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MINI REVIEW

Mitocans as anti-cancer agents targeting mitochondria: lessons from studies with vitamin E analogues, inhibitors of complex II

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Abstract Recently mitochondria in cancer cells have emerged as the Achilles heel for tumour destruction. Anti-cancer agents specifically targeting cancer cell mitochondria are referred to as 'mitocans'. These compounds act by destabilising these organelles, unleashing their apoptogenic potential, resulting in the efficient death of malignant cells and suppression of tumour growth. Importantly, at least some mitocans are selective for cancer cells, and these are represented by the group of redox-silent vitamin E analogues, epitomised by α-tocopheryl succinate (α-TOS). This compound has proven itself in pre-clinical models to be an efficient anti-cancer agent, targeting complex II of the respiratory chain to displace ubiquinone binding. We propose that disrupting the electron flow of mitochondrial complex II results in generation of superoxide, triggering mitochondrial destabilisation and initiation of apoptotic pathways. Moreover, α-TOS is selective for cancer cells with their reduced antioxidant defenses and lower esterase activity than the normal (non-malignant) counterparts. In this mini-review we discuss the emerging significance of mitocans, as exemplified by α-TOS.

Keywords Mitocans · Mitochondria · Complex II · Coenzyme Q · Vitamin E analogues · Superoxide · Apoptosis · Cancer

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Introduction

Recent progress in molecular medicine has greatly contributed to the installation of novel therapeutic approaches for a variety of pathologies, particularly in the area of cancer. Albeit, neoplastic disease remains a significant challenge, particularly since continual mutations make malignant cells resistant to established therapeutics. Hence, new approaches resolving the current dilemma are to be sought and put into practice. Mitochondria are emerging as a novel target providing considerable promise for intervention and treatment of cancer (Fantin and Leder, 2006; Galuzzi et al., 2006), because they are essential for cell survival, providing sources of cellular energy, as well as central purveyors

of cell death, containing many of the important mediators of apoptosis (Newmeyer and Ferguson-Miller 2003). These seemingly opposing roles for mitochondria are reconcilable upon reflection. Under normal conditions, mitochondria promote requisite cellular functions by carrying out oxidative phosphorylation, culminating in ATP production required for many metabolic processes. Mitochondria can also be perceived as sensors for cellular damage, whereby unfavourable conditions within a cell might arise that if left unchecked would lead to the potential demise of the whole organism. In this situation, the organism benefits from the mitochondrially triggered death of the afflicted cell. Therefore, mitochondria are the trigger point by which danger signals including the generation of reactive oxygen species (ROS), such as superoxide, switch these organelles from 'pro-life' to become 'pro-death' agents, initiating intrinsic apoptotic pathways (Leonard and Schapira, 2000; McLennan and Degli-Esposti, 2000; Raha and Robinson, 2000).

We have recently defined a class of compounds, referred to as 'mitocans' (Neuzil et al., 2006; Ralph et al., 2006). This group of compounds encompasses structurally distinct molecules that share one unifying feature: they all exert anticancer properties based on their ability to induce apoptosis in malignant cells by targeting mitochondria. Most importantly, a number of mitocans are selective for cancer cells, while exerting minimal toxicity towards normal cells and tissues (Ralph et al., 2006). Well documented examples include 3-bromopyruvate (3BP), an inhibitor of hexokinase and mitochondrial oxidative phosphorylation (Geschwind et al., 2002; Ko et al., 2004), as well as the family of vitamin E analogues, epitomised by α-tocopheryl succinate (α-TOS) (Neuzil et al., 2001), affecting the electron flow from complex II (unpublished data).

We review the vitamin E analogues, represented by the prototypic α-TOS, for their action on mitochondria, in particular their recently discovered activity in binding to the complex II ubiquinone site(s). We discuss the merits of a general approach whereby different compounds, as shown thus far in most detail for vitamin E analogues, could efficiently act as agents triggering apoptosis in cancer cells.

