PERGAMON

European Journal of Cancer 39 (2003) 1318–1329

European Journal of Cancer

www.ejconline.com

Trail-induced apoptosis and interaction with cytotoxic agents in soft tissue sarcoma cell lines

S. Tomek*, W. Koestler[a], P. Horak[a], T. Grunt[a], T. Brodowicz[a], I. Pribill[a], J. Halaschek[a], G. Haller[a], C. Wiltschke[a], C.C. Zielinski[a,b], M. Krainer[a]

[a]*Clinical Division of Oncology, Department of Medicine I, Waehringer Guertel 18-20, A-1090 Vienna, Austria*
[b]*Ludwig Boltzmann Institute for Clinical Experimental Oncology, Vienna University Hospital, Waehringer Guertel 18-20, A-1090 Vienna, Austria*

Received 23 September 2002; received in revised form 10 February 2003; accepted 27 February 2003

Abstract

Five human soft tissue sarcoma (STS) cell lines (HTB-82 rhabdomyosarcoma, HTB-91 fibrosarcoma, HTB-92 liposarcoma, HTB-93 synovial sarcoma and HTB-94 chondrosarcoma) were analysed for their sensitivity to tumour necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) and the function of the TRAIL apoptotic pathway in these cells. TRAIL induced significant apoptosis (>90%) in HTB-92 and HTB-93 cells, whereas no effect was observed in HTB-82, HTB-91 and HTB-94 cells. TRAIL-Receptor 1 (TRAIL-R1) was expressed in TRAIL-sensitive HTB-92 and HTB-93 cell lines, but not in TRAIL-resistant HTB-91 and HTB-94 cells. HTB-82 cells, which expressed the long (c-FLIP(L)) and short (c-FLIP(S)) splice variants of the FLICE-like inhibitory protein (FLIP), were resistant to TRAIL in spite of the presence of TRAIL-R1. TRAIL-R2,-R3,-R4 and osteoprotegerin (OPG) expression did not correlate with TRAIL sensitivity. Coincubation of TRAIL and doxorubicin led to the overexpression of TRAIL-R2 resulting in a synergistic effect of doxorubicin and TRAIL in TRAIL-sensitive cell lines and in the overcoming of TRAIL-resistance in all of the TRAIL-resistant cell lines, except HTB-91, which lacked caspase 8 expression. These data suggest that TRAIL, either as a single agent or in combination with cytotoxic agents, might represent a new treatment option for advanced STS, which constitutes a largely chemotherapy-resistant disease.

Keywords: TRAIL; Soft tissue sarcoma; FLIP; Caspase-8; Apoptosis; Doxorubicin; Paclitaxel

1. Introduction

Soft tissue sarcoma (STS) encompasses an abundance of malignant tumours of the connective tissue with various histological subtypes exhibiting similar biological characteristics and response to treatment. The efficacy of chemotherapeutic agents upon control of proliferation and/or induction of apoptosis in STS is limited [1,2]. Therefore, based on improved knowledge of tumour biology with respect to dysregulation of apoptosis, new agents with novel and specific mechanisms of action are being sought. Previous investigations from our laboratory have shown that retinoid-induced inhibition of proliferation and induction of apoptosis were associated with intact p53 within a panel of STS cell lines [3].

Members of the tumour necrosis factor (TNF) cytokine family including TNF-alpha and Fas ligand (FasL) are capable of inducing apoptosis independently from p53 through interaction with their cognate death receptors. TNF-alpha was previously shown to induce apoptosis in STS cells *in vitro* [4–7]. However, due to severe systemic toxicity including a potential nuclear factor Kappa B-mediated lethal inflammatory response [8,9], *in vivo* TNF-alpha administration is restricted to isolated limb perfusion [10–17]. Thus, the therapeutic potential of TNF-alpha in metastatic STS is to be regarded as limited at best.

TNF-related apoptosis-inducing ligand (TRAIL; Apo2L) represents a type II transmembrane protein exerting sequence homology to TNF-alpha and FasL [18–20]. Previous studies demonstrated TRAIL-mediated apoptosis in a variety of human cancer cell lines [21–27]. However, in contrast to other TNF-family members, TRAIL did not exert cytotoxic effects upon normal cells *in vitro* as well as *in vivo* [18,20,22,28,29]. Thus, TRAIL

* Corresponding author. Tel.: +43-1-40400-4445; fax: +43-1-40400-4452.
E-mail address: sandra.tomek@akh-wien.ac.at (S. Tomek).

0959-8049/03/$ - see front matter © 2003 Elsevier Science Ltd. All rights reserved.
doi:10.1016/S0959-8049(03)00227-2

merits further investigation as a possible safe alternative and potential candidate for cancer treatment.

TRAIL interacts with the pro-apoptotic death receptors TRAIL-R1 (DR4) [30] and the p53 regulated TRAIL-R2 (KILLER/DR5, TRICK2) [31–35], as well as with the inhibitory decoy receptors TRAIL-R3 (TRID, DcR1, or LIT) [33,36,37] and TRAIL-R4 (TRUNDD or DcR2) [38,39], both of which lack a cytoplasmatic death domain.

It is not unanimously agreed upon whether a certain pattern of TRAIL-receptor expression predicts sensitivity or resistance to the ligand: TRAIL-induced apoptosis was shown to be partly regulated by relative levels of TRAIL-R1, TRAIL-R2 and TRAIL-R3 [30,36,37,40] as well as by the expression level of TRAIL-R1 [41] or TRAIL-R4 [21] solely or the absence of decoy TRAIL receptors [22]. Other authors suggested that the expression profile of the TRAIL receptors did not completely follow the apoptotic potency of TRAIL [21,24,27,42,43]. Furthermore, the presence of anti-apoptotic proteins including the caspase activation inhibitor FAAD-like IL-1ß-converting enzyme (FLICE)-like inhibitory protein (FLIP) and the newly identified secreted TRAIL-receptor osteoprotegerin (OPG) may play a critical role in resistance to TRAIL-induced apoptosis [21,27,41,42,44,45].

TRAIL-mediated tumouricidal effects were shown in a wide range of malignant cell lines [21–27]. However, to our knowledge, only few data regarding the activity of TRAIL in STS have been published so far [24]. In the present model, we have investigated TRAIL-mediated effects upon five STS cell lines of various origins (chondrosarcoma, fibrosarcoma, liposarcoma, rhabdomyosarcoma and synovial sarcoma) in terms of induction of apoptosis and expression of TRAIL-R1,-R2,-R3 and-R4 as well as of apoptosis-modulating proteins caspase 8, FLIP and OPG. In addition, we have examined the cytotoxic effect of DNA-damaging agents, doxorubicin and paclitaxel, on STS cells and whether coincubation of TRAIL with doxorubicin or paclitaxel could produce additive effects in inducing apoptosis. Finally, the correlation of TRAIL sensitivity with p53 status was investigated.

2. Materials and methods

2.1. Cell lines

HTB-91 (fibrosarcoma, SW 684), HTB-92 (liposarcoma, SW 872), HTB-93 (synovial sarcoma, SW 982), HTB-94 (chondrosarcoma, SW 1353) and HTB-82 (rhabdomyosarcoma, A-204) STS cell lines were obtained from the American Type Culture Collection (ATCC, Rockville, Maryland, USA). The HTB-91, HTB-92 and HTB-94 cell lines were cultured in Leibowitźs L-15 medium with L-glutamine (PAA Laboratories Gmbh, Linz, Austria) supplemented with 10% heat-inactivated fetal calf serum (FCS) (Invitrogen, Lofer, Austria), 50U penicillin and 50μg streptomycin (Margaritella/Merck, Vienna; Austria) per ml. The human umbilical cord endothelial cells (HUVEC*) were a gift from Dr. Zyhdi Zhegu (Institute of Physiology/University of Vienna) and were obtained by a primary culturing technique. The normal human chondrocytes were purchased from PromoCell bioscience alive GmbH (Heidelberg, Germany). Both benign cell lines were cultured in the cell type-specific appropriate medium supplied by PromoCell bioscience alive GmbH (Heidelberg, Germany). Experiments with primary culture cells were performed within two to four passages.

