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Resveratrol-mediated sensitisation to TRAIL-induced apoptosis depends on death receptor and mitochondrial signalling

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Abstract

Natural food products such as resveratrol have gained considerable attention as cancer chemopreventive agents. In the present study, we investigated the potential of resveratrol to overcome the resistance of tumour cells against TRAIL. While resveratrol enhanced TRAIL-induced apoptosis through G1 cell cycle arrest and survivin depletion, resveratrol failed to sensitise cells with high expression levels of Bcl-2 or FADD-DN. Interestingly, overexpression of Bcl-2 or FADD-DN did not interfere with resveratrol-mediated cell cycle arrest or survivin depletion, but blocked release of cytochrome *c* and Smac from mitochondria into the cytosol, enhanced caspase activation and apoptosis upon combined treatment with resveratrol and TRAIL indicating that overexpression of Bcl-2 or FADD-DN decoupled the effect of resveratrol on the cell cycle and apoptosis. Similarly, cell cycle arrest at G1 using the cell cycle specific inhibitor mimosine or downregulation of survivin expression by antisense oligonucleotides failed to enhance TRAIL-induced apoptosis in Bcl-2- or FADD-DN-transfected cells. Likewise, inhibition of caspase activity using the broad range caspase inhibitor zVAD.fmk did not interfere with resveratrol-mediated cell cycle arrest and survivin depletion, while blocking apoptosis upon combined treatment with resveratrol and TRAIL. Thus, resveratrol is a potent sensitiser for TRAIL in certain tumours. However, it may be ineffective in others, e.g. in tumours with enhanced Bcl-2 expression or defective death receptor signalling.

Keywords: Apoptosis; Resveratrol; TRAIL; Cancer; Resistance

1. Introduction

Cell death by apoptosis plays a pivotal role in the regulation of various physiological or pathological conditions and has also been implicated in mediating therapy-induced cytotoxicity, e.g. in response to cytotoxic drug treatment, γ-irradiation or cytotoxic cytokines [1–3]. Apoptotic pathways may be initiated through different entry sites, such as death receptors (receptor pathway) or mitochondria (mitochondrial pathway) resulting in activation of effector caspases [3]. Stimulation of death receptors of the tumour necrosis factor (TNF) receptor superfamily such as CD95 (APO-1/Fas) results in receptor aggregation and recruitment of the adaptor molecule Fas-associated death domain (FADD) and caspase-8 [4]. Upon recruitment, caspase-8 becomes activated and initiates apoptosis by

Abbreviations: AIF, apoptosis-inducing factor; FACS, fluorescence-activated cell-sorting; FADD, Fas-associated death domain; FADD-DN, dominant-negative Fas-associated death domain; IAPs, inhibitor of apoptosis proteins; Smac, second mitochondria-derived activator of caspase; TRAIL, TNF-related apoptosis-inducing ligand; zVAD.fmk, benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone.

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direct cleavage of downstream effector caspases. A second pathway is initiated at the mitochondrial level. Upon induction of apoptosis, apoptogenic factors such as cytochrome *c*, apoptosis-inducing factor (AIF) or Smac/DIABLO (direct IAP binding protein with low pI) are released from mitochondria into the cytosol. Cytochrome *c* triggers caspase-3 activation through formation of the cytochrome *c*/Apaf-1/caspase-9-containing apoptosome complex, while Smac/DIABLO promotes caspase activation through neutralising the inhibitory effects to (IAPs) [5]. Signals originating from the CD95 receptor may be linked to mitochondria by Bid, a BH3 domain-containing protein of the Bcl-2 family which assumes cytochrome *c*-releasing activity upon cleavage by caspase-8, thereby initiating a mitochondrial amplification loop [5].

The death receptor ligand (TRAIL) is a promising candidate for cancer therapy, because it induces apoptosis in a broad spectrum of cancer cell lines and also exhibits potent tumoricidal activity in several xenograft models *in vivo* [6]. Unlike the other death ligands, CD95 or TNFα, systemic administration of soluble human TRAIL does not cause systemic toxicity in mice or non-human primates, although further studies may be necessary to evaluate the possible cytoxicity of TRAIL on human normal tissues, e.g. hepatocytes [6]. However, many tumours remain resistant towards treatment with TRAIL, which may be caused by defects in apoptotic programmes [6]. IAPs, such as survivin, are expressed at high levels in many tumours including neuroblastoma and have been associated with a poor prognosis [7,8]. IAPs block apoptosis at the core of the apoptotic machinery by inhibiting effector caspases. Tumours also often harbour defects in the mitochondrial pathway by overexpression of anti-apoptotic proteins of the Bcl-2 family [9]. In addition, the death receptor pathway is frequently impaired in tumours, e.g. by overexpression of FLICE inhibitory protein (FLIP) or by loss of caspase-8 expression [10]. Blockade of the mitochondrial pathway by Bcl-2 or inhibition of the death receptor pathway by (FADD-DN) inhibited TRAIL-induced apoptosis in certain cell types, e.g. in type II cells [10,11].

Naturally occurring dietary compounds, such as resveratrol, have recently gained considerable attention as cardioprotective and canceropreventive agents and also exhibit antitumour activity [12,13]. We previously found that the chemopreventive agent resveratrol sensitised tumour cells to TRAIL-induced apoptosis through G1 arrest and associated survivin depletion [14]. Since cell cycle-dependent sensitivity of tumour cells to the induction of cell death has been exploited to overcome the resistance of tumours, we investigated the potential of resveratrol to overcome the resistance of Bcl-2- or FADD-DN-overexpressing tumour cells towards TRAIL-induced apoptosis.

2. Materials and methods

2.1. Cell culture

SHEP neuroblastoma or Jurkat T cell leukaemia cells transfected with vector control (Neo), Bcl-2 or FADD-DN or were maintained in Roswell Park Memorial Institute (RPMI) 1640 medium (Life Technologies, Inc., Eggenstein, Germany) as previously described in Ref. [15]. 0.5×10^5 cells/ml were cultured in 96-well, 24-well or 6-well plates or in 75 cm^2 flasks (Falcon, Heidelberg, Germany).

2.2. Determination of apoptosis, cell viability and clonogenic growth

Cells were incubated with resveratrol, mimosine, thymidine, nocodazole (all from Sigma, Deisenhofen, Germany), TRAIL (PeproTech, Rocky Hill, NJ), anti-APO1 (CD95) monoclonal antibody, zVAD.fmk (Bachem, Heidelberg, Germany), TNFα (Biochrom, Berlin, Germany) at the concentrations and times indicated. Apoptosis was assessed by fluorescence-activated cell-sorting (FACS) analysis of DNA fragmentation of propidium-iodide stained nuclei or by annexin V staining (Roche Diagnostics, Mannheim, Germany) and cell viability was determined by the dimethylthiahzolyl-2,5-diphenyl-tetrazolium bromide (MTT) assay according to the manufacturer's instructions (Roche Diagnostics) as previously described in Ref. [15]. Clonogenic growth was assessed by crystal violet staining as previously described in Ref. [16].

2.3. Cell cycle analysis

Analysis of cell cycle and/ or apoptosis was performed by flow cytometric staining of permeabilised cells with propidium iodide for DNA content and with annexin-fluorescein isothiocyanate (FITC) for apoptosis as previously described in Ref. [14]. Briefly, 10^6 cells were stained with annexin-FITC for 20 min at 4 °C, fixed with 4% paraformaldehyde (Sigma) for 20 min at 4 °C, permeabilised with digitonin (Sigma, 50 μg/ml) for 5 min at room temperature and stained with propidium iodide (15 μg/ml) for 15 min at 37 °C in the presence of RNaseA (Sigma, 20 μg/ml).

