The Impact of $p53$ Status on Cellular Sensitivity to Antifolate Drugs

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ABSTRACT

The impact of $p53$ status on cellular sensitivity to antifolate drugs has been examined in seven human cell lines (A549, MCF7, T-47D, CCRF-CEM, COR-L23, A2780, and HCT-116) and $p53$ nonfunctional counterparts of two of the cell lines (HCT-116/N7 and A2780/CP70). $p53$ status was determined by sequencing and functional assays. The sensitivities of the cell lines to growth inhibition (sulphorhodamine B assay) produced by four antifolate drugs (Alimta, methotrexate, raltitrexed, and lometrexol) were studied. There was no clear relationship between functional $p53$ status and sensitivity to methotrexate or lometrexol, whereas a functional $p53$ status was possibly associated with resistance to Alimta- and raltitrexed-induced growth inhibition. In contrast, in the two pairs of related human tumor cell lines (HCT-116 and HCT-116/N7 and A2780 and A2780/CP70) cells with functional $p53$ were more sensitive to Alimta- and raltitrexed-induced growth inhibition ($P = 0.002$). Detailed studies were performed with the A2780 cell lines, and in the parental cells sensitivity to Alimta- and raltitrexed-induced cytotoxicity (clonogenic assay) was similar to the sensitivity determined in the sulphorhodamine B assay. However, in A2780/CP70 cells, 1 μM of drug resulted in only 40–60% growth inhibition yet >85% cytotoxicity. After Alimta and raltitrexed exposure for ≤72 h, there were no differences between the A2780 and A278/CP70 cell lines in cell cycle phase distribution, absolute cell number, or the induction of apoptosis. However, the cellular protein content of the A2780/CP70 cells was 3–6-fold higher than in A2780 cells after Alimta and raltitrexed treatment, suggesting that cells without functional $p53$ can maintain protein synthesis in the absence of cell division (unbalanced cell growth). In conclusion, the apparent impact of functional $p53$ status on sensitivity to antifolate drugs may depend upon the pheno-

typic/genotypic background as well as the assay used to measure cellular sensitivity.

INTRODUCTION

Antifolate anticancer drugs are inhibitors of folate-dependent enzymes required for the de novo synthesis of nucleotides for DNA replication and have been used for cancer therapy for >50 years. Among these inhibitors, some are potent enzyme-specific compounds, whereas others directly or indirectly inhibit multiple targets. DHFR is the primary intracellular target of classical antifolate drugs such as MTX. Inhibition of DHFR leads to depletion tetrahydrofolate pools and, hence, the cofactors required for TS and GARFT, enzymes involved in the de novo thymidylate and purine metabolic pathways, respectively (1). Recently, a number of the new antifolate drugs have been developed, including the TS inhibitors raltitrexed (ZD1694), the multitargeted antifolate Alimta (pemetrexed disodium, MTA, and LY231514) and the GARFT inhibitors lometrexol and LY309887 (2–5). Alimta is unique among these compounds in that it has TS as a primary target and also inhibits DHFR and GARFT (6). These new antifolate drugs are potent inhibitors of cell growth in vitro with antitumor activity in vivo (1–6). All of these new classical antifolate drugs, like MTX, are subject to the same biochemical determinants of activity as natural folates, notably membrane transport and intracellular polyglutamation (7–9).

Recently, a number of studies have suggested that loss of wt $p53$ function may be a major reason underlying failure to respond to radiotherapy and chemotherapy in various human cancers (reviewed in Refs. 10 and 11). Many, although not all, in vitro studies suggest that tumor cells lacking $p53$ function are resistant to cytotoxic agents and radiation compared with cells with wt $p53$ (10, 12, 13). For example, O’Connor et al. (12) reported that the presence of a functional wt $p53$ gene was associated with increased drug sensitivity, in comparison with tumor cell lines with mu $p53$ in the 60 cell lines of the National Cancer Institute anticancer drug screen. Thus, the activity of the majority of clinically used anticancer agents, including DNA cross-linking agents, antimetabolites, and topoisomerase I and II inhibitors, was influenced by $p53$ status, whereas the activity of antimiotic agents tended to be independent of $p53$ status. Consistent with the results of O’Connor et al., Ju et al. (14) found that restoration of wt $p53$ function in $p53$-null HL-60 cells conferred multidrug sensitivity. However, other studies have produced contradictory results on the relationship between $p53$ status and chemosensitivity (10, 11). For example, inactivation

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3 The abbreviations used are: DHFR, dihydrofolate reductase; TS, thymidylate synthase; GARFT, glycaminde ribonucleotide formyltransferase; HPV, human papillomavirus; Alimta, multitargeted antifolate or LY231514; MTX, methotrexate; LTX, lometrexol; RT-PCR, reverse transcription-PCR; wt, wild-type; mu, mutant; SRB, sulphorhodamine B.
of p53 function in normal human foreskin fibroblasts by ectopic expression of the HPV16 E6 gene resulted in enhanced sensitivity to DNA-damaging agents, such as cisplatin, rather than resistance (15).

