TRAIL and Taurolidine Enhance the Anticancer Activity of Doxorubicin, Trabectedin and Mafosfamide in HT1080 Human Fibrosarcoma Cells

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Abstract. Background: Disseminated fibrosarcoma still represents a therapeutic dilemma because of lack of effective cytostatics. Therefore we tested tumor necrosis factor related apoptosis-inducing ligand (TRAIL) and taurolidine, in combination with established and new chemotherapeutic agents on human fibrosarcoma (HT1080). Materials and Methods: Human fibrosarcoma cells (HT1080) were incubated with doxorubicin, mafosfamide and trabectedin both alone and in combination with taurolidine and TRAIL. Vital, apoptotic and necrotic cells were quantified using flow cytometric analysis. Cell proliferation was analysed using a bromodeoxyuridine (BrdU) ELISA assay. Results: Single application of doxorubicin and trabectedin induced apoptotic cell death and significantly reduced the proliferation of HT1080 cells. In combination treatment, the addition of taurolidine and TRAIL resulted in a stronger reduction in the degree of cell viability when compared to single treatment. Trabectedin and taurolidine displayed a greater potential for inhibiting proliferation than did doxorubicin alone. Conclusion: When combined with TRAIL and taurolidine, treatment with doxorubicin and trabectedin demonstrated stronger apoptosis-inducing and antiproliferative effects.

Fibrosarcomas are rare soft tissue sarcomas originating from the intra- and intermuscular fibrous tissues, fascia and tendons. They account for approximately 3% of all soft tissue sarcomas, which themselves represent about 1% of all new cancer cases in Europe and the United States (1-3). The therapy of choice involves surgical resection including a wide margin of healthy tissue. The role of chemotherapy in the management of fibrosarcomas remains controversial. Small retrospective analyses of all histological types of soft tissue sarcomas support the use of neoadjuvant therapy. To obtain more definitive results, these data need to be validated by randomized prospective studies restricted to single defined subtypes of cancer (4). A quantitative meta-analysis of data from 1,568 individual patients with localized resectable soft tissue sarcomas reported that doxorubicin-based adjuvant chemotherapy significantly improved the time to local and distant recurrence as well as the overall recurrence-free survival. However, there was no significant overall survival benefit at 10 years (5). Approximately 25% of all patients with soft tissue sarcoma in the extremities develop distant metastases, for which chemotherapy may be appropriate (6, 7). Chemotherapy is commonly used at many centres as part of the treatment plan for patients with metastatic or unresectable soft tissue sarcomas. 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 patients with disseminated disease (9, 10). The combination of doxorubicin with ifosfamide is more effective, exhibiting slightly higher response rates than when doxorubicin is used alone. This combination, however, is associated with severe short- and long-term toxicities, including bone marrow suppression (11, 12). Radiation treatment can reduce local...
recurrence but cannot improve overall survival or reduce the incidence of distant metastases (13). Thus, the optimal treatment, especially for metastatic or unresectable fibrosarcoma, has not been defined. The purpose of the present study was to determine the cytotoxic and potential synergistic effects of established and novel, promising chemotherapeutic agents on human fibrosarcoma cells (HT1080) in vitro, with the final aim of identifying suitable combination treatments for the treatment of fibrosarcomas. The tested drugs were doxorubicin, mafosfamide, trabectedin (also known as eetinascidin 743), tumour necrosis factor receptor apoptosis-inducing ligand (TRAIL) and taurolidine. Doxorubicin is an anthracycline antibiotic that halts DNA replication by intercalating with the DNA. Unfortunately, this drug produces reactive oxygen species that damage mitochondrial oxidative phosphorylation, especially in cardiac cells. Today, doxorubicin is commonly used to treat many malignancies, including fibrosarcomas. An encouraging study reported that doxorubicin sensitized sarcoma cells to TRAIL-induced apoptosis by increasing the expression of the TRAIL receptors DR4 and DR5 (14). Moreover, doxorubicin was efficient in sensitizing TRAIL-resistant tumour entities such as small-cell lung cancer, rhabdomyosarcoma and prostate cancer cells to TRAIL-mediated apoptosis (15-17). Thus, doxorubicin remains a valuable combination partner and is the subject of continued in vitro and in vivo studies.

Mafosfamide is an interesting member of the oxazophorine group that stops tumour growth by crosslinking guanine nucleotides in DNA. In various in vitro combination trials, mafosfamide demonstrated synergistic apoptosis-inducing effects with other cytostatics. Mafosfamide sufficiently increased the cytotoxic effect of fludarabine in B-chronic lymphocytic leukaemia cells (18). Furthermore, synergistic effects were observed by the addition of mafosfamide to medulloblastoma and neuroblastoma cell lines, incubated with the topoisomerase I inhibitor karenitecin (19). Mafosfamide spontaneously hydrolyses into the active metabolite 4-hydroxy-cyclophosphamide, whereas cyclophosphamide and ifosfamide require hepatic activation. Therefore, mafosfamide may represent a suitable agent for local therapy.

Trabectedin is a novel, promising drug of marine origin that was discovered in the Caribbean Sea squirt Ecteinascidia turbinata. It is a tetrahydroisoquinoline alkaloid that binds to the minor groove of DNA. It is currently produced using a semi-synthetic process (Pharma Mar, Madrid, Spain). Trabectedin received approval in 2007 from the European Medicines Agency and is indicated for use in patients with advanced or metastatic soft tissue sarcoma who have failed to respond to or who are not suitable for first-line therapy with anthracyclines and ifosfamide. In 2009, trabectedin received its second marketing authorisation from the European Commission for the treatment of relapsed platinum-sensitive ovarian cancer in combination with pegylated liposomal doxorubicin. Trabectedin is also being tested in clinical trials as a combination partner for the treatment of breast, prostate, and lung cancer as well as paediatric tumours.

