J Bioenerg Biomembr (2013) 45:165–173
DOI 10.1007/s10863-012-9493-5

Mitochondrial accumulation of a lipophilic cation conjugated to an ionisable group depends on membrane potential, pH gradient and pK_a: implications for the design of mitochondrial probes and therapies

Peter G. Finichiu · Andrew M. James · Lesley Larsen · Robin A. J. Smith · Michael P. Murphy

Received: 25 September 2012 / Accepted: 7 November 2012 / Published online: 22 November 2012

Abstract Mitochondria play key roles in a broad range of biomedical situations, consequently there is a need to direct bioactive compounds to mitochondria as both therapies and probes. A successful approach has been to target compounds to mitochondria by conjugation to lipophilic cations, such as triphenylphosphonium (TPP), which utilize the large mitochondrial membrane potential ($\Delta\psi_m$, negative inside) to drive accumulation. This has proven effective both in vitro and in vivo for a range of bioactive compounds and probes. However so far only neutral appendages have been targeted to mitochondria in this way. Many bioactive functional moieties that we would like to send to mitochondria contain ionisable groups with pK_a in the range that creates an assortment of charged species under physiological conditions. To see if such ionisable compounds can also be taken up by mitochondria, we determined the general requirements for the accumulation within mitochondria of a TPP cation conjugated to a carboxylic acid or an amine. Both were taken up by energised mitochondria in response to the protonmotive force. A lipophilic TPP cation attached to a carboxylic acid was accumulated to a greater extent than a simple TPP cation due to the interaction of the weakly acidic group with the pH gradient (ΔpH). In contrast, a lipophilic TPP cation attached to an amine was accumulated less than the simple cation due to exclusion of the weakly basic group by the ΔpH. From these data we derived a simple equation that describes the uptake of lipophilic cations containing ionisable groups as a function of $\Delta\psi_m$, ΔpH and pK_a. These findings may facilitate the rational design of additional mitochondrial targeted probes and therapies.

Keywords Mitochondria · Mitochondria-targeting · Triphenylphosphonium cation · Lipophilic cation · Mitochondrial pH gradient

Abbreviations

ACR	Accumulation ratio
$\Delta\psi_m$	Mitochondrial membrane potential
ΔpH	Mitochondrial pH gradient
FCCP	Carbonylcyanide *p*-trifluoromethoxyphenylhydrazone
TPMP	Triphenylmethylphosphonium cation
TPP	Triphenylphosphonium
RLM	Rat liver mitochondria
Δp	Protonmotive force
$TPP^+C_{10}CO_2H$	(10-Carboxydecyl)triphenylphosphonium cation
$TPP^+C_{10}NH_2$	(10-Aminodecyl)triphenylphosphonium cation

Introduction

Mitochondrial function and dysfunction are at the centre of a range of vital biomedical processes and contribute to numerous pathologies (Duchen and Szabadkai 2010; Smith

Electronic supplementary material The online version of this article (doi:10.1007/s10863-012-9493-5) contains supplementary material, which is available to authorized users.

P. G. Finichiu · A. M. James · M. P. Murphy (✉)
MRC Mitochondrial Biology Unit,
Wellcome Trust/MRC Building,
Cambridge CB2 0XY, UK
e-mail: mpm@mrc-mbu.cam.ac.uk

L. Larsen · R. A. J. Smith
Department of Chemistry, University of Otago, Box 56,
Dunedin, New Zealand

et al. 2011; Wallace et al. 2010). Consequently there is a need to develop mitochondria-targeted probes and therapies (Murphy 2009; Murphy and Smith 2007; Smith et al. 2011). One way in which the targeting of bioactive molecules to mitochondria has been achieved is by conjugation to lipophilic cations such as triphenylphosphonium (TPP) (Murphy and Smith 2007; Ross et al. 2005; Smith et al. 2011). The hydrophobic surface and the large ionic radius mean that the TPP cation can move directly and rapidly through biological phospholipid bilayers, without requiring a protein transporter (Ross et al. 2005). The positive charge facilitates TPP cations to be taken up into mitochondria driven by the large mitochondrial membrane potential ($\Delta\psi_m$) of~170 mV (negative inside) (Ross et al. 2005). The extent of this uptake, generally described by the Nernst equation, leads to their several hundred fold uptake into mitochondria in vivo (Fig. 1a) (Ross et al. 2005). This has led to the development of a wide range of bioactive molecules and probes that can be delivered to mitochondria in vivo, following oral, intravenous, intraperitoneal or subcutaneous administration (Porteous et al. 2010; Smith et al. 2011). These compounds include antioxidants such as MitoQ (Kelso et al. 2001; Smith and Murphy 2010) and MitoCP (Dhanasekaran et al. 2005), the *S*-nitrosating compound MitoSNO (Prime et al. 2009), the superoxide probe MitoSOX (Robinson et al. 2006) and the hydrogen peroxide probes MitoB (Cocheme et al. 2011) and MitoPY1 (Dickinson and Chang 2008). One of these compounds, MitoQ, has been progressed through phase II trials in humans, indicating the potential of this strategy to develop pharmaceuticals (Gane et al. 2010; Snow et al. 2010). However, all the TPP compounds that have been targeted to mitochondria to date by this strategy have contained a neutral bioactive component. Many bioactive compounds that we would like to target to mitochondria to act as drugs or probes have ionisable groups, typically carboxylic acid or

