Suppression of tumor growth *in vivo* by the mitocan α-tocopheryl succinate requires respiratory complex II

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Running title: Complex II is a mitocan target

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Statement of Translational Relevance

Vitamin E (VE) analogs have been proven efficient anti-cancer drugs in several experimental models, including the fatal mesotheliomas and the hard-to-treat HE2-positive breast carcinomas. The molecular target has not been unequivocally elucidated thus far, which is important before launching clinical trials. Here we show that the agents, epitomized by the redox-silent α-tocopheryl succinate, suppress progression of carcinomas with functional and reconstituted mitochondrial complex II (CII), while tumors with dysfunctional CII are resistant to the drug. Since CII rarely mutates in cancers, it is a promising target of clinical relevance for VE analogs and other, thus far unidentified agents.

Abstract

Purpose: Vitamin E (VE) analogs are potent novel anti-cancer drugs. The purpose of this study was to elucidate the cellular target by which these agents, represented by α-tocopoheryl succinate (α-TOS), suppress tumors *in vivo*, with the focus on the mitochondrial complex II (CII).

Experimental Design: Chinese hamster lung fibroblasts with functional, dysfunctional and reconstituted CII were transformed using H-Ras. The cells were then used to form xenografts in immunocompromized mice, and response of the cells and the tumors to α-TOS was studied.

Results: The CII-functional and –reconstituted cells, unlike their CII-dysfunctional counterparts, responded to α-TOS by ROS generation and apoptosis execution. Tumors derived from these cell lines reciprocated their responses to α-TOS. Thus, growth of CII-functional and –reconstituted tumors was strongly suppressed by the agent, and this was accompanied by high level of apoptosis induction in the tumor cells. On the other hand, α-TOS did not inhibit the CII-dysfuntional tumors.

Conclusions: We document in this report a novel paradigm, according to which the mitochondrial CII, which rarely mutates in human neoplasias, is a plausible target for anti-cancer drugs from the group of vitamin E analogs, providing support for their testing in clinical trials.

Mitochondria, organelles vital for cellular energy homeostasis as well as being central purveyors of cell death, have recently come into focus as promising targets for anti-cancer therapies (1-3). Mitocans are small compounds that exhibit anti-cancer activity by selectively inducing apoptosis by mitochondrial interference, comprising 7 classes of agents with different modes of action (4). The class 5 mitocans include drugs that act by targeting the mitochondrial electron transport chain (ETC), of which the prime examples are vitamin E (VE) analogs, epitomized by the ester α-tocopheryl succinate (α-TOS) (5, 6), 3-bromopyruvate (3BP) (7), and adaphostine (8). VE analogs also belong to the class 2 mitocans since, in addition to targeting the ETC, these agents act as Bcl-2 homology-3 (BH3) mimetics (9).

VE analogs hold substantial promise as selective anti-cancer drugs since they suppress tumor growth in several pre-clinical models, including mice with experimental breast (10, 11), lung (12), prostate (13) and colon carcinomas (6, 14), as well as mesotheliomas (15, 16). Due to its selectivity for cancer cells and low toxicity for non-malignant cells (5), the ester analog α-TOS is an agent that has considerable clinical potential. A similar level of anti-cancer efficacy and selectivity has also been reported for the ether analog of VE α-tocopheryloxyacetic acid (α-TEA) (17, 18).

The importance of mitochondria as target by which α-TOS induces apoptosis was proposed earlier (19-22). Although it is now established that the VE analog causes mitochondrial destabilization through the formation of Bax/Bak channels resulting in cytochrome c translocation and caspase activation (20-22), the precise molecular target for apoptosis induction by the agent was not fully understood. α-TOS has been recently identified as a compound that induces apoptosis by targeting the ubiquinone (UbQ)-binding sites in the mitochondrial complex II (CII; succinate dehydrogenase, SDH) (23). In this respect, we recently reported that the VE analog induces apoptosis by a novel mechanism via generation

of reactive oxygen species (ROS) upon displacing UbQ from the membrane subunits of CII

(23). Therefore, electrons that are generated during the conversion of succinate to fumarate by

subunit A of CII (SDHA, within the matrix part of the complex; 24) diffuse from CII and

reduce molecular oxygen to generate ROS (23, 25) that, in turn, induce apoptosis (21, 26, 27).