TRAIL and taurolidine are promising combination partners that exhibit synergistic apoptotic effects on a wide range of malignant cells in vitro, including fibrosarcoma, human colon and oesophageal carcinoma cells (20-22). TRAIL is a member of the tumour necrosis factor family and is particularly interesting because of its unique ability to selectively induce cell death in tumour cells by binding to the death receptors DR4 and DR5, while sparing most other normal cells (23). TRAIL has been also observed to exert apoptotic activity on fibrosarcoma cells (21). Taurolidine was originally used as an antimicrobial agent in patients with peritonitis. Meanwhile, its antitumour effects were also observed, with encouraging clinical results following intravenous treatment in patients with glioblastoma and advanced gastric cancer without systemic side-effects (24-26). Recent in vitro studies have revealed that the combination of TRAIL and taurolidine resulted in sustained cell death, which was superior to a single application of TRAIL or TRD. This is despite the use of lower concentrations of both substances in the combination trials (20-22). 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 (22). Inspired by these results, we examined the effects of doxorubicin, mafosfamide and trabectedin both separately and in combination with TRAIL and TRD on human fibrosarcoma cells.

Materials and Methods

Cell line. Human fibrosarcoma cells, HT1080, were purchased from the American Type Culture Collection (ATCC, cell line CCI 121, Wesel, Germany) and maintained in modified Eagle’s medium (MEM) and non-essential amino acids (NEAA) + 10% foetal bovine serum (FBS) supplemented with 1% penicillin (100 U/ml) and streptomycin (100 μg/ml), 1% sodium pyruvate and 1% L-glutamine. The cells were cultured in a humidified atmosphere at 37°C with 5% CO2 in 25 cm2 flasks.

Reagents. Doxorubicin was obtained from Pfizer AG (Zürich, Switzerland). Mafosfamide was obtained from Baxter Oncology (Halle/Westfalen, Germany). Trabectedin was obtained from Pharma Mar (Madrid, Spain). Taurolidine (Taurolin® 2%, Boehringer Ingelheim, Germany), containing 5% povidone, was used as supplied by the manufacturer. Recombinant human TRAIL/APO2L (Bender MedSystems, Vienna, Austria) was dissolved in distilled water according to the manufacturer’s instructions.

Cell treatment. For every drug experiment, 80 μl of 3×10⁵ cells/ml were placed in 6-well plates containing the medium. After 24 hours, the medium was replaced and the drugs were added to each well.
The cultivated human fibrosarcoma cells were incubated with doxorubicin (0.25 μg/ml), mafosfamide (1.5 μg/ml) and trabectedin (4 μg/ml) either alone or in combination with tauramide (250 μmol/l) and/or TRAIL (50 ng/ml) for 2, 6, 12 and 24 hours. Different time points were chosen to identify the possible time dependency of the effects. All experiments were repeated for each of the three consecutive passages.

Flow cytometric analysis. At the indicated incubation time, the floating cells were collected together with the supernatant and adherent cells, which were harvested by trypsinisation. The cells were pelleted by centrifugation, resuspended in 195 μl binding buffer (Bender MedSystems) and incubated with 5 μl annexin V (BD Biosciences, Heidelberg, Germany) and 10 μl propidium iodide (PI) (Bender MedSystems, Vienna, Austria), following the manufacturer’s instructions. The cells were analysed immediately using a FACS flow cytometer (FACS Calibur BD Biosciences, Heidelberg, Germany). For each measurement, 20,000 cells were counted. Dot plots and histograms were analysed using the CellQuest Pro software (BD Biosciences). Annexin V binds phosphatidylserine on the outer membranes of cells, which then becomes exposed on the surface of apoptotic cells. Thus, the annexin V-positive cells are 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. The morphology of the adherent and suspended cells was examined and documented using a phase contrast Zeiss Axiovert 25 microscope (Carl Zeiss, Jena, Germany).

Proliferation assay. In order to determine and quantify the effects of the different drugs on cell proliferation, a colourimetric cell proliferation 5-bromo-2’-deoxyuridine (BrdU)-ELISA (Roche Applied Science, Mannheim, Germany) assay was used according to the manufacturer’s instructions. For this experiment, the cells were incubated with the different compounds for 8 hours.

Silencing RNA (siRNA)-Targeted Fas-associated death domain (FADD) knockdown. To test the influence of FADD (Fas-associated-Death-Domain) on TRAIL- and tauramide-induced apoptosis in fibrosarcoma cells, FADD was down-regulated by transfection with siRNA. Prior to beginning the drug experiments with the FADD-knockdown cells, transfection efficiency was visually assessed using negative control siRNA labelled with the fluorescent dye Alexa Fluor 488 (AllStars Negative Control siRNA, Alexa Fluor 488 labelled; Qiagen, Hilden, Germany) and fluorescence microscopy (Leica DM4000B; Leica Microsystems, Nussloch, Germany). HiPerFect (Qiagen) was used as a transfection reagent in every siRNA experiment, according to the manufacturer’s protocol for long-term transfection. Cells were transfected with siRNA after splitting at day 1 when the cells became confluent. After 24 hours (day 2) of incubation under normal growth conditions, the medium was changed and the cells were retransfected. Twenty-four hours later (day 3), the extent of FADD knockdown was monitored using western blot analysis of FADD protein levels. To determine the extent of FADD knockdown, the cells were transfected separately and in combination with four pre-designed siRNAs (GeneSolution siRNA, Hs_FADD_8, Hs_FADD_9, Hs_FADD_5, Hs_FADD_2; Qiagen) against FADD, each targeting different sequences. The highest knockdown level was achieved using the FADD siRNA that targeted the sequence 5’-AGCGGGATCTCGATCTTTAAG-3’ (Hs_FADD_8, 5 μM), which was used in the subsequent drug experiments. Following transfection, TRAIL (50 ng/ml) and tauramide (250 μmol/l) were added at day 2 to the FADD-knockdown cells after medium change, and their influence on apoptosis was determined using FACS analysis after twenty-four hours (day 3). Apoptosis was assessed in this manner for each of three consecutive passages.