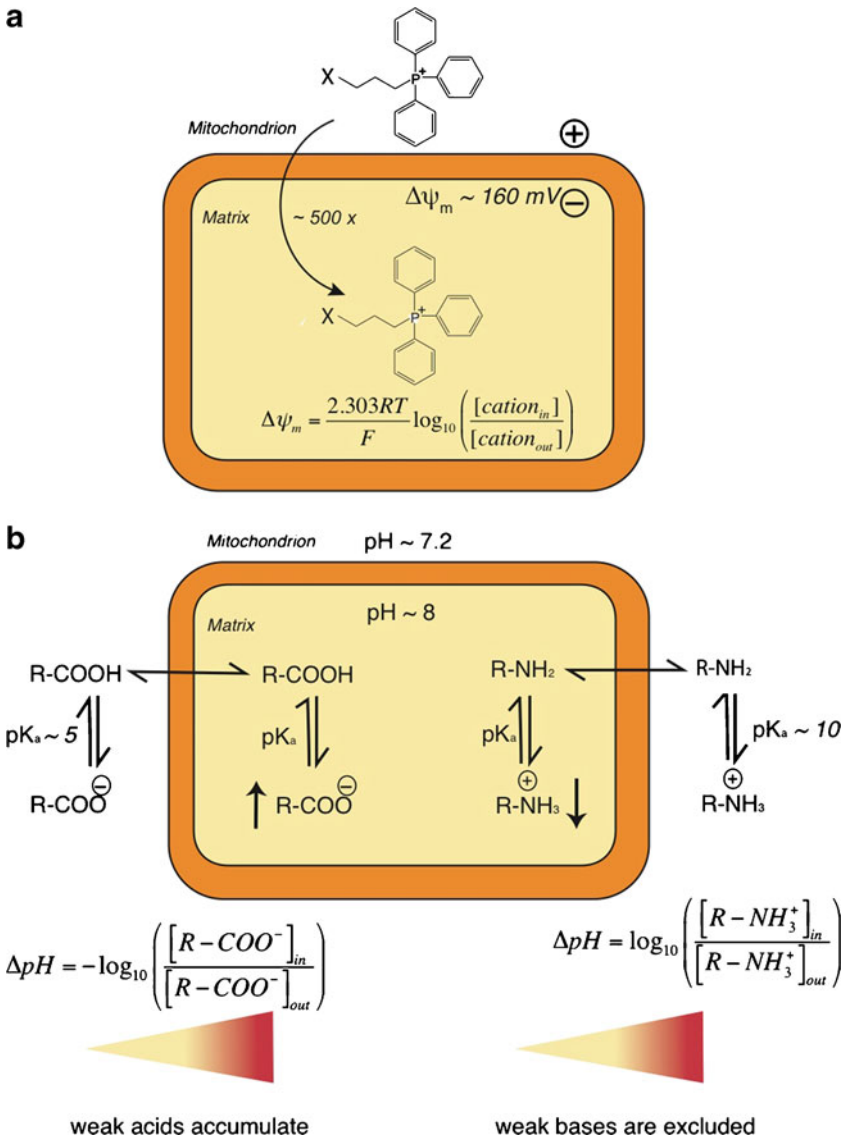

Fig. 1 Mitochondrial uptake of lipophilic cations, acids and bases. **a** Uptake of a lipophilic TPP cation conjugated to an uncharged moiety (X). The uptake of this compound into mitochondria is driven by the $\Delta\psi_m$ and the extent of uptake is given by the Nernst equation. **b** The distribution of lipophilic weak acids and bases across the mitochondrial inner membrane in response to ΔpH. Only the neutral forms are membrane permeant. Equilibration with the local pH means that the lipophilic weak acids are accumulated into the basic mitochondrial matrix while lipophilic amines are excluded. The extents of this uptake or exclusion are given by the indicated equations derived from the Henderson-Hasselbalch equation. For both acid and base the charged form is >>99 % of the total present, consequently these equations describe the total distribution of weak acids and bases across the mitochondrial inner membrane

amine functions. Under physiological conditions, conjugation of these moieties to the TPP cation, will generate compounds with a range of protonation and charge states. For example, a carboxylic acid (pK_a~5) attached to a TPP cation will be largely present as a neutral zwitterion, while a TPP attached to an amine (pK_a~10) would be predominantly a dication. A neutral zwitterion would not be expected to be taken up into mitochondria driven by $\Delta\psi_m$, while a dication may be too highly charged to cross the mitochondrial inner membrane (Ross et al. 2006). Furthermore, lipophilic weak acids and bases themselves distribute across the mitochondrial inner membrane in response to the pH gradient across the mitochondrial inner membrane (ΔpH, basic inside) (Fig. 1b) (Azzone et al. 1984; Brand 1995). Only the uncharged form crosses the membrane and then equilibrates with the local pH, consequently weak lipophilic acids are accumulated by mitochondria due to the higher pH of the matrix, while weak lipophilic bases are excluded (Fig. 1b). It is unclear how the conjugation of an ionisable group to a TPP cation affects its uptake in response to ΔpH. Together these issues are a significant impediment to the rational development of further mitochondria-targeted probes and therapies incorporating carboxylic acid or amine functions. To address this gap in our knowledge, here we report on the uptake by mitochondria of a TPP cation conjugated to a carboxylic acid (10-carboxydecyl)triphenylphosphonium ($TPP^+C_{10}CO_2H$), or to an amine (10-aminodecyl)triphenylphosphonium ($TPP^+C_{10}NH_2$) (Fig. 2). In both cases the ionisable function was attached to the TPP by a hydrophobic alkyl chain in order to mimic many widely used mitochondria-targeted compounds. From this, we develop a general model for how these molecules are accumulated into mitochondria and derive an equation that describes the dependence of this uptake on $\Delta\psi_m$, ΔpH and pK_a. These findings provide a rational basis for the design of further mitochondria-targeted probes and therapies.

Materials and methods

Chemical Syntheses

(10-Carboxydecyl)triphenylphosphonium bromide ($TPP^+C_{10}CO_2H$) was prepared as described (Wube et al. 2011). (10-Aminodecyl)triphenylphosphonium bromide as the hydrobromide salt (Lopez et al. 2009) was prepared by heating 10-bromo-1-decanamine, hydrobromide (0.915 g, 2.89 mmol) (Klayman et al. 1969) with triphenylphosphine (7 g, 26.7 mmol) in a sealed tube at 100 °C for 24 h under argon. The mixture was then cooled, dissolved in a minimum of ethanol and precipitated by adding to diethyl ether (100 mL) to give the product as a white solid (1.20 g, 2.07 mmol, 72 %). HPLC: Column Phenomenex Prodigy 250 ×3mm, gradient elution 10 % acetonitrile/H_2O (0.1 % trifluoroacetic acid) to 100 % acetonitrile over 12.5 min at 0.5 mLmin^{-1}. Detection at 210 and 254 nm. Retention time

