

HA14-1 is able to reconstitute the impaired mitochondrial pathway of apoptosis in renal cell carcinoma cell lines

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Abstract. Renal cell carcinomas (RCCs) exhibit a marked resistance towards apoptosis. Although most apoptotic stimuli converge at the level of the mitochondria, little is known about the mitochondrial apoptosis pathway in renal cell carcinoma cell lines by exposure to TRAIL, etoposide, HA14-1 and betulinic acid activating the mitochondria by different mechanisms. Sensitivity to TRAIL-induced apoptosis correlated with cleavage of the initiator caspase-8, but the mitochondrial apoptosis pathway was not induced. Similarly, etoposide and betulinic acid could not induce mitochondrial damage. In contrast, HA14-1 was able to activate mitochondrial apoptosis, thereby demonstrating functionally inducible signalling pathways downstream of the mitochondria. The intactness of the pathways upstream of the mitochondria was shown by pretreatment of TRAIL-sensitive cell lines with HA14-1, which could reconstitute TRAIL-induced mitochondrial damage and resulted in a synergistic apoptosis induction.

Our results demonstrate that the apoptotic pathways upstream and downstream of the mitochondria are intact and inducible in renal cell carcinoma cell lines. However, resistance towards mitochondrial apoptosis is located on the level of the mitochondria themselves.

Keywords: Apoptosis, renal cell carcinoma, anticancer drugs, TRAIL, BCL-2

1. Introduction

Renal cell carcinomas (RCCs) are known to exhibit a marked resistance towards a multitude of apoptotic stimuli contributing to the “multidrug resistance phenotype”. Thus, differences in apoptosis-sensitivity in RCC cell lines can be found towards ionizing irradiation [1], DNA-damaging drugs like Topotecan [2] as well as death receptor mediated apoptosis by TNF-related apoptosis inducing ligand (TRAIL) or CD95 [3,4]. However, the molecular mechanisms responsible for these differences in apoptosis-susceptibility are yet not very well known.

Over the last years two main apoptosis signalling pathways were described: the extrinsic pathway of apoptosis is mediated by death receptors on the cell

surface – like CD95 or DR5. Binding of the death ligand to their corresponding receptors results in formation of the death inducing signalling complex (DISC) consisting of the death receptor, the adaptor protein FADD and the initiator caspases-8 or -10 leading to an initiation of the apoptotic signal [5].

The intrinsic – or mitochondrial [6] – pathway is activated by oncogenes, withdrawal of growth factors or DNA damage, which induces transcription of p53-dependent BH3-only proteins like Noxa and Puma [7–9]; in addition, caspase-3 as well as caspase-2 are activated which in turn leads to cleavage of the BH3-only protein Bid [10,11]. The activated BH3-only proteins integrate into the outer mitochondrial membrane interacting and thereby inhibiting the antiapoptotic members of the BCL2-family like BCL-2 or BCL-XL which preserve the mitochondrial potential. This finally results in depolarisation of the mitochondria with release of cytochrome C and other proapoptotic proteins, like AIF [6,12,13]. Cytochrome C interacts with caspase-9 and APAF-1

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forming the apoptosome complex resulting in caspase-9 cleavage.

The intrinsic as well as extrinsic signalling pathways result in the activation of the effector caspases-3 and -7 – which in turn can lead to additional caspase-8 and caspase-9 processing and finally apoptosis [14].

This separation of the extrinsic and intrinsic pathways of apoptosis, however, is not strict since both pathways share the same players, like caspase-3, caspase-7, caspase-8, caspase-2 and Bid. Thus, Lakhani and coworkers [15] could attribute both death receptor and DNA-damage derived mitochondrial events of apoptosis to an early activation of caspase-3 and -7 lying upstream of their function as executioner caspases. Additionally, in certain cell types, caspase-2 is necessary for caspase-8 mediated Bid cleavage [11]. On the other hand, the anticancer drug etoposide can induce apoptosis by a sequential activation of first caspase-2 and then caspase-8, which in turn leads to apoptosis by Bid cleavage [16]. Cleaved Bid binds to two other proapoptotic members of the BCL-2 family, the BH3-only proteins Bax and Bak, which integrate into the mitochondrial membrane transmitting the death signal by depolarisation of the mitochondria [17]. This Bid derived mitochondrial damage can be inhibited by overexpression of the antiapoptotic members of the BCL-2 family, like BCL-2 and BCL-XL [6].

The aim of the present study was to determine the level of resistance in the mitochondrial apoptosis cascade: upstream of the mitochondria, on the level of the mitochondria themselves, or downstream of the mitochondria. For this reason we used four agents targeting the mitochondrial pathway by different, in some respect opposite, molecular mechanisms: TRAIL disrupts the mitochondrial membrane potential via cleavage of caspase-8 and subsequent activation of the BH3-only protein Bid in so called type II cells [18]. The anticancer drug etoposide, a DNA-damaging topoisomerase II inhibitor, also leads to Bid-cleavage by activation of caspase-2 in lymphoma cell lines [16]. Furthermore, etoposide targets the mitochondrion by p53-dependent induction of the BH3-only proteins Noxa and Puma in a mouse model and different human cell lines [19]. Thus, TRAIL as well as etoposide can activate different pathways upstream of the mitochondrion. In contrast, betulinic acid disrupts the mitochondria of cells expressing no or just low levels of the antiapoptotic members of the BCL-2 family [20,21] and high expression of BCL-2 or BCL-XL leads to betulinic acid resistance. In contrast, one of the mechanisms, by which

HA14-1 exerts its proapoptotic function is binding to the antiapoptotic members of the BCL-2 family by interacting at the BH3-binding site and displacing BH3-only proteins, thereby antagonizing their anti-apoptotic properties [22,23]. Thus, HA14-1 and betulinic acid target at the mitochondria themselves and are able to activate downstream pathways. However, the role of HA14-1 as a selective BCL-2 inhibitor has recently been questioned and results are conflicting [24,25], so that its exact mechanism of action is not clear. Nevertheless, our findings could provide a way of overcoming the well known chemotherapy resistance of RCCs.

By means of these agents, we could show for the first time that the level of resistance towards the mitochondrial apoptosis pathway lies on the level of the mitochondria themselves, whereas the apoptotic pathways upstream and downstream of the mitochondria are functionally intact and not impaired.

2. Material and methods

2.1. Cells and culture

The permanent cell lines clearCa-2, clearCa-6, clearCa-7 and clearCa-11 were derived from typical representatives of the clear cell type of RCCs [26,27]. These cell lines were maintained with Dulbecco's modified Eagle's medium (DMEM, Gibco, Karlsruhe, Germany), supplemented with 10% fetal calf serum (FCS), penicillin and streptomycin, aspartate/asparagines and HEPES buffer (standard growth medium) and cultivated at 37°C in an atmosphere with 5% CO₂.

2.2. Assessment of cell viability

Tumour cells in the exponential growth phase were transferred to a 24-well-plate (GIBCO, Karlsruhe, Germany) and seeded at 2.5×10^5 cells/cm² in standard growth medium. After 24 h the tumour cells were exposed to recombinant human soluble (rhs) TRAIL (25 and 100 ng/ml), the anticancer drug etoposide (25 and 50 µg/ml), the plant derived pentacyclic triterpene betulinic acid (20 and 40 µM), the small BCL-2 inhibiting polypeptide HA14-1 [23] (25 and 50 µM) or a combination of HA14-1 (25 µM) and TRAIL (25 respectively 100 ng/ml). The assessment of cell number was performed in three independent experiments ($n = 3$). The number of cells harvested was counted by the Trypan-blue exclusion method.

2.3. Morphological assessment of TRAIL-, etoposide-, HA14-1 and betulinic acid-induced apoptosis

2.5×10^5 cells/cm² of the cell line clearCa-11 were seeded on a 24-well-plate (Nunc, Wiesbaden, Germany). After 24 h, cells were treated either with 100 ng/ml rhs TRAIL, 50 µg/ml etoposide or 40 µM betulinic acid and cultured for another 24 h or treated with 50 µM HA14-1 and cultured for another 6 h. As control, cells were exposed to standard growth medium. Apoptosis or necrosis-like cell death were determined by light microscopy of haematoxylin–eosin (HE)-stained cells showing the typical morphological signs of apoptosis, i.e. chromatin condensation and/or fragmentation into apoptotic bodies, or necrosis-like cell death, i.e. cellular swelling with vacuolisation and swelling of the organelles.

2.4. RNA extraction

Total RNA was isolated by the RNeasy minikit with DNase treatment according to the manufacturer's instructions (Qiagen, Hilden, Germany) with on column DNA digestion to exclude DNA contamination. The integrity of all tested total RNA samples was verified by intact 18S/28S rRNA bands in agarose gel electrophoresis.

2.5. Reverse transcription

cDNA synthesis was performed with the "Reverse transcription system" of Promega (Heidelberg) according to the manufacturer's protocol. In short, for cDNA synthesis, 1 µg of total RNA was reverse transcribed in a final volume of 20 µl containing 0.5 µg Oligo (dt) Primer, 1 mM dNTP mix, 0.5 U of recombinant RNasin RNase inhibitor, 5 mM MgCl₂ and 15 U of AMV reverse transcriptase with the corresponding 2 µl of 10 × RT buffer. Reverse transcription (RT) was performed for 15 min at 42°C. Synthesized cDNA was diluted 1:5 for real-time PCR.

