Fine-tuning the hydrophobicity of a mitochondria-targeted antioxidant ☆

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Abstract The mitochondria-targeted antioxidant MitoQ comprises a ubiquinol moiety covalently attached through an aliphatic carbon chain to the lipophilic triphenylphosphonium cation. This cation drives the membrane potential-dependent accumulation of MitoQ into mitochondria, enabling the ubiquinol antioxidant to prevent mitochondrial oxidative damage far more effectively than untargeted antioxidants. We sought to fine-tune the hydrophobicity of MitoQ so as to control the extent of its membrane binding and penetration into the phospholipid bilayer, and thereby regulate its partitioning between the membrane and aqueous phases within mitochondria and cells. To do this, MitoQ variants with 3, 5, 10 and 15 carbon aliphatic chains were synthesised. These molecules had a wide range of hydrophobicities with octan-1-ol/phosphate buffered saline partition coefficients from 2.8 to 20 000. All MitoQ variants were accumulated into mitochondria driven by the membrane potential, but their binding to phospholipid bilayers varied from negligible for MitoQ3 to essentially total for MitoQ15. Despite the span of hydrophobicites, all MitoQ variants were effective antioxidants. Therefore, it is possible to fine-tune the degree of membrane association of MitoQ and other mitochondria targeted compounds, without losing antioxidant efficacy. This indicates how the uptake and distribution of mitochondria-targeted compounds within mitochondria and cells can be controlled, thereby facilitating investigations of mitochondrial oxidative damage.

Keywords: Mitochondria; Antioxidant; MitoQ; Lipophilic cation; Oxidative damage

1. Introduction

Mitochondrial oxidative damage contributes significantly to a range of degenerative diseases [1–5] and the production of reactive oxygen species (ROS) by mitochondria may be an important redox signal [6,7]. Consequently, there is considerable interest in selectively blocking mitochondrial oxidative damage and ROS production to determine the role of mitochondria in redox signalling and to develop therapies [3]. Towards these goals, we developed mitochondria-targeted antioxidants that selectively block mitochondrial oxidative damage [8–11]. These compounds comprise antioxidants covalently linked to triphenylphosphonium, a lipophilic cation that accumulates several-hundred fold within mitochondria driven by the large membrane potential (negative inside) [12]. The most effective is MitoQ, an antioxidant ubiquinol moiety attached to a triphenylphosphonium cation by an aliphatic carbon chain [13] (Fig. 1A). The selective accumulation of MitoQ prevents mitochondrial oxidative damage far more effectively than untargeted antioxidants [13–15]. Mitochondria-targeted antioxidants have potential as therapies, because they are taken up into mitochondria in vivo following oral administration [16], and they have also been used to probe the role of mitochondrial ROS production in redox signalling and metabolic regulation [17–19].

The uptake into mitochondria and binding to phospholipid bilayers of alkyl triphenylphosphonium cations such as MitoQ have been explored in detail [8,20,21]. To enter mitochondria, alkyltriphenylphosphonium cations first bind to the outer surface of the inner membrane, then permeate the hydrophobic potential energy barrier of the phospholipid bilayer, before binding to the inner surface of the membrane from where they desorb into the mitochondrial matrix [8,20] (Fig. 1A). In the presence of a membrane potential there is 100 to 1000-fold accumulation of the cation into the matrix, the extent of which is determined by the Nernst equation [22] (Fig. 1A). Alkyltriphenylphosphonium cations are in equilibrium between those free in solution and those adsorbed as a monolayer on the membrane surface, with the more hydrophobic the alkyl chain the greater the proportion of cation that is membrane bound [22]. This is illustrated in Fig. 1B, which shows how [10-(4,5-dimethoxy-2-methyl-3,6-dioxo-1,4-cyclohexadien-1-yl)decyl]-triphenylphos-phonium ($MitoQ_{10}$) is thought to interact with phospholipid bilayers. The membrane-bound triphenylphosphonium cation is located in a potential energy well at the level of the phospholipid carbonyls, while the alkyl chain inserts

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Abbreviations: CoQ_0, 2,3-dimethoxy-5-methyl-1,4-benzoquinone; FCCP, carbonylcyanide-p-trifluoromethoxyphenylhydrazone; MDA, malondialdehyde; MitoQ, ubiquinone linked to a triphenylphosphonium cation by an alkyl chain of unspecified length; $MitoQ_3$, [3-(4,5-dimethoxy-2-methyl-3,6-dioxo-1,4-cyclohexadien-1-yl)propyl]triphenylphosphonium; $MitoQ_5$, [5-(4,5-dimethoxy-2-methyl-3,6-dioxo-1,4-cyclohexadien-1-yl)pentyl]triphenylphosphonium; $MitoQ_{10}$, [10-(4,5-dimethoxy-2-methyl-3,6-dioxo-1,4-cyclohexadien-1-yl)decyl]triphenylphosphonium; $MitoQ_{15}$, [15-(4,5-dimethoxy-2-methyl-3,6-dioxo-1,4-cyclohexadien-1-yl)pentadecyl]triphenylphosphonium; PBS, phosphate buffered saline; ROS, reactive oxygen species; TBARS, thiobarbituric acid reactive species; THF, tetrahydrofuran; TPMP, methyltriphenylphosphonium cation

