α-Tocopheryl succinate causes mitochondrial permeabilization by preferential formation of Bak channels

Lubomir Prochazka[1†], Lan-Feng Dong[2], Karel Valis[3], Ruth Freeman[2], Stephen J. Ralph[4], Jaroslav Turanek[1], and Jiri Neuzil[2,3†]

[1]Veterinary Research Institute, Brno, Czech Republic

[2]Apoptosis Research Group, School of Medical Science, Griffith University, Southport, Qld, Australia

[3]Molecular Therapy Group, Institute of Biotechnology, Czech Academy of Sciences, Prague, Czech Republic

[4]Genomic Research Centre, School of Medical Science, Griffith University, Southport, Qld, Australia

Running head: Vitamin E succinate and Bak channel

Address correspondence to: Jiri Neuzil, Apoptosis Research Group, School of Medical Science and the Griffith Institute of Health and Medical Research, Griffith University, Parklands Avenue, Southport, 4222 Qld, Australia; phone: +61-2-555-29109; fax: +61-2-555-28804; e-mail: j.neuzil@griffith.edu.au; or Lubomir Prochazka, Veterinary Research Institute, Hudcova 70, 621 00 Brno, Czech Republic; phone: +420-53331333; fax: +420-541211229; e-mail: lubomir.pr@gmail.com.

Abstract

Mitocans are drugs selectively killing cancer cells by destabilizing mitochondria and many induce apoptosis via generation of reactive oxygen species (ROS). However, the molecular events by which ROS production leads to apoptosis has not been clearly defined. In this study with the mitocan α-tocopheryl succinate (α-TOS) the role of the Bcl-2 family proteins in the mechanism of malignant cell apoptosis has been determined. Exposure of several different cancer cell lines to α-TOS increased expression of the Noxa protein, but none of the other proteins of the Bcl-2 family, an event that was independent of the cellular p53 status. α-TOS caused a profound conformational change in the pro-apoptotic protein, Bak, involving oligomerization in all cell types, and this also applied to the Bax protein, but only in non-small cell lung cancer cells. Immunoprecipitation studies indicated that α-TOS activates the two BH1-3 proteins, Bak or Bax, to form high molecular weight complexes in the mitochondria. RNAi knockdown revealed that Noxa and Bak are required for α-TOS-induced apoptosis, and the role of Bak was confirmed using Bak- and/or Bax-deficient cells. We conclude that the major events induced by α-TOS in cancer cells downstream of ROS production leading to mitochondrial apoptosis involve the Noxa-Bak axis. It is proposed that this represents a common mechanism for mitochondrial destabilization activated by a variety of mitocans that induce accumulation of ROS in the early phases of apoptosis.

Keywords: Vitamin E succinate; apoptosis; Bak; Noxa; mitochondria; permeabilization

Introduction

α-Tocopheryl succinate (α-TOS) is a tumor-selective drug activating intrinsic apoptosis [1, 2] by targeting the mitochondrial complex II (CII; succinate dehydrogenase) triggering subsequent production of reactive oxygen species (ROS) [3, 4]. The importance of ROS accumulation in apoptosis induction by α-TOS was established in that higher levels of apoptosis in cancer cell lines were directly associated with greater ROS production [5]. In addition, antioxidants, such as the mitochondrially targeted coenzyme Q, suppressed the anti-cancer cell toxicity of the vitamin E (VE) analog [6, 7].

Several mechanisms have been suggested to explain α-TOS induced apoptosis, mostly involving mitochondrial destabilization [8]. It has also been reported that the VE analog acts as a Bcl-2 homology-3 (BH3) mimetic, since it interacted with the BH3 domain of the Bcl-2 family proteins, disrupting the interaction between Bak, Bcl-x_L and Bcl-2 in prostate cancer cells [9]. Another report suggested α-TOS induced translocation of Bax into mitochondria in breast cancer cells, although the mechanism of this process was not defined [10]. These results led to the proposal that ROS production induced the dimerization of Bax, followed by its mitochondrial mobilization [11], perhaps helping to explain the events occurring in α-TOS-challenged cells [8, 12]. However, the precise mechanisms of mitochondrial translocation and/or activation of apoptogenic Bcl-2 family proteins triggered by α-TOS remained unclear.

A hallmark of mitochondrial apoptosis is the permeabilization of the mitochondrial outer membrane (MOM) involving two Bcl-2 protein family members, the BH1-3 domain proteins, Bak and Bax with at least one of these two proteins shown to be necessary for pore formation in the MOM [13-15]. In most cases, MOM permeabilization is triggered by activation of BH1-3 proteins, either by increasing their expression or via the BH3-only proteins Noxa, Puma, Bim, or Bid. It has been proposed that the BH1-3 proteins are liberated from their

heterodimeric association with members of the pro-survival Bcl-2 family or they can be directly activated [16, 17, 18]. From this point of view, it is of interest that α-tocopheryloxyacetic acid (α-TEA), an ether analog of VE, was previously shown to kill breast cancer cells via a pathway involving upregulation of the BH3-only protein, Noxa [19].

Activation of the Bak and Bax proteins corresponds to conformational changes, which involve exposure of the N-terminus of these proteins [20, 21] and their subsequent oligomerization in the MOM [22]. ROS-dependent dimerization of Bax protein via disulfide bond formation has also been proposed as one mechanism for Bax activation [11]. According to another hypothesis, the BH1-3 proteins may polymerize at their ends or interact with lipids of the MOM, with ensuing MOM destabilization or lipid pore formation [13, 23-25]. In addition to Bax and Bak, the BH1-3 domain protein, Bok (also known as Diva) can also cause MOM permeabilization. However, since Bok is restricted to a small number of malignant tissues, it is irrelevant for most types of cancer cells [26].

Because individual types of cancers are complex and can differ considerably in their array of DNA mutations, harboring different sets of genetic changes [27, 28], it will be very unlikely to cure cancer with drugs targeted to only a few gene products or single pathways involved in tumor survival [29]. The importance of mitocans, epitomized by VE analogs (in particular the prototypic α-TOS), as anti-cancer agents that target mitochondria to trigger apoptosis [3, 4, 8, 12] is that mitochondrial function is a universal cellular requirement. Thus, mitochondria are prime targets and transmitters of apoptosis which if selectively activated in cancer cells would provide an effective treatment for a variety of different tumors. It is this imperative which makes it critical to understand in greater detail the molecular mechanisms by which VE analogs cause permeabilization of the MOM, a paradigm that may be utilized for efficient therapy of many different cancers [30, 31].

Materials and Methods

Cell Culture and Treatment

Jurkat T lymphoma cells, the Bak$^-$/Bax$^-$ and Bax$^-$ Jurkat cells [32], the p53-deficient H1299 non-small cell lung cancer cell line [33], as well as the MCF7 breast adenocarcinoma cell line and its p53-transcriptionally inactive sub-line (MCF7$_{DD9}$) [34] were grown in RPMI-1640 medium supplemented with antibiotics and 10% FBS. Jurkat cells were treated with α-TOS (Sigma) (for structure see Fig. 1) at 5x10^5 cells per ml, and the adherent H1299, MCF7 and MCF7$_{DD9}$ cells at 80-90% confluency. α-TOS was always freshly prepared in ethanol as a stock solution that was added to the cell cultures so that the concentration of ethanol in the medium did not exceed 0.1% (v/v). In some cases, the cells were pre-incubated (1 h) with 1 μM mitochondrially targeted coenzyme Q (MitoQ; for structure, see Fig. 1) [35].

Western Blot Analysis

The following antibodies were used: anti-Bak IgG (clone Ab-1) (Calbiochem); anti-Bak IgG (NT) and anti-Bax IgG (NT) (both from Upstate Biotechnology); anti-Bax IgG (6A7), anti-p53 IgG (DO-1) (both from Sigma); anti-Noxa IgG (Alexis); anti-Mcl-1 IgG (clone 22) (BD Pharmingen); anti-actin IgG (C-2), anti-cytochrome c IgG (A-8), anti-Bcl-2 IgG (C-2), anti-Bid IgG (5C9), anti-Bad IgG (C-7) were all from Santa Cruz; anti-Bcl-x$_L$ IgG, anti-Bim IgG, anti-Cox IV IgG and anti-Puma IgG were from Cell Signaling).