In this communication, we show the importance of CII as the major target for the mitocan,

α-TOS, using a mouse model with genetically modified tumors expressing either wild type,

defective or reconstituted CII. This study highlights the importance of CII as a target for anti-

cancer drugs as well as its essential role in the activity of the mitocan, α-TOS, for promoting

apoptosis.

Materials and methods

Cell culture, transfection and treatment. Parental Chinese hamster (ch) lung fibroblasts

(B1 cells) (28), CII-defective cells (B9 cells with mutant *CybL*, *SDHC*) (28), CII-reconstituted

cells (B9$_{SDHC}$ cells) (23), and CI-defective cells (B10 cells) (29), were grown in DMEM with

10% FCS, antibiotics, 5 mg/ml glucose and 1% (w/v) non-essential amino acids.

B1, B9 and B10 cells were transformed with H-Ras. Briefly, the cells were transfected

with *pEGFP-C3-H-Ras* (30) and the positive cells selected with G418. Single cell-derived

colonies were obtained by limiting dilution except for B10$_{Ras}$ cells. Clone 6 of B1$_{Ras}$ cells and

clone 1 of B9$_{Ras}$ cells were chosen for further experiments based on the level of the H-Ras

protein.

SDHC-mutant B9$_{Ras}$ cells were reconstituted by transfection with a vector encoding a

functional human (h) *SDHC* gene. First, the *pEFIRES-P-SDHC* plasmid was constructed.

SDHC was digested from *pCR3.1-SDHC* (31) using *Nhe1* and *Not1*. *SDHC* and *pEFIRES-P*

(32) were separately extracted from the gel using the QIAEX II kit (Qiagen). *SDHC* was then

inserted into *pEFIRES-P* and the resulting *pEFIRES-P-SDHC* digested with *BamHI* to

confirm the correct orientation of the cloned *SDHC*. The construct was amplified and purified using the QIAfilter plasmid purification kit. B9$_{Ras}$ cells were then reconstituted with *hSDHC* by transfection with *pEFIRES-P-SDHC*. G418 (100 μg/ml) and puromycin (4 μg/ml) were used for double selection; single clones were obtained by limiting dilution. Clone 5 of B9$_{Ras-SDHC}$ cells with high *SDHC* and H-Ras-EGFP expression was chosen for further studies.

For treatment, cells were grown to ~60% confluency before exposure to α-TOS or thenoyltrifluoroacetone (TTFA) (both Sigma). In some experiments, cells were pre-treated with MitoQ (33, 34).

Colony-forming assay. The colony-forming activity of the non-transformed B1, B9 and B9$_{SDHC}$ cells and their H-Ras-transformed counterparts was assessed as described (35). The number of colonies >50 μm in diameter in each dish was then counted using light microscopy.

Assessment of apoptosis, ROS accumulation and oxygen consumption. Apoptosis was estimated routinely with flow cytometry using the annexin V-FITC or annexin V-PE binding method(s) as detailed elsewhere (21).

Cellular ROS were detected by electron paramagnetic resonance (EPR) spectroscopy in cells loaded with the radical scavenger 5,5-dimethyl-1-pyrroline N-oxide (10 mM, DMPO; Sigma) (21, 23) or by flow cytometry using the redox-sensitive probe dihydroethidium (DHE) (21).

Western blotting and RT-PCR. Western blotting was performed using anti-SDHC IgG (clone 3E2; Novus Biologicals), anti-H-Ras IgG (BD Biosciences) and anti-GFP IgG with anti-β-actin IgG (both Santa Cruz) as a loading control.