Mechanisms of resistance to antifolate drugs include increased target enzyme activity usually accompanied by enzyme gene amplification and/or reduced intracellular drug uptake and polyglutamation (1). However, relatively little is known about gene amplification and/or reduced intracellular drug uptake and increased target enzyme activity usually accompanied by enzyme gene amplification (15).

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The manner in which p53 status affects cellular sensitivity to antifolate drugs is complex and is influenced by many factors, including the presence of specific p53 point mutations and the ability of p53 to bind DNA and activate the expression of p53-responsive genes. Indeed, p53 status has been shown to be a critical determinant of cellular sensitivity to antifolate drugs, and the presence of p53 alterations has been associated with resistance to these drugs in a variety of tumor types.

**MATERIALS AND METHODS**

Reagents and Chemicals. All of the chemicals and MTX were obtained from Sigma Chemical Co. (Poole, United Kingdom). Alimta and lometrexol were gifts from Lilly Research Laboratories, Indianapolis, IN. Raltitrexed was purchased as the clinical product from Astra-Zeneca Ltd., Cheshire, United Kingdom.

Cell Culture. A549 cells were obtained from the National Cancer Institute (NIH, Bethesda, MD). MCF7, T-47D, and CCRF-CEM cells were purchased from the American Type Culture Collection (Rockville, MD). COR-L23 cells were kindly supplied by Dr. Peter Twentyman (Medical Research Council Clinical Oncology and Radiotherapeutics Unit, Cambridge, United Kingdom). HCT-116 and HCT-116/N7 cells were available in the authors' laboratory. HCT-116/N7 cells are a stably transfected HCT-116 cell line in which a plasmid containing a cytomegalovirus promoter driving inserted HPV16 E6 cDNA has been incorporated, such that the function of the p53 protein is disrupted by the E6 protein through the ubiquitin-proteasome pathway (19). A2780 and A2780/CP70 cells were kindly provided by Prof. R. Brown (Cancer Research Campaign Beatson Laboratories, Glasgow, Scotland). The A2780/CP70 cell line is 5-fold resistant to cisplatin relative to the parental A2780 cell line (20). All of the cell lines were adapted for growth in RPMI 1640 (Life Technologies, Inc., Paisley, United Kingdom) supplemented with 10% (v/v) dialyzed fetal bovine serum at 37°C in 5% CO2. The doubling times for the cell lines (determined by daily cell number estimation) were 17 h (HCT-116), 18 h (HCT-116/N7 and A2780/CP70), 20 h (A2780), 24 h (A549), 30 h (COR-L23), 38 h (CCRF-CEM), 48 h (MCF7), and 60 h (T-47D).

**p53 cDNA Sequencing.** To determine the p53 status of the cells, total RNA was extracted from human tumor cells using an oligo dT primer and reverse-transcriptase II (Life Technologies Ltd., Paisley, United Kingdom) according to the manufacturer’s instructions. Two p53-specific PCR primers were used to amplify the PCR product covering exons 4–10 of p53 cDNA in which the majority of p53 point mutations are found. RT-PCR product (~1 kb) was excised from the gel, and the sequence of the PCR product was determined by 4–6 overlapping p53-specific PCR primers using CEQ dye terminator cycle sequencing kit on a CEQ 2000 DNA analysis system (Beckman Coulter UK Ltd., High Wycombe, United Kingdom).

**Measurement of p53 and WAF1 Expression.** Induction of p53 and WAF1 protein after γ-irradiation was determined using total cell extracts prepared from control or γ-irradiated cells at 3, 6, and 24 h after treatment according to standard procedures (12). Protein concentration was determined using the BCA protein assay kit (Pierce, Rockford, IL) according to the manufacturer’s instructions. Thirty μg of protein from each sample was electrophoresed on 12.5% (w/v) SDS-PAGE gels and electroblotted on to nitrocellulose membrane (Amersham Pharmacia Biotech UK Ltd., Buckinghamshire, United Kingdom) in transfer buffer [20% (v/v) ethanol, 25 mM Tris, and 191 mM glycine] for 1 h at 65 V. The filter was cut into two parts at a molecular weight ~M, 33,000 and incubated with monoclonal antibodies against p53 (clone DO-7; Novacastra Laboratories, Newcastle upon Tyne, United Kingdom) or WAF1 (Oncoogene Research Products, Calbiochem-Novabiochem Ltd., Nottingham, United Kingdom), respectively. The signal was detected using the enhanced chemiluminescence (ECL +Plus; Amersham) system after addition of antimum IgG-HRP conjugate (DAKO, Ely, United Kingdom).

