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 (array-CGH). Both cell lines were cross-resistant to 2'-deoxy-5-fluorouridine (>170-fold). Exposure to IC{sub 75}-TFT increased the S/G{sub 2}/M-phase of H630 cells, while 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 2-3 fold decreased, resulting in a 5-10 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 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.

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 chemotherapeutic regimen\(^1\). Upon uptake into the cells, TFT can be converted to its monophosphate derivative (TF-TMP) by thymidine kinase (TK) (Figure 1A). TF-TMP binds covalently to the active site of thymidylate synthase (TS) thereby inhibiting its activity\(^1,2,3\). TS catalyzes the methylation of 2'-deoxyuridine-5'-monophosphate (dUMP) to 2'-deoxythymidine-5'-monophosphate (dTMP), in which 5,10-
methylene-tetrahydrofolate (CH2-THF) 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. Inhibition of TS results in depletion of dTTP and an increase in dUTP in the cell (thymine-less state), resulting in misincorporation of dUTP into the DNA. 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.

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-2'-deoxyuridine-5'-monophosphate (FdUMP) and antifolates. In colon cancer cells TS protein is often overexpressed, resulting in possible drug resistance, which in turn is associated with poor response and/or survival rates in patients. Other antifolate resistance mechanisms include decreased transport into the cell, such as reduced expression or mutated forms of the reduced folate carrier (RFC). Resistance to nucleoside analogues is in general conferred by direct alterations in (expression of) enzymes involved in fluoropyrimidine metabolism. This means that next to an increased protein expression of the target enzyme TS, decreased activation by TK or increased degradation by thymidine phosphorylase (TP) are possible mechanisms responsible for (induced) resistance to TFT. Taken together, the involvement of metabolic enzymes, such as TS, TK and OPRT, but also transporter enzymes are often involved in drug resistance. Interestingly, TFT was active in some 5-FU resistant cell lines. This raises the question whether induction of TFT resistance would induce different resistance profiles, compared to 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 performed by (1) classical continuous exposure to increasing low concentrations of TFT, or (2) 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, while microarray RNA expression and Comparative Genome Hybridization (array CHG)-analysis were used to gain a more in depth insight in the mechanisms underlying TFT resistance.
MATERIALS & METHODS

Drugs and biochemicals

TFT and TPI (5-chloro-6-[1-(2-iminopyrrolidinyl)methyl]-uracil hydrochloride) were kindly provided by Taiho Pharmaceutical Co. Ltd. (Tokushima, Japan). GW1843 was obtained from GlaxoSmithKline Inc. (Research Triangle Park, NC, USA). 4-bromophenacyl bromide (4-BPB), 5-FU, FdUrd, 5'-deoxy-5-fluourouridine (5’DFUR; doxifluridine), 2',3'-didehydrodideoxythymidine (d4T; stavudine), thymidine (TdR), deoxycytidinetriphosphate (dCTP), CH2-THF, sulforhodamine B (SRB) and propidium iodide (PI) were purchased from Sigma-Aldrich Chemicals (Zwijndrecht, The Netherlands). 3'-deoxy-3'-fluorothymidine (FLT; alovudine) was kindly provided by Dr. Carla Molthoff (Dept. of Nuclear Medicine and PET Research, VUmc, Amsterdam, the Netherlands). [6-\(^3\)H]-FdUMP (specific activity 10.7 Ci/mmol) was purchased from Moravek Biochemicals Inc. (Brea, CA, USA). [5-\(^3\)H]-dUMP (specific activity 16.2 Ci/mmol), [2-\(^14\)C]-TdR (specific activity 57.0 mCi/mmol) and Hybond Enhanced ChemoLuminescence (ECL) detection kit were purchased from Amersham Biosciences Int. (Buckinghamshire, UK). The primary monoclonal antibodies mouse-anti-human TS and mouse-anti-human TK were purchased from NeoMarkers (clone TS106; Fremont, CA, USA) and QED Bioscience (San Diego, CA, USA), respectively. All other chemicals were of analytical 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, USA). Resistance to TFT was induced using 2 different exposure schedules for 12 months. First, by an intermittent schedule, where cells were exposed for 4 h every 7 days (starting at 5 \(\mu\)M). This resulted in the resistant variant H630-4TFT, grown in 250 \(\mu\)M TFT/4hr/week. Second, resistance was induced by exposing cells continuously (starting at 0.5 \(\mu\)M) to stepwise increasing concentrations of TFT (depending on the growth rate observed). This resulted in the resistant variant H630-cTFT, cultured in 20 \(\mu\)M 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 \(\mu\)M 5-FU\(^{16}\). The cell lines were cultured in DMEM medium (without antibiotics) supplemented with 10% heat-inactivated foetal bovine serum (FBS; Greiner Bio-One, Frickenhausen, Germany) and 20 mM Hepes buffer (Lonza, Basel, Switzerland). The cell lines were grown as monolayers at 37 °C in a humidified atmosphere containing 5% CO\(_2\) and were maintained in exponential growth with doubling times of 27 and 25 h, respectively.
TFT resistance by decreased TK, ENT or sPLA2 overexpression

