Synergistic apoptotic effects of taurolidine and TRAIL on squamous carcinoma cells of the esophagus

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Abstract. The treatment of choice for esophageal cancer is considered surgical resection, but a median survival of around 20 months after treatment is still discouraging. The value of adjuvant or neoadjuvant radiation or chemotherapy is limited and to date, benefits have only been described for certain tumor stages. Therefore, new therapeutic options are required. As alternative chemotherapeutics, we tested the antibiotic taurolidine (TRD) on KYSE 270 human esophageal carcinoma cells alone and in combination with rhTRAIL (TNF related apoptosis-inducing ligand). Viability, apoptosis and necrosis were visualized by TUNEL assay and quantitated by FACS analysis. Gene expression was analysed by RNA microarray. The most effective concentration of TRD as single substance (250 μmol/l) induced apoptosis to a maximum of 40% after 12-h dose dependently, leaving 4% viable cells after 48 h; by comparison, rhTRAIL did not have a significant effect. The combination of both substances doubled the effect of TRD alone. Gene expression profiling revealed that TRD downregulated endogenous TRAIL, TNFRSF1A, TRADD, TNFRSF1B, TNFRSF21, FADD, as well as MAP2K4, JAK2 and Bcl2, Bcl2l1, APAF1 and caspase-3. TNFRSF25, cytochrome-c, caspase-1, -8, -9, JUN, GADD45A and NFKBIA were upregulated. TRAIL reduced endogenous TRAIL, Bcl2l1 and caspase-1 expression. BIRC2, BIRC3, TNFAIP3, and NFKBIA were upregulated. The combined substances upregulated endogenous TRAIL, NFKBIA and JUN, whereas DFFA and TRAF3 were downregulated compared to TRD as single substance. We conclude that TRD overcomes TRAIL resistance in KYSE 270 cells. Synergistic effects are dependent on the same and on distinct apoptotic pathways which, jointly triggered, result in an amplified response. Several apoptotic pathways, including the TNF-receptor associated and the mitochondrial pathway, were differentially regulated by the substances on gene expression level. Additionally transcription factors seem to be influenced, NFKB in particular. Endogenous TRAIL expression is increased by the combination of substances, whereas it is reduced by each single substance. Taking into consideration that the non-toxic TRD was able to reduce rhTRAIL toxicity and dose, combined therapy with TRD and rhTRAIL may offer new options for treatment in esophageal cancer.

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Introduction

The carcinoma of the esophagus with a stage dependent survival of rarely >50% still represents a therapeutic challenge especially concerning the fact that its incidence is rising. Treatment of esophageal carcinoma is based on individualised and stage specific multimodal approaches which comprise esophagectomy as well as neoadjuvant, adjuvant or palliative chemotherapy and radiation. Despite this broad multidisciplinary armamentarium, the overall survival is still poor and problems such as primary or acquired resistance against chemotherapy or side effects remain unsolved (1-8). New treatments such as immunotherapy and hyperthermia so far have also failed to improve the outcome significantly (9-14). For unresectable tumors the effects of chemotherapy and radiotherapy may prolong survival in some cases, but side effects are severe and life quality remains poor (15-18).

To elucidate new options for treatment we tested two promising agents rhTRAIL (recombinant human tumor necrosis factor associated apoptosis inducing ligand) and taurolidine (TRD) seperately and in combination on esophageal squamous cancer cells (KYSE 270) *in vitro*.

TRAIL as a physiological apoptosis inducing molecule of 33 kD belongs to the TNF super-family and seems to be involved in the natural antitumoral and antiviral immunoresponse (19). TRAIL initiates cell death by binding to the transmembraneous death receptors 4 and 5 (DR4, DR5), thereby inducing the binding of the intracellular adaptor molecule FADD (Fas associated death domain) and starting a cascade involving caspase-8 to form the so-called DISC (death-inducing signalling complex) which is activating several effector caspases. Another type of cell death induction by TRAIL via the intrinsic, mitochondrial pathway leading to a cytochrome-c efflux from mitochondria, formation of apoptosomes and activation of the effector caspases has also been described (19-26). In contrast to many other chemotherapeutics, TRAIL induces apoptosis independently from p53 (21,27,28). Binding of TRAIL to the decoy receptors 1 and 2 (DcR1, DcR2) that, in contrast to DR4 and DR5, lack a transmembraneous death domain, probably results in a competitive inhibition of apoptosis (19,29). Several recombinant versions of TRAIL have been generated and some were shown to induce apoptosis in healthy hepatocytes and keratinocytes (30,31). In contrast, a new recombinant human TRAIL (rhTRAIL) without any added exogenous sequences has been shown to be well tolerated by mice and non-human primates without relevant side effects (20,32). Many substances, including established chemotherapeutics such as 5-Fluorouracil, cisplatin, doxorubicin, etoposide, have been shown to sensitize tumor cells including esophageal cancer cells to TRAIL-induced apoptosis probably by increased DR4 and DR5 expression, improved DISC formation and other mechanisms (33-39).

Taurine is a semi-essential amino acid and is not incorporated into proteins. In mammalian tissues, taurine is ubiquitous and is the most abundant free amino acid in the heart, retina, skeletal muscle, brain and leukocytes. It protects normal tissue from oxidant-induced injury and apoptosis (40). TRD, derived from Taurine, originally was used as an antimicrobial and antiinflammatory substance in the treatment of peritonitis and blood stream infections without any observed short- or long-term toxicity (41-43). Recent studies revealed an apoptotic effect on a variety of malignant cells *in vitro* and *in vivo*, including colon, ovarian, prostate carcinoma, melanoma, mesothelioma, osteosarcoma and leukemia (44-52). First reports of successful treatments of glioblastoma and gastric cancer without systemic side effects in humans are promising (53-55). The detailed mechanism of action is still unclear, but inhibition of Bcl-2 and an increased efflux of cytochrome-c, activation of the caspases, and an increased PARP (poly(ADP-ribose) polymerase) cleavage seem to be involved (44,48,49,51,52,56). By comparison, other authors found Fas-ligand dependent mechanisms or inhibition of tumor angiogenesis to be responsible for the inhibition of tumor growth (57-59).

The absence of toxicity and its apoptosis inducing potential make TRD a candidate for co-treatment with TRAIL, hypothetically increasing its apoptotic effects. To our knowledge, a combination of rhTRAIL and TRD has not yet been investigated on esophageal cancer cells.

Materials and methods

Cell line. Human esophageal squamous cancer cells, Kyse 270, were purchased from DSMZ (Braunschweig, Germany) and maintained with 48% RPMI-1640 + 48% Ham's F12 + 2% FBS supplemented with 1% penicillin (100 U/ml) and streptomycin (100 μg/ml) and 1% L-Glutamine. Cells were cultured in a humidified atmosphere with 5% CO_2 at 37˚C on 6-well plates with $1x10^6$ cells/well (subconfluent).

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

Dose-finding study. Cells were incubated with TRD (10, 50, 100, 250 and 500 μmol/l) or recombinant human TRAIL (50, 100, 250 and 500 ng/ml) and the respective controls (Povidon/H_2O) for 1, 3, 6, 12, 24 and 48 h to identify the most effective single concentrations and the time dependency of the effects. All experiments were repeated with 3 passages.

Additionally different concentrations of TRAIL (10, 100, 250 and 500 ng/ml) were combined with TRD 100 μmol/l in another experiment for 1, 3, 6, 12, 24 and 48 h to quantitate the effects of different TRAIL concentrations when combined with TRD. All experiments were repeated with 3 passages.

The most effective single concentrations of TRD and TRAIL were then used as single substances and in combination to identify a possibly synergistic effect. Three, 6 and 12 h were chosen as time points. All experiments were repeated with 3 passages. Cells for gene expression were harvested after 12 h, representing the time of maximal apoptosis.

