Comparison of Thymidylate Synthase (TS) Protein Up-Regulation after Exposure to TS Inhibitors in Normal and Tumor Cell Lines and Tissues

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ABSTRACT

Thymidylate synthase (TS) is an important target for cancer chemotherapy. However, several mechanisms of resistance to TS inhibitors have been described. One mechanism that may be relevant to short-term exposure to TS inhibitors occurs as a result of disruption of the autoregulatory loop, which allows TS to control its own translation. This disruption leads to up-regulation of TS protein and is generally thought to decrease efficacy. This study has investigated TS protein up-regulation using a range of TS inhibitors in both tumor and nonmalignant cell lines in vitro and in vivo.

Up-regulation of TS protein showed a time-, dose-, and cell-type-specific response to treatment with ZD9331. This response was observed in W1L2 cells treated for 24 h at equitoxic doses of raltitrexed (6-fold), ZD9331 (10-fold), fluorouracil (5-fold), LY231514 (7-fold), AG337 (7-fold), and BW1843U89 (3-fold). Up-regulation was observed over a range of doses. Elevation of TS protein only persisted up to 12 h after removal of drug. The extent of induction does not depend on basal TS levels. Nontransformed human fibroblasts showed significantly greater up-regulation of TS protein than tumor cells exposed to an equitoxic dose of ZD9331. In vivo experiments using the L5178Y thymidine kinase −/− mouse lymphoma implanted into DBA2 mice also showed greater up-regulation of TS protein in normal intestinal epithelial cells compared with tumor cells.

These results confirm that TS up-regulation is a common feature of TS inhibition in tumor cells and that it may occur to a greater extent in normal tissues, although the clinical implications of these findings remain to be determined.

INTRODUCTION

TS is a folate-dependent enzyme that catalyzes the reductive methylation of dUMP using 5,10-methylenetetrahydrofolate as a one-carbon donor to dTMP. Because TS represents the sole de novo source of thymidylate (dTTP), which is essential for DNA replication and repair (1), the enzyme has become an important target for cancer chemotherapy (2).

The importance of TS as a chemotherapeutic agent is now well established. 5-FU, a fluoropyrimidine drug, is widely used in the treatment of breast, gastrointestinal, and head and neck cancers (3). Several TS inhibitors have recently been clinically evaluated. These include the quinazoline antifolates, raltitrexed (Tomudex, ZD1694; Ref. 4) and ZD9331 (5, 6), MTA (7), a multitargeted antifolate, BW1843U89 (8), and the nonclassical lipophilic TS inhibitor, AG337 (9).

Several acquired mechanisms of resistance to TS inhibitors have been reported: (a) overexpression of the target enzyme, resulting in elevated levels of TS protein (10, 11, 12), (b) impaired drug uptake, e.g., because of down-regulation or mutation of reduced folate carrier (11, 13), and (c) diminished polyglutamation (11, 14, 15).

However, another mechanism by which transient resistance to TS inhibitors can occur has been described and may have important consequences to therapeutic outcome (16, 17). In the G1 phase of the cell cycle, TS protein binds tightly to TS mRNA preventing translation of TS protein. On entering the S phase when thymidylate is required for DNA replication, TS undergoes a conformational change on binding its substrate dUMP and the folate cofactor. TS is no longer able to bind to TS mRNA, thereby allowing translation. A similar effect is seen when TS binds to an inhibitor with the result that TS can no longer bind to TS mRNA in any phase of the cell cycle leading to up-regulation of TS protein. Recently, an alternative model proposed that the up-regulation of TS protein after exposure to TS inhibitors is attributable to a stabilization of the TS protein rather than an increase in translation (18). Regardless of the mechanism, the acute increase in TS observed after exposure to TS inhibitors may have important consequences to therapeutic outcome because induction of the target may result in decreased inhibition, although this may only be relevant if normal cells do

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3 The abbreviations used are: TS, thymidylate synthase; 5-FU, fluorouracil; MTA, LY231514; TK, thymidine kinase; DFM, drug-free medium; ODN, oligodeoxynucleotide.
not up-regulate TS to a similar or greater extent. Indeed, previous studies have suggested that induction of TS in normal cells is generally more responsive to the effects of 5-FU, particularly at higher doses (19). In addition, normal mammary epithelial cells demonstrated a 40-fold up-regulation compared with only 10-fold in cancer mammary epithelial cells after treatment with raltitrexed (17).

