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## **Mitochondrial targeting of $\alpha$ -tocopheryl succinate enhances its anti-mesothelioma efficacy**

Malignant mesothelioma (MM) is a fatal neoplastic disease with no therapeutic option.

Therefore, the search for novel therapies is of paramount importance.

**Methods:** Since mitochondrial targeting of  $\alpha$ -tocopheryl succinate ( $\alpha$ -TOS) by its tagging with triphenylphosphonium enhances its cytotoxic effects to cancer cells, we tested its effect on MM cells and experimental mesotheliomas.

**Results:** Mitochondrially targeted vitamin E succinate (MitoVES) was more efficient in killing MM cells than  $\alpha$ -TOS with  $IC_{50}$  lower by up to two orders of magnitude.

Mitochondrial association of MitoVES in MM cells was documented using its fluorescently tagged analogue. MitoVES caused apoptosis in MM cells by mitochondrial destabilization, resulting in the loss of mitochondrial membrane potential, generation of reactive oxygen species and destabilization of respiratory supercomplexes. The role of the mitochondrial complex II in the activity of MitoVES was confirmed by the finding that MM cells with suppressed succinate quinone reductase were resistant to MitoVES. MitoVES suppressed mesothelioma growth in nude mice with high efficacy.

**Discussion:** MitoVES is more efficient in killing MM cells and suppressing experimental mesotheliomas compared to the non-targeted  $\alpha$ -TOS, giving it a potential clinical benefit.

## Introduction

Malignant mesothelioma (MM), a fatal type of neoplasia,<sup>1</sup> is associated with asbestos inhalation. The lack of early diagnosis together with the aggressive nature of MM is the main reason for short survival of patients after diagnosis.<sup>2-4</sup> It is therefore important to search for novel therapeutic approaches that would be efficient against MM while being selective for tumour cells. In this context, mitochondria have emerged as an intriguing target for anti-cancer agents.<sup>5,6</sup> We have defined mitocans, a group of compounds with anti-cancer activity acting via mitochondrial destabilization, often in a cancer-specific manner, and classified them into several groups based on their molecular mode of activity.<sup>7</sup> Of these, group five mitocans target the electron transfer chain<sup>8</sup> with vitamin E (VE) analogues as prime examples.<sup>9</sup>

VE analogues are epitomized by the redox-silent agent  $\alpha$ -tocopheryl succinate ( $\alpha$ -TOS), which suppresses several types of tumours in animal models.<sup>9-13</sup> The agent acts by mitochondrial destabilization, resulting in the generation of reactive oxygen species (ROS).<sup>14,15</sup> The molecular targets of  $\alpha$ -TOS are ubiquinone (UbQ)-binding sites of the mitochondrial complex II (CII).<sup>16,17</sup> Interaction of  $\alpha$ -TOS with CII's UbQ sites results in the generation of superoxide anion radicals that activate the Mst1 kinase, followed by phosphorylation of the transcription factor FoxO1 and upregulation of the Noxa protein with ensuing departure of Mcl-1 from Bak, the latter forming pores in the mitochondrial outer membrane.<sup>18,19</sup> The notion that  $\alpha$ -TOS targets the UbQ sites in CII prompted us to modify it by tagging with the mitochondria-targeting triphenylphosphonium (TPP<sup>+</sup>) group, as initially accomplished for anti-oxidants like UbQ.<sup>20</sup> We have generated mitochondrially targeted VE succinate (MitoVES) that is considerably more efficient in killing cancer cells as well as suppressing experimental carcinomas compared to the parental compound.<sup>21,22</sup>

Given the grim prognosis of MM patients and the finding that  $\alpha$ -TOS is efficient against experimental MM,<sup>12,13</sup> we now tested the effect of MitoVES on a range of MM cell lines and on experimental mesotheliomas. Here we show that the agent is considerably more efficient than its untargeted counterpart, giving it clinical significance.

