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MINIREVIEW

Vitamin E Analogs, a Novel Group of "Mitocans," as Anticancer Agents: The Importance of Being Redox-Silent

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ABSTRACT

The search for a selective and efficient anticancer agent for treating all neoplastic disease has yet to deliver a universally suitable compound(s). The majority of established anticancer drugs either are nonselective or lose their efficacy because of the constant mutational changes of malignant cells. Until recently, a largely neglected target for potential anticancer agents was the mitochondrion, showing a considerable promise for future clinical applications. Vitamin E (VE) analogs, epitomized by α-tocopheryl succinate, belong to the group of "mitocans" (mitochondrially targeted anticancer drugs). They are selective for malignant cells, cause destabilization of their mitochondria, and suppress cancer in preclinical models. This review focuses on our current understanding of VE analogs in the context of their proapoptotic/anticancer efficacy and suggests that their effect on mitochondria may be amplified by modulation of alternative pathways operating in parallel. We show here that the analogs of VE that cause apoptosis (which translates into their anticancer efficacy) generally do not possess antioxidant (redox) activity and are prototypical of the mitocan group of anticancer compounds. Therefore, by analogy to Oscar Wilde's play *The Importance of Being Earnest*, we use the motto in the title "the importance of being redox-silent" to emphasize an essentially novel paradigm for cancer therapy, in which redox-silence is a prerequisite property for most of the anticancer activities described in this communication.

Despite advances in molecular medicine, the third millennium has borne witness to neoplastic disease becoming a major cause for mortality in developed countries. Moreover, fast-growing economies in countries like India and China are likely to be severely affected by cancer in a decade or so as a result of heavy industrialization. Certain types of cancer, such as malignant mesothelioma (MM), seem to remain beyond the realms of treatment. In many other cases, mutations arise in the tumors, seriously compromising the outcome of the therapy. For example, in breast cancer, in which a high frequency of overexpression of the tyrosine receptor kinase erbB2 occurs, this is often associated with resistance to chemotherapy (Xia et al., 2006). We are therefore in need of treatment modalities that would overcome these problems and that are efficient, selective, and readily available to all

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ABBREVIATIONS: MM, malignant mesothelioma; BH, Bcl-2 homology; DHFR, dihydrofolate reductase; DR, death receptor; ERK, extracellular signal-regulated protein kinase; FLIP, Fas-associated death domain-like interleukin-1β-converting enzyme-inhibitory protein; IAP, inhibitor of apoptosis protein; IκB, inhibitory subunit of nuclear factor κB; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MPTP, mitochondrial permeability transition pore; MTX, methotrexate; NFκB, nuclear factor-κB; OS, osteosarcoma; PKC, protein kinase C; PP2A, protein phosphatase 2A; ROS, reactive oxygen species; SAR, structure-activity relationship; SMase, sphingomyelinase; TNF, tumor necrosis factor; α-TOS, α-tocopheryl succinate; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; UbQ, ubiquinone; VDAC, voltage-dependent anionic channel; VE, vitamin E; CD437, 6-[3-(1-adamantyl)-4-hydroxyphenyl]-2-naphthalene carboxylic acid; 2DG, 2-deoxyglucose; DR4, tumor necrosis factor-related apoptosis-inducing ligand receptor 1; DR5, tumor necrosis factor-related apoptosis-inducing ligand receptor 2.

patients. Mitochondria have emerged as a novel target for cancer treatment (Fantin and Leder, 2006; Galluzzi et al., 2006), and it is possible that targeting mitochondria may provide the means for achieving the long sought after universal cancer treatment.

Mitochondria are organelles important for life and death (Newmeyer and Ferguson-Miller, 2003). They are major sources of cellular energy. However, mitochondria are also central to processes resulting in the induction of apoptosis. The exact mechanism(s) by which mitochondria act is not known in detail, but there are drugs that are known to induce death of cancer cells by targeting these organelles. Such agents, called "mitocans" (Ralph et al., 2006, Neuzil et al., 2006), destabilize mitochondria, thereby causing the cytosolic release of modulators of apoptosis (Green and Reed, 1998). Although the importance of mitocans as novel anticancer agents for selective apoptotic killing via mitochondrial destabilization has been the topic of several reviews (Don and Hogg, 2004; Fantin and Leder, 2006; Galluzzi et al., 2006), we present the case that the indirect pro-oxidant activity of these agents in cancer cells is essential for their actions as anticancer agents.

Analogs of VE, epitomized by α-tocopheryl succinate (α-TOS), present one group of mitocans (Neuzil et al., 2004, 2006; Dong et al., 2006; Ralph et al., 2006; Wang et al., 2006). α-TOS selectively kills malignant cells at levels at which it exerts no toxicity (or minimal toxicity) to normal cells or tissues (Neuzil et al., 2001b; Weber et al., 2002). It also overrides mutations leading to loss of tumor suppressor genes (Weber et al., 2002). VE analogs inhibit a diverse range of tumors in experimental animals, including melanomas, colorectal carcinomas, mesotheliomas, and erbB2-positive breast carcinomas (Neuzil et al., 2001c; Malafa et al., 2002; Tomasetti et al., 2004a; Stapelberg et al., 2005) (Fig. 1).

According to our current understanding, the selectivity of VE analogs for malignant cells arises as a result of at least two mechanisms. One is based on the ester structure of the VE agents and is due to the higher esterase activities in normal cells, cleaving α-TOS and similar agents to produce the nonapoptogenic α-tocopherol (Fariss et al., 2001; Neuzil et al., 2004). The selective tumor cell toxicity of VE analogs is also related to the property of certain drugs that induce apoptosis, leading to accumulation of reactive oxygen species (ROS) in cancer cells, resulting in activation of downstream proapoptotic signaling pathways (Simon et al., 2000). VE analogs, particularly α-TOS, cause ROS (superoxide) accumulation in different cancer cell lines (Kogure et al., 2001, 2002; Stapelberg et al., 2005; Swettenham et al., 2005). It is known that cancer cells feature decreased antioxidant defenses, such as expression of the mitochondria-specific manganese superoxide dismutase (Borrello et al., 1993).

Mitochondrial Targeting as a Novel Paradigm of Cancer Therapy: the Emergence of Mitocans. Mitochondria provide beneficial targets by which one can selectively kill cancer cells, with the advantage of limited side effects on normal cells. In addition, cancer cell death by apoptosis acts to restrict the spread of dying cellular debris, ensuring that the process of removal is contained. As a consequence of their selectivity, many diverse mitochondria-targeted drugs are currently in clinical trials to determine their effectiveness as anticancer therapies. However, many are still only in phase I studies such that it is too early to report on their relative efficacy. The predominant mechanism of action whereby mitochondria-targeted anticancer drugs kill cancer cells relies on the ability of these drugs to disrupt the energy-producing systems of cancer cells and particularly those in the mitochondria, leading to increased accumulation of ROS and the activation of the mitochondria-dependent death signaling pathways. Because of their mitochondrial targeting and anticancer roles, these drugs have been termed "mitocans" (Neuzil et al., 2006; Ralph et al., 2006). Mitocans include compounds that affect mitochondria-associated activities such as hexokinase inhibition, activation of the mitochondrial permeability transition pore (MPTP), inhibition of the Bcl-2 antiapoptotic proteins, and blocking of the electron transport/respiratory chain. The proposed classification of mitocans is shown in Table 1, and examples of some of the more characterized members of the group are discussed below.

