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Induction of resistance to the multitargeted antifolate Pemetrexed (ALIMTA) in WiDr human colon cancer cells is associated with thymidylate synthase overexpression

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Abstract

Pemetrexed (ALIMTA, MTA) is a novel thymidylate synthase (TS) inhibitor and has shown activity against colon cancer, mesothelioma and nonsmall-cell lung cancer. We induced resistance to Pemetrexed in the human colon cancer cell line WiDr by using a continuous exposure to stepwise increasing Pemetrexed concentrations (up to 20 μM) as well as a more clinically relevant schedule with intermittent exposure (up to 50 μM) for 4 hr every 7 days, resulting in WiDr variants WiDr-cPEM and WiDr-4PEM, respectively. However, using the same conditions, it was not possible to induce resistance in the WiDr/F cell line, a variant adapted to growth under low folate conditions. Mechanisms of resistance to Pemetrexed were determined at the level of TS, folylpolyglutamate synthetase (FPGS) and reduced folate carrier (RFC). WiDr-4PEM and WiDr-cPEM showed cross-resistance to the polyglutamatable TS inhibitor Raltitrexed (6- and 19-fold, respectively) and the nonpolyglutamatable TS-inhibitor Thymitaq (6- and 42-fold, respectively) but not to 5-fluorouracil. The ratios of TS mRNA:β actin mRNA in WiDr-4PEM and WiDr-cPEM were 5-fold ($P = 0.01$) and 18-fold ($P = 0.04$) higher, respectively, compared to WiDr (ratio: 0.012). In addition, TS protein expression in the resistant WiDr variants was elevated 3-fold compared to WiDr, while the catalytic activity of TS with 1 μM dUMP increased from 30 pmol/hr/10^6 cells in WiDr cells to 2201 and 7663 pmol/hr/10^6 cells in WiDr-4PEM and WiDr-cPEM, respectively. The activity of FPGS was moderately decreased, but not significantly different in all WiDr variants. Finally, no evidence was found that decreased catalytic activity of RFC was responsible for the obtained Pemetrexed resistance. Altogether, these results indicate that resistance to Pemetrexed in the colon cancer cell line WiDr was solely due to upregulation of TS of which all related parameters (mRNA and protein expression and TS activity) were increased, rather than alterations in FPGS or RFC activity.

Keywords: Antifolate; Colon cancer; Pemetrexed; ALIMTA; Thymidylate synthase; Reduced folate carrier; Folylpolyglutamate synthetase

1. Introduction

The enzyme thymidylate synthase (TS) catalyses the methylation of 2′-deoxyuridine-5′-monophosphate (dUMP) to 2′-deoxythymidine-5′-monophosphate (dTMP), an essential precursor during DNA synthesis [1]. 5,10-Methylene tetrahydrofolate (CH_2-THF) is the limiting methyl donor during this reaction. TS is usually elevated in tumours [2] and is therefore an interesting target for anticancer agents such as the antifolate Pemetrexed (multitargeted antifolate, MTA, ALIMTA, LY231514) (Fig. 1) [3], which inhibits activity of TS by competition with the binding site of CH_2-THF of TS. Other targets of Pemetrexed include glycinamide ribonucleotide formyltransferase (GARFT) which is a

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Abbreviations: Pemetrexed, *N*-[4-(2-(2-amino-4,7-dihydro-4-oxo-3H-pyrrolo[2,3-D]pyrimidin-5-yl)-ethyl)-benzoyl]-L-glutamic acid (LY231514, ALIMTA, MTA, multitargeted antifolate); TS, thymidylate synthase; FPGS, folylpolyglutamate synthetase; RFC, reduced folate carrier; dUMP, 2′-deoxyuridine-5′-monophosphate; dTMP, 2′-deoxythymidine-5′-monophosphate; CH_2-THF, N^5,N^{10}-methylene-5,6,7,8-tetrahydrofolate; GARFT, glycinamide ribonucleotide formyltransferase; DHFR, dihydrofolate reductase; ZD1694, *N*-[5-(*N*-(3,4-dihydro-2-methyl-4-oxoquinazolin-6-yl-methyl)-amino)-2-thenyl]-L-glutamic acid (Raltitrexed, Tomudex); GW1843U89, (*S*)-2-(5-(((1, 2-dihydro-3-methyl-1-oxobenzo(*f*)quinazolin-9-yl)-methyl)-amino)-1-oxo-2-isoindolinyl)-glutaric acid; AG337, 3,4-dihydro-2-amino-6-methyl-4-oxo-5-(4-pyridylthio)-quinazoline (Thymitaq); FPGH, folylpolyglutamate hydrolase.

