Resistance to multiple novel antifolates is mediated via defective drug transport resulting from clustered mutations in the reduced folate carrier gene in human leukaemia cell lines

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We have studied the molecular basis of resistance of multiple human leukaemia CCRF-CEM sublines to the novel antifolates ZD9331, GW1843, AG2034, PT523 and edatrexate, which use the reduced folate carrier (RFC) as their main cellular uptake route and that target different folate-dependent enzymes. Antifolate-resistant sublines established by stepwise and single-step selections displayed up to 2135-fold resistance to the selection drug, and up to 2323-fold cross-resistance to various hydrophilic antifolates. In contrast, these sublines were up to 17- and 20-fold hypersensitive to the lipophilic antifolates AG377 and trimetrexate, respectively. The total reduced folate pool of these antifolate-resistant sublines shrunk by 87–96%, resulting in up to 42-fold increased folic acid growth requirement. These sublines lost 92–97% of parental [³H]methotrexate influx rates. Genomic PCR single-strand conformational polymorphism analysis and sequencing revealed that most of these drug-resistant sublines harboured RFC mutations that surprisingly clustered in two

confined regions in exons 2 and 3. The majority of these mutations resulted in frame-shift and/or premature translation termination and lack of RFC protein expression. The remaining mutations involved single amino acid substitutions predominantly residing in the first transmembrane domain (TMD1). Some RFC-inactivating mutations emerged during the early stages of antifolate selection and were stably retained during further drug selection. Furthermore, some sublines displayed a markedly decreased or abolished RFC mRNA and/or protein expression. This constitutes the first demonstration of clustering of multiple human RFC mutations in TMD1, thereby suggesting that it plays a functional role in folate/antifolate binding and/or translocation. This is the first molecular characterization of human RFC-associated modalities of resistance to various novel antifolates in multiple leukaemia sublines.

Key words: drug resistance, mutation, transporter.

INTRODUCTION

Reduced folates are essential vitamin cofactors that function as donors of one-carbon units in the *de novo* biosynthesis of purines, thymidylate and glycine in mammalian cells [1]. Unlike prokaryotes, mammalian cells cannot synthesize their own folates and therefore must obtain these vitamins from exogenous sources [1]. In this respect, the mammalian reduced folate carrier (RFC) has long been known for its major role in mediating the cellular uptake of both reduced folate cofactors and the folic acid antagonist, methotrexate (MTX) [2,3]. The human RFC (hRFC) is an integral polytopic plasma membrane protein containing 591 amino acids, with 12 transmembrane domains (TMDs), a short N-terminus and a long C-terminus, both of which reside in the cytoplasm [4]. The human RFC contains a single N-linked glycosylation site at the first extracellular loop (L1), and it undergoes heavy glycosylation thereby rendering it a broadly migrating protein with a molecular mass of 70–120 kDa [5,6]. RFC is a classic facilitative transporter that functions as a bidirectional anion exchanger [7,8], taking up folate cofactors and extruding various organic anions [8], including thiamine pyrophosphate [9]. RFC is a member of the major facilitator superfamily, a large group of carriers that transport diverse organic and inorganic compounds in both prokaryotes and eukaryotes [10]. Within the major facilitator superfamily is a small family of facilitative solute carriers consisting of RFC (SLC19A1), SLC19A2 [11–14] and SLC19A3 [15]. At the amino

acid level, the hRFC shares 39–40% identity and 55–58% similarity with SLC19A2 and SLC19A3 [16]. It has been recently shown that both SLC19A2 [11–14] and SLC19A3 [15] function as thiamine transporters and have therefore been termed ThTr1 [11–14] and ThTr2 [16]. The central physiological role attributed to the mammalian RFC in the uptake of folate cofactors recently gained major support from targeted disruption studies of the murine RFC locus [17]. Zhao et al. [17] have recently demonstrated that homozygous disruption of the murine RFC gene causes embryonic lethality. They further showed that rescue of embryonic lethality in RFC-deficient mice by maternal folic acid supplementation results in an early neonatal failure of haematopoietic organs [17].

Over recent years it has become evident that RFC transport activity plays a central role in the uptake and thereby cytotoxicity of various rationally designed new-generation folate-based chemotherapeutic drugs [18–21]. These novel antifolate anticancer drugs (reviewed in [22]) include (i) the dihydrofolate reductase inhibitors edatrexate and PT523, (ii) the thymidylate synthase inhibitors Tomudex (Raltitrexed, ZD1694), ZD9331, GW1843 and Alimta (LY231514), as well as (iii) the glycinamide ribonucleotide transformylase inhibitors DDATHF (lometrexol), LY309887, AG2034 and AG2037. Recent studies from our laboratory [23,24] as well as others' laboratories [25,26] have shown that RFC-based defective transport is a major and frequent mechanism of resistance to MTX in cultured human tumour cell lines. The molecular bases of resistance to MTX

Abbreviations used: 10-CHOTHF, 10-formyltetrahydrofolate; CH₂THF, N⁶,N¹⁰-methylenetetrahydrofolate; hRFC, human RFC; MRP1, multidrug-resistance protein 1; MTX, methotrexate; RFC, reduced folate carrier; RT, reverse transcriptase; SSCP, single-strand conformational polymorphism; TMD, transmembrane domain.

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included premature translation termination of the hRFC [25] as well as single amino acid substitutions that impair antifolate transport (i.e. decreased $V_{\max}$ and/or increased K_m), sometimes associated with a drastic decrease in the transport K_m for folate cofactors [23,24]. Furthermore, we have shown recently that a cluster of three mutations occurred in the TMD1 of the RFC in GW1843-resistant human leukaemia cells [24]. These antifolate-resistant cells displayed a marked increase in the transport affinity for folic acid while exhibiting a prominently increased transport K_i for GW1843 and other antifolates [24]. Extending on these studies we have here isolated multiple human CCRF-CEM leukaemia cell lines that are resistant to various novel antifolates that target different folate-dependent enzymes. We then studied the molecular basis of resistance to these novel hydrophilic antifolates. We show that the majority of the antifolate-resistant sublines displayed a transport-defective phenotype with residual transport activities of 3–8%. This was a result of multiple transport-inactivating mutations that clustered in two highly confined regions in exon 2 and 3 of the hRFC gene. Furthermore, some of these mutations occurred during the early stages of antifolate selection (low-nanomolar concentration) and were stably retained during further selection to high drug levels. We also show that markedly decreased or loss of RFC mRNA and protein expression can be present in certain sublines, some of which lack RFC mutations. These results demonstrate that multiple transport-inactivating mutations frequently occur in highly confined regions of the hRFC gene and play a key role in the conferring of resistance to various new-generation, rationally designed novel antifolate anticancer drugs.