To obtain whole cell lysates, cells were centrifuged at 300 x *g* for 5 min and washed twice with ice-cold PBS. The pellet was resuspended in whole cell lysis buffer (10 mM TRIS at pH 7.4, 1 mM NaF, 1 mM Na_3VO_4, 1 mM PMSF, 0.1% SDS, 1% Triton X-100, plus protease inhibitor cocktail), frozen, thawed and sonicated. Aliquots were used to estimate the level of total protein using the BCA method (Pierce). The samples were then diluted in loading buffer and boiled for 4 min. Proteins were resolved by SDS-PAGE and transferred to

PVDF or nitrocellulose membranes (GE Healthcare). After probing with a specific primary antibody and horseradish peroxidase-conjugated secondary antibody, the bands were detected by the enhanced chemiluminiscence (ECL) kit or the Advanced ECL kit (both GE Healthcare) using either a gel documentation system (Bio-Rad) or X-ray film (AGFA).

Isolation of Mitochondria and Gel Filtration Chromatography

Cells (1.2×10^8) were left untreated or treated with an appropriate concentration of α-TOS, human recombinant TNF-related apoptosis-inducing ligand (TRAIL) [36] or staurosporine (STS) for different periods, harvested and washed twice with ice-cold PBS. The final pellet was resuspended in 500 µl of ice-cold hypotonic fractionation buffer (25 mM Tris at pH 7.4, 2 mM EDTA, 5 mM $MgCl_2$, 10 mM KCl, 125 mM sucrose, 1 mM PMSF, plus protease inhibitor cocktail), and left on ice for 10 min. The swollen cells were broken using a glass homogenizer (Kontes Glass Co.). The isotonicity of each sample was achieved by addition of 250 µl of ice-cold hypertonic fractionation buffer containing 500 mM sucrose. Organelles and unbroken cells were centrifuged at 900 x *g* for 10 min, followed by centrifugation of the supernatant at 1,700 x *g* for 5 min. The remaining supernatant was then centrifuged at 15,000 x *g* for 10 min and the mitochondrial pellet lysed in a buffer comprising 25 mM HEPES, pH 7.5, 300 mM NaCl and 2% CHAPS. The mitochondrial lysate was centrifuged at 19,000 x *g* for 5 min and loaded onto the Superdex-200 10/300 Preparation Grade column (Amersham Biosciences). The column was equilibrated with the 2% CHAPS lysis buffer (see above). The proteins were eluted at 0.3 ml/min, and fractions of 0.5 ml were collected and mixed with 3-times concentrated Laemmli reducing sample buffer and boiled. The samples were then analyzed by SDS-PAGE and western blotting.

Assessment of Apoptosis and ROS accumulation

Apoptosis was evaluated by flow cytometry (FACS Calibur, BD Bioscience) using the annexin-V FITC and propidium iodide method, essentially as described [2]. ROS accumulation was assessed using the fluorescent probe dihydroethidium (DHE) essentially as detailed elsewhere [2].

Analysis of Bak and Bax Conformational Changes

Drug-induced exposure of the N-terminus of Bak was assessed using the epitope-specific antibody directed against the amino acid sequence 1-57 of the Bak protein (clone Ab-1) [37]. The active conformation of Bax was estimated using anti-Bax IgG (clone 6A7) directed against amino acids, 12-24, which only cross-reacts with the active/mitochondrially localized Bax protein domain [20]. Cells grown in a 6-well plate were exposed to various concentration of α-TOS, STS (positive control for inducing Bak and Bax conformational changes) [37, 38] or TRAIL, after which they were harvested, washed with ice-cold PBS, and fixed with 0.25% paraformaldehyde. The cells were then washed with ice-cold PBS and incubated with primary antibody diluted 1:50 in digitonin (100 μg/ml of PBS) for 30 min, which was followed by a 30 min incubation with an FITC-labeled secondary antibody diluted 1:75 in digitonin-PBS, and analyzed by flow cytometry using 'live gating' to exclude cellular debris.

Immunoprecipitation

Pelleted mitochondria were resuspended in 1 ml of immunoprecipitation lysis buffer (1% CHAPS, 150 mM NaCl, protease inhibitors cocktail, 25 mM HEPES, pH 7.4), left on ice for 30 min before centrifugation at 16,000 x *g* for 3 min. A 20 μl aliquot of the supernatant was used for the BCA protein level assay while the rest of the lysate was pre-cleared with 1 μl of mouse serum (protein concentration 4 mg/ml) and 20 μl of Protein G-conjugated agarose

beads (Santa Cruz) at 4ºC for 30 min. The Protein G agarose beads with the bound immunoglobulins were spun down at 1,000 x *g* for 5 min and the remaining supernatant comprised pre-cleared lysate. The lysate was then diluted to the final protein concentration of 300 μg/ml. A 1 ml aliquot of the lysate was incubated with 2 μg of mouse monoclonal anti-Mcl-1 or rabbit polyclonal anti-Bcl-x_L or anti-Bim IgG at 4ºC for 3 h under gentle agitation followed by addition of 20 μl of Protein G-conjugated agarose beads and incubated overnight at 4ºC. The Protein G beads were then pelleted at 1,000 x *g* for 5 min, washed 4 times with the immunoprecipitation lysis buffer, resuspended in 40 μl of 1.5 times concentrated reducing sample loading buffer and boiled for 5 min. The samples were then centrifuged to remove the Protein G beads and the supernatant was loaded onto a gel for SDS/PAGE. Co-immunoprecipitated proteins bound to Mcl-1, Bcl-x_L or Bim were detected using specific antibodies and western blotting. Pure lysis buffer handled in parallel with the real samples, pre-cleared and containing antibodies was used as a negative control.

Quantitative Real-Time PCR (Q-PCR)

Total RNA was extracted with the TRI reagent (MRC) and reverse-transcribed to cDNA using oligo dT primers and the M-MLV reverse transcriptase (Invitrogen). Q-PCR was performed using the LightCycler 480 instrument (Roche) operated using 95°C for 15 min; 50 x: 95°C for 15 s, 58°C for 30 s, 72°C for 30 s and melting analysis was performed from 60°C to 95°C. Each PCR reaction mixture of 10 μl contained 5 μl of the SYBRgreen Master Mix (Invitrogen), 0.5 μl cDNA, and forward and reverse primers at the concentration of 0.8 μM. Relative quantification of target genes expression was performed using the formula described elsewhere [39]. *β-Actin* was used as a house-keeping gene.

PCR primers were as follows; *Noxa*: forward 5'CTG TCC GAG GTG CTC CAG TT 3' and reverse 5'TCC TGA GTT GAG TAG CAC AC 3'; *Puma*: forward 5'CCA AAC GTG

ACC ACT AGC CT 3’ and reverse 5’ACA GGA TTC ACA GTC TGG GC 3’; *Mcl-1*: forward 5’TAA GGA CAA AAC GGG ACT GG 3’ and reverse 5’ACC AGC TCC TAC TCC AGC AA 3’; *Bcl-2*: forward 5’TGC ACC TGA CGC CCT TCA C 3’ and reverse 5’AGA CAG CCA GGA GAA ATC AAA CAG 3’; *Bcl-x_L*: forward 5’GTA AAC TGG GGT CGC ATT GT 3’ and reverse 5’TGC TGC ATT GTT CCC ATA GA 3’; *Bak*: forward 5’GCT ATG ACT CAG AGT TCC AGA CCA 3’ and reverse ‘CAA TTG ATG CCA CTC TCAAAC AG 3’; *Bax*: forward 5’AGA GGA TGA TTG CCG CCG T 3’ and reverse 5’CAA CCA CCC TGG TCT TGG ATC 3’; *Bim*: forward 5’GCA CAT TTC CCT CTG GCC TG 3’ and reverse 5’CCC ACG GGA GGC ATA CTT TCT G 3’*β-actin*: forward 5’AAT CTG GCA CCA CAC CTT CT 3’ and reverse 5’AG CAC AGC CTG GATAGC AAC 3’.

