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Multiple mechanisms of resistance to methotrexate and novel antifolates in human CCRF-CEM leukemia cells and their implications for folate homeostasis

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Abstract

We determined the mechanisms of resistance of human CCRF-CEM leukemia cells to methotrexate (MTX) vs. those to six novel antifolates: the polyglutamatable thymidylate synthase (TS) inhibitors ZD1694, multitargeted antifolate, pemetrexed, ALIMTA (MTA) and GW1843U89, the non-polyglutamatable inhibitors of TS, ZD9331, and dihydrofolate reductase, PT523, as well as DDATHF, a polyglutamatable glycinamide ribonucleotide transformylase inhibitor. CEM cells were made resistant to these drugs by clinically relevant intermittent 24 hr exposures to 5–10 μM of MTX, ZD1694, GW1843U89, MTA and DDATHF, by intermittent 72 hr exposures to 5 μM of ZD9331 and by *continuous* exposure to stepwise increasing concentrations of ZD9331, GW1843U89 and PT523. Development of resistance required only 3 cycles of intermittent drug exposure to ZD1694 and MTA, but 5 cycles for MTX, DDATHF and GW1843U89 and 8 cycles for ZD9331. The predominant mechanism of resistance to ZD1694, MTA, MTX and DDATHF was impaired polyglutamylation due to ~10-fold decreased folylpolyglutamate synthetase activity. Resistance to intermittent exposures to GW1843U89 and ZD9331 was associated with a 2-fold decreased transport via the reduced folate carrier (RFC). The CEM cell lines resistant to intermittent exposures to MTX, ZD1694, MTA, DDATHF, GW1843U89 and ZD9331 displayed a depletion (up to 4-fold) of total intracellular reduced folate pools. Resistance to continuous exposure to ZD9331 was caused by a 14-fold increase in TS activity. CEM/GW70, selected by continuous exposure to GW1843U89 was 50-fold resistant to GW1843U89, whereas continuous exposure to PT523 generated CEM/PT523 cells that were highly resistant (1550-fold) to PT523. Both CEM/GW70 and CEM/PT523 displayed cross-resistance to several antifolates that depend on the RFC for cellular uptake, including MTX (95- and 530-fold). CEM/GW70 cells were characterized by a 12-fold decreased transport of [^{3}H]MTX. Interestingly, however, CEM/GW70 cells displayed an enhanced transport of folic acid, consistent with the expression of a structurally altered RFC resulting in a 2.6-fold increase of intracellular folate pools. CEM/PT523 cells displayed a markedly impaired (100-fold) transport of [^{3}H]MTX along with 12-fold decreased total folate pools. In conclusion, multifunctional mechanisms of resistance in CEM cells have a differential impact on cellular folate homeostasis: decreased polyglutamylation and transport defects lead to folate depletion, whereas a structurally altered RFC protein can provoke expanded intracellular folate pools. © 2002 Published by Elsevier Science Inc.

Keywords: Folate homeostasis; Reduced folate carrier; Thymidylate synthase; Polyglutamylation; Folylpolyglutamate synthetase; Antifolate resistance

1. Introduction

The classical antifolate MTX disrupts cellular folate metabolism by potent inhibition of its target enzyme, dihydrofolate reductase (DHFR) [1,2]. In addition, polyglutamate forms of MTX have also a marked inhibitory effect on TS and enzymes in the *de novo* biosynthesis of purines [3,4].

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Abbreviations: MTX, methotrexate; MTA, multitargeted antifolate, pemetrexed, ALIMTA; ZD1694, raltitrexed; AG337, nolatrexed; DDATHF, lometrexol; DHFR, dihydrofolate reductase; GARFT, glycinamide ribonucleotide formyltransferase; TS, thymidylate synthase; FPGS, folylpolyglutamate synthetase; FPGH, folylpolyglutamate hydrolase; IC_{50}, drug concentration required to inhibit cell growth to 50% of untreated controls.

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Intrinsic and acquired resistance to MTX has been studied extensively in *in vitro* and animal model systems [5,6]. The major causes of resistance to MTX are: (a) qualitative and quantitative alterations in DHFR activity [7–9]; (b) impaired membrane transport of MTX into the cell via the RFC [10–13] or increased efflux via members of the multidrug resistance protein family [14,15]; and (c) decreased polyglutamylation either due to decreased activity of folylpolyglutamate synthetase (FPGS) [16–18] or increased activity of folylpolyglutamate hydrolase (FPGH) [19,20]. More recently, MTX resistance has been associated with the loss of functional retinoblastoma protein, which can lead to increased levels of free E2F-1, which is involved in the transcriptional regulation of the DHFR and TS genes [21].

In recent years, the number of studies concerning clinical resistance to MTX has increased steadily [1,5,6,10,13,20, 22,23]. To overcome resistance to MTX, a great deal of effort has been put into the development of novel antifolates that were rationally designed to (a) inhibit crucial DHFR-independent enzymes in folate metabolism, such as TS; (b) exhibit a different route of cellular uptake; and/or (c) display either none or, in contrast, high FPGS substrate affinity [24–26]. ZD1694 (raltitrexed; Tomudex®) is approved for clinical use [27,28], whereas other novel antifolates including GW1843U89 [29,30] and MTA (pemetrexed, LY231514 ALIMTA) [31–34] are currently being evaluated in advanced clinical trials. ZD1694 and GW1843U89 are potent TS inhibitors that are superior to MTX with respect to transport via the RFC and substrate specificity for FPGS [27,29,35]. The pyrrolopyrimidine antifolate MTA has multiple cellular targets, including DHFR, TS and glycinamide ribonucleotide formyltransferase (GARFT) and it is an excellent substrate for FPGS [31]. In contrast, PT523 and ZD9331 are non-polyglutamatable inhibitors of DHFR and TS, respectively; both enter the cell via the RFC [24,36–38]. DDATHF is a GARFT inhibitor that is a better substrate for FPGS than MTX [39].