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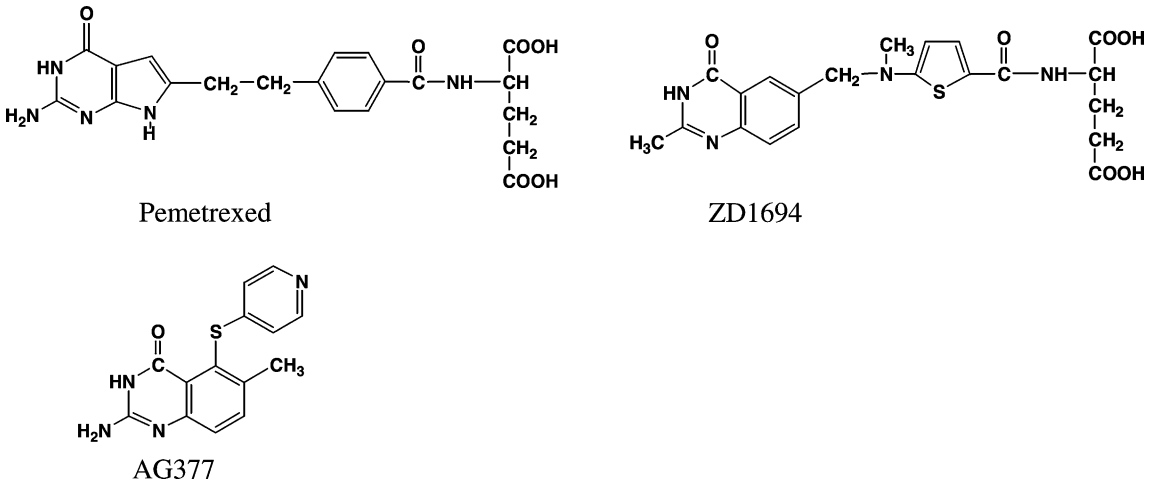

Fig. 1. Chemical structures of Pemetrexed, Tomudex and Thymitaq.

component of the *de novo* purine synthesis pathway, and dihydrofolate reductase (DHFR) which is responsible for recycling of tetrahydrofolates. Pemetrexed is currently being evaluated as an anticancer agent for treatment of colorectal, non-small-cell lung cancer and mesothelioma [4,5]. Resistance to this agent might develop similarly to other antifolates, and might be due to elevated activity or mutations of the target enzyme TS, impaired polyglutamation of antifolates, decreased transport into the cells [3,4], but also apoptosis regulating proteins may be involved [6,7]. Pemetrexed and other clinically active antifolates such as ZD9331 [8], ZD1694 (Tomudex, Raltitrexed) (Fig. 1) [9], GW1843U89 [10] and methotrexate (MTX) [11] require transport into the cell by the reduced folate carrier (RFC). The antifolate AG337 (Thymitaq; Fig. 1) [12,13] enters the cell by passive diffusion, circumventing a possible defect in the function of RFC in cancer cells. Mutated forms of RFC have been detected in cancer, leading to resistance to antifolates [14]. When the antifolates ZD1694, GW1843U89, MTX and Pemetrexed enter the cell, they are polyglutamated by the enzyme folylpolyglutamate synthetase (FPGS) for which most are excellent substrates. This enzyme catalyses the addition of glutamic acid residues to the glutamate residue of antifolates [15]. Polyglutamated antifolates are retained longer in cells, which may enhance antitumour activity. The enzyme folylpolyglutamate hydrolase (FPGH) is responsible for metabolic breakdown of polyglutamates of antifolates leading to decreased retention in cells [16]. The dynamic equilibrium between FPGS and FPGH will determine the polyglutamated form of antifolates [17]. The antifolates AG337 and ZD9331 can not be polyglutamated and may thus be exploited to circumvent resistance to antifolates due to impaired polyglutamation [8,12,18].