MATERIALS AND METHODS

Materials

[^3H]MTX (23.4 Ci/mmol) was obtained from Moravek Biochemicals (Brea, CA, U.S.A.), purified prior to use by thin-layer chromatography [27] and stored at -80°C . MTX was from Teva Pharmaceuticals, whereas folic acid and aminopterin were purchased from Sigma. The various non-radioactive antifolate drugs were generous gifts from the following sources: GW1843U89 was from Dr Gary Smith (Glaxo Wellcome, Research Triangle Park, NC, U.S.A.); Raltitrexed (Tomudex, ZD1694) and ZD9331 were from Dr A. Jackman (Institute of Cancer Research, Sutton, U.K.); PT523 was from Dr W. T. McCulloch (Sparta Pharmaceuticals); Alimta (LY231514, multi-targeted antifolate; Eli Lilly), AG377, AG2034 and AG2037 were from Dr T. Boritzki (Agouron Pharmaceuticals); trimetrexate was from Dr D. Fry (Warner-Lambert Parke-Davis) and edatrexate was from Dr J. H. Schornagel (Netherlands Cancer Institute, Amsterdam, The Netherlands).

Tissue culture and antifolate drug selections

Human CCRF-CEM leukaemia cells were maintained in RPMI-1640 medium containing $2.3\ \mu\text{M}$ folic acid (Biological Industries, Beth-Haemek, Israel) supplemented with 10% fetal calf serum (Gibco), 2 mM glutamine, 100 units/ml penicillin G (Sigma) and 100 $\mu\text{g}/\text{ml}$ streptomycin sulphate (Sigma). Stepwise selections were performed in gradually increasing antifolate concentrations over a period of 4–7 months as follows: parental CEM cells ($2 \times 10^5/25\ \text{cm}^2$ flask containing 5 ml of growth medium) were first exposed to an antifolate concentration equal to the IC_{50} (defined as the drug concentration at which cell growth is inhibited by 50%). Following 5–7 days of growth at 37°C , during which time the total cell number doubled, the antifolate con-

centration was gradually raised by 25–100% increments and selection was terminated at 60–428-fold of the IC_{50} depending on the specific antifolate (see Table 1).

Single-step selections were performed as single-dose exposures as follows: parental CEM cells were seeded ($3 \times 10^5/\text{well}$) in 24-well plates in growth medium (1 ml/well) containing 24 nM PT523 (26 times the IC_{50}). Following 2–3 weeks of incubation at 37°C , individual clones were picked and recloned by the limiting dilution method. The following sublines were obtained by stepwise selection: MTX $^{\text{R}0.6}$ (using selection with MTX, where $\text{R}^{0.6}$ refers to cells grown in a $0.6\ \mu\text{M}$ solution), PT $^{\text{R}0.08}$ (using PT523), EDX $^{\text{R}0.03}$ (using edatrexate), GW $^{\text{R}0.3}$ (using GW1843), ZD9331 $^{\text{R}1.5}$ (using ZD9331), AMT $^{\text{R}0.12}$ (using aminopterin) and AG2034 $^{\text{R}2}$ (using AG2034), whereas PT C-3 and PT C-7 were generated by single-step selection to 24 nM PT523. Due to the prolongation of the doubling time in the early stages of antifolate growth, sublines were considered established only when they resumed the doubling time ($\approx 20\ \text{h}$) of their parental CEM cells. CCRF-CEM 7A cells that highly overexpress RFC were cultured in folic acid-free medium containing 10% dialysed fetal calf serum and supplemented with 0.25 nM leucovorin [28].

Folate growth requirement

Exponentially growing cells in normal growth medium were washed three times with PBS and transferred to a folic acid-free RPMI-1640 medium (Biological Industries) supplemented with 10% dialysed fetal calf serum. Following 4–6 days of growth in folic acid-deficient medium, cellular growth was arrested. Growth-arrested parental cells and their various antifolate-resistant sublines were seeded ($3 \times 10^4/\text{well}$) in medium (0.15 ml) containing increasing concentrations of folic acid ranging from 0.1 nM to 30 μM in 96-well plates. Following 3 days of incubation at 37°C , viable cell numbers were determined by haemocytometer count using Trypan Blue exclusion. EC_{50} is defined as the folic acid concentration necessary to produce 50% of maximal cell growth.

Analysis of intracellular folate pools

Suspension cell cultures were grown for at least three passages in antifolate-free medium. Thereafter, exponentially growing cells were harvested and washed three times in PBS, counted, centrifuged, snap-frozen in liquid nitrogen as cell pellets and stored at -80°C until analysis. Intracellular reduced folate pools were analysed according to a method based on enzymic cycling of reduced folates to N^5,N^{10} -methylenetetrahydrofolate (CH_2THF) followed by entrapment into a stable ternary complex with *Lactobacillus casei* thymidylate synthase and [6- ^3H]5-fluorodeoxyuridylate monophosphate (20 Ci/mmol; Moravek Biochemicals) [29]. Frozen cell pellets were thawed (2.5×10^7 cells/ml) in extraction buffer containing 50 mM Tris/HCl, 50 mM sodium ascorbate and 1 mM EDTA, pH 7.4. Cells were lysed by boiling for 3 min and centrifuged at 15000 g for 10 min at 4°C . Aliquots (20–60 μl) of the supernatant were used to determine CH_2THF , tetrahydrofolate, dihydrofolate, 5-formyltetrahydrofolate (leucovorin) and 5-methyltetrahydrofolate [30]. The levels of 10-formyltetrahydrofolate (10-CHOTHF) were assayed in the presence of 0.4 m-unit of 10-CHOTHF dehydrogenase and 10 μM NADP.

Antifolate growth inhibition

Parental cells and their various antifolate-resistant sublines were first grown in normal growth medium lacking antifolates for between five and seven cell doublings. Thereafter, cells were

Table 1 Antifolate-growth inhibition and folic acid growth requirement in parental CCRF-CEM cells and their antifolate-resistant variants

IC₅₀ values were measured following a 72 h antifolate drug exposure. Results depicted are the means $\pm$ S.D. from 3–7 experiments. Numbers in parentheses represent the fold of antifolate resistance relative to parental CCRF-CEM cells. For folic acid, numbers in parentheses represent the fold increase in folic acid growth requirement relative to parental cells. N.D., not determined.