Discussion

The emergence of mitocans

Anti-cancer drugs that target mitochondria act by interfering with the energy-generating processes in the mitochondria of malignant cells. This often leads to elevated formation and accumulation of ROS that in turn induce the intrinsic, mitochondria-dependent pro-apoptotic pathways. The term 'mitocan' refers to the mitochondrial targeting and anticancer roles of such drugs (Ralph et al., 2006; Neuzil et al., 2006). The mitocan class of drugs includes compounds that affect such mitochondria-associated activities as hexokinase inhibition, activation of the mitochondrial permeability transition pore (MPTP), inhibition of the Bcl-2 anti-apoptotic proteins and blocking of the electron transport/respiratory chain. Examples of mitocans are discussed below.

Hexokinase-inhibiting compounds including glucose metabolites, 2-deoxyglucose, oxamate and 3BP form a class of mitocans selectively inducing apoptosis in cancer cells. In clinical and pre-clinical models of cancer, this mitocan class showed selective toxicity against tumour cells metabolizing anaerobically (Geschwind et al., 2002; Robey and Hay, 2005; Xu et al., 2005; Pelicano et al., 2006). These drugs are likely to enhance the efficacy of the current standard cancer chemotherapeutics and radiation regimens because they act in a different manner from the current therapies to kill cancer cells. In addition, hexokinase inhibitors may be made more effective when used in conjunction with anti-angiogenic agents to limit available oxygen supplied to the tumour.

Another form of mitocan therapy aims to obviate the protective effects of the Bcl-2 family of anti-apoptotic and pro-survival proteins over-expressed in cancer cells. For example, anti-sense oligonucleotide therapy against Bcl-2 and Bcl-x_L has been used to inhibit expression of these two pro-survival proteins in cancer cells, increasing the effectiveness of other anti-cancer chemotherapies (Cory and Adams, 2002). The discovery of the relationship between the BH4 (Bcl-2 homology 4) helix-containing anti-apoptotic proteins Bcl-2 and Bcl-x_L and their ability to form complexes by binding to the BH3 helix of the pro-apoptotic Bcl-2 family members has lead to development of a novel class of mitocans as small molecule inhibitors of Bcl-2 and Bcl-x_L (O'Neill et al., 2004; Reed and Pellecchia, 2005). Bcl-2 and Bcl-x_L both share a hydrophobic groove on their surface that binds the BH3 amphipathic helix of the pro-apoptotic family members, thereby preventing them from forming oligomers and initiating apoptosis. The hydrophobic groove of Bcl-2 and Bcl-x_L binds a range of small molecules, including natural compounds gossypol and the green tea polyphenol epigallocatechin-3-gallate, blocking BH3 binding and enabling BH3 pro-apoptotic proteins to then freely bind to their relevant targets and induce apoptosis (Degterev et al., 2001; Yin et al., 2005).

Another class of mitocans includes arsenites, used medically for many years to treat cancers including hematological malignancies. Arsenic oxides and derivatives have been used 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 modulate key cysteine residues of the adenine nucleotide translocator (ANT) channel in the

mitochondrial inner membrane of cancer cells (Belzacq et al., 2001), thereby inhibiting its activity. An ANT-inhibiting mitocan that differs from arsenites is lonidamine (an indazole carboxylate derivative), inducing activation of the MPTP and causing mitochondrial membrane permeabilization (Ravagnan et al., 1999; Belzacq et al., 2001). Although lonidamine's action on cancer cells has been well documented, clinical trails revealed it to be of little or no additional benefit over conventional therapies (Di Cosimo et al., 2003). Another group of mitocans targeting the MPTP are jasmonates that may be useful in treatment of chronic lymphocytic leukaemia and other cancers (Rotem et al., 2005).

Mitocans from the group of vitamin E analogues target complex II on mitochondria

Ester analogues of vitamin E, such as α-TOS as well as a number of other ethers (Birringer et al., 2004) and amides of vitamin E (Tomic-Vatic et al., 2005), have shown significant promise as anti-cancer agents. These compounds exert a number of activities that act by inhibiting cancer cell growth and inducing apoptosis (Wang et al., 2006). Exposure of cancer cells to α-TOS causes rapid accumulation of ROS, an event that subsequently translates into induction of apoptosis (Ottino and Duncan, 1997; Kogure et al., 2002; Weber et al., 2003; Kang et al., 2004; Stapelberg et al., 2005). The propensity for cells to accumulate ROS is a prerequisite before they can be killed by α-TOS (Kogure et al., 2002). In fact, ROS generation in response to α-TOS is not only essential for its pro-apoptotic activity but is also at least one of the reasons for the selectivity of vitamin E analogues for cancer cells (Neuzil et al., 2004). This is consistent with the situation whereby cancer cells commonly express lower levels of anti-oxidant enzymes, such as the manganese superoxide dismutase (MnSOD), than normal cells (Borello et al., 1993).