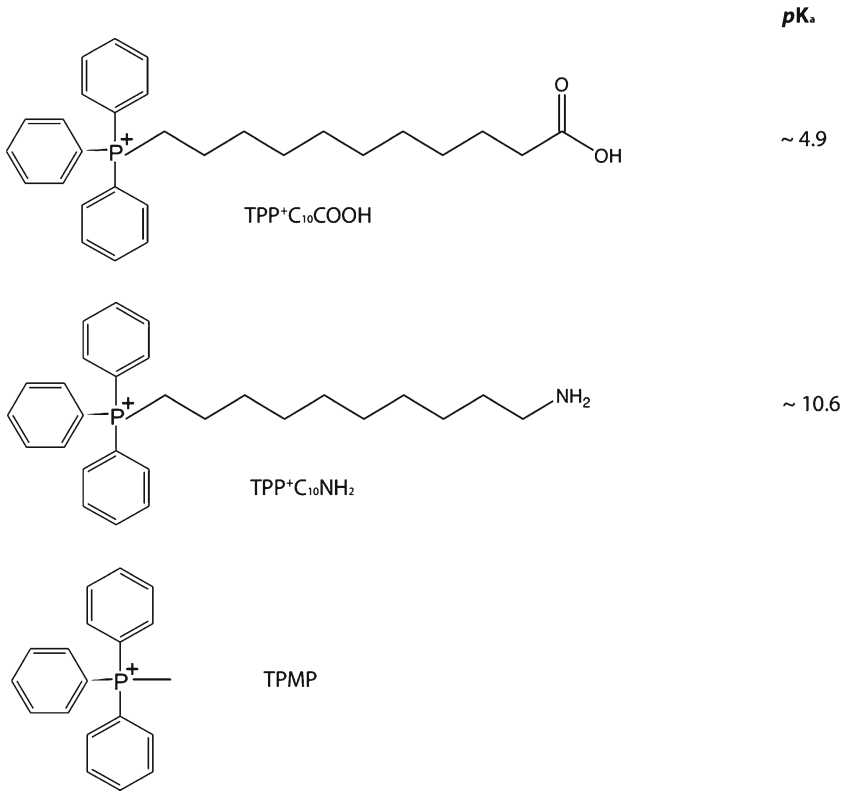

Fig. 2 Structures and pK_a values of the TPP compounds assessed. The pK_a values are estimated from literature values of similar compounds: $TPP^+C_{10}CO_2H$ (Kanicky and Shah 2003); $TPP^+C_{10}NH_2$ (Hoerr et al. 1943)

9.07 min 100 % pure. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.1 (3H, broad, NH), 7.8–7.4 (15H, m, ArH,), 3.75 (2H, m, CH_2P), 3.05 (2H, m, CH_2N), 1.9 (2H, m, CH_2), 1.7–1.2 (14H, m, CH_2) ppm. ^{31}P NMR: δ 24.4 ppm. Stock solutions of the TPP compounds used (Fig. 2) were made up in absolute ethanol, flushed with argon and stored at −20 °C prior to use.

Mitochondrial preparation

Liver mitochondria (RLM) were prepared from 6 to 13 weeks old female Wistar rats (300–500 g) by homogenisation followed by differential centrifugation in ice-cold 250 mM sucrose, 5 mM Tris–HCl, 1 mM EGTA, pH 7.4 (Chappell and Hansford 1972). The mitochondrial suspension was stored on ice until use. Mitochondrial protein concentration was determined by the biuret assay using bovine serum albumin as a standard (Gornall et al. 1949). Incubations were carried out in KCl buffer (120 mM KCl, 1 mM EGTA, 10 mM HEPES, adjusted to pH6, 7.2 or 8).

Ion-selective electrode measurements

Ion-selective electrodes sensitive to the TPP cation moiety of methyltriphenylphosphonium (TPMP), $TPP^+C_{10}CO_2H$ and $TPP^+C_{10}NH_2$ were constructed as previously described (Asin-Cayuela et al. 2004) and placed into a thermostated (37 °C) and stirred 3 mL incubation chamber containing rotenone (4 $\mu g mL^{-1}$) supplemented KCl buffer.

HPLC measurements

RLM (0.5 mg protein/mL) were incubated in 4 mL KCl buffer supplemented with rotenone (4 μg/mL) in open 15 mL plastic tubes in a shaking water bath at 25 °C. Respiration was initiated by addition of succinate (4 mM) and in some cases carbonylcyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) (1 μM) was added 4 min later. For the malonate incubations, the TPP compounds were added after RLM had been energized with succinate (4 mM, supplemented with 0.6 mM or 2.3 mM malonate). After a further 5 min the mitochondria were pelleted by centrifugation (7,500×g for 10 min). Supernatants were removed and stored at 4 °C in eppendorf tubes until reverse phase (RP)-HPLC analysis. The mitochondrial pellets were extracted by vortexing in 250 μL Buffer B (100 % acetonitrile (ACN), 0.1 % trifluoroacetic acid (TFA)) followed by centrifugation (7,500×g for 10 min). Supernatant (250 μL) was removed and stored at 4 °C in glass vials (Chromacol®). All samples were diluted to 25 % ACN by addition of Buffer A (100 % H_2O, 0.1 % TFA), filtered through a Milipore® Millex® syringe-driven filter unit (0.22 μm) and then injected into a 2 mL sample loop and separated by RP-HPLC. For this a C18 column (Jupiter 300A, Phenomenex®) with a Widepore C18 Guard Column (Phenomenex®) and a Gilson 321 pump were used. A gradient of buffer A and B was run at 1 mL/min (% B): 0–5 min, 5 – 15 %; 5 – 31 min, 15 – 100 %; 31–35 min, 100 – 5 %. Peaks were detected at 220 nm using a Gilson UV/VIS 151 spectrophotometer. TPMP, $TPP^+C_{10}CO_2H$ and $TPP^+C_{10}NH_2$ standards were used to determine elution times, and the Chart 5 software (ADInstruments®) was used to calculate peak areas. Accumulation ratios of the TPP cations between the mitochondrial matrix and the extra-mitochondrial environment were calculated by normalizing the ratios of the TPP cation peak areas to their corresponding volumes: 0.6 μL/mg protein for the mitochondrial matrix (Brand 1995) and 4 mL for the extra-mitochondrial environment.

Partition coefficients

The 1-octanol/phosphate buffered saline (PBS) partition coefficients of the TPP compounds were measured as described previously (Kelso et al. 2001). In brief, TPP cation (400 nmol) was added to 2 mL PBS-saturated 1-octanol and mixed for 60 min at 37 °C with 2 mL 1-octanol saturated PBS in a sealed 10 mL Kimax® glass tube. After phase separation by centrifugation (5 min, 870×g at RT) the concentrations in the two phases were measured at 268 nm relative to standard curves in 1-octanol saturated PBS or PBS saturated 1-octanol. For pH dependent partition coefficients, PBS was adjusted with HCl or KOH prior to 1-octanol saturation.

Results

Mitochondrial uptake of a TPP conjugated carboxylic acid or amine

We first assessed if the TPP conjugated carboxylic acid ($TPP^+C_{10}CO_2H$) and amine ($TPP^+C_{10}NH_2$) were taken up by isolated mitochondria in response to the generation of a protonmotive force (Δp), using a TPP-selective electrode (Fig. 3). When mitochondria incubated at the intracellular pH of 7.2 were energized with succinate this led to the significant uptake of the simple TPP cation TPMP, and also of $TPP^+C_{10}CO_2H$ and $TPP^+C_{10}NH_2$. All three were released from mitochondria upon abolishing the Δp by uncoupling with FCCP. The accumulation ratios (ACRs) were ~1,000–1,500 and ~3,000–4,000, for the $TPP^+C_{10}NH_2$ and $TPP^+C_{10}CO_2H$ cations, respectively (Fig. 3). To see if the ΔpH component of Δp contributed to uptake, we abolished the ΔpH by addition of the K^+/H^+ exchanger nigericin (Fig. 3). This led to an increase of $\Delta\psi_m$ as the magnitude of the Δp is retained but is now only in the form of $\Delta\psi_m$, as is demonstrated by the increased accumulation of TPMP. Nigericin significantly increased the accumulation