2.2. TRAIL-, paclitaxel-, and doxorubicin-mediated cytotoxicity and apoptosis

Recombinant human TRAIL (soluble 19.6 kDa protein, comprising the full-length of the extracellular domain of human TRAIL) was purchased from CHEMICON International Inc. (Temecula, CA, USA). Cells were plated in 6-well plates (Costar, Cambridge, MA, USA) at a density of 1×10^6 cells/well and allowed to adhere to the plate overnight. To assess the tumouricidal activity of TRAIL, recombinant human TRAIL was added (final concentrations: 100, 300 and 1000 ng/ml) and cells were then incubated for an additional 6, 12, 24 and 48 h. Cell viability was determined by the dimethylthiazolyl-2,5-diphenyltetrazolium bromide (MTT) dye reduction assay as previously described in Ref. [46] and apoptosis was measured by a DNA fragmentation assay as described below. In order to investigate the effect of TRAIL in the presence of paclitaxel or doxorubicin, co-incubation experiments were carried out: cells were plated as described above and eventually allowed to adhere overnight. Subsequently, TRAIL (final concentrations: 100, 300 and 1000 ng/ml) and doxorubicin (final concentrations: 0.5, 5 and 50 μM; Pharmacia & Upjohn, Kalamazoo, MI, USA) or paclitaxel (final concentrations: 0.5, 5 and 50 μM; Bristol-Myers Sqibb Co., Princeton, NJ, USA) were added and coincubated for 6, 12 and 24 h, respectively. Cell death was determined by the MTT dye reduction assay [46] and apoptosis was assessed by DNA fragmentation assay as described below.

2.3. DNA fragmentation assay analysed by flow cytometry

Using the Apo-Direct™ Kit (Phoenix Flow Systems, San Diego, CA, USA) the 3′OH termini in DNA breaks were measured by attaching fluorescent tagged deoxyuridine triphosphate nucleotides, fluorescein isothiocyanate (FITC)-dUTP, in a reaction catalysed by

terminal deoxynucleotidyl transferase (TdT) as previously described in Ref. [3]. The amount of incorporated fluorescein was detected by flow cytometry.

2.4. RT-PCR*

The expression of TRAIL-R1,-2,-3,-4, FLIP, OPG and caspase 8 was investigated by reverse transcriptase-polymerase chain reaction (RT-PCR). Total RNA was isolated from cell lines by standard procedures. First-strand cDNA was generated from total RNA using SuperScript II Rnase H Reverse Transcriptase (Life Technologies Inc., Gaithersburg, MD, USA). Oligo $(dt)_{12-18}$ were incubated with 200 units SuperScript II and 2 μg of total RNA for 50 min at 42 °C. PCR reaction was performed with 5 μl of cDNA template in a final volume of 25 μl containing all 4 deoxynucleotide triphosphates (dNTPs) (2 μl 10 mM dNTP Mix), 2.5 μl 10× PCR puffer, 0.2 μl *Taq* DNA polymerase (5 U/μl), and 1 μl (10 pmol) of each primer. PCR conditions were as follows: 5 min denaturation at 94 °C followed by 35 cycles, 30 s/94 °C; 30 s/60 °C; 30 s/72 °C. The PCR-amplified products were run on a 1% agarose gel containing ethidium bromide and were visualised under ultraviolet light. Amplification of β-2 microglobulin served as a positive control.

The sequence of specific primers used in this experiment were as follows:
DR4-RT5, CGATGTGGTCAAAGCTGGTACAGC;
DR4-RT3, GGACACGGCAGAGCCTGTGCCATC;
DR5-RT5, GGAGCCGCTCATGAGGAAGTTGG;
DR5-RT3, GGCAAGTCTCTCTCCCAGCGTCTC;
DcR1-RT5, ACCCTAAAGTTCGTCGTCATC;
DcR1-RT3, TTATCCACACCCTCTGTGCA;
DcR2-RT5, CTTTTCCGGCGGCGTTCATGTCCTTC;
DcR2-RT3, GTTTCTTCCAGGCTGCTTCCCTTTGTA;
FLIP-RT5, AATTCAAGGCTCAGAAGCGA;
FLIP-RT3, GGCAGAAACTCTGCTGTTCC;
OPG-RT5, GTGACGAGTGTCTATACTGCA;
OPG-RT3, ATCCTCTCTACACTCTCTGCG;
CASP-8-RT5, TCTGGAGCATCTGCTGTCTG;
CASP-8-RT3, CCTGCCTGGTGTCTGAAGTT.

2.5. TRAIL-R1 sequence analysis

PCR products were subjected to cycle sequencing using an automated fluorescence based cycle sequencer (model 310; PE Applied Biosystems, Foster City, CA, USA) and Taq dye terminator chemistry. Primers used in amplifying the death domain of DR-4 were DR4-11 CTCTGATGCTGTTCTTTGAC and DR4-12 TCACTCCAAGGACACGGCAGA. The sense and antisense strands of the sequenced products were aligned with that of the wild-type sequence using the Sequence Navigator program (PE Applied Biosystems, Foster City, CA, USA).

2.6. Western Blot analysis

Protein expression of TRAIL-R1,-2,-3,-4, FLIP and caspase 8 was determined by Western Blot analysis. Cells were lysed in 1 ml of lysis buffer containing 10 mM Tris–HCl pH7.4, 150 mM NaCl, 1 mM ethylene diamine tetra acetic acid (EDTA), 1% Triton X-100, 1% Na-Deoxycholate, 0.1% sodium dodecyl sulphate (SDS) and 1/100 Phosphatase Inhibitor Cocktail II (Sigma-Aldrich, St. Louis, MO, USA), left at 4 °C for 10 min and centrifuged at 4 °C and 15 000 rpm. The supernatant was boiled for 10 min in SDS loading buffer, separated by SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. Primary antibodies used were: rabbit polyclonal anti-DR4 (NT) (1:200) (ProSciInc, Poway, CA, USA), goat polyclonal anti-human TRAIL R2/DR5/TNFRSF10B (1:500) (R&D Systems, Inc. Minneapolis, MN, USA), goat anti-human TRAIL R3/TNFRSF10C antibody (1:1000) (R&D Systems, Inc. Minneapolis, MN, USA), goat polyclonal anti-human TRAIL R4 antibody (1:1200) (R&D Systems, Inc. Minneapolis, MN, USA), mouse monoclonal Caspase 8 (FLICE) Ab-1 (1:200) (clone 8CSP01) (NeoMarkers, Fremont, CA, USA), goat polyclonal $FLIP_L$ (C-19) (1:500), goat polyclonal $FLIP_S$ (F-20) (1:900) and goat polyclonal Actin (I-19) (1:300) (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). Secondary antibodies used were anti-goat IgG peroxidase conjugate (1:20000) (Sigma-Aldrich, St. Louis, MO, USA), anti-mouse (NA 931) and anti-rabbit (NA 934) IgG HRP-linked (1:50000) (Amersham Biosciences, Piscataway, NJ, USA). Protein determination was done by Protein Assay ESC (Roche Diagnostics, IN, USA). In cases of quantitative experiments, 40 μg of total protein was loaded and the loading control performed by β-actin staining. We used ChemiGlow™ (Alpha Innotech Corp, San Leandro, CA, USA) to detect chemi-fluorescence on the Western Blots.

To focus observations on TRAIL receptors located on the cellular membranes, the Mem-PER Eukaryotic Membrane Extraction Reagent Kit (Pierce Biotechnology, IL, USA) was used. Briefly, harvested cells were lysed with a proprietary detergent and then a second proprietary detergent was added to solubilise membrane proteins. The cocktail was incubated at 37 °C to separate hydrophobic from hydrophilic proteins through phase portioning. The hydrophobic fraction was used for Western Blot analysis as described above.

2.7. p53 Immunohistochemistry and p53 sequence analysis

Immunohistochemical staining of p53 protein and p53 sequence analysis were performed as previously described in Ref. [3].

2.8. Statistical analysis

The data were expressed as means ± standard error of the means (S.E.M.s) calculated from three separate experiments, each performed in triplicate.

2.8.1. Data analysis for combination treatment

Synergism was determined by calculating the combination index (CI) using the median effect analysis for fixed drug dose combinations [47–50].

$$CI_x = (D)_1/(Dx)_1 + (D)_2/(Dx)_2 + \alpha(D)_1(D)_2/(Dx)_1(Dx)_2$$

where CIx is the CI-value for x% effect. $(Dx)_1$ and $(Dx)_2$ are the doses of agents 1 and 2 required to exert x% effect alone, whereas $(D)_1$ and $(D)_2$ are the doses of agents 1 and 2 that elicit the same x% effect in combination with the other agent, respectively. α describes the mode of interaction: $\alpha = 0$ for mutually exclusive (similar modes of action), $\alpha = 1$ for mutually non-exclusive drugs (independent modes of action). CI = 1 indicates additivity, CI < 1 synergism and CI > 1 antagonism.

