# Chapter 5

## Synergistic interaction between trifluorothymidine and docetaxel is sequence dependent

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## ABSTRACT

Docetaxel is a microtubule inhibitor that has actions in the S and $G_2$/M-phase of the cell cycle. The pyrimidine trifluorothymidine (TFT) induces DNA damage and an arrest in the $G_2$/M-phase. TFT, as part of TAS-102, is clinically evaluated as an oral chemotherapeutic agent in colon and gastric cancer. The aim of the study was to determine the optimal administration sequence of TFT and docetaxel and to investigate the underlying mechanism of cytotoxicity. Drug interactions were examined by SRB-assays and subsequent combination index (CI) analyses and for long term effects the clonogenic assay was used. A pre-incubation with docetaxel was synergistic in SRB- (CI:0.6-0.8) and clonogenic assays, and was accompanied by a time dependent cell death induction (17-36%), the occurrence of polynucleation (22%) and mitotic spindle inhibition as determined by flow cytometry and immunostaining. Interestingly, administration of TFT followed by the combination displayed strong antagonistic activity, and was accompanied by less polynucleation and cell death induction than the synergistic combinations. Western blotting showed that the $G_2$/M-phase arrest (25-50%) was accompanied by phosphorylation of Chk2 and dephosphorylation of cdc25c in the synergistic combinations. Together, this indicates that synergistic activity requires docetaxel to initiate mitotic failure prior to the activation of TFT damage signaling, whereas antagonism is a result of TFT cell cycle arrested cells being less susceptible to docetaxel. Caspase 3 activation was low after docetaxel, suggestive of caspase independent mechanisms of cell death. Taken together, our models indicate that combination treatment with docetaxel and TFT displays strong synergy when docetaxel is given first thus providing clues for possible clinical studies.


## INTRODUCTION

Docetaxel (Taxotere®), a taxane, is one of the most important anticancer drugs today. It is part of the standard therapy of various types of solid tumors, such as breast, ovarian, non-small cell lung cancer, androgen independent metastatic prostate cancer and advanced gastric cancer[1,2]. Docetaxel promotes tubulin assembly in microtubules and inhibits their depolymerization[3]. In this way, docetaxel interferes with the dynamic changes that occur during the formation of the mitotic spindle. Docetaxel exhibits greater affinity to ß-tubulin, targeting centrosome organization and acting on cells in three phases of the cell cycle (S/$G_2$/M-phase)[4]. Treatment with taxanes often leads to side effects as well as drug resistance[5]. Therefore, it is important to develop new strategies that will lead to fewer side

effects and improved activity. Various approaches to enhance the efficacy of the taxanes have been investigated[6,7] and several taxane combinations are standardly used for various malignancies[3]. The mechanism of action is not always very well understood and may depend on the type of drug or disease. For combinations to be successful a mechanism-based interaction is preferable.

Trifluorothymidine (TFT) is a cytotoxic agent that might enhance the effect of docetaxel. TFT is part of the novel anti-tumor drug formulation TAS-102, consisting of the combination of TFT with a specific inhibitor of thymidine phosphorylase (TP), TPI[8]. Addition of TPI to TFT enhances its bioavailability to tumor cells, since TP can breakdown TFT to an inactive form. TAS-102 is currently tested as an oral formulation in phase II clinical studies against both colon and gastric cancer. Murakami *et al.* previously reported that TFT may be more effective in colorectal cancer cells to overcome (acquired) 5-FU and/or FdUrd resistance caused by amplification and subsequent overexpression of TS[9]. One mechanism of action consists of thymidylate synthase (TS) inhibition by its monophosphorylated form (TFT-MP) through a covalent binding to the active site of TS[10,11]. TS is one of the major rate-limiting enzymes in DNA synthesis, and inhibition induces a series of downstream events, eventually leading to cell death[12]. When TFT is further activated to its tri-phosphorylated form (TFT-TP), it can be incorporated into the DNA[13], which will subsequently result in DNA damage[14]. This may be the major mechanism of action of TFT compared to 5-FU. This possibly explains why, in contrast to 5-FU and antifolate based TS-inhibitors, TFT induced a $G_2$/M-arrest in cancer cells[15]. Moreover, it is not yet clear whether and how TFT inhibits the various DNA polymerases.

5-FU and the 5-FU prodrug capecitabine have been combined with docetaxel in various studies and have been reported to be effective in terms of objective response and overall survival[6,16,17]. In a phase II study where docetaxel was combined with the novel oral fluoropyrimidine formulation S-1, this combination was shown to be very active and well tolerated[18]. This synergism was probably due to a biochemical modulation of activating and inactivating enzymes of 5-FU[19]. However, the exact mechanism remains to be determined.

Both docetaxel and TFT show cell cycle dependent activity. Therefore, we tested sequential and concurrent combination schedules of the two drugs. Sequential administration of drugs can decrease the side effects, which is also an advantage. Moreover, drug combinations with docetaxel and other taxanes were more effective when given sequentially[20].

The aim of the present study was to determine whether TFT can enhance docetaxel sensitivity in colon cancer cells, using various treatment schedules, and to examine the

mechanism underlining the synergism to better understand how they can be combined in the clinic.