The glucose metabolites, 2-deoxyglucose (2DG), oxamate, and 3-bromopyruvate form the first class of hexokinase-inhibiting mitocans selectively inducing apoptosis in cancer cells. These mitocans have been found in clinical and preclin-

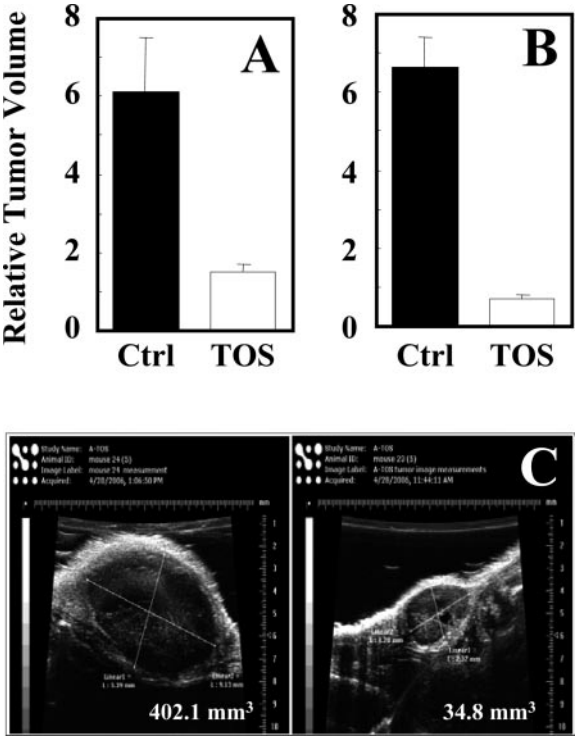


Fig. 1. α-Tocopheryl succinate efficiently suppresses experimental mesotheliomas and breast carcinomas. A, immunocompromised (nude) mice were inoculated subcutaneously with human MM cells and treated every third day by intraperitoneal injection with 100 μl of 200 mM α-TOS in dimethyl sulfoxide or with dimethyl sulfoxide only (control). Tumor volume was calculated after measuring its dimensions with digital calipers. The data shown are from day 14 of treatment and are expressed relative to the initial tumor volume (at the onset of the treatment) (adapted from Stapelberg et al., 2005). B, transgenic *FVB/N c-neu* mice with spontaneous breast carcinomas were subjected to intraperitoneal administration of 100 μl of 150 mM α-TOS in corn oil or the same volume of corn oil alone (control) every third day. The tumors were visualized and quantified by ultrasound imaging using the Vevo770 instrument (VisualSonics, Toronto, ON, Canada) allowing 40-μm resolution. The data are expressed relative to the volume at the onset of treatment. C, the images show representative visualization of a control and a treated tumor taken from experiment shown in B at day 14 of the treatment using the ultrasound imaging technique (J. Neuzil, unpublished data).

ical models to exert selective toxicity toward tumor cells that metabolize anaerobically (Geschwind et al., 2002; Robey and Hay, 2005; Xu et al., 2005; Pelicano et al., 2006). A phase I clinical trial of 2DG in combination with docetaxel is underway in patients with advanced solid tumors (Threshold Pharmaceuticals Ltd., Redwood City, CA). 2DG has been administered to patients with single intravenous doses as high as 200 mg/kg not producing any serious adverse events, although higher doses caused hypoglycemia and neurotoxicity (Tidmarsh, 2005). Use of hexokinase inhibitory drugs should enhance the efficacy of standard cancer chemotherapeutics and radiation regimens that focus on aerobic cancer cells. In addition, hexokinase inhibitors may be used in conjunction with antiangiogenic agents to limit the oxygen supply to the tumor.

The second class of mitocans is aimed at obviating the protective effects of the Bcl-2 family of antiapoptotic and prosurvival proteins that are overexpressed in cancer cells. For example, antisense oligonucleotide therapy against Bcl-2 and Bcl-x_L has been used to inhibit expression of these two prosurvival proteins in cancer cells, thereby increasing the effectiveness of anticancer chemotherapy (Cory and Adams, 2002). The discovery of the relationship between the BH4 (Bcl-2 homology 4) helix-containing antiapoptotic proteins Bcl-2 and Bcl-x_L and their ability to form complexes by binding to the BH3 helix of the proapoptotic Bcl-2-related members has resulted in the development of novel small-molecule inhibitors of Bcl-2 and Bcl-x_L (O'Neill et al., 2004; Reed and Pellecchia, 2005). Thus, Bcl-2 and Bcl-x_L both share a hydrophobic groove on their surface whose function is to bind the BH3 amphipathic helix of the proapoptotic family members, thereby preventing apoptosis. This hydrophobic groove also binds a range of small molecules, including the natural compound gossypol, that result in the blocking of BH3 binding. Thus, small molecules blocking Bcl-2 and Bcl-x_L will enable the BH3 family of proapoptotic inducers to then freely bind to their relevant targets and induce apoptosis (Degterev et al., 2001; Yin et al., 2005). The BH3 mimetics are only recently entering clinical trial in patients with cancer.

Mitocans from the arsenite class of compounds have been used medically for many years to treat cancers and are effective against hematological malignancies. Hence, arsenic oxides and their derivatives have become established as effective treatments for acute promyelocytic leukemia and are in trials for other hematological cancers, including myelodysplastic syndromes, multiple myeloma, and chronic myelogenous leukemia (Amadori et al., 2005). The arsenite compounds are likely to modulate critical cysteine residues in the adenine nucleotide translocator channel (Belzacq et al., 2001), thereby inhibiting its activity.

Lonidamine (an indazole carboxylate derivative) induces activation of the MPTP and causes mitochondrial membrane permeabilization by binding and affecting the adenine nucleotide transporter in the mitochondrial inner membrane of cancer cells (Ravagnan et al., 1999; Belzacq et al., 2001). This type of the adenine nucleotide transporter channel-inhibiting drug represents another group of mitocans. Although lonidamine's action as a potent antiproliferative drug acting on cancer cells has been well documented, recent clinical trials have shown it has little or no additional benefit over conventional therapies (Di Cosimo et al., 2003) and, as a result, has not been pursued further as a broad-spectrum anticancer therapy.

The redox-silent analogs of VE are an exciting new group of mitocans. Unlike the antioxidant VE, these analogs (represented by the prototypic α-TOS) selectively induce apoptosis in malignant cells via mitochondria-dependent apoptotic signaling (Neuzil et al., 2004). Mitochondrial DNA-deficient cells (ρ^0 phenotype) are resistant to α-TOS compared with the parental cells (Weber et al., 2003; Wang et al., 2005), indicating that mitochondria are major transmitters of apoptotic signaling induced by the esterified VE analog.

It is intriguing that α-TOS was recently shown to complex with the BH3 binding hydrophobic groove of Bcl-2 and Bcl-x_L (Shiau et al., 2006), thereby inhibiting their function, leading to the induction of apoptosis in prostate cancer cells. In particular, the hemisuccinate and two proximal isoprenyl units of the side chain were shown to play a critical role in ligand anchoring and Bcl-2 protein-ligand complex formation. Given the relationship of α-TOS to UbQ, we speculate that Bcl-2 and Bcl-x_L bind both ubiquinone and quinone-related structures. In fact, many of the small-chemical molecules that have been found recently to bind these proteins are likely to mimic ubiquinones. For example, antimycin A, a well-described inhibitor of the quinone-binding site on cytochrome bc1 in the mitochondrial respiratory chain (Gao et al., 2003; Huang et al., 2006), has also been shown to compete for binding with BH3 for the hydrophobic groove of either Bcl-2

