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Cross-Resistance to Antifolates in Multidrug Resistant Cell Lines with P-glycoprotein or Multidrug Resistance Protein Expression

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ABSTRACT. Resistance to some (lipophilic) antifolates has been associated with P-glycoprotein (P-gp)-mediated multidrug resistance (MDR). A possible relationship with non-P-gp MDR has not been established. We studied resistance to antifolates in SW-1573 human lung carcinoma cells, a P-gp overexpressing variant SW-1573/2R160 and a multidrug resistance protein (MRP) overexpressing variant SW-1573/2R120. In this study, thymidylate synthase (TS) inhibitors with different properties concerning the efficiency of membrane transport and the efficiency of polyglutamylation were tested for cross-resistance in SW-1573/2R120 and SW-1573/2R160 cells. Growth inhibition patterns in this cell line panel were measured by the Sulforhodamine B (SRB) assay. Resistance factors for TS inhibitors were: 2.4 and 0.4 for 5-fluorouracil (5FU), 18.8 and 8.8 for ZD1694, 17 and 0.7 for AG337, and 40 and 8.3 for BW1843U89 in SW-1573/2R160 and SW-1573/2R120, respectively. This study showed changes in the TS enzyme kinetics during the induction of doxorubicin resistance in both SW-1573 variants, resulting in 2-fold lower K_m values for 2′-deoxyuridine-5′-monophosphate (dUMP) in both resistant variants compared to the parental cell line. TS activity, TS protein induction and TS mRNA expression all had 2-fold increased in the SW-1573/2R120 compared to the SW-1573/2R160. ^{3}H-MTX influx was 2-fold lower in SW-1573/2R160 cells compared to SW-1573/2R120 and SW-1573 cells. In the SW-1573/2R160 cell line, an aberrant intracellular trafficking towards the target TS was observed, compared to SW-1573/2R120 and SW-1573 cells as measured by the TS in situ assay. The rate of TS inhibition by the TS inhibitors used in this study was similar in all cell lines. In conclusion, collateral sensitivity to 5FU and the lipophilic AG337 and cross-resistance to other antifolates were observed in non-P-gp MDR SW-1573/2R120 cells, as well as resistance to all antifolates in P-gp SW-1573/2R160 cells. The mechanism of resistance in SW-1573/2R160 cells possibly involves reduced influx and changes in intracellular trafficking routes. For the SW-1573/2R120 cell line, several changes related to the TS enzyme possibly play a role in the observed cross-resistance and collateral sensitivity pattern. BIOCHEM PHARMACOL 53;12:1855–1866, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. thymidylate synthase; 5-fluorouracil; antifolates; P-glycoprotein; multidrug resistance protein; lung resistance protein

Drug resistance is an important clinical problem in the treatment of cancer. In general, several mechanisms for drug resistance have been described at different cellular levels: the cell membrane (decreased drug uptake, increased drug efflux), drug metabolism (decreased drug activation, increased drug inactivation), the drug target level (decreased formation of drug-target complexes) and, finally, enhanced DNA repair mechanisms [1]. Specific mechanisms of resistance to 5-fluorouracil (5FU) are associated with the target enzyme thymidylate synthase (TS), a *de novo* enzyme in the pyrimidine nucleotide synthesis: aberrant enzyme kinetics, increased 2′-deoxyuridine-5′-monophosphate (dUMP) levels, decreased 5-fluoro-2′-deoxyuridine-5′-monophosphate (FdUMP) levels, depletion of intracellular folates, decreased polyglutamylation of folates and gene amplification, but also decreased incorporation into RNA [2]. Mechanisms of resistance to folate antagonists include transport defects, impaired polyglutamylation and increased activity or altered properties of the target enzymes dihydrofolate reductase (DHFR) and TS [2, 3, 4]. These resistance mechanisms for antimetabolites in gen-

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Abbreviations: 5FU, 5-fluorouracil; TS, thymidylate synthase; FdUMP, 5-fluoro-2′-deoxyuridine-5′-monophosphate; dUMP, 2′-deoxyuridine-5′-monophosphate; dTMP, 2′-deoxythymidine-5′-monophosphate; P-gp, P-glycoprotein; MDR, multidrug resistance; MRP, multidrug resistance protein; LRP, lung resistance protein; FPGS, folylpolyglutamate synthetase; FPGH, folylpolyglutamate hydrolase; DHFR, dihydrofolate reductase; DDATHF, 5,10-dideaza-5,6,7,8-tetrahydrofolic acid; MTX, methotrexate; RT-PCR, reverse transcriptase polymerase chain reaction.

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eral, and antifolates in particular, appear to be different from drug resistance associated with the multidrug resistance (MDR) phenotype. Classical MDR has been defined as cross-resistance to structurally and mechanistically unrelated antineoplastic drugs, e.g. anthracyclines and taxol which have different targets [5], resulting from decreased drug accumulation. Antimetabolites usually do not display MDR characteristics. Besides MDR, the well-known mechanism of P-glycoprotein (P-gp), another member of the ATP-binding cassette superfamily of transporter proteins, may contribute to the MDR phenotype, i.e. the multidrug resistance protein (MRP) [6, 7]. The elucidation of the clinical role of MDR remains an issue of continuing research [8].

5FU is one of the most important antimetabolites in the clinic and is active either by incorporation into RNA, incorporation into DNA or inhibition of the enzyme TS, by its metabolite FdUMP [9]. TS is the rate-limiting enzyme in the *de novo* synthesis of 2′-deoxythymidine-5′-monophosphate (dTMP) and its inhibition by FdUMP has been associated with response to 5FU therapy [10, 11, 12]. More recently, specific folate-based TS inhibitors, such as ZD1694 [13], BW1843U89 [14] and AG337 [15, 16] have been developed [17]. All of these antifolates have different properties concerning the efficiency of transport across the cell membrane and the efficiency of intracellular polyglutamylation. ZD1694 (Tomudex) and BW1843U89 require the reduced folate carrier for cellular uptake whereas they are good substrates for folylpolyglutamate synthetase (FPGS). AG337 is a lipophilic compound which is transported by passive diffusion and is not polyglutamylated.

Chu *et al.* [18] and Zhang *et al.* [19] reported cross-resistance to 5FU in MDR human breast and human colon cell lines. Both studies exhibited that an elevation in TS activity was responsible for the cross-resistance in the MDR cell lines without making a distinction between P-gp-mediated or non-P-gp-mediated MDR. Since several tumors (e.g. colon, liver, pancreas) frequently treated with 5FU display a high P-gp expression, we were interested in a possible effect of the MDR phenotype on 5FU and antifolate sensitivity [20].

