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The idebenone metabolite QS10 restores electron transfer in complex I and coenzyme Q defects

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A B S T R A C T

Idebenone is a hydrophilic short-chain coenzyme (Co) Q analogue, which has been used as a potential bypass of defective complex I in both Leber Hereditary Optic Neuropathy and *OPA1*-dependent Dominant Optic Atrophy. Based on its potential antioxidant effects, it has also been tested in degenerative disorders such as Friedreich's ataxia, Huntington's and Alzheiemer's diseases. Idebenone is rapidly modified but the biological effects of its metabolites have been characterized only partially. Here we have studied the effects of quinones generated during *in vivo* metabolism of idebenone with specific emphasis on 6-(9-carboxynonyl)-2,3-dimethoxy-5-methyl-1,4-benzoquinone (QS10). QS10 partially restored respiration in cells deficient of complex I or of CoQ without inducing the mitochondrial permeability transition, a detrimental effect of idebenone that may offset its potential benefits [Giorgio et al. (2012) Biochim. Biophys. Acta *1817*: 363–369]. Remarkably, respiration was largely rotenone-insensitive in complex I deficient cells and rotenone-sensitive in CoQ deficient cells. These findings indicate that, like idebenone, QS10 can provide a bypass to defective complex I; and that, unlike idebenone, QS10 can partially replace endogenous CoQ. In zebrafish (*Danio rerio)* treated with rotenone, QS10 was more effective than idebenone in allowing partial recovery of respiration (to 40% and 20% of the basal respiration of untreated embryos, respectively) and allowing zebrafish survival (80% surviving embryos at 60 hours post-fertilization, a time point at which all rotenone-treated embryos otherwise died). We conclude that QS10 is potentially more active than idebenone in the treatment of diseases caused by complex I defects, and that it could also be used in CoQ deficiencies of genetic and acquired origin.

Keywords: Idebenone, electron transfer, complex I, ubiquinone, respiration

Abbreviations: CoQ, coenzyme Q; CRC, Ca^{2+} retention capacity; FCCP, carbonylcyanide-*p*-trifluoromethoxyphenyl hydrazone; Cs, cyclosporin; DMEM, Dulbecco's modified Eagle's medium; hpf, hours post-fertilization; idebenone, 2-(10-hydroxydecyl)-5,6-dimethoxy-3-methyl-cyclohexa-2,5-diene-1,4-dione; NQO1, NAD(P)H:quinone oxidoreductases; OCR, oxygen consumption rate; PTP, permeability transition pore; QS4, 6-(9-carboxypropyl)-2,3-dimethoxy-5-methyl-1,4-benzoquinone; QS6, 6-(9-carboxypentyl)-2,3-dimethoxy-5-methyl-1,4-benzoquinone; QS8, 6-(9-carboxyheptyl)-2,3-dimethoxy-5-methyl-1,4-benzoquinone; QS10, 6-(9-carboxynonyl)-2,3-dimethoxy-5-methyl-1,4-benzoquinone; TMRM, tetramethylrhodamine methyl ester.

1. Introduction

Ubiquinone, or coenzyme Q (CoQ), is a key electron carrier in the mitochondrial respiratory chain [1]. CoQ is reduced by respiratory chain complexes I and II [2,3] and can also receive electrons from glycerol-3-phosphate dehydrogenase and from flavoprotein dehydrogenase [4]. Mutations in *COQ* genes involved in CoQ biosynthesis cause primary CoQ deficiency, a clinically heterogeneous mitochondrial disorder [5]. Due to its reported antioxidant properties, CoQ has also been suggested as a possible treatment in a wide variety of pathological conditions including Parkinson's disease, Huntington's disease, amyotrophic lateral sclerosis, Friedreich's ataxia and other conditions associated to mitochondrial dysfunction [5-7]. However, there is little evidence that CoQ therapy may be beneficial outside of CoQ deficiencies in a strict sense [6], possibly also because of the very low solubility and therefore bioavailability of the native CoQ molecule [5-7].

The quinone 2-(10-hydroxydecyl)-5,6-dimethoxy-3-methyl-cyclohexa-2,5-diene-1,4-dione (idebenone) is the prototype of short-chain analogues of CoQ, and was developed in Japan in the 1980s for the treatment of neurodegenerative conditions due to its ability to interact with the respiratory chain [8,9]. Idebenone itself cannot substitute for endogenous CoQ [10] but it appeared particularly promising as a bypass to defective complex I in Leber Hereditary Optic Neuropathy (LHON) [11-17] and *OPA1*-dependent Dominant Optic Atrophy [18,19]. Indeed, upon reduction by cytosolic NAD(P)H oxidoreductase I (NQO1) it can be oxidized by complex III thus reactivating electron flow, proton pumping and ATP synthesis [20]. Many quinones have been developed and studied with the aim of improving hydrophilicity and pharmacokinetics for patient treatment [21,22]. Through the use of a large number of analogues it was shown that effective electron transfer to the respiratory chain also requires an appropriate partition coefficient, with an inverse correlation between efficacy and ability to induce lipid peroxidation [21].

Idebenone as such lacks antioxidant activity, a feature that is possessed only by its reduced hydroquinone form idebenol [23]. Thus, it is not surprising that idebenone can act both as an antioxidant, inhibiting lipid peroxidation [8,9,24] and hydrogen peroxide production from glycerophosphate [25], and as a prooxidant [26,27]. Potentially toxic effects of idebenone are inhibition of complex I [9,28-30] and sensitization of the permeability transition pore (PTP) [31], an inner membrane high-conductance channel whose opening requires matrix Ca^{2+} and is favored by oxidative stress [32]. Interestingly, the PTP-inducing feature of idebenone is not shared by idebenol, which is also quite effective in restoring ATP levels in a variety of rotenone-treated cells as well as in mediating electron transfer to complex III in hepatocytes and HepG2 cells [20], in

complex I-deficient cells and in cybrids harboring the G3460A/MT-ND1 mutation of LHON [33].