Exposure of cancer cells *in vitro* to antifolates has been demonstrated to induce DNA damage, cell cycle arrest and programmed cell death [19]. Furthermore, it is known that antifolates affect the metabolism of methionine. Tetrahydrofolate serves as a methyl donor for synthesis of purines and thymidine as well as in the remethylation cycle of homocysteine to methionine. It has been shown *in vivo* that MTX treatment reduces intracellular pools of 5-methyltetrahydrofolate leading to a reduced conversion of homocysteine to methionine by methionine synthetase [20]. For Pemetrexed, a strong correlation was observed between plasma levels of homocysteine and the subsequent development of serious drug-related toxicities [21]. Decrease of homocysteine levels via nutritional supplementation (folic acid) led to a better safety profile of Pemetrexed.

In several studies on leukemic and solid tumour cell lines, resistance to antifolates including Pemetrexed was frequently associated with either RFC or FPGS but less frequently with TS [22–25]. In H630, HCT-8 and CEM cells, resistance to ZD1694 was associated with a decrease in FPGS activity [22,23,25]. Defective polyglutamation was also shown in Pemetrexed-resistant L1210 and CEM cells [22,24]. Impaired RFC transport as a mechanism of resistance was observed in ZD1694-resistant H630 cells, Pemetrexed-resistant L1210 cells and CEM cells resistant to GW1843U89 or ZD9331 [22,24,25]. Increased expression of TS was only shown in MCF-7 cells resistant to ZD1694 due to a 20-fold gene amplification [25]. Since Pemetrexed is also developed for treatment of colorectal cancer, we characterised the mechanisms underlying resistance in human colon cancer WiDr cells. For this purpose, we induced resistance to the antifolate Pemetrexed in the colon cancer cell line WiDr either by a short exposure to Pemetrexed concentrations every week, or by a continuous exposure to lower concentrations. The resistant WiDr variants were studied on the level of TS, FPGS and RFC.

2. Materials and methods

2.1. Drugs and biochemicals

Dulbecco's Modified Eagle's Medium (DMEM) was obtained from Flow Laboratories (Irvine, Scotland). Both

(nondialysed) fetal calf serum (FCS) and Hank's balanced salt solution (HBSS) were purchased from Gibco Europe. 2-[4-(2-Hydroxyethyl)-1-piperazinyl]-ethanesulfonic acid (HEPES) was obtained from Biowhitaker. 5-FU was provided by Sigma Chemical Co. ZD1694 was obtained from Zeneca Pharmaceuticals (now Astra Zeneca). MTX was from Pharmachemie. Pemetrexed (LY231514, ALIMTA, MTA) was provided by Eli Lilly Research Laboratories, and AG337 (Thymitaq) was a gift from Dr. R.C. Jackson, Agouron Pharmaceuticals (now Pfizer Inc). Bovine Serum Albumin (BSA) fraction V was purchased from Boehringer Mannheim. RNAzol was provided by Campro Scientific. Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV-RT) and RNAse inhibitor (25 IU/μL) were obtained from Promega. Deoxyribonucleotides (dNTPs), random hexamers and Taq polymerase (5IU/μL) were purchased from Pharmacia Biotech. TS primers and β actin primers were provided by Amersham Pharmacia. Acrylamide-bisacrylamide (29:1) was provided by Amresco Research. Hybond-Enhanced ChemoLuminescence (ECL) nitrocellulose membranes, Hyperfilm ECL plus and ECL detection kit were obtained from Amersham International. Polyclonal rabbit antihuman TS (batch R31) was a gift from Dr. G.W. Aherne and the secondary antibody donkey-anti-rabbit-horseradish peroxidase (HRP) was purchased from Amersham Life Science. [6-^{3}H]FdUMP (MT-692; specific activity 19 Ci/mmol) was obtained from Moravek Biochemicals Inc. and [5-^{3}H]dUMP (TRK-287; specific activity 19 Ci/mmol) from Amersham International. DL-Tetrahydrofolic acid was provided by Sigma Chemical Co. Cytidine-5′-monophosphate (·6H_2O, Na) (CMP) was from Merck. Sep-pack C_{18} cartridges were provided by Millipore Waters Associates. L-[2,3-^{3}H]Glu- Ci/mmol; 0.01 N HCl formulation) was obtained from New England Nuclear. [3′,5′,7-^{3}H]MTX (23 Ci/mmol) was obtained from Moravek Biochemicals. Unless otherwise specified, all other chemicals were of analytical grade and commercially available.

