Chapter 8

Effects of TRAIL, doxorubicin and 4-hydroxy-ifosfamide in a panel of soft-tissue sarcoma cell lines with different sensitivity to tumor necrosis factor-family cytokines

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Abstract

Doxorubicin (DOX) and ifosfamide (IFO) are the most active single agents in soft tissue sarcomas (STS). Still, the response rate in metastatic disease is only 20-30%. Tumor necrosis factor-α (TNF-α) is used for STS only in the setting of isolated limb perfusions. Like TNF-α, TNF-related apoptosis-inducing ligand (TRAIL) also induces apoptosis, and preliminary studies indicate that TRAIL lacks systemic side effects. Resistance to TRAIL has been demonstrated, but can be circumvented by combinations of TRAIL with conventional cytotoxic agents, in vitro. The effects of TRAIL alone and in combination with DOX or 4-hydroxy-IFO (i.e. the active metabolite of IFO, 4-OH-IFO) were evaluated in a panel of TNF-α sensitive and resistant human soft tissue sarcoma cells.

The rhabdomyosarcoma cell line KYM-1, its five-fold TNF-α sensitive subline KD4 and its >150-fold TNF-α resistant subline 37B8R were used. Membrane expression of the TRAIL-receptors DR4, DR5 (pro-apoptotic) and DcR1, DcR2 (anti-apoptotic) was assessed by flow cytometry. Drug-induced cytotoxicity was determined by a microculture tetrazolium assay. Apoptosis assays using acridine orange were conducted for the combination that was most potent of inducing cytotoxicity.

DOX and 4-OH-IFO decreased survival in all cell lines; a two-fold resistance was observed for both drugs in 37B8R. All three cell lines expressed DR4 and DR5, but no or very low levels of DcR1 or DcR2. TRAIL single agent decreased survival in KYM-1 and was even more cytotoxic in KD4 and induced massive apoptosis, while 37B8R was >500-fold resistant to TRAIL compared to KYM-1 and little apoptosis could be observed. The combination of TRAIL plus DOX showed synergistic cytotoxic effects in KYM-1 and 37B8R. The combination of TRAIL plus 4-OH-IFO showed to be additive in all three cell lines. DOX plus TRAIL induced more cytotoxicity as well as apoptosis in all three cell lines compared to TRAIL alone. In 37B8R, DOX overcame resistance to TRAIL.

In KYM-1 and its sublines KD4 and 37B8R, sensitivity and resistance to TNF-α and TRAIL parallels. TRAIL resistance was independent from expression of TRAIL receptors. DOX with TRAIL could overcome TRAIL-resistance in 37B8R cells, suggesting a therapeutic potential for this combination for TNF-α and TRAIL refractory STS cells.
Introduction

Soft tissue sarcomas (STS) are the group of malignancies of mesenchymal origin. In case of metastatic disease, curaion is difficult to reach with standard treatment, including doxorubicin (DOX) and ifosfamide (IFO). Therefore, finding alternative agents is critical.

A potential innovative treatment implies the use of cytokines of the tumor necrosis factor (TNF) superfamily. Some members of this growing family have drawn attention as anticancer agents, including the prototype TNF-α that rapidly induces apoptosis in many cancer types. TNF-α in combination with melphalan is used in the setting of a hyperthermic isolated limb perfusion, resulting in a local response rate up to almost 90% in locally advanced STS. However, this regional treatment has no impact on the metastasis-free and overall survival of patients, while the systemic use of TNF-α is hampered by severe toxic side-effects.

TNF-related apoptosis-inducing ligand (TRAIL) is a more recently identified member of the TNF superfamily that selectively induces apoptosis in malignant cells. Native TRAIL is safe in non-human primates, while human tissues are spared at tumoricidal concentrations, suggesting that TRAIL is a candidate for systemic cancer treatment. TRAIL is a type II transmembrane protein and so far, four membrane-bound receptors for TRAIL have been described: DR4, DR5, DcR1 and DcR2. Apoptosis is mediated by the two death receptors DR4 and DR5, while the two decoy receptors DcR1 and DcR2 interrupt apoptosis.