Although idebenone is generally well-tolerated, the complexity of its effects may explain the contradictory results obtained in disease models and in patients [11-19]. A puzzling feature related to the *in vivo* effects is its rapid metabolism through oxidation and shortening of the side chain, so that within 2-3 hours of administration parent idebenone is no longer detectable in the serum where it is replaced by the QS10, QS8, QS6, QS4 metabolites [34,35]. To what extent the decreased idebenone levels reflect cellular uptake of the parent drug is not easy to assess, but it is legitimate to ask whether the pharmacological effects of idebenone may also depend, in part at least, on its metabolites. We have reinvestigated this question by testing the effect of idebenone metabolites on electron transfer and respiration in cellular models of complex I insufficiency, CoQ deficiency and in zebrafish (*Danio rerio*) embryos treated with rotenone.

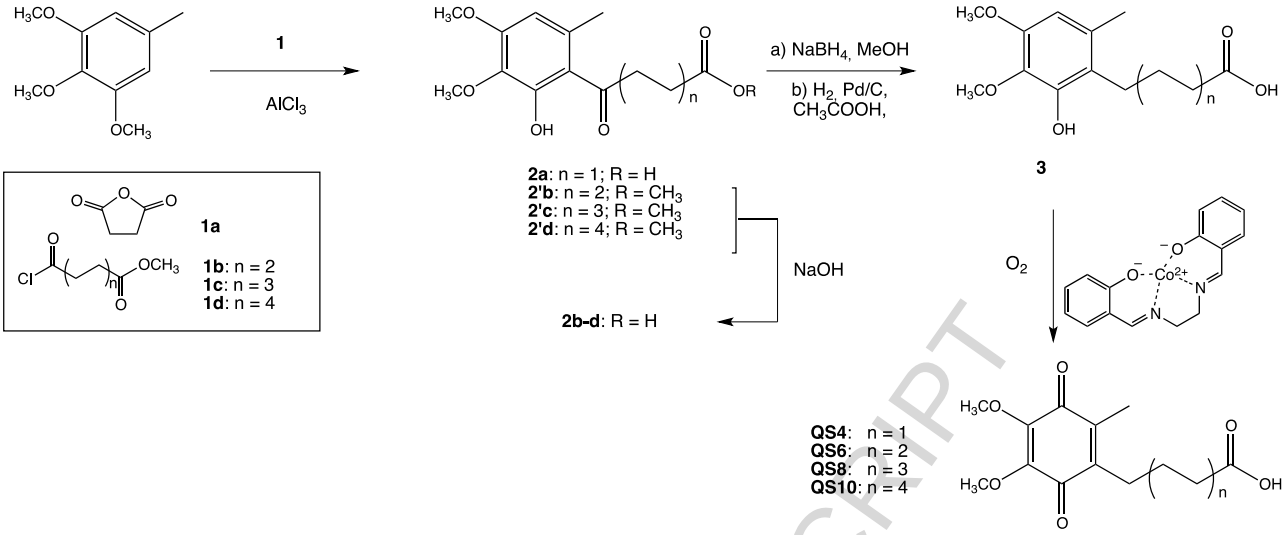
2. Materials and Methods

2.1. Chemicals

Oligomycin, rotenone, antimycin A, pyruvate, carbonylcyanide-*p*-trifluoromethoxyphenyl hydrazone (FCCP), dithiothreitol, dimethyl sulfoxide and protease inhibitors were from Sigma (Milan, Italy). Idebenone was from Apin Chemicals LTD (Oxon, UK). Cyclosporin (Cs) H was a generous gift of Dr. Urs Ruegg, Geneva. Calcium Green-5N was from Invitrogen (Milan, Italy). Tetramethylrhodamine methyl ester (TMRM) was purchased from Molecular Probes (Eugene, OR).

2.2. Synthesis of quinones

The preparation of derivatives QS4, QS6, QS8 and QS10 was performed following the synthetic approaches reported by Okamoto et al. [36]. The procedures have been adapted to each compound, depending on the commercial availability of the starting materials. In general, 3,4,5-trimethoxy toluene was allowed to react with an activated form of carboxylic acid (as succinic anhydride **1a** or acyl chloride **1b-d**) in presence of $AlCl_3$ (see scheme 1). When the acyl chloride was used, the second carboxylic acid was protected as methyl ester, which was removed by basic hydrolysis *in situ*. Later the corresponding ketones **2** were reduced using sodium borohydride, and converted to **3** in presence of acetic acid, hydrogen and Pd/C. To better purify derivatives **3c** and **3d**, it was necessary to perform supplementary steps of protection, chromatography and deprotection, to achieve the pure products. Derivatives **3** were later oxidized in presence of oxygen and selen-Co complex. All the characterizations were in agreement with the literature [36].



Scheme 1. Synthesis of QS4, 6, 8 and 10

2.3. Isolation of mitochondria and Ca^{2+} retention capacity

Mouse liver mitochondria were isolated in 250 mM sucrose, 10 mM Tris, 0.1 mM EGTA, pH 7.4, as described previously [37]. The mitochondrial Ca^{2+} retention capacity (CRC) was determined by measuring external Ca^{2+} following addition of 10 µM Ca^{2+} pulses to medium containing 0.5 mg/ml of mitochondria. Mitochondria were incubated in 130 mM KCl, 10 mM Mops-Tris, 5 mM succinate-Tris, 1 mM Pi-Tris, 10 µM EGTA, 1 µM rotenone, 1 µM Calcium Green-5N (see [33] for further details), pH 7.4.

2.4. Cell culture and growth conditions

Cybrids generated by fusion of enucleated fibroblasts derived from one control (HQB17) and one LHON patient (RJ206, harboring the G3460A/MT-ND1 LHON mutation causing the A52T amino acid substitution in ND1, a kind gift of Anthony H. Schapira) into osteosarcoma 143B.Tk– cells deprived of their own mtDNA [38,39]. XTC.UC1 bear a C insertion at bp3571 in MT-ND1, generating a premature stop codon at amino acid 101 of ND1 subunit that prevents complex I assembly [40]. The bioenergetics properties of all cells have been characterized described in some detail in a previous paper [33]. COQ4 null cells were generated from HEK-293 cells using TALEN technology to harbor a duplication of 25 nucleotides in exon 2 of *COQ4* (c.84_108dup). This insertion causes a frameshift at codon 37 with a predicted truncation at codon 39. CoQ was measured by HPLC and electrochemical detection [41]. CoQ biosynthesis rate was evaluated by measuring incorporation of radiolabelled 4-hydroxybenzoate [42], which was virtually zero.

