Mitochondria-targeted molecules comprising the lipophilic TPP (triphenylphosphonium) cation covalently linked to a hydrophobic bioactive moiety are used to modify and probe mitochondria in cells and in vivo. However, it is unclear how hydrophobicity affects the rate and extent of their uptake into mitochondria within cells, making it difficult to interpret experiments because their intracellular concentration in different compartments is uncertain. To address this issue, we compared the uptake into both isolated mitochondria and mitochondria within cells of two hydrophobic TPP derivatives, [3H]MitoQ (mitoquinone) and [3H]DecylTPP, with the more hydrophilic TPP cation [3H]TPMP (methyltriphenylphosphonium). Uptake of MitoQ by mitochondria and cells was described by the Nernst equation and was ∼5-fold greater than that for TPMP, as a result of its greater binding within the mitochondrial matrix. DecylTPP was also taken up extensively by cells, indicating that increased hydrophobicity enhanced uptake. Both MitoQ and DecylTPP were taken up very rapidly into cells, reaching a steady state within 15 min, compared with ∼8 h for TPMP. This far faster uptake was the result of the increased rate of passage of hydrophobic TPP molecules through the plasma membrane. Within cells MitoQ was predominantly located within mitochondria, where it was rapidly reduced to the ubiquinol form, consistent with its protective effects in cells and in vivo being due to the ubiquinol antioxidant. The strong influence of hydrophobicity on TPP cation uptake into mitochondria within cells facilitates the rational design of mitochondria-targeted compounds to report on and modify mitochondrial function in vivo.

Key words: lipophilic cation, membrane potential, mitochondria, mitochondrial targeting, mitoquinone (MitoQ).

INTRODUCTION

Mitochondria are central to many aspects of biomedical science; consequently there is considerable interest in the development of small molecules that can be targeted to mitochondria in cells and in vivo to report on and modify mitochondrial function [1]. Lipophilic cations such as TPP (triphenylphosphonium) have been linked covalently to small-molecule thiol probes, spin traps, peptide nucleic acid oligomers, superoxide probes and anti-oxidants to deliver them to mitochondria, driven by the Δψm (mitochondrial membrane potential) [2–13]. The delocalized positive charge on the lipophilic TPP cation promotes their Δψm-dependent accumulation into mitochondria and their direct passage through phospholipid bilayers; consequently, these molecules are useful in a range of mitochondrial studies [1,14].

Mitochondrial oxidative damage contributes to many pathologies because mitochondria are a source of ROS (reactive oxygen species) and are also susceptible to oxidative damage [15,16]. To prevent oxidative damage, mitochondria-targeted antioxidants have been developed [17–19]. The prototypical mitochondria-targeted antioxidant is MitoQ (mitoquinone) (Figure 1), which comprises a ubiquinone moiety linked covalently by an aliphatic ten-carbon chain to the TPP cation [13,20,21]. MitoQ protects cells in culture against oxidative damage [13,17,22], is taken up and converted into the ubiquinol form that prevents lipid peroxidation [13,25]. However, little is known about the rate and extent of MitoQ uptake into mitochondria within cells and whether MitoQ is substantially reduced to its ubiquinol form.

Most mitochondria-targeted TPP probes are similar to MitoQ, therefore the rate and extent of their uptake into cells is also unknown. Basic questions remain, such as whether increasing hydrophobicity increases or decreases the rate and extent of uptake relative to simple TPP cations such as TPMP (methyltriphenylphosphonium) (Figure 1). The uptake of simple cations such as TPMP in response to a membrane potential is unknown. Basic questions remain, such as whether increasing hydrophobicity increases or decreases the rate and extent of uptake relative to simple TPP cations such as TPMP (methyltriphenylphosphonium) (Figure 1). The uptake of simple cations such as TPMP in response to a membrane potential is described by the Nernst equation, where cation uptake increases

Abbreviations used: ACR, accumulation ratio; CoQ, coenzyme Q; Δψm, mitochondrial membrane potential; Δψp, plasma membrane potential; ESI-MS, electrospray ionization MS; FCCP, carbonylcyanide p-(trifluoromethoxy)phenylhydrazone; FCS, fetal calf serum; IDTP, 10-iiododecyltriphenylphosphonium iodide; MitoQ, mitoquinone; MitoQH2, mitoquinol; PMPI, plasma membrane potential indicator; RP-HPLC, reverse-phase HPLC; TFA, trifluoroacetic acid; TMA, tetramethylammonium; TPB, tetraphenylborate; TPMP, methyltriphenylphosphonium; TPP, triprenylphosphonium.

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15 min. Once the solution volume was less than 10 ml, the mixture was evaporated by bubbling with nitrogen for 10 min, then 54–72 h. The absorbance (\(A_{220}\)) of the column eluant was detected using a Gilson UV/VIS 151 spectrophotometer, and 

\[
\Delta \psi (\text{mV}) = 61.5 \times \log_{10} \left( \frac{[\text{TPP cation}]_{\text{in}}}{[\text{TPP cation}]_{\text{out}}} \right)
\]

where [TPP cation]_{in} is the concentration inside the membrane-enclosed compartment and [TPP cation]_{out} is the concentration outside the membrane. However, it is unclear if the significant potential-independent binding to membranes of hydrophobic TPP cations causes deviations from Nernstian behaviour [13]. These uncertainties hamper the interpretation of results obtained using TPP molecules and restrict the rational development of further mitochondria-targeted compounds. In this present paper, we report on the uptake of the hydrophobic TPP cations MitoQ and DecylTPP (Figure 1) into mitochondria and cells and compare these with TPMP, a simple hydrophilic TPP cation. We found that the uptake of hydrophobic TPP cations into mitochondria is described by the Nernst equation, and that their uptake into cells is more extensive and rapid than TPMP. These findings facilitate interpretation of experiments using mitochondria-targeted molecules and indicate how these compounds can be modulated rationally to optimize their properties.

**EXPERIMENTAL**

**Synthesis and purification of radiochemicals**

To synthesize \(^{3}H\)MitoQ, a solution of idebenol mesylate (900 mg, 2.15 mmol) [26] was prepared by reacting idebenone with methane sulfonyl chloride and triethylamine, followed by catalytic hydrogenation. This was added to dichloromethane (5 ml) in a Nalgene tube which contained \(^{3}H\)triphenylphosphine [27] (220 mg, 0.84 mmol, 19.3 mCi), stirred to give an homogeneous solution and then evaporated under nitrogen flow at 40 °C. The tube was flushed with argon, sealed and stirred for 29 h at ∼85 °C. The tube was then cooled and the contents dissolved in dichloromethane (~2 ml) and stirred vigorously while ethyl acetate was added in three portions (2, 3 and 5 ml) to precipitate the product. The mixture was stirred for ~5 min and then the dichloromethane was evaporated by bubbling with nitrogen for 15 min. Once the solution volume was less than 10 ml, the mixture was allowed to settle for 10 min. The ethyl acetate layer was decanted, and the semi-solid residue dissolved in dichloromethane (~2 ml) and precipitated with ethyl acetate (10 ml) as before. This precipitation process was repeated once more. After the ethyl acetate layer was decanted, the remaining solvent was removed by a nitrogen stream to produce MitoQH\(_2\) mesylate as a white powder (380 mg, 10.3 mCi, 66 % yield). Oxygen gas was then bubbled through a solution of tritiated MitoQH\(_2\) mesylate (200 mg, 0.29 mmol) in 5 ml of water/acetonitrile [1:2 (v/v)] for 30 min. The solution was then stirred in air for ~3 days at room temperature (~20 °C). The solution was evaporated under nitrogen at ~30 °C to ~5 ml. Water (~3 ml) was added and it was then freeze-dried which produced a sticky orange solid (~200 mg). Column chromatography on silica gel (5 g) and elution with acetonitrile produced MitoQ mesylate (118 mg, 0.17 mmol, 60 % yield, 93 % radiopurity as determined by HPLC). All \(^{3}H\)MitoQ preparations were further HPLC-purified to >97 % radiopurity, as described below, before use in experiments.

