Review

Vitamin E analogues as mitochondria-targeting compounds: From the bench to the bedside?

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Despite considerable effort focusing on designing and finding efficient anti-cancer drugs over the last decade, little progress has been achieved, in particular in case of highly recalcitrant malignancies. Also, since there is a trend suggesting that deaths from cancers may be more frequent than from cardiovascular diseases, it is important to look for novel efficient and selective therapeutic approaches to gradually start winning the battle with cancer. Redox-silent vitamin E analogues, epitomised by α-tocopheryl succinate, give some hope in the quest for drugs with such properties. Thus far, these agents have been successfully tested in experimental animals with different types of cancer, showing high efficacy against malignancies including HER2-positive breast carcinomas or malignant mesotheliomas. Further research will provide additional, necessary data to launch clinical trials, possibly in near future, translating into development of innovative anti-cancer drugs acting by targeting mitochondria selectively in cancer cells.

Keywords: Apoptosis / Cancer / Pre-clinical models / Signalling / Vitamin E analogues

Received: January 31, 2008; revised: April 10, 2008; accepted: April 14, 2008

1 Introduction

In the past 20 years, deaths from chronic noncommunicable diseases have surpassed those from acute infectious diseases in all regions of the world, except for Africa [1, 2]. Based on the GLOBOCAN database, there were 10.9 million new cases of cancer in the world in the year 2002. In the same year, 6.7 million cancer patients died and 24.6 million patients lived with the disease [3]. Despite a great advance in molecular medicine and understanding the intricate pathways that can be utilized in fight against cancer, the neoplastic disease remains a leading killer worldwide and continues to be the growing global burden [4]. Therefore, pursuing cancer control strategies is one of the major tasks for scientists these days. In the recent period, molecular genetic evidence in favour of a direct implication of mitochondria in oncogenesis and the use of these organelles to combat cancer has been accumulating [5–7].

Mitochondria are unique organelles essential for life and death of eukaryotic cells. Apart from their crucial role as a power-plant of bioenergetics, mitochondria participate in numerous metabolic reactions including growth, division and apoptosis [8–11]. Mitochondrial defects have long been speculated to play a central role in the development and progression of tumourigenesis [12–14]. More than 70 years ago, Warburg first hypothesized that cancer cells actively metabolize glucose *via* anaerobic glycolysis and produce excessive lactate, in concomitance with the impairment in mitochondrial respiration, which is referred to as the Warburg effect [14, 15].

Although the exact mechanisms are not clear, an increasing number of anticancer drugs are being discovered to induce the cell demise by targeting mitochondria [16, 17]. These agents, referred to as 'mitocans', function as key reg-

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Abbreviations: CL, cardiolipin; **DRs,** death receptors; **IAPs,** inhibitors of apoptosis proteins; **IκB,** inhibitory κB; **i.p.,** intraperitoneal; **JNK,** c-Jun N-terminal kinase; **MIM,** mitochondrial inner membrane; **MAPK,** mitogen-activated protein kinase; **MOM,** mitochondrial outer membrane; **MOMP,** mitochondrial outer membrane pore; **MPT,** mitochondrial permeability transition; **ODN,** oligodeoxynucleotide; **PKC,** protein kinase C; **ROS,** reactive oxygen species; **α**-TOB, α-tocopheryloxybutyric acid; **TNFα**, tumour necrosis factor-α; **-TOH,** α-tocopherol; **α**-TOS, α-tocopheryl succinate; **TRAIL,** TNF-related apoptosis-inducing ligand

ulators of apoptosis by destabilizing the mitochondrial outer membrane permeabilization (MOMP), consequently resulting in the release of soluble apoptosis modulators [6, 10, 17, 18].

The redox-silent analogues of vitamin E belong to one of seven groups of mitocans [18–20]. α-Tocopheryl succinate (α-TOS), representative of these analogues, selectively induces apoptosis in a variety of types of malignant cells through mitochondria-dependent apoptotic signalling [21–24]. Mitochondrial DNA-deficient cells (ρ^0 phenotype) are resistant to α-TOS compared with their parental cells [22, 23], indicating that mitochondria are key signalling transmitters of apoptosis induced by this ester analogue of vitamin E.

2 Preclinical anticancer activity of vitamin E analogues – Relation to their proapoptotic activity

Vitamin E analogues have been extensively explored as potential anticancer drugs in the past decade. This is quite rational, since compounds of the vitamin E group, redox-active micronutrients, are consumed in the regular diet and also used as food additives. Importantly, they are of general benefit and, as a rule, do not exert secondary deleterious effects.

