Trifluorothymidine Resistance Is Associated with Decreased Thymidine Kinase and Equilibrative Nucleoside Transporter Expression or Increased Secretory Phospholipase A2

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Abstract

Trifluorothymidine (TFT) is part of the novel oral formulation TAS-102, which is currently evaluated in phase II studies. Drug resistance is an important limitation of cancer therapy. The aim of the present study was to induce resistance to TFT in H630 colon cancer cells using two different schedules and to analyze the resistance mechanism. Cells were exposed either continuously or intermittently to TFT, resulting in H630-cTFT and H630-4TFT, respectively. Cells were analyzed for cross-resistance, cell cycle, protein expression, and activity of thymidine phosphorylase (TP), thymidine kinase (TK), thymidylate synthase (TS), equilibrative nucleoside transporter (hENT), gene expression (microarray), and genomic alterations. Both cell lines were cross-resistant to 2'-deoxy-5-fluorouridine (>170-fold). Exposure to IC75-TFT increased the S/G2-M phase of H630 cells, whereas in the resistant variants, no change was observed. The two main target enzymes TS and TP remained unchanged in both TFT-resistant variants. In H630-4TFT cells, TK protein expression and activity were decreased, resulting in less activated TFT and was most likely the mechanism of TFT resistance. In H630-cTFT cells, hENT mRNA expression was decreased 2- to 3-fold, resulting in a 5- to 10-fold decreased TFT-nucleotide accumulation. Surprisingly, microarray-mRNA analysis revealed a strong increase of secretory phospholipase-A2 (sPLA2; 47-fold), which was also found by reverse transcription-PCR (RT-PCR; 211-fold). sPLA2 inhibition reversed TFT resistance partially. H630-cTFT had many chromosomal aberrations, but the exact role of sPLA2 in TFT resistance remains unclear. Altogether, resistance induction to TFT can lead to different mechanisms of resistance, including decreased TK protein expression and enzyme activity, decreased hENT expression, as well as (phospho)lipid metabolism.

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Introduction

The fluorinated pyrimidine analogue 5-trifluoro-2'-deoxythymidine (TFT; trifluridine) is part of the novel drug combination TAS-102 (1). In this formulation, TFT is combined with a thymidine phosphorylase inhibitor (TPI) to increase the bioavailability of TFT. TAS-102 is currently tested in phase II clinical trials as an oral chemotherapy regimen (1). Upon uptake into the cells, TFT can be converted to its monophosphate derivative (TF-TMP) by thymidine kinase (TK; Fig. 1A). TF-TMP binds covalently to the active site of thymidylate synthase (TS) thereby inhibiting its activity (1–3). TS catalyzes the methylation of 2'-deoxyuridine-5'-monophosphate (dUMP) to 2'-deoxythymidine-5'-monophosphate (dTMP), in which 5,10-methylene-tetrahydrofolate serves as the methyl-donor. TS is a rate-limiting enzyme in the pyrimidine de novo deoxynucleotide synthesis; therefore, it is an excellent target for chemotherapeutic strategies (4). Inhibition of TS results in the depletion of dTTP and an increase in dUTP in the cell (thymine-less state), resulting in the mis-incorporation of dUTP into the DNA (5, 6). The triphosphate form of TFT (TF-TTP) can be incorporated into the DNA, leading to DNA strand breaks. The dTTP/dUTP imbalance and DNA damage induction will result in cell death induction (7).

TS can be inhibited by several cytotoxic agents that are active against colon cancer, such as the 5-fluorouracil (5-FU)–derived metabolite 5-fluoro-dUMP (FdUMP) and antifolates. In colon cancer cells, TS protein is often over-expressed, resulting in possible drug resistance, which in turn is associated with poor response and/or survival rates in patients (8–11). Other antifolate resistance mechanisms include decreased transport into the cell, such as...
reduced expression or mutated forms of the reduced folate carrier (12). Resistance to nucleoside analogues is in general conferred by direct alterations in (expression of) enzymes involved in fluoropyrimidine metabolism (1, 13). This means that next to an increased protein expression of the target enzyme TS, decreased activation by TK or increased degradation by TP are possible mechanisms responsible for (induced) resistance to TFT (Fig. 1A). Decreased cellular uptake through nucleoside transporters and increased export by multidrug resistance proteins might play a role as well (14, 15). Taken together, the involvement of metabolic enzymes, such as TS, TK,
and orotate phosphoribosyltransferase (OPRT), but also transporter enzymes are often involved in drug resistance (11, 16–18). Interestingly, TFT was active in some 5-FU–resistant cell lines (19). This raises the question whether the induction of TFT resistance would induce different resistance profiles, compared with other nucleoside analogues.

