Determinants of Activity of the Antifolate Thymidylate Synthase Inhibitors Tomudex (ZD1694) and GW1843U89 against Mono- and Multilayered Colon Cancer Cell Lines under Folate-restricted Conditions

Godefridus J. Peters, Evelien Smitskamp-Wilms, Kees Smid, Herbert M. Pinedo, and Gerrit Jansen

Department of Oncology, University Hospital Vrije Universiteit, 1007 MB Amsterdam, the Netherlands

ABSTRACT

The cytotoxicity and metabolic effects of two thymidylate synthase (TS) inhibitors, Tomudex (Raltitrexed, ZD1694) and GW1843U89, were studied in WiDr colon cancer cells under four different growth conditions: as standard monolayers and as postconfluent multilayers grown under either high (WiDr, 8.8 μM folic acid) or low (WiDr/F, 1 nM leucovorin) folate conditions. Both GW1843U89 and ZD1694 were 13-15-fold more active against WiDr/F than WiDr cells when cultured as monolayers (IC50s in WiDr/F cells were 0.22 and 0.39 nM, respectively). WiDr cells were markedly less sensitive to the drugs when grown as multilayers (4-15-fold), in contrast to the WiDr/F cells, which were equally sensitive. However, total growth inhibition could not be achieved in WiDr multilayers (concentration causing total growth inhibition > 10,000 nM), whereas in WiDr/F multilayers, it could be achieved at 0.42 nM ZD1694 and 150 nM GW1843U89. Growth conditions markedly affected the TS levels when using different enzyme assays. At nonsaturating substrate concentrations, the catalytic activity of TS was similar in mono- and multilayers grown under high folate conditions but lower in multilayers at saturating concentrations. In cells grown under low folate conditions, TS catalytic activity was 3-6-fold lower in multilayers than in monolayers. This was consistent with a decrease in the number of S-phase cells in multilayers. Western blotting revealed less pronounced (2-3-fold) differences in the TS protein content. Exposure of the cells for 24 h to the drugs increased the TS levels by 4-fold. Because this increase in TS levels might explain the decrease in sensitivity to the TS inhibitors, we measured TS inhibition (TSI) by the drugs in intact cells using the TS in situ assay. GW1843U89 was more active than ZD1694. However, after 4 h of exposure in WiDr/F mono- and multilayers, TSI was in the same range for both drugs [50% TSI (TSI50) 0.5-1.7 nM]. In WiDr cells, the TSI50 for ZD1694, but not GW1843U89, was 10 times higher in the multilayers as compared to the monolayers. Despite the increase in TS protein levels, the extent of TSI was similar or even more pronounced in both cell lines grown as either multi- or monolayers. Because the cells were grown under depleted and folate-rich conditions that may affect folate uptake, we measured folate transport using methotrexate (MTX) as the reference drug for the activity of the reduced folate carrier. MTX uptake was 4-fold lower in multilayers compared to monolayers in both WiDr and WiDr/F cells. Uptake of MTX was 5-fold more effective in WiDr/F cells than in WiDr cells in both mono- and multilayers. In conclusion, the resistance of WiDr multilayers to the novel antifolates ZD1694 and GW1843U89 may be due to the high folate medium concentrations, which may be responsible for impaired drug uptake along with less effective TSI. In contrast, WiDr/F monolayers and multilayers were very sensitive to these antifolates. These effects of folate homeostasis may explain some of the variable results seen in treatment of solid tumors with new antifolate TS inhibitors.

INTRODUCTION

Three-dimensionally cultured tumor cells offer better models for the study of human solid malignancies than monolayers (1, 2). Models such as multicellular spheroids (3), collagen gels (4), histocultures (5, 6), and cells grown as postconfluent multilayers in V-bottomed microwell plates (7-10) show a much greater mimicry of the complexity of human neoplastic cells than monolayered cell cultures. These factors include drug penetration barriers, cell proliferation gradients, and microenvironmental conditions such as hypoxia and acidic pH. Our in vitro model in V-bottomed microwell plates enabled us to perform large-scale drug screening with automatic data handling (7-10). In this system, all tested cell lines reached confluence after 5 days of growth, each with a specific pattern of cell stacking from 2-10 layers. Growth and cytotoxicity, as evaluated with cell counting and the SRB1 and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assays, demonstrated for most drugs that multilayered plateau phase cultures rather than monolayers showed a resistant phenotype (7, 8, 10, 11) that is more representative to the in vivo situation in animals and patients.

