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Trail receptor-targeted therapy : strategies to enhance DR4- and DR5-induced apoptosis

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THE NOVEL THYMIDYLATE SYNTHASE INHIBITOR TRIFLUOROTHYMIDINE (TFT) AND TRAIL SYNERGISTICALLY ERADICATE NON-SMALL CELL LUNG CANCER CELLS

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ABSTRACT

Purpose

TRAIL, a tumour selective anticancer agent, may be used for the treatment of non-small cell lung cancer (NSCLC). However, TRAIL resistance is frequently encountered. Here, the combined use of TRAIL with TFT, a thymidylate synthase inhibitor, was examined for sensitizing NSCLC cells to TRAIL.

Methods

Interactions between TRAIL and TFT were studied in NSCLC cells using growth inhibition and apoptosis assays. Western blotting and flow cytometry were used to investigate underlying mechanisms

Results

The combined treatment of TFT and TRAIL showed synergistic cytotoxicity in A549, H292, H322 and H460 cells. For synergistic activity the sequence of administration was important; TFT treatment followed by TRAIL exposure did not show sensitization. Combined TFT and TRAIL treatment for 24 h followed by 48 h of TFT alone was synergistic in all cell lines, with combination index (CI) values below 0.9. The treatments affected cell cycle progression, with TRAIL inducing a G1 arrest and TFT a G2/M arrest. TFT activated Chk2 and reduced Cdc25c levels known to cause G2/M arrest. TRAIL-induced caspase-dependent apoptosis was enhanced by TFT, whereas TFT alone mainly induced caspase-independent death. TFT increased the expression of p53 and p21/WAF1, and p53 was involved in the increase of TRAIL-R2 surface expression. TFT also caused down-regulation of c-FLIP and XIAP and increased Bax expression.

Conclusions

TFT enhances TRAIL-induced apoptosis in NSCLC cells by sensitizing the apoptotic machinery at different levels in the TRAIL pathway. Our findings suggest a possible therapeutic benefit of the combined use of TFT and TRAIL in NSCLC.

INTRODUCTION

Lung cancer is the leading cause of cancer-related death world-wide [1]. Non-small cell lung cancers (NSCLC) are epithelial tumours that represent around 80% of all lung carcinomas. In NSCLC almost half of all cases have locally advanced or widespread metastatic disease at diagnosis, with an overall 5-year survival rate of approximately 1 to 5% [2]. Whereas surgery is the mainstay treatment for localized disease, most patients are candidates for systemic or adjuvant chemotherapy. It has become clear in recent years that a therapeutic plateau has been reached for patients with advanced stage NSCLC treated with conventional chemotherapeutic agents. Therefore, novel chemotherapeutics or targeted therapeutics are required to improve prognosis of these patients.

A potential useful group of agents are the tumour necrosis factor apoptosis-inducing ligand (TRAIL) receptor targeting agents that can activate apoptosis directly in tumour cells, while healthy cells are not affected [3]. TRAIL activates the extrinsic apoptotic pathway after binding cell surface membrane-localized TRAIL death receptors (DRs) that include TRAIL-R1 (DR4), TRAIL-R2 (DR5) and two decoy receptors TRAIL-R3 (Dc1) and TRAIL-R4 (Dc2) that do not possess functional death domains [4]. After TRAIL binding to TRAIL-R1 or TRAIL-R2 several proteins are recruited to the intracellular receptor death domain, forming the death-inducing signalling complex (DISC) in which caspase-8 is activated. Caspase-8 on its turn can activate downstream caspases, such as caspase-3, or cross-activate mitochondrial apoptosis via the cleavage of the Bcl-2 family member Bid. The mitochondrial or intrinsic pathway involves mitochondrial outer membrane permeabilization (MOMP), which is regulated by the Bcl-2 family of proteins [5]. Cleaved Bid can induce MOMP, leading to the release of pro-apoptotic factors, such as cytochrome c and Smac/DIABLO into the cytosol. Cytochrome c facilitates the formation of the apoptosome in which pro-caspase-9 is activated, and Smac/DIABLO can sequester X-linked inhibiter of apoptosis (XIAP) thereby preventing XIAP-mediated binding and inhibition of caspase-9 and -3 [6;7]. At the DISC level, cellular FLICE-inhibitory protein (c-FLIP) is a potent inhibitor of pro-caspase-8 activation. Two variants, c-FLIP$_L$ and c-FLIP$_S$ representing inactive pro-caspase-8 analogues, have been found to prevent pro-caspase-8 activation [8]. Currently, several TRAIL-receptor targeting drugs are evaluated in clinical phase I/II trials alone or in combinations for the treatment of NSCLC [9]. However, preclinical research indicated that approximately 50% of tumour cells are resistant to TRAIL and combination with other agents can sensitize tumour cells for TRAIL [9].

Trifluorothymidine (TFT) is a thymidylate synthase (TS) inhibitor that interferes with thymidylate production and in its triphosphate form can be incorporated into the DNA causing DNA damage [10]. TFT has been found to induce apoptotic cell death in both colon and lung cancer cell lines. Moreover, apart from activating intrinsic and/or extrinsic apoptotic pathways also caspase-independent modes of cell death can be induced by TFT [11]. The lysosomal protease cathepsin B is known to be involved in caspase-independent cell death upon various stress stimuli [12]. Previously, we found that cathepsin B plays a

role in TFT-mediated cell death in colorectal cancer [11]. TFT is part of the formulation TAS-102, in which TFT is combined with the thymidine phosphorylase inhibitor (TPI). By inhibition of thymidine phosphorylase, which inactivates TFT, TPI will increase TFT's *in vivo* activity [10]. TAS-102 is active in tumour cells resistant to the anti-metabolite 5-fluorouracil (5-FU) suggesting at least partially non-overlapping mechanisms of action [13]. Currently, TAS-102 is being evaluated in phase II studies for the treatment of several solid tumours [14] and it has shown clinical activity in 5-FU resistant colon cancer patients [15].