A comparison of the responses of proliferating versus growth arrested/confluent endothelial cells (ECs) to α-TOS revealed that the proliferating ECs underwent rapid cell death while the quiescent cells were surprisingly resistant (Neuzil et al., 2001). This intriguing observation may be explained by greater levels of expression of MnSOD as well as Cu,ZnSOD in the arrested ECs (J.N. et al., unpublished data), and would provide an additional benefit of α-TOS in blocking active angiogenesis in tumours. Interestingly, selectivity in killing of angiogenic ECs via ROS accumulation has also been observed for mitocans of the arsenite group of compounds (Don et al., 2003), suggesting that targeting mitochondria in angiogenic but not arrested ECs may be shared by several types of mitocans. Hence, generation of ROS is likely to be an important early event in the pro-apoptotic pathway triggered by vitamin E analogues.

Superoxide is most likely the radical generated rapidly in cells after exposure to α-TOS (Kogure et al., 2001). In agreement with this proposal, we have found that addition of SOD to cells resulted in greatly reduced levels of ROS accumulation and significantly suppressed apoptosis induced by α-TOS (Wang et al., 2004; Swettenham et al., 2005). In addition, pre-treatment of cells with the mitochondrially targeted coenzyme Q (MitoQ) (Kelso et al., 2001; James et al., 2005) also suppressed ROS accumulation and apoptosis induction by α-TOS, pointing to mitochondria as the most likely source for superoxide generation (Wang et al., 2004; Stapelberg et al., 2005).

Until recently, the mechanism by which α-TOS caused generation of superoxide was unclear. We focused our study on the complexes of the mitochondrial electron redox chain, since the evidence for their involvement as the source of ROS was significant (Leonard and Schapira, 2000; McLennan and Degli-Esposti, 2000; Raha and Robinson, 2000). In particular, complex II, exerting the succinate dehydrogenase (SDH) activity, appeared a likely target for α-TOS, as the pro-apoptotic and ROS-generating activity of α-TOS was negated by MitoQ as an agent known to target complex II (James et al., 2005). This proposal was also supported by biochemical evidence revealing that α-TOS suppressed SDH activity but not that of complex I (NADH oxidoreductase) in isolated mitochondrial fractions (L.F.D. et al., unpublished data).

Hence, the biochemical evidence identified the specific target for α-TOS binding to complex II as a competing agent for ubiquinone (UbQ) binding. This was further supported by molecular modelling based on the recently published crystal structure of porcine complex II (Sun et al., 2005). Autodock identified α-TOS binding to both the proximal (Q_P) and distal (Q_D) UbQ sites on complex II. Analysis of α-TOS binding to the UbQ sites on complex II revealed that the binding energy for the Q_P site was comparable to that of UbQ, but α-TOS bound much stronger than UbQ to the Q_D site (Fig. 1A) (L.F.D. et al., unpublished data). Upon further examination, α-TOS was found to be stabilised in the Q_P site via hydrogen bonding to Ser42 of the CybL (SDHC) subunit, and in the Q_D site via hydrogen bonding to Lys128 and Lys134 of the CybS (SDHD) subunit (L.F.D. et al., unpublished data). The importance of complex II in ROS generation and apoptosis induction was confirmed when cells lacking complex II ($CybL^{-/-}$ cells) (Oostven et al., 1995) were compared with their CybL-proficient counterparts. $CybL^{-/-}$ cells failed to accumulate radicals and showed limited apoptosis when challenged with α-TOS. On the other hand, complex I-deficient cells (Seo et al., 1998) were fully responsive to α-TOS (L.F.D. et al., unpublished data). Interestingly, complex II, including the CybL (SDHC) subunit, has been classified as a tumour suppressor gene (Albayrak et al., 2003; Ishii et al., 2005; Gottlieb and Tomlinson, 2005).