Western blot. To monitor siRNA-induced gene silencing at the protein level, western blots were performed using a sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel and a human antibody against FADD (FD19), purchased from Santa Cruz Biotechnology Inc. (Heidelberg, Germany). Total protein was purified from the cells following transfection. The floating cells were collected together with the supernatant, and the adherent cells were harvested by trypsinisation and added to the solution. The cells were pelleted by centrifugation. Following the removal of the supernatant, each of the probes was incubated with 50 μl Cell Culture Lysis Reagent (Promega Corporation, Mannheim, Germany) for 1 hour on ice. The cell remnants were then pelleted by centrifugation and the supernatant containing the purified protein was frozen until further use.

Pancaspase inhibition. To determine the contribution of caspase activity to TRD- and TRAIL-induced cell death, cells were co-incubated with TRD or TRAIL and the pancaspase inhibitor z-VAD-FMK (Alexis Biochemicals, Enzo Live Sciences, Lörrach, Germany) for 24 hours at the highest possible dosage, without toxicity effects (1 μM) (27, 28). Cell viability, apoptosis and necrosis were assessed for each of three consecutive passages by FACS-analysis.

Statistical analysis. The results of FACS analysis were used to determine the percentages of viable, apoptotic and necrotic cells, which are expressed as the means±SD from at least three independent experiments and consecutive passages. In this study, comparisons between the experimental groups (single agents vs. combination treatments at various time points) were performed using one-way measurements of variance (one way ANOVAs) over all time points (Tukey test). Results were considered statistically significant for p-values ≤0.05.

Oligonucleotide microarray analysis. Total RNA was purified from the cells following incubation with the different compounds for 6 hours using the RNeasy KIT from Qiagen, as specified by the manufacturer. RNA integrity was assessed using standard denaturing agarose gel electrophoresis. For the microarray analyses, we used the Agilent Array platform, employing the manufacturer’s standard protocols for sample preparation and microarray hybridisation. Briefly, total RNA (500 ng) from each sample was amplified and transcribed into fluorescent cRNA following the manufacturer’s Quick Amp Labelling protocol (Version 5.7; Agilent Technologies, Boeblingen, Germany). The labelled samples were hybridised to the Whole Genome Oligo Array (4x44Kk, product no. G4412F; Agilent Technologies). Following the washing steps, the arrays were scanned using the Agilent Scanner G2505B. The Agilent’s Feature...
group, 62.2%±3.0% of the cells (with the doxorubicin-treated cells. After 6 hours, reached a maximum more rapidly than what was observed increased only moderately. With trabectedin alone, apoptosis apoptotic cells, whereas the number of necrotic cells 89.2%±2.8% in the control group. Exposure to trabectedin reduced to 77.8%±2.0% (viability was observed; the percentage of viable cells was 12 hours of incubation, but only a slight decrease in cell the degree of cell viability. This combination led to a significant increase in the percentage of necrotic cells to 57.7%±7.4%. Interestingly, the addition of taurodilone alone led to the same adverse effects as described for the doxorubicin-treated group, resulting in a significant diminution of the apoptotic response associated with trabectedin. Slight but significant synergistic effects were observed in the mafosfamide-treated group when combined with TRAIL, taurolidine or both TRAIL and taurolidine. The strongest cytotoxic effect was observed with the combination of mafosfamide with TRAIL and taurolidine. In comparison to cells treated with mafosfamide alone, the addition of TRAIL and taurolidine significantly reduced cell viability after 12 (p<0.001) and 24 hours (p=0.005). However, all the combinations with mafosfamide were less effective than was the treatment with trabectedin or doxorubicin alone.

The addition of taurodilone induced morphological changes and cell detachment. Trabectedin, mafosfamide and TRAIL

Results

Single applications of trabectedin, doxorubicin and mafosfamide induced apoptosis in HT1080 fibrosarcoma cells. Single treatment with doxorubicin led to the highest apoptosis rates after 24 hours, significantly reducing the population of the viable cells to 21.7%±5.1% (mean ± SD, p<0.001, Figure 1). In contrast, the viability of the control cells was 91.9%±3.8%. In the doxorubicin-treated cells, the first significant apoptotic response was observed after 12 hours of incubation, but only a slight decrease in cell viability was observed; the percentage of viable cells was reduced to 77.8%±2.0% (p<0.001), compared to 89.2%±2.8% in the control group. Exposure to trabectedin alone also led to a marked increase in the number of apoptotic cells, whereas the number of necrotic cells increased only moderately. With trabectedin alone, apoptosis reached a maximum more rapidly than what was observed with the doxorubicin-treated cells. After 6 hours, 62.2%±3.0% of the cells (vs. 10.6%±3.0% in the control group, p<0.001) were observed to be apoptotic, 19.3%±6.2% were necrotic and only 18.3%±3.9% were viable. As shown in Figure 2, the percentage of apoptotic cells remained at this high level throughout the later time points. Mafosfamide caused significant apoptosis after 24 hours of treatment and reduced the percentage of viable cells to 76.4%±8.1% (p=0.005, Figure 3). However, mafosfamide was significantly less effective in reducing the percentage of viable cells than were trabectedin and doxorubicin. The comparison of doxorubicin and trabectedin exhibited no statistically significant effect with respect to cell death induction (p>0.05) at 24 hours, although a higher apoptotic effect was observed with trabectedin at 2, 6 and 12 hours (p<0.001).