It is well established that α-tocopherol (α-TOH) exhibits the highest vitamin E bioactivity among the eight natural forms of the vitamin, as shown by the rat foetal resorption assay. α-TOH is also the form of vitamin E present at the highest concentration in serum and in dietary supplements. Although the best understood function of vitamin E is its antioxidant activity, cell culture, animal and epidemiological studies show that certain vitamin E-related compounds exhibit antitumour properties. In the α-TOH, Beta-Carotene Cancer Prevention trial, α-TOH supplements lowered the incidence and mortality of prostate cancer in male Finnish smokers [25], but had no significant effects on other types of tumours [26, 27]. On the other hand, the epidemiological evidence supporting a link between α-TOH or other forms of vitamin E and cancer is limited and intervention studies are scarce. Numerous attempts to illustrate antitumourigenic activity of dietary vitamin E give no conclusive answer, since studies completed thus far have provided rather divergent results.

Certain structural modifications of the vitamin E molecule, however, may enhance the antitumour activity of the agents, especially in the case of those which exert apoptogenic effects (Fig. 1). α-TOS, an efficient apoptogenic analogue of vitamin E, may be an optimal choice within such group of compounds. Experimental studies have clearly pointed to α-TOS as a promising anticancer agent. This paper reviews the anticancer potential of vitamin E analogues, in particular the prototypic α-TOS.

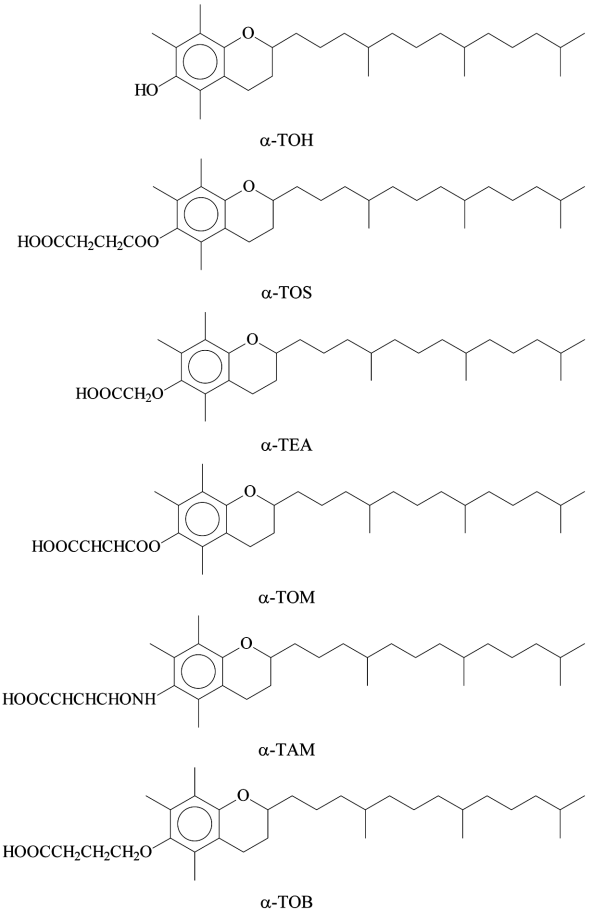

Figure 1. Structure of pro-apoptotic analogues of vitamin E and α-TOH.

2.1 Selective antitumour activity of vitamin E analogues

Many of the established chemotherapeutic agents (*e.g.* doxorubicin and cisplatin) kill not only tumour cells but also normal cells, resulting in serious side-effects. α-TOS, however, shows unique selectivity in killing of tumour cells, while not harming normal cells and tissues [28, 29]. α-TOS is derived from α-TOH *via* substitution of the hydroxyl group responsible for the redox activity with succinic acid at position C6 of the chromanol ring in the Functional Domain. This relatively small change in the molecular structure greatly alters its biological activity. While, α-TOH is a major chain-breaking antioxidant in the lipid phase, α-TOS, as a redox-silent analogue, significantly inhibits tumour progression *via* inhibition of tumour cell proliferation, blockage of the cell cycle, arrest of DNA synthesis as well as by induction of differentiation and, in particular, apoptosis [30–35].

The molecular basis underlying the selectivity of α-TOS for malignant cells results from at least two mechanisms. One is related to the ester structure of α-TOS: due to the

generally higher level of esterases in normal cells, such as hepatocytes, colonocytes, fibroblasts or cardiomyocytes, α-TOS is hydrolysed to produce vitamin E (α-TOH) [36]. The other reason for the cancer cell-specific toxicity of α-TOS is associated with the inherent property of some apoptosis inducers, including α-TOS, to trigger programmed cell death by initial induction of accumulation of reactive oxygen species (ROS), which in turn cause a cascade of subsequent reactions leading to transition into the apoptosis commitment phase [37].