In this study, we examined the interaction between TFT and TRAIL in NSCLC cells as a possible novel combination treatment. The effects on cell cycle progression and cell death activation, and underlying molecular mechanisms were explored.

MATERIALS AND METHODS

Cell lines and chemicals

Human NSCLC cell lines A549, H292, H322 and H460 were obtained from the American Type Culture Collection (ATCC, Teddington, UK) and were grown as monolayers in 25 cm^2 culture flasks (Greiner Bio-One, Frickenhansen, Germany) at 37 °C in a humidified 5% CO_2 atmosphere. The cells were cultured in RPMI, supplemented with 10% foetal calf serum, and 100 units/mL penicillin and streptomycin (Lonza, Verviers, Belgium). TFT (Taiho Pharmaceuticals Co., Ltd, Tokushima, Japan) was dissolved in PBS as a stock solution of 20 mM and was stored in aliquots at -20 °C. Aliquots of TRAIL (Peprotech, Rocky Hill, NJ, USA) were stored at -80 °C. The synthetic pan-caspase inhibitor zVAD-fmk was obtained from Bachem AG (Bubendorf, Switzerland), and was dissolved in DMSO (Sigma-Alderich, Steinheim, Germany) at 10 mM stock solutions and stored at -20 °C. Anti-caspase-3 (#9662), anti-caspase-8 (#9746), anti-caspase-9 (#9502), anti-cleaved caspase-3 (#9661), anti-Chk1 (#2345), anti-phosphorylated Chk1 (Ser345; #2341), anti-Chk2 (#2662), anti-phosphorylated Chk2 (Thr68; #2661), anti-Cdc25c (#4688) and anti-phosphorylated Cdc25c (Ser216; #4901), anti-p53 (#9282), anti-FLIP (#3210), anti-XIAP (#2042) antibodies were all purchased from Cell Signaling Technology (Danvers, MA, USA) and anti-p21 (#sc-756) was from Santa Cruz (Santa Cruz Biotechnology, Inc. Santa Cruz, California, USA). Anti-cathepsin B antibody was purchased from Oncogene Research Products (Boston, MA, USA), anti-β-actin antibody from Sigma-Aldrich Chemicals, Goat-a-mouse-IRDye (800CW;#926-32210 and 680;#926-32220) and goat-a-rabbit-IRDye (800CW;926-32211 and 680;#926-32221) were obtained from Licor (Westburg, Leusden, The Netherlands).

Growth inhibition assay

Drug cytotoxicity was determined by the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) assay [16]. Cells (2000/well) were seeded in 96-well plates (Greiner Bio-One, Frickenhausen, Germany). After 24 h, enabling attachment, cells were exposed to increasing concentrations of TFT for 72 h or a fixed concentration of TRAIL for 24 h

(based on the IC_{50}). Two combination schedules were evaluated (figure 1A). First, cells were exposed for 24 h to both TFT and TRAIL, followed by 48 h exposure to TFT alone (Schedule A). Second, cells were exposed for 48 h to TFT, after which the combination of TFT with TRAIL was added for 24 h (Schedule B). After drug exposure (72 h in total), the medium was removed and the cells were incubated for 3 h with 50 µL/well of 1 mg/mL MTT solution in phenol red free DMEM (Lonza) at 37 °C. Subsequently, 150 µL DMSO was added to each well and the optical density (OD) was measured at 540 nm (Tecan, Männedorf, Switzerland). Differences between OD of the treated and untreated controls were compared to calculate cell growth. From the growth inhibition curves, a combination index (CI) was calculated using CalcuSyn software from Biosoft (Cambridge, UK), based on the median-drug-effect method as described previously [10]. A CI < 0.9 indicates synergism and > 1.1 antagonism. For calculation of the CI, only values above a fraction affected (FA) of 0.5 were used, equivalent to 50 – 100% growth inhibition. FA values below 0.5 are considered to be irrelevant, because these represent only a minor growth inhibition. Per experiment the CI values at FA higher than 0.5 were averaged and the mean was used for comparison of separate experiments.

Cell cycle and cell death analyses

Cell cycle analysis and cell death measurements were performed by FACS analysis as described previously [17]. Cells were seeded in 6-well plates at a density of 150,000 cells/well. After drug exposure, cells were trypsinised, 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) on ice in the dark. DNA content of the cells was analysed by FACSCalibur flow cytometer (Becton Dickinson, Immunocytometry Systems, San Jose, CA, USA) with an acquisition of 10,000 events. The sub-G_1 peak was used to determine the extent of cell death.

5

Western blotting

Western blotting was performed as described previously [18]. Cells were exposed to TFT, TRAIL or the combination (schedule A) for 24, 48 or 72 h, after which cells were washed twice with ice-cold PBS and disrupted in lysis buffer (Cell Signalling Technology Inc.) supplemented with 0.04% protease inhibitor cocktail (Roche, Almere, the Netherlands). Cell lysates were scraped, transferred into a vial and centrifuged at 11,000 g at 4 ºC for 10 min. Protein concentrations were determined by the Bio-Rad assay, according to the manufacturer's instruction (Bio-Rad Laboratories, Veenendaal, the Netherlands). From each condition 30-80 µg of protein was separated on an 8-12% SDS-PAGE and electroblotted onto polyvinylidenedifluoride (PVDF) membranes (Millipore Immobilon™ –FL PVDF, 0.45 µm). Subsequently, the membranes were blocked for 1 h at room temperature (RT) in Odyssey blocking buffer (Odyssey blocking buffer #927-40003, Westburg, Leusden, the Netherlands) and incubated overnight at 4°C with the primary

antibodies (dilution 1:1,000-10,000 in Odyssey blocking buffer 1:1 diluted with PBS-T (PBS with 0.05% Tween-20). The membrane was washed 5 times in PBS-T and incubated with the secondary antibody (1:10,000 goat-α-mouse-IRDye (800CW;#926-32210 and 680;#926-32220) or goat-α-rabbit-IRDye (800CW;926-32211 and 680;#926-32221), Westburg) for 1 h at RT in the dark. After incubation, the membrane was washed in PBS-T and once with PBS followed by imaging using an Odyssey Infrared Imager (Westburg), at a 84 μm resolution, 0 mm offset and with high quality [19].

