

Effects of TRAIL and taurolidine on apoptosis and proliferation in human rhabdomyosarcoma, leiomyosarcoma and epithelioid cell sarcoma

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Abstract. Soft tissue sarcomas (STS) are a heterogeneous group of malignant tumours representing 1% of all malignancies in adults. Therapy for STS should be individualised and multimodal, but complete surgical resection with clear margins remains the mainstay of therapy. Disseminated soft tissue sarcoma still represents a therapeutic dilemma. Commonly used chemotherapeutic agents such as doxorubicin and ifosfamide have proven to be effective in fewer than 30% in these cases. Therefore, we tested the apoptotic and anti-proliferative *in vitro* effects of TNF-related apoptosis-inducing ligand (TRAIL) and taurolidine (TRD) on rhabdomyosarcoma (A-204), leiomyosarcoma (SK-LMS-1) and epithelioid cell sarcoma (VA-ES-BJ) cell lines. Viability, apoptosis and necrosis were quantified by FACS analysis (propidium iodide/Annexin V staining). Gene expression was analysed by DNA microarrays and the results validated for selected genes by rtPCR. Protein level changes were documented by western blot analysis. Cell proliferation was analysed by BrdU ELISA assay. The single substances TRAIL and TRD significantly induced apoptotic cell death and decreased proliferation in rhabdomyosarcoma and epithelioid cell sarcoma cells. The combined use of TRAIL and TRD resulted in a synergistic apoptotic effect

in all three cell lines, especially in rhabdomyosarcoma cells leaving 18% viable cells after 48 h of incubation ($p < 0.05$). Analysis of the differentially regulated genes revealed that TRD and TRAIL influence apoptotic pathways, including the TNF-receptor associated and the mitochondrial pathway. Microarray analysis revealed remarkable expression changes in a variety of genes, which are involved in different apoptotic pathways and cross talk to other pathways at multiple levels. This *in vitro* study demonstrates that TRAIL and TRD synergise in inducing apoptosis and inhibiting proliferation in different human STS cell lines. Effects on gene expression differ relevantly in the sarcoma entities. These results provide experimental support for *in vivo* trials assessing the effect of TRAIL and TRD in STS and sustain the approach of individualized therapy.

Introduction

Soft tissue sarcomas (STS) are a heterogeneous group of malignant neoplasms. They represent 15% of all malignancies in children and 1% in adults (1). The therapy of choice involves surgical resection with a wide margin of healthy tissue, usually followed by radiation treatment in order to decrease local recurrence (2,3). Unfortunately, about 50% of all patients develop distant metastases and are ineligible for surgical treatment (4,5). In cases of advanced metastatic disease the median survival time from the time of diagnosis with and without chemotherapy treatment is less than 12 months (6,7). Few agents such as doxorubicin, ifosfamide and dacarbazine have proven to be effective in the therapy of soft tissue sarcomas (2). However, the results of these treatments are poor and often exhibit no significant improvements in overall survival (8). Doxorubicin, which has been the most frequently used chemotherapeutic agent in the treatment of soft tissue sarcomas, demonstrates response rates of 20 to 30% in disseminated disease (9,10). The combination of doxorubicin with ifosfamide is more effective, exhibiting slightly higher response rates than doxorubicin alone, but is associated with

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severe short- and long-term toxicities, including bone marrow suppression (11,12). To date, most large trials have not distinguished between histological subtypes of soft tissue sarcomas. One example of such an early trial is the EORTC 62771 trial which was conducted in 1994 and involved 317 patients with several histological types of sarcomas (malignant fibrous histiocytoma, synovial sarcoma, liposarcoma and leiomyosarcoma) (13). Local recurrence was reduced in the chemotherapy arm, but there was no significant benefit in overall survival. The Sarcoma Meta-Analysis Collaboration in 1997 published a quantitative meta-analysis of 1,568 patients with localized resectable soft tissue sarcomas and reported that doxorubicin-based adjuvant chemotherapy significantly improved the time to local and distant recurrence and overall recurrence-free survival. However, there was no significant overall survival benefit at 10 years (14). Unfortunately, most of the large meta-analyses did not differentiate between histological subtypes because of the overall rarity of soft tissue sarcomas. However, differentiation is an important prognostic factor because the soft tissue sarcoma subtypes are differentially sensitive to several agents (2). In recent phase II trials, paclitaxel has proven to be effective in the treatment of angiosarcomas, whereas trabectedin has demonstrated promising activity in leiomyosarcomas and liposarcomas (2,15,16).

Within the scope of this trial, we investigated the effects of TRAIL and taurolidine on three different STS cell lines. Two common subtypes of soft tissue sarcomas, rhabdomyosarcoma (A-204) in children and leiomyosarcoma (SK-LMS-1) in adults (1), and epithelioid cell sarcoma (VA-ES-BJ), which is a rare subtype affecting mostly young patients and has a poor long-term prognosis (17), were examined.

TRAIL and taurolidine are promising combination partners that exhibit synergistic apoptotic effects on a wide range of malignant cells *in vitro*, including carcinoma cells of the oesophagus, pancreas, colon and liver (18-20) as well as fibrosarcoma (21).

Since the discovery of TRAIL, a member of the TNF-superfamily, its apoptosis-inducing effects were documented in several types of malignant cells (22-25). TRAIL binds to its receptors DR4 and DR5 (death receptor 4 and 5) resulting in receptor oligomerization and recruitment of FAS-associated protein with death domain (FADD) and caspase 8, forming a functional death-inducing signalling complex (DISC). Subsequently, DISC leads to the activation of the extrinsic pathway of apoptosis via caspase 8 (26-28). However, associations between TRAIL and the intrinsic mitochondrial pathway have been also described (20). In this pathway, formation of the apoptosome is a key regulatory point following the release of mitochondrial cytochrome *c* and thus leading to apoptosis (29).

Taurolidine (TRD) is an antiseptic agent derived from the amino acid taurine. It has been used to treat peritonitis and catheter-related infections (18). Recently, TRD was used effectively to treat malignant diseases (30-33). In a variety of malignant cell lines including carcinomas of the gastrointestinal tract as well as glioblastoma, fibrosarcoma, prostate and melanoma cancer cell lines, TRD caused the inhibition of proliferation (31,34-36), the inhibition of angiogenesis (30) and the induction of cell death (20,30-32,37-39). The precise mechanism of action is still not clear, but translational inhibition (35) and several pathways of programmed cell death (38) have been implicated. Some groups suggest that the extrinsic pathway (19,20,31,39,40) is involved, whereas others report involvement of the intrinsic

pathway (37,41). Furthermore, first clinical use of TRD was associated with remarkable low toxicity which could be a decided advantage over established chemotherapeutic agents (30,42). TRD was well tolerated after intravenous application in patients with advanced melanoma or glioblastoma (43,44).

