ZD1839 (Iressa) Modifies the Activity of Key Enzymes Linked to Fluoropyrimidine Activity: Rational Basis for a New Combination Therapy with Capecitabine

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ABSTRACT

Purpose: The efficacy of new oral fluoropyrimidines, including capecitabine, is improved in cells expressing high levels of thymidine phosphorylase (TP) and low levels of thymidylate synthase (TS) and dihydropyrimidine dehydrogenase. We used a human head and neck cancer cell line (CAL33) to examine the influence of cell cycle modifications on TS, TP, and dihydropyrimidine dehydrogenase activity.

Experimental Design: Cells were exposed to the epidermal growth factor receptor tyrosine kinase inhibitor ZD1839 (Iressa2) and 5'-deoxy-5-fluorouridine (5'-DFUR), alone and in combination, for up to 96 h, and modifications in cell cycle, enzyme activity, and gene expression were examined.

Results: ZD1839 (24- to 72-h exposure) markedly reduced proliferation and caused a rapid increase in G0-G1 and a decrease in S phase; a 40-fold decrease in TS activity at 24 h and a 2.5-fold increase in TP activity at 48 h were observed. A significant link between TP activity and expression was observed (r² = 0.98; P = 0.0068). Additional investigations pointed out an increased cellular production of 5-fluorouracil anabolites from 5'-DFUR when cells were preincubated with ZD1839.

Conclusions: These data demonstrate a strong synergistic interaction between ZD1839 and 5'-DFUR when ZD1839 is applied before or concurrently with 5'-DFUR. Such a drug combination would have two advantages: (a) the theoretical advantage of tumor selectivity of epidermal growth factor receptor-targeted therapy; and (b) the practical advantage of a combination therapy that could be administered p.o.

INTRODUCTION

Several recent clinical studies have shown that the rationally designed, tumor-selective, p.o.-administered fluoropyrimidine derivative capecitabine (N4-pentoxycarbonyl-5'-deoxy-5-fluorocytidine; Xeloda) is an effective and well tolerated treatment for head and neck (1), colorectal (2–4), and breast (4–6) cancer. After oral administration, capecitabine passes rapidly and extensively through the intestinal membrane as an intact molecule. After absorption, it is first transformed to 5'-deoxy-5-fluorocytidine by hepatic carboxylesterase and then to 5'-DFUR3 by cytidine deaminase, which is found in high concentrations in many human tumor tissues as well as in healthy liver tissue. As a key step, 5'-DFUR is then converted into 5FU by TP, which is present at higher concentrations in tumors than in healthy tissues (7). 5FU is catabolized by the enzyme DPD, which is expressed in liver tissue and tumors (8). Both experimental (9) and clinical data (10) have shown that elevated tumor DPD levels confer resistance to 5FU-based treatment. Experimental data suggest that the cytotoxic efficacy of capecitabine is improved in cells expressing high levels of TP and low levels of TS and DPD (11). Clinical data have recently confirmed this experimental observation by demonstrating the role of tumor TP and DPD for predicting the efficacy of 5'-DFUR in the treatment of patients with colorectal cancer (12). An assessment of tumor up-regulation of TP may help optimize capecitabine antitumor effects (13–15). Taxanes were found to up-regulate the tumoral activity of TP and have shown synergistic cytotoxic activity when combined with capecitabine (13, 14). This synergy has recently translated into interesting clinical results with the docetaxel-capecitabine combination (15).

TP and TS cellular levels are dependent on tumor prolif-

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2 Iressa is a trademark of the AstraZeneca group of companies.