## MATERIALS AND METHODS

*Cell culture and chemicals*

Human colon carcinoma cell lines Colo320 and WiDR were obtained from the American Type Culture Collection (ATCC) and were cultured as monolayers in DMEM supplemented with 10% heat inactivated FCS and 20 mM Hepes in 25 $cm^2$ culture flasks (Greiner Bio-One). Cells were maintained in a humidified 5% $CO_2$ atmosphere at 37℃. TFT was provided by Taiho Pharmaceuticals Co. Ltd. (Tokushima, Japan) and was dissolved in phosphate buffered saline (PBS) in stock solutions of 20 mM. Docetaxel (Sigma-Aldrich Chemicals, Zwijndrecht, The Netherlands) was dissolved in dimethylsulfoxide (DMSO) in a stock solution of 20 mM. The stock solutions were stored aliquoted at -20℃.

*Drug cytotoxicity assays*

Drug cytotoxicity was determined by the sulforhodamine B (SRB)-assay[21]. 5000 cells/well were seeded in 96-wells plates (Greiner Bio-One, Frickenhausen, Germany). After 24 h enabling attachment, cells were exposed to increasing concentrations of drugs for 72 h. For the simultaneous combination, cells were exposed to both TFT and docetaxel for 72 h. Sequential combinations consisted of a 24 h pre-incubation with either TFT or docetaxel, followed by a 48 h exposure to the combined drugs. Cells were precipitated with trichloro-acetic acid (TCA) for 1 h at 4ºC, colored with SRB, solubilized with Tris and the optical density (OD) was measured at 495-540 nm. For calculation of the growth inhibition curves, OD values were corrected for readings at the day of drug addition. $IC_{50}$ values were subsequently estimated from graphs and are given in means ± SEM. For simultaneous combinations, fixed ratios based on $IC_{50}$ values were used to determine the interaction with the multiple drug effect method, in which a combination index (CI) was calculated (Calcusyn, Biosoft, Cambridge UK), based on the method of Chou and Talalay as described previously[14]. A CI < 0.9 indicates synergism and > 1.1 antagonism. Per experiment the combination index (CI) values at fraction affected (FA) 0.5, 0.75 and 0.9 were averaged and used for calculation of means between experiments. Values below 0.5 are considered to be irrelevant, because they represent only a minor growth inhibition.

*Clonogenic assay*

Cell survival was determined using the clonogenic assay[22]. Single cells were diluted serially to appropriate concentrations (150-2000 cells) and seeded in 6-wells plates in 3 ml medium. After 24 h, cells were exposed to the $IC_{10}$, $IC_{25}$ and $IC_{50}$ values (as determined with the SRB-assay) of TFT, docetaxel and the tested combinations. 96 h after seeding cells, drug containing medium was replaced by fresh medium. After 10 days in drug free medium, cells were fixed in ethanol (15 min) and stained with 10% giemsa (Sigma). Surviving fractions (SF) were calculated by dividing the plating efficiency (PE) of exposed cells to the PE of control cells. Theoretical additivity was calculated by multiplication of the SF of TFT alone with that of docetaxel alone[23].

*Western blotting*

Colo320 and WiDR cells were seeded per 1.5 X $10^6$ in 25 $cm^2$ culture flasks (Greiner Bio-One). After 24 h cells were exposed to $IC_{50}$ concentrations of TFT alone, docetaxel alone or the various combination schedules as described in the drugs cytotoxicity assay. After treatment, cells were lysed in lysis buffer (10% glycerol, 5 mM EDTA, 10 mM Tris-HCl pH 7.5, 150 mM NaCl, 50 mM β-glycerophophate, 1% Triton X-100, 0.04% protease inhibitor cocktail, 1 mM $NaVO_3$) and centrifuged at 14,000 rpm at 4℃ for 10 min. Protein concentration in the supernatant was determined by performing a Bio-rad protein assay according to the manufacturer's instructions (Bio-rad Laboratories). From each condition 30 µg of protein was separated on a 10% SDS-PAGE gel and electroblotted onto a PVDF-membrane (Millipore Corp., Billerica, MA, USA). Subsequently, the membranes were blocked for 1 h (RT) in TBST (10 mM Tris-HCl, 0.15 M NaCl, 0.05% Tween-20) with 5% milk-powder and subsequently incubated overnight at 4°C with the primary antibodies directed against: CDK2 (#2546), phosphorylated CDK2 (Thr160; #2561), Chk1 (#2345), phosphorylated Chk1 (Ser345; #2341), Chk2 (#2662), phosphorylated Chk2 (Thr68; #2661), cdc25c (#4688) and phosphorylated cdc25c (Ser216; #4901) (1:1000; Cell Signaling Technology, Inc.; Danvers, MA, USA). Following this, membranes were incubated with the secondary antibody (1:2000 goat-α-mouse-HRP or donkey-α-rabbit-HRP, DAKO Cytomation, Denmark) in TBST (2% milk powder) for 1 h at room temperature. ß-actin served as a loading control for protein amount.

*Fluorimetric assay for caspase activity*

Caspase 3-like activity was determined using a spectrofluorimetric assay of proteolytic cleavage of fluorogenic DEVD-AFC substrates (Clontech Laboratories, Inco, Palo Alto, CA)[24]. 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 (FU)/h/million cells.

