Chapter 4

Differential activation of cell death and autophagy results in an increased cytotoxic potential for trifluorothymididine compared to 5-fluorouracil in colon cancer cells

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ABSTRACT

Trifluorothymidine (TFT) is part of the oral drug formulation TAS-102. Both 5-fluorouracil (5-FU) and TFT can inhibit thymidylate synthase and be incorporated into DNA. TFT shows only moderate cross-resistance to 5-FU. Therefore, we examined whether mechanistic differences in cell death could underlie their different modes of action in colorectal cancer cell lines (WiDR, Lovo92 and Colo320). Drug cytotoxicity was determined by SRB- and clonogenic assays, cell death by flow cytometry (PI and annexin V), caspase cleavage by Western blotting and activity assays and in vivo activity in the hollow fiber assay. The IC$_{50}$ values of TFT were 1-6 fold lower than for 5-FU, and clonogenic survival was less than <0.9% at 3 µM TFT, while 2-20% of the cells still survived after 20 µM 5-FU. In general, TFT was a more potent inducer of apoptosis than 5-FU, although the contribution of caspases varied between the used cell lines and necrosis-like cell death was detected. Accordingly, both drugs induced caspase (Z-VAD) independent cell death and lysosomal cathepsin B was involved. Activation of autophagy recovery mechanisms was only triggered by 5-FU, but not by TFT as determined by LC3B expression and cleavage. Inhibition of autophagy by 3-MA in 5-FU exposed cells reduced cell survival. Also, in vivo TFT (as TAS-102) caused more cell death than a 5-FU formulation. We conclude that TFT and 5-FU induce cell death via both caspase-dependent and independent mechanisms. TFT was more potent than 5-FU, because it induces higher levels of cell death and does not elicit an autophagic survival response in the cancer cell lines. This provides a strong molecular basis for further application of TFT in cancer therapy.

INTRODUCTION

5-Fluorouracil (5-FU) based therapy is part of the standard therapy for colorectal (CRC) and breast cancer. In order to exert its cytotoxic effect, 5-FU has to be activated to its monophosphate form, FdUMP. FdUMP is a strong irreversible inhibitor of thymidylate synthase (TS) by forming a stable ternary complex. In its triphosphate forms, FUTP and FdUTP, 5-FU can be incorporated into the RNA and DNA, respectively. The effect of 5-FU in combination with leucovorin has increased survival, but resistance is often encountered. 5-FU-resistance is related to a decreased level of 5-FU-activating enzymes, including orotate phosphoribosyl-transferase and uridine kinase, and an increased expression of the target TS. Recent improvements have been made by replacing bolus injections and inconvenient continuous infusions by oral fluoropyrimidine formulations, such as Xeloda.
TFT vs 5-FU: cell death and autophagy

(capecitabine) and UFT\(^5\). Another type of oral formulation, TAS-102, has recently been introduced into the clinic and consists of trifluorothymidine (TFT), in combination with a specific inhibitor of thymidine phosphorylase (TP), TPI. TAS-102, is currently tested in phase II studies against colorectal and gastric cancer\(^8\). Similar to 5-FU, TFT can inhibit TS in its monophosphate form (TF-TMP)\(^7\), however TFT does not form a ternary complex as for 5-FU, but binds covalently to the active site of TS, thereby inhibiting its activity\(^8,9\). Murakami et al. showed that TFT is active in colorectal cancer cells with (acquired) 5-FU and/or FdUrd resistance caused by amplification and subsequent overexpression of TS\(^10\), suggesting differences in their modes of action. When further activated to its tri-phosphate form, TFT can be incorporated into the DNA\(^11,12\), which results in DNA damage\(^13\) and subsequent cell death induction. TFT is not dependent on p53 to be active\(^14\) and can induce an arrest in the G2/M-phase of the cell cycle\(^15\). On the other hand, 5-FU induces an arrest in the G1/S-phase of cell cycle and cytotoxicity appeared to be more dependent on p53, although there is some controversy on this issue\(^4,16,17\). Moreover, the enzymatic activation route of these drugs is different, since 5-FU is a uracil analog and TFT a thymidine analog. Taken together, these mechanistic differences between TFT and 5-FU may contribute to the circumvention of resistance.