Flow cytometry analysis. At the indicated incubation time, floating cells were collected together with the supernatant and adherent cells which were harvested by trypsinization. Cells were sedimented by centrifugation, resuspended in 195 μl binding buffer (Bender MedSystems) and incubated with 5 μl Annexin V-FITC (BD Biosciences, Heidelberg, Germany) and 10 μl propidium iodide (PI) (Bender MedSystems) following the manufacturer's manual. Cells were analyzed immediately using a FACS flow cytometer (FACS Calibur BD Biosciences). For each measurement, 20,000 cells were counted. Dot plots and histograms were analyzed by CellQuest Pro software (BD Biosciences). Annexin V positive cells were considered apoptotic; Annexin V and PI positive cells were identified as necrotic. Annexin V and PI negative cells were termed viable.

Cell morphology. Morphology of adherent cells and cells suspended in culture medium was studied and documented using a phase contrast microscope, Zeiss Axiovert 25 (Karl Zeiss, Jena, Germany).

TUNEL assay. Apoptosis was evaluated by terminal deoxynucleotidyl transferase-mediated dUTP-nick end-labeling (TUNEL) using the *in situ* cell Death detection Kit® (Roche Applied Science, Mannheim, Germany) according to the manufacturer's instructions and analyzed by fluorescence microscopy (Leica DM4000B, Leica Microsystems, Nussloch, Germany).

Statistical analysis. Results of FACS analysis for percentages of viable, apoptotic and necrotic cells are expressed as means ± SEM of at least 3 independent experiments with consecutive passages. In this study, comparisons between experimental groups (single agent application in different doses and single agents versus combined treatment at various time-points) were performed using repeated measures analysis of variance (ANOVA) over all time points. p-values ≤0.05 were considered as statistically significant and indicated in the figures as follows: $^{***}p \leq 0.001$, $^{**}p \leq 0.005$ and $^{*}p \leq 0.05$.

Oligonucleotide microarray analysis. Total RNA was purified from the cells after incubation with the different substances for 12 h using the RNeasy KIT from Qiagen (Hilden, Germany), as specified by the manufacturer. RNA integrity was assessed using the Agilent 2100 Bioanalyzer (Agilent Technologies).

For microarray analyses we used the Affymetrix GeneChip platform employing a standard protocol for sample preparation and microarray hybridization. Total RNA (5 μg) was converted into biotinylated cRNA according to the Affymetrix standard protocol version 2, purified, fragmented and hybridized to HG-U133Plus_2.0 microarrays (Affymetrix). The arrays were washed and stained according to the manufacturer's recommendation and finally scanned in a GeneChip scanner 3000 (Affymetrix).

Array images were processed to determine signals and detection calls (Present, Absent, Marginal) for each probe set using the Affymetrix GCOS1.4 software (MAS 5.0 statistical algorithm). Arrays were scaled across all probesets to an average intensity of 1000 to compensate for variations in the amount and quality of the cRNA samples and other experimental variables of non-biological origin. Pairwise comparisons of treated versus control samples were carried out with GCOS1.4, which calculates the significance (change p-value) of each change in gene expression based on a Wilcoxon ranking test. To limit the number of false positives, we restricted further target identification to those probe sets, which received at least one present detection call in the treated/control pair. Probe sets exhibiting a significant increase or decrease were identified by filtering using the Affymetrix Data Mining Tool 3.0.

Results

TRD induces apoptotic cell death in esophageal cancer cells. Concentrations below 100 μmol/l (10 and 50 μmol/l) did not lead to significant apoptosis or necrosis, even after incubation for 48 h. The higher concentrations (100, 250 and 500 μmol/l) initiated apoptotic cell death after 3-6 h of incubation, reaching a maximum of detectable apoptosis between 12 and 24 h. The highest and most early rates of apoptosis were found for a concentration of 250 μmol/l with 40% apoptotic cells after 12 h. This concentration also led to the highest necrosis rates, with >80% after 48 h. At this time, only 4% of the cells were left viable (Fig.1).

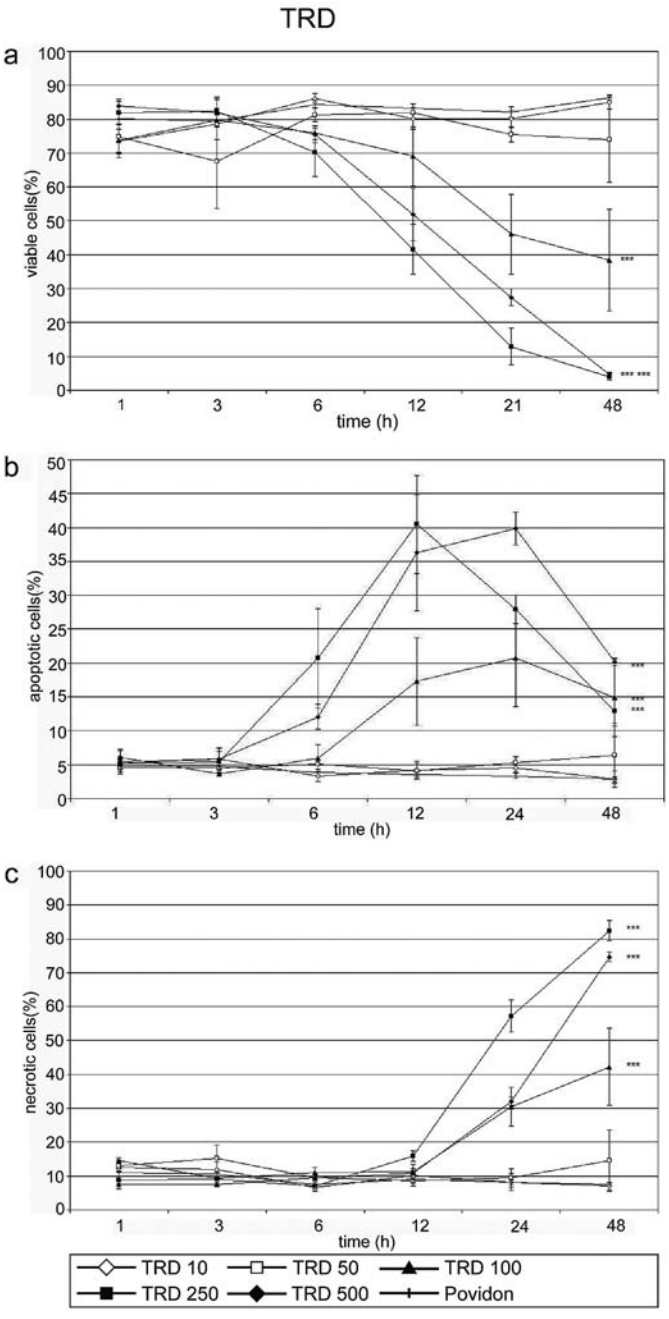

Figure 1. Effects of taurolidine (TRD) on viability, apoptosis and necrosis in KYSE 270 cells measured by FACS analysis: KYSE 270 cells were incubated with TRD in the concentrations indicated and with Povidon 5% (control) for 1-48 h. The percentages of viable (a), apoptotic (b) and necrotic cells (c) were determined by FACS analysis for Annexin V-FITC and propidium iodide. Values are means ± SEM of 3 independent experiments with consecutive passages ($^{***}p \leq 0.001$ compared to the control group, repeated measures ANOVA).

