RESEARCH ARTICLE

Affinity of vitamin E analogues for the ubiquinone complex II site correlates with their toxicity to cancer cells

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Scope: Vitamin E (VE) analogues, epitomised by the prototypic α-tocopheryl succinate (α-TOS), are potent anti-cancer agents. α-TOS has recently been shown to trigger apoptosis by targeting the ubiquinone (UbQ) binding site(s) of the mitochondrial complex II (CII) and to cause excessive production of reactive oxygen species.
Methods and results: We have modelled, using two approaches, a range of VE analogues into the proximal UbQ (Q_p) binding site of CII. This study reveals that in both cases, the calculated interaction energies of individual VE analogues correlate (R^2 value >0.8) with their toxicity to cancer cells.
Conclusion: These data further support the UbQ site(s) of CII as an important target determining the anti-cancer activity of VE analogues as well as other emerging anti-cancer drugs.

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1 Introduction

Vitamin E (VE) analogues, epitomised by α-tocopheryl succinate (α-TOS), are potent, cancer cell-selective inducers of apoptosis [1–4]. This notion stems from studies that proved them to be efficient anti-cancer agents in pre-clinical studies with a host of experimental carcinomas [5–11], including the highly recalcitrant malignant mesotheliomas [12, 13] and HER2-positive breast carcinomas [14].

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Abbreviations: CII, mitochondrial complex II; **IE,** interaction energy; **MIM,** mitochondrial inner membrane; **ROS,** reactive oxygen species; **SCC-DFTB-D,** self-consistent-charge density functional tight binding method with dispersion; **SDH,** succinate dehydrogenase; **α-TOS,** α-tocopheryl succinate; **UbQ,** ubiquinone; **VE,** vitamin E

The molecular mechanism of apoptosis induction by VE analogues has been studied [15]. It has been unequivocally documented that these agents can be referred to as mitocans, i.e. small molecules with anti-cancer efficacy acting by mitochondrial destabilisation [16, 17]. Mitocans are classified into eight groups, containing compounds with different molecular targets. Thus, group I of mitocans is presented by hexokinase inhibitors, group II comprises agents interfering with the BH3 function of Bcl-2 family proteins, group III thiol redox inhibitors, group IV VDAC/ANT-targeting drugs, group V electron transport chain targeting drugs, group VI lipophilic cations targeting the inner membrane, group VII agents targeting mtDNA and group VIII drugs with thus far unknown target [16, 17].

Within mitocans, VE analogues belong to group II, which includes the BH3 mimetics and related compounds functioning to block the Bcl-2 family of pro-survival proteins. This activity helps to explain how these agents sensitise cancer cells to other chemotherapeutic drugs [18]. VE analogues also belong to the group V of mitocans comprising compounds that interfere with the mitochon-

drial electron redox chain [19]. This activity is the main reason why α-TOS causes generation of reactive oxygen species (ROS) as an early event triggering the apoptotic signalling cascade [13, 20–24].

We have recently documented that VE analogues act by interfering with the mitochondrial complex II (CII) with the succinate dehydrogenase (SDH) enzymatic activity [25–27]. More specifically, VE analogues displace ubiquinone (UbQ), the natural acceptor of electrons that are generated by SDH during the conversion of succinate into fumarate. With UbQ not available, the electrons can be no longer intercepted and are likely to recombine with molecular oxygen. The net result of UbQ substitution by α-TOS or its analogues is the formation of increased levels of superoxide. We have previously presented evidence for this mechanism using cells containing genetic mutations in CII (the SDHC subunit) [26] as well as tumours with malfunctional CII (impaired SDH activity) [27]. Thus, the UbQ site(s) of CII present novel targets for anti-cancer drug therapy [19].

Many analogues of VE with apoptogenic activity have been synthesised and tested in cell culture and, in some instances, also in animal models [28–31]. To determine whether the various analogues (some of which show potential clinical relevance) act by targeting CII, we correlated their cytotoxicity towards cancer cells with the calculated affinity with which they bind to the well-characterised proximal UbQ-binding (Q_p) site determined from the crystal structure of mammalian CII [32].