**Cell Cycle Arrest Studies.** To determine whether induction of p53 and WAF1 has functional consequences, cell cycle arrest studies were carried out as described by O’Connor et al. (12). Briefly, adherent exponentially growing cells were seeded into 60-mm plates at 2–10 × 105 cells/5 ml/plate. For CCRF-CEM cells, exponentially growing cells were diluted to 2.5 × 106 cells/5 ml/plate. After 20–24 h at 37°C, cells were irradiated at room temperature with 6.3 Gy of γ-rays using a 137Cs source (3.82 Gy/min) and then incubated at 37°C for 17–20 h in the presence or absence of the microtubule inhibitor nocodazole (0.4 μg/ml). Nocodazole primarily blocks cells in G2-M and does not allow them to re-enter G1 of a second cell cycle. The medium containing floating cells was combined with cells detached from the plates by trypsin digestion and then centrifuged (300 g, 5 min, room temperature). The cell pellets were washed once with ice-cold PBS and fixed in 70% ethanol/30% PBS (v/v) for ≥2 h. The fixed cells were rehydrated and incubated in PBS containing RNase (0.1 mg/ml) and propidium iodide (40 μg/ml) at 37°C for 30 min. Cell cycle analysis was performed on a Becton Dickinson FACSscan flow cytometer (San Jose, CA) using the LYSIS II program. At least 10,000 cells were used for each analysis. G1 arrest was expressed as percentage of G1 arrest as calculated by the formula:

\[
\% \text{G}_1 \text{ arrest} = \frac{G_1 \ % \text{ control with nocodazole}}{G_1 \ % \text{ control with nocodazole}} \times 100
\]

**Growth Inhibition Assays.** The sensitivity of the cell lines to antifolate-induced cell growth inhibition was determined using the SRB assay as described previously (21). Briefly, adherent exponentially growing cells were seeded into...
96-well plates at 2–5 × 10^3 cells/100 µl/well. After 20–24 h at 37°C, the medium was replaced with fresh medium containing antifolate drugs at the appropriate drug concentration. After a drug exposure period equivalent to three cell population doubling times, the cells were fixed with Carnoy’s fixative (methanol:acetic acid 3:1, v/v), washed, air dried, and stained with SRB, a cellular protein stain (22). The absorbance per well was measured at 570 nm on a Dynatech MR 7000 Plate Reader (Dynex, Billingshurst, United Kingdom).

Inhibition of CCRF-CEM cell growth was determined by cell counting. Cells were seeded in 24-well plates at 1 × 10^5 cells/500 µl/well. After 20–24 h incubation, an equal volume of fresh medium containing either PBS or antifolate drugs at 2 × final concentrations was added. The incubation was continued for the equivalent of three doubling times (114 h). The total cell number in each well was counted after fixation with Carnoy’s fixative on a model Z1 Coulter Counter (Coulter Electronics, Lytton, United Kingdom).

**Clonogenic Assays.** The cytotoxicity of antifolate drugs was determined in the A2780 and A2780/CP70 cell lines. Exponential growing cells were seeded into 100-mm Petri dishes at densities ranging from 150 to 500 cells/dish, the cell-seeding density being adjusted to give an estimated 20–300 colonies/dish after drug exposure. The cells were allowed to attach for 24 h, and Alimta or raltitrexed was added to give a final concentration of 1 µM or 10 µM. Three dishes were used for each drug concentration, and duplicate experiments were carried out for each set of experimental conditions. The cells were exposed to drugs for 24, 48, and 72 h, after which time the medium was aspirated and fresh drug-free medium containing 10% (v/v) fetal bovine serum added. The cells were incubated at 37°C for 7 days, after which time the medium was changed and fresh medium containing either PBS or antifolate drugs at 3 × final concentrations was added. The incubation was continued for the equivalent of three doubling times (114 h). The total cell number in each well was counted after fixation with Carnoy’s fixative on a model Z1 Coulter Counter (Coulter Electronics, Lytton, United Kingdom).

**Evaluation of the Effects of Alimta and Raltitrexed on Apoptosis, Cell Numbers, and Cellular Protein Levels in A2780 and A2780/CP70 Cells.** Exponentially growing cells were seeded into 90-mm Petri dishes at 2.5–3 × 10^6 cells/7 ml/dish. After 20–24 h incubation at 37°C, Alimta or raltitrexed were added to a final concentration of 1 µM. The cells were exposed to the drugs for 72 h, then washed, harvested, and the number of cells counted using a Coulter counter. Half of the cell population was centrifuged, resuspended in 20 µl of Carnoy’s fixative, and stained with 20 µl of Hoechst 33242 (8 µg/ml) prior to viewing under a fluorescent microscope for determination of apoptosis. Cellular protein levels were determined using the other half of the cell population and the Pierce BCA protein assay kit. Apoptotic cells were expressed as a percentage of the total cell population and protein as ng of protein/cell.

