22.1	–
7	–	7p22.1–pter; q11.22–q11.23	–
8	8qter	8p23.3–pter; p22–p21.3; qter	8qter
9	9qter	9qter	9qter
10	10p11.22–p12.1; qter	10p11.22–p12.1; qter	10qter
12	–	12q13.11–q14.1	12q21.33–qter
14	14q32.2–q32.33	14q32.2–q32.33	14q32.2–q32.33
16	16p13.3–pter; q23.3–qter	16p13.3–pter; q23.3–qter	16p13.3–pter; q24.2–qter
17	17q25.1–qter	17p13.1–pter; q12–q21.33; q25.1–qter	17p13.1–pter
18	–	18qter	–
19	19p12–pter; q13.31–q13.33	19p13.11–pter; q13.2; q13.31–qter	–
20	20pter–qter	20pter–qter	20pter–qter
21	21q22.3–qter	21q22.3–qter	–
22	22q13.1–q13.2; qter	22q11.1–q11.23; q12.3–qter	22qter

Homozygous losses hz are indicated in italic and bold; Chr. = Chromosome.

Table 3
Expression levels of selected genes and copy number of cytoband involved.

Gene symbol		Expression-array		Cytoband concerned	Array-CGH		
		MTX	7-OHMTX		Parental	MTX	7-OHMTX
FPGS	Folylpolyglutamate synthetase	1.0	1.0	9q34.11	**Gain**	**Gain**	**Gain**
FPGH/GGH	Folylpolygammaglutamyl hydrolase, gamma-glutamyl hydrolase	1.2	**3.0**	8q12.3	n.a.	n.a.	n.a.
MTR	5-Methyltetrahydrofolate-homocysteine methyltransferase,	**2.8**	1.3	1q43	**Loss**	**Loss**	n.a.
AICART	5-Aminoimidazole-4-carboxamide ribonucleotide formyltransferase	1.0	1.0	2q35	n.a.	n.a.	n.a.
RFC/SLC19A1	Solute carrier family 19 (folate transporter) member 1	0.4	0.9	21q22.3	**Gain**	n.a.	n.a.
GART	Phosphoribosylglycinamide formyltransferase	1.6	1.8	21q22.11	n.a.	n.a.	n.a.
MTHFR	5,10-Methylenetetrahydrofolate reductase (NADPH)	**4.3**	**4.8**	1p36.22	**Gain**	**Gain**	**Gain**
MRP4	ATP-binding cassette, sub-family C (CFTR/MRP) member 4	1.4	0.9	13q32.1	n.a.	n.a.	**Loss**
MRP5	ATP-binding cassette, sub-family C (CFTR/MRP) member 5	1.6	1.2	3q27.1	n.a.	n.a.	n.a.
MTHFD1	Methylenetetrahydrofolate dehydrogenase 1	1.4	1.5	14q23.2	n.a.	n.a.	n.a.
MTHFD2	Methylenetetrahydrofolate dehydrogenase 2	1.2	1.8	2p13.1	n.a.	n.a.	n.a.
DHFR	Dihydrofolate reductase	1.4	1.0	18q11.2	n.a.	n.a.	n.a.
TS/TYMS	Thymidylate synthetase	0.6	0.7	18p11.32	n.a.	n.a.	n.a.
MTRR	5-Methyltetrahydrofolate-homocysteine methyltransferase reductase	1.1	0.9	5p15.31	n.a.	n.a.	n.a.
MTHFS	5,10-Methenyltetrahydrofolate synthetase	1.0	0.9	15q25.1	n.a.	n.a.	n.a.
GCH1	GTP cyclohydrolase 1 (dopa-responsive dystonia)	1.4	0.9	14q22.2	n.a.	n.a.	n.a.
SLC19A2	Solute carrier family 19 (thiamine transporter) member 2	1.4	**2.1**	1q24.2	**Loss**	**Loss**	n.a.
SHMT1	Serine hydroxymethyltransferase 1	1.1	2.0	17p11.2	n.a.	n.a.	n.a.
BCRP/ABCG2	ATP-binding cassette, sub-family G (WHITE) member 2	1.7	0.4	4q22.1	n.a.	n.a.	n.a.
PFAS	Phosphoribosylformylglycinamidine synthase (FGAR amidotransferase)	1.9	**2.5**	17p13.1	n.a.	**Gain**	n.a.
GCHFR	GTP cyclohydrolase I feedback regulator	0.6	**7.7**	15q15.1	n.a.	n.a.	n.a.
ITPA	Inosine triphosphatase (nucleoside triphosphate pyrophosphatase)	0.6	0.9	20p13	**Gain**	**Gain**	**Gain**
dGK/DGUOK	Deoxyguanosine kinase, dGK/ DGUOK	1.0	1.8	2p13.1	n.a.	n.a.	n.a.
APRT	Adenine phosphoribosyltransferase	**0.4**	0.6	16q24.3	**Gain**	**Gain**	**Gain**
ADA	Adenosine deaminase	0.9	**0.1**	20q13.2	**Gain**	**Gain**	**Gain**
PPAT	Phosphoribosyl pyrophosphate amidotransferase	1.1	1.3	4q12	n.a.	n.a.	n.a.
MRP1/ABCC1	ATP-binding cassette, sub-family C (CFTR/MRP) member 1	1.5	0.7	16p13.11	n.a.	n.a.	n.a.
MRP2/ABCC2	ATP-binding cassette, sub-family C (CFTR/MRP) member 2	0.8	0.8	10q24.2	n.a.	n.a.	n.a.
MRP3/ABCC3	ATP-binding cassette, sub-family C (CFTR/MRP) member 3	1.0	1.0	17q21.33	n.a.	**Gain**	n.a.
P-glycoprotein	ATP-binding cassette, sub-family B (MDR/TAP) member 1	1.2	0.9	7q21.12	n.a.	n.a.	n.a.
CBS	Cystathionine β-synthase	0.5	**0.0**	21q22.3	**Gain**	**Gain**	n.a.