Residual CoQ was detected, however, and it was proportional to the serum content, suggesting that it is taken up by cells. Specifically, cellular CoQ content was 5 and 15% of the wild-type value at serum concentrations of 3% and 10%, respectively, while CoQ levels were not affected by serum in wild-type cells. Cells were grown in Dulbecco's modified Eagle's medium (DMEM; Lonza, Basel, Switzerland) containing 10% fetal bovine serum, 2 mM L-glutamine, 100 units/ml penicillin, and 100 g/ml streptomycin (Thermo Fisher Scientific, Waltham, MA, USA) in a humidified incubator at 37 °C with 5% CO_2.

2.5. Mitochondrial membrane potential and respiration

Mitochondrial membrane potential was monitored based on mitochondrial accumulation of TMRM by epifluorescence microscopy, and oxygen consumption rate (OCR) with an XF24 Extracellular Flux Analyzer (Seahorse Agilent) exactly as described previously [33].

2.6. Zebrafish maintenance and treatment

Adult zebrafish were maintained in aerated, 28 °C-conditioned saline water according to standard protocols. Fish were kept under a 14 hour light/10 hour dark cycle. For mating, males and females were separated in the late afternoon, and were freed to start courtship the next morning, which ended with egg deposition and fertilization. Eggs were collected, washed with fish water (0.5 mM NaH_2PO_4, 0.5 mM $NaHPO_4$, 0.2 mg/l methylene blue, 3 mg/l Sea Salt (Instant Ocean, Blacksburg, VA, USA) and embryos were maintained at 28 °C. Embryos were dechorionated at 19 hours post-fertilization (hpf), divided in 6 groups and treated as follows: group 1, group 2 and group3 were treated in fish water at 20 hpf with 0.06% DMSO (vehicle control), 3 µM idebenone or 3 µM QS10, respectively; group 4 and group 5 were pretreated at 20 hpf with 3 µM idebenone or 3 µM QS10, respectively, and treated at 24 hpf with 0.1 µM rotenone; group 6 was treated only with 0.1 µM rotenone at 24 hpf. A second experiment was performed in which groups 1-3 were treated as above, while groups 4 and 5, after rotenone, were treated with 3 µM idebenone or 3 µM QS10 every 8 hours. Morphological changes were observed at 28 hpf using a Leica S8AP0 optical microscope (Leica microsystems GmbH, Wetzlar, Germany) equipped with Nikon Digital Sight Camera DS-L1 (Nikon, Tokyo, Japan). All protocols and manipulations with zebrafish were approved by the Ethics Committee of the University of Padova and authorized by the Italian Ministry of Health.

2.7. Motor ability and survival analysis in zebrafish embryos

To assess the effects of QS10, idebenone and rotenone during early stages of embryo development, the number of spontaneous coiling events observed in 15 s were recorded at 28 hpf using the Leica S8AP0 microscope. To evaluate survival rate, 75 embryos from each group were observed every 10 hours at 20, 30, 40, 50, 60, 70, 80 hpf. The number of surviving embryos was counted at each time point.

2.8. Statistics

Unless otherwise stated, each experiment was repeated at least 3 times. Data are presented as average ± s.e.m. or, for clarity, as representative experiments (see figure legends for details). *p* values indicated in the figures were calculated with GraphPad or Origin software.

3. Results

We synthesized the idebenone metabolites QS10, QS8, QS6 and QS4, which are detected in the plasma of patients after administration of idebenone [34,35] (Fig. 1*A*), and we tested whether these compounds have an effect on the CRC, i.e. the threshold matrix Ca^{2+} required to cause opening of the PTP in isolated mitochondria. At variance from idebenone, which decreased the CRC with a sensitizing effect on the PTP [33], none of the QS compounds significantly affected the CRC when added either as purified without further manipulations (Fig. 1*B*, *open columns*) or after reduction with DTT (Fig. 1*B*, *grey colums*). Although a decreased CRC was observed with QS10 this effect was not statistically significant (Fig. 1). Thus, PTP sensitization by idebenone is not a shared feature of its metabolites, irrespective of whether they are reduced.

We next tested whether, as is the case for idebenol [33], reduced QS metabolites could support electron transfer in mitochondria treated with rotenone. Mitochondrial membrane potential was measured in HQB17 cells based on TMRM accumulation. It should be recalled that in order to observe depolarization by rotenone it is essential to inhibit the F-ATP synthase, which would otherwise maintain the membrane potential by proton pumping coupled to the hydrolysis of ATP [43]. Consistent with our previous report, addition of DTT alone could not maintain the membrane potential after addition of oligomycin to rotenone-treated HQB17 cells (Fig. 2*A*). QS4, the species with the shortest side chain, which is the main metabolic fraction of idebenone in plasma and its main excreted metabolite [34,35], was likewise unable to allow maintenance of the mitochondrial membrane potential in the same protocols (Fig. 2*B*), and a similar negative result

was obtained with QS6 and QS8 (results not shown). On the other hand, oligomycin did not cause depolarization when QS10 was added in the presence of DTT (Fig. 2*C*) or after it had been reduced with $NaBH_4$ (Fig. 2*D*). Depolarization could be induced by antimycin A, indicating that QS10 was feeding electrons into complex III (Fig. 2*C*,*D*). It should be mentioned that the slight effect of QS10 was not significant, while the differential effect of oligomycin in cells treated with reduced QS10 and DTT alone (Panels 2*C,D* – compare with 2*A*) was highly reproducible, and became statistically significant within 1 minute of the addition of oligomycin (omitted for clarity).