Recent *in vitro* studies have revealed that the combination of TRAIL and TRD resulted in sustained cell death, which was superior to single application of TRAIL or TRD. This is despite the use of lower concentrations of both substances in the combination trials (19,20,40). Experimental findings have demonstrated that combined treatment with taurolidine reduces the potential toxic side-effects of TRAIL, not only by reducing the required optimal dose of TRAIL but also by modulating TRAIL's effector pathways without affecting its antitumour efficacy (20). Inspired by these results, we examined the effects of TRAIL and TRD on rhabdomyosarcoma (A-204), leiomyosarcoma (SK-LMS-1) and epithelioid cell sarcoma (VA-ES-BJ) cells.

Materials and methods

Cell lines and cell culture. Three different cell lines were used for this study. The human rhabdomyosarcoma cell line A-204 was purchased from DSMZ (Braunschweig, Germany, DSMZ no. ACC 250) and cultivated in McCoy's 5A with glutamine and 10% fetal bovine serum (FBS). Human leiomyosarcoma cells, SK-LMS-1, were purchased from ATCC (Manassas, USA) and maintained in MEM with Earle's Salts supplemented with 10% FBS, 1% non-essential amino acids, 1 mM sodium pyruvate and 2 mM L-glutamine. The human epithelioid sarcoma cell line VA-ES-BJ also was purchased from DSMZ (DSMZ no. ACC 328) and was cultured in Dulbecco's MEM with sodium pyruvate, supplemented with 20% FBS, 1% non-essential amino acids and 2 mM L-glutamine. All culture media were supplemented with 100 U/ml penicillin and 100 µg/ml streptomycin. The cells were grown to a sub-confluent monolayer and maintained at 37°C and 5% CO₂ in a humidified atmosphere.

Reagents. TRD (Taurolin® 2%, Boehringer Ingelheim, Ingelheim, Germany) containing 5% Povidon was used as supplied by the manufacturer. A 5% Povidon solution (K16 Povidon, generously provided by Geistlich Pharma AG, Wolhusen, Switzerland) was applied in equal volume and served as a control for the TRD group. Recombinant human TRAIL/Apo2L (Bender MedSystems GmbH, Vienna, Austria) was dissolved in distilled water according to the manufacturer's instructions. Distilled water served as a control for TRAIL and was applied in equal volume.

Dose-finding study and application of reagents. To determine the most effective single concentrations and the time dependency of the effects, cells were incubated with various concentrations of TRAIL (50, 100, 250 and 500 ng/ml), TRD (50, 100, 250 and 500 µmol/l) and the respective controls (distilled water or Povidon) for 2, 6, 12, 24 and 48 h. All experiments were repeated with three consecutive cell passages. All cell lines showed highest apoptotic response to 250 ng/ml TRAIL and 250 µmol/l TRD as single substances. These most effective single concentrations of TRD and TRAIL were then used as single substances and in combination to identify a possibly synergistic effect. We chose 2, 6, 12, 24 and 48-h time points.

Flow cytometry analysis. After the defined incubation time, the supernatant with floating cells was collected and the adherent cells were harvested by trypsinisation. These cells were centrifuged and subsequently resuspended with Binding Buffer (Bender MedSystems GmbH) to an absolute cell count of 1×10^5 . Next, the cells were incubated with Annexin V-FITC (BD Biosciences, Heidelberg, Germany) and propidium iodide (PI, Bender MedSystems GmbH) following the manufacturer's instructions. Cells were analysed using a FACS flow cytometer (BD FACSCalibur, BD Biosciences). Cells (20,000) were counted for each measurement. Dot plots and histograms were analysed by CellQuest Pro Software (BD Biosciences). Annexin V binds phosphatidylserine on the outer membranes of cells, and phosphatidylserine becomes exposed on the surfaces of apoptotic cells. Thus, the Annexin V-positive cells were considered apoptotic. PI is an intercalating agent that cannot permeate through the cell membranes of viable or early apoptotic cells. Therefore, PI stains only the DNA of necrotic or very late apoptotic cells. In this study, Annexin V- and PI-positive cells were termed necrotic. Annexin V- and PI-negative cells were counted as viable.

Cell morphology. Morphology of cultured cells was observed and documented using a phase contrast microscope (Zeiss Axiovert 25, Carl Zeiss, Göttingen, Germany).

TUNEL assay. To stain apoptotic cells, we used terminal deoxynucleotidyl transferase-mediated dUTP nick end-labelling consistent with the manufacturer's protocol (In Situ Cell Death Detection Kit, Fluorescein, Roche Applied Science, Mannheim, Germany). Cells were incubated with the appropriate reagents (TRD/TRAIL) for 12 h.

Proliferation assay. To evaluate the proliferation of the cells, we used a colorimetric cell proliferation BrdU-ELISA (Roche Applied Science) according to the manufacturer's instructions. An ELISA-Reader (Sunrise™, Tecan Trading AG, Männedorf, Switzerland) was used to detect the amount of newly synthesised DNA. We plated 10,000 cells per well in a 96-well plate. The incubation time was 6 h.

Statistical analysis. SPSS Version 17.0 for Windows was used for statistical analysis. The results of FACS analysis for percentages of viable, apoptotic and necrotic cells are given as the means \pm SEM of three independent experiments with consecutive passages. In this study, comparisons between experimental groups were performed using one-way analysis of variance (one-way ANOVA) and a post hoc test (Tukey's) over all time points and at singular time points. P-values ≤ 0.05 were considered statistically significant and indicated in the figures as follows: *** $p \leq 0.001$, ** $p \leq 0.005$ and * $p \leq 0.05$.

Oligonucleotide microarray analysis. To identify the changes in gene expression levels caused by the treatment with TRAIL and TRD, total-RNA was purified from the cells after incubation with the appropriate agent for 6 h using a RNeasy kit from Qiagen (Hilden, Germany) as specified by the manufacturer. RNA integrity was assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). For microarray analyses, we applied the methods previously described by Daigeler *et al* (40). We used the Affymetrix GeneChip platform,

employing a standard protocol for sample preparation and microarray hybridisation. A one-way ANOVA model followed by Tukey's HSD (Honestly Significant Difference)-test was used to verify the hypothesis that there were no differences in expression between the drug-treated group and the control group. The multiplicity correction was performed using Benjamini and Hochberg procedure to control the false discovery rate (FDR) at 0.05%. In a pair-wise comparison of the differentially expressed genes between the control and the drug-treated cells identified by the ANOVA analyses, a subset of genes was identified that displayed a conjoint regulation in the treated cells. Genes were placed in this latter group if they exhibited a mean ≥ 2 -fold increase or decrease compared to the control cells. This subset of genes was subjected to the GeneTrail (45) software to identify any over-representation of genes associated with the regulatory pathways that are represented in the Kyoto Encyclopaedia of Genes and Genomes (KEGG) and TRANSPATH databases. Microarray data are deposited in the GEO public database (accession no. GSE36572). These methods fulfilled the MIAME criteria (<http://www.mged.org/miame>).

