

Mitochondria-targeted antioxidants protect Friedreich Ataxia fibroblasts from endogenous oxidative stress more effectively than untargeted antioxidants

Matthias L. Jauslin,* Thomas Meier,* Robin A. J. Smith,[†] and Michael P. Murphy[‡]

*MyoContract Ltd., CH-4410 Liestal, Switzerland; [†]Chemistry Department, University of Otago, Dunedin, New Zealand; and [‡]MRC-Dunn Human Nutrition Unit, Cambridge CB2 2XY, United Kingdom

Corresponding author: Michael P. Murphy, MRC-Dunn Human Nutrition Unit, Wellcome Trust/MRC Building, Hills Road, Cambridge CB2 2XY, United Kingdom.
E-mail: mpm@mrc-dunn.cam.ac.uk

ABSTRACT

Friedreich Ataxia (FRDA), the most common inherited ataxia, arises from defective expression of the mitochondrial protein frataxin, which leads to increased mitochondrial oxidative damage. Therefore, antioxidants targeted to mitochondria should be particularly effective at slowing disease progression. To test this hypothesis, we compared the efficacy of mitochondria-targeted and untargeted antioxidants derived from coenzyme Q₁₀ and from vitamin E at preventing cell death due to endogenous oxidative stress in cultured fibroblasts from FRDA patients in which glutathione synthesis was blocked. The mitochondria-targeted antioxidant MitoQ was several hundredfold more potent than the untargeted analog idebenone. The mitochondria-targeted antioxidant MitoVit E was 350-fold more potent than the water soluble analog Trolox. This is the first demonstration that mitochondria-targeted antioxidants prevent cell death that arises in response to endogenous oxidative damage. Targeted antioxidants may have therapeutic potential in FRDA and in other disorders involving mitochondrial oxidative damage.

Key words: idebenone • mitochondrial oxidative stress • MitoQ • frataxin

Friedreich Ataxia (FRDA) is the most common recessively inherited ataxia, with onset in early adulthood leading to the progressive loss of neuromuscular function that usually culminates with death in the fourth or fifth decade (1, 2). FRDA is caused by an intronic GAA triplet expansion in the gene for the mitochondrial protein frataxin that significantly decreases the amount of frataxin (1). This causes mitochondrial dysfunction in FRDA patients, increasing oxidative stress, decreasing the activity of iron-sulfur cluster containing enzymes, and causing the accumulation of iron within mitochondria (2, 3). Deletion of the yeast frataxin homologue and tissue-specific deletions of frataxin in mice also lead to defects in iron-sulfur proteins and increased mitochondrial oxidative stress and iron accumulation (4–7). Although the function of frataxin is uncertain, it is clear that its impaired expression in FRDA patients increases mitochondrial oxidative stress, which is a major cause of the pathophysiology of FRDA (4, 8).

Antioxidant therapies that decrease mitochondrial oxidative damage should help to delay the onset or to slow the progression of FRDA (8). Supporting this, idebenone, a short-chain analog of coenzyme Q₁₀, decreases oxidative damage and improves disease-related parameters in FRDA patients (9, 10). However, a limit to the effectiveness of these antioxidants is that they distribute through the extracellular and intracellular compartments, with only a small proportion accumulating in mitochondria, the principal site of damage in FRDA. Thus, targeting these molecules to mitochondria may improve their effectiveness. Consequently, we developed mitochondria-targeted antioxidants by covalently coupling antioxidants to the triphenylphosphonium cation (11). These lipophilic cations easily permeate lipid bilayers by a noncarrier-mediated process and accumulate several hundredfold within mitochondria due to the large membrane potential (150–170 mV, negative inside; [Fig. 1](#); refs 11, 12). Furthermore, these molecules are also accumulated by cells driven by the plasma membrane potential (30–60 mV, negative inside; [Fig. 1](#)). The accumulation into the heart and central nervous system after feeding to mice (13–15) indicates that this procedure is an efficient way of increasing the antioxidant content of mitochondria in those tissues most affected by oxidative damage in FRDA.