In combination treatment, the addition of taurodilone and TRAIL resulted in a strong synergistic effect on cell death induction. The combination of doxorubicin and TRAIL caused a marked increase in apoptosis at all time points when compared to treatment with doxorubicin alone. The strongest difference in apoptosis induction was observed after 12 hours, with 15.3%±1.3% of cells remaining viable (p<0.001), compared to treatment with doxorubicin alone (77.8%±2.0% of cells remaining viable). Moreover, the combination of doxorubicin and TRAIL led to a significantly higher maximum degree of apoptosis than what was observed with doxorubicin alone. Co-treatment with TRAIL reduced cell viability to 10.8%±0.8% after 24 hours. Thus, the addition of TRAIL increased the maximum apoptotic effects of doxorubicin by more than 10% (p=0.004) after 24 hours of incubation. As expected, the combination of doxorubicin with TRAIL and taurolidine was more effective in reducing cell viability than was doxorubicin alone. However, this combination did not exhibit any additive effect when compared with doxorubicin plus TRAIL at 12 and 24 hours and was significantly less effective at 2 hours (p<0.001) and at 6 hours (p<0.001). Strikingly, the addition of taurolidine alone attenuated doxorubicin-induced apoptosis so significantly that cell viability was restored to 61.1%±8.8% at 24 hours. When compared to treatment with trabectedin alone, only the combination of trabectedin and both TRAIL and taurolidine was associated with a stronger reduction in the degree of cell viability. This combination led to a significant decrease in the percentage of viable cells to 13.6%±0.8% (p=0.023) after 24 hours, with a dramatic increase in the percentage of necrotic cells to 57.7%±7.4%. Interestingly, the addition of taurodilone alone led to the same adverse effects as described for the doxorubicin-treated group, resulting in a significant diminution of the apoptotic response associated with trabectedin. Slight but significant synergistic effects were observed in the mafosfamide-treated group when combined with TRAIL, taurolidine or both TRAIL and taurolidine. The strongest cytotoxic effect was observed with the combination of mafosfamide with TRAIL and taurolidine. In comparison to cells treated with mafosfamide alone, the addition of TRAIL and taurolidine significantly reduced cell viability after 12 (p<0.001) and 24 hours (p=0.005). However, all the combinations with mafosfamide were less effective than was the treatment with trabectedin or doxorubicin alone.

The addition of taurodilone induced morphological changes and cell detachment. Trabectedin, mafosfamide and TRAIL
did not alter cell morphology, as observed using bright-field microscopy. However, trabectedin treatment reduced cell density, indicating reduced rates of cell division (Figure 4). Slight morphological changes were induced by doxorubicin, with cells exhibiting enlarged and prominent nucleoli. Additional treatment with taurolidine resulted in obvious morphological aberrations, including the disintegration of the subconfluent cell groups and cell shrinkage followed by complete cell detachment.

Figure 1. Effects of doxorubicin on viability and apoptosis. HT1080 cells were incubated for 24 hours with doxorubicin, both alone and in combination with taurolidine (TRD), with tumor necrosis factor related apoptosis-inducing ligand (TRAIL) or with both (TT). The percentages of viable (a), apoptotic (b) and necrotic cells were determined using fluorescence activated cell sorting (FACS) analysis for annexin V-FITC and propidium iodide. Since doxorubicin is inherently fluorescent at a similar wavelength to propidium iodide, a differentiation between apoptosis and necrosis was not possible in the doxorubicin-treated cells. Here, cells were classified as annexin-negative (non-apoptotic) or Annexin-positive (apoptotic). The values indicate the mean±SD of three independent experiments and consecutive passages. Significantly different at ***p≤0.001, **p≤0.005, *p<0.05; one-way ANOVA. The indicators of significance refer to the differences between doxorubicin alone and doxorubicin with TRAIL.

Trabectedin, doxorubicin and the addition of taurolidine significantly reduced the number of proliferating cells compared to the control (p<0.001). As indicated by the BrdU assay, the antiproliferative potency of trabectedin and taurolidine was similar, but significantly higher than that of doxorubicin (p<0.001, Figure 5). Moreover, all drug combinations that included taurolidine had the same strong antiproliferative potency, whereas TRAIL was not able to reduce cell proliferation.
Figure 2. Effects of trabectedin on viability, apoptosis and necrosis. HT1080 cells were incubated for 24 hours with trabectedin both alone and in combination with taurolidine (TRD), tumor necrosis factor related apoptosis-inducing ligand (TRAIL) or with both (TT). The percentages of viable (a), apoptotic (b) and necrotic (c) cells were determined using fluorescence activated cell sorting (FACS) analysis for annexin V-FITC and propidium iodide. The values indicate the means±SD of three independent experiments and consecutive passages. Significantly different at ***p≤0.001, **p≤0.005, *p<0.05; one-way ANOVA. The indicators of significance refer to the differences between trabectedin alone and trabectedin with TT.
Figure 3. Effects of the mafosfamide group on viability, apoptosis and necrosis. HT1080 cells were incubated for 24 hours with mafosfamide both alone and in combination with taurolidine (TRD), with tumor necrosis factor related apoptosis-inducing ligand (TRAIL) or with both (TT). The percentages of viable (a), apoptotic (b) and necrotic (c) cells were determined using fluorescence activated cell sorting (FACS) analysis for annexin V-FITC and propidium iodide. The values indicate the means±SD of three independent experiments and consecutive passages. Significantly different at ***p≤0.001, **p≤0.005, *p<0.05; one-way ANOVA. The indicators of significance refer to the differences between mafosfamide alone and mafosfamide with TT.
Figure 4. Morphological changes after treatment. Representative photographs demonstrating morphological changes in HT1080 cells induced by the drug combinations following 24 hours of treatment. TRAIL: Tumor necrosis factor related apoptosis-inducing ligand; TT: TRAIL plus taurolidine.
Taurolidine- and TRAIL-induced apoptosis were not mediated by FADD. As shown in Figure 6 a-c, the down-regulation of FADD by siRNA targeting had no influence on the effect of taurolidine or TRAIL. After 24 hours of incubation, taurolidine and TRAIL reduced the number of viable cells equivalently, in cells transfected with FADD-targeting siRNA or non-targeting siRNA. Moreover, reduced FADD expression alone did not affect cell viability. Taurolidine-induced cell death was partially reversible by z-VAD-FMK-dependent caspase inhibition, whereas TRAIL-induced cell death remained unaffected by pancaspase-inhibition. HT1080 cells responded to z-VAD-FMK co-incubation with a partial protective effect characterized by a significantly increased cell viability of 69.3%±4.3% compared to 48.2±1.6% for taurolidine alone (p<0.001). However, cell viability after co-incubation with z-VAD-FMK was still significantly lower compared to untreated controls (69.3%±4.3% vs. 87.9%±2.1%, p<0.001) (Figure 6d). The partial protection by z-VAD-FMK was mainly achieved by a significant reduction in necrosis from 19.5%±1.0% for taurolidine alone to 6.4%±0.8% for taurolidine with z-VAD-FMK co-incubation (Figure 6f) and a small but not significant reduction in apoptosis (Figure 6e). Interestingly, pancaspase inhibition via co-incubation with z-VAD-FMK did not lead to a rescue effect in TRAIL-treated cells.