We next directly assessed the effects of QS10 on respiration. When added to HQB17 cells, reduced QS10 did not significantly affect basal respiration, oligomycin sensitivity or maximal respiration induced by FCCP (Fig. 3*A*). This is an important finding because it indicates that QS10 does not decrease electron transfer at complex I, which would result in respiratory inhibition. On the other hand, reduced QS10 allowed maintenance of oxygen consumption after the addition of rotenone; the effect was significant when rotenone was added shortly after QS10 (Fig. 3*A*) while the effect decreased at later time points (results not shown), suggesting that in HQB17 cells QS10 may not be efficiently reduced. When assayed in RJ206 cells (bearing the missense G3460A/MT-ND1 mutation of LHON) and in XTC.UC1 cells (that do not fully assemble complex I due to a C insertion at bp3571 in MT-ND1, generating a stop codon at amino acid 101 of ND1 subunit) QS10 promoted a sizeable rotenone-insensitive respiration (Fig. 3*B* and *C*, respectively).

Idebenone cannot replace endogenous CoQ in restoring ATP levels in fibroblasts from patients affected by primary CoQ deficiencies [10]. We reasoned that this negative result could have been due, in part at least, to inhibition of complex I [28], an effect that according to our current results is not observed with QS10. We therefore tested the effects of QS10 in a cell line where synthesis of CoQ had been prevented by genetic ablation of *COQ4.* The basal respiratory activity was expectedly very low, and corresponded to about 10% of the rate of cells reexpressing *COQ4* (not shown) and to 10% of the rate displayed by HQB17 cells (Fig. 3*D*, compare with *A*). Addition of QS10 significantly increased the rate of basal respiration, and this effect was not due to uncoupling because the increased OCR was inhibited by oligomycin (Fig. 3*D*). Another quite remarkable result of this experiment is that QS10-dependent respiration was rotenone-sensitive, i.e. it was due to the redox interactions of QS10 with complexes I/II and complex III of the respiratory chain. Thus, QS10 can be reduced by complex I and reconstitute electron flux within the respiratory chain of CoQ-deficient cells, although recovery of respiration was only partial.

The predictive value of the efficacy of drugs in cell-based assays is inevitably limited. In order to further explore their effects in a living organism, we treated with idebenone and QS10 developing zebrafish embryos, and assessed whether they could be protected from toxicity of 0.1 μM rotenone administered at 24 hpf (Fig. 4*A*). Analysis of respiration of individual embryos 4 hours later revealed that rotenone reduced the OCR to about 10% (Fig. 4*B*), while a 4hr pretreatment with idebenone or QS10 maintained respiration to 20% and 40% of the vehicle-treated value, respectively (Fig. 4*B*). Rotenone caused marked developmental abnormalities starting from the posterior tail region, where the hypoblast (mesodermal tissue progenitor cells that will generate the nothocord), skeletal muscle precursors and intermediate cell mass (containing progenitors of hematopoietic and vascular tissues) are located [44,45]. We observed signs of progressive tissue necrosis and degradation (dark zone indicated by arrows in Fig. 4*C*) which would spread rostrally as the embryo degradation proceeds. After treatment with idebenone embryos where protected only partially from the degenerative process, as demonstrated by a curly tail phenotype (Fig. 4*C*, arrowheads) while treatment with QS10 was quite effective at preventing the developmental abnormalities (Fig. 4*C*, bottom panels). Living fish were also tested for their motor ability by monitoring coiling events, spontaneous movements that normal embryos perform. Rotenone completely abolished motility, and this effect was rescued by pretreatment with QS10 but not idebenone (Fig. 4*D*). It should be mentioned that treatment with each quinone in the absence of rotenone caused a mild but not significant motor impairment.

We also monitored the effects of idebenone and QS10 on survival of rotenone-treated zebrafish. Within 6-10 hours of the addition of rotenone 50% of the embryos were dead, and no fish survived beyond 60-70 hpf (Fig. 5*A,B* closed circles; compared to vehicle, all values were significant with *p* of at least 0.001). Administration of a single dose of idebenone caused zebrafish cell death (Fig. 5*A*, open squares; compared to vehicle, all values were significant with *p* of at least 0.001), which blunted the rescue from rotenone toxicity (Fig. 5*A*, closed squares; compared to rotenone alone values were significant only up to 50 hpf with *p* of at least 0.001). A single dose of QS10 instead did not cause major toxicity (Fig. 5*A*, open triangles; compared to vehicle all values were not significant) and prevented rotenone-induced death up to 70 hpf (Fig. 5*A*, closed triangles; compared to rotenone alone values were significant with *p* of at least 0.001 and 0.05 up to 60 and 70 hpf, respectively). Repeated additions of idebenone worsened its toxicity (Fig. 5*B*, open squares; compared to vehicle, all values were significant with p of at least 0.001); and if

anything made rotenone toxicity at 40 hpf even worse (Fig. 5*B*, closed squares, compare with rotenone alone, closed circles). Remarkably, repeated additions of QS10 affected survival only slightly (Fig. 5*B*, open triangles; compared to vehicle, values were significantly different only at or above 60 hpf with *p* of 0.05) while they improved protection from rotenone toxicity with 50% embryos still surviving at 80 hpf (Fig. 5*B*, closed triangles; compared to rotenone alone all values were significant with *p* of at least 0.001).

4. Discussion

The main finding of the present manuscript is that QS10, which is generated during *in vivo* metabolism of idebenone [34,35], can provide a useful bypass of the respiratory defect in cells treated with rotenone or possessing deficient complex I, as reported previously for the parent compound [20,21]. Unlike idebenone [10], however, QS10 was also able to partially restore respiration in cells lacking endogenous CoQ and to rescue zebrafish embryos from rotenone acute toxicity *in vivo*.