Two of the targeted antioxidants developed to date are derivatives of ubiquinone (MitoQ) and of vitamin E (MitoVit E). They have been shown to be far more effective at protecting isolated mitochondria and mitochondria within cells from exogenous pro-oxidants than their untargeted equivalents (16, 17). The efficacy of MitoQ is of particular interest because its antioxidant moiety is the same as idebenone and coenzyme Q₁₀, which have shown some efficacy in FRDA patients (9, 10). Here we set out to test whether mitochondria-targeted antioxidants were more effective than their untargeted equivalents in a cell model of FRDA. For this we chose fibroblasts that had been isolated from FRDA patients and then exposed to the glutathione synthesis inhibitor L-buthionine-(S,R)-sulfoximine (BSO; ref 18). Previously it had been shown that although BSO treatment leads to a 70% decrease in the glutathione (GSH) content of both FRDA and control cells, only the FRDA fibroblasts die (18). This differential susceptibility to GSH depletion is due to elevated endogenous oxidative stress in the FRDA cells, so the cause of cell death is similar to that within FRDA patients. Most interestingly, this oxidative damage-induced death of FRDA fibroblasts was blocked by exogenous antioxidants such as idebenone (18). Therefore, this is a good system to compare the potency of mitochondria-targeted and untargeted antioxidants at preventing endogenous oxidative damage. Here we show that the mitochondria-targeted compounds tested are indeed several hundredfold more potent at preventing cell death than untargeted analogs.

MATERIAL AND METHODS

Cell culture

Human fibroblasts from FRDA patients were obtained from the Coriell Cell Repositories (Camden, NJ; catalog number GM04078). FRDA was confirmed at the molecular level by measurement of the intronic GAA triplet repeat length using a PCR-based method (1), which revealed 400–450 repeats. For both propagation and experiments, cells were grown in 25% (v/v) M199 with Earle's Balanced Salts (EBS) and 64% (v/v) Minimal Essential Medium-EBS without phenol red (Bioconcept, Allschwil, Switzerland) supplemented with 10% (v/v) fetal calf serum (PAA Laboratories, Linz, Austria), 100 U/ml penicillin, 100 µg/ml streptomycin (PAA Laboratories), 10 µg/ml insulin (Sigma, Buchs, Switzerland), 10 ng/ml epidermal growth factor

(Sigma), 10 ng/ml basic fibroblast growth factor (PreproTech, Rocky Hill, NJ), and 2 mM glutamine (Sigma).

Chemicals

BSO, carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP), methyltriphenylphosphonium (TPMP) bromide, Trolox, vitamin E (α -tocopherol, added as the acetate ester), and decylubiquinone were from Sigma. MitoVit E and MitoQ were synthesized as described previously (16, 17). Idebenone was from Chemo Iberica.

Experimental incubations

For experiments, cells were plated in 96-well microtiter plates at a density of \sim 3000 cells/well and allowed to attach overnight. Compounds and FCCP were dissolved in DMSO (both 5 mM) and stored at -20°C ; BSO was dissolved in cell culture medium (10 mM) and stored at 4°C . Immediately before the experiment, the compounds were diluted in fresh cell culture medium at a final concentration of 150 μM . This 3x stock solution was used to create a concentration gradient from 0.1 pM up to 50 μM . When used, the uncoupler FCCP was added 30 min before the antioxidants at a final concentration of 1 μM and was maintained for the duration of the incubation. Twenty-four hours later this culture medium was supplemented with 1 mM BSO. Cell viability was assessed 48 h after addition of BSO. For this the medium was aspirated and the plates were lightly tapped on a paper towel to remove residual medium. Cells were then stained for 60 min at room temperature in the dark with 1.2 μM calcein-AM (Molecular Probes, Eugene, OR) in phosphate-buffered saline. This probe is converted to the green fluorescent product calcein by cellular esterases within live cells but is unaffected by dead cells (19). Fluorescence intensity was measured with a Gemini Spectramax XS spectrofluorimeter (Molecular Devices, Sunnyvale, CA) using excitation and emission wavelengths of 485 nm and 525 nm, respectively. The fluorescence of the non-BSO treated cells on each plate was set as reference of 100% cell viability, and the viabilities of treated cells were calculated relative to this value. All compounds were tested in at least three independent experiments and for each experiment results for all concentrations assayed are the means of quadruplicate determinations. Dose-response curves from at least three independent experiments were used to calculate EC_{50} concentrations. All data are expressed as means \pm SD. Student's unpaired t test was used to determine significance, with $P < 0.05$ taken as significant.