For that purpose, we investigated whether induction of resistance to doxorubicin also has implications for the sensitivity pattern for 5FU and folate-based TS inhibitors in P-gp and MRP MDR cells. In order to study this phenomenon, we used MDR cell lines derived from the same parent non-small cell lung carcinoma cell line SW-1573 by exposure to stepwise increasing concentrations of doxorubicin, the SW-1573/2R160[1] variant which expresses P-gp and the SW-1573/2R120 variant with a MDR phenotype independent of P-gp, so-called non-P-gp MDR [21]. In the 2R120 cell line MRP overexpression was detected by immunocytochemical staining [22, 23]. Functionally, the overexpression of MRP can have consequences for transport of several drugs across the cell membrane [7, 24]. The lung resistance protein (LRP) is also overexpressed in the 2R120 cell line as measured by immunocytochemistry [25]. The 110 kilodalton LRP protein was shown to be a human major vault protein [26] with contribution to the MDR phenotype by cytoplasmic redistribution and nucleocytoplasmic transport of drugs [27]. In contrast, the 2R160 cell line has a classical MDR phenotype resulting from overexpression of P-gp, a plasma membrane glycoprotein of 170 kilodalton which acts as an energy-dependent efflux pump leading to a decreased intracellular drug accumulation [28]. Not only does the overexpression of different proteins make this cell line panel of interest, but the difference in the level of resistance is also of importance. The 2R120 cells are low resistant to doxorubicin compared to the highly resistant 2R160 cells. One can speculate about the implications of differences in resistance levels for relevance in the clinic.

In this study, we demonstrated that the 2R120 cells showed a mixed pattern of collateral sensitivity to 5FU and AG337 and cross-resistance to ZD1694 and BW1843U89 which could be related to factors at the level of TS resistance. The 2R160 cells were resistant to all compounds. The latter could be partially associated with limited drug transport across the cell membrane or intracellular transport.

MATERIAL AND METHODS

Drugs and Chemicals

5FU was obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). The antifolates used in this study were generously provided by the following persons/institutions: ZD1694 (Tomudex; Dr. F. T. Boyle, Zeneca Pharmaceuticals, Macclesfield, U.K.), ZD1694 pentaglutamate (Dr. A. L. Jackman, Cancer Research Campaign, Sutton, U.K.), BW1843U89 (Dr. B. Ferone from the Glaxo/Welcome Company), AG337 (Dr. R. C. Jackson, Agouron Pharmaceuticals, San Diego, CA, U.S.A.), Methotrexate (MTX) (Pharmachemic, Haarlem, The Netherlands), MTX-Glu_2 (Schricks Company, Iona, Switzerland), 5,10-dideaza-5,6,7,8-tetrahydrofolic acid (DDATHF) (the late Dr. G. B. Grindey from the Lilly Research Laboratories in Indianapolis, IN, U.S.A.) and doxorubicin hydrochloride (Laboratoire Roger Bellon, Neuilly sur Seine, France). Dulbecco's modified Eagle's medium was purchased from Flow Laboratories, Irvine, Scotland and foetal calf serum (FCS) from GIBCO (Paisley, U.K.). The 96-well flat bottom plates were purchased from Greiner Labortechnik (Solingen, Germany). [6-^{3}H]-FdUMP (MT-692, specific activity 19 Ci/mmol), [5-^{3}H]-2′-deoxyuridine (MT-648, specific activity 22 Ci/mmol) and [3′,5′,7′-^{3}H]-MTX (MT-701, specific activity 23 Ci/mmol) were obtained from Moravek Biochemicals Inc. (Brea, CA, U.S.A.), [5-^{3}H]-dUMP (TRK-287, specific activity 19 Ci/mmol) was from Amersham International (Buckinghamshire, U.K.), *l*-[U-^{14}C]-glutamic acid (NEC-290E, 296 mCi/mmol) from Du Pont NEN Research products (Boston, MA, U.S.A.). *dl*-Tetrahydrofo-

[1] In this report, SW-1573/2R120 and SW-1573/2R160, the SW-1573 doxorubicin resistant variants, are referred to as 2R120 and 2R160.

lic acid (Sigma Chemical Co, St. Louis, MO, U.S.A.) was converted to 5,10-methylenetetrahydrofolic acid (CH_2-THF) by addition of formaldehyde [29]. HPLC analysis of MTX was performed on a Partisphere SAX anion exchange column (Whatman Ltd., Maidstone, England) with a length of 110 mm, internal diameter of 4.7 mm and particle size of 5 μm. The liquid scintillation fluid Ultima Gold was from Packard, Tilburg, the Netherlands. RNAzol^{tm}B was obtained from Cinna/Biotecx Laboratories, Inc. The Bio-Rad protein assay was used for protein determination [30]. Unless otherwise specified, all other chemicals were of the highest quality grade available.

Cell Culture

The human non-small cell lung tumor cell line SW-1573 was originally isolated and characterized by Dr. A. Leibovitz (Scott and White Clinic, Temple, TX, U.S.A.). The two resistant sublines were selected by increasing the doxorubicin concentrations in a stepwise fashion. This resulted in a P-gp expressing MDR cell line 2R160 and a non-P-gp MDR cell line 2R120. Extensive characterisation of the parental and resistant sublines has been published [21, 22, 23, 25, 31]. Cell doubling times were 23, 42 and 30 hr for the SW-1573, 2R120 and 2R160 cell lines, respectively. Cells were routinely cultured in Dulbecco's modified Eagle's medium supplemented with 7.5% foetal calf serum, 2 mM L-glutamine and doxorubicin at 120 nM and 160 nM for the 2R120 and 2R160 cells, respectively. All experiments were carried out after a doxorubicin-free period of at least 7 days.

Growth Inhibition Studies

Cells in a subconfluent phase were transferred to 96-well flat bottom plates. SW-1573 cells were plated at a density of 8,000 cells/well in 100 μL medium, while 2R120 and 2R160 cells were plated at a density of 15,000 cells/well. Twenty-four hr later (day 0), 100 μL drug-containing medium was added. Drug exposure time was 72 hr (day 3). The drugs were tested in triplicate in different concentration ranges. Growth inhibitory effects were evaluated with the standard sulforhodamine B (SRB) assay [32, 33]. The IC_{50} was the drug concentration at which cell growth is 50% inhibited based on the difference of optical density values on day 0 and day 3 of drug exposure. The resistance factor is the IC_{50} value of the resistant cell line divided by the IC_{50} value of the parent cell line.

Thymidylate Synthase Assays
FdUMP Binding Assay

The binding assay with [6-^{3}H]-FdUMP as a ligand to determine the number of free FdUMP binding sites of TS was carried out as described previously [34, 35]. Briefly, the reaction mixture contained 50 μL enzyme suspension from 14000 *g* supernatants, 50 μL 6.5 mM CH_2-THF, 135 μL Tris-HCl buffer (200 mM, pH 7.4) and was started by adding 15 μL 570 nM [6-^{3}H]-FdUMP (specific activity 19 Ci/mmol), incubated at 37°C for 1 hr and stopped by adding 500 μL 10% neutralized activated charcoal (to remove free FdUMP). After centrifugation radioactivity was estimated by liquid scintillation counting of 250 μL supernatant.