In the present in vitro study, we aimed to characterize whether different protocols to induce resistance to TFT would also result in various types of resistance genotypes and phenotypes. Induction of resistance was either done by (a) classic continuous exposure to increasing low concentrations of TFT or (b) by intermittent short exposure to high TFT concentrations every week, which is in general a more clinically relevant schedule. The TFT-resistant H630 variants were characterized for protein expression and activity levels of the major enzymes involved in TFT metabolism, whereas microarray RNA expression and comparative genome hybridization analysis were used to gain a more in-depth insight in the mechanisms underlying TFT resistance.

Materials and Methods

Drugs and biochemicals
TFT and 5-chloro-6-[1-(2-iminopurrolidinyl)methyl]-uracil hydrochloride (TPI) were kindly provided by Taiho Pharmaceutical Co. Ltd. GW1843 was obtained from GlaxoSmithKline Inc. 4-Bromophenacyl bromide (4-BPB), 5-FU, 5-fluoro-2′-deoxyuridine, 5′-deoxy-5-fluorouridine (5′DFUR; doxifuridine), 2′,3′-didehydrodi-deoxythymidine (d4T; stavudine), thymidine (dTh), dCTP, 5,10-methylene-tetrahydrofolate, sulforhabdamine B (SRB), and propidium iodide (PI) were purchased from Sigma-Aldrich Chemicals. 3′-Deoxy-3′-fluorothymidine (FLT; alovudine) was kindly provided by Dr. Carla Molthoff (Department of Nuclear Medicine and PET Research, VUmc, Amsterdam, the Netherlands). [6-3H]-FdUMP (specific activity, 10.7 Ci/mmol) was purchased from Moravek Biochemicals Inc. [5-3H]-dUMP (specific activity, 16.2 Ci/mmol), [2-14C]-dTh (specific activity, 100 mCi/mmol), and the Hybond Enhanced Chemoluminescence detection kit were purchased from Amersham Biosciences Int. The primary monoclonal antibodies mouse-anti-human TS and mouse-anti-human TK were purchased from NeoMarkers (clone TS106) and QED Bioscience, respectively. All other chemicals were of analytic grade and commercially available.

Cell lines
The H630 cell line is derived from a human colorectal carcinoma and was a kind gift of Dr. P.G. Johnston (at that time at the National Cancer Institute, Bethesda, MD). Resistance to TFT was induced using two different exposure schedules for 12 mo. First, by an intermittent schedule, in which cells were exposed for 4 h every 7 d (starting at 5 μmol/L). This resulted in the resistant variant H630-cTFT, grown in 250 μmol/L TFT/4 h/wk. Second, resistance was induced by exposing cells continuously (starting at 0.5 μmol/L) to stepwise increasing concentrations of TFT (depending on the growth rate observed). This resulted in the resistant variant H630-cTFT, cultured in 20 μmol/L TFT. As reference cell line, we also used H630-R10, which is a 5-FU–resistant cell line derived from H630 and grows in the presence of 10 μmol/L 5-FU (16). The cell lines were cultured in DMEM (without antibiotics) supplemented with 10% heat-inactivated fetal bovine serum (Greiner Bio-One) and 20 mmol/L HEPES buffer (Lonza). The cell lines were grown as monolayers at 37°C in a humidified atmosphere containing 5% CO2 and were maintained in exponential growth with doubling times of 27 and 25 h, respectively, compared with 24 h for the parental H630 cells. Upon removal of TFT, the acquired resistance was maintained at least 2 wk for both H630 variants.

Growth inhibition studies
Sensitivity of the cell lines to TFT (±TPI), GW1843, 5-FU, 5-fluoro-2′-deoxyuridine, 5′DFUR, d4T, and FLT (Fig. 1B) was determined with the SRB cytotoxicity assay (20). 4-BPB was used to determine the role of phospholipase A2 in TFT resistance. Cells (5,000 cells/well) were exposed to increasing drug concentrations for 72 h. Subsequently, the cells were fixed with trichloro-acetic acid and stained with SRB. The IC50 values were defined as the concentrations that correspond to a reduction of cellular growth by 50% when compared with the values of nontreated control cells.

Flow cytometry analysis
Cell cycle distribution was measured of the cell lines exposed to TFT, as previously described (21). Briefly, 2 × 106 cells per well were seeded in six-well plates. After 24 h, the cells were exposed to 10 and 250 μmol/L TFT for 48 h. The cells were harvested, resuspended in PI solution (0.5 mg/mL RNase A, 0.05 mg/mL PI, 1 mg/mL sodium citrate, and 1 μL/mL Triton X-100), and chilled on ice (in the dark, at least 15 min), after which cell cycle distribution was measured by means of flow cytometry (FACScan, Becton Dickinson Immunocytometry Systems). For each measurement, 20,000 cells were counted and each cell line was assayed in duplicate. The percentage of cells in the G0+G1, S, or G2-M phase of the cell cycle was determined with the CellQuest software (Becton Dickinson). The total number of cells in these three cell cycle fractions was set at 100%.