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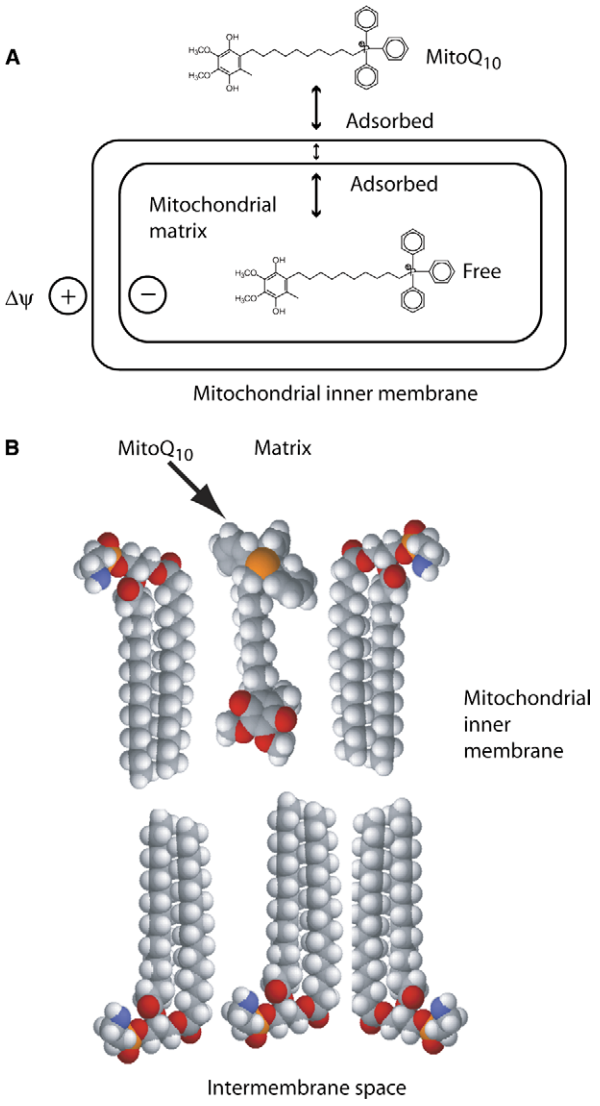

Fig. 1. Uptake of MitoQ by mitochondria. (A) The uptake of $MitoQ_{10}$ into an energised mitochondrion in response to the membrane potential ($\Delta\psi$) is shown schematically. (B) The predicted position of $MitoQ_{10}$ adsorbed to the matrix surface of the inner membrane is modelled relative to a simple phospholipid with the triphenylphosphonium moiety adsorbed to the membrane surface at the level of the fatty acid carbonyls, and the alkyl chain and ubiquinol moiety inserted into the hydrophobic core of the phospholipid bilayer.

into the phospholipid bilayer [22,23] (Fig. 1B). Consequently, increasing the length and hydrophobicity of the alkyl side chain probably increases both the extent of binding of alkyltriphenylphosphonium cations to phospholipid bilayers and the depth of side chain penetration into the membrane [22,23].

While MitoQ shows promise as both a therapy and as an experimental tool, its ten carbon alkyl chain makes it significantly more hydrophobic than the simple lipophilic cation methyltriphenylphosphonium (TPMP) [13]. Consequently, much of the MitoQ taken up into mitochondria is bound to the matrix-facing surface of the inner membrane with little free in the matrix [8,13]. The hydrophobicity of MitoQ also affects its intracellular distribution as it increases its binding to non-mitochondrial membranes, decreases its oral bioavailability [16], lowers its water solubility and renders it hygroscopic and difficult to crystallise. Therefore, it may be beneficial to manipulate the hydrophobicity of MitoQ to see how its ability to prevent mitochondrial oxidative damage is affected. Knowledge of alkyltriphenylphosphonium cations suggests that altering the length of the alkyl chain linking the triphenylphosphonium and the ubiquinol is a good way to change the hydrophobicity of MitoQ. Such an alteration is predicted to modulate the ability of MitoQ to prevent lipid peroxidation by changing both its extent of membrane adsorption and also how far the antioxidant inserts into the phospholipid bilayer (Fig. 1B). However, it is not known if altering the length of the alkyl chain would change MitoQ membrane binding sufficiently, or whether it would be counterproductive by disrupting its antioxidant efficacy and mitochondrial uptake.

For all these reasons, we sought to alter the hydrophobicity of MitoQ by changing the length of the alkyl chain so as to fine-tune its membrane binding, free concentration in the mitochondrial matrix and its depth of penetration into the membrane. Here, we report on the synthesis and characterisation of MitoQ variants with 3, 5, 10 and 15 carbon alkyl chains. These MitoQ variants had widely differing hydrophobicities and extents of binding to phospholipid bilayers. However, they retained their antioxidant efficacy and ability to be taken up into mitochondria driven by the membrane potential. These findings indicate that the properties of MitoQ, and of other mitochondria-targeted molecules, can be manipulated rationally in order to fine-tune their membrane binding and distribution within mitochondria and cells.

2. Materials and methods

2.1. Chemical syntheses

$MitoQ_{10}$ was synthesised as the bromide salt as previously described [13]. The chemical syntheses of [3-(4,5-dimethoxy-2-methyl-3,6-dioxo-1,4-cyclohexadien-1-yl)propyl]triphenylphosphonium ($MitoQ_3$), [5-(4,5-dimethoxy-2-methyl-3,6-dioxo-1,4-cyclohexadien-1-yl)pentyl]triphenylphosphonium ($MitoQ_5$) and [15-(4,5-dimethoxy-2-methyl-3,6-dioxo-1,4-cyclohexadien-1-yl)pentadecyl]triphenylphosphonium ($MitoQ_{15}$) are outlined in Fig. 2 and are described in the on-line supplementary material. Nuclear magnetic resonance spectra were acquired using a Varian 300 MHz instrument. For ^1H NMR, tetramethylsilane was the internal standard in $CDCl_3$. For ^{31}P NMR, 85% phosphoric acid was the internal standard. Chemical shifts (δ) are in ppm relative to the internal standard. Elemental analyses were done by the Campbell Microanalytical Laboratory, University of Otago. Electrospray mass spectrometry was done using a Shimadzu LCMS-QP800X liquid chromatography mass spectrometer. Stock solutions were prepared in absolute ethanol and stored at −20 °C in the dark.