2.2. Cell lines

WiDr is a nonclonal solid human colon cancer cell line which originated from a moderately differentiated adenocarcinoma (ATCC CLL218). Resistance to Pemetrexed was induced by a 4-hr exposure every 7 days (starting at 2 μM) and continuous exposure (starting at 0.2 μM) to stepwise increasing concentrations of Pemetrexed for a period of 19 months, resulting in WiDr variants WiDr-4PEM and WiDr-cPEM resistant to 50 and 20 μM, respectively. Cells were maintained under these conditions with similar doubling times of 32 and 33 hr, respectively, compared to 34 hr for parent WiDr cells. On removal of Pemetrexed, the acquired resistance of WiDr cells in culture was stable for at least 3 months. The adherent WiDr cell line and the resistant variants grow as monolayers and were cultured in DMEM, containing 10% FCS and 20 mM HEPES at 37° under an atmosphere of 5% CO_2.

2.3. Growth inhibition studies

Growth inhibitory effects of Pemetrexed, ZD1694, AG337 and 5-FU were evaluated with the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) assay [26] which measures mitochondrial activity. Cells of WiDr variants (5000 cells/well) were exposed to various drug concentrations ranging from 10 nM to 50 μM for 72 hr. Thereafter, medium was removed and cells were incubated for 3 hr at 37° in 50 μL/well MTT (final concentration 0.42 mg/mL). Formazan crystals were dissolved in 150 μL/well dimethylsulfoxide (DMSO) and the optical density was measured at 540 nm by means of an automated spectrophotometric microplatereader (Titertek Multiskan MCC/340 of Tecan Spectrafluor).

2.4. Competitive template RT-PCR to determine TS mRNA expression

RNA was extracted from 5×10^6 cells by the RNAzol method and reversed transcribed by M-MLV-RT and random hexamers as described by the manufacturer with minimal modifications [27]. To ensure accurate quantification of native target (NT), coamplification of competitive templates (CTs) was used. TS expression was normalised to the expression of β actin. PCRs were performed by adding different primer pairs to different aliquots of the same mastermix containing cDNA of cell lines and CT mixture. PCR products (cDNA, CT and heterodimers) were separated on agarose gel and measured by densitometry.

2.5. Western blot analysis

Frozen pellets of WiDr variants were lysed in buffer containing 0.1% Triton X-100, 150 mM NaCl, 10 mM Tris–HCl pH 7.6, and 5 mM EDTA. Protein content was measured using the BioRad assay [28]. For determination of protein expression of TS, 25 μg protein was loaded and separated on a 10% SDS–PAGE gel (acrylamide:bis 29:1), followed by blotting on a nitrocellulose membrane. Antibodies directed to the target are visualised with an HRP-conjugated secondary anti-rabbit antibody by means of ECL(+) detection solution and autoradiography, as described [29].

2.6. TS activity

Pellets were thawed by addition of 1 mL/20×10^6 cells of ice-cold Tris–HCl assay buffer (200 mM, pH 7.4) containing 20 mM β-mercaptoethanol, 100 mM NaF and 15 mM CMP. After sonification (3×5 s with intervals of 10 s) and centrifugation (14,000 *g* for 15 min at 4°),

the supernatant was used for the assays and a sample was taken for measurement of the protein content. The FdUMP binding assay was used to determine the number of free FdUMP binding sites of TS by using [^{3}H]FdUMP as described by Van Triest *et al.* [29].