**Statistical Analyses.** Analyses of the relationships between the growth inhibition after three cell doubling times and p53 status were performed using a nonparametric t test (Mann-Whitney test), and relationships between antifolate sensitivity in the seven human cell lines were examined using linear regression analysis (GraphPad PRISM; Intuitive Software for Science, San Diego, CA).

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**RESULTS**

**Determination of p53 Status in the Human Tumor Cell Lines.** To investigate the impact of p53 status on cellular sensitivity to antifolate drugs, p53 status was examined by RT-PCR/PCR-based sequencing and functional assays (G1 phase arrest and p53 and WAF1 protein induction in response to γ-irradiation) in seven human tumor cell lines (A549, MCF7, T-47D, CCRF-CEM, COR-L23, A2780, and HCT-116). Functional wt p53 status was confirmed in A2780, A549, HCT-116, and MCF7 cells as strong G1 phase arrest (>60%), and the induction of p53 and WAF1 protein expression was observed after γ-irradiation in these cell lines (Figs. 1 and 2). T-47D and CCRF-CEM cells, however, lacked these responses to γ-irradiation indicative of a nonfunctional p53 status. cDNA sequencing confirmed the mu p53 status of these cell lines (Table 1). Moderate G1 phase arrest (22%) was found in COR-L23 cells (Fig. 1). However, no p53 or WAF1 protein expression was observed in either untreated or γ-irradiated COR-L23 cells (Fig. 2). Sequence analysis revealed a 402-bp deletion corresponding to p53 gene exon/intron boundaries from exon 2 to exon 4 including the putative p53 protein start codon and NH2-terminal 125 amino acids in this cell line (Table 1).

In addition to the seven human tumor cell lines described above, related counterparts of two of the p53 wt cell lines with nonfunctional p53 were studied, namely HCT-116/N7 (p53 disrupted) and A2780/CP70 (p53 mu). Vikhanskaia et al. (19) have reported that the function of p53 protein in the HCT-116/N7 cell line had been disrupted by binding to the HPV16 E6 protein and degradation through the ubiquitin-proteasome pathway. In cisplatin-resistant A2780/CP70 cells, a mixture of point mu (GTT → TTT) and wt p53 sequences were detected in the p53 cDNA sequence, the mutation being at codon 172 (Val → Phe), which suggests that this cell line has a heterozygous mu p53 status (Table 1). In response to γ-irradiation, there was only weak G1 arrest in both the A2780/CP70 and HCT-116/N7 cell lines compared with their p53 wt parental counterparts (Fig. 1). Furthermore, Western blotting showed that in contrast to the parental cell lines, there was no induction of p53 or WAF1.
proteins in the A2780/CP70 and HCT-116/N7 cells after γ-irradiation (Fig. 2).

Fig. 2 Basal levels of p53 and WAF1 protein expression and induction after γ-irradiation. Exponential growing cells were γ-irradiated at 6.3 Gy and lysed at times indicated. Western blotting was carried out by using monoclonal antibody against p53 and WAF1 proteins as described in “Materials and Methods.”

Comparison of Antifolate Sensitivities in Human Tumor Cell Lines. Four mechanistically distinct antifolate drugs were selected for investigation: Alimta, MTX, lometrexol, and raltitrexed. Growth inhibition was determined by the SRB assay or cell counting in seven human tumor cell lines, including six adherent solid tumor cell lines (A549, MCF7, T-47D, COR-L23, A2780, and HCT-116) and one suspension hematological cell line (CCRF-CEM), each treated with the four antifolate drugs for three cell doubling times. A summary of the IC50s, defined as the concentration of antifolate required to inhibit growth by 50%, is given in Table 2. In general, HCT-116 and A549 cells were the least sensitive to all of the four antifolate drugs compared with the other five cell lines. In addition, lometrexol and Alimta were less potent (mean IC50s: 39 and 67 nM, respectively) than raltitrexed and MTX (mean IC50s: 3.5 and 7.5 nM, respectively; Table 2). There were significant linear

Table 1 p53 gene status in human tumor cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Cancer type</th>
<th>p53 sequence codon change</th>
<th>p53 sequence amino acid change</th>
<th>p53 status</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCF7</td>
<td>Breast</td>
<td>wt/wt</td>
<td></td>
<td>wt</td>
</tr>
<tr>
<td>T-47D</td>
<td>Breast</td>
<td>194 CTT→TTT</td>
<td>Leu→Phe</td>
<td>mu</td>
</tr>
<tr>
<td>A549</td>
<td>Lung</td>
<td>wt/wt</td>
<td></td>
<td>wt</td>
</tr>
<tr>
<td>COR-L23</td>
<td>Lung</td>
<td>exon 2–4 del</td>
<td>125 aa del</td>
<td>mu (del)</td>
</tr>
<tr>
<td>CCRF-CEM</td>
<td>Leukemia</td>
<td>248 CGG→CAG</td>
<td>Arg→Gln</td>
<td>mu</td>
</tr>
<tr>
<td>HCT-116</td>
<td>Colon</td>
<td>wt/wt</td>
<td></td>
<td>E6-degraded</td>
</tr>
<tr>
<td>HCT-116/N7</td>
<td>Colon</td>
<td></td>
<td></td>
<td>wt</td>
</tr>
<tr>
<td>A2780</td>
<td>Ovary</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A2780/CP70</td>
<td>Ovary</td>
<td>172 GTT→G/T7T</td>
<td>Val→Val/Phe</td>
<td>heterozygous mu</td>
</tr>
</tbody>
</table>