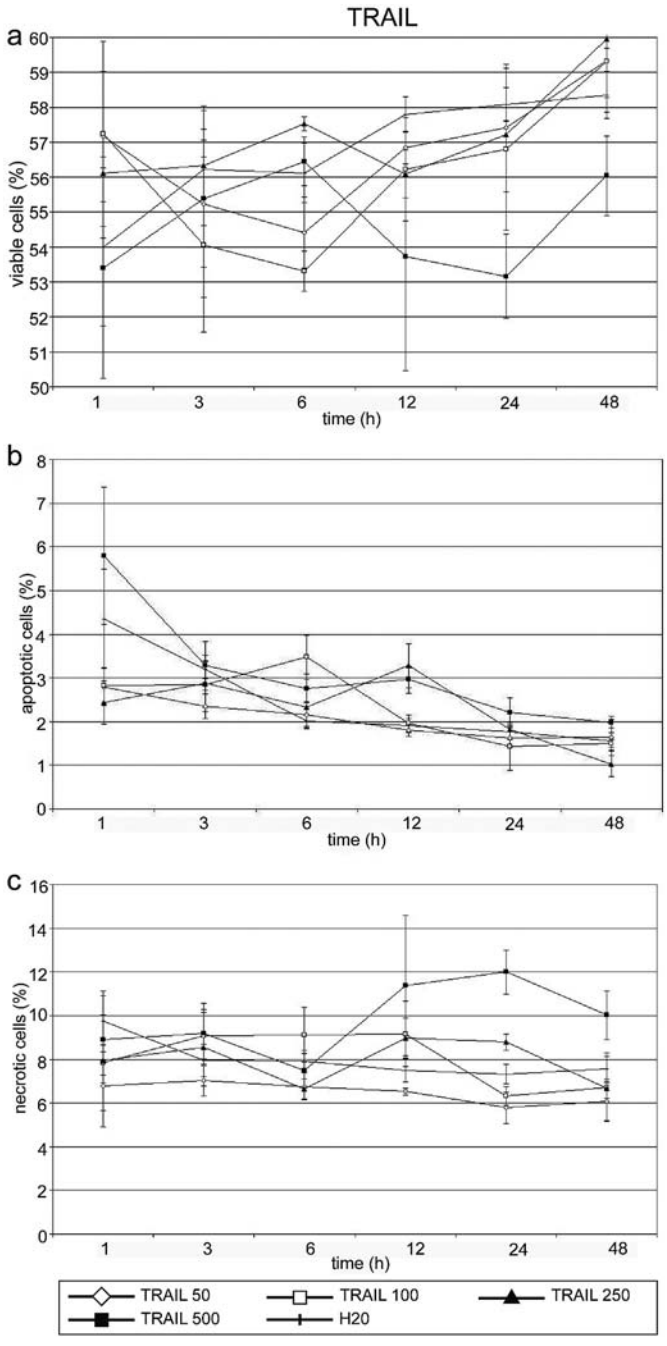

Figure 2. Effects of TRAIL on viability, apoptosis and necrosis in KYSE 270 cells measured by FACS analysis: KYSE 270 cells were incubated with TRAIL in the concentrations indicated and with H_2O (control) for 1-48 h. The percentages of viable (a), apoptotic (b) and necrotic cells (c) were determined by FACS-analysis for Annexin V-FITC and propidium iodide. Values are means ± SEM of 3 independent experiments with consecutive passages. No significant changes were detected.

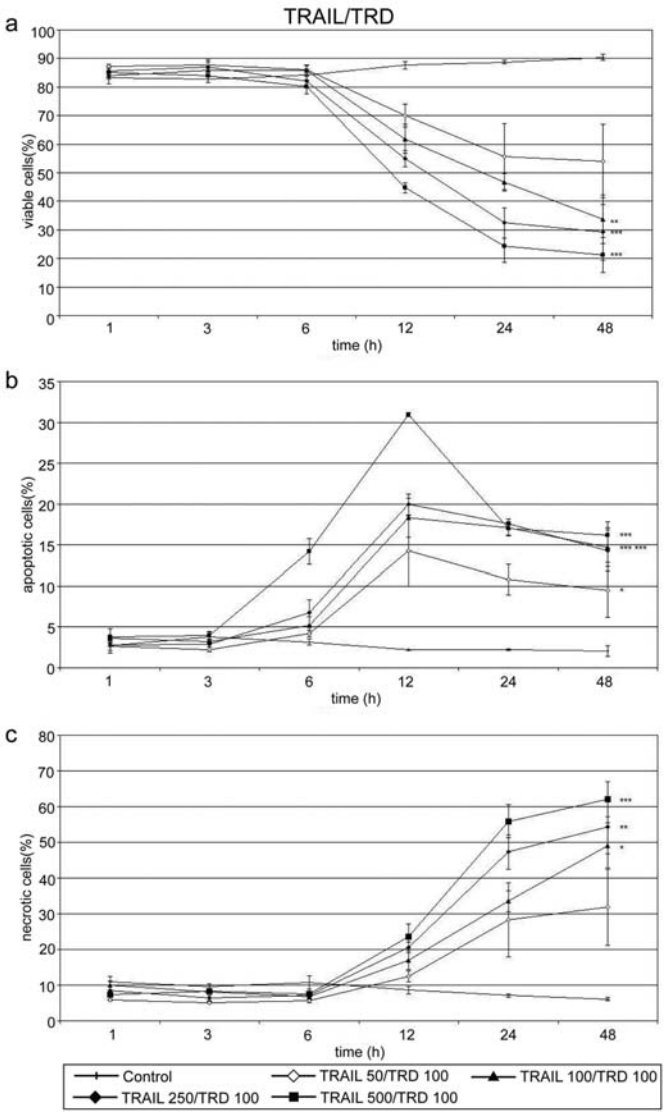

Figure 3. Effects of TRD 100 μmol/ml and different concentrations of TRAIL on viability, apoptosis and necrosis in KYSE 270 cells measured by FACS analysis: KYSE 270 cells were incubated with 100 μmol/l TRD and 50, 100, 250 and 500 ng/ml TRAIL as well as with Povidon 5% + H_2O (control) for 1-48 h. The percentages of viable, apoptotic and necrotic cells were determined by FACS analysis for Annexin V-FITC and propidium iodide. Values are means ± SEM of 3 independent experiments with consecutive passages ($^{***}p\leq0.001$, $^{**}p\leq0.005$ and $^{*}p\leq0.05$; repeated measures ANOVA compared to control).

TRAIL as single agent does not induce apoptotic cell death in esophageal cancer cells. The concentrations of TRAIL that were used (50, 100, 250 and 500 ng/ml) did not lead to any detectable significant increase in apoptosis. After 6 h, an increase of necrotic cells to a maximum of 12% at 24 h was detected with a TRAIL concentration of 500 ng/ml; however, this effect was not significant. The proportion of viable cells remained above 86% after 48 h of incubation with the highest concentration of TRAIL (Fig. 2).

A combination of TRAIL and TRD amplifies apoptotic effects. When the lowest TRD concentration that was found to be effective (100 μmol/l) in the previous experiment was combined with different concentrations of TRAIL (50, 100, 250 and 500 ng/ml), 500 ng/ml of TRAIL was the most potent, reaching a maximum of almost 29% apoptotic cells after 12 h. After 48 h, 62% of the cells were found to be necrotic and 21% were still viable (Fig. 3).

A combination of the most effective single doses (250 μmol/l TRD and TRAIL 500 ng/ml) reduces viable cells to 25% within 12 h. Compared to the single use of 250 μmol/l TRD, the addition of TRAIL 500 ng/ml doubles the effect. The reduction of viable cells as well as the induction of apoptosis was significantly increased by TRD