## **Materials and methods**

### *Cell culture and treatment*

Human Ist-Mes-2, MM-BI cells, Meso-2 and H28 cells,<sup>23</sup> and murine AE17 MM cells<sup>24</sup> were maintained in DMEM or RPMI media supplemented with 10% FCS and antibiotics at 37°C in a humidified atmosphere of 5% CO<sub>2</sub>. Cells were seeded and allowed to reach 60-70% confluency before addition of VE analogues (Fig. 1). They include  $\alpha$ -TOS (Sigma), MitoVES with different length of the chain spanning the TPP<sup>+</sup> group and the tocopheryl group, and an analogue of  $\alpha$ -TOS with the TPP<sup>+</sup> group esterified to the free carboxylate of the succinyl moiety via a 4-C spacer.<sup>21,22</sup> Unless specified, MitoVES refers to the 11-C chain homologue. Fluorescently labelled MitoVES (MitoVES-F) and  $\alpha$ -TOS ( $\alpha$ -TOS-F) were synthesized as described.<sup>21,22</sup>

### *Cell death and viability assay*

Cell death was assessed as detailed elsewhere<sup>14</sup> using the annexin V-FITC/propidium iodide (PI) method. Cell death is expressed as the sum of cells in the stage of apoptosis and post-apoptotic necrosis. Viability was estimated using the crystal violet method according to a standard procedure. In brief, MM cells were seeded in 96-well plates at 10<sup>4</sup> cells per well, allowed to adhere and treated with VE analogues for 24 h. The cells were then fixed with formalin and stained with 0.05% crystal violet for 1 h at room temperature. Absorbance at 595 nm was determined using the Tecan Infinity plate reader.

### *Assessment of mitochondrial potential ( $\Delta\Psi_{m,i}$ ) and generation of ROS*

The  $\Delta\Psi_{m,i}$ -sensitive fluorescent probe tetramethylrhodamine methyl ester (TMRM, Sigma) was utilized, and the cells evaluated by flow cytometry. The mitochondrial uncoupler carbonyl cyanide 4-(trifluoromethoxy)phenyl-hydrazine (CCCP, Sigma) was used as a control to determine non-specific TMRM loading.

ROS were evaluated using the probes dihydroethidium (DHE), 2',7'-dichlorodihydrofluorescein diacetate (DCF) or MitoSOX (Sigma). The cells were incubated with either probe for 15 min under normal culture conditions, harvested and the fluorescence of the oxidized probes assessed by flow cytometry.

### *Preparation of SDHC-deficient cells*

Ist-Mes-2 cells were seeded in 24-well plates at  $2 \times 10^5$  cells/well in antibiotic-free DMEM with 10% FBS and allowed to recuperate overnight. The SureSilencing human SDHC shRNA or non-silencing (NS) plasmid (SuperArray Biosciences) was combined with the Fugene HD transfection reagent and added to the cells. After 48 h, the cells were incubated with 4  $\mu\text{g/ml}$  of puromycin for 2 weeks to select the transfected cells. Successful transfection was detected by assessing the level of the SDHC protein using western blotting.

### *Native blue electrophoresis (NBE) and western blotting (WB)*

Cells were harvested, resuspended in the Tris/EGTA buffer containing 200 mM sucrose and homogenized on ice with a glass-Teflon homogeniser. Mitochondria were isolated by differential centrifugation and solubilized in the extraction buffer (1.5 M aminocaproic acid, 50 mM Bis-Tris, 0.5M EDTA, pH 7) with 1.3% lauryl maltoside. Samples containing 30-60  $\mu\text{g}$  of protein were combined with the sample buffer (0.75 M aminocaproic acid, 50 mM Bis-

Tris, 0.5 M EDTA, pH 7, 5% Serva-Blue G-250, 12% glycerol), loaded on pre-cast NativePAGE Novex 4-16% Bis-Tris gels and run overnight at 25 V. Separated protein complexes were transferred to PVDF membranes using the Transblot system (Invitrogen). Individual complexes were detected using the following antibodies (Abcam): CI, anti-NDUFS1 (79 kDa); CII, anti-SDHA (70 kDa); CIII, anti-UQCRC2 (48 kDa); CIV, anti-MTCO2 (26 kDa); CV, anti-ATP5A (60kDa). Anti-VDAC IgG (Abcam) was used as a loading control.

For WB following SDS-PAGE, the anti-SDHC IgG (Santa Cruz) was used. Anti-actin IgG (Sigma) was used as a loading control.