To synthesize \(^{3}H\)DecylTPP, a solution of 1-bromodecane (5.49 mg, 24.8 µmol; Aldrich) in N,N-dimethylformamide (13 µl) was added to \(^{3}H\)triphenylphosphine [27] (1.3 mg, 5 µmol, 11.9 µCi) in a 300 µl reaction vial. The solution was sparged with argon, stirred at 120 °C for 24 h, allowed to cool to room temperature and the residue dissolved in minimum dichloromethane (~60 µl) and transferred to a pre-weighed 2.5 ml screw-cap glass vial. Addition of diethyl ether (2 ml) gave a precipitate and the tube was then centrifuged at 600 g for 10 min at ~20 °C and the supernatant decanted. The residue was re-dissolved in a minimum amount of dichloromethane and precipitated with diethyl ether again. This process was repeated a total of four times. The final solid (~1 mg, 0.58 µCi) was dried under a stream of nitrogen.

\(^{3}H\)MitoQ, \(^{3}H\)DecylTPP and \(^{3}H\)TPMP iodide (60 Ci/mmol, American Radiolabeled Chemicals, St Louis, MO, U.S.A.) were further purified by RP-HPLC (reverse-phase HPLC) before use. \(^{3}H\)MitoQ (<100 µmol; 10–20 Ci/µmol) was loaded in 1 ml of 45 % (v/v) acetonitrile/0.1 % (v/v) TFA (trifluoroacetic acid) on to a C18 column (Jupiter 5 µm 300 Å, 25 mm × 4.6 mm; Phenomenex, Torrance, CA, U.S.A.) and was eluted using a gradient of acetonitrile/0.1 % TFA (4.5–54 % acetonitrile over 5 min, then 54–72 % acetonitrile over 20 min) at a flow rate of 1 ml/min. The absorbance (\(A_{220}\)) of the column eluant was detected using a Gilson UV/VIS 151 spectrophotometer, and 0.5 ml fractions were collected around the \(^{3}H\)MitoQ peak, transferred to scintillation vials containing 3 ml of Fluorosafe (BDH) and the radioactivity quantified using a Packard Tri-Carb 2800TR liquid-scintillation analyser (PerkinElmer) with appropriate quench corrections. Radioactive MitoQ fractions were pooled, freeze-dried, resuspended in ethanol at a concentration of 0.5–2 mM and stored at ~20 °C. Radiopurity was determined by RP-HPLC as above by comparing the radioactivity of product fractions with 0.5 ml fractions collected over the entire gradient. Greater than 97 % of the eluted radioactivity co-eluted with MitoQ, with less than 0.2 % of the eluted radioactivity present in any other fraction. The specific activity of \(^{3}H\)MitoQ was determined within each sample by liquid-scintillation counting of 2 × 200 µl aliquots of suspension immediately after the addition of \(^{3}H\)MitoQ, \(^{3}H\)DecylTPP and \(^{3}H\)TPMP were purified using the same method.

**Synthesis of IDTP (10-iododecytriphenylphosphonium iodide)**

1,10-Di-iododecane (2.4 g, 6.1 mmol) and triphenylphosphine (0.262 g, 1 mmol) were stirred in a Kimax tube at 75–80 °C overnight. The tube was cooled, the reaction dissolved in a minimum amount of dichloromethane (4 ml) and added dropwise

Figure 1  Structures of MitoQ, MitoQH\(_2\), DecylTPP, TPMP, IDTP and a sultated metabolite of MitoQ

Although MitoQ is sulfated on only one phenolic oxygen, it is not known which one is sulfated. 10-fold for every 61.5 mV at 37 °C. The equation is shown below:

\[
\Delta \psi (\text{mV}) = 61.5 \times \log_{10} \left( \frac{[\text{TPP cation}]_{\text{in}}}{[\text{TPP cation}]_{\text{out}}} \right)
\]
to diethyl ether (100 ml). The crude product was isolated by filtration and purified by silica-gel chromatography. Elution with acetonitrile/dichloromethane [1:1 ratio (v/v)] and acetonitrile gave a product (0.559 g, 0.85 mmol, 85% yield) that was 97.6% pure as judged by HPLC. 1H NMR: δ 7.67–7.87 (m, 15 H, PPh3), 3.74 (m, 2 H, CH2PPh3), 3.171 (t, 2 H, 7 Hz, CH2I), 1.52–1.83 (m, 6 H) and 1.18–1.39 (m, 10 H) p.p.m. 31P NMR: δ 25.57 p.p.m. (+)ESI-MS (positive ion electrospray ionization MS) found m/z = 529.153 (C39H35PI3+ requires m/z = 529.152).

**Uptake of [3H]MitoQ by isolated mitochondria**

Rat liver mitochondria were prepared by homogenization followed by differential centrifugation at 4 °C in 250 mM sucrose, 5 mM Heps and 1 mM EGTA (pH 7.2) [28]. For the [3H] uptake experiments, the buffer pH was adjusted using TMA (tetramethylammonium) hydroxide in place of KOH. The protein concentration was determined by the biuret assay using BSA as a protein standard [29].

The simultaneous accumulation of 86Rb+ and [3H]MitoQ or [3H]TPMP was measured as described previously [30]. Briefly, mitochondria (2 mg·ml−1 of protein) were incubated for 5 min at 30°C in 250 µl of 250 mM sucrose, 5 mM Heps and 1 mM EGTA (pH 7.2, adjusted using TMA hydroxide) supplemented with 4 µg·ml−1 rotenone, 0.2 nmol valinomycin per mg of protein, 10 mM succinate (TMA salt), 86RbCl (5 nCi·ml−1; PerkinElmer) and 1 µM [3H]MitoQ or [3H]TPMP (3–10 nCi·ml−1). The membrane potential was varied by the addition of KCl (0–20 mM) with or without FCCP [carbonylcyanide p-(trifluoromethoxy)phenylhydrazone; 0.5 µM]. Experiments to determine mitochondrial binding in the absence of Δψm were carried out in the presence of 0.5 µM FCCP/20 mM KCl and 0.3–1 µM [3H]MitoQ. After incubation, mitochondria were pelleted by centrifugation (14000 g for 1 min at room temperature), and 400 µl of supernatant and pellet [H] and 86Rb content assayed [30]. In parallel experiments, the non-mitochondrial sucrose space in the pellets was determined by incubation for 5 min at 30°C with [14C]sucrose (50 nCi·ml−1; PerkinElmer) and was used to correct the apparent pellet spaces for non-mitochondrial 86Rb+ or [3H]. These values were then divided by the mitochondrial volume under these conditions (0.6 µl per mg of protein) to give the ACR (accumulation ratio) [31] as shown below:

\[
ACR = \frac{\left(\frac{400 \times DPM_{\text{pellet}}}{DPM_{\text{supernatant}}} - \text{sucrose space}\right)}{\text{mitochondrial volume}}
\]

where DPM is disintegrations per min. Mitochondrial volume and sucrose space are both measured in µl per mg of protein.