TS Catalytic Assay

This assay determines the catalytic activity of TS by means of the ^{3}H-release during the TS catalyzed conversion of [5-^{3}H]-dUMP to TMP [36]. Briefly, the assay consisted of 25 μL enzyme suspension (in different dilutions), 5 μL 6.5 mM CH_2-THF, and 10 μL TRIS-HCl buffer (pH 7.4). The reaction was started by adding 10 μL [5-^{3}H]-dUMP (1 or 10 μM final concentration, specific activity 50 mCi/mol and 5 mCi/mol, respectively), incubated for 30 min at 37°C and stopped by adding 50 μL ice-cold 35% trichloroacetic acid and 250 μL 10% neutral activated charcoal. After centrifugation, 150 μL of the supernatant was collected and counted by liquid scintillation.

The potency of TS inhibition by the specific TS inhibitors was measured by adding appropriate concentrations of FdUMP, ZD1694, ZD1694 pentaglutamate, AG337 or BW1843U89. With purified human TS, we previously established inhibition of TS by FdUMP according to Lineweaver–Burk, demonstrating competitive inhibition of TS by FdUMP (data not shown). Since Dixon plots gave similar results, we applied this to calculate enzyme kinetic parameters when appropriate. The following final drug concentrations were chosen: 0.01 μM FdUMP, 10 μM ZD1694, 0.2 μM ZD1694 pentaglutamate, 0.01 μM AG337 or 0.001 μM BW1843U89.

TS in situ Assay

This assay was based on the assay as described previously by Yalowich and Kalman [37] and gives a qualitative indication of the potential intracellular inhibition of TS intracellularly. TS activity in intact cells was measured after a short-term drug exposure (4 hr) and a long-term drug exposure (24 hr): the retention of the TS inhibition was measured after a 4 hr drug exposure followed by a 3 hr drug-free period. For this purpose, $0.4 \cdot 10^6$ cells were plated in 6-well plates (Costar, Cambridge, U.K.) and after a recovery period of 24 hr exposed to the drugs (5FU, ZD1694, AG337) at different concentrations. The TS activity was determined by exposure to 2.5 μM [5-^{3}H]-2′-deoxyuridine (specific activity 0.16 μCi/nmol) during the last 2 hr of the assay, and medium was taken off and processed as described for the catalytic assay.

Western Blot Analysis

SW-1573, 2R120 and 2R160 cells were harvested during the logarithmic growth phase, washed, and centrifuged, and the pellet was immediately frozen in liquid nitrogen. Sam-

ples were kept at −80°C. Treated samples were from cells exposed to the drugs for 24 hr at their IC_{50} concentration. Before the Western blot analysis, cells were resuspended in Tris-HCl buffer (50 mM, pH 7.4) and sonicated three times for 5 sec at 10 sec intervals at 4°C. From each sample, an equivalent amount of protein was processed by a 12% SDS-polyacrylamide gel electrophoresis followed by electroblotting onto nitrocellulose PVDF membranes. Human recombinant TS (partially purified from the TS overexpressing lymphoblastoid cell line, W1L2:C1, kindly provided by Dr. A.L. Jackman, Sutton, U.K.) was applied to each gel to verify the TS position on the gel. Thereafter, the membranes were blocked with blocking buffer for 2 hr followed by incubation with the primary antibody rabbit-anti-human TS (1/3000 dilution in blocking buffer, kindly provided by Dr. G. W. Aherne, Sutton, U.K.) [38]. Subsequently, the blot was incubated with the secondary antibody (1/300 dilution), peroxidase-conjugated goat-anti-rabbit antibody. Finally, the blot was stained using 3-amino-9-ethylcarbazole. The density of the bands was quantified using a densitometric scanner.

Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

The PCR procedure was performed on the basis of the semi-quantitative method described by Horikoshi *et al.* [39]. RNA isolation of the cell lines was performed with RNAzol™B. Thereafter, cDNA was synthesized by reverse transcriptase by the use of random hexamers. Different cDNA concentrations of the samples prepared by serial dilutions in sterile water were used in the PCR assay. The PCR was carried out in a thermal cycler (Omnigene, Thermal cycler Master, HB-TR3-CM, Biozym, Landgraaf, The Netherlands). β-actin was used as an internal standard to quantify the TS mRNA and had to be used at 100 to 1000-fold higher dilutions. The sequences of the TS and β-actin primers were the same as described by Horikoshi *et al.* [39]. The PCR cycles were 1 min at 94°C, 1 min at 57°C and 1 min at 72°C. After completion of 35 PCR cycles, the tubes were incubated for 1 min at 72°C. The PCR procedure was followed by electrophoresis of the PCR products on a 16% agarose gel containing ethidium bromide. Quantification was performed by densitometric scanning. The slope of the linear region of TS cDNA dilutions was compared to that of the β-actin cDNA dilutions and used to calculate the relative TS mRNA expression. RNA samples without reverse transcriptase processing were used as a control for chromosomal DNA interference. In our hands, with this cell line panel, amplification of the signal by transcription with radioactive [α-^{32}P]CTP [39] or the use of a radiolabelled nucleotide [α-^{32}P]dATP [40] was not necessary.

FPGS Assay

The FPGS assay was performed as described by Van der Wilt *et al.* [41]. Briefly, the cell pellets were resuspended in ice-cold Tris/HCl buffer (200 mM, pH 8.5), supplemented with 30 mM $NaHCO_3$ and 45 mM β-mercaptoethanol at a concentration of 20.10^6 cells/mL and lysed using sonication (3 times 5 sec, at 10 sec intervals) followed by centrifugation (7000 *g*, 15 min). FPGS activity was assayed in the presence of 1 mM *l*-[U-^{14}C]-glutamate (0.4 μCi/mmol), 5 mM MgATP and 500 μM aminopterin as the antifolate substrate. In contrast to the other cell lines tested [41], the assay did not appear to be linear over a 30 min period and much shorter incubation times (15 min at 37°C) had to be used. The reaction was terminated by denaturation of the enzyme by heating the samples at 95°C for 3 min. After chilling on ice, the samples were centrifuged to precipitate denatured proteins. Product and substrates were separated after spotting onto PEI-cellulose TLC sheets; 0.5% (w/v) NH_4Cl and 0.5% (v/v) β-mercaptoethanol were used as eluents. Spots of AMT-[^{14}C]-Glu_2 (R_f = 0) and unreacted [^{14}C]-Glu (R_f = 0.6) were cut out and radioactivity was counted.

FPGH Assay

Folylpolyglutamate hydrolase (FPGH) was assayed by measuring the conversion of the substrate MTX-Glu_2 to MTX-Glu_1 in a 60 min assay, as described by O'Connor *et al.* [42]. Briefly, cells were harvested during logarithmic growth. The cells were extracted by sonication (3 times 5 sec, at 10 sec intervals) with a Tris/HCl buffer (100 mM, pH 6.9) and then centrifuged (14000 *g*, 15 min). The reaction was initiated by adding 20 μL MTX-Glu_2 (100 μM final concentration) to the supernatant containing 25 μg protein, bringing the total reaction volume to 200 μL. MTX-Glu_2 breakdown was linear for at least one hour at 37°C; blanks were kept at 4°C. The reaction was stopped by boiling for 3 min followed by chilling on ice and centrifugation. The substrate and product were separated using HPLC with an anion exchange column, essentially as described previously [43] with KH_2PO_4 and 20% MeOH (pH 4.9) as a buffer. UV detection at 306 nm was used for identification and quantification. The FPGH activity in the cell extracts was defined as nmol of MTX-Glu_1 produced per 10^6 cells per hour.