compared to 24 h for the parental H630 cells. Upon removal of TFT, the acquired resistance was maintained at least two weeks for both H630 variants.

**Growth inhibition studies**
Sensitivity of the cell lines to TFT (± TPI), GW1843, 5-FU, FdUrd, 5’DFUR, d4T and FLT (Figure 1B) was determined with the sulphorhodamine B (SRB)-cytotoxicity assay\(^{20}\). 4-BPB was used to determine the role of phospholipase A2 in TFT resistance. Cells (5000 cells/well) were exposed to increasing drug-concentrations for 72 h. Subsequently, the cells were fixed with trichloroacetic acid (TCA) and stained with SRB. The IC\(_{50}\) values were defined as the concentrations that correspond to a reduction of cellular growth by 50% when compared to the values of untreated control cells.

**Flow cytometry analysis**
Cell cycle distribution was measured of the cell lines exposed to TFT, as described previously\(^{21}\). Briefly, 2x10^5 cells/well were seeded in 6-wells plates. After 24 h, the cells were exposed to 10 µM and 250 µM TFT for 48 hr. The cells were harvested, resuspended in PI-solution (0.5 mg/ml RNase A, 0.05 mg/ml PI, 1 mg/ml sodium citrate, 1 µl/ml Triton X-100) and chilled on ice (in the dark, at least 15 min), where after cell cycle distribution was measured by means of flow cytometry (FACScan, Becton Dickinson Immunocytometry Systems, San Jose, CA, USA). For each measurement 20000 cells were counted and each cell line was assayed in duplicate. The percentage of cells in the G0/G\(_1\)-, S-, or G2/M-phase of the cell cycle was determined with 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 \textit{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 performed to determine the number of free FdUMP binding sites of TS (using [6-3H]-FdUMP), and the TS catalytic assay was performed to measure the catalytic activity of TS by measuring the release of tritiated water in the TS catalyzed conversion of [5-\(^3\)H]-dUMP into dTMP\(^{22}\). TS activity was measured at saturating substrate concentration (10 µM dUMP) and at approximate half-saturating substrate concentration (1 µM dUMP). Total TK activity consisting of cytosolic TK1 and mitochondrial TK2 was measured as described previously\(^{22}\). Using [2-\(^{14}\)C]-TdR we measured phosphorylation of TdR to dTMP. To determine TK1 activity
only, a specific inhibitor of TK2 (dCTP) was added to the substrate solution (final concentration 10 mM). TP activity was determined, as previously described\textsuperscript{22}. In this assay, TdR was used as a substrate to measure TP activity. Enzyme activity was calculated by the conversion of TdR into thymine, which were both detected by high performance liquid chromatography (HPLC). TFT-phosphorylation levels after exposure to 100 µM TFT were determined as described previously\textsuperscript{23}.