*FACS analysis of cell cycle distribution*

Cell cycle analysis and apoptosis measurements were performed as described previously[25]. In brief, 100000 cells were seeded in 6-wells plates. After treatment, cells were trypsinized, resuspended in medium collected from the matching sample and centrifuged for 5 min at 1200 rpm. Subsequently, cells were stained with propidium iodide buffer (0.1 mg/ml with 0.1% RNAse A) in dark on ice. DNA content of the cells was analyzed by FACS (Becton Dickinson) with an acquisition of 10,000 events. The sub-$G_1$ peak was used to determine the extent of cell death.

*Immunofluorescent staining*

Cells were exposed to $IC_{50}$ concentrations of TFT and docetaxel alone or the various combination schedules. After 72 h exposure, cells were fixed for 30 min in methanol, permeabilized 10 min with 0.2% Triton X100 in PBS, blocked 1 h with 3% BSA (RT) and stained for α-tubulin (1:200; Santa Cruz Biotechnology) overnight (O/N) at 4ºC. Subsequently, the secondary antibody (α–mouse-FITC; 1:200; Dako) was added together with Hoechst 33342 to stain the nuclei (1:400) for 1 h at RT. Subsequently, the cover slip was mounted onto microscope slides using Vectashield (Vector, Burlingame, CA, USA). Fluorescence microscopy analysis was carried out using an inverted Leica DMIRB/E fluorescence microscope. Images were collected using the Q500MC Quantimet software V01.01 (Leica Cambridge Ltd, Cambridge, UK).

## RESULTS

*Growth inhibition*

The cytotoxic effects of docetaxel and TFT were assessed after 72 h of continuous drug exposure in the two cell lines; WiDR and Colo320[14,15]. Docetaxel and TFT inhibited growth of both cell lines in a concentration dependent manner (Figure 1, Table 1). Docetaxel inhibited cell growth at nanomolar concentrations, TFT at micromolar concentrations. The $IC_{10}$, $IC_{25}$ and $IC_{50}$ values were determined from the graphs and were used as a basis for the other experiments.

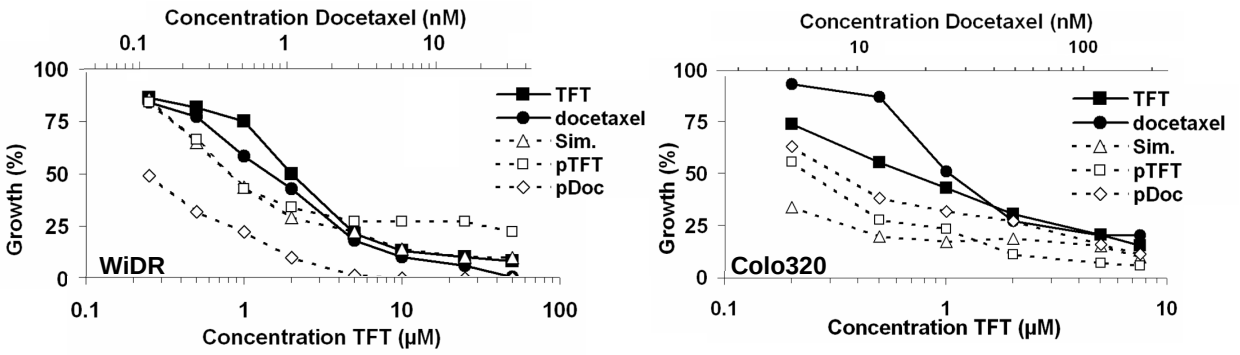


**Figure 1 -** Growth inhibition curves of WiDR and Colo320 after 72 h exposure to TFT, docetaxel or either 72 h concurrently (Sim.) to TFT and docetaxel, or 24 h pre-incubated with TFT (pTFT) or docetaxel (pDoc), followed by a 48 h combination of TFT with docetaxel. All combinations are based on $IC_{50}$:$IC_{50}$ ratios. All SEM values were < 10%.

**Table 1 - Growth inhibition of TFT and docetaxel in colorectal cancer cells; combination analysis of various treatment schedules of TFT combined with docetaxel**

| | Growth inhibition ($IC_{50}$) | | Combination index (CI) | | |
|---|---|---|---|---|---|
| | TFT (µM) | Docetaxel (nM) | Simultaneous | pTFT | pDoc |
| WiDR | 3.2 ± 0.3 | 1.7 ± 0.3 | 1.95 ± 0.13 | >10 | 0.62 ± 0. 04 |
| Colo320 | 0.6 ± 0.1 | 16 ± 1.4 | 0.76 ± 0.18 | 0.77 ± 0.10 | 0.80 ± 0.20 |

$IC_{50}$ values were estimated from graphs (Figure 1) and are given in means of at least 3-5 independent experiments ± SEM. Average CI values were calculated from fraction affected (FA) from data points with a FA of 0.5, 0.75 and 0.9. CI values < 0.9 indicates synergy; CI = 0.9-1 means additive; CI > 1.1 means antagonism. Values represent mean CI values ± SEM of 3-4 independent experiments.

*Evaluation of combinations*

The effect of the combinations was analyzed by determining the CI values from growth inhibition curves (Figure 1). In WiDR cells, the simultaneous combination (Sim) was antagonistic and strongly antagonistic after pre-exposure to TFT (pTFT) (Table 1). On contrary, when WiDR cells were 24 h pre-exposed to docetaxel (pDoc), synergistic activity was detected. In Colo320 cells, all tested combination schedules were synergistic (Table 1).

