

**$\alpha$ -Tocopheryl succinate induces apoptosis by targeting ubiquinone-binding sites in mitochondrial respiratory complex II**

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**Running title:** Complex II as an anti-cancer drug target

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**$\alpha$ -Tocopheryl succinate ( $\alpha$ -TOS) is a selective inducer of apoptosis in cancer cells, which involves the accumulation of reactive oxygen species (ROS). The molecular target of  $\alpha$ -TOS has not been identified. Here we show that  $\alpha$ -TOS inhibits succinate dehydrogenase (SDH) activity of complex II (CII) by interacting with the proximal and distal ubiquinone (UbQ) binding site ( $Q_P$  and  $Q_D$ , respectively). This is based on biochemical analyses and molecular modelling, revealing similar or stronger interaction energy of  $\alpha$ -TOS compared to that of UbQ for the  $Q_P$  and  $Q_D$  sites, respectively. CybL-mutant cells with dysfunctional CII failed to accumulate ROS and undergo apoptosis in the presence of  $\alpha$ -TOS. Similar resistance was observed when CybL was knocked down with siRNA. Reconstitution of functional CII rendered CybL-mutant cells susceptible to  $\alpha$ -TOS. We propose that  $\alpha$ -TOS displaces UbQ in CII causing electrons generated by SDH to recombine with molecular oxygen to yield ROS. Our data highlight CII, a known tumour suppressor, as a novel target for cancer therapy.**

## Introduction

Cytotoxic drugs acting by selectively affecting mitochondria in cancer cells, ‘mitocans’ (Ralph *et al.*, 2007; Neuzil *et al.*, 2006; Neuzil *et al.*, 2007), are attractive for the treatment of cancer (Neuzil *et al.*, 2007; Ko *et al.*, 2004; Bonnet *et al.*, 2007; Dong *et al.*, 2007). Prime examples of such drugs are  $\alpha$ -tocopheryl succinate ( $\alpha$ -TOS) (Neuzil *et al.*, 2001a, 2001b), 3-bromopyruvate (3BP) (Ko *et al.*, 2004; Geschwind *et al.*, 2002) and dichloroacetate (DCA) (Bonnet *et al.*, 2007). 3BP inhibits the glycolytic pathway enzyme hexokinase, and succinate dehydrogenase (SDH), suppressing ATP production and mitochondrial respiration (Ko *et al.*, 2001; Xu *et al.*, 2005). DCA targets cancer cells by inhibiting pyruvate dehydrogenase kinase (Bonnet *et al.*, 2007). Finally,  $\beta$ -phenylethyl isothiocyanate selectively kills cancer cells by eliciting the generation of reactive oxygen species (ROS) (Trachootham *et al.*, 2006).

$\alpha$ -TOS, a redox-silent analogue of vitamin E (VE), causes rapid production of ROS in cancer cells, triggering apoptosis (Weber *et al.*, 2003; Stapelberg *et al.*, 2005; Swettenham *et al.*, 2005; Wang *et al.*, 2005).  $\alpha$ -TOS also inhibits the anti-apoptotic function of Bcl-2 and Bcl-x<sub>L</sub> by blocking their BH3 domains (Shiau *et al.*, 2006). This may explain, in part, how  $\alpha$ -TOS sensitizes cancer cells to other anti-cancer drugs. However, it does not explain how the agent causes accumulation of ROS, obligatory mediators of  $\alpha$ -TOS-induced apoptosis.

Antioxidants like superoxide dismutase (SOD) or the mitochondrially targeted coenzyme Q (MitoQ) (James *et al.*, 2005, 2007) negate the apoptotic action of  $\alpha$ -TOS (Weber *et al.*, 2003; Stapelberg *et al.*, 2005; Alleva *et al.*, 2001). Cancer cells that accumulate low levels of ROS in response to  $\alpha$ -TOS are less susceptible to apoptosis (Stapelberg *et al.*, 2005; Swettenham *et al.*, 2005; Kogure *et al.*, 2002; Kang *et al.*, 2004).

The effects of  $\alpha$ -TOS on cancer cells, combined with a reduced toxicity toward normal cells, results from a greater antioxidant defence of the latter (Church *et al.*, 1993; Safford *et*

*al.*, 1994; Huang *et al.*, 2006; Allen and Balin, 2003) and/or increased levels of esterases that hydrolyse the pro-oxidant  $\alpha$ -TOS by hydrolysis to the redox-active, non-apoptogenic  $\alpha$ -TOH (Fariss *et al.*, 2001; Neuzil and Massa, 2005).

The target of  $\alpha$ -TOS in cancer cells that results in ROS production has not previously been defined. One possibility how the VE analogue could give rise to ROS and ensuing apoptosis is by interfering with the ROS-generating centres along the mitochondrial electron redox chain. Of particular interest is complex II (CII), since previous data showed that toxicity of  $\alpha$ -TOS on cancer cells can be suppressed by MitoQ (Stapelber *et al.*, 2005; Swettenham *et al.*, 2005) that is known to interact with the ubiquinone (UbQ)-binding sites of CII (James *et al.*, 2005, 2007). Here we examined the effect of  $\alpha$ -TOS on the respiratory chain, identifying the UbQ-binding pockets in CII as the key molecular target for the agent, and propose that its interaction with the UbQ-binding sites results in ROS generation. The UbQ-binding sites of CII represent a novel, thus far unrecognized target for anti-cancer drugs.