**Western blot analysis**

Frozen cell pellets were lysed in TBS buffer (10 mM Tris/HCl, 5 mM EDTA, 150 mM NaCl; pH 7.6) containing 0.1% Triton X-100 (2x10^7 cells/ml). Protein content was measured in supernatants, after sonification and centrifugation, using the Bradford protein assay. A total of 20 µg protein separated on a 10% SDS-PAGE gel, followed by blotting on a nitrocellulose membrane (Amersham). To prevent aspecific antibody binding, the membranes were pre-incubated overnight at 4°C with blocking buffer (TBS buffer containing 0.05% Tween-20 (TBS-T), 5% milkpowder). Membranes were subsequently incubated with the primary antibodies for 1 hr at room temperature. After washing the membranes with TBS-T, the secondary horseradish peroxidase-conjugated antibodies were added (diluted in blocking buffer containing 1% milkpowder). After washing, the antibody binding was detected by means of ECL and autoradiography. Quantification of the protein bands was performed by densitometric scanning.

**RT-PCR**

RNA was extracted using an RNeasy kit (Qiagen, Westburg B.V., Leusden, The Netherlands). Each extract was checked for DNA contamination and subsequently reverse transcribed by M-MLV-RT using random hexamers (Amersham, USA). Oligonucleotide primers were designed for β-actin and sPLA2 (F:GGGGCAGAAGTTGAGACCAC; R:CACAGTGGCAGCCGTAAG) using Primer 3 Output\textsuperscript{24}, hENT and hCNT\textsuperscript{25,26}. cDNA samples were amplified using a LightCycler (Roche Diagnostics, Almere, The Netherlands) with 10 sec 95°C denaturation, 5 sec 60°C primer annealing and 23 sec 72°C DNA elongation for 45 cycles starting with a 10 min hot start at 95°C. RNA expression levels were quantified using LightCycler software (Roche, 4.05), using calibration curves and β-actin for determining the expression ratios.
Arachidonic acid measurement by LC-MS/MS
Samples were analysed based on the method published by Carrascal et al.\textsuperscript{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
Total RNA isolations from separate harvests of H630, H630-4TFT, H630-cTFT cells were performed using the TriZol method according to the manufacturer's protocol (Invitrogen, Leek, The Netherlands). Concentrations were measured by A260 (Nanodrop Spectrophotometer) and quality was judged on a 1.2% agarose gel. Samples were dissolved in DMPC-treated H$_2$O and stored at –80°C prior to use in either the microarray hybridizations or RT-PCR confirmation. The human OligoLibrary (Compugen, Sigma-Aldrich) containing 30K 60-mer oligonucleotides was resuspended to a concentration of 10 $\mu$M in 150 mM sodium phosphate buffer (pH 8.5), using the SpotArray 72 (PerkinElmer, Zaventem, Belgium), as described before\textsuperscript{28}. Slides were processed according to the manufacturer's protocol. Single stranded cDNA was synthesized from 30 $\mu$g of total RNA by reverse transcription using aminally-labeled dUTP (Ambion Ltd., Huntingdon, UK). Labeling with fluorolink monofunctional Cy5 or Cy3 dye (Amersham, Roosendaal, The Netherlands) was described in detail previously\textsuperscript{29}. Qiaquick PCR purification columns (Qiagen, Westburg B.V., Leusden, The Netherlands) were used to remove the uncoupled dyes. (Pre)hybridization was done using a hybridization station (HybArray 12, Perkin-Elmer, Zaventem, Belgium). After hybridization, the slides were washed in the HybArray 12, with 50% formamide (Fluka, Sigma-Aldrich Chemie), 2x SSC (pH 7) at 35°C for 15 minutes followed by PI buffer (0.1 mol/L sodium phosphate, 0.1% Igepal Ca630 (pH 8)) at room temperature and three washes of 0.2x SSC, 0.1x SSC, and 0.01x SSC at RT followed by centrifugation. Arrays were scanned using a laser scanner (ScanArray Express, PerkinElmer, Zaventem, Belgium). Analysis was performed using BlueFuse software version 2.0 (BlueGnome, Cambridge, UK). Cy3/Cy5 ratios were calculated by taking the log$_2$ of the 'signal mean' of each spot, followed by a standard normalization for spot intensity and calculation of the ratios. 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. Pathway analysis of genes that were 2 fold up- or downregulated was performed using pathway-explorer.
Chapter 2

(https://pathwayexplorer.genome.tugraz.at/). Microarray data are available from Gene Expression Omnibus (GEO, 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 SAM (Significance Analysis of Microarrays; version 3.0, Stanford, CA, USA).