2.2 Apoptogenic properties of α-TOS

α-TOS has been proven to possess high apotogenic activity for a variety of cancer cell lines of different origin regarding the species (human, murine and avian) and tissue type (breast, prostate, lung, stomach, ovary, monocyte, colon, bone, mesothelium, *etc.*) [29–31, 34, 35, 38–47], whereas equivalent amounts of α-TOH or α-tocopheryl acetic acid (α-TOA) exert no such induction of apoptosis [39, 48, 49]. Different malignant cells also show diverse susceptibility to α-TOS. Neuzil *et al.* [29] demonstrated that the level of apoptosis induced by α-TOS (50 μM, 12 h exposure) varied from 30 to 60% in different malignant cells. ∼50% apoptosis was induced in the MDA-MB-435 human breast cancer cells exposed to α-TOS at 20 μg/mL for 48 h [21], and α-TOS at 20 μg/mL for 48 h efficiently triggered ∼90% apoptosis in the SGC-7901 human stomach cancer cells [35]. In summary, α-TOS is a potent apoptosis inducer selective for malignant cells, while inducing, in general, less than 5% apoptosis in normal cells.

Data have been published revealing that the nonantioxidant analogues of vitamin E strongly suppress cancer cell growth *in vivo* as well. Helson *et al.* [50] found for the first time that intraperitoneal (i.p.) administration of α-TOS (50 mg/kg body weight) markedly inhibited the growth of human neuroblastoma cells in athymic mice. Following studies demonstrate that exposure to α-TOS efficiently reduced the incidence of breast, colon, stomach and prostate cancer and melanomas. Intraperitoneal administration of α-TOS (150 mg/kg body weight) lowered the growth of human breast cancer, colon carcinoma and murine melanoma cells [29, 51–53]. α-TOS (100 mg/kg body weight) caused a significant reduction in the volume of human colorectal cancer xenografts [54], reduced prostate tumour burden in BALB/c nude mice fed with soybean oil [55], and increased survival of immunocompromised mice with experimental human peritoneal mesotheliomas [56]. α-TOS (200 mg/kg body weight) also suppressed the number of tumours and their volume in the benzo(a)pyrene-induced forestomach carcinoma model in female thymus-bearing mice [57]. In addition, it has been reported that α-TOS suppressed colon cancer metastases into the liver and mammary tumour metastases into the lungs in nude mice [53, 58], which further strengthens and extends the prospects for α-TOS as an anticancer drug. α-TOS may also exert its anticancer activity *via* down-regulation of vascular endothelial growth factor [51, 59] and sensitization of resistant cells to other inducers of apoptosis, such as the immunological tumour necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) [54].

Modified analogues of vitamin E can be designed to target cancer cells overexpressing certain receptors. For example, the newly synthesized α-TOS-LTVSPWY conjugate efficiently killed breast cancer cells with high levels of the receptor tyrosine kinase erbB2 (HER2), such that the peptide conjugate at 5 μmol reduced the initial volume of breast carcinomas in the *c-neu* transgenic mouse (with spontaneous erbB2-high tumours) by ∼70%, more effectively than α-TOS alone at 15 μmol which suppressed the tumour progression by ∼50% [60, 61]. The peptide conjugate induced higher level of apoptosis in erbB2-overexpressing cells than α-TOS, and the extent of apoptosis induced by free α-TOS was, more-or-less, independent of the level of erbB2 expression [60].

The results mentioned above imply that α-TOS is effective only when administered by an i.p. injection, not by oral administration, since it is efficiently hydrolysed to α-TOH when applied using the latter mode. It is assumed that the ester link in α-TOS is hydrolysed by nonspecific esterases in the intestinal tract before entering the bloodstream, ruling out oral administration for application of biologically active α-TOS [62].

2.3 Other proapoptotic analogues of vitamin E

A number of other vitamin E analogues in which modifications have been made to the functional domain also exhibit antiproliferative and proapoptotic properties in tumour cells. For example, an analogue of α-TOH with ether-linked acetic acid (α-TEA) shows similar anticancer and apoptogenic properties as does α-TOS in human breast, prostate, colon, lung and endometrial cancer cells, as well as angiogenic endothelial cells, and in mice xenografted with mammary tumour cells [58, 63]. α-TEA is more efficient than α-TOS in apoptosis induction in human ovarian and cervical cancer cells and in mouse mammary tumour cells *in vivo*, regardless of the administration method, *i.e.* whether the analogue is given by i.p. injection, by oral gavage or incorporated in the diet [58, 63, 64]. Ether analogues of vitamin E analogues can be administered orally, since the ether bond is resistant to hydrolysis, endowing the analogue with stability superior to its ester counterparts.