3 The abbreviations used are: 5'-DFUR, 5'-deoxy-5-fluorouridine; CI, combination index; DPD, dihydropyrimidine dehydrogenase; EGFR, epidermal growth factor receptor; 5FU, 5-fluorouracil; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; PSAU, phe- nylenediaminolycyclouridine; TP, thymidine phosphorylase; TS, thymidy- late synthase; HPLC, high-performance liquid chromatography; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
eration and growth (16, 17). Thus, modifying tumor cell proliferation might help optimize the activity of capcitabine through a modulation of TP and TS. The EGFR cellular pathway regulates cell growth as well as survival (18), and EGFR-targeting agents are currently in clinical development (19). Because EGFR is more highly expressed in tumors than normal tissue (20), tumor selectivity is a possible benefit of EGFR-targeting agents. The use of EGFR-directed drugs could be an interesting approach to modulate TS and TP. ZD1839 (Iressa) is a p.o.-active and -selective EGFR-tyrosine kinase inhibitor that blocks signal transduction pathways implicated in proliferation and survival of cancer cells, and other host-dependent processes promoting cancer growth. ZD1839 exhibits a broad spectrum of antitumor activity against many human solid tumor xenografts including breast, pancreas, lung, colorectal, and head and neck cancer (21, 22).

The aim of the present study was to examine the effects of ZD1839 on TS, TP, and DPD in CAL33, a head and neck tumor cell line that expresses high levels of EGFR (23). In addition, the cytotoxic effects of combining ZD1839 with 5'-DFUR were examined. This preclinical work was undertaken to assess the potential for clinical investigations of ZD1839 combined with the oral fluoropyrimidine capcitabine as a new strategy of p.o.-administered anticancer treatment.

MATERIALS AND METHODS

Chemicals. ZD1839 and 5'-DFUR were kindly provided by AstraZeneca and Roche Laboratories Inc., respectively. A 50-nm working solution in DMSO was prepared before use. DMEM and glutamine were purchased from Whittaker (Ver- vieurs, Belgium). Fetal bovine serum was obtained from Dut- scher (Brumath, France). Penicillin and streptomycin were from Meyrieux (Lyons, France). Transferrin and insulin were purchased from Flow (Irvine, Scotland). BSA, MTT, and DMSO were purchased from Sigma (St. Quentin Fallavier, France). Tritiated 5'-DFUR (5.1 Ci/mmol) was purchased from Moravek Biochemicals (Brea, CA).

Drug Administration Schedule. The human head and neck cancer cell line model CAL33 was used in the present study, as in our previous work (23). CAL33 cells carry a p53 mutation (codon 175, CGC -> CAC) and exhibit high EGFR expression (34,000 fmol/mg protein). CAL33 has no intrinsic mitogen-activated protein kinase activity or K-ras mutation (23).

Cells were routinely cultured in DMEM supplemented with 10% fetal bovine serum, 2 mm glutamine, 50,000 units/liter penicillin, and 80 μM streptomycin in a humidified incubator (Sanyo, Japan) at 37°C with an atmosphere containing 8% CO2.

The first part of the study consisted of the exploration of the impact of ZD1839 on the enzyme activities (TS, TP, DPD). For these experiments, CAL33 cells were seeded in 175-cm2 tissue culture flasks at 1.86 x 106 cells to obtain exponential growth for the duration of the experiments. Forty-eight hours after seeding, CAL33 cells were incubated with ZD1839 at 6 μM (IC50) alone for 96 h. Enzyme activities and gene expression were analyzed 24, 48, 72, and 96 h after the onset of drug application. All investigations were tripled in separate experiments for all cellular parameters studied. Attention was paid to the time- and concentration-related effects of ZD1839 on TP activity. For these experiments, CAL33 cells were seeded as described previously. Forty-eight hours after seeding, CAL33 cells were incubated with increasing doses of ZD1839 ranging from 8 x 10^-8 to 2 x 10^-6 M and TP activity was analyzed 24, 48, 72, and 96 h after the onset of ZD1839 application.