**Cell culture and incubations**

Cells were cultured at 37°C under humidified 95% air/5% CO2, in medium supplemented with penicillin (100 units·ml−1) and streptomycin (100 µg·ml−1). The Jurkat human T lymphocyte cell line was grown in suspension in RPMI 1640 medium (Invitrogen) supplemented with 10% (v/v) FCS (fetal calf serum), and cells were routinely maintained at densities of 0.2 × 106–2 × 106 cells/ml. C2C12 cells ([mouse myoblast cell line; ECACC (European Collection of Animal Cell Cultures)] were maintained at subconfluence in DMEM (Dulbecco’s modified Eagle’s medium; Invitrogen) supplemented with 10% (v/v) FCS.

To test the acute toxicity of MitoQ, Jurkat cells were seeded at 104 cells/ml (in 2 ml of medium) in six-well plates, and incubated with vehicle [0.1% (v/v) DMSO] or mitochondria-targeted TPP compound (0–50 µM) for 24 h. Cell integrity was measured by propidium iodide exclusion [32]. MitoQ and DecyITPP began to affect Jurkat cell viability at a concentration of 1–2 µM, therefore concentrations of 0.5 µM were used for most experiments. For uptake studies, cells were seeded at 3 × 105 cells/ml and supplemented with 500 nM [3H]MitoQ, [3H]DecyITPP or [3H]TPMP in the presence or absence of FCCP (5 µM), oligomycin/myxothiazol (2 µM of each; to abolish Δψm selectively) (Sigma) or oligomycin (2 µM)/myxothiazol (2 µM)/gramicidin (10 µg·ml−1; Sigma) [to abolish both Δψm (plasma membrane potential) and Δψm]. All additions were from stock solutions in DMSO or ethanol, and the final concentration of DMSO or ethanol in all incubations was <0.2% (v/v). For oligomycin/myxothiazol ± gramicidin incubations, cells were incubated with the inhibitors for 30 min at 37°C before the addition of MitoQ. Cell density was measured in parallel incubations containing 500 nM unlabelled MitoQ, and showed negligible cell growth over the incubations shown, therefore cell density at the zero time point was applied at all time points to calculate cellular uptake. Samples (1 ml) were taken at various time points and cells were pelleted by centrifugation (16000 g for 1 min at room temperature). Cell uptake was quantified as described previously [30]. The amount of [3H]MitoQ in the pellet was expressed in pmol per million cells, or as an ACR, calculated assuming a water volume of 0.2 pl for a Jurkat cell [33].

**Measurement of Δψm**

Jurkat cells (3 × 106 cells/ml) in Dulbecco’s PBS supplemented with 9 mM CaCl2 and 4.9 mM MgCl2 (Invitrogen), 25 mM glucose and 0.5% FCS were incubated for 1 h at 37°C with the proprietary PMPI (plasma membrane potential indicator, a component of the FLIPR Membrane Potential Assay Kit, Explorer format – blue; Molecular Devices), added to a final concentration of 0.5 µl·ml−1 from a stock solution reconstituted according to the manufacturer’s instructions. PMPI fluorescence in stirred cell suspensions in a 3 ml cuvette was detected over time in a Shimadzu RF 5301-PC fluorimeter (λex = 450 nm, slit width 10 nm; λem = 535 nm, slit width 5 nm), and the effect of adding oligomycin, myxothiazol (both at 5 µM) and gramicidin (10 µg·ml−1) was measured.

**Extraction of MitoQ from cells and mitochondria**

To determine the redox state of [3H]MitoQ in cells, Jurkat cells (3 × 105 cells/ml) were incubated in 6 ml of medium supplemented with 500 nM [3H]MitoQ. Where the effect of respiratory inhibitors was tested, cells were incubated with inhibitors (concentrations as above) or vehicle (0.2% ethanol) for 30 min at 37°C prior to [3H]MitoQ addition. Aliquots (4 ml) of the cell suspension were then transferred to two 2 ml Eppendorf tubes and pelleted by centrifugation (16000 g for 1 min at room temperature). The supernatant was aspirated, pellets were briefly centrifuged, and the residual supernatant removed with a piece of rolled-up tissue paper, then 250 µl of 90% (v/v) acetonitrile/0.1% TFA was added and the mixture was immediately vortex-mixed vigorously. Samples were centrifuged (16000 g for 1 min at room temperature) and 200 µl of the supernatant removed to fresh tubes, pooling within samples to give a total volume of 400 µl, which was then re-centrifuged and samples (300 µl) were snap-frozen on dry ice. For HPLC, samples were diluted with 0.1% TFA to give 45% (v/v) acetonitrile/0.1% TFA, and analysed immediately by RP-HPLC. Fractions (0.25 ml) were collected over a window where both redox forms of MitoQ eluted. MitoQH2 eluted at ~14.5 min/64% (v/v) acetonitrile and MitoQH2 eluted at ~16.5 min/67% (v/v) acetonitrile. Fractions were
transferred to a scintillation vial containing 3 ml of Fluoransafe scintillant and the radioactivity was determined. To determine the redox state of MitoQ in the extracellular medium, 1 ml of the supernatant obtained after pelleting cells (see above) was added to 1 ml of acetonitrile/0.2% TFA, vortex-mixed vigorously and centrifuged (16000 g for 5 min at room temperature). The supernatant (1.96 ml) was removed to a fresh tube, centrifuged again and a sample (1.92 ml) was analysed immediately by RP-HPLC. Mock extractions of chemically reduced [1H]MitoQH2 added to cell pellets immediately after the addition of 250 µl of 90% (v/v) acetonitrile/0.1% TFA showed negligible oxidation of [1H]MitoQH2 during extraction and freeze–thawing.

To determine the redox state of [1H]MitoQ after incubation with mitochondria, rat liver mitochondria (250 µg of protein per ml) were incubated for 5 min at 37°C in 250 mM sucrose, 5 mM Heps, and 1 mM EGTA (pH 7.4) supplemented with 500 nM [1H]MitoQ and with other substrates and inhibitors as indicated. The mitochondria were then pelleted by centrifugation (16000 g for 5 min at room temperature) and the pellets were extracted as described for cells, except that all centrifugation steps were 1 min; under these conditions there was negligible oxidation of added [1H]MitoQH2 during extraction, but freeze–thawing caused some oxidation of [1H]MitoQH2, so mitochondrial extracts were analysed by HPLC immediately after extraction.