RNA Interference (RNAi)

H1299 and MCF7 cells were modified by silencing the expression of the Noxa, Bim, Mcl-1, Bax and Bak proteins using short interfering RNA (siRNA) essentially as described elsewhere [40]. In brief, cells were grown at 30% confluence in the absence of antibiotics and incubated with individual siRNAs pre-incubated with Lipofectamine-2000-supplemented OptiMEM medium. *Bak* siRNA, *Bim* siRNA and *Mcl-1* siRNA (Ambion), and *Bax* siRNA (Santa Cruz Biotechnology) were used at 60 nM, *Noxa* siRNA (Santa Cruz Biotechnology) at 20 nM and the control (scrambled) siRNA (Santa Cruz Biotechnology) at 20 or 60 nM. After 7 h, the OptiMEM medium was replaced with complete RPMI medium without antibiotics. After an additional 24 h, the cells were transferred into a 96-well flat bottom test plate or into 12-well plates and grown for another 24 h, and assessed for the levels of individual proteins and for cytotoxicity induced by α-TOS.

MTT Cytotoxicity Test

The MTT colorimetric assay [41] was used to assess the cytotoxic effects of α-TOS on siRNA-transfected cells. MTT (Sigma) was dissolved in PBS at 2.5 mg/ml and sterile-filtered. 20 μl of the MTT solution were added into each well of the 96-well plate with siRNA-transfected cells treated with various concentrations of α-TOS for 24 h. The plates were then incubated at 37ºC in 5% CO_2 for 3 h, 100 μl of 10% SDS was then added into each well, and the plates were then stored overnight in the dark at room temperature, after which they were analysed for absorbance at 540 nm using a microplate reader (Synergy II; Biotek). All experiments were performed in triplicate. The value of IC_{50} and the 95% confidence interval of the mean were calculated using GraphPad PRISM 5.00 software (GraphPad Software) and non-linear regression to establish dose-response inhibitory curves. The data sets were compared by Extra sum-of-squares F test based on traditional statistical hypothesis testing.

Statistics

The acquired data, unless otherwise stated, were analyzed using the GraphPad PRISM 5.00 software. The data represents the mean ± SEM of three independent experiments. The symbol '*' denotes $p<0.05$. Images are representative of at least three independent experiments.

RESULTS

We had previously shown that cancer cells treated with α-TOS died independently of their p53 status as exemplified by the $p53^{-/-}$ HCT116 colorectal carcinoma cell line [36]. These studies have been extended here to p53 null cancer cells of different origin confirming that the p53 protein is redundant for apoptosis induced by the VE analog (Fig. 2). For these studies, the cell lines expressing wild type p53 protein included Jurkat (T lymphoma) and MCF7 (breast carcinoma) cell lines, whereas the non-small cell lung carcinoma cell line, H1299, is

p53-deficient and the breast carcinoma cell line, $MCF7_{DD9,}$ has a transcriptionally silent p53. α-TOS induced apoptosis at concentrations ranging between 50 and 80 μM within 24 h in all the cell lines tested, with only slight differences in the capacity of α-TOS towards the p53 null cell lines with all showing cytochrome c release (Fig. 2).

To obtain a greater understanding of the mechanism leading to activation of apoptosis by α-TOS and the role of mitochondria in the process [2-4, 9, 37, 42-45], the role of the Bcl-2 family proteins indispensable for apoptosis induction/progression was examined [13, 14]. First, Q-PCR was used to estimate mRNA levels for the pro- and anti-apoptotic Bcl-2 family members, including *Bax*, *Bak*, *Noxa*, *Puma*, *Bim*, *Mcl-1*, *Bcl-2* and *Bcl-*x_L. The only genes showing a significant response to α-TOS-induced stress with increased mRNA levels were *Noxa* and *Puma* (Fig. 3). We next examined α-TOS-induced protein expression of Noxa and Puma by western blot analysis, which confirmed increased levels of Noxa, but not the Puma protein and no changes in the levels of other Bcl-2 family proteins (Fig. 3). Importantly, the increase in expression of Noxa occurred independently of the p53 status of the cells, as the single BH3 only member of the Bcl-2 family upregulated in response to α-TOS in both the p53-null H1299 cells and in the $MCF7_{DD9}$ cells with transcriptionally silent p53 (Fig. 3).

In the next set of experiments we tested the premise that apoptosis induced by α-TOS and Noxa upregulation are a result of generation of ROS. Further, we tested the possibility that the increase of Noxa expression is downstream of ROS generation. This is suggested by our previous results showing that ROS are generated within 1 h in cancer cells exposed to α-TOS [2, 3, 7] and we documented it in this study by experiments in which MitoQ was used as a scavenger of ROS. Results presented in Fig. 4 document high levels of ROS in Jurkat cells exposed to α-TOS for 1 h, which was suppressed by MitoQ (Fig. 4A). The ROS scavenger also suppressed apoptosis induction (Fig. 4B) and Noxa protein upregulation (Fig. 4C). These

results therefore support a causal relationship in α-TOS-challenged cells between early ROS accumulation, Noxa upregulation and apoptosis induction.

Since Noxa protein upregulation is accepted as one of several possible cellular processes that precede MOM permeabilization [17], pore formation was examined. Before the MOM pore can be formed, its components (usually Bak and/or Bax) must be conformationally activated. In accordance with earlier reports [20, 37, 38], flow cytometry of permeabilized cells using antibodies directed against epitopes exposed on the surface of 'activated' Bax and Bak proteins can be utilized to assess the extent of conformational changes of Bak and Bax proteins in individual cell lines. STS (0.5 μM) was used as a positive control for the activation of Bak and Bax proteins in Jurkat cells, and TRAIL (250 ng/ml) was used for similar purposes in the other cell lines [45]. All the cell lines tested in this study responded to α-TOS treatment by undergoing conformational changes in Bak protein, whereas the Bax conformation remained unchanged in Jurkat cells, was slightly altered in MCF7 and was highly modified in H1299 cells (Fig. 5). The observed conformational changes of Bax protein were not regulated by p53, since we observed prominent activation of the protein in the p53-null H1299 cells and only slight changes in the $MCF7_{DD9}$ cells with transcriptionally silent p53 (Fig. 5).

Given the conformational changes detected in Bak protein from all cell lines tested and in Bax protein from some of the cell lines, we next investigated the extent of oligomerization of Bak or Bax in response to α-TOS. Gel filtration chromatography and mild buffer conditions were used to separate purified mitochondrial lysates from control cells and cells treated with the VE analog using a Superdex-200 column to separate macromolecules from 3 to 600 kDa. Individual fractions were collected and analyzed by western blotting for the presence of anti-Bak IgG- and anti-Bax-IgG-reactive proteins. In agreement with the results above showing conformational change (Fig. 5), we observed anti-Bak IgG-reactive proteins in high molecular

weight (HMW) fractions in all cell lines studied following their exposure to α-TOS, whereas only H1299 cells showed anti-Bax IgG-reactive proteins in HMW fractions when exposed to the VE analog (Fig. 6). Further, we observed very weak Bax-positive bands in the HMW fractions from mitochondrial lysates of MCF7 and $MCF7_{DD9}$ cells (Fig. 6), consistent with low levels of conformational change detected in these cells (Fig. 5). The results show that mitochondria from cells treated with α-TOS, as well as with TRAIL or STS contain significant amounts of Bak protein in HMW fractions of up to 260 kDa. In the control cell samples, Bak protein was present only in the low MW fractions (50 to 80 kDa), consistent with the proposal that Bak remains functionally inactive, bound in heterodimeric complexes with Mcl-1 or Bcl-x_L in these cells [17]. On the other hand, Bax protein was detected in HMW fractions in the cell lines (except for H1299 cells) only after treatment with TRAIL or STS. Bax protein in its inactive state largely resides in the monomeric form in the cytosol. However, initial analysis of fractions of mitochondrial lysates from non-treated and α-TOS treated cells did not contain sufficient Bax protein present to allow detection using standard ECL reagents. Consequently, Advanced ECL detection kits were used and with greater sensitivity revealed the presence of the Bax protein in the low MW fractions corresponding to 20-50 kDa in control cells as well as in α-TOS-treated cells. These low MW forms likely correspond to either the monomeric form of Bax loosely attached to the mitochondrial membrane or the heterodimer in complex with anti-apoptotic members of the Bcl-2 family.