Caspase activity

Effects of treatment on the activity of caspase-3, -8 and -9 were determined by fluorometric assay kits (Zebra Bioscience, Enschede, Netherlands), according to manufacturer's instructions. In brief, after drug exposure cell pellets were made in ice-cold PBS containing $1*10^6$ cells, which were stored at -80 °C until analysis. Fluorescence was detected at 350 nm excitation and 460 nm emission (Spectra Fluor Tecan, Salzburg, Austria). Relative caspase activity was calculated in ratio compared to the untreated control (set to 1).

TRAIL receptor expression

The levels of TRAIL-R1 and TRAIL-R2 expression on cellular membranes were determined by FACS analysis [20]. One million cells untreated or treated with TFT for 24 h were added to a FACS tube and stained with receptor-specific mAbs (TRAIL-R1 mouse anti human Alexis (Alx-804-297) and TRAIL-R2 (Alx-804-298) for 1 h at 4 °C. An IgG1 antibody (DAKO) was used as negative control. After washing, cells were incubated with Goat anti-mouse PE labelled (Alexa-488) for 30 min on ice in the dark. Next, the cells were washed and the fluorescence was measured on a FACSCalibur flow cytometer using CELLQuest software (Becton Dickinson, MountainView, CA).

5

RNA interference

Silencing p53 was performed as described previously [21]. The following p53 siRNAs were used, 5'GCAUGAACCGGAGGCCCAU-dTdT3' (sense) and control 5'AUGGGCCUCCGGUU CAUGC-dTdT3' (anti-sense). The negative control siRNA used was from Invitrogen (Breda, the Netherlands). Cells seeded in 6-wells plates were incubated in unsupplemented Optimem® medium and transfected with 133 nM siRNA using Oligofectamine® reagent according to the manufacturer's protocol (Invitrogen, Breda, the Netherlands). The next day, cells were treated with TFT for 24 h, and used for receptor expression analysis with FACS experiments and western blotting.

RESULTS

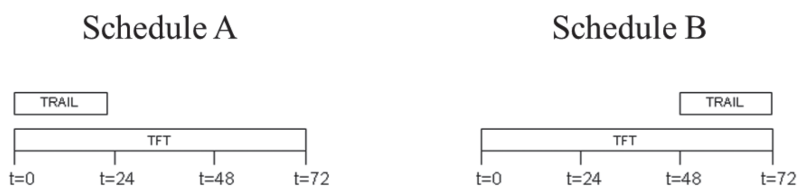
TRAIL, TFT and combined treatment

The sensitivities of the NSCLC cell lines to TFT and TRAIL are summarized in Table 1. IC_{50} values between 2.9 μM and 6.3 μM were observed for TFT. H292 and H460 cells were

TRAIL-sensitive, H322 cells were moderately sensitive and A549 cells were resistant to TRAIL up to the highest tested concentration of 1500 ng/mL. The IC_{50} values of TRAIL for a particular cell line were used in the combination experiments, except for resistant A549 cells where we used a TRAIL concentration of 150 ng/mL. Synergistic effects are illustrated as fraction affected (FA)-combination index (CI) plots obtained in H460 and A549 cells (figure 1).

The combination schedule of 24 h TFT and TRAIL followed by 48 h of TFT alone (Schedule A) was synergistic in all tested cell lines (CI < 0.9), including resistant A549 cells. In contrast, a schedule of 48 h TFT treatment followed by 24 h TFT and TRAIL (Schedule B) appeared to have antagonistic activity in all cell lines examined (Table 1). As TRAIL exerts its apoptotic function quite rapidly, the cells were incubated for just 24 h. TFT, on the other hand, has a long term effect; an incubation time of 72 h of this compound was therefore chosen. In the experiments described below the schedule A (24 h combination + 48 h TFT) was selected to further explore the mechanism underlying synergism of TRAIL and TFT.

A



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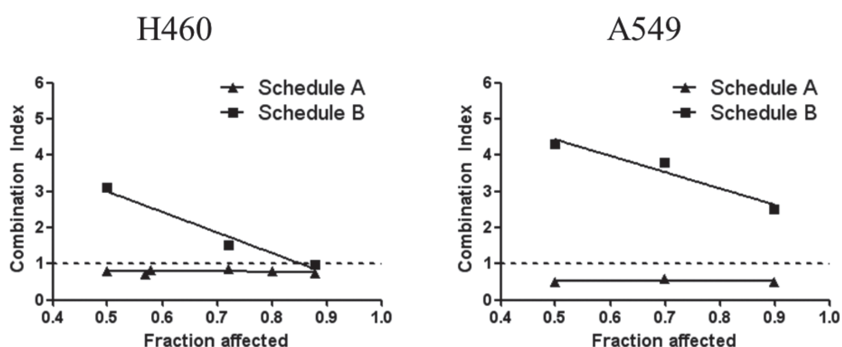


Figure 1. Representative FA-CI plot of combined TFT and TRAIL treatment in H460 and A549 cells using two different exposure schedules as indicated. (**A**) Cells were exposed to increasing concentrations of TFT for 72 h including 24 h exposure to a fixed IC_{50} concentration of TRAIL. TRAIL was added either the first 24 h, or the last 24 h in the presence of TFT and cytotoxicity was determined by MTT assays. (**B**) An average CI was calculated for data-points with FA higher than 0.5.