## Results

### *Cancer cells accumulate ROS and undergo apoptosis when exposed to $\alpha$ -TOS*

We first tested if exposure of breast cancer cells to  $\alpha$ -TOS caused generation of ROS. The results obtained by flow cytometry (Figure 1a,b,e) show that both erbB2-low MCF7 and erbB2-high MDA-MB-453 cells accumulated ROS and underwent apoptosis when challenged with  $\alpha$ -TOS. EPR spectroscopy confirmed ROS generation occurring in cells exposed to  $\alpha$ -TOS (Figure 1c,d). Pre-treatment of the cells with MitoQ or their co-treatment with SOD suppressed ROS accumulation and decreased the extent of apoptosis (Figure 1). MCF7  $\rho^0$  cells with dysfunctional electron redox chain, when exposed to  $\alpha$ -TOS, showed relatively low levels of ROS accumulation and the induction of apoptosis was reduced (Figure 1f,g).

*$\alpha$ -TOS targets the UbQ-binding pockets on CII*

Studies with  $\alpha$ -tocopherol ( $\alpha$ -TOH) indicated that it could bind to the UbQ site in CII where it acts as a competitive inhibitor of succinate dehydrogenase (SDH) (Yu and Yu, 1982). We thus investigated whether  $\alpha$ -TOS, with its related structure but opposing biological activity, interferes with UbQ binding to CII. Using high levels of succinate (20 mM) that is capable of entering cells (Spencer, 1976; Maehara *et al.*, 1988) to promote both SDH activity (Maehara *et al.*, 1998; Berridge and Tan, 1993) and mitochondrial respiration proceeding via CII, NeuTL cells were assayed for SDH activity in the presence or absence of  $\alpha$ -TOS. The inhibitors of SDH activity, 3-bromopyruvate (3BP) (Sanborn *et al.*, 1971) and 3-nitropropionic acid (3NPA) (Scallet *et al.*, 2003), were employed as positive controls. To establish the relationship between inhibition of SDH and MTT reduction, initial experiments with 3BP and 3NPA were performed in the absence of succinate that might otherwise compete for binding to the enzyme, and the results confirmed that 3BP and 3NPA inhibited CII-mediated MTT reduction and that SDH activity and MTT reduction were closely related (Figure 2a).

Thenoyltrifluoroacetone (TTFA) is an inhibitor of CII that blocks the succinate-dependent MTT reduction by targeting the UbQ-binding sites rather than the SDH enzyme catalytic site (Sun *et al.*, 2005). The CII UbQ sites are situated beneath the active site of SDH, in the transmembrane region of the CII. With high succinate levels as the substrate driving CII-mediated MTT reduction in whole cells, treatment with 3BP,  $\alpha$ -TOS or TTFA inhibited MTT reduction in a dose-dependent manner (Figure 2b-d). Pre-incubation of cells with MitoQ, a form of UbQ that preferentially localizes to mitochondria where it binds to CII but not CIII (James *et al.*, 2005, 2007; Kelso *et al.*, 2001), overcame the inhibition in MTT reduction by TTFA and  $\alpha$ -TOS, but not by 3BP (Figure 2b-d). These results indicate that  $\alpha$ -TOS inhibits SDH activity in a similar manner to TTFA.

### *Verification that $\alpha$ -TOS inhibits mitochondrial respiratory CII activity*

Additional support for the role of  $\alpha$ -TOS in inhibiting CII activity was obtained with mitochondrial preparations from rat liver and membrane fractions from *P. denitrificans*. SDH activity decreased rapidly after treatment with  $\alpha$ -TOS (Figure 3). These data strongly indicate that  $\alpha$ -TOS, acting directly on the UbQ site(s) of CII, interferes with the electron flow to PMS and DCPIP. In contrast,  $\alpha$ -TOS had no effect on NADH dehydrogenase (CI) activity (data not shown), consistent with selective targeting of CII.

Next we tested whether  $\alpha$ -TOS initiated apoptosis in parental Chinese hamster lung fibroblasts (B1 cells), CI dysfunctional cells (B10 cells) and CII-dysfunctional cells (B9 cells, with a mutation in the gene encoding the succinyl dehydrogenase subunit C (SDHC subunit, CybL)). B9 cells were less responsive to  $\alpha$ -TOS, with lower levels of ROS accumulation (Figure 4a) and diminished SDH activity (Figure 4b) compared to the parental (B1) or B10 cells. B9 cells were also relatively resistant to apoptosis induced by  $\alpha$ -TOS (Figure 4c). Reconstitution of CII in the CybL-mutant (B9) cells normalised the SDH activity (Figure 4b), and also restored cell sensitivity to  $\alpha$ -TOS-induced killing (Figure 4c).