Folate analogue	IC ₅₀ (nM)	CCRF-CEM (parental)	MTX ^{R0.6}	PT ^{R0.08}	EDX ^{R0.03}	GW ^{R0.3}	ZD9331 ^{R1.5}	AMT ^{R0.12}	AG2034 ^{R2}	PT C-3	PT C-7
MTX	1.4 ± 0.2 (1)	768 ± 32 (549)	988 ± 66 (706)	1797 ± 16 (1284)	618 ± 62 (441)	819 ± 18 (585)	67 ± 14 (48)	748 ± 203 (534)	606 ± 60 (433)	192 ± 13 (137)	
PT523	0.92 ± 0.2 (1)	1335 ± 71 (1451)	735 ± 41 (799)	556 ± 51 (604)	2051 ± 191 (2229)	2090 ± 156 (2272)	70 ± 17 (76)	864 ± 147 (939)	1921 ± 230 (2088)	639 ± 40 (695)	
Edatrexate	0.5 ± 0.1 (1)	589 ± 53 (1178)	354 ± 38 (708)	173 ± 6 (346)	302 ± 12 (604)	409 ± 33 (818)	15 ± 1 (30)	69 ± 3 (138)	N.D.	84.6 ± 4.6 (169)	
GW1843	0.9 ± 0.3 (1)	871 ± 79 (968)	464 ± 11 (516)	394 ± 88 (438)	625 ± 23 (695)	562 ± 38 (625)	44 ± 12 (49)	375 ± 12 (417)	396 ± 49 (440)	212 ± 22 (236)	
ZD9331	17.4 ± 3 (1)	2801 ± 200 (161)	3018 ± 126 (173)	3805 ± 572 (219)	4014 ± 318 (231)	3021 ± 262 (174)	1480 ± 213 (85)	2434 ± 200 (140)	2779 ± 230 (160)	2066 ± 125 (119)	
Alimta (LY231514)	31.3 ± 3.9 (1)	6994 ± 95 (224)	7520 ± 155 (240)	3137 ± 158 (100)	7736 ± 1054 (247)	4566 ± 237 (146)	563 ± 36 (18)	2044 ± 241 (65)	3357 ± 140 (107)	1653 ± 134 (53)	
Trimetrexate	15 ± 2.7 (1)	2.5 ± 0.4 (0.17)	1.9 ± 0.2 (0.13)	4.5 ± 0.4 (0.3)	1.2 ± 0.3 (0.08)	1.1 ± 0.1 (0.07)	4.7 ± 1 (0.31)	1.2 ± 0.03 (0.08)	0.8 ± 0.1 (0.05)	1.1 ± 0.2 (0.07)	
AG377	8.4 ± 2.8 (1)	1.7 ± 0.4 (0.2)	1.6 ± 0.3 (0.2)	1.8 ± 0.3 (0.21)	1.4 ± 0.1 (0.17)	1.6 ± 0.2 (0.19)	2.2 ± 0.2 (0.26)	0.5 ± 0.1 (0.06)	0.7 ± 0.05 (0.08)	0.9 ± 0.3 (0.11)	
Folic acid	54 ± 8 (1)	884 ± 83 (16)	2270 ± 279 (42)	331 ± 85 (6)	815 ± 91 (15)	1557 ± 31 (29)	N.D.	N.D.	1380 ± 13 (26)	488 ± 22 (9)	

seeded in 96-well plates (3×10^4 /well) in growth medium (0.15 ml/well) containing various concentrations of the different antifolates. After 3 days of incubation at 37 °C, cell numbers were determined by haemocytometer count using Trypan Blue exclusion.

Genomic PCR-single-strand conformational polymorphism (SSCP) analysis

High-molecular-mass genomic DNA was extracted from parental CEM cells and their various antifolate-resistant sublines as follows. Exponentially growing cells (2×10^7) were lysed, nuclei isolated by sucrose gradient centrifugation and genomic DNA was extracted as described previously [31]. Genomic PCR was performed using nine oligonucleotide primer pairs spanning the entire RFC coding region that contains five coding exons (exons 2–6; Drori, S., Kaufman, Y., Rothem, L., Rots, M. G., Matherly, L. H., Jansen, G. and Assaraf, Y. G., unpublished work). Genomic DNA samples (10–100 ng) were amplified with *Taq* polymerase (Promega) in a reaction buffer (50 μ l) consisting of 10 pmol of each primer, 0.2 mM non-radioactive dNTPs and 10 μ Ci of [³²P]dCTP in 1 $\times$ reaction buffer (Promega) supplemented with 1.5 mM MgCl₂ and 5% DMSO. The conditions of the PCR reactions as well as those of the SSCP analysis were detailed previously [23,24].

Reverse transcriptase (RT)-PCR of RFC and β -actin

Exponentially growing cells ($1-3 \times 10^7$) were harvested by centrifugation, washed with PBS and total RNA was isolated using the Biological Industries EZ-RNA kit according to the instructions of the manufacturer. A portion of total RNA (20 μ g in a total volume of 20 μ l) was reverse-transcribed using Moloney-murine-leukaemia virus (180 units; Promega) in a reaction buffer containing random hexamer primers, dNTPs and the ribonuclease inhibitor Rnasin, and the first cDNA strand was synthesized according to the instructions of the manufacturer (Promega).

To estimate the expression of RFC mRNA in the various antifolate-resistant cell lines we used RT-PCR of RFC and β -actin as a control. Portions of cDNA ($\approx$ 50 ng) synthesized from parental CCRF-CEM cells and their various antifolate-resistant sublines were amplified using Expand polymerase (Boehringer Mannheim) in a reaction buffer (total volume, 50 μ l) containing 10 pmol of each primer, 0.2 mM dNTPs, 1.5 mM MgCl₂ and 3% DMSO according to the instructions of the manufacturer. The PCR reaction was performed as follows: initial denaturation at 95 °C for 5 min, followed by 35 cycles each of 1 min at 95 °C, annealing at 62 °C (56 °C for β -actin) for 45 s, elongation at 72 °C for 1 min, followed by 10 min extension at 72 °C. Then the PCR products were resolved on 2% agarose gels. The primers used for hRFC PCR were 5'-CTGGCCTTCCTCACCTTCCA-3' (upstream) and 5'-CCGAGTTGAAGACCCACCAGA-3' (downstream), whereas those used for human β -actin were 5'-AAGAGAGGCATCCTCACCT-3' (upstream) and 5'-TACA-TGCTGGGGTGTGAA-3' (downstream).