2.2. Partition coefficients

To measure the octan-1-ol/phosphate buffered saline (PBS) partition coefficients of TPMP, $MitoQ_3$ and $MitoQ_5$, we used our previously published method [13]. This was done by adding 400 nmol of the compound to 2 ml PBS-saturated octan-1-ol and mixing for 30 min at 37 °C with 2 ml octan-1-ol saturated PBS in a glass Kimax tube. The concentrations of the compound in the two phases were measured by UV absorption at 268 nm and quantitated from standard curves of the compound in octan-1-ol saturated PBS, or PBS-saturated octan-1-ol [13,28]. For the more hydrophobic compounds, this procedure was found to be inaccurate due to the low concentrations in the aqueous phase, insolubility in aqueous buffer causing adsorption during analysis and the need for standard curves in PBS and octan-1-ol. Therefore, for $MitoQ_{10}$ and $MitoQ_{15}$ 100 μl of a 4

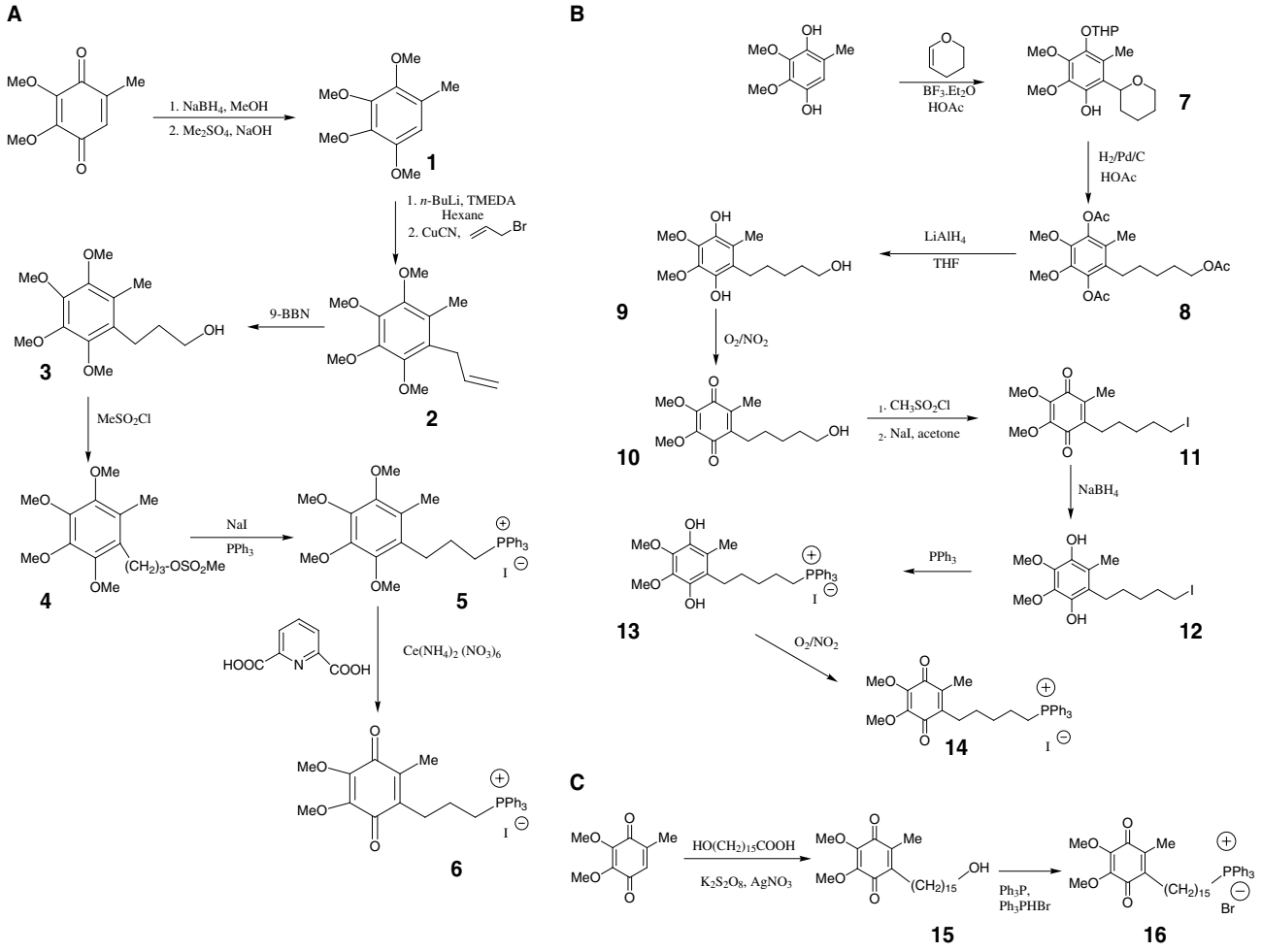

Fig. 2. Synthetic pathways. (A) MitoQ$_3$. (B) MitoQ$_5$. (C) MitoQ$_{15}$.

mM solution in PBS-saturated octanol was added to 10 ml octan-1-ol-saturated PBS in a glass Kimax tube at 37 °C and shaken for 5 min, then centrifuged at 1000 × *g* for 1 min to separate the phases. A portion (25 μl) of the upper octan-1-ol phase was diluted with 975 μl of PBS-saturated octan-1-ol and the absorbance measured at 275 nm. The aqueous phase (10 ml) was removed to a clean Kimax tube and the MitoQ was re-extracted into 1 ml octan-1-ol. One re-extraction was sufficient to ensure that >99% of the MitoQ was removed from the aqueous phase, and as both measurements were in octan-1-ol no standard curves were required. The values obtained for MitoQ$_5$ were the same by both procedures, however, for determining the partition coefficient of MitoQ$_5$ by the second method further octan-1-ol extractions were required.