*Cell cycle effects and cell death induction*

The sequence of the exposing cells to the combinations was important, which may be related to the cell cycle dependency of the drugs to exerting their activity. Therefore, we analyzed the mechanisms underlining the synergistic and antagonistic combinations by determining cell death and cell cycle distribution by FACS analysis on PI stained cells

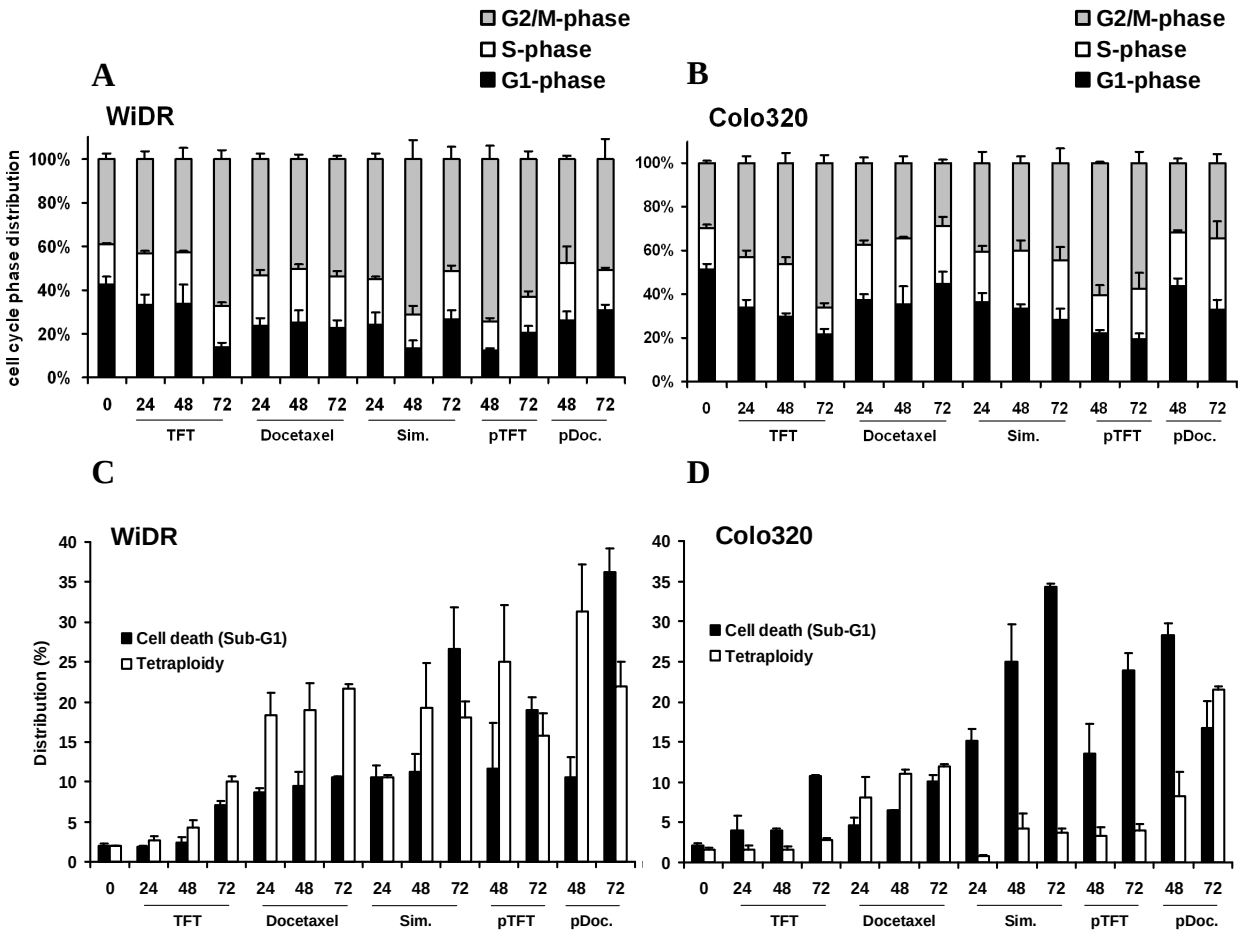


**Figure 2 -** Effect of TFT ($IC_{50}$) and docetaxel ($IC_{50}$) and the combinations ($IC_{50}$-ratio) on cell cycle distribution (A-B), cell death and polyploidy (C-D) in WiDR and Colo320 cells. Values are means of at least 3 independent experiments ± SEM.

(Figure 2). In WiDR cells, TFT induced a time dependent arrest in the $G_2$/M-phase and polyploidy. Docetaxel induced an immediate arrest in the $G_2$/M-phase, with cells progressing to polyploidy and cells undergoing cell death (Figure 2A,C). Under antagonistic combinations (simultaneous and pTFT) an increased number of cells accumulated in the $G_2$/M-phase, and a higher portion of cells died after 72 h, whereas similar levels of polyploidy were detected. For the synergistic pDoc combination, the $G_2$/M arrest was less pronounced, but clearly higher levels of polyploid and dead cells were detected.