2 Materials and methods

CII contains four proteins, of which three are relevant to the binding of UbQ. These are the iron-sulphur protein (subunit B, SDHB), the large (subunit C, SDHC; cytochrome b large, CybL) and small (subunit D, SDHD, CybS) transmembrane proteins. The flavoprotein containing the SDH enzymatic domain (subunit A, SDHA) with its associated FAD was not used for the docking studies. VE analogues, used in the modeling studies, comprise three domains (Fig. 1) and their structures are shown in Fig. 2.

2.1 Modelling approach I

Modelling of interaction of the prototypic VE analogue, α-TOS (compound 1), into the Q_p site of CII was based on the crystal structure data published earlier for the porcine CII and deposited in the Brookhaven Protein Databank (code 1ZOY) [32], basically as described earlier [26]. A BLAST search from the NCBI website showed that the sequence identity between porcine and human CII is very high: 97% for the iron-sulphur protein subunit, 90% for the large trans-membrane subunit and 94% for the small trans-membrane subunit.

The protein structure was prepared for docking by first adding hydrogen atoms using the program Reduce [33], which also optimised the internal hydrogen-bonding network of the protein. Atom types and partial charges for the cysteines involved in the iron-sulphur clusters and the haeme group were taken from the Amber 7 distribution [34]. The iron–sulphur clusters were treated in a simple way with the following overall charges being assigned to them, [2Fe-2S] = +2, [4Fe-4S] = +2 and [3Fe-4S] = +1. AutoDock Tools [35] was then used to prepare the rest of the protein by merging non-polar hydrogens and then adding Kollman United Atom charges and Stouten solvation parameters. UbQ5 was built from the crystal structure coordinates of the bound UbQ (1ZOY) using InsightII [36]. α-TOS was built from the crystal structure MOPHLB01 retrieved from the Cambridge Structural Database [37] by a substructure search for the ring system of α-TOS, again using InsightII. The same software was used to model the structures of remaining compounds/VE analogues based on the α-TOS structure. Both ligands were then prepared for docking by AutoDock Tools, which included merging non-polar hydrogens, assigning Gasteiger charges and defining the rotatable bonds.

Docking was performed using the Lamarckian Genetic Algorithm as implemented in Autodock 3.0.5 [38]. A docking grid was prepared of the size of 126 × 126 × 126 points with a grid spacing of 0.375 A?, centred on Tyr173 (Chain B) in the Q_p binding site. Default AutoDock parameters were used except for the following, which were increased due to the relatively high number of rotatable bonds present in the ligands of interest (UbQ5 = 16, α-TOS = 17):- ga_run = 250, ga_pop_size = 250, ga_num_evals = 10 000 000. Also, the parameter rmstol was increased to 2.5, to produce more manageable clusters during the analysis phase of the calculation. The docking calculation took just over 24 h on a 2.2-GHz AMD Opteron-based computer. Analysis of the results was performed using scripts provided with AutoDock and the docked structures were viewed using Astex Viewer [39].

Modelling of binding of the other VE analogues to the Q_p site of CII and calculations of their interaction energies (IE) with the site were from the best ranked conformation in the Q_p site.

2.2 Modelling approach II

To support the suggested correlation, an independent theoretical procedure was used. The IE was calculated using the accurate [40] self-consistent-charge density functional tight binding method [41] augmented with the empirical dispersion term [42] (SCC-DFTB-D) level of theory. The geometry of the ligands was obtained from 2 ns molecular dynamics simulation of the mitochondrial respiratory CII protein.