Inhibition of TS has been associated with the antitumor activity of 5FU (12). 5FU acts through its metabolite, FdUMP, which inhibits TS by the formation of a ternary complex of TS, FdUMP, and CH2THF (13, 14). TS expression has been reported to be cell cycle dependent (15, 16). TS protein and TS activity levels are higher in proliferating cells than in nonproliferating cells and vary 14-24-fold between exponential and confluent cell populations (17). Several antifolates are potent TS inhibitors and have been evaluated in the clinic (18, 19). In general, these folate analogues act by forming a tightly bound nonactive complex with TS and the natural substrate dUMP (19, 20). ZD1694 (Tomudex, Raltitrexed) and GW1843U89 are soluble, very potent, third-generation TS inhibitors (K1 60 or 0.09 nM, respectively; Refs. 20-24). Cytotoxic activity is dependent on active cellular uptake via the RFC and subsequent metabolism to polyglutamylated forms by FPGS (25). The predominant polyglutamate for ZD1694 is ZD1694-Glu4, which is ~60-fold more potent as a TS inhibitor than the parent drug (21-22). Intracellular metabolism of GW1843U89 usually proceeds only to the diglutamate derivative (GW1843U89-Glu2; Ref. 24). In addition, polyglutamated derivatives are not readily effluxed from the cells; for GW1843U89, polyglutamation resulted in a better retention, rather than a higher TSI.

Both compounds have shown activity against xenografts of human ovarian and colonomic origin (19, 26); Tomudex showed a response rate comparable to that of 5FU + LV in Phase III clinical studies (colonic cancer), but with a different toxicity profile (27, 28). For GW1843U89, only a Phase I study has been performed (29).

GW1843U89 has been tested against multicellular spheroids of the colon cancer cell line WiDr (30), which can be destroyed after

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2 To whom requests for reprints should be addressed, at Department of Oncology, University Hospital Vrije Universiteit, P.O. Box 7057, 1007 MB Amsterdam, the Netherlands. Phone: 31-20-444-2633; Fax: 31-20-444-3844; E-mail: g.peters@azvu.nl.
long-term exposure to 10–100 nM GW1843U89. Much higher concentrations of other antifolates, such as the dihydrofolate reductase inhibitor MTX (30-fold) or the glycaminide ribonucleotide formyl transferase inhibitor 5-deaza-acyclo-tetrahydrofolate (3000-fold) were required to achieve a similar effect. Shorter exposures or lower concentrations of G1843U89 led only to growth delay (30).

In this study, we determined the cytotoxicity of both ZD1694 and GW1843U89 against multilayers of the human colon carcinoma cell line WiDr in comparison to monolayers. Multilayered cell cultures are considered to be a better model system to screen for activity of new anticancer agents. Because we have demonstrated previously that low folate levels in medium affect sensitivity to antifolates, we also used a subline of WiDr, WiDr/F (31), which was adapted to low folate concentrations (1 nM LV), approximating the physiological folate levels in man. The differences in sensitivity between mono- and multilayers were related to the effects of culturing, drug-target enzyme interaction (TS enzyme activity and TS protein levels), and drug uptake.

MATERIALS AND METHODS

Chemicals. 5-[^3]HdUMP (specific activity, 18.6 Ci/mmol) was purchased from Amersham International plc (Buckinghamshire, United Kingdom), and 5-[^3]HdUrd (specific activity, 24.9 Ci/mmol) was purchased from Moravek Biochemicals Inc. (Brea, CA). 6-[^3]HdUMP (specific activity, 20.0 Ci/mmol) and [3',5',7-[^3]H]MTX ([^3]H]MTX; specific activity, 15.0 Ci/mmol) were obtained from Moravek Biochemicals Inc., DMEM was obtained from Flow Laboratories (Irvine, United Kingdom), RPMI 1640 without folate acid and FCS were obtained from Life Technologies, Inc. (Grand Island, NY), ZD1694 was provided by Zeneca (Macclesfield, United Kingdom), and GW1843U89 was provided by Dr. R. Ferone (then at Burroughs Wellcome Co., Research Triangle Park, NC). All other chemicals were of analytical grade. ZD1694 and GW1843U89 were both dissolved in 50 nM NaHCO₃ (adjusted pH to 7.4; stock solutions of 10⁻³ M were stored at −20°C) and diluted in culture medium for the growth inhibition studies.