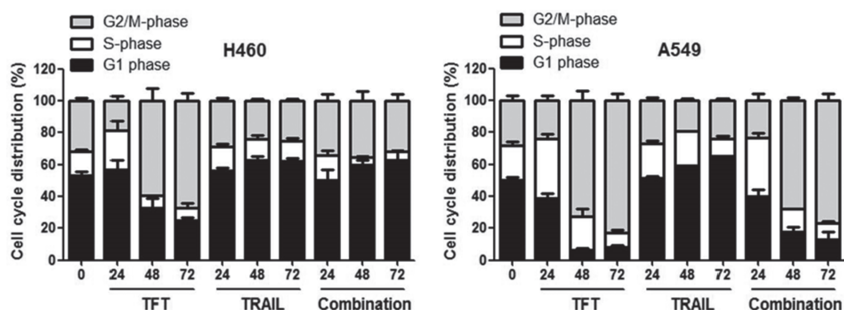
Table 1. Growth inhibition and synergy analyses for different treatment schedules of TFT combined with TRAIL. Combination index (CI) values were calculated from the fraction affected data points from 0.5-0.9. CI values lower than 0.9 specify synergism; CI between 0.9-1.0 indicate an additive effect; CI values greater than 1.1 denote antagonism.

	Growth inhibition (IC_{50})		**Combination index (CI)**	
Cell line	**TFT (μM)**	**TRAIL (ng/ml)**	**24 h Combination + 48 h TFT (schedule A)**	**48 h TFT + 24 h Combination (schedule B)**
A549	6.3 ± 1.5	>1500	0.6 ± 0.2	3.7 ± 1.3
H292	3.3 ± 0.8	25 ± 7	0.6 + 0.2	1.6 ± 0.3
H322	2.9 ± 0.2	80 ± 10	0.7 ± 0.3	1.3 ± 0.4
H460	4.2 ± 0.9	10 ± 2	0.6 ± 0.1	2.8 ± 0.9

Effects on cell cycle progression

The effects on cell cycle distribution were analysed in time-course experiments using flow cytometry on PI-stained cells (figure 2A). In both H460 and A549 cells, 24 h exposure to TFT alone induced the accumulation of cells in the S-phase while longer treatment, up to 72 h, was accompanied by an increase of cells in G2/M and a decrease in G1. This effect was most prominent in A549 cells. At the same time a strong rise in the percentage (up to around 35%) of death (sub-G1) cells was found for both cell lines. TRAIL alone mildly affected the cell cycle distribution of H460 cells with a small increase of cells in G1. This pattern was more pronounced in A549 cells. When TRAIL was combined with TFT following schedule A, in H460 cells an increase in G2/M cells was seen although lower than found in TFT-treated cells. In A549 cells, the cell cycle profile after combined treatment resembled more that of TFT-treated cells, with increased S-phase after 24 h followed by a pronounced accumulation of G2/M cells. In addition, only a very small percentage of A549 cells was detected in the S-phase after 48 and 72 h of combined treatment.

Next, the treatments were examined for affecting the cell cycle regulatory proteins Chk1, Chk2, and Cdc25c. Chk1 and Chk2 are known to become phosphorylated after DNA damage resulting amongst others in inactivation of Cdc25c phosphatase by stimulating its proteasome-dependent degradation leading to G2/M and G1/S phase arrest [22]. TFT treatment induced strong Chk2 phosphorylation in a time-dependent manner, which correlated with a decrease in Cdc25c levels and G2/M arrest (figure 2A). Total Cdc25c levels decreased after 48 and 72 h treatment with TFT and even more rapidly after combination treatment reflecting its Chk-induced phosphorylation-dependent degradation. In H460 and A549 cells exposure to TRAIL slightly increased the phosphorylation of Chk1, whereas Chk2 and Cdc25c phosphorylation levels remained similar (figure 2B) reflecting an increase of cells in the G1 phase (figure 2A).



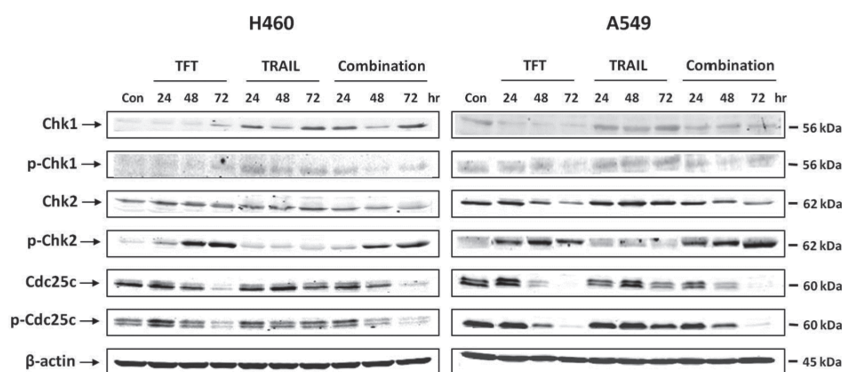
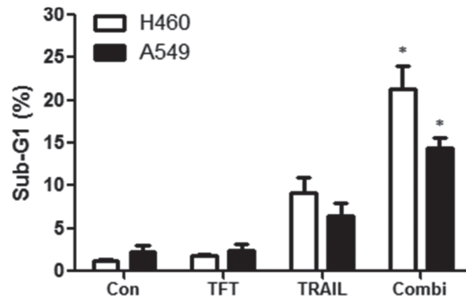


Figure 2. Effect of TRAIL, TFT and combined treatment on the cell cycle progression. (**A**) The percentages of cells in G1, S and G2/M phase are depicted after the indicated treatments in H460 and A549 cells. Values are means of three independent experiments ± S.E.M. After 24 h or 48 h incubation with TRAIL, medium was refreshed with drug free medium. (**B**) Western blots showing the expression and phosphorylation of cell cycle regulatory proteins Chk-1, Chk-2 and Cdc25c. Cells were exposed to 5 μM TFT, and 10 ng/mL TRAIL in H460, and 5 μM TFT, 150 ng/mL TRAIL in A549 or the combinations for the indicated time points.