DNA sequencing

Genomic PCR products obtained with DNA from antifolate-resistant sublines showing an altered electrophoretic mobility pattern of the RFC gene in three independent experiments compared with parental CEM cell DNA were fractionated on 1% agarose gels and purified using the Qiagen gel-purification kit. These genomic PCR reactions were performed with non-radioactive dNTPs. DNA sequencing was performed on purified

RFC DNA fragments by the fluorescence-based dideoxy chain-termination method using an ABI 377 sequencer (DNA Sequencing Unit, Weizmann Institute, Rehovot, Israel). At least three independent genomic PCR and sequencing reactions, performed on different days, were undertaken in order to determine a definitive mutation.

[³H]MTX transport

In order to determine the ability of the various antifolate-resistant sublines to take up antifolates we measured the influx rates of [³H]MTX relative to parental CEM cells. Cells (2×10^7) from the mid-log phase of growth were washed three times in transport buffer consisting of Hepes-buffered saline solution [32] and incubated at 37 °C for 3 min in Hanks balanced salt solution (1 ml suspensions) containing 2 μ M [³H]MTX. Transport controls contained a 500-fold excess (1 mM) of unlabelled MTX. The transport of [³H]MTX was stopped by the addition of 10 ml of ice-cold Hanks balanced salt solution. Then the cell suspension was centrifuged at 500 *g* for 5 min at 4 °C and the cell pellet was washed twice with 10 ml of ice-cold transport buffer. The final cell pellet was suspended in water and processed for scintillation counting.

Western blot analysis

To determine the levels of RFC expression in the various antifolate-resistant cell lines we first isolated microsomes as described previously [33]. Microsomal proteins were resolved by electrophoresis on 10 % polyacrylamide gels containing SDS and electroblotted on to nitrocellulose nylon membrane (Schleicher & Schuell). The blots were then blocked for 1 h at room temperature in TBS buffer (150 mM NaCl, 0.5 % Tween 20 and 10 mM Tris/HCl at pH 8.0) containing 1 % skimmed milk. The blots were reacted with a polyclonal antiserum (diluted 1:700) prepared in mice against a C-terminal hRFC peptide. Blots were then rinsed in the same buffer for 10 min at room temperature and reacted with horseradish peroxidase-conjugated goat anti-mouse IgG (1:40 000 dilution; Jackson Immunoresearch Labs, West Grove, PA, U.S.A.) for 1 h at room temperature. Following three 10 min washes in TBS at room temperature, enhanced chemiluminescence detection was performed according to the manufacturer's instructions (Biological Industries). To correct for loading differences, we determined the levels of the multidrug-resistance protein 1 (MRP1) using the rat anti-human MRP-r1 monoclonal antibody (1:500 dilution of supernatant) kindly provided by Professor R. Scheper (Free University Hospital, Amsterdam, The Netherlands). The second antibody was horseradish peroxidase-conjugated goat anti-rat IgG (1:40 000; Jackson Immunoresearch Labs). Protein content was determined using the Bio-Rad protein assay according to Bradford [34].

RESULTS

We studied the role of RFC alterations in the conferring of resistance to multiple novel antifolates that use RFC as their primary uptake route and target different folate-dependent enzymes. Towards this end, multiple drug-resistant variants were established by single-step and stepwise selection with the dihydrofolate reductase inhibitors PT523, edatrexate, aminopterin and MTX, the thymidylate synthase inhibitors, ZD9331 and GW1843, and the glycinamide ribonucleotide transformylase inhibitor AG2034. We first determined the patterns of resistance to the selection agent and cross-resistance to different hydrophilic antifolates. Regardless of whether the

Figure 1 Antifolate growth inhibition in parental human leukaemia CCRF-CEM cells and their various antifolate-resistant sublines

Logarithmically growing parental CEM (wild-type) cells and their different antifolate-resistant sublines were exposed to various concentrations of MTX (**A**) and ZD9331 (**B**) as detailed in the Materials and methods section. Following 3 days of incubation at 37 °C, viable cell counting was performed using Trypan Blue exclusion. Values depicted represent means $\pm$ S.D. of 3–7 independent experiments.

various antifolate-resistant sublines were selected to nanomolar or micromolar drug concentrations (Table 1), they displayed a disproportionately high level of resistance (173–2135-fold) to the selection antifolates at or above the micromolar range (Figure 1 and Table 1). For example, although the clonal subline PT C-3 was obtained by exposure to only 24 nM PT523 (26-fold the antifolate IC_{50}), the level of resistance to the selecting agent (i.e. PT523) was as high as 2135-fold (Table 1). Consistently, the different drug-resistant sublines displayed superimposable cross-resistance curves to various hydrophilic antifolates in the micromolar range (Figure 1 and Table 1). In contrast, these highly drug-resistant sublines were 3–20-fold hypersensitive to the lipid-soluble antifolates trimetrexate and AG377 (Figure 2 and Table 1) that do not depend on RFC for cellular uptake and enter cells by diffusion [35].