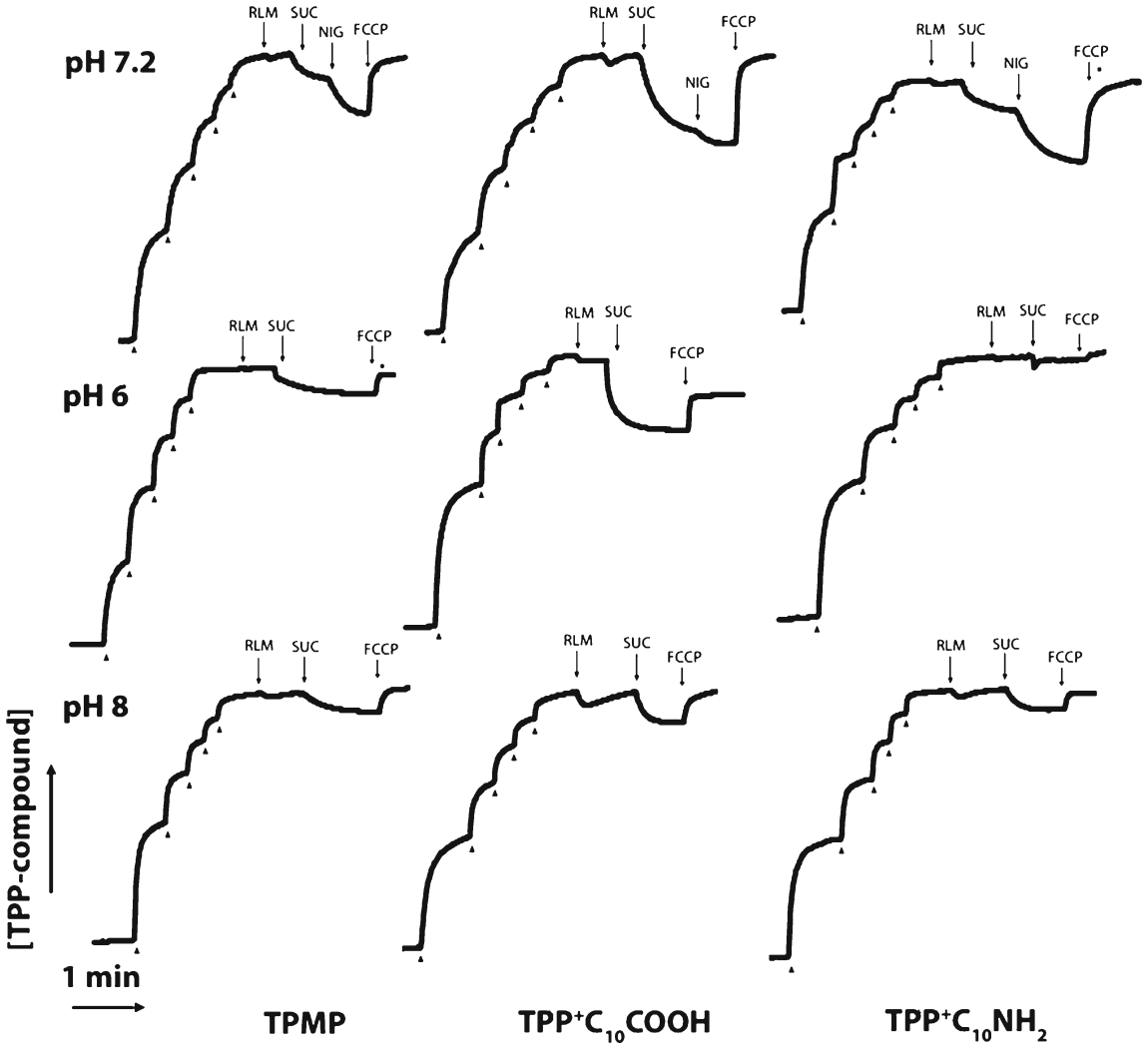

Fig. 3 Mitochondrial accumulation of $TPP^+C_{10}CO_2H$, $TPP^+C_{10}NH_2$ and TPMP assessed by ion-selective electrode (ISE) measurements at different pH values. The ISE electrode response was calibrated by 5×1 μM additions of the investigated compound. Then RLM (0.5 mg protein/mL) were added followed by succinate (10 mM). Nigericin (100 nM) or FCCP (1 μM) were added where indicated. Data are typical traces that were repeated 3–4 times with the same result

of $TPP^+C_{10}NH_2$, but only slightly increased that of $TPP^+C_{10}CO_2H$. This suggests that ΔpH enhanced the uptake of $TPP^+C_{10}CO_2H$, but decreased that of $TPP^+C_{10}NH_2$, relative to the uptake driven by $\Delta\psi_m$ alone.

To assess further the influence of pH on the mitochondrial uptake of these TPP compounds, we incubated mitochondria in media at pH6 or 8 (Fig. 3). The uptake of TPMP was similar at all pH values, indicating that mitochondria were functional under these conditions. The rate of uptake of $TPP^+C_{10}CO_2H$ was increased at pH 6 but decreased at pH8. As the pK_a for a C_{10} carboxylic acid is ~4.9 (Kanicky and Shah 2003), the proportion of $TPP^+C_{10}CO_2H$ in the singly (positively) charged state will decrease as the pH increases and will be~10 %, ~ 1 % and~0.1 % at pH6, 7 and 8, respectively. For $TPP^+C_{10}NH_2$ at pH 6 there was hardly any uptake while the profile of uptake at pH7.2 was similar to that at pH8. The pK_a of decylamine is ~10.6 (Hoerr et al. 1943), so the free amine fractions ($TPP^+C_{10}NH_2$) at pH6, 7.2 and 8 are ~0.001 %, ~ 0.01 % and ~0.1 %, respectively. To assess the effect of pH on the hydrophobicity of $TPP^+C_{10}CO_2H$ and $TPP^+C_{10}NH_2$, we measured their 1-octanol:PBS partition coefficients over the same pH range (Table 1). This showed that pH had little

Table 1 pH dependent 1-Octanol/PBS partition-coefficients

Compound	pH6	pH7.2	pH8
TPMP	2.3±0.2	2.2±0.5	2.0±0.1
$TPP^+C_{10}COOH$	17.3±1.6	14.8±1.7	13.2±0.9
$TPP^+C_{10}NH_2$	2.1±0.7	3.5±0.2	3.1±0.2

Data are means ± SEM (n=3 to 5)

effect on the partition coefficient of TPMP and that $TPP^+C_{10}CO_2H$ is significantly more hydrophobic than $TPP^+C_{10}NH_2$ at all pH values. The hydrophobicity of $TPP^+C_{10}CO_2H$ increases when pH decreased, consistent with greater partition into 1-octanol of the protonated monocationic form over the zwitterion. This was strongly supported by measurements of its partition coefficient at pH=2, where it was 108±24 indicating that the monocation form is considerably more soluble in a hydrophobic phase than the zwitterion and is likely the predominant form that passes through the membrane. For $TPP^+C_{10}NH_2$ the negligible change of the partition coefficient with pH is presumably because at all pH values the majority (>99.9 %) is in the dicationic form. Together these findings suggest that the TPP conjugated carboxylic acid is taken up through the mitochondrial inner membrane in its monocationic, protonated form, $TPP^+C_{10}CO_2H$ and that $TPP^+C_{10}NH_2$ is taken up as the monocationic, free amine form with negligible uptake of the dication, consistent with the constrained mitochondrial uptake of dications compared to monocations (Ross et al. 2006).