Cell Culture. The wild-type human colon cancer cell line WiDr was gradually adapted to grow at physiologically (low) folate concentrations, resulting in the WiDr/F cell line (31). The parental WiDr cells were routinely cultured in DMEM with 5% heat-inactivated (30 min at 56°C) FCS, which contains high concentrations (8.8 μM) of folic acid. During the adaptation process, medium with dialyzed serum was used to remove endogenous folates, whereas the DMEM was gradually substituted for the folate-free RPMI 1640. Exogenous LV was added as folate source and was also decreased gradually. The whole process required several months. The adapted cell line WiDr/F was cultured in folate-free RPMI 1640 with 10% dialyzed FCS, 2 mM glutamine, and 1 mM LV as the folate source. The doubling time of exponentially growing WiDr monolayer cells was 22 h, and that of WiDr/F cells was 33 h. In postconfluent multilayers, the doubling time increased to more than 3 days for both variants. These cultures grow slowly. Because of the three-dimensional structure, there is an equilibrium between cell death and growth similar to tumors.

Cell Cycle Analysis. Monolayer or multilayered cells were exposed for 1, 4, or 24 h to 50 μM BrdUrd in the culture medium (32). Cells were harvested by trypsinization and fixed by a 15-min incubation in 0.25% paraformaldehyde followed by a 1-h incubation in 70% methanol on ice. DNA was denatured by treatment with 1 N HCl for 30 min at room temperature. Cells were washed with medium containing 1% BSA and stained for 30 min at 4°C with a mouse-anti-BrdUrd antibody followed by a FITC-labeled rabbit antirabbit secondary antibody (both derived from DAKO and diluted 1:100). DNA was stained with propidium iodide (50 μg/ml) after RNA degradation by RNase treatment (0.1 μg/ml; Ref. 32). Cells were measured on a FACSSTAIR (Becton Dickinson Medical Systems, Sharon, MA) detecting propidium iodide at FL1 and FITC at FL2. The results were analyzed using the Cell Fit program (Becton Dickinson). In separate experiments, BrdUrd staining was performed in plastic (Techne, Waltham, MA) or glass (Heraeus, Wehrem, Germany)-embedded multilayer cells. The staining pattern revealed that cells at the tip of multilayer were also stained, demonstrating that BrdUrd could penetrate the cells.

CHEMOSENSITIVITY ASSAYS. Exponentially growing cells were harvested by trypsinization and resuspended in fresh medium; single cell suspensions were seeded at 15,000 cells/50 μl per well in V-bottomed 96-well plates on day 0 (D0). Chemosensitivity experiments were performed using triplicate wells for each condition and seven different concentrations for each drug. For monolayer testing (drug addition after 24 h, ∼D1), the drug concentrations ranged from 10⁻⁴ to 10⁻¹ M; for multilayer testing (drug addition after 120 h at D5), the drug concentrations ranged from 5.10⁻⁴ to 10⁻¹⁰ M. Because of the differences (see “Cell Culture”) between multilayers and monolayers, we chose for a constant exposure time of 24 h rather than an exposure time based on the same number of doublings. During the chemosensitivity assays, gentamicin (40 μg/ml) was added to the media to prevent bacterial infection.

At D1, 24 h after the initial plating of the cells, monolayers were exposed to drugs for 24 h until D2. Thereafter, the cells were rinsed once with culture medium and incubated in the presence of drug-free medium for another 72 h (until D5), during which the medium was refreshed once on D4. On D1 (growth control plate) and on D5 (plates treated with drugs), the SRB assay was performed (8, 33). For that purpose, the cells were fixed with 50 μl of cold 50% trichloroacetic acid and subsequently dyed with 0.4% (w/v) in 1% acetic acid SRB. The excess of SRB was washed away with 1% acetic acid. The plates were read on a titerTec Multiskan MCC/340 microplate reader at 450 or 492 nm, after dissolving the SRB in 150 μl of 10 N Tris. Treatment of multilayers was initiated 5 days after plating (D5), after medium renewal at D1, D2, and D4. At D6, after 24 h of drug exposure, the cells were rinsed once with fresh medium and then incubated for another 72 h in drug-free medium with daily medium renewal until D9. The SRB assay was performed on D5 and on D9 as described above.

The IC₅₀S were extrapolated from the resulting dose-response curves by correcting for the number of cells on the moment of drug addition, which was set at 0% (growth control plates, D1 for monolayers and D5 for multilayers); D5 and D9 represent 100% growth for mono- and multilayers, respectively. TGI is defined as the drug concentration that resulted in the same number of cells at the end of the assay (D5 or D9) as at the moment of drug addition (D1 or D5). The LC₅₀ is the drug concentration resulting in a 50% decrease of the number of cells at D1 and D5.