TABLE 1
Classification of mitocans

Class Number	Type	Examples	References
I	Hexokinase inhibitors	3-Bromopyruvate	Ko et al., 2004; Xu et al., 2005
		2-Deoxyglucose	Ko et al., 2004
II	Bcl-2/Bcl-x_L mimetics	Gossypol	Kitada et al., 2003
		Antimycin A	Tzung et al., 2001
		α-Tocopheryl succinate	Shiau et al., 2006
III	Thiol redox inhibitors	Isothiocyanates	Xu and Thornalley, 2001
		Arsenic trioxide	Miller, 2002
IV	VDAC/ANT targeting drugs	Lonidamine, arsenites	Belzacq et al., 2001
		Steroid analogs such as CD437	
V	Electron transport chain targeting drugs	4-OH retinamide	Hail and Lotan, 2001
		Tamoxifen	Moreira et al., 2006
		Antimycin A	Wolvetang et al., 1994
VI	Lipophilic cations targeting inner membrane	Rhodamine-123	Lampidis et al., 1983
		F16	Fantin et al., 2002
		$(KLAKKLAK)_2$ peptide	Ellerby et al., 1999
VII	Drugs targeting other (unknown) sites	Resveratrol (ATPase ?)	Zheng and Ramirez, 1999
		Betulinic acid	Fulda et al., 1998

or Bcl-x_L (O'Neill et al., 2004). Furthermore, a 2-methoxy antimycin A derivative with no inhibitory effects on the respiratory chain retains selectivity for Bcl-x_L. Hence, a role for quinones such as UbQ in binding the antiapoptotic Bcl-2-related family members, affecting their ability to form dimers with the BH3 pro-apoptotic family members, seems very likely. The significance of UbQ binding and preference in terms of the semiquinone or other form to cellular function and redox states still remains to be defined, although it may be that Bcl-2-related proteins act in this capacity as redox sensors regulating apoptosis.

Not only do ubiquinones bind to Bcl-2-related family members, but they may also bind directly to a common binding site involved in regulating the MPTP (Walter et al., 2002) affecting the flow of Ca^{2+} into mitochondria during induction of apoptosis. In addition, MPTP inhibition induced by specific ubiquinones like UbQ and decylubiquinone can be reversed by increasing $[Ca^{2+}]_i$ (Martinucci et al., 2000). Thus, radioactive compounds structurally resembling ubiquinones have been shown to specifically bind to the voltage-dependent anionic channel (VDAC) (Cesura et al., 2003). It is interesting that Bcl-2 family proteins, including Bax and Bcl-x_L, also bind to the outer regions of VDAC1 (Shi et al., 2003). This raises the possibility of an interrelationship between Bcl-2-binding BH3 peptides or related hydrophobic structures like ubiquinones or VE analogs, and VDAC binding the same. Whether the two proteins, VDAC and Bcl-2 family members, share such similar recognition and binding sites, and their significance, remain to be resolved.

Structure-Function Relationship of Proapoptotic/Antineoplastic VE Analogs

Naturally occurring VE consists of a mixture of eight compounds that differ by the methylation patterns of the chromanol ring (α-, β-, γ-, and δ-tocopherol) and the presence or absence of double bonds of the phytyl side chain (α-, β-, γ-, and δ-tocotrienol). The role of these molecules as lipophilic antioxidants in vitro and in vivo is widely accepted. In addition, the nonantioxidant properties of VE family members have also been investigated (Azzi et al., 2002).

The VE molecule can be divided into three different domains. The functional domain (I) arises from the substitution pattern at position C6 of the chromanol ring. This position determines whether the molecule behaves as redox-active or redox-silent. The well-documented antioxidant properties of the four tocopherol isomers resulted in their application in cancer clinical trials. None of these studies showed a positive outcome concerning the use of free tocopherols in cancer prevention (Pham and Plakogiannis, 2005). However, certain chemical modifications at C6 led to ethers (RO-), esters (RCOO-), and amides (RCONH-) that proved to be potent antineoplastic agents (Table 2).

The second, signaling domain (II), exhibits activities 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).

The lipophilic side chain of VE isomers distinguishes between tocopherols with saturated isoprenyl units and tocotrienols with unsaturated isoprenyl units. The hydrophobic domain (III) determines whether the molecule can bind to lipoproteins and membranes or be degraded by phase I enzymes (Birringer et al., 2002; Neuzil and Massa, 2005).

Redox-Silent Tocopherol Derivatives: Modifications of the Functional Domain

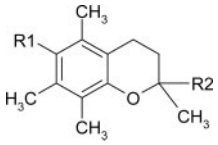
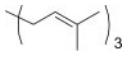
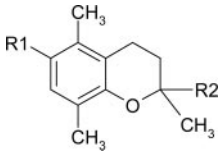
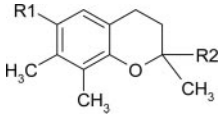
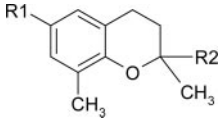
Tocopherol derivatives with a modified hydroxyl group have been tested for their proapoptotic activity (Table 2, Number column). The most prominent derivative has been α-TOS (**1**), bearing a succinyl ester at position C6 of the chromanol ring. Because of its low pK_a (<6), α-TOS is fully deprotonated under physiological conditions, leading to a detergent-like molecule, which destabilizes mitochondrial membranes, and our recent data point to an effect on the mitochondrial complex II (J. Neuzil, unpublished data). Dicarboxylic esters of tocopherols present the best studied compounds for structure-activity relationship (SAR). Strong apoptogens include α-tocopheryl succinate (**1**), oxalate (**10**), and malonate (**11**), the latter two inducing nonselective 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 (**3**) (Birringer et al., 2003) and α-tocopheryl fumarate (J. Neuzil, unpublished data). Increasing the chain length of the dicarboxylic acid led to decreased activity as shown for glutaric acid (**5**) and methylated glutaric acids (**6**-**8**) (Birringer et al., 2003), with pimelic acid (**24**) (Kogure et al., 2004) exhibiting no activity at all.

It has been established that the whole α-TOS molecule is necessary for its apoptogenic activity (Fariss et al., 1994; Birringer et al., 2003). Methylation of the free carboxyl group leads to noncharged derivatives without proapoptotic activity (compounds **9** and **25**). Aliphatic carboxylic acid esters, such as tocopheryl acetate and propionate (**19**), respectively, were inactive, as was the methyl ether (**18**).

Oral administration of α-TOS is not effective because the compound is cleaved by intestinal esterases (Cheeseman et al., 1995; Wu et al., 2004b). To overcome the problem of ester bond cleavage, compounds **20** and **21** and a side chain-truncated derivative (**42**) have been synthesized, replacing the ester bond with an ether bond, because the latter is resistant to hydrolysis (Nishikawa et al., 2003; Wu et al., 2004b; Shiau et al., 2006). It should be noted that the replacement of the ether bond by a methylene group is sufficient to accelerate apoptosis (**22**) (Sanders et al., 2001).

When the ester bond is replaced by an amide bond, further enhancement of proapoptotic activity was observed (**12**, **13**, **37**, and **38**) (Tomic-Vatic et al., 2005). Again, the unsaturated amides (**13** and **38**) were superior to their saturated counterparts. The rationale for introducing an amide bond in place of the ester was based on the well-established fact that anilinic amides are much less prone to hydrolysis than the corresponding phenolic esters. Enhancing the stability of these tocopheryl ester derivatives would protect these molecules in vivo, allowing them to stay intact longer, thereby increasing their bioavailability. The isosteric replacement of the esters by amides makes that linkage less prone to enzymatic hydrolysis as well. Several nonspecific esterases exist in the intes-

TABLE 2
Antiproliferative activity of vitamin E analogs
Compounds are sorted by the signaling domain.