Enzyme assays
TS, TK, and TP enzyme activities in the cell lines were determined according to previously described methods, which were summarized by van der Wilt et al. (22). For each assay, frozen cell pellets were suspended into their appropriate assay buffer and aliquots were taken for measuring protein content using the Bradford protein assay. The FdUMP binding assay was done to determine the number of free FdUMP binding sites of TS (using

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1050 RNA expression levels were quantified using the LightCycler software (Roche), using calibration curves and β-actin for determining the expression ratios.

**Arachidonic acid measurement by liquid chromatography-mass spectrometer**

Samples were analyzed based on the method published by Carrascal et al. (27). Supernatants from 0.5 million cells were prepared and analyzed using an API SCIEX3000 system.

**Statistical analysis**

The Student’s t test for paired data was used for the differences between H630 and the TFT-resistant variants for level in cytotoxicity, enzymatic activity, mRNA expression (RT-PCR), and protein expression levels. Differences were considered significant when P < 0.05.

**RNA expression microarray procedures**

The extended protocol of the RNA expression microarray procedure is described in the Supplementary Data. The resistant cell lines were hybridized to the parental H630 cells to find differences between the RNA expression profiles of the TFT-resistant and parental cell line. 30 K 60-mer oligonucleotides were used and labeled with fluorolink monofunctional Cy5 or Cy3 dye (Amersham; refs. 28, 29). Pathway analysis of genes that were 2-fold upregulated or downregulated was done using pathway-explorer (https://pathwayexplorer.genome.tugraz.at/). Microarray data are available from Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) with the accession number GSE18137. Statistical significance of sPLA2 upregulation was calculated using the R-software package of Significance Analysis of Microarrays (version 3.0).

**Array comparative genomic hybridization**

The extended protocol of the array comparative genomic hybridization (CGH procedures is described in the Supplementary Data. In Brief, 500 ng of DNA were labeled and hybridized on 44 k arrays (Agilent Technologies; ref. 30). Array data are available from Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) with the accession number GSE18137.

**Results**

**Resistance induction and levels of cross-resistance to TS inhibitors**

Resistance to TFT was induced in H630 cells by gradually increasing TFT concentrations, starting from 0.5 μmol/L (continuously) or 5 μmol/L (4 hours/7 days). Over a period of several months, this resulted in H630-cTFT and H630-4TFT cells, which were grown with 50 μmol/L TFT. For H630-cTFT cells, the concentrations were increased using only 0.5 μmol/L steps, which required ~5 months to induce resistance to 10 μmol/L TFT. For H630-4TFT cells, the
concentrations were increased using steps of 5 μmol/L, which required ~3 months to induce resistance to 50 μmol/L TFT, after which 20 μmol/L steps were used to further increase TFT resistance. The cell lines were tested (SRB-assay) for TFT sensitivity several times during the procedure.

H630-4TFT cells were resistant to TFT with a resistance factor of 1320 (Table 1). H630cTFT cells were ~336-fold resistant (Table 1). These resistance factors were higher than the level of TFT cross-resistance in the 5-FU-resistant cell line H630-R10 (Table 1). TFT sensitivity was maintained for at least 2 weeks, when the cell lines were grown in drug-free medium, and decreased ~35% after growing in TFT-free medium for 30 days (Table 1). The TP inhibitor TPI did not affect TFT sensitivity (data not shown), which agrees with earlier experiments showing that TPI did not affect TFT sensitivity of colon cancer cells even with high TP expression (1).

To get initial insight in the mechanisms of resistance, the cell lines were tested for cross-resistance to other drugs, which either have a related molecular structure or mechanism of action compared with TFT (Fig. 1D), such as TS inhibitors and drugs dependent on TP or TK for activation [GW1843, 5-FU, 2′-deoxy-5-fluorouridine (FdUrd), 5′DFUR, d4T, and FLT]. H630-4TFT and H630-cTFT cells were not cross-resistant to the specific and potent folate-based TS inhibitor GW1843 (Table 1). The H630-cTFT cells were ~2-fold resistant to 5-FU and 5′DFUR. On the other hand, both cell lines were clearly cross-resistant to FdUrd (>145-fold; P < 0.05), which needs to be activated by TK, and is targeted to TS. H630 cells were relatively insensitive to the anti-HIV drug d4T, which is also activated by TK1 (31). The H630 variants were comparably insensitive to the d4T. FLT is also a substrate for TK1 (31) and was not toxic to the cells with IC50 of >1 mmol/L (data not shown). As expected, H630-R10 with its high TS levels was resistant to 5-FU, FdUrd, and 5′DFUR (at least 8-fold; all P < 0.01).