Some of these novel antifolates have shown promising activity in clinical trials [25,28,30,32]. However, limited information is available about how rapidly and by what mechanism(s) resistance to these drugs develops. In the present study, we characterized the mechanisms underlying resistance of human CCRF-CEM leukemia cells that were selected for resistance to this panel of drugs using clinically relevant exposure schedules. Moreover, we analyzed the impact of these antifolate resistance mechanisms on cellular folate homeostasis.

2. Materials and methods

2.1. Drugs and biochemicals

RPMI 1640 cell culture medium, with and without folic acid, and (dialyzed) fetal calf serum (FCS) were obtained from Life Technologies, Inc. Folic acid was purchased from Sigma. MTX was a gift from Pharmachemie. ZD1694 (raltitrexed (*N*-[5-[*N*-(3,4-dihydro-2-methyl-4-oxoquinazolin-6-ylmethyl)-*N*-methylamino]-2-thenoyl]-L-glutamic acid), Tomudex®, property of Zeneca Limited (part of AstraZeneca)) and ZD9331 ((2*S*)-2-[*o*-fluoro-*p*-[*N*-(2,7-dimethyl-4-oxo-3,4-dihydroquinazolin-6-yl)methyl)-*N*-(prop-2-ynyl)amino]benzamido-4-(tetrazol-5-yl)]butyric acid were a gift from A.L. Jackman, Institute of Cancer Research. GW1843U89 ((*S*)-2[5-[[(1,2-dihydro-3-methyl-1-oxobenzo[*f*]quinazolin-9-yl)methyl]-amino]-1-oxo-2-isoindolinyl]-glutaric acid) was provided by G.K. Smith, Glaxo–Wellcome Research Laboratories. MTA (pemetrexed (*N*-[4-[2-(2-amino-3,4-dihydro-4-oxo-7*H*-pyrrolo[2,3-d]pyrimidine-5-yl)ethyl]-benzoyl]-L-glutamic acid)), ALIMTA®, and DDATHF (lometrexol (5,10-dideaza-5,6,7,8-tetrahydrofolic acid)) were supplied by Lilly Research Laboratories. PT523 (N^{α}-(4-amino-4-deoxypteroyl)-N^{δ}-hemiphthaloyl-L-ornithine) was a gift from A. Rosowsky, Dana-Farber Cancer Institute. TMQ (trimetrexate) was supplied by Warner-Lambert/Parke Davis. AG337 (nolatrexed (3,4-dihydro-2-amino-6-methyl-4-oxo-5-(4-pyridylthio)quinazoline)), Thymitaq®) was a gift of Agouron Pharmaceuticals, Inc.

[3′,5′,7-^{3}H]MTX (23 Ci/mmol) and [3′,5′,7,9-^{3}H]folic acid (28 Ci/mmol) were obtained from Moravek Biochemicals. [2,3-^{3}H]-L-glutamic acid (22 Ci/mmol, 0.01 N HCl formulation) was obtained from NEN Life Science Products. MTX-diglutamate was obtained from Schircks Chemical Co. All other chemicals were of the highest purity available.

2.2. Cell lines

CCRF-CEM, a human T cell leukemia cell line, and its antifolate-resistant sublines were maintained in RPMI-1640 medium (containing 2.2 μM folic acid), supplemented with 10% fetal calf serum, 2 mM glutamine, penicillin (100 unit/mL) and streptomycin (100 μg/mL), at 37° in a 5% CO_2 humidified atmosphere.

2.3. Establishment of antifolate-resistant cell lines

Cell cultures (20 mL) of exponentially growing CCRF-CEM leukemia cells were prepared at a density of 3×10^5 cells/mL and were exposed to various antifolate drugs. For development of resistance against polyglutamatable drugs (MTX, ZD1694, GW1843U89, MTA and DDATHF), a schedule of intermittent 24 hr drug exposures was applied with fixed drug concentrations: 5 μM of MTX, ZD1694 and GW1843U89, and 10 μM of MTA and DDATHF.

Since 24 hr exposure to 5 μM of the non-polyglutamatable drugs PT523 and ZD9331 did not result in growth inhibition (results not shown), a different schedule was used to induce resistance to these compounds. CCRF-CEM cells were made resistant to GW1843U89, PT523 and ZD9331 by continuous exposure to stepwise increasing concentrations of these

drugs up to 70 nM for GW1843U89, 500 nM for PT523 and 1 μM for ZD9331. Furthermore, resistance to ZD9331 was induced by intermittent drug exposures (5 μM) of 72 hr.

After drug exposure, cells were harvested by centrifugation, washed twice with drug-free medium, and resuspended in either drug-free medium (intermittent drug exposure schedule) or drug-containing medium (dose escalation schedule) at a density of 3×10^5 cells/mL. Cells were passaged twice weekly and, when cell numbers fell below 3×10^5 mL^{-1}, the cells were concentrated to a density of 3×10^5 cells/mL. Following recovery (i.e. restoration of wild type growth rate), another cycle of drug exposure (intermittent drug exposure schedule) or a 1.5-fold dose escalation was applied to the cells.

2.4. Growth inhibition studies

The inhibitory effects of the antifolate drugs on the growth of parental and antifolate-resistant CCRF-CEM cell lines were determined as described previously by Westerhof *et al*. [24]. Briefly, exponentially growing cells were prepared at a density of 1×10^5 cells/mL, after which 1 mL aliquots were distributed into 24-well plates and exposed to various concentrations of drugs. The cells were incubated for either 72 hr continuously or 24 hr followed by a 72 hr period in drug-free medium at 37° in a 5% CO_2 humidified atmosphere. Cell counts and viability were determined in a hemocytometer by trypan blue exclusion. IC_{50} values were defined as the drug concentrations at which cell growth is inhibited by 50% as compared to untreated controls.