2.3. Preparation and incubation of mitochondria and mitochondrial membranes

Rat liver mitochondria were prepared by homogenisation followed by differential centrifugation in ice-cold buffer containing 250 mM sucrose, 5 mM Tris–HCl, and 1 mM EGTA, pH 7.4 [29]. The protein concentration was determined by the biuret assay using BSA as a standard [30]. Mitochondrial membrane potential was measured by adding 500 nM TPMP supplemented with 50 nCi [^{3}H]TPMP (American Radiolabelled Chemicals Inc, MO, USA) to mitochondria suspended in KCl medium (120 mM KCl, 10 mM HEPES, pH 7.2, and 1 mM EGTA) at 25 °C [31]. After incubation, the mitochondria were pelletted by centrifugation and the amounts of [^{3}H]TPMP in the supernatant and pellets were quantitated by scintillation counting and the membrane potential calculated assuming a mitochondrial volume of 0.5 μl/mg mitochondrial protein and a TPMP binding correction of 0.4 [32].

2.4. Measurement of MitoQ uptake using an ion-selective electrode

To measure the uptake of MitoQ cations by energised mitochondria, an ion-selective electrode was constructed [8,33,34]. The electrode and a Ag/AgCl reference electrode were inserted through the air-tight Perspex lid of a stirred and thermostatted 3 ml incubation chamber at 30 °C, provided with an injection port for the addition of substrates. To measure MitoQ uptake, rat liver mitochondria (1 mg protein/ml) were incubated at 30 °C in KCl medium (120 mM KCl, 10 mM HEPES, pH 7.2, and 1 mM EGTA) and nigericin (1 μg/ml), rotenone (8 μg/ml), succinate (10 mM) and carbonylcyanide-*p*-trifluoromethoxyphenylhydrazone (FCCP) (500 nM) were added where indicated. The output from the ion-selective electrode was passed to a PowerLab Data acquisition system via a front-end pH amplifier and analysed using Chart software, all from ADInstruments.

2.5. Lipid peroxidation assays

To quantitate lipid peroxidation, the thiobarbituric acid reactive species (TBARS) assay was used. Rat liver mitochondria (2 mg protein/ml) were incubated in 0.8 ml medium containing 100 mM KCl, 10 mM Tris–HCl and pH 7.6, at 37 °C, supplemented with either 10 mM succinate and 8 μg/ml rotenone, or an ATP regenerating system of 2.5 mM ATP, 1 mM phosphoenolpyruvate and 4 U/ml pyruvate kinase. The appropriate MitoQ (5 μM) was then added and after 30 s the mitochondria were exposed to oxidative stress by addition of 50 μM $FeCl_2$/300 μM H_2O_2 for 15 min at 37 °C. After the incubation, 64 μl of 2% (w/v) butylated hydroxytoluene in ethanol was added, followed by 200 μl of 35% (v/v) $HClO_4$ and 200 μl of 1% (w/v) thiobarbituric acid. Samples were then incubated for 15 min at 100 °C, centrifuged (5 min at 12 000 × *g*) and the supernatant transferred to a glass tube. After addition of 3 ml water and 3 ml butan-1-ol, samples were vortexed,

and the two phases allowed to separate. Aliquots (200 μl) of the organic layer were then analysed in a fluorometric plate reader ($\lambda_{Ex} = 515$ nm; $\lambda_{Em} = 553$ nm) for TBARS and compared with a malondialdehyde (MDA) standard curve prepared from 0.01 to 5 μM 1,1,3,3-tetraethoxypropane [13].

3. Results and discussion

3.1. Synthesis and characterisation of MitoQ variants

A series of MitoQ variants with alkyl chains of 3, 5, 10 and 15 carbons linking the TPP cation and the ubiquinol were synthesised as described in Section 2. $MitoQ_{10}$ was synthesised as previously described [13]. An overall synthetic route to $MitoQ_3$ (**6**) is shown in Fig. 2A and is based on reducing 2,3-dimethoxy-5-methyl-1,4-benzoquinone (CoQ_0) to the hydroquinone [24] then methylating to give 2,3,4,5-tetramethoxytoluene (**1**) [25]. In our hands, the three carbon side chain was best introduced by metallation of **1** and reaction with allyl bromide to give **2** rather than the reported process [26] involving bromination and Grignard formation. Hydroboration of **2** gave the alcohol **3** which was activated as a mesylate **4** and displaced with triphenylphosphine to give the aromatic phosphonium iodide **5**. In this case, it was found that the phosphonium salt formation was best carried out in the absence of solvent using sodium iodide to enhance the reaction. Conversion of the tetramethoxyaromatic ring in **5** into the required dimethoxyquinone **6** was achieved using ceric ammonium nitrate on the nitrate salt of **5** (Fig. 2A). The methanesulfonate salt of $MitoQ_3$ was then obtained by anion exchange.

The synthesis of $MitoQ_5$ (**14**) is outlined in Fig. 2B. This synthesis was an improvement from initial work based on the addition of 5-bromohexyl radicals to CoQ_0 [27]. Here, the 5-carbon chain was introduced by substitution of CoQ_0 with an electrophile derived from dihydropyran [35] followed by hydrogenolysis and deacetylation. Selective functionalisation of the primary alcohol in **9** was achieved by oxidation [36,37] of the hydroquinone to give **10** [27], followed by net overall substitution with iodine to produce **11**. Reduction of **11** followed by phosphonium salt formation gave an oxidatively sensitive hydroquinol phosphonium salt **13**, which was converted to $MitoQ_5$ (**14**) by oxidation. The synthesis of $MitoQ_{15}$ (**16**) involved the radical addition of a hydroxyalkyl radical to CoQ_0 followed by phosphonium salt formation (Fig. 2C). The necessary hydroxyalkyl radical was obtained by oxidative decarboxylation of 16-hydroxyhexadecanoic acid with $K_2S_2O_8$ in the presence of $AgNO_3$ [38].