Compound Number	Functional Domain I (R1)	Signalling Domain II	Hydrophobic Domain III (R2)	IC_{50}	Cell Type	References
				μM		
1	$^{-}O_2CCH_2CH_2COO$-			43	Jurkat, HBT11, MCF7, MCF7-C3	Birringer et al., 2003
2	CH_3COO-			—[a]		
3	$^{-}O_2CCH{=}CHCOO$-			22		
4	$^{-}O_2CCH_2CH(CH_3)COO$-			—[b]		
5	$^{-}O_2CCH_2(CH_2)_2COO$-			—[b]		
6	$^{-}O_2CCH_2CH(CH_3)CH_2COO$-			—[b]		
7	$^{-}O_2CCH_2C(CH_3)_2CH_2COO$-			—[b]		
8	$^{-}O_2CC(CH_3)_2CH_2CH_2COO$-			—[b]		
9	$H_3COOCCH_2CH_2COO$-			—[b]		
10	$^{-}O_2CCOO$-			—[c]	B16-F1/nude mice	Kogure et al., 2005
11	$^{-}O_2CCH_2COO$-					
12	$^{-}O_2CCH_2CH_2CONH$-			13	Jurkat, U937, Meso-2	Tomic-Vatic et al., 2005
13	$^{-}O_2CCH{=}CHCONH$-			2		
14	$H_3COOCCH_2CH_2CONH$-			>100		
15	$^{+}NH_3$-CH_2COO-			—[a]	MCF7	Arya et al., 1998
16	$^{+}NH_3Lys(NH_3)COO$-			12		
17	Lys-Lys(Lys)COO-			—[a]		
18	CH_3O-			—[a]	Jurkat	Neuzil et al., 2001c
19	CH_3CH_2COO-			—[d]	A549	Yano et al., 2005
20	$^{-}O_2CCH_2CH_2CH_2O$-			—[e]	LNCaP, PC-3 MDA-MB-453	Wu et al., 2004; Nishikawa et al., 2003
21	$^{-}O_2CCH_2O$-			—[f]	MDA-MB-435, MCF7	Shun et al., 2004
22	$^{-}O_2CCH_2$-			15–20[g]	MCF7	Shiau et al., 2006
23	(PEG)$O_2CCH_2CH_2COO$-			—[h]	Lung carcinoma cells/ nude mice	Youk et al., 2005
24	$^{-}O_2C(CH_2)_5COO$-			—[a]	C127I	Kogure et al., 2004
25	$C_2H_5OOCCH_2CH_2COO$-			—[a]		
26	Nicotinic acid			—[a]		
27	$^{-}O_2CCH_2CH(SePh)COO$-			Unknown	Prostate	Vraka et al., 2006
28	all-*trans* retinoic acid			0.1–1	NB4, HT93	Makishima et al., 1996, 1998
29	9-*cis* retinoic acid			—[b]		
30	$HOPO_2O$-			—[b]	RASMC, THP-1	Munteanu et al., 2004
31	Toc-OPO_2O-			—[b]		
32	$^{-}O_2CCH_2CH_2COO$-			50% of α-TOS	Jurkat, HBT11, MCF7, MCF7-C3, U937, Meso-2	Birringer et al., 2003; Tomic-Vatic et al., 2005
33	$^{-}O_2CCH_2CH_2COO$-			—[b]	Jurkat, HBT11, MCF7, MCF7-C3	Birringer et al., 2003; Vraka et al., 2006
34	$^{-}O_2CCH_2CH(SePh)COO$-			—[b]	Prostate	Vraka et al., 2006
35	$^{-}O_2CCH_2CH_2COO$-			66	Jurkat, HBT11, MCF7, MCF7-C3	Birringer et al., 2003; Tomic-Vatic et al., 2005
36	$^{-}O_2CCH{=}CHCOO$-			49	Jurkat, U937, Meso-2	Tomic-Vatic et al., 2005
37	$^{-}O_2CCH_2CH_2CONH$-			20		
38	$^{-}O_2CCH{=}CHCONH$-			9		
39	$H_3COOCCH_2CH_2COO$-			—[a]		Birringer et al., 2003
40	HO-			—[b]	PC-3	Galli et al., 2004

[a] No effect.
[b] Inhibition of cell proliferation.
[c] Much more cytotoxic than α-TOS.
[d] Less effective than compound 54.
[e] The ether analog is less effective than α-TOS itself.
[f] Comparable with α-TOS.
[g] EC_{50} value in micrograms per milliliter.
[h] More efficient than α-TOS.

tinal mucosal cells and in the blood, whereas peptidases exhibit a much narrower specificity. For example, prodrugs with an amide linkage are more stable in the intestine and blood than their corresponding ester analogs (Sugawara et al., 2000).

Finally, the last group of compounds consisted of a series of lysine α-tocopheryl esters with a positively charged N terminus (**15**-**17**). It is interesting that the hydrophilic ammonium functionality exerted proapoptotic effects similar to those of its carboxylate counterpart, suggesting that a general motif is required for activity that consists of a lipophilic side chain and a hydrophilic head group. However, succinyl esters of long-chain aliphatic alcohols (e.g., phytol and oleol) did not show any activity (Birringer et al., 2003).

A general SAR can be drawn from the data in Table 2: 1) to gain apoptotic activity, modifications of the functional domain require a hydrophilic head group consisting of a dissociated acid or a charged ammonium group; 2) the chain length and the degree of unsaturation of the functional domain determine the activity. Conformational restrictions seem to potentiate the activity; and 3) the chemical linkage of the functional domain is not limited to esters, and other functionalities prevent enzymatic degradation of the analogs.

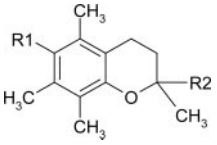
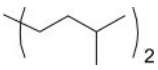
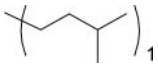
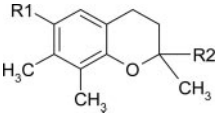
Influence of the Substitution Pattern of the Signaling Domain

The substitution pattern of the chromanol ring is often not merely related to the antioxidant properties of tocopherols (Azzi et al., 2002). Different biochemical observations emphasize the role of α-tocopherol in signaling and metabolic processes. α-Tocopherol is selectively recognized in the liver by α-tocopherol transfer protein. The relative affinities for α-tocopherol transfer protein decrease with the loss of methylation of the chromanol ring (Hosomi et al., 1997). The recently discovered tocopherol-associated proteins show similar preferences in tocopherol binding (Yamauchi et al., 2001). In endothelial cells, thrombin-induced PKC activation and endothelin secretion are inhibited by α-tocopherol but not by β-tocopherol (Martin-Nizard et al., 1998). At the transcriptional level, α-tocopherol causes up-regulation of α-tropomyosin expression (Aratri et al., 1999) and down-regulation of low-density lipoprotein scavenger receptors SR-A and CD36, whereas β-tocopherol is ineffective (Ricciarelli et al., 2000; Devaraj et al., 2001). In addition, the substitution pattern is probably responsible for the rate of side-chain degradation because γ- and δ-tocopherol are degraded much faster than α- or β-tocopherol (Birringer et al., 2001). Succinylation of the four tocopherol isomers produces the compounds **1**, **32**, **33**, and **35**. Of these, α-TOS (**1**) possesses the highest apoptogenic activity, followed by β-TOS (**32**), γ-TOS (**33**), and δ-TOS (**35**) (Birringer et al., 2001). In general, the more highly methylated members of the tocopherol family are the most potent, but this trend is reversed for tocotrienols (see below).