To extend the semi-quantitative ion-selective electrode experiments and to quantify the effects of $\Delta\psi_m$, ΔpH and pH on the accumulation of the TPP-cations, we next assessed their uptake into energized mitochondria by RP-HPLC (Fig. 4). Mitochondria were incubated with both $TPP^+C_{10}CO_2H$ and $TPP^+C_{10}NH_2$ simultaneously, to avoid inter-incubation variations, and their accumulation ratios (ACRs) determined by measuring the amounts in the mitochondrial pellets and supernatants (Fig. 4a). This analysis showed that the ACR for $TPP^+C_{10}CO_2H$ was unaffected by nigericin, while that of $TPP^+C_{10}NH_2$ increased, consistent with the presence of a ΔpH enhancing the uptake of $TPP^+C_{10}CO_2H$ but decreasing that of $TPP^+C_{10}NH_2$. When the ACRs for the two cations were measured simultaneously over a range of pH values the uptake of $TPP^+C_{10}CO_2H$ decreased as the pH was raised, while that of $TPP^+C_{10}NH_2$ increased (Fig. 4b), consistent with the electrode measurements (Fig. 3). The minimal effect of nigericin on $TPP^+C_{10}CO_2H$ uptake may be due to two opposing effects: by decreasing ΔpH nigericin lowers the uptake of $TPP^+C_{10}CO_2H$ that is due to weak acid accumulation, while in parallel increasing the $\Delta\psi_m$ will enhance uptake due to the TPP^+ component. To assess this possibility, we measured the ACR for the uptake of $TPP^+C_{10}CO_2H$ and TPMP in the same mitochondrial incubations ± nigericin (Fig. 4c). We then normalised the uptake of $TPP^+C_{10}CO_2H$ to that of TPMP to distinguish between the effects of nigericin due to changes in the ΔpH from those due to altering $\Delta\psi_m$ (Fig. 4c). This analysis showed that the TPMP-normalised uptake of $TPP^+C_{10}CO_2H$ was decreased significantly by abolishing the ΔpH and confirms that the extensive mitochondrial uptake of $TPP^+C_{10}CO_2H$ is due in part to the presence of a ΔpH across the mitochondrial inner membrane.

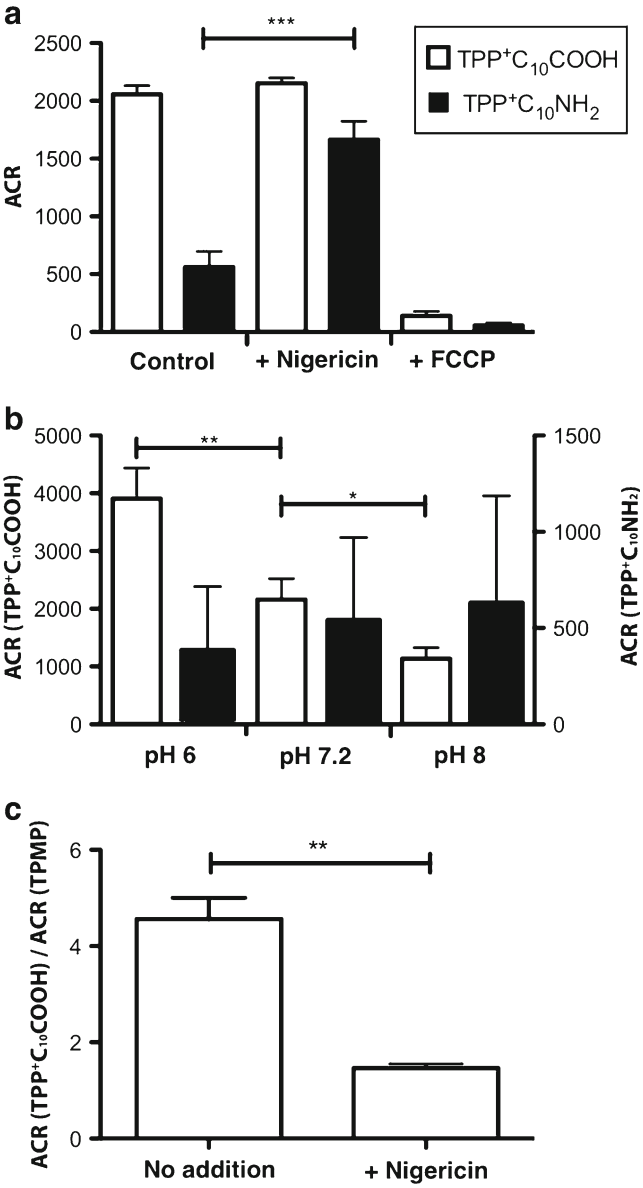

Fig. 4 Mitochondrial accumulation of $TPP^+C_{10}CO_2H$, $TPP^+C_{10}NH_2$ and TPMP assessed by RP-HPLC. **a** The accumulation ratios (ACRs) of $TPP^+C_{10}CO_2H$ and $TPP^+C_{10}NH_2$ were measured simultaneously in mitochondrial incubations ± nigericin at pH 7.2. **b** ACR of $TPP^+C_{10}CO_2H$ and $TPP^+C_{10}NH_2$ measured simultaneously in mitochondrial incubations at different pH values. **c** The ACRs of $TPP^+C_{10}CO_2H$ and TPMP were measured simultaneously in mitochondrial incubations ± nigercin and the ACR of $TPP^+C_{10}CO_2H$ was normalised to that of TPMP. Data are means ± SEM of three independent incubations. Statistical significance was determined by two-tailed Student's t-tests (*P<0.01, **P<0.001, ***P<0.0001)

Model for the mitochondrial uptake of TPP conjugated carboxylic acids and amines

Together these findings are consistent with a simple model for the uptake of TPP^+-conjugated carboxylic acids and amines by mitochondria (Fig. 5). In this only the monocationic ($TPP^+C_{10}CO_2H$ or $TPP^+C_{10}NH_2$) forms of the molecules cross