[^{3}H]-MTX Uptake

[^{3}H]-MTX uptake was performed as described by Van der Laan *et al.* [44]. The cells were harvested in the logarithmic growth phase by trypsinization and resuspended in a HEPES balanced saline solution (HBSS) containing 107 mM NaCl, 20 mM HEPES, 26.2 mM $NaHCO_3$, 5.3 mM KCl, 1.9 mM $CaCl_2$, 1.0 mM $MgCl_2$, 7 mM D-glucose and adjusted to pH 7.4 with NaOH. Influx was determined over an incubation period of 3 or 10 min at 37°C with 2 μM [^{3}H]-MTX (0.5 Ci/mmol). Uptake of [^{3}H]-MTX at 4°C served as a control. Retention of MTX was determined after a 1 hr drug free period. The reaction was stopped by adding 9 volumes ice-cold HBSS. Cells were centrifuged and

TABLE 1. Growth inhibitory effects of 5FU, various antifolates and doxorubicin in MDR cell lines

	IC_{50} (μM)	Resistance factors		
Drug	SW-1573	SW-1573	2R120 MRP	2R160 P-gp
5FU	3.7 ± 0.65	1	0.4 *	2.4 *
AG337	4.5 ± 0.46	1	0.7	17 *
BW1843	0.004 ± 0.001	1	8.3	40 *
ZD1694	0.036 ± 0.003	1	8.8 *	19 *
MTX	0.052 ± 0.005	1	23 *	6.4 *
DDATHF	0.52 ± 0.17	1	2.4 *	1.8
DOX	0.069 ± 0.001	1	8.9 *	73 *

The cell lines were exposed to the drugs for 72 hr. IC_{50} values (means ± SE) are from at least 3 separate experiments performed in triplicate and are statistically significant ($P < 0.05$) when * is indicated. Resistance factors were calculated for each separate experiment.

washed with 10 mL ice-cold HBSS. The final cell pellet was resuspended in 0.5 mL water and the amount of ^{3}H-MTX counted with liquid scintillation.

Cell Cycle Analysis

SW-1573, 2R120 and 2R160 cells were harvested during the logarithmic growth phase, washed and centrifuged. Fixation of the cells was performed by resuspending the pellets of $0.5 \cdot 10^6$ cells in 1 mL methanol and maintaining them at 4°C. For analysis the cells were centrifuged and 30 μL phosphate-buffered saline was added to the pellet. The DNA was stained with propidium iodide following a standard procedure described by Vindelov *et al.* [45]. Human lymphocytes were used as a standard control. DNA-cell-cycle distribution analysis was performed on a FACSSTAR (Becton Dickinson Medical Systems, Sharon, MA, U.S.A.). The results were analyzed by the Cell Fit program (Becton Dickinson).

RESULTS

Growth Inhibition Studies

The growth inhibitory effects (IC_{50} values and resistance factors) of doxorubicin and of the panel of antifolate drugs against the SW-1573, 2R120 and 2R160 cell lines are shown in Table 1. 2R120 and 2R160 cells were 9-fold and 73-fold resistant to doxorubicin as compared to the parental cells, respectively. These results are in agreement with data reported by Kuiper *et al.* [21]. The experiments with 5FU revealed a 2.4 cross-resistance in the 2R160 cell line, but a 2.5-fold enhanced sensitivity in the 2R120 cells. The lipophilic compound AG337 also showed a 17-fold cross-resistance in the 2R160 cells, but a collateral sensitivity in the 2R120 cell line. Reversal experiments with 32 μM verapamil to block the P-gp-mediated efflux could not reverse the resistance to AG337 in the 2R160 cell line (data not shown). Both 2R120 and 2R160 cells showed cross-resistance to BW1843U89: 8.5- and 40-fold, respectively. For ZD1694, the same cross-resistance pattern in these MDR cell lines was observed as for BW1843U89. For comparison, growth inhibition experiments with two non-TS-directed antifolates were included: MTX, an inhibitor of the enzyme DHFR, and DDATHF, a specific inhibitor of GAR transformylase which inhibits purine *de novo* synthesis [4, 46]. Both MTX and DDATHF showed resistance in the 2R120 and 2R160 cell lines. The growth inhibition experiments with MTX showed a higher degree of resistance in 2R120 cells (23-fold) than in 2R160 cells (6.4-fold). For DDATHF, a minor cross-resistance factor was noted for 2R120 and 2R160 cells (2.4- and 1.8-fold, respectively).

TS Levels
FdUMP Binding and TS Catalytic Assay

Since TS is a known target for FdUMP, one of the metabolites of 5FU, and for some of the antifolates tested (BW1843U89, ZD1694 and AG337), we determined whether differences in TS levels could explain the observed cross-resistance pattern. TS levels were measured by an FdUMP binding assay and TS catalytic assay. TS catalytic activity (conversion of dUMP to dTMP) was slightly higher in the 2R120 cell line than in the parental cell line SW-1573 at a substrate concentration of 10 μM dUMP. At physiological 1 μM dUMP, both 2R120 and 2R160 cells showed a 2-fold increase in TS catalytic activity (Table 2). The change in ratio between TS activity at 10 and 1 μM dUMP in 2R120 and 2R160 cells in comparison with the parental cell line indicates a change in kinetic properties of the enzyme after the induction of resistance by doxorubicin in the resistant cell lines. Another indication of kinetic changes is the difference in K_m values for dUMP, which are 2- to 3-fold higher in the parental compared to the 2R120 and 2R160 cell lines (Table 2). FdUMP binding was somewhat higher in the 2R120 cell line compared to the wild type and 2R160 cell lines; comparable FdUMP binding was found in 2R160 cells and the parental cell line (Table 2). Thus, changes in enzyme levels and enzyme kinetics occurred during the process of resistance induction to doxorubicin in both SW-1573 variants, although the changes were not identical in the 2R120 and 2R160 cells.

TABLE 2. TS levels in MDR cell lines as measured with various TS assays

Assay	SW-1573	2R120 MRP	2R160 P-gp
Catalytic activity (pmol/hr/10^6 cells)			
1 μM dUMP	27 ± 1.7	79 ± 12*	61 ± 3*
10 μM dUMP	209 ± 2	253 ± 32	202 ± 25
ratio 10 μM/1 μM	8	3	3
K_m dUMP (μM)	14.8 ± 2.4	7.1 ± 1.3*	5.9 ± 1.0*
FdUMP binding (fmol/10^6 cells)	328 ± 84	477 ± 80	237 ± 63
TS expression ratio TS/β-actin ($\cdot 10^{-3}$)	12.2 ± 2.5	23.2 ± 3.9	8.5 ± 1.7

Results are means ± SE of three experiments. Protein content of the cells was approximately 100 μg/10^6 cells. In the RT-PCR assay, β-actin was used as an internal standard and the TS mRNA is expressed relatively to the β-actin expression. Statistical significance ($P < 0.05$) is indicated by *.