Modifications of the Hydrophobic Domain and Tocotrienols

Succinylation of 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid, a water-soluble VE derivative, yields a compound with no apoptogenic activity. SAR experiments of various tocopherol succinates bearing truncated phytol side chains (**43**-**45**) revealed that the highest level of apoptogenic activity in prostate cancer cells was obtained with derivatives in which the side-chain length was two isoprenyl units (**43** and **44**). Computer-assisted molecular modeling and coimmunoprecipitation experiments showed that the binding of Bak BH3 peptide to Bcl-x_L and Bcl-2 was inhibited by the tocopherol analogs (Shiau et al., 2006). Central requirements

TABLE 3
Antiproliferative activity of vitamin E analogs with a modified hydrophobic domain

Compound Number	Functional Domain I (R1)	Signalling Domain II	Hydrophobic Domain III (R2)	IC_{50}	Cell Type	References
				μM		
41	$^{-}O_2CCH_2CH_2COO$-		COO^{-}	—[a]	Jurkat, HBT11, MCF7, MCF7-C3	Birringer et al., 2003
42	HO-			—[a]	LNCaP, PC-3	Shiau et al., 2006
43	$^{-}O_2CCH_2CH_2COO$-			4–9		
44	$^{-}O_2CCH_2CH_2O$-			4–8		
45	$^{-}O_2CCH_2CH_2COO$-			8–19		
46			CH_3	>100		
47	$^{+}NH_3Lys(NH_3)COO$-		CH_2-OH	194	MCF7	Arya et al., 1998
48			CH_2-O-nC_5H_{11}	22		
49			CH_2-OC(O)nC_4H_9	15		
50			CH_2-O-cholic acid	4		
51	HO-		$CH_2CH_2COO^{-}$	—[b]	PC-3	Galli et al., 2004
52	HO-		$CH_2CH_2COO^{-}$	—[c]		

[a] No effect.
[b] Weak inhibition at 50 μM.
[c] 82% inhibition at 10 μM.

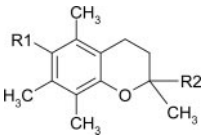
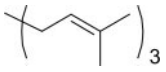
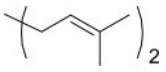
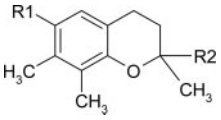
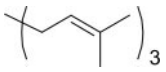
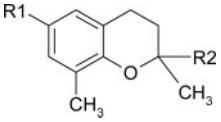
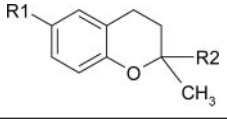
for antineoplastic activity were succinylation of the chromanol ring and a minimum chain length of one isoprenyl unit (**42** and **46**). A series of tocopheryl lysine esters with ether/ester-linked domain III side chains showed a negative correlation between chain length and IC_{50} values (**47**-**50**) (Table 3) (Arya et al., 1998).

Tocotrienols are efficient anticancer agents, and their proapoptotic property may be related to inactivation of the Ras family of proteins. Tocotrienols exhibit their proapoptotic activity without modifications of the functional domain. The hierarchy in the signaling domain is reversed, making δ-tocotrienol (**59**) the most potent agent, followed by γ- (**56**) and α-tocotrienol (**53**) (Table 4) (He et al., 1997). It is interesting that desmethyl tocotrienol (**60**), lacking all aromatic methyl groups, shows even higher activity with an IC_{50} value of 0.9 μM. This compound has been isolated from rice bran (Qureshi et al., 2000). A direct inhibitory action of tocotrienols has been proposed because the membrane-anchoring cysteine residue of Ras proteins is modified by a common structural element, a farnesyl chain. Thus, Ras farnesylation and RhoA prenylation was inhibited by tocotrienols in A549 cells containing an activating *ras* mutation (Yano et al., 2005). To expand the short in vivo half-life of tocotrienols, functional domains have been introduced. These modifications also enhanced the antiproliferative activity of the molecules (**54**, **57**, and **58**). Truncation of the side chain also improved activity, similar to that found for compound **55**.

Other Tocopherol Derivatives with Antiproliferative Activity

A number of compounds in which modifications have been made to the functional domain exhibit antiproliferative activity and provide additional specialized properties. For example, α-tocopheryl polyethylene glycol succinate (**23**) has

TABLE 4
Antiproliferative activity of vitamin E analogs
Compounds are sorted by the signaling domain.

Compound Number	Functional Domain I (R1)	Signalling Domain II	Hydrophobic Domain III (R2)	IC_{50}	Cell type	References
				μM		
53	HO-			210	MDA-MB-435	Guthrie et al., 1997
				14	MCF7	
				110	B16(F10)	He et al., 1997
54	CH_3CH_2COO-			—[a]	A549	Yano et al., 2005
55	HO-			—[b]	Jurkat, HBT11, MCF7, MCF7-C3	Birringer et al., 2003
56	HO-			4	Neoplastic + SA mammary epithelial cells	Shah and Sylvester, 2005
				15[c]	MCF7	He et al., 1997
				—[d]	Jurkat, HBT11, MCF7, MCF7-C3	Birringer et al., 2003
				20	B16(F10)	He et al., 1997
57	$^{-}O_2CCH_2CH_2COO$-			—[e]	Jurkat, HBT11, MCF7, MCF7-C3	Birringer et al., 2003
				—[f]	Prostate	Vraka et al., 2006
58	$^{-}O_2CCH_2CH(SePh)COO$-			—[f]	Prostate	Vraka et al., 2006
59	HO-			10	B16(F10)	He et al., 1997
				—[b]	MDA-MB-435, MCF7	Shun et al., 2004
				15[c]	MCF7	Nesaretnam et al., 1998
60	HO-			0.9	B16(F10)	He et al., 1997

[a] Cytotoxic in 0 to 40 μM range.
[b] Very potent.
[c] Complete inhibition.
[d] Comparable with α-TOS.
[e] 2-Fold more potent than γ-tocotrienol.
[f] Inhibition of cell proliferation.

been used as a vehicle for drug delivery systems. This compound was shown to possess anticancer activity against human lung carcinoma cells implanted in nude mice. The apoptosis-inducing efficacy of the compound was not due to its increased uptake into cells but rather to an increased ability to generate ROS (Youk et al., 2005). α-Tocopheryl phosphate (**30**) is believed to result from metabolism occurring during tocopherol-associated signaling (Negis et al., 2005). Mixtures of compound **30** and di-α-tocopheryl phosphate (**31**) inhibited proliferation in rat aortic smooth muscle cells and in the THP-1 monocytic leukemia cells (Munteanu et al., 2004). The authors proposed that tocopheryl succinate and tocopheryl maleate may act in cancer cells by mimicking and substituting for tocopheryl phosphate and causing permanent activation of cellular signaling.

Two experimental α-tocopheryl esters of all-*trans* retinoic acid (**28**) and 9-*cis* retinoic acid (**29**), respectively, have been used to reduce proliferation of acute promyelocytic leukemia cells (Makishima et al., 1998). Transactivation experiments with retinoid receptor-responsive reporter constructs revealed that both compounds acted as agonists for retinoic acid receptors. γ-Carboxyethyl hydroxychroman (**52**), a degradation product of γ-tocopherol often found secreted in the urine, reduces cell proliferation of PC-3 prostate cancer cells by inhibiting cyclin D1 expression (Galli et al., 2004).

Molecular Mechanism of Apoptosis Induced by VE Analogs

The Role of Mitochondrial Signaling

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 pathogeneses. There are many triggers of apoptosis, including increased levels of oxidants within the cell, damage to DNA by these oxidants or other agents (such as ultraviolet light, X-rays, and chemotherapeutic drugs), accumulation of proteins that fail to fold properly, or signaling by molecules binding to death receptors. Mitocans induce apoptosis by initiating the mitochondrial (intrinsic) pathway.

ROS generation is important in apoptosis induction involving mitochondria. Treatment of cells with α-TOS causes generation of ROS (Ottino and Duncan, 1997; Kogure et al., 2001, 2002; Weber et al., 2003; Stapelberg et al., 2005; Swettenham et al., 2005; Wang et al., 2005). Generation of ROS is an early event occurring in cells in response to VE analogs, and we have observed the accumulation of ROS in Jurkat T lymphoma cells within 1 h of treatment with α-TOS. The major form of ROS generated by cells in response to α-TOS is superoxide, because addition of superoxide dismutase removes the radicals and inhibits apoptosis (Kogure et al., 2001; Wang et al., 2005). Moreover, the site of superoxide generation and the target of ROS are very likely to be the mitochondria, as suggested by experiments in which the mitochondrially targeted coenzyme Q (Kelso et al., 2001) suppressed ROS accumulation and inhibited apoptosis induced by α-TOS (Stapelberg et al., 2005; Wang et al., 2005). It has been reported that α-TOS-induced apoptosis was more pronounced in cancer cells with reduced antioxidant capacity (Kogure et al., 2002). One of the major contributors to cellular ROS production seems to be the mitochondrial complex II in the respiratory chain (McLennan and Degli-Esposti, 2000), and we are currently exploring its role in apoptosis induced by VE analogs.