TS Assays. TS was assayed using various methods, each addressing different functional aspects. With the catalytic assay, the enzymatic conversion of the substrate dUMP to dTMP was determined. The ligand binding assay determined the number of dUMP binding sites, whereas the TS in situ assay was used to determine the functional enzyme activity in intact cells. The amount of TS protein was determined with Western blotting, whereas a semiquantitative difference between the cell lines is given with immunohistochemistry.

TS Ligand Binding Assay and TS Catalytic Activity in Cell-free Extracts. The TS catalytic assay measures the conversion of the substrate 5-[^3]HdUMP to dTMP and H₂O in cell-free extracts. The assay was performed as described previously (34, 35) at two substrate concentrations: (a) 1 μM (around K_m); and (b) 10 μM (saturating concentration). Linearity for protein and time was ascertained for both monolayers and multilayered cells. During isolation of the cells and preparation of the cell extract, endogenous folates and nucleotides (including dUMP) were diluted several hundred times, which would minimize interference in the TS assays. The ligand binding assay was performed with 34 nM 6-[^3]HdUMP as substrate, as described previously (34, 35).

TS in Situ Assay. The assay is based on that described by Yalowich and Kalman (36) and was essentially performed as described previously for monolayers (37). It gives a quantitative indication of the potential intracellular inhibition of TS activity. After TS-catalyzed conversion of 5-[^3]HdUrd via 5-[^3]HdUMP to dTMP and H₂O, the amount of tritiated water released into the cellular cytosol and subsequently into the culture medium was determined with liquid scintillation counting. For monolayers, cells were plated in 6-well plates at a density of 0.5 × 10⁶ cells in 2 ml of medium per well; for multilayers, cells were plated as described for the chemosensitivity assays. Drugs were added at D2 and D5. For ZD1694 and GW1843U89, a concentration range of 2 × 10⁻⁶ to 2 × 10⁻¹¹ M was used. The cells were exposed to the drugs for 4 or 24 h. For the last 2 h of the incubation, 5-[^3]HdUrd (0.16 Ci/mmol; final concentration, 2.5 μM) was present. After incubation, 150 μl of the medium from the wells were collected, added to an ice-cold suspension containing 750 μl of charcoal (with 0.5% T-70 dextran and 2.5% BSA) and
150 μl of 35% trichloroacetic acid, mixed thoroughly, and chilled on ice for 15 min. After centrifugation for 15 min at maximum speed (1500 × g) at 4°C, a mixture of 450 μl of the supernatant and 4.5 ml of scintillation fluid was counted by liquid scintillation counting. Enzyme activities were expressed as pmol 3H2O formed/h/10^6 cells and as a percentage of the drug-free control. A 50% inhibition of the in situ TS activity was termed TSI_{50}.

**Western Blot Analysis.** To study the effect of drug addition on TS protein levels in monolayers, an equal number of cells (2.10^{10} cells/5 ml) from each cell line was transferred into 75-cm^2 flasks. After 24 h, the cells were exposed for 24 h to the drugs (2.10^{-8} M). The cells were harvested, and the pellets were stored at −80°C. Cells for multilayers were plated in 96-well, V-bottomed plates as described above. On D5, drugs were added (final concentration, 2.10^{-8} M), and after 24 h of exposure, the cells were harvested and pelleted as described above. Cell samples from D2, D3, D4, D5, D7, and D9 cells (nontreated) were collected in the same way. The amount of TS protein was determined by Western blotting essentially as described previously (37, 38). Protein was extracted with lysis buffer [50 mM Tris-HCl (pH 7.4), 2 mM DTT, 20% (v/v) glycerol, 5 mM benzamidine, 0.5 mM phenylmethylsulfonyl fluoride, and 0.5% (v/v) NP40]. The amount of protein in the supernatant was estimated using the Bio-Rad protein assay as described by Bradford (39). Thirty μg of the protein extracts and 2 ng of purified TS protein (as standard) were separated on 12% SDS-polyacrylamide gels and transferred to a microporous polyvinylidene difluoride membrane by electroblotting. The blots were blocked with milk powder overnight at 4°C and then incubated for 2 h with a 1:3000 dilution of rabbit polyclonal antihuman TS antiserum [RahTS31; kindly provided by Dr. G. W. Aherne (Sutton, Institute of Cancer Research, United Kingdom); Refs. 37, 38, and 40] followed by incubation with swine antirabbit peroxidase for 1 h and staining with 3,3′-diaminobenzidine and hydrogen peroxide. TS protein was quantitated by densitometric scanning, and the amount of TS protein could be calculated according to the standard.