To identify metabolites of [1H]MitoQ, cells (3 × 106 cells/ml) were incubated with 500 nM [3H]MitoQ for up to 72 h at 37°C, before pelleting the cells (centrifuged at 16000 g for 5 min at room temperature) and extracting MitoQ and its metabolites from the pellet. RP-HPLC was performed as above, except that 60 fractions of 0.5 ml were collected, spanning the initial flow-through and the entire gradient. The radioactivity in each fraction was quantified by scintillation counting of 50 µl of the eluant. Any fractions other than [1H]MitoQH2 were identified by comparison of retention times and UV absorption spectra with authentic standards.

Measurement of serum binding of MitoQ and TPMP

Binding of [1H]MitoQ or [1H]TPMP to serum, which was present at 10% (v/v) for all cell incubations, was determined by equilibrium dialysis [34]. Two dialysis cassettes (Pierce; molecular-mass cut-off 3500 Da) containing 2 ml of 10% (v/v) FCS/PBS were placed in 500 ml of PBS supplemented with [1H]MitoQ (specific activity 5.6 Ci/mol; 500 nM) or [1H]TPMP (specific activity 10.4 Ci/mol; 200 nM), and incubated with stirring at 37°C for up to 48 h. Samples (500 µl) were taken from the PBS medium and from the dialysis cassettes, mixed with 3 ml of Fluoransafe scintillant and the radioactivity was quantified and compared in order to determine the binding to serum. [1H]MitoQ and [1H]TPMP uptake into the dialysis cassettes stabilized within 48 h. At this time point, 78% of [1H]MitoQ was bound to serum, whereas there was negligible binding of [1H]TPMP to serum.

Confocal microscopy

Fibroblasts were grown to semi-confluence on 22-mm-diameter glass coverslips in six-well culture plates. Cells were incubated with IDTP (1 µM) for 3.5 h at 37°C in the presence or absence of FCCP (5 µM). MitoTracker Orange (25 nM; Molecular Probes) was added at 3 h and incubated for a further 30 min. Immunocytochemistry was carried out as described previously [35], and cells were visualized on a Zeiss LSM 510 Meta inverted confocal microscope using a ×63 objective lens, 488 and 543 nm laser lines and 515/30 and 560LP filter sets.

RESULTS

The uptake of MitoQ by mitochondria is described by the Nernst equation

Hydrophobic lipophilic cations such as MitoQ (Figure 1) are extensively internalized by mitochondria, driven by the membrane potential (Δψm), but it is unclear if their significant potential-independent binding to membranes causes deviations from Nernstian behaviour [13]. To address this we measured the uptake and binding of [1H]MitoQ by isolated mitochondria over a range of Δψm values while simultaneously measuring 86Rb+ uptake (Figure 2). In the presence of valinomycin, 86Rb+ uptake is solely determined by Δψm [31,36]. Dual-isotope counting enabled the apparent pellet spaces of 86Rb+ and [1H]MitoQ to be determined simultaneously over a Δψm range set by various amounts of KCl [30,36]. From the known mitochondrial volume, and parallel measurement of the distribution of membrane-impermeant [13][35] suro to calculate the non-mitochondrial pellet space, the ACRs of 1000 [compound]mitochondria/[compound]supernatant for [1H]MitoQ and 86Rb+ were calculated [30]. Applying the Nernst equation to the 86Rb+ ACR enabled the MitoQ ACR to be plotted over a range of known Δψm values.

In Figure 2(A), the ACR of the simple lipophilic cation [1H]TPMP is plotted against that for 86Rb+ (lower x-axis) over a range of Δψm values (upper x-axis). As expected, the relationship is linear up to the highest 86Rb+ ACR (~900, Δψm ~ 174 mV), indicating that [1H]TPMP uptake is described by the Nernst equation. The slope is greater than unity because a fixed proportion of matrix TPMP is bound. A slope of ~2.1 means that ~53% of intramitochondrial TPMP is membrane-bound and the intercept with the y-axis close to the origin indicates that its binding to unenergized mitochondria is negligible [31,36]. Plotting the [1H]MitoQ ACR against that of 86Rb+ also gave a straight line (Figure 2B), but this line passed through the y-axis well above the origin (ACR ~ 8600) as a result of substantial binding of [1H]MitoQ to unenergized mitochondria (Figure 2B). The slope was 10.3, indicating that >90% of intramitochondrial MitoQ is bound, with <10% free in the matrix. This is consistent with the greater hydrophobicity of MitoQ (the octan-1-ol/PBS partition coefficients for MitoQ and TPMP are ~3000 and ~0.35 respectively [25]) and with its high affinity for phospholipid bilayers [20]. Thus the Δψm-dependent uptake of MitoQ by mitochondria is fully described by the Nernst equation.

To separate Δψm-independent and -dependent binding, we incubated unenergized mitochondria with 0.3–1 µM [1H]MitoQ and measured free and bound [1H]MitoQ, which gave a binding constant of 5.1 (pmol per mg of protein) per nM (Figure 2C). As the mitochondrial volume is 0.6 µl per mg of protein, this corresponds to an ACR of 8500 for uncoupled mitochondria, consistent with the intercept with the y-axis in Figure 2B. The TPMP binding constant was <1% of that for MitoQ (~0.038 (pmol per mg of protein) per nM) (Figure 2C). From this binding constant and the known free concentration of MitoQ, we used the data from Figure 2(B) to calculate the Δψm-independent mitochondrial binding of MitoQ, which was subtracted from the total accumulation to generate the Δψm-dependent mitochondrial accumulation (Figure 2D). This plot was also a straight line with a slope of 10.3, further confirming that MitoQ uptake was Nernstian. Note that the y-axis intercept in Figure 2(B)
Figure 2  Uptake of [3H]MitoQ and [3H]TPMP by mitochondria over a range of $\Delta \psi_m$ values

