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MECHANISMS OF RESISTANCE TO *N*-[5-[*N*-(3, 4-DIHYDRO-2-METHYL-4-OXOQUINAZOLIN-6-YLMETHYL)-*N*-METHYLAMINO]-2-THENOYL]-L-GLUTAMIC ACID (ZD1694), A FOLATE-BASED THYMIDYLATE SYNTHASE INHIBITOR, IN THE HCT-8 HUMAN ILEOCECAL ADENOCARCINOMA CELL LINE

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Abstract—*N*-[5-[*N*-(3,4-Dihydro-2-methyl-4-oxoquinazolin-6-ylmethyl)-*N*-methylamino]-2-thenoyl]-L-glutamic acid (ZD1694) is a folate-based thymidylate synthase (TS; EC 2.1.1.45) inhibitor. Metabolism to higher chain length polyglutamates is essential for its optimal cytotoxic effect. A ZD1694-resistant (300-fold) human ileocecal carcinoma cell line (HCT-8/DW2) was developed, and its mechanism of resistance was evaluated. TS activities *in situ* and TS protein levels in the HCT-8 parental line and HCT-8/DW2 were similar (168 ± 47 vs 137 ± 25 pmol/hr/10^6 cells and 2.05 ±0.28 vs 2.07 ± 0.19 pmol/mg protein, respectively). The IC_{50} values of ZD1694 for TS inhibition in cell-free extracts were similar in both lines, but the IC_{50} of ZD1694 for TS inhibition *in situ* in HCT-8/DW2 cells was 27- and 268-fold higher than that in HCT-8 cells at 0 and 24 hr, respectively, after a 2-hr drug exposure. Folylpolyglutamate synthetase (FPGS; EC 6.3.2.17) activity was significantly lower in resistant HCT-8/DW2 cells as compared with parental HCT-8 cells (88 ± 40 vs 1065 ± 438 pmol/hr/mg protein when ZD1694 was used as substrate). The combined endogenous pool of methylenetetrahydrofolate and tetrahydrofolate in HCT-8/DW2 cells was also decreased. In addition, HCT-8/DW2 cells accumulated lower levels of methotrexate (MTX) in a 2-hr period, although the initial velocity of MTX transport was similar to that in parental HCT-8 cells. The lower level of FPGS activity and the lower level of (anti)folate accumulation in HCT-8/DW2 correlated with drug resistance and with the higher IC_{50} of ZD1694 for *in situ* TS inhibition. In addition, drug resistance was also correlated with the rapid recovery of *in situ* TS activity after drug treatment. In brief, in this highly ZD1694-resistant HCT-8 cell line, resistance is associated with decreased FPGS activity, which, in turn, affects the metabolism of ZD1694 and consequently the extent and duration of *in situ* TS inhibition by the drug.

Key words: drug resistance; antifolate; thymidylate synthase; folylpolyglutamate synthetase

TS§ catalyzes the *de novo* synthesis of thymidylate from dUMP and the folate cofactor 5,10-CH_2H_4PteGlu. Inhibition of this enzyme leads to the depletion of TTP pools and results in inhibition of DNA synthesis. Preclinical and clinical results obtained with metabolic modulation of 5-FU by LV have demonstrated the important role of TS in cancer chemotherapy [1]. Based on the recognition that TS is an important target in cancer chemotherapy, a number of new and specific antifolate-type TS inhibitors were developed, such as ZD1694, AG-331, LY231514, and 1843U89. Because of their therapeutic activity in preclinical model systems [2–7] and because of the demonstrated activity of ZD1694 in patients with advanced colorectal cancer, these agents may offer therapeutic opportunities over existing treatment.

The TS inhibition and cytotoxic activity of ZD1694 were related to its rapid uptake and intracellular metabolism to higher chain length polyglutamates [8]. Synthetic polyglutamates of ZD1694 were up to 100-fold more potent than the monoglutamate as inhibitors of isolated TS [3]. Several determinants of resistance to ZD1694 have been described [9, 10], including decreased drug uptake by the reduced-folate/MTX carrier, impaired intracellular polyglutamylation, and TS elevation. In general, defective

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§ Abbreviations: 5-FU, 5-fluorouracil; FdUrd, 5-fluoro-2′-deoxyuridine; dUrd, 2′-deoxyuridine; FdUMP, 5-fluoro-2′-deoxyuridine monophosphate; FPGS, folylpolyglutamate synthetase; LV, [6*R*,*S*]-5-formyltetrahydrofolate; MTX, methotrexate; SRB, sulforhodamine B; 5,10-CH_2H_4PteGlu, 5,10-methylenetetrahydrofolate; TS, thymidylate synthase; and ZD1694, *N*-[5-[*N*-(3,4-dihydro-2-methyl-4-oxoquinazolin-6-ylmethyl)-*N*-methylamino]-2-thenoyl]-L-glutamic acid.

membrane uptake of ZD1694 causes cross-resistance to other antifolates that utilize reduced-folate/MTX carrier [9]. Failure to polyglutamylate ZD1694 causes cross-resistance to other TS inhibitors that require metabolism by FPGS for their activation [9, 10].