2.4. Western blotting analysis

Western blotting analysis were performed as previously described in Ref. [15], using mouse anti-caspase-8 monoclonal antibody C15 (1:10 dilution of hybridoma supernatant, kindly provided by P. Krammer), mouse anti-caspase-3 monoclonal antibody (1:1000, BD Biosciences, Heidelberg, Germany), mouse anti-caspase-2 monoclonal antibody (1:1000, BD Biosciences), rabbit

anti-caspase-9 polyclonal antibody (1:1000, BD Biosciences), rabbit anti-cleaved caspase-6 polyclonal antibody (1:1000, Cell Signaling, Beverly, MA), mouse anti-X-linked inhibitor of apoptosis (XIAP) monoclonal antibody (1:1000, H62120, BD Biosciences), mouse anti-p53 monoclonal antibody (1:1000, BD Biosciences;

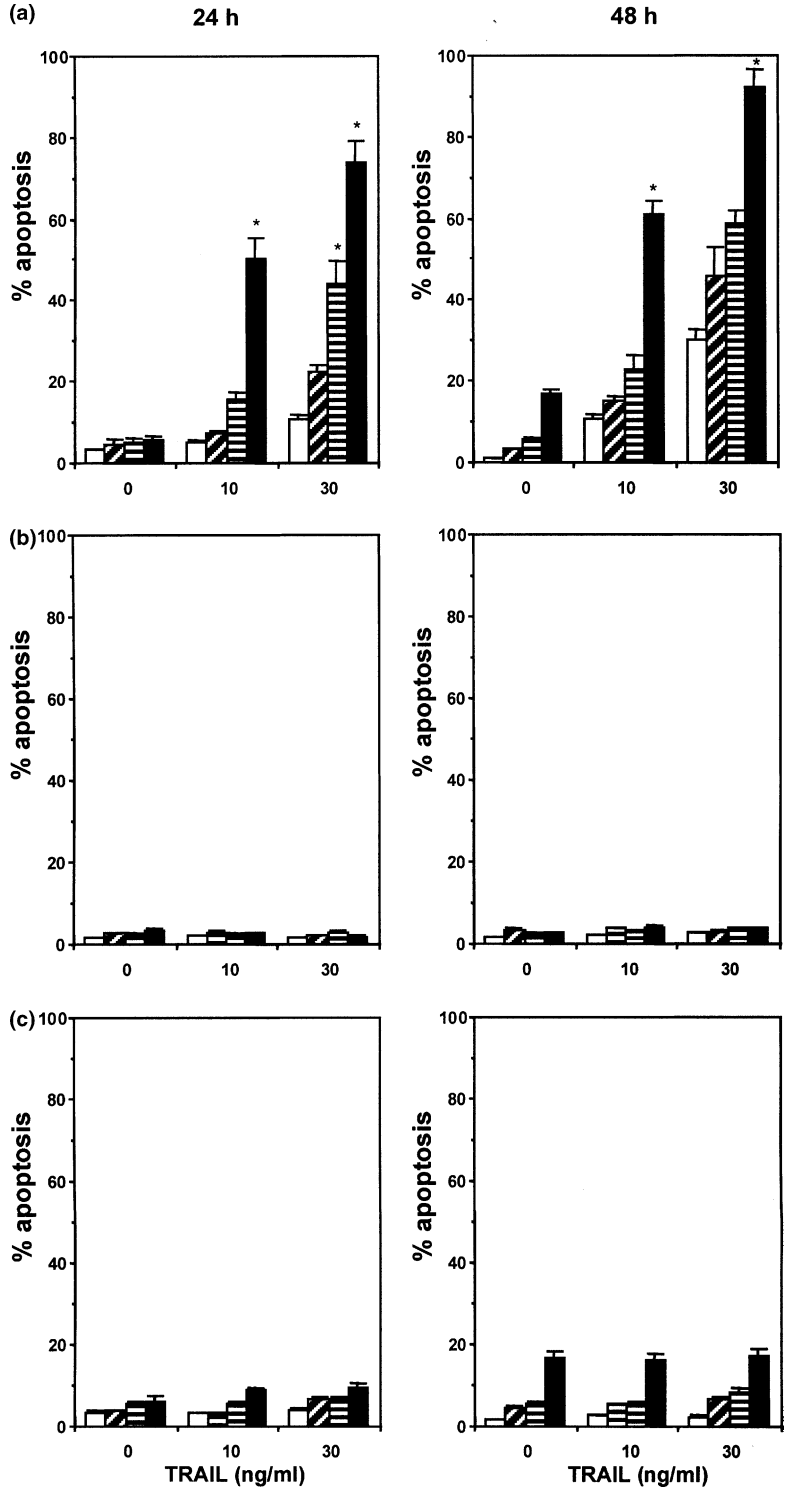

Fig. 1. Effect of Bcl-2 or FADD overexpression on resveratrol-mediated sensitisation to TRAIL-induced apoptosis. SHEP neuroblastoma cells transfected with vector control (a), Bcl-2 (b) or FADD-DN (c) were treated with 0-100 μM resveratrol (0 μM: white bars, 10 μM: vertically hatched bars, 30 μM: horizontally hatched bars, 100 μM: black bars) and/or 0–30 ng/ml TRAIL for 24 h (left panel) or 48 h (right panel). Apoptosis was determined by fluorescence-activated cell-sorting (FACS) analysis of propidium-iodide stained DNA. Mean and standard deviation (SD) of three independent experiments done in triplicate are shown. $^{*}P < 0.01$.

PharMingen), rabbit anti-survivin polyclonal antibody (1:1000, R&D Systems, Lake Placid, NY), rabbit anti-Bid polyclonal antibody (1:1000, R&D Systems), rabbit anti-p21 polyclonal antibody (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-poly (ADP-Ribose) polymerase (PARP) polyclonal antibody (1:1000, Roche Diagnostics, Penzberg, Germany), rabbit anti-cIAP2 polyclonal antibody (1:1000, Santa Cruz Biotechnology), β-actin monoclonal antibody (1:5000, Sigma) followed by goat anti-mouse IgG or goat anti-rabbit IgG (1:5000, Santa Cruz Biotechnology). Enhanced chemiluminescence (ECL, Amersham Pharmacia, Freiburg, Germany) was used for detection. Expression of β-actin was used to control for equal gel loading.

2.5. Antisense oligonucleotides

To inhibit survivin expression, phosphothiorate antisense oligonucleotides against survivin expression and

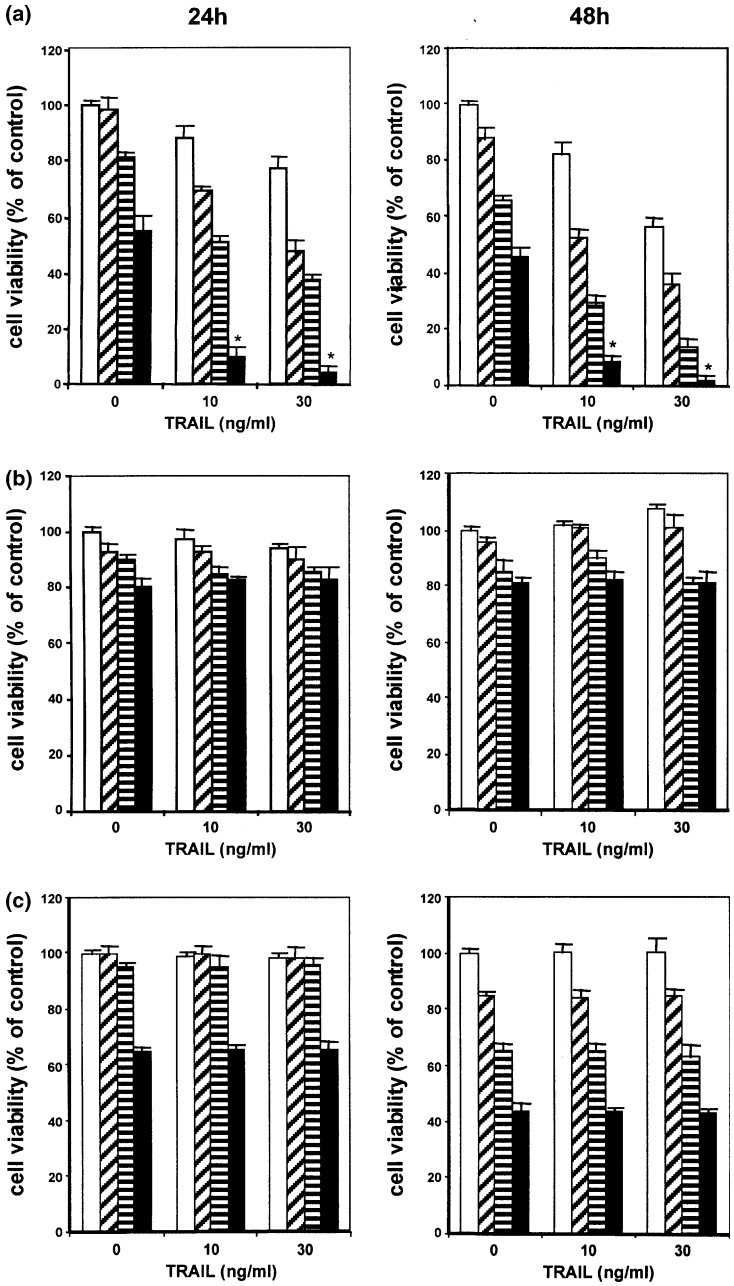

Fig. 2. Effect of Bcl-2 or FADD overexpression on resveratrol-mediated sensitisation to TRAIL-induced cytotoxicity. SHEP neuroblastoma cells transfected with vector control (a), Bcl-2 (b) or FADD-DN (c) were treated with 0-100 μM resveratrol (0 μM: white bars, 10 μM: vertically hatched bars, 30 μM: horizontally hatched bars, 100 μM: black bars) and/or 0–30 ng/ml TRAIL for 24 h (left panel) or 48 h (right panel). Cell viability was determined by the dimethylthiazolyl-2,5-diphenyltetrazolium bromide (MTT) assay and expressed as a percentage of untreated control cells. Mean and SD of three independent experiments done in triplicate are shown. $^{*}P < 0.01$.

mismatch control oligonucleotides (Thermo Hybaid, Ulm, Germany) with published sequences were used as previously described in Ref. [17].