The requirements for an effective bypass of defective complex I by idebenone and other short chain quinones are beginning to emerge. Besides cell permeation, an efficient reducing system is necessary, and this can be provided by NQO1 [20]. Through the analysis of a panel of 70 quinones it was later discovered that efficacy also critically depends on the physicochemical properties of the entire molecule rather than on the quinone moiety as such [21]. The most important issue appears to be whether the compound induces lipid peroxidation since an inverse correlation was found between this effect and quinone efficacy [21]. Another issue is stability, and this is an intriguing aspect of idebenone pharmacology. As already mentioned, parent idebenone is rapidly metabolized by oxidation and side chain shortening so that within 2-3 hours of administration it is no longer detectable in the serum [34,35]. Our observation that QS10 (but not QS8, QS6 and QS4) can restore electron transfer through inhibited or defective complex I is therefore a step forward in understanding the basis for the *in vivo* effects of idebenone. Importantly, QS10 was found to be way more stable in plasma than parent idebenone (which was virtually undetectable after 1 hour of administration), and QS10 concentrations were intermediate between those of QS4 (highest) and of QS6 (lowest). Given that QS4 and QS6 were inactive in promoting electron transfer in the presence of rotenone and oligomycin, we suspect that the pharmacological effects of idebenone are actually mediated by QS10. This would also explain lack of idebenone toxicity *in vivo* in spite of inhibitory effect of idebenone on electron transfer at

complex I [28]. Our data suggest that the QS10 metabolite is not an inhibitor of complex I, as indicated by its ability to partially reconstitute coupled electron transfer in cells lacking endogenous CoQ. This finding may pave the way to treatment of both complex I and CoQ deficiencies with short-chain quinones, which do not present the solubility and bioavailability problems encountered with CoQ supplementation. Major open questions remain, particularly as to whether QS10 will be absorbed, reaching pharmacologically relevant concentrations in mammals; and whether cellular uptake and reduction by NQO1 or other donor systems will be adequate to support bypass of defective complex I in relevant tissues. In this respect, the results obtained in zebrafish are encouraging.

Zebrafish has been used as an *in vivo* model of rotenone toxicity both in low-dose exposure studies modeling the features of Parkinson's disease [46-48] and as a surrogate of mitochondrial respiratory chain dysfunction [49-51]. Depending on dose and time of treatment observed phenotypes were body deformities (such as small head and eyes, trunk and tail deformation) with progressive necrosis and tissue degradation [49,50], motor function deficits reminiscent of those of Parkinson's disease [46,48] and early, severe developmental delays with arrest at the tail-bud stage [51]. Our studies generally agree with the morphological and functional alterations described in these previous studies, with detection of morphological abnormalities, motor deficits and progressive tissue necrosis and degradation. Of relevance is the lack of protection by idebenone treatment on fish survival after treatment with rotenone, which was also noted in a previous study [49]. However, our study is the first to have assessed the effect of rotenone on respiration of individual zebrafish embryos, which adds a relevant mechanistic basis to the protective effects of QS10. Indeed, the rescue by QS10 correlates with its ability to partially restore respiration in zebrafish treated with rotenone.

An obvious question is whether the results obtained in zebrafish are predictive of the potential beneficial/toxic effects of QS10 in mammals [52]. Within 5 days of fertilization zebrafish larvae develop functional pancreas, liver, bowel, hematopoietic tissue, cardiovascular system, kidney and blood-brain barrier that perform the same functions as their human counterparts, with a highly conserved integrated physiology [53-56]. Furthermore, 82% of disease-causing human proteins have an obvious orthologue in zebrafish with strong similarity of functional domains [57], and the zebrafish permeability transition is indistinguishable from that of mammals [58]. Although less is known about biotransformation of xenobiotics, recent studies demonstrate that zebrafish does possess a full complement of cytochrome P450 genes that have orthologues in humans, with

50-90% homology of protein sequence and conservation of enzymatic activity [57,59,60]. Comparison of liver microsomes from zebrafish and humans showed that some drugs and hormones (paracetamol, dexamethasone, dextromethorphan and diclofenac) underwent similar modifications, while others (testosterone and midazolam) either generated different metabolites or were not metabolized at all [61]. However, three model quinones underwent phase I reactions similar to those observed in humans [62]. Since zebrafish embryos and larvae have negligible or low biotransformation capacity compared to adults, we suspect that the effects we observed are indeed due to QS10. Thus, the present results in human cell models and zebrafish represent a strong rationale for the testing of QS10 in mammalian disease models of complex I and CoQ deficiencies.

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Figure legends

Fig. 1. Structure of CoQ10, idebenone, QS10, QS8, QS6, QS4 and their effects on the mitochondrial Ca^{2+} retention capacity. *A*, Chemical structures of the indicated quinones and of idebenone metabolites generated from idebenone *in vivo*. *B*, Ca^{2+} retention capacity of mitochondria isolated from mouse liver and treated with 50 μM Idebenone (IdB), QS10, QS8, QS6 or QS4 in their oxidized form (open bars) or reduced with 0.5 mM DTT (gray bars). Error bars denote s.d. Mitochondria were resuspended at 0.5 mg/ml and the Ca^{2+} retention capacity (in nmol Ca^{2+} x mg^{-1} protein) was determined following additions of 5 μM Ca^{2+}. The incubation medium contained 130 mM KCl, 10 mM MOPS-Tris, 5 mM succinate, 1 mM Pi-Tris, 10 μM EGTA, 1 μM Calcium Green-5N, 1 μM rotenone. $^{**}p = 0.0061$.

Fig. 2. Effects of reduced QS4 and QS10 on mitochondrial TMRM fluorescence in HQB17 cybrids. HQB17 cells were loaded with 10 nM TMRM in the presence of 2 μg/ml cyclosporin H. Where indicated 1 mM DTT, 50 μM QS4 or 50 μM QS10 preincubated with DTT, or QS10 reduced with $NaBH_4$ (Red QS10) were added. Further additions were 4 μM rotenone (R), 5 μM oligomycin (O), 1 μM antimycin A (AA) and 4 μM FCCP (F). Data in each panel are presented as mean ± s.e.m. of 3 independent experiments.

Fig. 3. Effects of reduced QS10 on cellular respiration *in situ*. *A*, HQB17 cells (40,000/well); *B*, RJ206 cells (40,000/well); *C*, XTC.UC1 (50,000/well); *D*, COQ4KO cells (50,000/well) were plated in Seahorse 24-well plates and respiration was measured in the absence (open bars) or presence (grey bars) of 50 μM QS10 prereduced with $NaBH_4$. After recording basal respiration (Basal), 1 μg/ml oligomycin (Oligo), 0.2 μM (*A,B*) 0.1 μM (*C*) or 0.05 μM (*D*) FCCP were sequentially added. Rotenone (Rot, 1 μM) was added right after recording the basal rate of respiration for 21 minutes in a separate set of experiments (panel *A*) or after FCCP (panels *B-D*). Antimycin A-insensitive (non mitochondrial) oxygen consumption rate (OCR) was subtracted. Data are mean of at least 3 independent experiments run in triplicate ± s.e.m. (*) *p* values ≤ 0.05; (***) *p* values ≤ 0.001, *Student t* test, Graphpad Software.