**Immunohistochemistry of TS on Cytospins.** Cytospins of exponentially growing cells and cells with longer culturing periods (isolated from 96-well, V-bottomed plates on D5, D7, and D9) were stained using the avidin-biotin-peroxidase technique (VECTA stain ABC staining kits). A 1:100 dilution of the rabbit antihuman TS31 antibody was used to detect TS (40). Total incubation time was 60 min. Controls were handled simultaneously, substituting the specific antibody for rabbit normal serum (1:400). Intensity was expressed as +, ++, or ++++, where ++++ was the value given to the cells with the highest intensity (40).

**Drug Uptake via RFC (1′[3H]MTX Accumulation).** RFC transport capacity was determined using MTX as the reference drug (38). Monolayer cells and multilayered cells (pooled from 10 individual wells of the V-bottomed, 96-well plates) were brought into a single cell suspension by trypsinization, after which the cells were washed with HBBS (pH 7.4) and resuspended to a density of 5 × 10^6 cells/ml in 37°C HBBS. One ml of cells was incubated at an extracellular concentration of 2 μM [1′H]MTX (specific activity, 1 Ci/mmol) for 10 min at 37°C. At the end of this period, 9 ml of ice-cold HBSS was added to the cell suspension. The cells were centrifuged and washed once more with 10 ml of ice-cold HBSS. The final cell pellet was resuspended in 0.5 ml of H2O and analyzed for radioactivity using a Packard scintillation counter (1900 TR). As controls, the same reactions were performed at 4°C and at 37°C with an excess of unlabeled MTX (final concentration, 200 μM; ref. 41).

**RESULTS**

**Growth Inhibition Studies.** Both ZD1694 and GW1843U89 were very effective against monolayers of the human colon cancer cell line WiDr and its variant, WiDr/F, which was adapted to low-folate medium (Table 1). IC_{50} values for the two antifolates were 3–6 μM for WiDr cells and about 15-fold lower for WiDr/F cells. WiDr multilayers were resistant to both drugs, as illustrated by only a slight growth inhibition (IC_{50} = 3–17-fold higher than that in monolayers) and no clear reduction of cell mass (no TGI was reached in the concentration range used; Fig. 1). Similar to the WiDr/F monolayers, the antifolates were more effective against the WiDr/F multilayers, in which a TGI could be reached. The IC_{50} for ZD1694 was even 2.5–3-fold lower for WiDr/F multilayers than for monolayers.

In the case of WiDr/F multilayers, a plateau in the chemosensitivity curves was seen at high drug concentrations at absorbance readings similar to or lower than the control plates, indicating that the same number of cells was present as at the moment of drug addition or that the cell number actually decreased. In addition, the chemosensitivity curves were generally not very steep, resulting in a large variation in IC_{50}s and TGIs between the experiments. Cell kill was only observed at high concentrations, and LC_{50} values (when evaluable) were more than 1 μM.

The relative resistance of WiDr multilayers may be related to the slower growth in the period from D5–D9 when compared to the period D1–D5. This slower growth was confirmed by fluorescence-activated cell-sorting analysis of the growth fraction of mono- and multilayers. A short exposure (4 h) to BrdUrd gives an indication of the S-phase fraction, which was 62% for WiDr monolayers and only 10% for multilayers. A longer exposure (24 h) shows the total number of proliferating cells, which was 97% for monolayers and 49% for multilayers. In plastic-embedded monolayers and multilayered cells, a similar qualitative difference was also observed (data not shown).

**TS Assays.** Because TS is the main target for both ZD1694 and GW1843U89, we determined whether differences in TS levels could explain the resistance of WiDr multilayers to TS inhibitors. TS levels were evaluated using different methods (Table 2).

With all assays, clear differences were observed in the TS levels in WiDr cells cultured under various conditions. These assays also indicated changes in enzyme characteristics. The TS catalytic activity was generally lower in multilayered cells than in monolayers. This difference was most pronounced at a saturating substrate concentration of 10 μM dUMP (6-fold for WiDr/F), but at 1 μM dUMP, activities were similar (WiDr) or only 2-fold different. This indicates a change in the characteristics of the enzyme. The ratio of activity at 10 and 1 μM dUMP was also different: in WiDr/F cells, it was 5.5 for monolayers and 2.2 for multilayers; for WiDr cells, these values were 4.0 and 1.6, respectively. This indicates that the K_{m} for dUMP will be lower for multilayers.