Array Comparative Genomic Hybridization (array CGH)
The genomic DNA of H630, H630-cTFT and H630-4TFT were isolated using the Wizard Genomic DNA Purification Kit (Promega, A1120)\(^3\). As common reference, genomic DNA of a pool of healthy volunteers was used. 500 ng of DNA was labeled using the Enzo Genomic DNA Labeling kit (AMADID#014950, Enzo Life Sciences, Farmingdale, USA). Hybridizations were performed on 44k arrays (Agilent Technologies, Palo Alto, USA) by using the Across array comparative genomic hybridization method. Images of the arrays were acquired using the Agilent microarray scanner G2505B and image analysis was performed using feature extraction software version 9.5 (Agilent Technologies). The Agilent CGH-v4_95 protocol was applied using default settings. Oligonucleotides were mapped according to the human genome build NCBI 35. For both Cy3 and Cy5 channels, local background was subtracted from the median intensities. The \(\log_2\) tumor to normal ratio was calculated for each spot and normalized against the median of the ratios of all autosomes. Array data are available from Gene Expression Omnibus (GEO, 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 \(\mu\)M (continuously) or 5 \(\mu\)M (4 hr/7 days). Over a period of several months, this resulted in H630-cTFT and H630-4TFT cells, which were grown with 20 \(\mu\)M and 250 \(\mu\)M TFT, respectively. For H630-cTFT cells, the concentrations were increased using only 0.5 \(\mu\)M steps, which required about 5 months to induce resistance to 10 \(\mu\)M TFT. For H630-4TFT cells, the concentrations were increased using steps of 5 \(\mu\)M, which required about 3 months to induce resistance to 50 \(\mu\)M TFT, where after 20 \(\mu\)M steps were used to further increase TFT resistance. The cell lines were tested (SRB-assay) for TFT sensitivity several times during the procedure.
TFT resistance by decreased TK, ENT or sPLA2 overexpression

H630-4TFT cells were resistant to TFT with a resistance factor of 1320 (Table 1). H630cTFT cells were about 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 resistance was maintained for at least two weeks when the cell lines were grown in drug-free-medium, and decreased about 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 is in agreement with earlier experiments showing that TPI did not affect TFT sensitivity of colon cancer cells even with high TP expression.

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>RF</th>
<th>GW1843 at day 0</th>
<th>5-FU at day 0</th>
<th>FdUrd at day 0</th>
<th>5dFUR at day 0</th>
<th>d4T at day 0</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 1*</td>
<td>431 ± 33 1</td>
<td>593 ± 34 1</td>
<td>1230 3.4 ± 0.1</td>
<td>4.2 ± 0.6</td>
<td>7.1 ± 1.0 *</td>
<td>69 ± 7</td>
</tr>
<tr>
<td>H630-cTFT</td>
<td>168 ± 30 *</td>
<td>107 ± 4.4 *</td>
<td>49 ± 9 1</td>
<td>336 7.5 ± 0.4</td>
<td>6.5 ± 1.0</td>
<td>5.9 ± 1.6 *</td>
<td>112 ± 22</td>
</tr>
<tr>
<td>H630-R10</td>
<td>149 ± 9 1*</td>
<td>nd</td>
<td>nd</td>
<td>298</td>
<td>nd</td>
<td>167 ± 26 1</td>
<td>15 ± 2.7 *</td>
</tr>
</tbody>
</table>

Values (IC\(_{50}\) in µM; in nM for GW1843) are means ± SEM of at least 3 experiments. Sensitivity to TFT was determined directly after continuous exposure of cells to TFT (at day 0), or after 30 days growing in TFT-free medium (at day 30). H630-R10 is a 5-FU-resistant cell line. RF = Resistance Factor (average IC\(_{50}\) variants at day 0/average IC\(_{50}\) H630), nd = not done. Compared to H630: * p<0.05; ** p<0.01. \(^{1}\) previously published.