Real-time PCR for microarray data validation. Microarray data validation was performed for selected candidate genes (BAG5, EGFR, FADD, FYN, GADD45A, HSPA1B/HSP70, NEU1, PPM1D, PPP1R15A/GADD34, PPP1R3D, SIAH1, WEE1). RNA isolation was performed from cells harvested after 6 h of treatment. Total-RNA (2 μ g) was reverse transcribed using the High Capacity cDNA Archive kit (Applied Biosystems, Carlsbad, CA, USA). Real-time PCR was performed with a 7900 HT SDS system (Applied Biosystems) in a 20 μ l reaction volume containing 1X Master Mix, 1 μ l assay and cDNA equivalent to 2 ng total-RNA. All reagents and real-time PCR assays (BAG5 Hs00191644_m1, EGFR Hs01076092_m1, FADD Hs00538709_m1, FYN Hs00941604_m1, GADD45A Hs00169255_m1, HSPA1B Hs01040501_sH, NEU1 Hs00166421_m1, PPM1D Hs01013293_m1, PPP1R15A Hs00169585_m1, PPP1R3D Hs00901540_s1, SIAH1 Hs00361785_m1, WEE1 Hs01119388_m1) were purchased from Applied Biosystems. Reactions were performed in duplicate and analysed by the $\Delta\Delta$ Ct method. Human GAPD was used for normalisation.

Western blot analysis. Western blot analyses were performed to validate the effects of alterations in gene expression on protein levels using an SDS-PAGE gel and the following antibodies (except BAG5, which was purchased from Abcam PLC, Cambridge, UK); all other antibodies were purchased from Santa Cruz Biotechnology Inc. (Heidelberg, Germany): BAG5 (mouse, ab56738), EGFR (rabbit, 1005), FADD (rabbit, H-181), GADD34/PPP1R15A (rabbit, S-20), GADD45 α (rabbit, H-165), HSP70 (mouse, C92F3A-5) and Wee1 (rabbit, C-20). Western blot analyses were not performed for PPP1R15A, FYN and PPP1R3D because functional antibodies were not available. Total protein was purified from the cells after incubation with the appropriate substances for 8 h. Floating cells were collected together with the supernatant; adherent cells were harvested using a cell scraper and added to the solution. Cells were gathered by centrifugation. After removal of the supernatant, pellets were incubated with 100 μ l Cell Culture Lysis Reagent (Promega Corporation, Mannheim, Germany) each for 1 h on

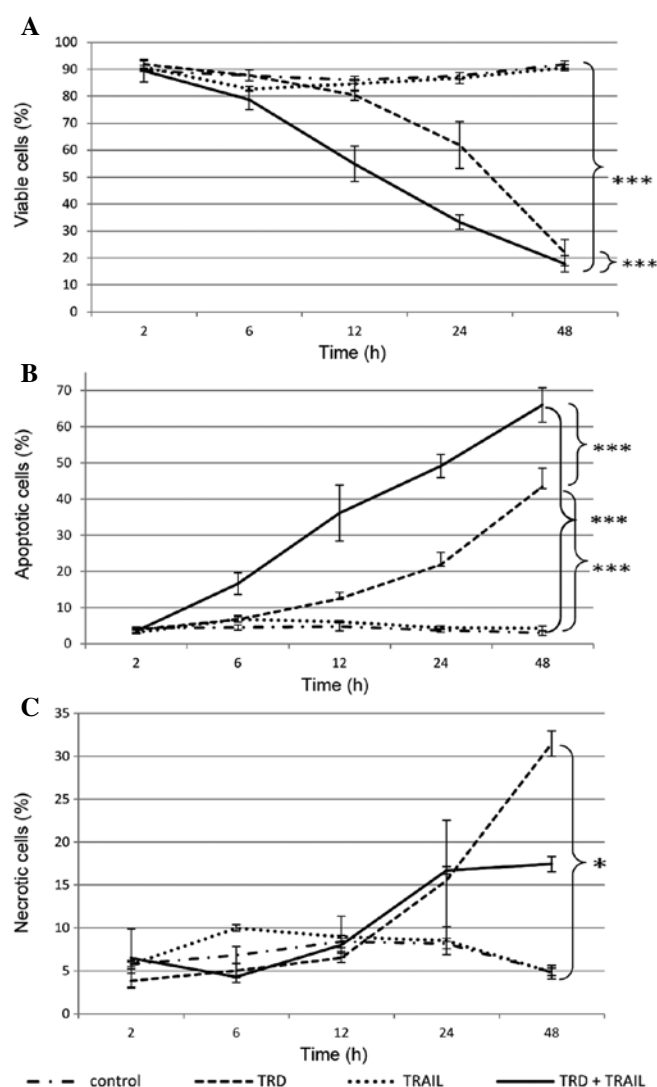


Figure 1. Effects of TRAIL, TRD and combination of both reagents on rhabdomyosarcoma (A-204) cells. A-204 cells were incubated for 2-48 h with TRAIL, TRD and combination of both reagents. The percentages of (A) viable, (B) apoptotic and (C) necrotic cells were determined using fluorescence activated cell sorting (FACS) analysis for Annexin V-FITC and propidium iodide. The values indicate the means \pm SD of three independent experiments and consecutive passages. Significantly different at *** $p \leq 0.001$, ** $p \leq 0.005$, * $p < 0.05$; one-way ANOVA.

ice. The cell remnants were then separated by centrifugation, and the supernatant containing the purified protein was frozen at -80°C until further use.

Results

The combination of TRAIL and TRD amplifies apoptotic effects in A-204 human rhabdomyosarcoma and VA-ES-BJ human epithelioid sarcoma cells. Single application of TRD induced significantly apoptotic and necrotic cell death in A-204 human rhabdomyosarcoma cells. Viable cells were decreased to 22.0% after 48 h of incubation compared to 91.9% in the control and 90.6% in the TRAIL group ($p < 0.001$) (Fig. 1). Addition of TRAIL promoted the apoptotic influence of TRD in rhabdomyosarcoma cells. The combined treatment of TRD and TRAIL resulted in a marked increase in cells undergoing