#### *In-gel CI and CII activity assay*

Isolated mitochondria were lysed and subjected to clear native electrophoresis, run as above but without the blue dye. CI activity was assessed on 3-12% gel strips using the NADH:NTB reductase assay. This gel strip was evaluated in freshly prepared NADH:NTB reductase assay buffer (25 mg NTB and 100  $\mu$ l of NADH, 10 mg/ml, added to 10 ml 5 mM Tris/HCl, pH 7.4); after 10-15 min the gel was transferred to 5 mM Tris/HCl, pH 7.4 and scanned. CII activity was evaluated on 4-16% gel strips using the succinate:NTB reductase assay. The gel strip was incubated for 10-20 min in freshly prepared succinate:NTB reductase assay buffer (25 mg NTB, 200  $\mu$ l 1 M sodium succinate, 8  $\mu$ l of 250 mM phenazine methosulfate dissolved in DMSO, added to 10 ml 5 mM Tris/HCl, pH 7.4). The gel was immediately transferred to 5 mM Tris/HCl, pH 7.4 and scanned.

#### *Mitochondrial respiration and CII activity*

Cellular respiration was evaluated using the Oxygraph-2 k apparatus (Oroboros Instruments) as published.<sup>25</sup> Experiments evaluating intact cell respiration were performed with MM cells

suspended in the complete RPMI medium. Experiments evaluating digitonin-permeabilized cell oxygen consumption were performed with MM cells suspended in the mitochondrial respiration medium MiR05. Respiration via CI and CII was evaluated in the presence of the proper substrates and inhibitors of the other complex. Increasing concentrations of the tested compounds were added to the Oxygraph chamber and the total oxygen concentration and consumption recorded. The results were related to the number of cells.

The succinate quinone reductase (SQR) and succinate dehydrogenase (SDH) activity of CII was assessed as detailed elsewhere.<sup>21</sup>

#### *Confocal microscopy*

Cells were seeded at  $10^5$  cells per chamber slide (Nunc) and allowed to attach overnight. The cells were then incubated with 100 nM TMRM and 5  $\mu\text{g/ml}$  DAPI for 1 h. The cells were then incubated with 5  $\mu\text{M}$  MitoVES-F or  $\alpha$ -TOS-F for 5 min. Imaging was performed using the Olympus FV1000 fluorescent confocal microscope. In some cases, the cells were pre-treated with 2  $\mu\text{M}$  CCCP.

#### *Animal experiments*

Athymic Balb-c *nu/nu* mice were injected subcutaneously with freshly harvested  $2 \times 10^6$  Ist-Mes-2 cells suspended in 100  $\mu\text{l}$  of saline. After about 15 days when tumours established ( $\sim 30 \text{ mm}^3$ ), the mice were injected into the peritoneum twice per week with 15  $\mu\text{mol}$   $\alpha$ -TOS or 1  $\mu\text{mol}$  MitoVES dissolved in corn oil after solubilization in EtOH (the final concentration of EtOH, 4% v/v).<sup>26</sup> Tumours were visualized and quantified non-invasively using the ultrasound imaging (USI) instrument Vevo770 (VisualSonics) fitted with a 30  $\mu\text{m}$  resolution 80 MHz scan-head.<sup>26</sup> All experiments 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 Griffith University Animal Ethics Committee.

### *Statistical analysis*

Experimental data are presented as mean  $\pm$  S.D. Comparisons between groups were performed using the Mann-Whitney-U-test for unpaired samples and Kruskal-Wallis analysis for multiple comparisons. Statistical calculations were performed using the SPSS statistical package version 12.0F. Statistical differences of at least  $p < 0.05$  were considered statistically significant.