The above results indicated a prominent role for the Bak protein in α-TOS-induced cell death. Next, the α-TOS induced increase in levels of Noxa was examined for its role in Bak channel formation leading to apoptosis. For these studies, samples of mitochondrial lysates prepared from α-TOS-treated or control H1299 cells were immunoprecipitated with antibodies against the anti-apoptotic Bcl-2 family protein Mcl-1 or Bcl-x_L, both of which are known to interact with BH1-3 and BH3-only proteins. The Noxa protein was found to co-

immunoprecipitate with Mcl-1 in samples from α-TOS-treated cells but not control cells (Fig. 7). No Noxa protein was detected in the Bcl-x_L immunoprecipitates. Surprisingly, the amount of Bak protein co-immunoprecipitated with Mcl-1 was significantly higher in α-TOS-treated cells than in the control cells (Fig. 7). A similar or even greater difference between treated and non-treated cells was observed in Bcl-x_L immunoprecipitates, where the amount of associated Bak was also higher in α-TOS treated cells. Moreover, Bcl-x_L was also co-immunoprecipitated with Bax protein from treated cells and not from the control cells.

Bim, as another BH3 protein of interest was analyzed for its interaction following α-TOS treatment and was found associated with Bak, but not Bax after α-TOS treatment (Fig. 7). The two splice variants of Bim protein, BimEL and BimL, were detected in the mitochondria of the treated cells at significantly greater levels than in control cells (Fig. 7). From these results, it is concluded that α-TOS upregulates the Noxa protein that binds Mcl-1 and also causes translocation of Bim into mitochondria that together leads to activation of Bak to form pores in the MOM.

Although the results presented above documented the involvement of the Noxa-Bak axis in apoptosis [46] induced in various cancer cell lines by α-TOS, more definitive evidence was sought identifying the role of the BH3 proteins. For this, use was made of Bax^-/Bak^- Jurkat cells as well as those lacking only the Bax protein. Exposure of these cells as well as their Bax/Bak-proficient counterparts revealed that $Bax^{-/-}$ and the parental Jurkat cells were equally susceptible to α-TOS-induced apoptosis, while the Bax^-/Bak^- cells were resistant, at least over the first 24 h of treatment. Similar observations were made when the three different Jurkat cell lines were exposed to TRAIL (Fig. 8), consistent with a previous report [47]. While these results confirm the importance of Bak and/or Bax proteins for fast induction of apoptosis, we observed delayed cell death in the Bax^-/Bak^- cells (Fig. 8). The nature and the molecular

mechanism of the delayed death of the double-deficient cells are unclear at this stage and under investigation in our laboratory.

To further determine the roles of Bcl-2 proteins in apoptosis induced in cancer cells by α-TOS, RNAi was used to knock down expression of proteins of interest and the resulting change in the IC_{50} values for cellular responses to α-TOS in the RNAi-treated cells assayed using MTT. Exposure of cells to individual siRNA duplexes resulted in substantially reduced levels of the targeted proteins, i.e. Bak, Bax, Bim, Noxa and Mcl-1 (Fig. 9A). Downregulation of the Bak protein in MCF7 cells shifted the 24 h IC_{50} value from ~65 to ~100 μM (Fig. 9B) and the 40 h IC_{50} value from ~45 to ~70 μM (data not shown). Combining *Bak* and *Noxa* siRNA treatment or using either siRNA alone produced similar effects in the H1299 cells becoming more resistant to α-TOS (Fig. 9C). Knocking down Bax had no effect on the susceptibility of the cells to α-TOS (Fig. 9C). Surprisingly, downregulation of Bim did not yield statistically significant difference in 24 h IC_{50}. Importantly, downregulation of the anti-apoptotic Mcl-1 protein significantly increased susceptibility of H1299 cells to α-TOS (Fig. 9C). These results confirm the importance of the Noxa-Bak-Mcl-1 axis in apoptosis induced in cancer cells by α-TOS.

DISCUSSION

Redox-silent VE analogs epitomized by the prototypic α-TOS belong to the anti-cancer drugs termed ‘mitocans’ [1, 8, 48] which induce apoptosis in cancer cells, often in a selective manner by mitochondrial destabilization. Mitocans comprise 8 groups of compounds that destabilize mitochondria by utilizing different mechanisms [48, 49]. We have placed VE analogs into group 2 [9], containing BH3 mimetics, and group 5 comprising agents interfering with the electron redox chain [3, 4]. We and others have documented that VE analogs cause high level accumulation of ROS in the early stages of apoptosis [2-7, 42, 44, 50] leading to

mitochondrial destabilization, involving regulation of the Bcl-2 family proteins [2, 10, 51]. However, the precise molecular bases for the link between ROS accumulation and mitochondrial destabilization have not been identified. Although many inducers of apoptosis cause ROS accumulation followed by pore formation in the MOM, surprisingly, how these events link together has until now not been clear [52].

In this study, the role of the Bcl-2 family proteins in permeabilization of the MOM during α-TOS-induced apoptosis of cancer cell lines has been determined. Results with different wild-type and inactive p53 cancer cells were consistent and extended our previous findings that the VE analog induced apoptosis independently of the p53 status in the HCT116 colon cancer cell line [36]. Recently, it was reported that α-TEA, an ether analog of α-TOS, also induced apoptosis independently of p53, by upregulating the BH3-only protein Noxa, although the precise mechanism of MOM formation was not determined [19]. We show here that α-TOS causes increased expression of the *Noxa* and *Puma* mRNA, both independently of p53. However, only the *Noxa* gene is upregulated at the protein level, while that of Puma is not increased, indicating possible differences in the post-transcriptional regulation of the Noxa and Puma proteins. Although Puma upregulation at the mRNA and not protein level to the best of our knowledge has not been reported before, the underlying mechanism for these events will require further studies.

The ‘indirect model’ [17] for pore formation in the MOM involving the Bcl-2 family proteins proposes a role for BH3-only proteins (*e.g*. Noxa, Puma, Bim), in apoptosis induction in eukaryotic cells, that are upregulated leading to the activation by subsequently displacing and liberating the pro-apoptotic Bak or Bax proteins from their complexes with anti-apoptotic Bcl-2 family members (such as Bcl-2, Bcl-x_L or Mcl-1). The Mcl-1 protein, which lacks the BH4 domain but contains a PEST sequence, has been proposed to undergo proteasomal degradation in the course of liberating Bak and/or Bax proteins that then form pores in the

MOM [53, 54]. However, the results presented here suggest that degradation of the Mcl-1 protein following Noxa upregulation did not occur in any of the cell lines tested. These results are similar to those from other studies documenting that the Mcl-1 protein does not need to be degraded in order to be functionally inactivated during the apoptotic process. For example, this has been reported for apoptosis induction by campthotecin involving Noxa upregulation in H1299 and HeLa cells [55], Bim-induced apoptosis in MCF7 cells [56], γ-secretase inhibitor GSI-induced apoptosis in HCT116 cells [46], or the proteosomal inhibitor bortezomid in B-cell lymphoma [57].

It was proposed that destabilization of the Mcl-1 protein may involve its JNK-dependent phosphorylation [58]. We tested this premise in our system using the JNK inhibitor SP600125. Since we found that this did affected α-TOS-induced apoptosis in neither Jurkat nor H2199 cells (data not shown), we did not pursue this pathway any further in our studies.