TS mRNA Expression Measured by RT-PCR

Since TS gene amplification has been described as a mechanism of resistance for 5FU and antifolates, we also determined whether the cross-resistance pattern shown in this cell line panel could be explained by the degree of TS gene expression. TS mRNA expression was measured by a semi-quantitative RT-PCR assay. TS mRNA expression, depicted as a ratio of TS mRNA/β-actin mRNA, was approximately 2-fold higher, although not significantly, in the 2R120 cell line than in the parental and 2R160 cell lines (Table 2).

Western Immunoblot Analysis

Since both 5FU and ZD1694 have been reported to induce overexpression of TS, we determined whether the effect of treatment with 5FU and folate-based TS inhibitors (ZD1694, AG337) on the TS protein level were similar in each cell line (Table 3). Treatment with 5FU increased TS protein level only in the 2R120 cells. ZD1694 increased TS protein in all cell lines, the enhancement being most pronounced in the 2R120 cell line. In contrast, AG337 decreased TS protein levels in the parental cell line and failed to affect TS levels in 2R160 cells, while the TS protein level increased in the 2R120 cells. Thus, with all compounds the TS induction was more pronounced in the 2R120 cell line.

TABLE 3. Western immunoblot analysis of TS after treatment with 5FU and the folate-based TS inhibitors ZD1694 and AG337

Drug	SW-1573	2R120 MRP	2R160 P-gp
5FU	100 ± 2	155 ± 27*	111 ± 29
ZD1694	143 ± 9	336 ± 25*	148 ± 14*
AG337	70 ± 8	189 ± 28*	96 ± 9

The amount of protein was related to that of the untreated cells and depicted in percentage. The values are the means ± SE of at least three experiments and statistically significant ($P < 0.05$) when indicated with *. The cells were exposed to the drugs for 24 hr at IC_{50} concentration. Quantitation was performed by densitometric scanning. In untreated cells, protein levels were comparable for all three cell lines (data not shown).

DNA Distribution Analysis

Since TS has been reported to be cell-cycle associated and able to be influenced by TS inhibitors [47], we determined the effect of treatment with 5FU, ZD1694 and AG337 at their IC_{50} concentration on the cell-cycle distribution of asynchronised cells. These experiments showed a similar S-phase distribution in all three untreated cell lines but a lower G_2/M distribution (Table 4). Exposure to 5FU and ZD1694 decreased the S-phase percentage in the parental and 2R120 cell lines, resulting in a higher G_1/G_0-phase. Exposure to AG337 decreased S-phase distribution in both MDR cell lines, but not in the parent cell line. Thus, drug-induced effects on DNA distribution were most pronounced in the 2R120 cells.

FPGS Activity

Since all antifolates used in this study, except for AG337, are dependent on polyglutamylation for their biological activity, and decreased FPGS activity is associated with resistance to antifolates, we determined FPGS activity in

TABLE 4. DNA distribution analysis and drug-induced effects in MDR cell lines

	G_1-phase %	S-phase %	G_2/M-phase %
SW-1573			
Control	52 ± 7.5	28 ± 6.9	20 ± 4.0
5FU	67 ± 2.9	14 ± 2.3	19 ± 2.9
ZD1694	63 ± 3.5	20 ± 6.9	16 ± 7.5
AG337	50 ± 9.8	34 ± 1.2	16 ± 8.7
2R120 (MRP)			
Control	57 ± 5.2	35 ± 3.5	8 ± 4.0
5FU	68 ± 3.5	24 ± 1.2	8 ± 2.3
ZD1694	69 ± 1.2	23 ± 4.6	8 ± 3.5
AG337	65 ± 9.2	27 ± 8.7	8 ± 1.2
2R160 (P-gp)			
Control	54 ± 5.6	27 ± 4.6	19 ± 2.9
5FU	51 ± 5.6	31 ± 5.2	18 ± 1.7
ZD1694	53 ± 9.2	28 ± 13.2	19 ± 5.2
AG337	57 ± 8.1	21 ± 10.4	22 ± 1.7

Values are means ± SE of at least three separate experiments. Cells were exposed to 5FU, ZD1694 and AG337 for 24 hr at IC_{50} concentration.

TABLE 5. FPGS and FPGH activity in MDR cell lines

Enzyme	SW-1573	2R120 MRP	2R160 P-gp
FPGS	1510 ± 83	2046 ± 39*	2687 ± 208*
FPGH	1700 ± 300	1300 ± 350	1300 ± 300

The activities are given in pmol/10^6 cells/hr. Values are means ± SE of at least three experiments and statistically significant ($P < 0.05$) when indicated with *.

the cell line panel. A 1.3- and 1.8-fold higher level of FPGS activity was measured in the 2R120 and 2R160 cell lines, respectively, compared to the parental cell line (Table 5).

FPGH Activity

The polyglutamylated status of antifolates is an equilibrium between synthesis by FPGS and catabolism by FPGH. Resistance to antifolates can be correlated with an increase in FPGH activity. Therefore, FPGH activity was measured. The FPGH level was not significantly altered in 2R120 and 2R160 cells (Table 5).

^{3}H-MTX *Transport*

Since resistance to antifolates can be caused by defects in transport across the cell membrane, we determined folate in- and efflux by using [^{3}H]-MTX, one of the best substrates for the reduced folate carrier (Table 6). The influx of [^{3}H]-MTX was decreased in the 2R160 cell line in comparison with the parental and 2R120 cell lines. The retention of [^{3}H]-MTX in the cells was similar for all cell lines. The addition of 32 μM verapamil, which blocks P-gp-mediated drug efflux, did not affect the retained levels of MTX in the three cell lines (data not shown).

TS *Inhibition Experiments*

Since potential differences in TS inhibition by TS inhibitors could be related to the differences in drug sensitivity to the antifolates tested, we determined inhibition of TS by these compounds in cellular extracts. TS inhibition was measured at 1 μM or 10 μM dUMP concentration as shown in Fig. 1. Since FdUMP is a competitive inhibitor with regard to dUMP, inhibition by FdUMP was more pronounced at 1 μM dUMP than at the saturated concentration of 10 μM dUMP. K_i values for FdUMP as determined by Dixon plots were: 10.5 ± 2.2, 4.5 ± 2.5 and 4.2 ± 3.2 nM FdUMP for the parental, 2R120 and 2R160 cell lines, respectively. Besides FdUMP, other TS inhibitors were used in these experiments at concentrations specified in Fig. 1. For all these antifolates, more TS inhibition at 10 μM dUMP was observed. As expected, the ZD1694 pentaglutamate was a better inhibitor than the ZD1694 monoglutamate. AG337, BW1843U89 and ZD1694 showed the same pattern of inhibition in all cell lines. However, BW1843U89 was a relatively better TS inhibitor in 2R120 and 2R160 cells compared to the parental cell line. Using this approach for the antifolates, no K_i values could be determined, since both dUMP and the antifolates have different binding sites at the TS enzyme.