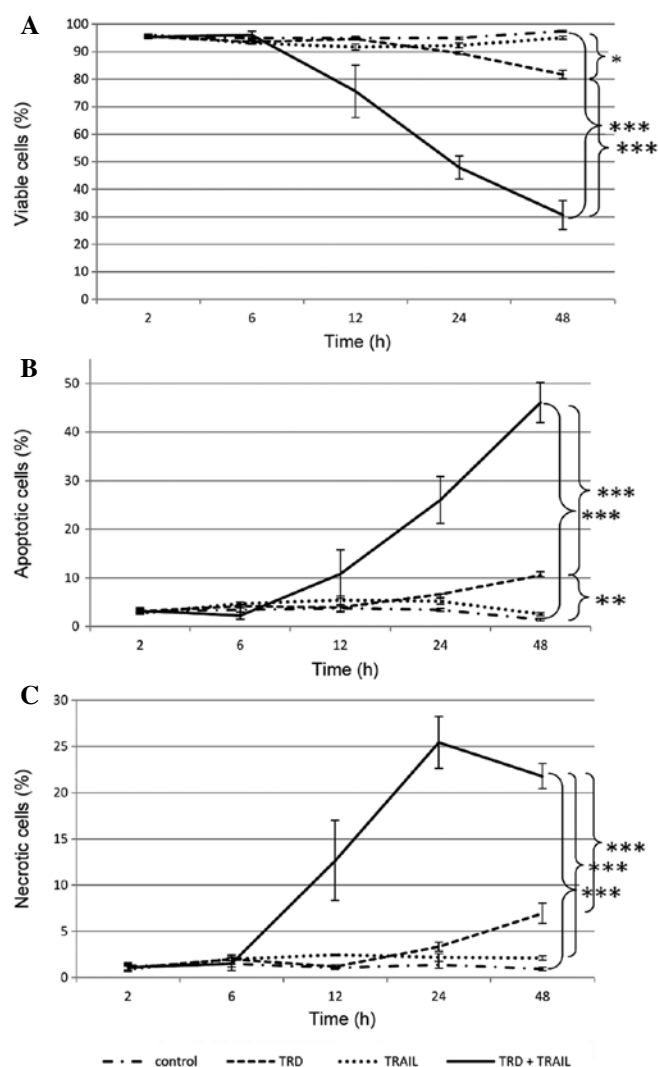


Figure 2. Effects of TRAIL, TRD and combination of both reagents on human epithelioid sarcoma (VA-ES-BJ) cells. VA-ES-BJ cells were incubated for 2-48 h with TRAIL, TRD and combination of both reagents. The percentages of (A) viable, (B) apoptotic and (C) necrotic cells were determined using fluorescence activated cell sorting (FACS) analysis for Annexin V-FITC and propidium iodide. The values indicate the means \pm SD of three independent experiments and consecutive passages. Significantly different at *** $p \leq 0.001$, ** $p \leq 0.005$, * $p < 0.05$; one-way ANOVA.

apoptosis over all time points ($p \leq 0.001$). After 6 h of incubation first apoptotic effects were seen. The combination with TRAIL and TRD exhibited highest apoptosis rates after 48 h with 66.1% apoptotic cells (3.0% in control group, $p \leq 0.001$). Thus, combination treatment was most effective in reducing cell viability with 17.9% remaining viable cells after 48 h (vs. 91.9% in the control group, $p < 0.001$) (Fig. 1A).

The viability of the VA-ES-BJ human epithelioid sarcoma cells was moderately but significantly reduced by single treatment with TRD (Fig. 2). A total of 81.8% of the cells was detected as viable after 48 h treatment with TRD (vs. 97.5% in the control group, $p = 0.015$). Single application of TRAIL had no significant effect on cell viability. The combined treatment with TRAIL and TRD led to a significant increased apoptotic cell death after 12 h. The population of viable cells

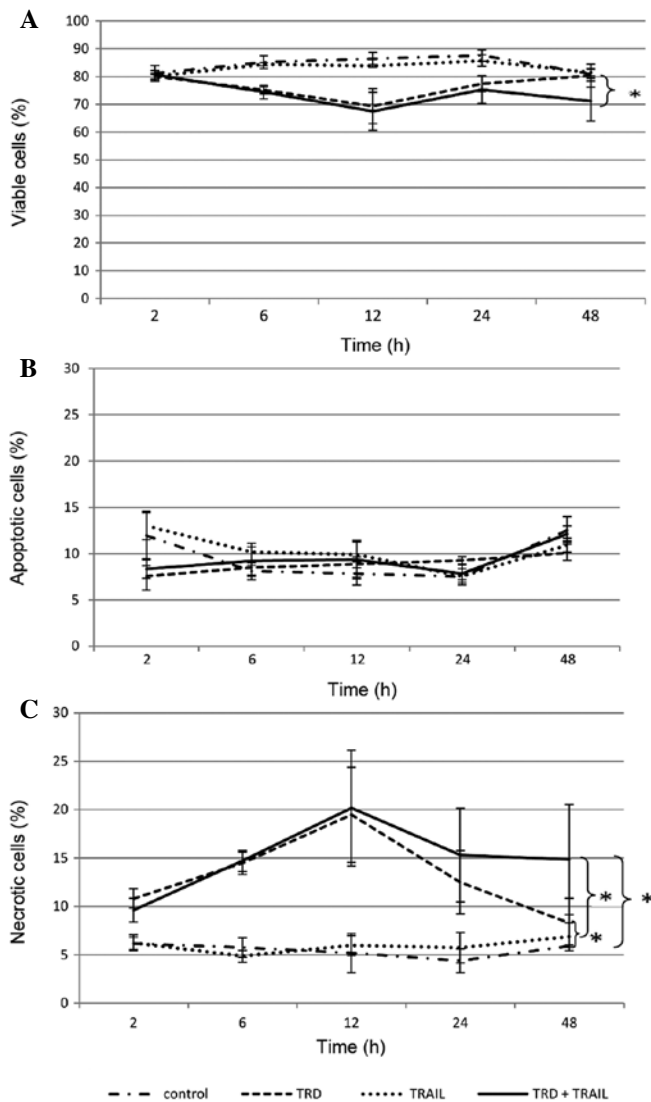


Figure 3. Effects of TRAIL, TRD and combination of both reagents on leiomyosarcoma (SK-LMS-1) cells. SK-LMS-1 cells were incubated for 2-48 h with TRAIL, TRD and combination of both reagents. The percentages of (A) viable, (B) apoptotic and (C) necrotic cells were determined using fluorescence activated cell sorting (FACS) analysis for Annexin V-FITC and propidium iodide. The values indicate the means \pm SD of three independent experiments and consecutive passages. Significantly different at *** $p \leq 0.001$, ** $p \leq 0.005$, * $p < 0.05$; one-way ANOVA.

was reduced to 30.7% after 48 h of incubation whereas 97.5% were left viable in the control group ($p < 0.001$). Apoptosis also peaked at this time point, reaching a maximum of 46.1% in the combination group compared to only 1.5% in the control group ($p < 0.001$) (Fig. 2A).

