Chapter 7
Vitamin E Analogues as Prototypic Mitochondria-Targeting Anti-cancer Agents

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Contents

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Abstract Mitochondria have emerged recently as a novel, intriguing target for anti-caner drugs, owing largely to their importance for proper growth of cancer cells. Destabilization of mitochondria often results in the induction of apoptosis in cancer cells that, consequently, may translate into suppression of tumour growth. A class of mitochondria-targeting compounds, mitocans, comprises several groups of agents with different targets within the mitochondrion. Of these, vitamin E analogues have been recently promoted as agents that disrupt mitochondria by several modes of action. These compounds hold substantial promise as potential anti-cancer drugs of clinical relevance.

Keywords Vitamin E analogues • Cancer • Mitochondria • Redox-silent • Functional domain • Anti-cancer effect • Apoptosis • Signaling pathways • α-tocopherol succinate • α-tocopheryloxyacetic acid • α-tocopheryl maleamide • Mitochondrially targeted vitamin E succinate • Complex II

1 Introduction

In spite of the recent unprecedented advancement in molecular medicine, there has been only limited progress in the search for efficient, selective anti-cancer drugs (Siegel et al. 2013). Although the hallmarks of cancer have been defined and re-defined (Hanahan and Weinberg 2000, 2011), recent findings indicate that cancer is more complex than thought, and, accordingly, the successful treatment may be harder to find than anticipated. This grim notion stems from the findings of high level of genetic and mutational differences amongst patients with the same or very similar type of tumour (Jones et al. 2008; Parsons et al. 2008), as well as within one tumour and the derived metastases in the same patient (Gerlinger et al. 2012). This heterogeneity considerably complicates the treatment (Hayden 2008), indicating that current therapies may be efficient only for some patients and, even worse, may kill only a part of the tumour cells.

Therefore, it is imperative to search for an invariant target that could be utilised across the landscape of different cancers and also could be used to selectively kill cancer cells with low or no effect on normal cells and tissues. Such a plausible target is presented by mitochondria, essential organelles that are the major source of energy as well as purveyors of cell death. Recently, there has been a focus on mitochondria as potential targets for cancer therapy, with numerous original papers and several excellent reviews (see e.g., Wallace 2012; Galluzzi et al. 2010; Fulda et al. 2010; Ralph et al. 2010a, b; Gogvadze et al. 2008). We have been coining the term 'mitocans' (standing for 'mitochondria' and 'cancer'), encompassing a class of compounds that exert anti-cancer activity by way of targeting and destabilising mitochondria, causing apoptosis, often selectively in cancer cells (Ralph et al. 2006; Neuzil et al. 2006, 2013). Of these agents, we and others have focused on intriguing types of mitocans, vitamin E (VE) analogues, that act via mitochondria, causing apoptosis selectively in cancer cells, and featuring anti-tumour activity (*e.g.* Neuzil

et al. 2007; Zhao et al. 2009; Angulo-Molina et al. 2014). In this review, we focus on VE analogues, discussing their structural features, molecular mode of activity as well as their promise as efficient anti-cancer agents.

2 Structure of Vitamin E and Its Analogues

VE is a generic term that refers to a family of eight different naturally occurring phenolic compounds (Sen et al. 2000; Elnagar et al. 2010). These agents belong to two categories, tocopherols and tocotrienols. Both groups share the aromatic chromanol group with two rings (one phenolic and one heterocyclic) and an isoprenoid chain (a 16-carbon tail) attached at the C2 position. Tocopherols differ from tocotrienols only in the aliphatic tail (Kamal-Eldin and Appelqvist 1996). Tocopherols refer to VE compounds with a saturated phytyl side chain, tocotrienols refer to compounds containing poly-unsaturated side chain. Each group includes four isoforms, named α-, β-, γ-, and δ-tocopherols or tocotrienols, which differ by the number and position of methyl groups on the chromanol ring (see the structures in Fig. 7.1) (Aggarwal et al. 2010; Elson and Yu 1994). The role of these molecules as lipophilic antioxidants *in vitro* and *in vivo* is widely accepted (Neuzil et al. 2007). It has been reported that both tocopherol and tocotrienol isoforms display a wide-range of antioxidant-related activities (McIntyre et al. 2000), with the level of phytyl chain saturation and/or chromanol ring methylation critical for the differential bioactivity of the individual isoforms (Elson and Yu 1994; McIntyre et al. 2000). In addition, non-antioxidant properties of VE family members have also been investigated (Akazawa et al. 2002).

While α-tocopherol (α-TOH) was the first VE analogue to be recognized, and was named in 1924 and synthesized in 1938 (Sen et al. 2007), the term tocotrienol was first suggested by Bunyan and colleagues (Bunyan et al. 1961), and tocotrienols were first isolated from the latex of the rubber plant in 1964 (Whittle et al. 1996). Furthermore, among the VE isomers, α-tocopherol is the most common form found in human diets; it also has the highest bioavailability. So far, α-tocopherol is the best characterized VE isomer and has been examined in a large number of studies (Ling et al. 2012). There is evidence suggesting that human tissues can convert tocotrienols to tocopherols (Qureshi et al. 2001, 2002). The differences in the aliphatic side chain may account for the differences in the efficacy and potency both *in vitro* and *in vivo*. To date, over 30,000 papers have been published on tocopherols, and about 600 on tocotrienols (Aggarwal et al. 2010). The most studied functions of these two groups of compounds are related to their antioxidant (Lee et al. 2009; Cai et al. 2012), anti-proliferative and anti-survival (Shun et al. 2004; Mudit et al. 2010), anti-inflammatory (Bachawal et al. 2010; Ivanova et al. 2011), anti-angiogenic (Inokuchi et al. 2003; Weng-Yew et al. 2009), pro-apoptotic and anti-cancer (Wu and Ng 2010; Parker et al. 1993), cardio-protective (Koba et al. 1992) and neuro-protective activity (Aggarwal et al. 2010; Sen et al. 2000).

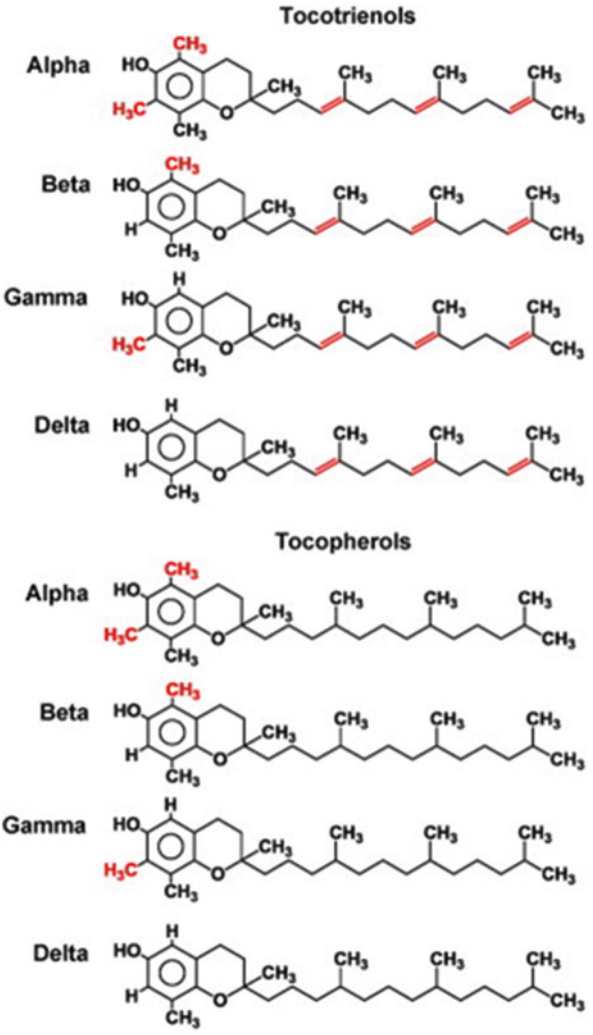

Fig. 7.1 Chemical structures of naturally occurring forms of vitamin E, tocotrienols and tocopherols