We describe in this paper the characterization of a ZD1694-resistant HCT-8 subline. This subline was cross-resistant to short-term exposure (2 hr) to folate-based TS inhibitors that require polyglutamylation (e.g. LY231514) for their action, but not to MTX or AG-331. The roles of changes in FPGS activity and alterations of *in situ* TS activity inhibition have been evaluated and emphasized as the central components of resistance mechanisms.

MATERIALS AND METHODS

Chemicals. ZD1694 was supplied by Zeneca Pharmaceuticals (Macclesfield, England), LY231514 by Eli Lilly & Co. (Indianapolis, IN) and AG-331 by Agouron Pharmaceuticals, Inc. (San Diego, CA). MTX, FdUrd, 5-FU, dUrd, LV, H_4PteGlu and SRB were purchased from the Sigma Chemical Co. (St. Louis, MO). [^{3}H]MTX (17 Ci/mmol), [5-^{3}H]dUMP (22 Ci/mmol), [6-^{3}H]FdUMP (24 Ci/mmol) and [5-^{3}H]dUrd (22 Ci/mmol) were purchased from Moravek Biochemicals, Inc. (Brea, CA). L-[2,3-^{3}H]-Glutamic acid (25 Ci/mmol) was obtained from DuPont NEN (Boston, MA). Sephadex G-25 fine was purchased from Pharmacia Biotechnology Inc. (Piscataway, NJ).

Cell culture studies. Mycoplasma-free HCT-8 (ATCC CCL 244) human ileocecal adenocarcinoma cells were maintained in monolayer culture in RPMI 1640 medium containing 10% dialyzed horse serum and 1 mM sodium pyruvate. Cell growth inhibition was measured by the SRB assay as described [11, 12]. For enzyme assays in cell extracts, exponentially growing cells were harvested by trypsin treatment, washed twice with PBS, and frozen as cell pellets at −70°.

Development of the ZD1694-resistant cell line. The ZD1694-resistant HCT-8 subline, HCT-8/DW2, was established by repeated, intermittent exposure to escalating concentrations of ZD1694. HCT-8 cells (1×10^6) were seeded in a 75-cm^2 flask, and 24 hr after seeding the cells were treated with 1 μM ZD1694 for 2 hr. The cells were rinsed twice with medium and allowed to grow in drug-free medium. Eighteen days after exposure to 1 μM ZD1694, the surviving cells in the original 75-cm^2 flask were treated with 5 μM ZD1694 for 2 hr. After 6 days, the surviving cells were treated with 50 μM ZD1694 for 2 hr, and it took 28 days for the surviving cells to grow to confluence. After subculture, 1×10^6 cells were selected once more with 50 μM ZD1694 for 2 hr. The above-established HCT-8/DW2 cells were maintained in drug-free medium, and the resistance was stable for up to 50 passages (≈ 180 generations).

TS enzyme assays. TS catalytic activity and TS protein levels in cell extracts were measured as described by Zhang *et al.* [13]. In brief, a cell pellet corresponding to 2×10^6 cells was extracted with 200 μL extraction buffer (20 mM Tris–HCl, pH 7.5, 250 mM sucrose, 2 mM dithiothreitol, 1.5 mM $MgCl_2$ and 1 mM EDTA) by freezing in ethanol/dry ice and thawing at 37°. TS catalytic activity was measured at 37° by tritium release from [5-^{3}H]dUMP as [^{3}H]-H_2O in a 210-μL reaction mixture containing 75 μM [5-^{3}H]dUMP, 750 μM (6*R*,*S*)-H_4PteGlu and 20 mM HCHO. The reaction was stopped at different times (10–60 min) by mixing a 60-μL aliquot of the reaction mixture with 200 μL of 10% (w/v) charcoal in 2% trichloroacetic acid, and [^{3}H]H_2O was separated from adsorbed [5-^{3}H]dUMP by centrifugation at 8800 *g* for 15 min. Radioactivity in a 100-μL aliquot of supernatant was quantitated in 4 mL of Ecoscint A scintillation fluid (National Diagnostics) using a Beckman LS 1701 liquid scintillation counter. Enzyme activity is expressed as picomoles of ^{3}H released per minute per milligram of protein. Assay of the TS protein level was based on quantitating the stoichiometric formation of covalent ternary complexes of TS, [6-^{3}H]FdUMP and 5,10-CH_2H_4PteGlu. The mixtures (200 μL) containing cell extract, 0.12 μM (6*R*,*S*)-5,10-CH_2H_4PteGlu and 0.165 μM [6-^{3}H]FdUMP were incubated at 25° for 3 hr. Samples (50 μL) were mixed with 10 μL of 6% SDS and boiled for 3 min. Ternary complexes were separated from unreacted [6-^{3}H]FdUMP by centrifuging 25 μL of boiled sample through a Sephadex G-25 mini-column (400 μL). The radioactivity in the filtrate was quantitated. TS protein levels are expressed as picomoles per milligram of protein. Calculations of the data are based on the assumption that 1.7 mol of FdUMP are bound per mol of TS. Protein was determined with the Bio-Rad protein assay kit using bovine serum albumin as a standard.