RESULTS

Fibroblasts from Friedreich Ataxia patients, but not control cells, die after GSH depletion on incubation with BSO (18). This cell death is due to the increased endogenous mitochondrial oxidative stress in FRDA cells and can be blocked by antioxidants (18). We hypothesized that mitochondria-targeted antioxidants should be more effective at preventing this cell death than untargeted antioxidants. To test this hypothesis, we compared the ability to prevent cell death of MitoQ ([Fig. 2A](#)), a targeted antioxidant derived from coenzyme Q, with the untargeted coenzyme Q derivatives idebenone and decylubiquinone ([Fig. 2A](#)). Although both idebenone and decylubiquinone did protect FRDA cells, MitoQ was effective at far lower concentrations ([Fig. 2B](#)). In [Fig. 2C](#), the efficacy of the antioxidants at preventing cell death is expressed as the concentration required to rescue 50% of the FRDA cells (EC_{50}). These data show that MitoQ (EC_{50} : 0.51 ± 0.50 nM) is \sim 800-fold more effective than idebenone (EC_{50} : 426 ± 102 nM) and 50-

fold more effective than decylubiquinone (EC_{50} : 26.5 ± 19 nM). The lower concentration at which MitoQ afforded protection is likely to be due to its several hundredfold accumulation into mitochondria driven by the membrane potential. To see if this was the case, we abolished the mitochondrial membrane potential with the uncoupler FCCP, thereby preventing the selective uptake of MitoQ into mitochondria. The concentration of FCCP used ($1 \mu\text{M}$) was the highest level tolerated by the FRDA cells in the absence of BSO (data not shown) and is known to abolish the mitochondrial membrane potential in fibroblasts (20). [Figure 2B](#) shows that FCCP decreased the potency of MitoQ ~25-fold, making its antioxidant potency similar to the untargeted antioxidant decylubiquinone. FCCP did not affect the potency of idebenone or decylubiquinone, as is illustrated in [Fig. 2C](#) where the EC_{50} concentrations for prevention of cell death in the absence (filled bars) and presence (open bars) of FCCP are compared. In the presence of FCCP, the potency of MitoQ and the nontargeted decylubiquinone was indistinguishable ($P=0.192$). Together, the data in [Fig. 2](#) indicate that MitoQ is a far more effective antioxidant than idebenone or decylubiquinone and that this increased efficacy is due to its membrane potential-dependent accumulation by mitochondria within fibroblasts.

We next considered whether MitoVit E, a targeted version of vitamin E (α -tocopherol), was more effective than untargeted analogs. To do this we compared MitoVit E with the water-soluble vitamin E derivative Trolox, which lacks the hydrophobic side chain of vitamin E, and also with native vitamin E ([Fig. 3A](#)). MitoVit E did prevent cell death in FRDA cells (EC_{50} : 23.6 ± 14.0 nM), and it was ~350-fold more potent than Trolox (EC_{50} : $8.2 \pm 6.3 \mu\text{M}$) and ~20-fold more potent than vitamin E (EC_{50} : 416 ± 76 nM; [Fig. 3B](#)). However, the EC_{50} of MitoVit E did not increase on dissipating the mitochondrial membrane potential with FCCP ([Fig. 3B](#)).

DISCUSSION

We have shown that the mitochondria-targeted antioxidant MitoQ is effective at preventing cell death in FRDA patients' fibroblasts on treatment with BSO, an inhibitor of GSH biosynthesis. In this model, GSH depletion leads to the death of cells from FRDA patients, but not controls, consistent with the pathology of FRDA being due to increased endogenous mitochondrial oxidative stress. The prevention of this cell death by antioxidants confirms their potential in treating FRDA, in particular because these results indicate that the antioxidants used block endogenously produced, disease-specific oxidative stress. We were particularly interested in whether antioxidants targeted to mitochondria were more effective at preventing cell death than those that distributed throughout the cell. This is the case for MitoQ, which was ~800-fold more effective than the untargeted antioxidant idebenone. That this greater potency was due to the accumulation into the mitochondria driven by the membrane potential is strongly suggested by our finding that the potency of MitoQ was decreased ~25-fold when the mitochondrial membrane potential was abolished and under these conditions the efficacy of MitoQ was indistinguishable from decylubiquinone. These data indicate that mitochondria-targeted antioxidants may be therapeutically more effective than untargeted antioxidants, particularly as they are rapidly taken up by the brain and heart (13, 15). This finding of greater efficacy of mitochondria-targeted antioxidants also confirms that the oxidative stress in FRDA arises primarily from mitochondria.