All the MitoQ compounds are shown in Fig. 3. A subscript indicates the number of carbons in the alkyl chains. In previous work, the MitoQ variant used had a ten carbon alkyl chain and is here described as $MitoQ_{10}$, while MitoQ is used generically to describe all chain length variants.

To determine the hydrophobicities of the MitoQ compounds, we measured their octan-1-ol/PBS partition coefficients. For the more hydrophobic compounds, we were concerned that the usual method was inaccurate; therefore, we devised a new procedure in which we re-extracted and concentrated the small amount of hydrophobic compound in the aqueous phase back into octan-1-ol before measurement, to facilitate its quantitation. As both samples were measured in octan-1-ol, a standard curve was not required. This procedure gave the same partition coefficient for $MitoQ_5$ as the published method. However, the new method gave a significantly higher partition coefficient for $MitoQ_{10}$ than the one reported previously, due to systematic errors in the earlier method [13]; therefore, the new procedure was used for $MitoQ_{10}$ and $MitoQ_{15}$. From their octan-1-ol/PBS partition coefficients (Fig. 3), it is clear that $MitoQ_3$, $MitoQ_5$, $MitoQ_{10}$ and $MitoQ_{15}$ span a wide range of hydrophobicities. That of $MitoQ_3$ is similar to the simple, relatively water soluble TPMP cation, while that of $MitoQ_{15}$ indicates that it has very low water solubility. Alkyltriphenylphosphonium cations such as MitoQ are thought to adsorb onto phospholipid bilayers with the cation at the level of the carboxylic acid groups, while the hydrophobic alkyl group penetrates into the hydrophobic core of the membrane (Fig. 1B). Consequently, the longer the alkyl chain the deeper the antioxidant ubiquinol is likely to penetrate into the hydrophobic core of the membrane. The maximum extent to which penetration into one leaflet of the membrane will occur for these compounds is illustrated in Fig. 3, which shows a molecular dimension comparison of the MitoQ variants with a typical phospholipid. From this it is clear that the ubiquinol moiety of $MitoQ_3$ only penetrates close to the membrane surface, while those of $MitoQ_{10}$ and $MitoQ_{15}$ will penetrate close to the core of the phospholipid bilayer. Therefore, we have synthesised a series of MitoQ variants with a range of hydrophobicities and depths of penetration into the phospholipid bilayer. Below we test their interactions with mitochondria and their antioxidant properties.

3.2. Binding and uptake of MitoQ variants by mitochondria

To see if the MitoQ variants were taken up by mitochondria in response to the membrane potential, an ion-selective electrode was used to measure their steady-state concentrations [8,33,34]. The response of this electrode to simple triphenylphosphonium cations such as TPMP is Nernstian, with a linear response of electrode voltage to $\log_{10}$[cation concentration] and a slope of ~60 mV at 30 °C [33,34]. The most hydrophilic compound, $MitoQ_3$, also gave a Nernstian electrode response with a slope close to 60 mV at concentrations above 10 μM. This is illustrated by the logarithmic electrode response to sequential additions of 1 μM $MitoQ_3$ in the absence of mitochondria (Fig. 4B). The electrode output was stable and also responded rapidly to sequential additions of $MitoQ_5$, $MitoQ_{10}$ and $MitoQ_{15}$ in the absence of mitochondria (Fig. 4D, F and H, respectively). However, in these cases the electrode responses were not Nernstian, presumably due to the greater hydrophobicity of the compounds. Even so, for all four compounds the ion-selective electrode enabled us to measure the free concentrations of the compounds and thus assess their uptake by mitochondria in real time.

To measure MitoQ uptake, we first added mitochondria to the electrode chamber in the presence of rotenone to prevent formation of a membrane potential (Fig. 4A, C, E and G). We then made five sequential 1 μM additions of MitoQ to calibrate the electrode response, followed by the respiratory substrate succinate to generate a membrane potential. Mitochondrial energisation led to the rapid uptake of all the MitoQ variants by the mitochondria, and subsequent addition of the uncoupler FCCP abolished the membrane potential and led to their rapid release from the mitochondria (Fig. 4A, C, E and G). These experiments clearly show mitochondrial membrane potential-dependent uptake of $MitoQ_3$, $MitoQ_5$, and $MitoQ_{10}$ (Fig. 4A, C and E). While $MitoQ_{15}$ was also taken up