In situ TS activity (in intact cells) was measured by the release of tritium from [5-^{3}H]dUMP (anabolized from [5-^{3}H]dUrd added to the growth medium) as described by Yalowich and Kalman [14] with modifications. HCT-8 cells were plated into T-25 Falcon flasks at a density of 5×10^5 cells/flask and incubated overnight for attachment before the *in situ* TS activity assay. Culture medium was aspirated, and 0.5 mL of pre-warmed (37°) reaction medium containing 1 μM [5-^{3}H]dUrd (1.25 μCi/mL) was added. After incubation at 37° for 15 min, the reaction was terminated by inserting the flask into ice for 30 sec and mixing a 200-μL aliquot of medium with 200 μL of 15% charcoal (in 4% trichloroacetic acid). The charcoal mixture was centrifuged at 8800 *g* for 15 min, and 200 μL of supernatant was removed and the radioactivity quantitated as described above. Attached cells in the flask were trypsinized and counted with a ZBI Coulter counter. *In situ* TS enzyme activity is expressed as picomoles [^{3}H]H_2O released per hour per 10^6 cells.

Thymidylate synthase inhibition by ZD1694. TS activity inhibition by ZD1694 was studied *in situ* and in cell extracts. For studies of TS inhibition *in situ*, 5×10^5 HCT-8 cells were plated in T-25 flasks and allowed to attach for 24 hr and then treated with different concentrations of ZD1694 for 2 hr. TS activity in intact cells with or without ZD1694 treatment was assayed (as above) at different times following drug removal. For study of TS inhibition in cell extracts, 10^7 cells were extracted in 1 mL of extraction buffer, and the cell extracts were passed through Sephadex G-25 columns to remove endogenous nucleotides. TS activity inhibition was

assayed with equal amounts of TS activity ($[^3H]H_2O$ release: 1 pmol/min) from extracts of HCT-8 or HCT-8/DW2 cells, in the presence of 60 μM [^{3}H]-dUMP, 100 μM (6*R*,*S*)-5,10-CH_2H_4PteGlu and various concentrations of ZD1694. From ZD1694 concentration (logarithm)–TS activity curves, IC_{50} values for TS activity inhibition were obtained as the concentrations of ZD1694 producing 50% of inhibition of TS activity *in situ* or in cell extracts.

FPGS activity assay. FPGS activity in crude cell extracts was analyzed [15] using 4 μM ZD1694 or 200 μM MTX as substrate.

Analysis of FPGS mRNA expression by northern blot. HCT-8 and HCT-8/DW2 cells were washed three times with PBS and harvested from 225-cm^2 tissue culture flasks with a rubber policeman. Total cellular RNA was extracted using the guanidine thiocyanate method [16] and purified by centrifugation through a cushion of 5.7 M CsCl [17]. For northern blot analysis, total cellular RNA was fractionated on a 1.2% agarose gel containing 2.2 M formaldehyde and transferred to a Hybond N membrane as described by Dolnick [18]. The cDNA of human FPGS was from Dr. Shane [19] obtained through Dr. Dolnick in this department. The β-actin cDNA was generated by polymerase chain reaction as previously described [20]. The levels of mRNA were determined by densitometric scanning of the autoradiographs.

5,10-$CH_2H_4PteGlu_n$/$H_4PteGlu_n$ level and polyglutamate distribution assay. The method was based on the stoichiometric formation of a covalent ternary complex between $CH_2H_4PteGlu_n$, [6-^{3}H]FdUMP and *Lactobacillus casei* TS as described [21]. Cells (2×10^6) were extracted with 200 μL of reagent mixture containing 65 mU/mL *L. casei* TS, 0.165 μM [6-^{3}H]FdUMP, 6.5 mM formaldehyde, 50 mM sodium ascorbate, 213 mM sucrose, 1 mM disodium-EDTA, and 50 mM Tris-HCl, pH 7.5. Cell lysates were centrifuged at 8800 *g* for 15 min, and the supernatants were incubated at 30° for 1 hr. The combined 5,10-$CH_2H_4PteGlu_n$ and $H_4PteGlu_n$ pool was determined by quantitating ternary complex formation as described in the TS level assay. To determine polyglutamate chain length distribution within the pool, undenatured ternary complexes were resolved on nondenaturing discontinuous polyacrylamide slab gels (9% polyacrylamide for separating gel and 4.5% polyacrylamide for stacking gel). After electrophoresis, the gel was fixed with 10% acetic acid/30% methanol and impregnated with EN^{3}HANCE (DuPont). The dried gel was exposed to Kodak X-ray film, and the polyglutamate chain length was read from the autoradiograph with reference to the migration of ternary complex standards (5,10-$CH_2H_4PteGlu_{1-7}$). The relative level was quantitated by densitometric scanning.