In order 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 to TFT (Figure 1B), such as TS inhibitors and drugs dependent on TP or TK for activation (GW1843, 5-FU, 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 about 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 TK\(^{31}\). The H630 variants were comparably insensitive to the d4T. FLT is also a substrate for TK\(^{31}\) and was not toxic to the cells with IC\(_{50}\)>1mM (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

In order 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 untreated...
Figure 1 - Mechanism of action of trifluorothymidine (TFT) and chemical structures of related compounds. A, TP: thymidine phosphorylase, TK: thymidine kinase, TS: thymidylate synthase, TF-Thy: trifluorothymidine, TF-TMP/TDP/TTP: trifluorothymidine monophosphate/ diphosphate/ triphosphate, dTMP/dTTP: deoxythymidine mono/triphosphate, dUMP/UTP: deoxyuridine mono/triphosphate. TFT together with a potent inhibitor of TP (thymidine phosphorylase inhibitor: TPI) forms the novel drug combination TAS-102. TFT incorporation into the DNA results in DNA damage and cell death. Upon TS inhibition, dUTP accumulates which can be misincorporated into the DNA resulting in DNA damage. B, Trifluorothymidine (TFT), the antifolate GW1843, 5-fluorouracil (5FU), 2’-deoxy-5-fluorouridine (FdUrd), 5’-deoxy-5-fluorouridine (5’dFUR; doxifuridine), 2',3'-didehydrodideoxythymidine (d4T; stavudine) and 3'-deoxy-3'-fluorothymidine (FLT; alovudine). C, Effect of TFT on cell cycle distribution in H630 and the TFT-resistant variants H630-4TFT and H630-cTFT. The cell lines were exposed for 48 hr to TFT. Values are Means ± SEM of 3 separate experiments. Compared to control: *p<0.05; **p<0.01. D, TK and TS protein levels in the H630 cell lines. Equal amounts of protein from unexposed cells were used for Western blotting (as checked with β-actin loading), as described in the ‘materials and methods’ section.
parental and resistant cells was observed, although the resistant cells tended to have less cells in the G2/M-phase (Figure 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 µM or 250 µM 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 µM 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 µM 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 µM) and saturating (10 µM) 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 (Figure 1D) showed no significant change in TS protein levels in the TFT-resistant cell lines.

TK protein and TK activity levels were significantly decreased by more than 95% (p<0.01) in H630-4TFT (Figure 1D; Table 2). This clearly explained the resistance to TFT, and also the resistance to FdUrd. Remarkably, in H630-cTFT 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 dCTP decreased total TK activity about 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 human equilibrative nucleoside transporter (hENT) for entering the cell, since dipyridamole caused a 29.5 fold (± 1.25) increase in IC50 concentration in H630 cells. To examine whether the levels of the nucleoside transporters were changed, RT-PCR analysis of mRNA expression of hENT and human concentrative nucleoside transporter
(hCNT) were determined. ENTs transport nucleoside substrates such as adenosine into the cell. CNTs 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 to H630 (Table 2). hCNT mRNA levels were slightly lower in H630-4TFT. hCNT mRNA levels were 16 fold increased in H630-cTFT compared to 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 to H630 cells, TFT
accumulated at much lower levels in H630-cTFT and H630-4TFT cells. Take together, these data demonstrate 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. In order to elucidate this resistance mechanism, we performed a whole human genome microarray analysis to identify changes in mRNA levels. Functional classification revealed a frequent deregulation of genes encoding signalling proteins in both resistant derivatives. In H630-4TFT cells, hardly any major alterations were found in the expression profile (Figure 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).