In Colo320 cells, TFT also induced a time dependent $G_2$/M arrest. The $G_2$/M arrest induced by docetaxel was less pronounced (Figure 2 B,D). Combined treatments arrested cells in both the $G_2$/M and the S-phase of cell cycle. pDoc combination resulted in the induction of both cell death and polyploidy, whereas for the other combinations, cell death induction alone could account for the synergism. The apparent difference between WiDR and Colo320 cells in polyploidy seems to be related to the extent of cell kill, which was time dependent. The pDoc combination, which was synergistic in both cell lines, mostly worked by the accumulation in polyploidy after which cell death was induced.

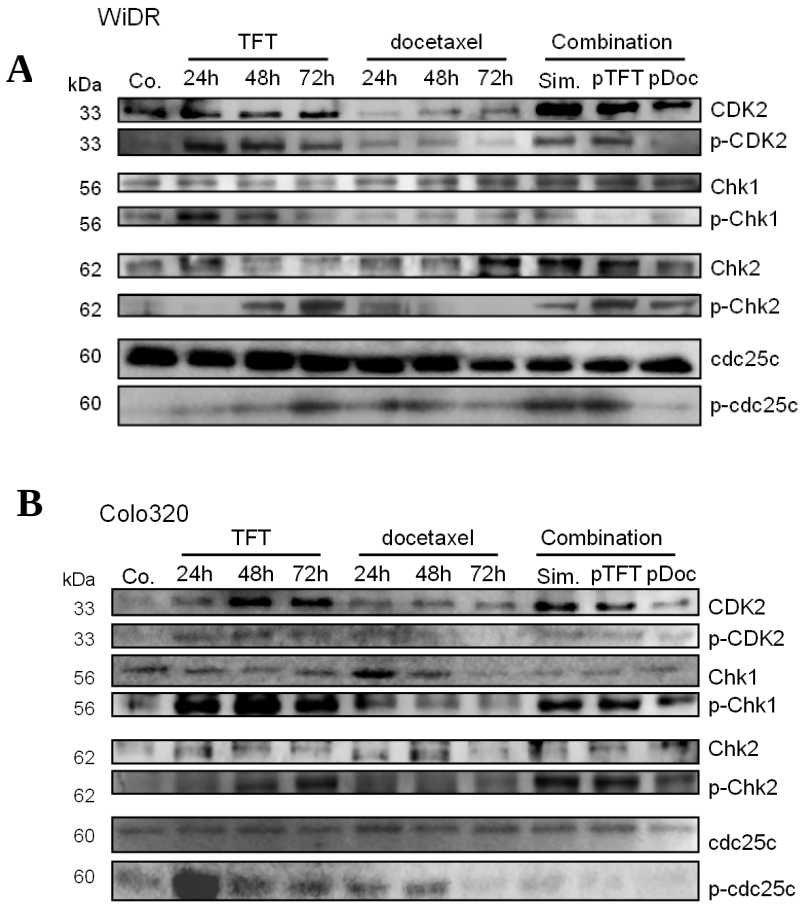


**Figure 3 -** Representative western blot (n=3) of the expression of cell cycle regulating proteins in WiDR (A) and Colo320 (B). Cells were exposed to $IC_{50}$ concentrations of TFT and docetaxel alone and to the $IC_{50}$ ratio based combinations for 72h.

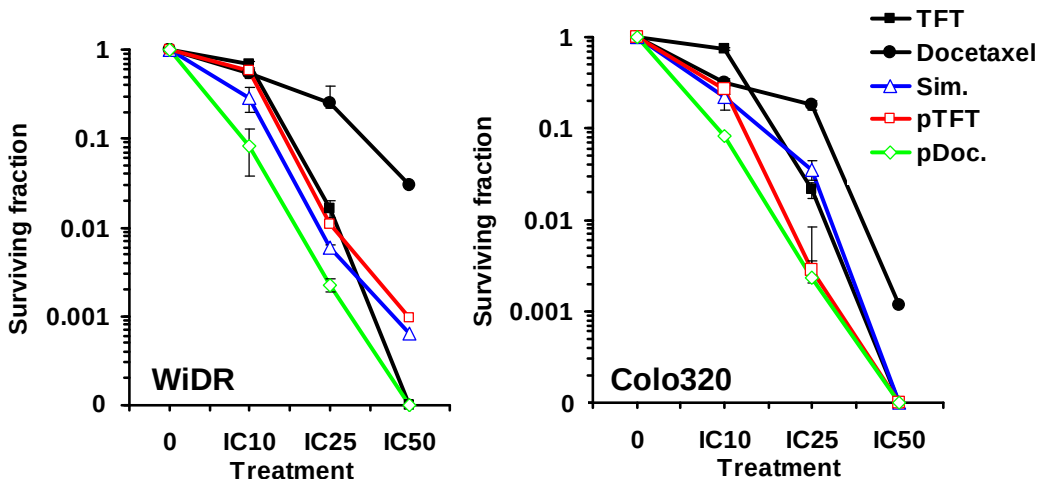


**Figure 4 -** Clonogenic cell survival after exposure to TFT, docetaxel and the combinations. Combination schedules used were $IC_{10}$:$IC_{10}$ and $IC_{25}$:$IC_{25}$ ratios, based on the SRB-data. Values represent means of at least 3 independent experiments ± SEM.