The combination of TRAIL with cytotoxic agents offers several possibilities. First, TRAIL can increase sensitivity to cytotoxic agents, or even restore sensitivity in resistant cells. Second, cytotoxic agents can increase sensitivity to TRAIL-mediated apoptosis. Third, when combinations proof to be more effective than the single agents, lower amounts of the cytotoxic agents and TRAIL can be applied.

The aim of the present study was to analyze the effect of cytotoxic agents (DOX and activated IFO) with the new apoptosis-inducing agent TRAIL in a panel of isogenic soft-tissue sarcoma cell lines. The panel consisted of the rhabdomyosarcoma cell line KYM-1, its TNF-α sensitive subline KD4 and its TNF-α resistant subline 37B8R. Cytotoxicity of the cytotoxic agents and TRAIL was tested alone and in combination. In addition, TRAIL receptor status of the cell lines was evaluated and apoptosis studies were performed under conditions of the most effective cytotoxic combinations.
**Materials and Methods**

**Chemicals.** RPMI 1640 medium, L-glutamine and sodium pyruvate were obtained from Invitrogen (Merelbeke, Belgium), fetal calf serum (FCS) from Bodinco BV (Alkmaar, The Netherlands). TNF-α was kindly provided by Boehringer-Ingelheim (Ingelheim am Rhein, Germany). Recombinant human soluble TRAIL was made according Ashkenazi et al. and was dissolved in medium with 10% FCS at a stock concentration of 423 µg/ml. The active metabolite of IFO, 4-hydroxy-IFO (4-OH-IFO) was a gift from Asta Medica (Frankfurt, Germany). DOX (Adriablastina™) was obtained from Pharmacia & Upjohn (Woerden, The Netherlands). Sodium azide and acridine orange (AO) were purchased from Sigma (St Louis, MO, USA); AO was dissolved in demineralized water at a 1-mg/ml concentration. The ready-to-use tetrazolium dye solution Cell Proliferation Reagent WST-1 was purchased from Roche Diagnostics GmbH (Mannheim, Germany). Anti-DR4, -DR5, -DcR1, -DcR2 antibodies were a gift from Amgen (formerly Immunex; Thousand Oaks, CA, USA). FITC-labelled rabbit-anti-mouse antibodies were bought from DAKO (Glostrup, Denmark).

**Cell lines and culturing.** KYM-1, an embryonal rhabdomyosarcoma cell line and the two sublines KD4 and 37B8R were kindly provided by A. Meager (National Institute for Biological Standards and Control, Potters Bar, UK). KYM-1 has been described as a TNF-α sensitive cell line. KD4 was established after limited dilution cloning of KYM-1 cells and has increased sensitivity to TNF-α. 37B8R cells are resistant to TNF-α, established after exposure to gradually increasing concentrations of TNF-α. The cell lines were grown in RPMI 1640 medium supplemented with 7% FCS, 1mM L-glutamine and 1mM sodium-pyruvate. Cells were incubated at 37°C with saturated humidity and 5% CO₂. All three cell lines grow in suspension under increasing medium viscosity due to the production of hyaluronic acid. To avoid reproducibility problems due to handling cells under viscous conditions, cells were harvested for experiments at latest two days after addition of fresh medium. New passages of all three cell lines were started every three months.

**Cell viability assays.** For cytotoxicity measurements, a modified microculture tetrazolium assay was used. This colorimetric assay is based on cleavage of the tetrazolium dye WST-1 (4-[3-(4-iodophenyl)-2-(4-
nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate) to water-soluble formazan by mitochondrial dehydrogenases in viable cells. The linear association between cell concentrations to the production of the formazan was confirmed and cell growth studies were performed to assure exponential cell growth at the time of measuring cell survival. For KYM-1 and KD4, 1.2 × 10^4 cells per well and for 37B8R, 1.0 × 10^5 cells per well were incubated with single agent or combinations of DOX, 4-OH-IFO and TRAIL in a total volume of 200 µl, all in triplo. After 96 hours, 20 µl of the WST-1 solution was added and incubated for 3 hours at 37 °C. Formazan production was assessed by measuring the absorbance at a wavelength of 450 nm on a scanning microplate spectrophotometer (Benchmark™ Microplate Reader, Bio-Rad Laboratories).