While the naturally occurring VE compounds have been a focus of numerous studies for many years since they were first recognized in 1924, semisynthetic VE analogues, a group of compounds which are derived from VE and share the same core structure with VE, have recently emerged as very important compounds with even higher biological activity, especially the anti-cancer effect. This group of compounds have been represented by agents like α-tocopheryl succinate (α-TOS), α-tocopheryl ether-linked acetic acid (α-TEA), or mitochondrially targeted vitamin E succinate (MitoVES). Their anti-cancer activity and the molecular mechanism of their biological action have been studied profoundly in recent years and will be highlighted in this paper.

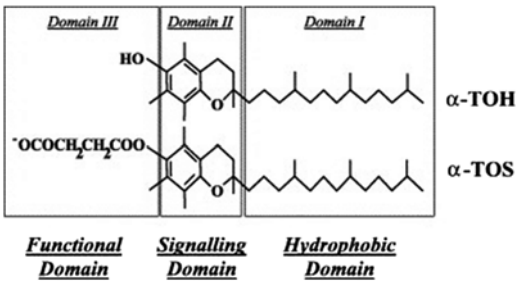

Fig. 7.2 Structures and major domains of α-TOH and α-TOS

2.1 Functional Domains of Vitamin E Analogues

VE analogues, including α-tocopheryl succinate, α-tocopherol ether-linked acetic acid, α-tocopheryl polyethylene glycol 1000 succinate, tocopheryl nicotinate, α-tocopheryl ferulate, tretinoin tocoferil or α-tocopheryl phosphate have been synthesized and their cellular effects investigated. They are examples of VE analogues modified at the tocopheryl-C6 position that do not have antioxidant activity in their intact form, unless they are hydrolysed, suggesting that individual 'regions' of the molecule of VE and its analogues can be divided into functionally different groups. Thus, the VE molecule comprises three different domains (Fig. 7.2): the hydrophobic domain (I), the signalling domain (II) and the functional domain (III) (Neuzil et al. 2007).

(i) The functional domain is the group attached at C6 of the chromanol ring. This position determines whether the molecule behaves as a redox-active or a redox-silent compound. The well documented antioxidant properties of the four tocopherol isomers and their bioavailability (in particular that of α-tocopherol) led to their testing in cancer clinical trials. In the two of the largest chemoprevention trials, VE (α-tocopherol) consumption was found to have no beneficial effect on cancer incidence, indicating little if any positive outcome concerning the use of free tocopherols in cancer prevention (Pham and Plakogiannis 2005). However, certain chemical modifications at C6, such as those resulting in the generation of ether, ester and amide analogues (with a free carboxylate) proved to endow the parental agents with pro-apoptotic and anti-neoplastic activity, which resulted in a concerted research on the anticancer efficacy of these agents (Neuzil et al. 2007).

(ii) The signalling domain is responsible for activities of the agents that are independent of the antioxidant nature of tocopherols and are given by the methylation pattern of the aromatic ring. For example, α-tocopherol has been reported to inhibit protein kinase C (PKC) by decreasing diacylglycerol levels, whereas other tocopherols with similar antioxidant efficacies do not inhibit the kinase activity. Thus, the PKC inhibitory activity of α-tocopherol is independent of its antioxidant capacity (Kunisaki et al. 1995; Tasinato et al. 1995).

(iii) The lipophilic side chain of VE compounds distinguishes between tocopherols with saturated isoprenyl units and tocotrienols with unsaturated isoprenyl units. The hydrophobic domain determines whether the molecule can bind to lipoproteins and membranes or will be degraded by phase I enzymes (Birringer et al. 2002; Neuzil et al. 2007). It has been reported that tocotrienols can be degraded to a larger extent than their counterparts with saturated side chains, and there are quantitative differences in the metabolism between individual tocopherols as well as between tocotrienols and tocopherols (Kogure et al. 2005).

2.2 *Redox-Silent Analogues of Vitamin E*

VE analogues with a modified hydroxyl group of α-TOH have been tested for their pro-apoptotic activity. The most prominent derivative of this group (and certainly the most studied one) is α-TOS (Fig. 7.2), bearing a succinyl group at position C6 of the chromanol ring. Because of its low pKa (<6), α-TOS is fully deprotonated under physiological conditions, leading to a detergent-like molecule, which destabilizes mitochondrial membranes (Neuzil et al. 2007). Dicarboxylic esters of tocopherols present the best studied compounds for structure-activity relationship (SAR). Strong apoptogens include α-TOS, α-tocopheryl oxalate and α-tocopheryl malonate, the latter two inducing non-selective cytotoxicity in mice inoculated with B16-F1 melanoma cells (Kogure et al. 2005). Even greater apoptogenic activity has been observed for unsaturated dicarboxylic acids like α-tocopheryl maleate (Birringer et al. 2003). Increasing the chain length of the dicarboxylic acid led to decreased activity as shown for α-tocopheryl glutarate and α-tocopheryl methyl glutarate (Birringer et al. 2003), or α-tocopheryl pimelate (Kogure et al. 2004) exhibiting no activity at all. The substitution pattern of the chromanol ring is often not merely related to the antioxidant properties of tocopherols (Azzi et al. 2002). An important feature of compounds with α-tocopheryl structure is their selective recognition by the hepatic α-tocopherol transfer protein (TTP). The relative affinities of the homologues of VE for TPP decrease with the loss of methylation of the chromanol ring (Hosomi et al. 1997).

2.3 *Other Vitamin E Analogues with Anti-proliferative Activity*

A number of compounds with modifications in the functional domain exhibit anti-proliferative activity and exert additional properties. For example, α-tocopheryl polyethylene glycol succinate (α-TPGS) has been used as a vehicle for drug delivery systems. As one of the components of the nano-emulsion vehicles (NEs), it showed significant pro-apoptotic effect for both murine breast and colon carcinoma cell lines, indicating built-in anti-cancer properties for such NE platforms, potentially enhancing overall anti-neoplastic effects of incorporated chemotherapeutic agents (Jordan et al. 2012). α-TPGS also showed enhanced chemosensitization and

anti-tumour efficacy against non-small cell lung cancer cell lines (Gill et al. 2012). In addition, this compound has been shown to possess anti-cancer activity against human lung carcinoma cells implanted in nude mice. The apoptosis-inducing efficacy of the compound was not due to its increased uptake by cells but rather due to increased ability to generate ROS (Youk et al. 2005).