The binding of different VE analogues to the mitochondrial respiratory CII protein was studied theoretically by

means of empirical force field molecular modelling/molecular dynamics and SCC-DFTB-D methods. The initial geometry of the protein was taken from the available crystal structure of the porcine heart CII (PDB code 1ZOY). The initial position of the ligands was modelled by aligning the ligand molecules with the molecule of mitochondrially targeted VE succinate in its previously predicted Q_p binding position [43]. The protein residues found within 10 A? from the aligned ligands were selected for the calculations. The final selection contained residues 76–81, 103–104, 164–179 and 209–224 from chain B, 29–50, 100–111 and 130 from chain C and 83–98 from chain D. Terminal residues of the selection were capped with ACE and NME groups. The molecular dynamics study was performed employing the AMBER force field. The parm99 force field [44] was used for the standard protein residues, while for the ligand molecules the general AMBER force field (GAFF) [45] parameters were used. The point charges were determined by a restrained fit to the electrostatic potential (RESP) according to recommended procedures [46]. The modelled complexes were placed in a periodic rectangular box extending by 1 nm the complex dimensions along all three axes. The box was filled with TIP3P water molecules. Chlorine ions were added to neutralise the system placing them at the positions with the lowest electrostatic potential. An MD simulation was then conducted employing the GROMACS suite of programs [47]. The used equilibration procedure consists of heating the water molecules separately to 300 K during 20 ps with the solute at 10 K, followed by a 20-ps heating of the whole system to 300 K, while applying position restraints on the heavy atoms of the solute. After heating, the position restraints only on the carbonyl carbons of protein were used (allowing for side-chain rearrangement while keeping the backbone fixed), and the 2-ns simulation at constant temperature of 300 K and constant pressure of 1 atm was performed. A time step of 2 fs with van der Waals and electrostatics cut-offs of 1 nm were used throughout the simulations. The equilibrated last 500 ps of the trajectory was used to obtain the averaged geometry of the complexes. The geometries were further optimised with the same empirical force field parameters as used for the simulation. The solvent molecules were removed and the IE were calculated using the SCC-DFTB-D method [40–42] by subtracting the calculated total energies of the selected protein residues and the ligand from the energy of complex.

The IC_{50} values for individual VE analogues were obtained from publications referred in Table 1.

Table 1. IC_{50} values and interaction energies for the CII Q_p site of selected VE analogues

Compound number	IC_{50} (μM)[a)]	Interaction energy (kcal/mol)[b)]	
		AutoDock	SCC-DFTB-D
4	2 (31)	−15.34	−137.4
13	6 (18)	−13.89	−127.9
12	6.5 (18)	−13.67	−129.7
11	9 (32)	−15.01	−130.5
5	12 (28)	−13.89	−141.2
3	13 (31)	−14.38	−136.4
14	13.5 (18)	−12.38	−144.3
18	15 (28)	−13.74	−129.2
6	17.5 (18)	−14.23	−141.4
10	20 (31)	−14.71	−136.4
2	22 (29)	−14.55	−133.7
17	22 (28)	−13.29	−141.6
1	43 (29)	−14.04	−137.9
9	49 (29)	−14.08	−126.1
8	66 (29)	−14.64	−132.1
7	86 (29)	−13.58	−131.3
15	100 (18)	−10.97	−123.7
16	194 (28)	−11.24	−94.7
19	200 (30)	−8.66	−75.0
20	200 (30)	−8.66	−80.3

a) The IC_{50} values were compiled based on the publications shown in brackets at each compound, and are derived from experiments with the following cell lines: Jurkat (compounds 1, 2, 3, 4, 7, 8, 9, 10, 11), MCF-7 (compounds 5, 6, 16, 17, 18), PC-3 cells (compounds 12, 13, 14, 15, 19, 20).

b) The IE values for binding of VE analogues to the Q_p site of CII were calculated using the AutoDock or SCC-DFTB-D approach as detailed in Section 2.