3. Results

3.1. TRAIL-induced apoptosis in HTB-93 and HTB-92 cells

Five human STS cell lines (HTB-82, HTB-91, HTB-92, HTB-93 and HTB-94) as well as two benign cell lines, human chondrocytes and human umbilical cord endothelial cells (HUVEC), were tested for their sensitivity to TRAIL-mediated apoptosis. TRAIL sensitivity was defined as a <75% cell viability and/or >25% apoptosis at 12 h after the initiation of incubation with TRAIL, compared with control cells and quantitated by the MTT dye reduction assay and a DNA fragmentation analysis. STS cell lines showed a variable response to TRAIL (Fig. 1; Table 1): TRAIL-mediated apoptosis was observed in HTB-92 and HTB-93 cells in a dose- and time-dependent manner, with up to 90 and 100% apoptotic cells after 24 or 48 h incubation with 1000 or 300 ng/ml TRAIL, respectively (Fig. 2a and b). In addition, cell viability was reduced to less than 5% (Fig. 2c). In contrast, HTB-82, HTB-91 and HTB-94 cells were found to be resistant to TRAIL after an incubation period of up to 48 h and with final TRAIL concentrations of up to 1000 ng/ml. The benign cell lines tested, HUVEC and human chondrocytes, were resistant to TRAIL after any incubation time and at any final TRAIL concentration.

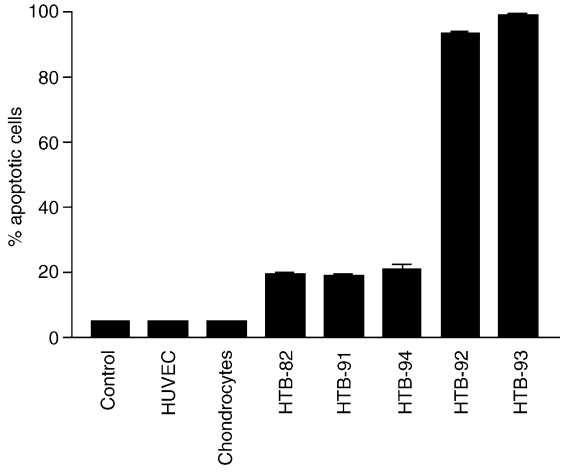

Fig. 1. Sensitivity of cell lines to treatment with TRAIL. Cells were incubated for 24 h in the presence of TRAIL (1000 ng/ml) and apoptosis was evaluated by DNA-fragmentation assay. Each treatment was performed in triplicate and the plotted values represent the means ±standard error of the means (S.E.M.) of three independent experiments. HUVEC, human umbilical cord endothelial cells.

Table 1
TRAIL sensitivity and p53 genotype characterised in five human soft tissue sarcoma cell lines. Wild-type p53 was found in HTB-93 cells whereas p53 mutations were detected in HTB-91, HTB-92 and HTB-94 cell lines. Sensitivity of STS cell lines to TRAIL did not correlate with p53 status

Cell line	Base change	Exon/codon	Amino acid change	TRAIL-sensitivity
HTB-82 (Rhabdomyosarcoma)	CGA to CGG	6/213	Arg.-Arg; silent	TRAIL-resistant
HTB-91 (Fibrosarcoma)	ATC to AAC	7/251	Ile-Asn	TRAIL-resistant
HTB-92 (Liposarcoma)	GTG to TTG	6/203	Val-Leu	TRAIL-sensitive
HTB-93 (Synovial sarcoma)	Wild-type	–	–	TRAIL-sensitive
HTB-94 (Chondrosarcoma)	CGA to TGA	6/213	Arg-STOP	TRAIL-resistant
HTB-94 (Chondrosarcoma)	TTC to TTT	10/341	Phe-Phe; silent	TRAIL-resistant

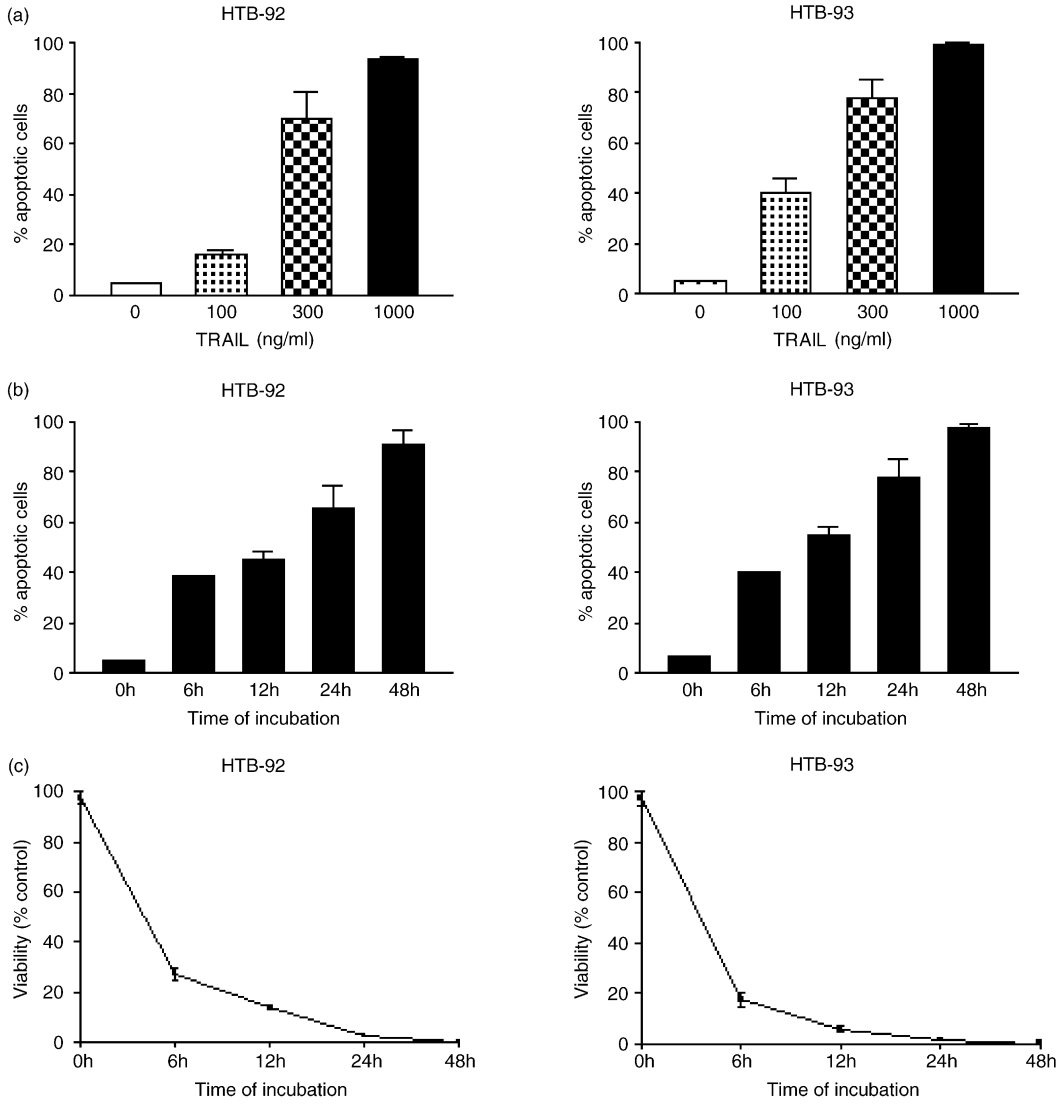

Fig. 2. HTB-92 and HTB-93 cells were incubated with TRAIL at 100, 300 and 1000 ng/ml for 24 h (a) and with a fixed concentration of TRAIL (300 ng/ml) for 6, 12, 24 and 48 h (b, c). Apoptosis was measured by a DNA-fragmentation analysis (a, b) and cell viability was evaluated by the MTT assay (c). Each treatment was performed in triplicate and the plotted values represent the means±S.E.M of three independent experiments.

3.2. TRAIL receptor expression and sensitivity to TRAIL

TRAIL-R1, TRAIL-R2, TRAIL-R3 and TRAIL-R4 expression were analysed by RT-PCR and Western Blotting in STS cell lines in order to determine whether a certain pattern of TRAIL-receptor expression correlated with TRAIL-sensitivity or-resistance (Figs. 3 and 4, Table 2): TRAIL-R1 was expressed in TRAIL sensitive HTB-92 and HTB-93 cells, but not in TRAIL-resistant HBT-91 and-94 cell lines and thus, appeared (on the whole) to correlate with the sensitivity to TRAIL. Yet this observation was not confirmed in HTB-82 cells, which expressed TRAIL-R1, but were highly resistant to TRAIL, indicating different mechanisms of resistance.