TABLE 6. ^{3}H-MTX influx and retention in MDR cell lines

^{3}H-MTX	SW-1573	2R120 MRP	2R160 P-gp
Influx	0.91 ± 0.08	0.90 ± 0.02	0.42 ± 0.06*
Retention	29% ± 1.45	32% ± 5.9	29% ± 2.4

Values (means ± SE) are of 10 min ^{3}H-MTX uptake of at least three experiments. Influx values in pmol/min/10^6 cells. The retention of ^{3}H-MTX was measured after a drug-free period of 1 hr and is the % of radioactivity retained in the cell after 1 hr. Statistical significance ($P < 0.05$) is indicated by *.

TS *in situ Experiments*

In order to obtain additional information on the interaction of TS inhibitors with TS, we measured the effect of AG337, ZD1694 and 5FU on TS activity in intact cells using the TS *in situ* assay in addition to the catalytic TS assay in cell extracts (Fig. 2). The TS *in situ* assay is semi-quantitative and provides more information concerning limitations of membrane transport, intracellular trafficking and metabolism to other active forms before the drug reaches the target, TS. This was studied by using different drug exposure times (4 vs 24 hr) and by measuring the retention of TS inhibition after 4 hr drug exposure followed by a 3 hr drug-free period. For 5FU, no clear differences between the cell lines could be observed, neither after 4 and 24 hr exposure nor for the retention of TS inhibition. In all cell lines, inhibition after 4 hr exposure was very poor, whereas only 30% of TS activity was uninhibited following 24 hr incubation with 5FU; no retention of TS inhibition was observed. For ZD1694, a completely different pattern was manifest. In the 2R160 cells, the drug could not reach the target TS adequately to inhibit TS completely, resulting in only 40% TS inhibition after 4 hr ZD1694 exposure and no complete inhibition after 24 hr drug exposure. In contrast, the parent and 2R120 cells showed a complete TS inhibition after 24 hr ZD1694 exposure. However, AG337, a lipophilic compound, showed less inhibition in the TS *in situ* assay in the 2R160 cells compared to the 2R120 and parental cell lines (Figure 2). After 24 hr drug exposure, only 30% of the TS enzyme was inhibited by AG337 in the 2R160 cell line, whereas the enzyme was almost completely inhibited in the other cell lines. In all cell lines, recovery of TS inhibition by AG337 was complete and comparable with the retention after 5FU treatment. The TS *in situ* experiments showed clear differences between the 2R160 cells, and the 2R120, and parental cell lines. ZD1694 and AG337 failed to inhibit the TS target efficiently, presumably because of difficulties in the intracellular trafficking pathway. The 2R120 cells showed a complete TS inhibition after 5FU, ZD1694 or AG337 exposure.

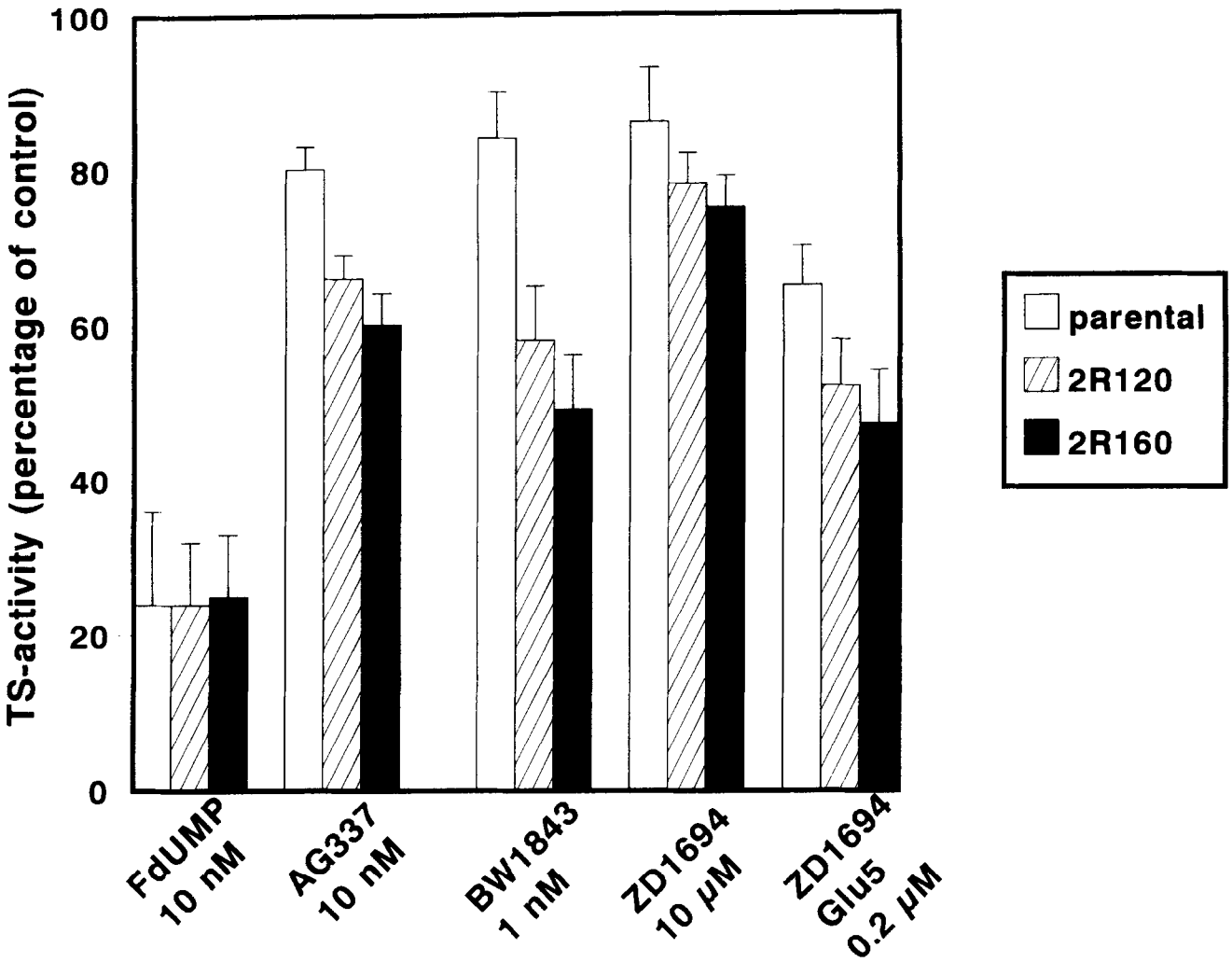

FIG. 1. TS inhibition by FdUMP, ZD1694, ZD1694-Glu_5, BW1843U89 and AG337 in MDR cells. Values are means ± SE of at least three separate experiments. TS inhibition was measured by simultaneous addition of ^{3}H-dUMP (1 μM) and a TS inhibitor (FdUMP, AG337, BW1843U89, ZD1694 and the ZD1694 pentaglutamate).