Background absorbance was corrected by subtracting the absorbance measured for the culture medium in absence of cells. The percentage of cell survival was calculated as:

\[
% \text{ survival} = \left( \frac{\text{absorbance of experimental well}}{\text{absorbance of untreated well}} \right) \times 100
\]

Each experiment was performed in triplicate, with a minimum of three individual experiments per cell line per drug or combinations of drugs.

**Apoptosis assays.** AO staining was used to distinguish apoptotic cells from vital cells. To analyze apoptosis induction, 6.0 × 10^4 cells per well were incubated in triplo with different concentrations of cytotoxic agent and/or TRAIL in a total volume of 200 µl into a 96-wells plate. Untreated cells were used as controls. After 24 hours of exposure, AO (final concentration: 5 ng/ml) was added and incubated at 37 °C for 10 minutes. Then cells were centrifuged at 900 rpm for 15 minutes. Three-quarter of the supernatant was removed and checked first for unintentional aspiration of cells before proceeding to counting cells under a fluorescent microscope (Olympus IM, Japan; wavelength 525 nm). The apoptotic index was calculated as:

\[
% \text{ apoptosis} = \left( \frac{\text{number of apoptotic cells}}{\text{total number of cells}} \right)
\]

A minimum of three independent experiments per single agent and combination per cell line was performed.

**Analysis of cell viability and apoptosis assays.** To analyze the potential enhancing effects of the cytotoxic agents on TRAIL-mediated cytotoxicity
and apoptosis, the enhancement ratio (ER) for each experiment was calculated. ER was calculated by dividing the effect of TRAIL alone by the effect of TRAIL combined with the cytotoxic agent, the latter corrected for the effect of cytotoxic agent alone. In concordance with previous studies, an ER-value between 0.8-1.2 was considered as indicative for additivity, ER lower than 0.8 as antagonistic and ER higher than 1.2 as synergistic.

**Expression of TRAIL receptors.** Cells (1.0 × 10^5) were analyzed for DR4, DR5, DcR1 and DcR2 expression by flow cytometry, using receptor NH2-terminal-specific monoclonal antibodies. First, harvested cell were washed in phosphate buffered saline (PBS) with 2% FCS and 0.1% sodium azide. Cells were then incubated with the primary antibodies at 37 °C for 30 minutes. After washing in PBS with 2% FCS and 0.1% sodium azide, cells were incubated with FITC-labelled rabbit-anti-mouse antibody for 30 minutes while kept on ice. As control for auto-fluorescence, cells that were not incubated with the primary and secondary antibody were analyzed. As control for a-specific binding of the FITC-labelled secondary antibody, cells that were not incubated with the primary antibody were analyzed. Data from KD4 and 37B8R cells were compared with those of the parental KYM-1 cells. Three individual experiments were performed.

**Results**

**Effect of DOX, 4-OH-IFO and TRAIL on cell survival.** The sensitivity profiles of the three cell lines to TNF-α as previously described were confirmed by a pilot study (data not shown).

Table 1 shows the sensitivity of the three cell lines to the tested drugs: DOX, 4-OH-IFO and TRAIL. The IC50 (concentration inhibiting cell survival by 50%) level for KYM-1 and KD4 was reached at similar concentrations of both DOX and 4-OH-IFO, while 37B8R was a factor 2 resistant compared to KYM-1 for both drugs.