2.6. Quantitative real-time detection PCR

Amplification and quantification of the genes PED/PEA-15, Flip short, Flip long and the house-keeping gene SDHA were carried out in a LightCycler (Roche Diagnostics, Mannheim, Germany) by using a total volume of 20 µl, including 10 µl Platinum SYBR Green qPCR SuperMix UDG (Roche Diagnostics, Mannheim, Germany), 4 µl 5× diluted cDNA, 1 µl Bovine Serum Albumin (1 mg/ml stock solution) and

1 µM each sense and antisense primers (PED/PEA-15 forward primer 5'-GAA GAC ATC CCC AGC GAA AAG A-3', PED/PEA-15 reverse primer 5'-GGC ACT GGG GAT ACG GGT TAG-3'; cFlip short forward primer 5'-GTT CAA GGA GCA GGG ACA AGT TA-3, cFlip short reverse primer 5'-CCA AGA ATT TTC AGA TCA GGA CAA-3'; cFlip long forward primer 5'-GGC TCC CCC TGC ATC ACA TC-3, cFlip long reverse primer 5'-CCG CAG TAC ACA GGC TCC AGA-3'; SDHA forward primer 5'-TGG GAA CAA GAG GGC ATC TG-3, SDHA reverse primer 5'-CCA CCA CTG CAT CAA ATT CAT G-3'). Real-time PCR was carried out in glass capillaries with an initial incubation step at 55°C for 2 min for reduction of dUTP-containing amplifications, followed by a denaturation step of 5 min at 95°C, followed by 50 cycles for 5 s at 95°C, annealing for 10 s at 62°C for PED/PEA-15, 55°C for Flip short, 67°C for Flip long and 60°C for SDHA followed by extension for 20 s at 72°C. Melting curve was directly drawn after amplification.

PCR products were additionally checked by electrophoresis on 2% agarose gels containing ethidium bromide and visualised under UV transillumination. PCR products were confirmed by DNA sequencing. Briefly, products were excised from agarose gels and isolated by QIAquick gel extraction kit (Qiagen, Hilden, Germany). The purified PCR products were sequenced using the ABI-Prism BigDye Terminator Cycle Sequencing kit (ABI, Weiterstadt, Germany) with the respective forward and reverse primers used in the real-time PCR according to the manufacturer's protocol. Sequence analysis was carried out using an ABI-Prism 310.

2.7. Western Blot analysis

RCC cell lines were lysed by exchanging cell culture medium with ice cold Lysis Buffer (100 mM NaCl, 10 mM Tris-HCl, pH: 7.6, 1 mM EDTA, 1% NP40, protease inhibitors). Then cells were scraped off the cell culture dish and the lysate was incubated for 10 min at 4°C with occasional vortexing. Then the lysates were centrifuged at 4°C at 13,000 × g for 10 min. Protein concentration of the supernatant was determined by the Bradford method (Biorad, Germany). 50 µg of protein lysate were separated under denaturing conditions in 7–12% polyacrylamid-gels. After blotting, the proteins were transferred to a reinforced nitrocellulose membrane (Schleicher and Schuell, Germany) and equal loading of the proteins was checked by Ponceau Red staining. The membranes were then blocked with TBS containing 5% non-fat dry milk and 0.2% Tween 20

for 24 h at 4°C. Afterwards the membranes were incubated for 12 h with human specific monoclonal respectively polyclonal primary antibodies (caspase-8, mouse monoclonal, clone 1C12, Cell Signalling; Bid, rabbit polyclonal, Cell Signalling; caspase-9, rabbit polyclonal, Cell Signalling; PARP, rabbit polyclonal, Cell Signalling; caspase-2, mouse monoclonal, clone C2, Cell Signalling; BCL-XL, rabbit polyclonal, Cell Signalling; Flip, rabbit polyclonal, Cell Signalling; PEA-15, rabbit polyclonal, Cell Signalling; BCL-2, mouse monoclonal, clone 124, DAKO) and then washed with TBS-Tween for 30 min. The blots were incubated with a horseradish peroxidase conjugated anti-mouse respectively anti-rabbit secondary antibody (Cell Signalling) for another 12 h at 4°C, then washed for 30 min. with TBS-Tween and developed with the chemiluminescence method according to the manufacturer's protocol (Roche, Germany) at room temperature.

2.8. Determination of cytochrome C release

The release of cytochrome C from mitochondria was analysed by the previously described selective digitonin permeabilization method [28]. It was established to avoid possible artefacts resulting from mechanical breakage of the outer mitochondrial membrane by Dounce homogenization. In short, cell culture medium was exchanged by 500 µl permeabilization buffer (210 mM D-mannitol, 70 mM sucrose, 10 mM Hepes, 5 mM succinate, 0.2 mM EGTA, 0.15% BSA, 80 µg/ml digitonin, pH 7.2, 4°C) at the indicated time points. Then cell culture plates were gently shaken for 10 min at 4°C on an orbital shaker. Afterwards the permeabilisation buffer was removed and centrifuged for 10 min at 13,000 × *g*. Protein concentration was measured by the Bradford method. Western Blotting was performed as described above with 200 µg total protein loaded upon each lane. Cytochrome C was detected by chemiluminescent detection after blotting on reinforced nitrocellulose membrane with a monoclonal antibody against cytochrome C (mouse monoclonal, clone 6H2B4, Pharmingen).

3. Results

3.1. TRAIL-sensitivity correlates with cleavage of caspase-8 but does not induce the mitochondrial pathway

Since it is not known, which apoptotic pathways participate in TRAIL-induced apoptosis in RCC cell

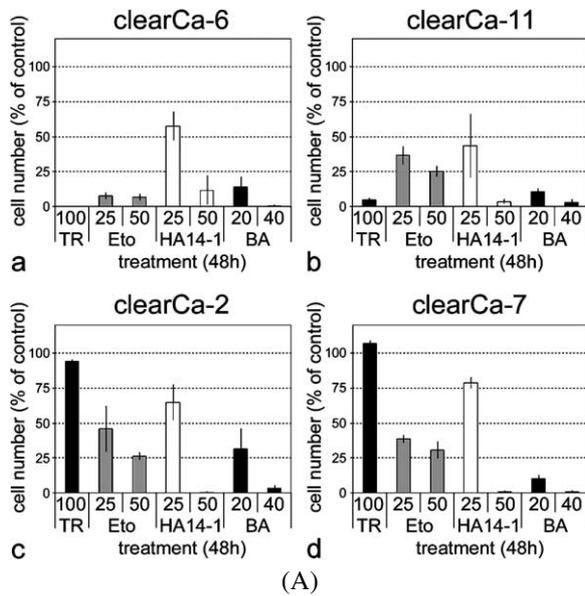
lines, we, therefore, examined in the first step, which pathways were activated by TRAIL and analysed, if the cross-talk between extrinsic and intrinsic apoptosis pathway was functional.

To evaluate the extent of TRAIL-induced apoptosis, we exposed four RCC cell-lines (clearCa-2, clearCa-6, clearCa-7 and clearCa-11) to soluble recombinant (rhs) TRAIL. Exposure to 100 ng rhs TRAIL resulted in a significant decrease of cell number (Fig. 1(A): a, b) in two RCC cell lines (clearCa-6: 0% of the control after 48 h; clearCa-11: $4 \pm 2.5\%$ of the control after 48 h), whereas the two other RCC cell lines (clearCa-2 and clearCa-7) were TRAIL resistant (Fig. 1(A): c, d). Decrease in cell number was paralleled by an increase in apoptotic figures as became evident from light microscopic evaluation of HE-stained cells (Fig. 1(B): b). As determined by Western Blot analysis (Fig. 2), the marked reduction of cell number was accompanied by a strong increase in caspase-8- and PARP-cleavage as markers of apoptosis after 3, 6 and 12 h, respectively. Interestingly, weak cleavage of caspase-9, however, occurred only in clearCa-11 after 12 h. Moreover, reduction in Bid protein-levels as marker of increased Bid cleavage could only be detected in clearCa-6. Though using an antibody capable of detecting Bid and tBid, we could never show tBid by Western Blot. Obviously tBid is subjected to rapid degradation. Thus, we had to take reduction of Bid level as measure for cleavage of Bid. In the TRAIL-resistant cell lines (clearCa-2 and clearCa-7) neither a reduction of cell number (Fig. 1(A): c, d), nor caspase-8, -9 and PARP-cleavage, nor reduction of Bid protein-levels were detectable after exposure to TRAIL (Fig. 2).

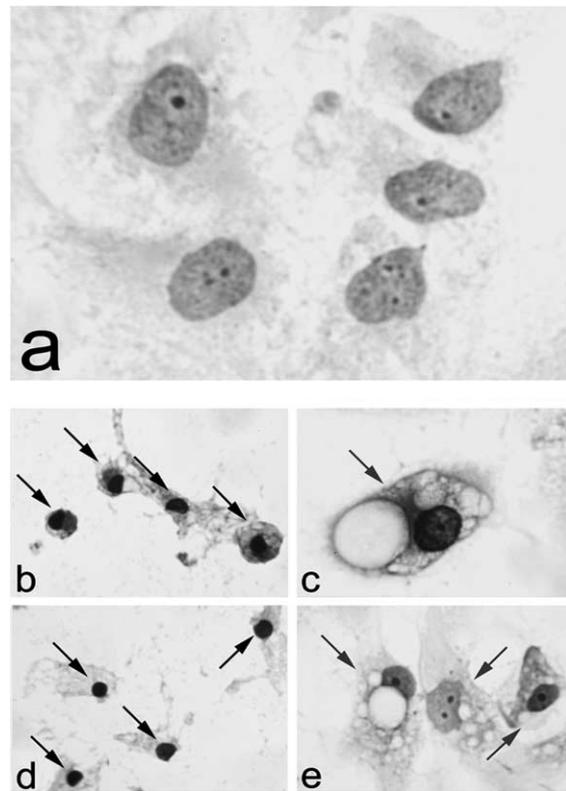
3.2. TRAIL-sensitivity does not correlate with expression of caspase-8 inhibiting genes

Before analysing the obvious impairment in the mitochondrial apoptosis pathway in RCC cell lines we first tried to determine mechanisms for the observed differences in caspase-8 cleavage.