2.5. FPGS activity

The activity of FPGS was determined as described previously [40,41]. Briefly, a frozen pellet of 2×10^7 cells was suspended in 0.5 mL extraction buffer (50 mM Tris–HCl, 20 mM KCl, 10 mM $MgCl_2$, and 5 mM dithiotreitol, pH 7.5). Crude cell extracts were obtained after sonication (MSE Soniprep, amplitude 14 microns, 3×5 s with 10 s intervals, at 4°) followed by centrifugation at 12,000 *g* for 15 min at 4°. The protein content was determined using the Bio-Rad protein assay according to Bradford [42]. The assay mixture contained 200 μg protein, 4 mM [2,3-^{3}H]-L-glutamic acid (specific activity, 6.6 Ci/mol) and 250 μM MTX in a buffer of: 100 mM Tris, 10 mM ATP, 20 mM $MgCl_2$, 20 mM KCl, and 10 mM dithiotreitol at a final volume of 250 μL and pH of 8.5. After 2 hr incubation at 37°, the reaction was stopped by the addition of 1 mL of an ice-cold solution containing 5 mM unlabeled L-glutamic acid. Sep-Pack C_{18} cartridges (Millipore, Waters Associates) were used for the separation of free, unreacted [^{3}H] glutamate from MTX-[^{3}H]diglutamate [40]. The amount of MTX-[^{3}H]diglutamate was measured by β-scintillation counting. Controls lacking MTX were included to correct for polyglutamylation of endogenous folates present in the samples.

2.6. FPGH activity

The activity of FPGH was determined according to O'Connor *et al*. [43]. Briefly, pellets of 5×10^6 cells were suspended in 100 μL extraction buffer (0.1 M Tris–HCl, pH 6.9) and crude cell extracts were obtained by sonication as described above. The reaction mixture contained 25 μg protein from the crude cell extract and 100 μM MTX-diglutamate in 200 μL of 0.1 M Tris–HCl, pH 6.9 and was incubated for 1 hr in a 37° water bath. The reaction was stopped by heating the samples for 3 min at 95°. After cooling on ice for 15 min, the samples were centrifuged (15 min, 12,000 *g*, 4°) and the supernatant was stored at −20° until analysis. MTX-diglutamate was separated from the product MTX (monoglutamate) by HPLC [20,44].

2.7. TS activity

The catalytic activity of TS was assayed by measurement of tritium release during the conversion of [5-^{3}H]dUMP to dTMP, as described in detail by Van der Wilt *et al*. [45]. In brief, a cell pellet of 2×10^7 cells was suspended in 1 mL assay buffer (0.2 M Tris, 100 mM NaF, 15 mM cytidine-5′-monophosphate, pH 7.5) and disrupted by sonication as described above. The reaction mixture consisted of 25 μL cell extract in different dilutions, 5 μL of 6.5 mM CH_2-THF, and 10 μL assay buffer pH 7.4. The reaction was initiated by the addition of 10 μL [5-^{3}H]dUMP (1 or 10 μM final concentration). After an incubation of 30 min at 37°, the reaction was terminated by the addition of 50 μL ice-cold 35% trichloroacetic acid and 250 μL of 10% neutral activated charcoal. After centrifugation, 150 μL of the supernatant was counted by liquid scintillation.

2.8. Transport of [^{3}H]MTX and [^{3}H]folic acid

Logarithmically growing cells were harvested and washed in Hepes-buffered saline solution (HBSS) pH 7.4 [46]. For measurement of 3 min influx rates of MTX and folic acid, cells were resuspended in HBSS buffer at a density of 10^7 cells/mL and incubated with [^{3}H]MTX and [^{3}H]folic acid at an extracellular concentration of 2 μM at 37°. Transport was stopped at the indicated times by the addition of 10 mL of ice-cold HBSS buffer. Cells were then centrifuged (500 *g*, 5 min, 4°) and washed once more with ice-cold HBSS buffer. The final cell pellet was dissolved in water and radioactivity was determined by liquid scintillation counting. Folic acid transport studies were performed with HBSS buffer supplemented with 5 μM TMQ in order to block metabolism of folic acid [47].

2.9. Determination of MTX polyglutamate formation

Cells were incubated in 10 mL culture medium in 25 cm^2 tissue flasks (density: 1×10^6 mL^{-1}) in the presence of 1 μM [^{3}H]MTX (specific activity, 0.45 Ci/mmol).

After 24 hr, cells were washed twice with ice-cold HBSS pH 7.4 by centrifugation (5 min at 300 *g*, 4°) and the pellet was resuspended in 1 mL HBSS. A sample of 90 μL was counted for radioactivity, 10 μL was used to determine the cell number; the remaining suspension was centrifuged (5 min at 12,000 *g*, 4°) and the pellet was kept at −20° until extraction. The extraction of [^{3}H]MTX polyglutamates and HPLC analysis were performed as described previously [20].

2.10. Analysis of intracellular folate pools

Prior to harvesting cells for folate pool analysis, resistant cell lines were cultured in the absence of drugs for at least three passages. Intracellular folate pools were analyzed according to a method based on enzymatic cycling of reduced folates to N^5,N^{10}-methylenetetrahydrofolate (CH_2THF) followed by entrapment into a stable ternary complex with excess *Lactobacillus casei* TS and [6-^{3}H]-F-dUMP (20 Ci/mmol, Moravek Biochemicals) [48]. Frozen cell pellets were thawed (25×10^6 cells/mL) in an 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 15,000 *g* for 10 min at 4°. Aliquots (20–60 μL) of the supernatant were used to determine CH_2THF, THF, DHF, leucovorin and 5-CH_3THF [49]. The 10-CHO-THF pool was assayed in the presence of 0.4 mU 10-CHO-THF dehydrogenase and 10 μM NADP.

3. Results

3.1. Resistance to intermittent exposures to MTX, ZD1694, MTA, GW1843U89, DDATHF and ZD9331

In order to develop antifolate-resistant sublines, human CCRF-CEM leukemia cells were repeatedly exposed to MTX, ZD1694, MTA, GW1843U89 and DDATHF for 24 hr and to the non-polyglutamatable antifolate ZD9331 for 72 hr (Table 1). We noted a marked difference in the number of drug exposures that was required for the development of resistance; only 3–4 cycles of 24 hr pulse exposures for the polyglutamatable drugs MTX, ZD1694, MTA, GW1843U89 and DDATHF, but 8 cycles of 72 hr pulse exposures for ZD9331 (Table 1). The resistant phenotypes were stable for more than 4 months after maintenance in drug-free medium.

The levels of resistance are listed in Table 1 and are based on either 24 or 72 hr drug exposure. Levels of resistance obtained by intermittent exposure varied between 125- and 4550-fold. In particular, the cells made resistant to ZD1694 and GW1843U89 displayed the highest degree of resistance.