The amide analogues, such as α-tocopheryl maleyl amide (α-TAM), are very potent inducers of apoptosis in a number of cancer cell lines, including the erbB2-over-expressing breast cancer cells [23], as well as lymphoma and neuroblastoma cells [65, 66]. However, α-TAM was found extremely toxic *in vivo*, when injected into the peritoneum of mice as a free compound. On the other hand, when for-

Table 1. Effects of vitamin E analogues in selected experimental cancer models

Animal	Inoculated cell line or tumour inducer	Applied dose	Duration of treatment and effect on tumour growth	Reference
Nude mouse	MDA-MB-231 human breast cancer cells	150 mg/kg/day in sesame oil	2 wk; 80–90% tumour dormancy	[51]
Nude mouse	B16F10 murine melanoma cells	100 mg/kg/day in sesame oil	2 wk; 80–90% tumour dormancy, inhibition of liver metastases	[59]
Nude mouse	CT-26 colon cancer cells	100 mg/kg/day in 20% DMSO	2 wk; ~75% inhibition of liver metastasis	[53]
Nude mouse	B16F10 murine melanoma cells	150 mg/kg/day in sesame oil	2 wk; ~70% tumour growth inhibition	[54]
Nude mouse	HCT116 human colon cancer cells	100 mg/kg in DMSO every third day	10 days; ~75% tumour growth inhibition	[29]
Nude mouse	HCT116 human colon cancer cells	50 mg/kg in DMSO every third day plus 20 μg/mouse of hrTRAIL	10 days; ~70% tumour growth inhibition	[54]
Nude mouse	Human mesothelioma Ist-Mes2 cells (peritoneal grafts)	100 mg/kg in DMSO every third day	21 wk; >3-fold increase in survival	[56]
Nude mouse	Human mesothelioma Ist-Mes2 cells (s.c. xenografts)	100 mg/kg in DMSO every second day	16 days; >90% tumour growth inhibition	[102]
Female Kunming mouse	Benzo(a)pyrene-induced forestomach tumours	200 mg/kg in corn oil twice *per* week	4 wk; ~85% tumour growth inhibition	[57]
Nude mouse	MDA-MB-435-FL-breast cancer cells	36 μg/mouse of α-TEA daily in aerosol	31 days; ~60% tumour growth inhibition	[103]
C57BL/6 mouse	3LLD122 murine Lewis lung carcinoma cell line	200 mg/kg α-TOS in ethanol or 200 mg/kg vesiculated α-TOS	20 days; >70% tumour growth inhibition	[104]
Transgenic *c-neu* mouse	Spontaneous breast carcinomas	15 μmol/mouse α-TOS or 5 μmol/mouse α-TOS-LTVSPWY in corn oil every 3-4 days	3 wk; 50% reduction in tumour size by 15 μmol α-TOS or 70% by 5 μmol peptide conjugate	[60]
Transgenic *c-neu* mouse	Spontaneous breast carcinomas	15 μmol/mouse in corn oil every third day	3 wk; 30% reduction in original tumour size	[61]
Nude mouse	MCF-7 human breast cancer cells	100 mg/kg in DMSO every third day	4 wk; >30% reduction in original tumour size	[61]
Nude mouse	LNCaP human prostate cancer cells	100 mg/kg in sesame oil every day	2 wk of 7 wk in total; ~70% reduction in original tumour size	[55]
Nude mouse	4T1 murine breast cancer cells	4 mg/mouse α-TOS or α-TEA by i.p. every 4 days or daily oral gavage, or 5.5 mg/mouse α-TEA in diet	30 days; 60% reduction in tumour size by α-TOS by i.p. or α-TEA by both i.p. and orally; inhibition of lung metastasis by α-TEA	[58]

mulated into liposomes, α-TOM efficiently suppressed breast carcinomas while exerting no discernible general toxicity (Neuzil *et al*., submitted for publication). α-Tocopheryl oxalate and α-tocopheryl malonate are two strong apoptogens *in vitro* among α-tocopheryl esters with a terminal dicarboxylic moiety, but they were shown to induce nonselective cytotoxicity in mice inoculated with B16-F1 melanoma cells [67, 68]. α-Tocopheryloxybutyric acid (α-TOB), a nonhydrolysable ether form of α-TOS, is also capable of suppressing proliferation and inducting apoptosis in breast and prostate cancer cells, however this occurs to a lower extent than with α-TOS on equimolar basis [69–71]. α-TOB has been demonstrated to efficiently reduce in mouse lung tumours *via* the suppression of the ERK cascade [72]. Besides analogues of α-TOH, tocotrienols display relatively strong proapoptotic and anticancer activity *in vitro* and *in vivo*, with δ-tocotrienol being the most robust apoptogen of this group of vitamin E analogues [73, 74].

These vitamin E analogues, epitomised by the prototypic α-TOS, may provide new strategies to develop and establish novel anticancer agents. This is well exemplified by the anticancer effect of the compounds in preclinical cancer models, of which some recent ones are summarized in Table 1.

Taken together, vitamin E analogues with potent apoptogenic activity show efficient anticancer activity *in vitro* and *in vivo* using experimental animal models. However, it is still unclear whether they are also effective in the case of human cancer patients and what the mechanisms under such setting would be. More data from experimental, epidemiological and finally clinical studies are necessary for further investigation of these intriguing and highly promising agents, so that they may be established as routine anticancer drugs.