Data obtained with the CII-dysfunctional (B9) and the reconstituted B9 cells were independently verified by treatment of MCF7 cells with four different short-interfering RNAs (siRNA) against *CybL*. Duplexes 1, 3 and 4 substantially suppressed CII activity (Figure 4d), as well as ROS generation (Figure 4e) and apoptosis induction in response to  $\alpha$ -TOS (Figure 4f).

Using a human monoclonal SDHC antibody, we probed the B1, B9 and B10, and the CII-reconstituted Chinese hamster lung fibroblasts (B9<sub>rec</sub>) for the presence of SDHC. Western blotting analysis (Figure 4h,i) revealed high levels of human SDHC in the B9<sub>rec</sub> cells and its low level in MCF-7 cells treated with the SDHC siRNAs. The B9 cells with reconstituted CII

revealed re-appearance of SDHC. Densitometric evaluation, showed that siRNA treatment of MCF7 cells lowered the level of the SDHC protein by 50-80% (Figure 4j). These data are consistent with the RT-PCR results showing presence of human *SDHC* mRNA in CII-reconstituted cells and low levels of the transcript in MCF7 cells treated with SDHC siRNA (Figure 4g). The siRNA approach was specific for SDHC, since we did not observe any changes in the level of the SDHB subunit protein in the SDHC-treated MCF7 cells (data not shown).

#### *Molecular modelling reveals strong binding of $\alpha$ -TOS to UbQ sites on CII*

To rationalize our results, suggesting that  $\alpha$ -TOS interacts with CII via the UbQ-binding sites, we performed molecular modelling using AutoDock (Morris et al., 1998). The crystal structure of porcine heart mitochondrial CII has been reported (Sun *et al.*, 2005). Since it exhibits high sequence identity with human CII (95-97% for the individual subunits), we used this structure (1ZOY) and the related structure (1ZP0) with the inhibitor TTFA bound as the basis for our study. Structural analysis revealed the proximal UbQ-binding site ( $Q_P$ ) and also identified the position of the proposed distal UbQ-binding site ( $Q_D$ ). To test the feasibility of using AutoDock for this system, we first simulated the binding of UbQ5 to both the  $Q_P$  and the proposed  $Q_D$  sites. UbQ5 was chosen as it is of similar size to  $\alpha$ -TOS (Figure 5e) and contains a similar number of rotatable bonds (16 and 17, respectively). Figure 5a indicates that UbQ5 docked in  $Q_P$  to a deeper position than that observed for the portion of UbQ resolved in the published crystal structure (Sun *et al.*, 2005). UbQ5 was also found to dock into the proposed  $Q_D$  site, with the UbQ ring positioned in front of the binding site and the hydrophobic tail located inside the site (Figure 5c).

In the  $Q_P$  site the ring system of  $\alpha$ -TOS sits in the same binding pocket as the UbQ ring but is tilted to one side. The succinate ester moiety extended deeper into the binding site

protruding down towards the location of the prosthetic heme group. The carboxyl group fits neatly into this pocket and is involved in a bidentate hydrogen bond with Ser42(C) (Figure 5b). Ser42(C) also interacts with the ester oxygen of  $\alpha$ -TOS. As befits the hydrophobic nature of the remainder of the  $\alpha$ -TOS molecule, all the other interactions with the protein are hydrophobic. Thus, the hydrocarbon side chain loops around and extends out of the Q<sub>P</sub> site and along the same channel where the isoprenoid side chain of UbQ5 is located.

In the Q<sub>D</sub> site the phenyl ring system of  $\alpha$ -TOS sits towards the bottom of the binding site with the succinate ester moiety extending out the bottom of the site in a similar way to that observed for the head group of the phospholipid visible in the original crystal structure (Figure 5c). The succinate moiety hydrogen-bonds to Lys135(D) and Lys128(D) (Figure 5d), while the hydrocarbon side chain loops around the inside of the binding site. The calculated energy of interaction for the docked conformations of  $\alpha$ -TOS (Figure 5e) suggested that it can bind at either site. While the binding energy of  $\alpha$ -TOS at the Q<sub>P</sub> site is slightly less than that of UbQ5,  $\alpha$ -TOS would certainly compete with UbQ5. At the Q<sub>D</sub> site,  $\alpha$ -TOS shows a much stronger binding energy than UbQ5 and would be expected to easily displace it from this site (Figure 5e).

We had to use UbQ5 in the modelling study because of the relatively high number of rotatable bonds in  $\alpha$ -TOS (17) and UbQ5 (16); adding the full isoprene tail of UbQ10 (with the extra rotatable bonds) makes the AutoDock calculations intractable. Also, data analysis from the published crystal structure only shows electron density for the very start of the isoprenoid chain suggesting that the rest of the tail is not particularly tightly bound at this site (Sun *et al.*, 2005). Therefore, the expected interaction energies of UbQ10 for the two sites will be similar to those calculated for UbQ5.