Another example is α-tocopheryl phosphate (α-TOP), a water-soluble molecule, which has been found in animal and plant tissues (Gianello et al. 2005). It has been shown to exert more potent cellular effects than α-tocopherol itself in terms of inhibition of cell proliferation and regulation of gene expression. It may directly modulate intermediate steps of signal transduction or gene regulation (Negis et al. 2009). At a higher concentration, α-TOP induces apoptosis, as documented, for example, by its high anti-proliferative and apoptogenic effect on MG-63 cancer cells (Rezk et al. 2007). The reason for α-TOP being more efficient than α-tocopherol may be due to its better penetration across the plasma membrane (Gianello et al. 2005), and it may also be possible that the phosphorylated forms of tocopherol can be transported to the sites of action more efficiently than the non-phosphorylated ones. It is believed that α-TOP can act as a signalling molecule, modulating cell functions such that it may be a source of α-TOH that can undergo enzymatic dephosphorylation and phosphorylation reactions, modulating cell signalling pathways (Negis et al. 2005). Mixtures of α-TOP and di-α-TOP were shown to inhibit proliferation of rat aortic smooth muscle cells as well as THP-1 monocytic leukemia cells (Munteanu et al. 2004). It was proposed that α-TOS and α-TOM act in cancer cells by mimicking and substituting for α-TOP, causing permanent activation of cellular signalling (Neuzil et al. 2007).

Furthermore, two experimental α-tocopheryl esters, the all-trans retinoic acid α-tocopoheryl ester and 9-cis retinoic acid α-tocopoheryl ester, have been documented to reduce proliferation of acute promyelocytic leukemia (APL) cells. The 9-cisretinoic acid α-tocopheryl ester also inhibited proliferation of APL-derived NB4 and HT93 cells and induced their differentiation, as documented by biomarkers, such as granulocytic maturation, nitroblue tetrazolium reduction and enhanced CD11b expression (Makishima et al. 1998). Transactivation experiments with a retinoid receptor-responsive reporter construct revealed that both compounds acted as agonists of retinoic acid receptors. γ-Carboxyethyl hydroxychroman, a degradation product of γ-tocopherol often found secreted in the urine, reduces proliferation of PC-3 prostate cancer cells by inhibiting cyclin D1 expression (Galli et al. 2004).

2.4 Tocotrienol and Its Analogues

Tocotrienols are forms of VE with potent anti-proliferative activity against different types of cancer cells with little or no effect on normal cell growth or functions. Previous studies suggested that tocotrienol treatment is associated with significantly lower tumour incidence, lower tumour numbers and longer tumour latency in mammary carcinoma models (Whittle et al. 1996). However, physico-chemical and pharmacokinetic properties greatly limit their use as therapeutic agents. Chemical

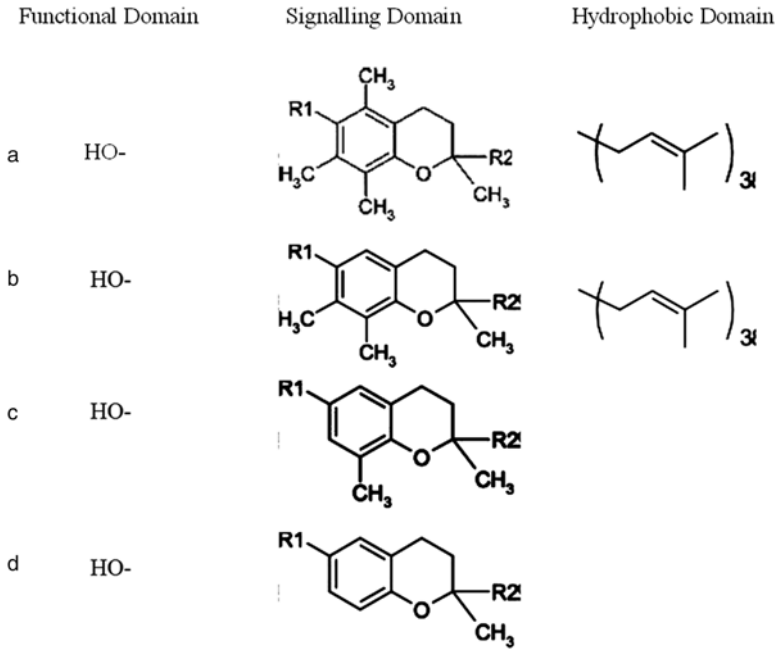

Fig. 7.3 Modification of the hydrophobic domain of tocotrienols

instability, poor water solubility, and rapid metabolism of tocotrienols are examples of obstacles that hinder the therapeutic use of these natural products and their semi-synthetic analogues (Behery et al. 2010). Tocotrienols exhibit their pro-apoptotic activity without modifications of the functional domain. The hierarchy in the signalling domain is reversed, making δ-tocotrienol (Fig. 7.3c) the most potent agent, followed by γ-tocotrienol (Fig. 7.3b) and α-tocotrienol (Fig. 7.3a) (He et al. 1997). The pro-apoptotic property of tocotrienols may be related to the inactivation of the Ras family of proteins. Recent studies indicate that both γ- and δ-tocotrienols have potent anti-proliferative and pro-apoptotic activity in several different types of pancreatic cancer cells, Panc-28, MIA PaCa-2, Panc-1 and BxPC-3. The mechanisms of the anti-cancer effects of the two tocotrienols mainly involve the Akt and the Ras/Raf/MEK/ERK signalling pathways (Shin-Kang et al. 2011). A direct inhibitory action of tocotrienols has been proposed, since the membrane-anchoring cysteine residue of Ras proteins is modified by a common structural element, a farnesyl chain. Accordingly, Ras farnesylation and RhoA prenylation were inhibited by tocotrienols in A549 cells with an activating Ras mutation (Yano et al. 2005).

It was reported that γ-tocotrienol displays potent anti-cancer activity associated with suppression of HER/ErbB receptor signalling (Ayoub et al. 2011), and a recent study showed that γ-tocotrienol enhances chemo-sensitivity of human oral cancer cells to docetaxel through the down-regulation of nuclear factor-κB (NF-κB)-regulated anti-apoptotic gene products (Kani et al. 2013). Interestingly, the number and position of methyl substituents in tocotrienols affect their hypocholesterolemic, antioxidant, and anti-tumour properties. The desmethyl tocotrienol (Fig. 7.3d),

lacking all aromatic methyl groups, shows even higher activity with the IC_{50} value of 0.9 μM. This compound has been isolated from rice bran, giving it the status of a native product (Qureshi et al. 2000).

3 Molecular Mechanism of Anti-cancer Function of Vitamin E Analogues

Advanced sequencing and bioinformatics lead to the discovery that tumours are heterogeneous. This is demonstrated by recent reports revealing the extraordinary variability of mutations in the same type of tumour from different patients (Jones et al. 2008; Parsons et al. 2008; Hayden 2008). This grim notion has been further accentuated by the finding that there are differences in the mutational signatures of different regions even in a single tumour and its metastases (Gerlinger et al. 2012). These findings underscore the low possibility of the discovery of an efficient cancer cure that would target only a single gene or a single signalling pathway. Currently, the majority of established anti-cancer drugs are either non-selective or gradually lose their efficacy because of the constant mutational changes in malignant cells. This is also likely to be the major reason why cancer incidence is either stagnating or on the rise (Siegel et al. 2012). Therefore, pursuing cancer control strategies is one of the major tasks for scientists, and discovery and development of novel selective and efficient anticancer agents for treating neoplastic diseases is of paramount importance.

VE analogues have been studied extensively for their anti-cancer potential and have been shown to possess strong pro-apoptotic and anti-cancer efficacy in different types of cancer. They are selective for malignant cells, cause destabilization of cancer cell mitochondria, and suppress cancer in pre-clinical models. The feature of selectivity for malignant cells, different molecular mechanisms of anti-cancer activity as well as their unique targets via which they exert anti-cancer activity endow VE analogues agents with a great promise to be developed into novel anti-cancer drugs to benefit cancer patients. Anti-cancer molecular mechanisms of VE analogues are summarized below.