This is the first demonstration that mitochondria-targeted antioxidants are able to reduce cell death arising from endogenous oxidative stress. FRDA is in many ways a paradigm for degenerative diseases that involve mitochondrial oxidative stress, such as Parkinson disease and

Huntington disease (21). Therefore, the finding of efficacy in a FRDA cell model involving endogenous oxidative stress suggests that it may be worthwhile extending this approach to other diseases associated with mitochondrial oxidative damage. However, there are still uncertainties to be resolved. The targeted version of vitamin E was 350-fold more potent than Trolox and 20-fold more potent than vitamin E; however, its potency was unaffected by dissipating the mitochondrial membrane potential, in contrast to the dramatic effect of uncoupling on the potency of MitoQ. The reasons for this are not known, but factors that may contribute include the greater hydrophobicity of MitoQ relative to MitoVit E (octanol/phosphate-buffered-saline partition coefficients of 160 and 7.4, respectively) and the ability of the mitochondrial respiratory chain to recycle MitoQ directly but not MitoVit E. These differences may also suggest why MitoQ is ~50-fold more potent an antioxidant than MitoVit E in this system. It is likely that exploration of these findings will lead to the development of further targeted antioxidants and to a deeper understanding of mitochondrial oxidant production and oxidative damage in general.

In conclusion, we have shown that targeting a coenzyme Q derivative to mitochondria improves protection against cell death caused by endogenous mitochondrial oxidative stress. This increased protection is due to the accumulation of the antioxidant within mitochondria. This is the first demonstration of specific protection against endogenous mitochondrial oxidative damage in cells. Furthermore, when these molecules are administered orally to mice they accumulate in the mitochondria of the heart and brain (15). Together with the finding reported here, this suggests that mitochondria-targeted antioxidants may have potential as therapies for FRDA and for other diseases associated with mitochondrial oxidative damage.

ACKNOWLEDGMENTS

We thank Dr. J. Magyar, Meredith F. Ross, and Jordi Asin-Cayuela for helpful comments on this manuscript. This work was supported by Grant No. QLRT-CT-1999-00584 from the 5th framework program of the European Commission and by the Swiss Network for Research on Friedreich's Ataxia to Thomas Meier. Michael P. Murphy is grateful to the Friedreich's Ataxia Research Alliance for support.

REFERENCES

1. Campuzano, V., Montermini, L., Molto, M. D., Pianese, L., Cosee, M., Cavalcanti, F., Monros, E., Rodius, F., Duclos, F., Monticelli, A., et al. (1996) Friedreich's ataxia: autosomal recessive disease caused by an intronic GAA triplet repeat expansion. *Science* **271**, 1423–1427
2. Kaplan, J. (1999) Friedreich's ataxia is a mitochondrial disorder. *Proc. Natl. Acad. Sci. USA* **96**, 10948–10949
3. Lodi, R., Cooper, J. M., Bradley, J. L., Manners, D., Styles, P., Taylor, D. J., and Schapira, A. H. (1999) Deficit of in vivo mitochondrial ATP production in patients with Friedreich ataxia. *Proc. Natl. Acad. Sci. USA* **96**, 11492–11495
4. Puccio, H., and Koenig, M. (2000) Recent advances in the molecular pathogenesis of Friedreich ataxia. *Hum. Mol. Genet.* **9**, 887–892