*Apoptosis induced by  $\alpha$ -TOS is independent of its BH3 mimetic activity*

Shiau *et al.* (2005) reported that VE analogues possess BH3 mimetic activity, interacting with Bcl-2 and Bcl-x<sub>L</sub>. To ascertain whether the apoptogenic activity of  $\alpha$ -TOS is dependent on this activity, we utilized the BH3 mimetic BH3I-2' (Calbiochem) (Degterev *et al.*, 2001; Milanesi *et al.*, 2006). Cytotoxicity of  $\alpha$ -TOS was assessed in MCF7 cells exposed to increasing concentrations of BH3I-2'. We found that levels >5  $\mu$ M caused mitochondrial destabilization (not shown). Combining BH3I-2' (5  $\mu$ M) with  $\alpha$ -TOS significantly enhanced the sensitivity of the cells to the VE analogue (Figure 6). These data indicate a synergistic effect of BH3I-2' and  $\alpha$ -TOS, and suggest that the VE analogue induces apoptosis predominantly by other modes than acting as a BH3 mimetic.

#### *$\alpha$ -TOS inhibits tumour growth irrespective of erbB2 status*

To demonstrate anti-cancer activity of  $\alpha$ -TOS, we first determined whether the VE analogue could suppress tumour growth in an animal model of breast cancer with low erbB2 expression, given that over two thirds of human breast cancers express low levels of the receptor tyrosine kinase (Slamon *et al.*, 1989). Nude mice were xenotransplanted with MCF7 cells and treated with  $\alpha$ -TOS. Data in Figure 7a show that  $\alpha$ -TOS repressed tumour growth with a significant decrease in the tumour size. We then tested treatment of erbB2-high breast carcinomas using the transgenic *FVB/N c-neu* mice (Guy *et al.*, 1992). Compared to the controls, the tumours in the  $\alpha$ -TOS-treated mice showed a 30-40% reduction in size over the several weeks of treatment. These results suggest a high level of efficacy for  $\alpha$ -TOS therapy against breast carcinomas regardless of their erbB-2/HER2 status.

## **Discussion**

It is increasingly recognized that mitochondria are emerging as effective targets that may

provide the selectivity needed for potent anti-cancer therapy (Neuzil *et al.*, 2001b; Don and Hogg, 2004). Studies with mitocans have shown exciting potential for cancer therapy since these drugs have limited side-effects, and some of these compounds possess specificity for cancer cells (Neuzil *et al.*, 2007).

This study supports a role for  $\alpha$ -TOS as a competitive inhibitor of the UbQ site(s) in CII. Our data reveal that both the Q<sub>P</sub> and Q<sub>D</sub> sites of CII are targets for  $\alpha$ -TOS. Binding of the VE analogue at CII promotes ROS generation in cancer cells. The validity of the molecular modelling approach is supported by our identification of several other compounds with strong interaction energies for the CII UbQ site(s) (not shown). Of them, pyridoxal phosphate has been previously identified as a potent inhibitor of SDH activity by competing with UbQ (Choudhry *et al.*, 1985, 1986).

Molecular modelling strongly supports the notion that the binding of  $\alpha$ -TOS to CII within the Q<sub>P</sub> and Q<sub>D</sub> sites is a key to the apoptogenic activity of the agent. These observations were verified by experiments with cells where a mutation in *CybL* disrupts the CII function and the ability of cells to survive when growing in the presence of succinate. First, *CybL*-mutant cells, unlike their CI-deficient and parental counterparts, failed to accumulate ROS and induce apoptosis when exposed to  $\alpha$ -TOS, while reconstitution of functional CII reinstated susceptibility to the agent. These findings can be reconciled with a study reporting resistance of *CybL*-mutant cells to apoptosis when challenged with various cytotoxic agents (Albayrak *et al.*, 2003). Hence, CII is an important driver of ROS production mediated by cytotoxic drugs. Second, we demonstrate that knocking down *CybL* rendered cancer cells resistant to  $\alpha$ -TOS-triggered ROS accumulation and apoptosis induction (Figure 4). This is important evidence, since the siRNA approach is an acute insult to the cells, while a knock-out cell line can undergo adaptive differential expression of genes, potentially obscuring the direct effect of the knock-out genotype.

We and others have previously shown  $\alpha$ -TOS to be a potent inducer of ROS in cancer cells (Weber *et al.*, 2003; Kang *et al.*, 2004). The  $\alpha$ -TOS drug target we have identified as CII of the respiratory chain has been proposed as a contributor to cellular ROS production (McLennan and Degli-Esposti, 2000). Addition of MitoQ to cells did not overcome the 3BP-mediated inhibition of SDH activity since 3BP acts upstream at the site of the SDH catalytic centre. MitoQ overcame both the TTFA- and  $\alpha$ -TOS-mediated inhibition of SDH activity. While TTFA binds to both  $Q_P$  and  $Q_D$  sites in CII (Sun *et al.*, 2005) like  $\alpha$ -TOS, it displays less selective toxicity (Zhang *et al.*, 2001) than the VE analogue for killing cancer cells (Neuzil *et al.*, 2001b). Further studies are required to understand this differential specificity.