Cell death activation by TFT and TRAIL

For the cell death assays, 10 ng/mL TRAIL was used in the H460 cell line and 150 ng/mL TRAIL in A549 cells. The combination of TFT and TRAIL resulted in a 2-fold increased cell death indicating synergistic activity already after 24 h incubation (figure 3A). Since TFT alone did not induce cell death at 24 h, the cell death detected upon combining drugs can likely be attributed to sensitization for TRAIL. The contribution of caspase-dependent cell death was investigated by adding the broad-range caspase inhibitor zVAD to the cultures. This resulted in a partial inhibition of around 30 to 40 % of TFT-induced cell death, indicating that both caspase-dependent and -independent mechanisms contribute to cell death (figure 3B,C). TRAIL-induced apoptosis, as expected, was completely prevented by

A



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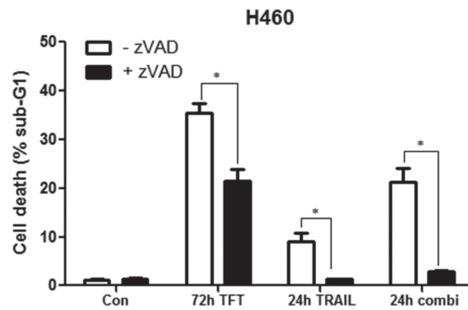

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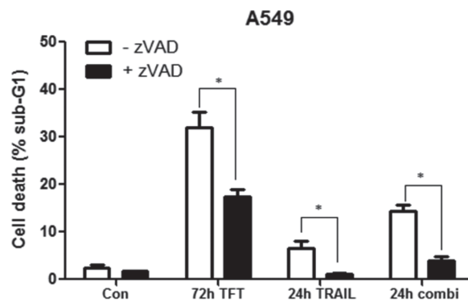


Figure 3. Caspase-dependent and -independent cell death activation. (**A**) TRAIL (10 ng/mL in H460 and 150 ng/mL in A549), TFT (5 μM) and the combination were examined for cell death activation by determining the percentage of sub-G1 cells in PI-stained cells after 24 h incubation with these drugs (*p<0.05, t test compared to TFT and TRAIL alone). (**B**) Comparison of cell death activation in the presence or absence of zVAD-fmk. TFT at 5 μM and TRAIL at 10 ng/mL were used for single and combined treatments in H460 cells. (**C**) Cell death activation in the presence or absence of zVAD-fmk. In A549 cells, 5 μM TFT at 5 and 150 ng/mL TRAIL were used, both single and in the combination treatments. Values are means of three independent experiments ± S.E.M. (*p<0.05, t-test).

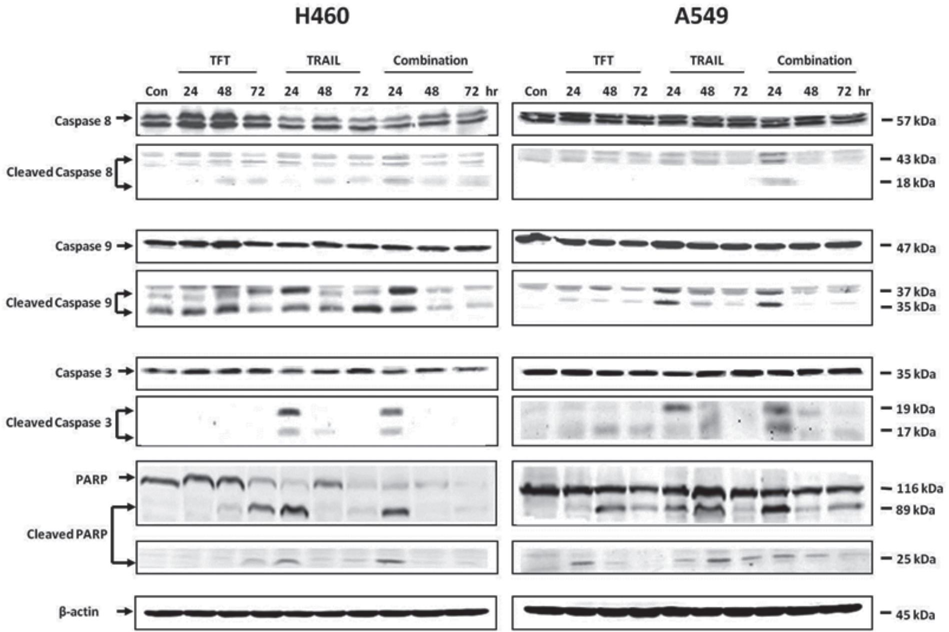
zVAD. In the first 24 h of combined treatment, the induction of cell death was completely inhibited by zVAD further indicating that at this time-point TFT enhanced or sensitized TRAIL-dependent cell death in both H460 and A549 cells.

Caspase and cathepsin B activation by TRAIL and TFT

The effects of TFT and TRAIL on caspase-dependent apoptosis activation were analysed in more detail by western blotting and fluorescent substrate-based activity assays (figure 4A,B). Cleavage of the downstream caspase substrate, PARP, an indicator of apoptotic cell death was also determined. Exposure of H460 and A549 cells to the IC_{50} concentration of TFT hardly resulted in cleavage of any of the tested caspases as indicated by no apparent decrease in the levels of pro-caspases and lack of detectable cleaved forms (figure 4A). This was in line with the unchanged caspase activity and the absence of PARP cleavage (figure 4A,B). In sensitive H460 cells, 24 h exposure to TRAIL induced caspase-8, -9 and -3 cleavage and activation. Resistant A549 cells showed low levels of caspase cleavage and activation, however not sufficient to trigger an apoptotic response. When cells were exposed to combined TRAIL and TFT for 24 h, strong caspase and PARP cleavage was seen in both cell lines in Western blots, although in H460 cells this appeared not stronger than after treatment with TRAIL alone, whereas caspase-3-like activity was enhanced upon combined exposure. In caspase activity assays most notably caspase-3/7-like and caspase-6/8-like activity were enhanced after combination treatment compared to either drug alone. In conclusion, TFT did not trigger effective caspase activation, but it sensitized for TRAIL-induced caspase cleavage in A549 cells and enhanced caspase activity in H460 resulting in strong PARP cleavage and apoptosis. Since TFT-induced cell death was to a large extent caspase-independent a possible role of cathepsin B was investigated. Cathepsin B is expressed as an inactive pro-enzyme (43 kDa), which is cleaved into active cathepsin B (25 kDa or 31 kDa) upon lysosomal activation. TFT exposure induced cathepsin B cleavage at 48 and 72 h post-treatment following schedule A (figure 4C). This cleavage is much stronger in A549 than H460 cells. Furthermore, TRAIL treatment increased cathepsin B cleavage progressively during time in A549 cells, whereas no cleavage was detected in H460 cells. When combining TRAIL and TFT, cathepsin B cleavage was also observed at later time points in A549 cells. In contrast, in H460 cells no cathepsin B cleavage is found after combined treatment. Finally, the effect of the cathepsin B inhibitor, CA074-me, on TRAIL and TFT-induced cell death was examined. Pre-treatment with CA074-me for 4 h had no effect on the level of cell death induction of any treatment, suggesting that cathepsin B was not directly involved in mediating TFT and/ or TRAIL-induced cell death (figure 4D).