2.6. Preparation of mitochondria or cytosolic extracts

Preparation of mitochondria or cytosolic extracts was performed using the ApoAlert cell fractionation kit (BD Biosciences) according to the manufacturer's instructions.

2.7. Assessment of mitochondrial transmembrane potential

The cationic lipophilic fluorochrome 3,3′-dihexyloxacarbocyanide iodide ($DiOC_6(3)$) (460 ng/ml, Molecular

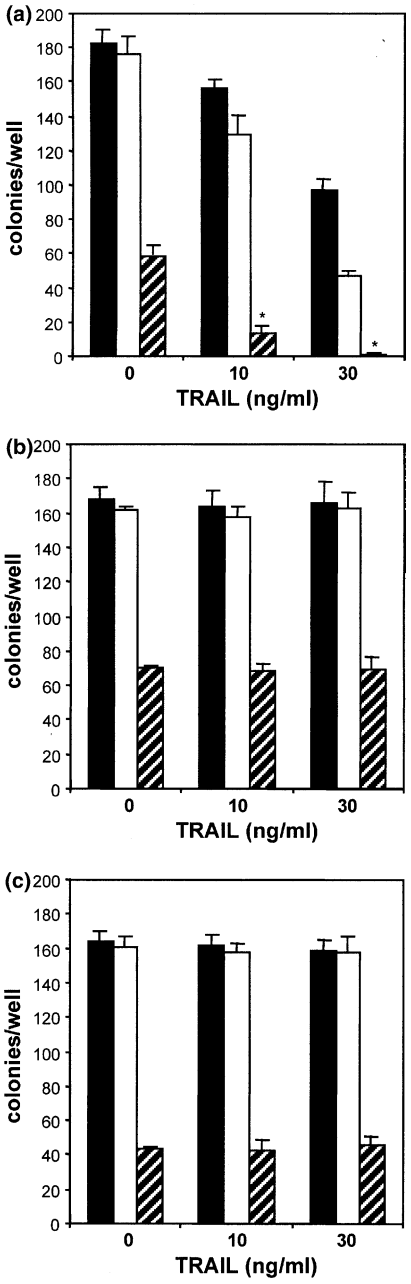

Fig. 3. Effect of Bcl-2 or FADD overexpression on resveratrol-mediated sensitisation to TRAIL-induced inhibition of clonogenic survival. SHEP neuroblastoma cells transfected with vector control (a), Bcl-2 (b) or FADD-DN (c) were treated with 0–30 μM resveratrol (0 μM: black bars, 10 μM: white bars, 30 μM: vertically hatched bars) and/or 0–30 ng/ml TRAIL. Clonogenic survival was determined by crystal violet staining and colony numbers per well of a 24-well plate are indicated. Mean and SD of three independent experiments done in triplicate are shown. $^*P < 0.01$.

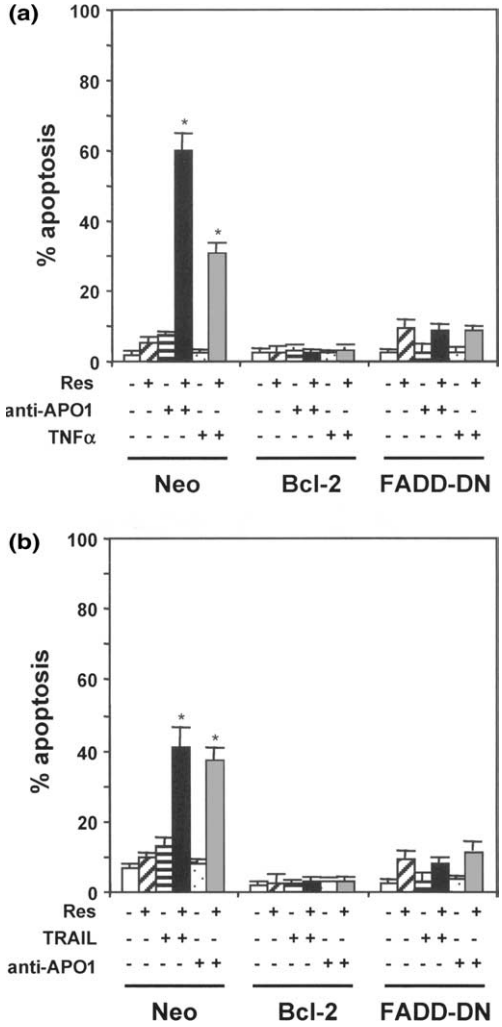

Fig. 4. Effect of Bcl-2 or FADD overexpression on resveratrol-mediated sensitisation to death receptor-induced apoptosis. SHEP neuroblastoma cells (a) or Jurkat T cell leukaemia cells (b) transfected with vector control (Neo), Bcl-2 or FADD-DN were left untreated or were treated with 100 μM (a) or 30 μM (b) resveratrol and/or 1 μg/ml (a) or 0.01 μg/ml (b) anti-CD95 antibody, 10 ng/ml TRAIL or 100 ng/mL tumour necrosis factor α (TNFα). Apoptosis was determined by FACS analysis of propidium-iodide stained DNA. Mean and SD of three independent experiments done in triplicate are shown. $^*P < 0.01$.