TRAIL-R2 was expressed in both TRAIL-sensitive (HTB-92 and HTB-93) as well as TRAIL-resistant (HTB-82,-91 and-94) cell lines indicating no correlation of TRAIL-R2 expression with TRAIL-sensitivity. Likewise, expression of the decoy receptors TRAIL-R3 and TRAIL-R4 failed to predict for TRAIL sensitivity or resistance, which is in contrast to previous findings [21,22,37,38,40], where expression of theses non-functional receptors correlated inversely with sensitivity to TRAIL.

3.3. Expression of genes regulating TRAIL-induced apoptosis

Searching for other factors which might influence TRAIL-induced apoptosis, we investigated the

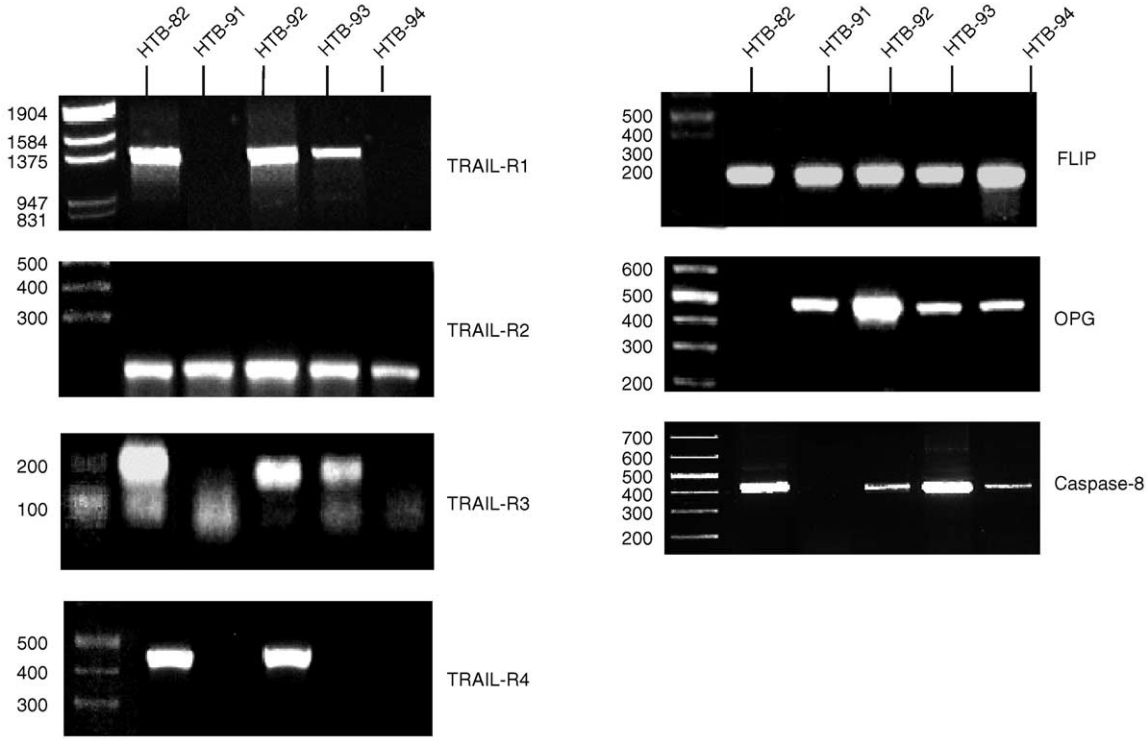

Fig. 3. Expression of TRAIL-receptors (TRAIL-R1,-2,-3,-4), FLIP, OPG and caspase-8 was assessed by reverse transcriptase-polymerase chain reaction (RT-PCR) in the five soft tissue sarcoma (STS) cell lines as described in "Materials and methods".

Table 2
Expression of mRNA and protein of TRAIL-receptors (TRAILR1-R4), caspase 8, FLIP and OPG in five STS cell lines (HTB-82, HTB-91, HTB-92, HTB-93, HTB-94), as assessed by RT-PCR and Western Blotting (W), and sensitivity to TRAIL

Cell line	TRAIL-sensitivity	TRAIL-R1		TRAIL-R2		TRAIL-R3		TRAIL-R4		Caspase 8		FLIP				OPG
		RT-PCR	W	RT-PCR	W	RT-PCR	W	RT-PCR	W	RT-PCR	W	RT-PCR	W			RT-PCR
													c-FLIP(L)	c-FLIP(S)		
HTB-82	–	+	+	+	+	+	+	+	+	+	+	+	+	+	–	
HTB-91	–	–	–	+	+	–	–	–	–	–	–	+	–	–	+	
HTB-92	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	
HTB-93	+	+	+	+	+	+	+	–	–	+	+	+	–	+	+	
HTB-94	–	–	–	+	+	–	–	–	–	+	+	+	–	–	+	

(+) Detectable, (–) Not detectable.

expression of caspase 8, FLIP and OPG in STS cell lines (Figs. 3 and 4, Table 2). Caspase 8, which has been described to be crucial in TRAIL-induced apoptosis [51], was present in all cell lines with the exception of the TRAIL-resistant HTB-91 cells. mRNA of the caspase activation inhibitor FLIP could be detected in all cell lines, but Western Blotting revealed that protein expression of the short (c-FLIP(S)) and long (c-FLIP(L)) splice variants of FLIP varied between cell lines. HTB-93 cells (TRAIL-sensitive) expressed c-FLIP(S), HTB-92 cells (TRAIL-sensitive) expressed c-FLIP(L) and HTB-82 cells (TRAIL-resistant) showed expression of both c-FLIP variants (Fig. 4). With the exception of one cell line (HTB-82), both TRAIL-sensitive (HTB-92, HTB-93) as well as TRAIL-resistant (HTB-91, HTB-94) STS cell lines were found to express OPG mRNA.

3.4. A-to-G alteration in the death domain of TRAIL-R1

In SKOV3 and J82 cells, an A-to-G alteration in nucleotide 1322 in the death domain of TRAIL-R1 has previously been demonstrated to lead to TRAIL resistance in spite of TRAIL-R1 expression. The A-to-G sequence change was heterozygous in both cell lines and resulted in the conversion of the amino acid lysine (codon 441) to arginine [41]. Similarly, an A-to-G

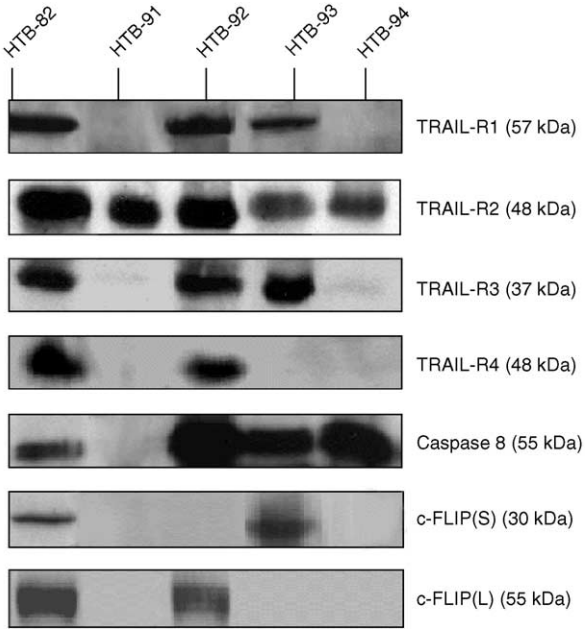

Fig. 4. Expression of TRAIL-receptors (TRAIL-R1,-2,-3,-4), caspase 8, c-FLIP(S) and c-FLIP(L) in STS cell lines was determined by Western Blotting. To ascertain that the TRAIL-receptors investigated were located on the cellular membranes, membrane protein extraction was performed as described in "Materials and methods".

sequence change was detected in HTB-92 and HTB-93 STS cell lines both of which were heterozygous. However, HTB-92 as well as HTB-93 cells showed high sensitivity to TRAIL-induced apoptosis.

3.5. Induction of apoptosis by TRAIL and cytotoxic agents in STS cell lines

Cytotoxic agents, doxorubicin and paclitaxel, were tested for their ability to induce apoptosis in HTB-82, HTB-91, HTB-92, HTB-93 and HTB-94 cells in a time- and concentration-dependent manner. While the cytotoxicity of paclitaxel was high, doxorubicin failed to induce significant apoptosis in STS cells *in vitro* (Fig. 5a and b).