Co-immunoprecipitation of Mcl-1 complexed with Noxa or Bak, Bcl-x_L with Bak and of Bim with Bak in H1299 cells showed that Mcl-1/Noxa and, rather surprisingly, Mcl-1/Bak and Bcl-x_L/Bak interactions were more abundant in the α-TOS-treated than in the control cells. This kind of result was very surprising, since co-IP experiments of this type of proteins usually give opposite results. Explanation of this, however, may lie in the definition of the 'direct model' for Bax and Bak activation [18, 59-61]. Central to this model is the concept that Bak and Bax sequestered by the anti-apoptotic Bcl-2 proteins represent only a fraction of the total Bak and Bax protein pools when these pro-apoptotic proteins are activated either spontaneously or by some unspecified mechanisms [18, 62]. Co-immunoprecipitation experiments presented here indicate that α-TOS-induced activation of Bak and Bax may cause their sequestration by Mcl-1 and Bcl-x_L, respectively, until a saturation point is surpassed allowing accumulation of the active forms of Bak and Bax. Moreover, the Noxa protein, upregulated by treating cancer cells with α-TOS not only could compete with

activated Bak for the hydrophobic BH3 binding groove of Mcl-1 [63] or Bcl-x_L [64] making Bak freely available, but could also possibly displace Bim from its complex with Mcl-1, allowing Bim to act as a direct co-activator of Bak or Bax [46]. Unexpectedly, we did not detect any interaction between Bim and Mcl-1, although Bim accumulated in mitochondria and interacted with the Bak protein, which was the first time observed (and also tested in H1299 cells) direct interaction of the wt Bim and Bak protein to the best of our knowledge. The co-IP results suggest that increased level of the Bim protein associated to the mitochondria of α-TOS treated H1299 cells probably directly relates to the increased number of the Bak protein detected in co-IP WB. α-TOS perhaps aims Bim at mitochondria without any other direct effect on the self-interaction of Bim and Bak. Similarly, Noxa does not seem to disrupt, at least in our study in H1299 cells, the pre-formed anti-apoptotic Bak/Mcl-1, Bak/Bcl-x_L or even Bax/Bcl-x_L complexes and rather competes with the BH1-3 proteins for the Mcl-1 protein.

The results obtained in this study better fit a model where the excessive unsequestered monomers of activated Bak protein (and Bax protein in the case of H1299 cells), are then free to self-interact, forming a pore triggering the apoptotic cascade downstream of mitochondria. In the case of the non-small cell lung carcinoma H1299 or A549 cells, which express high levels of the Mcl-1 protein, Mcl-1 can significantly prolong the apoptotic process induced by various pro-apoptotic stimuli including cisplatin, etoposide, UV irradiation or calcium ionophores [65] with similar results also shown for hematopoietic cells [66]. We propose that α-TOS sensitizes Mcl-1-overexpressing cells to a variety of pro-apoptotic stimuli, in a similar manner to that shown for cells treated with the topoisomerase I inhibitor CPT-11 which also acted to increase expression of the Noxa protein [67].

Both the indirect and direct activation models share a common denominator: any major disruption of the dynamic balance between the anti-apoptotic Bcl-2 family proteins and the

BH3 pro-apoptotic proteins favoring unsequestration of the latter triggers the intrinsic apoptotic process. It has been published that a conformational change of the BH1-3 proteins is important for aggregation of these proteins and for their targeting to the MOM, where they eventually form a pore that allows the apoptotic cascade to proceed [21, 22]. In this study, we used antibodies recognizing epitopes exposed only on the activated states of BH1-3 proteins. However, the detection of the activation epitope on the Bak protein (documented by using the specific antibody Ab-1) unlike that found for the Bax protein (utilizing the 6A7 antibody) does not necessarily mean that the Bak MOM pore will be formed [20, 21]. Thus the recognition of the N-terminal activation epitope of the Bak protein by the Ab-1 antibody may detect only the 'priming' stage for Bak functional activation. We have observed the activation or 'priming' of Bak in all cell lines tested, and this correlated with the process of α-TOS-induced Bak MOM pore formation. By contrast, the Bax protein was fully activated only in the H1299 cells suggesting a relatively similar importance for these two proteins to mediate α-TOS-induced apoptosis in the non-small cell lung carcinoma cell line. The MCF7 and $MCF7_{DD9}$ cells showed only slight levels of Bax activation, occurring after most of the Bak protein had been activated which suggests a higher level of functional importance for the Bak protein in breast cancer cells.

After epitope activation or 'priming' of the BH1-3 proteins, they oligomerize due to interaction of activated monomers via their BH3 domains within the MOM, becoming detectable in higher molecular weight fractions of mitochondrial lysates [68]. Our results demonstrate that the Bak protein oligomerizes in mitochondria after α-TOS treatment in all tested cell lines, since it was only then detected in fractions >160 kDa. Our data from FPLC analysis of the mitochondrial lysates of cells treated with α-TOS (Fig. 6) indicate that the Bak protein complexes formed are homo-oligomeric, since Bax protein was not found in these same fractions. Conversely, the results observed in the H1299 cells indicate that the Bax

protein oligomers do not contain Bak protein. These observations rule out hetero-oligomerization of the Bak and Bax proteins during cancer cell apoptosis induced by VE analogs.

Our data for Bak oligomerization are consistent with and further explained by recent reports, documenting that following an apoptogenic signal, two Bak monomers initially form a homodimer by way of generation of disulphide bridges, which is followed by higher homo-oligomeric structures that results in formation of a Bak channel in the MOM [69, 70]. We propose that this step occurs following Noxa upregulation in cancer cells exposed to α-TOS, which gives initially higher levels of ROS that themselves not only cause increased expression of the Noxa protein but, following Noxa-mediated departure of Bcl-2 family anti-apoptotic proteins from Bak, but also catalyse formation of the Bak-Bak dimers.

Conformational changes of Bak protein has been shown to occur during apoptosis induced by VE analogs in prostate cancer cells via a BH3 mimetic like mode of action [9]. However, we did not observe a similar activity induced by α-TOS in the cell lines used in our experiments. Unlike the report by Shiau and colleagues, who observed disruption of Bak-Mcl-1 or Bak-Bcl-x_L interaction in prostate cancer cells treated with α-TOS [9], we found that the Bak protein did immunoprecipitate with Mcl-1 and Bcl-x_L in MCF7 (data not shown) and in H1299 cells exposed to α-TOS. Therefore, the BH3 mimetic-like activity of VE analogs may be cell-type specific, dose related or both. Notwithstanding, we suggest that the BH3 mimetic-like activity of VE analogs should make a significant contribution by sensitizing cancer cells to apoptosis induction by other agents, particularly in cell lines overexpressing the anti-apoptotic Bcl-2 family proteins. However, the major role of VE analogs in apoptosis induction can be ascribed to the accumulation of high levels of ROS in the exposed cells, which translates to mitochondrial destabilization, as delineated in this report.

The requirement of the Bak protein and involvement of Mcl-1/Bak axis in α-TOS-induced apoptosis was confirmed by RNAi against Bak and Mcl-1 while knocking down of the Bax protein did not significantly affect the IC_{50} values of α-TOS, at least in H1299 cells. We observed an increase in the IC_{50} value in cells exposed to the VE analogue following knock down of the Bak or Noxa protein levels. Probably the most direct evidence for the importance of the Bak protein in apoptosis induced by α-TOS, at least for the Jurkat T lymphoma cells, comes from experiments comparing wild-type cells with cells deficient in both Bak and Bax or either one of these two proteins. The results clearly indicate that Jurkat cells containing the Bak protein are susceptible to apoptosis while the Bax protein appears not to be critical.

We have recently documented that the target of VE analogs is the mitochondrial CII [3, 4, 44], and this has been supported by others (Gogvadze et al., personal communication). Thus, α-TOS displaces ubiquinone from CII, such that electrons, generated due to conversion of succinate to fumarate at CII [71], cannot be intercepted and give rise to superoxide [72]. The resulting ROS then cause mitochondrial destabilization by a mechanism that has previously not been clarified. According to the data shown here, we propose a model whereby ROS leads to transcriptional upregulation of the BH3-only protein Noxa that may cooperate with Bim and results in unsequestration, activation and oligomerization of the BH1-3 protein Bak, with ensuing formation of a pore in the MOM. The α-TOS-triggered ROS accumulation that causes transcriptional upregulation of Noxa is dependent on the FOXO family of transcription factors and is the subject of ongoing research (K.V. et al., unpublished results).