### Cell cycle distribution

To determine whether induction of resistance led to different response of cells on the cell cycle, the cell cycle distribution was analyzed after exposing cells to various concentrations of TFT. No clear difference in cycle distribution between the nontreated parental and resistant cells was observed, although the resistant cells tended to have less cells in the G2-M phase (Fig. 1C). In H630 cells, after exposure to the IC50 concentration of TFT, the cell cycle hardly redistributed. After exposure to higher concentrations, e.g., 10 or 250 μmol/L TFT (IC75 and IC90, respectively), the parental H630 cells were strongly arrested in the S and G2-M phase (P < 0.01). In H630-4TFT cells, the cell cycle distribution hardly changed when exposed to 10 or 250 μmol/L TFT, which were subtoxic concentrations for this cell line. In contrast, an S-phase arrest was induced in the less resistant H630-cTFT after exposure to 250 μmol/L TFT, probably because this concentration exceeds the IC50 concentration.

### Changes in enzyme levels involved in TFT metabolism

Based on the cross-resistance patterns and known targets of TFT, we determined the activities of rate-limiting and target enzymes. Elevated TS might be a mechanism responsible for the acquired TFT resistance. Surprisingly, TS activity was decreased in the H630-4TFT cells both at half-saturating (1 μmol/L) and saturating (10 μmol/L) substrate concentrations (>50%; P < 0.05), but no change in TS activity was seen in the H630-cTFT cells (Table 2). The number of FdUMP binding sites remained unchanged in both cell lines (Table 2). Western blot analysis (Fig. 1D) showed no significant change in TS protein levels in the TFT-resistant cell lines.

### Table 1. Growth inhibition by different drugs for the TFT-resistant colon cancer cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>TFT At day 0</th>
<th>At day 30</th>
<th>+ 4-BPB RF</th>
<th>GW1843</th>
<th>5-FU</th>
<th>FdUrd 5dFUR</th>
<th>d4T</th>
</tr>
</thead>
<tbody>
<tr>
<td>H630</td>
<td>0.5 ± 0.1†</td>
<td>0.4 ± 0.2</td>
<td>5.6 ± 0.9</td>
<td>3.3 ± 0.7</td>
<td>0.04 ± 0.003</td>
<td>60 ± 8</td>
<td>384 ± 2</td>
</tr>
<tr>
<td>H630-4TFT</td>
<td>660 ± 70†</td>
<td>431 ± 33‡</td>
<td>593 ± 34</td>
<td>320</td>
<td>3.4 ± 0.1</td>
<td>4.2 ± 0.6</td>
<td>7.1 ± 1.0‡</td>
</tr>
<tr>
<td>H630-cTFT</td>
<td>168 ± 30†</td>
<td>107 ± 4.4‡</td>
<td>49 ± 9‡</td>
<td>336</td>
<td>7.2 ± 0.4</td>
<td>8.5 ± 1.0</td>
<td>5.9 ± 1.6‡</td>
</tr>
<tr>
<td>H630-R10</td>
<td>149 ± 9†</td>
<td>ND</td>
<td>298</td>
<td>ND</td>
<td>167 ± 26‡</td>
<td>15 ± 2.7†</td>
<td>497 ± 50†</td>
</tr>
</tbody>
</table>

NOTE: Values (IC50 in μmol/L; in nmol/L for GW1843) are means ± SEM of at least three experiments. Sensitivity to TFT was determined directly after continuous exposure of cells to TFT (at day 0), or after 30 d growing in TFT-free medium (at day 30). H630-R10 is a 5-FU-resistant cell line.

Abbreviations: RF, resistance factor (average IC50 variants at day 0/average IC50 H630); ND, not done.

†Previously published (50).

‡P < 0.01 compared with H630.

*P < 0.05 compared with H630.
cells, total TK activity (including both the cytosolic TK1 and the mitochondrial TK2) was increased over 2-fold \((P < 0.05)\), thereby possibly increasing TFT activation. The TK2-inhibitor deoxycytidinetriphosphate decreased total TK activity \(\sim 75\%\) in all three cell lines; the TK1 activities showed a similar pattern as the total TK activity values. The activity of the TFT-degrading enzyme TP did not change in the TFT-resistant cell lines (Table 2). Taken together, these data indicate that for H630-cTFT cells, another mechanism is responsible for the observed TFT and FdUrd resistance, besides changes in expression levels and activity of activating and inactivating enzymes.