Antifolate resistance is frequently associated with an increased requirement for folate cofactors. We therefore examined the folic acid growth requirement of parental CCRF-CEM cells and their various antifolate-resistant sublines (Table 1). The folic acid concentration that produced 50 % of maximal cell growth (EC_{50})

Figure 2 Trimetrexate and AG377 growth inhibition of parental CCRF-CEM cells and their various antifolate-resistant sublines

Exponentially growing parental CEM cells and their different antifolate-resistant sublines were exposed for 3 days at 37 °C to various concentrations of the lipophilic antifolates trimetrexate (TMQ; black bar) and AG377 (grey bar), after which viable cell counting was performed as described in Figure 1. To generate a measure of lipophilic antifolate sensitivity, values are presented as the antifolate IC_{50} ratio of parental CEM/resistant subline. Numbers shown were obtained from 3–4 independent experiments with S.D. that did not exceed 15%.

in parental CCRF-CEM cells was 54 nM but was drastically shifted towards the micromolar range (0.33–2.3 μ M) in the different antifolate-resistant variants (Table 1). Consequently, the antifolate-resistant sublines required up to 42-fold more folic acid for their growth than parental CEM cells (Table 1). In order to characterize the basis for this markedly increased folate growth requirement, we first determined the intracellular folate pools in the various sublines (Table 2). The cellular folate pools of the vast majority of the antifolate-resistant sublines were only 4–13% that of parental CEM cells (Table 2). Furthermore, whereas 10-CHOTHF comprised more than 50% of the total folate pool in parental cells, this reduced folate cofactor was below the level of detection in all antifolate-resistant sublines (Table 2). As these results were consistent with a defective

Figure 3 Genomic PCR-SSCP analysis of RFC exon 2 (A and B) and exon 3 (C and D) in parental CEM cells and their various antifolate-resistant sublines

High-molecular-mass genomic DNA (50–100 ng) isolated from parental CEM cells and their various antifolate-resistant sublines was PCR amplified in the presence of [32 P]dATP using oligonucleotide primers targeting exon 2 (A and B) and exon 3 (C and D). The PCR products were resolved by electrophoresis on 8% polyacrylamide gels as described in the Materials and methods section. The short bars shown on the left-hand side of each panel represent the positions of the bands of wild-type electrophoretic mobility.

transport of (anti)folates in the various sublines, we measured the initial rates of [3 H]MTX uptake. The various antifolate-resistant sublines displayed severely depressed influx rates of [3 H]MTX with residual transport activities of only 3–8%, relative to parental CEM cells (Table 2). In order to characterize the molecular basis underlying this markedly impaired transport of MTX we used a genomic PCR-SSCP assay capable of a reliable screening of the entire hRFC coding region for mutations ([23,24]

Table 2 MTX influx and intracellular reduced folate pools in parental CCRF-CEM cells and their antifolate-resistant sublines

Values represent the means $\pm$ S.E.M. from six determinations performed on three different separate samples. For MTX influx, data presented were obtained from 3–4 independent experiments performed on different days. The influx rate of [3 H]MTX in parental CEM cells was 4.11 ± 0.56 pmol of MTX/min per 10^7 cells. DHF, dihydrofolate; 5-CH₃THF, 5-methyltetrahydrofolate.

Cell line	[3 H]MTX influx (% of control)	Reduced folates (pmol/mg of protein)					Percentage of CCRF-CEM
		CH ₂ THF + THF	5-CH ₃ THF	10-CHOTHF	DHF	Total	
CCRF-CEM	100	15.4 $\pm$ 1.3	7.9 $\pm$ 1.2	29.5 $\pm$ 2.5	4.1 $\pm$ 1.4	56.6 $\pm$ 3.4	100
MTX ^{R0.6}	5.6 $\pm$ 1.4	3.7 $\pm$ 0.4	0.7 $\pm$ 0.3	< 0.3	3.3 $\pm$ 0.6	7.6 $\pm$ 1.0	13.42
PT ^{R0.08}	5.7 $\pm$ 0.6	2.3 $\pm$ 0.4	< 0.3	< 0.3	2.0 $\pm$ 0.5	4.2 $\pm$ 0.5	7.42
EDX ^{R0.03}	3.5 $\pm$ 0.6	3.8 $\pm$ 0.6	< 0.3	< 0.3	3.5 $\pm$ 0.3	7.3 $\pm$ 0.7	12.9
GW ^{R0.3}	8.2 $\pm$ 4.3	1.5 $\pm$ 0.3	< 0.3	< 0.3	0.8 $\pm$ 0.5	2.5 $\pm$ 0.5	4.42
ZD9331 ^{R1.5}	6.5 $\pm$ 1.4	< 0.3	< 0.3	< 0.3	4.7 $\pm$ 0.4	4.7 $\pm$ 0.4	8.3
PT C-3	4.8 $\pm$ 1.1	< 0.3	< 0.3	< 0.3	4.1 $\pm$ 0.3	4.1 $\pm$ 0.3	7.24
PT C-7	5.2 $\pm$ 0.6	4.7 $\pm$ 1	< 0.3	< 0.3	4.6 $\pm$ 1.0	9.6 $\pm$ 0.4	16.96
AG2034 ^{R2}	3.0 $\pm$ 1.1	< 0.3	< 0.3	< 0.3	3.2 $\pm$ 1.1	3.2 $\pm$ 1.1	5.65
AMT ^{R0.12}	5.9 $\pm$ 0.5	10.1 $\pm$ 1.7	1.6 $\pm$ 0.7	< 0.3	17.2 $\pm$ 3.9	29.0 $\pm$ 2.8	51.24

Table 3 Summary of RFC mutations identified in the various antifolate-resistant sublines

aa, amino acids.

Cell line	Exon no.	Nucleotide position and change	Amino acid position and change	Transporter location	Phenotype	Remarks
CEM (parental)	2	G175 (wild type)	Arg-27*	TMD1	Arg-27	Homozygous Arg-27
	3-3	C791 → T	Silent Pro-232*	L6	Arg-27	Heterozygous Pro-232
MTX ^{R0.6}	2	G175 → A	Arg-27 → His*	TMD1	His-27/Stop 225	Homozygous His-27, truncated RFC of only 225 aa
	3-3	C769 → A	Stop Ser-225†	L6	His-27/Stop 225	Homozygous His-27, truncated RFC of only 225 aa
		C791 → T	Silent Pro-232*	L6	His-27/Stop 225	Homozygous Pro-232
		G854 → A	Silent Arg-253	L6	His-27/Stop 225	Homozygous Arg-253
		G887 → A	Silent Arg-264	TMD7	His-27/Stop 225	Homozygous Arg-264
EDX ^{R0.03}	2	G175 → A, C228 → T	Arg-27 → His, Glu-45 → Lys	TMD1	His-27/Lys-45	Homozygous His-27
	3-3	C791 → T	Silent Pro-232*	L6	His-27/Lys-45	Homozygous Pro-232
PT ^{R0.08}	3-3	G810 → T	Stop Gly-239§	L6	Stop 239	Truncated RFC of only 239 aa
GW ^{R0.3}	3-3	C791 → T	Silent Pro-232*	L6	Stop 393	Homozygous Pro-232
		Insertion of A827	Stop Lys-393‡	L6	Stop 393	Homozygous Pro-232
PT C-3	2	Deletion of C254	Stop Leu-203¶	L1	Stop 203	Truncated RFC of only 203 aa
PT C-7	2	C225 → T	Gly-44 → Arg	TMD1	Arg-44	—
		C254 → T	Silent Leu-53	L1	Arg-44	—
ZD9331 ^{R1.5}	3-3	G864 → A	Stop Glu-257	L6	Stop 257	Truncated RFC of only 257 aa

* Parental CEM cells are homozygous or heterozygous for Arg-27 and Pro-232 respectively.