Previous studies revealed that sensitivity to TRAIL in our RCC cell lines did not correlate with expression of DR4 and DR5 [4]. However, cleavage of caspase-8 can be inhibited by multiple antiapoptotic regulators. In addition to the long and the short form of cellular Flip [29], PED/PEA-15 (protein enriched in diabetes/astrocytes-15) has also been reported to inhibit caspase-8 [30,31]. Therefore, we performed light cycler analysis of these genes to de-



(A)



(B)

Fig. 1. Cell number and morphological aspects of RCC cell lines after exposure to TRAIL, etoposide, HA14-1 and betulinic acid. (A): Significant reduction of cell number ($p < 0.05$) after exposure to rhs TRAIL (TR) (100 ng/ml) for 48 h in clearCa-6 and clearCa-11, whereas cell number in clearCa-2 and clearCa-7 remained constant. Significant reduction of cell number ($p < 0.05$) after exposure with etoposide (Eto) (25 and 50 $\mu\text{g/ml}$), HA14-1 (25 and 50 μM) and betulinic acid (BA) (20 and 40 μM) in all RCC cell lines after 48 h. (Data represent median \pm standard deviation in three independent experiments.) (B): (a) Untreated control of clearCa-11 cells. (b and d) Marked increase of apoptotic figures after exposure to TRAIL (b) and HA14-1 (d) (black arrows). (c and e) Marked increase of necrotic cellular changes with disintegration as well as intracellular swelling of organelles and without chromatin condensation after exposure to etoposide (c) and betulinic acid (e) (grey arrows).

termine their expression levels in these four RCC cell lines (Fig. 3(A)). For quantitative analysis, expression levels were normalized to the house keeping gene SDHA. Light cyclase analysis revealed expression of all these caspase-8 inhibiting genes in every RCC cell line with different expression levels (Fig. 3(A)). Be it as it may, mRNA expression levels did not correlate with susceptibility towards TRAIL-induced apoptosis or cleavage of caspase-8. Be it as it may, mRNA expression levels do not necessarily correlate with the protein expression levels. We, therefore, additionally analyzed protein expression of PED/PEA-15, cFlip_L and cFlip_S by Western Blot (Fig. 3(B)). Hereby, cFlip_S expression could not be detected on the protein level, though using an antibody capable of detecting both cFlip_L and cFlip_S. Concerning cFlip_L and PED/PEA-15 protein expression slight differences between mRNA expression levels and pro-

tein expression levels could be detected. However, neither protein expression levels nor mRNA expression levels correlated with susceptibility towards TRAIL-induced apoptosis in RCC cell lines with the TRAIL-sensitive cell line clearCa-11 exhibiting a relatively strong and the TRAIL-resistant cell line clearCa-2 exhibiting a relatively weak PED/PEA-15 and cFlip_L expression.

3.3. HA14-1 induces the mitochondrial pathway, in contrast to etoposide and betulinic acid

In the following experiments we focussed on the analysis of the impaired mitochondrial pathway. Since our investigation revealed, that cleavage of caspase-9 was repressed after exposure to TRAIL, we analysed in the next step, whether the mitochondrial pathway of apoptosis was nevertheless functional and inducible in

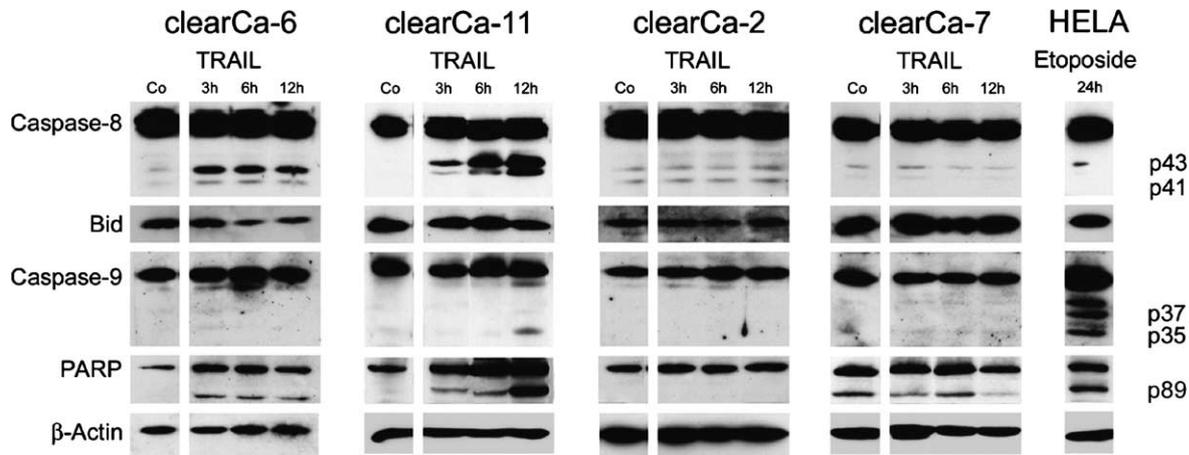


Fig. 2. Western Blot analysis of caspase-8-, caspase-9- and PARP-cleavage as well as Bid reduction after exposure to TRAIL. Increased detection of active cleavage products of initiator caspase-8 (p43 and p41) as well as PARP (p89) after exposure to rhs TRAIL in the sensitive cell lines clearCa-6 and clearCa-11 after 3, 6 and 12 h as markers for increased apoptosis. Weak reduction of Bid protein concentration only in clearCa-6. Weak and late caspase-9 cleavage (after 12 h) only in clearCa-11. No caspase-8-cleavage or PARP activation as well as Bid cleavage in the resistant cell lines clearCa-2 and clearCa-7. (HELA cells treated with 25 $\mu\text{g}/\text{ml}$ etoposide for 24 h were used as positive control for caspase-9 and PARP cleavage.)

RCC cell lines by etoposide, HA14-1 or betulinic acid. Furthermore, we investigated by means of these agents on which level of the mitochondrial pathway resistance was located.

Etoposide as a classical DNA-damaging agent activates the mitochondria by p53-mediated transcriptional induction of proapoptotic members of the BCL-2 family like Noxa and Puma [19]. Additionally, it can activate caspase-2 which in turn mediates Bid-cleavage and mitochondrial damage [16]. As demonstrated in Fig. 3(B), caspase-2 is expressed in all cell lines.

Taking advantage of betulinic acid and HA14-1, we were able to investigate the relevance of the antiapoptotic BCL-2 family members in mitochondrial apoptosis. Thus, betulinic acid can activate the mitochondrial pathway of apoptosis in cells expressing no or just small levels of BCL-2 and BCL-XL [20,21]. In contrast, HA14-1 induces apoptosis in BCL-2 overexpressing cells [23].

Exposure of the RCC cells to etoposide (25 and 50 $\mu\text{g}/\text{ml}$), HA14-1 (25 and 50 μM) and betulinic acid (20 and 40 μM) resulted in a significant decrease in cell number of different extent in all RCC cell lines after 48 h (Fig. 1(A): a-d). Importantly, as demonstrated in Fig. 4, caspase-8, caspase-9, Bid- and PARP-cleavage occurred only after administration of HA14-1 (50 μM) in all RCC cell lines indicating an activation of the mitochondrial apoptosis pathway. Interestingly, caspase-activation and PARP-cleavage was weakest in

clearCa-6 expressing the lowest levels of BCL-2 and BCL-XL (Fig. 3(C)). In contrast, no unequivocal caspase-8, caspase-9, Bid- or PARP-cleavage could be detected after exposure to betulinic acid and etoposide in most of the cell lines. Only in clearCa-6, a weak and late activation of caspase-8, caspase-9 and PARP after treatment with etoposide was detectable after 12 h. Moreover, morphological analysis of HE-stained cells revealed an increase in apoptotic figures only in HA14-1 treated cells (Fig. 1(B): d), whereas etoposide (Fig. 1(B): c) and betulinic acid (Fig. 1(B): e) induced an increase in typical necrotic cellular changes when compared to the control (Fig. 1(B): a).

3.4. HA14-1 induces cytochrome C release from mitochondria in contrast to TRAIL, etoposide and betulinic acid

The next step was to determine the exact level of resistance in the mitochondrial apoptosis pathway. Deficient caspase-9 cleavage upon stimuli activating the intrinsic pathway of apoptosis can be due to mechanisms lying upstream of the mitochondria, on the level of the mitochondria themselves, or downstream of the mitochondria. Thus, loss or defective proteins of the apoptosome complex can prevent an adequate activation of caspase-9. Moreover, caspase-9-cleavage can be inhibited by a splice-variant of caspase-9, caspase-9s, or members of the IAP protein family.