Microarray analysis revealed differential gene expression patterns of HT1080 cells treated with the compounds. Based on the MAS5 comparison analysis algorithm, all three substances and their combinations remarkably altered the expression levels of different combinations of probe sets. Single treatment with trabectedin and doxorubicin, the combination of doxorubicin with TRAIL, and the combination of trabectedin with TRAIL/taurolidine led to expression changes in a wide range of apoptosis-associated genes. In trabectedin-treated cells, microarray analyses identified noticeable expression changes with at least a ≥2-fold change in 1772 genes, among which 122 were apoptosis-related (Table I). Out of these, 95.08% (116) were up-regulated. In comparison, the combination treatment with trabectedin and TRAIL/taurolidine for 6 hours led to changes in expression in a smaller number of probe sets (941) than did trabectedin alone, and only 57.2% (538) of these probesets were up-regulated. Following single treatment with doxorubicin for 6 hours, 329 probe sets, 22 of which were apoptosis-related, exhibited differential expression levels. Among these, 145 were up- and 184 were down-regulated. Co-treatment with doxorubicin and TRAIL resulted in expression changes in 405 genes (209 up- and 196 down-regulated), affecting 37 apoptosis-associated genes. Mafosfamide, the agent with the lowest effect on apoptosis-induction in FACS analysis, had a relevant impact on gene expression in 1538 altered probe sets. Based on the FACS results, however, mafosfamide exhibited no significant over-representation in apoptosis-related probesets. Interestingly, the combination of mafosfamide and taurolidine altered the expression of 85 apoptosis-related genes, revealing a slight correlation to its apoptotic effect.
Figure 6. Effect of taurolidine (TRD) on viability and tumor necrosis factor related apoptosis-inducing ligand (TRAIL) on Fas-associated death domain (FADD) knockdown of HT1080 cells (a-c) and cells co-incubated with the pancaspase-inhibitor Z-VAD-FMK (d-f). Control cells were incubated with HiPerFect transfection reagent (a-c) or the pancaspase-inhibitor Z-VAD-FMK (d-f). The percentages of viable, apoptotic and necrotic cells were determined using fluorescence activated cell sorting (FACS) analysis for annexin V-FITC and propidium iodide following 24 hours of treatment. The indicators of significance refer to the difference between the inhibited (FADD-siRNA or Z-VAD-FMK) and non-inhibited series. Data are presented as the mean±SD and the scales of the y-axes were adjusted to different values for clarity and are therefore variable.
effectiveness in the FACS analysis. To obtain an overview of the biological processes affected by the tested drugs, we analysed lists of regulated targets of the pathways that were over-represented in our data set using the GeneTrail application (29). Significant overrepresentation was detected in several pathway categories that included apoptosis, cell cycle, JAK-STAT signalling, p53 signalling, pathways in cancer and cytokine-cytokine receptor interaction, depending on which chemotherapeutic agent was used (Table I). To understand the molecular details underlying the diverse modes of cell death in fibrosarcoma cells, we focused on the differentially expressed apoptosis-associated genes that were altered by the four most effective treatment groups with respect to apoptosis induction based on FACS analysis (Table II). In this experiment, we observed notable candidates for further investigations: Jun B proto-oncogene (JUNB), dual specificity phosphatase 1 (DUSP1), cyclin-dependent kinase inhibitor 1B (CDKN1B), superoxide dismutase 2 (SOD2), nitric oxide synthase 1 (NOS1), suppressor of cytokine signaling 3 (SOCS3), growth arrest and DNA-damage-inducible beta 45 (GADD45B), nuclear factor of kappa light poly-peptide gene enhancer in B-cells inhibitor alpha (NFKBIα), tumor necrosis factor receptor-associated factor (TNFAIP3), colony stimulating factor 2 (CSF-2), FADD and baculoviral IAP repeat-containing protein 3 (BIRC3).

Table I. Microarray analysis, using the GeneTrail software, identified significant changes in the following computed Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway categories, with p-values below 0.05 and false discovery rate (FDR) adjustments (Benjamini-Hochberg). The analysed genes in the subcategories were significantly up- or down-regulated with a ≥2-fold mean change (FC) in the various treatment groups compared to the control treatment.