## **Results and Discussion**

We report on the effect of a novel anti-tumour agent MitoVES<sup>21,22</sup> on MM cells and experimental mesotheliomas. This follows from earlier findings that the parental compound of MitoVES,  $\alpha$ -TOS, suppresses tumours in multiple models of cancer<sup>9-13</sup> and that it relays its effect via mitochondria by the interaction with the UbQ sites of CII.<sup>16,17</sup> Addition of the TPP<sup>+</sup> group to the parental compound caused a higher killing of tumour cells, considerably enhancing its efficacy.<sup>21,22</sup> Since MM is a fatal disease,<sup>1-3,27</sup> MitoVES has now been tested as a potentially useful MM therapeutic. MM cell lines H28, Meso-2, Ist-Mes-2, MM-BI and AE17 were exposed to  $\alpha$ -TOS and MitoVES. Table I documents that the IC<sub>50</sub> values were by up to two orders of magnitude lower for MitoVES than for  $\alpha$ -TOS. While MitoVES killed almost all cells within 24 h at  $>2.5 \mu\text{M}$ , similar efficacy of killing was achieved with 50-100  $\mu\text{M}$   $\alpha$ -TOS (Fig. 2A, B).

$\alpha$ -TOS and MitoVES exert their effect on cancer cells largely via mitochondria.<sup>11,14,15,21,22</sup>

We tested whether the agents cause generation of ROS and dissipation of  $\Delta\Psi_{m,i}$  in MM cells.

At 5  $\mu\text{M}$ , MitoVES generated high levels of ROS within  $<10$  min, consistent with earlier data



for neuroblastoma cells transfected with a redox-sensitive plasmid.<sup>22</sup>  $\alpha$ -TOS generated very low levels of ROS in MM cells at 100  $\mu$ M and at 60 min (Fig. 2C-E). MitoVES caused rapid dissipation of  $\Delta\Psi_{m,i}$  in MM cell lines (Fig. 2F). However, the drop of  $\Delta\Psi_{m,i}$  was mild in MMBI cells (Fig. 2F) and it increased in Meso-2 cells (not shown). We found that exposure of AE17 cells to the uncoupler CCCP (2  $\mu$ M) resulted in a decrease of  $\Delta\Psi_{m,i}$  from the initial relative level of 1 to 0.34, for MM-BI, H28 and Ist-Mes-2 cells to 0.76, 0.24 and 0.31, respectively, which corresponds to its maximum decrease when cells were exposed to MitoVES. In Meso-2 cells, CCCP caused a mild increase in  $\Delta\Psi_{m,i}$ , from the relative level of 1 to 1.09.  $\alpha$ -TOS caused only low and transient dissipation of  $\Delta\Psi_{m,i}$  (Fig. 2F). The differences in the effect of MitoVES and  $\alpha$ -TOS on the level of ROS generation and  $\Delta\Psi_{m,i}$  indicate potentially different modes of action of the two agents. While MitoVES is directly targeted to mitochondria,  $\alpha$ -TOS associates with a variety of lipophilic structures (*cf* refs 21 and 22, and see below).  $\alpha$ -TOS may trigger apoptosis both by a direct effect on mitochondria and on lysosomes.<sup>28</sup> The effect of  $\alpha$ -TOS on signalling pathways, such as shown for the FGF2 autocrine loop in MM cells<sup>12</sup> or the c-jun pathway in leukemia and breast cancer cells<sup>29,30</sup> can also play a role. Finally, it appears that MitoVES is capable of causing ROS generation even at initial low  $\Delta\Psi_{m,i}$  as long as it can be modulated by the agent.

Since the TPP<sup>+</sup> group targets small molecules to mitochondria, we tested mitochondrial association of MitoVES in MM cells. Cells grown on cover slips were incubated with fluorescent analogues of MitoVES and  $\alpha$ -TOS together with TMRM. Fig. 3A-D shows exclusive mitochondrial localization of MitoVES while  $\alpha$ -TOS localized to non-mitochondrial compartments as well as to mitochondria. Dissipation of  $\Delta\Psi_{m,i}$  with 2  $\mu$ M CCCP prior to incubation of Ist-Mes-2 cells with MitoVES caused a failure of exclusive

mitochondrial localization of the agent (Fig. 4E) and suppressed apoptosis induced by MitoVES (Fig. 4F). This documents the importance of  $\Delta\Psi_{m,i}$  for efficient killing of MM cells.