The TS activity measured in the in situ assay followed a slightly different pattern; it was 1.5-fold higher (WiDr) in monolayers than in multilayers or similar (WiDr/F) in both monolayers and multilayers (Table 2). The activity in WiDr/F cells was lower than that in the corresponding WiDr cells, which may be related to the depletion of folates essential for the TS reaction. The absolute in situ TS activities on D1 and D5 cannot be compared to the catalytic activities partly because the substrate (5-[3H]dUrd) was different (5-[3H]dUMP) in the catalytic assay. In addition, the tritiated deoxyuridine has to be taken up by the cells before it can be intracellularly converted by thymidine kinase into tritiated dUMP, which is the substrate for TS. The absolute in situ TS activities are thus a result of transport, thymidine kinase and TS activities, and the intracellular availability of folates, which are required for the catalytic conversion of dUMP to dTMP.
Another change in the characteristics of TS is the aberrant pattern of FdUMP binding (Table 2). In contrast to the catalytic activity, the number of FdUMP binding sites is 3–4-fold higher in WiDr and WiDr/F multilayers compared to monolayers, whereas in the WiDr/F monolayers, the number of binding sites is 2–3-fold lower. Together, these data demonstrate that culture conditions clearly influence TS levels and characteristics.

To gain more insight into the nature of these changes, we stained cytospins of mono- and multilayers for TS with a TS antibody. In both cell lines, under all culture conditions, there was diffuse cytosolic staining. A qualitative decrease in time was visible. The intensity of the staining was ++ for monolayers, + for D5 multilayers, and + for D7 and D9 multilayers. The assay was not sensitive enough to detect differences between the WiDr and WiDr/F cells.

Table 2 Comparison of TS levels in WiDr mono- and multilayered cells measured by different methods

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<tr>
<th>TS assay</th>
<th>WiDr cells</th>
<th>WiDr/F cells</th>
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<tr>
<td>1 μmol/dUMP</td>
<td>10 μmol/dUMP</td>
<td>ratio, 10:1</td>
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<tr>
<td>TS catalytic activity (pmol/h/10⁶ cells)</td>
<td>33 ± 2</td>
<td>36 ± 12</td>
</tr>
<tr>
<td>10 μmol/dUMP</td>
<td>131 ± 2</td>
<td>58 ± 24</td>
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<td>ratio, 10:1</td>
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<td>5.6</td>
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<tr>
<td>TS in situ (fmol/h/10⁶ cells)</td>
<td>171 ± 16</td>
<td>117 ± 2</td>
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<tr>
<td>FdUMP binding (fmol/10⁶ cells)</td>
<td>65 ± 2</td>
<td>205 ± 61</td>
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<tr>
<td>Western blot (ng/10⁶ cells)</td>
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TS protein levels, measured by Western blot, were around 2-fold lower in WiDr multilayers than in the monolayers (Fig. 2; Table 2). The difference between both WiDr/F cultures was smaller. The decrease during culturing seems to be related to the decrease in proliferation, because the fluorescence-activated cell-sorting analysis indicated that multilayers contain fewer proliferating cells than monolayers.

Effect of ZD1694 and GW1843U89 on TS Protein Levels and Inhibition. The effect of both drugs on TS was studied using two approaches: (a) protein levels; and (b) intracellular enzyme inhibition. With Western blot analysis, an increase in TS was observed (Fig. 2) after exposure of the cells for 24 h to 2 and 20 nM ZD1694 or GW1843U89. The increase in TS levels was 3–5-fold in both variants when grown as mono- or multilayers. This increase seems to be a clear regulatory effect of the drugs on TS expression, apparently similar to that described by Keyomarsi (16), because the increase was independent of the extent of growth inhibition or the presence of folates.

The observed increase in TS protein levels can be an explanation for the resistance to GW1843U89 and ZD1694; however, increased protein levels do not necessarily correlate with the potential enzyme inhibition by these drugs. The latter was studied by measuring the in situ TS activity in intact cells, with and without drugs. TS was inhibited very rapidly because a 4-h exposure to GW1843U89 resulted in a 50% TSI at (sub)nanomolar levels in WiDr and WiDr/F cells grown as either mono- or multilayers (Fig. 3). ZD1694 was clearly less effective in WiDr cells than in WiDr/F cells, whereas multilayers of WiDr were about 10-fold less sensitive than monolayers (Table 3).