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<th>Substance</th>
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<th>Selection of significant subcategories (p&lt;0.05, FDR adjusted)</th>
<th>p-Value</th>
<th>Expected number of regulated genes</th>
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<td>422/189</td>
<td>No significant subcategories</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Mafosfamide</td>
<td>223/1306</td>
<td>No significant subcategories</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Mafosfamide+TRD</td>
<td>863/538</td>
<td>Apoptosis</td>
<td>0.013</td>
<td>60.0</td>
<td>85</td>
</tr>
<tr>
<td>Mafosfamide+TRAIL</td>
<td>133/1197</td>
<td>No significant subcategories</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Mafosfamide+TT</td>
<td>306/538</td>
<td>Pathways in cancer</td>
<td>0.024</td>
<td>6.9</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td></td>
<td>p53 signaling pathway</td>
<td>6.70e-3</td>
<td>1.5</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cell cycle</td>
<td>0.024</td>
<td>2.6</td>
<td>8</td>
</tr>
</tbody>
</table>

JAK: Janus kinase; STAT: signal transducer and activator of transcription, TRD: taurolidine; TRAIL: tumor necrosis factor related apoptosis-inducing ligand; TT: TRAIL plus taurolidine.

Discussion

Fibrosarcomas are a rare tumour within the heterogeneous group of soft tissue sarcomas and respond poorly to conventional treatments, such as chemotherapy and radiation. Despite excellent rates of local disease control, treatment options in distant metastatic disease, especially in pulmonary locations, are very limited and have an associated median survival of only 11 months (10, 33). Due to the diversity and rarity of fibrosarcomas, the development of new therapeutics has been difficult, and the lack of novel chemotherapy protocols remains a major problem. For these reasons, there is increasing interest in assessing whether the results obtained with doxorubicin alone could be improved by combining it with other drugs. Among the novel agents for the management of soft tissue sarcomas, trabectedin is one of the most promising candidates that have been tested over the last two decades. It received approval in 2007 by the European Medicines Agency as a second-line treatment for advanced soft tissue sarcomas in patients who have received doxorubicin and ifosfamide treatments and whose disease continues to progress. Furthermore, trabectedin has been proven to be efficacious in a variety of other human malignancies, including ovarian and breast cancer, indicating a general antineoplastic potential (34-36). Some common
Histotypes of soft tissue sarcomas appear to be highly sensitive to trabectedin, although the sensitivity of fibrosarcoma to this new agent is poorly understood due to the rarity of this tumour. In this regard, studies elucidating the efficacy of trabectedin on human fibrosarcoma cells in vitro would be highly valuable prior to the use of this agent in clinical practice. The basis for combination treatments with cytostatics is their additive or synergistic effects. In this in vitro study, we analysed the apoptotic and antiproliferative activities of doxorubicin, trabectedin and mafosfamide alone and in combination with TRAIL and taurolidine. The results of this study demonstrate synergism in the combinations of doxorubicin and trabectedin with TRAIL and taurolidine on human fibrosarcoma cells. In addition, the study demonstrates that the apoptotic effect of these combinations is accompanied by expression changes in a wide range of apoptosis-regulating genes. Our data demonstrate that trabectedin is highly effective in reducing cell viability and in inhibiting proliferation. The addition of TRAIL and taurolidine enhanced the apoptotic activity of trabectedin, suggesting synergistic cytotoxicity associated with this combination. Furthermore, our results demonstrate that doxorubicin is more potent when combined with TRAIL, resulting in a greater decrease in the percentage of viable cells at earlier time points. Neither the antiproliferative effect of trabectedin nor the synergistic apoptotic effect of trabectedin combined with TRAIL and taurolidine have been reported. Moreover, only a few reports have investigated the activity of trabectedin on

### Table II. Summary of the expression changes of apoptosis-related genes (+, increase; -, decrease) for the single substances and the most effective combination treatments (based on fluorescence activated cell sorting analysis), compared to untreated cells.