The second part of the study consisted of the examination of the sequence-dependent cytotoxic effects resulting from the combination of ZD1839 and 5'-DFUR. Cells were seeded in 96-well microtitration plates (100 μl/well) to obtain exponential growth for the duration of the experiments (initial cell density was 3000 cells/well). After 48 h, cells were exposed to ZD1839 and/or 5'-DFUR in a sequence based on the previously published synergistic interaction observed between ZD1839 and SFU (24). ZD1839 was given alone for 48 h, then 5'-DFUR was added for 48 h, giving a total length of exposure of 96 h for ZD1839 and 48 h for 5'-DFUR. Eleven concentrations were tested for each drug: ZD1839 up to 200 μM and 5'-DFUR from 0.035 to 1000 μM. Thereafter, growth inhibition was assessed 48 h after the end of the drug exposure (in medium alone) by the MTT test (25), as follows. Cells were washed with PBS and incubated with MTT; after 2 h of exposure, DMSO (100 μl) was added to terminate the reaction, and absorbance at 540 nm was measured using a microplate reader (Labsystems, Helsinki, Finland). Results were expressed as the relative percentage of absorbance compared with controls without drug. Cell sensitivity to ZD1839 and 5'-DFUR was expressed as IC50 (25). Experimental conditions were tested in sextuplicate (six wells of the 96-well plate per experimental condition) and in three separate experiments. Dose-effect curves were analyzed using Prism software (GraphPad Software, San Diego, CA).

CI Calculations. The cytotoxic effects obtained with the different ZD1839/5'-DFUR combinations were analyzed, according to the method of Chou and Talalay (26), on Calcsyn software ( Biosoft, Cambridge, United Kingdom). Interaction between ZD1839 and 5'-DFUR was assessed by means of an automatically computed CI. The CI was determined at 50% and 75% cell death and was defined as:

\[ CI_{A+B} = [(D_{A} + D_{B}) / D_{A} + D_{B}] + [\alpha(D_{A} + D_{B}) / D_{A} D_{B}], \]

where CI_{A+B} is the CI for a fixed effect (F) for the combination of cytotoxic A and cytotoxic B; D_{A+B} is the concentration of cytotoxic A in the combination A + B, giving an effect F; D_{B/A+B} is the concentration of cytotoxic B in the combination A + B, giving an effect F; D_{A} is the concentration of cytotoxic A alone, giving an effect F; D_{A+B} is the concentration of cytotoxic B alone, giving an effect F; α is the parameter with value 0 when A and B are mutually exclusive and 1 when A and B are mutually nonexclusive.

Synergism is indicated by CI < 0.8, additivity by CI values between 0.8 and 1.2, and antagonism by CI > 1.2; slight synergistic and additive cytotoxic activity are indicated by CI values of 0.8 and 1.2, respectively.

For the application of the Chou and Talalay model (26), it is recommended that the cytotoxic agents are used at a fixed dose ratio (for example, ratio of drug IC50).
Cell Cycle Analysis. The cell cycle was analyzed by fluorescence-activated cell sorting according to the Vindelov model. For flow cytometry analysis, 10⁶ CAL33 cells in exponential growth were treated with graded concentrations of ZD1839 for 24 h and then washed three times with citrate buffer. Cell pellets were incubated with 250 μl of trypsin-containing citrate buffer for 10 min at room temperature and then incubated with 200 μl of citrate buffer containing a trypsin inhibitor and RNase (10 min) before adding propidium iodide (200 μl at 125 μg/ml). Samples were analyzed on a Becton Dickinson FACScan flow cytometer using Modfit software, which was also used to determine the percentage of cells in the different phases of the cell cycle. Propidium iodide was excited at 488 nm, and fluorescence was analyzed at 620 nm (fluorescence channel 3).

TS Activity Assay. TS activity was measured according to the tritium-release assay described by Spears and Gustavsson (27). Cytosol (25 μl) was incubated with [3H] 2'-deoxyuridine-5'-monophosphate (final concentration, 1 μM) and 5,10-methylenetetrahydrofolate (final concentration, 0.62 mM) in a total volume of 55 μl. After 0 (for blank subtraction), 10, 20, and 30 min of incubation at 37°C, the reaction was stopped in ice. Excess [3H]2'-deoxyuridine-5'-monophosphate was removed by adding activated charcoal (300 μl, 15%) containing 4% trichloroacetic acid before a 5-min centrifugation at 14,000 × g at room temperature. The [3H]2O formed during the incubation was then counted in the supernatant by a liquid scintillation counter (Wallac 1409 DSA; Wallac, Turku, Finland). Results were expressed as femtomoles of [3H] 2 O formed per minute per milligram of protein, based on the linear regression obtained from the incubation times. The sensitivity limit was 1 fmol/min/mg protein. The interassay reproducibility (pooled cell suspension) gave a coefficient of variation of 7.08%.