For RT-PCR, the primers were as follows; *hSDHC* primers (31): sense 5'-CAC TTC CGT CCA GAC CGG AAC-3', anti-sense 5'-ATG CTG GGA GCC TCC TTT CTT CA-3'; *chSDHC* primers: sense 5'-CGT CCT GTT TCT CCC CAC CTC-3', anti-sense 5'-CAG CAA GCA TCA AGA CAG CCA C-3'; *chGAPDH* primers (36): sense 5'-GCA AGT TCA AAG

GCA CAG TCA A-3'; anti-sense 5'-CGC TCC TGG AAG ATG GTG AT-3'. For RT-PCR

ex vivo, total RNA was prepared from mouse tumors (see below) by homogenizing with

TRIZOL in a glass mortar placed on ice (Dual Size 22/5 ml, Kontes Glass).

   MTT and SDH activity analysis. The MTT viability assay was used to assess the

proliferative activity of the parental and H-Ras-transformed B1, B9, B10 and $B9_{SDHC}$ cell lines.

   SDH activity was measured using a short term (2 h) modified MTT assay with succinate as

the sole source of electrons, driving the respiratory system specifically via CII, as described (11,

23).

   Animal experiments. Immunocompromized, athymic Balb c/nu-nu mice were injected

subcutaneously with either $B1_{Ras}$, $B9_{Ras}$ or $B9_{Ras-SDHC}$ cells at $5x10^6$ cells per animal. After 2-3

weeks ($B1_{Ras}$ and $B9_{Ras-SDHC}$ cells) and 3-4 weeks ($B9_{Ras}$ cells), tumors were observed in the

animals and further inspected using the Vevo770 ultrasound imaging (USI) device equipped

with the RMV708 probe (frequency, 80 MHz; resolution, 30 μm) (VisualSonics) (10, 11, 23).

Mice with tumors were treated by intraperitoneal (i.p.) injection of α-TOS dissolved in corn

oil/4% ethanol (v/v) twice a week, at 10 μmol α-TOS for the first two doses and 6 μmol α-

TOS subsequently. The kinetics of tumor progression was quantified non-invasively twice a

week by measuring the tumor size using the USI device and the volumetric analysis software.

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.

   Histochemistry. Tumors were excised and either snap-frozen (-80°C) or fixed in neutral-

buffered formalin. The frozen tissue was used for RT-PCR and the fixed tissue was paraffin-

embedded and cut into 5 μm-thick serial sections, which were evaluated following

hematoxylin-eosin (H&E) staining. For fluorescence microscopy, nuclei were stained with

DAPI present in the Vectashield mounting medium (Vector Laboratories), and the tumor cells

detected on the basis of green fluorescence resulting from expression of *H-Ras-EGFP*. The confocal images were taken using the Olympus IX81 microscope operated by the Fluoroview v. 1.6A software. Tumor sections were also inspected for apoptotic nuclei. Three sections were evaluated for every type of tumor and condition.

Statistical analyses. Between-group comparisons were made using mean $\pm$ SD and the unpaired Student's *t* test. Differences in the mean relative tumor size ($\pm$ SEM) were examined using analyses of covariance (ANCOVA) with days as the covariate. Statistical analyses were performed using SPSS® 10.0 analytical software. Statistical significance was accepted at $P{<}0.05$.

Results

Our previous study with cells either defective in CII due to a mutation in *SDHC* (B9 cells) or reconstituted with CII (B9$_{SDHC}$ cells) and with cells defective in complex I (B10) supported the possibility that CII in cancer cell mitochondria could act as a potent target for anti-cancer drugs (23). In order to define the importance of CII as a target for mitocan activity on tumors *in vivo*, we first prepared a series of transformed Chinese hamster lung fibroblast cell lines by transfection with oncogenic *H-Ras*. We then showed that these cells were capable of growing and forming tumors *in vivo*, unlike the parental non-transformed cells. For the reconstitution, the *hSDHC* gene was cloned from the original *pCR3.1-SDHC* plasmid into *pEFIRES-P*, and the resulting *pEFIRES-P-SDHC* plasmid was transfected into B9$_{Ras}$ cells to produce the B9$_{Ras-}$ $_{SDHC}$ cell line. Individual clones from B1/B1$_{Ras}$, B9/B9$_{Ras}$, B9$_{Ras-SDHC}$ and B10/B10$_{Ras}$ cells were tested for the level of *hSDHC* mRNA as well as the expression of the EGFP and H-Ras proteins (Fig. 1A and B). Data in Figure 1c documents the levels of expression of the Chinese hamster *chSDHC* mRNA and human *hSDHC* mRNA in the B1$_{Ras}$ (lane 1), B9$_{Ras}$ (lane 2), B9$_{Ras-SDHC}$ (lane 3) and B10$_{Ras}$ cells (lane 4). Based on these results, B1$_{Ras}$ clone 6, B9$_{Ras}$

clone 1 and $B9_{Ras-SDHC}$ clone 5 were selected for further experiments.