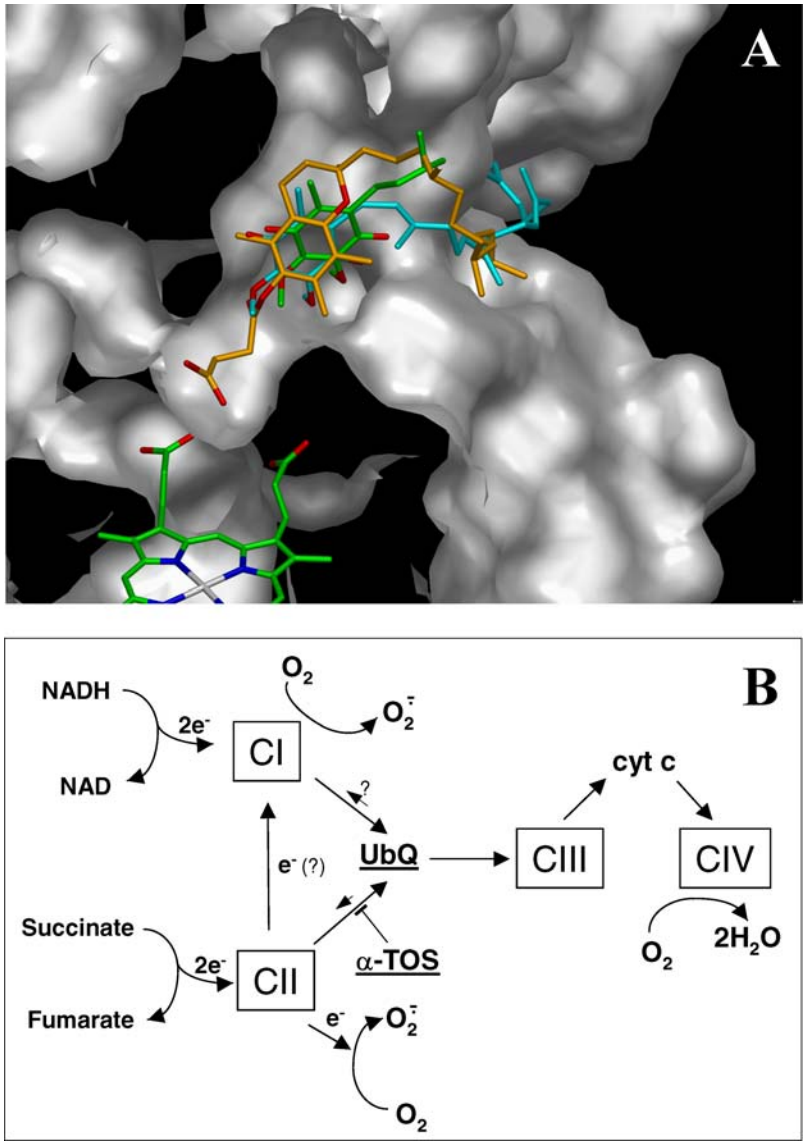

Fig. 1 α-TOS binds in the Q_P site of complex II and causes generation of superoxide **A**. A cutaway view of the Q_P binding site of complex II showing the heme group in the bottom left hand corner (green carbons), also the position of the UbQ from the crystal structure is shown with green carbons The best docked conformations of UbQ5 (cyan carbons) and α-TOS (orange carbons) are also shown (adapted from LFD et al., unpublished data) **B**. The flow chart indicates the suggested effect of α-TOS It shows the branching of the electron transport chain in its upstream region and clarifies the point of inhibition by α-TOS by specific interaction with complex II It also suggests the possible site of superoxide generation, being complex II and/or complex I.

It was proposed after elucidation of the crystal structure of bovine complex II (Sun et al., 2005) that electrons move from the FAD prosthetic group in SDH on to the two matrix subunits via a series of [Fe-S] redox clusters and possibly also via a heme group to end up at the terminal acceptor, UbQ, residing within the membrane subunits CybL and CybS. We propose that displacement of UbQ in either or both of the Q_P and Q_D sites by α-TOS will result in recombination of electrons with molecular oxygen, a reaction that will give rise to superoxide.