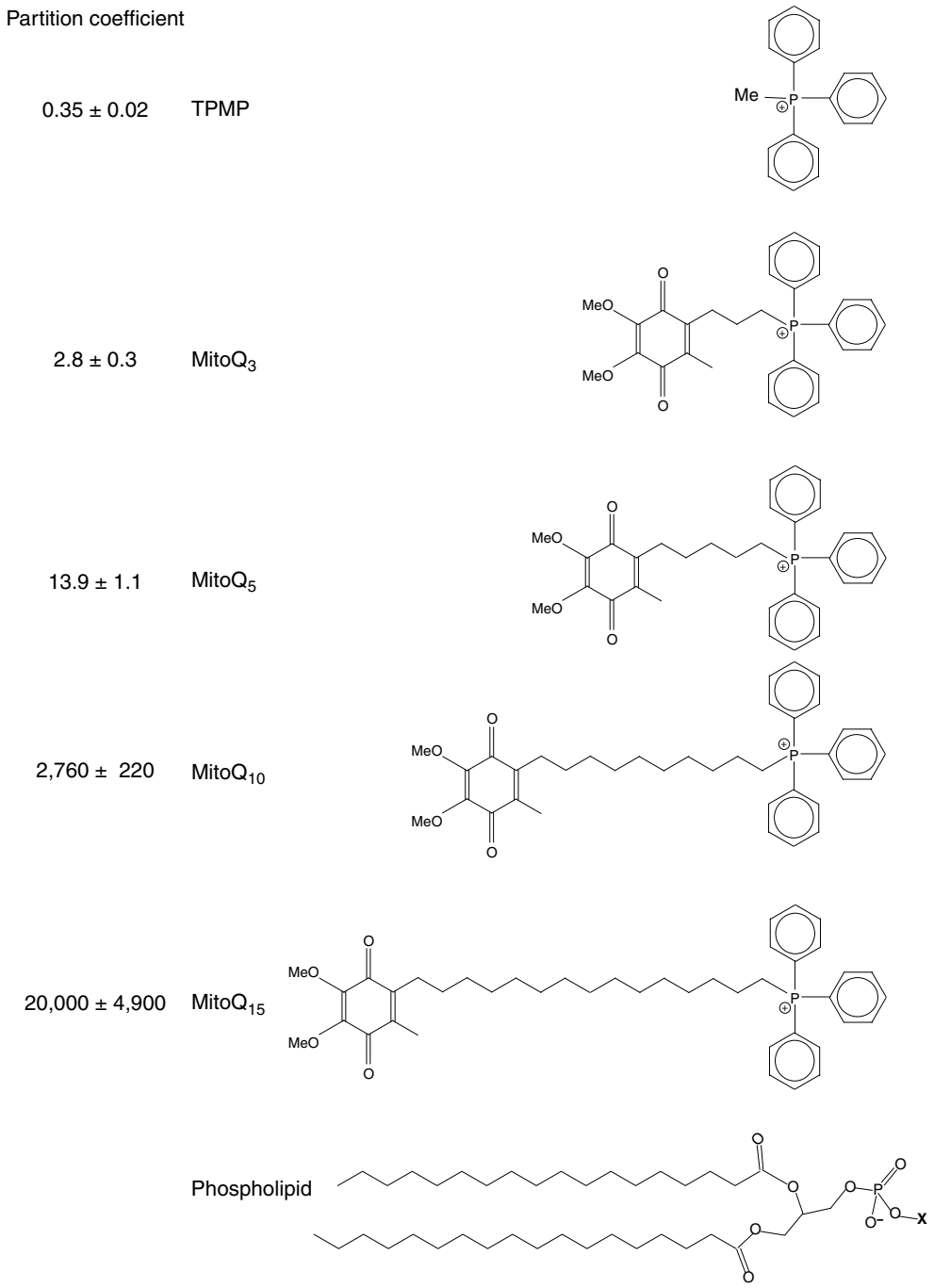

Fig. 3. Structure and octan-1-ol partition coefficients of MitoQ variants. The structures of TPMP and the MitoQ variants are shown. A phospholipid drawn to the same scale is aligned with the MitoQ variants to indicate potential maximum depths of penetration of the ubiquinol side chain into one leaflet of a phospholipid bilayer. The partition coefficients are the means ± S.E.M. of three determinations. The value for TPMP is from [13], that for $MitoQ_3$ is from [16], while $MitoQ_5$, $MitoQ_{10}$ and $MitoQ_{15}$ are from this work.

by mitochondria on induction of a membrane potential, the electrode response to $MitoQ_{15}$ in the presence of mitochondria was less reliable and prone to drift (Fig. 4G). This contrasts with the electrode response to $MitoQ_{15}$ in the absence of mitochondria (Fig. 4H) and is presumably due to low concentrations of free $MitoQ_{15}$ in the presence of mitochondria. Therefore, we next estimated the extent of MitoQ binding to deenergised mitochondria (Fig. 4B, D, F and H). For these experiments, the MitoQ variants were first added to the electrode chamber and then mitochondria were introduced in the presence of rotenone to prevent formation of a membrane potential. The decrease in MitoQ concentration on adding mitochondria is due to binding of MitoQ to the deenergised mitochondria. (Note that this binding of hydrophobic MitoQ variants to mitochondria means that it is not possible to compare electrode calibrations done in the presence and absence of mitochondria.) The subsequent addition of succinate to generate a membrane potential indicates the membrane