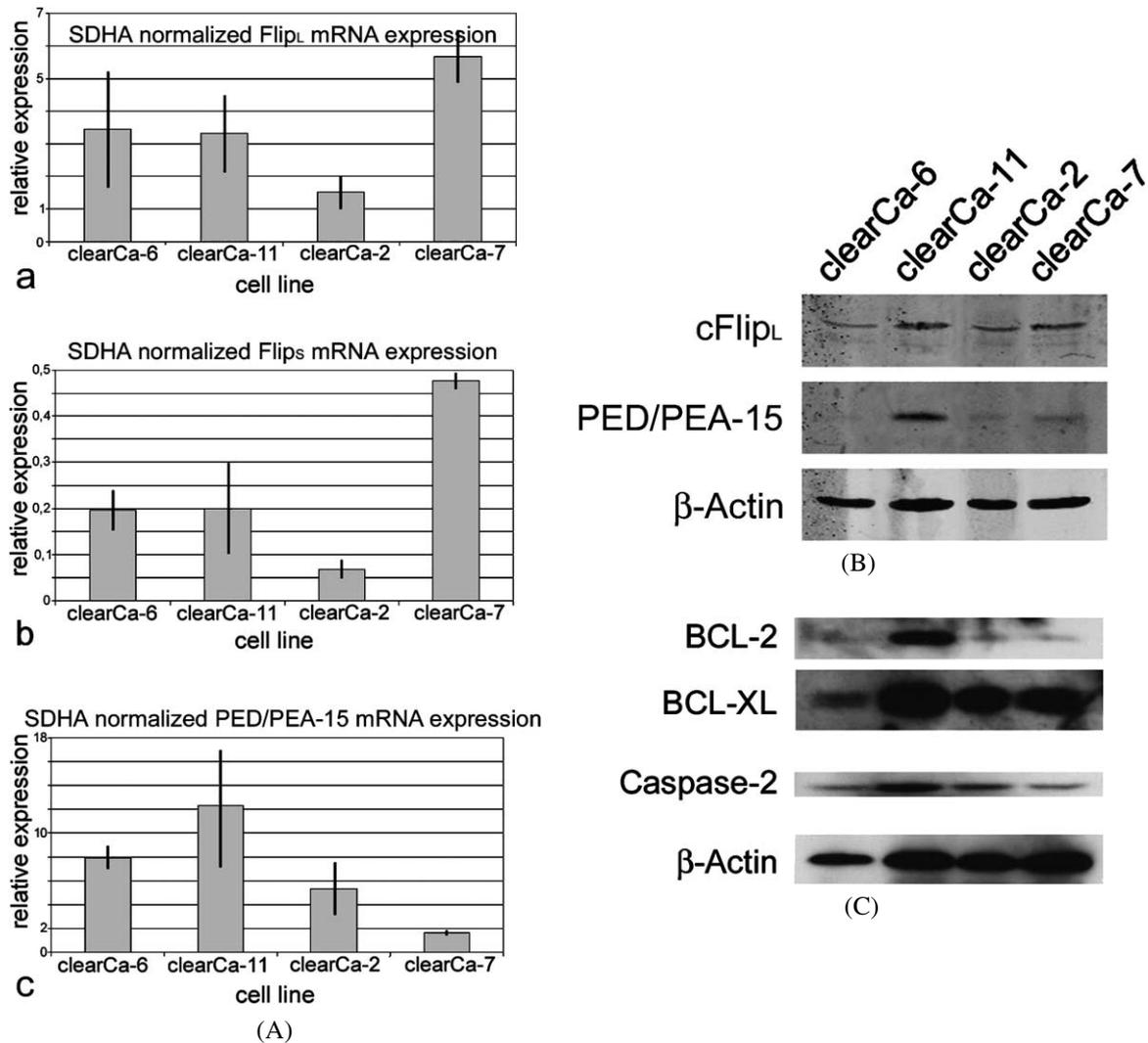


Fig. 3. Real time PCR analysis of caspase-8 inhibiting genes and Western Blot analysis of pro- and anti-apoptotic proteins in RCC cell lines. (A): Expression of the caspase-8 inhibiting genes cFlip_L (a), cFlip_S (b) and PED/PEA-15 (c) in RCC cell lines does not correlate with susceptibility towards TRAIL-induced apoptosis (shown is the median \pm standard deviation of two independent experiments). (B): Expression of the caspase-8 inhibiting proteins cFlip_L and PED/PEA-15 could be detected in all RCC cell lines whereas cFlip_S protein expression could not be shown by Western Blot. Protein expression levels slightly differed from the relative mRNA expression levels comparing the RCC cell lines. However, no correlation between the protein expression level of cFlip_L and PED/PEA-15 and apoptosis-susceptibility could be observed. (C): Expression of BCL-XL and BCL-2, inhibitors of the mitochondrial pathway of apoptosis, in all RCC cell lines with lowest expression in clearCa-6. Detection of caspase-2, which is responsible for transduction of DNA damage signals to the mitochondria in all RCC cell lines.

To exclude these more downstream events as resistance mechanisms responsible for deficient caspase-9 cleavage after administration of TRAIL, etoposide and betulinic acid, we directly measured cytochrome C release from the mitochondria into the cytosol by the Digitonin method. These experiments revealed cytochrome C release from the mitochondria in every RCC cell line after 1 h, respectively 3 h only after exposure to HA14-1 (Fig. 5). In contrast, no cy-

tochrome C release was detectable after exposure to TRAIL, etoposide or betulinic acid in any cell line. Concerning TRAIL, cytochrome C release was determined at 12 h, as we could show (Fig. 2) that apoptosis was strongly induced at that time point in the sensitive cell lines and we wanted to avoid effects that were due to disintegration of the cells in advanced stages of apoptosis. We determined cytochrome C release at 12 h after etoposide treatment since it is well known from

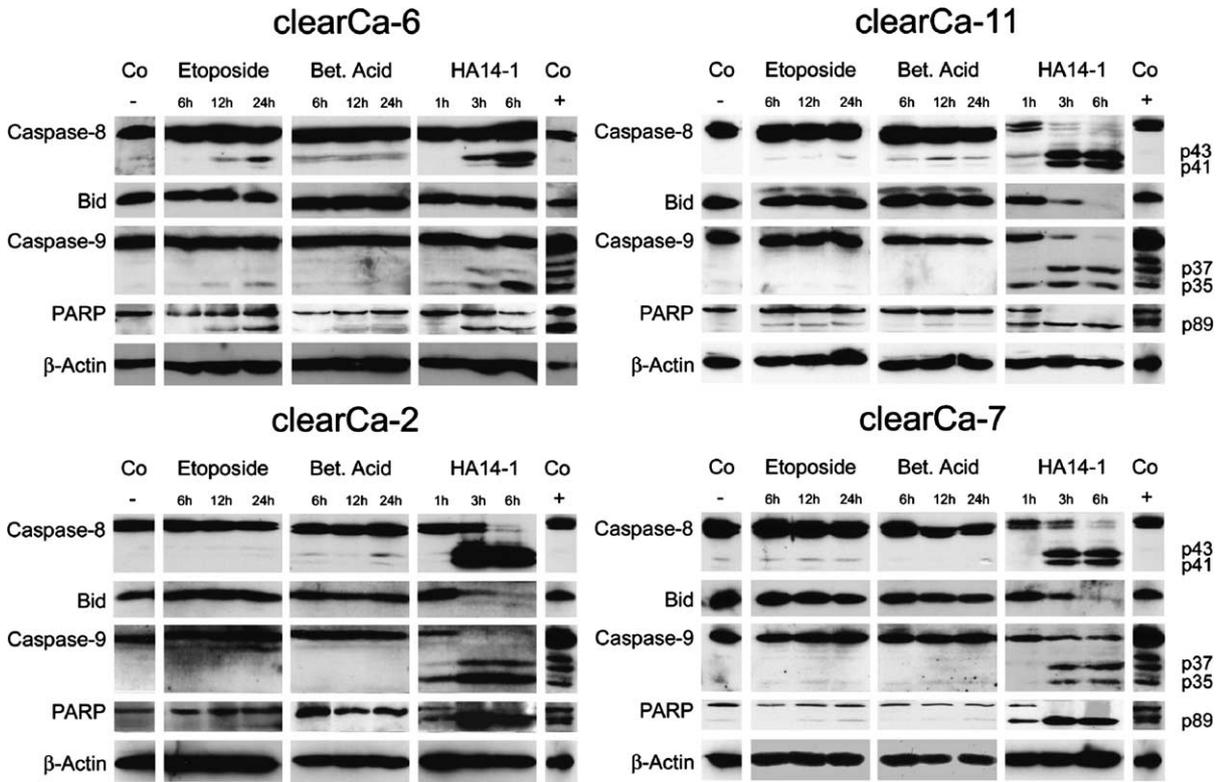


Fig. 4. Western Blot analysis of caspase-8-, caspase-9- and PARP-cleavage after exposure to etoposide, HA14-1 and betulinic acid. Caspase-8, caspase-9 and PARP-cleavage as well as reduction of Bid whole protein only after exposure to the BCL-2-antagonist HA14-1 in all cell lines after 1–3 h indicating an increased apoptosis-induction with activation of the mitochondrial pathway. Only weak detection of active cleavage products of the initiator caspase-8 with minimal caspase-9- and PARP-cleavage after exposure to etoposide in clearCa-6. No caspase-, Bid- or PARP-cleavage in all cell lines after exposure to betulinic acid. (HELA cells treated with 25 μ g/ml etoposide for 24 h were used as positive control for caspase-9 and PARP cleavage.)

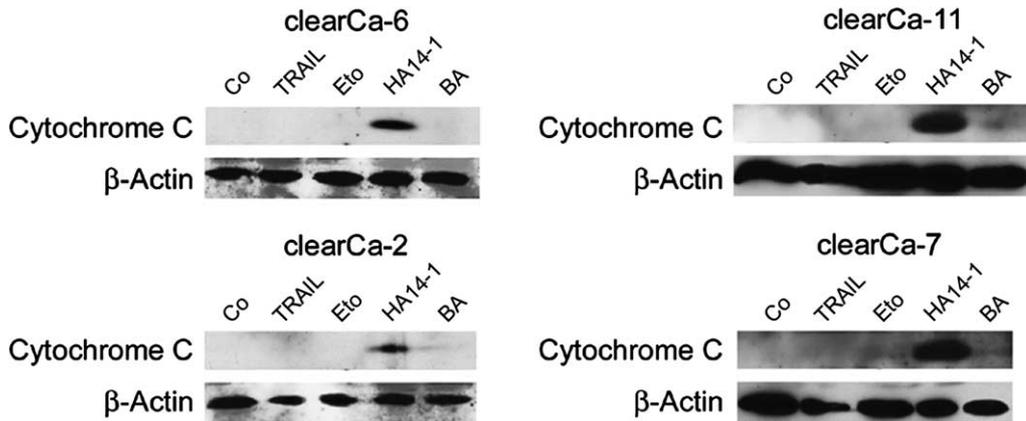


Fig. 5. Western Blot analysis of cytochrome C release after exposure to TRAIL, etoposide, HA14-1 and betulinic acid. Exposure of RCC cell lines to 100 ng/ml TRAIL for 12 h, 50 μ g/ml etoposide for 12 h, 50 μ M HA14-1 for 1 h (clearCa-2, clearCa-7, clearCa-11) respectively 3 h (clearCa-6) and 40 μ M betulinic acid (BA) for 12 h resulted in a cytochrome C release only after exposure to the BCL2-antagonist HA14-1. No detectable cytochrome C release after exposure to TRAIL, etoposide or betulinic acid.

other cell lines that it induced caspase-9 activation at 12 h and earlier [32]. Activation of effector caspases after administration of betulinic acid has also been reported as early as 12 h after administration of 5 $\mu\text{g/ml}$ betulinic acid [33].