TP Activity Assay. There are two distinct pyrimidine nucleoside phosphorylases present in normal and neoplastic cells: TP, for which the major substrate is thymidine, and uridine phosphorylase, which is responsible for the reversible catalysis of uridine to uracil. Because TP is primarily responsible for the catalysis of 5'-DFUR into 5FU, TP activity was measured in the analyzed samples. Specific inhibitors for TP (TP inhibitor) and uridine phosphorylase (PSAU) were applied to determine the specific activity of TP in the reaction mixture. PSAU was kindly provided by Dr. M. El Kouni (University of Alabama at Birmingham, Birmingham, AL).

The analytical method used for the determination of TP activity was derived from Kubota et al. (16). Cultured cells (10⁷) were homogenized in 500 μl of lysis buffer [50 mM Tris-HCl (pH 6.8), 1% Triton X-100, 2 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride, and 0.02% mercaptoethanol]. The samples were centrifuged at 105,000 × g for 30 min at 4°C. Protein concentration was determined by using the method of Bradford. Supernatants (0.8 mg of protein/ml) were incubated for 4 h at 37°C with 10 mM 5'-DFUR and 180 mM potassium phosphate (pH 7.4) ± 100 μM TP inhibitor or PSAU. The reaction was stopped by the addition of 360 μl of ice-cold methanol to the 120-μl reaction mixture. After removal of the precipitate by centrifugation, an aliquot (dilution, 1:5) of the reaction mixture was applied to the HPLC column (Licrospher 100-RP 18). The elution buffer consisted of 50 mM phosphate buffer (pH 6.8) containing 10% methanol. The amount of 5FU produced was monitored by UV absorbance (262 nm). TP activity was expressed as nanomoles of 5FU converted/milligram of protein per hour.

DPD Activity Assay. DPD activity was measured according to the method described by Harris et al. (28) and under experimental conditions previously described by us (29). DPD activity determination consisted of the quantification of 14C-dihydro-5-fluorouracil. 14C-fluoro-β-alanine, and 14C-α-fluorouracil via a previously reported HPLC method (29). DPD activity was determined by summing the dihydrofluorouracil, fluoro-β-alanine, and α-fluorouracil peaks. DPD activity was expressed as picomoles of (14C-5-fluorouracil) catalyzed per minute and per milligram of protein. Each sample was assayed in duplicate, and DPD activity was measured in three independent experiments. The sensitivity limit was 1 pmol/min/mg protein. The interassay reproducibility (pooled cell suspension) gave a coefficient of variation of 12% (n = 8).

TS, TP, and DPD Expression. mRNA levels of TS, TP, and DPD were measured by real-time quantitative RT-PCR on the samples obtained at 24, 48, 72, and 96 h after the onset of ZD1839 application on CAL33 cells.

RNA Extraction and Reverse Transcription. DPD, TS, and TP expressions were determined in cell line pellets stored at −80°C. Total RNA was isolated from 4 × 10⁶ cells using the RNA NOW kit from Biogentex (Ozyme, Montigny-le-Bretonneux, France) based on a method derived from Chomczynski and Sacchi (30). RNA quality was checked by agarose gel electrophoresis. Quantification was performed by densitometric analysis at 260 nm. Total RNA (100 ng) was incubated for 10 min at 25°C in a 20-μl final volume of 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM MgCl₂, containing 0.4 μM of each deoxycytidinucleotide triphosphate, 0.08 A₂₆₀ units of random hexamers, 20 units of avian myeloblastosis virus reverse transcriptase, and 40 units of RNase inhibitor. This preparation was then incubated for 1 h at 42°C, followed by 5 min at 94°C in a classical Biometra thermocycler (all of the reagents for the RT reaction were included in the mRNA amplification kit from Roche Diagnostics, Meylan, France).