Since H-Ras is an oncogene that alone is capable of causing malignant transformation (37), we compared the phenotype of non-malignant versus H-Ras-transformed cells after selecting clones by the limiting dilution method. Parental B9 cells showed elongated and relatively uniform morphology (Fig. 2A, left image) that differed from the H-Ras-transformed ($B9_{Ras}$) cells which were irregular and mainly non-polarized (Figure 2A, middle image). Fluorescence microscopy revealed that all of the $B9_{Ras}$ cells were positive for the H-Ras protein, as assessed by EGFP expression (Fig. 2A, right image). Similar observations of altered morphology were made for the $B1/B1_{Ras}$ and $B9_{SDHC}/B9_{Ras-SDHC}$ cells (data not shown). None of the non-malignant Chinese hamster lung fibroblast cell lines formed colonies in soft agar (Fig. 2B), whereas prominent colony formation was observed for the H-Ras-transformed cells, and the malignant cells showed increased proliferation rates compared to the parental cells (Fig. 2C). Importantly, the *SDHC*-mutant $B9_{Ras}$ cells maintained significantly lower levels of SDH enzymatic activity than the $B1_{Ras}$, $B10_{Ras}$ and $B9_{Ras-SDHC}$ cells (Fig. 2D).

We next tested the selected clones of $B1_{Ras}$, $B9_{Ras}$ and $B9_{Ras-SDHC}$ cells, as well as $B10_{Ras}$ cells for their response to α-TOS and to the CII inhibitor, TTFA (24). The data (Fig. 3A and B) revealed that $B1_{Ras}$, $B9_{Ras-SDHC}$ and $B10_{Ras}$ cells, unlike the $B9_{Ras}$ cells, responded to α-TOS by early generation of ROS and apoptosis induction. The mitochondrially targeted antioxidant MitoQ, a UbQ analog that can reach the UbQ-binding sites in CII, but not those in CI or CIII (33), significantly suppressed apoptosis induction by α-TOS in the $B1_{Ras}$, $B9_{Ras-SDHC}$ and $B10_{Ras}$ cells but not the $B9_{Ras}$ cells. TTFA exerted comparable effects to those of the VE analog with $B9_{Ras}$ cells again showing resistance. ROS accumulation in response to α-TOS was further documented by EPR spectroscopy analysis (Fig. 3C), providing similar results to those obtained from using fluorescent probes and flow cytometry for ROS

measurement. The specificity of the drug, α-TOS, targeting CII but not CI was confirmed in other studies using rotenone to inhibit CI (data not shown). No significant differences in ROS production by cells treated with α-TOS were detected, even at high (~300 μM) levels, and comparing with or without rotenone inhibition. Hence, the data is entirely consistent with α-TOS targeting CII, but not CI in tumor cell mitochondria.