The earliest effect observed upon exposure of cells to α-TOS is activation of sphingomyelinase (SMase), an enzyme that converts sphingomyelin to the apoptogenic ceramide (Ogretmen and Hannun 2004). Treatment of Jurkat cells resulted in activation of SMase within 15 to 30 min, and this action was not suppressed by a caspase inhibitor, suggesting a caspase-independent, possibly direct targeting of the VE analog affecting SMase (Weber et al., 2003). It is possible that activation of SMase is caused by a change in the plasma membrane fluidity upon incorporation of the lipophilic α-TOS and would be consistent with a recently suggested mechanism for chemotherapy-induced cell death (Dimanche-Boitrel et al., 2005). Generation of the lipid second-messenger ceramide as a very early response to α-TOS may also provide an explanation for the activation of protein phosphatase 2A (PP2A) and the ensuing hypophosphorylation of PKCα in cells exposed to α-TOS, because the agent does not directly target PP2A (Neuzil et al., 2001c). This is consistent with the previous finding that long-chain ceramides are activators of PP2A (Ruvolo et al., 1999).

Initiation of apoptotic pathways leading to mitochondria-dependent events are likely to result from the actions of α-TOS directly on mitochondria and/or via ceramide formation, with both processes having a net effect of destabilizing the mitochondrial membrane. The apoptotic action of α-TOS may also be initiated and/or amplified by ROS, generated during the cellular response to the agent (Ottino and Duncan, 1997; Kogure et al., 2001).

During apoptosis induced by VE analogs, down-stream events after mitochondrial destabilization involve mobilization of apoptotic mediators, including cytochrome *c*, the apoptosis-inducing factor, and Smac/Diablo (Neuzil et al., 2004). Cytochrome *c*, upon cytosolic translocation, triggers activation of the caspase cascade, transferring the cells into the commitment phase of apoptosis (Yamamoto et al., 2000; Neuzil et al., 2001a; Weber et al., 2003). It is now clear that this particular pathway is critically important in apoptosis induced by α-TOS in a variety of cancer cells (Neuzil et al., 2004).

Smac/Diablo is an important agonist of the caspase-dependent apoptotic signaling, because it antagonizes the caspase-inhibitory members of the family of inhibitors of apoptosis proteins (IAPs) (Du et al., 2000; Verhagen and Vaux, 2002). The expression of IAPs is under control of the transcriptional factor nuclear factor-κB (NFκB), whose activity is inhibited by α-TOS (Erl et al., 1997; Neuzil et al., 2001a; Dalen and Neuzil, 2003). Thus, cytosolic translocation of Smac/Diablo may promote inhibition of the survival pathways in apoptosis induced by α-TOS, which may maximize the apoptogenic potential in resistant cells (Wang et al., 2005).

The mitochondrial pro- and antiapoptotic proteins, including Bax, Bcl-2, Mcl-1, and Bcl-x_L, are important modulators of apoptotic signaling (Cory et al., 2003). Generation of the MPTP has also been suggested in cells exposed to α-TOS (Yamamoto et al., 2000). It is likely that this is modulated by a cross-talk between the mitochondrial pro- and antiapoptotic proteins (Yamamoto et al., 2000; Weber et al., 2003). Overexpression of Bax results in cells becoming sensitized to α-TOS-induced apoptosis (Weber et al., 2003; Yu et al., 2003), whereas overexpression of Bcl-2 or Bcl-x_L protected them

from α-TOS. Likewise, down-regulation of Bcl-2 by antisense oligodeoxynucleotide treatment sensitized cells to the VE analog (Neuzil et al., 2001a,c; Weber et al., 2003).

Probably the most compelling evidence for mitochondria as major transmitters of apoptotic signaling induced by VE analogs stems from experiments in which ρ^0 cells were found to be resistant to α-TOS (Weber et al., 2003; Wang et al., 2005). We 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 (Weber et al., 2003). Similar resistance of ρ^0 cells has been found for other inducers of apoptosis, including tumor necrosis factor-α (TNFα) (Higuchi et al., 1997). Although mitochondria are central to apoptosis induction by VE analogs, a number of nonmitochondrial pathways seem to amplify the process, as reviewed below.

Nonmitochondrial Signaling Pathways and Apoptosis Induction by VE Analogs

Activation of Death Receptors by VE Analogs. Activation of the extrinsic cell death pathway is initiated by ligation of cell surface death receptors (DRs), which include 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 preclinical models. Impaired apoptotic signaling pathways endow some types of malignant cells with resistance to DR-mediated apoptosis, and such tumors are difficult to treat (O'Connell et al., 2000; Srivastava, 2001; Cretney et al., 2002). It has been reported that α-TOS-mediated apoptosis involves DR signaling. 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; Yu et al., 1999). In a separate study, expression of Fas, the Fas-associated death domain, and caspase-8 was enhanced after α-TOS treatment in gastric cancer cells, whereas Fas antisense oligonucleotide inhibited 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. Tumor cells escape from TRAIL-modulated killing when the balance between DRs and the nonapoptogenic decoy receptors is altered, and expression of the latter predominates. 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 showed a synergistic proapoptotic activity with TRAIL both in vitro and in experimental colon cancer (Weber et al., 2002). α-TOS also sensitized to TRAIL the resistant MM and osteosarcoma (OS) cells. The IC_{50} value for TRAIL was greatly decreased by treating MM cells with sublethal doses of α-TOS, whereas an antagonistic effect of α-TOS on TRAIL sensitivity was found in the case of nonmalignant mesothelial cells (Tomasetti et al., 2004b). 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 expression of DR4 and DR5 without modulation of expression of the decoy receptors in MM cells (Tomasetti et al., 2004b, 2006). α-TOS also enhanced sensitivity of Jurkat T lymphoma cells to apoptosis induced by TRAIL by suppression of NFκB activation (Dalen and Neuzil, 2003). Thus, VE analogs may play a role in adjuvant therapy of DR-resistant cancers. These analogs 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 tumor surveillance.

Involvement of the MAPK Pathway in Apoptosis Induced by VE Analogs. 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 that is related to many disorders (Fang and Richardson, 2005). 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 (Nishina et al., 2004). Kline's group first reported a role of JNK and c-jun in α-TOS-induced apoptosis. The VE analog up-regulated c-jun expression in different types of cancer cells (Qian et al., 1996; Yu et al., 1997a, 1998). 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 (Yu et al., 2001). Increased phosphorylation and transactivation of c-jun and ATF-2 were 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 protein expression of phospho-JNK was also noticeably increased by α-TOS in prostate cancer cells (Zu et al., 2005). In addition, JNK and c-jun were important in α-TOS-induced apoptosis in SGC-7901 gastric cancer cells (Zhao et al., 2002; Wu et al., 2004a). Dominant-negative JNK significantly reduced c-jun expression and apoptosis triggered by α-TOS. 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 (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 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 for apoptosis induced by α-TOS. However, it is not clear at this stage how this signaling pathway is linked to destabilization of mitochondria by the VE analog.

The Role of Protein Kinase C in α-TOS-Triggered Apoptosis. PKC, a multigene family of phospholipid-dependent serine/threonine protein kinases, is involved in modulation of divergent biological functions (Spitaler and Cantrell, 2004). 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 (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.