† Truncated RFC protein containing only 225 amino acids.

‡ Altered RFC protein from amino acid 244 to 392 and translation termination at 393 amino acids.

§ Truncated RFC protein containing only 239 amino acids.

¶ Altered RFC protein from amino acid 53 to 203 and translation termination at 203 amino acids.

|| Truncated RFC protein containing only 257 amino acids.

and Drori, S., Kaufman, Y., Rothen, L., Rots, M. G., Matherly, L. H., Jansen, G. and Assaraf, Y. G., unpublished work). The genomic RFC fragments obtained with DNA isolated from the various antifolate-resistant sublines and their parental cells were then subjected to SSCP analysis on non-denaturing polyacrylamide gels ([23,24] and Drori, S., Kaufman, Y., Rothen, L., Rots, M. G., Matherly, L. H., Jansen, G. and Assaraf, Y. G., unpublished work). DNA isolated from EDX^{R0.03}, MTX^{R0.6}, PT C-3 and PT C-7 displayed altered electrophoretic mobility patterns in RFC exon 2 when compared with parental CEM cells (Figures 3A and 3B). Furthermore, the sublines PT^{R0.08} (Figure 3C) as well as EDX^{R0.03}, MTX^{R0.6}, GW^{R0.3} and ZD9331^{R1.5} also displayed aberrant electrophoretic profiles in RFC exon 3 (Figure 3D).

As these aberrant SSCP profiles were indicative of mutations in exons 2 and/or 3 of the RFC gene, the altered genomic PCR RFC fragments were then purified and subjected to direct DNA sequencing. All fragments showing an electrophoretic mobility shift harboured single or multiple mutations in the RFC gene (Table 3). The majority of these RFC genetic alterations involved nonsense (i.e. stop codons) or deletion/insertion mutations resulting in frame-shifts and premature translation termination (Table 3). The remaining genetic alterations were either missense mutations resulting in single amino acid substitutions, or silent mutations (Table 3). In contrast, AMT^{R0.12} and AG2034^{R2} displaying a parental electrophoretic mobility pattern did not harbour any mutations in the RFC gene (Figures 3B and 3C). An analysis of the distribution of the mutations identified in the antifolate-resistant sublines revealed a striking pattern of clustering in two highly confined regions of the hRFC cDNA (Figure 4). One consisting of the 3'-end of exon 2 and the 5'-end of exon 3, as well as another cluster at the 3'-end of exon 3 (Figure 4). Furthermore, an analysis of the distribution of all previous genetic alterations of the hRFC (a total of 33 mutations; see [23,24] and Drori, S., Kaufman, Y., Rothen, L., Rots, M. G., Matherly, L. H., Jansen, G. and Assaraf, Y. G., un-

Figure 4 Distribution of the different mutations identified in the hRFC gene in the various antifolate-resistant sublines

The mutations (a total of 33) identified in the various human antifolate-resistant sublines in the present study (●), as well as in previous studies including Drori, S., Kaufman, Y., Rothen, L., Rots, M. G., Matherly, L. H., Jansen, G. and Assaraf, Y. G., unpublished work (◆), Drori et al. [24] (▲), Wong et al. [26] (○), Jansen et al. [23] (■) and Gong et al. [25] (□) were plotted as a function of their position in the hRFC cDNA. Also shown are the positions of the different exons as well as the translation-initiation (ATG) and -termination (TGA) sites.

published work) revealed a clustering of the mutations to these two cDNA regions (Figure 4). In contrast, distribution analysis of the previously published mutations obtained with the mouse RFC in the various antifolate-resistant murine leukaemia L1210 sublines [36–38] revealed that these mutations were rather scattered in exons 3, 4 and 5, with somewhat higher frequencies in exons 3 and 4 (results not shown). Consistently, analysis of the distribution of the single amino acid substitutions obtained in the present study as well as those in previous studies ([23,24,26] and Drori, S., Kaufman, Y., Rothen, L., Rots, M. G., Matherly, L. H., Jansen, G. and Assaraf, Y. G., unpublished work) revealed a cluster of mutations in TMD1 of the hRFC (results not shown). Thus 11 of the 13 single amino acid substitutions

Figure 5 Western blot analysis of RFC (A) and MRP1 (B) expression in parental CEM cells and their antifolate-resistant sublines

Microsomes were isolated from exponentially growing cells and microsomal proteins were extracted using a Triton X-100-containing buffer as detailed in the Materials and methods section. Aliquots of detergent-soluble proteins (50 μ g) from RFC-overexpressing CEM-7A (lanes a), parental CEM (lanes b), EDX^{R0.03} (lanes c), PT C-7 (lanes d), MTX^{R0.6} (lanes e), PT^{R0.08} (lanes f), ZD9331^{R1.5} (lanes g), GW1843^{R0.3} (lanes h), PT C-3 (lanes i), AG2034^{R2} (lanes j) and AMT^{R0.12} cells (lanes k) were resolved by SDS/PAGE (10% gels) and electroblotted on to a Nytran nylon membrane (Schleicher and Schuell). Then, the blots were reacted with a peptide antiserum against a C-terminal peptide of hRFC (A) or a monoclonal antibody (MRP-1) directed to MRP1 (B) and detected by enhanced chemiluminescence (Biological Industries). On denaturing polyacrylamide gels the human RFC appears as a broadly migrating $\approx$ 85 kDa glycoprotein whereas MRP1 is a $\approx$ 190 kDa glycoprotein. The lines on the left denote molecular-mass markers in kDa.

clustered in TMD1, of which seven mutations involved three consecutive amino acids: Gly-44, Glu-45 and Ser-46.