When  $\alpha$ -TOS displaces UbQ from its binding site(s) on CII, electrons may no longer be tunneled down the SDH hydrophilic head on to FAD and relayed via the [Fe-S] centres and the heme group to UbQ (Tran *et al.*, 2006; Cheng *et al.*, 2006). Instead, they recombine with molecular oxygen to enhance superoxide anion radical ( $O_2^{\cdot-}$ ) production that leads to induction of apoptosis in the cancer cell. Although it is possible that ROS generation occurs via the FAD site (Yankowskaya *et al.*, 2003; Gottlieb and Tomlinson, 2005), it cannot be excluded that electrons in the case of a dysfunctional CII, interact with oxygen, which is dissolved in the membrane part of CII (Slane *et al.*, 2006). Another possibility is that electrons move to CI by reverse electron transport, where they form  $O_2^{\cdot-}$  (Adam-Vizi and Chinopoulos, 2006). Blocking UbQ binding promotes ROS generation by removing the opportunity of electrons to react with its acceptor (Figure 8). This is strongly supported by studies with the *C. elegans* *mev-1* mutant that can oxidize succinate to fumarate but cannot transfer electrons to UbQ, resulting in increased ROS levels and premature aging (Ishii *et al.*, 1998). This can be reconciled with a recent study suggesting that the  $Q_P$  site is important for electron channelling between UbQ and the juxta positioned heme group (Zhao *et al.*, 2006), which contributes to stabilization of the transient ubisemiquinone radical (Tran *et al.*, 2006).

Interestingly, several human cancers, such as paragangliomas, resulting from genetic disorders due to mutations in CII subunits, including SDHC, may also be induced by increased ROS formation (Gottlieb and Tomlinson, 2005; Niemann and Muller, 2005; Ishii *et al.*, 2005).

Our results are not dissimilar to a recent report documenting selective molecular mechanism of apoptosis induction in cancer cells by adaphostin, a compound that binds to the UbQ reduction ( $Q_i$ ) site of CIII, resulting in ROS accumulation (Le *et al.*, 2007). That CII is of importance in ROS formation is further evidenced by a recent report showing that non-steroid anti-inflammatory drugs caused ROS generation in cells, of which the majority was derived from CII (Soller *et al.*, 2007). Moreover, ferulenol, a coumarine derivative, has been reported to affect the succinate ubiquinone reductase by interfering with the UbQ cycle (Lahouel *et al.*, 2007). Given these recent results, more drugs affecting CII are likely to be found in near future.

Although anti-cancer drugs have been described that may target the UbQ sites on the oxidoreductase complexes along the mitochondrial respiratory chain to increase cellular ROS levels and activate apoptosis (Le *et al.*, 2007; Dias and Bailly, 2005; Hall, 2005),  $\alpha$ -TOS has an additional feature in that it also disrupts the anti-apoptotic function of Bcl-2 and Bcl-x<sub>L</sub> by blocking their BH3-binding domain (Shiau *et al.*, 2006). Hence,  $\alpha$ -TOS may prove superior for inducing cancer cell death because of its double action on two important targets,  $Q_P$  and  $Q_D$  sites of CII, and the Bcl-2 family proteins, promoting the induction of mitochondrially mediated apoptosis. This notion is further corroborated by our data revealing sensitization of cancer cells to  $\alpha$ -TOS by the BH3 mimetic BH3I-2'.

We also show that  $\alpha$ -TOS suppresses tumour progression in mouse models of breast cancer regardless of their erbb2 status. This could be explained by  $\alpha$ -TOS blocking the CII UbQ-binding sites, thereby causing ROS generation with ensuing induction of apoptosis, acting in a dominant manner downstream of any anti-apoptotic pro-survival activity resulting from the

erbB2 receptor tyrosine kinase signalling. This makes the VE analogue a potentially useful anti-cancer agent when dealing with the challenge of breast cancers with high levels of expression of erbB2/HER2 that are rather recalcitrant to therapy (Slamon *et al.*, 1989). Similarly, we recently demonstrated that a peptide conjugate of  $\alpha$ -TOS (with relatively high affinity for the erbB2 receptor) effectively kills breast cancer cells (Wang *et al.*, 2007).

To conclude, we present the molecular targets of the intriguing anti-cancer agent  $\alpha$ -TOS with low toxicity to non-cancerous tissues (Weber *et al.*, 2002), a compound that acts by selectively affecting mitochondria, organelles that are essential both for life and for unleashing the apoptosis machinery (Newmeyer and Ferguson-Miller, 2005). We identify the UbQ-binding sites of the mitochondrial CII as a new target for anti-cancer drugs, and propose that these results will not only lead to the establishment of VE analogues as efficient anti-cancer agents but also will increase the interest in mitochondrial components as targets that are yet to be fully exploited for selective cancer therapy. Work is presently underway, using *in silico* modelling, to identify lead compounds that induce apoptosis by interfering with the UbQ-binding site(s) of CII and develop them into efficient and selective anti-cancer drugs. Our results on the molecular mechanism of apoptosis induced in cancer cells by  $\alpha$ -TOS may be reconciled with our clinical outcome from a mesothelioma patient who has been treated with the VE analogue. The data reveals a significant clinical benefit with  $\alpha$ -TOS therapy, causing a reduction in tumour volume and improved the well-being of our subject who had a lethal type of neoplastic pathology (Robinson *et al.*, 2005). We are currently preparing to set up a larger clinical trial in which a cohort of mesothelioma patients will be treated with the mitocan  $\alpha$ -TOS.