The TS catalytic assay measured the catalytic activity of TS by means of [^{3}H]H_2O-release during the TS catalysed conversion of [^{3}H]dUMP into dTMP. The activity of TS was measured at a saturating substrate concentration (10 μM dUMP) and at the approximate half-saturating substrate concentration (1 μM dUMP). The enzyme extract of cells was prepared as described in the [^{3}H]FdUMP binding assay. This assay was performed as described previously [29]. For this assay, the assay buffer was supplemented with 1% bovine serum albumin (BSA) to stabilise the TS enzyme. The reaction mixture consisted of 25 μL of enzyme suspensions, 5 μL 6.4 mM CH_2-THF, 10 μL 50 μM or 5 μM [^{3}H]dUMP in a total volume of 50 μL in assay buffer. The reaction vials were incubated at 37°. The reaction was stopped after addition of 50 μL cold TCA 35% and 250 μL 10% activated charchoal and the vials were left for at least 20 min on ice to stabilise the TCA–protein complex. Subsequently, they were centrifuged at 14,000 *g* and 4° for 15 min. Linearity of [^{3}H]dUMP conversion with respect to both the amount of protein and time was established. Thereafter, 150 μL of the supernatant is transferred to 5 mL of LSC fluid and ^{3}H-radioactivity is counted for 5 min.

2.7. FPGS activity

Cell pellets were resuspended in 1 mL/40 × 10^6 cells extraction buffer (50 mM Tris–HCl, 20 mM KCl, 10 mM $MgCl_2$, 5 mM dithiotreitol, pH 7.5) and sonicated (3 × 5 s with intervals of 10 s at 4°) and subsequently centrifuged at 14,000 *g* for 15 min at 4°. The supernatant was transferred to another eppendorf tube and a sample was taken for estimation of protein content. The FPGS assay is based on a reversed phase chromatographic separation of [^{3}H]-Glutamate from polyglutamated substrate via a Sep-pack C_{18} cartridge as described by Jansen *et al.* [30].

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

For measurement of 3 min influx rates of MTX, logarithmically growing WiDr cells were harvested by trypsinisation, washed in HEPES-buffered saline solution (HBSS) pH 7.4 and subsequently resuspended in HBSS buffer at a density of 10^7 cells/mL [22,29]. Thereafter, cells were incubated with [^{3}H]MTX at an extracellular concentration of 2 μM at 37°. After 3-min incubation, MTX transport was stopped by addition of 10 mL of ice-cold HBSS buffer. After a centrifugation (500 *g*, 5 min, 4°) and a washing step with 10 mL ice-cold HBSS, the final cell pellet was resuspended in MQ and radioactivity was measured using LSC.

2.9. Statistical analysis

To analyse differences in levels of TS mRNA, TS protein, activity of TS and FPGS between wild-type WiDr cell line and resistant WiDr variants, the two-tailed unpaired Student's *t*-test was used. Correlative data were analysed using Pearson correlation coefficient. Differences between WiDr variants were considered significant when $P < 0.05$.

3. Results

3.1. Growth inhibition studies

Resistance to Pemetrexed was induced by gradually increasing Pemetrexed concentrations from 2.0 μM for the 4-hr exposure and from 200 nM for the continuous exposure over a period of 19 months resulting in WiDr-4PEM and WiDr-cPEM, respectively. For the WiDr-4PEM cell line, increasing concentrations using steps of 2 μM required 15 months to induce resistance to 10 μM. Thereafter, steps of 20 μM were tolerated to result in resistance to 50 μM which only required 4 months. For WiDr-cPEM, increasing concentrations using steps of 200 nM required 15 months to result in resistance to 1.2 μM. The following concentrations that were used were 1.6, 5 and 10 μM over a period of 4 months, resulting in stable resistance to 10 μM Pemetrexed. For both cell lines, the first step in elevated concentrations required 3 months to result in stability of growth. Simultaneously, we attempted to induce resistance to Pemetrexed in WiDr/F cells, which are adapted to grow in folate-free medium supplemented with 2.5 nM LV [31], which reflects a more physiological folate homeostasis. Concentrations of 1 and 0.1 μM were used for the 4 hr and continuous exposure, respectively. However, we did not manage to increase the concentration of Pemetrexed tolerated by these cells to a similar extent as with WiDr cells. Over a period of 19 months, several attempts were performed to increase tolerance of cells to a higher concentration. However, no viability of cells exposed to concentrations higher than the starting level was obtained in both treatments. The final level of resistance to Pemetrexed of WiDr-4PEM and WiDr-cPEM was 9- and >25-fold, respectively (Table 1). Also using a clonogenic assay [32], a similar pattern of resistance was found. Eventual cross-resistance of the MTA-resistant WiDr variants to antifolates and 5-FU was measured. Sensitivity to ZD1694 in WiDr-4PEM and WiDr-cPEM was 6-fold ($P = 0.024$) and 19-fold ($P = 0.002$) lower, respectively, compared with parental WiDr in which an IC_{50} value of 8 nM was measured. Furthermore, IC_{50} values for AG337 in WiDr-4PEM and WiDr-cPEM were 6-fold ($P = 0.009$) and 42-fold ($P = 0.002$) higher, respectively, when compared to parental WiDr (4840 nM). For 5-FU, no cross-resistance was observed.