Fig. 4. Effects of idebenone, QS10 and rotenone on zebrafish embryo respiration, morphology and coiling. *A*, Scheme of embryo treatment. Embryos were treated with 3 μM idebenone (IdB) or 3 μM QS10 at 20 hours post-fertilization (hpf) and with 0.1 μM of rotenone at 24 hpf, and all analyses were performed at 28 hpf. *B*, Oxygen consumption rate (OCR) of individual embryos treated with vehicle (open circles), rotenone (closed circles), rotenone in combination with idebenone (closed squares) or rotenone in combination with QS10 (closed triangles). Where indicated 1 μM antimycin A (AA) was added. Traces represent mean values ± s.e.m. of 3 independent experiments. Respiration of rotenone-treated embryos supplemented with idebenone and QS10 was significantly different from respiration of rotenone-treated embryos (*p* was lower

than 0.001). *C*, Morphology of zebrafish embryos was analyzed at 28 hpf and the corresponding images are shown. Left rows, vehicle; right rows, rotenone. Note the developmental zebrafish alteration due to rotenone toxicity (arrows) and the residual abnormal morphology (curly tail) of embryos treated with rotenone plus idebenone (arrowheads). *D*, Spontaneous coiling events of embryos treated with rotenone (Rot), idebenone (IdB) and QS10 are indicated (+). Bars represent mean values ± s.e.m. of 5 independent experiments. ** $p = 0.0082$, *** $p = 0.0022$.

Fig. 5. Effects of idebenone, QS10 and rotenone on zebrafish embryo survival. *A,B* the percentage of surviving fish treated as described in Fig. 4A was monitored. Open circles, vehicle; closed circled, 0.1 µM rotenone added where indicated (arrow); open squares, idebenone; open triangles, QS10; closed squares, idebenone plus rotenone; closed triangles, QS10 plus rotenone. Treatment with idebenone and QS10 (arrows) was done only once at 20 hpf in the experiments of panel *A*, while it was repeated every 8 hours in the experiments of panel *B*. Statistical data (omitted for clarity in the figure) are reported in the main text, refer to the s.e.m. and were obtained with two way *ANOVA*, Origin 8 Software. Each group of treatment included 75 embryos.