Role of Nuclear Factor-κB in Apoptosis Induced by VE Analogs. Activation of the multicomplex transcription factor NFκB is crucial for a wide variety of cellular responses. In nonstimulated cells, NFκB is sequestered in the cytoplasm by the inhibitory κB (IκB). Upon activation by a number of stimuli, IκB proteins are 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 antiapoptotic activities. Thus, NFκB activation stimulated by TNFα 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 -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 by modulating the degradation of IκB, sensitizing cells to TRAIL (Dalen and Neuzil, 2003).

Although there are a number of signaling pathways involved in apoptosis induced by VE analogs, mitochondria are still the major target. The various pathways are probably triggered via the initial effect of VE analogs on mitochondria and may contribute to the main, intrinsic apoptogenic pathway, thereby maximizing the outcome; or, as discussed below, VE analogs by inducing the various signaling pathways may sensitize cancer cells to other unrelated apoptogens.

Synergism of VE Analogs with Other Inducers of Apoptosis

Resistance to chemotherapy is the principal cause of cancer treatment failure. Development of cancer involves the acquisition of multiple genetic aberrations that reduce cellular susceptibility to apoptosis and confers resistance to therapy (Mow et al., 2001; Kaufmann and Vaux, 2003). Multiple drug resistance has long been considered to be multifactorial, and numerous mechanisms have been shown to confer changed sensitivity to both chemotherapeutic and immunological agents in vitro (Zhang et al., 2000; LeBlanc et al., 2002; Rippo et al., 2004). Considerable effort has been devoted to reversing multiple drug resistance mechanisms by using resistance modulators, which are either functional blockers or expression modulators of the ATP-binding cassette family of drug efflux pumps, such as the P-glycoprotein and the multidrug resistance protein-1 (Borst et al., 2000). It has been shown that α-TOS synergistically enhanced the cytotoxic effect of etoposide (V-16) in glioblastoma cells expressing multidrug resistance protein-1 (Kang et al., 2005). The ability of α-TOS to decrease the intracellular concentration of glutathione seems to have multiple effects on the etoposide response, including enhanced intracellular accumulation of VP-16 (i.e., decreased VP-16 efflux) and potentiation of VP-16-induced ROS generation and consequently induction of apoptosis.

α-TOS induces a variety of concentration-dependent cellular events. More specifically, it modulates signaling pathways in various in vitro models, in general in the 10- to 30-μM range, whereas its cytotoxic effect becomes prominent at higher concentrations (You et al., 2001). α-TOS enhances the growth-inhibitory effect of several chemotherapeutic agents on cancer cells in culture. For instance, it augments the effects of doxorubicin (Adriamycin; Pfizer, New York, NY) on prostate carcinoma cells (Ripoll et al., 1986); the effects of cisplatin, tamoxifen, and decaprazine on melanoma cells (Prasad et al., 1994) and parotid acinar carcinoma cells (Prasad and Kumar, 1996); and the effects of doxorubicin on leukemia cells (Fariss et al., 1994).

Cell cycle arrest during S/G_2 transition was observed in OS cell lines exposed to sublethal doses of α-TOS, which sensitized them to methotrexate (MTX) (Alleva et al., 2006). MTX is a cell cycle-specific chemotherapeutic agent currently used to treat human osteosarcoma, acting via its inhibitory effect on dihydrofolate reductase (DHFR) (Serra et al., 2004). Although MTX is one of the most important drugs in OS therapy, a considerable number of patients develop drug resistance and die (Bruland and Pihl, 1997). Intrinsic resistance to MTX can occur through impaired transport of drugs into cells via the reduced folate carrier, an increase in DHFR due to gene amplification or increased transcription (Guo et al., 1999; Cole et al., 2002; Serra et al., 2004), and circumvention of the inhibition of de novo nucleotide biosynthesis via the salvage of extracellular nucleosides and bases (Serra et al., 2004). The E2F family of transcription factors is known to be involved in the transcriptional regulation of several DNA synthesis enzymes and common chemotherapeutic targets (Wells et al., 1997). E2F1 is the transcription factor most closely associated with thymidylate synthase expression (Wells et al., 1997), whereas E2F4 has been shown to be a regulator of DHFR expression (DeGregori et al., 1995). However, data from a population study suggest that E2F1 could be an important regulator of DHFR expression (Sowers et al., 2003).

It has been observed that α-TOS induced cytostasis or cell death in OS cells involving the transcription factor E2F1 (Alleva et al., 2005). The VE analog can increase expression of E2F1 in the presence of functional p53, which in turn induces apoptosis. However, α-TOS induced down-regulation of E2F1 and subsequent S/G_2 transition arrest in the $p53^{-/-}$ MG63 and the SAOS OS cells that contain a truncated form

of the retinoblastoma protein. Down-regulation of E2F1 could inhibit expression of genes involved in DNA synthesis, sensitizing OS cells to drugs destabilizing DNA during its replication. Combining MTX with α-TOS induced cell death in SAOS and MG63 cells that were otherwise resistant to MTX. MTX/α-TOS treatment was shown to induce apoptosis and decreased cell viability by caspase activation.

α-TOS can induce cancer cells to undergo apoptosis by modulating several signaling pathways, including the transforming growth factor-β, JNK, MAPK, and TNF routes (Yu et al., 1997b, 1998, 1999). Among the TNF ligand members, TRAIL has recently drawn interest as a potential effective antitumor therapeutic agent. TRAIL is largely selective for malignant cells, whereas the Fas ligand is toxic to normal cells (Pitti et al., 1996; French and Tschopp, 1999). Although both DRs are widely expressed in human tissues, some cancer cells are insensitive to TRAIL-mediated killing (Degli-Esposti et al., 1997; Ashkenazi and Dixit, 1998; Rippo et al., 2004). Heterogeneous sensitivity of tumor cells to TRAIL-induced apoptosis has been observed in MM cells, which may lead to a persistent growth of TRAIL-resistant cells, limiting successful treatment of neoplastic diseases by the ligand. A synergistic and cooperative effect was observed in MM cells by combining α-TOS and TRAIL, and the effect was selective for cancer cells (Tomasetti et al., 2004a). Impaired apoptotic pathways contribute to render MM cells resistant to TRAIL-induced apoptosis. Sublethal doses of α-TOS significantly decrease the high IC_{50} values for TRAIL by a factor of ~10 to 100. The observation that α-TOS and TRAIL synergize in $p53^{wt}$ MM but not in the $p53^{-/-}$ cells suggests a role of p53 in transactivation of the proapoptotic genes involved in the drug synergism (Tomasetti et al., 2006).

At low concentrations, α-TOS induces expression and activation of p53, which in turn induces expression of DR4 and DR5. Studies using small interfering RNA directed at p53 revealed that the p53 protein contributes significantly to the expression of TRAIL DRs. It was observed that α-TOS-induced expression and activation of the p53 protein was enhanced in the presence of antioxidants with a high reducing potential, such as *N*-acetylcysteine, which changes the cell's redox state. Regulation of activity of transcription factors by redox modulators has been described previously (Sun and Oberley, 1996). Thus, a novel mode of action of α-TOS has been proposed as follows: reduction of p53 leads to an increase in the efficiency of TRAIL's DR expression, sensitizing MM cells to TRAIL-induced apoptosis.