In order to determine whether TRAIL-induced apoptosis could be augmented by cytotoxic agents, STS cells were incubated with doxorubicin (0.5, 5 and 50 μM) or paclitaxel (0.5, 5 and 50 μM) and TRAIL (100, 300 and 1000 ng/ml) for 6, 12 and 24 h, respectively. The resulting data demonstrated that coincubation of TRAIL with doxorubicin was able to overcome apoptotic resistance to either agent alone, leading to apoptosis up to 90–95% and reducing cell numbers of HTB-94 and HTB-82 cells by 5–10% (Fig. 6; Table 3). In contrast, no similar effect was observed in HTB-91 cells lacking caspase 8. The combination of TRAIL and paclitaxel did not reverse resistance to either agent alone.

In TRAIL-sensitive cell lines, TRAIL and doxorubicin were synergistic (Table 4) and co-incubation with TRAIL and paclitaxel produced an additive response (Fig. 7).

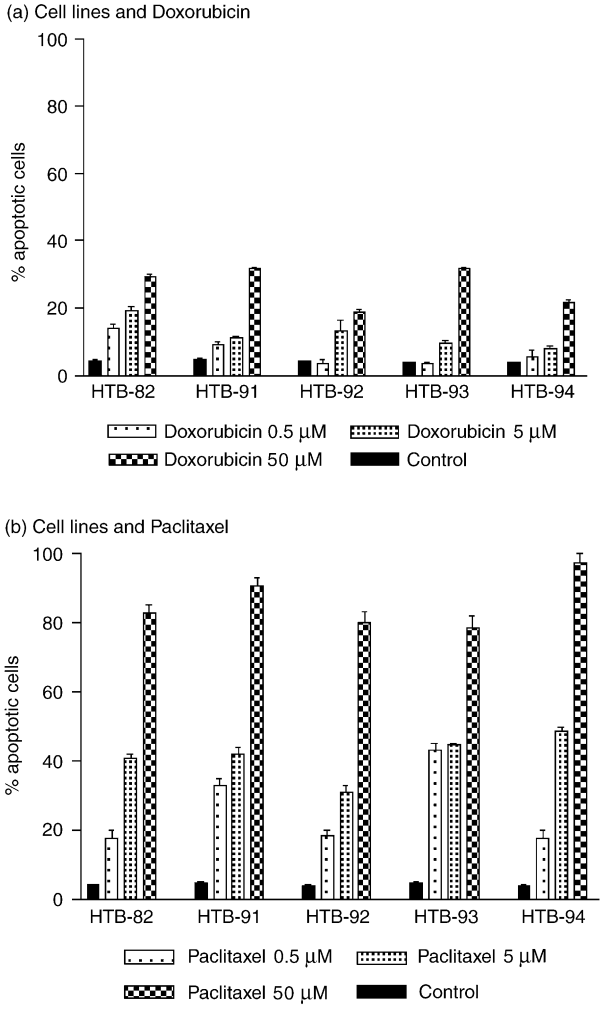

Fig. 5. Induction of apoptosis by doxorubicin and paclitaxel in STS cells. Cells were incubated for 24 h in the presence of doxorubicin (0.5, 5 or 50 μM) (a) or paclitaxel (0.5, 5 or 50 μM) (b). The percentage of apoptosis was measured by DNA-fragmentation assay. Each treatment was performed in triplicate and the plotted values represent the means±S.E.M of three independent experiments.

3.6. Effect of doxorubicin and TRAIL on TRAIL-receptor expression

TRAIL-R1 and-R2 mRNA and protein were measured after exposure of STS cells to doxorubicin and TRAIL in order to investigate, whether a subsequent change in TRAIL receptor expression was responsible for the augmentation of TRAIL-induced apoptosis by doxorubicin. A strong upregulation of TRAIL-R2 protein was detected in all TRAIL-sensitive cell lines as well as in TRAIL-resistant HTB-82 cells in response to doxorubicin treatment. In contrast, not the anthracycline, but TRAIL itself led to an increased expression of the proapoptotic receptor in TRAIL-resistant HTB-91 and-94 cells (Fig. 8). No induction of TRAIL-R1 mRNA or protein was observed in both TRAIL-sensitive and TRAIL-resistant STS cell lines (data not shown).

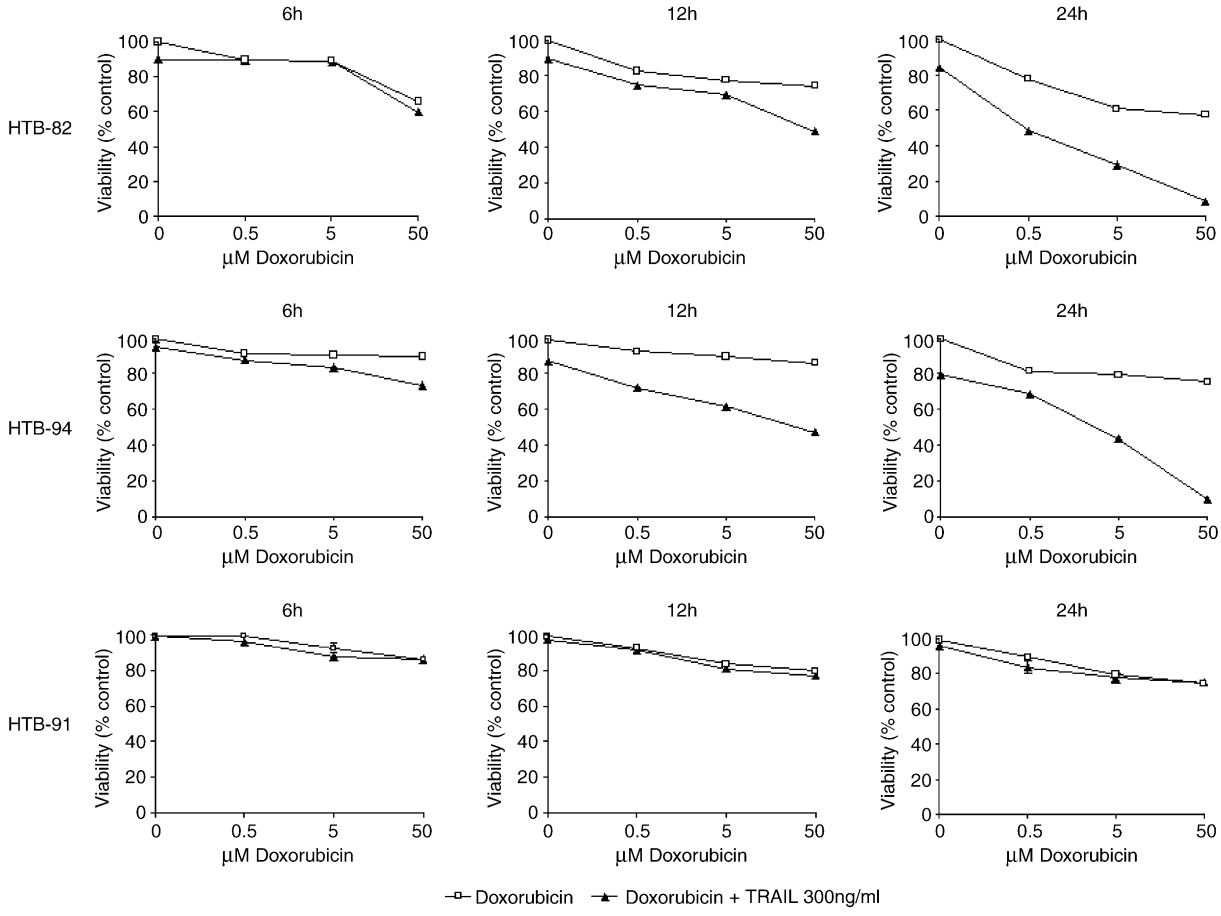

Fig. 6. Viability of TRAIL-resistant cell lines HTB-94, HTB-82 and HTB-91 incubated with doxorubicin (0.5, 5 or 50 μM) plus or minus 300 ng TRAIL for 6, 12 and 24 h. Each treatment was performed in triplicate and the plotted values represent the means ± S.E.M. of three independent experiments.

3.7. TRAIL sensitivity and p53 status

HTB-91, HTB-92, HTB-93 and HTB-94 cells were screened for their p53 genotype and p53 protein expression. No apparent correlation of STS cell line sensitivity to TRAIL with p53 status was detected (Table 1). Likewise, upregulation of TRAIL-R2 by doxorubicin appeared to be independent of the p53 status.