TRAIL by itself had a strong cytotoxic effect on both KYM-1 and KD4. At the highest concentrations tested (5µg/ml), the cytotoxic effect of TRAIL on KYM-1 was 60%, while for KD4 over 90% cytotoxicity was reached. On the contrary, TRAIL alone had limited effect on 37B8R, never capable of inducing more than 25% cytotoxicity. The drug concentrations used in the combination experiments were titrated on a range of 25 to 75% cytotoxicity (when feasible).
<table>
<thead>
<tr>
<th></th>
<th>KYM-1 mean</th>
<th>KYM-1 SD</th>
<th>KD4 mean</th>
<th>KD4 SD</th>
<th>37B8R mean</th>
<th>37B8R SD</th>
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<tr>
<td>DOX [nM]</td>
<td>3.10 ± 0.63</td>
<td></td>
<td>3.84 ± 0.45</td>
<td></td>
<td>6.1 ± 2.20</td>
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<tr>
<td>4-OH-IFO [nM]</td>
<td>254 ± 126</td>
<td></td>
<td>172 ± 150</td>
<td></td>
<td>538 ± 261</td>
<td></td>
</tr>
<tr>
<td>TRAIL [µg/ml]</td>
<td>0.0065 ± 0.0018</td>
<td>0.0047 ± 0.0037</td>
<td>&gt;5</td>
<td></td>
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</table>

Abbreviations: IC50, concentration inhibiting cell survival by 50%; DOX, doxorubicin; 4-OH-IFO, 4-hydroxy-ifosfamide; TRAIL, tumor necrosis related apoptosis-inducing ligand; SD, standard deviation

Figure 1 shows the effects of combining DOX with TRAIL in the KYM-1 cell line. Both DOX and TRAIL individually decreased cell survival. At all tested concentrations, DOX and TRAIL were able to induce extra cell death. The enhanced cytotoxic effect became also evident in TRAIL-resistant 37B8R cells (Figure 2). For the tested combinations of DOX, 37B8R cells were sensitized to TRAIL-mediated cytotoxicity. When comparing absolute cell survival at isomolar concentrations, KYM-1 was found to be more sensitive to this combination than 37B8R at DOX [3nM] and TRAIL [0.001 µg/ml], but these cell lines were equally sensitive at DOX [15nM] and TRAIL [0.001 µg/ml].
Figure 2. Sensitivity of 37B8R to DOX, TRAIL and combinations of these two drugs (96-hours continuous incubation) as measured in a modified microculture tetrazolium assay.

Table 2 summarizes the enhancement ratio (ER) found for the combination of DOX and TRAIL in the three cell lines. Combining TRAIL with DOX shows synergy (ER > 1.20) in especially KYM-1 and 37B8R, with the effect being most stable in the 37B8R cell line.

The combined effects of 4-OH-IFO and TRAIL have been depicted in Table 3, showing the calculated ER’s. For all combinations and all cell lines, additivity was observed.

**Table 2. DOX and TRAIL**

<table>
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<th>KYM-1</th>
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<td>3.69 (1.85)</td>
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<td>TRAIL [µg/ml]</td>
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<td>0.01</td>
<td>0.97 (0.12)</td>
<td>1.64 (0.42)</td>
<td>2.25 (1.57)</td>
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**Table 3. 4-OH-IFO and TRAIL**

<table>
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<tr>
<th>KYM-1</th>
<th>4-OH-IFO [nM]</th>
<th>20</th>
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<th>200</th>
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<td></td>
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<td>TRAIL [µg/ml]</td>
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<td>0.95 (0.08)</td>
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<td>0.01</td>
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<td>0.97 (0.26)</td>
<td>1.12 (0.32)</td>
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</table>
Enhancement ratio’s of modified microculture tetrazolium assays for KYM-1, KD4 and 37B8R cell lines at indicated concentrations of DOX and TRAIL (Table 2) and 4-OH-IFO and TRAIL (Table 3).

