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Irinotecan-induced cytotoxicity to colon cancer cells *in vitro* is stimulated by pre-incubation with trifluorothymidine

Olaf H. Temmink[a], *Eveline K. Hoebe*[a], *Masakazu Fukushima*[b], *Godefridus J. Peters*[a,*]

[a]*Department of Medical Oncology, VU University Medical Center, De Boelelaan 1117, P.O. Box 7057, 1007 MB Amsterdam, The Netherlands*
[b]*Tokushima Research Center, Taiho Pharmaceutical Co., Tokushima, Japan*

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ABSTRACT

SN38 is the active metabolite of the anti-cancer agent irinotecan (CPT-11) and is a potent inhibitor of topoisomerase-I (topo-I), leading to DNA strand breaks and eventually cell death. The pyrimidine analog trifluorothymidine (TFT) is part of the anti-cancer drug formulation TAS-102, which was developed to enhance the bioavailability of TFT *in vivo*, and is currently being evaluated as an oral chemotherapeutic agent in phase I clinical studies. In this study, the combined cytotoxic effects of dual-targeted TFT with SN38 were investigated in a panel of human colon cancer cell lines (WiDr, H630, Colo320, SNU-C4, SW1116). We used different drug combination treatment schedules of SN38 with TFT, and possible synergism was evaluated using median drug effect analysis resulting in combination indexes (CI), in which CI < 0.9 indicates synergism, CI = 0.9–1.1 indicates additivity and CI > 1.1 indicates antagonism. Drug target analysis was performed to investigate the effect of TFT on SN38-induced DNA damage, cell cycle delay and apoptosis. Simultaneous exposure to SN38 in combination with TFT was not more than additive, whereas pre-incubation with TFT resulted in synergism with SN38 (CI = 0.3–0.6). Only for Colo320 synergism could be induced for both simultaneous and sequential drug combinations. SN38 and TFT induced most DNA damage in H630 and Colo320 cells, which was increased in combination. TFT pre-incubation further enhanced SN38-induced DNA strand breaks in H630 and Colo320 (>20%), which was most pronounced in H630 cells ($p < 0.01$). Exposure to SN38 alone induced a clear cell cycle G2M-phase arrest and pre-incubation with TFT enhanced this effect in WiDr and H630 ($p < 0.05$). Both drugs induced significant apoptosis; SN38-induced apoptosis increased significantly in the presence of TFT ($p < 0.01$), either when added simultaneously (about 3-fold) or at pre-incubation (about 2-fold). Topo-I protein levels varied among the cell lines and TFT hardly affected these. In conclusion, TFT pre-incubation can enhance SN38-induced cytotoxicity to colon cancer cells resulting in synergism between the drugs, thereby increasing DNA damage and apoptosis induction.

1. Introduction

Combination chemotherapeutic regimens for treatment of different solid tumours often include antimetabolites.[1] For the treatment of colorectal cancer (CRC), the fluoropyrimidine 5-fluorouracil (5FU) has been the standard chemotherapeutic agent used for decades.[2,3] In order to improve response and survival rates for patients with colorectal tumours, 5FU is usually combined with leucovorin (LV) and several DNA-damaging agents, such as the DNA topoisomerase-I (topo-I) inhib-

* *Corresponding author:* Tel.: +31 20 4442633; fax: +31 20 4443844.
E-mail address: gj.peters@vumc.nl (G.J. Peters).
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itor irinotecan (CPT-11; Camptosar®, Pfizer Pharmaceuticals Inc.), which significantly improved results of first-line CRC chemotherapy treatment when given in various schedules: bolus IFL or infusional FOLFIRI regimens.[4–6]

CPT-11 is cleaved by a carboxylesterase (CE) into the active metabolite SN38,[7] which is present in the liver and serum.[8] SN38 is a potent inhibitor of topo-I by binding to the enzyme-DNA cleavable complex, thereby leading to the induction of DNA strand breaks.[9–11] The subsequent arrest of DNA replication (S-phase arrest) eventually results in cell death. Mechanisms of resistance to CPT-11 include low expression or mutated gene of the target enzyme topo-I, reduced metabolic activation of CPT-11, increased inactivation by cytochrome P450 enzymes, increased glucuronidation and increased efflux of the drug mediated by multidrug resistance proteins.[10,12]

CPT-11 has potent anti-tumour activity against a wide range of tumour types, and has been investigated in several combination studies.[13] The increased understanding of the intracellular interactions of CPT-11 led to combination of the drug with other metabolites, such as the nucleoside analogs. This has also led to a the understanding that various antimetabolites such as gemcitabine (dFdC; 2′,2′-difluoro-2′-deoxycytidine) and the 5FU-derived nucleotide polymer of 5-fluoro-2′-deoxyuridine-5′-monophosphate FdUMP[10] can act by poisoning of topo-I.[14–16] Possibly these effects play a role in the interaction of, *e.g.* dFdC with CPT-11 regarding the formation of cleavable complexes.[16]