3.1 Mitochondrial Apoptotic Pathways Induced by Vitamin E Analogues

Mitochondria are organelles important for both life and death of a cell. They are membrane-enclosed structures distributed in the cytosol of most eukaryotic cells, with the mitochondrial outer membrane (MOM) defining the entire organelle and the mitochondrial inner membrane (MIM) containing the fluid-filled matrix. Mitochondria are the major source of cellular energy with a crucial role in cellular bioenergetics and are vital for signalling of mammalian cells. However they are also central actors in cell death, critically contributing to the process of apoptosis induction and progression (Ralph et al. 2006; Neuzil et al. 2006). Furthermore,

mitochondria are also a major source of intracellular reactive oxygen species (ROS); as such, they are themselves potentially vulnerable to oxidative stress. When ROS production exceeds the capacity for detoxification and repair, oxidative damage to proteins, DNA, and phospholipids can occur, disrupting mitochondrial OXPHOS and eventually leading to the impairment of cell function and death. Accordingly, mitochondrial ROS production and oxidative damage are attractive targets for pharmacological intervention.

3.1.1 Initiation of Mitochondrial Membrane Permeabilization

Apoptosis, an organized sequence of events controlled by a network of genes, is an essential process during development and plays a key role in a variety of pathologies. There are many triggers of apoptosis, including increased levels of oxidants within the cell, damage to DNA by these oxidants or other agents, accumulation of proteins that fail to fold properly, or signalling by molecules binding to death receptors. Apoptosis induction is also a major process by which VE analogues kill cancer cells.

Various apoptotic stimuli may trigger the formation of the MOM pore (MOMP). This is a complex process that involves numerous molecular players and also serves as a target for anti-cancer drugs. During this event, both MOM and MIM are permeabilized, resulting in the "rupture" of the MOM and the release of soluble proteins from the inter-membrane 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, which, together, contribute to the cell demise. The fact that MOMP represents or is close to the commitment point in the process of cell death cascade has prompted efforts to develop agents capable of efficiently eliciting these events (Neuzil et al. 2007; Alirol and Martinou 2006).

ROS generation is important in apoptosis induction involving mitochondria. The generation of ROS is an early event occurring in response to VE analogues. 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 the MOM. α-TOS is able to induce ROS accumulation in many different cancer cell lines, most probably originally in the form of superoxide anion radicals (Weber et al. 2003). 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 (CII) in the respiratory chain when the cells were challenged with α-TOS (Weber et al. 2003; Dong et al. 2008).

3.1.2 Apoptotic Signalling Downstream of Mitochondria

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

analogues, down-stream events of mitochondrial destabilization comprise mobilization of apoptosis mediators, including cytochrome c, 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 both 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 OXPHOS 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 (Kogure et al. 2002). 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 (Kakkar and Singh 2007), and MTP-independent mechanisms involving the voltage-dependent anion channel in the MOM or an oligomeric form of Bax (Petrosillo et al. 2003).

The mitochondrial pro- and anti-apoptotic proteins, including Bax, Bak, Bcl-2, Mcl-1 and Bcl-x_L, are important modulators related to apoptotic signalling pathway, regulating the formation of a mega-channel across the MOM (Cory et al. 2003). Generation of the MPT pore and translocation of Bax from the cytosol to the mitochondria have also been suggested as an important event upon exposure of cancer cells to α-TOS. This process is likely modulated by a balance between the Bcl-2 family of pro- and anti-apoptotic proteins (Yamamoto et al. 2000; Yu et al. 2003). Over-expression of Bax results in sensitization of cells to α-TOS-induced apoptosis, whereas over-expression of Bcl-2 or Bcl-x_L protects them from α-TOS. Likewise, down-regulation of Bcl-2 by antisense oligodeoxynucleotide treatment sensitized cells to the VE analogue (Weber et al. 2003).

Probably the most compelling evidence for mitochondria as major transmitters of apoptotic signalling induced by VE analogues stems from experiments in which ρ^0 cells were found to be resistant to α-TOS (Weber et al. 2003; Wang et al. 2005). It was found that cancer cells lacking mitochondrial DNA failed to translocate cytochrome c when challenged with α-TOS, unlike the apoptosis-sensitive parental cells, and also showed low levels of phosphatidyl serine externalization and caspase-3 activation. Similar resistance of ρ^0 cells has been found for other inducers of apoptosis, including tumour necrosis factor-α (TNF-α) (Higuchi et al. 1997).

Collectively, mitochondria are the critical intracellular organelles that relay the initial apoptotic signals downstream to the apoptosis commitment stage. It needs to be noticed, though, that other organelles may also be involved in apoptosis induced by VE analogues, such as lysosomes (Neuzil et al. 2002).

3.2 *Mitochondria-Independent Apoptosis Induction by Vitamin E Analogues*

Although mitochondria are central to apoptosis induction by VE analogues via the intrinsic signalling pathways, a number of extrinsic non-mitochondrial or cytoplasmic signalling pathways have also been implicated to play a role in apoptosis induction by VE 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 (Yu et al. 2001).

3.2.1 Activation of Death Receptors by Vitamin E Analogues

Activation of the extrinsic cell death pathway is initiated by ligation of cell surface death receptors (DRs) including Fas, the TNF receptor, and the TNF-related apoptosis-inducing ligand (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 against carcinogenesis in pre-clinical models. Impaired apoptotic signalling pathways endow some types of malignant cells with resistance to DR-mediated apoptosis, and such tumours are rather difficult to treat (O'Connell et al. 2000; Cretney et al. 2002). It has been reported that α-TOS-mediated apoptosis involves DR signalling. For example, Fas-resistant breast cancer cells were sensitized by α-TOS via mobilization of the cytosolic Fas protein to the cell surface (Turley et al. 1997). In a separate study, expression of Fas, Fas-associated death domain and caspase-8 was enhanced after α-TOS treatment in gastric cancer cells, whereas Fas antisense oligonucleotides inhibited the expression of the Fas-associated death domain protein and decreased caspase-8 activity (Wu et al. 2002).

TRAIL has attracted attention as a selective immunological apoptogen with anticancer activity. Tumour cells escape from TRAIL-induced death signalling when the balance between DRs and the 'anti-apoptogenic' decoy receptors is altered, and the latter are expressed at a higher level. It was found that the combination of TRAIL with chemotherapeutics or radiation resulted in a synergistic apoptotic response proceeding via caspase-activating signals. α-TOS and TRAIL showed a synergistic pro-apoptotic activity both *in vitro* and *in vivo* for experimental colon cancer (Weber et al. 2002). α-TOS also sensitized the resistant MM and osteosarcoma cells to TRAIL. The IC_{50} value of TRAIL treatment was greatly decreased when treating MM cells with sub-lethal doses of α-TOS, whereas an antagonistic effect of α-TOS on TRAIL sensitivity was found in the case of non-malignant mesothelial cells (Tomasetti et al. 2004). Combination of α-TOS and TRAIL resulted in enhanced apoptosis in a caspase- and p53-dependent manner (Weber et al. 2003; Tomasetti et al. 2006), and α-TOS elevated the expression of DR4 and DR5 without modulating the expression of the decoy receptors in MM cells (Tomasetti et al. 2004, 2006).

α-TOS also enhanced the sensitivity of Jurkat T lymphoma cells to TRAIL-induced apoptosis by suppression of NFκB activation (Dalen and Neuzil 2003). 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.