<table>
<thead>
<tr>
<th>Functional category</th>
<th>Up-regulated genes</th>
<th>Down-regulated genes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>H630-4TFT</td>
<td>H640-cTFT</td>
</tr>
<tr>
<td>Metabolism</td>
<td>46.4% (39)</td>
<td>57.1% (8)</td>
</tr>
<tr>
<td>carbohydrate</td>
<td>14.3% (12)</td>
<td>7.1% (1)</td>
</tr>
<tr>
<td>lipid</td>
<td>4.8% (4)</td>
<td>50% (7)</td>
</tr>
<tr>
<td>DNA</td>
<td>4.8% (4)</td>
<td>0% (0)</td>
</tr>
<tr>
<td>Communication</td>
<td>10.7% (9)</td>
<td>0% (0)</td>
</tr>
<tr>
<td>Cell cycle</td>
<td>8.3% (7)</td>
<td>0% (0)</td>
</tr>
<tr>
<td>Development</td>
<td>2.4% (2)</td>
<td>0% (0)</td>
</tr>
<tr>
<td>Endocrine system</td>
<td>6.0% (5)</td>
<td>14.3% (2)</td>
</tr>
<tr>
<td>Signal transduction</td>
<td>14.3% (12)</td>
<td>21.4% (3)</td>
</tr>
<tr>
<td>Ligand receptor interaction</td>
<td>6.0% (5)</td>
<td>7.1% (1)</td>
</tr>
<tr>
<td>Translation</td>
<td>6.0% (5)</td>
<td>0% (0)</td>
</tr>
<tr>
<td>Total</td>
<td>100% (84)</td>
<td>100% (14)</td>
</tr>
</tbody>
</table>

Gene expression in important cellular pathways using pathway analysis. Of the pathways analyzed, the total up or down regulated genes was set to 100%. Total genes that were changed in the indicated pathway are indicated in relative numbers of total affected genes and the absolute number of changed genes is given in parentheses.

Although the number of altered genes was higher in H630-4TFT cells, compared to 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 IIA (sPLA2 IIA), which could easily be judged by eye (Figure 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 performed. A 211 fold difference was found in the sPLA2/ß-actin ratio between H630-cTFT and H630 cells (p<0.05). This ratio was not more than 1.2 fold for H630-4TFT cells. sPLA2 protein was highly increased in both H630-cTFT and H630-4TFT cells compared to 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 to 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
In order 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 to reference DNA (Figure 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 to 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).
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#### Table 4

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Gene ID</th>
<th>Gene description</th>
<th>Ratio*</th>
<th>Gene function</th>
<th>Chromosome</th>
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<tbody>
<tr>
<td><strong>H630-cTFT cells</strong></td>
<td></td>
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<td></td>
<td></td>
<td></td>
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<tr>
<td>PLA2G2A</td>
<td>NM_000300</td>
<td>Phospholipase A2 group II A</td>
<td>47.83</td>
<td>Hydrolysis of fatty acids; lipid catabolism</td>
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<tr>
<td>UGT1A6</td>
<td>NM_001072</td>
<td>UDP glucuronosyltransferase 1 family, polypeptide A6</td>
<td>6.45</td>
<td>Metabolism</td>
<td>2q37</td>
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<tr>
<td>PLA2G4A</td>
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<td>phospholipase A2, group IVA</td>
<td>4.85</td>
<td>Lipid catabolism, phospholipid catabolism</td>
<td>1q25</td>
</tr>
<tr>
<td>HMGCS1</td>
<td>NM_002130</td>
<td>3-hydroxy-3-methylglutaryl-Coenzyme A synthase 1 (soluble)</td>
<td>3.48</td>
<td>Acetyl-CoA metabolism, cholesterol biosynthesis, lipid metabolism</td>
<td>5p14-p13</td>
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<td>AKR1C3</td>
<td>NM_003739</td>
<td>Aldo-keto reductase family 1</td>
<td>2.83</td>
<td>Cell proliferation, lipid and prostaglandin metabolism</td>
<td>10p15-p14</td>
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<tr>
<td>FDT1</td>
<td>NM_004462</td>
<td>Farnesyl-diphosphate farnesyltransferase 1 aldehyde dehydrogenase 9 family, member A1</td>
<td>2.65</td>
<td>Cholesterol and isoprenoid biosynthesis</td>
<td>8p23.1-p22</td>
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<td>ALDH9A1</td>
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<td>Hormone metabolism, oxidation reduction</td>
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<td>CPT1A</td>
<td>NM_001876</td>
<td>carnitine palmitoyltransferase 1A</td>
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<td>Androgen metabolism, estrogen biosynthesis, oxidation reduction</td>
<td>9q13.2</td>
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<tr>
<td>OGT</td>
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<td>O-linked N-acetylgalactosamine (GlcNAc) transferase</td>
<td>0.41</td>
<td>Response to nutrients, signal transduction</td>
<td>Xq13</td>
</tr>
<tr>
<td><strong>H630-4TFT cells</strong></td>
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<td></td>
<td></td>
<td></td>
</tr>
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<td>TPI1</td>
<td>NM_000365</td>
<td>Triosephosphate isomerase 1</td>
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<td>EHHADH</td>
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<td>enoyl-Coenzyme A, hydratase</td>
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<td>DGKE</td>
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<td>diacylglycerol kinase</td>
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<td>Phospholipid biosynthesis, intracellular signaling cascade</td>
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<tr>
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<td>O-linked N-acetylgalactosamine (GlcNAc) transferase</td>
<td>0.41</td>
<td>Response to nutrients, signal transduction</td>
<td>Xq13</td>
</tr>
</tbody>
</table>