In the present study, a variety of human tumor cell lines and nontransformed fibroblasts were used to determine the extent of TS up-regulation in vitro after treatment with a range of TS inhibitors. In addition, in vivo experiments using a mouse lymphoma model were carried out. The results confirmed that TS up-regulation is a common feature of TS inhibition in tumor cells and that it may also occur to a greater extent in normal tissues.

**MATERIALS AND METHODS**

**Cell Lines.** The following cell lines were used in this study: A549 (human lung carcinoma), W1L2 wild-type (human lymphoblastoid) and drug-resistant variants [W1L2R<sup>299331</sup> (5), W1L2R<sup>D1694</sup> (11), W1L2C1 (20), W1L2R<sup>865</sup> (21), and W1L2R<sup>D179</sup> (12)], HT29, SW480 (human colon carcinomas), HX62, A2780 (human ovarian carcinomas), LS178Y TK −/− and +/− (mouse lymphoma), and normal nontransformed human fibroblasts. All cell lines except fibroblasts were obtained from “in-house” tissue stores (Cancer Research Campaign Center for Cancer Therapeutics, Institute of Cancer Research, Sutton, United Kingdom). Fibroblasts were kindly supplied by Dr. John Eady (Institute of Cancer Research, Sutton, United Kingdom). All cells were maintained as exponentially growing cultures in DMEM (Life Technologies, Inc., United Kingdom; attached cells) or RPMI (Life Technologies, Inc., United Kingdom; suspension cells) supplemented with 10% heat-inactivated dialyzed FCS and antibiotics (both Life Technologies, Inc., United Kingdom) at the time of this study.

**Compounds.** All standard laboratory chemicals were AnalR grade purchased from either British Drug Houses (BDH, Poole, United Kingdom) or Sigma (Poole, United Kingdom).

ZD9331, raltitrexed, and AG337 were synthesized at Zeneca Pharmaceuticals (Macclesfield, United Kingdom). MTA and BW1843U89 were generously supplied by Eli Lilly and Company (Indianapolis, IN) and Glaxo-Welcome Pharmaceuticals (Stevenage, Hertfordshire, United Kingdom), respectively.

5-FU was purchased from Sigma. All compounds except AG337 were dissolved at 10 mM in 100% DMSO. The dissolved compounds were then passed through a 0.22-µm filter and stored at −20°C for a maximum of 3 months.

**Drug Exposure.** To measure changes in TS levels, cell lines were exposed to TS inhibitors for 16 or 24 h at doses standardized to IC<sub>50</sub> values. IC<sub>50</sub> values were determined using 72-h (all cell lines except A549) or 120-h (A549) 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assays as described previously (Ref. 22; adherent cell lines) or by cell counting using a Coulter counter (suspension cell lines). At the end of drug exposure, cells were harvested and washed with PBS, and protein concentrations for Western blotting were measured using the bicinchoninic acid protein assay according to the manufacturer’s instructions (Pierce, United Kingdom).

**Western Blotting.** Analysis of TS protein levels was performed as described previously (23). Briefly, total cellular protein was isolated from cells in lysis buffer containing proteinase inhibitors, and equal amounts (50 µg) of protein were separated by SDS-PAGE (8–16% tris/glycine gels purchased from NOVEX, Germany) and electroblotted to a nitrocellulose membrane. Recombinant human TS (generously supplied by Agouron, San Diego, CA) was used as a positive control. Immunoblotting was performed using a rabbit polyclonal antibody to recombinant human TS (23), followed by horse radish peroxidase-conjugated antirabbit secondary antibody. Proteins were detected using enhanced chemiluminescence according to the manufacturer’s instructions (Amersham Life Sciences, Bucks, United Kingdom).