*del, deleted p53; aa, amino acids.
Table 2  Antifolate-induced growth inhibition in human tumor cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>p53 status</th>
<th>IC_{50} nM&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Alimta</th>
<th>MTX</th>
<th>Lomotrexol</th>
<th>raltitrexed</th>
</tr>
</thead>
<tbody>
<tr>
<td>A549</td>
<td>wt</td>
<td>156 ± 108 (4)</td>
<td>11 ± 7 (4)</td>
<td>71 ± 9 (4)</td>
<td>7.8 ± 1.0 (4)</td>
<td></td>
</tr>
<tr>
<td>HCT-116</td>
<td>wt</td>
<td>134 ± 82 (4)</td>
<td>5.5 ± 0.4 (3)</td>
<td>54 ± 9 (3)</td>
<td>6.3 ± 1.1 (3)</td>
<td></td>
</tr>
<tr>
<td>A2780</td>
<td>wt</td>
<td>59 ± 28 (3)</td>
<td>4.4 ± 1.1 (3)</td>
<td>32 ± 21 (3)</td>
<td>2.8 ± 0.9 (3)</td>
<td></td>
</tr>
<tr>
<td>MCF7</td>
<td>wt</td>
<td>31 ± 9 (3)</td>
<td>3.4 ± 0.4 (3)</td>
<td>21 ± 16 (4)</td>
<td>1.4 ± 0.7 (3)</td>
<td></td>
</tr>
<tr>
<td>COR-L23</td>
<td>del</td>
<td>20 ± 3 (3)</td>
<td>7.3 ± 0.7 (3)</td>
<td>26 ± 17 (3)</td>
<td>0.6 ± 0.2 (3)</td>
<td></td>
</tr>
<tr>
<td>T-47D</td>
<td>mu</td>
<td>15 ± 13 (4)</td>
<td>18 ± 11 (4)</td>
<td>44 ± 16 (4)</td>
<td>0.4 ± 0.1 (4)</td>
<td></td>
</tr>
<tr>
<td>CCRF-CEM</td>
<td>mu</td>
<td>54 ± 6 (3)</td>
<td>2.8 ± 1.0 (3)</td>
<td>28 ± 8 (4)</td>
<td>5.5 ± 0.5 (3)</td>
<td></td>
</tr>
<tr>
<td>A2780/CP70</td>
<td>heterozygous mu</td>
<td>&gt;10 μM (3)</td>
<td>10 ± 4 (3)</td>
<td>46 ± 9 (3)</td>
<td>30 ± 18 (3)</td>
<td></td>
</tr>
<tr>
<td>HCT-116/N7</td>
<td>E6-degraded</td>
<td>850 ± 96 (3)</td>
<td>7.3 ± 0.7 (3)</td>
<td>66 ± 7 (3)</td>
<td>9.4 ± 0.5 (3)</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Data are mean ± SD with the number of the experiments given in parenthesis. IC_{50}s were calculated by fitting a sigmoidal concentration/inhibition curve to the results using nonlinear least square regression (GraphPad PRISM) to data generated by direct cell counting (CCRF-CEM) or the SRB assay.

was the only suspension cell line, and, thus, the IC_{50} for this cell line was determined by cell counting rather than by the SRB assay. Omitting the CCRF-CEM data, lack of functional p53 may be associated with sensitivity to Alimta- or raltitrexed-induced growth inhibition, although additional cell lines would need to be investigated to confirm this observation. It is interesting to note that in cells with wt p53, there was a consistent rank order of sensitivity to all of the four antifolate drugs, i.e., MCF7 > A2780 > HCT-116 > A549 (Fig. 4).


Antifolate sensitivity was then investigated in the two pairs of related cell lines (HCT-116:HCT-116/N7 and A2780:A2780/CP70). Growth inhibition produced by the four antifolate drugs (Alimta, raltitrexed, MTX, and LTX) was studied by using the SRB assay (Fig. 5). Cells with wt p53 (A2780 and HCT-116) were more sensitive to both Alimta- and raltitrexed-induced growth inhibition than those with mu or disrupted p53 (A2780/CP70 and HCT-116/N7; P = 0.002). In wt p53, A2780 cell growth was completely arrested after exposure to high concentrations of Alimta (>1 μM) and raltitrexed (>10 μM). In contrast, A2780/CP70 cells appeared resistant to high concentrations of Alimta (>1 μM) and raltitrexed (>10 μM), with growth remaining at ~50% of control after exposure to Alimta and 40% of control after exposure to raltitrexed (Fig. 5). In contrast to Alimta and raltitrexed, A2780/CP70 cells were not markedly less sensitive to MTX or lometrexol (Fig. 5) than A2780 cells.