3 Results and discussion

VE analogues, represented by α-TOS, are cancer cell-selective mitocans. We have recently identified their key molecular target in cancer cells as the UbQ sites of the mitochondrial CII [26]. By interacting with the UbQ-binding site(s) formed by the SDHC and SDHD subunits of CII [32], the agents most likely displace UbQ, the natural acceptor of electrons generated by the conversion of succinate into fumarate at the SDHA subunit of CII. In the presence of VE analogues, the electrons generated by conversion of succinate to fumarate can no longer be intercepted by their natural ligand and recombine with molecular oxygen to yield superoxide [26, 27]. The ROS then trigger a cascade of reactions that culminate in formation of Bax and/or Bak channels in the mitochondrial outer membrane allowing for mitochondrial apoptotic regulators to translocate to the cytosol, activating caspases that results in the entry of the cell into the commitment phase of apoptosis [48–50]. Additional supporting evidence is provided by the observation that various anti-cancer drugs do not induce efficient apoptosis in cell lines with a mutation in the SDHC subunit of CII [51]. Another mitocan, 3-bromopyruvate, has been reported to induce apoptosis, also inhibiting the SDH activity of CII like α-TOS, but by interfering with the catalytic centre at the level of the SDHA subunit [52].

The UbQ sites of CII provide an intriguing target for anticancer drugs like α-TOS, since these agents at doses at which they induce efficient apoptosis in cancer cells suppress the SDH activity of CII by ∼50% [26]. Thus,

conversion of succinate into fumarate still occurs, albeit at a lower rate, in the presence of α-TOS, and ROS can form at a high level. CII/SDH is an integral component of mitochondria that couples the tricarboxylic acid cycle and the electron redox chain [32]. By interfering with the UbQ sites on CII, VE analogues cause high levels of ROS generation, while the level of ATP is still sufficient to enable apoptotic cell death to proceed (suppressed by ~40%; J. N. et al., unpublished data). This can be contrasted with situations whereby 'metabolic poisons' act as compounds that cause sudden depletion of ATP, resulting in necrosis [53].

Using modelling studies, we identified the Q_p and Q_d sites on CII as targets for the prototypic VE analogue, α-TOS. The initial modelling studies revealed strong hydrogen bonding of the oxo groups of the succinyl moiety of this VE analogue, co-ordinating linkages with Ser42 (Ser68 in the whole SDHC polypeptide) on the SDHC subunit for the Q_p site (Fig. 3) and Lys128 and Lys135 of the SDHD subunit for the Q_d site [26]. Also, the initial calculations of the IE values of α-TOS with the Q_p and Q_d sites revealed similar or better values than those for the natural ligand, UbQ [26]. We found that α-TOS and another analogue of VE, the ether α-tocopheryloxyacetic acid [9–11], suppressed angiogenesis by causing apoptosis in proliferating but not arrested endothelial cells by interfering with CII, resulting in ROS accumulation [25]. Although the exact target was not studied in this context, we think it highly plausible that the CII UbQ-binding sites present a target also in this case. To better document the interaction of VE analogues with CII, a 3-D model of the binding of α-TOS in the Q_p site of CII is presented in Fig. 4.

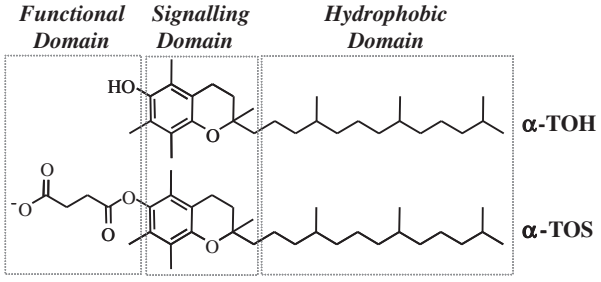

Figure 1. Schematic structure of VE analogues documenting the three major functional domains.

A number of VE analogues have been synthesised to date [16]. For some of them, toxicity to cancer cells recorded as IC_{50} values has been reported [18, 27–30]. Since we can expect that these compounds, in particular those with high pro-apoptotic activity, act by targeting the UbQ sites of CII, we calculated their IE values for the UbQ sites of CII. To do this, we chose the Q_p site, which is more clearly defined than the Q_d site from the crystal structure atomic coordinates of the porcine CII [32]. The structure of VE analogues comprises three domains, the *Functional*, the *Signalling* and the *Hydrophobic Domain* (Fig. 1), and their modification results in lower or higher apoptotic activity relative to the prototypic α-TOS [16]. We chose different VE analogues for this study, varying their substituents in the individual domains; their structures are shown in Fig. 2.