4. Discussion

As chemotherapeutic agents in metastatic STS have been shown to be of limited efficacy, novel treatment strategies based on a better understanding of the multistep tumourigenic process in STS need to be explored. Unlike chemotherapeutic agents which frequently require intact p53 to drive tumour cells into

Table 3
Combination of 'TRAIL' with 'Doxorubicin' at the dose ratio of 1:2.7 ('TRAIL': 'Doxorubicin') in HTB-82 and -94 cells. Determination of the combination index (CI) for 30% (CI_{30}) and 50% (CI_{50}) growth inhibition[a]

	Combination ratio	CI Values at			
		IC_{30}		IC_{50}	
		Excl.	Non-excl.	Excl.	Non-excl.
HTB-82					
TRAIL (ng/ml) + DOXO (ng/ml)	1:2.7	0.114	0.115	0.017	0.017
HTB-94					
TRAIL (ng/ml) + DOXO (ng/ml)	1:2.7	0.042	0.042	0.021	0.021

CI < 1, = 1 or > 1 indicates synergism, additivity or antagonism. Representative values of one out of four determinations.

[a] Calculated as described in Materials and methods for mutually non-exclusive (non-excl.) effects (independent modes of action, $\alpha = 1$) and mutually exclusive (excl.) effects (similar modes of action, $\alpha = 0$).

Table 4
Combination of 'TRAIL' with 'Doxorubicin' at the dose ratio of 1:2.7 ('TRAIL': 'Doxorubicin') in HTB-82 and -94 cells. Determination of the combination index (CI) for 30% (CI_{30}) and 50% (CI_{50}) apoptosis[a]

	Combination ratio	CI Values at			
		IC_{30}		IC_{50}	
		Excl.	Non-excl.	Excl.	Non-excl.
HTB-92 TRAIL (ng/ml) + DOXO (ng/ml)	1:2.7	0.135	0.135	0.119	0.119
HTB-93 TRAIL (ng/ml) + DOXO (ng/ml)	1:2.7	0.946	0.950	0.448	0.448

CI <1, $=1$ or >1 indicates synergism, additivity or antagonism. Representative values of one out of four determinations.
[a] Calculated as described in Materials and methods for mutually non-exclusive effects (independent modes of action, $\alpha=1$) and mutually exclusive effects (similar modes of action, $\alpha=0$).

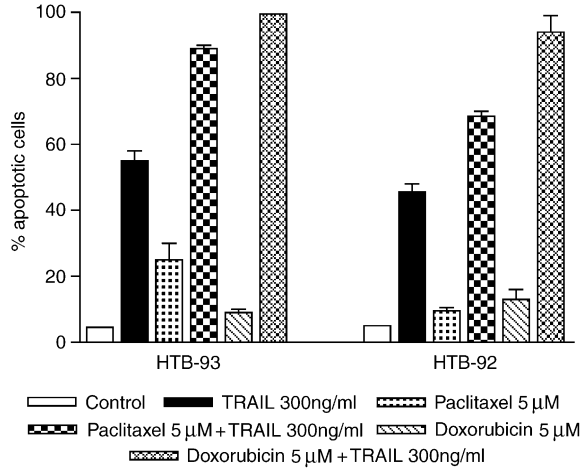

Fig. 7. Induction of apoptosis in TRAIL-sensitive cells by chemotherapeutic agents and TRAIL. Cells were incubated for 12 h in the presence of TRAIL (300 ng/ml), doxorubicin (5 µM), paclitaxel (5 µM) or the combination of chemotherapeutic agent and TRAIL (300 ng/ml). The percentage of apoptosis was measured by a DNA-fragmentation assay. Each treatment was performed in triplicate and the plotted values represent the means±S.E.M. of three independent experiments.

programmed cell death, members of the tumour necrosis factor (TNF)-family of cytokines including TNF-alpha and FasL are capable of inducing apoptosis independently from p53. TNF-alpha was previously shown to exert clinical efficacy in patients with locally advanced STS of the extremities [10–17]. However, due to severe systemic toxicity, TNF-alpha administration could be performed via isolated limb perfusion only. Similarly, the therapeutic usefulness of FasL was hampered by Fas expression on hepatocytes resulting in lethal hepatic apoptosis upon intravenous administration [52].

TRAIL is a type II transmembrane protein recently discovered on account of its sequence homology to TNF-alpha and FasL [18–20]. In contrast to other TNF-family members, TRAIL is capable of inducing apoptosis in a variety of malignant cells without affecting normal tissue thus having potential for application in tumour treatment *in vivo*. Although data have been generated for the induction of apoptosis in a variety of cancer cell lines [21–23,25–27] including rhabdomyosarcoma [24], only a little is known about the biological effects of TRAIL in STS. Our investigations have attempted to elucidate the latter aspect and concentrated upon the induction of apoptosis by TRAIL, its interaction with appropriate receptors, its regulation and dependence upon the presence of wild-type p53, and finally, the potential additive effect upon apoptosis exerted by cytotoxic drugs *in vitro*.

In the present study, we have shown that TRAIL was capable of inducing apoptosis in liposarcoma (HTB-92) and synovial sarcoma (HTB-93) cell lines, whereas no effect was seen in rhabdomyosarcoma, (HTB-82), fibrosarcoma (HTB-91) and chondrosarcoma (HTB-94) cells. In continuation of these experiments, we assessed expression profiles of TRAIL-receptors (TRAIL-R1,-2, 3-, and-4) and genes regulating TRAIL-induced apoptosis. Our results indicate that TRAIL-R1, which was expressed in TRAIL-sensitive HTB-92 and HTB-93 cell lines, but not in TRAIL-resistant HTB-91 and HTB-94 cells, was an important predictor of TRAIL-sensitivity. In contrast, expression of proapoptotic TRAIL-R2 and of decoy receptors TRAIL-R3 and-R4 demonstrated no correlation with TRAIL sensitivity. mRNA of the caspase activation inhibitor FLIP was detectable in all STS cell lines and thus did not serve as a useful predictor for TRAIL resistance. In contrast, Western Blotting revealed a heterogenous expression profile of the long (c-FLIP(L)) and short (c-FLIP(S)) splice variant of FLIP. The exclusive presence of one c-FLIP variant in HTB-92 and -93 cells did not confer TRAIL resistance. Our data suggest, however, that expression of both c-FLIP variants might lead to TRAIL resistance in spite of the presence of TRAIL-R1 and caspase 8, as demonstrated in HTB-82 cells. Expression of the secreted TNF homologue OPG was investigated in order to determine whether OPG inhibited TRAIL-induced apoptosis in STS cells, as previously described in Jurkat

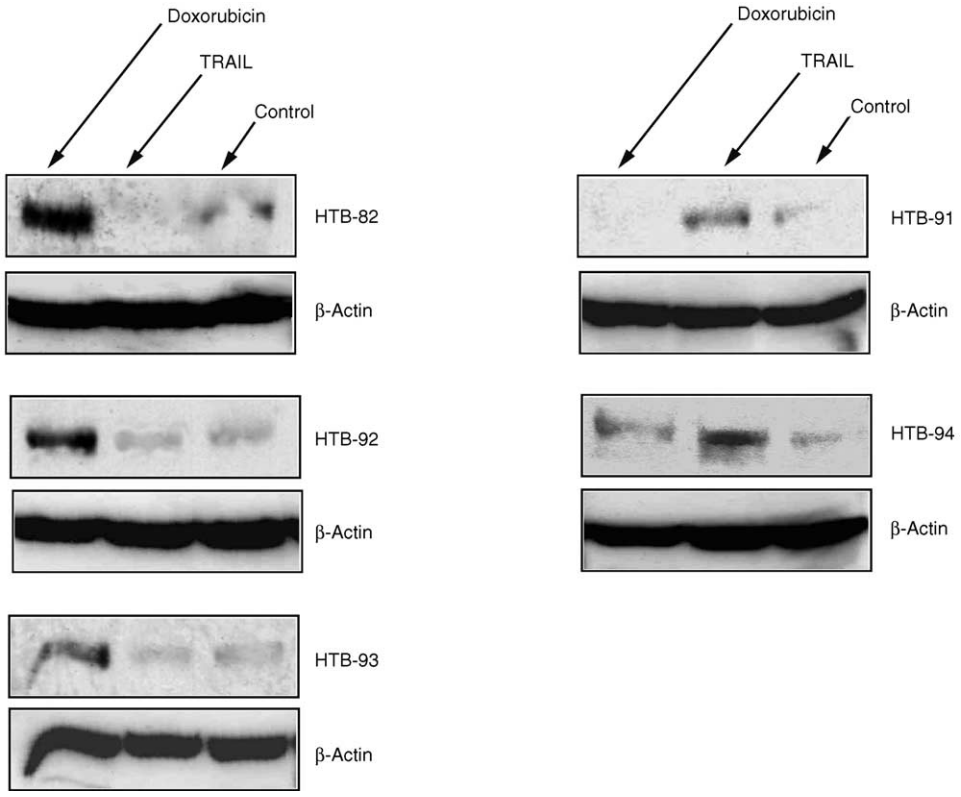

Fig. 8. STS cells were cultured for 12 h in the presence of doxorubicin (5 μM) or TRAIL (300 ng/ml) and the expression of TRAIL-R2 (48 kDa) after treatment was analysed by Western Blotting as described in "Materials and methods".

cells [45]. However, OPG presence did not predict TRAIL-resistance in the present model.