Comparison of Alimta- and Raltitrexed-induced Cytotoxicity, Cell Cycle Arrest, and Apoptosis in A2780: A2780/CP70 and HCT-116:HCT-116/N7 Cell Lines. Potential mechanisms underlying the apparent differences in sensitivity to Alimta and raltitrexed between related and unrelated cell lines with functional or nonfunctional p53 were additionally investigated. Clonogenic assays were undertaken to examine whether the differences in sensitivity observed in the SRB growth inhibition assay reflected differences in sensitivity to Alimta- and raltitrexed-induced cytotoxicity. As the biggest differences in Alimta- and raltitrexed-induced growth inhibition were found in the A2780 and A2780/CP70 cell lines (Fig. 5), this pair of cell lines was investigated additionally using clonogenic assays and cell counting. A2780 and A2780/CP70 cells were treated with 1 μM and 10 μM of Alimta or raltitrexed for...
24, 48, and 72 h, after which, medium was replaced with drug-free medium containing nondialyzed FCS for an additional 10–14 days. The A2780 cell line was more sensitive than A2780/CP70 cells to Alimta and raltitrexed cytotoxicity at all of the three time points studied (P, 0.05), and cytotoxicity increased with increasing exposure time in both cell lines (data not shown). In A2780 cells, sensitivity to a 72-h exposure to Alimta or raltitrexed determined by clonogenic assay (99% inhibition of survival) was similar to that measured in the SRB growth inhibition assay (100% inhibition of growth; Table 3). In contrast, in the A2780/CP70 cells a 72-h drug exposure produced survival to <15% of control yet inhibited growth by only 40–60% (Table 3). Thus, the differential sensitivity of the A2780 and A2780/CP70 cell lines to Alimta and raltitrexed was far less marked when sensitivity was measured using a clonogenic cell survival assay as compared with the SRB growth inhibition assay.

The SRB assay is an indirect measure of cell growth, which uses total cellular protein as a surrogate for cell number. To determine whether or not the SRB assay actually reflected the effect of drug treatment on cell number, cell numbers and the corresponding protein content of cells were measured after 72-h drug exposures. As shown in Table 3, in contrast to the SRB assay data, there were similar levels of growth inhibition of A2780 and A2780/CP70 cells after drug treatment when cell number was measured by counting. However, the protein content of A2780/CP70 cells was higher (3-fold for Alimta and 6-fold for raltitrexed) than in A2780 cells after 72 h of treatment. This result suggests that the cells without functional p53 may become enlarged in response to antifolate exposure and as a consequence have a higher cellular protein content. In comparison with untreated control cells, protein content decreased by ≤50% in A2780 cells, whereas it increased ~2-fold in A2780/CP70 cells after a 72-h Alimta or raltitrexed exposure.

Cell cycle effects after treatment with Alimta or raltitrexed were examined by flow cytometry in both A2780:A2780/CP70 and HCT-116:HCT-116/N7 cell lines. Cells were exposed to Alimta and raltitrexed at concentrations equivalent to 1-3, 10-3, and 100-3 IC50s; harvested at 0, 24, and 48 h; and analyzed by flow cytometry. The percentage of cells in G1, S, G2 + M, or sub-G1 fractions was calculated for each treatment as shown in Table 3. After a 24-h exposure to Alimta or raltitrexed, both A2780 cells and A2780/CP70 cells accumulated in the G1 phase. At 48 h, cells treated with raltitrexed remained arrested in G1, whereas cells treated with Alimta gradually re-entered S-phase (Fig. 6). Even after 72-h exposure to Alimta or raltitrexed, only a very small proportion of apoptotic cells was detected by microscopic evaluation of Hoechst 33242-stained cells in either cell line (Table 3).

In the pair of HCT-116 cell lines, no G1 phase accumulation was observed in HCT-116 cells (wt p53) after treatment with either Alimta or raltitrexed, whereas G2 + M phase arrest was apparent in HCT-116/N7 cells (disrupted p53 function). It was noted that the population of sub-G1-phase events in HCT-116 cells was much higher after either Alimta (~25%) or raltitrexed (~50%) treatment than in HCT-116/N7 (~<20%), suggesting that HCT-116 cells may have a greater ability than A2780 cells to engage an apoptotic cell death pathway in response to antifolate drugs, engagement which is initially p53 dependent.