Nucleoside analogues are often transported into the cell by nucleoside transporters. A decreased nucleoside transporter expression may be related to drug resistance. TFT is indeed dependent of hENT for entering the cell because dipyridamole caused a 29.5-fold \((\pm 1.25)\) increase in IC\(_{50}\) concentration in H630 cells. To examine whether the levels of the nucleoside transporters were changed, RT-PCR analysis of mRNA expression of hENT and hCNT were determined. Equilibrative nucleoside transporters transport nucleoside substrates such as adenosine into the cell. Concentrative nucleoside transporters transport nucleosides in an active, concentrative, and Na\(^+\)-dependent manner. hENT mRNA was over 2-fold lower in both H630-4TFT and H630-cTFT cells, compared with H630 (Table 2). hCNT mRNA levels were slightly lower in H630-4TFT. hCNT mRNA levels were 16-fold increased in H630-cTFT compared with H630 cells. To examine whether these lowered levels also resulted in a lower accumulation of TFT inside the cells, TFT nucleotides were determined (Table 2). Compared with H630 cells, TFT accumulated at much lower levels in H630-cTFT and H630-4TFT cells. Taken together, these data show a general decrease in TFT activation and also indicate that hCNT does not play a role in TFT uptake and hence sensitivity. The very low phosphorylation rate in H630-4TFT cells are consistent with the decreased TK1 and possibly hENT expression levels.

RNA expression profiles of the resistant cells
The analysis of the investigated resistance variables for TFT revealed a clear logic explanation for H630-4TFT cells, but the exact mechanism of resistance in H630-cTFT remained unclear. To elucidate this resistance mechanism, we performed a whole human genome microarray analysis of the investigated resistance variables for TFT.

### Table 2. Levels of target proteins, nucleoside transporter genes, and TFT nucleotide accumulation in H630 cells and the TFT-resistant variants H630-4TFT and H630-cTFT

<table>
<thead>
<tr>
<th>Cell line</th>
<th>H630</th>
<th>H630-4TFT</th>
<th>H630-cTFT</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>TS</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FdUMP binding sites*</td>
<td>0.52 ± 0.13</td>
<td>0.34 ± 0.08</td>
<td>0.77 ± 0.23</td>
</tr>
<tr>
<td>TS activity: at 1 μmol/L dUMP†</td>
<td>0.53 ± 0.03</td>
<td>0.23 ± 0.08‡</td>
<td>0.56 ± 0.03</td>
</tr>
<tr>
<td>TS activity: at 10 μmol/L dUMP†</td>
<td>1.93 ± 0.23</td>
<td>0.58 ± 0.14‡</td>
<td>1.89 ± 0.23</td>
</tr>
<tr>
<td>TS protein expression (%)§</td>
<td>100</td>
<td>122.3 ± 21.2</td>
<td>88.7 ± 9.1</td>
</tr>
<tr>
<td><strong>TK</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total TK activity†</td>
<td>9.04 ± 1.09</td>
<td>0.57 ± 0.06¹</td>
<td>21.28 ± 0.25²</td>
</tr>
<tr>
<td>TK1 activity†</td>
<td>2.96 ± 0.35</td>
<td>0.16 ± 0.01‡</td>
<td>4.07 ± 1.13</td>
</tr>
<tr>
<td>TK protein expression (%)§</td>
<td>100</td>
<td>&lt;5</td>
<td>154 ± 28.6</td>
</tr>
<tr>
<td><strong>TP</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TP activity†</td>
<td>18.96 ± 2.93</td>
<td>16.76 ± 2.6</td>
<td>20.68 ± 2.72</td>
</tr>
<tr>
<td><strong>Transporters</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>hENT mRNA expression (%)¶</td>
<td>100</td>
<td>40 ± 8</td>
<td>46 ± 14²</td>
</tr>
<tr>
<td>hCNT mRNA expression (%)¶</td>
<td>100</td>
<td>93 ± 7</td>
<td>1,600 ± 265¹</td>
</tr>
<tr>
<td><strong>TFT nucleotides (nmol/million cells)</strong></td>
<td>29.1 ± 3.2</td>
<td>6.8 ± 2.1</td>
<td>3.2 ± 1.4</td>
</tr>
<tr>
<td><strong>sPLA2 IIA</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intracellular (pg/million cells)</td>
<td>41.2 ± 5.0</td>
<td>&gt;10,000</td>
<td>&gt;10,000</td>
</tr>
<tr>
<td>Extracellular (pg/million cells)</td>
<td>7.0 ± 0.9</td>
<td>235.3 ± 25.1</td>
<td>333.6 ± 16.8</td>
</tr>
</tbody>
</table>