Table 1
Sensitivity of WiDr cells and Pemetrexed-resistant sublines to Pemetrexed (PEM), ZD1694, AG337 and 5-FU

Drugs	IC_{50} (nM; 72-hr drug exposure)				
	WiDr	WiDr-4PEM	Resistance factor	WiDr-cPEM	Resistance factor
PEM	393 ± 77	3600 ± 586	9*	>10000	>25*
ZD1694	8 ± 1	51 ± 11	6*	147 ± 9	19**
AG337	4840 ± 392	32333 ± 3930	6**	203333 ± 11667	42**
5-FU	3530 ± 537	5100 ± 1795		3400 ± 208	

Sensitivity to the drugs was determined using the MTT assay with 72-hr exposure to the drugs as described in the Section 2. The fold resistance compared to the parental WiDr is given. Values are means ± SE of at least three separate experiments.
* Significantly different from the parental cell line; $P < 0.05$.
** Significantly different from the parental cell line; $P < 0.01$.

Table 2
Levels of TS, FPGS and RFC in WiDr parental cells and the resistant variants WiDr-4PEM and WiDr-cPEM

	WiDr	WiDr-4PEM	Fold	WiDr-cPEM	Fold
TS protein expression (O.D. × mm^2)	2.1 ± 1.2	6.2 ± 1.0	3.0*	7.1 ± 2.4	3.4*
Ratio TSmRNA/β actin	0.012 ± 0.0038	0.057 ± 0.0067	5*	0.22 ± 0.0586	18*
FdUMP binding sites (fmol/10^6 cells)	74 ± 9	1425 ± 109	19**	4926 ± 639	67*
TS activity (pml/hr/10^6 cells) at 1 μM dUMP	30 ± 3	2201 ± 371	73**	7663 ± 1533	255*
TS activity (pmol/hr/10^6 cells) at 10 μM dUMP	233 ± 51	5811 ± 905	25*	17958 ± 2610	77*
FPGS activity (pmol/hr/10^6 cells)	51 ± 26	33 ± 10		30 ± 15	
RFC activity (pmol/min/10^7 cells)	2.1 ± 0.4	2.3 ± 0.9		2.6 ± 0.4	

TS protein and mRNA expression were measured using Western blotting and competitive template RT-PCR, respectively. TS catalytic activity was measured in presence of 1 and 10 μM dUMP in WiDr variants. RFC activity in WiDr variants was determined with [^{3}H]MTX influx assay as described in Section 2. The fold resistance compared to the parental WiDr is given. Values are means ± SE of at least three separate experiments.
* Significantly different from the parental cell line; $P \leq 0.05$.
** Significantly different from the parental cell line; $P < 0.01$.