The growth inhibitory effects of 72 hr exposures to MTX, ZD1694, MTA, GW1843U89, ZD9331, AG337, DDATHF, TMQ and PT523 against the parental CCRF-CEM cell line and the various antifolate-resistant sublines are shown in Table 2. Because of the longer exposure time, levels of resistance changed such as the wild-type sensitivity of CEM/MTX^{R24} to MTX. Comparable cross-resistance patterns were observed in the cells that were made resistant to 24 hr exposures to MTX, ZD1694, MTA, and DDATHF. All these cell lines were characterized by a high level of resistance to antifolates that depend on polyglutamylation for their cytotoxic activity, such as ZD1694, MTA and DDATHF, while retaining wild-type sensitivity to MTX, GW1843U89, ZD9331, TMQ, PT523 and the lipophilic TS inhibitor AG337. CEM/ZD1694^{R24} and CEM/MTA^{R24} displayed collateral sensitivity to the lipophilic DHFR inhibitor TMQ. CEM/GW1843U89^{R24} and CEM/ZD9331^{R72} were 3- and 11-fold resistant to MTX, respectively, and also resistant to different types of antifolates such as ZD1694, ZD9331 and AG337. CEM/ZD9331^{R72} was also 8-fold resistant to the non-polyglutamatable but RFC-dependent drug PT523.

Table 1
Characteristic features of antifolate-resistant sublines

Cell line	Selection condition		No. of cycles	IC_{50} (nM) of selection drugs in wild type CCRF-CEM cells[a]	Level of resistance to selection drug in resistant cells[b]
CEM/MTX^{R24}	Intermittent 24 hr	5 μM MTX	4	94	>530
CEM/ZD1694^{R24}	Intermittent 24 hr	5 μM ZD1694	3	11	>4550
CEM/MTA^{R24}	Intermittent 24 hr	10 μM MTA	3	98	>1020
CEM/GW1843U89^{R24}	Intermittent 24 hr	5 μM GW1843U89	4	7	>3570
CEM/GW70	Continuous exposure	70 nM GW1843U89	–	2.4	51
CEM/DDATHF^{R24}	Intermittent 24 hr	10 μM DDATHF	4	245	>410
CEM/ZD9331^{R72}	Intermittent 72 hr	5 μM ZD9331	8	16	125
CEM/ZD9331^{cont}	Continuous exposure	1 μM ZD9331	–	16	1280
CEM/PT523	Continuous exposure	500 nM PT523	–	1.1	1550

[a] For CEM/MTX^{R24}, CEM/ZD1694^{R24}, CEM/MTA^{R24}, CEM/GW1843U89^{R24} and CEM/DDATHF^{R24}, the IC_{50} values for 24 hr exposure to the selection drug for CCRF-CEM cells are shown; for CEM/GW70, CEM/ZD9331^{R72}, CEM/ZD9331^{cont} and CEM/PT523 cells, the IC_{50} values for 72 hr of drug exposure are shown.

[b] For CEM/MTX^{R24}, CEM/ZD1694^{R24}, CEM/MTA^{R24}, CEM/GW1843U89^{R24} and CEM/DDATHF^{R24}, the level of resistance represents the ratio of IC_{50} values for 24 hr exposure to the selection drug relative to the corresponding IC_{50} value for CCRF-CEM cells, whereas for CEM/GW70, CEM/ZD9331^{R72}, CEM/ZD9331^{cont} and CEM/PT523 cells the resistance levels were determined on basis of IC_{50} values for 72 hr of drug exposure.

Table 2
Growth inhibitory effects of antifolates against human CCRF-CEM leukemia cells and antifolate-resistant sublines[a]

	MTX	ZD1694	MTA	GW1843U89	ZD9331	AG337	DDATHF	TMQ	PT523
RFC substrate	+	+	+	+	+	−	+	−	+
FPGS substrate	+	+	+	+	−	−	+	−	−
Target enzyme	DHFR	TS	TS[b]	TS	TS	TS	GARFT	DHFR	DHFR
Cell line	IC_{50} (nM), 72 hr drug exposure								
CCRF-CEM	8.1 ± 2.2	3.5 ± 0.6	23 ± 7	2.4 ± 0.4	16 ± 1	1800 ± 340	11 ± 4	7.5 ± 2.1	1.1 ± 0.3
CEM/MTX^{R24}	9.9 ± 4.1	79 ± 33	177 ± 35	3.2 ± 0.5	11 ± 2	1125 ± 55	155 ± 65	6.7 ± 1.8	2.6 ± 0.8
CEM/ZD1694^{R24}	8.1 ± 1.0	3450 ± 2165	1790 ± 1120	3.3 ± 2.7	19 ± 9	975 ± 280	443 ± 363	3.8 ± 2.9	2.1 ± 0.4
CEM/MTA^{R24}	7.4 ± 1.6	4135 ± 2250	6015 ± 3922	2.3 ± 0.8	22 ± 10	1300 ± 320	866 ± 7	2.3 ± 0.8	2.1 ± 2.1
CEM/DDATHF^{R24}	15 ± 4	220 ± 173	301 ± 190	3.9 ± 1.9	19 ± 3	1200 ± 370	441 ± 171	7.0 ± 5.3	3.1 ± 2.1
CEM/ZD9331^{R72}	86 ± 35	214 ± 61	322 ± 67	60 ± 31	1985 ± 1085	4035 ± 580	160 ± 35	3.1 ± 1.2	8.9 ± 1.9
CEM/ZD9331^{cont}	39 ± 3	665 ± 76	9060 ± 4050	106 ± 33	20450 ± 3530	38670 ± 5890	50 ± 5	2.4 ± 1.2	3.2 ± 1.6
CEM/GW1843U89^{R24}	26 ± 9	71 ± 31	235 ± 127	50 ± 18	92 ± 42	3240 ± 870	111 ± 63	7.2 ± 1.7	2.4 ± 0.9
CEM/GW70	775 ± 81	515 ± 224	351 ± 73	123 ± 48	479 ± 67	974 ± 241	360 ± 106	5.3 ± 1.3	81 ± 16
CEM/PT523	4300 ± 500	2100 ± 310	2520 ± 370	408 ± 45	2350 ± 630	430 ± 40	7720 ± 850	1.7 ± 0.7	1710 ± 110

[a] Cells were plated at an initial density of 1×10^5 cells/mL in 24-well tissue culture plates with drugs at various concentrations. The cell counts and viability were determined after 72 hr by trypan blue exclusion. IC_{50} values were determined in at least three different experiments. Results are the mean ± SEM.