(A) The TPMP ACR within isolated mitochondria is a linear function of the $^{86}$Rb$^+$ ACR: TPMP ACR = 2.1 x $^{86}$Rb$^+$ ACR. (B) The MitoQ ACR within isolated mitochondria is also a linear function of the $^{86}$Rb$^+$ ACR: MitoQ ACR = 10.3 x $^{86}$Rb$^+$ ACR + 8600. (C) Quantification of MitoQ binding to mitochondria in the absence of $\Delta \psi_m$. The MitoQ bound to mitochondria in the absence of $\Delta \psi_m$ is proportional to the concentration of MitoQ free in solution, and is described by the relationship: MitoQ (bound; pmol per mg of protein) = 5.1 x MitoQ (free; nM). The bound/free ratio for 1$\mu$M TPMP (0.038 (pmol per mg of protein) per nM) is also shown. (D) Relationship between MitoQ ACR and $^{86}$Rb$^+$ ACR when $\Delta \psi_m$-independent binding is accounted for. The concentration of free MitoQ (i.e. MitoQ in the supernatant after isolation of mitochondria) in the samples shown in (B) was used to quantify $\Delta \psi_m$-independent binding of MitoQ using the equation from (C). This was subtracted from the total amount of mitochondria-associated MitoQ in the samples shown in (B), and a new ACR calculated. The results for the TPMP ACR from (A) are included for comparison. (A–D) show representative results from single experiments that were repeated at least three times. Lines of best fit were determined using Microsoft Excel. (E) Variation of mitochondrial uptake of TPMP and MitoQ with $\Delta \psi_m$ at a fixed extramitochondrial concentration of 10 nM. (F) Variation of mitochondrial uptake of TPMP and MitoQ with extramitochondrial concentration at a fixed $\Delta \psi_m$ of 150 mV. For (E) and (F), mitochondrial uptake was modelled using the equations generated in (D).

depends solely on the free concentration of MitoQ and the mitochondrial amount as described by Figure 2(C), whereas the ACR-dependence on $\Delta \psi_m$ is independent of [MitoQ].

From these results, it was possible to model the $\Delta \psi_m$-dependent uptake of MitoQ and TPMP as a function of $\Delta \psi_m$, at an external free concentration of 10 nM (Figure 2E), and as a function of external free concentration at $\Delta \psi_m$ = 150 mV (Figure 2F). These show that the $\Delta \psi_m$-dependent MitoQ uptake by mitochondria was always ~5-fold greater than that of TPMP, even though the Nernst equation predicts identical ratios of free MitoQ and TPMP inside and outside mitochondria at a given $\Delta \psi_m$. This occurs because the greater binding of MitoQ within the mitochondrial matrix increases its $\Delta \psi_m$-dependent uptake. Therefore the $\Delta \psi_m$-dependent uptake of hydrophobic TPP compounds such as MitoQ...
is fully described by the Nernst equation and is greater than more hydrophilic TPP compounds.

Rapid and extensive uptake of MitoQ and DecylTPP by cells

It was unknown if the greater hydrophobicity of TPP cations such as MitoQ increases or decreases the rate and extent of their uptake into cells compared with more hydrophilic cations, such as TPMP. To address this we compared the uptake of MitoQ and DecylTPP with that of TPMP into Jurkat T lymphocytes. Use of a suspension cell line facilitates uptake studies because they can be rapidly isolated from the incubation medium by centrifugation [33]. We incubated cells with [3H]MitoQ and isolated cells at various times over 24 h and measured the [3H]MitoQ content in the cell pellet and supernatant (Figure 3A). This experiment was repeated over a shorter time scale to show uptake over the first 40 min of incubation (Figure 3B). [3H]MitoQ uptake into cells was rapid, with a steady state reached within ∼12 min and remaining stable for at least 24 h (Figures 3A and 3B). In contrast, uptake of TPMP was slow, taking ∼6–8 h to reach a steady state (Figures 3A and 3B). To see if these differences in uptake were due to hydrophobicity or were specific for the ubiquinone moiety of MitoQ, we measured the uptake of another hydrophobic TPP derivative, DecylTPP, which lacks a ubiquinone moiety (Figure 1). Uptake of DecylTPP was as rapid as that of MitoQ, quickly reaching a steady state (Figure 3B) and thereafter remaining stable (results not shown). Therefore increasing the hydrophobicity of TPP cations greatly increases their rate of uptake into cells.

To see if increasing the hydrophobicity of TPP cations also affected their efflux rate, we incubated cells with MitoQ or TPMP for 3 or 6 h respectively and then abolished Δψₘ with FCCP and measured efflux over 90 min (Figure 3C). Efflux of MitoQ was rapid and was complete within 10 min, whereas efflux of TPMP took 1 h (Figure 3C). Efflux showed first-order kinetics, enabling rate constants and half-lives (t₁/₂) to be calculated: k = 0.16 min⁻¹ and t₁/₂ = 4 min for MitoQ; k = 0.03 min⁻¹ and t₁/₂ = 24 min for TPMP (Figure 3C, inset). This 6-fold faster efflux of MitoQ compared with TPMP is consistent with the relative rates of cell uptake of the two compounds and suggests that the rate of movement of hydrophobic TPP compounds into and out of cells is significantly faster than for more hydrophilic compounds such as TPMP.

Substantially more MitoQ and DecylTPP were taken up by cells than TPMP (Figures 3A and 3B). As the volume of Jurkat cells is ∼0.2 pl per cell [33], the uptake of compounds can be expressed as an ACR ([TPP cation]cell/[TPP cation]supernatant), and for MitoQ the ACR was 921 ± 104 at 3 h, 4.75-fold greater than that for TPMP (192 ± 56) (Figure 3D). When the uncoupler FCCP was added, the ACRs of MitoQ, DecylTPP and TPMP decreased by 81%, 87% and 79% respectively (Figure 3D). These results are consistent with Δψₘ-dependent uptake of all three TPP compounds into mitochondria after the initial uptake into cells.
Therefore hydrophobic TPP compounds such as MitoQ and DecylTPP are more rapidly taken up into cells than the hydrophilic cation TPMP. These hydrophobic molecules are also 4–10-fold more extensively accumulated by cells than TPMP, consistent with their greater uptake into isolated mitochondria (Figure 2).

Within cells hydrophobic TPP cations predominantly localize to mitochondria

FCCP led to the release of the TPP compounds from the cell, indicating that uptake of intracellular MitoQ, DecylTPP and TPMP is driven by $\Delta\psi_m$ (Figure 3D). However, directly demonstrating the mitochondrial localization of TPP compounds within cells is challenging, as they rapidly redistribute on subcellular fractionation. To circumvent this, we investigated the uptake of a MitoQ analogue, IDTP (Figure 4). Like MitoQ and DecylTPP, IDTP contains the TPP targeting moiety and a ten-carbon aliphatic alkyl chain, but also has an iodo moiety that reacts slowly with protein thiols to form a stable thioether bond [11] (Figure 1). Thus the extent of covalent binding of IDTP to intracellular proteins is a reflection of its steady-state concentration within a cytosolic compartment. The TPP moiety of IDTP can be detected using anti-TPP serum, enabling the localization of IDTP binding by immunocytochemistry [11]. IDTP was taken up rapidly into isolated mitochondria in a $\Delta\psi_m$-dependent manner, similar to MitoQ and DecylTPP (results not shown). In fibroblasts, IDTP localized exclusively to mitochondria and this uptake was prevented by FCCP (Figure 4). In the C2C12 myoblast cell line, IDTP localization was also mitochondrial and was prevented by FCCP and by oligomycin/mxyothiazol (results not shown). The reactivity of protein thiols (pK $\approx 8$–9 typically) with IDTP within the mitochondrial matrix (pH $\approx 8$) is likely to be higher than in the cytosol (pH 7.2), because it is the thiolate form that reacts with IDTP [11]. However, there was negligible cytoplasmic IDTP binding in fibroblasts after 6 h incubation, suggesting that the steady state concentration of IDTP in the cytosol is far lower than in mitochondria. Together these results strongly suggest that within cells hydrophobic TPP compounds are predominantly concentrated within mitochondria, driven by the $\Delta\psi_m$.