3.2.2 MAP Kinase Pathway and Apoptosis Induction by Vitamin E Analogues

The mitogen-activated protein kinase (MAPK) pathway is one of the most frequently studied pathways in cancer. It plays an important role in the regulation of many processes that are critical to the malignant phenotype, and a variety of genetic alterations that activate the pathway have been detected in different types of cancer. As a result, the search for compounds that inhibit the pathway has been an active area of investigation for many years (Davies and Kopetz 2013). The importance of the MAPK pathway is in the control of cellular responses to the environment and in the regulation of gene expression, cell growth, and apoptosis, making it a priority for research related to many disorders (Fang and Richardson 2005). The c-Jun N-terminal kinases (JNKs), along with Erk and p38, constitute the principle members of the MAPK family (Johnson and Lapadat 2002; Wagner and Nebreda 2009). JNKs function primarily through the activator protein-1 (AP-1) family of transcription factors to regulate a plethora of cellular processes, including cell proliferation, differentiation, survival and migration (Zhang and Selim 2012). There is also a cross-talks and integration with other signalling pathways in a cell context-specific and cell type-specific manner (Wagner and Nebreda 2009). JNKs were originally identified as the major kinase responsible for the phosphorylation of c-jun, leading to increased transcriptional activity of AP-1, again largely in response to multiple cellular stimuli, including stress events, growth factors, and cytokines (Nishina et al. 2004).

Kline's group was the first to report a role of JNK and c-jun in α-TOS-induced apoptosis. The VE analogue up-regulated c-jun expression in different types of cancer cells (Qian et al. 1996; Yu et al. 1997). α-TOS-triggered apoptosis involves a prolonged increase in c-jun expression, and AP-1 trans-activation 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 (Yu et al. 2001). Increased phosphorylation and trans-activation of c-jun and ATF-2 were also observed in cells exposed to α-TOS.

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 level of phospho-JNK was also noticeably increased in prostate cancer cells in response to α-TOS (Zu et al. 2005). Dominant-negative JNK significantly reduced c-jun expression and apoptosis triggered by the agent. On the other hand, α-TOS stimulated early activation of ERK1/2

and, in turn, reduced the ERK activity concomitant with the activation of PKC in HL60 cells. However, blockage of ERK activity showed no significant effects on α-TOS-triggered apoptosis (Bang et al. 2001). Conversely, it was reported that α-TOS and α-tocopheryloxybutyric acid inhibited ERK phosphorylation and activated p38 in breast cancer cells (Akazawa et al. 2002). The discrepancy in the role of ERK activity may result from differences in the treatment time in that ERK can be rapidly and transiently induced by α-TOS, but longer exposures may lead to the suppression of ERK activation. Thus, there is strong 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 the destabilization of mitochondria by the VE analogue.

3.3 Role of Protein Kinase C in Apoptosis Induced by Vitamin E Analogues

Protein kinase C (PKC) has been a tantalizing target for drug discovery ever since it was first identified as the receptor for the tumour promoter phorbol ester in 1982 (Mochly-Rosen et al. 2012). It forms a multi-gene family of phospholipid-dependent serine/threonine protein kinases involved in modulation of divergent biological functions (Spitaler and Cantrell 2004). Much of the interest in PKC began with the discovery that members of this family of isozymes are activated in various diseases (Mochly-Rosen et al. 2012), including diabetes (Geraldes and King 2010), cancer (Totoń et al. 2011), ischaemic heart disease (Inagaki et al. 2006) and heart failure (Ferreira et al. 2011). PKC is normally present in an inactive form. Binding of cofactors to the regulatory domain induces conformational changes that result in the activation of the enzyme, which is usually associated with its membrane translocation (Basu 2003). Treatment of Jurkat cells with α-TOS caused a decrease in PKC activity by activation of PP2A, leading to hypophosphorylation of PKCα and decreased phosphorylation of Bcl-2 on Ser70 (Ruvolo et al. 1998; Neuzil et al. 2001c). 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 (Neuzil et al. 2001c).

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 (Endo et al. 2000; Smith et al. 2003). It was shown that α-TOS induced apoptosis via activation of PKCβII and promoted PKCα membrane translocation, concomitant with a decline in ERK activity (Bang et al. 2001). 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.

3.4 Role of Nuclear Factor-κB in Apoptosis Induced by Vitamin E Analogues

NFκB signalling plays a critical role in cancer development and progression (Karin 2006). NFκB provides a mechanistic link between inflammation and cancer, and is a major factor controlling the ability of both pre-neoplastic and malignant cells to resist apoptosis-based tumour-surveillance mechanisms. NFκB might also regulate tumour angiogenesis and invasiveness, and the signalling pathways that mediate its activation provide attractive targets for new chemopreventive and chemotherapeutic approaches (Karin 2006). Activation of the multi-complex transcription factor NFκB is crucial for a wide variety of cellular responses. In non-stimulated cells, NFκB 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 NFκB into the nucleus and binding to cognate-response elements. In addition to its fundamental role in regulation of immune and inflammatory responses, NFκB also exerts anti-apoptotic activities.

In line with the above, it was found that NFκB activation stimulated by TNF-α was inhibited by α-TOS in Jurkat and endothelial cells (Suzuki and Packer 1993; Neuzil et al. 2001a), possibly sensitizing them to apoptosis induction. Because activation of NFκB is negatively associated with apoptosis induced by TRAIL in multiple cancer cells, agents that inhibit NFκB activation may convert TRAIL-resistant to TRAIL-sensitive cells. TRAIL may transiently activate NFκB, thereby delaying the onset of apoptosis. We found that α-TOS has the capacity to overcome this resistance by suppressing TRAIL-stimulated NFκB activation. This is based on modulating the degradation of IκB, which sensitizes cells to the effect of TRAIL (Dalen and Neuzil 2003).

4 Vitamin E Analogues, Anti-cancer Agents Targeting Mitochondria

Cancer is an ever-increasing neoplastic disease that has been a major threaten of the mankind for centuries. In 2012, over a million of Americans, representing an industrialized nation, were diagnosed with cancer, and most of them suffered more from metastatic tumours and drug side-effects rather than from the initial tumours. Despite great advances in molecular medicine and increasing understanding of molecular signalling pathways related to cancer as well as great efforts to develop better treatments, currently there is still no selective and efficient cure for most types of cancer.

As mentioned before, mitochondria have come into the focus of current research of cancer biologists, since they have emerged as an intriguing target for anti-cancer drugs, inherent to vast majority if not all cancer types. The exceptional potential of mitochondria as an emerging and perspective target for anti-cancer agents has been reinforced by the discouraging finding that there are different mutations in individual areas even in the same tumour in one patient (Gerlinger et al. 2012). This is consistent

with the idea of personalised therapy, an elusive goal at this stage, in line with the notion that tumours are unlikely to be treated by agents that target only a single gene or a single pathway. This endows mitochondria, an invariant target present in all tumours, with an exceptional momentum. Drugs that target mitochondria and exert anti-cancer activity have become a focus of recent research due to their great clinical potential. Mitochondria, whilst being the 'powerhouse' of the cell, are also reservoirs of a number of apoptosis-promoting proteins that are essential for apoptosis induction and its progression downstream of these organelles, in order for the cancer cell to shift into the commitment phase and to undergo the final demise (Galluzzi et al. 2010). It is also important to take into consideration the aberrant mitochondrial metabolism of malignant cells (Koppenol et al. 2011; Ward and Thompson 2012). Thus, the recent decade has witnessed an unprecedented focus on and discovery of novel agents that target mitochondria to induce cancer cell death, which opened a new paradigm of efficient cancer therapy and is likely to benefit cancer patients in the (near) future.