After establishing that the Ras-transformed cells with defective CII were less responsive to the drug, we proceeded to address whether CII would also be critical as a target for the anti-cancer drugs in tumor models growing *in vivo*. We assessed the ability for α-TOS to suppress the growth of tumors produced by either of the $B1_{Ras}$, $B9_{Ras}$ and $B9_{Ras-SDHC}$ cells using immunocompromized, athymic Balb/c nu/nu mice. We found that the H-Ras-transformed cells, injected subcutaneously at $5x10^6$ cells per mouse, all successfully developed into tumors, albeit at different growth rates. Of the three cell lines, the slower-proliferating $B9_{Ras}$ cells were less efficient, requiring about twice as long to establish tumors from the same number of initial cells injected per mouse when compared to animals receiving the $B1_{Ras}$ or $B9_{Ras-SDHC}$ cells. Before assessing the response of the tumors to α-TOS, we analyzed tumor tissue derived from the different cell lines for differences in SDHC expression to validate their CII status of the cells growing as tumors *in vivo.* Firstly, sections of tumors were assessed for morphology and were found exhibit characteristics of poorly differentiated sarcomas. Secondly, the sections were assessed for levels of green fluorescence, characteristic of H-Ras-EGFP expression and this revealed the majority of cells within the tumors to be of malignant origin (Fig. 4A). We also inspected tumor sections prepared from the α-TOS-treated mice by fluorescence microscopy (see below). Evaluation of apoptosis in the sections by the appearance of blebbed and fragmented nuclei on DAPI staining showed a high level of sensitivity to α-TOS of the $B1_{Ras}$ and $B9_{Ras-SDHC}$ but not the $B9_{Ras}$ cell-derived tumors (Fig. 4B). Parallel RT-PCR analysis validated the presence of the *chSDHC* mRNA in $B1_{Ras}$ cell-

derived tumors and *hSDHC* mRNA in the B9$_{Ras-SDHC}$ cell-derived tumors. Neither the

chSDHC nor the murine *mSDHC* mRNA was detected in the B9$_{Ras}$ cell-derived tumors.

Hence, the pattern of *SDHC* mRNA expression in the different types of tumors matched the

same distribution in the cell lines from which the tumors were derived (Fig. 4C).

Since the different types of tumors maintained the characteristics of the source cells, we

next assessed the effect of the VE analog on tumors with fully functional, dysfunctional or

reconstituted CII. Fig. 5 reveals that B1$_{Ras}$ and B9$_{Ras-SDHC}$ cell-derived tumors were highly

responsive to α-TOS, whereas the B9$_{Ras}$ cell-derived tumors were relatively resistant to the

same dose of the VE analog. This was also consistent with the reduced apoptotic index

revealed by DAPI-staining nuclei in sections from α-TOS-treated mice with B9$_{Ras}$ cell-

derived tumors (*cf*. Fig. 4). Thus, the results consistently supported the importance of CII as a

target for the effectiveness of the mitocan, α-TOS, as an anticancer drug working *in vivo*.

Discussion

Mitochondria have emerged as attractive targets for anti-cancer drugs (1-3, 38). We have

recently proposed to classify these drugs by the term mitocans, referring to small molecules

with anti-cancer activity that target mitochondria and induce apoptosis (4, 25, 39-41). VE

analogs, selectively inducing apoptosis in cancer cells (5), initiate the cascade of reactions that

stimulate ROS accumulation and that culminates in cancer cell death (11, 21, 23). We have

placed VE analogs into group 5 of mitocans, since they act by targeting the mitochondrial

ETC (11, 23), similar to 3BP, adaphostin or tamoxifen. Of these mitocans, VE analogs, such

as α-TOS and α-TEA, and 3BP affect CII of the ETC (7, 11, 23), while adaphostin acts at the

level of CIII (8) and tamoxifen inhibits the activity of CI (42).

Mitochondrial CII, endowed with SDH activity, comprises 4 subunits with the catalytic

subunit (the FAD-containing subunit F$_P$ or SDHA) and the Fe-S cluster subunit (the I$_P$ subunit

or SDHB) within the mitochondrial matrix, and trans-membrane subunits, cytochrome b large (CybL or SDHC) and cytochrome b small (CybS or SDHD) within the mitochondrial inner membrane. The SDHC and SDHD subunits contain the proximal (Q_P) and distal UbQ-binding sites (Q_D). UbQ acts as the terminal acceptor of electrons formed during the conversion of succinate to fumarate at SDHA, and electrons are channeled to the UbQ sites via the Fe-S clusters of SDHB (24). 3BP acts at the SDHA subunit, inhibiting the conversion of succinate to fumarate (43). α-TOS targets the Q_D and Q_P sites, effectively displacing UbQ (22), in a similar way to the metabolic poison, TTFA (24). The net effect of displacing UbQ is enhanced recombination of electrons with molecular oxygen and generation of superoxide anion radicals (44-47), leading to the induction of apoptosis by destabilizing the mitochondrial outer membrane (45).