Mammalian cells comprise two complex II UbQ-binding sites, the proximal ( $Q_p$ ) and distal ( $Q_d$ ) site.<sup>31</sup> We proposed that while  $\alpha$ -TOS can interact with both sites,<sup>16</sup> MitoVES, due to its localization with the charged TPP<sup>+</sup> group in the matrix and the tocopheryl succinyl group in the mitochondrial inner membrane (MIM), can reach only the  $Q_p$  site.<sup>21</sup> To assess importance of the length of the aliphatic chain for efficient activity of MitoVES towards MM, we tested homologues of MitoVES by shortening the aliphatic chain by two carbons at a time. The agents were evaluated for their effect on the SQR activity as well as their efficacy to cause ROS generation,  $\Delta\Psi_{m,i}$  dissipation and apoptosis induction. Fig. 3G-J documents that the short-chain homologues were less active, with the C-9 homologue almost as efficient as the full length MitoVES. We also tested the premise that a free carboxylate of the succinyl moiety of MitoVES is important for its activity.<sup>9</sup> Fig. 3G-J shows that MitoVES4TPP was inefficient, documenting that the free carboxyl group is needed for the interaction of MitoVES with the  $Q_p$  site (serin 68)<sup>31</sup> of CII, as proposed earlier.<sup>16,21</sup>

The effect of small anti-cancer compounds on mitochondria can involve suppression of their respiration.<sup>7</sup> We tested this for  $\alpha$ -TOS and MitoVES in MM cells, first assessing the effect of the two agents on routine respiration, *ie* total oxygen consumption by whole cells in the cultivation medium in the absence of an uncoupler. Fig.4A documents much higher efficacy for MitoVES than that of  $\alpha$ -TOS, which was relatively inefficient. Interestingly, low levels of MitoVES (1-2  $\mu$ M) caused an initial increase in respiration, most likely due to mitochondrial uncoupling. This is consistent with experiments documenting uncoupling activity of MitoVES at low concentrations in intact cells and isolated mitochondria, with inhibition of respiration at its higher levels.<sup>32</sup> This inhibition is related to ROS generation in response to MitoVES and is compatible with CII as a source of radicals.<sup>21,22,33,34</sup> We next

tested the effect of MitoVES and  $\alpha$ -TOS on the contribution of CI and CII to mitochondrial respiration in permeabilised cells. Fig. 4B indicates that MM cells respire predominantly via CII and reveals a superior effect of MitoVES, which at lower concentrations uncoupled CII-dependent respiration in several of the cell lines, followed by its sharp inhibition. The agent also exerted an effect on CI at higher levels, reminiscent of a recent paper showing inhibition of the CI activity by  $\alpha$ -TOS in leukemia cells.<sup>35</sup>

In next experiments, we tested the effect of the VE analogues on the assembly of mitochondrial (super)complexes. Fig. 5A reveals that mitochondrial complexes form supercomplexes in Ist-Mes-2 and H28 cells, referred to as respirasomes.<sup>36</sup> While the assembly of CII, the target for MitoVES, was not affected, the agent destabilized at 5  $\mu$ M and in 1 h in particular the higher forms of supercomplexes. This is obvious for the CI+CIII<sub>2</sub>+CIV<sub>n</sub> respirasome in H28 cells, which is thought to allow for direct transfer of electrons from CI to CIII and to CIV, and is the most active form of the three complexes,<sup>36,37</sup> and the CI+CIII<sub>2</sub> supercomplex in Ist-Mes-2 cells.  $\alpha$ -TOS was much less efficient (Fig. 5A). Further, we found that MitoVES suppressed CII and CI activity using the in-gel assay following the separation of the mitochondrial fraction by clear native electrophoresis (Fig. 5B). Thus, MitoVES efficiently affects the assembly of mitochondrial supercomplexes in MM cells. This may be explained by the interaction of MitoVES with CII, which results in ROS generation and subsequent effect on mitochondrial (super)complexes, although the exact mechanism is yet to be established.

To unequivocally determine the importance of CII for the activity of MitoVES, we prepared MM cells in which its function was compromised by down-regulating the SDHC subunit of CII in Ist-Mes-2 cells by SDHC shRNA. Fig. 6A reveals a very low level of the SDHA protein and low SQR activity in the SDHC knock-down cells, while the SDH activity was only marginally lower. As expected, the CII-compromised cells respired less via CII

compared to the control cells (Fig. 6B). Importantly, Fig. 6C shows low level of apoptosis in the SDHC knock-down cells, clearly pointing to CII as the molecular target of MitoVES in MM cells.