Mitochondria are proving to be worthy targets for activating specific killing of cancer cells in tumours and a diverse range of mitochondrially targeted drugs are currently in clinical trial to determine their effectiveness as anti-cancer therapies. The mechanism of action of many mitochondrially targeted anti-cancer drugs relies on their ability to disrupt the energy producing systems of cancer cell mitochondria, leading to increased generation of ROS and activation of the mitochondrially dependent cell death signalling pathways within cancer cells. We believe that targeting mitochondria for tumour treatment may lead to a potential future breakthrough in the management of neoplastic pathologies. To better classify the various group of agents that act by mitochondrial destabilisation, we proposed the term mitocans (Ralph et al. 2006; Neuzil et al. 2006).

Mitocans, an acronym for 'mitochondria and cancer', are small compounds that in many cases selectively kill cancer cells via mitochondrial destabilisation, whereby inducing apoptosis, often via generation of ROS. They are classified into eight subgroups based on the site of action of the individual agents, from the surface of the MOM to the mitochondrial matrix (Fig. 7.4). The selection of the sites also stems from their importance as targets for the development of drugs that hold substantial promise to be utilised in the clinical practice. VE analogues that act by targeting mitochondria to cause ROS production and, as well, to boost the immune surveillance, exemplify a group of mitocans. The role of ROS production and the events leading to the activation of the inflammasome and pro-inflammatory mediators induced by dying cancer cell mitochondria are later on discussed along with the evidence for their contribution to promoting immune responses against cancer (Hahn et al. 2013).

4.1 α-Tocopheryl Succinate

α-Tocopheryl succinate (α-TOS), bearing a succinyl ester at position C6 of the chromanol ring (Fig. 7.4), belongs to class 2 (BH3 mimetics group) and class 5 (electron transport chain targeting drugs) sub-groups of mitocans. It has been shown

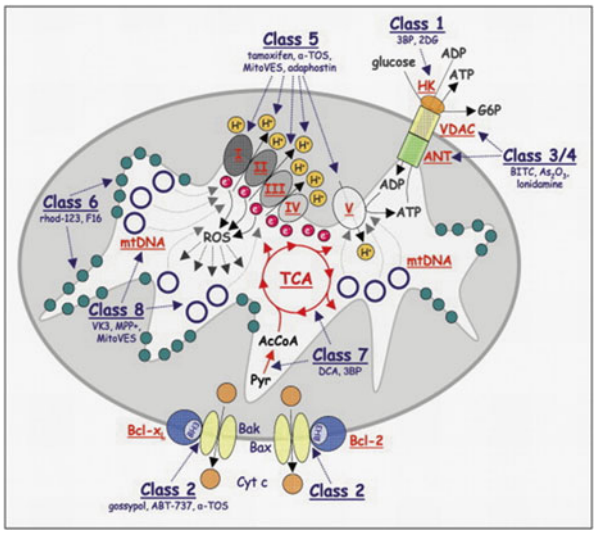

Fig. 7.4 Schematic illustration of molecular targets of individual class of mitocans. Vitamin E analogues belong to both class 2 (BH3 mimetics and related agents that impair the function of the anti-apoptotic Bcl-2 family proteins), class 5 (compounds targeting the mitochondrial electron transport chain) and class 8 (compounds targeting mtDNA)

to be a potent apoptosis inducer and growth inhibitor in a variety of cancer cells. This agent has the selectivity to kill malignant cells at concentrations non-toxic to normal cells and tissues (Neuzil et al. 2001b) and regardless whether the cells feature mutations in key tumour suppressor genes, such as p53 (Weber et al. 2002). Importantly, α-TOS also has been shown to exert anti-cancer activity in a wide range of solid tumours in pre-clinical models, including the difficult-to-treat melanomas, mesotheliomas, HER2-positive breast carcinomas, as well as prostate and colorectal tumours. So far, α-TOS has been shown to suppress tumour growth in up to 15 types of cancer (Wang et al. 2005; Neuzil et al. 2001c; Malafa et al. 2002; Stapelberg et al. 2005).

The molecular mechanism underlying the selectivity of α-TOS and similar agents for malignant cells results from at least two mechanisms. One relates to the ester structure of α-TOS and is due to the generally higher levels of esterases in normal cells such as hepatocytes, colonocytes, fibroblasts or cardiomyocytes that cleave α-TOS to produce vitamin E (Don and Hogg 2004; Fariss et al. 2001). Another reason for the cancer cell-specific toxicity of α-TOS may be related to the inherent property of many inducers of apoptosis to trigger programmed cell death by initially inducing cells to accumulate ROS that, in turn, cause a cascade of subsequent reactions leading to the transition of the cell into the apoptosis commitment phase (Simon et al. 2000). α-TOS has been reported to induce ROS accumulation in many different cancer cell lines, most probably resulting in the generation of superoxide anion radicals (Kogure et al. 2001, 2002; Malafa et al. 2002; Swettenham et al. 2005). In addition, it has been reported that cancer cells feature lower antioxidant defences than normal cells. For example, malignant cells express lower levels of manganese superoxide dismutase (MnSOD) compared to non-malignant cells. Moreover, we have found that proliferating endothelial cells,

unlike their confluent (growth-arrested) counterparts, are susceptible to α-TOS-induced apoptosis (Neuzil et al. 2001a), endowing α-TOS the ability to selectively kill proliferating endothelial cells and inhibit angiogenesis in mouse tumour models (Dong et al. 2007).

An important discovery demonstrated that α-TOS has a unique target to perform its biological (apoptogenic) activity. Research from Neuzil's group found that α-TOS induces cancer cell apoptosis by targeting the mitochondrial complex II (CII). More specifically, α-TOS inhibits the succinate quinone reductase (SQR) activity of CII by interacting with the proximal and distal ubiquinone (UbQ) binding site (Q_P and Q_D, respectively) (Dong et al. 2008). Additional work documented that CII is also a target for α-TOS in a pre-clinical tumour model, since tumours with mutant CII (a stop codon mutation in the CII's subunit SDHC) were not responsive to the agent unlike the wild-type or reconstituted CII tumours (Dong et al. 2009). This is the first time that a molecular target of α-TOS has been defined. CII as the target indicates that the agent may be an efficient anti-cancer drug since genes coding for the four subunits of CII only rarely mutate (for example there is only one mutation in CII per one million breast cancer patients).

Recent studies from Gogvadze's group also emphasized the importance of mitochondria as a target for α-TOS. The agent was shown by these researchers to trigger cancer cell apoptosis via targeting mitochondria, more specifically by stimulating rapid entry of Ca^{2+} into the cytosol, compromising the Ca^{2+} buffering capacity of mitochondria and sensitising them to mitochondrial permeability transition and subsequent apoptotic cell death. This mechanism was reported for neuroblastoma cells that were found to be killed by α-TOS irrespective of their *MycN* oncogene expression level and amplification (Kruspig et al. 2012).

Collectively, there is ample evidence for α-TOS (and similar agents) to kill cancer cells by destabilising mitochondria. Therefore, it seems a logical next step to modify the drugs in such a way that they accumulate in mitochondria, i.e. in the proximity of their molecular target.