**Confocal Microscopy.** A549 cells were grown to 60% confluency on coverslips. The cells were then treated for 24 h with varying doses of ZD9331. After treatment, the cells were fixed (4% paraformaldehyde in PBS, room temperature, 30 min), washed three times with PBS, and permeabilized (0.1% Triton X-100, 10 min). The cells were washed three times with PBS, nonspecific binding was blocked with PBS/0.5% BSA, and the cells were incubated for 1 h in primary antibody [anti-TS (23)] diluted 1:150 in PBS/0.5% BSA for 30 min, and the cells were incubated for 1 h in primary antibody [anti-TS (23)] diluted 1:150 in PBS/0.5% BSA. After a further three washes in PBS, the cells were incubated for 1 h with secondary antibody (Alexa 488 goat antirabbit IgG conjugate, Molecular Probes, Cambridge, United Kingdom) diluted 1:200 in PBS/0.5% BSA, and nuclei were labeled with propidium iodide after treatment with RNase. Slides were then mounted using Vectashield (Vector Laboratories, Peterborough) and examined at 488 nm on a TCS-SP confocal microscope (Leica, Milton Keynes, United Kingdom).

**ELISA.** TS protein concentrations in W1L2 cells (wild-type and acquired resistant variants; Refs. 5, 11, 20, and 21) were determined using ELISA (23). Information generated from this method was used to validate and quantify the flow cytometry method described below.

**Flow Cytometry to Determine TS Protein Concentrations.** A flow cytometry method was evaluated to measure TS protein in cells. Appropriately drug-treated cells (1 × 10<sup>6</sup>) were washed with PBS + 1% FCS + 0.01% Na<sub>2</sub>CO<sub>3</sub> (sodium azide; 5 min, 1500 rpm, 25°C), fixed (1 ml 2% paraformaldehyde, 10 min), and permeabilized (50 µl 0.1% Triton X-100, 10 min). The cells were split into two aliquots and washed twice (as above). One aliquot of cells was then treated with 100 µl of TS antibody (Ref. 23; 1/60 in PBS, 1 h), the other was treated with 100 µl of normal rabbit serum (1/60 in PBS, 1 h). The optimal dilutions were found by titration of the antibodies. One hundred µl of second antibody [goat antirabbit FITC (Amersham, Buckinghamshire, United Kingdom) 1/100 in PBS] were added to each aliquot after washing (as above), and samples were analyzed within 2 h by flow cytometry after two washes (as above). To quantify the difference in TS between samples, the following ratio was calculated:

\[
\text{Ratio} = \frac{\text{mean fluorescence of anti-TS peak}}{\text{mean fluorescence of isotype peak}}
\]
TS Protein Up-Regulation in Cell Lines and Tissues

Snap-frozen in liquid nitrogen. Samples were stored at −70°C before analysis for TS protein using Western blotting.

Statistical Analysis. An ANOVA two-way statistical analysis was performed using Graphpad Prism.

RESULTS

Validation of a Novel Flow Cytometry Method to Measure TS Protein. Several methods are presently in use to measure TS protein, including Western blotting and ELISA (23, 24, 25). However, these methods require large numbers of cells and are relatively time consuming. A flow cytometry method was therefore evaluated to enable reproducible quantitation of TS protein in cells. A standard curve was constructed by plotting the arbitrary ratio obtained from flow cytometry against protein concentrations measured in a series of cell lines with acquired

Table 1: Up-regulation of TS protein using Western blotting

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Average densitometry reading above background</th>
<th>Average fold up-regulation compared with control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (untreated)</td>
<td>13014 ± 101</td>
<td>1</td>
</tr>
<tr>
<td>24 h ZD9331</td>
<td>46151 ± 256</td>
<td>3.6</td>
</tr>
<tr>
<td>48 h ZD9331</td>
<td>49065 ± 278</td>
<td>3.8</td>
</tr>
</tbody>
</table>

In Vivo Studies. Six-week-old female DBA2 mice were obtained from Harlan UK Ltd (Bicester, Oxon) and randomly allotted to one of three treatment groups and allowed free access to a standard diet (RM1). The experiment was approved by the local ethical committee. Animals were treated when 8 weeks old. L5178Y TK−/− cells were grown to 60% confluency in RPMI medium. The cells were then harvested (1500 rpm, 5 min), counted using a hemocytometer, and diluted to 1 × 10⁶ cells/ml in sterile saline. Cells (5 × 10³; 50 μl) were implanted into the gastrocnemius muscle of 17 mice. The tumor was allowed to reach 8–9 mm in diameter after which 10 mice were treated with ZD9331 (50 mg/kg i.p. bolus). Five drug-treated mice and three (7 h) or four (16 h) untreated mice were sacrificed by cervical dislocation either 7 or 16 h after injection. Tumors and gut scrapes were collected immediately. Fifteen cm of gut starting 5 cm from the stomach were removed, flushed with ice-cold PBS, slit along its longitudinal axis, and scraped using the back of a scalpel blade. All tissue harvested was immediately homogenized in 0.3 ml of lysis buffer (23) and snap-frozen in liquid nitrogen. Samples were stored at −70°C before analysis for TS protein using Western blotting.