3 Mitochondrial apoptogenic pathways targeted by vitamin E analogues

Mitochondria are membrane-enclosed organelles distributed through the cytosol of most eukaryotic cells. They consist of the mitochondrial outer membrane (MOM) that defines the entire structure and the mitochondrial inner membrane (MIM) that envelopes the fluid-filled matrix. Mitocans trigger tumour cell apoptosis through the mitochondrial pathway as a consequence of cellular damage, also referred to as the intrinsic apoptotic pathway. The role of mitochondria has been well documented for induction of apoptosis by vitamin E analogues [18–22].

3.1 Initiation of mitochondrial membrane permeabilization: The central event of apoptosis

Various apoptotic stimuli may trigger formation of the MOM pore (MOMP). This is a complex process that involves numerous molecular players and may serve as a target for anticancer drugs. During this event, the MOM and MIM are, finally, both permeabilized, resulting in the 'rupture' of the MOM and the release of soluble proteins from the intermembrane space into the cytosol. This process is accompanied by the loss of the mitochondrial inner *trans*-membrane potential ($\Delta\psi_{m,i}$), depletion of the cellular ATP pool and increase in the ROS levels that, together, contribute to the cell demise. The fact that MOMP represents or is close to the commitment point in the process of cell death has prompted efforts to develop agents capable of efficiently eliciting the process [6, 10, 11, 19].

It appears that the initiation of apoptotic pathways leading to mitochondria-dependent events might result from the direct actions of α-TOS and/or *via* ceramide formation, with both processes destabilizing the mitochondrial membrane. The earliest event observed in response to α-TOS is the activation of neutral sphingomyelinase (SMase), an enzyme that converts sphingomyelin to the apoptogenic ceramide. The activation of SMase and the formation of the lipid second messenger ceramide occured within 15–30 min after addition of α-TOS to Jurkat cells, and was not suppressed by a pan-caspase inhibitor, suggesting a caspase-independent process, possibly direct targeting of SMase by the vitamin E analogue [22]. Switching on SM metabolism can be caused by a change in the plasma membrane fluidity upon incorporation of the lipophilic α-TOS and might be consistent with a suggested mechanism for chemotherapy-induced cell death [75].

Generation of ROS is an early event occurring in response to vitamin E analogues. The mitochondrial respiratory chain within the MIM is a major intracellular source of ROS, which cause damage to lipids, proteins and DNA, leading to alteration or loss of cellular function and, consequently, trigger or amplify the destabilization of mitochondrial membrane. α-TOS is able to induce ROS accumulation in many different cancer cell lines, most probably resulting in the generation of superoxide anion radicals [22, 23, 66, 76, 77]. Substantial accumulation of ROS in Jurkat T lymphoma cells was observed within 1 h, most likely as a result of disrupting the electron flow within the mitochondrial complex II in the respiratory chain when the cells were challenged with α-TOS [20, 78].

3.2 Apoptotic signalling downstream of mitochondria

Although the initial apoptosis triggers have not been completely resolved, the events in apoptosis induced by vitamin E analogues downstream of mitochondria are relatively well understood.

During the apoptotic process induced by vitamin E analogues, down-stream events of mitochondrial destabilization comprise mobilization of apoptosis mediators, including cytochrome *c*, endonuclease G, the apoptosis-inducing factor (AIF), and Smac/Diablo. In turn, they set in motion a series of biochemical events that mediate the execution phase of the cell death programme resulting in the degradation of key proteins by caspases and of genomic DNA by endonucleases.

Cytochrome *c* is a key player in mitochondria-dependent apoptosis, leading to caspase activation. The soluble protein is anchored to the MIM *via* its affinity to the mitochondria-specific phospholipid cardiolipin (CL), and the binding is disrupted upon oxidation of CL by ROS derived from the oxidative phosphorylation complexes. Increasing evidence suggests that ROS play a key role in promoting cytochrome *c* release from the mitochondria upon exposure of cancer cells to α-TOS, and the protein in the cytoplasm triggers activation of the caspase cascade that ultimately leads to apoptosis [22, 23, 76, 77]. ROS induce dissociation of cytochrome *c* from CL by way of causing CL hydroperoxidation, which lowers the affinity of the phospholipid for cytochrome *c*, and the protein may then be released *via* the mitochondrial permeability transition (MPT)-dependent or MPT-independent mechanisms. ROS also promote Ca^{2+}-dependent MPT, with swelling of the mitochondrial matrix and rupture of the MOM [7, 8]. A recent report suggested MPT-independent mechanisms involving the voltage-dependent anion channel in the MOM or an oligomeric form of Bax [79].