MTX transport study. The method was adopted from Pizzorno *et al.* [22] with modifications [23]. Cells were plated at 2×10^5 cells/well into 24-well plates in folate-free medium at 37° for 24 hr before MTX transport studies. Culture medium was aspirated and uptake was initiated by adding 1 mL of pre-warmed (37°) folate-free RPMI 1640 medium containing 1 μM [^{3}H]MTX (0.5 μCi/mL). After incubation at 37°, uptake was stopped by putting the plate on ice and washing the cells quickly with 5 mL of ice-cold PBS (pH 7.4). To remove cell surface bound [^{3}H]MTX, cells were further rinsed with 5 mL of ice-cold saline (pH 3.0 adjusted with acetic acid) for 1 min on ice and washed once with PBS. Cells in each well were lysed with 0.25 mL of 1 N NaOH for 1 hr and neutralized with 0.25 mL of 1 N HCl. Radioactivity in cell lysates was quantitated as described above. Cell number per well was determined by counting trypsinized cells in three representative wells with a ZBI Coulter counter. Non-specific uptake of [^{3}H]MTX was determined by adding 1000-fold excess unlabeled MTX (1 mM) in uptake medium at each time point and was subtracted from the total uptake.

RESULTS

Growth inhibition. The *in vitro* response of HCT-8 and HCT-8/DW2 cells to a 2-hr exposure of ZD1694 was evaluated (Fig. 1). The concentration–response curve of HCT-8 cells was steeper than that observed with HCT-8/DW2 with an IC_{50} (μM) of 0.08 ± 0.001 and 23.7 ± 7.7, respectively. The data in Table 1 indicate that although HCT-8/DW2 cells were also resistant to 72 hr continuous ZD1694 exposure, the relative degree of resistance was lower, 66- vs 296-fold in the 2-hr exposure. In addition, *in vitro* potency of ZD1694 was increased significantly with prolonged cellular exposure to the drug. Table 1 summarizes the *in vitro* sensitivities of the HCT-8 and HCT-8/DW2 cells to other TS inhibitors and to MTX. In either a 2-hr or 72-hr exposure, HCT-8/DW2 cells were cross-resistant to LY231514, but not to MTX, AG-331, 5-FdUrd or 5-FU.

TS inhibition: Extent. HCT-8 and HCT-8/DW2 cells showed similar TS protein levels and enzyme activity in cell extracts (2.07 ± 0.32 vs 2.05 ± 0.28 pmol/mg protein and 53.4 ± 15 vs 38.9 ± 9 pmol/min/mg protein, respectively; mean ± SD, N = 3), similar TS activity *in situ* (168 ± 47 vs 137 ± 25 pmol/hr/10^6 cells; mean ± SD, N = 3), and similar TS mRNA levels (data not shown). Inhibition of TS activity by ZD1694 *in situ* and in a cell-free system of HCT-8 and HCT-8/DW2 cells was evaluated (Table 2). Before assay of TS inhibition *in situ*, cells were treated with ZD1694 for 2 hr and then incubated in drug-free medium for 0 or 24 hr. Immediately after 2 hr of ZD1694 treatment, HCT-8/DW2 cells showed an approximately 27-fold higher concentration requirement for ZD1694 to achieve the same extent of inhibition of TS activity as HCT-8 cells (Table 2). At 24 hr post 2 hr ZD1694 exposure, both HCT-8/DW2 and HCT-8 cells required higher drug concentrations than at 0 hr to inhibit TS by 50% (Table 2), but the relative resistance of TS inhibition in HCT-8/DW2 was much higher than that at 0 hr post-treatment (268-fold resistance vs 27-fold). Notably, the IC_{50} concentration of ZD1694 for TS activity inhibition *in situ* right after 2 hr of exposure was subcytotoxic and was lower than the IC_{50} concentration required for growth inhibition after the same 2-hr treatment in HCT-8 and HCT-8/DW2 cells, respectively (comparing the IC_{50} concentration in Table 2 with growth inhibition concentrations in Fig. 1). The relative degree of