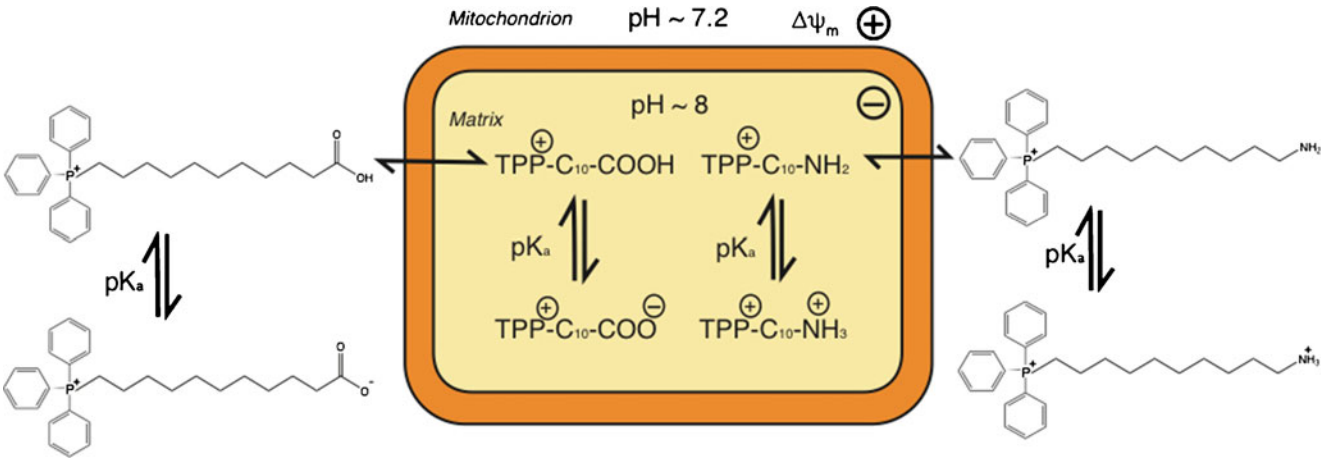

Fig. 5 Model for the uptake by energised mitochondria of a lipophilic cation conjugated to a carboxylic acid or amine function. A TPP conjugated to an acidic function generates a membrane-impermeant zwitterion and a membrane permeant singly charged species which is accumulated in response to $\Delta\psi_m$. Within the mitochondrial matrix the ΔpH leads to greater net accumulation of the lipophilic cation. For a TPP conjugated to a basic function, a membrane-impermeant dication is generated along with a membrane permeant species that is accumulated in response to $\Delta\psi_m$. Within the mitochondrial matrix the ΔpH leads to the decreased formation of the membrane-impermeant dication and hence to a decreased overall accumulation of the lipophilic cation

the mitochondrial inner membrane and thereby equilibrate with the $\Delta\psi_m$, as described by the Nernst equation. The monocationic forms then equilibrate with the local pH in the cytosol and matrix to form a dication ($TPP^+C_{10}NH_3^+$) or a zwitterion ($TPP^+C_{10}CO_2^-$), neither of which is membrane permeant. Consequently, for $TPP^+C_{10}CO_2H$ a conventional mitochondrial ΔpH will lead to a greater uptake of the compound into the matrix compared to a simple TPP cation at that $\Delta\psi_m$. In contrast, for $TPP^+C_{10}NH_2$ the presence of a ΔpH will decrease the uptake relative to that of a simple TPP cation at that $\Delta\psi_m$.

Measurement of the overall ACR of these compounds does not distinguish between protonation states, therefore the mitochondrial uptake of lipophilic cations conjugated to amines or carboxylic acids will depend on both the $\Delta\psi_m$ and the ΔpH. Furthermore, as the interaction of the molecules with ΔpH depends on their protonation states, the pK_a of the carboxylic acid or amine will also contribute. Using this model we generated a simple equation to describe the experimental ACR for lipophilic cations conjugated to acids or bases as a function of $\Delta\psi_m$, ΔpH and pK_a (Eq. 1: see Supplementary Material for the derivation).

$$ACR = \left\{ \frac{1 + 10^{i(pH_m - pK_a)}}{1 + 10^{i(pH_{cyt} - pK_a)}} \right\} 10^{\left(\frac{F\Delta\psi_m}{2.3RT}\right)} \quad (1)$$

For a lipophilic cation-conjugated to an acid i is +1 and for a base i is −1. F = Faraday constant, R = universal gas constant, T = absolute temperature. Note that lipophilic cations adsorb extensively to the matrix-facing surface of the mitochondrial inner membrane, consequently the experimentally observed ACR will be greater than predicted by Eq. 1 and will be related to the predicted ACR by a binding correction (Brown and Brand 1985).

Two general cases illustrate the implications of this equation: a TPP^+ conjugated to an acidic function, where a membrane-impermeant zwitterion is generated at high pH, and a TPP^+ conjugated to a basic function, where a membrane-impermeant dication is generated at low pH. At plausible values for $\Delta\psi_m$ (170 mV) and ΔpH (0.8; pH matrix=8, pH cytosol=7.2) Eq. 1 generates the ACR for a TPP cation conjugated to an acidic function (Fig. 6a) or to a basic function (Fig. 6b) as a function of the pK_a. These ACR values are given as a percentage of that for a simple TPP cation lacking an exchangeable group. For a TPP cation conjugated to an acidic function the uptake into mitochondria increases compared to that of a simple TPP cation as the pK_a drops below ~8. This is due to the extra accumulation driven by the ΔpH, in addition to that by $\Delta\psi_m$ (Fig. 6a). In contrast, for a lipophilic cation conjugated to a basic function the uptake into mitochondria decreases relative to that of a simple TPP cation as the pK_a increases (Fig. 6b). This is due to the exclusion of weak bases from the mitochondrial matrix by the ΔpH acting counter to the accumulation driven by $\Delta\psi_m$. These predictions of Eq. 1 are consistent with the experimental measurements of the uptake of $TPP^+C_{10}CO_2H$ and $TPP^+C_{10}NH_2$ into mitochondria seen in Figs. 4 and 5.