Upon cytosolic translocation, cytochrome *c* complexes with the adaptor protein Apaf-1 and procaspase-9 form the so-called apoptosome, triggering the activation of the initiator caspase-9 with ensuing activation of the effector caspase-3, -6 or -7. At this stage, the cell enters the commitment phase, an irreversible stage of the apoptotic signalling cascade [80]. Mitochondrial permeabilization is therefore recognized as a crucial checkpoint in the programmed cell death of both normal and cancer cells. It is now clear that

this particular pathway is critically important in α-TOS-induced apoptosis in a variety of cancer cells [22, 23, 33, 81].

Smac/Diablo is an important agonist of the caspase-dependent apoptotic signalling, since it antagonizes the caspase-inhibitory members of the family of inhibitors of apoptosis proteins (IAPs) [82]. The expression of IAPs is under the control of the transcriptional factor nuclear factor-κB (NF-κB), whose activity is depressed by α-TOS [83, 84]. Thus, cytosolic relocalization of Smac/Diablo from mitochondria may promote inhibition of the survival pathways in apoptosis induced by α-TOS, which might maximize the apoptogenic potential in resistant cells [23].

Another mitochondrial apoptogenic protein is AIF. Exposure to α-TOS leads to the direct translocation of AIF from mitochondria into the nuclei, thereby bypassing the caspase cascade. Once in the nucleus, AIF triggers cleavage of chromatin in a caspase-independent manner [22]. In this way, AIF can bypass mutations in the caspase-dependent signalling or situations where IAPs are overexpressed, and may mediate α-TOS-induced apoptosis in cells resistant to conventional anticancer drugs that rely solely on caspase activation [30].

The mitochondrial pro and antiapoptotic proteins, including Bax, Bak, Bcl-2, Mcl-1 and Bcl-x_L, are important modulators related to apoptotic signalling pathway, regulating formation of a megachannel across the MOM [85]. Generation of the MPT pore and translocation of Bax from the cytosol to the mitochondria have also been suggested upon exposure of cancer cells to α-TOS. This process is likely modulated by a balance between the Bcl-2 family pro and antiapoptotic proteins [22, 86]. Overexpression of Bax results in sensitization of cells to α-TOS-induced apoptosis, and Bcl-2 plays a crucial role in stabilizing mitochondria against such damage, whereas knocking down of Bax with antisense oligodeoxynucleotide (ODN) or siRNA or overexpression of Bcl-2 or Bcl-x_L protected the cells from α-TOS-induced MPT and apoptosis [21, 22, 24, 66]. Likewise, down-regulation of Bcl-2 with antisense ODN rendered the cells more susceptible to the analogue [32]. Also, α-TOS was able to disrupt the binding of Bak BH3 peptide to Bcl-x_L and Bcl-2 in line with its potential role in antiproliferation, suggesting that the effects of α-TOS on apoptosis were partially mediated through the inhibition of Bcl-x_L function by disrupting its heterodimerization with the MOM channel-forming protein Bak [24].

Convincing evidence for mitochondria as major conductors of apoptotic signalling by vitamin E analogues follows from experiments in which the ρ^0 cybrids deficient in mtDNA were found resistant to α-TOS when compared to their wild-type and revertant counterparts [22, 23]. Cancer cells devoid of mtDNA failed to translocate cytochrome *c* in response to α-TOS challenge, unlike the apoptosis-sensitive parental and revertant cells, and also showed decreased apoptotic effects [22]. Similar resistance of ρ^0 cells has been found for other inducers of apoptosis, including TNFα [87].

Collectively, mitochondria are the critical intracellular organelles that relay the initial apoptotic signals downstream to the apoptosis commitment stage. It needs to be noted, though, that other organelles may also be involved in apoptosis induced by vitamin E analogues, such as lysosomes [33, 88].

4 Nonmitochondrial signalling involved in apoptosis induced by vitamin E analogues

In addition to the major intrinsic pathways, extrinsic, nonmitochondrial or cytoplasmic signalling pathways have also been implicated to play a role in apoptosis induced by vitamin E analogues in many types of tumour cells. The extrinsic signalling pathways comprise a number of mediators, and it has been recently established that death receptor, mitogen-activated protein kinase (MAPK), protein kinase C (PKC) and NF-κB signalling pathways are all related to α-TOS-triggered apoptosis [29, 34, 48, 84].

4.1 Activation of death receptor (DR) by vitamin E analogues

Activation of the extrinsic cell death pathway is initiated by ligation of DRs, which include Fas, the TNF receptor, and the TRAIL receptor 1 (DR4) and TRAIL receptor 2 (DR5). DRs are constitutively expressed on the surface of mammalian cells, and both the Fas and TRAIL systems are effective for cancer immune surveillance. Impaired apoptotic signalling pathways endow some types of malignant cells with resistance to DR-mediated apoptosis, and such tumours are difficult to treat [89]. It has been reported that α-TOS-mediated apoptosis involves DR signalling. For example, the Fas-resistant breast cancer cells were sensitized by α-TOS *via* mobilization of the cytosolic Fas protein to the cell surface [40, 90]. In a separate study, expression of Fas, the Fas-associated death domain (FADD), and caspase-8 was enhanced after α-TOS treatment in gastric cancer cells, whereas Fas antisense ODN inhibited expression of FADD and caspase-8 activity [91].