Complex II is the only membrane bound enzyme of the Krebs cycle, and it feeds electrons into the electron transport chain from the oxidation of succinate. The activity of complex II can be impaired by α-TOS binding, and as a result of blocking the Qp/Q_D sites electrons can escape (presumably from the flavin free radical) to form superoxide that in turn acts as an inducer of down-stream apoptotic signalling (see Fig. 1B). Although it appears that NADH oxidation by complex I and hence oxidative phosphorylation should not be affected, it should be noted that *in vivo* an inhibition of complex II stops the Krebs cycle and hence the production of NADH in the mitochondrial matrix. Under such conditions it is likely that cancer cells can compensate with an increased flux through the glycolytic pathway to generate ATP and to sustain the apoptotic cascade leading to cell death.

Superoxide generated from complex II may activate Bax

How ROS generated in mitochondria are transmitted to relay the initial stress (e.g. blocking the UbQ-binding sites on complex II) to activation of apoptosis is still uncertain. Upon exposure to inducers of apoptosis leading to generation of ROS, a chain of events is set in motion including processes such as activation of the caspase cascade and mobilization of the two

pro-apoptotic Bcl-2 family members, Bax and Bak (either or both of them) to the mitochondrial outer membrane (MOM), resulting in its destabilisation (Reed, 2006). α-TOS typifies such an apoptotic inducer, since it results in Bax movement to the MOM in cancer cells (Weber et al., 2003; Yu et al., 2003). In healthy cells, Bax is normally sequestered in the cytosol in a conformation that prevents exposure of the BH3 and trans-membrane (TM) domains, but when apoptosis is induced, these regions become exposed (Wolter et al., 1997; Wang et al., 1998; Makin et al., 2001; Cartron et al., 2005). In death receptor-induced apoptosis in cells undergoing mitochondrially mediated apoptosis, truncated Bid (tBid) causes a conformational change of the 'inactive' Bax, so that the TM domain is exposed and the 'activated' protein moves to the MOM, where individual molecules form homo- or hetero-octamers (the latter including Bak molecules) (Korsmeyer et al., 2000). However, it has not been documented how ROS mobilises molecules like Bax to the MOM. Therefore, an important link between early upstream events of mitocan-induced apoptosis, generation of ROS, and mobilisation of Bax to the MOM still remains to be defined.

An intriguing report was published recently by d'Alessio et al. (2005), who revealed that incubation of cytosolic lysates with hydrogen peroxide resulted in Bax dimerisation via disulfide bridges. The dimerised Bax then translocated to mitochondria, where it assembled to form megachannels in the MOM. These authors also modelled the conformational changes during Bax dimerisation, examining the most thermodynamically favourable route for inter-molecular disulfide bridges to occur, linking the surface-exposed Cys62 and Cys162 residues and exposing the TM domain required for Bax mitochondrial recognition. As a result of their observations, a plausible explanation was conceived by the present authors for ROS acting to 'mobilise' Bax (Neuzil et al., 2006).

Several assumptions are implicit in our proposal that should first be taken into consideration. Thus, the radical species produced in mitochondria takes the form of the charged superoxide molecule that cannot cross membranes. Most cells possess the enzyme MnSOD that rapidly converts superoxide to the highly diffusible hydrogen peroxide – a non-reactive species that most likely ought to be converted within the cytosolic fraction into more reactive radicals with the propensity of catalysing disulfide bonds. Such 'activation' of hydrogen peroxide can occur via the redox activity of catalytic iron present within the crude cytosolic fraction (in line with the experiments conducted by d'Allesio and colleagues) existing either as free iron in lysosomes or as heme iron present on various proteins. The mechanism of Bax dimerisation by the formation of disulfide bridges would explain the translocation of Bax molecules to the MOM under oxidative stress and also implicates a possible anti-apoptotic role for proteins capable of reducing disulfide bridges, such as thioredoxin, a protein with known anti-apoptotic activity (Patenaude et al., 2004; Freeman and Neuzil, 2006) and whose activity is enhanced in cancer cells, probably to counteract the apoptogenic potential of their increased generation of ROS (Yoo et al., 2006; Schumacker, 2006). It follows that the net result of hydrogen peroxide activity on Bax will be regulated by the level of the hydrogen peroxide 'activating' agents, in particular catalytic iron versus the disulfide bond-reducing counterparts as well as the levels of catalase that convert hydrogen peroxide into water.