<table>
<thead>
<tr>
<th>GeneID</th>
<th>Gene title</th>
<th>Oncological relevance (ref)</th>
<th>Trabecedtin</th>
<th>Trabecedtin +TT</th>
<th>Doxorubicin</th>
<th>Doxorubicin +TRAIL</th>
</tr>
</thead>
<tbody>
<tr>
<td>JUNB</td>
<td>Jun B proto-oncogene</td>
<td>Increases invasive potential of HT1080 fibrosarcoma cells (90)</td>
<td>4.50</td>
<td>–10.53</td>
<td>4.02</td>
<td>–3.96</td>
</tr>
<tr>
<td>DUSP1</td>
<td>Dual specificity phosphatase 1</td>
<td>Inhibits proliferation and induces apoptosis in human hepatocellular carcinoma (91). Induced in fibroblasts by oxidative/heat stress (92)</td>
<td>371.57</td>
<td>4.53</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CDKN1B</td>
<td>Cyclin-dependent kinase inhibitor 1B</td>
<td>Functions as a tumor suppressor in prostate cancer (93), neuroblastoma (94), acute myeloid leukemia and myelodysplastic syndromes (95)</td>
<td>38.01</td>
<td>2.40</td>
<td>-</td>
<td>3.61</td>
</tr>
<tr>
<td>SOD2</td>
<td>Superoxide dismutase 2</td>
<td>Enhances radiation resistance of murine sarcoma tumours (46). Increased expression through activated NFkB (96)</td>
<td>–9.15</td>
<td>–37.50</td>
<td>2.31</td>
<td>–11.58</td>
</tr>
<tr>
<td>NOS1</td>
<td>Nitric oxide synthase 1</td>
<td>Associated with lower overall survival in chondrosarcomas and angiogenesis in various tumours (97, 98)</td>
<td>–2.80</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>SOCS3</td>
<td>Suppressor of cytokine signaling 3</td>
<td>Suppresses the growth of malignant fibrous histiocytoma (MFH) (62)</td>
<td>21.55</td>
<td>3.21</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>GADD45B</td>
<td>Growth arrest and DNA-damage-inducible, beta</td>
<td>Induces apoptosis and growth arrest after UV irradiation and other genotoxic stresses (71). Associated with reduced invasiveness of chondro-sarcomas (73)</td>
<td>24.60</td>
<td>5.89</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>NFKBIA</td>
<td>Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha (IkappaB-alpha)</td>
<td>Inhibits NF-κB which is an enhancer of proliferation and invasiveness in HT1080 cells (54, 55, 99). High activity of NF-κB leads to doxorubicin resistance (100)</td>
<td>25.63</td>
<td>2.55</td>
<td>3.86</td>
<td>4.90</td>
</tr>
<tr>
<td>TNFAIP3</td>
<td>Tumor necrosis factor receptor-associated factor</td>
<td>Inhibits NF-κB-activation (101)</td>
<td>3.09</td>
<td>-</td>
<td>3.58</td>
<td>–4.74</td>
</tr>
<tr>
<td>CSF 2</td>
<td>Colony-stimulating factor 2</td>
<td>Reduces tumor growth in murine fibrosarcoma (102, 103). Induces T-cell-mediated apoptosis in various solid tumors (104)</td>
<td>3.42</td>
<td>–4.56</td>
<td>37.16</td>
<td>–32.33</td>
</tr>
<tr>
<td>FADD</td>
<td>Fas-associated death domain</td>
<td>Induces apoptosis by recruiting caspases (105, 106)</td>
<td>4.86</td>
<td>2.10</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

TRAIL: Tumor necrosis factor related apoptosis-inducing ligand; TT: TRAIL plus taurolidine.
fibrosarcomas. An encouraging in vitro study conducted in 2001, using HT1080 fibrosarcoma cells and a sulforhodamine B assay detected trabectedin-induced cytotoxic cell death (37). However, the data obtained from this study met quality but not quantity requirements, as the study did not consistently and reliably compare trabectedin and doxorubicin regarding their apoptotic activity. In another recent case report on a patient with metastatic fibrosarcoma, trabectedin treatment was unsuccessful (38). Nevertheless, in some of the most common subgroups among the soft tissue sarcomas, trabectedin has the potential to be a front-line treatment for advanced or metastatic disease. Thus far, various trabectedin regimens have demonstrated significant antitumour activity in patients with liposarcomas and leiomyosarcomas, with a comparable toxicity profile to that of standard treatments (39, 40). Taken together, these published results and our study indicate that trabectedin and its combination with TRAIL and taurolidine should be considered for in vivo studies, which in turn may justify the use of these drugs in clinical practice.