TRAIL has attracted attention as a selective immunological apoptogen with anticancer activity. Tumour cells escape from TRAIL-modulated killing when the balance between DRs and the nonapoptogenic decoy receptors is altered, and expression of the latter predominates. Combination of TRAIL with chemotherapeutics or radiation resulted in a synergistic apoptotic response proceeding *via* caspase-activating signals. α-TOS showed a synergistic proapoptotic activity with TRAIL both *in vitro* and in experimental colon cancer [54]. α-TOS also sensitized to TRAIL the resistant malignant mesothelioma (MM) and osteosarcoma (OS) cells. Combination of α-TOS and TRAIL resulted in

enhanced apoptosis in a caspase- and p53-dependent manner [22, 92], and α-TOS elevated expression of DR4 and DR5 without modulation of expression of the decoy receptors in MM cells [56, 92]. α-TOS also enhanced the sensitivity of Jurkat T lymphoma cells to apoptosis induced by TRAIL by suppression of NF-κB activation [84]. Thus, VE analogues may play a role in adjuvant therapy of DR-resistant cancers. These analogues can also be used alone, because they are expected to sensitize cancer cells to endogenous immunological inducers of apoptosis by cells of the immune system, thereby potentiating the natural tumour surveillance.

4.2 Involvement of MAPK pathway in vitamin E analogues-modulated apoptosis

The importance of MAPKs in the control of cellular responses to the environment and in the regulation of gene expression, cell growth, and apoptosis has made them a priority for research related to many disorders [93]. The c-Jun N-terminal kinase (JNK) was originally identified as the major kinase responsible for the phosphorylation of c-jun, leading to increased activity of the AP-1 transcription factor. JNK-regulated transcription factors contribute to the modulation of gene expression in response to multiple cellular stimuli, including stress events, growth factors, and cytokines [94]. Kline's group first reported a role of JNK and c-jun in α-TOS-induced apoptosis. The vitamin E analogue up-regulated c-jun expression in different types of cancer cells [95–97]. α-TOS-triggered apoptosis induced a prolonged increase in c-jun expression, and AP-1 transactivation, and transfection of dominant-negative c-jun reduced α-TOS-mediated apoptosis. It was subsequently demonstrated that α-TOS enhanced ERK1/2 and JNK activity but not the p38 kinase activity [34, 48].

Three upstream components of the JNK cascade, apoptosis signal-regulating kinase 1, growth arrest DNA damage-inducible 45β and stress-activated protein kinase/ERK kinase-1 were all induced, and the protein expression of phospho-JNK was also noticeably increased by α-TOS in prostate cancer cells [81]. In addition, JNK and c-jun were important in α-TOS-induced apoptosis in SGC-7901 gastric cancer cells. Dominant-negative JNK significantly reduced c-jun expression and apoptosis triggered by α-TOS [39, 98]. On the other hand, α-TOS stimulated early activation of ERK1/2 and then reduced the ERK activity concomitant with the activation of PKC in HL60 cells. Blockage of ERK activity, however, showed no significant effects on α-TOS-triggered apoptosis [41]. Conversely, it was reported that α-TOS and α-TOB inhibited ERK phosphorylation and activated p38 in breast cancer cells [71]. The discrepancy in the role of ERK activity may result from differences in treatment time in that ERK can be rapidly and transiently induced by α-TOS, but longer exposures may lead to suppression of ERK activation. There is overwhelming evidence that the JNK cascade is an important modulator of apoptosis induced by α-TOS. However, it is not clear at this stage how this signalling pathway is linked to destabilization of mitochondria by the VE analogue.

4.3 The role of PKC in α-TOS-triggered apoptosis

PKC, a multigene family of phospholipid-dependent serine/threonine protein kinases, is involved in modulation of divergent biological functions. PKC is normally present in an inactive form. Binding of cofactors to the regulatory domain induces conformational changes that result in activation of the enzyme, which is usually associated with membrane translocation [99]. Treatment of Jurkat cells with α-TOS caused a decrease in PKC activity by activation of protein phosphatase 2A, leading to hypophosphorylation of PKCα and decreased phosphorylation of Bcl-2 on Ser70. Phorbol-12-myristate-13-acetate, a PKC activator, efficiently protected the cells from apoptosis induced by α-TOS, indicating an inhibitory role of PKC in the regulation of apoptosis [29].

PKC isozymes can also be activated by proteolytic separation of the regulatory and the catalytic domain. Several members of the PKC family have now been identified as substrates for caspases. During apoptosis, activation of caspases results in the cleavage of PKC isozymes, followed by PKC activation [100]. It was shown that α-TOS induced apoptosis *via* activation of PKCβII and promoted PKCα membrane translocation, concomitant with a decline in the ERK activity [41]. The differences in the effects of α-TOS on PKC in relation to apoptosis might be due to the presence of specific PKC isozymes in cells of different origin, resulting in different or even opposing effects on the outcome of apoptosis.