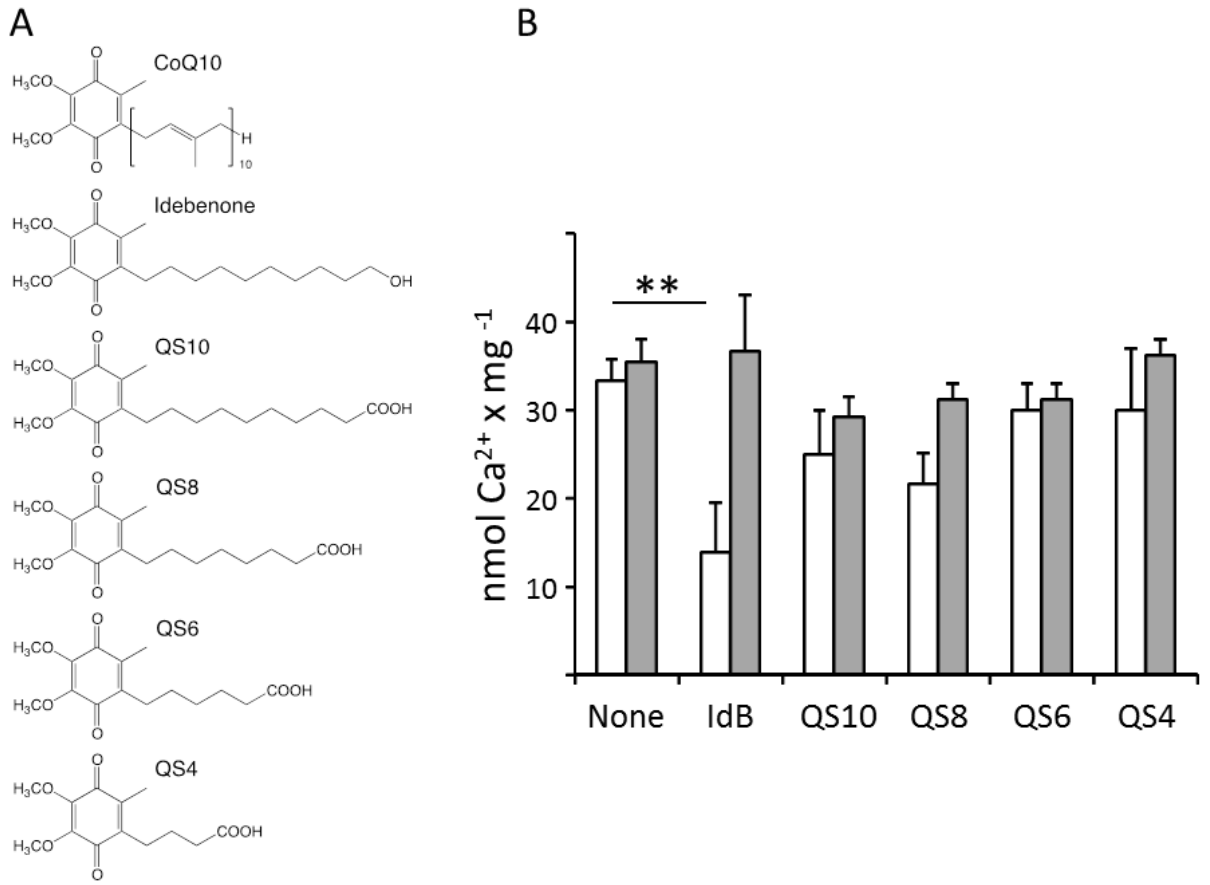


Fig. 1

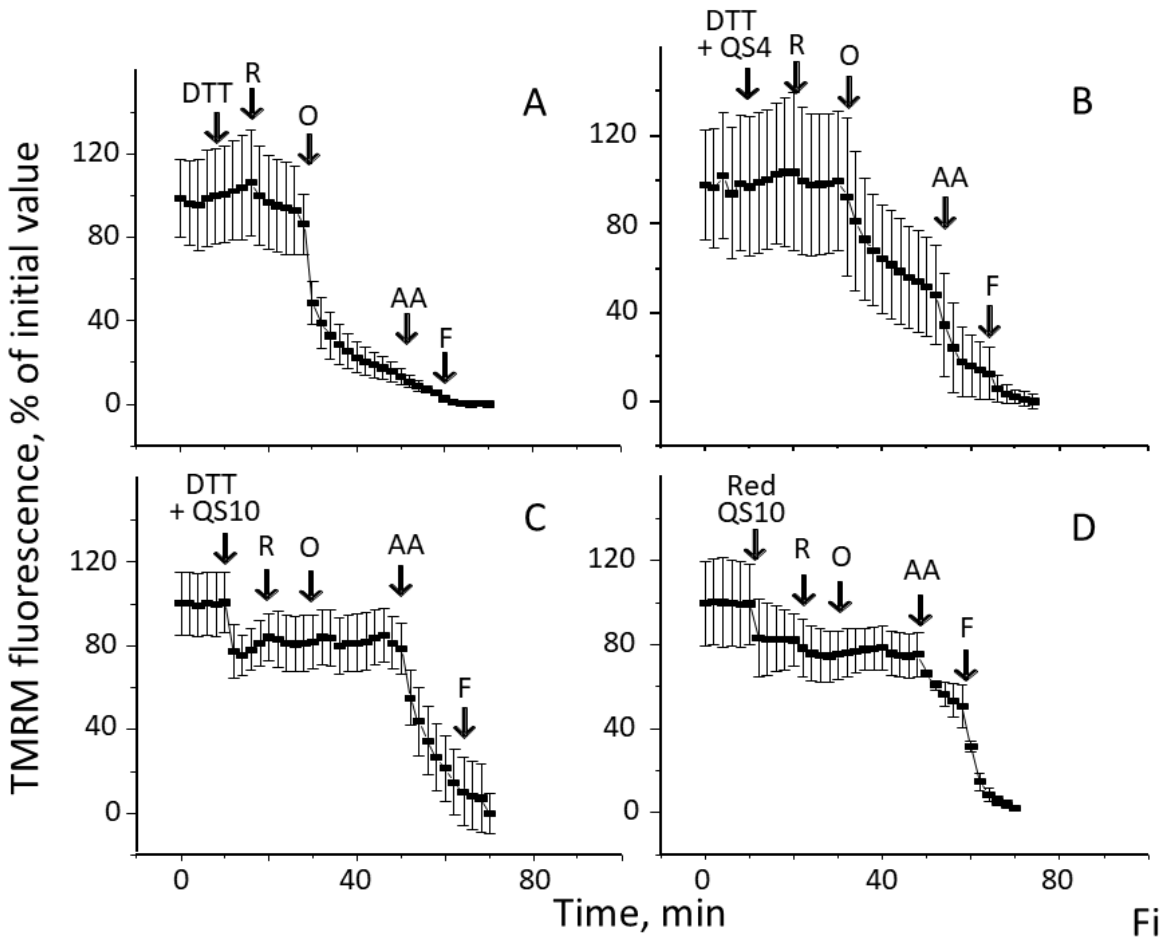


Fig. 2