**NOTE:** All values are means ± SEM of at least three experiments.
*FdUMP binding sites in fmol/mg protein.
†Enzyme activities in nmol/hr/mg protein.
‡Significantly different compared to parental cells \((P < 0.05)\).
§Relative density compared to H630.
∥Significantly different compared to parental cells \((P < 0.01)\).
¶Values are given as a percentage in which H630 mRNA expression levels were set to 100%.
analysis to identify changes in mRNA levels. Functional classification revealed a frequent deregulation of genes encoding signaling proteins in both resistant derivatives. In H630-4TFT cells, hardly any major alterations were found in the expression profile (Fig. 2A) and genes that were differentially expressed were involved in cell metabolism, cell communication, and signal transduction, although genes were not statistically differently expressed due to the low sample size (Table 3). Although the number of altered genes was higher in H630-4TFT cells, compared with H630-cTFT cells, the major relative difference between the two resistant cell lines was the large number of genes involved in lipid metabolism in H630-cTFT cells (Table 4). In H630-cTFT cells, the most pronounced alteration was the 47-fold ($P < 0.05$) upregulation in transcript coding for secretory phospholipase A2 (sPLA2) IIA, which could easily be judged by eye (Fig. 2A). Other genes that were differentially expressed were involved in cell metabolism and signal transduction. Interestingly, genes that are directly or possibly involved in TFT metabolism (TK, TS, TP, and dUTPase) did not show any significant change in mRNA levels.

To confirm the increased sPLA2 mRNA expression levels in H630-cTFT found in the expression microarray, RT-PCR was done. A 211-fold difference was found in the sPLA2/β-actin ratio between H630-cTFT and H630 cells ($P < 0.05$). This ratio was 1.2-fold for H630-4TFT cells. sPLA2 protein was highly increased in both H630-cTFT and H630-4TFT cells compared with wild-type cells, leading to an increased secretion (Table 2). sPLA2 mediates the conversion of phospholipids to arachidonic acid and subsequent prostaglandins. Therefore, we determined intracellular arachidonic acid levels in H630-cTFT cells. The level of arachidonic acid was increased (138%) in H630-cTFT, compared with the parental cell line (data not shown), indicating that sPLA2 increased the conversion to arachidonic acid. To determine whether sPLA2 plays a role in the resistance to TFT, we added the PLA2 inhibitor 4-BPB and measured a 70% reduction ($P < 0.01$) in TFT resistance, although resistance was not completely reversed back to parental sensitivity (Table 1). 4-BPB did not change TFT sensitivity in H630 and H630-4TFT cells.

**DNA copy number alterations by array CGH**

To determine whether the differences in gene expression were associated with alterations in gene copy numbers in TFT-resistant cells, we used array CGH and compared the cell lines with each other. In the parental H630 cells, homozygous losses and gains were found in almost all chromosomes compared with reference DNA (Fig. 2B). These alterations included the often observed 18q loss and 20q gain in colon cancer (32, 33). Several chromosomes containing genes involved in TFT metabolism showed losses in 22q13.33 (TP), 18p11.32 (TS), and...
15q15-q21.1 (dUTPase; Table 2) compared with reference DNA. However, H630 cells had normal levels of these enzymes (Table 1); thus, the gene is functionally expressed. In addition, a chromosomal loss was found in the genes containing the nucleoside transporters hENT (6p21.2-p21.1) and hCNT (9q22.2).

In H630-4TFT cells, three large genomic alterations were found compared with the parental H630 cells, consisting of a loss within chromosome 1 and a gain within chromosomes 17 and 19 (Fig. 2B). Interestingly, the chromosomal location of TK1 showed a gain. H630-cTFT cells were distinguished from the parental cells by many losses within chromosome 3, 5, 6, 7, 9, 11, and 12 and a gain within chromosome 1 (Fig. 2B). Chromosomal regions containing the metabolic enzymes were not changed in this cell line, compared with the parental H630 cells, which agrees with the functional activity and protein expression of these enzymes (Table 2; Fig. 1D).

Discussion

In the present study, we describe the induction of acquired resistance to TFT in H630 colon cancer cells. The use of alternative exposure schedules (intermittent, continuous) yielded two TFT-resistant cell lines with different mechanisms of resistance. This emphasizes the importance of drug scheduling in inducing in vitro drug resistance, but also indicates that scheduling of drugs in vivo and in the clinic may lead to different resistance mechanisms. Resistance mediated by intermittent exposure was predominantly associated with a decreased expression in the key activating enzyme TK. The up-regulation of sPLA2 has to our knowledge never been reported before in relation to resistance to a nucleoside analogue. The exact role of sPLA2 remains unknown.