Mitochondrial CII as a molecular target for α-TOS, and also other VE analogs (11), was previously proposed at the level of cell culture (23). However, before testing any potential therapeutic anti-cancer applications, it is important to first establish the molecular target in animal cancer models. To do this, we generated tumors in immunocompromized mice using CII-functional (B1), CII-defective (B9) and CII-reconstituted (B9$_{SDHC}$) Chinese hamster lung fibroblasts (23), which had been transformed with H-Ras. The transformed cells exerted a malignant phenotype, as evidenced by high colony-forming activity, higher proliferation rates and a malignant/transformed morphology compared to their non-transformed counterparts. We exposed the malignant cells to α-TOS and TTFA. Both agents caused accumulation of ROS and induction of apoptosis in the malignant CII-proficient and SDHC-reconstituted cells (as well as the CI-dysfunctional cells), while the *SDHC*-mutant cells were resistant to both CII-targeting agents. Moreover, α-TOS-induced apoptosis was inhibited by MitoQ, which has been shown to interact with CII but not CI or CIII (34).

H-Ras-transformed cells with a malignant phenotype were previously used to form tumors

in immunocompromized mice and to compare the anti-neoplastic efficacy of various agents (48). However, to the best of our knowledge, the use of transformed cells with selected genetic mutations (49, 50) in components of the respiratory chain to prove the molecular action of an anti-cancer agent has not been previously reported. Subcutaneous injection of $B1_{Ras}$ and $B9_{Ras\text{-}SDHC}$ cells caused generation of ultrasound-detectable tumors within 2-3 weeks, while this required 3-4 weeks in the case of the slower proliferating $B9_{Ras}$ cells. Since H-Ras was expressed in the tumor cells as a fusion protein with EGFP, we were able to use the green fluorescence protein to document that virtually all cells within the three tumor types maintained H-Ras expression. The tumors also exerted similar patterns of expression of *SDHC* mRNA as the cells from which they were derived. These data suggest that the two transgenes, *H-Ras-EGFP* and *hSDHC*, became stably incorporated into the genome and were not lost upon propagation of the cells growing within the developing tumors. Hence, the effects of anti-cancer agents on the tumors can be effectively and accurately compared with those of the corresponding cell lines, providing a unique set of cancer cell models for examining the impact of respiratory chain targeting agents on cancers growing *in vivo*. They can also be used to evaluate the importance of additional *in vivo* confounding factors, such as the efficiency of tumor targeting and bioavailability. In this respect it is very promising that $B1_{Ras}$-, $B9_{Ras}$- and $B9_{Ras\text{-}SDHC}$-derived tumors growing in mice *in vivo* showed similar susceptibility to α-TOS as did the corresponding cultured cell lines growing *in vitro*. Thus, tumors with fully functional CII were responsive to α-TOS, while tumors with mutant *SDHC* were more resistant to the VE analog. These data document for the first time the importance of CII as a target for anti-cancer drugs *in vivo*, as exemplified by the vitamin E analog, α-TOS. The importance of CII as a target for α-TOS was further supported by the finding that the VE analog acts mainly at the level of CII, only marginally at CI requiring much higher concentrations of the agent, and does not target CIII, as documented by the O_2 consumption

assays using mitochondria isolated from hepatocarcinoma (data not shown).

The CII subunits SDHB, SDHC and SDHD have been proposed as tumor suppressors in several types of neoplastic diseases, including pheochromocytoma and paraganglioma (51-54) and, rarely, renal cell carcinoma (52, 54). With respect to highly widespread cancers, it has been shown that one in one million breast cancers features a CII mutation (54), further supporting the very low frequency of mutations in subunits of CII in neoplastic disease.