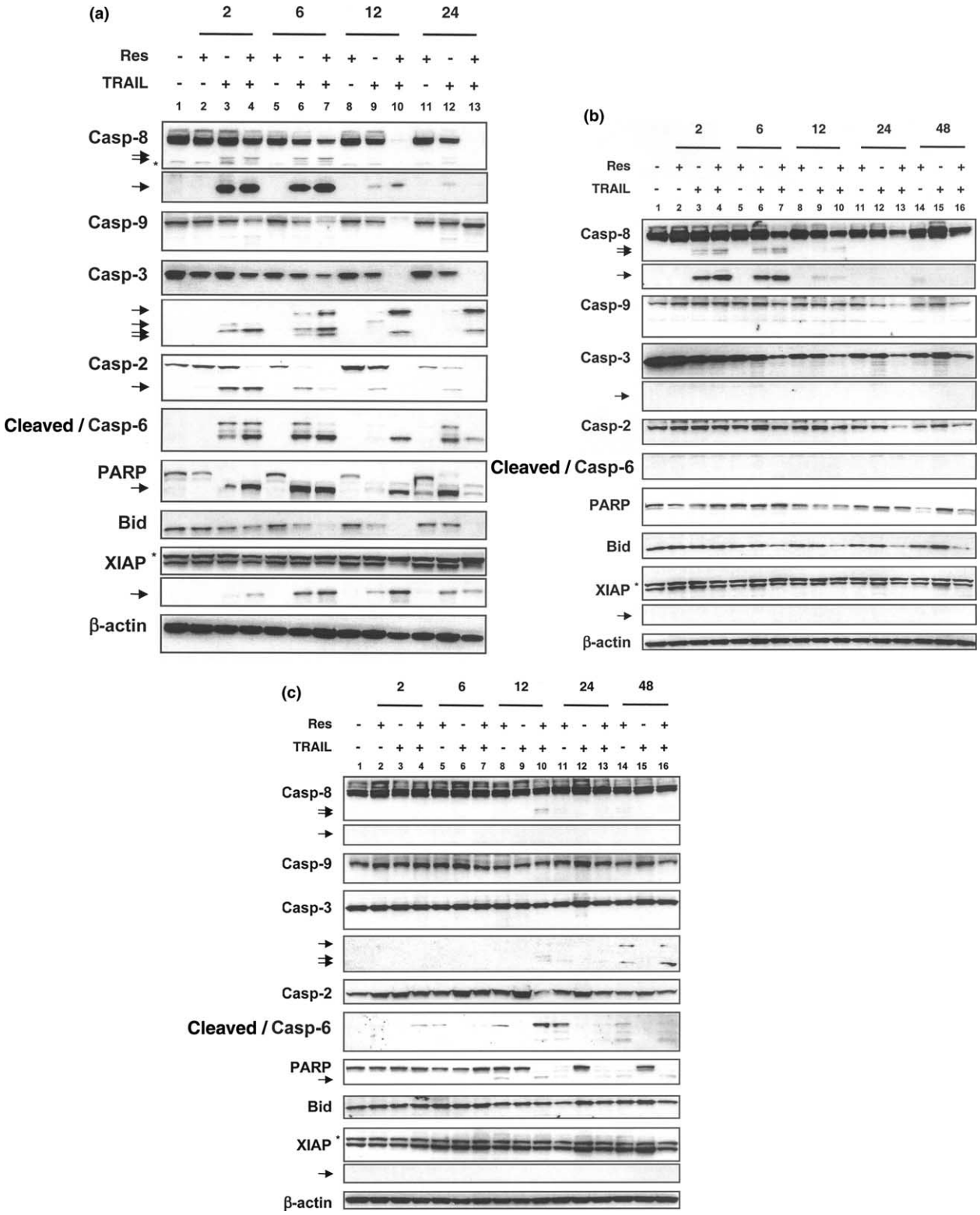

Fig. 5. Effect of Bcl-2 or FADD overexpression on resveratrol-mediated sensitisation to TRAIL-induced caspase activation. SHEP neuroblastoma cells transfected with vector control (a), Bcl-2 (b) or FADD-DN (c) were treated for 0–48 h with 100 μM resveratrol and/or 30 ng/ml TRAIL. Expression of caspase-8, -9, -3, -2, cleaved caspase-6, poly (ADP-ribose) polymerase (PARP), Bid, X-linked inhibitor of apoptosis (XIAP) and β-actin was assessed by Western blotting analysis. Cleavage products are indicated by arrows and non-specific immune-reactive bands are indicated by an asterix.

Probes, Eugene, OR) was used to measure the mitochondrial transmembrane potential ($\Delta\Psi_m$). Cells were incubated for 15 min at 37 °C in the presence of the fluorochrome, washed in phosphate-buffered saline (PBS)/1% fetal calf serum (FCS) and immediately analysed by flow cytometry (FACScan, Becton–Dickinson). $DiOC_6(3)$ was recorded in fluorescence 1.

2.8. Statistical analysis

The experiments were performed in triplicate and repeated at least three times. The significance of the observed effects was evaluated by the *t* test at $^*P < 0.01$.

3. Results

3.1. Inhibition of resveratrol-mediated sensitisation to TRAIL-induced apoptosis by Bcl-2 or FADD-DN overexpression

We previously found that the chemopreventive agent resveratrol sensitised tumour cells to TRAIL-induced apoptosis through cell cycle-mediated survivin depletion [14]. Since cell cycle-dependent sensitivity of tumour cells to cytotoxic therapies has been exploited to target resistant tumours, we investigated the interplay between the cell cycle and the sensitivity to apoptosis of tumour cells overexpressing Bcl-2 or FADD-DN, which were resistant TRAIL-induced apoptosis. Resveratrol

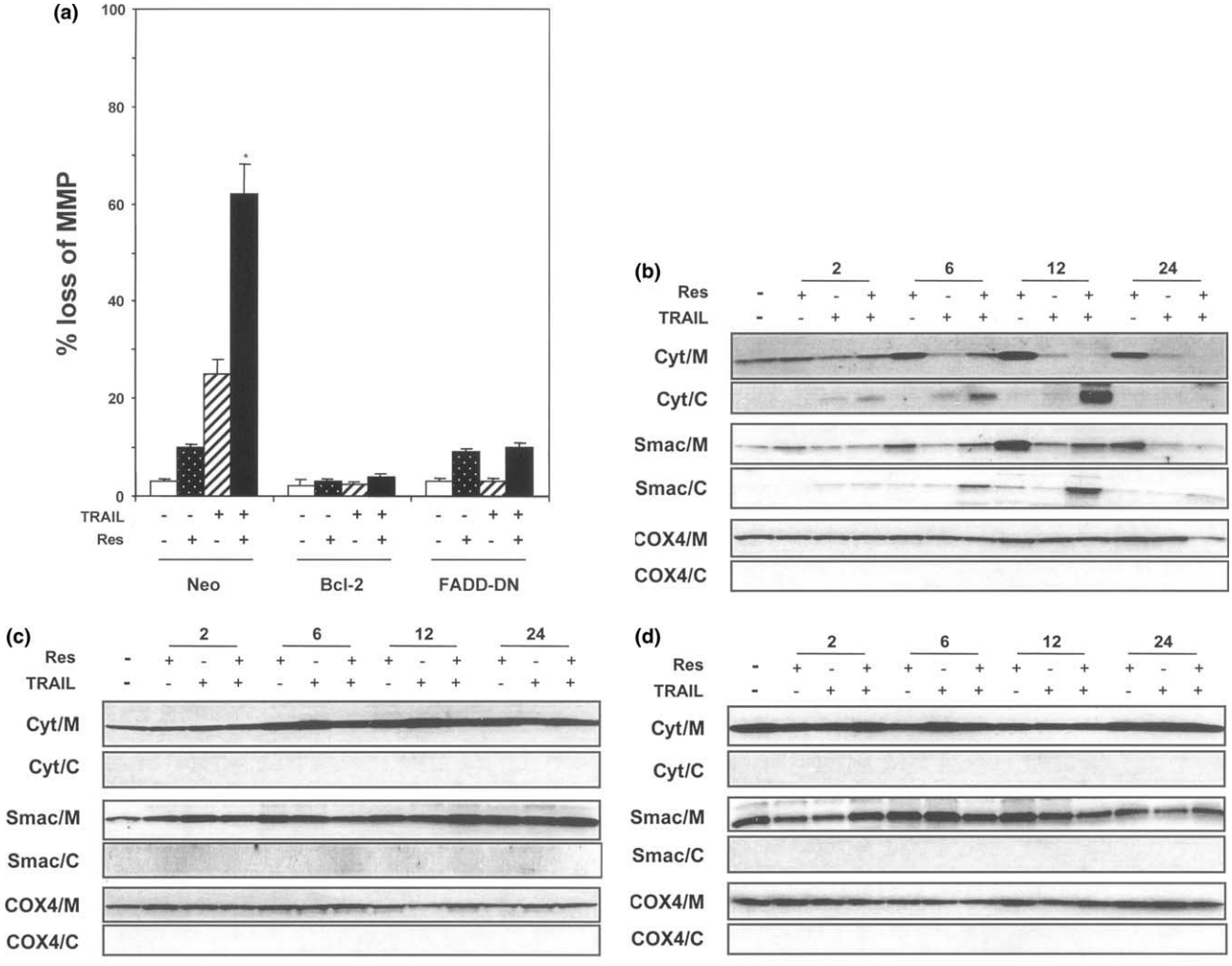

Fig. 6. Effect of Bcl-2 or FADD overexpression on resveratrol-mediated sensitisation to TRAIL-induced mitochondrial perturbations. (a) Effect of Bcl-2 or FADD overexpression on mitochondrial membrane potential (MMP). SHEP neuroblastoma cells transfected with vector control (Neo), Bcl-2 or FADD-DN were treated for 12 h with 100 μM resveratrol and/or 30 ng/ml TRAIL. Mitochondrial membrane potential was assessed by flow cytometry using the fluorescent dye $DiOC_6(3)$. Mean and SD of three independent experiments done in triplicate are shown.* $P < 0.01$ (b)–(d) Effect of Bcl-2 or FADD overexpression on the release of mitochondrial proteins. SHEP neuroblastoma cells transfected with vector control (b), Bcl-2 (c) or FADD-DN (d) were treated for 0–24 h with 100 μM resveratrol and/or 30 ng/ml TRAIL. Expression of Smac, cytochrome *c* or COX4 in the mitochondrial (M) or cytosolic (C) fraction was assessed by Western blotting analysis.

cooperated with the death ligand TRAIL to induce apoptosis in SHEP neuroblastoma cells transfected with empty vector control in a dose- and time-dependent manner (Fig. 1(a)). In contrast, overexpression of Bcl-2 blocked apoptosis upon treatment with TRAIL, resveratrol or upon combined treatment with TRAIL and resveratrol (Fig. 1(b)). In addition, resveratrol failed to sensitise FADD-DN overexpressing cells to TRAIL-induced apoptosis, whereas treatment with resveratrol alone induced apoptosis in FADD-DN overexpressing cells to a similar extent as in vector control cells (Fig. 1(c), compare with Fig. 1(a)). Similar results were obtained when cell viability was assessed by the MTT assay (Fig. 2). To investigate whether overexpression of Bcl-2 or FADD-DN simply delayed cell death or provided long-term protection, we performed a colony forming assay. Importantly, overexpression of Bcl-2 or FADD-DN blocked the cooperative effect of resveratrol and TRAIL to inhibit clonogenic tumour cell growth (Fig. 3). To see whether our observations were restricted to the death ligand TRAIL, we also tested the effect of resveratrol on CD95- or TNFα-induced apoptosis. Similarly, resveratrol sensitised vector control cells to CD95- or TNFα-induced apoptosis, while overexpression of Bcl-2 or FADD-DN blocked resveratrol-mediated sensitisation to CD95- or TNFα-induced apoptosis (Fig. 4(a)). Furthermore, we extended our studies to Jurkat T cell leukaemia cells to exclude that our findings were restricted to a particular tumour cell type. While resveratrol cooperated with TRAIL or agonistic anti-CD95 antibody to trigger apoptosis in Jurkat cells, overexpression of Bcl-2 or FADD-DN blocked apoptosis upon combined treatment with resveratrol and TRAIL or resveratrol and agonistic anti-CD95 antibodies (Fig. 4(b)). These findings indicate that overexpression of Bcl-2 or, FADD-DN blocked resveratrol-mediated sensitisation to death receptor-mediated apoptosis in different cell types.