Expression levels are in relation to parental cells (MTX/parental or 7-OHMTX/parental) giving an arbitrary value of 1.0 for parental cells.
DNA copy number are in relation to normal reference DNA; n.a. = no aberration detected.
Expression changes greater than two-fold and copy number aberrations above thresholds are indicted in bold.

Table 4
Expression of selected genes by array and qRT-PCR in resistant as compared to parental cells.

	By Affymetrix array			By qRT-PCR		
	Parental	MTX	7-OHMTX	Parental	MTX	7-OHMTX
FPGS	(1.0)	1.0	1.0	(1.0)	0.83 ± 0.02	1.0 ± 0.20
FPGH/GGH	(1.0)	1.2	3.0	(1.0)	0.76 ± 0.05	2.0 ± 0.38
MTR	(1.0)	2.8	1.3	(1.0)	1.60 ± 0.24	1.1 ± 0.10
ATIC	(1.0)	1.0	1.0	(1.0)	0.87 ± 0.03	1.0 ± 0.05
RFC/SLC19A1	(1.0)	0.4	0.9	(1.0)	0.20 ± 0.03	2.2 ± 0.37
GART	(1.0)	1.6	1.8	(1.0)	1.76 ± 0.25	1.6 ± 0.16
MTHFR	(1.0)	4.3	4.8	(1.0)	2.80 ± 0.40	2.3 ± 0.20
MRP4	(1.0)	1.4	0.9	(1.0)	1.30 ± 0.18	1.2 ± 0.08
MRP5	(1.0)	1.6	1.2	(1.0)	1.40 ± 0.11	1.9 ± 0.16

Expression values are in relation to parental cells (MTX/parental or 7-OHMTX/parental), giving an arbitrary value of 1.0 in parental cells as indicated.

Both resistant cell lines exhibited four- to five-fold up-regulation of *MTHFR*. In addition, pronounced up-regulation of more than two-fold of *MTR* was detected in MTX-resistant cells, while 7-OHMTX cells overexpressed *FPGH*, *SLC19A2*, *PFAS*, and *GCHFR*. Prominent under-expression involved *APRT* in MTX-resistant cells, whereas in 7-OHMTX-resistant cells, *ADA* and *CBS* were expressed at very low or undetectable levels.