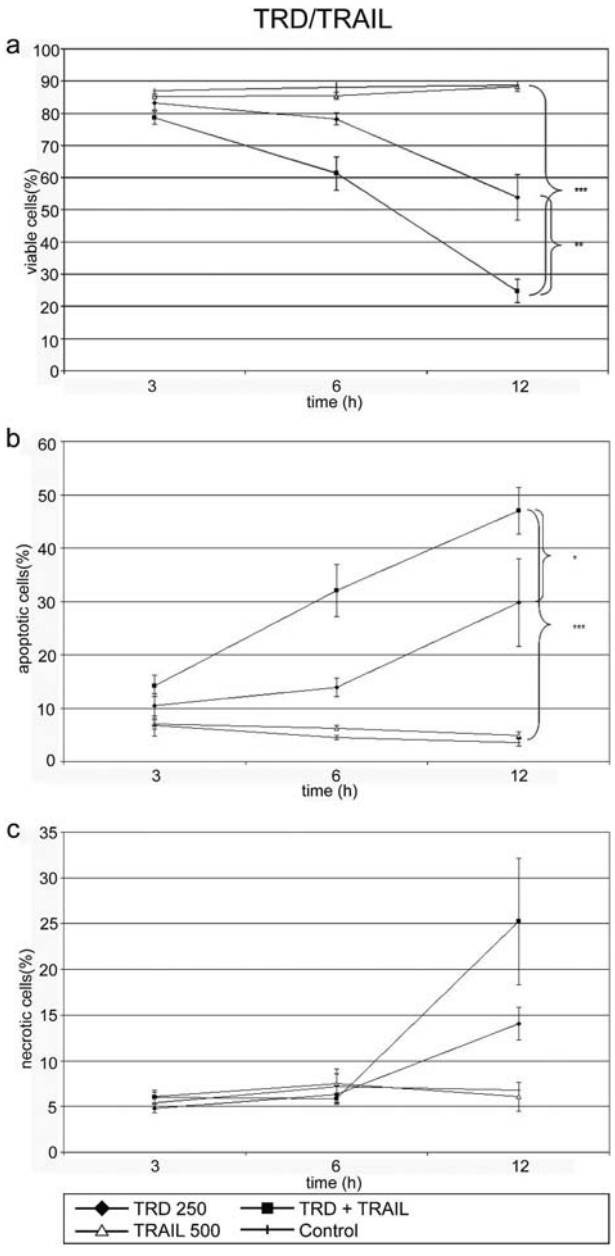

Figure 4. Effects of TRD, TRAIL and combination of both agents on viability, apoptosis and necrosis in KYSE 270 cells measured by FACS analysis: KYSE 270 cells were incubated with 250 μmol/l TRD and 500 ng/ml TRAIL alone and in combination as well as with Povidon 5% + H_2O (control) for 3 and 12 h. The percentages of viable, apoptotic and necrotic cells were determined by FACS analysis for Annexin V-FITC and propidium iodide. Values are means ± SEM of 3 independent experiments with consecutive passages ($^{***}p \leq 0.001$, $^{**}p \leq 0.005$ and $^{*}p \leq 0.05$; repeated measures ANOVA).

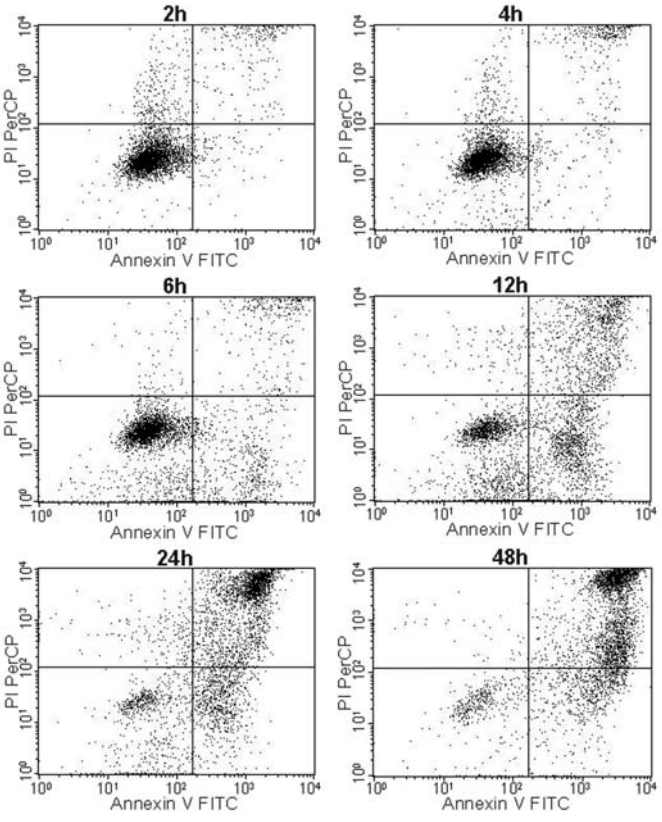

Figure 5. Representative dot plot of FACS analysis for Annexin V-FITC and propidium iodide after application of TRD 100 μmol/l and TRAIL 500 ng/ml in KYSE 270 cells: KYSE 270 cells were incubated with 100 μmol/l TRD and 500 ng/ml TRAIL for 2, 4, 6, 8 and 12 h (from upper right to lower left). Lower left quadrant: Annexin V and propidium iodide negative (viable), lower right quadrant: Annexin V positive and propidium iodide negative (apoptotic), upper right quadrant: Annexin V and propidium iodide positive (necrotic).

and TRAIL, compared to the control and compared to the single use of TRD (Fig. 4).

The dot blots of the FACS analysis demonstrated that cells were undergoing apoptotic cell death, showing Annexin V positivity in the early phases of incubation and an additional PI positivity at the later time points, representing a shift from early apoptosis to cell death and necrosis (Fig. 5).

Taurolidine and TRD/TRAIL induce morphological changes, cell detachment and TUNEL positivity. TRAIL incubation did not change the cell morphology, or cause a detachment of the cells from the ground neither did it influence the TUNEL staining results. The combination of TRD and TRAIL resulted in disintegration of the subconfluent cell groups and shrinkage of the cells, followed by complete cell detachment (Fig. 6). TUNEL staining was negative for the controls, showed a few positive cells in the TRD treated cultures, and a noticeable increase of stained cells in the TRD/TRAIL group. Nuclear fragmentation and apoptotic bodies were also detectable in this group (Fig. 7).

Gene expression. In this experiment we selectively focussed on apoptosis related probesets. Out of 621 of those probesets 266, representing 186 apoptosis related genes showed expression changes. TRD alone induced differences in the reading of 213 probe sets, representing 154 apoptosis related genes, of which 66 were 'upregulated'; whereas TRAIL as a single substance caused changes in 12 probe sets standing for 9 genes related to apoptotic pathways, 'upregulating' 6 of them. TRD and TRAIL in combination induced overexpression of 17 and 'downregulation' of 17 (22 probe sets each) compared to TRD as single substance (Fig. 8). Due to the multitude of differential regulated genes, only a representative selection of genes is mentioned in detail.

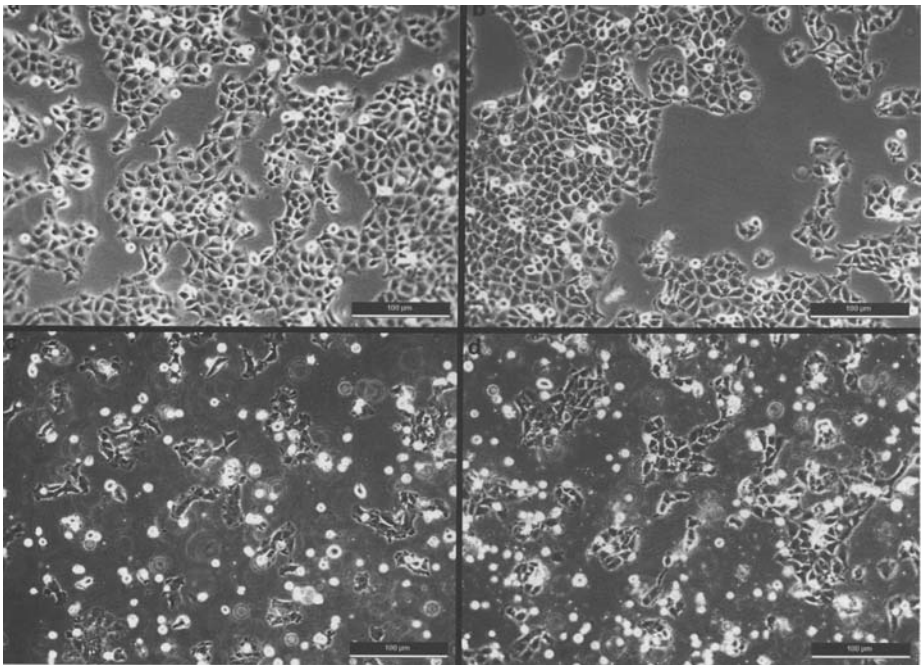

Figure 6. Representative microscopic photographs showing morphologic changes in KYSE cells induced by TRD, TRAIL and combination of both agents after 12 h: KYSE cells were incubated with Povidon 5% + H_2O (control) (a), 500 ng/ml TRAIL (b), 250 μmol/l TRD (c) and a combination of TRD/TRAIL (d) for 12 h. Phase contrast microscopy of cells at x20 magnification.