Statistical Analysis. An ANOVA two-way statistical analysis was performed using Graphpad Prism.

Table 2 shows results on W1L2 and A549 cells using flow cytometry. Greater induction of TS protein can be seen using flow cytometry than using Western blotting (9-fold compared with 3-fold up-regulation in A549 cells under similar conditions of ZD9331 exposure). A dose- and time-dependent response can be observed in both A549 and W1L2 cells after treatment for either 16 or 24 h with increasing concentrations of ZD9331. No statistically significant up-regulation is observed in either cell line below 7 × IC₅₀ values after 16-h treatment with ZD9331, but statistically significant up-regulation is observed with 1 × IC₅₀ values after 24-h treatment with ZD9331 (P < 0.001). Over a 24-h time course, W1L2 cells show significantly greater up-regulation of TS protein than A549 cells (5-fold up-regulation in A549 cells compared with 10-fold in W1L2 cells at 100 × IC₅₀ values of drug; P < 0.001).

TS Protein Is Up-Regulated after Exposure to ZD9331. The time course of up-regulation of TS protein was determined after treatment with a specific TS inhibitor using human tumor cell lines. A549 cells were treated with ZD9331 and showed a time-dependent increase in TS protein (up to 5-fold at 48 h compared with untreated controls measured using densitometry after Western blotting; Table 1). Confocal microscopy confirmed the up-regulation observed over 24 h of treatment in A549 cells with ZD9331 (Fig. 2). A dose response was also observed using this method. Treated cells were enlarged compared with untreated controls—a characteristic of thymineless death (26).

Table 2 shows results on W1L2 and A549 cells using flow cytometry. Greater induction of TS protein can be seen using flow cytometry than using Western blotting (9-fold compared with 3-fold up-regulation in A549 cells under similar conditions of ZD9331 exposure). A dose- and time-dependent response can be observed in both A549 and W1L2 cells after treatment for either 16 or 24 h with increasing concentrations of ZD9331. No statistically significant up-regulation is observed in either cell line below 7 × IC₅₀ values after 16-h treatment with ZD9331, but statistically significant up-regulation is observed with 1 × IC₅₀ values after 24-h treatment with ZD9331 (P < 0.001). Over a 24-h time course, W1L2 cells show significantly greater up-regulation of TS protein than A549 cells (5-fold up-regulation in A549 cells compared with 10-fold in W1L2 cells at 100 × IC₅₀ values of drug; P < 0.001).

TS Protein Is Up-Regulated in Response to a Range of TS Inhibitors. TS protein has been reported to be up-regulated in response to a number of TS inhibitors, including 5-FU and raltitrexed (17, 27, 28). Up-regulation of TS protein was observed using this method.
therefore investigated after treatment with a range of TS inhibitors to compare the extent of up-regulation (Table 3). Increased levels of TS protein were measured after 24-h treatment of W1L2 cells with all of the TS inhibitors tested. The specific TS inhibitors, ZD9331, AG337, and raltitrexed, caused greatest up-regulation of TS protein (9.8-fold, 7.3-fold, and 6.0-fold up-regulation with 100 IC$_{50}$ doses, respectively). BW1843U89, a specific polyglutamatable benzoquinazoline TS inhibitor, gave the least up-regulation (3.1-fold using 100 IC$_{50}$ dose).

**Up-Regulation of TS Protein Decreases on Resuspension in DFM.** The duration of elevation of TS protein was determined after resuspension of W1L2 cells in DFM after 24-h treatment with ZD9331 (ZD9331 is not polyglutamated within the cell; hence, it is not retained after washing and resuspension in DFM; Fig. 3). TS protein was up-regulated to a similar extent as in previous experiments, but a significant decrease in TS levels was observed within 4 h of resuspension of the cells in DFM ($P < 0.001$). After 12 h, the amount of protein was less than in untreated controls and remained at this level for up to 24 h after resuspension in DFM.