The failure of anticancer drugs to induce cell death may contribute to the development of drug resistance. Two classical apoptotic cell death pathways have been identified; the extrinsic (death receptor) and intrinsic (mitochondrial) pathway, although cross-talk between these and other pathways have been described\(^18,19\). The extrinsic pathway is activated upon binding of ligands to death receptors, such as tumor necrosis factor (TNF), TNF-related apoptosis-inducing ligand treatment (TRAIL) or Fas ligands\(^20,21\). This will lead to the cleavage and activation of initiator caspases 8 and 10 which in turn cleave and activate executioner caspases 3, 6 and 7, leading to apoptosis. The intrinsic pathway is activated when cytochrome c is released from the mitochondria. Subsequently, Apaf-1 will form the apoptosome with dATP and procaspase 9, leading to caspase 9 activation followed by the activation of the executioner caspases 3, 6 and 7. The extrinsic pathway is able to cross-activate the intrinsic pathway via caspase 8-dependent activation of Bid that can induce mitochondrial disruption via interactions with other Bcl2 family members, Bax and Bak. Cytotoxic agents that inhibit TS activity can induce cell death via the extrinsic pathway\(^22\), after which the intrinsic pathway can be cross-activated\(^23\). On the other hand, DNA damaging agents are often found to activate the intrinsic apoptotic pathway via activation of p53.

TFT and 5-FU can inhibit both TS and induce DNA damage, but the different resistance profiles suggest different modes of action that may involve differences in cell death.
activation. Caspases are well established as main players in apoptosis, however, other proteases such as cathepsins and calpains may also be involved in activation of alternative routes of cell death. Cathepsins, in particular cathepsin B, can act as the main executor of caspase-independent cell death induced by TNF-α in several systems\textsuperscript{24,25}. It has become evident that these caspase-independent cell death pathways are important in cell death induction after chemotherapy, including induced by microtubule-interacting agents\textsuperscript{24}. Another process important in life-death decisions is autophagy. Autophagy is a (reversible) state of the cell that involves a degradative lysosomal pathway and is known to enhance cell survival under conditions of nutrient starvation and other detrimental conditions, including chemotherapy\textsuperscript{26,27}.

Insight in the molecular mechanisms mediating drug cytotoxicity can facilitate the identification of biological markers to predict drug response and/or to the rationalized design of improved therapy. Therefore, in this study we explored whether differences in the mode of cell death activation, including autophagy, are at the basis of the differential activity between TFT and 5-FU in human colon cancer cell lines.

MATERIALS AND METHODS

Cell lines and chemicals
Human colon carcinoma cell lines Colo320 (wt p53, but impaired pathway)\textsuperscript{28}, WiDR (p53 mutant)\textsuperscript{21}, Lovo92 (wt p53)\textsuperscript{29}, were cultured as monolayers in DMEM supplemented with 10% heat inactivated FCS and 20 mM Hepes. Cells were maintained in a humidified 5% CO\textsubscript{2} atmosphere at 37°C. TFT was provided by Taiho pharmaceuticals Co. Ltd. (Tokushima, Japan). Z-VAD-FMK, IETD-FMK, LEDH-FMK were obtained from Bachem (Weil am Rhein, Germany) and CA-074me was obtained from Peptide Institute Inc. (Osaka, Japan). These inhibitors were used in previously reported non-toxic concentrations\textsuperscript{21,31}. 3-Methyladenine (3-MA; Sigma-Aldrich Chemicals, Zwijndrecht, The Netherlands) is an autophagy inhibitor, which was used in a 3 mM concentration, reported by others to inhibit autophagy\textsuperscript{32}.

Drug cytotoxicity assays
Drug cytotoxicity was determined using the sulforhodamine B (SRB)-assay. Cells were seeded in 96-wells plates (Greiner Bio-one, Frickenhausen, Germany) at 5000 cells/well. After 24 h enabling attachment, cells were exposed to increasing concentrations of drugs for 72 h. Thereafter, cells were fixed with trichloroacetic acid (TCA) for 1 h at 4°C, colored with
SRB and analyzed as described previously\textsuperscript{33}. Optical density was measured at 540 nm. IC\textsubscript{50} and IC\textsubscript{80} values were determined from graphs.

\textit{Clonogenic assay}

Cell survival was determined by the clonogenic assay\textsuperscript{34}. Depending on drug concentration, 150-5000 cells were seeded in 6 wells plates (Greiner Bio-one). After 72 h drug exposure, drug containing medium was replaced by drug free medium (DFM). After 8 days of growth, clones were fixed in formaldehyde (3.7\%) and stained with 10\% giemsa. Colonies containing 50 or more cells were counted and corrected for the plating efficiency (PE), after which the surviving fraction (SF) was calculated. The ratio between the SF at 0.1 (10\%) of TFT and 5-FU was calculated by dividing the concentration of 5-FU to obtain a SF of 0.1 through that concentration of TFT.