Another pyrimidine analog that might enhance the effect of topo-I inhibition is trifluorothymidine (TFT; trifluridine), which is part of the novel oral anti-tumour drug formulation TAS-102,[17–20] which consists of TFT and the anti-angiogenic thymidine phosphorylase inhibitor TPI.[21–23] TAS-102 is currently evaluated in different treatment schedules in clinical trials. TFT acts by incorporation into DNA leading to DNA strand breaks,[24] and by inhibition of thymidylate synthase (TS) by covalent binding to the active site of TS.[25] 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.[26] Because of these effects TFT might be an alternative in the combination with CPT-11, and in addition, TFT is able to exert cytotoxicity against 5FU resistant tumour cells.[27,28]

In this study, we aimed to elucidate the possible mechanisms of synergism between TFT and SN38 in colon cancer cells. For this purpose, we used colorectal cancer cell lines to investigate the effect of different combinations of TFT with SN38 on growth inhibition, the extent of DNA damage, cell cycle distribution and apoptosis induction.

2. Materials and methods

2.1. Materials

Dulbecco's modified Eagle's medium (DMEM) and Hepes buffer were purchased from Cambrex BioScience (Verviers, Belgium) and foetal bovine serum (FBS) from Greiner Bio-One (Frickenhausen, Germany). Bovine serum albumin (BSA) was obtained from Merck (Darmstadt, Germany). Sulforhodamine B (SRB) protein dye and propidium iodide were purchased from Sigma–Aldrich Chemicals (Zwijndrecht, The Netherlands). TFT and SN38 were synthesised and provided by Taiho Pharmaceuticals Co. (Tokushima, Japan) and Rhône-Poulenc Rorer (now Sanofi-Aventis Pharma) (Vitry sur Seine, France), respectively. The Enhanced ChemoLuminescence (ECL) Hybond nitrocellulose membranes, hyperfilms and detection kit were purchased from Amersham Biosciences (Buckinghamshire, UK). The primary polyclonal rabbit-anti-human topo-I and Lamin B1 antibodies were purchased from TopoGEN Inc. (Columbus, OH) and Abcam (Cambridge, MA), respectively. The secondary peroxidase-conjugated antibodies were purchased from Amersham. All other chemicals were of analytical grade and commercially available.

2.2. Cell culture

In this study, the colon cancer cell lines WiDr, H630, Colo320, SNU-C4 and SW1116 were used. WiDr, Colo320 and SW1116 were obtained from the American Type Culture Collection (ATCC); H630 and SNU-C4 were a kind gift of Dr. P.G. Johnston (at that time at the National Cancer Institute, Bethesda, MD, USA).[29] All these cell lines were cultured in DMEM supplemented with 10% heat-inactivated FBS and 20 mM Hepes buffer. They were grown as adherent monolayers in a humidified atmosphere containing 5% CO_2 at 37 °C and were maintained in exponential growth.

2.3. Growth inhibition experiments

To assess cytotoxicities of the cell lines to the drugs, the SRB cytotoxicity assay was used.[30,31] In brief, the cells were seeded in 100 μl medium in triplicate in 96 well flat-bottom plates (Greiner Bio-One, Frickenhausen, Germany) in different densities depending on their growth rate (5000–10,000 cells/well). After 24 h, 100 μl drug containing medium was added to the wells and the cells were incubated for another 72 h. The concentration series of the drugs were 0.025–250 μM TFT and 0.625–6250 nM SN38. After the incubation period, the cells were fixed using trichloroacetic acid and stained with the SRB dye. Differences in optical density (measured at 540 nm) between the treated cells and untreated control cells were compared to calculate the IC_{25} and IC_{50} values. These were expressed as the concentrations that corresponded to a reduction of cellular growth by 25% and 50%, respectively, when compared to values of the untreated control cells.

2.4. Median-drug effect analysis

The cell lines were exposed 72 h to the drugs alone or to a combination of TFT with SN38 in several treatment schedules. Two combination variants were used to test the interaction of the drugs: either both drugs were added in a fixed IC_{50}-based molar ratio, or one drug was added at a concentration that caused 15–25% growth inhibition and the other drug was added in a concentration range. The SRB assay was used to obtain the IC_{50}values and to generate the dose-effect curves, which were used to perform median-drug effect analysis.[1] To evaluate the effect of a combination of two drugs Calcusyn (Biosoft, Cambridge, UK) was used, a program based on the