Mutations resulting in malfunctional CII may lead to an increase in sustained generation of ROS, consequently causing conversion of the cell to a malignant phenotype over a relatively long period of time, ultimately contributing to the appearance of neoplastic tissue (55-58). Similar findings have also been demonstrated for other mutations occurring in the mitochondrial genome (59). However, blocking the activity of fully functional CII by acute pharmacological intervention, as documented herein for α-TOS, results in the generation of high levels of ROS. Under these conditions, with elevated ROS that are unsustainable by the malignant cell, the cell will succumbs and will die via apoptosis. These observations may lead to novel approaches to cancer therapy targeting this mitochondrial respiratory complex. Whether neoplasias of the peripheral nervous system with mutations in their CII subunits are resistant to α-TOS treatment remains to be confirmed. Notwithstanding, we believe that the data presented in this study have clearly identified CII as an essential and novel *in vivo* target required for the anti-cancer drugs epitomized by the malignant cell-selective mitocan, α-TOS (5). Our results are consistent with the renewed interest in mitochondria as a very promising target for selective killing of cancer cells (60).

In conclusion, we have documented the importance of the role played by the mitochondrial CII as a target for VE analogs in a relevant animal model, although it cannot be excluded that the agents also act by targeting other sites. The importance of this finding is two-fold. First, it will promote a search for other compounds that may exert similar anti-

cancer effects via CII, opening the possibility of identifying novel mitocans with even more potent activity than α-TOS. Second, since mutations in CII occur very rarely in human cancers, this complex appears an intriguing target for anti-cancer drugs, and based on the series of promising results from our pre-clinical studies, we are now planning to start clinical trials in cancer patients using the CII-targeting drug, α-TOS. The importance of our results can be also seen in the context of recent findings that individual types of cancers are complex and can differ considerably in their array of DNA mutations, harbouring different sets of genetic causes. This indicates that it will be very unlikely to cure cancer by drugs targeting only a few gene products or single pathways that are essential for tumor survival (61). What is needed then is an invariant target, common to all cells, but which is predominantly only affected by drugs when delivered inside the cancer cells. Mitocans, epitomised here by the VE analog α-TOS targeting the mitochondrial CII, may meet this demand.

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Legend to Figures

Fig. 1. Preparation of H-Ras-transformed cells. *A,* Following transfection of $B9_{Ras}$ cells with *pEFIRES-P-SDHC*, individual clones of the cells were tested for the level of expression of *hSDHC* mRNA (lanes 2-8). Lane 9 shows analysis of $B9_{Ras}$ cells as a negative control lacking full length *SDHC* expression. *chGAPDH* mRNA was used as a loading control. *B,* B1, $B1_{Ras}$, B9, $B9_{Ras}$, B10 and $B10_{Ras}$ cells were probed for the expression of the H-Ras protein by western blotting (top panel). Middle and bottom panels show the level of H-Ras and EGFP protein expression in individual clones of $B1_{Ras}$, $B9_{Ras}$ and $B9_{Ras-SDHC}$ cells selected by limiting dilution. β-Actin was used as a loading control. *Ci* Selected clones of $B1_{Ras}$ (clone 6; lane 1), $B9_{Ras}$ (clone 1; lane 2), $B9_{Ras-SDHC}$ (clone 5; lane 3) as well as $B10_{Ras}$ cells (lane 4) were analyzed for expression of *chSDHC* and *hSDHC* with *chGAPDH* mRNA as a loading control. Images shown are representative of at least 3 independent experiments.

Fig. 2. H-Ras-transfected cells feature a malignant phenotype. *A,* B9 and $B9_{Ras}$ cells were seeded on cover slips and observed by light and fluorescence microscopy ($B9_{Ras}$ cells). *B,* B1, $B1_{Ras}$, B9, $B9_{Ras}$, $B9_{SDHC}$ and $B9_{Ras-SDHC}$ cells were seeded in soft agar and allowed to form colonies for 14 d, and the colony number per dish was counted. *C,* B1, $B1_{Ras}$, B9, $B9_{Ras}$, $B9_{SDHC}$ and $B9_{Ras-SDHC}$ cells were assessed for their relative proliferation rate in culture based on MTT activity. *D,* $B1_{Ras}$, $B10_{Ras}$, $B9_{Ras}$ and $B9_{Ras-SDHC}$ cells were assessed for their SDH activity, as detailed in the Materials and Methods section. The images shown are representative of at least 3 independent experiments. The data in panels *B* and *C* are mean

values ± S.D. (n=3), with the symbol '*' denoting significant differences between parental and

H-Ras-transformed cells, and the symbol '**' indicating significant differences between $B1_{Ras}$

and $B9_{Ras}$ cells ($P<0.05$).