We calculated the IE values for the VE analogues presented in Fig. 2 as described in Section 2 using two modelling approaches. The data are presented in Table 1,

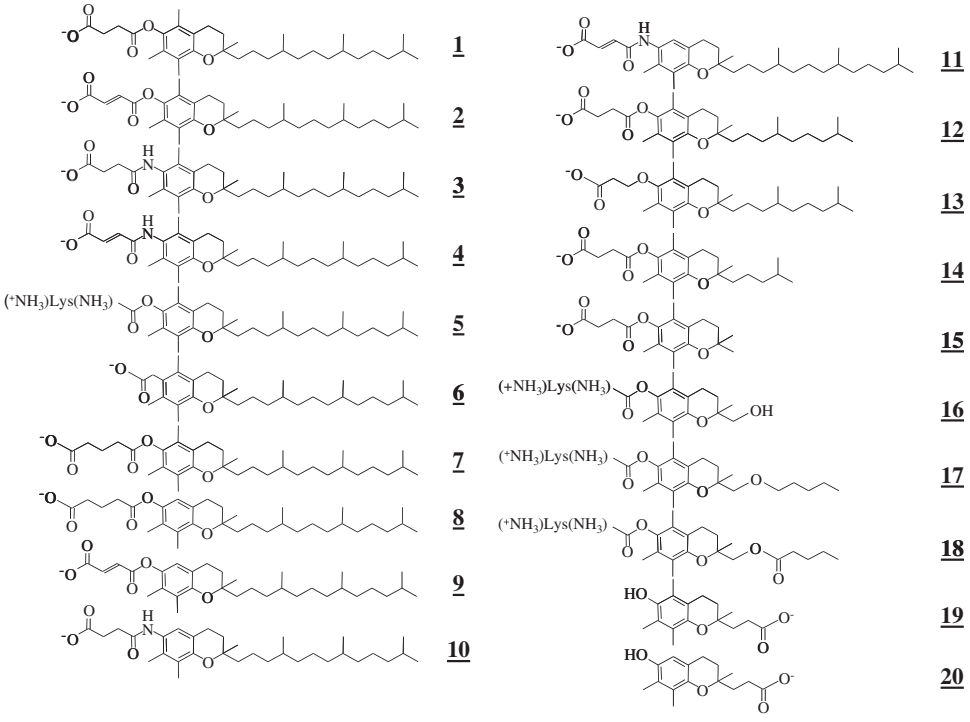

Figure 2. Vitamin E analogues used in this study.

together with the IC_{50} values compiled from several publications [18, 27–30]. The most toxic compound from this list is α-tocopheryl maleyl amide (α-TAM; **4**) with an IC_{50} value of 2 μM, with very high levels of apoptotic activity: 25–50 μM α-TAM induced almost 100% apoptosis in Jurkat cells within 6 h [30]. This compound also has the highest calculated IE value for the Q_p site of CII (−15.34 kcal/mol) by modelling method I, while method II showed strong, albeit relatively weaker binding of α-TAM to Q_p. Since the calculated IE value of the natural ligand, UbQ, for the Q_p site of CII is −14.7 kcal/mol using modelling method I [26], it can be expected that α-TAM will interfere with its binding and function in CII. Modelling method II provided a relatively low IE value for UbQ, which further supports the notion that a range of VE analogues will readily displace UbQ from the Q_p site of CII. On the other hand, in general, compounds with either a very short or no hydrophobic domain or a hydroxyl group in the position of the functional domain (compounds **15**, **16**, **19** and **20**) all exert very low toxicity [18, 27, 29] and their calculated Q_p IE values are also relatively low (Table 1). The graph of calculated IE values as a function of the IC_{50} values for the range of compounds shows a direct correlation (Fig. 5), with an R^2 value of ∼0.82 for modelling method I and ∼0.84 for modelling method II, indicating a close relationship between these two properties. In other words, the toxicity of VE analogues correlates with their affinity for the Q_p site of CII based on the calculated IE values (using two different methods) for a range of compounds tested in the different cancer cell lines. Although showing a very similar trend, the IE values calculated by the two approaches differ considerably for the following reasons. The 'energy' values are not directly comparable because of their different meanings. The values from AutoDock represent the predicted change in Gibbs free energy, including also the effects of water environment and temperature – the values correspond to the 'experimental conditions'. The second 'type' of IE is close (the ZPVE change is not included) to the interaction enthalpy at 0 K in vacuum. The similar correlation of both series suggests that the differences between studied compounds