\textit{Western blotting}

Western blot was performed as described previously\textsuperscript{35}. From each condition 60 µg of protein was separated on a 12\% SDS-PAGE gel and electroblotted onto polyvinylidenedifluoride (PVDF) membranes (Millipore Corp., Billerica, MA). Subsequently, the membranes were blocked for 1 h at room temperature (RT) in TBS-Tween (TBST) with 5\% milk and incubated overnight (O/N) at 4°C with the primary antibodies\textsuperscript{35}; anti-caspase 3, anti-caspase 8, anti-caspase 9, anti-cleaved caspase 3, LC3B, PARP (all from Cell Signaling Technology (Danvers, MA, USA; dilution 1:1000 in 5 \% BSA) and anti-cathepsin B antibody\textsuperscript{24} (Oncogene Research Products, Boston, MA, USA; dilution 1:1000 in 5 \% BSA) or 1 h with the primary antibody against β-actin (Sigma-Aldrich Chemicals; 1:10000 in 5 \% BSA). The membrane was washed 5 times in TBST and incubated with the secondary antibody (1:2000 goat-α-mouse-HRP or goat-α-rabbit-HRP, DAKO Cytomation, Glostrup, Denmark) in TBST (with 2 \% milk) for 1 h at RT. After incubation, the membrane was washed in TBST and developed using enhanced chemoluminscencence (ECL-plus and ECL for β-actin; Amersham Pharmacia Biotech, Roosendaal, The Netherlands). The chemoluminescent signal was detected using a hyperfilm (Bio-Rad laboratories, Veenendaal, the Netherlands). Digital scanning of the film was performed using Versadoc system (Versadoc 4000 Imaging System, and Quantity One Software, Bio-Rad).

\textit{Fluorimetric assay for caspase activity}

Caspase 3 like activity was determined using a spectrofluorimetric assay for proteolytic cleavage of fluorogenic DEVD-AFC substrates (Clontech Laboratories, Inco, Palo Alto, CA)\textsuperscript{36}. Experiments were performed according to the manufacturer’s instructions.
Fluorescence was detected at 400 nm excitation and 505 nm emission (Spectra fluor Tecan, Salzburg, Austria). Relative caspase activity was calculated in fluorescence units/ $10^6$ cells/h.

**Cell death analysis**

Cell death was analyzed using FACS analysis as described previously$^{15}$. In brief, 100000 - 200000 cells were seeded in 6-wells plates. After treatment with TFT or 5-FU, cells were trypsinized, resuspended in medium collected from the matching sample containing the dead cells, and centrifuged for 5 min at 1200 rpm. Subsequently, cells were stained with propidium iodide (PI) buffer (0.1 mg/ml PI, 0.1% RNase A) in dark on ice. DNA content of the cells was analyzed by fluorescence activated cell sorting (FACScan, Becton Dickinson Immunocytometry Systems, San Jose, CA, USA) with an acquisition of 10,000 events. The sub-G$_1$ peak was used as indication of the percentage cell death induction. For discriminating apoptotic from the necrotic fraction, living cells were stained with Annexin V-FITC and PI (#APOAF apoptosis detection kit, Sigma-Aldrich Chemicals) directly after exposure to IC$_{80}$ concentrations of TFT or 5-FU for 24, 48 and 72 h. According to the manufacturers’ instructions, Annexin V positive cells were defined as early apoptotic cells, and cells positive for both Annexin V and PI were defined necrotic.

**Immunofluorescent staining**

Immunofluorescent staining was performed according to manufacturer’s instructions. In brief, cells were seeded in 6-wells plates on a cover slip and exposed to IC$_{80}$ concentrations of TFT or 5-FU. After 72 h exposure, cells were fixed for 15 min in 4% formaldehyde and permeabilized with methanol for 10 min at -20ºC. Subsequently, cells were blocked and the LC3B antibody was added (1:200, Cell Signaling; O/N at 4ºC), after which the secondary antibody goat anti rabbit conjugated with Alexa Fluor was added together with Hoechst 33342 (1:800) for 1 h at RT. The coverslips were mounted onto microscope slides using Vectashield (Vector, Burlingame, CA, USA). Fluorescence microscopy was carried out using an inverted Leica DMIRB/E fluorescence microscope (Leica Cambridge, Cambridge, UK). Images were collected using Q500MC Quantimet software V01.01 (Leica Cambridge).

**Hollow fiber assay**

In order to evaluate the activity of the drugs in vivo, the hollow fiber assay was performed as described previously.$^{37}$ The protocol was approved by the local Animal Ethical Committee in accordance with the UKCCR guidelines. In brief, Colo320 cells ($7.5\times10^6$ cells/ml) were transplanted into a 2 cm hollow fiber in BALB/c mice. The fibers were 24 h pre-incubated in
culture medium prior to transplantation. Three fibers were transplanted in the flank of each mouse. Since TFT and 5-FU have a poor bioavailability, we used TFT as the formulation TAS-102 and 5-FU as the prodrug 5’DFUR (5’-deoxy-5-fluorouridine). Mice were treated on day 3 with TAS-102 or 5’DFUR at their maximum tolerable dose (MTD) by oral administration. The mice were sacrificed by CO₂ inhalation at day 10 and the fibers were collected. Cells were retrieved and growth inhibition was determined by the MTT-assay and cell death by the TUNEL assay as described previously. \(^{37}\)

**Statistical analysis**

Potential differences between TFT and 5-FU were evaluated using the two-tailed Student’s \(t\)-test for paired data. Changes were considered significantly different when \(p<0.05\).