[b] Beyond its primary target TS, MTA can also inhibit DHFR and GARFT.

3.2. Resistance to continuous exposure to ZD9331, GW1843U89 and PT523

CCRF-CEM cells were also made resistant to GW1843U89, PT523 and ZD9331 by continuous exposure to stepwise increasing concentrations of these drugs up to 70 nM for GW1843U89 (CEM/GW70), 500 nM for PT523 (CEM/PT523) and 1 μM for ZD9331 (CEM/ZD9331^{cont}).

CEM/GW70 cells were 51-fold resistant to GW1843U89 and CEM/PT523 cells were highly resistant (1550-fold) to PT523 (Table 2). Moreover, both CEM/GW70 and CEM/PT523 cells displayed cross-resistance to MTX (95- and 530-fold, respectively) and to various other antifolates that depend on the RFC for their cellular uptake, including ZD1694, MTA, ZD9331 and DDATHF, whereas collateral sensitivity to both TMQ and AG337 was observed (Table 2). CEM/ZD9331^{cont} cells were characterized by a 21-fold cross-resistance to the lipophilic TS inhibitor AG337.

3.3. Transport studies

To determine whether impaired RFC transport contributes to the resistant phenotypes, [^{3}H]MTX influx over 3 min was measured at a 2 μM extracellular concentration (Fig. 1). [^{3}H]MTX influx in CEM/MTX^{R24}, CEM/ZD1694^{R24}, CEM/MTA^{R24} and CEM/DDATHF^{R24} was similar to that obtained with parental CCRF-CEM cells. In contrast, a 2-fold decrease in [^{3}H]MTX influx was observed in CEM/GW1843U89^{R24} and CEM/ZD9331^{R72}. CEM/GW70 cells showed a 12-fold lower [^{3}H]MTX influx compared to parental cells (Fig. 1). Interestingly, the transport rate of folic acid in these cells was 3-fold increased compared to CCRF-CEM cells (0.66 ± 0.17 vs. 0.19 ± 0.05 pmol/min/10^7 cells). CEM/PT523 cells displayed a markedly reduced (>100-fold) [^{3}H]MTX influx compared to parental cells (Fig. 1).

3.4. MTX accumulation and polyglutamate formation

After a 24 hr incubation with [^{3}H]MTX at an extracellular concentration of 1 μM, the accumulation and distribution of MTX polyglutamate derivatives were analyzed. The total accumulation of MTX was 3-fold reduced in the cell lines resistant to MTX, ZD1694, MTA, GW1843U89 and DDATHF compared to parental cells (Fig. 2). A markedly decreased accumulation of [^{3}H]MTX was observed in CEM/GW70 and CEM/PT523 cells (10- and 30-fold, respectively). In contrast, CEM/ZD9331^{cont} cells showed a substantial increase in MTX accumulation (Fig. 2).

A profound polyglutamylation defect was observed in CEM/MTX^{R24}, CEM/ZD1694^{R24}, CEM/MTA^{R24} and CEM/DDATHF^{R24} cells and also in the cells that were made resistant to continuous exposure to ZD9331 (Fig. 3).

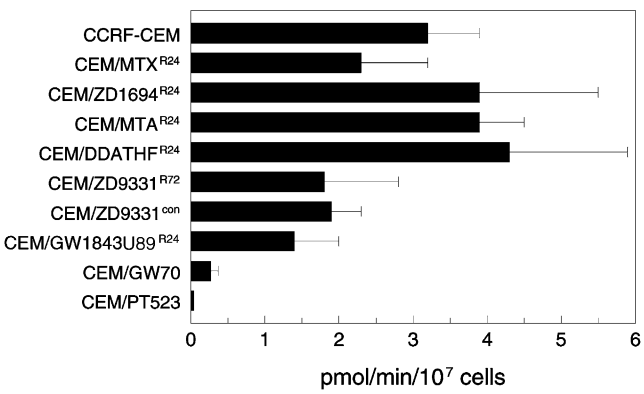

Fig. 1. Transport of [^{3}H]MTX in CCRF-CEM cells and antifolate-resistant sublines. Cells were washed and resuspended in HEPES buffered saline solution pH 7.4 and exposed to 2 μM [^{3}H]MTX for 3 min. Values for [^{3}H]MTX transport are depicted in pmol [^{3}H]MTX/min/10^7 cells and presented as the mean ± SEM of at least three separate experiments.

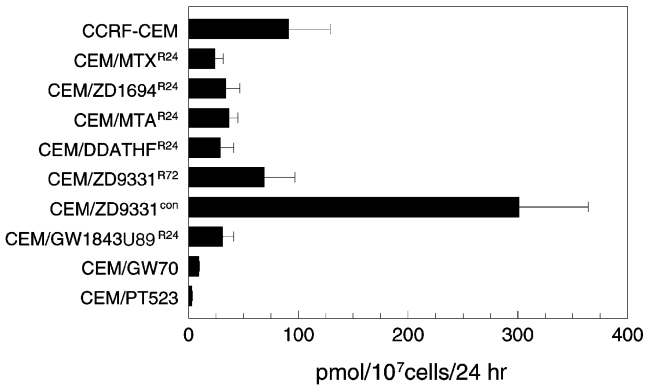

Fig. 2. Accumulation of [^{3}H]MTX in CCRF-CEM cells and antifolate-resistant sublines. Cells were incubated in culture medium (10^7 cells/10 mL) in the presence of 1 μM [^{3}H]MTX. After 24 hr, [^{3}H]MTX accumulation was depicted as pmol [^{3}H]MTX/10^7 cells. Results are presented as the mean ± SEM of at least three separate experiments.