There is another plausible scenario that may explain how molecules like Bak may be mobilised to the MOM as a consequence of ROS generated by mitochondria. It has been recently shown that p53, a transcription factor regulating expression of a number of crucial apoptosis-modulating genes, including Bax and the BH3-only Bcl-2 family proteins Puma and Noxa (Li et al., 2006; Ming et al., 2006), can be activated (stabilised) not only as a result of DNA damage, but also by oxidative stress (Sun and Oberley, 1996). We have observed such rapid activation of p53 in mesothelioma cells exposed to α-TOS (Tomasetti et al., 2006). A change in the cytosolic redox tone results in activation of the p53 transcriptional activity, leading to increase in the expression of Puma and Noxa, that are known to displace binding of Bak to the anti-apoptotic protein Mcl-1, which is then rapidly degraded by the proteasomal mechanism (Gelinas and White, 2005; Willis et al., 2005). Liberated Bak then forms in the MOM homo- or hetero-oligomers (the latter together with Bax) (Gelinas and White, 2005; Willis et al., 2005). Thus this is another possibility how the pro-apoptotic Bcl-2 family proteins may be 'activated' as a response to oxidative stress induced by mitocans like α-TOS (J.N. et al., unpublished data).

Incorporation of Bax dimers to the MOM (Antosson et al., 2001) is counteracted by mitochondrial anti-apoptotic proteins of the Bcl-2 family, including Bcl-2 or Bcl-x_L. These proteins also contain a BH3 domain by which they interact with proteins like Bax or Bak to preclude them from forming megachannels in the MOM (Zha et al., 1996; Cory and Adams, 2002). Over-expression of Bcl-2 or Bcl-x_L in cancers makes their treatment very difficult and as a result, BH3 mimetics are now being exploited as potential agents for adjuvant therapy (Degterev et al., 2001; Yin et al., 2005). A recent report revealed that α-TOS and its analogues act as BH3 mimetics (Shiau et al., 2006). In this interesting communication, the authors showed that interaction between Bak and Bcl-2/Bcl-x_L was blocked by α-TOS binding to the BH3 binding domain of the anti-apoptotic proteins Bcl-2/Bcl-x_L. Consequently, vitamin E analogues can now also be classified within the class of BH3 mimetics. However, in distinction from the BH3 mimetic-only compounds, vitamin E analogues have the added advantage of also being potent inducers of ROS production as detailed above. We propose