In order to examine the effect of the different mutations on RFC protein expression, we performed Western blot analysis using a mouse polyclonal antiserum raised against a C-terminal hRFC peptide (Ifeghan, I., Kaufman, Y., Liani, E., Rothem, L., Jansen, G. and Assaraf, Y. G., unpublished work; Figure 5A). An RFC band of $\approx$ 85 kDa was expressed at low levels in parental CEM cells (Figure 5A, lane b) and at high levels in CEM-7A cells (Figure 5A, lane a), which highly overexpress RFC [28,39]. In contrast, no RFC could be detected in any of the antifolate-resistant mutants that harboured premature translation-termination mutations (Figure 5A, lanes c-k). However, the sublines EDX^{R0.03} (Figure 5A, lane c) and PT C-7 (Figure 5A, lane d) that harboured missense mutations had detectable levels of RFC. Western blot analysis with a control monoclonal antibody against the human MRP1 revealed similar levels of MRP1 expression in parental CEM cells (Figure 5B, lane b) and their antifolate-resistant sublines (Figure 5B, lanes c-k), whereas CEM-7A cells expressed markedly decreased MRP1 levels (Figure 5B, lane a). Surprisingly, however, the severely transport-defective cell lines AG2034^{R2} and AMT^{R0.12} that did not harbour any RFC mutation had no detectable levels of the RFC protein (Figure 5A, lanes j and k, respectively). Thus in order to determine whether decreased RFC mRNA expression plays a contributing role to the MTX-transport-defective phenotype, we performed RT-PCR analysis (Figure 6). RFC mRNA was completely absent from ZD9331^{R1.5} and PT C-3 cells, and was poorly represented in AG2034^{R2} cells (Figure 6). In contrast, RFC mRNA was well expressed in all the remaining antifolate-resistant cell lines (Figure 6). However, AMT^{R0.12} cells had neither an RFC mutation nor decreased RFC mRNA levels.

We also explored the question of whether RFC mutations occurred during the early (nanomolar antifolate concentration) or late (micromolar antifolate concentration) stages of stepwise antifolate selection. Thus we examined two representative cell lines, MTX- and PT523-resistant cells, in their early and late stages of antifolate selection (MTX^{R0.06} versus MTX^{R0.6}, and PT^{R0.02} versus PT^{R0.08}, respectively) for mutations in the RFC

Figure 6 RT-PCR of RFC mRNA expression in parental CEM cells and their various antifolate-resistant sublines

Portions of cDNA ($\approx$ 50 ng) synthesized from total RNA isolated from parental cells and their various antifolate-resistant sublines were PCR amplified using oligonucleotide primers targeting RFC and β -actin as detailed in the Materials and methods section. The 267 and 199 bp RFC and β -actin products, respectively, were resolved on 2% agarose gels containing ethidium bromide. M.W., molecular mass.

gene. Genomic PCR-SSCP analysis revealed aberrant electrophoretic mobility profiles in both RFC exons 2 and 3 that were already present during the early stages of selection with MTX (MTX^{R0.06}) and PT523 (PT^{R0.02}; Figures 3A, 3C and 3D). Furthermore, the electrophoretic mobility profiles of the early and late MTX-resistant (MTX^{R0.06} versus MTX^{R0.6}; Figure 3A) and the PT523-resistant sublines (PT^{R0.02} versus PT^{R0.08}; Figure 3C) were identical. DNA sequencing confirmed that the complex pattern of five mutations present in RFC exons 2 and 3 of MTX^{R0.6} cells was already present in the early MTX^{R0.06} mutant (Table 3). Likewise, the exact mutations identified in RFC exon 3 of PT^{R0.08} cells were present in the early PT^{R0.02} subline (Table 3). Consequently, PT^{R0.02} and PT^{R0.08} as well as MTX^{R0.06} and MTX^{R0.6} cells displayed identical curves of a high-level resistance to the selecting agents PT523 (Figure 7A) and MTX (Figure 7D), and cross-resistance to other antifolates (Figures 7B, 7C, 7E and 7F).

DISCUSSION

In the present study we have isolated multiple human leukaemia sublines that display a marked level of resistance to a variety of novel antifolate anticancer drugs that use RFC as their primary route of entry and that target different cellular folate-dependent enzymes including dihydrofolate reductase (PT523, edatrexate, MTX and aminopterin), thymidylate synthase (GW1843 and ZD9331) and glycinamide ribonucleotide transformylase (AG2034). This study was aimed at determining the role that RFC alterations play in mediating resistance to novel hydrophilic antifolates. Thus sublines established by both stepwise and single-step selection harboured one or more mutation(s) in the RFC gene. Consequently, these sublines lost up to 97% of parental [³H]MTX-transport activity and displayed high-level resistance to all hydrophilic antifolates. These results establish that mutations disrupting the transport activity of the hRFC are a major mechanism of resistance to various novel hydrophilic antifolates.

An analysis of the distribution of the multiple mutations identified here and in previous studies ([23–26] and Drori, S., Kaufman, Y., Rothem, L., Rots, M. G., Matherly, L. H., Jansen, G. and Assaraf, Y. G., unpublished work) revealed a remarkable pattern of clustering in two confined regions of the hRFC gene. Thus both nonsense and missense mutations clustered in exons 2 and 3 of the hRFC. This clustering of mutations in the hRFC gene is different from the more dispersed pattern of mouse RFC

Figure 7 Antifolate growth inhibition of drug-resistant variants selected at low and high drug concentrations

Logarithmically growing parental CEM cells and their antifolate-resistant sublines, selected at low and high PT523 concentrations, including PT^{R0.02} and PT^{R0.08} (A–C), or selected at low or high MTX concentrations, including MTX^{R0.06} and MTX^{R0.6} (D–F), were exposed to various concentrations of PT523 (A, E), MTX (B, D) and GW1843 (C, F) as detailed in the Materials and methods section. Following 3 days of incubation at 37 °C, viable cell counting was performed using Trypan Blue exclusion. Values depicted represent means $\pm$ S.D. from 3–5 independent experiments.