From Eq. 1 the ACR for TPMP should be a linear function of that of $TPP^+C_{10}CO_2H$ or $TPP^+C_{10}NH_2$ with a slope given by the coefficient in Eq. 1, adjusted by a correction constant dependent on the extent of binding to the matrix face of the mitochondrial inner membrane of TPMP relative to $TPP^+C_{10}CO_2H$ or $TPP^+C_{10}NH_2$. To see if this was the case, we measured the ACRs of $TPP^+C_{10}CO_2H$ or $TPP^+C_{10}NH_2$ relative to those of TPMP over a range of $\Delta\psi_m$ values set by different amounts of the respiratory inhibitor malonate (Fig. 7). The measured ACRs of

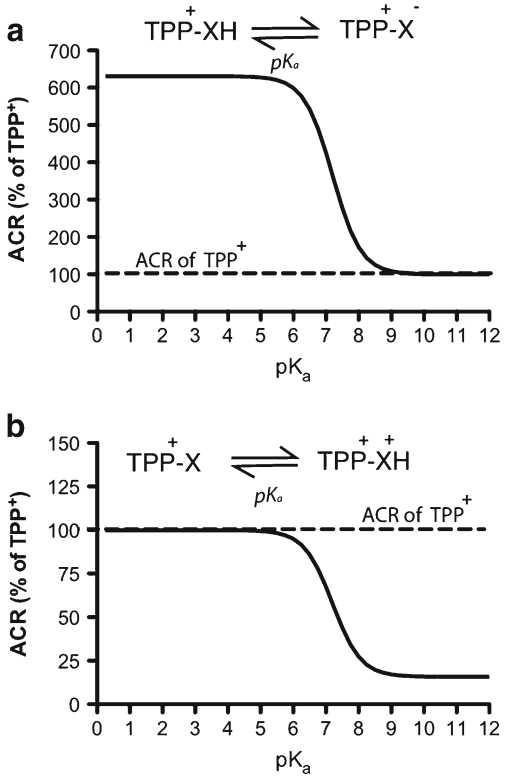

Fig. 6 Predicted uptake of a lipophilic cation conjugated to an acidic group or basic group as a function of pK_a. Equation 1 was used to calculate the ACR for a TPP^+ conjugated to an acidic function **a**, where a membrane-impermeant zwitterion is generated at high pH, and a TPP^+ conjugated to a basic function **b**, where a membrane-impermeant dication is generated at low pH. The mitochondrial $\Delta\psi_m$ was set at 170 mV, the ΔpH was set at 0.8, the mitochondrial matrix pH was set at 8 and that of the cytosol at 7.2. The calculated ACR is expressed as a percentage of that of a simple TPP cation that does not contain an ionisable group (*dashed line*)

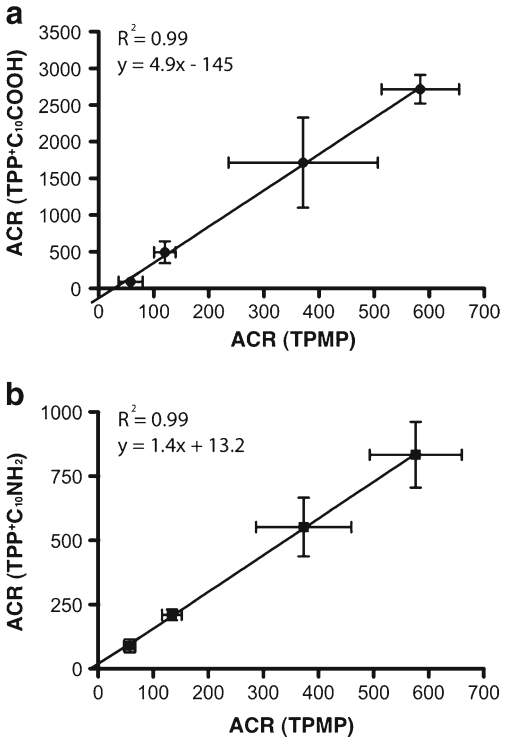

Fig. 7 Uptake of $TPP^+C_{10}CO_2H$ and $TPP^+C_{10}NH_2$ relative to TPMP over a range of $\Delta\psi_m$ values. Mitochondria were incubated with $TPP^+C_{10}CO_2H$ and TPMP **a** or with $TPP^+C_{10}NH_2$ and TPMP **b** and a range of membrane potentials was established by including different concentrations of malonate (0–2.3 mM) or FCCP (1 μM) in the incubations. The ACRs of $TPP^+C_{10}CO_2H$ **a** or $TPP^+C_{10}NH_2$ **b** were measured simultaneously with that of TPMP by RP-HPLC and are plotted against the ACR of TPMP. Data are means ± SEM of three independent incubations

$TPP^+C_{10}CO_2H$ (Fig. 7a) or $TPP^+C_{10}NH_2$ (Fig. 7b) were both linear functions of the measured TPMP ACR, consistent with Eq. 1. Furthermore, the slopes of the lines are consistent with the relative partition coefficients shown in Table 1.

Discussion

This study enables us to draw some important conclusions about the use of lipophilic cations to deliver therapeutic and probe compounds to mitochondria. The most important of these is that lipophilic cations containing weakly acidic or basic groups such as carboxylic acids or amines can be effectively targeted to mitochondria. This opens up many possibilities for the delivery of a wide range of further compounds to mitochondria as potential therapeutics, bioactives or probes. Interestingly, the incorporation of a weak acid significantly enhanced the extent of uptake of a lipophilic cation by energised mitochondria. This suggests that it may be possible to incorporate a weak acid function into lipophilic cations to further increase the extent of mitochondrial uptake. The combined response to a $\Delta\psi_m$ and to a ΔpH, and the fact that this can be quantified by Eq. 1, suggests that it may be possible to use a TPP cation conjugated to a weak acid to assess the mitochondrial ΔpH in vivo. This could be achieved by comparing the uptake of a compound such as $TPP^+C_{10}CO_2H$ with that of a closely related TPP cation lacking an exchangeable group. Their distribution in cell systems or in vivo could be measured by LC/MS/MS relative to deuterated internal standards, as we have done previously with MitoB (Cocheme et al. 2011) to quantify mitochondrial H_2O_2 production in vivo. Hence this work leads to new possibilities for assessing the mitochondrial ΔpH in vivo, something that is currently not possible. For lipophilic cations conjugated to weak bases the situation is somewhat different, with the presence of a weak base decreasing net uptake. Thus while mitochondria-targeted lipophilic cations containing weakly basic moieties can be directed to mitochondria, when possible they should be excluded to enhance uptake.

We have generated a simple model and equation for the uptake of lipophilic cations conjugated to weak acids and bases, the derivation of which is shown in the Supplementary Material. These gave a reasonable description of the behaviour

of the cations assessed here. However, the validity of this description depends on only the monocationic forms of the molecules being membrane permeant. Equation 1 would not hold for a more lipophilic dicationic form that was membrane permeant, or where the conjugate base of the weak acid was a delocalised anion that facilitated membrane permeation. Even so, Eq. 1 and Figs. 5 and 6 give a useful simplified description of the mitochondrial uptake of lipophilic cations conjugated to weak acids and bases under most biological conditions.

In summary, we have shown that lipophilic cations incorporating weak acids or bases can be targeted to mitochondria. By providing a foundation for the design of a greatly expanded range of compounds that can be taken up by mitochondria, this work opens the way for the rational design of many more mitochondria-targeted drugs and probes.