method of Chou and Talalay.[32] The absorbance values of drug treated wells were compared to the absorbance values of the control wells to calculate each fraction affected (FA). FA = 0.25 means a decrease in absorbance and growth inhibition of 25%. From the median drug effect plots, the dose that reduced absorbance by 50% (D_x) and the slope (m) were calculated (linear correlation coefficient >0.9). The program uses the formula $D_{1-FA} = D_x[FA/(1\text{-}FA)]^{1/m}$ to calculate the doses of the separate drugs and combination required to induce various levels of cytotoxicity. For each level of cytotoxicity, a mutually non-exclusive combination index (CI) was calculated using the formula: $CI = [(D)_1/(D_{1-FA})_1] + [(D)_2/(D_{1-FA})_2] + [\alpha(D)_1(D)_2/(D_{1-FA})_1(D_{1-FA})_2]$. The parameters $(D)_1$ and $(D)_2$ represent the doses of the separate drugs in the combination, whereas $(D_{1-FA})_1$ and $(D_{1-FA})_2$ are the doses of the individual drugs resulting in survival 1 − FA ($\alpha = 1$). In this method, the calculated CI indicates synergism (CI < 0.9), additivity (CI = 0.9–1.1) or antagonism (CI > 1.1). A mean CI was calculated from datapoints with FA > 0.5 for the combinations in which one drug was added at a constant concentration, and for the fixed ratio combinations a mean CI was calculated from the FA values 0.6, 0.75, 0.9. We considered FA < 0.5 as not relevant growth inhibition.[1]

2.5. FADU DNA damage assay

To determine the extent of DNA damage induced by the drugs, alone or in combination, the FADU (Fluorometric Analysis of DNA Unwinding) assay was used.[33,34] The assay is based on the principle that the unwinding rate of double stranded DNA (dsDNA) in an alkaline environment is related to the extent of dsDNA breaks induced by the drugs (detected with ethidium bromide). DNA with a high amount of strand breaks will unwind faster than DNA without strand breaks. DNA strand breaks were measured after 72 h exposure of cells to the drugs alone or in combination, with or without a 24 h TFT pre-incubation period. Concentrations of the single drugs were chosen so that at least 55% of the dsDNA was double stranded compared to the controls (the untreated cells). Two hours before harvesting separate cells were also exposed to 50 μM etoposide (VP16) as a positive control for DNA strand break formation. To predict the effect of a combination of drugs, fractional effect analysis was performed.[35]

2.6. Flow cytometry analysis

Flow cytometry was used for the determination of cell cycle distribution within the cell populations exposed to the drugs alone or in combination. A series of six-well plates were filled with cell suspensions at a concentration of 2×10^5 cells/well. After 24 h the cells were exposed to the drugs for 72 h, with or without a 24 h TFT pre-incubation period. After this incubation period, the percentage of cells in the different cell cycle phases (G1, S, G2M) was measured with FACScan (Becton Dickinson Immunocytometry Systems). For each measurement 20,000 cells were counted and each sample was assayed in duplicate. For calculation of the cell cycle distribution, the Becton Dickinson's CellQuest software was used. The complete procedure was previously described by Cloos and colleagues.[36]

2.7. Detection of apoptosis

The terminal deoxynucleotidyl transferase (TdT)-mediated dNTP-labelling method was used for the detection of cells undergoing apoptosis. For this purpose, we used the TdT-DNA-Fragment End Labeling Kit (FragEL™; Calbiochem, Oncogene Research Products, Cambridge, MA, USA). In this method, TdT binds to exposed 3′-OH ends of DNA fragments generated in apoptotic cells in order to add biotin-(un)labelled dNTPs, which are detected using a streptavidin-horseradish peroxidase conjugate. A part of the cell populations evaluated for cell cycle distribution was used for apoptosis detection. Using light microscopy, 1000 cells were counted twice for positive/negative staining on randomly selected areas on the glass slide, and the apoptotic index was calculated as the percentage of positive staining cells (for details of the procedure, see also[37]).

2.8. Western blot analysis

For determination of topo-I protein levels, nuclear protein extracts were prepared from living cells using a modified protocol, adapted from Van Hattum and colleagues.[38] Cell nuclei were harvested by centrifugation for 10 min at 150*g* (4 °C). After measuring protein content, the samples were loaded onto a 10% SDS–PAGE gel (without loading buffer) followed by blotting onto a PVDF membrane. The primary anti-topo-I (100 kDa) antibodies were diluted 1:5000 in blocking buffer, and Lamin B1 (0.1 μg/ml; 68 kDa) was used as a loading control for nuclear proteins. Detection of antibody-binding was measured with ECL, and protein levels were quantified by densitometric scanning (VersaDoc 4000 Imaging System and Quantity One software from BioRad).

2.9. Statistical evaluation

The (un)paired Student's *t*-test was used for statistical evaluation of the results. Changes were considered to be significant when $p < 0.05$.