4.4 Role of NF-κB in apoptosis induced by vitamin E analogues

Activation of the multicomplex transcription factor NF-κB is crucial for a wide variety of cellular responses. In nonstimulated cells, NF-κB is sequestered in the cytoplasm by the inhibitory κB (IκB). Upon activation by a number of stimuli, the IκB protein is rapidly degraded, allowing translocation of NF-κB into the nucleus and binding to cognate-response elements. In addition to its fundamental role in regulation of the immune and inflammatory responses, NF-κB also exerts antiapoptotic activities. Thus, NF-κB activation stimulated by TNFα was inhibited by α-TOS in Jurkat and proliferating endothelial cells [32, 83, 101], possibly sensitizing them to apoptosis induction.

Because activation of NF-κB is negatively associated with apoptosis induced by TRAIL in multiple cancer cells, agents that inhibit NF-κB activation may convert TRAIL-resistant to -sensitive cells. TRAIL may transiently activate NF-κB, thereby delaying the onset of apoptosis. α-TOS has

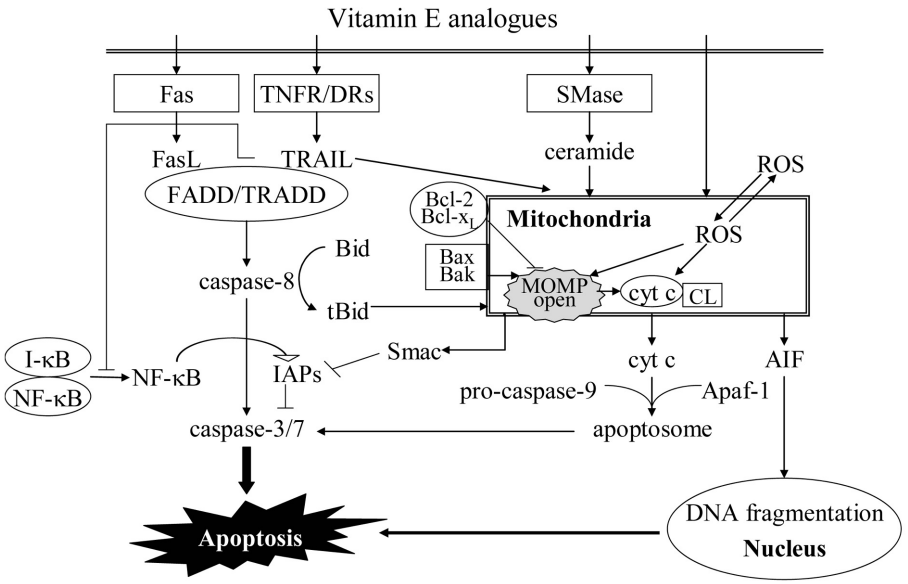

Figure 2. Vitamin E analogues-mediated signaling pathways.

the capacity to overcome such resistance by suppressing TRAIL-stimulated NF-κB activation by modulating the degradation of IκB, sensitizing cells to TRAIL [84].

Although there are a number of signalling pathways involved in apoptosis induced by vitamin E analogues, mitochondria are the major target. The various pathways are probably triggered *via* the initial effect of vitamin E analogues on mitochondria and may contribute to the main, intrinsic apoptogenic pathway, thereby maximizing the outcome, which is efficient elimination of a malignant cell. Figure 2 shows some of the possible signalling pathways mediated by vitamin E analogues.

Current knowledge of complex signalling pathways that control apoptosis in the different tumour types will be helpful for further understanding the molecular mechanism by which compounds like α-TOS exert their anticancer activity, and will promote preclinical or clinical trials using different types of tumours, some being resistant to therapy due to known mutations in genes necessary for eliciting efficient killing of the malignant cells.

5 Conclusion

The above evidence suggests that vitamin E analogues, epitomized by α-TOS, as a novel group of mitocans, are efficient anticancer agents with great promise for future clinical applications, and supports the intriguing idea of mitochondria as a potent target for cancer therapy.

The progress concerning our understanding of the central place of mitochondria as a regulator of cell death and of its role in the carcinogenesis has launched the search for new opportunities for therapeutic intervention. The compounds capable of inducing MOMP will withstand clinical evaluation in the future. Mitochondria will, if any, be expected to be used as biomarkers for early detection of cancer, or as unique cellular targets for novel and selective anticancer agents. Therefore, it can be envisaged that mitocans, anticancer agents that act by destabilizing mitochondria, will become drugs of choice in our quest against, in some cases thus far untreatable, neoplastic pathologies.

The authors have declared no conflict of interest.

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