We next tested the effect of MitoVES on experimental MM, *i.e.* xenografts prepared in nude mice subcutaneously grafted with Ist-Mes-2 cells. Fig. 6D documents the superior effect of MitoVES compared to  $\alpha$ -TOS, which was shown to inhibit MM earlier.<sup>12,13</sup> At 1  $\mu$ mol per dose per mouse given twice per week, MitoVES suppressed tumour progression by >90%, while  $\alpha$ -TOS at 15  $\mu$ mol was less efficient, with ~80% tumour growth inhibition. We did not observe any obvious toxicity of the agent during the course of the experiment. The data on the effect of MitoVES on MM cells and, in particular, its high cytotoxic efficacy compared to that of the parental  $\alpha$ -TOS, can be reconciled with our recent paper, in which we documented the development of resistance in cancer cells during their long-term exposure to  $\alpha$ -TOS.<sup>38</sup> We found that escalating doses of  $\alpha$ -TOS resulted in their resistance due to increased expression of the ABCA1 protein. This resistance was overcome by exposing the cells to MitoVES, which enters cells on the basis of their plasma membrane potential, bypassing the ABCA1 protein.<sup>38</sup> Recently, we treated a single MM patient with topical  $\alpha$ -TOS. The patient survived by 4 years following diagnosis (as opposed to the predicted ~6 months) with the tumour getting smaller. However, the tumour started to grow and the patient succumbed to the disease. It is possible that at this stage, the tumour developed resistance to  $\alpha$ -TOS, which could be potentially overcome by MitoVES. Given our findings on  $\alpha$ -TOS and MitoVES, we believe that the novel mitochondrial target, CII,<sup>8,16,17,21,22</sup> will be used in the future for such difficult-to-treat malignancies as is MM. In conclusion, since MM is a fatal malignancy with very few if any options beyond palliative therapy, our results on MitoVES are highly encouraging and of potential clinical benefit.

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**Table I.** IC<sub>50</sub> values for  $\alpha$ -TOS and MitoVES for MM cell lines.<sup>a</sup>

Cell line	IC <sub>50</sub>	
	$\alpha$ -TOS	MitoVES
H28	26.6	2.0
Ist-Mes2	37.7	0.25
Meso-2	32.9	0.9
MM-BI	25.6	0.75
AE17	37.5	0.5

<sup>a</sup>The IC<sub>50</sub> values were estimated from the killing curves of the individual cell lines exposed to  $\alpha$ -TOS and MitoVES.

## Legend to Figures

### Figure 1. Structures of compounds used in the study.

### Figure 2. MitoVES is toxic to MM cells and causes ROS generation and $\Delta\Psi_{m,i}$

**dissipation.** Ist-Mes-2, MM-BI, Meso-2, H28 and AE17 cells were seeded and allowed to reach 60-70% confluency. The cells were then incubated with  $\alpha$ -TOS or MitoVES for 24 h at the concentrations shown, and viability assessed by the crystal violet (A) and cell death by the annexin V/PI method (B). Panels C-E show increase in the level of ROS, evaluated as mean fluorescence intensity (MFI), assessed in MM cells exposed to MitoVES (5  $\mu$ M) and  $\alpha$ -TOS (100  $\mu$ M) using the fluorescent probes DHE, DCF and MitoSOX, respectively, and flow cytometry. Panel F reveals the relative levels of  $\Delta\Psi_{m,i}$  in MM cells exposed to MitoVES (5  $\mu$ M) and  $\alpha$ -TOS (100  $\mu$ M). The data are mean values  $\pm$  S.D. (n=3).