*Cell cycle regulation*

In order to link the drug-induced cell cycle effects with molecular mechanisms of cell cycle regulation, the major checkpoints and cell cycle dependent kinases, Chk1, Chk2, cdc25c and CDK2 were analyzed by western blotting (Figure 3)[26]. Chk1 and Chk2, when phosphorylated, prevent progression from $G_1$- to S-phase and $G_2$- to M-phase, respectively. Cdc25c phosphorylation prevents $G_2$ to M progression and upon phosphorylation of CDK2, $G_1$ to S-phase progression is stimulated.

In WiDR cells, CDK2 was phosphorylated after TFT exposure. In these cells, docetaxel exposure resulted in the downregulation of CDK2, but the phosphorylation levels of CDK2 did not change. The antagonistic combinations (Sim and pTFT) resulted in the phosphorylation of CDK2, while after the synergistic pDoc combination, CDK2 was not phosphorylated. Chk1 was phosphorylated after 24 h TFT exposure, which reduced in time. Docetaxel did not change Chk1 phosphorylation levels. Chk 1 levels hardly changed after the combination, and seemed to decrease after the pTFT combination. Chk2 was phosphorylated in time after TFT exposure, which is in relation to the $G_2$/M arrest found by FACS analysis (Figure 2). This, in contrast to docetaxel, after which these phosphorylation levels increased after 24 h, but reduced in time to control levels, which explains the less time dependent effect of this drug. This may be related to the induction of polyploidy and cell death. After all combination schedules, Chk2 was phosphorylated. Only after the pDoc combination, cdc25c was not phosphorylated.

In Colo320 cells, TFT exposure resulted in phosphorylation of CDK2, Chk1, Chk2 and cdc25c, which can be related to the time dependent induction of $G_2$/M arrest. Docetaxel exposure of Colo320 cells hardly affected CDK2 phosphorylation, activated Chk1, Chk2 and cdc25c after 24 h, which all reduced in time. The combinations resulted in activation of Chk1, Chk2, and dephosphorylated cdc25c. All the synergistic combinations resulted in dephosphorylation of cdc25c, which may explain why cells arrested to a lower extent in the $G_2$/M phase and could progress to polyploidy, resulting in mitotic failure. Probably the synergistic action of the pDoc combination in both cell lines was due to the induction of mitotic failure, after which cells died, while after the other combinations checkpoints were activated after cell cycle progression was inhibited in either the $G_2$/M phase or polyploidy.

*Clonogenic cell survival*

For the tested combination schedules there was a time dependent induction of cell death and polyploidy. Large polynucleated cells may mask the true growth inhibition as determined by the SRB-assay. Moreover, other reports about combination studies with taxanes indicated that multiple failed completed cell division cycles result in induction of

polynucleation (mitotic failure) after which cell death will be induced[27,28]. For determining the clonogenic survival, we used the $IC_{10}$, $IC_{25}$ and $IC_{50}$ concentrations as determined in the SRB-assay (Figure 1). At these concentrations, docetaxel had a higher clonogenic survival than TFT in both WiDR and Colo320 cells (Figure 4). In contrast to the difference in $IC_{50}$, both WiDR and Colo320 cells had a comparable clonogenic survival after exposure to TFT alone. After exposure to the $IC_{50}$ concentration of TFT and the some of the combinations ($IC_{50}$:$IC_{50}$), no colonies were formed. Similar to the SRB-assay, the pDoc combination resulted in strong additivity for both cell lines. The other combinations were less active. These long term survival data are in agreement with the combined induction of polyploidy and cell death (Figure 2), which were both strongly induced after the pDoc combination.

*Caspase 3 activation*

Caspase 3 is an important mediator of the execution phase of the apoptotic process. We determined caspase 3 enzymatic activity and found activation upon TFT exposure in WiDR cells, but only to some extent in Colo320 cells (Figure 5). Docetaxel moderately activated caspase 3 in both cell lines, although more in Colo320 cells than in WiDR cells. Exposure to any of the combinations did not result in increased activation when compared to exposure to the single agents. Apparently, caspase 3 is not key in triggering apoptosis by these agents, suggesting that other caspases or caspase-independent mechanisms may be involved.

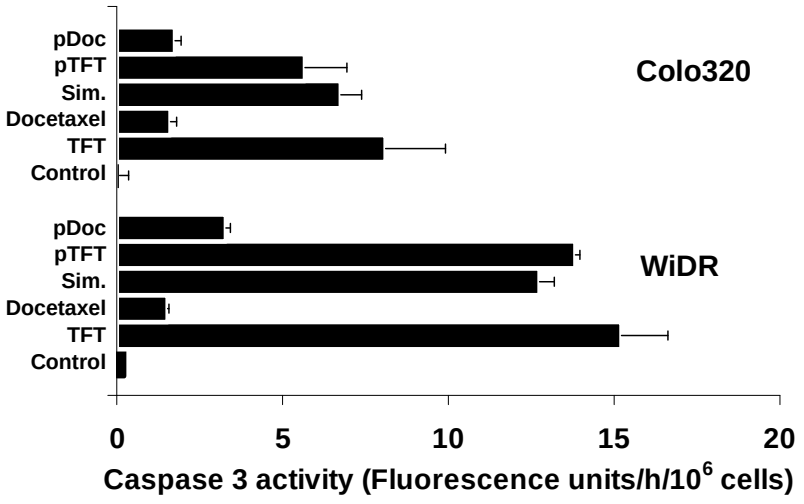


**Figure 5 -** Caspase 3 enzymatic activity after 72 h exposure to TFT or docetaxel alone or the (sequential) combinations. Values are means ± SEM of 2 independent experiments.