Cell uptake of MitoQ is consistent with the Nernst equation

The uptake of hydrophilic TPP cations such as TPMP into cells occurs in response to both the $\Delta\psi_p$ and the $\Delta\psi_m$, as described by the Nernst equation [37,38]. Uptake is determined experimentally as an ACR ([TPP cation]$_{cell}$/[TPP cation]$_{supernatant}$), and this is related to $\Delta\psi_p$ and $\Delta\psi_m$ by eqn (3) [37] where $V_c$ and $V_m$ are the volumes of the cytosol and mitochondria, and $a_s$, $a_c$ and $a_m$ are the activity coefficients of the compounds in the extracellular environment, cytosol and the mitochondrial matrix, respectively (the activity coefficient is the proportion of compound in that compartment that is free in solution and not bound).

$$\text{ACR} = \frac{[\text{TPP cation}]_{cell}}{[\text{TPP cation}]_{supernatant}} = \frac{a_s \times 10^{\Delta\psi_p/61.5} \left\{ \left\{ \frac{V_c}{a_c} + \frac{V_m}{a_m} \right\} \times 10^{\Delta\psi_m/61.5} \right\}}{V_c + V_m}$$

In Jurkat cells, the $\Delta\psi_p$ is 40 mV (negative inside) [33] and the volume is 200 nl per 10⁶ cells, with $\sim 5\%$ occupied by mitochondria [33], giving values for $V_m$ and $V_c$ of 10 nl per 10⁶ cells and 190 nl per 10⁶ cells respectively. We determined that the serum binding of TPMP in these experiments was negligible, so $a_s = 1$. The cytoplasmic binding of TPMP within hepatocytes, $a_c = 0.21$ [31], was taken as the value for $a_c$ in Jurkat cells. The binding of TPMP within mitochondria, determined from the reciprocal of the slope of Figure 2(A), gives $a_m = 0.47$. These parameters, together with the TPMP ACR of 192 from Figure 3(D), give $\Delta\psi_m = 157$ mV (from eqn 3), which is in the expected range [31]. Thus eqn (3) describes the Nernstian uptake of TPP cations by cells in our experiments.

Figure 4  IDTP localizes predominantly to mitochondria within cultured human fibroblasts

Cells were incubated with 1 µM IDTP for 3.5 h in the presence (+FCCP) or absence of 5 µM FCCP. MitoTracker Orange was added for the last 30 min. The cells were then fixed and treated with antiserum against the TPP moiety of IDTP and a secondary antibody linked to Oregon Green 488. Scale bar, 20 µm.
If the uptake of MitoQ into Jurkat cells is Nernstian then its ACR should also be described by eqn (3). To assess this, we required the activity coefficients of MitoQ in the three compartments. MitoQ was ∼78% bound to serum in the incubation medium, consequently \(a_c = 0.22\). The intramitochondrial binding of MitoQ, from the reciprocal of the slope in Figure 2(B), indicates that \(a_m = 0.097\). The extensive binding of MitoQ to de-energized cells makes it technically difficult to determine a reliable value for \(a_c\) [34]. This would require abolishing \(\Delta \psi_c\) and \(\Delta \psi_m\) and comparing the [3H]MitoQ ACR relative to that for 86Rb+ over a range of \(\Delta \psi_p\) values [34], as was done for mitochondria in Figure 2(B). However, for cells it is only possible to vary \(\Delta \psi_p\) over ∼60 mV [34]. As can be seen in Figure 2(B), the extensive non-specific binding of MitoQ would render any \(\Delta \psi_p\)-sensitive uptake negligible relative to random errors in binding below \(\Delta \psi_p\) of ∼120 mV.

To determine \(a_c\) by an alternative approach we assume that eqn (3) does describe MitoQ uptake, then we can use it to calculate \(a_c\), from \(\Delta \psi_m = 157\) mV, the known values for \(V_m\), \(V_c\) and \(\Delta \psi_p\), and the values of \(a_c\) and \(a_m\) that we have derived for MitoQ. Entering these values, along with the ACR for MitoQ of 921 from Figure 3(D), gives \(a_c = 0.002\). This indicates that our data on the uptake of MitoQ into cells are consistent with Nernstian uptake, provided ∼99.8% of the MitoQ in the cytosol is bound.

To check if this estimate for extensive (∼99%) cytosolic binding of MitoQ was valid, we calculated \(a_c\) by an alternative method based on different starting assumptions. For the Jurkat cell incubation in Figure 3, the total [MitoQ] added was 500 nM which, after equilibration, reached a steady state total [MitoQ] in the extracellular medium of 260 ± 5 nM (Figure 3D and results not shown). As ∼78% of extracellular MitoQ is bound to serum, the free concentration is 57 nM. Assuming Nernstian uptake into the cytosol and mitochondria driven by a \(\Delta \psi_p\) of 40 mV and a \(\Delta \psi_m\) of 157 mV, the cytosolic and mitochondrial free MitoQ concentrations will be 255 nM and 91 µM respectively. From the cytosolic and mitochondrial volumes (190 nl per 10⁶ cells and 10 nl per 10⁶ cells) this gives 0.048 and 0.91 pmol of MitoQ per 10⁶ cells free in the cytosol and mitochondria respectively. The total amount of cytosolic MitoQ can be estimated as that lost when cells incubated with myxothiazol and oligomycin to abolish \(\Delta \psi_m\) are further treated with gramicidin to abolish \(\Delta \psi_p\) (Figure 5C). The total amount of mitochondrial MitoQ can be estimated as that which is sensitive to the abolition of \(\Delta \psi_m\) with myxothiazol and oligomycin (Figure 5A). From these experiments the total amounts of MitoQ present in the cytosolic and mitochondrial compartments are 16 pmol and 29.3 pmol of MitoQ per 10⁶ cells respectively. Comparing these values with the free concentration of MitoQ estimated from the Nernst equation indicates that in the cytosol, free MitoQ is ∼0.3% of the total, with ∼99.7% bound. In mitochondria, ∼3% of the total MitoQ is free with ∼97% bound. This estimate of ∼99.7% of cytosolic MitoQ bound is comparable with our earlier estimate.
of 99.8% bound, suggesting that $a_e = 0.002$ for MitoQ is a reasonable estimate.

To summarize, uptake of MitoQ into cells is consistent with the Nernst equation, provided that binding of MitoQ in the cytosol is > 99%. It was not possible to directly determine MitoQ binding in the cytosol, but two different calculations suggest that cytosolic MitoQ is > 99% bound. MitoQ is far more hydrophobic than TPMP; octan-1-ol partition coefficients for MitoQ and TPMP are 2760 and 0.35 respectively [25]; affinity of MitoQ for unenergized mitochondrial membranes is 134-fold greater than that of TPMP (Figure 2C); and 78% of MitoQ was bound to serum in the incubation medium, whereas binding of TPMP was negligible. In hepatocytes ∼80% of cytoplasmic TPMP is bound [31], making > 99% binding of the far more hydrophobic MitoQ plausible. Therefore our findings are consistent with uptake of MitoQ into cells being described by the Nernst equation, with most of the intracellular compound being bound.