4.2 Mitochondrially Targeted Vitamin E Succinate

Although α-TOS acts on mitochondria, it does not discriminate between the different membranous intracellular compartments. Therefore, we modified the structure of α-TOS in order to generate a variant of the agent that would be directly targeted to mitochondria, so that its apoptogenic activity would be increased. Based on the previous work of synthesis and testing of a series of mitochondrially targeted antioxidants by tagging them with the positively charged triphenylphosphonium group (TPP^+) (Murphy and Smith 2007), producing very efficient redox-active compounds (Kelso et al. 2001; James et al. 2007), our group prepared the mitochondrially targeted vitamin E succinate (MitoVES) by adding the TPP^+ group to the hydrophobic chain of vitamin E succinate. The result of this modification was the preferential

localisation of the agent in mitochondria, greatly enhancing its pro-apoptotic and anti-cancer activity (Dong et al. 2011a, b; Rohlena et al. 2011).

MitoVES, besides accumulating in mitochondria, has another advantage: its mitochondrial accumulation is based on the $\Delta\Psi_{m,i}$, which is considerably higher (in negative values) in cancer cells than in normal cells (Modica-Napolitano and Aprille 2001; Fantin et al. 2002). Due to the chemical structure of MitoVES, the compound is expected to be positioned such that the positive charge of the TPP^+ group is adjacent to the interface of the MIM and matrix and its hydrophobic alkyl chain spans the MIM with the tocopheryl succinyl group juxtapositioned to its molecular target, the UbQ-site of CII. Our recent research documents that the prototypic compound of such a targeted VE analogue, MitoVES, is some 1–2 orders of magnitude more efficient in apoptosis induction than the untargeted parental compound α-TOS, while maintaining selectivity for malignant cells (Dong et al. 2011a, b). One reason is that at relatively low levels, high percentage of the MitoVES pool (90-95 %) accumulates in mitochondria, resulting in very fast generation of ROS (within minutes) and changes in mitochondrial morphology (10–15 min). This then causes the modulation of expression of Bcl-2 family proteins. More specifically, we found that the ROS generated in response to MitoVES activates the Mst1 kinase that, in turn, phosphorylates the transcription factor FoxO1. Phosphorylated FoxO1 then translocates to the nucleus where it activates transcription of the BH3-only protein Noxa that, in turn, diverts Mcl-2 from Bak that can then form a channel in the MOM (Dong et al. 2011a, b; Valis et al. 2011).

MitoVES proved to be superior in suppression of experimental tumours compared to the untargeted analogue, as we showed for breast cancer, colon cancer and mesothelioma (Dong et al. 2011a, b; Kovarova et al. 2014). Based on the prototypic MitoVES targeting the mitochondrial CII, we propose that mitochondrially targeted delivery of anti-cancer agents offers a new paradigm for increasing the efficacy of compounds with anti-cancer activity (Figs. 7.5, 7.6). Accordingly, preparation of novel anti-cancer agents by tagging other compounds that target mitochondrial complexes is imminent and we are conducting relevant experiments.

An interesting way how to possibly suppress tumour promotion avoiding the many mutations in cancer cells is targeting angiogenesis. In this regard, we found that MitoVES efficiently kills proliferating endothelial cells (ECs) but not contact-arrested ECs or ECs deficient in mitochondrial DNA. It also suppressed angiogenesis *in vitro* by inducing accumulation of ROS and induction of apoptosis in proliferating/angiogenic ECs. Resistance of arrested ECs was ascribed, at least in part, to lower $\Delta\Psi_{m,i}$ of quiescent ECs compared with their proliferating counterparts, resulting in the lower level of mitochondrial uptake of MitoVES. (Rohlena et al. 2011).

Additional studies on energy-related mitochondrial function using isolated mitochondria demonstrated that MitoVES stimulates basal respiration and ATP hydrolysis, but inhibits net state 3 (ADP-stimulated) respiration and Ca^{2+} uptake by collapsing $\Psi\Delta_{m,i}$ at low doses, acting as an uncoupler (1–5 μM). At higher doses (> 5 μM), MitoVES targets the SQR activity of the mitochondrial CII. Uncoupled

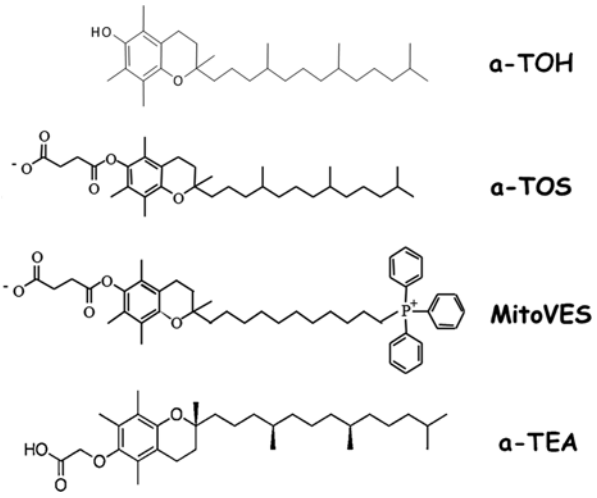

Fig. 7.5 Structures of mitochondria-untargeted and -targeted vitamin E analogues

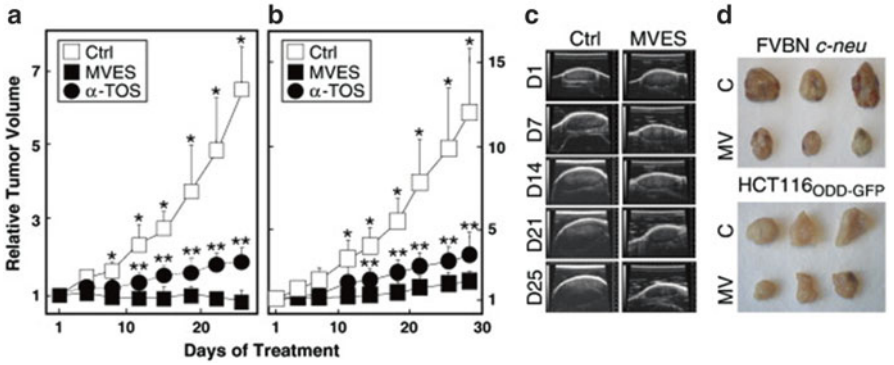

Fig. 7.6 MitoVES suppresses tumour progression. (**a**) FVB/N c-neu mice with breast carcinomas and (**b**) Balb c nu/nu mice with xenografts derived from HCT116 cells were treated by ip injection of 1–2 μmol MitoVES or 15 μmol α-TOS per mouse every 3–4 days, and tumors were visualized and their volume was quantified using ultrasound imaging (panel (**c**) shows representative ultrasound images of tumours acquired on given days, panel (**d**) documents representative tumours at the end of the experiment. (Adapted from Dong et al. 2011a, b)

mitochondrial respiration and basal respiration of SMPs were inhibited by VE analogues with the following efficacy: MitoVES > α-TEA > α-TOS (Rodríguez-Enríquez et al. 2012). The same authors also showed that MitoVES inhibited oxidative phosphorylation and induced ATP depletion in rodent and human cancer cells more potently than in normal rat hepatocytes. This is consistent with our recent data that *in vivo*, cancer cell mitochondria are a preferred target of MitoVES compared to normal cells (such as found for kidney, heart or liver; Truksa et al. unpublished data). Collectively, these findings are consistent with and corroborate the notion that targeting of anti-cancer agents to mitochondria enhances their anti-cancer efficacy and, as shown for MitoVES, maintains their selectivity for malignant cells.