TFT (as TAS-102) is currently under development as a new (oral) treatment option in 5-FU resistant cells, both in in vitro and in vivo studies (18, 19). Increased TS levels are often associated with resistance to TS inhibitors, including 5-FU-based (8) and antifolate-based TS-inhibitors (22); hence, TS activity is one of the best predictors for 5-FU sensitivity (4). 5-FU- or antifolate-resistant colorectal cancer cells with increased TS levels may show cross-resistance to TFT (1, 11, 18). Therefore, increased TS levels can cause TFT resistance, possibly only when TS is increased at very high levels. In the present study, no significant increase in TS level was detected in both TFT-resistant cell lines, and no cross-resistance to 5-FU, 5′DFUR, and the antifolate GW1843 was observed.

TP is one of the enzymes that can inactivate TFT. However, we previously showed that increased TP levels in the cancer cells were not directly associated with TFT resistance (1, 34). Only at a very short TFT exposure, inhibition of TP affected TFT cytotoxicity in Colo320TP1 cells, which express TP at very high levels (1). However, in the TFT-resistant cell lines in our study, TP activity also remained at control levels in both TFT-resistant cell lines, and can therefore not be considered a resistance marker for TFT. This is in contrast to 5′DFUR because increased TP levels may enhance 5′DFUR activation.

Previously, TFT resistance, developed after continuous TFT exposure by increasing drug concentrations, was related to a decreased TK activity (18). To convert TFT to its active forms, sufficient TK activity is essential (1).
study, only H630-4TFT cells had both decreased TK activity and decreased protein levels, which was also associated with cross-resistance toFdUrd.

Resistance mechanisms to nucleoside analogues may also be conferred by decreased expression of transporters proteins. RNA levels of hENT, which is necessary to transport TFT into the cells, were downregulated and TFT nucleotides accumulated at low levels in both H630-cTFT and H630-4TFT cells, indicating that a lowered hENT membrane expression or a lowered hENT function may also be one of the responsible mechanisms of TFT resistance. Enzyme and growth inhibition studies did not reveal a mechanism for the resistant phenotype of H630-cTFT, although a decreased hENT expression is likely to be responsible for the decreased accumulation of TFT nucleotides in the cells. To further study whether other pathways were involved in TFT resistance, we performed an expression microarray and array CGH analysis of the resistant cell lines. The expression microarray data analysis did not show a change in mRNA levels in the H630-cTFT cells of the genes involved in TFT metabolism. The most significant result was the enormous upregulation of sPLA2 type IIA, which is a novel finding. sPLA2 has a role in carcinogenesis, including that of gastrointestinal cancers (35–37). Phospholipases A2 determines most of the arachidonic acid release in cells, of which the concentration was indeed increased in H630-cTFT cells. Arachidonic acid can be converted