Effects of TFT on expression of TRAIL receptors and apoptosis regulatory proteins

The molecular mechanisms underlying the synergistic interaction between TFT and TRAIL were further investigated. First, western blotting showed that TFT increased the levels of



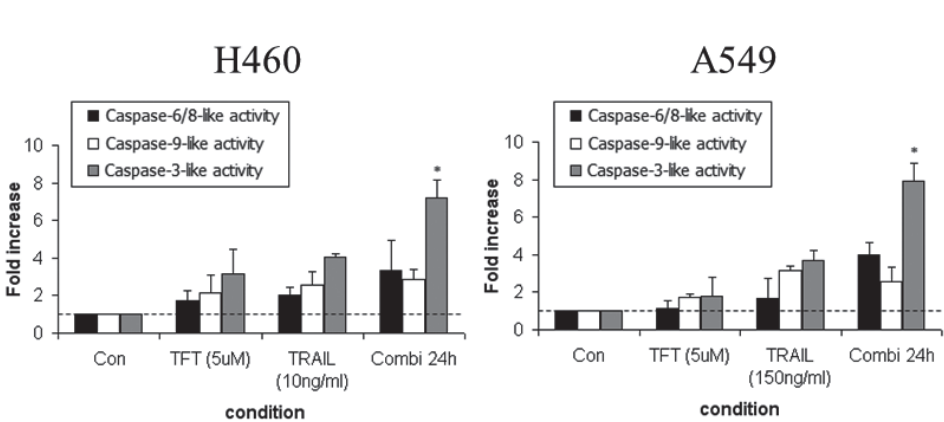
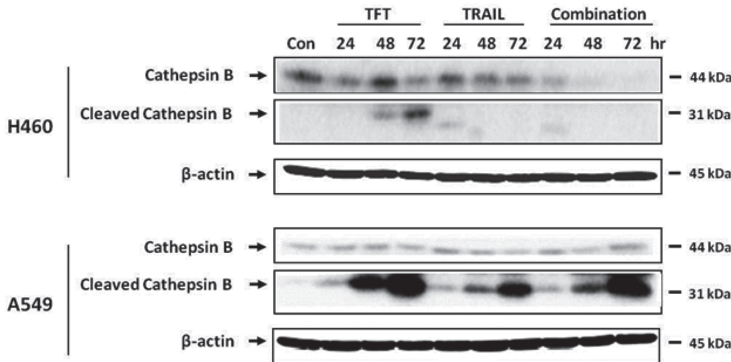


Figure 4. Caspase and cathepsin B activation in TRAIL- and TFT-treated H460 and A549 cells. (**A**) Western blot experiments showing caspase-8, -9, -3 and PARP cleavage. Pro-caspase and cleaved products are indicated. The cells were treated for the indicated time points following schedule A (**B**) Fluorescent substrate-based caspase activity assays following 24 h incubation with TFT, TRAIL or the combination. Fold increase in activity of caspase-3/7, caspase-6/8 and caspase-9 are compared to untreated controls. TFT at 5 μM, 10 ng/mL TRAIL (H460) and 150 ng/mL TRAIL (A549). Values are means of three independent experiments ± S.E.M. (*p<0.05, t-test, comparing the combination with either treatment alone). ▶

p53 within 24 h, which was also associated with p21/ WAF1 up-regulation in A549 and H460 cells (figure 5A). TFT reduced the expression of c-FLIP$_L$ and XIAP was down-regulated in both cell lines. Moreover, TFT increased the expression of pro-apoptotic Bax, whereas the expression of Bcl-2 and Bcl-XL did not alter (not shown). Second, the effect of TFT on the level of expression of the TRAIL receptors was determined by flow cytometry. As

C



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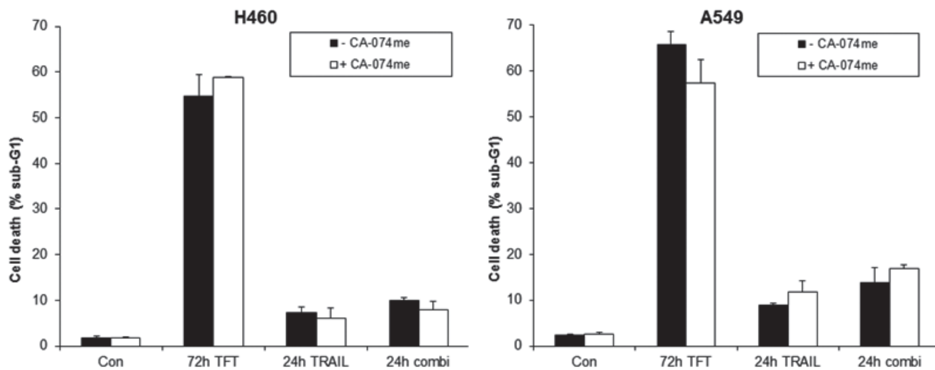