3.2. DNA copy number alterations by array-CGH

To characterize alterations in gene copy numbers in parental and antifolate-resistant cells, each of these cell lines were first compared to normal reference DNA using array-CGH. Homozygous deletions common to all three cells were recorded at several chromosomal locations including 7q34, 9p21.2–p21.3, 11p14.3–15.1 and 14q11.2, while gene amplifications were not found. The target interval in 14q11.2, spans the loci for *TRAV20* (gene segment for T-cell receptor alpha variable 20) and TRA genes. The region 9p21.3 encompasses the *CDKN2A* gene locus encoding p16 and p14. In addition, regular gains or losses involved almost all chromosomes and were in most situations present in parental as well as drug-resistant cells (Table 2). Alterations present in drug-resistant cells are of interest since they may be related to the mechanism by which drug resistance has emerged. MTX-resistant cells were distinguished from parental cells by chromosomal gains within 5p, 6p, 7p, 7q, 8p, 12q, 17p, 17q, 18q and 22q as well as by loss in 10p. Cells resistant to 7-OHMTX differed from parental cells by gains involving 5p, 12q, 17p and loss of 1p, 10p, and 13q. To further delineate acquired alterations, profiles were compared visually between parental and drug-resistant cells as a complementary approach to the scoring of copy number alterations by CGH plotter plugin. This revealed that several of the distinguishing alterations in resistant cells were present as close to borderline alterations at the threshold in parental cells. In addition, array-CGH experiments were carried out whereby resistant cells were hybridized against parental cells. This approach identified gene copy number alterations outside the ±0.25 threshold relative to parental cells including increase in 8pter–q24.3 in MTX-resistant cells, as well as increases in1q21.1–qter and 12q23.2–qter and decreases in 10p11.22–p11.23 and 13q22.1–qter in 7-OHMTX cells.

3.3. Corroboration of array expression levels by qRT-PCR

Analysis of the gene expression patterns of parental and resistant cells employing the Affymetrix HG-U133A microarray revealed consistent differences with respect to nine genes, some of which encode proteins involved in folate metabolism or purine biosynthesis. These differences were confirmed by qRT-PCR (Table 4). The levels were normalized against the endogenous housekeeping gene *GAPDH*, which showed comparable expression levels in parental and antifolate-resistant cells. These analyses revealed similar levels of expression determined by microarray as well as by qRT-PCR for all nine genes analysed. The results also confirmed the extensive up-regulation of *MTR* and *MTHFR* along with down-regulation of *RFC* in MTX-resistant cells, as well as up-regulation of *FPGH/GGH* and *MTHFR* in 7-OHMTX-resistant cells (Table 4).

4. Discussion

Concentrations of 7-OHMTX, the primary catabolite of MTX, as high as 30–40 μM, are readily attainable in the plasma of childhood ALL patients treated with high-dose MTX (Fig. 2) [12]. Furthermore, although MTX itself is efficiently cleared from the plasma, relatively high levels of 7-OHMTX (i.e. several μM), that exceed those of the parent compound MTX by approximately 10-fold, persist in the plasma for a long time. Accordingly, we herein explored the pattern of gene expression and gene dose alterations in human leukemia cells with acquired resistance to MTX or 7-OHMTX [11]. Copy number alterations were detected in parental cells as well as in both antifolate-resistant cell lines (Table 2). The genomic regions of acquired gains and losses do not include the genes whose products are known to be significant in response to MTX (Table 3). These findings suggest that drug-induced changes in gene expression do not stem from copy number alterations, but may instead be due to transcriptional regulatory alterations. Furthermore, gene amplifications were not detected, thereby demonstrating that increases in copy numbers of drug- resistance mediating genes were neither induced by treatment with MTX nor with exposure to 7-OHMTX. Expression of the *RFC/SLC19A1* gene was down-regulated more than two-fold in the MTX-resistant cells, thereby confirming the role of this influx transporter as an important determinant of antifolate resistance [18–21]. However, the major finding of the present investigation is that the dramatic loss of FPGS activity in the 7-OHMTX cells, presumably associated with decreased intracellular levels of folate, was accompanied by consistent alterations in the expression of genes capable of preserving intracellular pools of reduced folate and/or increase purine nucleotide biosynthesis. Thus, among the major differences observed between these cell lines were the following (Fig. 4): (a) Expression of *ADA* encoding the major enzyme of purine catabolism was down-regulated 10-fold in 7-OHMTX cells but unchanged in MTX cells. (b) Consistently, the cystathionine β synthase (*CBS*) gene was not expressed at all in 7-OHMTX cells, while being expressed two-fold lower than the normal levels present in MTX-resistant cells. (c) Expression of genes encoding for MTHFR and GART, enzymes involved in methyl-tetrahydrofolate and purine biosyntheses, respectively, were elevated 5- and 2-fold in 7-OHMTX-resistant cells and 4- and 1.5-fold in MTX-resistant cells. (d) Furthermore, the levels of mRNAs encoding for the ATP-driven folate efflux transporters *MRP1/ABCC1* and *BCRP/ABCG2* were decreased in gene expression by 30% and approximately