## Materials and methods

### *Cell culture and treatment*

Human breast cancer cells MCF7 with low and MDA-MB-453 with high levels of erbB, and the murine breast cancer erbB2-high NeuTL cells derived from the *c-neu* mice were cultured in DMEM with 10% FCS and antibiotics. Jurkat T lymphoma cells were cultured in RPMI-1640 medium with 10% FCS and antibiotics. MCF7 cells deficient in mtDNA ( $\rho^0$ ) were prepared as described (Weber *et al.*, 2003). CI-dysfunctional (B10 cells) (Seo *et al.*, 1997), CII-dysfunctional (B9 cells with mutant CybL (Oostveen *et al.*, 1995), and the parental Chinese hamster lung fibroblasts (B1 cells) (Oostveen *et al.*, 1995) were grown in DMEM with 10% FCS, antibiotics, 10 mg/ml glucose and non-essential aminoacids.

### *Assessment of apoptosis, ROS accumulation, and membrane potential*

Apoptosis was quantified using the annexin V-FITC kit (PharMingen) (Weber *et al.*, 2003). Cellular ROS were detected with the probe dihydroethidium (DHE) (Molecular Probes) by flow cytometry (Weber *et al.*, 2003), or by trapping with 5,5-dimethyl-1-pyrroline N-oxide (DMPO; Sigma) using electron paramagnetic resonance (EPR) spectroscopy (Weber *et al.*, 2003). Cells were also pre-treated for 1 h with 2  $\mu$ M MitoQ (James *et al.*, 2005) or co-incubated with SOD (PEG-SOD, 750 units/ml; Sigma). The mitochondrial inner transmembrane potential ( $\Delta\Psi_{m,i}$ ) was assessed using 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolyl-carbocyanino iodide (JC-1; Molecular Probes) (Weber *et al.*, 2003).

### *SDH activity assay*

MTT solutions were prepared by dissolving 2.5 mg/ml 3-(4,5-dimethyl-2,5-diphenyl 2H-tetrazolium bromide (MTT) (Sigma) in PBS alone or phenol red-free RPMI media with 20 mM succinic acid, pH 7.4. Solutions of 3NPA,  $\alpha$ -TOS and MitoQ in ethanol, TFA in DMSO

or 3BP in PBS were added to cells simultaneously with MTT. The drugs were tested in exponential growth phase of cells in 96-well plates using 4-8 replicates per dilution. Cells were also pre-incubated for 1 h with 2 or 5  $\mu$ M MitoQ. Final concentrations of ethanol or DMSO in cultures were  $\leq 0.1\%$  (v/v). Treated and control cells were exposed to MTT for 2 or 4 h at 37°C and 5% CO<sub>2</sub>. Supernatants were removed, except for 30  $\mu$ l, before adding 150  $\mu$ l DMSO to dissolve the formazan crystals, and absorbance measured at 570 nm.

#### *Preparation of mitochondrial particles*

Rat liver mitochondria were freshly prepared as described (Rice and Lindsay, 1997) and stored at -20°C until used. *P. denitrificans* CCM982 (NCIB 8944) was grown anaerobically at 30°C in a medium containing 50 mM succinate as the carbon source and 10 mM nitrate as the terminal acceptor. Membranes were prepared as published (Burnell *et al.*, 1975) and stored at -20°C until used.

#### *Measurement of mitochondrial CI and CII activity*

Reduction of the CII substrate DCPIP in the presence of cells or liver mitochondrial preparations was measured at 600 nm (Trounce *et al.*, 1996). Reaction mixtures contained 0.5 mM NADH, 5 mM succinate, 10 mM KCN, 50  $\mu$ M DCPIP, and 50  $\mu$ M PMS. For each assay point, 0.5 mg sample protein was used and  $\alpha$ -TOS added as indicated. When measuring the CI (NADH dehydrogenase) activity, PMS was omitted.

#### *RNA interference, cell transfection, Western blotting and RT-PCR*

The siGENOME ON-TARGETplus set of four duplexes of siRNA oligonucleotides (Dharmacon) targeting the *CybL* (*SDHC*) subunit of CII were used. Non-specific siRNA was used as a negative control. Transfections of MCF7 cells with siRNA were performed as

described (Stapelberg *et al.*, 2005). B9 cells were transfected using the Topo pCR3.1 Uni plasmid harbouring the *CybL* gene (Slane *et al.*, 2006) and selected as described (Weber *et al.*, 2003). Stably transfected and siRNA-treated cells were assessed for SDH activity and SDHC expression. Western blotting was performed as described (Wang *et al.*, 2007) using anti-SDHC IgG (clone 3E2; Novus Biologicals) with anti- $\beta$ -actin IgG (Santa Cruz) as a loading control. RT-PCR was performed using a standard protocol. The published human *CybL* (Slane *et al.*, 2006) and Chinese hamster *GAPDH* primers (Sever *et al.*, 2004) were used.