Primers. The oligonucleotides used for DPD, TP, and TS amplification are the property of Roche. Primers for DPD, TP, and TS gave products of 148 bp, 108 bp, and 111 bp, respectively.

The GAPDH primers (GAPDH is the reference gene in all three kits) gave a product of 123 bp.

PCR Conditions. LightCycler mRNA Quantification kits (Roche Molecular Biochemicals) were used to perform real-time RT-PCR for DPD, TP, and TS by using the LightCycler system. The manufactured kit contained 25 mM MgCl₂, LightCycler DNA Master SYBR Green I 10X containing deoxynucleotide triphosphate mix, 10 mM MgCl₂, SYBR Green I dye, specific primers for the target and the reference gene, and Hot Start TaqDNA polymerase. cDNA (4 μl), previously obtained by RT-PCR (see above), was amplified in duplicate (one incubation for the target gene, one incubation for the reference gene) in a 20-μl reaction capillary containing 2 μl of Light-
Cyclers DNA Master SYBR Green I 10X, 1.6 μl of 25 mM MgCl₂, and 6 μl of one couple of primers. In each experiment, a cDNA preparation obtained from a RNA sample, included in the kit, was introduced as the calibrator. Simultaneously, a blank was performed by incubating H₂O PCR grade instead of sample cDNA.

The PCR protocol programmed on the LightCycler software consisted of two steps. The first step was denaturation of cDNA and activation of the enzyme for 5 min at 95°C. In the second step, DNA was amplified for 40 cycles of 10 s at 95°C, 10 s at 62°C, and 10 s at 72°C. Results, calculated using ReLQuant software, were expressed as the ratio of concentration of TP (or DPD or TS) to GAPDH for a given sample, normalized by the same ratio for the calibrator.

**Determination of [³H]-5’-DFUR Intracellular Metabolites.** Separation and detection of [³H]-5’-DFUR metabolites was performed as described previously (31). Exponentially growing cells were exposed for 24 h to either tritiated 5’-DFUR alone or after a 48-h pretreatment period with ZD1839. Drug concentrations were set at their respective IC50. Cells were harvested after the 24-h exposure to 5’-DFUR, and cytosols were isolated for HPLC analysis. The HPLC consisted of a HP1090 (Hewlett Packard) system coupled with a A200 radioactive flow detector (Packard). Separation of tritiated metabolites was obtained using a Lichrospher 100 RP18 5-μm column (Hewlett Packard) eluted by 50 mM K₂HPO₄ (pH 6.8) containing 5 mM tetrabutyl ammonium nitrate and 5% (0.60) methanol. Results were expressed as dpm/μg protein.

**Statistical Analysis.** Differences between mean values were evaluated using either one-way ANOVA with Tukey test or one-way ANOVA on ranks with Dunnett’s test or Student-Newman-Keuls test, according to data distribution. Correlation coefficients (r) from linear regressions and respective Ps (P < 0.05 was regarded as statistically significant) were computed using the program SPSS (Paris, France).

**RESULTS**

The impact of ZD1839 on cell cycle organization is shown in Table 1. Compared with drug-free controls, ZD1839 increased the proportion of cells in G₀-G₁ and decreased the proportion in S phase. ZD1839 slowed down cell proliferation after drug exposure and caused a rapid increase in G₀-G₁ phase (with a maximum of 89% cells in G₀-G₁ phase at 24 h). This phenomenon was accompanied by a decrease in S phase (with a minimum of 7% of cells in S phase at 24 h) compared with control. Cells treated with ZD1839 presented a statistically significant maximum increase of almost 2-fold in TP at 48 h (P = 0.0001; Fig. 1) and a statistically significant maximum increase of almost 2-fold in TP at 48 h (P = 0.01; Fig. 2). ZD1839 treatment did not significantly modify DPD activity at any time of exposure (the ratio DPD activity in ZD1839-treated cells/control cells remained close to 1 from 24 h to 96 h after ZD1839 onset; data not shown). A more detailed examination of the effects of ZD1839 on TP activity revealed time- and concentration-related changes in TP in the presence of ZD1839 (Fig. 3). Although TP activity was significantly up-regulated as a function of both ZD1839 concentration and time of exposure to ZD1839 (P = 0.0001), it is clear that marked changes relative to controls occur above the micromolar level in ZD1839. In this situation, the maximal relative increase in TP at 48 h is again put into evidence.