DISCUSSION

In general, two sets of parameters can influence cellular sensitivity to antifolate drugs, i.e., processes prior to (e.g., antifolate transport and polyglutamation) or after (e.g., p53...
induction, cell cycle arrest, and apoptosis) target enzyme inhibition. In attempting to define the relative contributions of these two sets of parameters to the differential sensitivity of cell lines, it is important to study relationships between extracellular drug concentration, nucleotide pool depletion, and growth inhibition. For example, in the case of the antipurine antifolate LY309887, in four of five cell lines studied, cell growth inhibition and cytotoxicity were only observed once intracellular ATP concentrations fell below 1 mM despite markedly different extracellular LY309887 concentrations being required to induce this level of ATP depletion (22). This latter result suggests that antifolate transport, polyglutamation, target enzyme levels, and/or purine salvage are primary determinants of sensitivity to LY309887. In the studies described herein, the relationship between p53 status and sensitivity to four clinically used antifolate drugs was investigated in a panel of seven human tumor cell lines from a variety of tissues and in two pairs of related cell lines with or without disruption of p53 function.

Prior to undertaking chemosensitivity studies, the p53 status of the cell lines was determined by RT-PCR/PCR-based
sequencing and functional assays (G_1 phase arrest and p53 and WAF1 protein induction in response to g-irradiation). The p53 status of most of the cell lines was consistent with published results, i.e., A549, MCF7, T-47D, CCRF-CEM, A2780, and HCT-116 (Table 1; Refs. 12, 19, 23). A heterozygous mu p53 was detected in the A2780/CP70 cell line, which has lacked functional p53 because of its derivation (20), although its p53 sequence was reported previously to be wt (23). It is possible that genetic instability in the A2780/CP70 cell line attributable to mismatch repair deficiency (which confers resistance to cisplatin in these cells) has led to the acquisition of the p53 mutation reported here. Although a moderate G_1 phase arrest (22%) was found in COR-L23 cells, there was neither basal nor g-irradiation-induced p53 or WAF1 protein expression. Se-

Table 3  Growth inhibition, protein content and apoptosis in A2780 and A2780/CP70 cells after 72 hours incubation with Alimta or raltitrexed

<table>
<thead>
<tr>
<th>Antifolates (1 μM)</th>
<th>A2780 Cell line</th>
<th>A2780/CP70 Cell line</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Alimta</td>
<td>raltitrexed</td>
</tr>
<tr>
<td>Cell growth (% control)</td>
<td>0 ± 9.8</td>
<td>0 ± 6.8</td>
</tr>
<tr>
<td>SRB assay</td>
<td>5.5 ± 1.6</td>
<td>3.1 ± 1.5</td>
</tr>
<tr>
<td>Cell counting</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Protein content (ng/cell)</td>
<td>0.09 ± 0.09</td>
<td>0.05 ± 0.03</td>
</tr>
<tr>
<td>(% control)</td>
<td>84%</td>
<td>47%</td>
</tr>
<tr>
<td>Clonogenic survival (% control)</td>
<td>1.3, 3.0</td>
<td>0.0, 0.6</td>
</tr>
<tr>
<td>Apoptotic fraction (% total cells)</td>
<td>1.7 ± 0.4</td>
<td>2.4 ± 0.6</td>
</tr>
</tbody>
</table>

* Data are mean ± SD with three experiments.
* Control protein content (ng/cell): A2780, 0.10 ± 0.03; A2780/CP70, 0.15 ± 0.02.
* Data are from two experiments.

Fig. 6  Cell cycle effects of Alimta and raltitrexed treatment at 10×IC_{50}s in A2780, A2780/CP70, HCT-116, and HCT-116/N7 cells, harvested at 0, 24, and 48 h. Percentage of cells containing sub-G_1 (■), G_1 (□), S (■), and G_2 + M (●) phase DNA in each treatment population analyzed by flow cytometry. Data are from a single representative experiment.
sequencing data showed that a sequence from exons 2 to 4, including the putative start codon and NH₂-terminal 125 amino acids of the p53 protein, was deleted, indicative of a deleted and, hence, nonfunctional p53 status. This result is consistent with the observation that the exon 4 could not be amplified from COR-L23 genomic DNA.⁴

In growth inhibition assays, there was a consistent rank order of cellular sensitivity for the four wt p53 cell lines to the antifolate drugs, which may reflect the influence of well-defined determinants of antifolate sensitivity such as carrier-membrane transport and polyglutamation (Table 2). In addition, there was a relationship between the IC₅₀ values obtained with Alimta and those of raltitrexed (r² = 0.92, P < 0.003) and lometrexol (r² = 0.84, P < 0.02), which could reflect the ability of Alimta to inhibit both TS and GARFT (Table 2 and Fig. 3; Ref. 6). The relationship between Alimta and raltitrexed sensitivity was highly significant; however, the correlation between Alimta and lometrexol, although statistically significant, may not be biologically important, because there was relatively little variation in sensitivity to lometrexol (Fig. 3).