**Extent of Up-Regulation of TS Protein Does Not Correlate with Basal TS Protein Levels.** Results obtained thus far show different degrees of up-regulation in A549 cells and W1L2 cells. Because it is expected that these different cell lines will express different amounts of TS protein, we investigated whether the extent of up-regulation of TS protein correlates with basal levels of the enzyme. Four cell lines were selected from “in-house” panels of human colon and ovarian tumor cell lines. SW480 and A2780 express high levels of TS protein, whereas HT29 and HX62 express low levels of TS protein (Fig. 4A). All of these cell lines express higher levels of TS protein than W1L2 (lowest levels) or A549 cells. The extent of up-regulation of TS protein in the ovarian and colon cell lines was then determined after 24-h treatment with ZD9331 (Fig. 4B) and compared with basal TS levels. No correlation was found between basal TS protein levels and extent of up-regulation or with doubling times of the cell lines, which ranged from 18 to 24 h (data not shown).

**Up-Regulation of TS Protein in A549 Cells Shown Using Confocal Microscopy After Treatment with ZD9331 [IC$_{50}$ (120 h) = 0.07 μM].** A549 cells were grown on coverslips in 24-well plates. On reaching 60% confluency, the cells were treated with ZD9331 for 24 h as indicated: i, control (untreated); ii, 10 × IC$_{50}$; iii, 50 × IC$_{50}$; and iv, 100 × IC$_{50}$. The cells were then prepared for analysis using confocal microscopy.

**Table 2** Up-regulation of TS protein using flow cytometry

Up-regulation of TS protein was determined using flow cytometry as described in “Materials and Methods” after treatment with increasing doses of ZD9331 for 16 or 24 h. Data represent mean ± SD of three experiments carried out in duplicate.

<table>
<thead>
<tr>
<th>× IC$_{50}$</th>
<th>16 h</th>
<th>24 h</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A549</td>
<td>W1L2</td>
</tr>
<tr>
<td>1</td>
<td>0.9 ± 0.02</td>
<td>1.0 ± 0.1</td>
</tr>
<tr>
<td>4</td>
<td>1.4 ± 0.04</td>
<td>1.1 ± 0.1</td>
</tr>
<tr>
<td>7</td>
<td>2.4 ± 0.07$^{a}$</td>
<td>1.8 ± 0.1$^{a}$</td>
</tr>
<tr>
<td>10</td>
<td>3.3 ± 0.02$^{a}$</td>
<td>2.3 ± 0.1$^{a}$</td>
</tr>
<tr>
<td>50</td>
<td>4.4 ± 1.4$^{a}$</td>
<td>5.8 ± 1.6$^{a}$</td>
</tr>
<tr>
<td>100</td>
<td>3.8 ± 1.6$^{a}$</td>
<td>7.2 ± 1.5$^{a}$</td>
</tr>
</tbody>
</table>

$^{a}$ Values significantly different from control fold up-regulation (control = 1.0; $P < 0.001$).
Table 3  Up-regulation of TS protein after treatment with TS inhibitors

<table>
<thead>
<tr>
<th>Drug</th>
<th>IC_{50} (μM)</th>
<th>× 50 IC_{50}</th>
<th>× 100 IC_{50}</th>
</tr>
</thead>
<tbody>
<tr>
<td>ZD9331</td>
<td>0.01</td>
<td>7.2 ± 1.5</td>
<td>9.8 ± 2.7</td>
</tr>
<tr>
<td>Raltitrexed</td>
<td>0.004</td>
<td>6.2 ± 0.6</td>
<td>6.0 ± 0.6</td>
</tr>
<tr>
<td>AG337</td>
<td>8.0</td>
<td>6.6 ± 0.6</td>
<td>7.3 ± 0.5</td>
</tr>
<tr>
<td>BW1843U89</td>
<td>0.0023</td>
<td>2.3 ± 0.6</td>
<td>3.1 ± 0.4</td>
</tr>
<tr>
<td>MTA</td>
<td>0.042</td>
<td>7.0 ± 1.2</td>
<td>4.5 ± 0.2</td>
</tr>
<tr>
<td>5-FU</td>
<td>0.002</td>
<td>3.1 ± 0.5</td>
<td>4.3 ± 0.5</td>
</tr>
</tbody>
</table>