5. Puccio, H., Simon, D., Cossee, M., Criqui-Filipe, P., Tiziano, F., Melki, J., Hindelang, C., Matyas, R., Rustin, P., and Koenig, M. (2001) Mouse models for Friedreich ataxia exhibit cardiomyopathy, sensory nerve defect and Fe-S enzyme deficiency followed by intramitochondrial iron deposits. *Nat. Genet.* **27**, 181–186
6. Babcock, M., de Silva, D., Oaks, R., Davis-Kaplan, S., Jiralerspong, S., Montermini, L., Pandolfo, M., and Kaplan, J. (1997) Regulation of mitochondrial iron accumulation by Yfh1p, a putative homolog of frataxin. *Science* **276**, 1709–1712
7. Wilson, R. B., and Roof, D. M. (1997) Respiratory deficiency due to loss of mitochondrial DNA in yeast lacking the frataxin homologue. *Nat. Genet.* **16**, 352–357
8. Schulz, J. B., Dehmer, T., Schols, L., Mende, H., Hardt, C., Vorgerd, M., Burk, K., Matson, W., Dichgans, J., Beal, M. F., et al. (2000) Oxidative stress in patients with Friedreich ataxia. *Neurology* **55**, 1719–1721
9. Hausse, A. O., Aggoun, Y., Bonnet, D., Sidi, D., Munnich, A., Rotig, A., and Rustin, P. (2002) Idebenone and reduced cardiac hypertrophy in Friedreich's ataxia. *Heart* **87**, 346–349
10. Lodi, R., Hart, P. E., Rajagopalan, B., Taylor, D. J., Crilley, J. G., Bradley, J. L., Blamire, A. M., Manners, D., Styles, P., Schapira, A. H., et al. (2001) Antioxidant treatment improves in vivo cardiac and skeletal muscle bioenergetics in patients with Friedreich's ataxia. *Ann. Neurol.* **49**, 590–596
11. Murphy, M. P., and Smith, R. A. J. (2000) Drug delivery to mitochondria: the key to mitochondrial medicine. *Adv. Drug Deliv. Rev.* **41**, 235–250
12. Murphy, M. P. (1997) Targeting bioactive compounds to mitochondria. *Trends Biotechnol.* **15**, 326–330
13. Kelso, G. F., Porteous, C. M., Hughes, G., Ledgerwood, E. C., Gane, A. M., Smith, R. A., and Murphy, M. P. (2002) Prevention of mitochondrial oxidative damage using targeted antioxidants. *Ann. NY Acad. Sci.* **959**, 263–274
14. Murphy, M. P. (2001) Development of lipophilic cations as therapies for disorders due to mitochondrial dysfunction. *Expert Opin. Biol. Ther.* **1**, 753–764
15. Smith, R. A. J., Porteous, C. M., Gane, A. M., and Murphy, M. P. (2003) Delivery of bioactive molecules to mitochondria in vivo. *Proc. Natl. Acad. Sci. USA* **100**, 5407–5412
16. Smith, R. A. J., Porteous, C. M., Coulter, C. V., and Murphy, M. P. (1999) Targeting an antioxidant to mitochondria. *Eur. J. Biochem.* **263**, 709–716
17. Kelso, G. F., Porteous, C. M., Coulter, C. V., Hughes, G., Porteous, W. K., Ledgerwood, E. C., Smith, R. A. J., and Murphy, M. P. (2001) Selective targeting of a redox-active ubiquinone to mitochondria within cells. *J. Biol. Chem.* **276**, 4588–4596

18. Jauslin, M. L., Wirth, T., Meier, T., and Schoumacher, F. (2002) A cellular model for Friedreich Ataxia reveals small-molecule glutathione peroxidase mimetics as novel treatment strategy. *Hum. Mol. Genet.* **11**, 3055–3063
19. Levesque, A., Paquet, A., and Page, M. (1995) Measurement of tumor necrosis factor activity by flow cytometry. *Cytometry* **20**, 181–184
20. James, A. M., Wei, Y.-H., Pang, C.-Y., and Murphy, M. P. (1996) Altered mitochondrial function in fibroblasts containing MELAS or MERRF mitochondrial DNA mutations. *Biochem. J.* **318**, 401–407
21. Puccio, H., and Koenig, M. (2002) Friedreich ataxia: a paradigm for mitochondrial diseases. *Curr. Opin. Genet. Dev.* **12**, 272–277

Received April 4, 2003; accepted June 19, 2003.