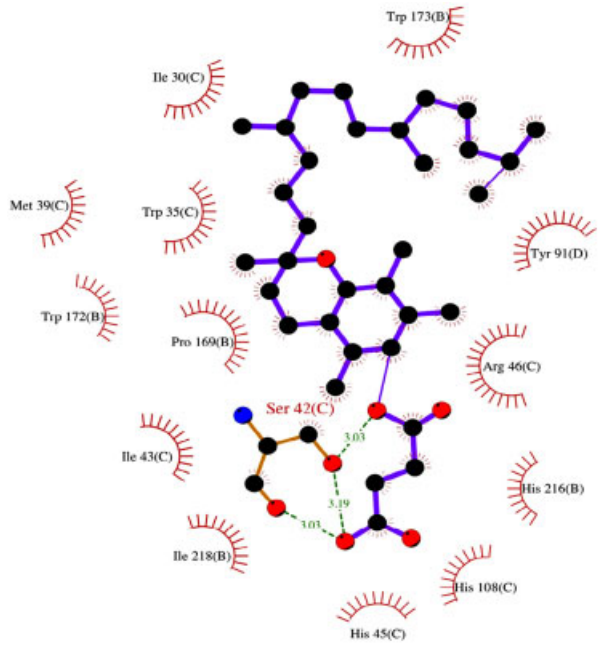

Figure 3. Lig plot of interaction of α-tocopheryl succinate with the Q_p site of CII. Adapted from [26].

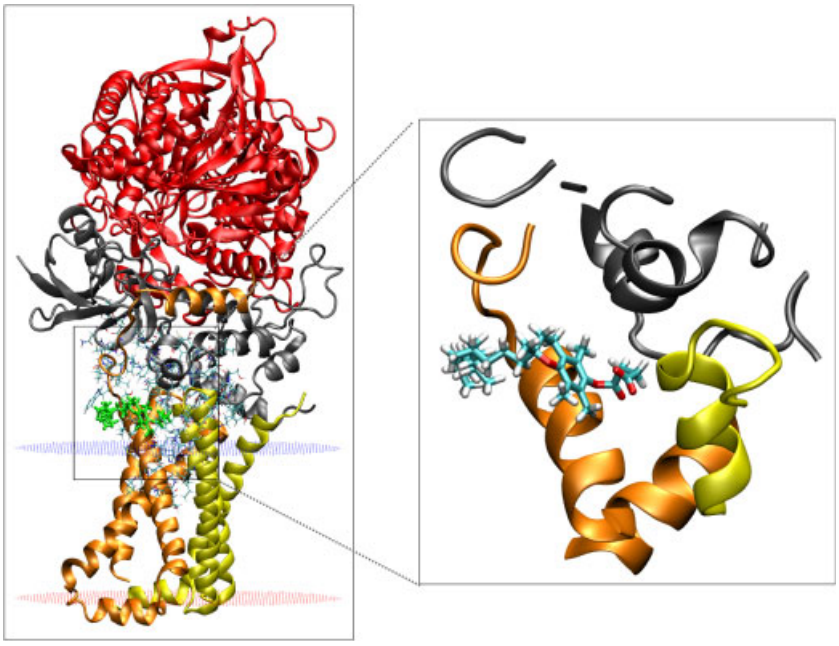

Figure 4. A 3-D model of the interaction of α-TOS with the Q_p site of CII.