**DISCUSSION**

Over the past decade, evidence has accumulated suggesting that insufficient inhibition of TS may be a major resistance mechanism to TS inhibitors both in preclinical models and patients (reviewed in Ref. 29). Chu et al. (27) demonstrated a 2.8-fold increase in TS enzyme activity in vitro after 24-h exposure to 5FU in H630 colon carcinoma cells. Swain et al. (28) also showed a 3-fold increase in TS activity in tumor biopsy specimens from patients with advanced breast cancer after 24-h treatment with 5FU. However, increased TS protein expression has also been shown to play an important role in the development of resistance to folate TS inhibitors in vitro (25), and recent studies examining the level of TS expression in clinical tumor samples suggest that TS expression predicts for overall clinical outcome and response to cytotoxic therapy (30, 31). Both high TS mRNA, as quantified by RT-PCR, and high TS protein expression have been shown to predict for a poor response to fluoropyrimidine-based therapy in colorectal (32–34), gastric (32, 35, 36), and head and neck cancer (37, 38). Amplification of the TS gene resulting in increased TS mRNA and protein content has also been found in cell lines intrinsically resistant to TS inhibitors (39, 40).

This study has demonstrated that “short-term” induction of TS occurs to a significant extent (up to 10-fold after treatment with ZD9331 compared with nontreated control samples in the human tumor cell lines W1L2 and A549; Fig. 2 and Table 2). Statistically significant up-regulation occurred by 16 h at doses of ZD9331 >0.07 μM and 0.7 μM (7 × IC_{50}) in W1L2 and A549 cells, respectively (P < 0.001). However, statistically significant up-regulation occurred at doses as low as 0.01 μM and 0.1 μM in the same cell lines (1 × IC_{50}) after 24-h exposure to ZD9331 (P < 0.001). ZD9331 has been shown to be present in the plasma of mice bearing the L5178Y TK−/− tumor at concentrations exceeding 0.5 μM for 12 h after bolus administration of ZD9331 (19 h following s.c. infusion; Refs. 41 and 42). Tumor drug levels were shown to be 2-fold higher than plasma levels (42). The concentration of free drug in human plasma has been estimated at 20 μM in humans, and pharmacokinetic data from Phase I trials have demonstrated a long terminal elimination half-life (83 h; Ref. 43). These results suggest that up-regulation could occur at drug concentrations relevant to pharmacologically active doses in both mice and man, although protein binding of the drug also needs to be taken into account.

The up-regulation was not retained in W1L2 cells after removal of drug (Fig. 3), and TS protein levels fell to below pretreatment levels within 12 h of resuspension in DFM, demonstrating the reversible nature of this response. The levels...
remained up to 4-fold less than pretreatment values 24 h later. This drop below control levels of TS protein could be explained in three ways. Firstly, it could be attributable to overcompensation of the regulatory mechanism when the cell is no longer exposed to a TS inhibitor. Alternatively, this may simply represent the lack of cell replication immediately after removal of the TS inhibitor when little TS activity is required pending downstream DNA repair or cell death. Lastly, TS levels and activity may vary 14- to 24-fold between the peak exponential and confluent growth phase (44), and this may account for the down-regulation observed because a doubling time has passed over the course of the experiment.

Up-regulation of TS protein was observed using a range of TS inhibitors of varying structure (Table 3). Up-regulation ranged between 3- and 10-fold in W1L2 cells treated for 24 h. Previous studies have demonstrated similar increases in TS activity after exposure to 5FU and raltitrexed (17, 25, 27). No correlation was observed between the extent of up-regulation and (a) whether drugs are specific TS inhibitors; (b) whether the drugs undergo polyglutamation within cells; or (c) the mechanism of uptake into the cell. Consistent with previous studies, no elevation in TS mRNA levels were measured using Northern blotting after treatment for up to 72 h (100 \times IC_{50}) with TS inhibitors (data not shown; Refs. 17 and 18).

Several researchers have reported a direct correlation between TS protein expression and response to 5FU (25, 30), with lower TS protein expression predicting for better response to TS inhibitors, although other studies failed to show any correlation (45). Considerable variation in TS expression has also been reported between sensitive and resistant cell lines in vitro (25).