**RESULTS**

*TFT and 5-FU induced cytotoxicity*

The cytotoxicity of TFT and 5-FU was examined in a panel of colon cancer cell lines (see Table 1). TFT had an equal or up to 6.6 fold lower \(IC_{50}\) value than 5-FU in the colorectal cancer cell lines as determined by the SRB-assay (Table 1). For both TFT and 5-FU, no relationship was found between p53 status and cytotoxicity.

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>p53 Expression</th>
<th>(IC_{50}) TFT (µM)</th>
<th>(IC_{80}) TFT (µM)</th>
<th>(IC_{50}) 5-FU (µM)</th>
<th>(IC_{80}) 5-FU (µM)</th>
<th>(IC_{50}) 5-FU/TFT</th>
</tr>
</thead>
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<tr>
<td>Lovo92</td>
<td>wt</td>
<td>0.5 ± 0.1</td>
<td>60 ± 3.8</td>
<td>1.1 ± 0.1</td>
<td>60 ± 4.1</td>
<td>2.2</td>
</tr>
<tr>
<td>Colo320</td>
<td>wt</td>
<td>0.4 ± 0.1</td>
<td>5.3 ± 0.3</td>
<td>2.3 ± 0.2</td>
<td>10 ± 1.4</td>
<td>5.8</td>
</tr>
<tr>
<td>WiDR</td>
<td>mut</td>
<td>2.5 ± 0.5</td>
<td>75 ± 1.3</td>
<td>2.5 ± 0.8</td>
<td>116 ± 3.5</td>
<td>1</td>
</tr>
</tbody>
</table>

\(IC_{50}\) and \(IC_{80}\) values (µM) of TFT and 5-FU were determined after 72 h exposure.

\(5-FU IC_{50}\) values and p53 expression levels were obtained from De Bruin et al.\(^{47}\) and Peters et al.\(^{4}\). Values represent means of at least 5 independent experiments ± SEM.

*p53 mutations were confirmed by sequencing.

\(^{†}\)Cells demonstrate high p53 expression indicative of a yet unknown defect in this pathway. Moreover, cells display lack of p21 activation (data not shown).
Next, the clonogenic assay was performed to study the effects of the TFT and 5-FU treatment on the long term, and to allow the detection of multiple cytotoxic effects, including different types of cell death (e.g. apoptosis, necrosis) and of irreversible growth inhibition (senescence). After TFT exposure, the clonogenic cell survival was much lower than observed after 5-FU exposure in all tested colon cancer cell lines (Figure 1A). In order to achieve a surviving fraction of 0.1 (SF 0.1), a 4.7 to more than 30 fold lower concentration of TFT was required compared to 5-FU. This indicates that TFT has a stronger antitumor activity than 5-FU in these cancer cells. For further experiments, we selected three cell lines, with different p53 status, Lovo92 (wt p53), WiDR (mutant p53; missense mutation at codon 273) and Colo320 (impaired p53 pathway).

To examine cell death activation and recovery of cells after treatment with TFT or 5-FU, PI stained cells were analyzed by flow cytometry at various time points after culturing cells in drug-free medium (Figure 1B). In this respect we chose 10 µM, a concentration at which cells do not survive after TFT exposure, while cells had a good survival after 5-FU. In general, cells that were exposed to 5-FU displayed a lower level of sub-G₁ cell fraction representing death cells, than seen after TFT. Furthermore, in particular in Colo320 and Lovo92, cells seemed to recover from the cytotoxic insults inflicted by 5-FU. This in contrast to TFT treatment, after which cells continued to die, displaying much less recovery. WiDR cells, although showing higher levels of death cells after TFT treatment showed apparently no or little recovery after treatment with the selected drug concentration.