Acknowledgments We thank Marina Roxburgh, Tracy A. Prime and Angela Logan for technical assistance and Judy Hirst for helpful discussions. This work was supported by the Medical Research Council (UK), and the Foundation for Research, Science and Technology (NZ).

References

Asin-Cayuela J, Manas AR, James AM, Smith RA, Murphy MP (2004) Fine-tuning the hydrophobicity of a mitochondria-targeted antioxidant. FEBS Lett 571(1–3):9–16

Azzone GF, Pietrobon D, Zoratti M (1984) Determination of the proton electrochemical gradient across biological membranes. Curr Top Bioenerg 13:1–77

Brand MD (1995) Measurement of mitochondrial protonmotive force. In: Brown GC, Cooper CE (eds) Bioenergetics - a practical approach. IRL, Oxford, pp 39–62

Brown GC, Brand MD (1985) Thermodynamic control of electron flux though mitochondria cytochrome bc_1 complex. Biochem J 225(2):399–405

Chappell JB, Hansford RG (1972) Preparation of mitochondria from animal tissues and yeasts. In: Birnie GD (ed) Subcellular components: preparation and fractionation. Butterworths, London, pp 77–91

Cocheme HM, Quin C, McQuaker SJ, Cabreiro F, Logan A, Prime TA, Abakumova I, Patel JV, Fearnley IM, James AM et al (2011) Measurement of H_2O_2 within living Drosophila during aging using a ratiometric mass spectrometry probe targeted to the mitochondrial matrix. Cell Metab 13(3):340–350

Dhanasekaran A, Kotamraju S, Karunakaran C, Kalivendi SV, Thomas S, Joseph J, Kalyanaraman B (2005) Mitochondria superoxide dismutase mimetic inhibits peroxide-induced oxidative damage and apoptosis: role of mitochondrial superoxide. Free Radic Biol Med 39(5):567–583

Dickinson BC, Chang CJ (2008) A targetable fluorescent probe for imaging hydrogen peroxide in the mitochondria of living cells. J Am Chem Soc 130(30):9638–9639

Duchen MR, Szabadkai G (2010) Roles of mitochondria in human disease. Essays Biochem 47:115–137

Gane EJ, Weilert F, Orr DW, Keogh GF, Gibson M, Lockhart MM, Frampton CM, Taylor KM, Smith RA, Murphy MP (2010) The mitochondria-targeted anti-oxidant mitoquinone decreases liver damage in a phase II study of hepatitis C patients. Liver Int 30 (7):1019–1026

Gornall AG, Bardawill CJ, David MM (1949) Determination of serum protein by means of the biuret reaction. J Biol Chem 177(2):751–766

Hoerr CW, McCorkle MR, Ralston AW (1943) Studies on the high molecular weoight aliphatic amines and their salts. X. Ioinzation constants of primary and symmetrical secondary amines in aqueous solution. J Am Chem Soc 65(3):328–329

Kanicky JR, Shah DO (2003) Effect of premicellar aggregation on the pK_a of fatty acid soap solutions. Langmuir 19:2034–2038

Kelso GF, Porteous CM, Coulter CV, Hughes G, Porteous WK, Ledgerwood EC, Smith RA, Murphy MP (2001) Selective targeting of a redox-active ubiquinone to mitochondria within cells: antioxidant and antiapoptotic properties. J Biol Chem 276(7):4588–4596

Klayman DL, Grenan MM, Jacobus DP (1969) Potential antiradiation agents. I. Primary aminoalkanethiosulfuric acids. J Med Chem 12 (3):510–512

Lopez M, Hardy MJ, Kalyanaraman B, Zhao M (2009) 99mTc-labeled triphenylphosphonium derivative contrasting agents and molecular probes for early detection and imaging of breast tumors (USA). Cancer Biother Radiopharm 24(5):579–587

Murphy MP (2009) Mitochondria–a neglected drug target. Curr Opin Investig Drugs 10(10):1022–1024

Murphy MP, Smith RA (2007) Targeting antioxidants to mitochondria by conjugation to lipophilic cations. Annu Rev Pharmacol Toxicol 47:629–656

Porteous CM, Logan A, Evans C, Ledgerwood EC, Menon DK, Aigbirhio F, Smith RA, Murphy MP (2010) Rapid uptake of lipophilic triphenylphosphonium cations by mitochondria in vivo following intravenous injection: implications for mitochondria-specific therapies and probes. Biochim Biophys Acta 1800(9):1009–1017

Prime TA, Blaikie FH, Evans C, Nadtochiy SM, James AM, Dahm CC, Vitturi DA, Patel RP, Hiley CR, Abakumova I et al (2009) A mitochondria-targeted S-nitrosothiol modulates respiration, nitrosates thiols, and protects against ischemia-reperfusion injury. Proc Natl Acad Sci USA 106(26):10764–10769

Robinson KM, Janes MS, Pehar M, Monette JS, Ross MF, Hagen TM, Murphy MP, Beckman JS (2006) Selective fluorescent imaging of superoxide in vivo using ethidium-based probes. Proc Natl Acad Sci USA 103(41):15038–15043

Ross MF, Kelso GF, Blaikie FH, James AM, Cocheme HM, Filipovska A, Da Ros T, Hurd TR, Smith RA, Murphy MP (2005) Lipophilic triphenylphosphonium cations as tools in mitochondrial bioenergetics and free radical biology. Biochemistry (Mosc) 70(2):222–230

Ross MF, Da Ros T, Blaikie FH, Prime TA, Porteous CM, Severina II, Skulachev VP, Kjaergaard HG, Smith RA, Murphy MP (2006) Accumulation of lipophilic dications by mitochondria and cells. Biochem J 400(1):199–208

Smith RA, Murphy MP (2010) Animal and human studies with the mitochondria-targeted antioxidant MitoQ. Ann N Y Acad Sci 1201:96–103

Smith RA, Hartley RC, Murphy MP (2011) Mitochondria-targeted small molecule therapeutics and probes. Antioxid Redox Signal 15(12):3021–3038

Snow BJ, Rolfe FL, Lockhart MM, Frampton CM, O'Sullivan JD, Fung V, Smith RA, Murphy MP, Taylor KM (2010) A double-blind, placebo-controlled study to assess the mitochondria-targeted antioxidant MitoQ as a disease-modifying therapy in Parkinson's disease. Mov Disord 25(11):1670–1674

Wallace DC, Fan W, Procaccio V (2010) Mitochondrial energetics and therapeutics. Annu Rev Pathol 5:297–348

Wube AA, Hufner A, Thomaschitz C, Blunder M, Kollroser M, Bauer R, Bucar F (2011) Design, synthesis and antimycobacterial activities of 1-methyl-2-alkenyl-4(1H)-quinolones. Bioorg Med Chem 19(1):567–579