Fig. 1

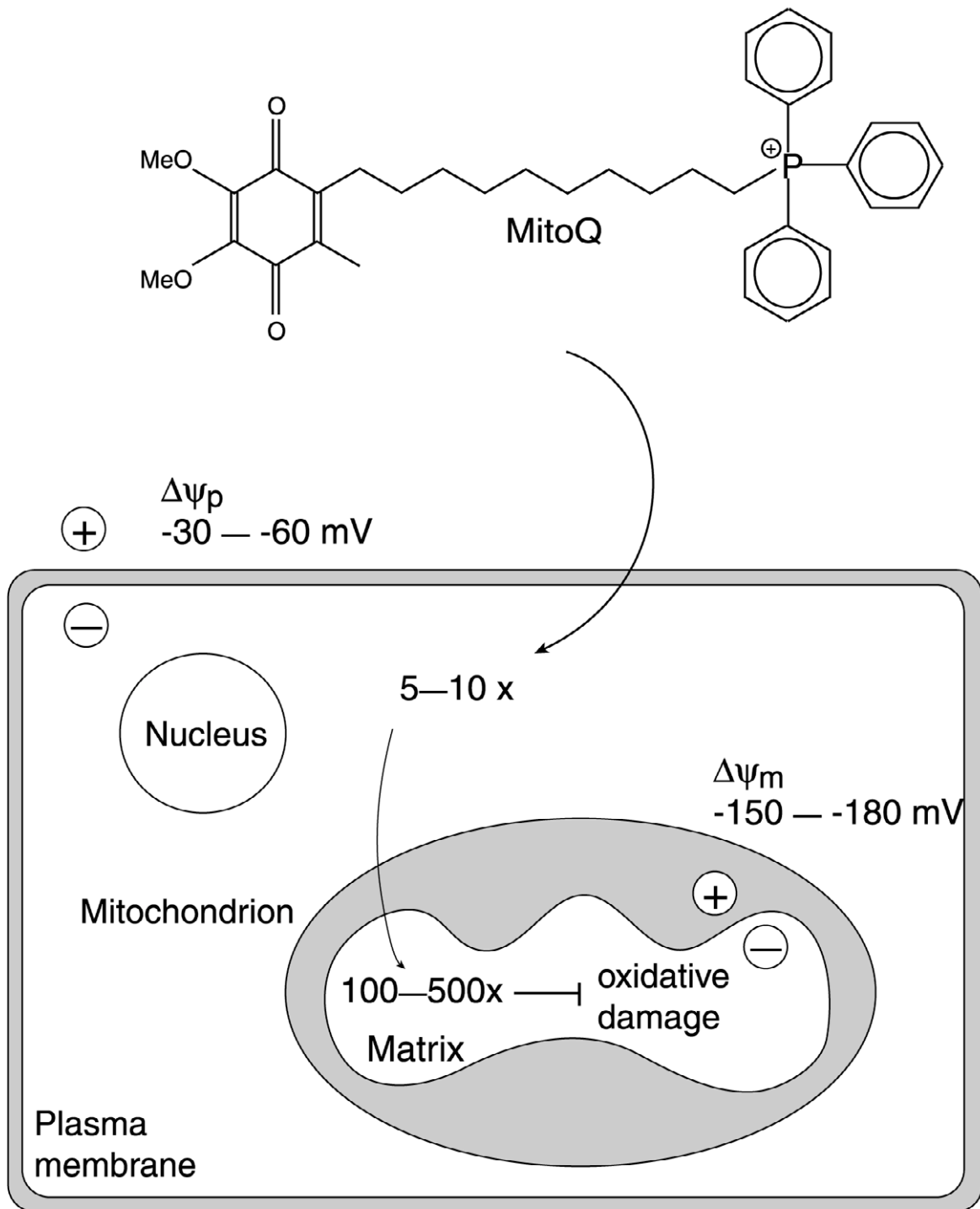


Figure 1. Schematic diagram illustrating the selective uptake of MitoQ into the cytoplasm driven by the plasma membrane potential ($\Delta\psi_p$) and its subsequent further accumulation by mitochondria driven by the mitochondrial membrane potential ($\Delta\psi_m$). Within mitochondria, this several-hundredfold accumulation of MitoQ relative to its concentration in the external fluid will protect the organelle from oxidative damage, such as that associated with Friedreich Ataxia, far more effectively than untargeted antioxidants.