(**C**) Cathepsin B cleavage in H460 and A549 cells after TFT, TRAIL and combination treatment. Western blots showing pro-cathepsin B and cleaved products after exposing cells to 5 μM TFT, 10 ng/mL TRAIL (H460), 150 ng/mL TRAIL (A549) or the combinations for the indicated time points following schedule A. (**D**) The amount of cell death was measured in H460 and A549 cells after treatment with TFT, TRAIL or the combination, with or without the cathepsin B inhibitor, CA-074-me (10 μM). Values are means of three independent experiments ± S.E.M.

depicted in figure 5C, 24 h incubation of TFT increased the cell surface expression of mainly TRAIL-R2 and to a lesser extent TRAIL-R1 in both cell lines. As TFT increased the expression of p53, and it is known that p53 can up-regulate the death receptors [23], we examined p53-dependency of this up-regulation. A siRNA specifically directed against p53 strongly reduced p53 expression in H460 and A549 cells, whereas control siRNA had no effect when compared to untransfected cells (figure 5B). Down-regulation of p53 expression followed by TFT exposure resulted predominantly in reduced TRAIL-R2 up-regulation and only A549 cells (figure 5C).

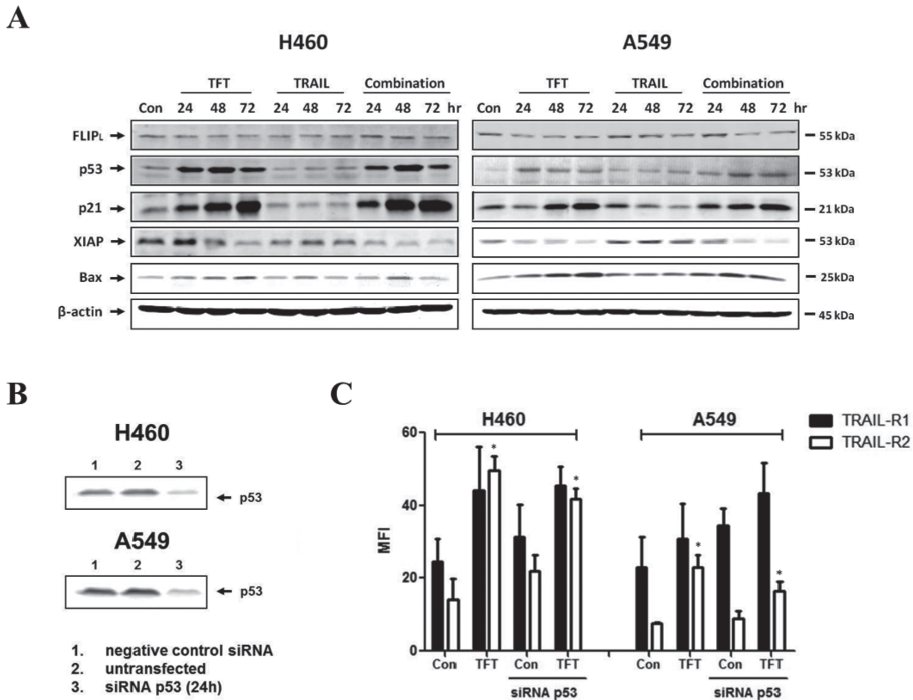

Figure 5. Mechanisms of TFT-dependent TRAIL sensitization. (**A**) Western blots showing the expression of c-FLIP$_L$, p53, p21, XIAP, and Bax after 24, 48 and 72 h exposure to TFT, TRAIL, and the combination following schedule A. Representative blots are shown. (**B**) Cells were transfected with negative control siRNA and p53 specific siRNA and p53 expression was determined by western blotting compared to non-transfected cells. (**C**) Effect of TFT on TRAIL-R1 and -R2 cell surface expression. Cells were exposed to 5 μM TFT for 24 h and TRAIL receptor expression was determined by FACS analysis. P53 was silenced with siRNA, incubating the cells for 24 h prior to TFT treatment. The graph depicts the changes in mean fluorescence intensity (MFI) of the TRAIL receptors compared to IgG1 isotype controls of at least two independent experiments ± S.E.M. (*p<0.05, t-test, comparing with control).

DISCUSSION

Resistance of cancer cells to apoptosis is one of the causes underlying resistance to anti-cancer therapy. Sensitization of cancer cells to apoptosis could therefore be a valuable strategy to define new treatment options for cancer in particular when using agents that aim to directly activate apoptotic pathways [24]. The induction of apoptosis by TRAIL is essentially dependent on the activation of caspases. The present study shows that the novel thymidylate synthase inhibitor TFT by its incorporation into DNA can synergistically enhance TRAIL-induced apoptosis in NSCLC cells. TFT enhanced TRAIL-induced caspase-dependent apoptosis in TRAIL sensitive NSCLC cells and could restore TRAIL sensitivity in resistant NSCLC cells. However, the sequence of administration determined the synergistic effect of combined TFT and TRAIL treatment. Concurrent TRAIL/TFT exposure sensitized for TRAIL dependent apoptosis (schedule A), whereas first TFT exposure followed by TRAIL treatment (schedule B) did not enhance apoptosis and even had antagonistic activity.

We identified a number of molecular mechanisms that are likely accountable for the sensitizing effect of TFT on TRAIL-induced apoptosis. TFT treatment resulted in the up-regulation of TRAIL-R2 and down-regulation of c-FLIP, which is known to increase DISC formation and subsequent caspase-8 activation. This is in line with the observed enhancement of caspase-8 cleavage by TRAIL/TFT. TFT treatment also led to the accumulation of p53 and p21 known to mediate cell cycle arrest and activation of mitochondrial apoptosis. Furthermore, the TFT-mediated increase in Bax expression and a decrease in XIAP will also facilitate the activation of the mitochondrial apoptosis by TRAIL. It is likely that all the above mentioned pro-apoptotic effects of TFT are contributing to the synergistic activity of TFT and TRAIL.