In conclusion, we document here that redox-silent VE analogs, epitomized by α-TOS, destabilize mitochondria by promoting formation of MOM pores. To the best of our knowledge, this is the first report defining the molecular mechanism triggered by mitocan induced ROS accumulation in cancer cells leading to mitochondrial activated apoptosis.

REFERENCES

1. Neuzil J, Dong LF, Ramanathapuram L et al (2007) Vitamin E analogues as a novel group of mitocans: Anti-cancer agents that act by targeting mitochondria. Mol Asp Med 28:607-645
2. Weber T, Dalen H, Andera L et al (2003) Mitochondria play a central role in apoptosis induced by alpha-tocopheryl succinate, an agent with antineoplastic activity: Comparison with receptor-mediated pro-apoptotic signaling. Biochemistry 42:4277-4291
3. Dong LF, Low P, Dyason JC et al (2008) α-Tocopheryl succinate induces apoptosis by targeting ubiquinone-binding sites in mitochondrial respiratory complex II. Oncogene 27:4324-4335
4. Dong LF, Freeman R, Liu J et al (2009) Suppression of tumor growth in vivo by the mitocan α-tocopheryl succinate requires respiratory complex II. Clin Cancer Res 15:1593-1600
5. Kang YH, Lee E, Choi MK et al (2004) Role of reactive oxygen species in the induction of apoptosis by α-tocopheryl succinate. Int J Cancer 112:385-392
6. Alleva R, Tomasetti M, Andera L et al (2001) Coenzyme Q blocks biochemical but not receptor-mediated apoptosis by increasing mitochondrial antioxidant protection. FEBS Lett 503:46-50
7. Stapelberg M, Gellert N, Swettenham E et al (2005) α-Tocopheryl succinate inhibits malignant mesothelioma by disrupting the fibroblast growth factor autocrine loop. J Biol Chem 280:25369-25376
8. Neuzil J, Wang XF, Dong LF, Low P, Ralph SJ (2006) Molecular mechanism of 'mitocan'-induced apoptosis in cancer cells epitomizes the multiple roles of reactive oxygen species and Bcl-2 family proteins. FEBS Lett 580:5125-5129

9. Shiau CW, Huang JW, Wang DS et al (2006) α-Tocopheryl succinate induces apoptosis in prostate cancer cells in part through inhibition of Bcl-x_L/Bcl-2 function. J Biol Chem 281:11819-11825
10. Yu WP, Sanders BG, Kline K (2003) RRR-α-tocopheryl succinate-induced apoptosis of human breast cancer cells involves Bax translocation to mitochondria. Cancer Res 63:2483-2491
11. D'Alessio M, De Nicola M, Coppola S et al (2005) Oxidative Bax dimerization promotes its translocation to mitochondria independently of apoptosis. Faseb J 19:1504-1506
12. Neuzil J, Dyason JC, Freeman R et al (2007) Mitocans as anti-cancer agents targeting mitochondria: lessons from studies with vitamin E analogues, inhibitors of complex II. J Bioenerg Biomembr 39:65-72
13. Wei MC, Zong WX, Cheng EHY et al (2001) Proapoptotic BAX and BAK: A requisite gateway to mitochondrial dysfunction and death. Science 292:727-730
14. Reed JC (2006) Proapoptotic multidomain Bcl-2/Bax-family proteins: mechanisms, physiological roles, and therapeutic opportunities. Cell Death Differ 13:1378-1386
15. Lindsten T, Ross AJ, King A et al (2000) The combined functions of proapoptotic Bcl-2 family members Bak and Bax are essential for normal development of multiple tissues. Mol Cell 6:1389-1399
16. Korsmeyer SJ, Wei MC, Saito M, Weller S, Oh KJ, Schlesinger PH (2000) Pro-apoptotic cascade activates BID, which oligomerizes BAK or BAX into pores that result in the release of cytochrome c. Cell Death Differ 7:1166-1173
17. Willis SN, Fletcher JI, Kaufmann T et al (2007) Apoptosis initiated when BH3 ligands engage multiple Bcl-2 homologs, not Bax or Bak. Science 315:856-859
18. Brunelle JK, Letai A (2009) Control of mitochondrial apoptosis by the Bcl-2 family. J Cell Sci 122:437-441

19. Wang P, Yu WP, Hu ZZ et al (2008) Involvement of JNK/p73/NOXA in vitamin E analog-induced apoptosis of human breast cancer cells. Mol Carcinogen 47:436-445
20. Hsu YT, Youle RJ (1997) Nonionic detergents induce dimerization among members of the Bcl-2 family. J Biol Chem 272:13829-13834
21. Griffiths GJ, Corfe BM, Savory P et al (2001) Cellular damage signals promote sequential changes at the N-terminus and BH-1 domain of the pro-apoptotic protein Bak. Oncogene 20:7668-7676
22. Zhang LL, Shimizu S, Sakamaki K, Yonehara S, Tsujimoto Y (2004) A caspase-8-independent signaling pathway activated by fas ligation leads to exposure of the Bak N terminus. J Biol Chem 279:33865-33874
23. Kuwana T, Mackey MR, Perkins G et al (2002) Bid, Bax, and lipids cooperate to form supramolecular openings in the outer mitochondrial membrane. Cell 111:331-342
24. Schafer B, Quispe J, Choudhary V et al (2009) Mitochondrial outer membrane proteins assist bid in bax-mediated lipidic pore formation. Mol Biol Cell 20:2276-2285
25. Hardwick JM, Polster BM (2002) Bax, along with lipid conspirators, allows cytochrome c to escape mitochondria. Mol Cell 10:963-965
26. Lee RM, Chen J, Matthews CP, McDougall JK, Neiman PE (2001) Characterization of NR13-related human cell death regulator, Boo/Diva, in normal and cancer tissues. Biochim Biophys Acta1520:187-194
27. Jones S, Zhang XS, Parsons DW et al (2008) Core signaling pathways in human pancreatic cancers revealed by global genomic analyses. Science 321:1801-1806
28. Parsons DW, Jones S, Zhang XS et al (2008) An integrated genomic analysis of human glioblastoma Multiforme. Science 321:1807-1812
29. Hayden EC (2008) Cancer complexity slows quest for cure. Nature 455:148

30. Reed JC, Pellecchia M (2005) Apoptosis-based therapies for hematologic malignancies. Blood 106:408-418.
31. Green DR, Kroemer G (2005) Pharmacological manipulation of cell death: clinical applications in sight? J Clin Invest 115:2610-2617
32. Wang GQ, Wieckowski E, Goldstein LA et al (2001) Resistance to granzyme B-mediated cytochrome c release in Bak-deficient cells. J Exp Med 194:1325-1337
33. Giaccone G, Battey J, Gazdar AF, Oie H, Draoui M, Moody TW (1992) Neuromedin-B is present in lung-cancer cell-lines. Cancer Res 52:2732s-S2736s
34. Kotala V, Uldrijan S, Horky M, Trbusek M, Strnad M, Vojtesek B (2001) Potent induction of wild-type p53-dependent transcription in tumour cells by a synthetic inhibitor of cyclin-dependent kinases. Cell Mol Life Sci 58:1333-1339
35. Kelso GF, Porteous CM, Coulter CV et al (2001) Selective targeting of a redox-active ubiquinone to mitochondria within cells: antioxidant and antiapoptotic properties. J Biol Chem 276:4588-9456
36. Weber T, Lu M, Andera L et al (2002) Vitamin E succinate is a potent novel antineoplastic agent with high selectivity and cooperativity with tumor necrosis factor-related apoptosis-inducing ligand (Apo2 ligand) in vivo. Clin Cancer Res 8:863-869
37. Griffiths GJ, Dubrez L, Morgan CP et al (1999) Cell damage-induced conformational changes of the pro-apoptotic protein bak in vivo precede the onset of apoptosis. J Cell Biol 144:903-914
38. Mandic A, Viktorsson K, Molin M et al (2001) Cisplatin induces the proapoptotic conformation of Bak in a Delta MEKK1-dependent manner. Mol Cell Biol 21:3684-3691
39. Pfaffl MW (2001) A new mathematical model for relative quantification in real-time RT-PCR. Nucl Acids Res 29:e45