Kim and colleagues [41] recently suggested that the presence of an A-to-G alteration at codon 441 in the death domain of TRAIL-R1 might contribute to TRAIL resistance. However, such an A-to-G transition was detected in HTB-92 and HTB-93 cell lines, both of which were highly sensitive towards TRAIL-induced apoptosis. Thus, an A-to-G sequence change of TRAIL-R1 did not reduce sensitivity to TRAIL in our model.

As STS are extremely heterogeneous tumours, the question of whether the histological subtype of STS predicts TRAIL sensitivity or resistance needs to be addressed by further, larger studies, preferably in cell lines deriving from primary tumours.

As doxorubicin continues to be the mainstay of cytotoxic chemotherapy for STS *in vivo*, we investigated its ability to induce apoptosis in STS cells. Doxorubicin was able to induce apoptosis in the tested STS cell lines only weakly (<40%). However, coincubation of TRAIL and doxorubicin resulted in significant apoptosis of up to 100% in TRAIL- resistant HTB-82 and -94 cells, leaving the HTB-91 cell line, which lacks caspase 8, as the only cell line, where the combination is ineffective.

On the search for the mechanism(s) underlying the ability of doxorubicin to sensitise for TRAIL, we assessed the TRAIL-R1 and-R2 status in terms of mRNA and protein after doxorubicin and TRAIL treatment. A strong induction of TRAIL-R2 was detected in all TRAIL-sensitive cell lines as well as in TRAIL-resistant HTB-82 cells in response to doxorubicin. This finding suggests that upregulation of TRAIL-R2 by doxorubicin stimulated sensitivity to TRAIL, an effect which led to a synergistic response in originally TRAIL-sensitive cells and might have even overcome the putative inhibitory effect of both c-FLIP variants in HTB-82 cells. In TRAIL-resistant HTB-91 and -94 cells, which both lack TRAIL-R1 expression, no induction of TRAIL-R2 was observed. However, doxorubicin reversed TRAIL-resistance in HTB-94 cells, suggesting a different mechanism of this phenomenon which requires further mechanistic studies. In HTB-91 and -94 cells, TRAIL-R2 was upregulated after incubation with its ligand, which seemed to have no effect on TRAIL-induced apoptosis. No change in TRAIL-R1 expression was observed in both TRAIL-sensitive as well as TRAIL-resistant cell lines (data not shown).

In contrast to doxorubicin, incubation of STS-cells with paclitaxel resulted in a high level of apoptosis in all cell lines, and coincubation of TRAIL-sensitive STS cells with paclitaxel and TRAIL revealed an additive effect of both agents. Due to the controversial data with respect to taxanes in the treatment of advanced STS [53–55] the present findings might influence therapeutic strategies within this patient population.

In conclusion, our data suggest that TRAIL might be evaluated as a new treatment option in STS, applied either as a single agent or, even more promisingly, in combination with anthracycline and/or taxane-containing regimens.

Acknowledgements

This project was funded in part by the Fonds zur Foerderung der Wissenschaftlichen Forschung (FWF), Austria. Project Nr.: P 14138.

References

1. Antman K, Crowley J, Balcerzak SP, *et al*. An intergroup phase III randomized study of doxorubicin and dacarbazine with or without ifosfamide and mesna in advanced soft tissue and bone sarcomas. *J Clin Oncol* 1993, **11**, 1276–1285.
2. Borden EC, Amato DA, Edmonson JH, Ritch PS, Shiraki M. Randomized comparison of doxorubicin and vindesine to doxorubicin for patients with metastatic soft-tissue sarcomas. *Cancer* 1990, **66**, 862–867.
3. Brodowicz T, Wiltschke C, Kandioler-Eckersberger D, *et al*. Inhibition of proliferation and induction of apoptosis in soft tissue sarcoma cells by interferon-alpha and retinoids. *Br J Cancer* 1999, **80**, 1350–1358.
4. Tomazic VJ, Farha M, Loftus A, Elias EG. Anti-tumor activity of recombinant tumor necrosis factor on mouse fibrosarcoma in vivo and in vitro. *J Immunol* 1988, **140**, 4056–4061.
5. Meager A. A cytotoxicity assay for tumour necrosis factor using a human rhabdomyosarcoma cell line. *J Immunol Methods* 1991, **144**, 141–143.
6. Schulze-Osthoff K, Bakker AC, Vanhaesebroeck B, Beyaert R, Jacob WA, Fiers W. Cytotoxic activity of tumor necrosis factor is mediated by early damage of mitochondrial functions. Evidence for the involvement of mitochondrial radical generation. *J Biol Chem* 1992, **267**, 5317–5323.
7. Claudio E, Segade F, Wrobel K, Ramos S, Bravo R, Lazo PS. Molecular mechanisms of TNFalpha cytotoxicity: activation of NF-kappaB and nuclear translocation. *Exp Cell Res* 1996, **224**, 63–71.
8. Nagata S. Apoptosis by death factor. *Cell* 1997, **88**, 355–365.
9. Tartaglia LA, Goeddel DV. Two TNF receptors. *Immunol Today* 1992, **13**, 151–153.
10. Eggermont AM, Schraffordt Koops H, Klausner JM, *et al*. Isolated limb perfusion with tumor necrosis factor and melphalan for limb salvage in 186 patients with locally advanced soft tissue extremity sarcomas. The cumulative multicenter European experience. *Ann Surg* 1996, **224**, 756–764 (discussion 64-5.).
11. Eggermont AM, Schraffordt Koops H, Lienard D, *et al*. Isolated limb perfusion with high-dose tumor necrosis factor-alpha in combination with interferon-gamma and melphalan for non-resectable extremity soft tissue sarcomas: a multicenter trial. *J Clin Oncol* 1996, **14**, 2653–2665.
12. Eggermont AM, Schraffordt Koops H, Klausner JM, *et al*. Isolated limb perfusion with high-dose tumor necrosis factor-alpha for locally advanced extremity soft tissue sarcomas. *Cancer Treat Res* 1997, **91**, 189–203.
13. Gutman M, Inbar M, Lev-Shlush D, *et al*. High dose tumor necrosis factor-alpha and melphalan administered via isolated limb perfusion for advanced limb soft tissue sarcoma results in a >90% response rate and limb preservation. *Cancer* 1997, **79**, 129–137.
14. Katsen AD, Vollmar B, Mestres-Ventura P, Menger MD. Cell surface and nuclear changes during TNF-alpha-induced apoptosis in WEHI 164 murine fibrosarcoma cells. A correlative light, scanning, and transmission electron microscopical study. *Virchows Arch* 1998, **433**, 75–83.
15. Lev-Chelouche D, Abu-Abeid S, Kollander Y, *et al*. Multifocal soft tissue sarcoma: limb salvage following hyperthermic isolated limb perfusion with high-dose tumor necrosis factor and melphalan. *J Surg Oncol* 1999, **70**, 185–189.
16. Ten Hagen TL, Van Der Veen AH, Nooijen PT, Van Tiel ST, Seynhaeve AL, Eggermont AM. Low-dose tumor necrosis factor-alpha augments antitumor activity of stealth liposomal doxorubicin (DOXIL) in soft tissue sarcoma-bearing rats. *Int J Cancer* 2000, **87**, 829–837.
17. van Der Veen AH, ten Hagen TL, de Wilt JH, van Ijken MG, Eggermont AM. An overview on the use of TNF-alpha: our experience with regional administration and developments towards new opportunities for systemic application. *Anticancer Res* 2000, **20**(5B), 3467–3474.
18. Wiley SR, Schooley K, Smolak PJ, *et al*. Identification and characterization of a new member of the TNF family that induces apoptosis. *Immunity* 1995, **3**, 673–682.
19. Marsters SA, Pitti RM, Donahue CJ, Ruppert S, Bauer KD, Ashkenazi A. Activation of apoptosis by Apo-2 ligand is independent of FADD but blocked by CrmA. *Curr Biol* 1996, **6**, 750–752.
20. Pitti RM, Marsters SA, Ruppert S, Donahue CJ, Moore A, Ashkenazi A. Induction of apoptosis by Apo-2 ligand, a new member of the tumor necrosis factor cytokine family. *J Biol Chem* 1996, **271**, 12687–12690.
21. Keane MM, Ettenberg SA, Nau MM, Russell EK, Lipkowitz S. Chemotherapy augments TRAIL-induced apoptosis in breast cell lines. *Cancer Res* 1999, **59**, 734–741.
22. Tanaka S, Sugimachi K, Shirabe K, Shimada M, Wands JR. Expression and antitumor effects of TRAIL in human cholangiocarcinoma. *Hepatology* 2000, **32**, 523–527.
23. Ozawa F, Friess H, Kleeff J, *et al*. Effects and expression of TRAIL and its apoptosis-promoting receptors in human pancreatic cancer. *Cancer Lett* 2001, **163**, 71–81.
24. Petak I, Douglas L, Tillman DM, Vernes R, Houghton JA. Pediatric rhabdomyosarcoma cell lines are resistant to Fas-induced apoptosis and highly sensitive to TRAIL-induced apoptosis. *Clin Cancer Res* 2000, **6**, 4119–4127.
25. Van Valen F, Fulda S, Truckenbrod B, *et al*. Apoptotic responsiveness of the Ewing's sarcoma family of tumours to tumour necrosis factor-related apoptosis-inducing ligand (TRAIL). *Int J Cancer* 2000, **88**, 252–259.
26. Wu M, Das A, Tan Y, Zhu C, Cui T, Wong MC. Induction of apoptosis in glioma cell lines by TRAIL/Apo-2l. *J Neurosci Res* 2000, **61**, 464–470.
27. Yamanaka T, Shiraki K, Sugimoto K, *et al*. Chemotherapeutic agents augment TRAIL-induced apoptosis in human hepatocellular carcinoma cell lines. *Hepatology* 2000, **32**, 482–490.
28. Walczak H, Miller RE, Ariail K, *et al*. Tumoricidal activity of tumor necrosis factor-related apoptosis- inducing ligand in vivo. *Nat Med* 1999, **5**, 157–163.
29. Ashkenazi A, Pai RC, Fong S, *et al*. Safety and antitumor activity of recombinant soluble Apo2 ligand. *J Clin Invest* 1999, **104**, 155–162.
30. Pan G, O'Rourke K, Chinnaiyan AM, *et al*. The receptor for the cytotoxic ligand TRAIL. *Science* 1997, **276**, 111–113.
31. Wu GS, Burns TF, McDonald ER, *et al*. Induction of the TRAIL receptor KILLER/DR5 in p53-dependent apoptosis but not growth arrest. *Oncogene* 1999, **18**, 6411–6418.
32. Wu GS, Burns TF, McDonald ER, *et al*. KILLER/DR5 is a DNA damage-inducible p53-regulated death receptor gene. *Nat Genet* 1997, **17**, 141–143.