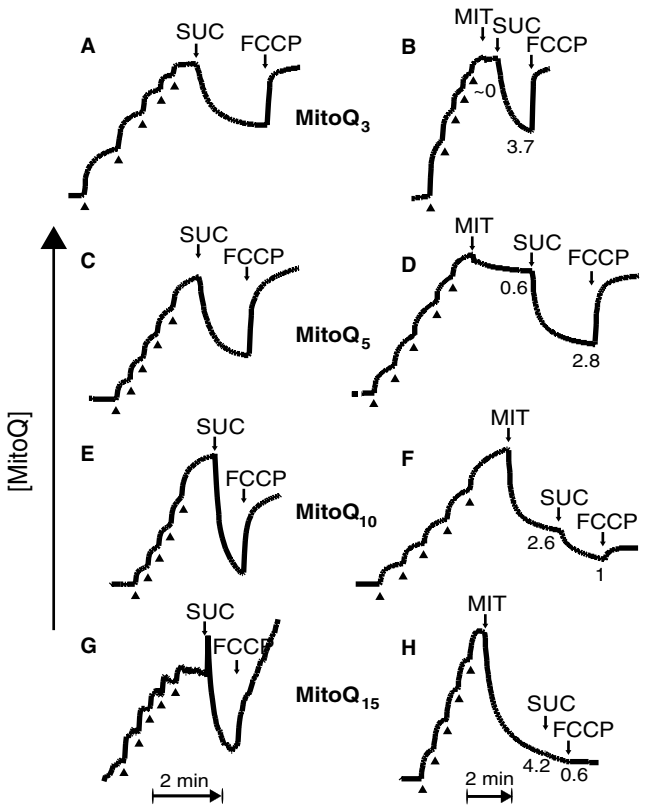

Fig. 4. Uptake and binding of MitoQ variants by mitochondria measured using an ion selective electrode. In the left hand panels (A, C, E and G), mitochondria (1 mg protein/ml) in the presence of rotenone were present and the MitoQ variants were then added as five sequential 1 μM additions (black arrowheads) to calibrate the electrode response. For the right hand panels (B, D, F and H), the electrodes were first calibrated by five sequential 1 μM additions (black arrowheads) and mitochondria (1 mg protein/ml) were then added. Note that on addition of the more hydrophobic compounds $MitoQ_{10}$ and $MitoQ_{15}$, much of the added compound will be bound to the mitochondria and will therefore not be free in solution. As the electrode responds to the free concentration, the electrode responses to additions of $MitoQ_{10}$ and $MitoQ_{15}$ in the presence or absence of mitochondria are not comparable and the scales have been adjusted to make each experimental trace roughly the same size. In all cases, succinate was added to generate a membrane potential and FCCP was added to dissipate it. Data are typical traces of experiments repeated at least 2–3 times. MIT, mitochondria; SUC, succinate.

potential dependent uptake of the compounds, which is then reversed by addition of FCCP to abolish the membrane potential. The free concentration of $MitoQ_3$ was unaffected by addition of mitochondria, indicating that negligible amounts of $MitoQ_3$ bind to deenergised mitochondria (Fig. 4B). The FCCP-sensitive uptake of $MitoQ_3$ on energisation with succinate was ~3.7 nmol $MitoQ_3$/mg protein, corresponding to an accumulation ratio of $\sim 2 \times 10^3$. This is consistent with that expected from the Nernst equation and a mitochondrial membrane potential of about 180 mV, allowing for corrections for intramitochondrial binding. For $MitoQ_5$, there was some binding of the compound to the deenergised mitochondria (~0.6 nmol/mg protein) (Fig. 4D). However, this was negligible compared to its subsequent uptake on energisation with succinate, of ~2.8 nmol $MitoQ_5$/mg protein, corresponding to an accumulation ratio of about 1.4×10^3. For $MitoQ_{10}$, there was significant binding to deenergised mitochondria of ~2.6 nmol $MitoQ_{10}$/mg protein, and this was followed by further uptake of ~1 nmol/mg protein on addition of succinate (Fig. 4F). Nearly all of the free $MitoQ_{15}$ was bound to the deenergised mitochondria, making further uptake on energisation difficult to detect (Fig. 4H). In contrast, the membrane potential-dependent uptake of $MitoQ_{15}$ was clearer when the electrode was calibrated in the presence of mitochondria (Fig. 4G) and consequently the electrode response was highly sensitive to the small amount of free $MitoQ_{15}$.

It is clear from Fig. 4 that the lengths of the alkyl chains of the MitoQ variant determine their extents of adsorption to mitochondrial membranes. The adsorption ranges from negligible for $MitoQ_3$ to almost complete binding for $MitoQ_{15}$. On addition of $MitoQ_{15}$ to deenergised mitochondria essentially all the compound binds, distributed across both surfaces of the inner and outer membranes. When a membrane potential is induced, there will be significant redistribution of the compound to the matrix-facing surface of the inner membrane from the outer surface of the inner membrane and from the outer membrane. In summary, all the MitoQ variants are taken up into mitochondria driven by the membrane potential and the longer the alkyl chain the greater their adsorption to phospholipid bilayers.

3.3. Antioxidant efficacy of MitoQ variants

We next determined how the different lengths of alkyl chains affected the antioxidant efficacy of the MitoQ variants. To do this we measured their abilities to prevent lipid peroxidation in mitochondria, measured from the accumulation of TBARS in mitochondria exposed to ferrous iron and hydrogen peroxide (Fig. 5). For mitochondria energised with succinate the background level of TBARS was negligible, but it increased to about 3.75 nmol MDA/mg protein on exposure to oxidative stress (Fig. 5A; black bars). High concentrations (5 μM) of any of the MitoQ variants largely prevented the accumulation of TBARS, while the simple cation TPMP did not. This confirms that it was the ubiquinol side chain of MitoQ that was responsible for the antioxidant action and not any non-specific interactions of the cation with mitochondria. In these experiments, succinate will both maintain a membrane potential to drive the uptake of the cations into mitochondria and also recycle the ubiquinone form of MitoQ to the active antioxidant ubiquinol form [13]. To see if reduction by the respiratory chain was required for the antioxidant efficacy of MitoQ, we incubated mitochondria in the presence of ATP and an ATP regenerating system. ATP hydrolysis and reversal of the mitochondrial ATP synthase led to extensive proton pumping, which built up a membrane potential similar to that generated by succinate (Fig. 5B). This leads to the same MitoQ uptake as for mitochondria energised by succinate, but now the MitoQ will no longer be recycled to their active ubiquinol forms by the respiratory chain. The MitoQ compounds were ineffective at preventing lipid peroxidation in mitochondria energised by ATP hydrolysis (Fig. 5A, white bars), compared with the dramatic protection seen in mitochondria energised by succinate (Fig. 5A, black bars). Therefore, reduction of MitoQ variants by the respiratory chain, as well as accumulation by the mitochondrial membrane potential are required for the antioxidant efficacy of the MitoQ variants. Interestingly, lower levels of lipid per-