3.2. Inhibition of resveratrol-mediated sensitisation to TRAIL-induced caspase activation by Bcl-2 or FADD-DN overexpression

To gain further insight into the mechanism(s) regulating the sensitivity or resistance towards treatment with resveratrol and TRAIL, we analysed activation of the caspase cascade. Western blotting analysis revealed that resveratrol and TRAIL cooperated to activate caspase-8, -9, -3, -2, -6 and cleavage of PARP, Bid and XIAP in SHEP neuroblastoma cells transfected with vector control (Fig. 5(a)). Overexpression of Bcl-2 inhibited activation of the effector caspase-3 into active fragments, and cleavage of caspase substrates such as PARP or XIAP in response to treatment with TRAIL and resveratrol (Fig. 5(b)). Interestingly, overexpression of Bcl-2 did not prevent initial cleavage of caspase-8 induced by TRAIL or the combination of TRAIL and resveratrol (Fig. 5(b), lanes 3,4,6,7), while Bcl-2 inhibited enhanced caspase-8 cleavage upon combined treatment with TRAIL and resveratrol for 120-48 h (Fig. 5(b), lanes 10,13, compare with Fig. 5(a), lanes 10,13). These findings indicate that overexpression of Bcl-2 blocked the full turnover of caspase-8 by inhibiting the activation of effector caspases, which may activate caspase-8 in a feedback amplification loop. In cells overexpressing FADD-DN, treatment with resveratrol alone or with the combination of resveratrol and TRAIL resulted in minimal cleavage of caspase-3 and PARP (Fig. 5(c)), in line with the minimal amount of apoptosis induced by resveratrol (Fig. 1(c)). These findings indicate that overexpression of Bcl-2 or FADD-DN inhibited the cooperative effect of resveratrol and TRAIL on the activation of effector caspases.

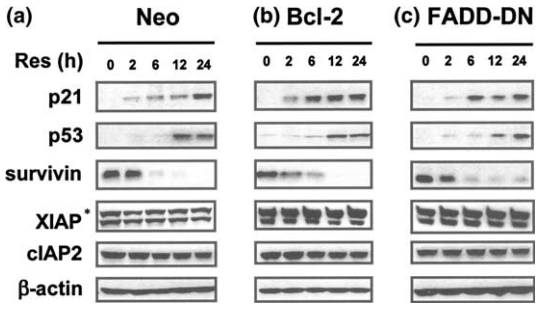

Fig. 7. Effect of Bcl-2 or FADD overexpression on resveratrol-induced modulation of apoptotic regulatory molecules. SHEP neuroblastoma cells transfected with vector control (a), Bcl-2 (b) or FADD-DN (c) were treated with 100 μM resveratrol for 0–24 h and assessed for protein expression of p21, p53, survivin, XIAP, cIAP2 and β-actin by Western blotting analysis. In addition to XIAP, a non-specific XIAP immune-reactive molecule migrating slightly larger than XIAP was detected using the mouse monoclonal antibody H62120, which is indicated by an asterix.

Table 1
Effect of Bcl-2 or FADD overexpression on resveratrol-induced cell cycle distribution

	G1 (%)	S (%)	G2/M (%)
Neo			
− resveratrol	58 ± 2.7	19 ± 1.2	23 ± 1.5
+ resveratrol	82 ± 2.1	11 ± 0.9	9 ± 1.4
Bcl-2			
− resveratrol	56 ± 2.4	21 ± 1.2	22 ± 1.1
+ resveratrol	80 ± 2.2	10 ± 1.6	9 ± 1.3
FADD-DN			
− resveratrol	59 ± 2.2	20 ± 2.8	24 ± 1.9
+ resveratrol	79 ± 1.9	10 ± 1.8	10 ± 1.8

SHEP neuroblastoma cells transfected with vector control (Neo), Bcl-2 or FADD-DN were left untreated (−resveratrol) or were treated with 100 μM resveratrol for 24 h (+resveratrol). Cell cycle distribution was assessed as described in Section 2. Mean and standard deviation (SD) of three independent experiments done in triplicate are shown.

3.3. Overexpression of Bcl-2 or FADD-DN inhibit the resveratrol-mediated increase in mitochondrial perturbations

We then tested the effect of resveratrol and TRAIL on mitochondrial function. Treatment with resveratrol and TRAIL resulted in enhanced loss of mitochondrial membrane potential (MMP) and release of cytochrome *c* or Smac from mitochondria compared with either treatment alone in SHEP neuroblastoma cells transfected with vector control (Figs. 6(a) and (b)). Overexpression of Bcl-2 inhibited the loss of MMP induced by resveratrol and /or TRAIL (Fig. 6(a)). In addition, overexpression of FADD-DN inhibited the cooperative effect of resveratrol and TRAIL to trigger loss of MMP, while it did not prevent loss of MMP upon treatment with resveratrol alone (Fig. 6(a)), consistent with the minimal activation of effector caspases and apoptosis induced by resveratrol in FADD-DN-transfected cells (Figs. 1 and 5(c)). In addition, overexpression of Bcl-2 or FADD-DN inhibited the release of cytochrome *c* or Smac from mitochondria into the cytosol upon treatment with resveratrol and/or TRAIL (Figs. 6(b) and (c)).

3.4. Modulation of regulators of apoptosis by resveratrol

To gain further insight into the molecular mechanism(s) of resveratrol to regulate the sensitivity of tumour cells to undergo apoptosis, we investigated several key regulatory molecules of the apoptotic pathway. Interestingly, upregulation of p53 and p21 and downregulation of survivin protein expression upon treatment with resveratrol was found to be similar in vector control-Bcl-2- or FADD-DN-transfected cells (Fig. 7). No detectable alterations in the expression levels of XIAP, cIAP2 or FLIP were seen (Fig. 7 and data not shown). In addition, no upregulation of TRAIL or CD95 ligand and no increased surface expression of agonistic TRAIL receptors or CD95 were found (data not shown).

3.5. Overexpression of Bcl-2 or FADD-DN do not interfere with resveratrol-mediated cell cycle arrest, but decouple the effect of resveratrol on the cell cycle and sensitivity to apoptosis

We analysed in more detail the effect of resveratrol on the cell cycle. Interestingly, treatment with resveratrol