40. Swettenham E, Witting PK, Salvatore BA, Neuzil J (2005) α-Tocopheryl succinate selectively induces apoptosis in neuroblastoma cells: potential therapy of malignancies of the nervous system? J Neurochem 94:1448-1456
41. Mosmann T (1983) Rapid colorimetric assay for cellular growth and survival - application to proliferation and cytotoxicity assays. J Immunol Meth 65:55-63
42. Neuzil J, Weber T, Schroder A et al (2001) Induction of cancer cell apoptosis by α-tocopheryl succinate: molecular pathways and structural requirements. FASEB J 15:403-415
43. Alleva R, Benassi MS, Tomasetti M et al (2005) α-Tocopheryl succinate induces cytostasis and apoptosis in osteosarcoma cells: the role of E2F1. Biochem Biophys Res Commun 331:1515-1521
44. Dong LF, Swettenham E, Eliasson J et al (2007) Vitamin E analogues inhibit angiogenesis by selective induction of apoptosis in proliferating endothelial cells: The role of oxidative stress. Cancer Res 67:11906-11913
45. Baksh S, Tommasi S, Fenton S et al (2005) The tumor suppressor RASSF1A and MAP-1 link death receptor signaling to bax conformational change and cell death. Mol Cell 18:637-650
46. Han J, Goldstein LA, Hou W, Rabinowich H (2007) Functional linkage between NOXA and Bim in mitochondrial apoptotic events. J Biol Chem 282:16223-16231
47. Han J, Goldstein LA, Gastman B et al (2004) Differential involvement of Bax and Bak in TRAIL-mediated apoptosis of leukemic T cells. Leukemia 18:1671-1680
48. Neuzil J, Tomasetti M, Zhao Y et al (2007) Vitamin E analogs, a novel group of "mitocans," as anticancer agents: The importance of being redox-silent. Mol Pharmacol 71:1185-1199

49. Ralph SJ, Neuzil J (2009) Mitochondria as targets for cancer therapy. Mol Nutr Food Res 53:9-28

50. Wang XF, Birringer M, Dong LF et al (2007) A peptide conjugate of vitamin E succinate targets breast cancer cells with high ErbB2 expression. Cancer Res 67:3337-3344

51. Zhao Y, Li R, Xia W, Neuzil J, Lu Y, Zhang H, Zhao X, Zhang X, Sun C, Wu K (2010) Bid integrates intrinsic and extrinsic signaling in apoptosis induced by α-tocopheryl succinate in human gastric carcinoma cells. Cancer Lett 288:42-49.

52. Gogvadze V, Orrenius S, Zhivotovsky B (2008) Mitochondria in cancer cells: what is so special about them? Trends Cell Biol; 18:165-173

53. Chen L, Willis SN, Wei A et al (2005) Differential targeting of prosurvival Bcl-2 proteins by their BH3-only ligands allows complementary apoptotic function. Mol Cell 17:393-403

54. Czabotar PE, Lee EF, van Delft MF et al (2007) Structural insights into the degradation of Mcl-1 induced by BH3 domains. Proc Natl Acad Sc USA 104:6217-6222

55. Mei YD, Xie CW, Xie W, Tian X, Li M, Wu M (2007) Noxa/Mcl-1 balance regulates susceptibility of cells to camptothecin-induced apoptosis. Neoplasia 9:871-881

56. Lee EF, Czabotar PE, van Delft MF et al (2008) A novel BH3 ligand that selectively targets Mcl-1 reveals that apoptosis can proceed without Mcl-1 degradation. J Cell Biol 180:341-355

57. Pérez-Galán P, Roué G, Villamor N, Montserrat E, Campo E, Colomer D (2006) The proteasome inhibitor bortezomib induces apoptosis in mantle-cell lymphoma through generation of ROS and Noxa activation independent of p53 status.Blood 107:257-264

58. Kang MH, Wan Z, Kang YH, Sposto R, Reynolds CP (2008) Mechanism of synergy of N-(4-hydroxyphenyl)retinamide and ABT-737 in acute lymphoblastic leukemia cell lines: Mcl-1 inactivation. J Natl Cancer Inst 100:580-595

59. Letai A, Bassik MC, Walensky LD, Sorcinelli MD, Weiler S, Korsmeyer SJ (2002) Distinct BH3 domains either sensitize or activate mitochondrial apoptosis, serving as prototype cancer therapeutics. Cancer Cell 2:183-192

60. Marani M, Tenev T, Hancock D, Downward J, Lemoine NR (2002) Identification of novel isoforms of the BH3 domain protein Bim which directly activate Bax to trigger apoptosis. Mol Cell Biol 22:3577-3589

61. Merino D, Giam M, Hughes PD et al (2009) The role of BH3-only protein Bim extends beyond inhibiting Bcl-2-like pro-survival proteins. J Cell Biol 186:355-362

62. Fletcher JI, Meusburger S, Hawkins CJ et al (2008) Apoptosis is triggered when prosurvival Bcl-2 proteins cannot restrain Bax. ProcNatl Acad Sci USA 105:18081-18087

63. Day CL, Chen L, Richardson SJ, Harrison PJ, Huang DCS, Hinds MG (2005) Solution structure of prosurvival Mcl-1 and characterization of its binding by proapoptotic BH3-only ligands. J Biol Chem 280:4738-4744

64. Sattler M, Liang H, Nettesheim D et al (1997) Structure of Bcl-x_L-Bak peptide complex: Recognition between regulators of apoptosis. Science 275:983-986

65. Song LX, Coppola D, Livingston S, Cress D, Haura EB (2005) Mcl-1 regulates survival and sensitivity to diverse apoptotic stimuli in human non-small cell lung cancer cells. Cancer Biol Ther 4:267-276

66. Zhou P, Qian LP, Kozopas KM, Craig RW (1997) Mcl-1, a Bcl-2 family member, delays the death of hematopoietic cells under a variety of apoptosis-inducing conditions. Blood 89:630-643

67. Okumura K, Huang SB, Sinicrope FA (2008) Induction of Noxa sensitizes human colorectal cancer cells expressing mcl-1 to the small-molecule Bcl-2/Bcl-x_L inhibitor, ABT-737. Clin Cancer Res 14:8132-8142

68. Dewson G, Kratina T, Sim HW et al (2005) To trigger apoptosis, Bak exposes its BH3 domain and homodimerizes via BH3 : Groove interactions. Mol Cell 30:369-380
69. Wang H, Takemoto C, Akasaka R et al (2009) Novel dimerization mode of the human Bcl-2 family protein Bak, a mitochondrial apoptosis regulator. J Struct Biol 166:32-37
70. Dewson G, Kratina T, Czabotar P, Day CL, Adams JM, Kluck RM (2009) Bak activation for apoptosis involves oligomerization of dimers via their α6 helices. Mol Cell 36:696-703
71. Sun F, Huo X, Zhai YJ et al (2005) Crystal structure of mitochondrial respiratory membrane protein complex II. Cell 121:1043-1057
72. Adam-Vizi V, Chinopoulos C (2006) Bioenergetics and the formation of mitochondrial reactive oxygen species. Trends Pharmacol Sci 27:639-645.