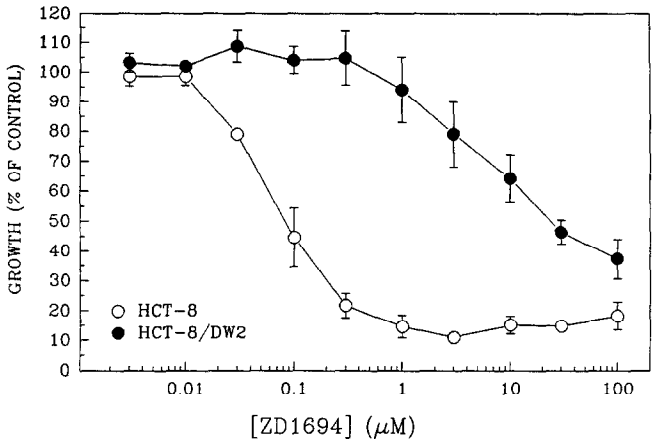

Fig. 1. Growth inhibition of HCT-8 and HCT-8/DW2 cells treated with ZD1694. Exponentially growing cells were treated with ZD1694 for 2 hr and then allowed to grow for an additional 72 hr in drug-free medium. Growth was assessed by the SRB total protein assay. Data represent the mean ± SD of three separate experiments.

Table 1. Growth inhibition (IC_{50}) of HCT-8 and HCT-8/DW2 cells by ZD1694, LY231514, MTX, AG-331, 5-FdUrd and 5-FU

	IC_{50} (μM)					
	2-hr Exposure			72-hr Exposure		
Drug	HCT-8	HCT-8/DW2	RDR*	HCT-8	HCT-8/DW2	RDR
ZD1694	0.08 ± 0.01†	23.7 ± 7.7	296	0.003 ± 0.001	0.2 ± 0.21	66
LY231514	2.0 ± 1.1	207 ± 13	103	0.03 ± 0.0045	1.8 ± 0.4	60
MTX	0.1 ± 0.02	0.15 ± 0.08	1.5	0.001 ± 0.0001	0.001 ± 0.0001	1.0
AG-331	20.3 ± 3.1	22 ± 2	1.1	1.4 ± 0.2	1.67 ± 0.41	1.2
5-FdUrd	0.11 ± 0.08	0.17 ± 0.07	1.5	0.003 ± 0.0004	0.005 ± 0.002	1.7
5-FU	156 ± 39	166 ± 50	1.0	3.6 ± 0.5	5.3 ± 0.3	1.5

IC_{50}, concentration that produces 50% inhibition of cell growth.
* RDR, relative degree of resistance (ratio of IC_{50} of HCT-8/DW2 to IC_{50} of HCT-8).
† Values are means ± SD of three experiments.

Table 2. ZD1694 concentration required for 50% inhibition of TS activity *in situ* and in a cell-free system

	IC_{50} (μM) for TS activity inhibition		
Cell line	*In situ* (0 hr)*	*In situ* (24 hr)*	In cell-free extract†
HCT-8	0.01 ± 0.001‡	0.066 ± 0.01	1.9 ± 0.8
HCT-8/DW2	0.27 ± 0.08	17.7 ± 11.5	1.8 ± 0.5
Fold of resistance	27	268	1.0

* Cells were incubated in ZD1694 for 2 hr and subsequently in drug-free medium for 0 hr and 24 hr before the *in situ* TS activity assay.
† Extracts of untreated cells were assayed for TS activity in the presence of ZD1694.
‡ Data represent means ± SD (N = 3).

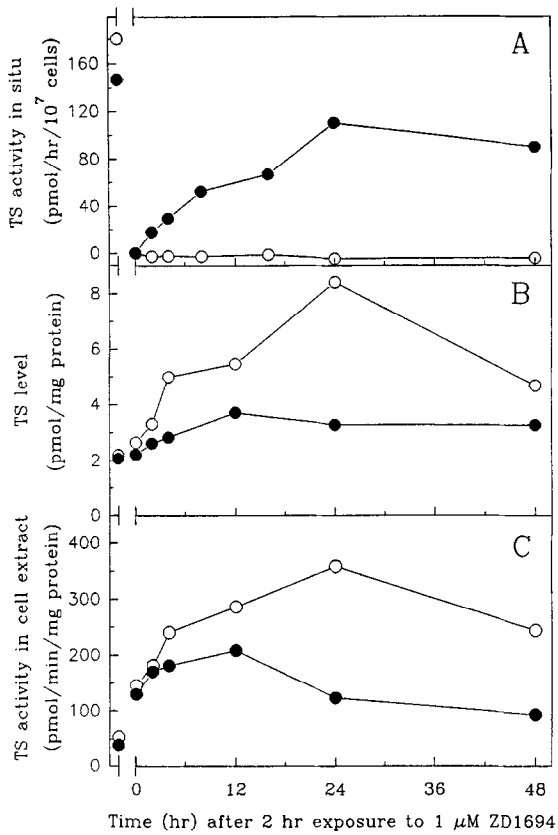

Fig. 2. Time courses of *in situ* TS activity inhibition (A), TS level (B) and TS activity (C) in cell-free extracts of HCT-8 (○) and HCT-8/DW2 (●) cells after a 2-hr exposure to 1 μM ZD1694. Data represent the averages of two experiments. Separated symbols represent respective control data of untreated cells.

resistance of TS activity inhibition *in situ* at 24 hr after 2 hr of ZD1694 exposure (268-fold) was comparable to that for cell growth inhibition (296-fold, Table 1).