(standard deviation indicated between brackets; synergistic interactions highlighted in bold)

**Induction of apoptosis.** The combination DOX plus TRAIL was further investigated in apoptosis assays, as this combination gave rise to the most pronounced effects in the cytotoxicity assays. AO staining was used to distinguish apoptotic cells from viable cells. After incubation with AO, apoptotic cells were recognized as cells with condensed chromatin, whether intact or already fragmented, intensely staining with AO. Membrane blebbing with shedding of apoptotic bodies was considered as an alternative hallmark of apoptosis. Viable cells had loosely packed (“extended”) chromatin with an intact cellular membrane. Because of the shorter incubation period for apoptosis assays, higher concentrations of TRAIL and DOX were used compared to the modified microculture tetrazolium assays.
Figure 3. Induction of apoptosis by DOX, TRAIL and combinations of these two drugs (24 h exposure): (A) KYM-1; (B) KD4; (C) 37B8R
Figures 3a, 3b and 3c show the results of apoptosis assays in KYM-1, KD4 and 37B8R, respectively. While single-agent induced apoptosis in KYM-1 and KD4 cells, the combination of the two agents increased the percentage of apoptotic cells. KYM-1 and KD4 revealed similar pattern in sensitivity to TRAIL and DOX; still, apoptosis was more pronounced in KD4. After 24 hours of exposure, more KD4 cells than KYM-1 cells were in an advanced stage of apoptosis, with fragmented chromatin and membrane blebbing. Contrary, a relatively small percentage of 37B8R cells revealed features of apoptosis after exposure to DOX and higher doses of TRAIL (Figure 3c). Still, the combination of TRAIL with DOX resulted in a marked increase of apoptosis (Figure 3c). A dramatic increase of apoptosis in 37B8R was observed when DOX [375 nM] was combined with TRAIL, either [0.05µg/ml] or [0.25µg/ml].

Similar to the analysis of modified microculture tetrazolium assays, ER’s were calculated for the induction of apoptosis by the combination of DOX and TRAIL and are show in Table 4. Synergistic apoptosis induction by DOX plus TRAIL was observed for all three cell lines at most concentrations tested. In KYM-1, synergistic ER-values were observed especially at the higher DOX concentrations. Noticeable, the apoptosis induction was quite variable at the highest concentrations. This variability was even higher in KD4 cells, with extremely high ER’s as a result of maximal apoptosis induction. Still, at the lower concentrations of DOX [75nM], the effect remains synergistic even though to a lesser degree, yet more stable as indicated by the narrower standard deviation. The synergistic effect of DOX and TRAIL on apoptosis induction was present as well in 37B8R, although less pronounced than in the other two cell lines. However, while TRAIL and DOX alone did scarcely induce apoptosis, their combination showed apoptosis induction comparable to the KYM-1 cell line. Moreover, the synergistic effect in 37B8R at the higher concentrations of DOX [375 nM] was found to be the most stable of all three cell lines. Higher concentrations of TRAIL were required for 37B8R to obtain similar levels of apoptosis compared to KYM-1, illustrative of a more resistant phenotype.
**Table 4.** Enhancement ratio’s of apoptosis induction in KYM-1, KD4 and 37B8R cell lines at indicated concentrations of DOX and TRAIL. (standard deviation indicated between brackets; synergistic interactions highlighted in bold; n.t. = not tested)

<table>
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<tr>
<th></th>
<th>KYM-1</th>
<th>DOX [nM]</th>
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<th>75</th>
<th>375</th>
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<tbody>
<tr>
<td></td>
<td>TRAIL [µg/ml]</td>
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<td></td>
<td>0.002</td>
<td>n.t.</td>
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<tr>
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<td>DOX [nM]</td>
<td></td>
<td></td>
<td></td>
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<tr>
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<td>TRAIL [µg/ml]</td>
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<tr>
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<td>0.002</td>
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<td>1.54 (0.21)</td>
<td>18.4 (18.7)</td>
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<td>2.59 (2.86)</td>
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<td>37B8R</td>
<td>DOX [nM]</td>
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<td></td>
<td>TRAIL [µg/ml]</td>
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<td>0.01</td>
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<td>1.06 (0.06)</td>
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<td>1.06 (0.03)</td>
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<td>0.25</td>
<td>n.t.</td>
<td>1.06 (0.02)</td>
<td>5.60 (2.77)</td>
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</table>

**TRAIL receptor membrane expression.** KYM-1, KD4 and 37B8R expressed DR4 and DR5, while no or very low levels of DcR1 or DcR2 could be detected. Compared to KYM-1, KD4 expressed factor 1.5 to 2 less DR4, and comparable levels of DR5. Compared to KYM-1, 37B8R showed similar expression of both DR4 and DR5.