**Table 1** Time-related influence of ZD1839 (6 μM) on proliferation of CAL33 cells

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>% G₀-G₁</th>
<th>% S</th>
<th>% G₂-M</th>
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<tbody>
<tr>
<td></td>
<td>mean (SD)</td>
<td>mean (SD)</td>
<td>mean (SD)</td>
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<tr>
<td>Control</td>
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<td></td>
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<tr>
<td>24</td>
<td>59 (11)</td>
<td>35 (11)</td>
<td>7 (1)</td>
</tr>
<tr>
<td>48</td>
<td>69 (6)</td>
<td>22 (2)</td>
<td>9 (5)</td>
</tr>
<tr>
<td>72</td>
<td>77 (4)</td>
<td>15 (5)</td>
<td>7 (2)</td>
</tr>
<tr>
<td>96</td>
<td>87 (3)</td>
<td>9 (3)</td>
<td>4 (1)</td>
</tr>
<tr>
<td>ZD1839</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>24</td>
<td>89 (10)</td>
<td>7 (3)</td>
<td>4 (1)</td>
</tr>
<tr>
<td>48</td>
<td>86 (6)</td>
<td>8 (2)</td>
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<td>87 (2)</td>
<td>7 (0)</td>
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<tr>
<td>96</td>
<td>87 (12)</td>
<td>7 (9)</td>
<td>7 (2)</td>
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* Results from three separate experiments.
To elucidate the origin of the modifications in enzyme activities observed, the respective gene expressions were examined in parallel. A significant link between TP activity and TP expression levels was demonstrated with an $r^2$ value of 0.98 ($P = 0.0068$; Fig. 4). Modifications in TS activity and TS expression levels after ZD1839 treatment were also positively correlated ($r^2 = 0.68$), but the correlation was not significant (data not shown).

Monitoring of 5'-DFUR metabolism showed a more efficient activation of this fluoropyrimidine, both quantitatively and qualitatively, when this later drug was combined with ZD1839 (Fig. 5). In particular, cells exposed to ZD1839 + 5'-DFUR displayed higher amounts of 5FU derivatives, FUR and fluorodeoxyuridine, as compared with those produced after 5'-DFUR alone. As a corollary, nuclear incorporation of triphosphorylated fluoronucleotides was found to be increased by up to 110% when cells were exposed to the combination 5'-DFUR + ZD1839 in comparison with 5'-DFUR alone (data not shown).

Dose-effect curves resulting from the application of ZD1839 alone, 5'-DFUR alone, or both drugs on the CAL33 cell line are shown in Fig. 6. Both drugs exhibited classic dose-response curves with IC$_{50}$ for ZD1839 and 5'-DFUR of 2.38 $\mu$M and 4.8 $\mu$M, respectively. CIs (mean ± SD) from 5'-DFUR-ZD1839 combinations were 0.58 ± 0.1 and 0.63 ± 0.1 for 50% survival (CI$_{50}$) and 25% survival (CI$_{75}$), respectively. An illustration of the synergistic cytotoxic effects between ZD1839 and 5'-DFUR is shown in Fig. 7. Altogether, these data point to a strong synergistic interaction between ZD1839 and 5'-DFUR when ZD1839 is applied before and during 5'-DFUR application.