* Genes that were up- 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.
In H630-4TFT cells, 3 large genomic alterations were found compared to the parental H630 cells, consisting of a loss within chromosome 1 and a gain within chromosomes 17 and 19 (Figure 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 (Figure 2B). Chromosomal regions containing the metabolic enzymes were not changed in this cell line, compared to the parental H630 cells, which is in agreement with the functional activity and protein expression of these enzymes (Table 2; Figure 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 upregulation of secretory phospholipase A2 (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 resistance in colorectal and gastric cancer. TFT has shown activity in 5-FU-resistant cells, both in in vitro and in vivo studies\textsuperscript{18,19}. Increased TS levels are often associated with resistance to TS inhibitors, including 5-FU\textsuperscript{8} and antifolate based TS-inhibitors\textsuperscript{22}, hence TS activity is one of the best predictors for 5-FU sensitivity\textsuperscript{4}. 5-FU- or antifolate-resistant colorectal cancer cells with increased TS levels may show cross-resistance to TFT\textsuperscript{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 demonstrated that increased TP levels in the cancer cells were not directly associated with TFT resistance\textsuperscript{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\textsuperscript{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, since 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\(^\text{18}\). To convert TFT to its active forms sufficient TK activity is essential\(^\text{1}\). In our study, only H630-4TFT cells had both decreased TK activity and decreased protein levels, which was also associated with cross-resistance to FdUrd.

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 H630-cTFT cells of the genes involved in TFT metabolism. The most significant result was the enormous upregulation of secretory phospholipase A2 type IIA (sPLA2 IIA), which is a novel finding. sPLA2 has a role in carcinogenesis, including that of gastrointestinal cancers\(^\text{35,36,37}\). 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 into prostaglandins, which are involved in a number of (patho)physiological processes, including cell survival, proliferation and Fas-mediated apoptosis\(^\text{36,38}\). Arachidonic acid produced by sPLA2 enzymatic activity promotes apoptosis in colon cancer cells\(^\text{36,39,40,41}\). In addition, Fas may be downregulated, although hardly any genes involving apoptosis were differentially expressed. TS inhibition can trigger Fas-dependent apoptosis\(^\text{42,43,44,45}\). 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\(^\text{44}\). TFT was previously shown to induce apoptosis via both the extrinsic and intrinsic pathway in CRC cells\(^\text{46}\). Since 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, since 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, where increased sPLA2 activity in the tumor microenvironment is used as a trigger for the release of anticancer etherlipids. 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-aminooxalyl-1-benzyl-2-ethyl-6-methyl-1H-indol-4-yloxy]-acetic acid methyl ester). 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.

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REFERENCES


6 Aherne GW, Hardcastle A, Raynaud F, Jackman AL. Immunoreactive dUMP and TTP pools as an index of thymidylate synthase inhibition; effect of tomodex (ZD1694) and a nonpolyglutamated quinazoline antifolate (CB30900) in L1210 mouse leukaemia cells. Biochem Pharmacol 1996;51:1293-1301.


36 Fijneman R, Cormier RT. The roles of sPLA2-IIA (Pla2g2a) in cancer of the small and large intestine. Front Biosci. 2008;13:4144-74.


41 Zhao S, Du XY, Chai MQ, Chen JS, Zhou YC, Song JG. Secretory phospholipase A(2) induces apoptosis via a mechanism involving ceramide generation. Biochim Biophys Acta 2002;1581:75-88.


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