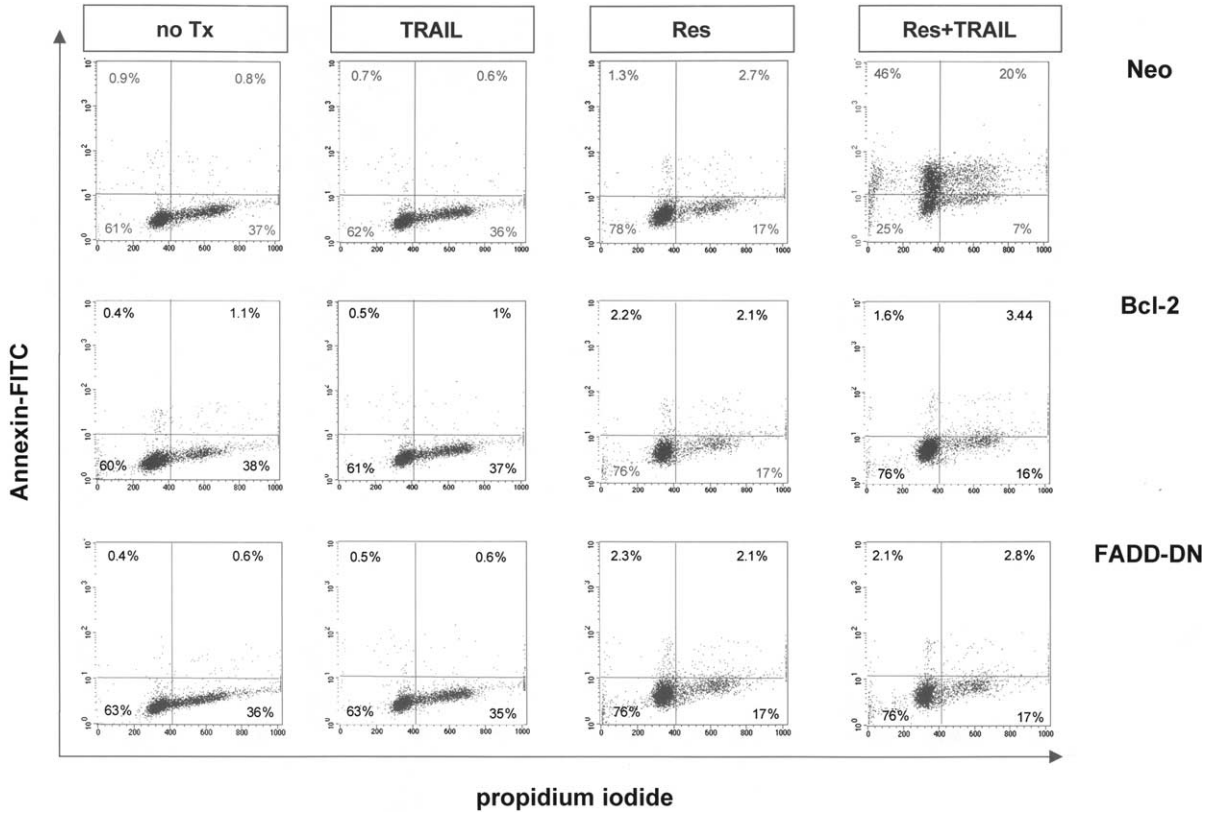

Fig. 8. Effect of Bcl-2 or FADD overexpression on resveratrol-induced cell cycle distribution and apoptosis. SHEP neuroblastoma cells transfected with vector control (Neo), Bcl-2 or FADD-DN were left untreated (no Tx) or were treated with 30 ng/ml TRAIL (TRAIL), 100 μM resveratrol (Res) or 100 μM resveratrol and 30 ng/ml TRAIL (Res + TRAIL). Concomitant analysis of cell cycle and apoptosis was performed by staining with propidium iodide for cell cycle distribution (DNA content, *x*-axis) and annexin-fluorescein isothiocyanate (FITC) for apoptosis (*y*-axis) as described in Section 2. The percentage of cells in each quadrant are indicated (lower left: G1 phase-annexin low, lower right: S/G2/M phase-annexin low, upper left: G1 phase-annexin high, upper right: S/G2/M phase-annexin high).

induced a G1 arrest regardless of the overexpression of Bcl-2 or FADD-DN (Table 1). Concomitant analysis of the cell cycle distribution by propidium iodide staining and apoptosis by annexin V revealed that combined treatment of vector control cells with resveratrol and TRAIL preferentially induced apoptosis in vector control cells arrested in G1 phase by resveratrol (Fig. 8). Interestingly, overexpression of Bcl-2 or FADD-DN did not prevent resveratrol-mediated cell cycle arrest in G1 phase, but inhibited apoptosis upon combined treatment with resveratrol and TRAIL (Fig. 8). Likewise, cell cycle arrest in G1 or S phase by the specific cell cycle inhibitors mimosine or thymidine, respectively, sensitised only vector control cells for subsequent treatment with TRAIL, whereas no sensitisation for TRAIL was found in Bcl-2- or FADD-DN-transfected cells (Figs. 9(a)–(c)). Of note, mimosine or thymidine similarly induced G1 or S phase arrest in vector control-Bcl-2- or FADD-DN-transfected cells (data not shown). These findings indicate that Bcl-2 or FADD inhibited apoptosis induced by the combination of resveratrol and TRAIL without interfering with resveratrol-mediated cell cycle arrest.

To test whether caspase activity was required for resveratrol-mediated cell cycle arrest, we used the broad range caspase inhibitor zVAD.fmk. Resveratrol similarly triggered G1 arrest in the presence or absence of zVAD.fmk (Fig. 10(a)). In addition, zVAD.fmk did not interfere with resveratrol-mediated upregulation of p53 and p21 expression or downregulation of survivin expression (Fig. 10(b)). In contrast, zVAD.fmk inhibited apoptosis induced by resveratrol, TRAIL or the combination of resveratrol and TRAIL (Fig. 10(c)). These findings indicate that caspase activity was required for resveratrol-mediated sensitisation for TRAIL-induced apoptosis, but not for resveratrol-induced cell cycle arrest or modulation of expression of p53, p21 or survivin protein.

3.6. Sensitisation to TRAIL-induced apoptosis by survivin depletion is inhibited by overexpression of Bcl-2 or FADD-DN

Since we found downregulation of survivin by resveratrol, a protein expressed in a cell cycle-dependent manner (Fig. 7), we further investigated the role of survivin as molecular link between resveratrol-induced cell cycle alterations and apoptosis. Importantly, downregulation of survivin expression by survivin antisense oligonucleotides sensitised vector control cells for TRAIL-induced apoptosis (Fig. 11(a)). In contrast, survivin antisense oligonucleotides failed to sensitise Bcl-2 or FADD-DN-transfected cells for TRAIL treatment (Figs. 11(b) and (c)) although survivin protein expression was similarly downregulated by survivin antisense oligonucleotides in all cell types (data not shown). This indicates that downregulation of survivin by resveratrol is not sufficient for the induction of apoptosis by TRAIL, when activation of effector caspases is blocked by overexpression of Bcl-2 or FADD-DN.

4. Discussion

Despite aggressive therapies, resistance to current treatment protocols has been a major obstacle in clinical oncology [18]. Thus, attempts to improve the survival of cancer patients largely depends on strategies that target

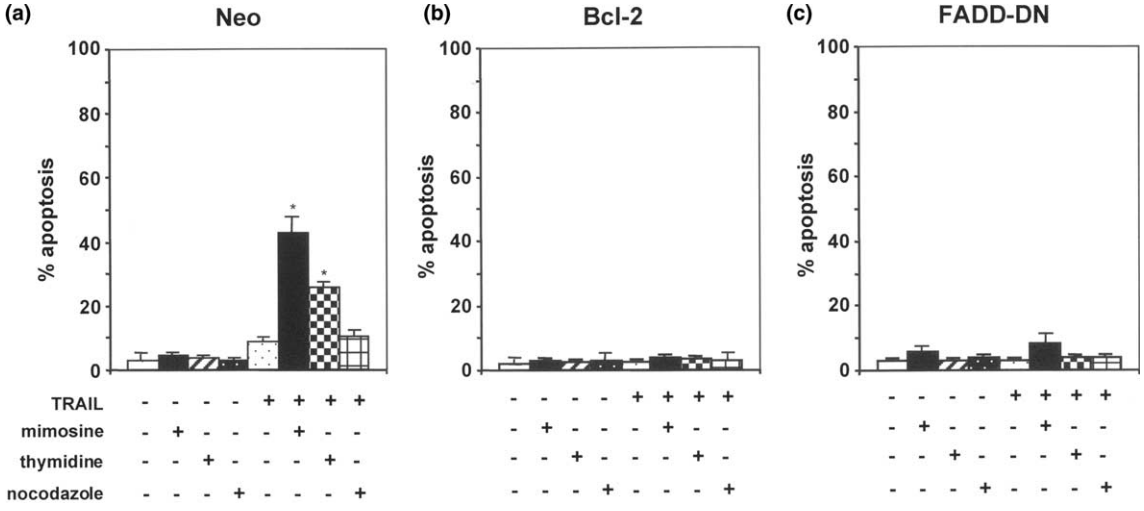

Fig. 9. Effect of cell cycle inhibitors on TRAIL-induced apoptosis. SHEP neuroblastoma cells transfected with vector control (a), Bcl-2 (b) or FADD-DN (c) were cultured in medium or were pretreated for 24 h with 0.4 mM mimosine, 2 mM thymidine or 0.4 μg/ml nocodazole and further cultured in the presence or absence of 30 ng/ml TRAIL for 24 h. Apoptosis was determined by FACS analysis of propidium-iodide stained DNA. Mean and SD of three independent experiments done in triplicate are shown.