Although MM cells express DR4 and DR5 on the cell surface, exogenous TRAIL was ineffective at inducing apoptosis. An apical receptor-mediated apoptotic block was observed in MM cells as a result of overexpression of the caspase-8 inhibitor FLIP (Rippo et al., 2004). Up-regulation of TRAIL DRs by α-TOS may contribute to a shift in the balance between the anti- and proapoptotic signals in favor of the latter, triggering apoptotic signals, which may then be amplified by

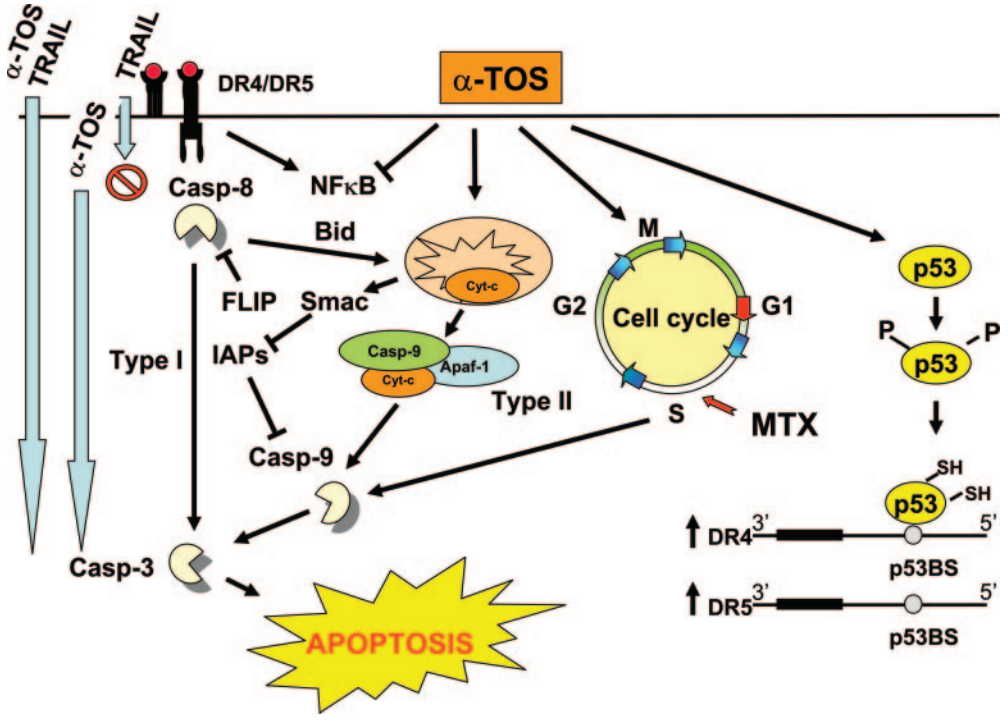


Fig. 2. Possible pathways of α-TOS-sensitized apoptosis. Immunological ligands induce apoptosis by trimerization of their cognate death DRs followed by caspase-8 activation. Active fragment of caspase-8 can directly cleave caspase-3 (type I cells) or indirectly activate caspase-9 through the intrinsic pathway by release of cytochrome *c* (type II cells). The final activation of caspase-3 leads to apoptosis, resulting in DNA fragmentation and chromosomal condensation. Various antiapoptotic proteins inhibit each signaling event, such as FLIP, which suppresses the activation of caspase-8, and IAP family proteins directly inhibiting caspase-9. Anti- and proapoptotic factors are regulated by the cellular transcription system. Two important transcriptional factors, NFκB and p53, are activated in response to the apoptotic stimulus. α-TOS directly induces expression and activation of the p53 protein. Phosphorylated p53 accumulates in the nucleus and directly regulates transcription of the DR4/DR5 gene via an intronic sequence-specific p53 binding site, which is regulated by a redox mechanism. Increased expression of DRs on cell surface overcomes the apoptotic pathway blocked at the death-inducing signaling complex level, triggering apoptotic signaling that is amplified by activation of the mitochondrial pathway (type II cells). Activation of NFκB negatively modulates apoptosis dependent on immunological inducers by transcription of survival factors, such as FLIP and the IAP proteins. α-TOS inhibits NFκB activation, thereby amplifying the cell susceptibility to immunological stimuli. α-TOS also induces cell cycle arrest by down-regulation of CDKs and cyclins. The S/G_2 transition arrest sensitizes otherwise resistant malignant cells to apoptosis inducers like MTX.

the intrinsic apoptotic pathway. Kinetic analysis of TRAIL-induced signaling revealed a transient activation of caspase-8, which resulted in induction, albeit low, of apoptosis. Caspase-8 activation was less pronounced in the presence of TRAIL plus α-TOS. Under this setting, activation of the mitochondria-dependent apoptotic pathway, including Bid cleavage, cytochrome *c* cytosolic mobilization and caspase-9 activation were observed. Bid cleavage may lead to mitochondrial translocation of Bax, as shown for α-TOS in other cancer models (Weber et al., 2003, Yu et al., 2003). Thus, there is a cross-talk between α-TOS and TRAIL in potentiation of apoptosis in the TRAIL-resistant MM cells, in particular in linking the receptor- and mitochondria-associated events.

A cooperative proapoptotic effect of α-TOS with immunological apoptogens has been also observed in breast (Yu et al., 1999) and colon cancer cells and in an animal model showing a combined effect against tumor growth (Weber et al., 2002). The study of Yu et al. (1999) showed that α-TOS converted Fas-resistant to -sensitive cells via mobilization of the Fas receptor from the cytosol to the plasma membrane. α-TOS enhanced the sensitivity of Jurkat T lymphoma cells to the induction of apoptosis by TRAIL, and the effect was not observed with α-TOH (Dalen and Neuzil 2003). A transient NFκB activation was found when Jurkat cells were exposed to TRAIL. It is known that NFκB controls expression of prosurvival genes, including FLIP (Kreuz et al., 2001) and the IAP family members (Degli-Esposti et al., 1997). Therefore, it is tempting to postulate that α-TOS inhibits NFκB activation induced by TRAIL, which in turn results in lower expression of survival proteins that confer resistance of cell to TRAIL-induced apoptosis.

In conclusion, combination of α-TOS with chemotherapeutic or immunological agents (e.g., TRAIL) efficiently enhances the apoptotic effect. The simultaneous delivery of different death signals may converge to promote apoptosis of tumor cells. These findings provide the molecular rationale for the use of α-TOS as anticancer agents alone or, in particular, in combination with other anticancer drugs currently used in clinical practice. The molecular bases for some of these effects are depicted in Fig. 2.

Conclusions and Perspectives

The above evidence suggests that mitocans from the group of VE analogs are efficient and anticancer agents with great promise for future clinical applications. One of the most intriguing aspects of VE analogs from the ester group of compounds is that they show at least two different bioactivities. Thus, compounds like α-TOS have to reach the circulation, within which they bind to circulating lipoproteins, which will transport them to the tumor, in which they exert their anticancer activity. They are then gradually cleared via the hepatic system, in which esterases cleave the compounds to yield VE (α-TOH in case of α-TOS). VE is partially resecreted into the bloodstream, boosting the antioxidant and anti-inflammatory defenses. Thus, not only are α-TOS and similar compounds metabolized into harmless products, but even more advantageous, they are converted to VE with a secondary beneficial bioactivity.

α-TOS and several other analogs of VE are effective against a variety of cancers, including the difficult-to-treat erbB2-high breast cancers and the thus-far-untreatable mesotheliomas. The highly apoptogenic α-tocopheryl maleyl amide is very toxic to mice when administered as a corn oil emulsion by intraperitoneal injection. However, when formulated into liposomes, it selectively suppresses experimental tumors without showing adverse effects on the animals (J. Neuzil, unpublished data).

Thus, these intriguing mitocans from the group of VE analogs are promising anticancer agents whose molecular mode of action is now better understood. We believe that the greatest problem at this stage is the logistics of their safe delivery to the tumor in sufficient amounts. Liposomal formulations for intravenous delivery or transdermal/transmucosal applications using formulations of the agents in a cream containing an appropriate drug carrier may be a suitable way for administering them to patients.

To summarize, a host of data on the mode of action and selectivity of the anticancer VE analogs has been amassed. This warrants a trial of the prototypic and very economical α-TOS in patients with recurring cancers such as the erbB2-high breast carcinomas (to potentially displace the costly Herceptin treatment) or the fatal mesotheliomas. Finally, we propose that we are witnessing the emergence of VE analogs from their "infancy years" to be fully recognized as potent anticancer drugs.

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