3. Results

3.1. Evaluation of the combination of SN38 with TFT

The sensitivities of the cell lines for TFT or SN38 are summarised in Table 1. The IC_{50} concentrations were also used to calculate the drug ratios for the fixed ratio combinations, and for the mechanistic studies. The cell lines were more sensitive to SN38 (all IC_{50} values <250 nM) than to TFT (all IC_{50} values >450 nM). The drug sensitivity for both drugs in the cell lines seems to be correlated, indicating that the cell lines are sensitive or more resistant to the drugs regardless of the drug used. WiDr and SW1116 were most resistant to both SN38 and TFT; H630 and Colo320 were most sensitive to both SN38 and TFT. Representative growth inhibition curves for SN38-induced growth inhibition for WiDr, H630 and Colo320 are shown in Fig. 1.

FA values were obtained after exposure of cells to a series of combinations of SN38 or TFT. To illustrate the synergistic effects at different FA values, FA–CI plots are shown in

Table 1 – Sensitivity to SN38 and TFT for the colon cancer cell lines

Cell line	SN38	TFT
WiDr	50.7 ± 11.0	2025 ± 527[a]
H630	7.8 ± 1.4	453 ± 114[a]
Colo320	4.3 ± 0.3	533 ± 133[a]
SNU-C4	10.0 ± 2.9	830 ± 214
SW1116	235.0 ± 59.2	7450 ± 1340

Growth inhibition expressed as IC_{50} values (in nM) after exposure for 72 h was determined as described in Section 2. Values are means ± SEM (n = 3–5).
a Previously published values [18].

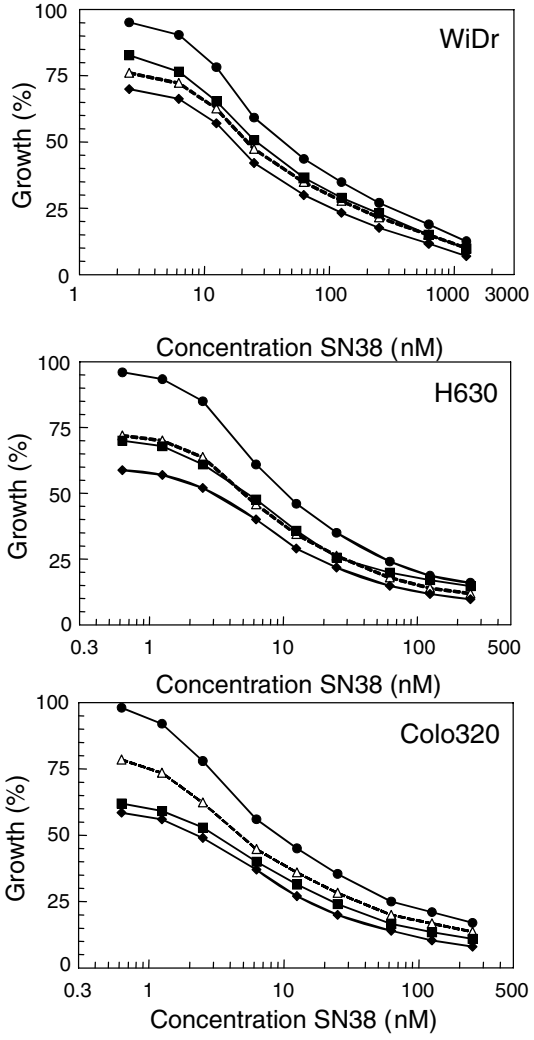

Fig. 1 – Representative growth inhibition curves for the combinations using a variable drug ratio. Cells were exposed 72 h to SN38, TFT or SN38 + TFT in which SN38 was added at variable concentrations (SN38v) and TFT was kept at a constant concentration (TFTc) inducing about 25% growth inhibition. (filled circles) SN38v; (open triangles) expected growth inhibition calculated from the single drug doses; (filled squares) TFTc + SN38v for 72 h; (filled diamonds) TFTc 24 h > SN38v 48 h. The growth inhibition curves were obtained from 3 to 4 separate experiments. Per experiment an average CI value was calculated from all data points with FA > 0.5. All SEM values <15%.

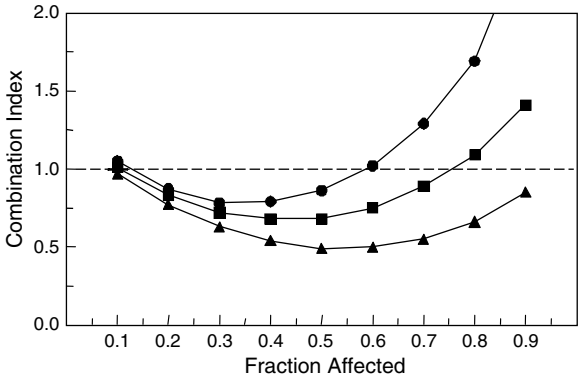

Fig. 2 – Illustrative FA-CI plots for combinations of SN38 with TFT at different schedules. H630 cells were exposed for 72 h to different combinations of SN38 with TFT. SN38 was added at variable concentrations (SN38v) and TFT was kept at a constant concentration (TFTc) inducing FA = 0.25. (circles) SN38v + TFTc 72 h; (squares) TFTc first 24 h > SN38v + TFTc last 48 h; (triangles) TFTc first 24 h > SN38v last 48 h. An average CI value was calculated from datapoints with FA > 0.5.