**Discussion**

STS comprise a heterogeneous group of malignancies, sharing their origin from primitive mesenchymal cells. As a whole, STS are notorious for early hematogenous spread. The presence of (micro-) metastases limits the curative use of local treatments (surgery and radiotherapy), rendering chemotherapy as the only tumor-directed therapy.

Anticancer drugs exert their cytotoxic effect through the induction of apoptosis in tumor cells. Blockades in the complex routes leading to apoptosis may consequently convey resistance to anticancer drugs. One means to circumvent the resistance to cytotoxic agents is combining them...
with alternative apoptosis-inducing agents. TRAIL is a newly described apoptosis-inducing member of the TNF superfamily of cytokines, which might be of value for these purposes.22

The aim of the present study was to analyze the combined effect of the two most active cytotoxic agents in STS, i.e. DOX and activated IFO, with the new apoptosis-inducing agent TRAIL in a panel of rhabdomyosarcoma cell lines.

The cell lines selected for the current study were initially reported for their sensitivity to TNF-α, the prototype of the TNF superfamily. In later studies, additional data became available for the sensitivity profile to other TNF family cytokines: TRAIL, Fas Ligand, TWEAK.23,24 This panel represents a unique model of related soft tissue sarcoma cell lines with different sensitivity to the TNF family of cytokines, including TRAIL. These characteristics allow exploring the effects of combining cytotoxic agents with TRAIL on TRAIL-sensitive, but even more importantly, TRAIL-resistant STS cells.

DOX and 4-OH-IFO were both capable of inducing cytotoxicity in all three cell lines. As the cell lines have been established in the absence of cytotoxic anticancer agents, no mechanisms of drug resistance driven by the exposure to such drugs could have been evolved. Still, the IC50 to DOX and 4-OH-IFO for the TNF-α/TRAIL resistant cell line 37B8R was higher than for the TNF-α/TRAIL sensitive cell lines KYM-1 and KD4. KYM-1 and KD4 were both sensitive to TRAIL-mediated cytotoxicity. However, while for KD4 nearly complete cell kill could be achieved, a survival of approximately 40% of KYM-1 cells was still observed at the highest tested concentration of TRAIL (5 µg/ml). Caron et al. reported that KD4 cells were resistant to TRAIL at concentrations up to 0.2 µg/ml.19 This difference with the current study might be attributed to molecular differences in TRAIL used (we used native TRAIL versus His-tag TRAIL in the study of Caron) and the method to detect apoptosis (morphological versus flow cytometric analysis of annexin V flip-flop to the external membrane leaflet). The concentrations of TRAIL applied in the present study are corresponding to those of former in vitro and in vivo studies, at which levels clearly identifiable anticancer results were achieved.6,22 We observed that KD4 was the most sensitive cell line to TRAIL; after 24 hr exposure to TRAIL, KD4 cells were already in an advanced stage of apoptosis. Contrary, TRAIL was able to induce 20% cytotoxicity maximum in 37B8R at the tested range of concentrations. Concerning this TRAIL resistance of 37B8R cells, the current study comes to similar
findings to that of the study by Caron et al. In an earlier study on single-agent TRAIL, Petak et al. reported that four out of a panel of seven rhabdomyosarcoma (not KYM-1) cell lines were sensitive to TRAIL. However, in the three TRAIL-resistant cell lines, an additional factor would have been required to achieve significant anticancer effects. Therefore, in the current study we tested TRAIL single agent, but we also tested the interaction of TRAIL with cytotoxic agents.

Combining DOX with TRAIL resulted in an increased effect on cell kill in all three rhabdomyosarcoma cell lines tested. In KYM-1 and KD4, in which substantial cell kill could be achieved with TRAIL single agent, DOX could still contribute to the cytotoxicity. Interesting, in 37B8R, TRAIL alone lacked a significant cytotoxic effect, whereas combination with DOX was able to overcome TRAIL-resistance. In 37B8R, the synergistic effect as determined by ER was the most stable in repeated experiments.