Our results, therefore, demonstrate that release of cytochrome C from the mitochondria correlates with cleavage of caspase-9 in RCC cells. Thus, it is reasonable to assume, that the apoptotic pathways downstream of the mitochondria are functional and intact, so that resistance towards the mitochondrial apoptosis pathway must be located on the level of the mitochondria or even upstream of the mitochondria.

3.5. All RCC cell lines express antiapoptotic members of the BCL-2 family

As demonstrated above, the putative BCL-2 antagonist HA14-1 was the only agent to activate the mitochondrial apoptosis pathway in RCC cell lines. We therefore examined expression of HA14-1 target proteins, BCL-2 and BCL-XL, important antiapoptotic members of the BCL-2 family in RCC cell lines. As shown in Fig. 3(B), BCL-XL was expressed in all cell lines with the weakest expression in clearCa-6, whereas BCL-2 was weakly expressed in all RCC cell lines with strongest expression in clearCa-11.

3.6. Combined treatment of TRAIL and HA14-1 in TRAIL-sensitive cell lines results in a synergistic activation of caspase-9 and apoptosis

So far, we could show that the mitochondrial pathway of apoptosis in RCC cell lines is impaired by resistance mechanisms lying on the level or upstream of mitochondrial activation. To further locate the level of

resistance, we treated the RCC cell lines with a combination of TRAIL and HA14-1. If HA14-1 was able to enhance TRAIL-induced apoptosis by additional activation of the mitochondrial pathway of apoptosis, resistance had to be located on the level of the mitochondria themselves. Therefore, one sensitive (clearCa-11) and one resistant RCC cell line (clearCa-7) were pre-treated with a dose of HA14-1 (25 μM), that only induced minimal apoptosis – as determined by PARP cleavage – before administration of a combination of TRAIL (25 ng/ml in clearCa-11 and 100 ng/ml in clearCa-7) and HA14-1 (25 μM). These investigations revealed in the TRAIL sensitive cell line clearCa-11 a synergistic reduction of cell number (Fig. 6). In contrast, in the TRAIL-resistant cell line clearCa-7 no synergistic reduction of cell number was observed. Administration of TRAIL alone resulted in a strong cleavage of PARP in the TRAIL-sensitive cell lines clearCa-6 and clearCa-11 after 12 h, without cleavage of caspase-9 (Fig. 7). HA14-1 alone in the dose of 25 μM induced no or just a weak cleavage of caspase-9 and only weak cleavage of PARP (Fig. 7). Combination treatment of HA14-1 and TRAIL, however, revealed a strong and synergistic activation of caspase-9 as well as PARP-cleavage (Fig. 7). Thus, our results clearly demonstrate that TRAIL is able to activate the mitochondrial apoptosis pathway in these RCC cell lines. It is reasonable to assume, that the effects of HA14-1 on the mitochondria are at least in part mediated by inhibition of the antiapoptotic BCL-2 family members. In addition, these experiments clearly demonstrate that resistance towards the mitochondrial apoptosis pathway is not located upstream of the mitochondria but on the level of the mitochondria themselves, since the death receptor mediated apop-

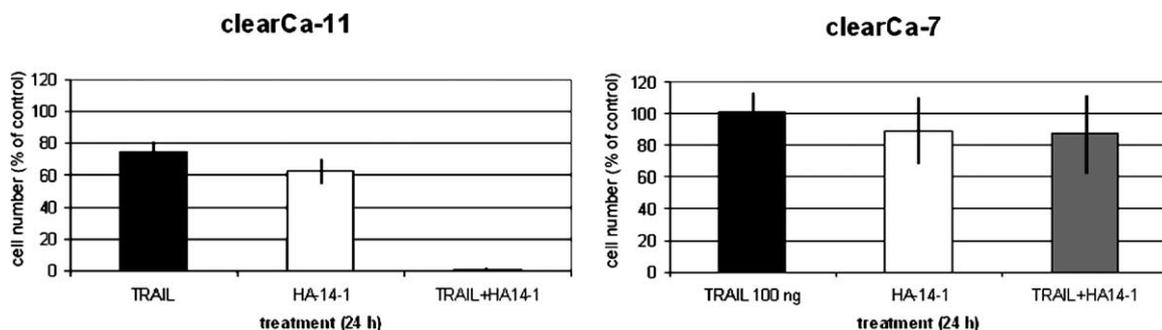


Fig. 6. Effect of combined treatment of TRAIL and HA14-1 on RCC cell number. Synergistic reduction of cell number in the TRAIL-sensitive cell line clearCa-11 after combined exposure to 25 μM HA14-1 and 25 ng TRAIL after 24 h. No effect in the TRAIL-resistant cell line clearCa-7 after combined treatment with HA14-1 (25 μM) and TRAIL (100 ng/ml) after 24 h. (Data represent median \pm standard deviation in three independent experiments.)

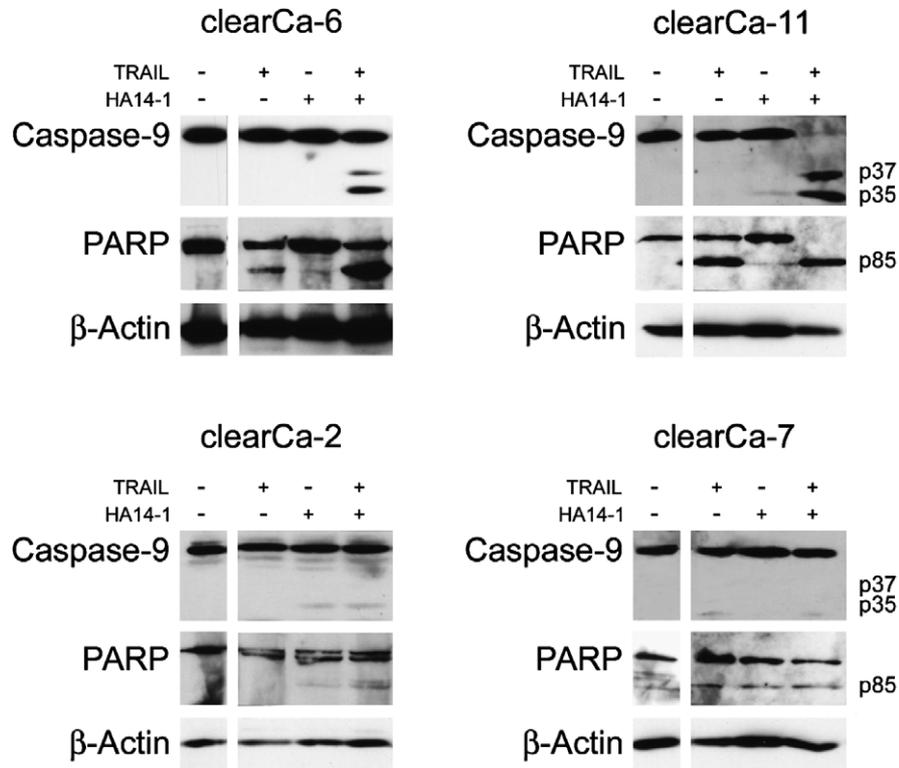


Fig. 7. Western Blot analysis of caspase-9- and PARP-cleavage after combined exposure to HA14-1 and TRAIL. Strong caspase-9 and PARP-cleavage in the TRAIL-sensitive cell lines clearCa-6 and clearCa-11 after combined treatment with TRAIL (100 ng) and HA14-1 (25 μ g) after 12 h as markers for synergistic activation of the mitochondrial pathway of apoptosis. No cleavage of PARP and caspase-9 in the resistant cell lines clearCa-2 and clearCa-7 after combined treatment with TRAIL (100 ng/ml) and HA14-1 (25 μ M) after 12 h.

otic signal can be conducted to the mitochondria as soon as the BCL-2 proteins are inactivated.

Another situation is found in the TRAIL resistant cell lines clearCa-2 and clearCa-7, where cotreatment with TRAIL and HA14-1 did not induce a synergistic activation of the mitochondrial apoptosis pathway (Figs 6 and 7). HA14-1 only resulted in a weak activation of caspase-9 and PARP in clearCa-2, which could not be further increased by exposure to TRAIL. TRAIL itself did not induce any caspase-9- or PARP-activation, confirming the results presented in Fig. 2. This can be explained by the observation that TRAIL was not able to induce caspase-8 cleavage in these cell lines (Fig. 2). Thus, the extrinsic apoptotic signalling pathway is not induced in the resistant cell lines and no signal is transduced to the mitochondria. As a result, inactivation of the antiapoptotic members of the BCL-2 family by HA14-1 is not able to synergistically activate the mitochondrial pathway of apoptosis, since the extrinsic apoptotic stimulus does not even reach the mitochondria.