Fig. 2. The mean calculated CI values of all combination variants are given in Table 2. The combinations of simultaneous SN38 with TFT were mainly additive for both combination variants with a fixed or a variable drug ratio. Moderate synergistic interactions were only seen in Colo320 (all CI $\leqslant$ 0.9). For WiDr and H630, the combination in which TFT was kept at a constant concentration was even moderately antagonistic. To investigate whether TFT pretreatment influenced SN38-induced cytotoxicity, sequential combinations were performed with WiDr, H630 and Colo320 cell lines (see Table 2). These cell lines were selected because they were more easy to handle for these experiments, and because the CI-values ranged from synergistic to antagonistic for all conditions, while WiDr is representative for a relatively insensitive cell line. The CI values decreased significantly when SN38 was only added to the medium, the last 48 h of the total 72 h incubation period (all $0.4 < CI < 0.9$; $p < 0.05$). Even more potent synergism was obtained when the cells were pre-incubated with TFT for 24 h followed by 48 h exposure to SN38 alone (all $0.3 < CI < 0.6$; $p < 0.03$). For Colo320 both drug schedules induced a strong synergistic interaction. The combinations for these cell lines are represented in Fig. 1. These cell lines were chosen for further evaluation because the drug combinations apparently induced different effects.

3.2. *Induction of DNA strand breaks*

To evaluate whether the combination of SN38 with TFT would result in more DNA damage, we measured the formation of DNA strand breaks, either after simultaneous exposure or TFT pre-exposure (Fig. 3). Less DNA damage was induced in WiDr exposed to SN38 with or without TFT, compared to H630, and Colo320. At IC_{50} values, SN38 induced a comparable extent of DNA strand breaks compared to TFT, and in the presence of TFT this was only enhanced in Colo320 cells from 56% to 38% dsDNA left ($p < 0.05$). When

Table 2 – Combination analysis of SN38 combined with TFT using different treatment schedules for the colon cancer cell lines

Cell line	Simultaneous combinations			Sequential combinations	
	1:1 Ratio	SN38c	TFTc	TFTc 72 h	TFTc first 24 h
WiDr	1.6 ± 0.4	1.4 ± 0.3	1.2 ± 0.2	0.6 ± 0.1	0.5 ± 0.1
H630	0.9 ± 0.1	0.9 ± 0.2	1.7 ± 0.3	0.9 ± 0.1	0.6 ± 0.1
Colo320	0.7 ± 0.1	0.9 ± 0.1	0.7 ± 0.1	0.4 ± 0.1	0.3 ± 0.1
SNU-C4	1.2 ± 0.2	1.1 ± 0.1	1.0 ± 0.1	nd	nd
SW1116	4.3 ± 2.1	0.8 ± 0.2	1.0 ± 0.2	nd	nd

Cells were incubated for a total period of 72 h and exposed to TFT or SN38 alone or in combination. 1:1 ratio: the two drugs were added in a fixed 1:1 IC_{50}-based molar ratio; SN38c: combination of the drugs in which SN38 was kept at a constant concentration; TFTc: combination of the drugs in which TFT was kept at a constant concentration. For the sequential combinations the SN38 concentration series was added only the last 48 h, where TFT remained in the medium (TFTc 72 h) or the cells were washed after 24 h and TFT was only present in the medium the first 24 h (TFTc first 24 h). Interpretation of CI values: CI < 0.9 means synergism; CI = 0.9–1.1 means additive; CI > 1.1 means antagonism. A mean CI was calculated from data points with FA > 0.5 for the combinations with variable drug ratio, and from the FA values 0.6, 0.75, 0.9 for the fixed ratio combinations. Values (mean CI ± SEM) depicted here are based on 3–4 separate experiments. See also Section 2. nd, not done.

the cells were pre-incubated with TFT before SN38 exposure the amount of DNA damage increased in H630 and Colo320 (>20%), but not in WiDr. This was most pronounced for H630 when the drugs were given sequentially at their IC_{50} values (<15% dsDNA left; $p < 0.01$).