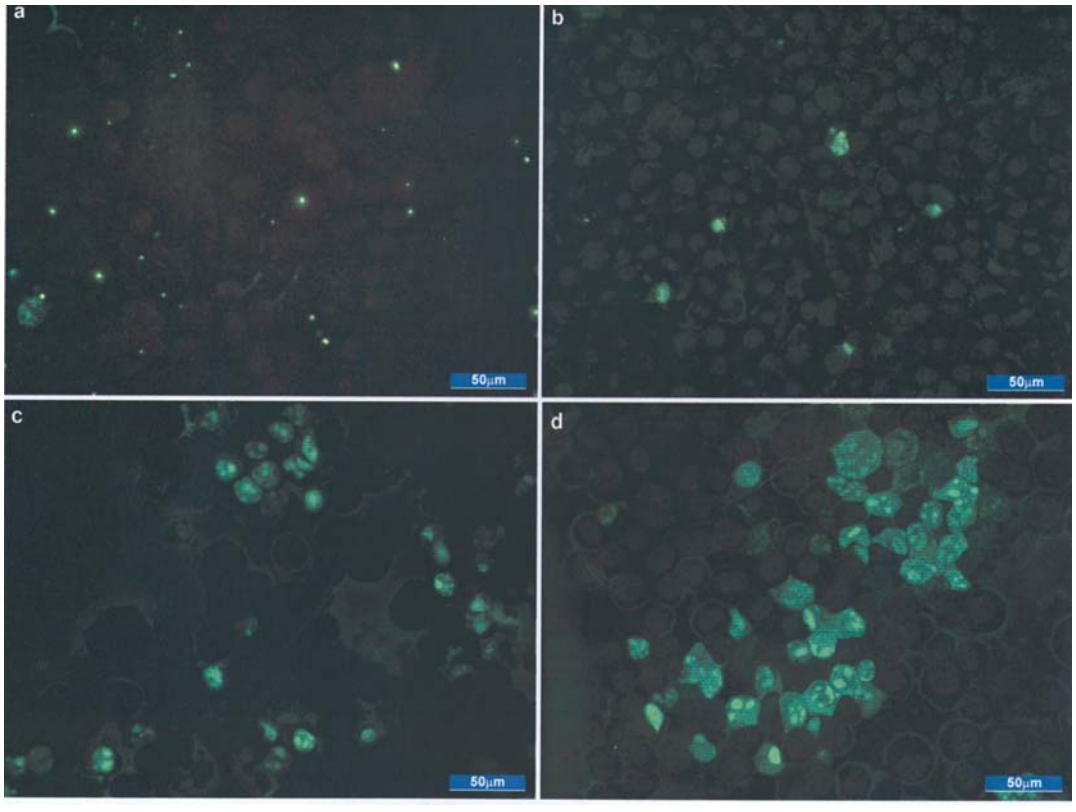

Figure 7. Representative TUNEL-assay for KYSE 270 cells after incubation with a control with Povidon 5% + H_2O (a), TRAIL 500 ng/ml (b), TRD 250 μmol/l (c), a combination of both agents (d) for 12 h.

In the cells treated with TRD alone, we found the tumor necrosis factor (ligand) super-family 10 (TNFSF10/ TRAIL), the tumor necrosis factor receptor super-family 1A (TNFRSF1A), TNFRSF1A-associated death domain (TRADD), tumor necrosis factor receptor super-family 1B (TNFRSF1B), tumor necrosis factor receptor super-family 21 (TNFRSF21), Fas-associated death domain (FADD), mitogen-activated protein kinase kinase 4 (MAP2K4/c-Jun

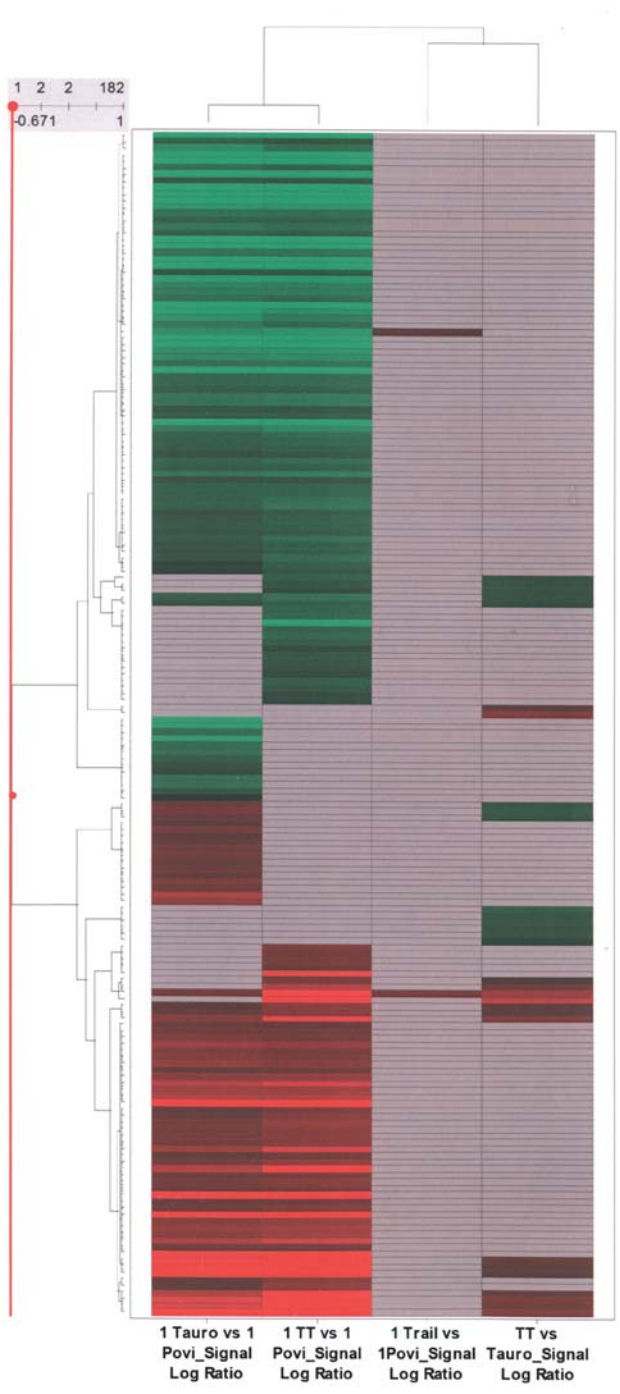

Figure 8. Overall expression patterns of reliably measured (ca+filter1) 266 probe sets associated with apoptosis out 621 apoptosis associated probe sets of the HG-U133A_2.0 chip. Horizontal rows represent individual probe sets/genes; vertical columns represent individual samples (from left to right: colour range: brightest red, signal log ratio (SLR)≥2 (indicates expression level above compared sample); brightest green, SLR≤2 (indicates expression level below compared sample); black, SLR=0 (indicates unchanged expression); grey, no reliable filter target. The dendogram at the top of the matrix indicates the degree of similarity between tumor samples; the dendogram at the left side indicates the degree of similarity among the selected genes according to their expression patterns.

N-terminal kinase kinase 1), Janus kinase 2 (JAK2), Bcl-2, Bcl2l1/ BclxL, apoptotic peptidase activating factor (APAF1) and caspase-3 'downregulated'.

Caspase-1, -8, -9, cytochrome-c, poly(ADP-ribose) polymerase family 1 (PARP1), tumor necrosis factor receptor super-family 25 (TNFRSF25), nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor alpha (NFKBIA), growth arrest and DNA-damage-inducible gene alpha (GADD45A) and v-jun sarcoma virus 17 oncogene homolog (JUN/AP-1) were 'upregulated'.