FOOTNOTES

Jurkat Bax^-/Bak^- and Jurkat Bax^- cells were provided by H. Rabinowicz (University of Pittsburgh, Pittsburgh, PA, USA), H1299 and $MCF7_{DD9}$ cells by Dr. B. Vojtesek (Masaryk Memorial Institute, Brno, Czech Republic), and human recombinant TRAIL by Dr. L. Andera (Institute of Molecular Genetics, Academy of Sciences of the Czech Republic, Prague, Czech Republic). This work was supported in part by grants from the Australian Research Council (to J.N. and P.K.W.), the Queensland Cancer Fund, the National Breast Cancer Foundation, the Grant Agency of the Academy of Sciences of the Czech Republic KAN200520703, IAA5005220602 and IAA5005200602 to J.N, by Concept Grant AV0Z50520514 awarded by the Academy of Sciences of the Czech Republic, by a grant from Ministry of Agriculture of the Czech Republic (Grant No. MZE 0002716202) to J.T. and by a grant from the Grant

agency of the Czech Republic 204/09/P632 to L.P. L.P. was supported in part by the Apoptosis Research Group (Griffith University).

The abbreviations used are: BH3, Bcl-2 homology domain-3; CII, complex II; Cox IV, cytochrome c oxidase subunit IV; DHE, dihydroethidium; ECL, enhanced chemiluminescence; MOM, mitochondrial outer membrane; RNAi, RNA interference; ROS, reactive oxygen species; Q-PCR, quantitative real-time PCR**;** siRNA, short interfering RNA; STS, staurosporin; α-TEA, α-tocopheryloxyacetic acid; α-TOS, α-tocopheryl succinate; TRAIL, TNF-related apoptosis-inducing ligand; VE, vitamin E;

FIGURE LEGENDS

Fig.1. Structures of α-tocopheryl succinate (α-TOS) and mitochondrially targeted coenzyme Q (MitoQ).

Fig. 2. α-TOS kills cancer cells regardless of their p53 status. The wild type p53 Jurkat and MCF7 cells, the p53-null H1299 cells and MCF_{DD9} cells with transcriptionally silent p53 were exposed to increasing concentrations of α-TOS for 24 h and the apoptosis levels analyzed by flow cytometry using the annexin V-FITC/propidium iodide method and the results are expressed as the percent cell viability relative to non-treated cells. The insert documents the level of cytochrome c release in control Jurkat cells (1) and cells exposed to 50 μM α-TOS for 7 (2) or 8 h (3). Actin was used as a loading control for the cytosolic fraction, while COX IV was used to document the purity of the cytosolic fraction. Sample 4 documents the presence of COX IV solely in the mitochondrial subfractions. Data shown represent mean ± SEM (n = 3), western blot images are representative of at least three independent experiments.

Fig. 3. α-TOS enhances expression of the BH3-only protein Noxa independently of cellular p53 status. Jurkat T lymphoma (a) and other cell lines (b) represented by MCF7 with wild-type p53, p53-deficient H1299 cells and $MCF7_{DD9}$ cells with transcriptionally silent p53 were treated with α-TOS (see below) and assessed for the expression of Bcl-2 family proteins (a) or only Noxa and Puma (b) using Q-PCR and Western blotting. Jurkat cells were exposed to 50 μM α-TOS for 6 h, H1299 cells to 80 μM α-TOS for 12 h, and MCF7 and $MCF7_{DD9}$ cells to 50 μM α-TOS for 15 h. (c) The control and α-TOS-treated cells were also probed by western blotting for p53 or p21 protein levels in the case of MCF7 wt and $MCF7_{DD9}$ cells and the cells were treated with 20 μM Roscovitine for 20 h as a control for p53 dependent upregulation of p21 protein. Actin was used as a marker of equal protein loading for western blotting and as a house-keeping gene for Q-PCR. The Q-PCR data represent mean ± SEM (n = 3). The symbol '*' denotes significant differences between control and treated cells with $p<0.05$. Western blot images are representative of at least three independent experiments.

Fig. 4. ROS play a role in Noxa upregulation and apoptosis induction by α-TOS. Jurkat cells were pre-incubated, as indicated, for 1 h with 1 μM MitoQ, exposed to 50 μM α-TOS, and assessed for accumulation of ROS using the fluorescent probe DHE (A; 1-h exposure), apoptosis using the annexin V-FITC kit (B; 12-h exposure) and Noxa protein expressing by western blotting (C; 4-h exposure). The data in panels A and B represent mean ± SEM (n = 3), the images in panel C are representative of three independent experiments.

Fig. 5. α-TOS activates BH1-3 proteins. Jurkat, MCF7 and $MCF7_{DD9}$ cells were treated with 50 μM α-TOS, and H1299 cells with 80 μM α-TOS for the indicated time points. The cells were then analyzed for conformational changes of the Bak and Bax proteins as described in

the Experimental section. Treatment with 500 nM STS for 4 h or 250 ng/ml TRAIL for 6 h was used as a positive control for conformational changes of the Bax protein. Following the treatment, permeabilized whole cells were reacted with anti-Bak IgG or anti-Bax IgG recognizing the activated form of the proteins due to their altered conformation, and the level of this change was assessed by flow cytometry. Filled histograms represent untreated cells, empty histograms represent α-TOS treated cells.

Fig. 6. α-TOS induces Bak oligomerization in the mitochondrial membrane. Proteins of mitochondrial lysates (100 μg) derived from control cells and cells treated with α-TOS (Jurkat cells, 50 μM, 12 h; H1299 cells, 80 μM, 20 h; MCF7 and $MCF7_{DD9}$ cells, 50 μM, 24 h) were fractionated using gel filtration through a Superdex 200 Preparation Grade column. Individual fractions were collected and probed by western blotting for the presence of Bak and Bax proteins. Treatment with 500 nM STS for 6 h was used as a positive control for Bak and Bax oligomerization in Jurkat cells. Treatment with TRAIL at 250 ng/ml for 10 h was used as a positive control for Bak and Bax oligomerization in H1299, MCF7 and $MCF7_{DD9}$ cells.

Fig. 7. Immunoprecipitation of selected Bcl-2 family members confirms α-TOS induced interactions among the Bcl-2 family. Mitochondrial lysates of H1299 cells were analyzed by antibody pull-down and western blotting as shown. Ctrl: non-treated cells; TOS: α-TOS-treated cell samples with the antibodies as indicated. LB: immunoprecipitation using lysis buffer as a control for antibody cross-reactivity. WCL: whole cell lysates as positive control for Bak, Bax or other proteins as indicated. a: Right hand panel shows proteins co-immunoprecipitating with Mcl-1. The left hand panel shows proteins co-immunoprecipitating with Bcl-x_L. b: Only Bak protein co-immunoprecipitated with Bim in mitochondrial lysates

from α-TOS treated cells. c: The levels of protein expressed in samples of whole mitochondrial lysates. d: Comparison of levels of Bim protein expressed in whole cell lysates from α-TOS treated versus non-treated cells.

Fig. 8. Susceptibility of Bax- and/or Bak-deficient cells to α-TOS-induced apoptosis. Panels a and b: Levels of apoptosis in the parental, Bax^- or Bax^-/Bak^- Jurkat cells treated for 12 h with 50 μM α-TOS or 20 ng/ml TRAIL as indicated in panels c and d: Time dependent increase in apoptosis of parental, Bax^- or Bax^-/Bak^- Jurkat cells treated with 50 μM α-TOS or 20 ng/ml TRAIL as indicated. The inset panel in D shows levels of proteins as indicated in the respective Jurkat cell populations. The extent of apoptosis was estimated by flow cytometry using the annexin V-FITC/propidium iodide assay. The data represent mean ± SEM of three independent experiments. The symbol '*' denotes significant differences with $p<0.05$.

Fig. 9. Knocking down Noxa and Bak desensitizes cancer cells to α-TOS-induced apoptosis. a: Efficacy of downregulation of the Bak, Bax, Bim, Noxa and Mcl-1 proteins in H1299 and MCF7 cells using siRNA knock down. Actin was used as a standard for protein loading. b: Change in the IC_{50} value for α-TOS of MCF7 cells pre-treated with scrambled (SC) siRNA or Bak siRNA. c: Changes in the IC_{50} value for α-TOS of H1299 control cells or cells pre-treated with scrambled (SC), Noxa, Bak, Bax, Bim or Mcl-1 siRNA, or with Bak plus Noxa siRNA. Data show the mean ± 95% confidence interval, and the symbol '*' denotes statistically significant differences from control cells with $p<0.05$.