#### *Molecular modelling of $\alpha$ -TOS interaction with UbQ binding in CII*

For details, see the Supplementary material.

#### *Animal experiments*

Balb/c nu/nu mice were inoculated s.c. with MCF7 cells ( $2 \times 10^6$  cells/mouse). After tumours developed, mice were injected with 10  $\mu$ moles  $\alpha$ -TOS in DMSO i.p. every 3 d. Control mice were injected with an equal volume of DMSO. Tumour size was estimated with digital callipers.

Transgenic *FVB/N c-neu* mice (Guy *et al.*, 1992) were used with ~70% of the female mice developing spontaneous ductal breast carcinomas within ~7 months. Tumours were quantified by ultrasound imaging (USI) using the Vevo770 device fitted with the RMV704 scan-head (VisualSonics) operated at 60 MHz and with 40  $\mu$ m resolution (Wang *et al.*, 2007; Dong *et al.*, 2007). Mice received 10  $\mu$ moles  $\alpha$ -TOS in corn oil/4% ethanol or the vehicle administered i.p. every 3 d.  $\alpha$ -TOS therapy of the animals commenced when the tumour volume was ~40 mm<sup>3</sup>. Animal studies were performed according to the guidelines of the Australian and New Zealand Council for the Care and Use of Animals in Research and Teaching and were approved by the local Animal Ethics Committee.

### *Statistical analyses*

Between-group comparisons were made using mean $\pm$ SD and the unpaired Student *t* test. Differences in the mean relative tumour size ( $\pm$ SEM) was examined using analyses of covariance (ANCOVA) with days as the covariate. Statistical analyses were performed using SPSS<sup>®</sup> 10.0 analytical software. Statistical significance was accepted at  $p < 0.05$ .

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## Figure Legends

**Figure 1** ROS generation and induction of apoptosis in cancer cells by  $\alpha$ -TOS. ErbB2-low MCF7 and -high MDA-MB-453 cells were exposed to  $\alpha$ -TOS at 50  $\mu$ M and for times shown, and assessed for DHE- (a) and annexin V-positive cells (%) (b). Jurkat cells were treated with 50  $\mu$ M  $\alpha$ -TOS for 2 h or as shown and assessed for ROS accumulation using EPR spectroscopy (c – evaluation of the DMPO-OH adduct level in nmol/mg protein; d – representative EPR spectra of cells exposed to  $\alpha$ -TOS in the absence (1) or presence of MitoQ (2) or to MitoQ only (3)). Panel e shows the kinetics of apoptosis induction in Jurkat cells exposed to 50  $\mu$ M  $\alpha$ -TOS in the absence or presence of MitoQ. Control (C) MCF7 parental and  $\rho^0$  cells exposed to 50  $\mu$ M  $\alpha$ -TOS for 2 h (T) were assessed for ROS accumulation (f) and apoptosis (g). Where indicated, the cells were pre-treated for 1 h with 2  $\mu$ M MitoQ (MQ) or co-treated with SOD (PEG-SOD, 750 units/mg). The data shown represent mean values  $\pm$  S.D. (n=3). The symbol ‘\*’ denotes significant difference ( $p<0.05$ ) in the level of apoptosis

and ROS of treated cells compared with the untreated control cell population.

**Figure 2** The effect of 3NPA, 3BP, TTFA and  $\alpha$ -TOS on the ability of NeuTL cells to reduce MTT. **(a)** MTT reduction in PBS was assessed after a 4 h co-incubation period in the presence of 3NPA or 3BP used at the concentrations ( $\mu$ M) as shown. **(b-d)** Cells were pre-incubated for 60 min with MitoQ at the concentrations indicated (Ctrl, 2 or 5  $\mu$ M; insert box) before addition of 3BP (0-100  $\mu$ M) **(b)** and assessed for their ability to reduce MTT after a 2 h incubation period. Cells pre-treated with MitoQ were then treated with TTFA **(c)** and with  $\alpha$ -TOS **(d)** at the concentration shown, and were assessed for their ability to reduce MTT in RPMI containing 20 mM succinic acid (pH 7.4) after a 2 h incubation period. Results are presented as mean reduction (%) of MTT relative to control (untreated)  $\pm$  S.D. The symbol ‘\*’ denotes significant differences with  $p < 0.05$ .

**Figure 3** Inhibition of SDH/CII activity in isolated rat liver mitochondria **(a, c)**, and *Paracoccus denitrificans* **(b, d)** by  $\alpha$ -TOS. Preparations of mitochondria from rat liver or membranes from *P. denitrificans* were incubated in a reaction mixture facilitating mitochondrial SDH/CII activity ( $\mu$ mol/min/mg protein) and containing DCPIP+PMS. Samples in **a** and **b** contained succinate and were both treated with  $\alpha$ -TOS as indicated. Dose-response curves displaying changes in the reaction rate ( $\mu$ mol/min) for DCPIP were measured as absorbance at 600 nm under different concentrations of succinate as indicated in the absence or presence of  $\alpha$ -TOS **(c, d)**. Results are represented as mean values  $\pm$  S.D. (n=3). The symbol ‘\*’ indicates values significantly different from the controls with  $p < 0.05$ .