3.3. *Induction of cell cycle arrest*

The combination of SN38-TFT was also evaluated using flow cytometric analysis to determine the cell cycle distribution after 72 h exposure to the drugs (Fig. 4). Cell cycle delay was predominantly induced by a G2M-phase arrest and was clearly schedule- and cell line-dependent. The G1-phase cell populations were significantly decreased ($p < 0.05$) for all cell lines when exposed to IC_{50} concentrations of TFT or/and SN38, probably as a result of DNA synthesis inhibition. In WiDr and Colo320 a stronger cell cycle arrest was induced at IC_{50} TFT than at IC_{25} TFT. SN38 induced a stronger G2M-phase arrest than TFT in the cell lines. The induction of growth arrest when exposed to both TFT and SN38 was more pronounced in WiDr compared to Colo320 and H630, although less DNA damage was induced in WiDr at these IC_{50} concentrations. Only in WiDr TFT enhanced SN38-induced cell cycle arrest at their IC_{50}s, which was about 20% ($p < 0.01$). TFT-pre-incubation hardly affected the induced G2M-phase arrests compared to the simultaneous combination of the drugs. The concentration of TFT seemed to be less relevant in the sequential combination schedules, because at both IC_{25} and IC_{50} concentrations an equal effect was seen.

3.4. *Induction of apoptosis*

Table 3 summarises the results of apoptosis induction when the cells were exposed to SN38 with or without TFT given in different schedules. The untreated controls contained on average less than 3% apoptotic cells. Both drugs induced

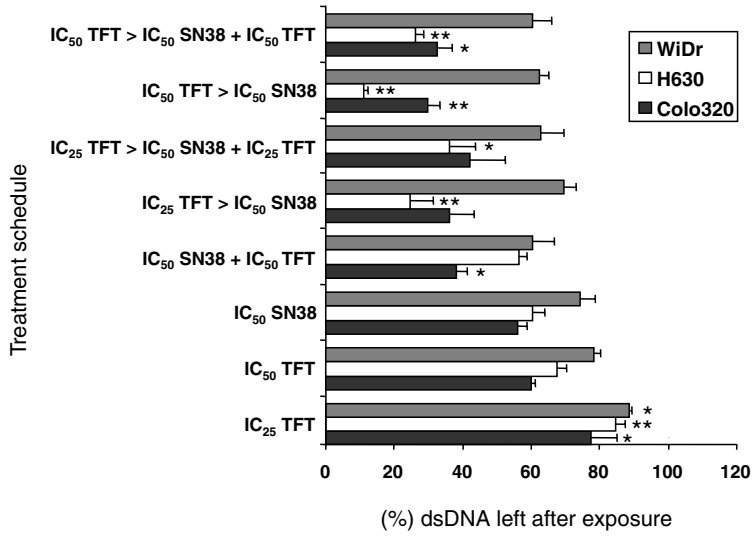

Fig. 3 – Effect of TFT on SN38-induced DNA damage in WiDr, H630 and Colo320 cells. The cells were exposed 72 h to TFT or SN38 alone or in combination, with or without 24 h TFT pre-exposure. VP16 served as a positive control with about 50% dsDNA left compared to control (all three cell lines). Values are means ± SEM ($n = 3$). Compared to IC_{50} SN38 alone: $^{*}p < 0.05$; $^{**}p < 0.01$.

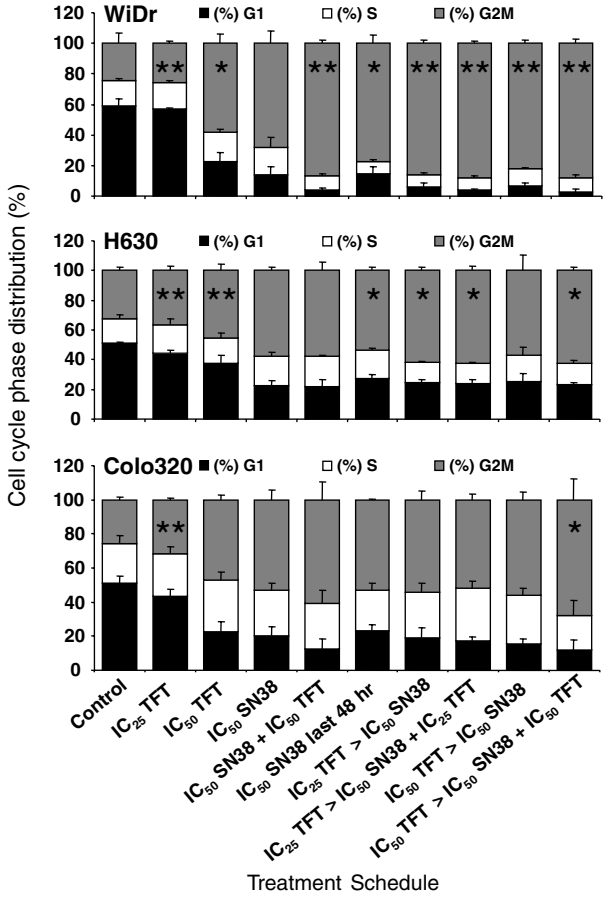

Fig. 4 – Effect of TFT and SN38 on cell cycle distribution of WiDr, H630 and Colo320 cells. The cells were exposed 72 h to TFT or SN38 alone or in combination, with or without 24 h TFT pre-exposure. Values are means ± SEM ($n = 4$). (%) G2M compared to IC_{50} SN38 alone: $^{*}p < 0.05$; $^{**}p < 0.01$.

significant apoptosis when added alone compared to the controls. Most apoptosis was induced in the WiDr cell population at equal growth-inhibiting concentrations. TFT hardly affected apoptosis induced by SN38 for WiDr and H630, but increased apoptosis for Colo320 almost 3-fold ($p < 0.01$) in the simultaneous combination. The sequential combination of TFT and SN38 at their IC_{50}s increased apoptosis induction in the cell lines compared to exposure to SN38 at IC_{50}.