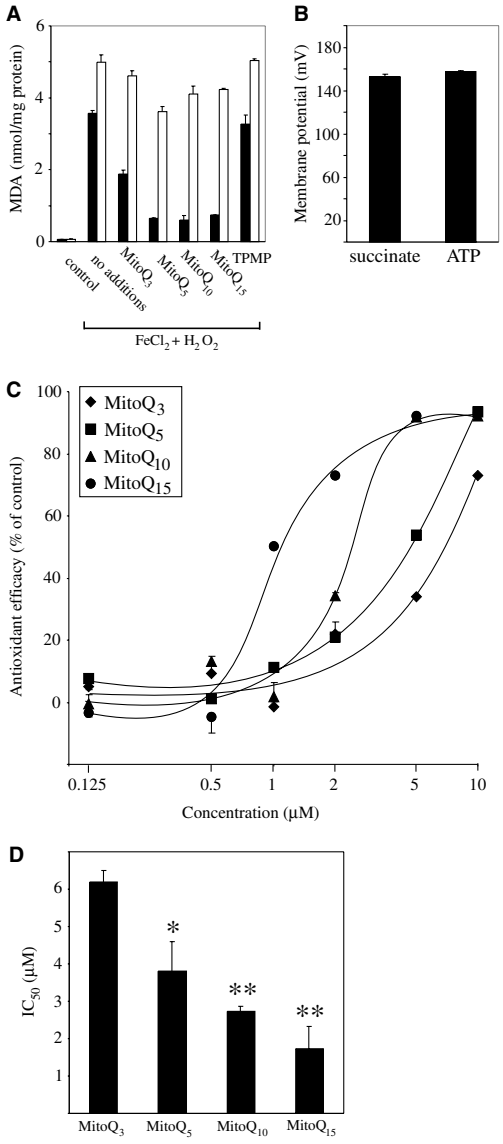

Fig. 5. Antioxidant efficacy of MitoQ variants. (A) Mitochondria were energised with succinate (black bars) or by incubation with an ATP regenerating system consisting of ATP, phosphoenol pyruvate and pyruvate kinase (white bars). After a 30 s preincubation with the various MitoQ variants (5 μM), TPMP (5 μM) or carrier, oxidative stress was induced by addition of 50 μM $FeCl_2$ and 300 μM H_2O_2. After 15 min incubation at 37 °C, lipid peroxidation was estimated by measuring TBARS. Data are means ± range of two independent experiments. (B) The mitochondrial membrane potential induced with succinate or with the ATP regenerating system was measured from the accumulation of [^{3}H]TPMP. Data are means ± range of duplicate determinations of a 25 min incubation. The membrane potentials after a 5 min incubation were the same (data not shown). (C) The concentration dependence of the prevention of the accumulation of TBARS by MitoQ variants was measured. All incubations were carried out in the presence of succinate as described for (A). Results are expressed as % inhibition of TBARS formation, taking the value of a sample exposed to $FeCl_2/H_2O_2$ in the absence of MitoQ variants as 0% inhibition, and a control sample (no $FeCl_2/H_2O_2$ added) as 100%. The data shown are a typical titration with each concentration determined in triplicate ± S.D. (D) The IC_{50} concentrations for prevention of lipid peroxidation. Data are means ± S.E.M., estimated from three independent titrations as shown in (C). The statistical significance relative to the IC_{50} for $MitoQ_3$ was determined using Student's two tailed *t*-test: $^*P < 0.05$; $^{**}P < 0.005$.

oxidation were observed in the control samples of mitochondria energised with succinate, compared to those energised with ATP (Fig. 5A). This is due to the protective antioxidant effect of the endogenous mitochondrial Coenzyme Q pool, which is held reduced in the presence of succinate but not in the presence of ATP [39,40]. In summary, all the MitoQ variants require activation by the respiratory chain to be effective antioxidants.

The data described in Fig. 5A were obtained using a uniform concentration of 5 μM for all the MitoQ variants. To compare their relative antioxidant efficacies, we titrated the compounds in the presence of succinate: a typical titration is shown in Fig. 5C. This experiment suggests that the antioxidant efficacy of these compounds correlates with the length of the alkyl chain. To quantitate this, we calculated the IC_{50} values for the prevention of lipid peroxidation by the four MitoQ variants (Fig. 5D). These measurements confirmed that the order of antioxidant efficacy was: $MitoQ_{15} > MitoQ_{10} > MitoQ_5 > MitoQ_3$. As ubiquinol reactivity with ROS will be unaffected by altering the length of the alkyl chain, this indicates that antioxidant efficacy correlated with both degree of adsorption to the membrane and depth of penetration into the phospholipid bilayer.

3.4. Conclusions

We have synthesised a series of variants of MitoQ with alkyl chains of 3, 5, 10 and 15 carbons. These molecules had a range of hydrophobicities leading to wide differences in their octan-1-ol/PBS partition coefficients from 2.8 to 20 000. All the MitoQ variants were accumulated into mitochondria driven by the mitochondrial membrane potential, although for the most hydrophobic compound, $MitoQ_{15}$, this effect was largely masked by the extensive binding of the compound to phospholipid bilayers. All of the compounds were effective antioxidants and for persistent antioxidant activity over 15 min all required the action of the respiratory chain to convert MitoQ to its active antioxidant form and to recycle MitoQ after having detoxified lipid peroxidation intermediates. The range of membrane binding indicates that the compounds will have different locations within mitochondria with most of the more hydrophobic compounds being membrane adsorbed, while more of the hydrophilic compounds will be free in the matrix. In addition, the non-specific binding of the less hydrophobic compounds will decrease non-mitochondrial binding throughout the cell. Thus, by using appropriate variants of MitoQ it should be possible to infer the relative importance of the ROS produced within the membrane or in the matrix. Finally, the increased hydrophilicity of many of these MitoQ variants will simplify their handling and may also lead to beneficial pharmacokinetic properties such as increased bioavailability.

These findings show that it is possible to alter the aliphatic chain linking an antioxidant moiety and a targeting lipophilic cation but still retain mitochondrial targeting and antioxidant potency. Furthermore, it is possible to fine-tune the hydrophobicity of MitoQ and related compounds in order to control their intracellular and mitochondrial location and penetration into the membrane. This will facilitate the design of further mitochondria-targeted compounds in order to optimise their use as probes of the role of mitochondrial oxidative damage in cell signalling and pathology.

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