4. Discussion

In this study we show for the first time that impairment of mitochondrial apoptosis in RCC cell lines is not due to defects in the signalling pathways upstream or downstream of the mitochondria: much more, the components of the mitochondrial apoptosis pathway are available and functionally inducible, though being repressed by the antiapoptotic members of the BCL-2 family. Thus, apoptosis resistance is located on the level of the mitochondria themselves. This repression can be overcome by HA14-1, showing that resistance mechanisms located on the level of the mitochondria (e.g., overexpression of antiapoptotic BCL-2 family members) are – at least in part – responsible for the well known apoptosis resistance of RCC cell lines and, in consequence, for the “multidrug-resistance” phenotype of RCCs (for a simplified diagram comprising the substances and apoptotic pathways investigated in this study see Fig. 8). However, the exact mechanisms by which HA14-1 overcomes this resistance remains obscure and has to be elucidated in future experiments.

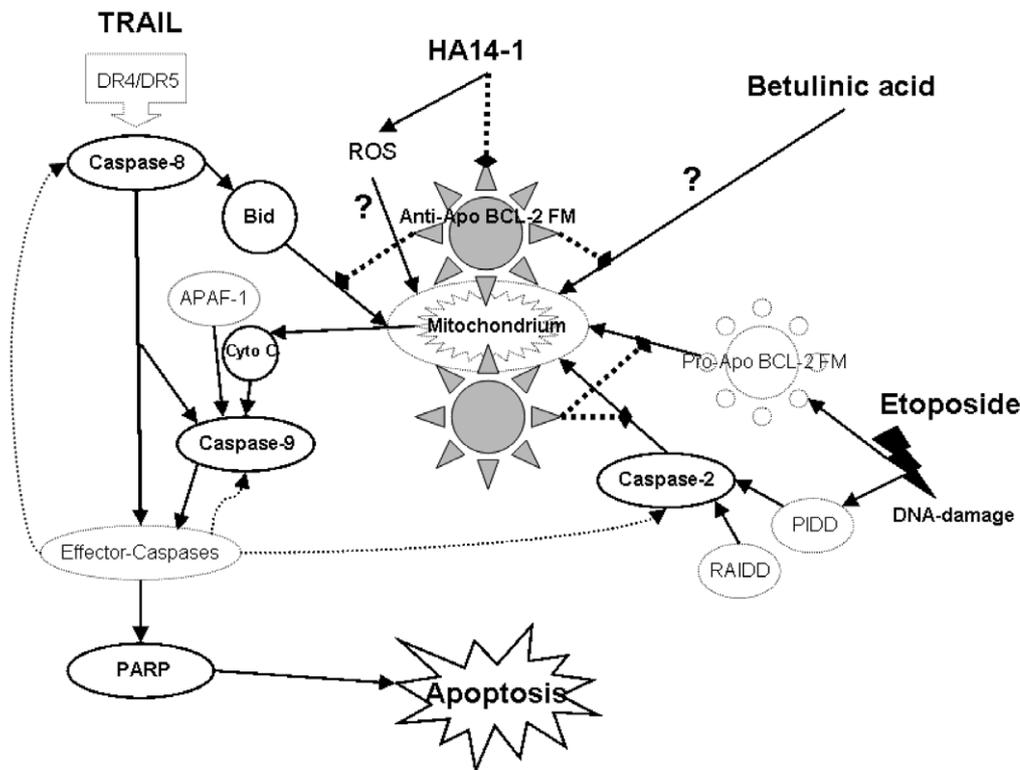


Fig. 8. Simplified diagram comprising the substances and apoptotic pathways investigated in this study. The substances and proteins analysed in this study are bold. The bold lines with arrow heads mean induction or activation, the bold dotted lines with diamond heads mean inhibition. Feedback loops are marked by thin curved dotted lines with arrow heads. (This diagram is simplified and makes no claim to be complete.) Abbreviations: Anti-Apo BCL-2 FM – antiapoptotic BCL-2 family members; Cyto C – cytochrome C; Pro-Apo BCL-2 FM – proapoptotic BCL-2 family members.

In our experiments we could show for the first time that sensitivity towards TRAIL-induced apoptosis correlated with cleavage of caspase-8, whereas no or just weak mitochondrial activation could be observed either in TRAIL-sensitive or -resistant RCC cell lines. Therefore, we first investigated resistance mechanisms possibly responsible for the observed differences in TRAIL-mediated caspase-8 activation, before we analysed the obvious impairment of the mitochondrial apoptosis pathway: we observed no correlation between susceptibility towards TRAIL and expression of the important caspase-8 inhibiting genes *cFLIP_L* and *cFLIP_S* [34,35] or *PED/PEA-15* [36]. Previous investigations on our cell lines revealed that TRAIL-sensitivity did not correlate with expression of DR4 or DR5 [4]. As a result, resistance mechanisms towards TRAIL-induced apoptosis based on the expression of these genes in RCCs could be excluded. Future experiments will have to identify the underlying resistance mechanisms: these could comprise mutations in the

DR4 or DR5 receptor [37–39], disturbances in DISC assembly etc.

In the following we focussed our analysis on the mitochondrial apoptosis pathway. Therefore, we examined mitochondrial apoptosis by exposing RCC cell lines to four agents – TRAIL, etoposide, HA14-1 and betulinic acid – known to target the mitochondrial pathway of apoptosis by different mechanisms. By means of these agents we were able to locate the level of resistance in mitochondrial apoptosis.

In certain cell types TRAIL can activate the mitochondrial pathway by Bid-mediated crosstalk between the extrinsic and mitochondrial pathway. Mitochondrial activation results in caspase-9 cleavage, which in turn results in activation of the executioner caspases. However, in our experiments no, respectively late and weak cleavage of caspase-9 in RCC cell lines could be observed after exposure to TRAIL. Additionally, no cytochrome C release from the mitochondria was detected in any cell line: concerning clearCa-11 this was surprising at first glance, since a weak caspase-9

cleavage after TRAIL administration was observed in this cell line; thus, at least a little cytochrome C release was expected. However, it is well known, that the caspase-cascade is not a one-way street and initiator caspases can be cleaved by effector caspases by feedback mechanisms [40,41]. Furthermore direct, cytochrome C independent caspase-9 activation by caspase-8 has been shown recently [42,43]. Finally the missing cytochrome C detection in the cytosolic fraction of clearCa-11 after TRAIL application could be due to the sensitivity of the method applied. Be it as it may, the missing or just minimal cleavage of caspase-9 in concordance with the lacking cytochrome C release into the cytosol after administration of TRAIL leads to the conclusion, that the mitochondrial pathway of apoptosis is functionally repressed in RCC cell lines. This repression of the mitochondrial pathway of apoptosis can occur on different levels of the signalling cascade: upstream of the mitochondria, transduction of the apoptotic signal can be impaired by inhibition of Bid-cleavage [44] or sequestration of Bax and Bak, necessary to transmit the proapoptotic signal from Bid to the mitochondria. On the level of the mitochondria, overexpression of antiapoptotic members of the BCL-2 family, like BCL-2 and BCL-XL, might be responsible for impaired apoptosis. Downstream of mitochondrial activation, impaired assembly of the apoptosome could be responsible for missing caspase-9 activation. However, it was shown in previous *in vivo* studies that RCCs own a functional apoptosome [45].

To further clarify the resistance-mechanisms towards the mitochondrial apoptosis pathway, we analysed the different death pathways induced by etoposide, HA14-1 and betulinic acid. Our experiments revealed that etoposide, betulinic acid and HA14-1 resulted in a significant reduction of cell number in all RCC cell lines. However, we could show for the first time that DNA-damaging etoposide could not increase cytochrome C efflux from mitochondria in any of the cell lines. Furthermore, in most cell lines, etoposide could not induce cleavage of caspase-8, caspase-9 or PARP and, moreover, morphological changes induced by etoposide were necrosis-like, indicating that the strong reduction of cell number was due to necrosis-like cell death.

In our experiments only the putative BCL-2 antagonist HA14-1 in a concentration of 50 μM was able to strongly induce the mitochondrial apoptosis pathway with release of cytochrome C into the cytosol as well as cleavage of caspase-9 and PARP in all cell lines and morphological induction of apoptosis. This

demonstrates that caspase-9-cleavage in RCC cell lines is in principle functional. However, the fast and strong activation not only of caspase-9 but also of caspase-8 by HA14-1 was surprising and pointed to the fact, that inhibition of the antiapoptotic family members of the BCL-2 family was not the only mechanism by which HA14-1 induced apoptosis [46,47]: on the one hand it is well known that apoptosis is not a one way street and multiple feedback mechanisms exist, by which executioner caspases (like caspase-3) can activate initiator caspases (like caspases-2, -8 and -9) [40,41]. On the other hand the very fast and strong caspase-activation after administration of HA14-1 in a concentration of 50 μM makes it arguable, that inhibition of the antiapoptotic BCL-2 family members is the only mechanism responsible for apoptosis induction by HA14-1. Much more, HA14-1 induced apoptosis has also been attributed to generation of reactive oxygen species (ROS) by rapid degradation of HA14-1 itself in the culture medium [46,47]. Thus, it is reasonable to assume that in our cell lines a combinatory effect of ROS-generation and inhibition of the antiapoptotic BCL-2 family members was responsible for the observed fast and strong apoptosis induction. The fact that the cell line expressing the weakest levels of BCL-2 and BCL-XL (clearCa-6) exhibited the "weakest" apoptosis induction upon HA14-1 administration makes it plausible that the HA14-1 mediated proapoptotic effects are at least in part mediated by inhibition of the antiapoptotic members of the BCL-2 family. Consistently, our experiments revealed that betulinic acid, which is known to induce the mitochondrial pathway only in BCL-2-deficient cells, did not lead to an increase in cytochrome C release or caspase-9- and PARP-cleavage in any RCC cell line, which expressed high levels especially of BCL-XL. Morphologically, betulinic acid induced typical necrosis-like cell death like etoposide.