3.5. *Topo-I protein levels*

The topo-I protein levels for the cell lines are depicted in Fig. 5. These levels did not directly correlate with the observed cytotoxicity of SN38. TFT hardly affected the topo-I levels in these cells, indicating a possible increase in the formation of cleavable complexes by TFT itself.

4. Discussion

This study showed that TFT pretreatment can enhance SN38 cytotoxicity to colorectal cancer cells significantly, which is related to increased DNA damage followed by cell cycle arrest,

Table 3 – Induction of apoptosis by TFT and SN38 for the colon cancer cell lines

Drug treatment	Apoptosis enrichment factor		
	Widr	H630	Colo320
IC_{25}TFT	1.7 ± 0.3**	2.3 ± 0.4	1.8 ± 0.2**
IC_{50}TFT	13.8 ± 2.7	3.1 ± 0.3*	9.5 ± 1.2
IC_{50}SN38	18 ± 3.0	5.8 ± 1.0	5.8 ± 0.6
IC_{50}SN38 + IC_{50} TFT	15 ± 1.8	6.9 ± 1.2	15.8 ± 2.4**
IC_{50}SN38 last 48 h	7.2 ± 1.7	2.8 ± 1.5	4.3 ± 1.4
IC_{25}TFT > IC_{50}SN38	17.7 ± 3.9	6.8 ± 1.6	5.5 ± 1.3
IC_{25}TFT > IC_{50}SN38 + IC_{25}TFT	16.5 ± 4.3	4.3 ± 1.9	7.9 ± 2.2
IC_{50}TFT > IC_{50}SN38	22.5 ± 5.8	6.7 ± 1.3	10.1 ± 1.1**
IC_{50}TFT > IC_{50}SN38IC_{50}TFT	12.5 ± 2.0	3.6 ± 1.1*	13 ± 0.9**

Cells were incubated for a total period of 72 h and exposed to TFT or SN38 alone or in combination, with or without 24 h TFT pre-exposure. In one set cells were washed after 24 h pre-exposure to TFT followed by 48 h exposure to SN38 (TFT > SN38); in the other set SN38 was added after 24 h pre-exposure to TFT, where TFT remained in the medium (TFT > SN38 + TFT). The apoptosis enrichment factor (AEF) is defined as [apoptotic cells treated]/[means ± SEM] ($n = 4$). AEF compared to IC50 SN38 alone: $^{*}p < 0.05$; $^{**}p < 0.01$.

resulting in increased cell death induction. Strongest synergism was seen in Colo320 cells, in which TFT pre-incubation significantly enhanced SN38-induced DNA damage and apoptosis. Combining SN38 and TFT simultaneously was not more than additive in the cell lines, for both combinations variants with a fixed or variable drug ratio.

Besides the importance of dose-scheduling and integration of the pharmacokinetic drug profiles, the metabolic and biological interactions of the drugs must be positively assessed in order to obtain potential clinical efficacy. The H630 and Colo320 cell lines are comparably sensitive to SN38 and TFT, while WiDr is more resistant to the drugs (Table 1), which can probably be explained by the different cellular characteristics of the cell lines,[39] such as drug transport mechanisms and metabolic activation of the drugs. However, it was previously shown that no correlation exists between CPT-11 activity and topo-I levels or relative S-phase distribution in human colon cancer cells[40] and cells of other tissue origin.[39,41] Topo-I intracellular levels are commonly upregulated in many types of cancer cells,[42] but are stable across the cell cycle.[43] Cells in late S-phase to early G2-phase of the cell cycle are most sensitive to CPT-11,[44] because ongoing DNA replication is an essential event for SN38-induced cytotoxicity. Our results showed an increase in G2M population in the cell lines after exposure to SN38 alone or in combination with TFT, which was most significant in WiDr. For the sensitive H630 and Colo320 cell lines the arrest was not enhanced when TFT was added, for both sequential and simultaneous combinations. These cell lines sustained more DNA damage than WiDr at equal growth inhibiting concentrations of the drugs, either alone or in combination, which suggests that DNA strand break formation is not directly related to the induced G2M-arrest.