**DISCUSSION**

In a study of mouse xenograft models of human breast and colon cancers, exposure to paclitaxel, docetaxel, or mitomycin C increased tumor concentrations of TP by 4- to 8-fold within 8 days of administration (13). Another study showed that daily oral administration of cyclophosphamide for 2 weeks resulted in a gradual increase in TP concentrations in tumor tissue, reaching concentrations 4-fold higher than in control tumors (32). In WiDr human colon cancer xenografts, vinblastine, vindesine, and cisplatin were found to up-regulate TP activity (14), and gemcitabine and vinorelbine have also been demonstrated to cause TP up-regulation (14). Similarly, TP concentrations were increased up to 10-fold after exposure to human recombinant IFN-$\gamma$ (14). Radiotherapy has also been shown to enhance TP expression in tumor tissue in a number of human cancer xenografts, with single-dose irradiation resulting in a 13-fold increase in intratumoral TP activity, whereas no up-regulation was observed in healthy liver tissue (33). However, it is not clear from these studies what the origin of the up-regulation in TP activity is; most of the effects on TP were observed in xenograft models, whereas up-regulation of TP by these drugs was hardly detectable in cultures of tumoral cells (13, 33). The hypothesis was that elevations in TP observed in vivo and not in vitro could be mediated by tumor necrosis factor $\alpha$ in tumor cells, which in turn may up-regulate TP (13). The present study clearly establishes that TP activity may be intrinsically up-regulated in the tumor cells by a change in proliferation status induced by ZD1839, a drug that specifically targets EGFR. The role of ZD1839 in TP up-regulation was further demonstrated by the clear time- and concentration-dependent effects of ZD1839 on TP activity (Fig. 3). Parallel to TP were the modifications observed in TS activity, but in an opposite sense: a decrease in TS activity after a ZD1839-induced reduction in cell proliferation. This decrease in TS activity induced by the drug-related inhibition in cell proliferation agrees well with previously reported data showing the link between tumor cell proliferation and TS activity (34–36). In the present study, a positive and significant relationship was put into evidence between TS activity and the percentage of cells in S phase ($r^2 = 0.87; P = 0.0008; n = 7$; data not shown). The fact that parallel changes occurred in enzyme activities and enzyme expressions (mRNA levels) means that the observed modifications are mainly under transcriptional control. It has been postulated that TS might regulate the transcription of cellular gene expression including p53 and perhaps other cell cycle-related proteins (37). It is, thus,
possible that changes in TS may participate in the final modification in TP activity with a possible translational repression of TP under the presence of TS; a decrease in TS protein would allow more TP mRNA to be translated into TP protein. A possible TS protein-TP mRNA interaction would be interesting to explore in additional experiments, which were beyond the scope of the current study. The next logical step in the present study was to analyze the cytotoxic effect resulting from the combination of ZD1839 with 5'-DFUR. A strong synergistic interaction in terms of final cytotoxic effect was observed when combining these two drugs (Fig. 6); this is in line with both a reduction in TS activity by ZD1839, which is in favor of fluoropyrimidine activity (38), and an increase in TP activity, which permits more 5FU to be intracellularly delivered from its prodrug 5'-DFUR (7). This last hypothesis was strongly supported by the monitoring of the intracellular metabolism of 5'-DFUR. Marked differences in cellular 5FU anabolites were observed in favor of the combination ZD1839–5'-DFUR as compared with 5'-DFUR alone (Fig. 5). Above all, these observations are fully consistent with previously published reports showing that higher levels of TP in the target cell are related to an increase in the cytotoxic effect of capecitabine, 5'-DFUR, and 5FU (39–41). The present data may serve as a rational basis for setting up clinical trials combining EGFR-targeting agents such as ZD1839 with the new oral fluoropyrimidine capecitabine, which is the prodrug of 5'-DFUR. Such strategy of drug combination would carry a double advantage: the theoretical advantage of tumor selectivity, because high EGFR expression/signaling plays a more important role in tumor tissue than normal tissue (20), and the oral administration route of capecitabine plus ZD1839 therapy.

REFERENCES


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