To further elucidate the actions of the tested drugs on a molecular basis, we analysed changes in gene expression using microarray technology. Interestingly, we found high correlations between apoptotic efficacy and decrease in SOD2 expression in cells treated with trabectedin or doxorubicin. SOD2 is recognized as an effective radioprotector in both normal and malignant tissues. It also is known to play a key role in the depletion of radiation-induced reactive oxygen species (ROS) (41-43). Elevated SOD2 activity was reported to result in lower ROS-mediated apoptosis and in consequent enhancement of radiation resistance of different malignancies, including murine sarcomas (44-46). Recent studies have indicated that gene silencing of SOD2 via siRNA targeting was able to improve radiosensitivity in both normal and malignant tissues (47, 48). However, these findings suggest that ROS-mediated cell damage and signalling pathways may play roles in apoptosis induction in human fibrosarcomas. Moreover, the combination of trabectedin with TRAIL and taurolidine may be valuable in the context of radiotherapy by improving radiosensitivity through the down-regulation of SOD2. Further investigation is required to conclusively demonstrate this hypothesis in soft tissue sarcomas and in other malignant tumours. Notably, the four most effective treatment groups with respect to apoptosis induction (trabectedin, trabectedin plus TRAIL/taurolidine, doxorubicin, and doxorubicin plus TRAIL) led to a differential up-regulation of NFκBIA, in treated cells which was up-regulated by more than two-fold compared to control cells, indicating the relevance of the NFκB pathway in the induction of HT1080 cell death. NFκBIA is an inhibitor of NFκB and plays a critical role in tumourigenesis, inducing an array of downstream anti-apoptotic genes (49). NFκBIA stabilises NFκB in the cytoplasm, preventing its translocation into the nucleus, where it activates downstream pathways. Recent oncological studies have suggested that the constitutive activation of NFκB is associated with poor prognosis and resistance to chemotherapy in many types of human cancer, including sarcomas (50-53). More specifically, it has been reported that the tumour invasiveness of HT1080 tumour cells can be significantly reduced by inhibiting NFκB activity (54). It has also been reported that the doxorubicin analogue DA-125 can reduce proliferation via a NFκB-dependent pathway (55). Pharmacological inhibition of NFκB signalling is well-known to enhance TRAIL-induced apoptosis (56, 57). However, there has been increased pharmacological interest in agents aiming at inhibiting the activation of the NFκB pathway. In our study, NFκBIA was observed to be highly overexpressed at the gene level following treatment with trabectedin. Accordingly, the inhibition of NFκB may be one of the mechanisms by which trabectedin mediates cell death in fibrosarcoma. Overall, the signalling mechanisms involved in NFκB-dependent apoptosis have not been completely elucidated. Understanding the complex role of NFκB in sarcoma cell death may provide new opportunities for rational pathway-based therapies and drug development. Considering that the overexpression of NFκBIA and the subsequent inhibition of NFκB were associated with increased apoptosis in our study, the proteasome inhibitor bortezomib may be an attractive therapeutic agent for human fibrosarcomas. As the only drug in a new class of proteasome inhibitors, bortezomib has already been approved for clinical use. It blocks the cellular degradation of NFκBIA, leading to impaired NFκB activation (58, 59). These findings indicate a possible synergistic effect between bortezomib and doxorubicin or trabectedin, all of which led to an up-regulation of NFκBIA. SOCS3, GADD45B, DUSP1 and FADD were differentially up-regulated following treatment with trabectedin or with trabectedin in combination with taurolidine and TRAIL. SOCS3 is a well-known negative regulator of the JAK/STAT signalling pathway (60, 61). The forced expression of SOCS3 was demonstrated to suppress the growth of malignant soft tissue tumour cells by blocking the activation of STAT3 in the JAK/STAT signalling pathway (62). Constitutive STAT3 activity has been demonstrated to cause tumourigenic inflammation and increased proliferation in a wide range of malignant diseases, including malignant fibrous histiocytoma (63-65). Blocking STAT3 in tumour cells was demonstrated to induce the expression of proinflammatory cytokines and chemokines which activate the innate immune system and dendritic cells (66). For example, STAT3 was demonstrated to protect prostate cancer cells from cytotoxic T-lymphocyte-induced apoptosis (67). The role of STAT3 in human fibrosarcoma warrants further research because the novel group of JAK kinase inhibitors are potentially useful therapeutic options for suppressing the JAK2/STAT3 pathway (68-70). GADD45 proteins cooperate
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 (71). The mechanisms by which GADD45 proteins function in negative growth control is not fully understood, although up-regulation of these proteins was reported to be associated with increased apoptosis and p53-independent cell cycle arrest in a variety of soft tissue sarcomas (72). In a recent immunohistochemical study, the high expression of GADD45B was associated with reduced invasiveness of chondrosarcomas, suggesting its potential diagnostic value in the histological grading of malignant chondrogenic tumours (73). DUSP1, also known as mitogen activated protein kinases (MAPK) phosphatase 1 (MKP1), is a stress-inducible dual-specific phosphatase that inactivates members of the MAPK family by dephosphorylation (74, 75). MAPKs play a major role in neoplastic cell transformation. Activated MAPK or elevated MAPK expression has been detected in a wide range of malignancies, where it promotes tumour growth and metastasis (76, 77). In a recent study, MAPK activation via MAPK kinase led to increased proliferation and vascular function in HT1080 fibrosarcoma cells by modulation of vascular endothelial growth factor (VEGF) signalling (78). FADD can be recruited by receptors of the TNF super family, which include the TRAIL receptors DR4 and DR5. Following FADD recruitment, apoptosis is induced by caspase-8 activation. However, the lack of effect of FADD-knockdown and pancaspase inhibition on TRAIL-mediated apoptosis in this study indicates that there are other mechanisms responsible for TRAIL-induced apoptosis in HT1080 cells. Our findings suggest an involvement of caspase-dependent pathways in taurolidine-induced cell death. Further studies are necessary to specify programmed cell death following taurolidine and TRAIL treatment. In recent years, TRAIL and taurolidine have demonstrated anti-neoplastic activity in several in vitro and in vivo studies. Taurolidine was demonstrated to induce cell death in a wide range of malignant cell lines derived from colon carcinoma (22, 24), squamous cell oesophageal carcinoma (21), glioblastoma (79, 80), melanoma (81, 82), mesothelioma (83, 84) and sarcoma (21). Moreover, in several case reports, the systemic application of taurolidine prevented disease progression in patients with glioblastoma and gastric carcinoma with almost no toxicity (24, 85, 86). TRAIL has emerged as a novel anticancer agent due to its tumour-selective apoptotic activity and lack of adverse effects on normal cells (23, 87-89). TRAIL and two agonistic antibodies against DR4 and DR5, mapatumumab and lexatumumab, are currently being tested in several clinical trials in patients with solid tumours. These studies must evaluate the clinical response, safety and tolerability of these agonistic TRAIL antibodies to determine their future efficacy as cancer treatment options.

In conclusion, this in vitro study demonstrates that TRAIL and taurolidine synergise with both well-established and novel chemotherapeutic agents in inducing apoptosis and inhibiting proliferation in HT1080 human fibrosarcoma cells. The single application of trabectedin and doxorubicin led to apoptosis and proliferation inhibition. A stronger apoptosis-inducing effect was observed with the combination of doxorubicin and TRAIL and the combination of trabectedin and TRAIL/taurolidine. In addition, trabectedin and taurolidine exhibited a higher potential to inhibit proliferation than did doxorubicin. Although a wide variety of genes and pathways were involved, the NFKB pathway appears to play a key role in mediating apoptosis of HT1080 cells. More detailed knowledge regarding the function of the genes affected by these drugs will aid in the understanding of the diverse modes of cell death in fibrosarcoma cells and in identifying new pathway-based therapy options. These results provide experimental support for in vivo trials assessing the effect of trabectedin combined with TRAIL/taurolidine and the combination of doxorubicin and TRAIL.

Competing Interests

The Authors declare that they have no competing interests

Authors’ Contributions

KH carried out the experiments, interpreted the data and drafted the manuscript. AC developed the study design, coordinated the work and participated in statistical analysis. DB coordinated the FACS analysis and interpreted the data. OG participated in the design of the study. SH carried out the microarrays. TH participated in the design of the study. LK helped to interpret the microarray data. ML and WU were helpful in preparing the manuscript and conceived the work. AD developed the idea and participated in its design and coordination and helped to draft the manuscript.

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