Cells exposed to TRAIL 'downregulated' TNFSF10 (TRAIL), Bcl2l1 and caspase-1. Baculoviral IAP repeat-containing 2 (BIRC2), BIRC3, tumor necrosis factor alpha-induced protein 3 (TNFAIP3) and NFKBIA were 'upregulated'.

When the TRD/TRAIL incubated cells were compared to the cells only receiving TRD, DNA fragmentation factor, 45 kDa, alpha polypeptide (DFFA), TNF receptor-associated factor 3 (TRAF3) were 'downregulated'. TNFSF10 (TRAIL), NFKBIA and JUN/AP-1 were 'upregulated'. A summary of all genes associated with apoptosis by Gene Ontology is shown in Table I.

Discussion

Taurolidine. A concentration of 100 μmol/l was the lowest effective to induce apoptotic cell death in squamous esophageal cancer cells. The most effective concentration (reaching the peak of apoptotic cells the earliest) was 250 μmol/l. Apoptosis was detectable after 3-6 h and reached its maximum at 12 h; it then decreased and gave way to necrosis that reached a maximum after 48 h with >80%. The fact that 250 μmol/l TRD was more effective than 500 μmol cannot be explained but may be interpreted as an indication for an apoptotic mechanism rather than a necrotic one.

Many of the factors, 'downregulated' by TRD, belong to the TNF receptor associated death signalling pathway, such as endogenous TRAIL, TNFRSF1A, TRADD, TNFRSF1B, TNFRSF21, FADD and downstream of that pathway MAP2K4.

The receptors TNFRSF1A and TNFRSF1B are transducers of TNF action and lead to programmed cell death via TRADD and FADD activation (60). Crosstalk between TNFRSF1A and TNFRSF1B (a regularly non-apoptotic receptor lacking a death domain) via endogenous TNF was shown to induce apoptosis and activate MAP2K4 and NFKB (61-63). TNFRSF21 (Death receptor 6, DR6) a recently identified member of the TNF receptor family, induces apoptosis via a FADD-independent mechanism and can be inhibited by increased levels of Bcl-2 (64).

MAP2K4/c-Jun N-terminal kinase kinase 1, which was also 'downregulated' by TRD, is a proapoptotic factor of the JNK transcription factors cascade also being activated by the TNF receptor system (65). Another anti-apoptotic tyrosine kinase JAK2, whose blockade has already been shown to inhibit tumor growth and induce apoptosis (66) and to sensitize cells to TRAIL induced apoptosis (67), was also 'downregulated' by TRD.

The inhibition of Bcl2 and Bcl2l1 expression by TRD may be responsible for an increased efflux of cytochrome-c and thereby positively correlates with increased complex-formation with APAF 1 and consecutive caspase-9 activation

Table I. The table summarizes the detailed expression changes identified for 266 apoptosis-associated probe sets, representing 186 genes.

Probe set	Gene symbol	TRD vs. control	TRAIL vs. control	TRD/TRAIL vs. control	TRD/TRAIL vs. TRD
212772_s_at	ABCA2	0.89		0.62	
202123_s_at	ABL1	-0.79		-1.18	
201715_s_at	ACIN1				-0.3
202820_at	AHR	-1.5		-1.23	
201012_at	ANXA1	0.38		0.4	
201301_s_at	ANXA4	0.79		0.75	
201302_at	ANXA4	0.69			
200782_at	ANXA5	0.4			
204859_s_at	APAF1	-1.46		-1.64	
201686_x_at	API5			-0.76	
203381_s_at	APOE	0.64		0.73	
203382_s_at	APOE	0.93		1.31	
200602_at	APP	0.54		0.79	
201167_x_at	ARHGDIA			-0.44	-0.49
202511_s_at	ATG5	-0.97		-1.02	
202512_s_at	ATG5	-1.42		-1.14	
202686_s_at	AXL	0.93		1.08	
209364_at	BAD	0.5			
202387_at	BAG1	0.68		0.52	
202985_s_at	BAG5	-1.65		-1.85	
202984_s_at	BAG5	-0.77			
203685_at	BCL2	-1.77		-1.99	
215037_s_at	BCL2L1	-1.56	-0.29	-1.02	
206665_s_at	BCL2L1	-1.17		-1.11	
212312_at	BCL2L1	-0.89		-0.78	
201084_s_at	BCLAF1	-0.26		-0.55	
201101_s_at	BCLAF1	-0.78		-1	
204493_at	BID			-0.64	
202076_at	BIRC2		0.35		
210538_s_at	BIRC3		1.3		
221478_at	BNIP3L	0.92		1.06	
221479_s_at	BNIP3L	1.47		1.4	
200921_s_at	BTG1	-1.13		-0.38	0.62
200920_s_at	BTG1			0.78	0.68
200935_at	CALR			-0.61	-0.65
209970_x_at	CASP1	0.95	-0.24	1.04	
211367_s_at	CASP1	0.92	-0.36	1.09	
211368_s_at	CASP1	0.9	-0.39	1.09	
211366_x_at	CASP1	0.96		0.89	
206011_at	CASP1		-0.63		
202763_at	CASP3	-1.07		-1.12	
213373_s_at	CASP8		0.33	-1.05	
203984_s_at	CASP9	0.43		0.56	
210916_s_at	CD44 /// MAPK10			-0.52	
202284_s_at	CDKN1A	0.87		1.03	
200021_at	CFL1	0.35		0.71	
201953_at	CIB1	0.68		0.64	
214683_s_at	CLK1	1.16		1.37	
203229_s_at	CLK2				0.83
203804_s_at	CROP	-0.29			
202329_at	CSK	-0.16			

Table I. Continued.

Probe set	Gene symbol	TRD vs. control	TRAIL vs. control	TRD/TRAIL vs. control	TRD/TRAIL vs. TRD
202468_s_at	CTNNAL1	0.38			
200838_at	CTSB	0.5		0.57	
200839_s_at	CTSB	0.61		0.84	
203079_s_at	CUL2	-1.11		-1.27	
201370_s_at	CUL3	-0.48		-0.72	
201371_s_at	CUL3	-0.55		-0.79	
201423_s_at	CUL4A	-0.67		-1.16	-0.41
201424_s_at	CUL4A			-0.34	
203532_x_at	CUL5	-0.69		-0.94	
203531_at	CUL5			-0.32	
208905_at	CYCS	0.43		0.6	
200046_at	DAD1	0.55		0.52	
203139_at	DAPK1			-0.99	
202262_x_at	DDAH2	1.19			
1007_s_at	DDR1	0.53		0.65	
207169_x_at	DDR1	0.63		0.92	
208779_x_at	DDR1	0.61		0.85	
210749_x_at	DDR1	0.56		0.74	
202480_s_at	DEDD	-0.61		-0.67	
203277_at	DFFA				-0.41
206752_s_at	DFFB			-0.42	
203187_at	DOCK1	0.49			
201041_s_at	DUSP1	2.01		2.33	0.37
221563_at	DUSP10	-2.22		-1.95	
202703_at	DUSP11	-1.03		-0.9	
218576_s_at	DUSP12	-0.63		-0.77	
204794_at	DUSP2	-1.12			
201537_s_at	DUSP3	-0.78		-1.2	
204014_at	DUSP4	-0.68		-0.39	
204015_s_at	DUSP4	-0.84		-1.02	
209457_at	DUSP5	-1.53	0.36	-1.68	
208892_s_at	DUSP6			0.7	
2028_s_at	E2F1	-0.39		-0.52	
201983_s_at	EGFR	0.56		0.45	
201984_s_at	EGFR				-0.23
210984_x_at	EGFR				-0.62
211607_x_at	EGFR				-0.46
201231_s_at	ENO1			0.62	0.33
202221_s_at	EP300	-1.56		-1.04	
203499_at	EPHA2			-0.42	
206070_s_at	EPHA3	-3.48			
1438_at	EPHB3	-1.06		-1.11	
202176_at	ERCC3			-0.68	
209009_at	ESD	0.93		0.83	
215096_s_at	ESD	1.2		1.24	
202535_at	FADD	-2.92		-2.98	
215719_x_at	FAS	-1.17		-1.05	
216252_x_at	FAS	-1.62		-1.27	
215404_x_at	FGFR1			1.75	1.33
222164_at	FGFR1	0.66		0.94	
210973_s_at	FGFR1			-0.32	
203638_s_at	FGFR2	-2.63		-2.01	

Table I. Continued.