33. Pan G, Ni J, Wei YF, Yu G, Gentz R, Dixit VM. An antagonist decoy receptor and a death domain-containing receptor for TRAIL. *Science* 1997, **277**, 815–818.
34. Walczak H, Degli-Esposti MA, Johnson RS, *et al*. TRAIL-R2: a novel apoptosis-mediating receptor for TRAIL. *Embo J* 1997, **16**, 5386–5397.
35. Screaton GR, Mongkolsapaya J, Xu XN, Cowper AE, McMichael AJ, Bell JI. TRICK2, a new alternatively spliced receptor that transduces the cytotoxic signal from TRAIL. *Curr Biol* 1997, **7**, 693–696.
36. Sheridan JP, Marsters SA, Pitti RM, *et al*. Control of TRAIL-induced apoptosis by a family of signaling and decoy receptors. *Science* 1997, **277**, 818–821.
37. Degli-Esposti MA, Smolak PJ, Walczak H, *et al*. Cloning and characterization of TRAIL-R3, a novel member of the emerging TRAIL receptor family. *J Exp Med* 1997, **186**, 1165–1170.
38. Pan G, Ni J, Yu G, Wei YF, Dixit VM. TRUNDD, a new member of the TRAIL receptor family that antagonizes TRAIL signalling. *FEBS Lett* 1998, **424**, 41–45.
39. Marsters SA, Sheridan JP, Pitti RM, *et al*. A novel receptor for Apo2L/TRAIL contains a truncated death domain. *Curr Biol* 1997, **7**, 1003–1006.
40. Schneider P, Thome M, Burns K, *et al*. TRAIL receptors 1 (DR4) and 2 (DR5) signal FADD-dependent apoptosis and activate NF-kappaB. *Immunity* 1997, **7**, 831–836.
41. Kim K, Fisher MJ, Xu SQ, el-Deiry WS. Molecular determinants of response to TRAIL in killing of normal and cancer cells. *Clin Cancer Res* 2000, **6**, 335–346.
42. Dejosez M, Ramp U, Mahotka C, *et al*. Sensitivity to TRAIL/APO-2L-mediated apoptosis in human renal cell carcinomas and its enhancement by topotecan. *Cell Death Differ* 2000, **7**, 1127–1136.
43. Ahmad M, Shi Y. TRAIL-induced apoptosis of thyroid cancer cells: potential for therapeutic intervention. *Oncogene* 2000, **19**, 3363–3371.
44. Zhang XD, Nguyen T, Thomas WD, Sanders JE, Hersey P. Mechanisms of resistance of normal cells to TRAIL induced apoptosis vary between different cell types. *FEBS Lett* 2000, **482**, 193–199.
45. Emery JG, McDonnell P, Burke MB, *et al*. Osteoprotegerin is a receptor for the cytotoxic ligand TRAIL. *J Biol Chem* 1998, **273**, 14363–14367.
46. Keane MM, Ettenberg SA, Lowrey GA, Russell EK, Lipkowitz S. Fas expression and function in normal and malignant breast cell lines. *Cancer Res* 1996, **56**, 4791–4798.
47. Chou TC, Talalay P. Analysis of combined drug effects: a new look at a very old problem. *Trends Pharmacol Sci* 1983, **4**, 450–454.
48. Chou TC, Talalay P. Quantitative analysis of dose-effect relationships: the combined effects of multiple drugs or enzyme inhibitors. *Adv Enzyme Regul* 1984, **22**, 27–55.
49. Sacks PG, Harris D, Chou TC. Modulation of growth and proliferation in squamous cell carcinoma by retinoic acid: a rationale for combination therapy with chemotherapeutic agents. *Int J Cancer* 1995, **61**, 409–415.
50. Grunt TW. Tyrphostins and retinoids cooperate during inhibition of in vitro growth of ovarian cancer cells. *Cancer Lett* 2003, **189**, 147–156.
51. Peter ME. The trail discussion: it is FADD and caspase-8!. *Cell Death Differ* 2000, **7**, 759–760.
52. Ogasawara J, Watanabe-Fukunaga R, Adachi M, *et al*. Lethal effect of the anti-Fas antibody in mice. *Nature* 1993, **364**, 806–809.
53. Brodowicz T, Kostler WJ, Wiltschke C, Zielinski CC. Efficacy of docetaxel in the second-line treatment of locally advanced or metastatic soft tissue sarcoma after failure of chemotherapy with anthracycline or ifosfamide. *Am J Clin Oncol* 1999, **22**, 535.
54. Verweij J, Lee SM, Ruka W, *et al*. Randomized phase II study of docetaxel versus doxorubicin in first- and second-line chemotherapy for locally advanced or metastatic soft tissue sarcomas in adults: a study of the European organization for research and treatment of cancer soft tissue and bone sarcoma group. *J Clin Oncol* 2000, **18**, 2081–2086.
55. Kostler WJ, Brodowicz T, Attems Y, *et al*. Docetaxel as rescue medication in anthracycline- and ifosfamide-resistant locally advanced or metastatic soft tissue sarcoma: results of a phase II trial. *Ann Oncol* 2001, **12**, 1281–1288.