Neither single application nor combination treatment with TRAIL and TRD affected viability of SK-LMS-1 leiomyosarcoma cells significantly. The effects when treating SK-LMS-1 leiomyosarcoma cells with TRD and TRAIL were only moderate (Fig. 3). The combined application of TRAIL and TRD led to a slight decrease of viable cells to 67.5% after 12 h (vs. 86.5% in the control group, $p = 0.088$). Single application of TRD had a similar effect on SK-LMS-1 cells (69.4% viable

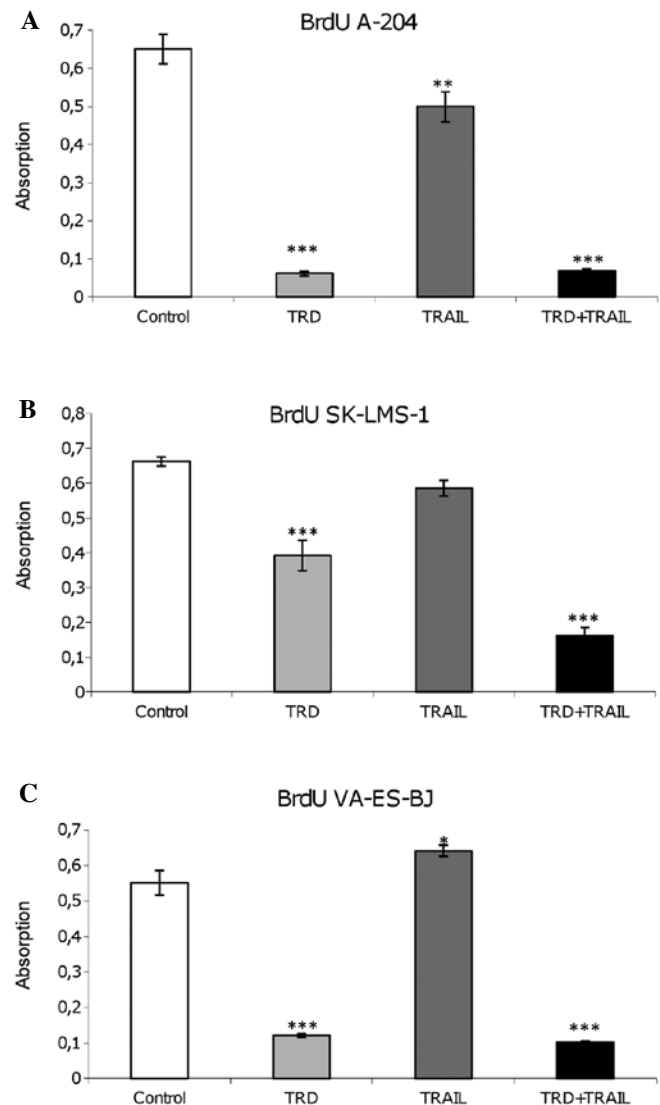


Figure 4. Bromodeoxyuridine (BrdU) proliferation assay. The effects of the indicated compounds on proliferation in (A) A-204, (B) SK-LMS-1 and (C) VA-ES-BJ cells were measured by BrdU cell proliferation assay. The assay was performed following 6 h of incubation with sodium pyruvate (control), 250 $\mu\text{mol/l}$ TRD, 250 ng/ml TRAIL or with a combination of TRD and TRAIL. Data are presented as the mean \pm SD of 8 measurements. ANOVA was used to demonstrate statistical significance between the different categories with a Tukey's multiple comparison post hoc test. Significantly different at *** $p \leq 0.001$, ** $p \leq 0.005$, * $p < 0.05$; ns, $p > 0.05$). The indicators of significance refer to the difference between the treatment groups and the control series.

cells after 12 h). Neither single application nor combination treatment with TRAIL and TRD had a significant influence on apoptosis.

TRD significantly inhibited proliferation of A-204 human rhabdomyosarcoma, VA-ES-BJ human epithelioid sarcoma and SK-LMS-1 leiomyosarcoma cells. TRD was able to inhibit cell proliferation in all examined cell lines ($p < 0.001$) as indicated by the BrdU-Assay (Fig. 4). In SK-LMS-1 cells, combination with TRAIL resulted in a stronger effect compared to incubation with TRD alone ($p < 0.001$). For the other cell lines, combination therapy did not increase the inhibition of proliferation. Administration of TRAIL as a single

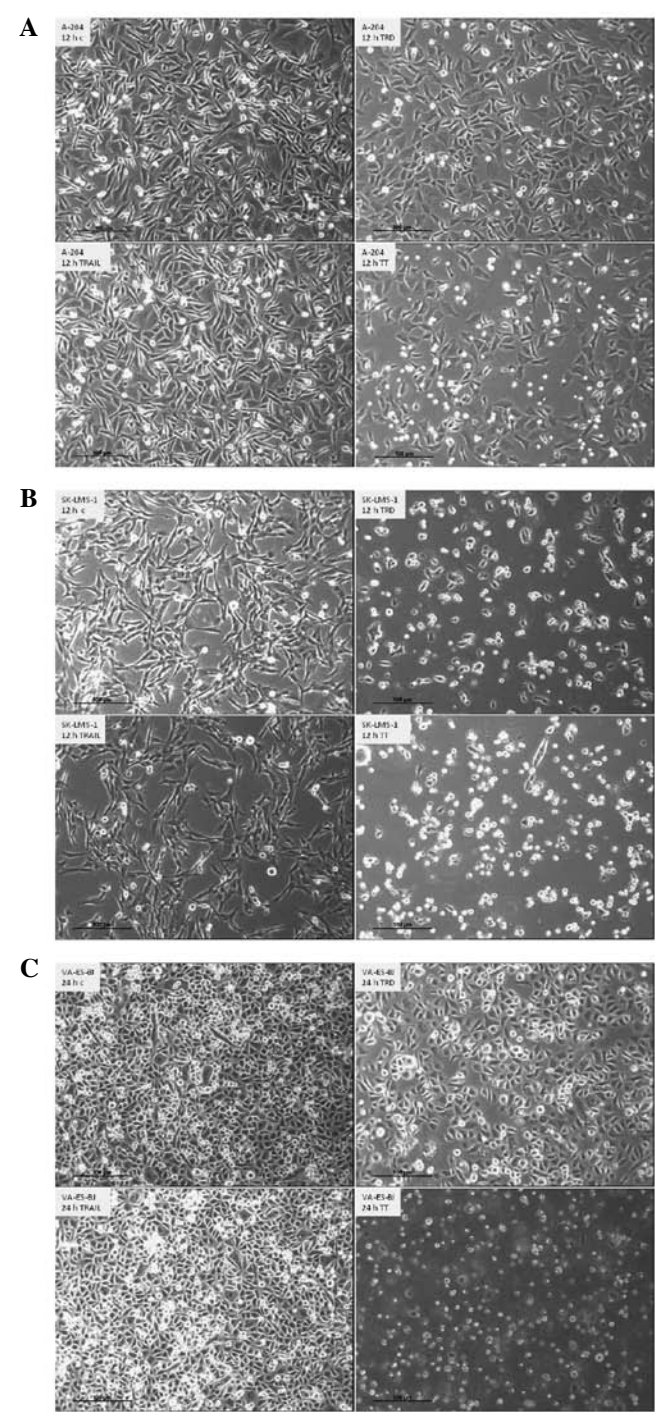


Figure 5. Morphological changes after treatment. Representative microscopic photographs showing morphological changes in (A) A-204, (B) SK-LMS-1 and (C) VA-ES-BJ cells induced by TRD, TRAIL and combination of both after 12 and 24 h, respectively.

agent reduced proliferation significantly only in A-204 cells. Strikingly, proliferation was increased in VA-ES-BJ cells after single application of TRAIL.