Conclusively, exposure to HA14-1 can activate the mitochondrial pathway downstream of the mitochondria resulting in apoptosis, in contrast to etoposide and betulinic acid.

To further clarify, if the impairment of the mitochondrial pathway in TRAIL-mediated apoptosis was located upstream of the mitochondria at the connection of extrinsic and intrinsic apoptotic pathway or on the level of the mitochondria themselves we cotreated the RCC cell lines with HA14-1 in a concentration which did not or just barely activate the mitochondrial pathway (25 μM) and TRAIL: synergism between HA14-1 treatment and anticancer therapies could be

demonstrated in different cell lines and with different substances including TRAIL in colon carcinoma and leukaemia cell lines [46]. Although the exact mechanism of action of HA14-1 remains obscure and has to be determined in further experiments, cotreatment of RCC cell lines with HA14-1 further sensitized the TRAIL-sensitive cell lines clearCa-6 and clearCa-11 synergistically towards TRAIL-induced apoptosis. This synergistic apoptosis-induction was due to an additional activation of the mitochondrial pathway as determined by caspase-9-cleavage. In conclusion, HA14-1 is able to synergistically activate the mitochondrial apoptosis pathway in TRAIL-induced apoptosis: it is well known that ROS can enhance TRAIL-induced apoptosis by Bax translocation and reduction of the mitochondrial membrane potential [48]. Furthermore, TRAIL itself induces oxidative stress in cancer cells [49], and inhibition of ROS can impair this TRAIL-mediated caspase-8 activation [50]. This goes in concert with our own observation that TRAIL strongly enhances expression of Glutathione peroxidase in TRAIL-sensitive RCC cell lines pointing at an increased cellular oxidative stress after TRAIL application [51].

These results clearly demonstrate that the proapoptotic pathways upstream of the mitochondria are functional: TRAIL-mediated caspase-8-cleavage is able to transduce the proapoptotic signal to the mitochondria with the BCL-2 family members inactivated. Obviously the proapoptotic members of the BCL-2 family responsible for transmitting the TRAIL mediated proapoptotic signal in RCC cell lines – like Bid – are intact and functional.

In the TRAIL-resistant cell lines clearCa-2 and clearCa-7, however, we could not observe an increase in apoptosis after treatment with only weakly apoptosis-inducing doses of HA14-1. Obviously, HA14-1 could not evolve its synergistic proapoptotic effects since the initial TRAIL-mediated caspase-8-cleavage did not occur and, as a result, no apoptotic signalling cascade could be induced and transmitted to the mitochondria.

Taken together, our results show for the first time that sensitivity to TRAIL-induced apoptosis correlates with caspase-8 cleavage in RCC cell lines without or just weak activation of the mitochondrial apoptosis pathway. We answer the question whether the mitochondrial pathway is defect and uninducible or just repressed by inhibitors: here we show that the components of the mitochondrial apoptosis pathway upstream and downstream of the mitochondria are present, avail-

able, intact and inducible in RCC cell lines. However, the mitochondrial apoptosis pathway is repressed on the level of the mitochondria and it is reasonable to assume that the antiapoptotic BCL-2 family members are – at least in part – responsible for the broad apoptosis resistance of RCC cell lines and the so called “multidrug-resistance-phenotype” which can be overcome by a combination of the HA14-1 mediated induction of ROS as well as its inhibitory effect on these BCL-2 family members. The exact mechanism, however, by which HA14-1 exerts its synergistic function on TRAIL-mediated apoptosis has to be determined in future experiments.

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References

- [1] U. Ramp, E. Caliskan, C. Mahotka, A. Krieg, S. Heikaus, H.E. Gabbert and C.D. Gerharz, Apoptosis induction in renal cell carcinoma by TRAIL and gamma-radiation is impaired by deficient caspase-9 cleavage, *British Journal of Cancer* **88** (2003), 1800–1807.
- [2] U. Ramp, C. Mahotka, T. Kalinski, E. Ebel, H.E. Gabbert and C.D. Gerharz, Topotecan (Hycamtin) responsiveness in human renal carcinoma cell lines of the clear cell and papillary types, *Anticancer Research* **21** (2001), 3509–3517.
- [3] U. Ramp, M. Dejosez, C. Mahotka, B. Czarnotta, T. Kalinski, M. Wenzel, I. Lorenz, M. Muller, P. Krammer, H.E. Gabbert and C.D. Gerharz, Deficient activation of CD95 (APO-1/Fas)-mediated apoptosis: a potential factor of multidrug resistance in human renal cell carcinoma, *British Journal of Cancer* **82** (2000), 1851–1859.
- [4] M. Dejosez, U. Ramp, C. Mahotka, A. Krieg, H. Walczak, H.E. Gabbert and C.D. Gerharz, Sensitivity to TRAIL/APO-2L-mediated apoptosis in human renal cell carcinomas and its enhancement by topotecan, *Cell Death and Differentiation* **7** (2000), 1127–1136.
- [5] C. Falschlehner, C.H. Emmerich, B. Gerlach and H. Walczak, TRAIL signalling: Decisions between life and death, *International Journal of Biochemistry and Cell Biology* **39** (2007), 1462–1475.
- [6] J. Skommer, D. Wlodkovic and A. Deptala, Larger than life: Mitochondria and the Bcl-2 family, *Leukemia Research* **31** (2007), 277–286.
- [7] A.G. Yakovlev, S. Di Giovanni, G. Wang, W. Liu, B. Stoica and A.I. Faden, BOK and NOXA are essential mediators of p53-dependent apoptosis, *Journal of Biological Chemistry* **279** (2004), 28367–28374.

- [8] M.T. Hemann, J.T. Zilfou, Z. Zhao, D.J. Burgess, G.J. Hannon and S.W. Lowe, Suppression of tumorigenesis by the p53 target PUMA, *Proceedings of the National Academy of Sciences of the United States of America* **101** (2004), 9333–9338.
- [9] N.G. Iyer, S.F. Chin, H. Ozdag, Y. Daigo, D.E. Hu, M. Cariati, K. Brindle, S. Aparicio and C. Caldas, p300 regulates p53-dependent apoptosis after DNA damage in colorectal cancer cells by modulation of PUMA/p21 levels, *Proceedings of the National Academy of Sciences of the United States of America* **101** (2004), 7386–7391.
- [10] E.A. Slee, S.A. Keogh and S.J. Martin, Cleavage of BID during cytotoxic drug and UV radiation-induced apoptosis occurs downstream of the point of Bcl-2 action and is catalysed by caspase-3: a potential feedback loop for amplification of apoptosis-associated mitochondrial cytochrome c release, *Cell Death and Differentiation* **7** (2000), 556–565.
- [11] K.W. Wagner, I.H. Engels and Q.L. Deveraux, Caspase-2 can function upstream of bid cleavage in the TRAIL apoptosis pathway, *Journal of Biological Chemistry* **279** (2004), 35047–35052.
- [12] C. Du, M. Fang, Y. Li, L. Li and X. Wang, Smac, a mitochondrial protein that promotes cytochrome c-dependent caspase activation by eliminating IAP inhibition, *Cell* **102** (2000), 33–43.
- [13] A.M. Verhagen, P.G. Ekert, M. Pakusch, J. Silke, L.M. Connolly, G.E. Reid, R.L. Moritz, R.J. Simpson and D.L. Vaux, Identification of DIABLO, a mammalian protein that promotes apoptosis by binding to and antagonizing IAP proteins, *Cell* **102** (2000), 43–53.
- [14] K. Kuribayashi, P.A. Mayes and W.S. El-Deiry, What are caspases 3 and 7 doing upstream of the mitochondria?, *Cancer Biology and Therapy* **5** (2006), 763–765.
- [15] S.A. Lakhani, A. Masud, K. Kuida, G.A. Porter Jr., C.J. Booth, W.Z. Mehal, I. Inayat and R.A. Flavell, Caspases 3 and 7: key mediators of mitochondrial events of apoptosis, *Science* **311** (2006), 847–851.
- [16] C.F. Lin, C.L. Chen, W.T. Chang, M.S. Jan, L.J. Hsu, R.H. Wu, M.J. Tang, W.C. Chang and Y.S. Lin, Sequential caspase-2 and caspase-8 activation upstream of mitochondria during ceramide and etoposide-induced apoptosis, *Journal of Biological Chemistry* **279** (2004), 40755–40761.
- [17] L. Lalier, P.F. Cartron, P. Juin, S. Nedelkina, S. Manon, B. Bechinger and F.M. Vallette, Bax activation and mitochondrial insertion during apoptosis, *Apoptosis* **12** (2007), 887–896.
- [18] S. Wang and W.S. El-Deiry, TRAIL and apoptosis induction by TNF-family death receptors, *Oncogene* **22** (2003), 8628–8633.
- [19] A. Villunger, E.M. Michalak, L. Coultas, F. Mullauer, G. Bock, M.J. Ausserlechner, J.M. Adams and A. Strasser, A p53- and drug-induced apoptotic responses mediated by BH3-only proteins puma and noxa, *Science* **302** (2003), 1036–1038.
- [20] S. Fulda, C. Friesen, M. Los, C. Scaffidi, W. Mier, M. Benedict, G. Nunez, P.H. Kramer, M.E. Peter and K.M. Debatin, Betulinic acid triggers CD95 (APO-1/Fas)- and p53-independent apoptosis via activation of caspases in neuroectodermal tumors, *Cancer Research* **57** (1997), 4956–4964.
- [21] S. Fulda, I. Jeremias, H.H. Steiner, T. Pietsch and K.M. Debatin, Betulinic acid: a new cytotoxic agent against malignant brain-tumor cells, *International Journal of Cancer* **82** (1999), 435–441.
- [22] J.L. Wang, D. Liu, Z.J. Zhang, S. Shan, X. Han, S.M. Srinivassula, C.M. Croce, E.S. Alnemri and Z. Huang, Structure-based discovery of an organic compound that binds Bcl-2 protein and induces apoptosis of tumor cells, *Proceedings of the National Academy of Sciences of the United States of America* **97** (2000), 7124–7129.
- [23] J.D. Lickliter, N.J. Wood, L. Johnson, G. McHugh, J. Tan, F. Wood, J. Cox and N.W. Wickham, HA14-1 selectively induces apoptosis in Bcl-2-overexpressing leukemia/lymphoma cells, and enhances cytarabine-induced cell death, *Leukemia* **17** (2003), 2074–2080.
- [24] M.F. van Delft, A.H. Wei, K.D. Mason, C.J. Vandenberg, L. Chen, P.E. Czabotar, S.N. Willis, C.L. Scott, C.L. Day, S. Cory, J.M. Adams, A.W. Roberts and D.C. Huang, The BH3 mimetic ABT-737 targets selective Bcl-2 proteins and efficiently induces apoptosis via Bak/Bax if Mcl-1 is neutralized, *Cancer Cell* **10** (2006), 389–399.
- [25] J.D. Lickliter, N.J. Wood, L. Johnson, G. McHugh, J. Tan, F. Wood, J. Cox and N.W. Wickham, HA14-1 selectively induces apoptosis in Bcl-2-overexpressing leukemia/lymphoma cells, and enhances cytarabine-induced cell death, *Leukemia* **17** (2003), 2074–2080.
- [26] C.D. Gerharz, R. Moll, S. Storkel, U. Ramp, W. Thoenes and H.E. Gabbert, Ultrastructural appearance and cytoskeletal architecture of the clear, chromophilic, and chromophobe types of human renal cell carcinoma *in vitro*, *American Journal of Pathology* **142** (1993), 851–859.
- [27] C.D. Gerharz, U. Ramp, J. Olert, R. Moll, S. Storkel, N. Marx and H.E. Gabbert, Cytomorphological, cytogenetic, and molecular biological characterization of four new human renal carcinoma cell lines of the clear cell type, *Virchows Archiv* **424** (1994), 403–409.
- [28] M. Leist, C. Volbracht, E. Fava and P. Nicotera, 1-Methyl-4-phenylpyridinium induces autocrine excitotoxicity, protease activation, and neuronal apoptosis, *Molecular Pharmacology* **54** (1998), 789–801.
- [29] W. Roth and J.C. Reed, FLIP protein and TRAIL-induced apoptosis, *Vitamines and Hormones* **67** (2004), 189–206.
- [30] C. Hao, F. Beguinot, G. Condorelli, A. Trencia, E.G. Van Meir, V.W. Yong, I.F. Parney, W.H. Roa and K.C. Petruk, Induction and intracellular regulation of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) mediated apoptosis in human malignant glioma cells, *Cancer Research* **61** (2001), 1162–1170.
- [31] C. Xiao, B.F. Yang, N. Asadi, F. Beguinot and C. Hao, Tumor necrosis factor-related apoptosis-inducing ligand-induced death-inducing signaling complex and its modulation by c-FLIP and PED/PEA-15 in glioma cells, *Journal of Biological Chemistry* **277** (2002), 25020–25025.
- [32] C.F. Lin, C.L. Chen, W.T. Chang, M.S. Jan, L.J. Hsu, R.H. Wu, Y.T. Fang, M.J. Tang, W.C. Chang and Y.S. Lin, Bcl-2 rescues ceramide- and etoposide-induced mitochondrial apoptosis through blockage of caspase-2 activation, *Journal of Biological Chemistry* **280** (2005), 23758–23765.
- [33] G.R. Jung, K.J. Kim, C.H. Choi, T.B. Lee, S.I. Han, H.K. Han and S.C. Lim, Effect of betulinic acid on anticancer drug-resistant colon cancer cells, *Basic & Clinical Pharmacology & Toxicology* **101** (2007), 277–285.