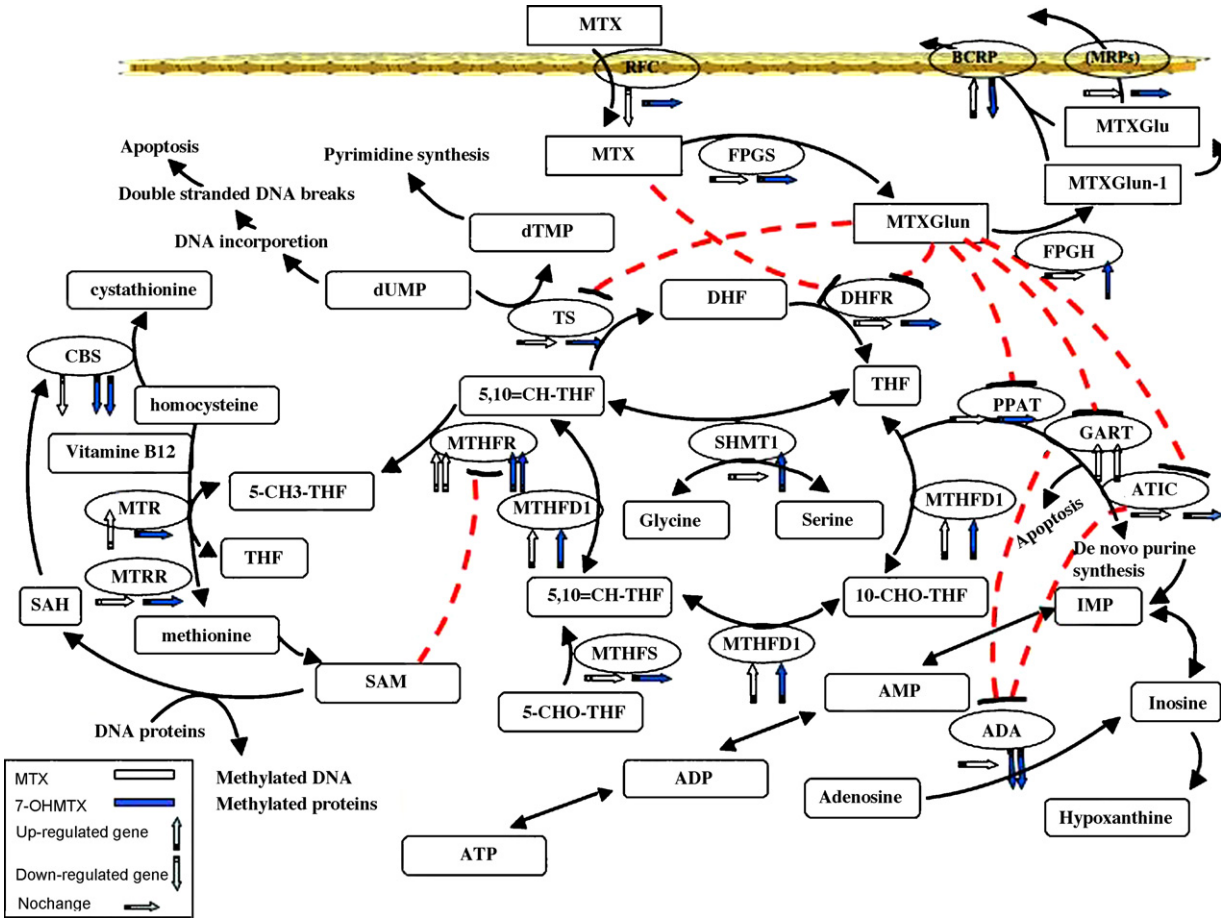

Fig. 4. Schematic illustration of dysregulated genes mediating the effects of MTX, as well as their relation to folate pathway and to purine, pyrimidine and methionine biosynthesis (modified from Ref. [2]). Abbreviations are as follows: dihydrofolate reductase (DHFR); dihydrofolate (DHF); tetrahydrofolate (THF); S-adenosylmethionine (SAM); thymidylate synthetase (TS); deoxyuridine monophosphate (dUMP); deoxythymidine monophosphate (dTMP); S-adenosylhomocysteine (SAH); methylenetetrahydrofolate dehydrogenase 1 (MTHFD 1); 5,10-methylenetetrahydrofolate reductase (MTHFR); phosphoribosylglycinamide formyltransferase (GARFT); 5,10-methenyltetrahydrofolate synthetase (MTHFS), 5-methyltetrahydrofolate-homocysteine methyltransferase (MTR); 5-methyltetrahydrofolate-homocysteine methyltransferase reductase (MTRR); 5-aminoimidazole-4-carboxamide ribonucleotide formyltransferase (ATIC or AICART); serine hydroxymethyltransferase 1 (SHMT 1); phosphoribosyl pyrophosphate amidotransferase (PPAT); cystathionine β-synthase (CBS); 10-formyltetrahydrofolate (10-CHO-THF); 5,10-methylentetrahydrofolate (5,10-CH-THF); 5-formyltetrahydrofolate (5-CHO-THF); 5-methyltetrahydrofolate (5-CH3-THF).