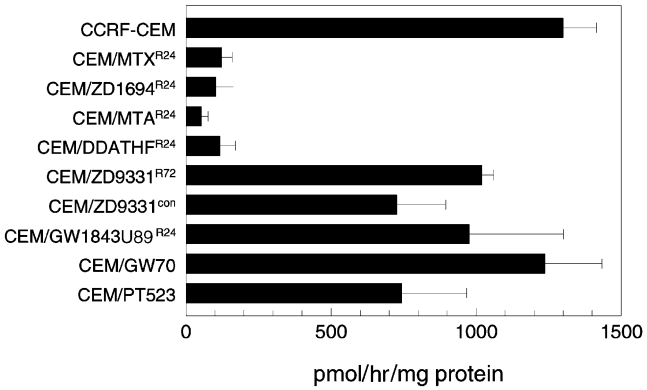

Fig. 4. Activity of FPGS in CCRF-CEM cells and antifolate-resistant sublines. FPGS activity was determined in cell extracts with MTX as a substrate at a concentration of 250 μM. Values for FPGS activity were given as pmol [^{3}H]Glu incorporated/hr/mg protein and presented as the mean ± SEM of at least three separate experiments.

Also in CEM/GW70 cells the distribution of polyglutamates showed a marked shift to short chain forms compared to parental cells while in CEM/PT523 cells the formation of MTX polyglutamates was entirely abolished (Fig. 3). MTX polyglutamylation in CEM/GW1843U89^{R24} and CEM/ZD9331^{R72} cells was similar to that in parental cells (Fig. 3).

3.5. FPGS and FPGH activity

The decreased polyglutamylation of MTX in CEM/MTX^{R24}, CEM/ZD1694^{R24}, CEM/MTA^{R24} and CEM/DDATHF^{R24} was associated with a 91–96% loss of FPGS activity as compared to parental cells (Fig. 4). CEM/ZD9331^{cont} cells displayed a 1.8-fold decrease in FPGS activity. No change in FPGS activity in CEM/GW70 and CEM/PT523 cells was observed (Fig. 4).

To test whether the decreased polyglutamylation was a result of an increased FPGH activity, we also measured the activity of this enzyme using MTX-diglutamate as a substrate. FPGH activity was unchanged in all antifolate-resistant cell lines except for CEM/ZD9331^{cont} which showed a 2-fold decreased FPGH activity (Fig. 5).

3.6. TS activity

Fig. 6 shows the TS activity of the different cell lines measured at a dUMP concentration of 10 μM. CEM/ZD9331^{cont} cells displayed a 14-fold increased TS activity compared to parental cells (Fig. 6). All the other cell lines did not show any elevation of TS activity. In addition, no difference in the ratio of TS activity at 1 μM over that at 10 μM dUMP was observed in any cell line (results not shown). CEM/GW70 cells did not show any change in TS activity compared to parental cells (Fig. 6). However, further augmentation of the concentration of GW1843U89 (above 70 nM) resulted in an increase in TS activity (results not shown).

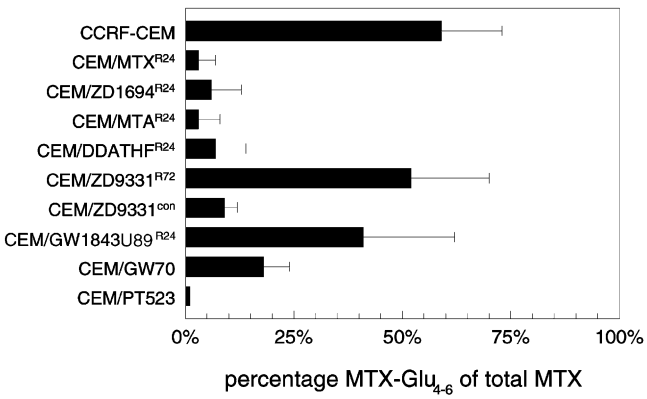

Fig. 3. Polyglutamylation of [^{3}H]MTX in CCRF-CEM cells and antifolate-resistant sublines. Cells were incubated in RPMI medium (10^7 cells/10 mL) in the presence of 1 μM [^{3}H]MTX. After 24 hr, cell extracts were analyzed for [^{3}H]MTX polyglutamates by HPLC. Results are presented as the mean ± SEM of at least three separate experiments.

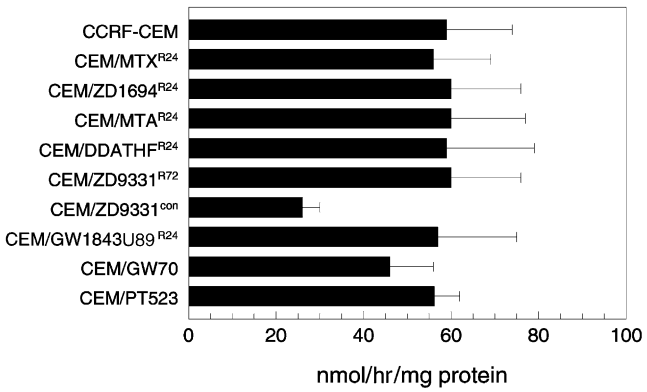

Fig. 5. Activity of FPGH in CCRF-CEM cells and antifolate-resistant sublines. FPGH activity was determined in cell extracts with MTX-Glu$_2$ (100 μM) as a substrate. Values for FPGH activity were given as pmol MTX formed/min/mg protein and presented as the mean ± SEM of at least three separate experiments.