*Effect on microtubules*

Since both drugs induce $G_2$/M arrest and docetaxel is known to stabilize microtubules cells were immunofluorescently stained for their cellular tubulin network. Docetaxel exposed cells showed the characteristic aberrant spindle formation, named aster spindles (Figure 6). TFT treatment resulted in the formation of large multinucleated (polyploidy) cells that seemed to occur prior to the formation of spindles, since mitotic spindles were not (clearly) detected. All combinations of TFT with docetaxel resulted in an increased cell size in both WiDR and Colo320. Multinucleated cells were detected upon treatment with the concurrent and pTFT combinations, also displaying increased cell size compared to normal cells. These phenomena were more clearly seen in WiDR cells and is reflected by the increased polyploid cell fraction in the FACS profiles (Figure 2). Abnormal multipolar aster spindles were seen after all treatment combinations.

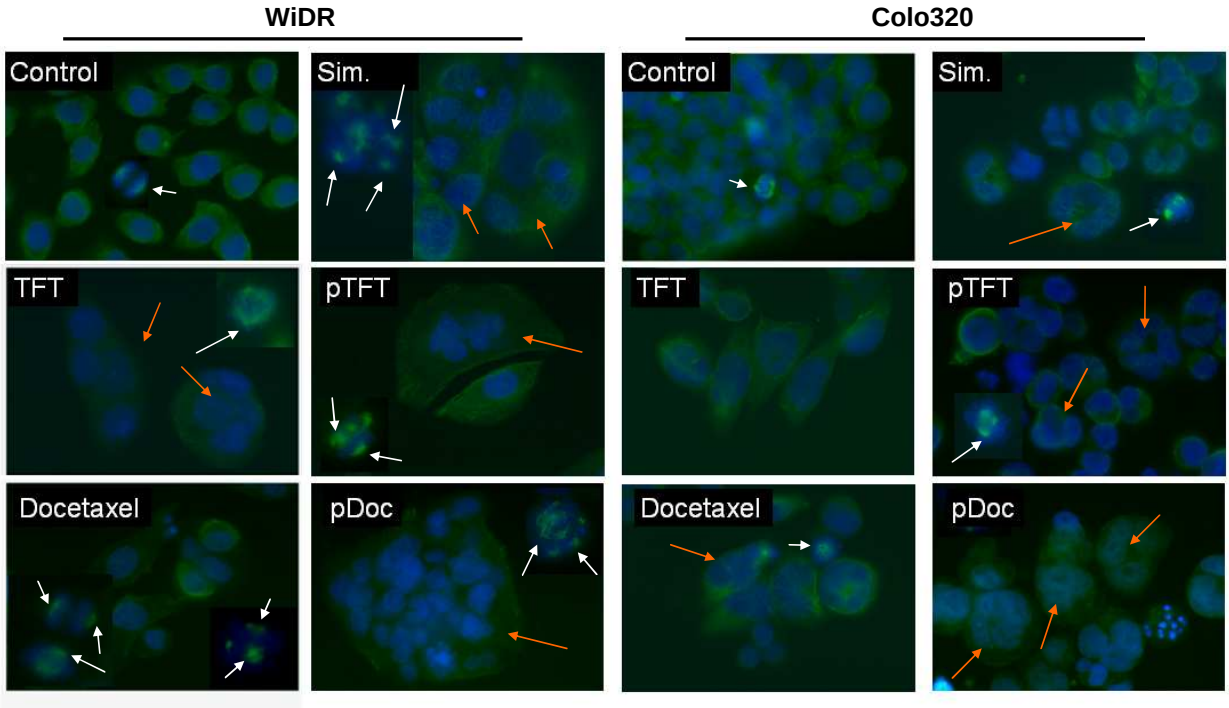


**Figure 6 -** Representative immunofluorescent staining of α-tubulin (mitotic spindle; green; white arrows) and Hoechst (DNA; blue; polynucleated cells are indicated with orange arrows) (n=4). Cells were exposed to TFT, docetaxel, simultaneous combination (Sim), pre-incubation with TFT (pTFT) or a pre-incubation with docetaxel (pDoc) for 72 h.

## DISCUSSION

This study shows that docetaxel and TFT have synergistic activity, which is most likely related to the combined induction of cell death and mitotic failure. The various combination

schedules resulted in different cellular responses. Especially when cells were pretreated with docetaxel before cells were exposed to the combination, WiDR and Colo320 cells both seem to have similar actions, e.g. the induction of both cell death and tetraploidy and inactivation of the cell cycle kinase cdc25c. However, for the other combinations, the response was more cell type dependent. Although TFT and docetaxel have different mechanisms of action, both drugs arrested cells in the $G_2$/M-phase of cell cycle[15], but with different effects on the mitotic spindles. Docetaxel caused the formation of aster spindles, while TFT seems to prevent or induced cell cycle arrest prior to the spindle formation. Apparently, these drug-dependent effects form the basis for synergism.