Different rates of uptake of TPP cations across the plasma membrane

The relatively hydrophobic TPP cations MitoQ and DecylTPP are far more rapidly taken up and released by cells than the hydrophilic TPMP cation (Figure 3). This discrepancy is not the result of differences in rates of uptake into mitochondria, as TPMP and MitoQ are both taken up rapidly into isolated mitochondria, with > 90% of uptake complete within 1 min [25,27]. This suggests that the different uptake rates into cells are the result of plasma membrane differences.

To measure the uptake of TPP compounds across the plasma membrane without the confounding effects of subsequent mitochondrial uptake, we abolished $\Delta \psi_m$ with oligomycin and myxothiazol. While this loss of $\Delta \psi_m$ decreased the extent of uptake of MitoQ (Figure 5A) and DecylTPP (results not shown), a steady state distribution was reached within 5 min (Figure 5A). Oligomycin and myxothiazol did not disrupt $\Delta \psi_m$ over the time scale of these experiments, as measured by the fluorescent $\Delta \psi_m$ indicator PMPI [39] (Figure 5B). Subsequent addition of the ionophore gramicidin collapsed $\Delta \psi_m$ (Figure 5B) and further decreased the uptake of MitoQ (Figure 5C) and DecylTPP (results not shown). Therefore the gramicidin-sensitive uptake of MitoQ and DecylTPP in the presence of myxothiazol and oligomycin is rapid and is primarily driven by $\Delta \psi_m$. In contrast, uptake of TPMP under these conditions was far slower than that of MitoQ or DecylTPP and was mostly prevented when $\Delta \psi_m$ was abolished with gramicidin (Figure 5D). It is concluded that hydrophobic TPP compounds are taken up more rapidly into cells than TPMP because they cross the plasma membrane far more quickly.

Redox state of MitoQ within mitochondria

The reduction of MitoQ to its ubiquinol form MitoQH$_2$ by the respiratory chain is thought to be essential for it to prevent lipid peroxidation [13,40]. In simple in vitro systems, MitoQ is rapidly reduced by complex II, but in cells and isolated mitochondria little is known about the redox state of MitoQ or its rate of equilibration with endogenous electron donors. We therefore set out to investigate reduction of $[^{3}H]$MitoQ by mitochondria (Figure 6).

After incubation of mitochondria with $[^{3}H]$MitoQ, the ubiquinone ($[^{3}H]$MitoQ) and ubiquinol ($[^{3}H]$MitoQH$_2$) forms were extracted into acidified acetonitrile, separated by HPLC and quantitated by scintillation counting (Figure 6A). The ratio of the area under the $[^{3}H]$MitoQH$_2$ peak to the total amount recovered gave the percentage reduction of MitoQ. Control experiments with exogenous $[^{3}H]$MitoQH$_2$ showed that this extraction and analysis procedure preserved the MitoQ redox state. In mitochondria energized with succinate, 75 ± 8% of $[^{3}H]$MitoQ was reduced (Figure 6B). Succinate-mediated reduction was largely abolished
Results are means ± S.D. (n ≥ 3), with each experiment performed in duplicate. The effect of respiratory inhibitors on the redox state of MitoQ in cells. [3H]MitoQ was incubated with Jurkat cells as described in (A), and after 1 h the ratio of reduced to oxidized MitoQ in the cells was determined by extraction, RP-HPLC and scintillation counting as described in Figure 6(A). The effect on this of the respiratory inhibitors rotenone (ROT; 4 µg·ml⁻¹) and antimycin A (ANT; 1 µM) are shown, with vehicle-incubated cells as a control. Results are means ± S.D. (n ≥ 3), with each experiment performed in duplicate. (C) Metabolism of MitoQ by cells. Jurkat cells were incubated with 500 nM [3H]MitoQ for 1, 24 and 48 h. At these times [3H]MitoQ and any metabolites were extracted and separated by RP-HPLC and all fractions counted for radioactivity. Over 24 and 48 h, a radioactive metabolite of MitoQ was detected on the HPLC profile (arrow), dpm, disintegrations per min. (D) Quantification of the MitoQ metabolite over time. The metabolite indicated by the arrow in (C) is expressed as a percentage of the total radioactivity recovered from the HPLC analysis shown in (C). Results at 48 h are means ± S.D. (n = 3).

Redox state and metabolism of MitoQ within cells

When [3H]MitoQ was added to cells, it was rapidly taken up and converted into the ubiquinol form within 10 min and then remained stable at 50–60 % reduced (Figure 7A), similar to the redox state in isolated mitochondria. The redox state of [3H]MitoQ in the extracellular medium also became reduced over time, equilibrating with the redox state of intracellular MitoQ within 2 h (Figure 7A), presumably by molecular exchange.

To see whether MitoQ reduction within cells was set by cell metabolism, we added the reduced form, [3H]MitoQH₂, [3H]MitoQH₂ was rapidly oxidized and within 20 min reached a stable steady state level of 40–60 % reduction, the same as that following addition of oxidized MitoQ (results not shown). The redox state of [3H]MitoQ was also unaffected when the amount of added MitoQ was varied from 125 nM to 1 µM, or when the [O₂] was decreased from 21 % to 3 % (results not shown). The reduction of exogenous MitoQ was not cell type-specific, as [3H]MitoQ added to C2C12 cells rapidly reached a steady state level of reduction similar to that of Jurkat cells (results not shown). When cells were incubated with [3H]MitoQ in the presence of rotenone, the [3H]MitoQ became less reduced (30 ± 4 %),
whereas addition of the complex III inhibitor antimycin caused the [3H]MitoQ to become more reduced (87 ± 7%) (Figure 7B). Together these results indicate that the redox state of MitoQ within cells responds to that of the mitochondrial CoQ pool, and that reduction of MitoQ occurs rapidly, consistent with its fast uptake into mitochondria within cells.

To see how stable MitoQ was within cells, we incubated Jurkat cells with [3H]MitoQ for 1, 24 and 48 h, and assayed for metabolites of [3H]MitoQ by separating cell extracts by RP-HPLC and measuring the radioactivity in all fractions (Figure 7C). Over 48 h [3H]MitoQ remained predominantly (60–80%) in the reduced form (results not shown). After 1 h, 99% of the radioactivity co-eluted with [3H]MitoQH2 or [3H]MitoQ (Figure 7C), with no detectable MitoQ metabolites. At 24 and 48 h one new peak that accounted for 1–2% of the [3H] radioactivity at 24 h and 12 ± 6% at 48 h appeared on the HPLC trace. To identify the metabolite, equivalent fractions from parallel 48 h incubations with unlabelled MitoQ were isolated by HPLC and analysed by ESI-MS. The metabolite peak contained a species of m/z = 665.3 that by tandem MS readily fragmented to MitoQH2 (m/z = 585.4) with a loss of 79.9 Da. The MitoQ mass was further fragmented to give a daughter ion of 262 Da, diagnostic of the TPP moiety, confirming that the metabolite was a derivative of MitoQ. These results are consistent with the metabolite being sulfated MitoQ (m/z = 665.2) which, on loss of a sulfate (80.05 Da), regenerates MitoQH2 (Figure 1). This is in agreement with animal and cell results where monosulfated MitoQ was a major metabolite [42,43]. Therefore within cells MitoQ is rapidly reduced and attains a stable steady state level of the ubiquinol form over long-term incubations. Over 48 h some MitoQ is metabolized to a sulfated derivative, but the majority remains in the active ubiquinol form.