DISCUSSION

In this paper, we report cross-resistance and collateral sensitivity to TS inhibitors and antifolates in two phenotypically different MDR cell lines: 2R160 cells with P-gp overexpression, and the 2R120 cell line, which has no detectable P-gp but high MRP and LRP expression. Previously, only cross-resistance to 5FU in P-glycoprotein expressing MDR cell lines had been observed, but non-P-gp cells were not included in these studies [18, 19]. Assaraf and co-workers reported cross-resistance of lipophilic antifolates in P-gp overexpressing MDR cell lines [48, 49], but antifolates have not been evaluated in non-P-gp MDR cell lines.

In the present study, we focused on mechanisms of TS resistance by measuring the following parameters: TS activity, TS induction, TS mRNA level and TS inhibition. Besides mechanisms of TS resistance, several other potential mechanisms for antifolate resistance have been investigated, such as polyglutamylation and transport of the antifolates.

The TS catalytic and FdUMP binding assays revealed a 2-fold increase in the 2R120 cell line compared to the parent line. In the 2R160 cells, catalytic activity showed a 2-fold increase at physiological dUMP concentration, whereas FdUMP binding was comparable to the parental cell line. This increase in TS levels cannot be explained by a higher proliferation rate of the cells, although a proliferation dependency has been postulated [2, 47]. Thus, these differences in TS levels are suggestive of changes at the enzyme kinetic level, such as the two-fold decrease in K_i values for FdUMP and the two-fold decrease in K_m values for dUMP in the 2R120 and 2R160 cells compared with the parent line. This improved TS inhibition pattern might be related to the higher sensitivity of the 2R120 cells compared to the parental cell line. For the 2R160 cells, other factors appear to play a role in 5FU resistance.

The folate-based TS inhibitors tested failed to reveal any additional differences between the 2R120 and 2R160 cells in their capacity to inhibit the enzyme (Fig. 1). However,

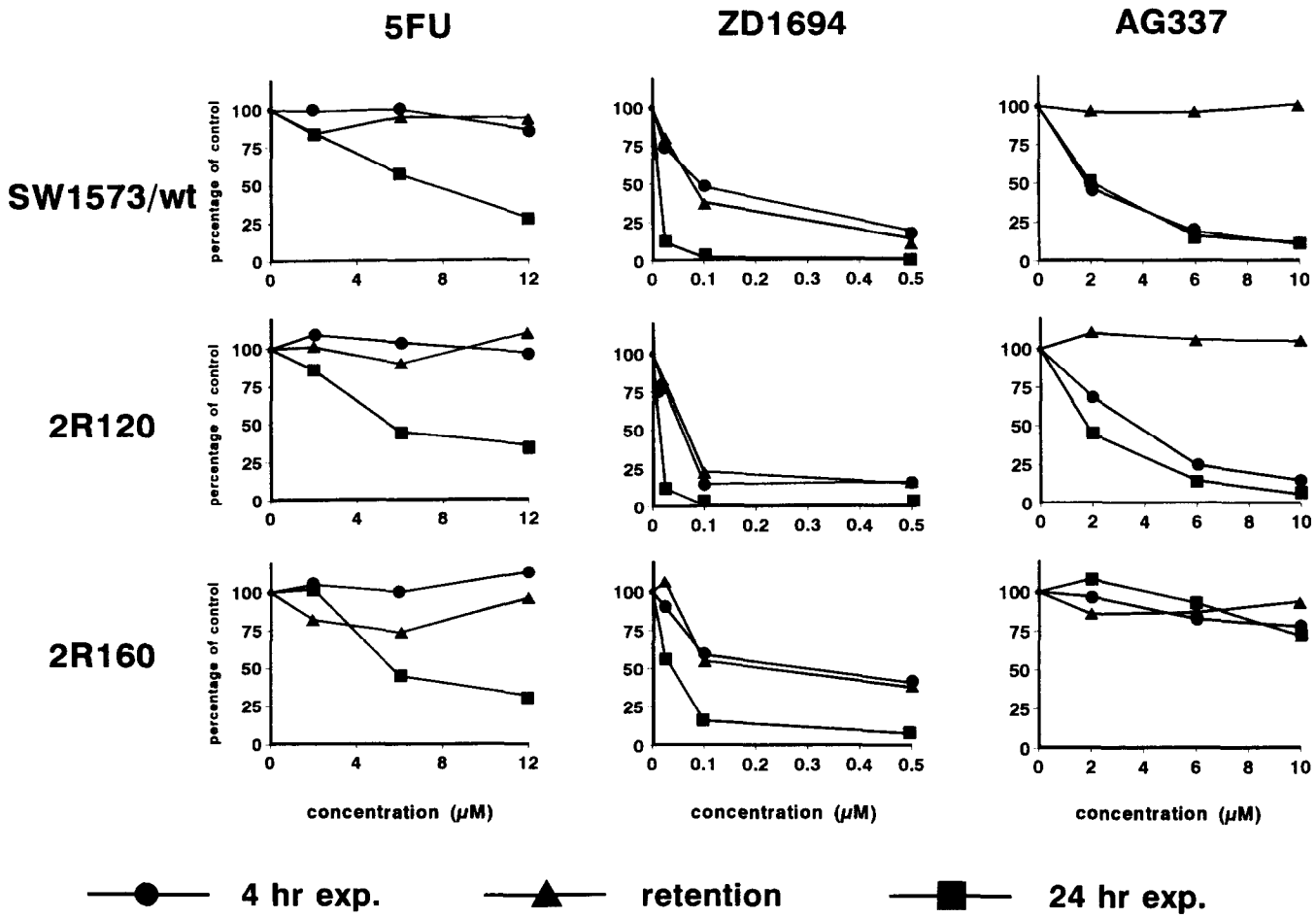

FIG. 2. Inhibition of TS and its retention in intact MDR cells. Values are means of at least three experiments. The SE was less than 30% of the mean. Inhibition of intracellular TS activity as measured with the *in situ* TS assay is expressed as % of control tritiated water release and calculated per experiment. The parental cells, the non-P-gp (2R120) and P-gp (2R160) cells were exposed to the drugs (5FU, ZD1694 and AG337) at several concentrations for 4 or 24 hr. The retention of TS inhibition was determined after a 4 hr drug exposure followed by a 3 hr drug-free period. ^{3}H-dUrd was present during the last 2 hr.

when compared to the parental cell line the MDR variants exhibited a greater capacity to inhibit TS. It appears that the induction of resistance to doxorubicin in the SW-1573 line is accompanied by alterations in TS enzyme kinetics, both in low level resistant 2R120 cells and in high level resistant 2R160 cells. In addition, other factors such as transport and drug distribution seem to play a more dominant role in antifolate resistance in both 2R120 and 2R160 cells. Differences between the 2R120 and 2R160 cells at the TS level were also observed in the TS *in situ* assay. These data clearly indicate that in the 2R160 cells, following a decreased membrane transport of ZD1694, sufficient drug concentration cannot be reached at the target to inhibit TS. Only after 24 hr exposure does the intracellular drug concentration seem to be high enough to almost completely inhibit TS. In contrast, the parental and 2R120 cells showed an almost complete TS inhibition after only 4 hr ZD1694 exposure. For AG337 as well, a decreased inhibition was observed in 2R160 cells compared to the 2R120 and parental cell lines. There are two possible explanations for this phenomenon. Either a reduced amount of AG337 enters the cell, or influx at the level of the plasma membrane is not reduced but an altered intracellular distribution of the drugs prevents interaction with the target TS.