Probe set	Gene symbol	TRD vs. control	TRAIL vs. control	TRD/TRAIL vs. control	TRD/TRAIL vs. TRD
203639_s_at	FGFR2	-4.77		-4.16	
208228_s_at	FGFR2	-2.09		-1.65	
211401_s_at	FGFR2	-3.49			
204579_at	FGFR4	0.89		0.94	
211237_s_at	FGFR4				-0.45
202723_s_at	FOXO1A	-0.45		-1.04	
202724_s_at	FOXO1A	-3.66		-2.14	
204132_s_at	FOXO3A	-0.87		-0.96	
204131_s_at	FOXO3A	-0.5			
201636_at	FXR1			-0.33	
210105_s_at	FYN	-0.36			
208926_at	FYN /// NEU1	1.33		1.43	
203725_at	GADD45A	2.44		2.53	
200824_at	GSTP1	0.48		0.83	
208018_s_at	HCK	1		0.98	
202389_s_at	HD	0.64		0.89	
201209_at	HDAC1	0.65		0.82	
220085_at	HELLS	0.54			
200679_x_at	HMGB1	0.49		0.52	
200680_x_at	HMGB1			0.64	
206864_s_at	HRK			1.2	0.52
200599_s_at	HSP90B1	0.41		0.66	
200598_s_at	HSP90B1	0.38			
200799_at	HSPA1A	1.11		1.6	0.48
200800_s_at	HSPA1A /// HSPA1B	1.61		2.05	0.42
202581_at	HSPA1B	1.76		2.15	0.35
200692_s_at	HSPA9B			-0.3	-0.34
200691_s_at	HSPA9B				-0.34
201841_s_at	HSPB1 /// MEIS3	0.92		1.08	
200806_s_at	HSPD1	-0.2		-0.57	
201631_s_at	IER3	1.44		2.04	0.65
203627_at	IGF1R	-0.67		-0.6	
203628_at	IGF1R	0.61			
202531_at	IRF1	-1.14			
203275_at	IRF2	-2.35		-2.64	
208436_s_at	IRF7	1.95		2.48	
205841_at	JAK2	-3.7			
201464_x_at	JUN	1.47		2.21	0.51
201465_s_at	JUN	2.16		3	0.71
201466_s_at	JUN	0.91		1.55	0.67
213281_at	JUN	2.16		2.9	
205051_s_at	KIT	-3.9		-5.07	
201030_x_at	LDHB			0.57	
201105_at	LGALS1	1.06		1.49	
202625_at	LYN	-1.1		-1.1	
202626_s_at	LYN	-0.43		-0.61	
210754_s_at	LYN	-0.62		-1.09	
203265_s_at	MAP2K4	-3.27		-3.04	
203266_s_at	MAP2K4	-3.37		-3.59	
203836_s_at	MAP3K5	-0.24		-0.75	
200796_s_at	MCL1	-1.86		-1.81	
200797_s_at	MCL1	-0.59		-0.78	

Table I. Continued.

Probe set	Gene symbol	TRD vs. control	TRAIL vs. control	TRD/TRAIL vs. control	TRD/TRAIL vs. TRD
200798_x_at	MCL1	-0.86		-0.96	
217373_x_at	MDM2	1.23		1.88	
203510_at	MET	-2.3		-2.15	
211599_x_at	MET	-2.47		-2.47	
213816_s_at	MET	-0.79		-0.72	
213807_x_at	MET			-2.98	
202431_s_at	MYC	-2.93		-2.71	
201502_s_at	NFKBIA	0.63	0.64	1.62	1
201577_at	NME1	0.49			
201865_x_at	NR3C1	-1.13		-1.33	
201866_s_at	NR3C1	-2.79		-2.76	
211671_s_at	NR3C1	-1.11		-1.37	
216321_s_at	NR3C1	-2.41		-2.03	
214680_at	NTRK2	-0.41		-0.76	
202153_s_at	NUP62	-1.43		-1.54	
201831_s_at	PAK1 /// VDP	0.66			-0.58
201832_s_at	PAK1 /// VDP	0.57			
208644_at	PARP1	0.47			
204004_at	PAWR			-0.5	-0.47
204005_s_at	PAWR			-0.28	
200787_s_at	PEA15	0.54			-0.5
200788_s_at	PEA15	0.81		0.72	
200659_s_at	PHB	-0.5		-0.51	
209019_s_at	PINK1	0.5		0.64	
203966_s_at	PPM1A			-0.86	
209296_at	PPM1B	-2.25		-2.4	
213225_at	PPM1B	-1.1			
204566_at	PPM1D	-1.46		-1.4	
37384_at	PPM1F	0.68		0.62	
203063_at	PPM1F	0.98			
200913_at	PPM1G	-0.62		-1.01	
201407_s_at	PPP1CB	1.04		1.07	
201408_at	PPP1CB	0.56			
201409_s_at	PPP1CB	0.74			
200726_at	PPP1CC	-0.8		-1.03	
202014_at	PPP1R15A	3.74		4.23	
205478_at	PPP1R1A	0.37		0.88	
204554_at	PPP1R3D	-1.82		-1.72	
208652_at	PPP2CA	-0.59		-0.68	
215628_x_at	PPP2CA			1.55	
202884_s_at	PPP2R1B	-0.52		-0.45	
202886_s_at	PPP2R1B	-0.69		-0.85	
202883_s_at	PPP2R1B			-0.96	
202313_at	PPP2R2A	-0.35		-0.73	-0.28
207749_s_at	PPP2R3A			-0.8	-0.39
202425_x_at	PPP3CA	0.82		0.98	
208932_at	PPP4C	0.49			-0.41
203460_s_at	PSEN1	-0.34		-0.42	
204053_x_at	PTEN	-0.44		-0.5	
204054_at	PTEN	-1.1		-1.01	
208820_at	PTK2	-0.44		-0.78	
207821_s_at	PTK2			-0.8	

Table I. Continued.

Probe set	Gene symbol	TRD vs. control	TRAIL vs. control	TRD/TRAIL vs. control	TRD/TRAIL vs. TRD
206482_at	PTK6			0.61	
207011_s_at	PTK7	0.54			
204021_s_at	PURA				0.41
203223_at	RABEP1			-0.72	
200607_s_at	RAD21	-0.73		-0.9	
201244_s_at	RAF1	-1.17		-1.51	
203749_s_at	RARA	-1.08			
209878_s_at	RELA	-0.4		-0.62	
209941_at	RIPK1	-1.61		-1.71	
209544_at	RIPK2	-0.73			
209545_s_at	RIPK2	-1.07			
201257_x_at	RPS3A	0.41		0.8	0.39
200099_s_at	RPS3A /// LOC439992	0.45		0.79	0.38
201628_s_at	RRAGA	-1.23		-0.96	
204197_s_at	RUNX3	-1.31		-1.26	
01844_s_at	RYBP	-1.01		-0.97	
201845_s_at	RYBP	-2.42		-2.49	
201846_s_at	RYBP	-0.98		-0.84	
216976_s_at	RYK			-0.76	-0.56
200051_at	SART1	0.58			
201819_at	SCARB1	0.41			-0.38
203528_at	SEMA4D	-0.81		-0.95	
214882_s_at	SFRS2				0.55
201739_at	SGK	-0.39			
202980_s_at	SIAH1	-2.01		-1.56	
202981_x_at	SIAH1	-2.23		-2.32	
203489_at	SIVA	-0.19		-0.22	
200071_at	SMNDC1	-0.88		-0.9	
201086_x_at	SON	-1.46		-1.83	
202693_s_at	STK17A	-2.22		-2.22	
202695_s_at	STK17A	-1.01		-1	
207540_s_at	SYK	-0.86		-0.59	
200804_at	TEGT	0.6			
202039_at	TIAF1 /// MYO18A	0.63			
202405_at	TIAL1			-0.87	
202406_s_at	TIAL1	-0.18			
201149_s_at	TIMP3			-0.88	
202644_s_at	TNFAIP3	1.5	0.68	1.5	
207643_s_at	TNFRSF1A	-2.84		-2.93	
203508_at	TNFRSF1B	-2.53		-1.9	
214581_x_at	TNFRSF21	-1.2		-1.3	
218856_at	TNFRSF21	-0.91		-1.12	
219423_x_at	TNFRSF25			1.13	
202688_at	TNFSF10	-0.14	-0.31	0.48	0.78
202687_s_at	TNFSF10	-0.36			0.81
214329_x_at	TNFSF10			0.58	0.85
203839_s_at	TNK2			1.23	
201746_at	TP53	-0.54			
203120_at	TP53BP2	-1.02		-0.84	
1729_at	TRADD	-0.45		-0.8	
221571_at	TRAF3			-0.29	-0.27