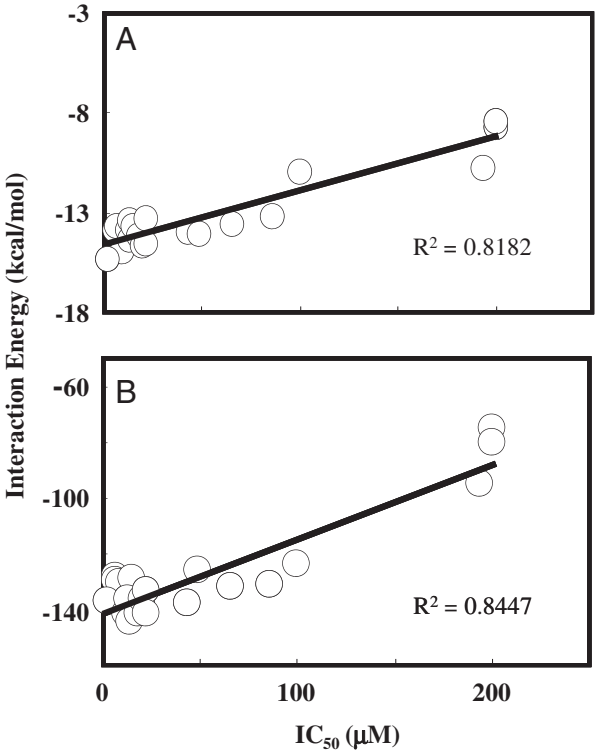

Figure 5. Correlation of the interaction energies of VE analogues with the Q_p site of CII and their toxicity to cancer cells. The interaction energies are derived either from the AutoDock modelling method (A) or from the SCC-DFTB-D method (B).

are caused mainly by specific non-covalent interaction, while the effect of solvation/desolvation is less dominant.

While we found a general correlation for the IE values as a function of their IC_{50} values, these values do not exactly agree for a few of the compounds, as follows from modelling method I. For example, the second and third most toxic VE analogues, compounds **13** and **12** with IC_{50} values of 6 and 6.5 μM, respectively [18], revealed relatively low Q_p IE values of −13.89 and −13.67 kcal/mol, respectively (Table 1). On the other hand, we found that the calculated IE value of −14.64 kcal/mol for compound **8** does not correlate with its toxicity, since its IC_{50} is only 66 μM [28]. Several possible reasons exist that might account for the discrepancies. One is that the IC_{50} values are derived from experiments in different cell lines, including Jurkat [28, 29], MCF7 [27] and PC3 lines [18, 30]. Jurkat cells are, generally, more susceptible to VE analogues than, for example, MCF7 cells. Another possibility is that we have based our calculations on the IE values for the Q_p site alone, since the Q_d site is less well defined from the crystal structure of porcine CII [32]. Certain inconsistencies were also found using modelling method II. Although we previously reported the predicted IE value of α-TOS for the Q_d site and the values were slightly higher than for the Q_p site [26], we decided not to include the Q_d site IE values here because the site is poorly defined structurally and would likely skew the calculated data. Notwithstanding, we propose that the toxicity of VE analogues correlates with their IE values for the Q_p site, i.e. the affinity with which they bind and will thereby displace the natural ligand, UbQ.

The evidence suggests that high-level cancer cell killing and binding affinities for CII by this class of anti-cancer drugs is associated with greater secondary toxicities. In this regard, a prime example among VE analogues is presented by the amide derivatives (compounds **3**, **4**, **10** and **12** in Fig. 4) [29]. Of these, α-TAM is highly apoptogenic for cancer cell lines (IC_{50} ∼2 μM [29]) but its administration to mice using DMSO or corn oil solutions as excipients results in extreme toxicity (J. N. et al., unpublished data). Thus, α-TAM can be lethal if administered as one to two doses of ∼10-fold lower levels than the effective doses of α-TOS that are well tolerated and cause substantial suppression of tumour progression [6, 12–15]. By contrast, administering α-TAM as a liposomal formulation preserved the cancer-suppressive activity of the drug while avoiding the toxicity to the treated animals [54]. This result clearly documents that high-level toxicity of VE analogues to cancer cells, as in the case of α-TAM, with a high IE value for the Q_p site of CII, may be associated with severe secondary toxicity, and appropriate formulation of the drug and its delivery must be considered carefully.