TS inhibition: Duration. Since the data in Table 2 show that the relative degree of resistance to TS inhibition in HCT-8/DW2 cells was higher at 24 hr post 2-hr exposure than at 0 hr post exposure, there seemed to be a difference in the duration of TS inhibition by ZD1694 between HCT-8 and HCT-8/DW2 cells. We used an equimolar concentration of ZD1694 (1 μM, an IC_{90} for HCT-8 and an IC_{10} for HCT-8/DW2 cells) to study the duration of TS inhibition *in situ* by 2-hr drug exposure. Under the same treatment, the time courses of TS activity and TS protein level in cell-free systems were also evaluated. The results are summarized in Fig. 2. While TS activity *in situ* of both cell lines was inhibited by > 98% immediately after the 2-hr drug exposure, persistent TS inhibition was observed in HCT-8 cells for up to 48 hr post drug treatment (Fig. 2A). In contrast, as compared with untreated control cells, 75% of TS activity was recovered in HCT-8/DW2 at 24 hr post drug treatment. The data in Fig. 2B demonstrate an increase in TS protein level after ZD1694 treatment. In HCT-8 cells, the TS protein level increased by about 4-fold at 24 hr, while TS activity *in situ* remained inhibited; in HCT-8/DW2 cells, the TS protein level increase was moderate (less than 2-fold) while TS activity *in situ* was recovering until 24 hr post drug treatment. Increased TS activity in cell extracts following drug treatment was also observed in HCT-8 and HCT-8/DW2 cells (Fig. 2C), and the magnitude and duration of the increase paralleled the time course of TS level changes in each cell line (compare panels C and B of Fig. 2). The growth rate of HCT-8/DW2 cells was lowered slightly, while complete inhibition of growth was observed in HCT-8 cells at 16 hr after drug treatment (data not shown).

Table 3. Folylpolyglutamate synthetase activity and mRNA expression in HCT-8 and HCT-8/DW2 cells

Cell line	FPGS activity (pmol/hr/mg protein) ZD1694	MTX	FPGS mRNA/ β-actin mRNA
HCT-8	1065 ± 438*	1354 ± 78	100
HCT-8/DW2	88 ± 40	82 ± 12	87

* Data represent means ± SD of two experiments with duplicates.

FPGS activity. FPGS activity was measured in crude extracts of HCT-8 and HCT-8/DW2 cells (Table 3). The specific activity of FPGS in extracts of HCT-8/DW2 cells was 8% of the value in HCT-8 cells, using either 4 μM ZD1694 or 200 μM MTX as substrate. FPGS mRNA levels were similar in both cell lines (Table 3).

5,10-$CH_2H_4PteGlu_n$/$H_4PteGlu_n$ pools and polyglutamate chain length distribution. The endogenous combined intracellular pools of $CH_2H_4PteGlu_n$ and $H_4PteGlu_n$ were measured in HCT-8 and HCT-8/DW2 cells maintained in culture medium containing 2.3 μM folic acid. Table 4 shows that the endogenous $CH_2H_4PteGlu_n/H_4PteGlu_n$ pool in HCT-8/DW2 cells was about 36% of that in HCT-8 cells. The presence of 20 μM LV in the culture medium for 24 hr expanded the endogenous $CH_2H_4PteGlu_n/H_4PteGlu_n$ pools in both cell lines, but HCT-8/DW2 cells showed only about 50% of the pool level of HCT-8. The polyglutamate chain length distribution within these pools was measured. In culture medium with 2.3 μM folic acid, the predominant polyglutamates of $CH_2H_4PteGlu_n/H_4PteGlu_n$ contained five and six glutamate residues in both cell lines, but in HCT-8/DW2 cells there was an obvious shift in chain length from six glutamates to five glutamates as compared with HCT-8 cells. In the presence of 20 μM LV for 24 hr, the chain lengths of polyglutamates shortened, with predominant polyglutamates of four and five glutamate residues in both cell lines. However, as compared with HCT-8 cells, HCT-8/DW2 cells showed a chain length shift from five glutamates to three glutamates.