3-fold, respectively, in 7-OHMTX-resistant cells, whereas in MTX-resistant cells these levels were actually elevated 1.5- and 1.7-fold, respectively. These results are in accord with our recent findings that folate deprivation leads to a dramatic loss of *BCRP/ABCG2* expression as well as retention of this folate influx transporter in the cytoplasm rather than sorting it to the plasma membrane [22,23]. Furthermore, these findings are consistent with those reported recently by Evans and colleagues [24] who documented differential patterns of gene expression in various subtypes of bone marrow cells from newly diagnosed patients with B- or T-ALL. These investigators found that 42–44 h after MTX administration (0.8 g/m^2), T-ALL bone marrow cells formed long-chain (Glu4-7) MTX polyglutamates at a rate of only 355 pmol/10^9 cells, while the corresponding rate for hyperdiploid B-cell leukemia was as high as 3170 pmol/10^9 cells. This difference was consistently associated with very low levels of *FPGS* mRNA in the T-ALL cells, as much as two orders of magnitude lower than the level present in B-lineage hyperdiploid cells, together with low level expression of the *BCRP/ABCG2* gene in T-ALL cells. In contrast, the levels of expression of the *AICART* and *GARFT* genes, encoding key enzymes involved in *de novo* purine biosynthesis were higher in the case of T-ALL than any other leukemia subtypes.