Fig. 2

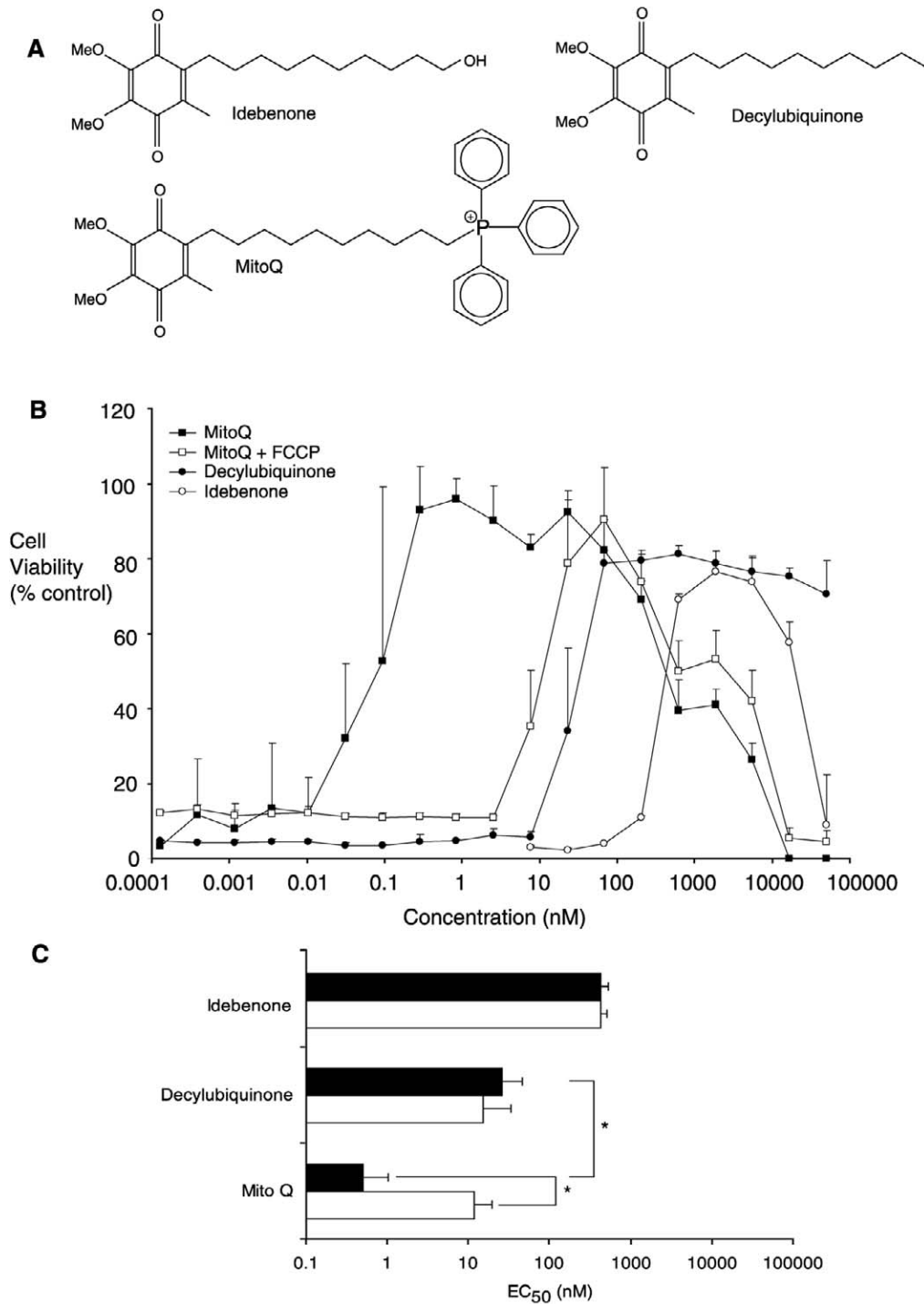


Figure 2. Prevention of cell death in FRDA fibroblasts by Coenzyme Q derivatives. **A)** Structures of the two untargeted coenzyme Q derivatives used, idebenone and decylubiquinone, and the structure of the mitochondria-targeted coenzyme Q derivative MitoQ. **B)** Dependence of protection against cell death on concentration of MitoQ (filled squares), idebenone (open circles), and decylubiquinone (filled circles). The effect of FCCP (1 μ M) in the presence of MitoQ is also shown (open squares). Data are shown from a typical experiment, and each point is means \pm SD of $n = 4$ values. Cell viability is shown as a percentage of that for incubations of FRDA patients' fibroblasts that were not treated with BSO. In control incubations, the simple lipophilic cation TPMP did not protect against cell death at concentrations up to at least 50 μ M (data not shown). **C)** Concentrations of antioxidants that prevent 50% of cell death (EC₅₀) in the absence (filled bars), or presence (open bars), of 1 μ M FCCP. Data are means \pm SD of at least 3 independent experiments (* $P < 0.01$).