| Table 4. Altered gene expression in lipid metabolism pathways of H630-cTFT versus H630 cells and H630-4TFT versus H630 cells |
|-----------------|-----------------|----------------|-----------------|-----------------|
| Gene name       | Gene ID         | Gene description                                      | Ratio* | Gene function | Chromosome   |
|-----------------|-----------------|-----------------|-----------------|-----------------|
| H630-cTFT cells |                 |                 |                 |                 |
| PLA2 G2A        | NM_000300       | Phospholipase A2 group IIA                              | 47.83  | Hydrolysis of fatty acids; lipid catabolism | 1p35 |
| UGT1A6          | NM_001072       | UDP glucuronosyltransferase 1 family, polypeptide A6    | 6.45   | Metabolism    | 2q37   |
| PLA2G4A         | NM_024420       | Phospholipase A2, group IVA                              | 4.85   | Lipid catabolism, phospholipid catabolism | 1q25   |
| HMGCS1          | NM_002130       | 3-Hydroxy-3-methylglutaryl-CoA synthase 1 (soluble)      | 3.48   | Acetyl-CoA metabolism, cholesterol biosynthesis, lipid metabolism | 5p14-p13 |
| AKR1C3          | NM_003739       | Aldo-keto reductase family 1                             | 2.83   | Cell proliferation, lipid and prostaglandin metabolism | 10p15-p14 |
| FDFT1           | NM_004462       | Farnesyl-diphosphate farnesyltransferase 1              | 2.65   | Cholesterol and isoprenoid biosynthesis | 8p23.1-p22 |
| ALDH9A1         | NM_000696       | Aldehyde dehydrogenase 9 family, member A1              | 2.44   | Hormone metabolism, oxidation reduction | 1q33.1 |
| CPT1A           | NM_001876       | Carnitine palmitoyltransferase 1A                        | 0.22   | Lipid metabolism, fatty acid metabolism, fatty acid β-oxidation, transport | 11q13.1-q13.2 |
| CYP2B6          | NM_000767       | Cytochrome P450, family 2, subfamily B, polypeptide 6   | 0.41   | Arachidonic acid metabolism, oxidation reduction | 9q13.2 |
| OGT             | NM_003605       | O-linked N-acetylglucosamine (GlcNAc) transferase       | 0.41   | Response to nutrients, signal transduction | Xq13 |
| H630-4TFT cells |                 |                 |                 |                 |
| TPI1            | NM_000365       | Triosephosphate isomerase 1                             | 2.60   | Fatty acid biosynthesis, glyceraldehyde-3-phosphate metabolic process, glycolysis, pentose-phosphate shunt | 12p13 |
| LYPAL1          | NM_006330       | Lysophospholipase I                                     | 2.17   | Fatty acid metabolism, lipid metabolism | 8q11.23 |
| HSD17B8         | NM_014234       | Hydroxysteroid (17-β) dehydrogenase 8                   | 2.30   | Androgen metabolism, estrogen biosynthesis, oxidation reduction | 6p21.3 |
| EHHADH          | NM_001966       | Enoyl-CoA hydratase                                     | 2.27   | Fatty acid β-oxidation, fatty acid metabolism, lipid metabolism, oxidation reduction | 3q26.3-q28 |
| DGKE            | NM_003647       | Diacylglycerol kinase                                   | 0.47   | Phospholipid biosynthesis, intracellular signaling cascade | 17q22 |
| OGT             | NM_003605       | O-linked N-acetylglucosamine (GlcNAc) transferase       | 0.41   | Response to nutrients, signal transduction | Xq13 |

*Genes that were upregulated or downregulated at least 2-fold were selected. Data are expressed as the ratio between mRNA levels of H630-cTFT or H630-4TFT compared to H630.
into prostaglandins, which are involved in several (patho)physiologic processes, including cell survival, proliferation, and Fas-mediated apoptosis (36, 38). Arachidonic acid produced by sPLA2 enzymatic activity promotes apoptosis in colon cancer cells (36, 39–41). In addition, Fas may be downregulated, although hardly any genes involving apoptosis were differentially expressed. TS inhibition can trigger Fas-dependent apoptosis (42–45). Deregulation of the Fas/FasL signaling pathway confers resistance to CRC, despite achievement of strong TS inhibition upstream. In patients, 5-FU treatment decreased Fas expression (44). TFT was previously shown to induce apoptosis through both the extrinsic and intrinsic pathway in CRC cells (46). Because H630-cTFT cells were resistant to all fluoropyrimidine drugs tested and TS was not upregulated, prevention of apoptosis induction may therefore also be a mechanism of resistance to TFT.

Upregulation of sPLA2 in H630-cTFT cells was associated with a strong disturbance in signal transduction and energy and lipid metabolism, possibly resulting in a growth advantage under stress conditions, such as high TFT levels. The role of sPLA2 in TFT resistance was evident because the sPLA2 inhibitor 4-BPB reversed resistance almost completely, although the exact role of sPLA2 remains to be identified. sPLA2 is currently also under investigation in liposome-mediated drug target–delivered therapies, in which increased sPLA2 activity in the tumor microenvironment is used as a trigger for the release of anticancer etherlipids (47, 48). In addition, TFT may also be combined with sPLA2 inhibitors, such as varespladib methyl (1-H-indole-3-glyoxamide; A-002), LY311727 (3-[1-benzyl-3-carbamoylmethyl]-2-ethyl-indol-5-yl]-oxypropylphosphonic acid), and LY374388 (3-aminoxyalyl-1-benzyl-2-ethyl-6-methyl-1H-indol-4-yloxy-acetic acid methyl ester; ref. 49). These agents are under evaluation in clinical trials against cardiovascular diseases. This provides potential advantages for the use of TFT in combinations with sPLA2 inhibitors and/or liposome-mediated drug target delivery systems.

We can conclude that a decreased TK protein and hENT expression are important mechanisms for TFT resistance. However, enzymes involved in TFT metabolism do not necessarily have to be related to induction of TFT resistance. The method to induce TFT resistance may lead to different mechanisms of resistance.

Disclosure of Potential Conflicts of Interest

M. Fukushima: employee, Taiho Pharmaceutical Co., Ltd. G.J. Peters: research grant support, Taiho Pharmaceutical Co., Ltd. No other potential conflicts of interest were disclosed.

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References


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