mutations induced by the mutagen *N*-methyl-*N*-nitrosourea in MTX-resistant murine L1210 leukaemia cells [38]. This difference can be easily reconciled by the fact that a mutagen like *N*-methyl-*N*-nitrosourea, although preferring certain nucleotide conversions [38], should not have a bias towards a certain region of the RFC gene. Furthermore, analysis of the distribution of the single amino acid substitutions from the present study as well as those from previous reports ([23,24,26] and Drori, S., Kaufman, Y., Rothen, L., Rots, M. G., Matherly, L. H., Jansen, G. and Assaraf, Y. G., unpublished work) revealed a pattern of mutation clustering in TMD1 of the hRFC. These findings are consistent

with our recent papers [23,24] suggesting that certain amino acid residues within TMD1 are involved in (anti)folate substrate binding and possibly in substrate translocation across the membrane. Of particular interest is the present finding that selection with edatrexate resulted in the emergence of the Glu-45 $\rightarrow$ Lys mutation, first identified in CEM-MTX human leukaemia cells [23] and in MTX-resistant mouse leukaemia cells [40]. Both the human and mouse Glu-45 $\rightarrow$ Lys mutations were shown to dramatically increase the affinity of RFC for folic acid while disrupting MTX influx capability [23,40]. Our present results support this conclusion as EDX^{R0.03} cells lost their MTX

transport (Table 2) but, among all antifolate-resistant sublines, these cells were (i) the least folic acid-growth-requiring and (ii) the least hypersensitive to trimetrexate (Table 1). However, one should bear in mind that mutation cluster regions can sometimes be cell- and/or gene-specific. Therefore, since the vast majority of mutations identified in the hRFC gene were obtained with a single human CCRF-CEM leukaemia cell line, one cannot exclude the possibility that this phenomenon of mutation clustering is cell-specific and may not necessarily represent other cell lineages.

The other frequently occurring mutation was the neighbour Gly-44 → Arg substitution in TMD1. Thus the Gly-44 → Arg mutation emerged in three MTX-transport-defective cell lines: in the PT C-7 cell line (the present study), in CCRF-CEM-Mtx1 cells [26] and in CEM-T cells (Drori, S., Kaufman, Y., Rothen, L., Rots, M. G., Matherly, L. H., Jansen, G. and Assaraf, Y. G., unpublished work). Furthermore, the Gly-44 residue was also targeted in MTX-resistant, transport-defective mouse L1210 D17 and C12 cell lines that harboured the Gly-44 → Glu mutation [38]. Thus PT C-7 lost their MTX-transport ability but surprisingly had folate pools that were not decreased to a very low level, as found in other cell lines with comparable transport loss (Table 2). Consistently, PT C-7 cells had only a 9-fold increase in the folic acid growth requirement compared with other cell lines, which required as much as 42-fold more folic acid (Table 1). Transfection of the Gly-44 → Arg mutant hRFC cDNA into MTX-transport-null cells resulted in a 12-fold increase in the transport K_m for MTX [26]. Similarly, the Gly-44 → Glu mutation in both D17 and C12 mouse leukaemia cell lines was associated with a 99% loss of MTX-transport activity [38]. Surprisingly, however, these cell lines had only a modestly increased folate growth requirement [38]. Clearly, all the Gly-44 and Glu-45 substitutions involved a net change in charge. Thus the various mutations in both the neighbouring residues Gly-44 and Glu-45 were associated with the loss of MTX transport but at the same time with preservation of sufficient folic acid uptake to sustain cell growth. We propose that within TMD1 of the mammalian RFC, the Gly-44 and Glu-45 residues play a functional role in the formation of the (anti)folate binding pharmacophore and possibly in substrate translocation.

Several lines of evidence indicate that a high level of resistance to various hydrophilic antifolates could be acquired during the early stages of exposure to low antifolate concentrations, as exemplified in the MTX^{R0.06}-MTX^{R0.6} and PT^{R0.02}-PT^{R0.08} sublines. First, an identical genomic PCR-SSCP pattern was obtained at low and high concentrations of antifolate selection. Secondly, the same mutations were obtained at low and high antifolate concentrations. Thirdly, superimposable resistance and cross-resistance patterns were obtained during selection with low and high antifolate concentrations. Fourthly, a similar folate growth requirement was obtained in the low and high antifolate selection and fifthly, a similar pattern of hypersensitivity to lipid-soluble antifolates was obtained in the low and high antifolate concentrations. These results strongly suggest, that during the early stages of exposure to low antifolate concentrations, mutations may arise which alter and/or inactivate hRFC transporter activity. These genomic alterations may be stably carried over to the high antifolate levels in the form of a dominant clonal variant that is equipped with a strong selective advantage.

We find no RFC protein expression in the antifolate-resistant sublines with premature translation-termination mutations. Consistently, these sublines contained intracellular folate pools that shrunk as much as 23-fold, reaching an extremely low cellular folate pool level of 2.5 pmol of reduced folates/mg of protein. These findings suggest that antifolate-resistant sublines could

successfully grow and proliferate under antifolate selective pressure while containing a cellular folate pool that is only $\approx 4\%$ of parental cells. This is consistent with our previous reports with the human leukaemia CEM-7A [28] and CEM-MTX-LF cell lines [23]. These cell lines highly overexpress RFC due to a gradual deprivation of leucovorin and folic acid from the growth medium. CEM-7A [28] and CEM-MTX-LF cells [23] contained an extremely low cellular folate pool of ≈ 1.2 pmol of reduced folate/mg of protein [23]. Thus it appears that the minimal cellular folate pool size necessary to support normal growth is $\approx 1\text{--}3$ pmol of reduced folate/mg of protein. This comprises $\approx 1.5\text{--}4.5\%$ of the normal cellular reduced folate pool.

All the antifolate-resistant human leukaemia sublines isolated in the present paper displayed a consistently high-level resistance to the selecting agent and cross-resistance of up to 2323-fold to the various hydrophilic antifolates. In contrast, all the antifolate-resistant sublines exhibited a marked hypersensitivity of up to 20-fold to the lipophilic antifolate trimetrexate. Since the hydrophilic antifolates use RFC as their primary route of entry into the cell, disruption of RFC transport activity will necessarily result in a high level of resistance to these hydrophilic antifolates. In contrast, trimetrexate and AG377, lipid-soluble antifolates that enter mammalian cells by diffusion [35], bypass the inactivated RFC thereby overcoming the resistance to hydrophilic antifolates. Furthermore, the profound hypersensitivity to these hydrophilic antifolates correlated favourably with the degree of folate pool depletion. This finding of the marked hypersensitivity to lipid-soluble antifolates has a potentially important bearing to the clinical setting; tumours displaying resistance that is elevated by over three orders of magnitude to various hydrophilic antifolates due to RFC transport defects could possibly be eliminated at extremely low concentrations of these lipophilic antifolates without harming healthy tissues.

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