**TFT and 5-FU induced cell death and caspase activation**

To determine whether the difference in clonogenic survival between TFT and 5-FU was related to a different activation of the two main apoptotic routes, the expression and cleavage of a number of key caspases were determined (Figure 2A). Therefore, cells were exposed to equitoxic IC₈₀ concentrations, as determined by the SRB-assay, to induce detectable amounts of cell death. WiDR, Lovo92 and Colo320 cells expressed procaspase 9 and 3, whereas procaspase 8 was expressed in WiDR and Lovo92, but hardly detectable in Colo320 cells. In WiDR cells, caspases 8, 9 and 3 were cleaved after 72 h TFT exposure, whereas 5-FU exposure triggered caspase 8 and 3 cleavage to some extent, but caspase 9 was hardly cleaved. In Lovo92 cells caspase 3 and to some extent caspase 8 were cleaved after TFT and 5-FU treatment, while no evidence of caspase 9 cleavage was observed. In Colo320 cells, caspase 3 was strongly cleaved, and low levels of cleaved forms of caspases 9 were observed after both TFT and 5-FU exposure. The caspase 3 substrate PARP showed similar cleavage patterns as caspase 3, with stronger cleavage detected after TFT.
**Figure 1 - Clonogenic survival and cell death activation by 5-FU and TFT.** **A**, Clonogenic cell survival was determined in WiDR, Colo320, H630 and Lovo92 cells after 72 h exposure to various concentrations of drugs. The ratio between 5-FU and TFT was calculated by dividing the concentration of 5-FU to obtain a SF of 0.1 through that concentration of TFT and are indicated in the graphs. *p < 0.05 **B**, Cell death induction in WiDR, Lovo92 and Colo320 cells, analyzed by FACS analysis of PI stained cells, after 10 µM TFT and 5-FU exposure for 72 h followed by removal of the drugs and subsequent incubation in drug free medium (DFM). * p<0.05, ** p<0.0005, *** p<0.000005, compared to the same time point.
Figure 2 - TFT and 5-FU induced caspase cleavage and activation. A, Western blots showing pro- and cleaved caspase 3, 8 and 9 and PARP after 0, 24, 48 and 72 h exposure to IC_{50} concentrations of TFT or 5-FU in WiDR, Lovo92 and Colo320 cell lysates. β-actin was used as loading control for protein amount. B, Relative caspase 3 activity after 72 h of TFT or 5-FU exposure as measured by DEVD-AFC cleavage in the indicated cell lines. C, Contribution of caspases in cell death activation was determined by use of the inhibitors Z-VAD (50 µM), IETD (30 µM) and LEHD (40 µM) after 72 h drug exposure. Cell death induced by TFT or 5-FU alone were set at 100%. Values represent means ± SEM of 3-4 independent experiments. Significant differences between TFT/5-FU alone are indicated with * (p<0.05).
compared to 5-FU. In Lovo92 cells, PARP cleavage was hardly detectable, which is in agreement with the observed low cleavage-levels of the caspases. In the Western blots, generally, caspases and PARP cleavage after TFT exposure was somewhat stronger than after 5-FU, even though cells were exposed to equitoxic concentrations.

To further monitor caspase 3 activation, an enzymatic activity assay was used. In WiDR cells, caspase 3 was strongly activated after exposure to TFT in a time dependent manner (Figure 2B), while 5-FU showed only limited activation. In Lovo92 and Colo320 cells, both TFT and 5-FU induced comparable levels of caspase 3 activity that increased in time. Overall these results are in agreement with the caspase 3 cleavage patterns detected in Figure 2A, although the enzymatic activity assays appeared to be more sensitive for detecting active caspase 3.

To determine to what extent caspase 8 and 9 contributed to the induction of cell death, cells were pre-treated with specific synthetic inhibitors (Figure 2C). In WiDR cells, inhibition of caspase 8 with IETD, caspase 9 with LEDH, and all caspases with the pan-caspase inhibitor Z-VAD, resulted in a similar reduction of TFT-induced cell death by approximately 50% and 5-FU induced cell death was decreased by 70%. In Lovo92 cells, caspase 8 appeared to be the major mediator of cell death after exposure to both TFT and 5-FU. This is in agreement with the Western blot, in which only cleaved caspase 8 was detected. In Colo320 cells, caspase 9 inhibition reduced TFT induced cell death by 20%, while 5-FU induced cell death was reduced by approximately 50%. Furthermore, Z-VAD was able to reduce TFT-induced cell death as monitored by the sub-G₁ fraction by approximately 45, 60 and 80% (Figure 2C). For 5-FU this reduction was approximately 70, 15 and 95% in WiDR, Colo320 and Lovo92, respectively. The incomplete inhibition predominantly found in WiDR and Colo320 cells indicate that caspase independent mechanisms were also involved. In both WiDR and Lovo92 cells, Z-VAD was more effective in reducing 5-FU-induced cell killing, indicating that 5-FU cytotoxicity may be more dependent on caspase activation than TFT.