Table I. Continued.

Probe set	Gene symbol	TRD vs. control	TRAIL vs. control	TRD/TRAIL vs. control	TRD/TRAIL vs. TRD
202871_at	TRAF4	-0.6			
201588_at	TXNL1	-1.19		-1.11	
205546_s_at	TYK2	0.65		0.74	
202316_x_at	UBE4B	-0.37		-0.58	-0.58
212533_at	WEE1	-1.39		-1.28	
215711_s_at	WEE1	-1.08		-1.19	
202932_at	YES1			-0.28	-0.32
201020_at	YWHAH	-1.22		-1.49	

Signal log ratios of the changes are given for the several samples (TRD vs. control, TRAIL vs. control, TRD/TRAIL vs. control, TRD/TRAIL vs. TRD), signal log ratio of 1 representing a 2-fold increase, one of -1, that the expression is half of the expression of the control group and so forth.

(68), which was 'upregulated' by TRD. These effects may outweigh the reduced expression of APAF 1, found in the TRD treated cells and represent a proapoptotic effect of TRD via the mitochondrial pathway.

TRD interacted with the mitochondrial pathway also by 'upregulation' of cytochrome-c expression, which is known to activate the caspase cascade as mentioned above. Within the TNF-receptor system TNFRSF25/DR3, a death receptor binding to FADD and thereby triggering apoptosis (69) was found to be 'upregulated'.

GADD45A, a potent inhibitor of the c-Jun N-terminal kinase (JNK) cascade and NFKB, inhibits transcription factors associated with tumor growth (65,70-72) and was 'upregulated' by TRD. Additionally GADD45 may antagonize TNF-receptor mediated cytotoxicity (70). The transcriptional factor JUN, that was 'upregulated' in the TRD and the TRAIL treated cells, is known to be a product of MAP2K4-activation and to mediate apoptosis by several chemotherapeutics (71,73).

TRAIL. No concentration of TRAIL as a single substance had a significant influence on the treated cells concerning apoptosis or necrosis within the observed time frame of 48 h, indicating that KYSE 270 squamous esophageal cancer cells are primary TRAIL resistant.

Accordingly, the influence of TRAIL as a single substance on gene expression was moderate. Only caspase-1 and Bcl2l1, which affect the cytochrome-c efflux in a similar way as Bcl-2 and endogenous TRAIL, were 'down-regulated'. Coinciding with our gene expression profile findings, TRAIL is known to increase the expression of anti-apoptotic mediators such as BIRC2/IAP2 and BIRC3/API2 in some malignant cells, probably via an inactivation of NFKB (74,75). TNFAIP3, that was found to be 'upregulated' by TRAIL in our study, is an inhibitor of NFKB and may thereby promote apoptosis, but on the other hand TNFAIP3 was shown by other authors to decrease TNF-mediated apoptosis (76), leaving its specific influence unclear.

As previously shown, the members of the TNF family, such as TRAIL, also have many non-apoptotic functions *e.g.* activating transcription through NFKB and JUN/AP-1 leading to induction of immunomodulatory and inflammatory genes (77), involving TRADD and FADD, which are also known as mediators of several apoptotic pathways (78-80). Crosstalking to these other pathways and activating transcription factors may be unselective and partly explain possible toxic effects of TRAIL on non-malignant cells (81). Interestingly, TRD was shown to reduce toxicity of TNF *in vivo* without reducing its antitumoral activity probably by interfering not with TNF directly but with its down-stream pathway which is largely the same for TRAIL (82,83). Accordingly, TRD, among other effects, inhibits the activation of NFKB, which is a potent signal trans-ducer for inflammatory cytokines, by oxidation of IκB-α at Met45 (40).

NFKB is activated TRADD-, TRAF3- and FADD-dependently (72) and also plays a key role in the survival of tumor cells by inducing expression of anti-apoptotic genes such as Bcl2, Bcl2l1, vascular endothelial growth factor (VEGF) and X-linked inhibitor of apoptosis (XIAP). In physiological conditions, NFKB is sequestered in inactive form by inhibitory proteins such as NFKBIA (84-86).

Another indication for the relevance of NFKB for the observed TRD and TRAIL mediated apoptosis is the fact that NFKBIA was 'upregulated' by combination of TRAIL and TRAIL/TRD.

TRD/TRAIL. When TRAIL was added to TRD, we detected a highly significant increase in apoptotic and non-viable cells compared to the control and to TRD alone. The kinetics remained unchanged compared to TRD alone. TRAIL (500 ng/ml) proved to be the most effective concentration for the combined use with TRD 100 μmol/l, leaving only 79% of the cells viable after 48 h. In the direct comparison of the most potent single concentrations as mono-substance and the combination-treatment, a synergistic effect of TRAIL (500 ng/ml) and TRD (250 μmol/ml) with a bisection of the remaining living cells after 12 h was observed.

The synergistic effects of the combination of TRD and TRAIL may be caused by an activation of the same and of distinct apoptotic pathways, such as the TNF-receptor and the mitochondrial one, which, jointly triggered, result

in an amplified response. However, the effects of the single substances and the gene expression profiles of the cells, treated with the combination TRD and TRAIL, point to NFKB as a key factor in apoptosis, mediated by these substances. When the TRD/TRAIL results were compared to TRD alone, we found endogenous TRAIL 'upregulated', whereas it was 'downregulated' by TRD and TRAIL as single substances. TRAF3 as an activator of NFKB was 'downregulated'; NFKBIA as an inhibitor of NFKB was 'upregulated'. JUN, that was 'upregulated' in the TRAIL and in the TRD/TRAIL treated cells, is known to be a product of MAP2K4-activation and to mediate apoptosis by several chemotherapeutics, but has also been associated with anti-apoptotic effects (73,87). While all death receptors as well as the described transcription factors can activate both apoptotic and non-apoptotic pathways, it has been widely assumed that the main physiological role of FADD-binding death receptors is to trigger apoptosis (69) and of NFKB to have mainly anti-apoptotic effects (85).

The 'downregulation' of DFFA may be associated with the TRD dependent 'downregulation' of caspase-3, which has been shown to be responsible for activation of DFFA (88). Altogether, as shown by the FACS analysis, the anti-apoptotic effects of the combination therapy detected in the microarray analysis were greatly outweighed by the cell death inducing ones, suggesting that important factors for the synergistic effects may be increased TRAIL and decreased NFKB activity.

We conclude that the increased activation of the mitochondrial and the TNF-receptor associated pathway as well as the inhibition of crucial transcription factors qualify the TRD/TRAIL combination therapy for further studies, especially taking into consideration that other chemotherapeutics that could amplify the effect of TRAIL or sensitize resistant cells to TRAIL show considerable toxicity (35,89). However, there is evidence that TRD may not only add to the apoptotic effect of TRAIL but also decrease its possible toxic side effects by pathway modulation.

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