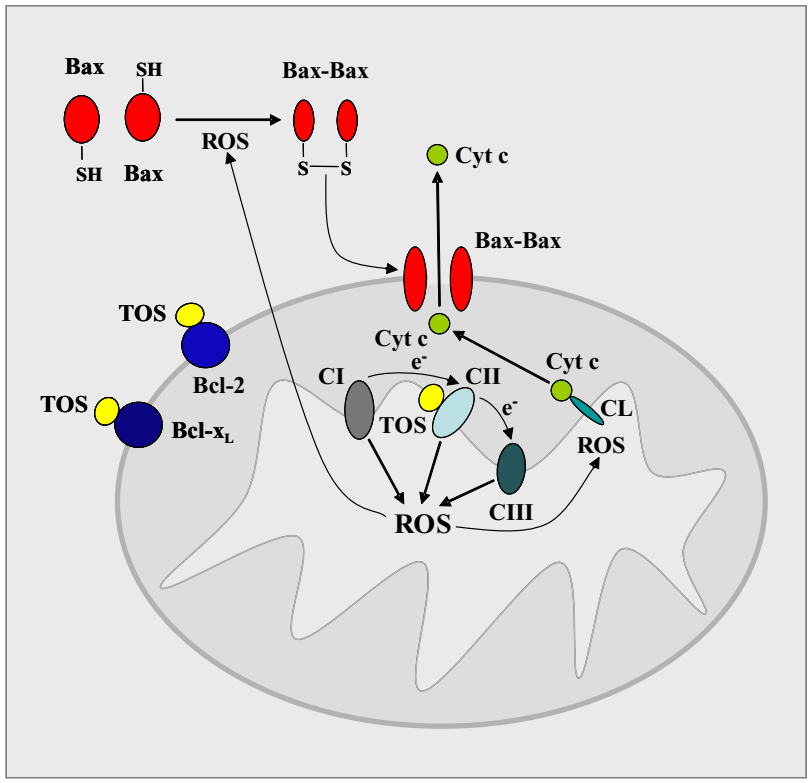

Fig. 2 Model of the role of superoxide in α-TOS-triggered apoptosis initiation. α-TOS binds to complex II of the mitochondrial redox chain, resulting in generation of ROS in mitochondria These, in the form of hydrogen peroxide, diffuse across the mitochondrial membrane, where they are 'activated' (probably by redox-active iron) so that they can catalyse formation of disulfide bridges between Bax monomers Such Bax dimerisation exposes the TM domain of the protein, which results in its recognition by the MOM Consequently, the dimers associate to form an octameric channel in the MOM α-TOS, being a BH3 mimetic, binds to the MOM proteins Bcl-2 and Bcl-x_L, therefore preventing their Bax inhibitory activity ROS, generated from complex II, also trigger the Cyt *c* oxidase activity, which results in oxidative modification of CL that will release Cyt *c*, which can then traverse via the Bax megachannel and activate the caspase cascade (adapted from Neuzil et al., 2006).

that the ROS-dependent generation caused by α-TOS (and its analogues) is the primary function of these agents, whereby they trigger the process of apoptosis, and that blocking Bax/Bak interaction with Bcl-2/Bcl-x_L is secondary, maximising the apoptogenic potential of the agent. The major trigger of apoptosis downstream of mitochondria is cytochrome *c* (Cyt *c*) that activates the caspase cascade (Zhivotovsky et al., 1998). Cyt *c* is a mitochondrial inter-membrane protein that has an essential role in the mitochondrial electron redox chain. It is attached to the inter-membrane face of the mitochondrial inner membrane (MIM) by affinity to the mitochondria-specific phospholipid cardiolipin (CL), which brings it to the proximity of complexes of the redox chain. The interaction of Cyt *c* with CL is interrupted by ROS at the onset of apoptosis (Petrosillo et al., 2001; Ott et al. 2002). It has been shown that ROS triggers the latent oxidase activity within Cyt *c*, based on its heme iron. As a result, CL peroxidation occurs, altering the lipid conformation with loss of affinity to and release of Cyt *c* (Kagan et al., 2005). Liberation of Cyt *c* from binding to CL in this manner can be counteracted by the addition of exogenous antioxidants (Zhao et al., 2004). Therefore, ROS generated in mitochondria of cancer cells exposed to α-TOS and acting to catalyse Cyt *c* release represent a third component of the complex mitochondria-based effects that the vitamin E analogue can exert in the early phases of the cascade of apoptogenic events.

The above scenario that we propose for the initial events in apoptosis induced by vitamin E analogues, a novel group of mitocans, is summarised in Fig. 2. We would like to stress that while this scenario has been devised based on results obtained largely with vitamin E analogues and on theoretical considerations, it is likely to be applicable, with some specific modifications, to other mitocans that trigger early generation of ROS in cancer cells.

Conclusion

To conclude, vitamin E analogues, represented by α-TOS, bind to the Q_P and, in particular, the Q_D sites on the mitochondrial complex II trans-membrane domain. Binding disrupts the flow of electrons that then combine with molecular oxygen to form superoxide. This ROS is converted to hydrogen

peroxide that diffuses across the mitochondrial membrane. Catalytic iron then 'activates' hydrogen peroxide, and the resulting radicals catalyse formation of disulfide bridges between two monomers of Bax in the cytoplasm. This results in Bax conformational change, exposing the TM domain, and the Bax dimer moves to the MOM, where it forms a megachannel. Vitamin E analogues, also acting as BH3 mimetics, bind to the critical domains on the anti-apoptotic Bcl-2 family proteins so that the activated Bax (or Bak) cannot be diverted from forming megachannels. Superoxide in mitochondria also causes liberation of CL-bound Cyt *c*, releasing Cyt *c* that traverses the MOM through the Bax (Bak) megachannel and activates the caspase cascade, whereby the cell enters the commitment phase of apoptosis. Such a mechanism of apoptosis initiation may be relevant to a variety of mitocans that trigger generation of ROS, and further supports the intriguing idea of mitochondria as a potent target for efficient and selective cancer therapy.

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