A longer exposure to the drugs (24 h) resulted in a similar inhibition for GW1843U89 but a more pronounced inhibition for ZD1694 as compared to the 4-h exposure. Exposure of 20 nM GW1843U89 for 24 h completely inhibited TS in situ (>97%) in both mono- and multilayers, despite the increase in TS protein (Fig. 2). ZD1694 was less active: 20 nM inhibited TS for more than 90% under all four growth conditions, but 2 nM ZD1694 only inhibited TS for 90% under low folate conditions. Under high folate conditions, TS was only inhibited by about 20%. Because the degree of TS protein increase was the same for both cell lines and did not differ between the two...
The relative pattern of TS in situ inhibition (Table 3) is not completely similar to that of growth inhibition (Table 1). This may be related to differences in the properties of the drugs. The TSI by GW1843U89 is very rapid and appears to be too potent to discriminate between cell lines or conditions. This may explain the lack of correlation with growth inhibition, which is also dependent on the retention of TSI. For ZD1694, the initial TSI observed after 4 h is more likely to predict differences in growth inhibition, but a 24-h exposure did not reveal differences in TSI between mono- and multilayers. Decreased interference of normal folates in cells cultured in folate-depleted medium clearly determines the extent of TSI because in WiDr/F cells, ZD1694 was as active as GW1843U89 after 24-h exposure.

**RFC-mediated Antifolate Transport.** An important factor in resistance to antifolates consists of defects in transport across the cell membrane (42). MTX, GW1843U89, and ZD1694 all depend on the RFC for cellular uptake (25). We used MTX as a reference drug for transport studies using the different growth conditions (25, 43). The uptake of MTX was more efficient under low folate conditions in WiDr/F cells than under normal folate conditions (WiDr cells; 5-fold) in both mono- and multilayers (Fig. 4). In addition, despite the use of single cell suspensions, the uptake of MTX is 4-fold higher in monolayers than in multilayers for both WiDr and WiDr/F cells. This pattern is in line with the chemosensitivity data and can contribute to the resistant phenotype in WiDr multilayers. In WiDr/F multilayers, more drug was taken up than in WiDr monolayers, which is also in line with the growth inhibition studies.

**DISCUSSION**

Both ZD1694 and GW1843U89 were very potent inhibitors of cellular growth of WiDr and WiDr/F monolayers. In contrast, WiDr multilayers were markedly resistant to these antifolates, whereas WiDr/F multilayers were much more sensitive as shown by the appearance of cell kill. Cytotoxicity of TS-targeted antifolates seems to depend very much on environmental conditions, including proliferation and folate status, which affect TS levels. These environmental factors may be important for clinical drug resistance.

Several resistance factors are related. The higher cytotoxicity of antifolates under low folate conditions may be due to: (a) a more efficient drug uptake via RFC because RFC expression and functional activity may be up-regulated under low folate conditions (41); and (b) less competition for polyglutamylation catalyzed by FPGS, which is increased in cells and tumors adapted to low folate levels (31, 41, 43, 44); this will lead to an accumulation of more and longer polyglutamates that are not readily effluxed and have a higher affinity for TS. In contrast, the levels of the target enzyme TS may increase (44, 45), possibly due to a hypomethylation of the TS gene, which may increase the expression of TS (45). Several studies with cell lines showed that acquired resistance toward ZD1694 in vitro is achieved by the induction of TS, decreased RFC transport (46), decreased FPGS (47), or a combination of these factors. In multilayers, the growth fraction is decreased, which may explain the lower activity of TS and the lower expression found with immunohistochemistry and Western blotting. The former aspect (proliferation) may explain the decreased efficacy of the S-phase-specific antifolates, whereas the latter aspect (TS level) may actually be associated with an increased sensitivity. The low proliferation status of multilayered postconfluent cultures is practically determined by an equilibrium between cell division and cell death. Indeed, we previously observed necrotic regions in colon cancer multilayers (8) that may be the source for the release of purines and thymidine due to nucleic acid breakdown (48). Together with the release of intracellular folates to the medium, the compounds may salvage TSI and prevent drug-induced cell kill (48). Thus (in)sensitivity to these TS-directed antifolates is unlikely to be determined by one single determinant. Therefore, we investigated several mechanisms of antifolate resistance to determine one or more factors with the largest impact on resistance of solid tumors.

As mentioned above, TS should be considered as a primary target but not as the only determinant factor leading to cell death. In addition, it is not clear which aspect of TS may be predominant for cytotoxicity. The results of four different TS assays (two activity measurements and two assays for the amount of protein) were not consistent. In three of the four assays, TS levels in multilayers were...
lower than or equal to TS levels in monolayers. Only the FuUMP binding assay showed higher values in multilayers. The induction of TS by treatment with antifolates is equal under all circumstances (3–4-fold) and is not dependent on folate status or the age of the culture. This induction does not seem to lead to resistance, because the values for TSlav were not much different after 4 h or 24 h incubation, although after 24 h, the amount of TS protein was increased 4-fold. Induction has now been described for several TS inhibitors such as ZD1694 in colon cancer, leukemia, and lung cancer cell lines (16, 37). AG337 (37) and SFU (37, 49) are also able to increase TS levels.