In agreement with the pronounced reduction observed in MTX uptake into MTX-resistant cells, diminished *RFC* mRNA expression was observed by microarray (two-fold) and qRT-PCT (five-fold) in MTX-resistant leukaemia cells as compared to their parental counterpart (Table 4). However, the expression of *RFC* was not altered in 7-OHMTX-resistant cells. Moreover, the level of *FPGH* mRNA was elevated more than two-fold in 7-OHMTX-resistant cells. In contrast, no difference in *FPGS* expression was detected in either of the resistant cell lines suggesting that other mechanisms, e.g. post-transcriptional modifications, may be responsible for the low activity of this enzyme in these cells. In a recent study it has shown [31] that *FPGS transcripts in antifolate-resistant ALL cell lines were highly aberrant as they underwent impaired splicing including exon skipping as well as intron retention.* Hence, these novel alterations result in premature translation termination of FPGS, thereby leading to lack of FPGS activity and resistance to polyglutamatble antifolates. This mechanism was also corroborated in specimens from patients harbouring ALL, both at diagnosis and relapse. These findings provide a novel mechanistic basis for the loss of FPGS function in the absence of quantitative changes (i.e. decrease) in FPGS levels.

Members of the family of multidrug resistance proteins, notably MRP1-5/ABCC1-5, and BCRP/ABCG2 are known to export the folate antagonist MTX out of cells [25–29]. Levels of *BCRP* and *MRP1–5* mRNAs were all slightly elevated (about 50%) in the MTX-resistant subline, but unchanged in 7-OHMTX cells (Table 3). Cells resistant

to 7-OHMTX displayed a dramatic up-regulation of *VAMP8* (>700-fold); VAMP8 is a vesicle-associated membrane protein involved in exocytosis, and it is hence tempting to speculate here that elimination of vesicle-sequestered 7-OHMTX could be facilitated by *VAMP8* overexpression. This finding warrants further studies to explore this interesting possibility.

Among the genes that encode proteins involved in the folate pathway, the expression of *MTHFR* was most profoundly altered in both of our antifolate-resistant cell lines (>four-fold up-regulation) (Fig. 4). MTHFR plays a key role in folate metabolism by channelling single-carbon units between nucleotide biosynthesis and methylation reactions. This enzyme converts 5,10-methylene-THF into 5-methyl-THF, the predominant circulating folate form that provides a methyl group for methylation of homocysteine to methionine. Moreover, 5,10-methylene-THF is required for TS activity and thymidylate biosythesis and its depletion could lead to dUTP misincorporation into DNA and a consequent increase in the frequency of chromosome damage, thereby facilitating the genotoxic effects of MTX. It has been reported that a deactivating *MTHFR* allele (the *MTHFR* C677T variant allele) can enhance the risk for ALL relapse potentially by increasing cellular levels of 5,10-methylene-THF and deoxythymidine monophosphate (dTMP), thereby enhancing DNA synthesis and counteracting MTX cytotoxicity. Thus, the significant increment in the expression of *MTHFR* mRNA in both of our antifolate-resistant cell lines may be related to cellular tolerance to MTX and 7-OHMTX. Although diminished transport and lack of polyglutamylation can readily explain the mechanisms underlying the significant increase in resistance to MTX and 7-OHMTX, respectively, some alterations in gene expression of resistant cells may have contributed to their tolerance.

In summary, the present characterization of the different patterns of gene expression profiles of MTX- and 7-OHMTX-resistant sublines of parental MOLT-4 leukemia cells selected by exposure to MTX and 7-OHMTX, provides a new perspective on differences in the pharmacodynamic consequences of HDMTX therapy. 7-OHMTX, the major metabolite of MTX exerts its cytotoxic effect by mechanisms that are distinct from those underlying the effects of the parent drug. Our current findings may facilitate the identification of the specific targets for MTX therapy and may provide additional insight into cellular responses to 7-OHMTX. The distinct patterns of gene expression profiles in cells that have acquired resistance to MTX or 7-OHMTX may indicate that part of the gene expression profile observed following exposure to high doses of MTX reported earlier [30] is related to exposure to 7-OHMTX, rather than to MTX itself. The differential expression of a relatively large number of genes by 7-OHMTX- and MTX-resistant cells indicates the occurrence of separate and independent effects. Thus, in relation with HDMTX therapy, where higher concentrations of 7-OHMTX can be detected in the plasma, the pattern of gene expression will be different from that when low concentrations of 7-OHMTX are present.

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