Notably, apart from the suspension cell line CCRF-CEM, there was a possible relationship between the sensitivity of the six adherent solid tumor cell lines to Alimta and raltitrexed and p53 status, with the mu cell lines being more sensitive (Fig. 4). However, tumor cell lines carry a multitude of molecular genetic abnormalities, altered expression of target proteins, as well as normal polymorphisms, such that any impact of p53 status alone may be difficult to identify. To address this difficulty, two pairs of related cell lines were investigated that have common genetic abnormalities, altered expression of target proteins, as well as normal polymorphisms, such that any impact of p53 status alone may be difficult to identify. To address this difficulty, two pairs of related cell lines were investigated that have common genetic backgrounds: the HCT-116 and HCT-116/N7 and A2780 and A2780/CP70 cell line pairs. The anticipated functional p53 status of the two pairs of related cell lines was confirmed (Fig. 1 and 2; Table 1) and sensitivity to antifolate-induced growth inhibition studied (Fig. 5). For MTX and lometrexol there was again no major impact of functional p53 status on cellular antifolate sensitivity. However, for both Alimta and raltitrexed, the cell lines with functional p53 were more sensitive as measured by the SRB growth inhibition assay. The effect was most notable in the case of A2780-A2780/CP70 cells where the maximum inhibition of cell growth in the p53 mu cells was only ~50%, as opposed to 100% in the p53 wt A2780 cells (Fig. 5). Conclusions regarding the impact of p53 must, however, be tempered with the observation that A2780/CP70 cells are a drug-selected cell line and may therefore also have other phenotypic or genotypic alterations in addition to alterations in p53 and mismatch repair. However, it is unlikely that the changes associated with cisplatin-resistance will also affect common determinants of classical antifolate activity.

Possible mechanisms underlying the apparent impact of p53 on the sensitivity of A2780 cells to Alimta and raltitrexed were additionally investigated using clonogenic cytotoxicity assays, which revealed that the magnitude of the differential was not as marked as in the SRB assay (Table 3). Thus, an additional explanation must underlie the effect observed in the SRB assay. The SRB assay measures cellular protein and, hence, can give misleading results if the protein level per cell is altered by drug treatment. After exposure to 1 μM of Alimta or raltitrexed (>IC₉₀), in A2780 and A2780/CP70 cells, there were marked differences in the cellular protein content of the two cell lines (Table 3). Protein levels increased by ~2-fold in the A2780/CP70 cells while decreasing (raltitrexed) or remaining the same (Alimta) in A2780 cells. Thus, the differential sensitivity of the A2780 and A2780/CP70 cells to Alimta and raltitrexed treatment seen in the SRB assay is primarily a reflection of the differential effects of drug treatment on cellular protein content. The phenomenon of “unbalanced growth,” i.e., cessation of DNA synthesis and, hence, cell replication but maintenance of protein synthesis, is well known after cytotoxic drug treatment. In contrast to A2780/CP70 cells, the effects of Alimta and raltitrexed as measured by the SRB, cell counting, and clonogenic assays were similar in A2780 cells (Table 3) and, hence, the lack of unbalanced growth in A2780 cells may be a p53-dependent phenomenon.

Apoptosis is one mechanism by which p53 could influence cellular protein levels after antifolate treatment; however, levels of apoptotic cells as measured either by morphological assessment (72 h; Table 3) or the appearance of cells with a sub-G₁ DNA content in flow cytometric analyses (24 and 48 h; Fig. 6) were similar in the A2780 and A2780/CP70 cell lines after Alimta and raltitrexed treatments. Interestingly, in HCT-116 cells, both Alimta and raltitrexed caused an increase in cells with a sub-G₁ DNA content, whereas in p53 nonfunctional HCT-116-N7 cells there was an accumulation of cells in G₁ (Fig. 6). Previously, cell lines with mu p53 have been reported to be both sensitive (CCRF-CEM) and resistant (HT29) to Alimta-induced apoptosis (24, 25).

In conclusion, on the basis of data generated using the SRB growth inhibition assay in the pairs of related cell lines (A2780 and A2780/CP70; HCT-116 and HCT-116/N7), cells with wt p53 status were more sensitive to both Alimta- and raltitrexed-induced growth inhibition. However, in contrast, in a panel of unrelated human tumor cell lines, functional p53 status was possibly associated with cellular resistance to Alimta and raltitrexed. Different conclusions regarding the relationship between functional p53 status and sensitivity to Alimta and raltitrexed can therefore be obtained dependent on the genetic background of the model system used. Furthermore, the assay system used, in particular the SRB method, and cellular factors other than p53, can influence the apparent impact of p53 on cellular sensitivity to antifolate drugs. p53 status alone is therefore unlikely to be a useful predictor of antifolate efficacy.

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REFERENCES


⁴ P. Rabbitts, personal communication.
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The Impact of $p53$ Status on Cellular Sensitivity to Antifolate Drugs

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