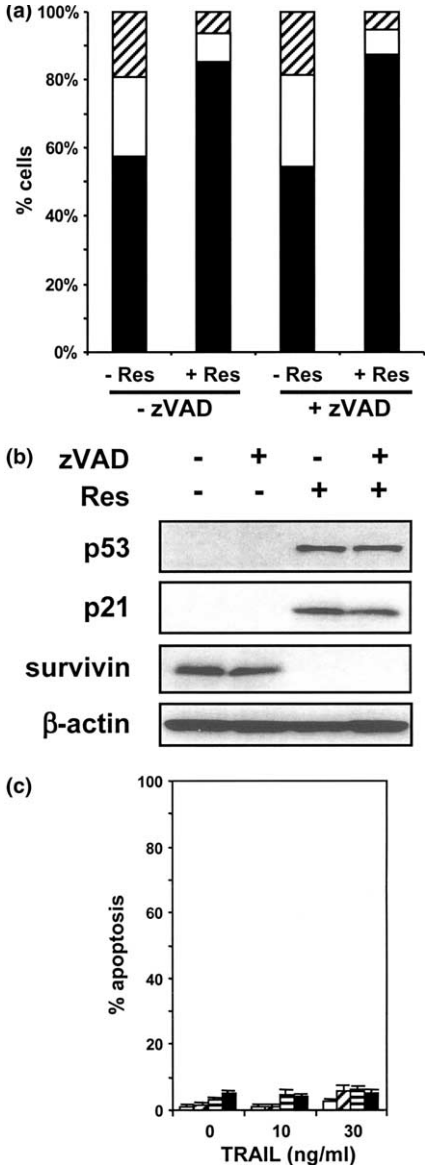

Fig. 10. Involvement of caspases in resveratrol-induced cell cycle alterations and apoptosis. (a) Effect of the caspase inhibitor zVAD.fmk on resveratrol-induced cell cycle distribution. SHEP neuroblastoma cells transfected with vector control were treated for 24 h with 100 μM resveratrol in the presence or absence of 50 μM zVAD.fmk. Cell cycle distribution (G1 phase: black bars, S phase: white bars, G2/M phase: hatched bars) was assessed as described in Section 2. The means of three independent experiments done in triplicate are shown, SD were <3% (b) Effect of the caspase inhibitor zVAD.fmk on resveratrol-induced modulation of apoptotic regulatory proteins. SHEP neuroblastoma cells transfected with vector control were treated for 24 h with 100 μM resveratrol in the presence or absence of 50 μM zVAD.fmk and assessed for protein expresspion of p53, p21, survivin or β-actin by Western Blotting analysis. (c) Effect of the caspase inhibitor zVAD.fmk on resveratrol-mediated sensitisation to TRAIL-induced apoptosis. SHEP neuroblastoma cells transfected with vector control were treated for 24 h with 0–100 μM resveratrol (0 μM: white bars, 10 μM: vertically hatched bars, 30 μM: horizontally hatched bars, 100 μM: black bars) and/or 10–30 ng/ml TRAIL in the presence of 50 μM zVAD.fmk. Apoptosis was determined by FACS analysis of propidium-iodide stained DNA. Mean and SD of three independent experiments done in triplicate are shown.

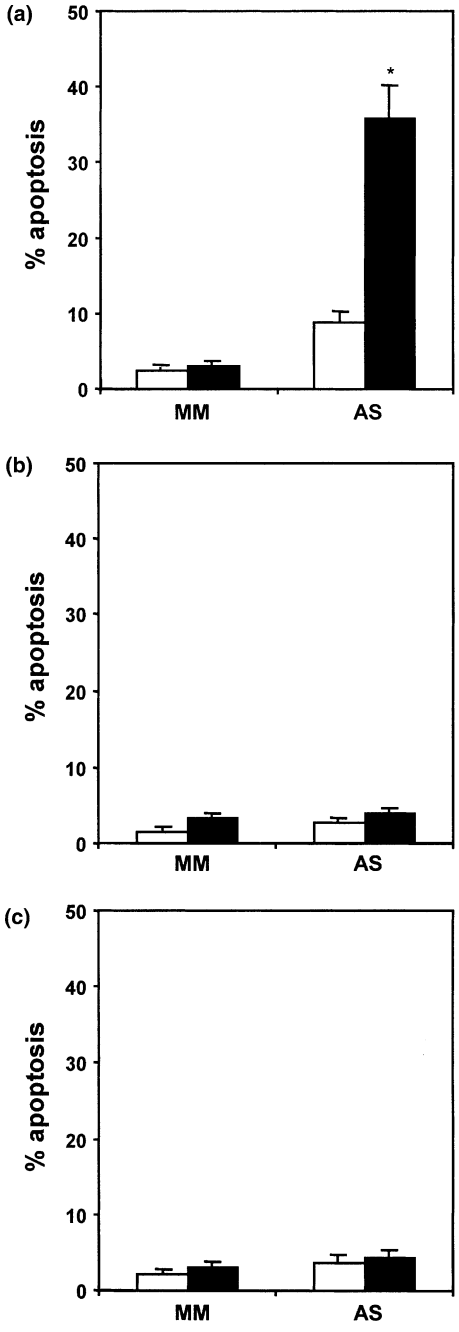

Fig. 11. Sensitisation to TRAIL-induced apoptosis by resveratrol involves survivin. SHEP neuroblastoma cells transfected with vector control (a), Bcl-2 (b) or FADD-DN (c) were treated with 600 nM survivin antisense oligonucleotides (AS) or mismatched oligonucleotides (MM) for 24 h and assessed for apoptosis after treatment with 30 ng/ml TRAIL for 24 h. Apoptosis was determined by FACS analysis of propidium-iodide stained DNA. Mean and SD of three independent experiments done in triplicate are shown.

tumour cell resistance. Since most anticancer agents act through the induction of apoptosis in target cells, defects in apoptotic cell programme may confer resistance [2]. One potential strategy to overcome resistance is direct induction of cell death by death receptors, e.g. using TRAIL [4]. However, many tumours remain resistant

towards treatment with TRAIL, which has been related to the dominance of anti-apoptotic signals [10]. IAPs such as survivin are expressed at high levels in many tumours including neuroblastoma and have been associated with a poor prognosis [7,8]. Naturally occurring antioxidants present in diet and beverages such as resveratrol have gained considerable attention because of their beneficial effects on health as cancer chemopreventive agents [12,13]. Since cell cycle-dependent sensitivity of tumour cells for the induction of cell death has been exploited to overcome the resistance of tumours [21], we investigated the interplay between the cell cycle and sensitivity to apoptosis upon combination therapy with resveratrol and TRAIL in tumour cells that were resistant to TRAIL-induced to apoptosis because of Bcl-2 or FADD-DN overexpression.

We found that resveratrol sensitised tumour cells to TRAIL-induced apoptosis through cell cycle-mediated survivin depletion (Fig. 12). However, resveratrol failed to sensitise cells with high expression of Bcl-2 or FADD-DN to TRAIL treatment. Overexpression of Bcl-2 or FADD-DN did not simply delay cell death, but provided long-term protection against the sensitisation effect of resveratrol to TRAIL treatment. Interestingly, overexpression of Bcl-2 or FADD-DN did not interfere with resveratrol-mediated cell cycle arrest and associated survivin depletion. However, Bcl-2 or FADD-DN inhibited the release of cytochrome *c* and Smac from mitochondria into the cytosol, activation of effector caspases and apoptosis upon combined treatment with resveratrol and TRAIL. Thus, resveratrol-mediated cell cycle arrest and survivin depletion did not translate into cell death upon TRAIL treatment when apoptosis pathways were blocked by overexpression of Bcl-2 or FADD-DN, indicating that of Bcl-2 or FADD-DN decoupled the effect of resveratrol on the cell cycle and apoptosis. Similarly, cell cycle arrest at G1 using the cell cycle-specific inhibitor mimosine or downregulation of survivin expression by antisense oligonucleotides failed

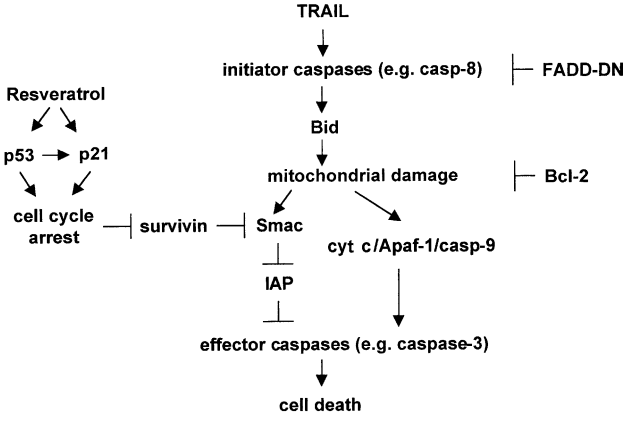

Fig. 12. Illustration of apoptosis pathways modulated by resveratrol, TRAIL, FADD-DN or Bcl-2. See text for details.

to enhance TRAIL-induced apoptosis in Bcl-2- or FADD-DN-transfected cells. Likewise, inhibition of caspase activity using the broad range caspase inhibitor zVAD.fmk blocked apoptosis upon combined treatment with resveratrol and TRAIL without interfering with resveratrol-mediated cell cycle arrest and survivin depletion. These findings indicate that enhanced sensitivity to TRAIL-induced apoptosis provided by resveratrol-mediated cell cycle arrest and survivin depletion required intact signalling through the death receptor or mitochondrial apoptotic pathways. In addition, the natural food product curcumin was found to enhance TRAIL-induced cytotoxicity [22]. Interestingly, resveratrol has recently been reported to sensitise tumour cells to CD95-induced apoptosis by triggering redistribution of the CD95 receptor in membrane rafts and also to chemotherapy-induced apoptosis [19,20]. Thus, natural food products may be exploited to enhance the susceptibility of tumour cells for the induction of cell death.