In the laboratory setting, the combination of DOX with TRAIL has proven to be effective in killing epithelial cancer cell lines: prostate, breast, liver, lung, ovarian, colon, squamous cell, melanoma, hematological cancer cell lines, and mesenchymal cancer cell lines. Clayer et al. described the effects of TRAIL and DOX in primary cultures obtained from three STS of different histological type (rhabdomyosarcoma, fibrosarcoma and malignant fibrous histiocytoma). In their study, TRAIL alone had no cytotoxic effect. The combination of TRAIL, however, led to overtly more cytotoxicity in the respective cell lines than DOX alone. The combined effect of TRAIL and DOX was more pronounced compared to the combinations of TRAIL with cisplatin, etoposide, methotrexate or cyclophosphamide.

To our knowledge, this is the first report on the effect of activated IFO and TRAIL. Overall, combinations showed an additive cytotoxic effect on the three tested cell lines. Evdokiou reported on TRAIL with cyclophosphamide, a drug that is structurally related to IFO, on osteogenic sarcoma cells. In these cells, cyclophosphamide alone did not induce cytotoxicity within the tested range of concentrations, nor did it enhance TRAIL-mediated cytotoxicity. However, it remains uncertain whether the investigators had used cyclophosphamide as a pro-drug or in its activated form. Of interest, drug resistant tumor cells can develop cross-resistance to pro-apoptotic cytokines. As mentioned, combining these cytokines with cytotoxic agents might still overcome this resistance. In the current model the combination of DOX and TRAIL prevailed over that of 4-OH-IFO and
TRAIL. The molecular mechanisms by which DOX and TRAIL aid each other in killing cancer cells remain to be investigated.

The underlying mechanism of sensitivity to TRAIL in tumor cells is poorly understood. Apoptosis is mediated by the two death receptors DR4 and DR5, while the two decoy receptors DcR1 and DcR2 interrupt apoptosis. The cell lines presented here expressed similar levels of DR4 and DR5, while DcR1 and DcR2 were absent or nearly detectable. While expression of the death receptors DR4 and DR5 is essential for TRAIL-induced apoptosis, the presented results suggest that their level of expression is not determinative for the sensitivity to TRAIL. Several factors are uncovered that can influence this machinery to reach this final goal. Amongst these are the pro-apoptotic factors caspases 8 and 10, and Bax, and the anti-apoptotic factor FLIP, the family of inhibitors of apoptosis proteins (IAP’s) and the Bcl-2 family. Much is yet unclear about the contribution of these individual factors and it appears conceivable that differences exist on their role between tumors. Laboratory studies continue to uncover the very multifactorial nature of resistance to TRAIL. In this context it is difficult to define a clinically applicable target to circumvent TRAIL-resistance. Meanwhile, as suggested by the current study and others, conventional cytotoxic agents appear a realistic approach to this purpose. Still, it remains a challenge to search for non-toxic agents with these properties.

In the rhabdomyosarcoma cell line KYM-1 and its sublines KD4 and 37B8R, sensitivity to TNF-α and TRAIL paralleled. TRAIL-sensitivity and -resistance was independent from TRAIL receptor expression, suggesting that one or more downstream apoptotic blocks exist in TRAIL resistant 37B8R cells. The data presented here show that in the TRAIL sensitive lines KYM-1 and KD4, enhanced cytotoxicity could be achieved when combining TRAIL with DOX or 4-OH-IFO. Importantly, in the TRAIL resistant cell line 37B8R, DOX with TRAIL could overcome TRAIL-resistance, suggesting a therapeutic potential for this combination for TNF-α and TRAIL refractory STS cells.

References

38. Jazirehi AR, Ng CP, Gan XH et al. Adriamycin sensitizes the adriamycin-resistant 8226/Dox40 human multiple myeloma cells to Apo2L/tumor necrosis factor-related
TRAIL and cytotoxic agents in soft tissue sarcoma cells