Table 4. Endogenous and LV expanded $H_4PteGlu_n/CH_2H_4PteGlu_n$ pools and their polyglutamate chain length distribution in HCT-8 and HCT-8/DW2 cells

Cell line	$H_4PteGlu_n/CH_2H_4PteGlu_n$ (%)						Total pool (pmol/mg protein)
	n = 1–2	n = 3	n = 4	n = 5	n = 6	n = 7	
HCT-8	0	1	3	49	43	4	19.2 ± 5.1*
HCT-8/DW2	0	1	6	67	25	1	6.8 ± 1.5
HCT-8 + LV†	0	2	33	58	7	0	68.8 ± 9.4
HCT-8/DW2 + LV†	0	18	38	38	6	0	32.8 ± 2.3

* Data represent means ± SD (N = 3).

† Cells were cultured for 24 hr in the presence of 20 μM LV and then collected for the assay of $H_4PteGlu_n/CH_2H_4PteGlu_n$ pool level and polyglutamate chain length distribution.

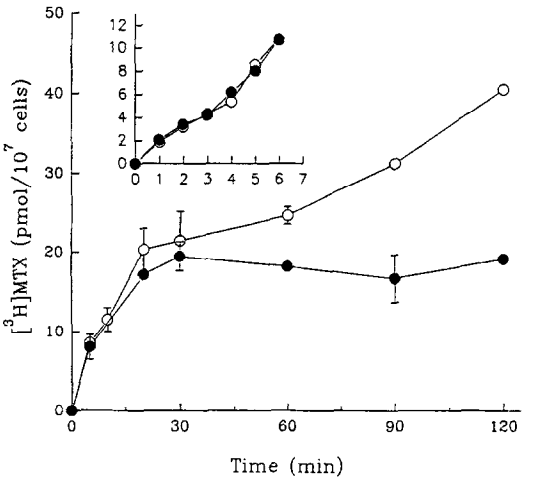

Fig. 3. Uptake of MTX in HCT-8 (○) and HCT-8/DW2 (●) cells. Cells were plated in folate-free medium for 24 hr and then incubated in folate-free medium containing 1 μM [^{3}H]MTX for various times; intracellular [^{3}H]MTX content was determined as described in Materials and Methods. The inset represents the initial rates of MTX uptake during the first 6 min. Data represent means ± SD of two experiments with duplicates.

Cellular accumulation of MTX. ZD1694 utilizes both the MTX/reduced folate carrier and membrane folate binding protein as routes of cellular uptake [24]. To determine whether the resistance to ZD1694 was a consequence of defective (anti)folate transport systems, the uptake and intracellular accumulation of [^{3}H]MTX in resistant HCT-8/DW2 and parental HCT-8 cells were measured. The initial rates of MTX uptake into HCT-8/DW2 and HCT-8 cells (Fig. 3, inset) were 1.67 ± 0.35 and 1.57 ± 0.26 pmol/min/10^7 cells (N = 2), respectively. The uptake reached a steady-state value of about 19 pmol/10^7 cells in resistant HCT-8/DW2 cells between 30 and 120 min. In contrast, a time-dependent increase in [^{3}H]MTX cellular accumulation was observed in HCT-8 cells, reaching a level of about 40 pmol/10^7 cells at 120 min.

DISCUSSION

The present study demonstrated that resistance to ZD1694 in HCT-8/DW2 is associated with decreased FPGS activity. Since polyglutamylation of ZD1694 increases its TS binding affinity and intracellular drug retention [2], decreased FPGS activity in HCT-8/DW2 cells is likely to be responsible for resistance. The results obtained with uptake of MTX (Fig. 3) and the data of 5,10-$CH_2H_4PteGlu_n/H_4PteGlu_n$ pools and polyglutamate chain length distribution (Table 4) support this hypothesis. Since the initial rates of MTX uptake (≤ 6 min) were similar in resistant and parental cell lines (Fig. 3), impaired membrane uptake appeared to be ruled out as a mechanism of ZD1694 resistance in HCT-8/DW2. The lower accumulation of MTX observed in HCT-8/DW2 relative to HCT-8 cells after a 2-hr exposure (Fig. 3) probably reflects the impaired intracellular retention of the drug secondary to the FPGS deficiency. Furthermore, FPGS-deficient HCT-8/DW2 cells showed a lower pool of $CH_2H_4PteGlu_n/H_4PteGlu_n$ relative to HCT-8 cells in the absence or presence of 20 μM LV and a down-shift of polyglutamate chain length distribution (Table 4). The FPGS mRNA level in HCT-8/DW2 was comparable to that in the parental cell line.