In the past, most concepts formed on cell cycle-dependent sensitivity to the induction of cell death were focused on the machinery for DNA synthesis and replication [21,23]. However, the key finding that the killing of tumour cells by cytotoxic therapies critically requires intact apoptosis signalling pathways may provide an alternative explanation [24]. Thus, a molecular link between the differential sensitivity of tumour cells towards the induction of apoptosis at distinct cell cycle phases may be attributed to the differential expression of apoptotic regulatory proteins such as survivin. Importantly, our findings indicate that the cell cycle-dependent sensitivity of tumour cells to the induction of cell death may depend critically on intact apoptosis pathways as effector systems for cell death execution, as shown here for resveratrol-mediated sensitisation to TRAIL.

Notably, treatment with resveratrol resulted in a substantial reduction in survivin expression, even in Bcl-2 or FADD-DN overexpressing cells, with no detectable changes in the expression levels of other IAP proteins, such as XIAP or cIAP2. Similar to treatment with resveratrol, downregulation of survivin expression by survivin antisense oligonucleotides only sensitised the vector control cells to TRAIL treatment, and not the Bcl-2- or FADD-DN-transfected cells. These findings indicate that the relatively selective downregulation of survivin expression, e.g. by resveratrol, may not be sufficient for activation of effector caspases and apoptosis in the absence of additional signals promoting caspase activation, such as the release of Smac and/or cytochrome *c* into the cytosol, which were blocked by overexpression of Bcl-2 or FADD-DN. In contrast, we recently found that antagonising IAPs by Smac agonists, which simultaneously antagonise several IAP proteins, strongly enhanced TRAIL-induced antitumour activity, even in cells overexpressing Bcl-2 [25].

Our findings may have implications for the potential application of resveratrol as a sensitiser in TRAIL-based protocols. TRAIL is a promising death receptor ligand for cancer therapy, because it induces apoptosis in a broad spectrum of cancer cell lines, but not in normal cells, and also exhibits potent tumoricidal activity in several xenograft models *in vivo* [6]. Since resveratrol significantly potentiated the cytotoxic activity of TRAIL, resveratrol may be included in TRAIL-based therapies to enhance the antitumour activity of TRAIL. To this end, the potential clinical implications of our studies will also depend on whether or not resveratrol can be given safely to humans at doses high enough to achieve pharmacologically active levels. Pharmacokinetic data for resveratrol, although limited at present, indicate that pharmacologically active plasma levels may be achievable in rodents or humans [26]. However, whether plasma levels of resveratrol, shown here to be required for sensitisation of tumour cells towards TRAIL-induced apoptosis, will be achievable in humans is perhaps doubtful. In addition, our findings demonstrate that resveratrol-mediated sensitisation to TRAIL treatment required intact apoptotic pathways. Bcl-2 is highly expressed in many tumours and confers protection against TRAIL in different tumour cell lines [9]. In addition, signalling by death receptors is frequently impaired in tumours, e.g. by loss of caspase-8 expression or high FLIP levels [10]. Thus, in terms of a clinical perspective, our findings indicate that resveratrol is a potent sensitiser for TRAIL in certain tumours. However, it may not be effective in others, e.g. in tumours with enhanced Bcl-2 expression or defective death receptor signalling.

Conflict of interest

The authors state that there is no conflict of interest.

Acknowledgements

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References

1. Lowe SW, Lin AW. Apoptosis in cancer. *Carcinogenesis* 2000, **21**, 485–495.
2. Johnstone RW, Ruefli AA, Lowe SW. Apoptosis: a link between cancer genetics and chemotherapy. *Cell* 2002, **108**, 153–164.
3. Hengartner MO. The biochemistry of apoptosis. *Nature* 2000, **407**, 770–777.
4. Ashkenazi A. Targeting death and decoy receptors of the tumour-necrosis factor superfamily. *Nat Rev Cancer* 2002, **2**, 420–430.
5. van Loo G, Saelens X, van Gurp M, *et al*. The role of mitochondrial factors in apoptosis: a Russian roulette with more than one bullet. *Cell Death Differ* 2002, **9**, 1031–1042.
6. LeBlanc HN, Ashkenazi A. Apo-2L/TRAIL and its death and decoy resceptors. *Cell Death Differ* 2003, **10**, 66–75.
7. Salvesen GS, Duckett CS. IAP proteins: blocking the road to death's door. *Nat Rev Mol Cell Biol* 2002, **3**, 401–410.
8. Altieri DC. Validating survivin as a cancer therapeutic target. *Nat Rev Cancer* 2002, **3**, 46–54.
9. Cory S, Adams JM. The Bcl2 family: regulators of the cellular life-or-death switch. *Nat Rev Cancer* 2002, **2**, 647–656.
10. Igney FH, Krammer PH. Death and anti-death: tumour resistance to apoptosis. *Nat Rev Cancer* 2002, **2**, 277–288.
11. Fulda S, Meyer E, Debatin KM. Inhibition of TRAIL-induced apoptosis by Bcl-2 overexpression. *Oncogene* 2002, **21**, 2283–2294.
12. Cal C, Garban H, Jazirehi A, *et al*. Resveratrol and cancer: chemoprevention, apoptosis, and chemo-immunosensitizing activities. *Curr Med Chem Anti-Canc Agents* 2003, **3**, 77–93.
13. Pervaiz S. Resveratrol: from grapevines to mammalian biology. *FASEB J* 2003, **17**, 1975–1985.
14. Fulda S, Debatin KM. Sensitization for tumor necrosis factor-related apoptosis-inducing ligand-induced apoptosis by the chemopreventive agent resveratrol. *Cancer Res* 2004, **64**, 337–346.
15. Fulda S, Sieverts H, Friesen C, *et al*. The CD95 (APO-1/Fas) system mediates drug-induced apoptosis in neuroblastoma cells. *Cancer Res* 1997, **57**, 3823–3829.
16. Fulda S, Debatin KM. IFNgamma sensitizes for apoptosis by upregulating caspase-8 expression through the Stat1 pathway. *Oncogene* 2002, **21**, 2295–2308.
17. Olie RA, Simoes-Wust AP, Baumann B, *et al*. A novel antisense oligonucleotide targeting survivin expression induces apoptosis and sensitizes lung cancer cells to chemotherapy. *Cancer Res* 2000, **60**, 2805–2809.
18. Peto J. Cancer epidemiology in the last century and the next decade. *Nature* 2001, **411**, 390–395.
19. Delmas D, Rebe C, Lacour S, *et al*. Resveratrol-induced apoptosis is associated with Fas redistribution in the rafts and the formation of a death-inducing signaling complex in colon cancer cells. *J Biol Chem* 2003, **278**, 41482–41490.
20. Jazirehi AR, Bonavida B. Resveratrol modifies the expression of apoptotic regulatory proteins and sensitizes non-Hodgkin's lymphoma and multiple myeloma cell lines to paclitaxel-induced apoptosis. *Mol Cancer Ther* 2004, **3**, 71–84.
21. Pucci B, Kasten M, Giordano A. Cell cycle and apoptosis. *Neoplasia* 2000, **2**, 291–299.
22. Deeb D, Xu YX, Jiang H, *et al*. Curcumin (diferuloyl-methane) enhances tumor necrosis factor-related apoptosis-inducing ligand-induced apoptosis in LNCaP prostate cancer cells. *Mol Cancer Ther* 2003, **2**, 95–103.
23. Smith DM, Gao G, Zhang X, *et al*. Regulation of tumor cell apoptotic sensitivity during the cell cycle. *Int J Mol Med* 2000, **6**, 503–507.
24. Mow BM, Blajeski AL, Chandra J, *et al*. Apoptosis and the response to anticancer therapy. *Curr Opin Oncol* 2001, **13**, 453–462.
25. Fulda S, Wick W, Weller M, *et al*. Smac agonists sensitize for Apo2L/TRAIL- or anticancer drug-induced apoptosis and induce regression of malignant glioma in vivo. *Nat Med* 2002, **8**, 808–815.
26. Gescher AJ, Steward WP. Relationship between mechanisms, bioavailibility, and preclinical chemopreventive efficacy of resveratrol: a conundrum. *Cancer Epid Biom Prev* 2003, **12**, 953–957.