Table 3
Intracellular reduced folate pools in CCRF-CEM and antifolate-resistant variants[a]

Cell line	Reduced folates (pmol/mg protein)				
	$CH_2THF + THF$	5-CH_3THF	DHF	10-CHO-THF	Total
CCRF-CEM	24 ± 1	9.9 ± 3.1	<0.3	37 ± 2	70 ± 3
CEM/MTX^{R24}	7.0 ± 0.8	7.1 ± 2.9	0.9 ± 0.5	12 ± 3	29 ± 2
CEM/ZD1694^{R24}	11 ± 4	4.1 ± 2.0	1.3 ± 0.9	15 ± 2	33 ± 4
CEM/MTA^{R24}	12 ± 5	2.8 ± 1.5	<0.3	7.0 ± 1.6	20 ± 4
CEM/DDATHF^{R24}	20 ± 5	4.9 ± 1.0	<0.3	33 ± 1	57 ± 4
CEM/ZD9331^{R72}	12 ± 3	3.4 ± 2.0	2.8 ± 1.8	2.9 ± 1.3	17 ± 2
CEM/ZD9331^{cont}	15 ± 2	4.6 ± 0.9	<0.3	30 ± 5	52 ± 6
CEM/GW1843U89^{R24}	12 ± 2	6.5 ± 3.0	<0.3	20 ± 4	38 ± 5
CEM/GW70	44 ± 3	37 ± 10	<0.3	82 ± 9	163 ± 16
CEM/PT523	1.6 ± 0.3	0.4 ± 0.2	3.0 ± 0.9	0.8 ± 0.3	5.8 ± 0.5

[a] Values represent the mean ± SEM from six separate determinations.

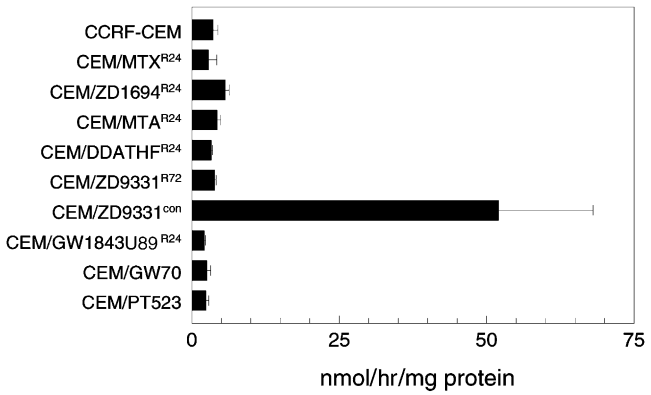

Fig. 6. Catalytic activity of TS in CCRF-CEM cells and antifolate-resistant sublines. TS activity was determined at saturating substrate concentrations of 10 μM [5-^{3}H]dUMP and 650 μM 5,10-methylene-THF. Values for FPGH activity were given as nmol [^3H_2O] formed/hr/mg protein and presented as the mean ± SEM of at least three separate experiments.

3.7. Folate pools in CCRF-CEM and antifolate-resistant sublines

In order to determine whether the various types of resistance affect the cellular folate pools, we measured the concentration of various reduced folates. In the cell lines that were selected by intermittent exposures to MTX, ZD1694, MTA, DDATHF, ZD9331 and GW1843U89, the total pools of reduced folates were decreased to 24–81% of that in parental cells (Table 3). A marked depletion of total intracellular folate pools was observed in CEM/PT523 (12-fold lower compared to CCRF-CEM cells).

Interestingly, CEM/GW70 cells showed 2.3-fold expanded total folate pool over wild type CEM cells. This was attributable to a 2–4-fold increased levels of all folate intermediates: 5-CH_3THF, 10-CHO-THF, CH_2THF and THF (Table 3).

4. Discussion

In this study, we demonstrated that acquired resistance to various novel antifolates may consist of different mechanisms (Table 4) depending on the drug and the method of induction of resistance. Beyond this, these antifolate resistance mechanisms can have a marked impact on cellular folate homeostasis.

Table 4
Summary of mechanisms of resistance in antifolate-resistant CEM cell lines

Cell line	↓ Transport	↓ FPGS activity	↑ TS expression
CEM/MTX^{R24}	−	+	−
CEM/ZD1694^{R24}	−	+	−
CEM/MTA^{R24}	−	+	−
CEM/DDATHF^{R24}	−	+	−
CEM/ZD9331^{R72}	+	−	−
CEM/ZD9331^{cont}	+	−	+
CEM/GW1843U89^{R24}	+	−	−
CEM/GW70	+	−	−
CEM/PT523	+	−	−

In human CCRF-CEM leukemia cell lines that were made resistant to MTX, ZD1694, MTA and DDATHF by intermittent drug exposure, the development of drug resistance was remarkably rapid. The underlying mechanism of resistance in these cell lines was defective polyglutamylation due to a marked decrease in FPGS activity. The results of the growth inhibition studies in these cell lines were consistent with a polyglutamylation defect, since the highest levels of resistance were observed against the antifolates that are most dependent on polyglutamylation for their pharmacologic activity, such as ZD1694 [50–54], MTA [55] and DDATHF [56]. Likewise, these polyglutamylation defective cell lines showed no reduced sensitivity to 72 hr exposure to MTX, thereby confirming previous results that show that polyglutamylation is not important for DHFR inhibition by this drug when given continuously as MTX monoglutamate is a potent DHFR inhibitor [17,18,44,57,58]. As shown before, short-term exposure to MTX results in resistance mechanisms based on RFC and FPGS defects rather than alterations in DHFR activity [17,18]. Increased DHFR activity is often observed after prolonged continuous exposure to stepwise increasing concentrations of MTX and is associated with DHFR gene amplification [8].

It was particularly noteworthy that CEM/ZD1694^{R24} and CEM/MTA^{R24} displayed near identical FPGS activities as CEM/MTX^{R24} cells, but showed a much higher level of resistance to ZD1694 and MTA than CEM/MTX^{R24} (Table 2, Fig. 4). A similar pattern as in CEM/MTX^{R24} was observed in polyglutamylation defective CCRF-CEM/R30dm cells, that were selected by short-term exposures to MTX [18,57]. Although the FPGS level in CEM/R30dm cells is even lower than in CEM/MTX^{R24} cells, the low levels of resistance to ZD1694 and MTA in CEM/R30dm were comparable to those in CEM/MTX^{R24} cells [57,58]. The higher level of resistance to ZD1694 and MTA in CEM/ZD1694^{R24} and CEM/MTA^{R24} compared to cells made resistant to MTX might be explained by: (a) possible differences in FPGS mutations resulting in altered K_m-values for either glutamic acid or antifolate substrate [59,60]; (b) differences in TS induction [51] or (c) downstream events, e.g. alterations at the level of E2F-1, that can potentially cause (differential) resistance to antifolates [21].