**Involvement of cathepsin B in TFT and 5-FU induced cell death**

The lysosomal protease cathepsin B is known to contribute to caspase-independent cell death upon various stress stimuli, including microtubule-interacting agents. Therefore, we explored the possible involvement of cathepsin B in TFT and 5-FU induced cell death. During activation, the inactive pro-enzyme (43 kDa) is processed into active cathepsin B (25 kDa or 31 kDa forms). In WiDR, Lovo92 and Colo320 cells, both TFT and 5-FU exposure resulted in cleavage of cathepsin B in a time dependent manner (Figure 3A), although cleavage
Figure 3 - Cathepsin B and cell death activation by TFT and 5-FU. A, Cathepsin B cleavage was
determined by Western blotting after exposing cells to IC₈₀ concentrations of the drugs. B, Effects on the
cathepsin B inhibitor CA074-me (10 µM) on cell death activation was determined by FACS (sub-G₁).
TFT and 5-FU induced cell death was set at 100%. Values represent means ± SEM of 3-4 independent
experiments. Significant differences between TFT/5-FU alone are indicated with * (p<0.05). C, Western
blot showing cleaved caspase 3 and cathepsin B with(out) Z-VAD (Z), CA074-me (CA) or both inhibitors
(Z+CA).

was only weakly detectable in Colo320 cells. Furthermore, in Lovo92 cells TFT was more
potent than 5-FU in triggering cathepsin B cleavage time dependently, although 5-FU
-treatment induced an earlier onset of cleavage.

To determine to what extent cathepsin B contributed to the final induction of cell death,
cells were pre-treated with the cathepsin B inhibitor CA074-me. In WiDR cells, inhibition of
cathepsin B reduced TFT and 5-FU induced cell death by about 60% and 40%, respectively
(Figure 3B). In Colo320 cells, the induction of cell death by TFT and 5-FU was hardly
inhibited after cathepsin B inhibition, which is in line with the almost not detectable cathepsin
B processing in these cells in Western blots (Figure 3A). Interestingly, in Lovo92 cells,
inhibition of cathepsin B decreased in particular 5-FU induced cell death by 75%, while a
reduction of 40% was seen after TFT exposure. Taken together, TFT and 5-FU can both activate cathepsin B, although TFT may be somewhat more potent in this respect in some cell types (Lovo92). However, the contribution of cathepsin B in TFT and 5-FU-induced cell death varied amongst the cell lines from almost no effect in Colo320 to approximately 75% in Lovo92.

To determine the relationship between the activation of caspases and cathepsin B, cells were exposed to Z-VAD, CA074-me or both inhibitors prior to TFT or 5-FU treatment, after which cathepsin B and caspase 3 cleavage were determined (Figure 3C). In WiDR and Lovo92 cells, Z-VAD prevented the cleavage of caspase 3, but did not block cathepsin B processing. Cathepsin B inhibition by CA074-me resulted in a strong decrease in a cleavage of both cathepsin B and caspase 3 in Lovo92 cells, whereas only cathepsin B was suppressed in WiDR cells. In Colo320 cells, Z-VAD inhibited caspase 3 cleavage, and as expected no role for cathepsin B could be found. In Lovo92 cells, both caspase-dependent routes as well as cathepsin B activation could lead to caspase 3 cleavage. Moreover, the hardly detectable levels of cathepsin B processing in Colo320 cells and lack of effect of CA074-me do not suggest an important role for this protease in this cell line. Together, these data indicate that in two of the three cell lines, cathepsin B activation significantly contributed to TFT and 5-FU-induced apoptosis.

**Apoptotic and necrotic cell death activation**

Cathepsin B cleavage has been linked with non-apoptotic routes of cell death. Therefore, the possible contribution of necrotic cell death was analyzed in more detail using PI-Annexin V staining (Figure 4). In WiDR cells, necrosis and apoptosis both contributed almost equally to the induction of cell death by 5-FU. After TFT exposure, the apoptotic fraction was somewhat more dominant compared to the necrotic fraction. When Z-VAD was added, the apoptotic component appeared to be suppressed, and the necrotic fraction was somewhat increased. CA074-me reduced both the apoptotic and necrotic fractions after TFT and 5-FU exposure. In Lovo92 cells, the largest portion of cells underwent apoptosis after TFT and 5-FU exposure. The increase in the necrotic-fraction during time may represent later stages of apoptosis, where cells become somewhat permeable for PI. Interestingly, in Lovo92 cells CA074-me addition decreased the apoptotic fraction after both TFT and 5-FU exposure. CA074-me hardly affected the (low) necrotic fraction, indicating that cathepsin B may cross-activate caspases. For this, evidence was also obtained in Figure 3C. In Colo320 cells, predominantly necrosis was induced after TFT and 5-FU exposure. Although fractions were to some extent decreased by Z-VAD or CA074-me, this is probably mediated by other pathway(s).
Figure 4 - 5-FU and TFT-induced apoptotic and necrotic cell death. Cells were stained with PI and Annexin V-FITC conjugate and measured by FACS. Apoptotic (AnV+) and necrotic (AnV+/PI+) cells were monitored at the indicated time points after treatment (0 h is untreated control). Percentage of cells of the total cell fraction was calculated. Values represent means of at least 3 independent experiments ± SEM. Significant differences are indicated with * (p<0.05).
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**Figure 5** - Activation of autophagy by 5-FU and TFT. A, Western blot analyses of LC3B (autophagy marker) expression and processing after 72 h of TFT and 5-FU exposure (10 µM). B, Immunofluorescent microscopic staining of LC3B following exposure to TFT or 5-FU (10 µM) for 72h. Localization of LC3B in vesicles is indicative for autophagy. C, Clonogenic survival after addition of 3-MA to 10 µM 5-FU or 1 µM TFT. Values represent means of at least 3 independent experiments ± SEM. Significant differences between with and without 3-MA addition are indicated with * (p<0.05) ** (p<0.005).