The addition of TRD induced morphological changes and cell detachment. As demonstrated in Fig. 5, addition of TRD resulted in morphological changes in all cell lines. TRD led to shrinkage of cells and dissolution of confluent cells groups. Longer incubation with TRD resulted in marked cell detachment.

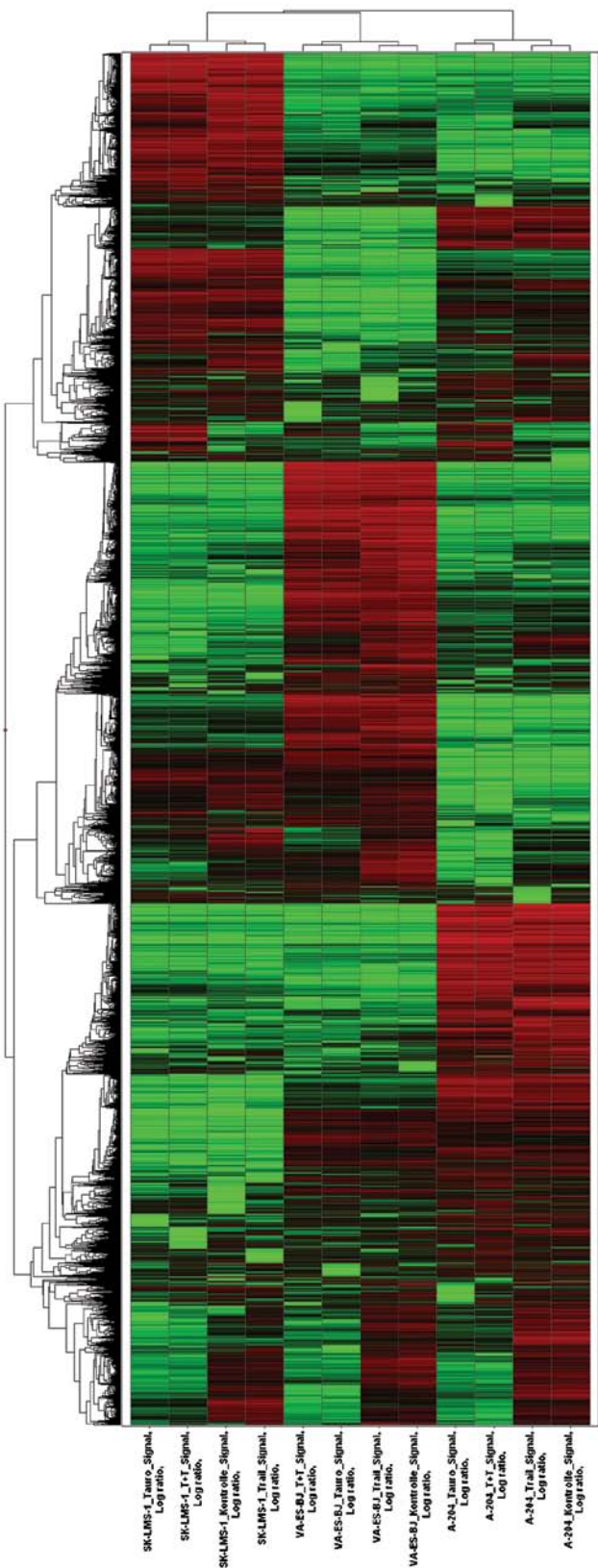


Figure 6. Hierarchical clustering: overall gene expression pattern of reliably measured probesets (24797). Horizontal rows represent individual probe sets/genes; vertical columns represent individual samples. Kontrolle, control; Taurolidine, TRD; Trail, TRAIL; T+T, combination treatment. Color scale: black, mean (indicates unchanged expression), brightest green, 0.25 x mean (indicates expression level below mean), brightest red, 4 x mean (indicates higher expression level than mean). The dendrogram at the top of the matrix indicates the degree of similarity between examined groups; the dendrogram at the left side indicates the degree of similarity among the selected genes according to their expression patterns.

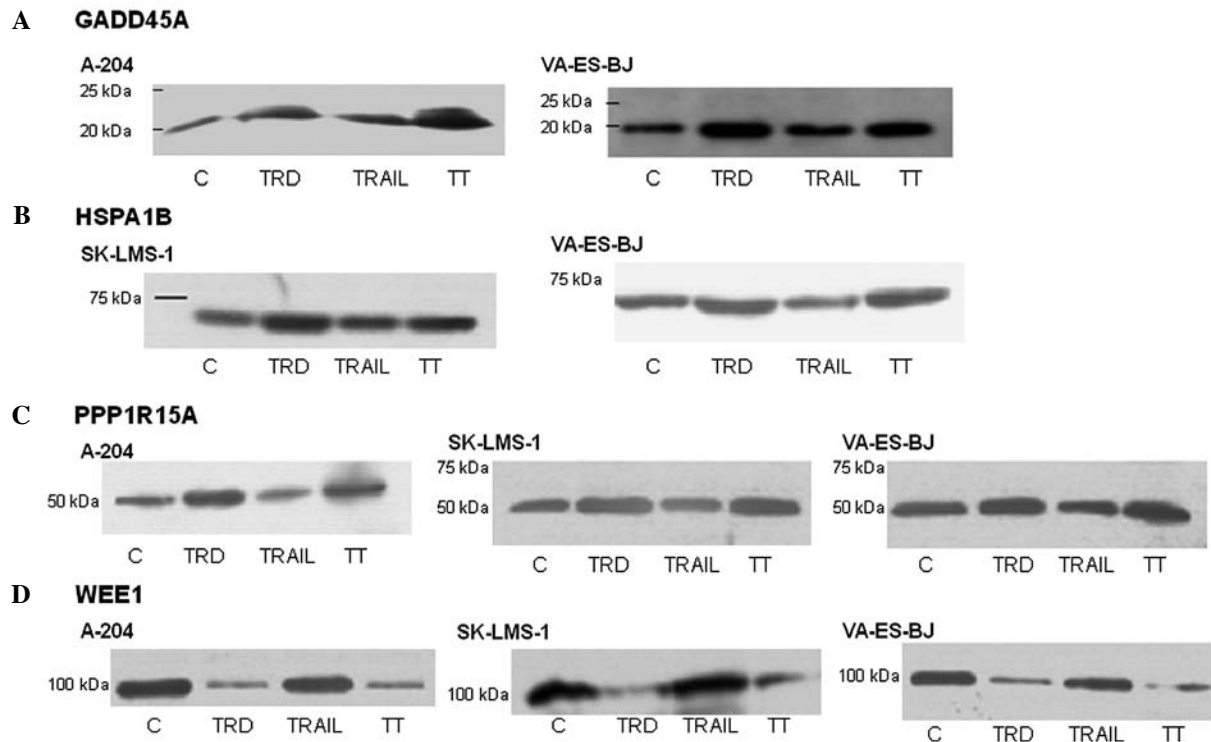


Figure 7. Selected western blot analysis results for candidate proteins. Protein isolation was performed after an incubation time of 8 h with the respective substances. Antibodies used: GADD34/PPP1R15A (rabbit, S-20), GADD45 α (rabbit, H-165), HSP70 (mouse, C92F3A-5) and Wee1 (rabbit, C-20). C, control; TRD, 250 μ mol/l taurilidine; TRAIL, 250 ng/ml TRAIL; TT, 250 μ mol/l TRD and 250 ng/ml TRAIL.