**Figure 4** Apoptosis induction by  $\alpha$ -TOS is suppressed in CII dysfunctional cells. Parental (B1), CI-dysfunctional (B10), CII-dysfunctional (*CybL*-mutant; B9), and *CybL*-mutant cells

following CII reconstitution by transfection with human *Cybl* (*B9<sub>rec</sub>*) were exposed to  $\alpha$ -TOS 50  $\mu$ M and for 24 h, unless shown otherwise, harvested and assessed for ROS accumulation (a), SDH activity assessed in whole cells on the basis of MTT reduction with succinate as a substrate (b), and apoptosis (c). MCF7 cells were pre-treated with CybL or non-specific (NS) siRNA, exposed to  $\alpha$ -TOS as shown, and assessed for ROS accumulation (d), SDH activity (e), and apoptosis induction (f). Panel g shows results of RT-PCR analysis of B1, B10, B9 or B9<sub>rec</sub> cells as well as *SDHC* siRNA-treated MCF7 cells using human *SDHC* primers. Panel h reveals results of Western blotting of B1, B10, B9 and B9<sub>rec</sub> cells using monoclonal IgG anti-human *SDHC*. Western blotting is also shown to document the levels of *SDHC* in MCF7 cells treated with different *SDHC* siRNA duplexes and with NS siRNA (i), which was evaluated relative to the actin band (j). Results are represented as mean values  $\pm$  S.D. (n=3), images are representative of three independent experiments. The symbol '\*' indicates values significantly different from the controls with  $p < 0.05$ .

**Figure 5** Molecular modelling reveals interaction of  $\alpha$ -TOS with UbQ-binding sites in CII. (a) Cut-away view of the Q<sub>P</sub> binding site showing the heme group (bottom left corner) and the position of UbQ (green carbon atoms) as indicated with arrows. The best-fit conformations of UbQ5 (cyan carbons) and  $\alpha$ -TOS (orange carbon atoms) are also shown. (b) Ligplot diagram showing the major interactions between the best docked conformation of  $\alpha$ -TOS and the Q<sub>P</sub> binding site. (c) View of the proposed Q<sub>D</sub> binding site (with overhanging bridge as a translucent surface) showing the best docked conformations of UbQ5 (cyan carbons) and  $\alpha$ -TOS (orange carbons). (d) Ligplot diagram showing the major interactions between the best docked conformation of  $\alpha$ -TOS and the Q<sub>D</sub> binding site. (e) Chemical structures of UbQ5 and  $\alpha$ -TOS and a table of interaction energies for the best ranked docking conformations for UbQ5 and  $\alpha$ -TOS in the Q<sub>P</sub> and Q<sub>D</sub> binding sites. Images in panels a and c were prepared

using Astex Viewer (Hartshorn, 2002), while those in panels **b** and **d** were prepared using Ligplot (Wallace *et al*, 1995).

**Figure 6**  $\alpha$ -TOS causes apoptosis independent of its BH3 mimetic activity. MCF7 cells were treated with 5  $\mu$ M BH3I-2' or exposed to 30  $\mu$ M  $\alpha$ -TOS following 10 min pre-treatment with 5  $\mu$ M BH3I-2'. Cells were then assessed for mitochondrial depolarization (**a**), ROS generation (**b**) and apoptosis induction (**c**). Results are represented as mean values  $\pm$  S.D. (n=3). The symbol '\*' indicates values significantly different from the controls with  $p < 0.05$ .

**Figure 7** Inhibition of breast cancer in mouse models by  $\alpha$ -TOS. (**a**) Nude mice were inoculated with MCF7 cells and once tumours became established, the animals were treated every 3 d with 10  $\mu$ moles per mouse of  $\alpha$ -TOS dissolved in DMSO or with DMSO alone, by i.p. injection. Tumour size was measured using callipers and was correlated to the size of the carcinomas at the onset of the therapy. Four animals were used in each group. (**b**) Female *FVB/N c-neu* mice with small tumours received either 10  $\mu$ moles  $\alpha$ -TOS solubilized in corn oil/4% ethanol (n=11) or the excipient alone (control, n=9) by i.p. injection once every 3 d. Tumour size was quantified using USI. Two independent experiments were conducted. The inset in **b** shows representative images of tumours acquired by USI in the control (upper image) and treated (lower image) *FVB/N c-neu* mice on day 22 of the experiment. The results shown are mean values  $\pm$  SEM. The symbol '\*' denotes significant differences ( $p < 0.05$ ).

**Figure 8** Model for the interference of  $\alpha$ -TOS with the mitochondrial electron chain. The scheme shows the proposed effects of  $\alpha$ -TOS with the branching of the electron transport chain in its upstream region and clarifies the point of inhibition by  $\alpha$ -TOS as specifically interacting with CII. It also suggests CII as the possible site of superoxide generation,

although its precise location within SDH has not been identified. The possible reverse electron transport from CII to CI is also shown.