- [34] M. Irmeler, M. Thome, M. Hahne, P. Schneider, K. Hofmann, V. Steiner, J.L. Bodmer, M. Schroter, K. Burns, C. Mattmann, D. Rimoldi, L.E. French and J. Tschopp, Inhibition of death receptor signals by cellular FLIP, *Nature* **388** (1997), 190–195.
- [35] C. Scaffidi, I. Schmitz, J. Zha, S.J. Korsmeyer, P.H. Krammer and M.E. Peter, Differential modulation of apoptosis sensitivity in CD95 type I and type II cells, *Journal of Biological Chemistry* **274** (1999), 22532–22538.
- [36] G. Condorelli, G. Vigliotta, A. Cafieri, A. Trencia, P. Andalo, F. Oriente, C. Miele, M. Caruso, P. Formisano and F. Beugnot, PED/PEA-15: an anti-apoptotic molecule that regulates FAS/TNFR1-induced apoptosis, *Oncogene* **18** (1999), 4409–4415.
- [37] M.J. Fisher, A.K. Virmani, L. Wu, R. Aplenc, J.C. Harper, S.M. Powell, T.R. Rebbeck, D. Sidransky, A.F. Gazdar and W.S. El-Deiry, Nucleotide substitution in the ectodomain of trail receptor DR4 is associated with lung cancer and head and neck cancer, *Clinical Cancer Research* **7** (2001), 1688–1697.
- [38] W.S. Park, J.H. Lee, M.S. Shin, J.Y. Park, H.S. Kim, Y.S. Kim, C.H. Park, S.K. Lee, S.H. Lee, S.N. Lee, H. Kim, N.J. Yoo and J.Y. Lee, Inactivating mutations of KILLER/DR5 gene in gastric cancers, *Gastroenterology* **121** (2001), 1219–1225.
- [39] L. Bin, J. Thorburn, L.R. Thomas, P.E. Clark, R. Humphreys and A. Thorburn, Tumor-derived mutations in the TRAIL receptor DR5 inhibit TRAIL signaling through the DR4 receptor by competing for ligand binding, *Journal of Biological Chemistry* **282** (2007), 28189–28194.
- [40] E.A. Slee, C. Adrain and S.J. Martin, Executioner caspase-3, -6, and -7 perform distinct, non-redundant roles during the demolition phase of apoptosis, *Journal of Biological Chemistry* **276** (2001), 7320–7326.
- [41] A.D. Guerrero, M. Chen and J. Wang, Delineation of the caspase-9 signaling cascade, *Apoptosis* **13** (2008), 177–186.
- [42] M.A. McDonnell, D. Wang, S.M. Khan, M.G. Vander Heiden and A. Kelekar, Caspase-9 is activated in a cytochrome c-independent manner early during TNF α -induced apoptosis in murine cells, *Cell Death and Differentiation* **10** (2003), 1005–1015.
- [43] M. Gyrd-Hansen, T. Farkas, N. Fehrenbacher, L. Bastholm, M. Høyer-Hansen, F. Elling, D. Wallach, R. Flavell, G. Kroemer, J. Nylandsted and M. Jäättelä, Apoptosome-independent activation of the lysosomal cell death pathway by caspase-9, *Molecular and Cellular Biology* **26** (2006), 7880–7891.
- [44] S. Desagher, A. Osen-Sand, S. Montessuit, E. Magnenat, F. Vilbois, A. Hochmann, L. Journot, B. Antonsson and J.C. Martinou, Phosphorylation of bid by casein kinases I and II regulates its cleavage by caspase-8, *Molecular Cell* **8** (2001), 601–611.
- [45] M.C. Gerhard, N. Zantl, G. Weirich, S. Schliep, B. Seiffert and G. Hacker, Functional evaluation of the apoptosome in renal cell carcinoma, *British Journal of Cancer* **89** (2003), 2147–2154.
- [46] J.M. Doshi, D. Tian and C. Xing, Ethyl-2-amino-6-bromo-4-(1-cyano-2-ethoxy-2-oxoethyl)-4H-chromene-3-carboxylate (HA 14-1), a prototype small-molecule antagonist against anti-apoptotic Bcl-2 proteins, decomposes to generate reactive oxygen species that induce apoptosis, *Molecular Pharmacology* **4** (2007), 919–928.
- [47] D. Tian, S.G. Das, J.M. Doshi, J. Peng, J. Lin and C. Xing, sHA 14-1, a stable and ROS-free antagonist against anti-apoptotic Bcl-2 proteins, bypasses drug resistances and synergizes cancer therapies in human leukemia cell, *Cancer Letters* **259** (2008), 198–208.
- [48] A.A. Chaudhari, J.W. Seol, S.J. Kim, Y.J. Lee, H.S. Kang, I.S. Kim, N.S. Kim and S.Y. Park, Reactive oxygen species regulate Bax translocation and mitochondrial transmembrane potential, a possible mechanism for enhanced TRAIL-induced apoptosis by CCCP, *Oncology of Reproduction* **18** (2007), 71–76.
- [49] K. Izeradjene, L. Douglas, D.M. Tillman, A.B. Delaney and J.A. Houghton, Reactive oxygen species regulate caspase activation in tumor necrosis factor-related apoptosis-inducing ligand-resistant human colon carcinoma cell lines, *Cancer Research* **65** (2005), 7436–7445.
- [50] I. Perez-Cruz, J.M. Cárcamo and D.W. Golde, Caspase-8 dependent TRAIL-induced apoptosis in cancer cell lines is inhibited by vitamin C and catalase, *Apoptosis* **12** (2007), 225–234.
- [51] S. Heikaus, E. Casliskan, C. Mahotka, H.E. Gabbert and U. Ramp, Differential gene expression in anticancer drug- and TRAIL-mediated apoptosis in renal cell carcinomas, *Apoptosis* **12** (2007), 1645–1657.



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