Fig. 3

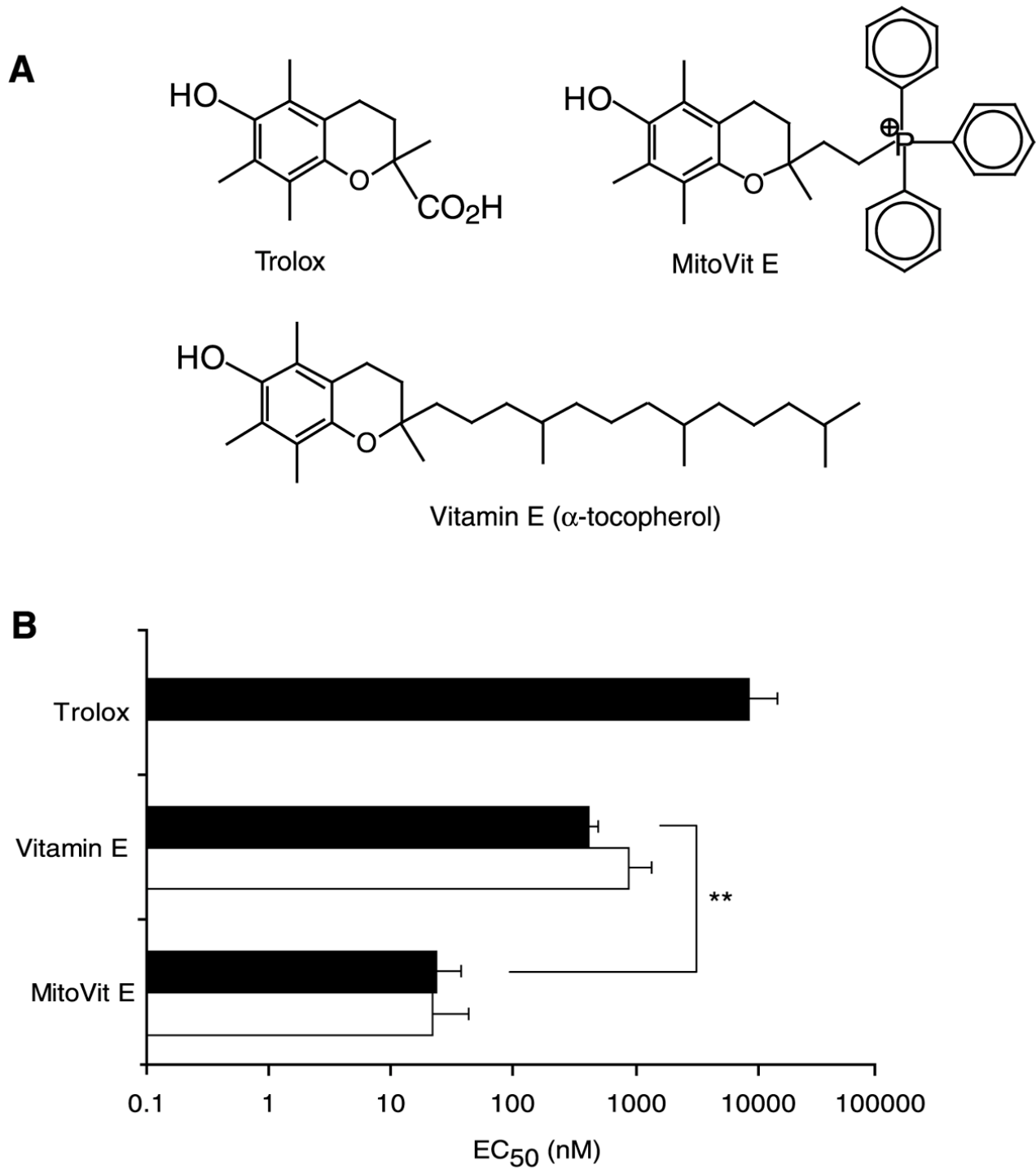


Figure 3. Prevention of cell death in FRDA fibroblasts by vitamin E derivatives. **A)** Structures of native vitamin E (α -tocopherol) and a water-soluble derivative, Trolox. Neither of these is targeted to mitochondria, in contrast to the mitochondria-targeted derivative MitoVit E. **B)** Concentrations of the antioxidants that prevent 50% of cell death (EC₅₀) in the absence (filled bars), or presence (open bars), of 1 μ M FCCP. Data are means \pm SD of at least 3 independent experiments (** $P < 0.001$).