**Autophagy induction after 5-FU exposure**

To further examine pathways involved in cell death regulation by TFT and 5-FU, we studied the ability of the drugs to activate autophagy. Therefore, we determined the expression and processing of LC3B, which is regarded as a key step in the induction of autophagy. Interestingly, Western blots showed that LC3B was strongly upregulated in WiDR, Lovo92
and Colo320 cells after exposure to 5-FU, which was not seen after TFT treatment (Figure 5A). The cleaved form of LC3B was only detectable in WiDR and Lovo92 cells and not in Colo320 cells. To confirm that LC3B was located in vesicles, immunofluorescent staining of LC3B was performed (Figure 5B). This clearly shows that after 5-FU exposure, and not TFT, LC3B is highly expressed in autophagic vesicles in WiDR and Lovo92 cells and to a lesser extent in Colo320 cells.

Finally, we determined whether inhibition of autophagy by 3-MA could result in a decreased cell survival by preventing an autophagic recovery process. The three cell lines were exposed to 1 µM TFT or 10 µM 5-FU in the presence or absence of 3-MA, after which the clonogenic potential of the cells was determined. We used a lower concentration of TFT, because after exposure to 10 µM no cells survived. 1 µM TFT had a comparable cytotoxicity to 10 µM 5-FU in this assay. 3-MA did not affect the clonogenic survival in cells exposed to 1 µM TFT. On the other hand, 3-MA decreased the cell survival after exposure to 10 µM 5-FU in WiDR, Lovo92 and Colo320 cells, respectively (Figure 5C).

**In vivo growth inhibition and cell death induction**

Finally, in order to determine whether TFT was more potent than 5-FU in an *in vivo* model, we used the hollow fiber assay. This model allows the determination of growth inhibition and cell death induction, upon treatment. Mice were treated with TFT (as TAS-102) and 5-FU (as the prodrug 5′-DFUR) at their MTD which resulted in 10-20% weight loss compared to untreated mice. TFT treatment caused higher levels of growth inhibition and cell death induction compared to 5′-DFUR (Figure 6). Thus, this *in vivo* model reflects the results obtained in the cell culture model, whereby TFT was more potent in inducing cell death than 5-FU and is much more active on the long term than 5-FU.

![Figure 6](image-url) *In vivo* cell growth inhibition and cell death induction after treatment of mice for 7 days with TAS-102 or 5′-DFUR. Fibers (3 per mouse) were collected from different mice and were assayed for the level of growth inhibition (MTT) and cell death (TUNEL). Values are means ± SEM and are expressed as %. Statistical differences between TAS-102 and 5′DFUR are indicated in the graph (* p < 0.01).
DISCUSSION

This study demonstrates that autophagy protects against 5-FU cytotoxicity, in contrast to TFT in human colon cancer cell lines. Cell death by TFT and 5-FU was induced via activation of multiple cell death pathways, although cathepsin B mediated caspase independent cell death might be more important for TFT. These differences may be related to different mechanistic actions of TFT, compared to 5-FU. The differences between TFT and 5-FU emphasize the potential importance of TFT as a new treatment strategy for colorectal and gastric cancer.

TFT (as TAS102) 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. Both TFT and 5-FU induced cell death that was partially mediated in a caspase dependent manner, however TFT activity seems to be less dependent on p53. Here, we found that the activation of caspase dependent cell death pathways varied between cell lines, but the pathway of cell death activation did not differ much between TFT and 5-FU. The cell line specific variation in cell death activation may be related to different expression levels and or regulation of cell death mediators that are currently not known. In general, TFT appeared to be somewhat more potent in inducing caspase-dependent cell death than 5-FU.