DISCUSSION

The targeting of probes and bioactive molecules to mitochondria by conjugation to lipophilic cations such as TPP plays an increasing role in reporting on and modifying mitochondrial function. These molecules generally comprise relatively bulky and hydrophobic groups attached to a TPP moiety that drives their selective uptake into mitochondria. However, it was unknown how these hydrophobic additions to the TPP molecule affected the rate and extent of their uptake into mitochondria within cells. In this present paper, by comparing the uptake of MitoQ and DecylTPP into mitochondria and cells with the hydrophilic TPP cation TPMP, we have shown how the hydrophobicity of TPP cations affects their uptake.

The uptake of MitoQ into mitochondria and cells was described by the Nernst equation. Previous preliminary studies suggested that saturation of binding might lead to deviations from Nernstian behaviour [13]. However, comparing the uptake of MitoQ with that of 86Rb+ showed that MitoQ uptake into mitochondria is fully described by the Nernst equation and that MitoQ bound extensively (> 90%) within the mitochondrial matrix. As a result of this binding, the total amount in mitochondria is about 10-fold greater than simple equilibration of the free compound with the Δψm, and was ~5-fold greater than that of TPMP. This binding is most likely to be to the matrix-facing surface of the inner membrane, with the TPP compounds adsorbed as a monolayer on the membrane with the TPP cation in a potential energy well close to the surface with the hydrophobic side chain inserted into the membrane [14,21]. Such a location for hydrophobic TPP derivatives is fortuitous, as many mitochondrial activities are associated with the inner membrane.

The extent of uptake of MitoQ into mitochondria within cells was also 4–5-fold greater than that of TPMP and was also consistent with the Nernst equation. In the cytosol, most of the MitoQ was bound. The uptake was selective for mitochondria, with the majority of MitoQ in the cell being concentrated within mitochondria as a result of the Δψm.

Increasing the hydrophobicity of TPP molecules increased their rate of uptake into cells severalfold. This increase was due to the far more rapid permeation of the plasma membrane by the hydrophobic TPP compounds. The reason for this is likely to be the greater hydrophobicity of MitoQ and DecylTPP lowering their activation energies for plasma membrane passage (octan-1-ol/PBS partition coefficients: MitoQ ~3000, DecylTPP ~5000 and TPMP = 0.35) [25]. The activation energy for transport of lipophilic cations through a phospholipid bilayer is made up of an unfavourable Born energy due to the charge of the TPP moiety, which is the same for all TPP cations, and a favourable hydrophobic force, which is the main difference between molecules such as MitoQ and TPMP [14]. That the plasma membrane acts as a significant kinetic barrier to movement of hydrophilic TPP cations is strongly supported by the fact that the lipophilic TPP (tetraphenylborate) anion is routinely added to speed up equilibration of TPMP into cells and does so by lowering the activation energy for membrane transport [14,38,44]. TPB is not required to assist the uptake of TPMP through the mitochondrial inner membrane, suggesting that the activation energy for transport across this membrane is lower. Lipophilic dications are also more readily taken up through the mitochondrial inner membrane than the plasma membrane, and increasing their hydrophobicity increases their uptake through the plasma membrane [30]. Several factors may contribute to this difference in activation energies between the mitochondrial and plasma membranes, including their different phospholipid compositions, lack of cholesterol and presence of cardiolipin in the inner membrane, the difference in membrane potential values and the very high protein content of the inner membrane which may increase its dielectric constant [41].

Once past the plasma membrane, MitoQ and DecylTPP were taken up rapidly into mitochondria, as demonstrated by the predominantly mitochondrial localization of the MitoQ analogue IDTP and by the fact that the uptake of MitoQ and DecylTPP into cells was greatly decreased by FCCP. MitoQ was rapidly reduced to its ubiquinol form by the mitochondrial respiratory chain, further supporting mitochondrial localization within cells. As this reduction had the same kinetic profile as uptake into cells, it is concluded that these compounds are rapidly accumulated by mitochondria once within cells.

In cells, MitoQ rapidly becomes 50–60% reduced to the ubiquinol form. MitoQ is stably retained in the reduced form within cells over at least 48 h. Over this time there was some metabolism to a sulfated derivative, but most of the added MitoQ persisted and was predominantly present in the reduced form. These findings are consistent with the protective effects seen for MitoQ in cell and animal models being the result of MitoQH2 preventing lipid peroxidation within mitochondria [40].

To summarize, uptake of lipophilic TPP cations into mitochondria is described by the Nernst equation, is rapid compared with its metabolism and it equilibrates rapidly with the CoQ pool. A model for the uptake of MitoQ by cells that is consistent with our findings is illustrated in Figure 8. The Nernst equation relates free, unbound pools of compound that equilibrate with the Δψm and Δψm. Extracellular MitoQ is ~78% bound to serum, and the free MitoQ then equilibrates with the free concentration within the cytosol in response to the Δψm. This free concentration is then in equilibrium with the >99% of cytosolic MitoQ that is bound.
The free MitoQ in the cytosol is in turn in equilibrium with the free concentration within the mitochondrial matrix, and this relationship is determined by the $\Delta \psi_p$. The free pool of MitoQ in the matrix is in equilibrium with a 10-fold excess that is bound to the matrix face of the mitochondrial inner membrane. These findings suggest that mitochondria-targeted compounds can be optimized by adjusting their hydrophobicity to modulate their rates of uptake into cells, and the extent of their uptake and concentration in the matrix modified by altering their binding to the mitochondrial inner membrane. Through such modifications it should be possible to design rationally further compounds to manipulate and report on particular mitochondrial functions.

This work was supported by the Medical Research Council (MRC), Antipodean Pharmaceuticalists (San Francisco, CA, U.S.A.) and by the European Community's 6th Framework Programme for Research, Priority 1 "Life sciences, genomics and biotechnology for health" contract LSHM-CT-2004-503116. We thank Dr Abdul-Rahman bin Manas and Pauline Bandeen (University of Otago) for technical assistance with the synthetic chemistry, Stephanie Brown (MRC Dunn Human Nutrition Unit) for technical assistance, and Dr Martin Brand (MRC Dunn Human Nutrition Unit) for helpful discussions on the manuscript.

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Received 8 January 2008/22 February 2008; accepted 25 February 2008
Published as BJ Immediate Publication 25 February 2008, doi:10.1042/BJ20080063