The extent of up-regulation of TS protein was shown to vary between cell lines in this study (Table 2). It was therefore proposed that the extent of up-regulation might depend upon basal TS protein levels. Four cell lines were selected based upon their basal TS protein levels. However, no correlation was observed between predrug exposure TS protein levels and extent of up-regulation of TS. Differences in doubling times of cell lines used during this study also did not correlate with the extent of up-regulation of TS (data not shown).

TS protein expression was also shown to be increased in normal, nontransformed human fibroblasts (Fig. 5). Up-regulation was significantly greater in these cells than in any of the tumor cell lines tested (up to 20-fold compared with 9-fold in W1L2 cells after 24 h treatment with equitoxic doses of ZD9331). Up-regulation was also significantly greater (P < 0.05) in fibroblasts than tumor cells when equimolar doses of ZD9331 are compared (10-fold up-regulation in W1L2 cells compared with 15-fold in fibroblasts after treatment with 1 \mu M ZD9331 for 24 h). This is in agreement with previous studies that have shown a 40-fold increase in TS activity in human normal mammary epithelial cells compared with a 10-fold increase in tumor cells after treatment with equimolar concentrations of raltitrexed for 36 h (17). Induction of TS in normal cells has also been reported to be generally more responsive to treatment with 5-FU compared with malignant cells (19).

Significantly greater up-regulation was observed in vivo in...
gut compared with lymphoma cells (2 and 1.2, respectively; \( P < 0.05 \)) after treatment for either 7 or 16 h. The extent of up-regulation observed both \( \text{in vitro} \) and \( \text{in vivo} \) in the tumor tissue was small compared with that observed in other tumor cell lines studied. Up-regulation of TS protein was identical at both time points, suggesting that a threshold level of TS protein up-regulation had been reached for this concentration of ZD9331.

These results indicate that normal proliferating cells may up-regulate TS protein to a greater extent than tumor cells upon treatment with a TS inhibitor, which could in turn lead to an increase in therapeutic index. Indeed, this is indicated by data showing levels of TS inhibition in solid human tumor and normal liver biopsies treated with 5-FU. Seventy to 80% inhibition of TS was observed in tumor tissues compared with 50% inhibition in histologically normal tissue. Also, patients whose tumors were responsive to 5-FU had greater inhibition of TS (46). This is consistent with our data.

Several studies have hypothesized that antisense oligonucleotide down-regulation of TS mRNA would decrease TS levels and enhance the cytotoxicity of inhibitors of TS. Indeed, these studies have demonstrated that TS mRNA levels are reduced, and growth of human colon and cervical cancer RKO, HT29, and HeLa cells may be inhibited after treatment with antisense oligonucleotides or ODNs (47–49). ODN 85 also enhanced the cytotoxicity of raltitrexed, 5-fluoro-2'-deoxyuridine, 5-FU, and methotrexate (49). These results suggest that the enhanced therapeutic efficacy suggested by the present study may be further exploited by the use of tumor-directed oligoribonucleotides or ODNs to enhance the selectivity of TS inhibitors by further increasing the difference in TS levels between tumor and normal cells after treatment with TS inhibitors.

In summary, these results confirm that up-regulation of TS protein occurs to a significant extent both \( \text{in vitro} \) and \( \text{in vivo} \) at pharmacologically active doses of ZD9331, which may be detrimental to response after treatment with TS inhibitors. Up-regulation occurs within 16 h of treatment with a range of TS inhibitors. However, significant decreases in protein levels are observed within 4 h of resuspension of W1L2 cells in DFM. Significantly greater up-regulation was apparent in normal compared with tumor cells both \( \text{in vitro} \) and \( \text{in vivo} \), suggesting that up-regulation of TS protein may be beneficial in normal tissues by providing protection from the cytotoxic effects of TS inhibitors, leading to increased therapeutic efficacy.

**ACKNOWLEDGMENTS**

We thank Drs. Lorraine Skelton and Lloyd Kelland (Institute of Cancer Research, Sutton, United Kingdom) for donating cell lines and Dr. Jean Sargent (Pembrey Hospital, Kent, United Kingdom) for advice on flow cytometry methods.
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