4.3 α-Tocopheryloxyacetic Acid

α-TEA is an ether analogue of VE with potent anti-cancer actions via activation of pro-apoptotic pathways and suppression of pro-survival pathways both *in vitro* and *in vivo*. It has been shown to suppress tumour growth in various murine and human xenograft tumour models, including melanoma, breast, lung, prostate, and ovarian cancers (Jia et al. 2008; Yu et al. 2006; Dong et al. 2012). α-TEA has also been shown to exhibit anti-metastatic activities in xenografts, syngeneic and spontaneous mouse models of breast cancer. Importantly, similar to α-TOS, α-TEA has the ability to possess anti-cancer properties that are selective for cancer cells, to reduce tumour burden and metastases *in vivo* with no or very low toxicity to normal tissues (Yu et al. 2006; Lawson et al. 2004; Latimer et al. 2009; Hahn et al. 2009; Shun et al. 2010). This gives α-TEA a great potential for future drug development for clinical use.

α-TEA is a stable semi-synthetic analogue of naturally occurring VE. It is derived from α-tocopherol by a chemical modification, replacing the hydroxyl group C6 of the phenolic ring with an acetic acid residue linked via an ether bond (Fig. 7.5). This modification makes α-TEA redox-silent, while making it active against tumours (Lawson et al. 2003; Hahn et al. 2006). The presence of the non-cleavable ether bond ensures the stability of α-TEA, allowing it to be delivered via the oral route in a biologically active form (Dong et al. 2012). An earlier report documented that α-TEA, when given orally (incorporated into mouse chow), significantly inhibited the progression of breast cancers and strongly reduced the incidence of spontaneous lung metastases before and after primary tumour establishment without general toxicity (Lawson et al. 2003). A number of reports from different groups have showed that apoptosis is a primary mode of α-TEA-induced tumour cell death (Kline et al. 2001; Neuzil et al. 2004). However, since the antitumor activity of α-TEA could not be completely blocked using pan-caspase or caspase-specific inhibitors (Jia et al. 2008), additional pathway(s) may be involved in α-TEA-mediated tumour cell killing, such as mitochondria-independent signalling pathways.

It has been reported that α-TEA induces apoptosis in MCF-7 and HCC-1954 breast cancer cells via TRAIL/DR5-induced activation of caspase-8 that is relayed to mitochondria-dependent pro-apoptotic pathway by increasing the DR5 and TRAIL protein levels, and via suppression of the anti-apoptotic protein c-FLIP by decreasing its levels. This mitochondria-dependent apoptosis signalling pathway involves the initial activation of caspase-8 followed by cleavage of Bid to tBid, activation of the Bax protein (its translocation to the MOM) and increased mitochondrial permeability transition. The data suggest that that α-TEA has the potential as a treatment for human breast cancer either as a stand-alone drug or in combination with the recombinant TRAIL protein or antagonistic antibodies to the TRAIL receptor (Yu et al. 2010). Mechanistic studies showed that the major events necessary and sufficient for inducing apoptosis of cancer cells with α-TEA include the activation of pro-apoptotic mechanisms such as signalling via the Fas receptor/Fas ligand, and endoplasmic reticulum (ER) stress involving JNK/CHOP/DR5 and p73/Noxa,

leading to caspase-8 activation followed by mitochondria-dependent apoptotic cascade of reactions (Hahn et al. 2009, 2011; Shun et al. 2010). One report documented that α-TEA disrupts the cholesterol-rich micro-domains, acting cooperatively with a selective estrogen-receptor modulator to reduce pro-survival mediators, and induces DR5-mediated mitochondria-dependent apoptosis via the ER stress-mediated pro-apoptotic pJNK/CHOP/DR5 amplification loop (Lawson et al. 2003; Tiwary et al. 2010).

A previous report suggested that α-TEA suppresses the phosphatidylinositol-3-kinase (PI3K)/Akt/ERK pathways via JNK-mediated down-regulation of insulin-receptor substrate (IRS-1) (Tiwary et al. 2011b). It induced apoptosis in human MCF-7 and HCC-1954 breast cancer cells by suppressing constitutively active basal levels of pAKT, pERK, p-mTOR, and their downstream targets. In addition, α-TEA increased levels of pIRS-1 (Ser-307), a phosphorylation site correlated with insulin receptor substrate-1 (IRS-1) inactivation, as well as of total IRS-1. Down-regulation of the IRS-1/PI3K pathways via JNK are critical for α-TEA and α-TEA + MEK or mTOR inhibitor-induced apoptosis in human MCF-7 and HCC-1954 breast cancer cells (Tiwary et al. 2011b). α-TEA has also been shown to induce cancer cell death, at least in part, by down-regulation of members of the EGFR family (Shun et al. 2010).

Collectively, there is ample data documenting the potential of α-TEA as an anti-cancer agent. Except for some specific activities in cancer cells, the ether can be considered as a stable 'homologue' of the prototypic anti-cancer VE analogue, α-TOS. From the application point of view, the great advantage of α-TEA is that it can be applied orally, while α-TOS has to reach the site of the tumour while bypassing the alimentary system, where it is completely hydrolysed to the non-apoptogenic vitamin E.

5 Vitamin E Analogues as Stimulants of the Immune System

Recent research demonstrated that, in addition to the direct cytotoxic effects, α-TEA stimulates anti-tumour immune responses, resulting in higher level of infiltration of activated T cells in the tumour microenvironment and increased ratios of $CD4^+$ and $CD8^+$ T cells to regulatory T cells in the tumour, respectively (Hahn et al. 2011; Tiwary et al. 2011a). Moreover, autophagy has been found to be involved in the immune system stimulation by α-TEA, since autophagy plays an essential role in the major histocompatibility complex (MHC) class II-restricted antigen presentation (Münz 2009), and recently its role in MHC class I–restricted stimulation of $CD8^+$ T cells (cross-presentation) has been documented (Li et al. 2008; Kepp et al. 2009). A recent report indicates that α-TEA triggers tumour cell autophagy and improves cross-presentation of tumour antigens to the immune system. α-TEA stimulated both apoptosis and autophagy in murine mammary and lung cancer cells. These findings suggest that both autophagy and apoptosis signalling programmes

are activated during α-TEA-induced tumour cell killing (Li et al. 2012), endowing the agent with a very interesting pattern of bioactivities.

6 Conclusions

Taken together, VE analogues are potent anti-cancer agents that selectively kill cancer cells with the advantage of limited side effects on normal cells, as also shown for high level of toxicity of the agents to cancer tissues and low (if any) deleterious effects on normal tissues. Mitochondria act as targets for VE analogues, relaying the selective apoptotic signals shifting cancer cells to the commitment phase of programmed cell death. The VE analogues α-TOS, α-TEA and MitoVES epitomize mitocans, a large group of compounds acting as anti-cancer agents by destabilizing mitochondria. These compounds present a source of very promising, potentially highly effective and selective lead structures hopefully leading to exciting new developments in cancer therapy. The predominant mechanism of action whereby mitochondria-targeted anticancer drugs kill cancer cells is linked to the ability of these drugs to disrupt the energy-producing systems of cancer cells (concentrated in mitochondria), leading to increased accumulation of ROS and the activation of the mitochondria-dependent death signalling pathways. There is little doubt that, given recent advances in anti-cancer research, mitocans will become an integral part of modern weaponry in the fight to eliminate cancer, although there is still a lot of work to achieve this goal. Notwithstanding this, the authors are optimistic and can 'see the light at the end of the (apparently) long tunnel'.

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