With respect to the involvement of TS in drug cytotoxicity, in a previous study Lovo92 (high TS expression) and WiDR (low TS expression) cells exposed to the specific TS inhibitor, AG337, demonstrated levels of caspase related cell death induction comparable to that induced by 5-FU in this study. This suggests that 5-FU acts mainly by TS inhibition in these cell lines. Previously it was reported that TFT, in its monophosphate form (TFT-MP), can inhibit TS very rapidly, however it does not form a stable complex, which is in contrast to 5-FU. Since TFT can also be incorporated into the DNA and induces DNA damage, TS inhibition may not be the main mechanism of action of TFT. Moreover, we reported previously that TFT induces an arrest in the G2/M-phase and polyploidy. Although these phenomena are known to be induced by microtubule inhibitors, such as taxanes, they are less expected to be associated with the activity of a nucleoside analog that targets the S-phase of the cell cycle, e.g. by TS inhibition and DNA damage induction. The difference between TFT and 5-FU induced cell death was also found in *in vivo* experiments, in which TFT (as TAS-102) induced more tumor growth inhibition and caused more cell death than the 5-FU prodrug 5’-DFUR. In other preclinical *in vivo* studies, TAS-102 also showed a better antitumor effect than various other 5-FU formulations, all at their MTD. Together
with these findings, our current study indicates that TFT and 5-FU elicit different cellular responses with respect to cell cycle arrest, cell death activation and induction of autophagy.

Autophagy can act as a rescue mechanism that cells can use to escape from cell death. However, autophagy may play a dual role that may be treatment and cell type dependent. Previously, it was reported that various anticancer compounds such as histone deacetylase inhibitors can induce autophagy, which possibly contributed to their anticancer activity. However, other histone deacetylase inhibitors triggered autophagy that did not result in an enhancement of cell death activation. The induction of autophagy in the latter studies, similar as we found for 5-FU, reflects a survival response in reaction to drug treatment whereby damaged intracellular material is stored and degraded in vesicles. For cancer therapy, this can be considered as an undesired effect of the mechanism of action of the anticancer agent, which counteracts the tumor cell killing ability of the drug. Since we found that inhibition of the autophagic response by 3-MA enhances 5-FU activity, inhibitors of autophagy may potentially act synergistic with compounds that induce autophagy. Recently, Li et al reported a similar effect, whereby inhibition of autophagy could increase the cytotoxicity of 5-FU in the colon cancer cell lines HT-29 and Colon26. Further investigation of the autophagy related cellular responses that are associated with anticancer drug activity should diminish some of the current controversy.

The last few years, a number of caspase independent cell death pathways have been described. Possible secondary mechanisms of cell death induction include lysosomal proteases, the cathepsins. Upon release from the lysosome into the cytoplasm, cathepsins can cleave a number of substrates, which will end in cell death. Cathepsin mediated cell death results in a necrotic type of cell death, since the regulation of the cathepsins outside the lysosomes is less precise. The caspase independent cell death induction by TFT and 5-FU was (partially) mediated by the activation of cathepsin B, which was associated with the occurrence of necrotic cells. Cathepsin B dependent death has been reported to partially mediate cell death activation by microtubule stabilizing agents, such as paclitaxel, epothilone B and discodermolide, which are also known to induce a G2/M arrest similar to TFT. Moreover, cathepsin B may also cross-activate the caspase-apoptotic pathway via bid, since inhibition clearly reduced the apoptotic-cell death. Our data indicate that nucleoside analogs such as TFT can also trigger cathepsin B, although the mechanism underlying its activation is yet unclear. Our finding that this contribution varies in a cell specific manner and that in some cases its activation can be either caspase-dependent or independent indicates that multiple mechanisms can induce this cell death pathway. Regardless of this, cathepsin B may be important for the sensitivity to these agents. Besides cathepsins, RIP1 kinase may be involved in a necrotic-type of regulated cell death, called
necroptosis. This type of cell death can be induced when apoptosis is blocked by inhibition of caspases. Since in Colo320 caspase 8 is not expressed and the exact mechanism of cell death is unclear, RIP1 kinase may be a potential regulator in this cell line.

The present data give more insight into the mechanism of TFT induced cell death, when compared to 5-FU. These differences between TFT and 5-FU can potentially be exploited in the clinic, e.g. TFT as second line treatment when patients are or have become resistant to 5-FU. Since the long term survival after exposure of cells to TFT was much lower than after 5-FU, clinical trials should be focused on overall survival and less on direct tumor responses (growth inhibition). Moreover, since TFT is clinically combined with the thymidine phosphorylase inhibitor (TPI) (TAS-102), angiogenesis may potentially also be inhibited, providing a strong basis for further development of TFT. Therefore, we conclude that the clinical development of TFT yields promising perspectives as therapy for gastrointestinal cancers.

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REFERENCES


47 Festjens N, Vanden Berghe T, Cornelis S, Vandenabeele P. RIP1, a kinase on the crossroads of a cell's decision to live or die. Cell Death Differ. 2007;14:400-10