In Colo320 we observed synergism for both SN38–TFT sequential and simultaneous combinations, thereby significantly increasing DNA damage and apoptosis induction compared to SN38-exposure alone, whereas in H630 cells only the

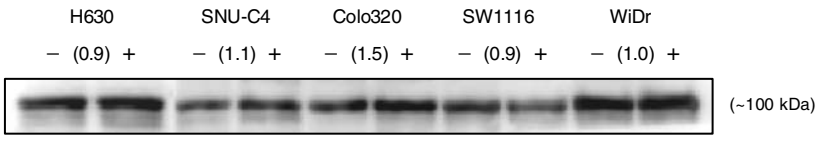

Fig. 5 – Effect of TFT on the basal topo-I protein levels of the colon cancer cell lines. Cells were exposed 24 h to IC_{50} TFT. Numbers between brackets indicate ratio [+TFT]/[−TFT], corrected for the loading controls.

sequential combination resulted in synergism with increased DNA strand break formation, but not apoptosis. The differences between the cell lines with respect to sensitivity to the combination of drugs are most likely at the level of SN38-induced DNA strand breaks, but not related to difference in topo-I levels or changes in these levels. SN38 acts by covalent binding to the enzyme-DNA cleavable complex, resulting in irreversible DNA replication arrest and DNA strand breaks with subsequent S- or G2M-arrest, and ultimately cell death.[9–11] Goldwasser and colleagues[40] previously determined that CPT-11-induced growth inhibition and cleavable complexes correlated well, although these ternary complexes are not directly cytotoxic to non-proliferating cells. TFT is able to enhance DNA strand break formation in the cells, especially in the sequential exposure schedule for H630. This can be explained by the fact that TFT induces strand breaks itself through direct incorporation into DNA,[24] and in addition, TFT might also have other indirect effects on topo-I activity. Downstream parameters from the cleavable complexes are probably also critical for CPT-11-induced growth inhibition, because differences in CPT-11 sensitivity show minimal differences in cleavable complexes and S-phase distribution.[40]

The topo-I cleavable complexes trapped by CPT-11 in cancer cells may be enhanced by nucleoside analogs, and therefore may contribute to the synergistic or additive effects of CPT-11 when in combination. A wide range of alterations in DNA can trap topo-I cleavable complexes,[45] including uracil misincorporation and incorporation of certain nucleoside analogs,[15] such as 5FU,[14] 1-beta-D-arabinofuranosyl cytidine (AraC)[46] and dFdC.[16] It is unknown whether TFT itself is able to form cleavable complexes or enhances the formation of CPT-11-induced cleavable complexes. TFT has overlapping mechanisms of action as 5FU and therefore it is likely that TFT also mediates comparable cytotoxic effects, and topo-I poisoning may partly contribute to the overall anti-cancer activity of TFT.

Synergism between CPT-11 and 5FU in colorectal cancer cells increased when a sequential dose-schedule was used, both *in vitro*[47,48] and *in vivo*,[49,50] in contrast to simultaneous drug exposure. The most cytotoxic schedule was SN38 followed by 5FU exposure, with increased DNA damage induction. We demonstrated that the anti-tumour activity of SN38 against colon cancer cells can be increased by TFT pretreatment, in contrast to 5FU pretreatment, which often results in additive or even antagonistic effects.[47] *In vivo* repeated scheduling led to more incorporation of TFT into DNA resulting in increased DNA damage;[19,24] therefore alternating TFT and CPT-11 administration (TFT first) would be the most suitable clinical treatment schedule. TFT concentrations used in the experiments are in the range found in phase I studies with TAS-102.[19] The SN38 concentrations are also in the range of repeated plasma SN38 concentrations at prolonged infusion,[51] while the 120 min infusions generate higher SN38 plasma concentrations. TFT is active against 5FU-resistant tumours[27,28] and therefore might be a potent alternative to be combined with CPT-11, also because SN38-induced DNA damage can be increased by TFT pretreatment.

DNA lesions induced by CPT-11 are mainly repaired by nucleotide excision repair (NER) and DNA double-strand break repair (DSBR)[45] and possibly DNA mismatch repair (MMR), which plays an important role in detecting fluoropyrimidine induced damage.[52] The CPT-11 plus 5FU combination is partly based on decreased repair of CPT-11 induced DNA damage,[47] possibly leading to increased induction of apoptosis. It seems that the combination of TFT and SN38 is also based on decreased DNA repair, such as in Colo320 in this study.

In conclusion, these *in vitro* results provide a rationale for the experimental use of TFT together with SN38, suggesting that the drug might be of potential value in the (second-line) treatment of patients with advanced colorectal cancer. The anti-tumour effects of this combination might even be increased *in vivo*, because TAS-102 also consists of TPI, which, besides improving the bioavailability of TFT, also has anti-angiogenic[23] and anti-metastatic properties.[22] Furthermore, the approval of the biological agents, bevacizumab (Avastin®) and cetuximab (Erbitux®), might also be of interest in potential TAS-102 involving combinations, with respect to enhanced inhibition of angiogenesis or EGFR targeting, respectively.

Conflict of interest statement

None declared.

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