3.2. Changes in enzyme levels in MTA-resistant WiDr variants

Since the data on cross-resistance to antifolates indicated that elevated TS expression might be responsible for the acquired resistance to Pemetrexed in the human colon cancer cell line WiDr, we measured expression of TS at the level of mRNA, protein and catalytic activity. TS mRNA expression was determined using CT-RT-PCR (Table 2) and was increased in the Pemetrexed-resistant variants WiDr-4PEM and WiDr-cPEM, from 0.057 and 0.22, respectively, compared to 0.012 in the parental WiDr cells. TS protein expression as measured using Western blot analysis was elevated 3–4-fold compared to WiDr (Fig. 2). Changes in TS protein expression levels were accompanied with differences in the number of FdUMP binding sites and TS catalytic activity (Table 2). At 1 μM dUMP, the TS catalytic activity was 70-fold higher in WiDr-4PEM and approximately 200-fold in WiDr-cPEM compared to parental WiDr cells. In line with these results, TS activity was 25-fold in WiDr-4PEM and 77-fold in WiDr-cPEM higher at the saturating substrate concentration of 10 μM dUMP. The ratio of 10 μM/1 μM dUMP in parent WiDr shifted from 7.8 to 2.6 and 2.3 in WiDr-4PEM and WiDr-cPEM, respectively. These ratios suggest more rapid saturation of TS in the resistant WiDr variants, possibly due to decreased K_m values.

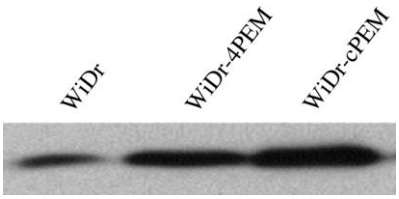

Fig. 2. Upregulation of TS protein in Pemetrexed resistant WiDr variants (WiDr-4PEM and WiDr-cPEM) compared to parent WiDr. Loading of the gels was controlled by staining for β actin, which was comparable for the three cell lines. Experiments were performed at least three times with identical results.

FPGS activity was also determined as a possible resistance factor due to impaired polyglutamation and thus activity of antifolates (Table 2). The FPGS activity was slightly lower in WiDr-4PEM and WiDr-cPEM compared to the parental cell line although not significantly. Impaired RFC transport is another mechanism which contributes to antifolate-resistant phenotypes. To determine whether decreased transport into the cells by the RFC was present in the Pemetrexed-resistant WiDr variants, the activity of RFC was measured by estimation of the [^{3}H]MTX influx rate at a 2-μM extracellular concentration (Table 2). No significant differences were seen in RFC activity between the WiDr variants.

In the WiDr variants, significant correlations were observed between IC_{50} values for MTA and TS expression on the level of mRNA and activity ($R^2 > 0.992$, $P < 0.05$). In addition, a clear correlation between TS mRNA and activity was observed ($R^2 > 0.994$, $P < 0.05$).

4. Discussion

The results in this study indicate that induction of resistance to Pemetrexed in colon carcinoma WiDr cells led to upregulation of TS. All TS related parameters were increased: mRNA and protein expression and TS activity. However, resistance was not related to alterations in the activity of the FPGS or RFC enzymes.

Consistent with the elevated TS activity, Pemetrexed-resistant WiDr variants displayed cross-resistance to other antifolate TS inhibitors such as ZD1694 and AG337. Schultz *et al.* [33] also observed cross-resistance of ZD1694 in induced Pemetrexed-resistant solid tumour and leukemia cell lines. A total period of 19 months was required to develop resistance in the WiDr cell line to 50 and 10 μM for 4 hr and continuous exposure, respectively. This observation was in contrast to the human CCRF-CEM leukemia cell line that was made resistant to a similar Pemetrexed concentration by intermittent exposure in just three cycles of intermittent 24-hr drug exposure, this resistance was >1020-fold [22]. However, the underlying mechanism of Pemetrexed resistance in the leukemia cells was defective polyglutamylation due to a marked decrease in FPGS activity. Development of resistance to ZD1694 in the adherent human ileocecal carcinoma cell line HCT-8/DW2 (300-fold), was also related to a decreased (<90%) FPGS activity and induction required only 2 months [23]. It is possible that induction of antifolate resistance in leukemic cell lines is more easily obtained because of different intrinsic properties of cells. However, induction of antifolate resistance in other solid tumour lines than WiDr to similar antifolate concentrations also required much less time varying from 2 to 4 months [23,25], indicating involvement of cell line specific properties which are responsible for initial impairment of induction of resistance. In addition, it was not possible to induce resistance in WiDr/F cells, which were adapted to grow under more physiologically low folate concentrations. It is, however, also possible that the nature of Pemetrexed, being multitargeted, is a limiting factor in the development of resistance, in contrast to, e.g. ZD1694, which is only directed against TS. Possibly induction of resistance to Pemetrexed in this cell line requires pretreatment with mutagens such as ethylmethane-sulfonate similar to the L1210 cell line as described by Wang *et al.* [24]. In this respect, pretreatment with mutagens facilitated induction of resistance to 5-FU and 5′-deoxy-5-fluorouridine in WiDr cells (unpublished results).