To reconcile the above findings with the fast growing field of cancer biology and the search for novel targets for anti-cancer drugs and their molecular targets, it should be pointed out that we have been developing novel agents that, in some cases, more specifically target the Q_p site of CII. For example, the non-apoptogenic lipid-lowering compound probucol, when modified by the addition of the succinyl moiety, acquires apoptogenic activity, which is relayed via CII (unpublished data). The fact that the target for VE analogues is localised in the mitochondrial inner membrane (MIM) can be utilised for designing novel and more efficient anti-cancer drugs. To this effect, we modified agents like VE succinate by the addition of a lipophilic cationic group triphenylphosphonium (TPP^+) to its lipophilic chain. This mitochondrially targeted VE succinate (MitoVES) proved superior to its untargeted counterpart both in apoptosis induction and in suppressing experimental tumours. Molecular modelling, structural considerations as well as biochemical analyses document that mitochondrially targeted VE succinate is positioned at the interface of the mitochondrial matrix and the MIM, with the triphenylphosphonium at the matrix face of the MIM and the bioactive group, tocopheryl succinate, inside the Q_p site of CII [43]. The importance of CII as a target for a growing number of compounds can be further accentuated by a report on atpenins, a group of compounds analogous to UbQs that suppress the succinate-ubiquinone oxidoreductase of CII [55]. These agents, initially isolated from a strain of the fungus *Penicillinum* as well as their synthetic analogues [56], widen the group of agents targeting CII, described initially for VE analogues [26, 27].

Our modelling, presented in this manuscript, indicates that the free carboxylate of agents like α-TOS is important for their apoptogenic activity, since its oxo groups interact via hydrogen bonding with the UbQ-binding Ser68 of the SDHC subunit of CII [26, 32, 43]. We found that masking this Q_p-interacting site of α-TOS by methylation suppresses its apoptogenic activity [29] as well as affinity for binding to Q_p (unpublished data). Therefore, it is of interest that metabolites of VE (such as α-tocopherol) with a carboxyl group at the terminus of the isoprenyl chain, but not those with a hydroxyl group, exert strong apoptogenic effect [53]. While no details have been reported, it cannot be excluded that such compounds may induce apoptosis by interacting with the UbQ-binding site(s) of CII.

The model we propose suggests that VE analogues interact with the Q_p sites of CII, which results in generation of radicals that then trigger apoptosis by way of activating the Mst1-FoxO1 pathway to transcriptionally upregulate the Noxa protein [50, 43], which then promotes formation of a Bak channel in the mitochondrial outer membrane with the ensuing demise of the cells [49]. The radicals, in the form of superoxide, are derived from CII via its interaction with the Q_p site(s). Although radicals have been shown to be released from CI or CIII and these complexes also contain UbQ, our data point to CII as a selective target for VE analogues. This stems from experiments in which we show that cells with dysfunctional CI exert susceptibility to α-TOS comparable to their parental counterparts [26]. Further, we showed that mutation of serine 68 of the SDHC subunit of CII that is important for binding UbQ in the Q_p resulted in resistance of cells with $SDHC_{S68A}$ or $SDHC_{S68L}$ to ROS generation and apoptosis induction when exposed to a mitochondrially targeted analogue of α-TOS [43] as well as to α-TOS (data not shown).

4 Concluding Remarks

We show here that a good correlation exists between the cytotoxic activity of VE analogues for cancer cells and their relative affinity for binding to the Q_p site of CII, further supporting the role for CII as an important intracellular target for VE analogues. Since CII is a novel, intriguing target for anti-cancer drugs, we are now setting up a high-throughput screening programme to identify compounds that kill cancer cells by interacting with the mitochondrial CII. This should result in the discovery of more compounds that may eventually be developed into clinically relevant anti-cancer drugs. Taken together, the UbQ sites of CII, in particular the Q_p site, present an intriguing target for VE analogues and probably a number of other, thus far unidentified compounds with potent and selective anti-cancer activity that may be tested in translational studies.

The authors have declared no conflict of interest.

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