Clinical data showed that docetaxel is active against various tumor types[3]. In this study, docetaxel alone could inhibit cell growth at clinically relevant concentrations. Docetaxel acts by interfering with the formation of the mitotic spindle. This is associated with an arrest in the S/$G_2$/M-phase of cell cycle and a subsequent cell death induction[4]. Docetaxel is currently studied in several clinical trials in combination with 5-FU and oral 5-FU formulations, such as S-1, against various cancer types[16,18,19]. Combining docetaxel with other chemotherapeutic agents has shown to improve overall survival and responses, but side effects and resistance are recurrent problems. Therefore, TFT (as TAS-102) can be a new strategy to circumvent resistance and decrease the side effects. TFT has overlapping actions with 5-FU, and might be a good alternative to 5-FU. TFT induces cell cycle arrest in the $G_2$/M-phase of cell cycle, while 5-FU has been described to arrest cells in the $G_1$ or S-phase[29].

An S-phase arrest is expected for TS inhibitors, such as nolatrexed and raltitrexed[29]. These drugs clearly showed a strong TS inhibition and an arrest in the S-phase. TFT can also inhibit TS in cancer cells, however, it may be that TFT has also other mechanistic actions than TS inhibition. Previously we showed that TFT can be incorporated into the DNA. Moreover, TFT could overcome drug resistance in 5-FU resistant cell lines[9,30]. Therefore, TFT may be active against 5-FU resistant colorectal cancer cells. TFT can be activated rapidly, since it only needs one activation step, while 5-FU needs two or more activation steps. These differences in response further point out that TS inhibition may not be the main mechanism of action TFT.

Induction of cell cycle arrest is regulated by the activation (phosphorylation) of cell cycle regulators[26]. Interestingly, TFT initially stimulates cells to progress from the $G_1$- to S phase (see Figure 2). An explanation for this is that TFT is a nucleoside analog. Exposing cells to nucleosides may stimulate cell cycle progression towards DNA synthesis. The differences in sensitivity between the SRB-assay and the clonogenic assay may indicate that TFT needs more cycles to cause enough damage to induce cell death. TFT incorporation into the DNA induces DNA strand breaks[14,15]. In addition, due to intracellular thymidine depletion after TS

inhibition, uracil can be misincorporated into the DNA thus enhancing the induction of DNA damage[31]. TFT-induced DNA damage leads to cell cycle arrest in $G_2$ followed by cell death activation in a p53-independent way, as we also have demonstrated recently[32]. Following activation of the checkpoints Chk1 and 2, probably in an ATM dependent manner[33], cdc25c is inactivated, thereby further preventing the entry to the M-phase, inducing an arrest in the $G_2$-phase of cell cycle after which cell death will be induced.

We speculate that the synergistic pDoc combination resulted in the activation of both mitotic catastrophe-induced cell death and TFT-induced cell death, while the other combinations triggered predominantly either one of the death mechanisms. The pDoc combination demonstrated less activation of the checkpoints and seems to have the highest activity after 48 h, which finally resulted in cell death induction in WiDR and an increase in polyploidy in Colo320 (see Figure 2). The decrease in cell death may be related to the increase in tetraploidy, delaying the cell death induction. Interestingly, all synergistic combinations were accompanied by low phosphorylation levels of cdc25c. Recent data suggest that cdc25c plays a complex role during mitosis. In a recent report, where HT29 colon cells were exposed to a cdc25 phosphatase inhibitor, the mitotic spindle was impaired and the segregation of chromosomes became aberrant[34]. The low phosphorylation of cdc25c in the synergistic combinations in our study implicates that this may be important for the mitotic catastrophe and subsequent induction of cell death. Apparently, for synergistic activity docetaxel is required to first inflict tubulin damage causing cell cycle arrest and mitotic failure, thereafter TFT-induced damage is able to further increase cell death activation. The antagonistic combinations in WiDR were probably due to TFT actions alone, preventing docetaxel to exert its activity, however the precise antagonistic mechanism remains unclear. Under these conditions TFT-induced damage causes activation of the cdc25-dependent checkpoint thereby pausing cells and preventing docetaxel to induce a mitotic catastrophe thus explaining antagonistic activity. Both the simultaneous and pTFT combination had comparable cell cycle actions, activation of the checkpoints, Chk1 and Chk2, a strong arrest in the $G_2$/M phase and an induction of cell death. These cell cycle dependent actions of the drugs may explain why the sequence of the combination is such important using these type of drugs.

Inhibition of microtubule function by docetaxel will lead to the induction of apoptosis[35]. One of the main executioner caspases is caspase 3, which is active in its cleaved form. Caspase 3 was activated upon TFT exposure and only weakly after docetaxel treatment. Furthermore, caspase 3 activation levels did not reflect the levels of cell death induction after combination treatments. Caspase independent apoptotic cell death has previously been reported for microtubule inhibitors including paclitaxel, epothilone B and

discodermolide and might involve cathepsin B or another mechanism[36]. Furthermore, in mice with implanted Ly-TH lymphoma, docetaxel caused tumor lysis, rather than apoptosis[37].

In conclusion, these *in vitro* results provide a rational for the experimental use of the combination of docetaxel with TFT, in which scheduling was important. Moreover, *in vivo* potential of this combination might be enhanced, because TAS-102 also contains TPI, which besides increasing TFT bioavailability, also has anti-angiogenic potential. This, combined with reports showing an anti-angiogenic potential of taxanes[38], provides further perspective for using this combination in the clinic.

## ACKNOWLEDGEMENT

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