Several mechanisms could be responsible for resistance to Pemetrexed. The cross-resistance to the polyglutamatable ZD1694 and lipophilic nonpolyglutamatable AG377 led us to speculate that TS levels might be increased and that the resistance might not be due to a defect in transport or polyglutamylation. Indeed, TS levels were increased at mRNA, protein and activity level in WiDr-4PEM and WiDr-cPEM compared to WiDr. Previous studies have also revealed TS protein upregulation in H630 colon and MCF-7 breast tumour cell lines resistant to the antifolate ZD1694 [25]. In addition, elevated TS catalytic activity was observed in Pemetrexed-resistant GC3 colon carcinoma cells [33]. TS amplification was present in ZD1694-resistant MCF-7 and Pemetrexed-resistant GC3 tumour cell lines. We wanted to be sure that the increase of TS protein expression was not a transient reaction to Pemetrexed as proposed by the TS autoregulation translation model of Chu *et al.* [34] and which was also observed in the WiDr cell line [35,36]. Therefore, TS levels were also determined in resistant WiDr variants after a drug-free period of a month. Similar TS levels were obtained, which indicated a stable upregulation of TS. It is unlikely that the use of nondialysed FCS is a source for thymidine rescue because of high Thymidine Phosphorylase (TP) levels measured in WiDr cells [37]. In addition, we observed previously that endogenous thymidine levels are depleted rapidly during culturing [38]. Because of a comparable resistance profile of ‘‘pure’’ TS inhibitors, antipurine effects are unlikely to be resistance related.

The increase of active TS protein is probably caused by increased transcription of TS DNA and translation of TS mRNA. The ratios between WiDr-cPEM and WiDr-4PEM are constant (about 3-fold) at the level of mRNA, FdUMP binding sites and TS activity in presence of the saturation level of dUMP (10 μM). This observation was not found for TS protein expression. It might be that saturation levels of autoradiography were exceeded in measurement of TS protein expression. In addition, the expected ratio is observed in measurement of FdUMP binding sites, another indicator for TS protein. In the resistant WiDr variants, it was shown that the upregulation of TS mRNA is higher than the upregulation of TS protein, compared to WiDr. This can be explained by the autoregulation model; when TS protein binds to its own mRNA it inhibits the translation leading to a decrease in protein levels. Furthermore, it should be noticed that kinetics of TS in the resistant variants are different from those of parent WiDr. A higher catalytic activity per mg protein indicates a posttranslational regulation. Genetic polymorphisms probably do not play a role since both in the parent and the resistant variants double repeats of the TS enhancer regions (TSERs) in the 3′UTR of the promotor were present (unpublished data). The Pemetrexed-resistant WiDr cells were not cross-resistant to 5-FU, confirming that in this cell line the main mechanism of action of 5-FU is its incorporation into RNA.

Notably, FPGS was not decreased in contrast to in the human leukemia CEM line with induced resistance to Pemetrexed [22]. Since the activity of FPGS was not related to resistance to Pemetrexed in the resistant WiDr variants, the polyglutamation level of Pemetrexed is unlikely to be a resistance factor because cross-resistance was also found for the nonpolyglutamable antifolate AG337. In addition, in the MTX-resistant cell line CCRF-CEM impaired antifolate transport into cells by RFC has shown

to be a resistance factor [39,40]. However, no differences in the RFC activity were measured.

Taken together, this study demonstrates that increased TS levels are responsible for resistance to the TS based antifolate Pemetrexed in WiDr colon cancer cells. Insight in all these resistance mechanisms of colon cancer cells to antifolates can be used to overcome the problem of resistance and will help to develop new effective chemotherapeutics for treatment of patients with cancer being treated with antifolates.

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