Further supporting evidence for the implication of FPGS in ZD1694 resistance comes from the TS activity inhibition studies. TS in cell-free extracts from HCT-8 and HCT-8/DW2 cells showed similar sensitivity to ZD1694 (Table 2). However, to achieve the same TS activity inhibition *in situ*, FPGS-deficient HCT-8/DW2 cells required up to 100 times more ZD1694 than parental HCT-8 cells (Table 2). At an equimolar concentration of ZD1694 (1 μM), > 98% inhibition of TS activity *in situ* was observed in both cell lines immediately after a 2-hr drug exposure, but the duration of TS inhibition was different in HCT-8 and HCT-8/DW2 (Fig. 2A). Persistent inhibition of TS activity in HCT-8 cells led to complete inhibition of cell growth, while the rapid recovery of TS activity in HCT-8/DW2 cells was associated only with slightly lowered growth rate. The decrease in the extent and duration of TS inhibition *in situ* by ZD1694 in HCT-8/DW2 cells can be the effects of FPGS deficiency on polyglutamylation and intracellular retention of the

drug. Quantitative determination of the extent and/or duration of *in situ* TS inhibition required to induce cell death is currently under study.

A rapid increase in TS protein level has been demonstrated in normal and tumor cells after treatment with ZD1694 [25] and 5-FU [26–29]. In this report (Fig. 2B), the extent of increase in TS protein level after ZD1694 treatment was not quantitatively related to the recovery of *in situ* TS activity. In HCT-8 cells, the TS level increased by about 4-fold, whereas TS activity *in situ* was inhibited completely. In contrast, in HCT-8/DW2 cells, the less significant increase (less than 2-fold) in TS protein level was accompanied by rapid recovery of TS activity *in situ*. Therefore, the extent of acute TS increase itself is not a determinant of drug resistance in this case, since the increased TS can be associated with drug and cannot exert its enzyme activity *in situ*. The recovery of TS activity *in situ* in HCT-8/DW2 cells following > 98% inhibition by ZD1694 treatment could be due to rapid dissociation of drug–TS complex and/or to new synthesis of TS. Since the FPGS activity in HCT-8/DW2 cells is relatively low, rapid recovery of TS activity *in situ* is likely due to more rapid dissociation of TS from hypopolyglutamylated ZD1694 and increased efflux of nonpolyglutamylated drug from the cells. The other possible contribution to rapid recovery of TS activity *in situ* in HCT-8/DW2 is newly synthesized TS, since the TS level was also elevated in HCT-8/DW2 cells after ZD1694 treatment. However, the acute increase of TS is inherent to the translational regulation of TS synthesis by TS inhibitors or substrates [30], and the contribution of increased TS to *in situ* TS activity recovery is dependent on its potential to increase the amount of free TS, which, in turn, is determined by intracellular drug availability and drug–TS interaction.

The data reported herein demonstrated that: (1) in ZD1694-sensitive cells, sustained complete inhibition of TS activity *in situ* is accompanied by a significant increase in TS level; (2) resistance to ZD1694 is associated with decreased FPGS activity and, consequently, with rapid recovery from TS inhibition; (3) the degree of TS inhibition immediately after drug treatment cannot predict the response to ZD1694, because TS inhibition can be achieved with subcytotoxic concentrations of the drug and the duration of TS inhibition depends on polyglutamylation and long-term retention of the drug; in contrast, the relative extent of TS activity inhibition *in situ* at 24 hr post drug treatment correlated well with relative *in vitro* drug sensitivity; and (4) the extent of TS level elevation after ZD1694 treatment is not an indicator of drug resistance *in vitro*. These data indicate that when TS activity is measured in clinical material as a determinant of response to TS inhibitors, duration of enzyme inhibition must be measured. Information gained on enzyme inhibition immediately after i.v. push drug administration could be of limited value.

Cross-resistance studies by growth assay (Table 1) and colony-forming assay (data not shown) revealed that the HCT-8/DW2 subline was not cross-resistant to MTX, a dihydrofolate reductase inhibitor to which drug resistance can also be associated with FPGS deficiency [22, 31–35]. The polyglutamates of MTX do not show enhanced affinity for isolated dihydrofolate reductase [36], but they are preferentially retained by cells. The mechanism of the lack of cross-resistance to MTX in FPGS-deficient HCT-8/DW2 cells during a 2-hr exposure is not clear to us. Recently, ZD1694-resistant/MTX-sensitive human leukemia MOLT-3 sublines were described [37], and these sublines had an apparent decrease in the long-term accumulation of ZD1694 without change of uptake rate [38]. In our study, HCT-8/DW2 cells similarly showed a decrease in 2-hr accumulation of MTX even though there was no difference in initial uptake rate (Fig. 3). HCT-8/DW2 cells also had a decrease in the reduced folate pool (Table 4). Furthermore, immediately after the 2-hr MTX exposure, TS inhibition *in situ* was similar in HCT-8 and HCT-8/DW2 cells (data not shown). Therefore, the intracellular ratio of reduced folate to MTX may play a role in MTX drug action and cytotoxicity. The mechanism underlying the lack of cross-resistance to MTX in the HCT-8/DW2 subline is now under further study.

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