Chemotherapy and radiation are known to cause an enhancement of TRAIL receptor cell surface expression, mostly affecting TRAIL-R2 expression and to a lesser extent TRAIL-R1 [27]. We also found that TFT specifically enhances TRAIL-R2 expression and to a lesser extent that of TRAIL-R1. The TRAIL-R2 gene has transcription factor binding sites for p53 that mediate cytotoxic agent-dependent enhancement of expression [23]. In A549 cells, the up-regulation of TRAIL-R2 by TFT was indeed largely p53-dependent. In H460 cells the TFT-mediated increases in TRAIL receptor expression appeared not to be dependent on p53. Alternative mechanisms of TRAIL receptor up-regulation have been described such as by NF-κB [23].

Chemotherapeutics are known to affect c-FLIP expression amongst others via transcriptional regulation by multiple factors and by posttranslational regulation involving rates of proteasomal degradation [8]. TFT down-regulated c-$FLIP_L$, a property that was also found for the well-studied nucleoside analogue 5-fluorouracil in colon cancer cells [28]. Accordingly we observed stronger caspase-8 cleavage after combined TRAIL/TFT treatment. However, Galligan and co-workers found no effect of 5-fluorouracil on TRAIL

receptor expression independent of p53 status [28], in contrast to our observations with TFT. In another study, an increase of TRAIL receptor expression was reported for another nucleoside analogue, fludarabine, in chronic lymphocytic leukaemia cells [29]. Comparable to TFT, fludarabine has been reported to decrease the expression of XIAP in chronic lymphocytic leukaemia (CLL) cells [30]. Furthermore, the nucleoside analogues, fludarabine, cladribine and clofarabine promote cell death by alterations in MOMP causing cytochrome c release, as a consequence of Bax and Bak translocation to the mitochondria, in a p53-dependent manner [31]. Together this illustrates that available nucleoside analogues have different or overlapping TRAIL sensitizing properties, depending on the tumour type and cellular context. The property of TFT to enhance TRAIL-R2 expression may indicate that combined treatment with TRAIL-R2 agonists may have beneficial effects.

We also explored possible drug interactions at the level of cell cycle progression. TRAIL caused an accumulation of cells in the G1 phase. A small increase in phosphorylated Chk1 after TRAIL exposure may be responsible for this arrest. An increase of p-Chk1 upon TRAIL treatment has not been reported before. However, phosphorylation of Chk2 by TRAIL has been described in colon and cervix carcinoma cells [32]. TRAIL was found to induce a DNA damage response pathway in which also DNA-PK (DNA-dependent protein kinase) and ATM (ataxia telangiectasia) were activated leading to Chk2 phosphorylation. In a similar way this may lead to Chk1 phosphorylation by TRAIL as we observed in this study. Alternatively, TRAIL-induced accumulation of p21 (WAF1), may lead to cell cycle arrest as has been reported previously in human T-cells [33]. However, we did not detect a TRAIL-dependent increase in p21 levels. Combined treatment resulted in similar cell cycle effects compared to TFT alone, indicating that drug interactions at the level of cell cycle regulation and checkpoint-dependent apoptosis activation are not likely to underlie the synergistic effects.

The mechanism of TFT-induced cell death was also further addressed in this study. Since cell death by TFT was largely caspase-independent we examined the possible involvement of the lysosomal protease cathepsin B in cell death activation. Previously, we found TFT as well as 5-fluorouracil to be able to activate cathepsin B in colon cancer cell lines and a variable contribution of cathepsin B cleavage to cell death activation was found [11]. Others have reported that TRAIL can induce apoptotic cell death in oral squamous cell carcinoma cells through activation of cathepsin B [34]. In the current study we also observed cleavage of cathepsin B induced by both TFT and TRAIL, in particular in A549 cells. However, inhibition of cathepsin B by a chemical inhibitor did not affect cell death activation. Thus, the more precise way by which TFT induces cell death in NSCLC cells remains to be identified. A number of alternative cell death mechanisms have been implicated in chemotherapy-induced cell death, such as amongst others necroptosis and autophagy [35;36], and their possible involvement in TFT-induced cell death remains to be explored.

The finding that pre-treatment with TFT followed by TRAIL exposure (schedule B) does not enhance apoptosis and even has antagonistic activity is intriguing, considering that exposure to TFT alone for 24 h increased TRAIL receptor expression, reduced XIAP expression and increased BAX levels. However, although speculative, the activation of caspase-independent cell death mechanisms by TFT following longer exposure times may counteract caspase-dependent apoptosis induced by TRAIL. Regardless of this, the property of TFT to kill NSCLC tumour cells partially via caspase-independent mechanisms may be an advantage compared to other nucleoside analogues with respect to bypassing apoptosis-resistance mechanisms.

TAS-102 (TFT/TPI) is clinically effective in 5-FU resistant colon cancer patients [15]. 5-FU acts predominantly by inhibition of TS. TAS-102, on the other hand, acts not only by inhibition of TS, but mainly by incorporation into the DNA [10]. Hence, TAS-102 is likely to be an alternative for 5-FU in colon cancer. In non-squamous NSCLC the anti-folate TS inhibitor, pemetrexed, is used as first line treatment [25]. By dual targeting, TAS-102 may be an alternative for pemetrexed resistant NSCLC. In NSCLC models, TAS-102 has also shown activity and no cross resistance to other anti-folates was observed [26]. Previously, both TAS-102 (TFT/TPI) and TRAIL receptor agonists have been tested in mouse models and clinical studies in patients and appeared to be well tolerated [37-40]. In addition, also TRAIL receptor agonists have been evaluated in combination with different types of chemotherapy in clinical studies [41;42]. Based on this the possible combined use of TRAIL and TFT seems a realistic approach, although this requires additional testing in animal models. In conclusion, the present study demonstrates synergistic interactions between TFT and TRAIL in NSCLC cells. As TFT is also able to sensitize TRAIL-resistant NSCLC cells, this combination of TFT and TRAIL may offer a potential novel regime for NSCLC treatment.

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