Table I. Number of altered genes with at least a ≥ 2 -fold change in gene expression.

	TRD vs. control	TRAIL vs. control	TT vs. control	TT vs. TRD	TT vs. TRAIL
A-204	128	10	113	19	125
SK-LMS-1	116	22	122	2	118
VA-ES-BJ	102	35	117	7	97
All three cell lines	56	3	53	1	49

TT, TRAIL and TRD.

Microarray analysis revealed differential gene expression patterns in all examined cell lines after the treatment with TRAIL and TRD. With respect to the overall gene expression patterns, there were differences between the individual cell lines (Fig. 6). In our gene expression study, we focused on apoptosis-related genes. For all three cell lines, we tested 621 probe sets as previously described by Daigeler *et al.* (20,40), which corresponded to 349 genes. TRAIL caused only a few differences in the expression of the analysed genes compared to the control, whereas TRD alone and in combination with TRAIL led to expression changes in a wide range of apoptosis-related genes. The number of altered genes with at least a ≥ 2 -fold change is shown in Table I.

Additional evaluations with real-time PCR for selected candidate genes yielded consistent results regarding changes in expression of several genes. A consistent increase of expression in all three cell lines was observed after TRAIL and TRD treatment for GADD45A (growth arrest and DNA damage A),

PPP1R15A (protein phosphatase 1, regulatory subunit 15A) and HSPA1B (heat shock 70 kDa protein 1B) (Table II). Further, microarray analysis revealed a downregulation of Wee1 (protein kinase wee1), FADD (Fas-associated protein with death domain), Fyn (proto-oncogene tyrosine-protein kinase Fyn) and PPM1D (protein phosphatase 1D) in several treatment groups.

Western blot analyses demonstrated consistent results for gene expression and protein levels for GADD45A, HSPA1B, PPP1R15A and WEE1. For GADD45A, the results of the western blot analyses corresponded to the gene expression changes in A-204 and VA-ES-BJ cells. Specifically, combination treatment with TRD and TRAIL caused increased protein expression compared to control and treatment with TRAIL (Fig. 7A). HSPA1B was upregulated in TRD-treated SK-LMS-1 and VA-ES-BJ cells corresponding to the results of the microarray and PCR analyses (Fig. 7B). Analogous to the

Table II. Summary of the microarray and rtPCR data of the selected candidate genes.

Gene symbol/ cell line	Signal log ratio control vs. TT		
	GADD45A	PPP1R15A	HSPA1B
Microarray data			
A-204	1.77	2.03	0.68
SK-LMS-1	1.77	1.96	2.13
VA-ES-BJ	1.19	2.17	1.04
rtPCR data			
A-204	2.05	1.81	0.82
SK-LMS-1	1.74	1.36	1.69
VA-ES-BJ	1.89	2.24	1.57
TT, TRD plus TRAIL.			

gene expression levels, protein expression of PPP1R15A was enhanced by treatment with the different agents in all cell lines, in which combination treatment led to the highest protein levels (Fig. 7C). In all treated cell lines, Wee1 was downregulated in the microarray analyses and in the protein analyses after treatment with TRD (Fig. 7D).

Discussion

Since the discovery of TRAIL as a cell death-inducing member of the TNF-superfamily, its effects on apoptosis have been demonstrated for several malignancies, including soft tissue sarcomas (46,47). In order to overcome TRAIL-resistance or enhance its apoptotic activity there is an increasing interest to find suitable combination partners for TRAIL (48-50). Recent trails unveiled the anti-neoplastic qualities of TRD (31,36,38,51). In this study, we chose to combine TRAIL with TRD to treat three different soft tissue sarcoma cell lines. In our study the combination of TRAIL and TRD induced apoptotic cell death in A-204 rhabdomyosarcoma and VA-ES-BJ epithelioid sarcoma cells but not in SK-LMS-1 leiomyosarcoma cells. For both treated cell lines, the apoptotic index could be increased significantly by addition of TRAIL compared to treatment with TRD alone, suggesting that TRD and TRAIL work synergistically in rhabdomyosarcoma and epithelioid sarcoma cell lines beyond the mere additive effect. Earlier trials also revealed synergistic effects of TRAIL and the chemotherapeutic agent melphalan in the rhabdomyosarcoma cell line Te-671 (52). Likewise, the combined application of TRAIL and doxorubicin was shown to be more effective than single treatment of TRAIL in several rhabdomyosarcoma cell lines (53). To our knowledge, there are no trials evaluating TRAIL in epithelioid sarcomas. Merely paclitaxel and 5-FU are known to induce apoptosis in different epithelioid sarcoma cell lines (54).

TRD inhibited proliferation in all tested sarcoma cell lines. Strikingly, the anti-proliferative effect of TRD was enhanced significantly in SK-LMS-1 leiomyosarcoma cells by addition of TRAIL. Meanwhile, recent studies revealed TRD-induced proliferation inhibition in a wide range of malignant cell lines,

including HT1080 human fibrosarcoma cells (30,34,35,40). Remarkably, proliferation inhibition was accompanied by a disruption of cell adherence and cytoskeleton which play a crucial role in tumour growth, metastasis and development. However, the exact mechanism of TRD-induced proliferation inhibition is still unknown and the appealing hypothesis that TRD inhibits cell proliferation by disruption of cell adhesion and cytoskeleton requires further experimental support.

RNA-microarray technology showed high correlations between apoptotic efficacy and upregulation of GADD45A (growth arrest and DNA damage-inducible protein 45), PPP1R15A (protein phosphatase 1 regulatory subunit 15A, synonym: GADD34) and HSPA1B (heat shock 70 kDa protein 1B).

GADD45A and PPP1R15A were upregulated with at least a ≥ 2 -fold change in all three cell lines after single treatment with TRD and combined treatment with TRD and TRAIL compared to untreated control. Gene alteration could be confirmed by quantitative real-time PCR and western blot analysis.

GADD45 proteins co-operate in the activation of S and G2-M checkpoints following the exposure of cells to UV irradiation and other genotoxic stresses, thereby inducing growth arrest and apoptosis (55). The mechanisms by which GADD45 proteins function in negative growth control is not fully understood, although upregulation of these proteins was reported to be associated with increased apoptosis and p53-independent cell cycle arrest in a variety of soft tissue sarcomas (56). In a recent immunohistochemical study, high expression of GADD45 was associated with reduced invasiveness of chondrosarcomas, suggesting its potential diagnostic value in the histological grading of malignant chondrogenic tumours (57). However, the increased expression of GADD45B in our experiments suggests a potential involvement of GADD45B in TRD-mediated cell death in soft tissue sarcoma cells and has to be addressed in further studies.