**Figure 3. Association of MitoVES with mitochondria and its cytotoxic activity in MM cells depend on their  $\Delta\Psi_{m,i}$  and on the structure of the agent.** MM-BI (A), Meso-2 (B), Ist-Mes-2 (C) and H28 cells (D) were seeded on cover slips and incubated for 30 min with TMRM and MitoVES-F or  $\alpha$ -TOS-F (both 5  $\mu$ M). E. Ist-Mes-2 cells were pretreated for 10 min with 2  $\mu$ M CCCP before the addition of VE analogues. The cells were then mounted in DAPI-containing Vectashield and images acquired in a confocal microscope. F. Ist-Mes-2 cells were seeded, allowed to reach 60-70% confluency, pre-treated (where shown) with 2  $\mu$ M CCCP for 10 min, exposed to  $\alpha$ -TOS or MitoVES for 24 h at the concentrations shown ( $\mu$ M) and evaluated for apoptosis using the annexin V/PI method. Ist-Mes-2 cells at 60-70% confluency were exposed to MitoVES analogues (5  $\mu$ M) as shown for 60 min and evaluated for SQR activity (G), ROS generation using the DHE probe (H) and dissipation of  $\Delta\Psi_{m,i}$

using TMRM (I). Cell death induction was evaluated using the annexin V/PI method after 24-h incubation (J). The images are representative of three independent experiments, data are mean values  $\pm$  S.D. (n=3); the symbol ‘\*’ indicates significant differences with  $p < 0.05$ .

**Figure 4. MitoVES suppresses mitochondrial respiration.** AE17, MM-BI, H28, Ist-Mes-2 and Meso-2 cells were seeded and allowed to reach 60-70% confluency. A. The cells were harvested and placed in the Oxygraph chamber at  $10^6$  per ml in the original medium. Oxygen consumption was assessed following addition of increasing concentration of  $\alpha$ -TOS or MitoVES. B. The cells were harvested, permeabilized and placed in the Mir05 medium in the Oxygraph chamber at  $2 \times 10^6$  per ml. Oxygen consumption linked to CI or CII was evaluated in the presence of increasing concentration of  $\alpha$ -TOS or MitoVES following inhibition of CII or CI, respectively, and using the appropriate substrates as detailed in Materials and methods. The data are mean values  $\pm$  S.D. (n=3).

**Figure 5. MitoVES affects the mitochondrial complexes assembly.** Ist-Mes-2 and H28 cells were allowed to reach 60-70% confluency, after which they were exposed to  $\alpha$ -TOS at 50  $\mu$ M for 0.5 h (1), 1 h (2) or 2 h (3) or at 25  $\mu$ M for 24 h (4), or to MitoVES at 5  $\mu$ M for 0.5 h (1), 1 h (2) or 2 h (3) or at 1  $\mu$ M for 24 h (4), and the mitochondrial fraction prepared. A. The mitochondrial fraction was subjected to NBE as detailed in Materials and methods. The text on the right indicates the position of (super)complexes and partially assembled complexes. B. The mitochondrial fraction was separated using clear native electrophoresis and the CI and CII in-gel activity assessed as detailed in Materials and methods. The activity related to the position of (super)complexes, as well as partially assembled complexes is indicated by the text on the right. The images are representative of three independent experiments.

**Figure 6. MitoVES kills MM cells by targeting CII and suppresses experimental MMs.**

Ist-Mes-2 cells were stably transfected with NS or SDHC shRNA as described in Material and methods, and evaluated for the level of the SDHC protein by WB and the activity of SQR and SDH (A), for respiration via CI and CII using the Oxygraph (B), and for the effect of MitoVES (5  $\mu$ M) on cell death using the annexin V/PI method (C). D. Nude mice were injected s.c. with  $2 \times 10^6$  Ist-Mes-2 cells per animal. The mice were treated intraperitoneally with 15  $\mu$ mol  $\alpha$ -TOS or 1  $\mu$ mol MitoVES dissolved in 4% EtOH in corn oil or with 100  $\mu$ l of the excipient (control mice) twice per week. Tumours were quantified using USI and expressed relative to the initial tumour volume ( $\sim 20$ - $30 \text{ mm}^3$ ). The images are representative of three independent experiments, the data in panels A-C are mean values  $\pm$  S.D. (n=3). The symbol ‘\*’ indicates significant differences with  $p < 0.05$ . The data in panel D are mean values  $\pm$  S.D. (n=3), the symbol ‘\*’ indicates significant differences between values from control and MitoVES-treated animals, ‘\*\*’ between  $\alpha$ -TOS- and MitoVES-treated animals, with  $p < 0.05$ .