The mechanism behind this phenomenon seems to be a down-regulation of the translation of TS in RNA by native TS protein. ZD1694 apparently induces a conformational change in TS protein, preventing down-regulation and leading to increased TS synthesis (16). The FuUMP metabolite FuUMP also induces a clear conformational change in TS protein, resulting in a ternary complex between FuUMP, TS, and CH3THF that is also unable to down-regulate TS mRNA translation (49). In addition, the ternary complex is more stable than native TS protein, preventing the degradation of TS protein. Passage of the cells also regulates TS mRNA translation, resulting in more TS protein in S-phase cells after being released from a synchronized state (15, 16). The effect of synchronizing cells in S phase apparently induces a larger effect on TS production than was found when a population of proliferating and nonproliferating cells were compared (17). The results of the latter studies were better comparable to our results than the effect seen in synchronized cells. Despite the increase in TS, both antifolates were very potent TS inhibitors in situ (Table 3), which was in line with the chemosensitivity profile in monolayers (Table 1). The final result of the TS in situ assay is the effect of a combination of differences in membrane transport and folate polyglutamylation (37) but seems to be independent of changes in TS. Lu et al. (47) also observed that the extent of the TS increase by TS inhibitors such as 5FU and antifolates is not an important determinant of drug resistance, because the extra TS molecules can still associate with the drugs and result in complete inhibition. Likewise, TSI immediately after drug treatment only predicts a possible potent cytotoxicity, not the final outcome. The duration of the inhibition seems to be of more importance, because only then may dTMP and, consequently, dTTP pools be depleted, bypassing a supply of dTMP via thymidine kinase by using thymidine in the medium. For the latter reason, mice fed a normal diet seem to be a poor model system for antifolates, because these animals are kept on a folate-rich diet, which also contains many nucleic acids, leading to high plasma and tissue levels of both folates and thymidine (up to 1 mM; Refs. 26 and 50). High thymidine levels, in contrast to high folate levels, do not affect the TS in situ assay (48), which may explain the relatively high sensitivity of the WiDr/W cell monolayer to both antifolates.

The relatively low sensitivity of WiDr cells as compared to WiDr/W cells and of the WiDr multilayers compared to WiDr monolayers led us to consider folate transport as a possible major resistance factor for both TS inhibitors. For that purpose, we used MTX as the reference drug to determine to determine the role of RFC; both ZD1694 and GW1843U89 are good substrates for human RFC (25). In addition, studies from our laboratory have shown that the growth of leukemic/solid tumor cells in low folate enhances the efficiency of carrier (25, 41, 46, 51), and that these and other differences between such cells can be attributed mainly to the folate status. Indeed, we observed a 4-fold higher activity of the RFC cells in WiDr/W cells as compared to WiDr cells in both mono- and multilayers. The decreased RFC activity in multilayers agrees very well with the lower sensitivity of multilayers to both TS inhibitors. Because slowly proliferating cells have lower rates of polyglutamylation (52) along with a decreased uptake, the drug is less efficiently polyglutamylated. Chemosensitivity to ZD1694 and its effect in the TS in situ assay were more influenced by the folate levels than the effect of GW1843U89 cytoxicity and TSI. TSI by the more potent GW1843U89 is less dependent on FPGS than TSI of ZD1694 (21–24). In a panel of CEM variants with aberrant RFC levels adapted to different folate conditions in the medium, the effect of GW1843U89 was not influenced or was hardly influenced by the level of FPGS, in contrast to ZD1694. In addition, intracellular folate pools were of less importance for the chemosensitivity to GW1843U89 than to ZD1694 (46, 51).

It can be concluded that multilayered postconfluent cultures of solid tumors are good models to study the effects of TS inhibitors in an environment more similar to human solid tumors than normal monolayers or mice fed on standard chow. The multilayers display characteristic features of tumors such as decreased drug uptake and emphasize the importance of three-dimensional cell culture (1, 53). However, the sensitivity to antifolates may also be affected by the release of potential rescue agents such as folates and thymidine (48, 54). In addition, the low proliferation rate is more in line with that of tumors. These effects of folate homeostasis may explain some of the variable results seen in the treatment of solid tumors with new antifolate TS inhibitors.

REFERENCES

THYMIDYLATE SYNTHASE INHIBITORS IN MULTILAYERED CELLS


Determinants of Activity of the Antifolate Thymidylate Synthase Inhibitors Tomudex (ZD1694) and GW1843U89 against Mono- and Multilayered Colon Cancer Cell Lines under Folate-restricted Conditions


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