In the 2R120 cell line not only were differences observed at the TS enzyme level, but divergences were also noted in the degree of TS protein induction and TS mRNA expression. While TS protein levels were comparable in the parent, 2R120 and 2R160 cell lines, drug (5FU, ZD1694, AG337)-exposed cells revealed that TS protein induction was 2- to 2.5-fold higher in the 2R120 cells than in the wild type and 2R160 cell lines. This is a common phenomenon: TS induction after 5FU exposure has been measured by several groups, which reported a 2- to 4-fold increase in TS level both in cell lines [50, 51, 52] and in tumors *in vivo*, both in mice and patients [53, 10, 11]. In the 2R120 cells, TS mRNA expression relative to β-actin mRNA expression was twice as high compared to the wild type and 2R160 cell lines. No increase in TS mRNA compared to

the parent cell line could be measured in the 2R160 cell line. Similar discrepancies in this relation were reported by Freemantle *et al.* [40] in lymphoid and ovarian cancer cell lines with acquired resistance to ZD1694. In the lymphoblastoid cell line no correlation was found between TS protein and TS mRNA, in contrast to the ovarian cancer cell line in which a similar, moderate increase in both TS protein and mRNA was observed. An interesting observation was made by Keyomarsi *et al.* [47] in a human breast cancer cell line treated with ZD1694, in which an increase in TS protein level was measured while the TS mRNA level remained unchanged. Comparing the TS data in the 2R120 cells, a relation between cross-resistance to TS inhibitors and a similarly increased level of TS activity, TS protein induction and TS mRNA can be observed. In the 2R160 cell line, an increased TS level and different properties in the TS inhibition were only observed in the functional *in situ* assay.

The enhanced 5FU sensitivity of the 2R120 cell line is remarkable and can be partially explained by the fact that the enzyme is inhibited at the nucleotide binding site by FdUMP in contrast to the folate binding site for the antifolates. Possible structural changes in the TS enzyme may have resulted in less favourable binding for antifolates. However, since the TS activity and TS mRNA levels are increased in this cell line, a TS directed cytotoxic effect of 5FU might be less likely to be successful. The results from the DNA distribution analysis demonstrated an enhanced RNA-directed effect following 5FU exposure by incorporation of FUTP into RNA rather than a DNA-directed effect, although the latter was to be expected after TS inhibition by FdUMP. The result of TS inhibition would be a disturbance of DNA synthesis because thymidylate, the essential nucleotide, is less available. DNA-directed effects on cell-cycle distribution after 5FU exposure would result in an accumulation of cells in the S-phase. In cells in which cytotoxicity of 5FU via FUTP incorporation into RNA is more predominant, flow cytometry of 5FU-exposed cells would show ablation of cells in the S-phase [54, 55]. Thus, the DNA distribution pattern indicates an effect through RNA. Resistance to 5FU in 2R160 cells might also be mediated by enhanced DNA repair enzyme activity, as reported by Chu *et al.* [56].

Disturbances in the intracellular folate pools could be another factor in the cross-resistance and collateral sensitivity pattern in the 2R120 cell line. FPGS and FPGH showed no significant differences between the 2R120 and 2R160 cells, but it is not clear to what extent these enzymes contribute to cellular polyglutamate synthesis and degradation. Both FPGS and FPGH have a different intracellular distribution, FPGS being located in mitochondria [57] and FPGH in the lysozymes [58]. Not only the enzymes, responsible for the polyglutamylated status of the cell, but also the polyglutamates are possibly restricted to certain regions in the cell. Schlemmer and Sirotnak described the possibility of an intracellular site or compartment at which MTX and its polyglutamates interact, resulting in a decreased efflux or possibly an inability to reach the right target [59].

Differences in the transport capacity of antifolates dependent on transport by the membrane-bound reduced folate carrier have been associated with resistance to antifolates, such as ZD1694, BW1843U89 and MTX [60, 61]. A decrease in MTX influx in 2R160 cells could be indicative of a transport defect across the cell membrane for drugs which require transport via the reduced folate carrier, such as ZD1694, BW1843U89 and MTX. This is in line with the resistance observed in the growth inhibition experiments for these compounds. Although the lipophilic compound AG337 is not dependent on membrane transport via the reduced folate carrier, resistance was observed in the P-gp overexpressing 2R160 cells. In this cell line, verapamil, a potent modulator of the MDR phenotype, failed to reverse AG337 resistance. Cross-resistance to AG337 cannot be explained by MDR gene amplification and overexpression of P-170 P-gp protein, indicating that the P-gp efflux pump is not responsible for AG337 efflux. When applied to two MDR cell lines with resistance to lipophilic antifolates, verapamil did reverse the resistance to the antifolate in one cell line, whereas it failed in the other. Apparently, mechanisms of lipophilic antifolate resistance other than the P-gp pathway exist in MDR cell lines [49]. Assaraf *et al.* postulated that intracellular trafficking and drug distribution may be responsible for the observed antifolate resistance. The results obtained in the TS *in situ* assay indeed point to the existence of such a phenomenon, since the drugs do not seem to reach the target TS.

In summary, a multimodal explanation was found for the cross-resistance and collateral sensitivity pattern in the MDR cell lines. The resistance mechanisms appear to be very different for the 2R120 cells compared to the 2R160 cells. In the former, the induction of resistance to doxorubicin probably altered the kinetics of TS, resulting in increased affinity of FdUMP, which is possibly responsible for the collateral sensitivity to 5FU. In addition, a higher TS catalytic activity and TS mRNA expression were observed, while TS protein induction in the 2R120 cell line increased more than in the wild type cell line. All these factors are likely to be responsible for the antifolate resistance in 2R120 cells. However, although the cross-resistance to 5FU and several antifolates may partially be related to these kinetic changes in TS in the 2R160 cells, differences in transport, both transmembrane transport and intracellular trafficking, seem to be more important. Recent findings indicate that efflux systems are possible mechanisms of MTX resistance [62] and that MTX can be a substrate for P-gp [63]. The roles of transmembrane and intracellular transport and of membrane proteins such as P-gp and MRP, in relation to antifolate resistance, will be a challenging field of research.

In conclusion, this paper demonstrates that induction of MDR, either associated with increased P-gp or MRP, may lead to a multi-modal complexity of acquired drug resis-

tance. It is clear that even in a relatively simple cell culture system, many factors play a role in the acquired drug resistance. This may also hold for the cancer patient observed in clinical practice.

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