Stressful growth conditions and exposure to DNA damaging agents lead to an upregulation of PPP1R15A in a wide range of human cell lines (58,59). High expression of PPP1R15A is known to promote apoptotic cell death in a p53-independent manner (59-61). A recent *in vitro* study detected enhanced gene expression of PPP1R15A during TRD-induced cell death in different malignant cell lines including human fibrosarcoma cells (62). Thus, PPP1R15A should be considered as a potential target of TRD in cancer cells.

Combination treatment with TRAIL and TRD enhanced HSPA1B protein level in SK-LMS-1 leiomyosarcoma and VA-ES-BJ epithelioid cell sarcoma cells. HSPA1B protein belongs to the heat shock protein family with a molecular weight of 70 kDa which acts as an important regulator of cell growth and survival in a wide range of cancer cells (63-65). These proteins are known to affect the intrinsic pathway by the inhibition of mitochondrial cytochrome *c* release and also by preventing apoptosome assembly (29). Upregulation of heat-shock proteins in different malignant cell lines was associated with an enhanced resistance towards hypoxia-induced apoptosis (66). More particularly, heat-shock 70 kDa proteins protected nucleus integrity in non-small cell lung carcinoma cells which were subjected to heat shock (67). In

patients with urothelial carcinoma high levels of HSPA1B protein were associated with early tumour progression and invasion (68). Furthermore, recent *in vitro* studies suggested that heat-shock 70 kDa proteins might be responsible for chemoresistance in different malignant cell lines, and increased levels were found in 5-fluorouracil-resistant colon carcinoma cells suggesting its involvement in colon cancer chemoresistance (69), whereas inhibition of heat shock 70 kDa protein helped to overcome resistance to etoposide and 5-fluorouracil in oral squamous carcinoma cells (70). Moreover, TNF- and TRAIL-induced apoptosis could be suppressed by heat-shock 70 kDa in many different cell types (71). We could not be sure which of the manifold effects of heat-shock proteins are relevant in sarcoma cell death. Their upregulation might only be a response to TRAIL- and TRD-induced cell stress. However, heat-shock proteins are essential to the survival of many cell types and might be one of the tools in chemoresistance in leiomyosarcoma or epithelioid cell sarcoma cells.

Microarray analysis revealed a decrease of FADD with a ≥ 2 -fold change in all three cell lines after combination treatment. FADD participates in death signalling in the extrinsic apoptotic pathway and can be recruited by several death receptors, including TRAIL-receptor. Subsequently this interaction leads to recruitment of caspase 8 and initiation of apoptosis. The role of FADD in TRAIL signalling is controversial (73). Some studies showed that the absence of FADD leads to partial TRAIL-resistance and concluded that FADD is necessary for TRAIL-induced apoptosis by the death receptors DR4 and DR5 (74,75). Other groups showed that the induction of apoptosis by TRAIL is independent of FADD in different cell lines (73,76,77). Additionally, FADD was described as a negative regulator of the transcription factor NF- κ B (nuclear factor κ -light chain-enhancer of activated B-cells), which promotes cell survival and tumour invasiveness of fibrosarcoma cells (78,79). In our study, we observed consistent downregulation of FADD mRNA in all cell lines and protein levels in the SK-LMS-1 cells. Repetitive western blot analyses in A-204 and VA-ES-BJ did not show significant changes in protein expression. Therefore, a meaningful interpretation whether the downregulation of FADD plays a role in activation of apoptosis in the tested soft tissue sarcoma cells is not possible based on our data.

Expression of the PPM1D gene was downregulated with a ≥ 2 -fold change in all three sarcoma cell lines. PPM1D, also known as Wip1, is a member of the nuclear type 2C protein phosphatase family and is known to be a negative regulator of cell stress response pathways (80). Previous studies have shown that PPM1D expression and phosphatase activity are required for the survival and progression of breast and ovarian carcinoma cells (81-83). Loss of PPM1D gene function sensitises mouse embryonic fibroblasts to stress- and DNA damage-induced apoptosis (84). PPM1D overexpression has been observed in neuroblastomas, medulloblastomas, pancreatic adenocarcinomas and gastric carcinomas (85-89). Since there has been evidence that PPM1D acts as an oncogene, efforts were made to find selective inhibitors of PPM1D. As expected, tumour cell lines that overexpress PPM1D have shown to be more sensitive to PPM1D inhibition and consecutive apoptosis than cell lines with normal levels (90). These

findings point to PPM1D as a regulator in tumour cell survival.

The cell cycle gene Wee1 was downregulated in all 3 cell lines after single treatment with TRD as well as combined treatment with TRD and TRAIL. Though PCR measurements were not performed to confirm gene expression, microarray analyses and western blot analyses results were consistent for all cell lines. The Wee1 protein kinase functions as key regulator of the G2/M-checkpoint and stabilizes the genome in the S phase (91). Overexpression of Wee1 has previously been reported in osteosarcoma, glioblastoma, breast cancer and malignant melanomas (92-95). Recent studies identified Wee1 as a potential molecular target in cancer cells and the selective small molecule Wee1-inhibitor MK-1775 demonstrated promising results in cancer cells with enhanced levels of Wee1 (96-98). However, MK-1775 has recently been included in a phase I clinical trial in patients with advanced solid tumours (95,99). In a present study, MK-1775 caused significantly apoptotic cell death in various sarcoma cell lines and patient-derived tumour explants *ex vivo* suggesting that Wee1 may represent a new potential target in the treatment of sarcomas (100).

Combined treatment with TRD and TRAIL led to an upregulation of the Fyn gene primarily in A-204 and VA-ES-BJ cells. Fyn belongs to the Src family of kinases and is involved in a variety of signalling pathways. It is particularly upregulated in prostate cancer cells and may have a pivotal role in cancer progression and metastasis (101). Furthermore, high levels of Fyn activity were correlated with a higher metastatic ability of human pancreatic carcinoma and murine fibrosarcoma cells (102,103). However, these findings would indicate an enhanced cancer activity with subsequent disease aggravation induced by TRAIL and TRD and require further investigations.

Taken together, all results described above arose from *in vitro* tests. To make more concrete conclusions, further *in vivo* studies are necessary to specify programmed cell death following TRD and TRAIL treatment in soft tissue sarcomas and the results should be validated with primary cultures. Finally, gene expression and cell viability differed remarkably in all three analysed sarcoma entities after exposure to TRD and TRAIL pointing out the pivotal cellular differences among the soft tissue sarcoma subtypes. Unfortunately, most of the large clinical trials did not differentiate between histological subtypes because of the overall rarity of soft tissue sarcomas resulting in generalized pharmaceutical references and therapy. However, our findings sustain the approach of individualized therapy and investigation. Future trials as well as clinical therapy should focus on histological subtypes and be more individualized in spite of the rarity and difficulties.

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