It is of interest to note that the development of resistance by pulsatile exposure to the non-polyglutamatable antifolate ZD9331 and the not strictly polyglutamylation-dependent GW1843U89, appeared to take longer time than the development of resistance to ZD1694 and MTA, which are highly dependent on polyglutamylation for their pharmacologic activity. McGuire *et al*. showed that defective polyglutamylation can rapidly emerge in CCRF-CEM cells following intermittent MTX exposure due to the selection of (pre-existing) clones with decreased FPGS activity [61]. It is conceivable that in CEM cells this feature also underlies the rapid evolution of resistance upon intermittent exposure to novel polyglutamatable antifolates as observed in the present study. In the case of tumors with a resistance phenotype based on defective FPGS, the use of analogues that are not dependent on polyglutamylation, e.g. ZD9331 and PT523, may be warranted.

Several tumor cell lines made resistant to ZD1694 by continuous exposure of the cells to this drug have been described previously. These studies have demonstrated that the most common mechanisms of resistance are increased TS activity [50,54,62] along with decreased polyglutamylation and impaired transport [50,52,53,62]. Consistent with previous reports [51,53], in the present study, selection of ZD1694-resistant cells using intermittent drug exposures (which mimics the clinical mode of treatment) resulted in diminished polyglutamylation as the mechanism of resistance to this drug. Resistance to DDATHF has only been studied in cell lines that were obtained upon prolonged exposures to increasing drug concentrations and appeared to be due to altered RFC transport [63] or impaired polyglutamylation [56]. The present study shows that intermittent DDATHF exposures can likewise lead to resistance based on impaired polyglutamylation.

Recently, a human MOLT-3 leukemia cell line was described that was made resistant to ZD9331 by continuous exposure and characterized by a decreased MTX transport and an overexpression of TS [64], which is comparable with the phenotype of CEM/ZD9331^{cont} cells. Remarkably, these CEM/ZD9331^{cont} cells displayed 2.5-fold increased MTX accumulation. The mechanistic basis for this needs to be elucidated although in another TS overproducing human WiL2/C2 cell line [65] a comparable 3.4-fold increase in MTX accumulation was observed (results not shown). Moreover, CEM/ZD9331^{cont} cells displayed decreased FPGH activity. It seems unlikely that this decreased activity plays a role since only increased FPGH activity has been associated with antifolate resistance [19].

Both CEM/PT523 and CEM/GW70 cells were characterized by defective RFC transport resulting in high levels of resistance to GW1843U89, PT523 and MTX as well as other RFC-dependent antifolates. The severe transport defect of the CEM/PT523 cells was at a molecular level associated with a 5-fold decreased level of RFC1 mRNA expression as measured with a reverse transcription PCR method using competitive templates (results not shown). Further investigations are ongoing to determine whether this phenotype is associated with a mutated *RFC1* gene.

The functionally altered RFC transport in CEM/GW70 cells was manifested by a marked decrease in transport of MTX while the transport of folic acid was enhanced. In a recent report from our laboratory, we have described a structurally altered RFC in another MTX-resistant CCRF-CEM subline CEM/MTX, that was also characterized by a markedly decreased MTX transport concurrent with an increased transport of folic acid [66]. This functional alteration of RFC transport in CEM/MTX cells appeared to be due to a point mutation that gives rise to a substitution of a lysine for glutamate at amino acid 45, E45K [66]. Mutation analysis of the RFC in CEM/GW70 cells revealed that the functional alterations of RFC transport in these cells were associated with the same mutation as was found in CEM/MTX cells along with two additional mutations (S46I and V29L) in CEM/GW70 cells [67]. The E45K mutation was also identified in murine MTX-resistant L1210 cells that exhibit a selectively enhanced transport of natural folates over antifolates [68]. A dramatic increase of folic acid transport due to RFC mutations was also detected in a DDATHF-resistant murine leukemia cell line [69]. Altogether, these observations point to a common feature in several cell lines after exposure to different antifolates that allow these cells to meet their folate growth requirements while their antifolate transport is abolished [70].

The observation that exposure of CEM/GW70 cells to concentrations of GW1843U89 higher than 70 nM resulted in increased TS activity points to a sequential effect in the development of resistance to this drug. This might have implications for the clinical applicability of GW1843U89 since a transport defect could be circumvented with lipophilic compounds, while drugs with other targets will be required to bypass increased activity of TS.

Analysis of intracellular reduced folate pools indicated that the implications of resistance to antifolates for folate homeostasis depend on the mechanism of resistance and thus on how resistant cell lines were selected. The total pools of intracellular reduced folates were diminished in the antifolate-resistant cell lines with decreased polyglutamylation. In addition, a marked depletion of folate pools was observed in CEM/PT523 cells with a functionally inactive RFC protein. The increased pool of dihydrofolate in these cells might contribute to their resistance by more effectively competing with PT523 for the DHFR active site. In contrast, the altered RFC transport in CEM/GW70 cells resulted in increased folate pools. As has been demonstrated previously by us and others, expanded intracellular folate pools resulted in resistance to antifolates that are dependent on polyglutamylation due to competition between antifolate drugs and natural folates at the level of FPGS [66,71,72].

The differential effects on the folate status in the antifolate-resistant cell lines can assist to distinguish specific antifolate drugs that retain cytotoxic activity, either at a low or a high folate status. The cell lines with a low FPGS activity and a decreased folate status were collaterally sensitive to the lipophilic DHFR inhibitor TMQ and displayed an almost unaltered sensitivity to the lipophilic TS inhibitor AG337. The increased TMQ sensitivity may be explained by the less effective circumvention of the folate-depleting effect that arises from inhibition of DHFR due to the decreased folate pools [72–74]. On the contrary, under conditions of high intracellular folate pools at least four antifolate drugs, i.e. GW1843U89, ZD9331, AG337 and PT523 retained substantial activity. In view of recent reports that the patient's folate status is known to influence the activity/toxicity of the antifolate MTA [75,76], it may be warranted to explore the clinical potential of antifolates whose pharmacodynamic effects are less affected by variations in folate status among different patients.

Acknowledgments

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