Fig. 3. $B1_{Ras}$, $B9_{Ras}$ and $B9_{Ras\text{-}SDHC}$ cells show different sensitivity to CII-targeting drugs.

$B1_{Ras}$, $B9_{Ras}$, $B9_{Ras\text{-}SDHC}$ and $B10_{Ras}$ cells were exposed to α-TOS (*A*) and TTFA (*B*) at the

levels indicated (μM) for 6 h or as shown (ROS), or for 12 h (apoptosis). Flow cytometry was

applied to analyze ROS accumulation using the fluorescent probe DHE and apoptosis levels

using annexin V-PE. Where shown, cells were pre-incubated for 1 h with 2 μM MitoQ. Panel

C shows ROS accumulation in $B1_{Ras}$ (1), $B9_{Ras}$ (2) and $B9_{Ras\text{-}SDHC}$ (3) cells exposed for 2 h to

50 μM α-TOS after loading with DMPO, as analyzed by EPR spectroscopy, with the area

under the quartet signal indicative of the levels of ROS accumulated. The data shown are

mean values ± S.D. (n=3). The symbol '*' denotes significant differences between levels of

ROS or apoptosis in treated cells compared to the controls, except for the left graph in panel

A, where it indicates significant differences between levels of ROS in $B1_{Ras}$, $B9_{Ras}$ and $B10_{Ras}$

cells, and the symbol '**' indicates significant differences in apoptosis between samples with

and without MitoQ ($P<0.05$). The EPR spectra in panel *C* are representative of three

independent experiments.

**Fig. 4. Tumors derived from H-Ras-transformed cells maintain features of the source

cells and show different apoptosis level when expose to α-TOS.** *A,* $B1_{Ras}$, $B9_{Ras}$ and $B9_{Ras\text{-}SDHC}$ cell-derived tumors from control and α-TOS-treated mice were sectioned, and were

assessed for morphology following H&E staining (left images) and by fluorescence

microscopy for H-Ras-EGFP expression (reflected by the extent of green fluorescence of the

plasma membrane). Nuclei were counterstained using DAPI. *B,* Level of apoptosis in tumors

from control and α-TOS-treated animals was assessed on the basis of presence of nuclei with apoptotic morphology. *C,* RNA was extracted form $B1_{Ras}$ (lanes 1), $B9_{Ras}$ (lanes 2) and $B9_{Ras\text{-}SDHC}$ (lanes 3) cell-derived tumors and assessed by RT-PCR for *hSDHC* and *chSDHC* mRNA, with *chGAPDH* mRNA as a loading control. The images shown are representative of at least 3 independent experiments, and the symbol '*' indicates significant differences in apoptosis between $B1_{Ras}$, $B9_{Ras}$ and $B9_{Ras\text{-}SDHC}$ cell-derived tumors (*P*<0.05), with 100 cells in three sections assessed for each tumor type.

Fig. 5. Tumors derived from CII-malfunctional cells are resistant to α-TOS. Mice with $B1_{Ras}$ (*A*), $B9_{Ras}$ (*B*) and $B9_{Ras\text{-}SDHC}$ (*C*) cell-derived tumors were treated by i.p. injection of α-TOS every 3-4 d as detailed in the Materials and Methods section, and assessed for tumor volume using the Vevo770 USI device. The images on the right document USI visualization of corresponding representative tumors on day 22 of the experiment in control and α-TOS-treated animals, with arrows indicating the position of the tumors. The data in graphs in panels *A-C* represent mean values ± S.E.M. (n=5-7). The symbol '*' denotes significant differences between tumor volumes of control versus the treated animals, with *P*<0.05. The images are derived from a single mouse from each group and are representative of all animals in the particular treatment group.