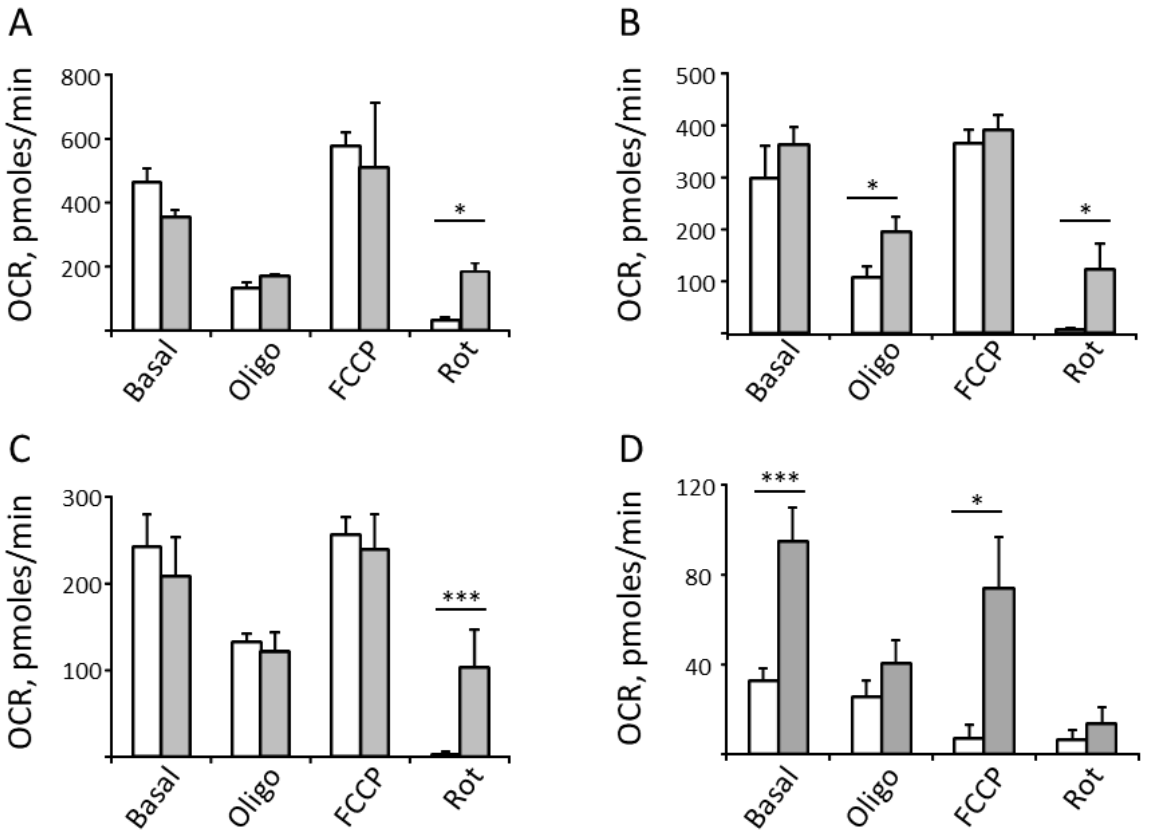


Fig. 3

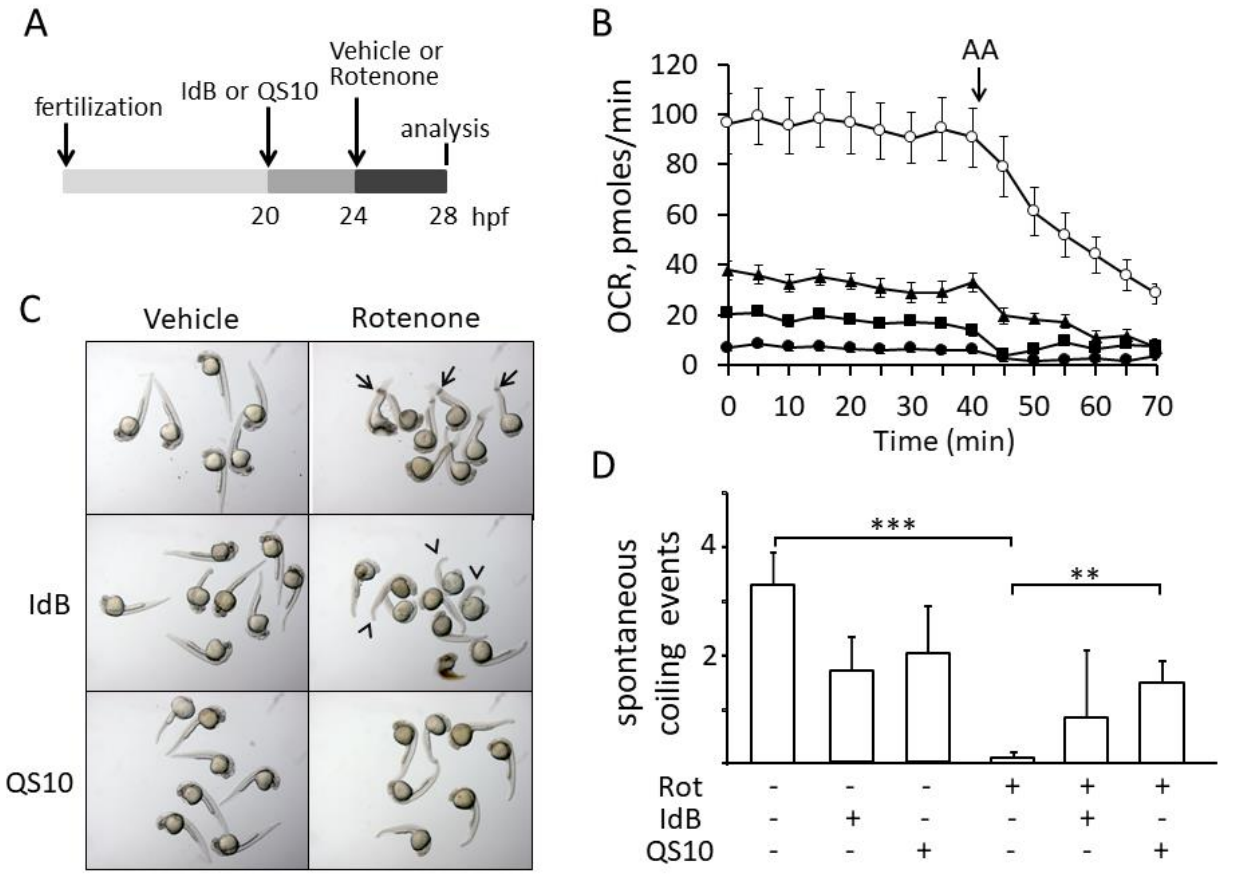


Fig. 4

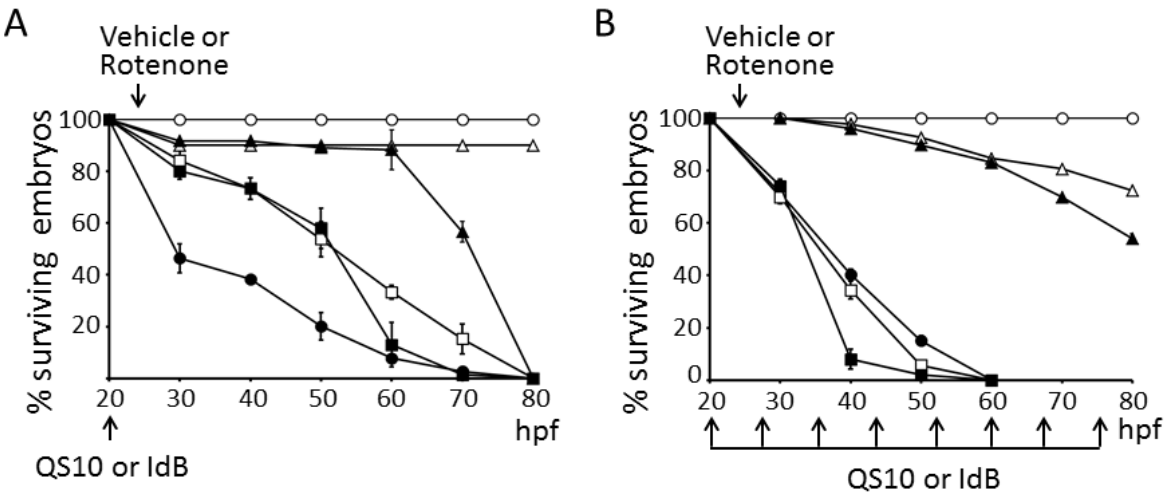
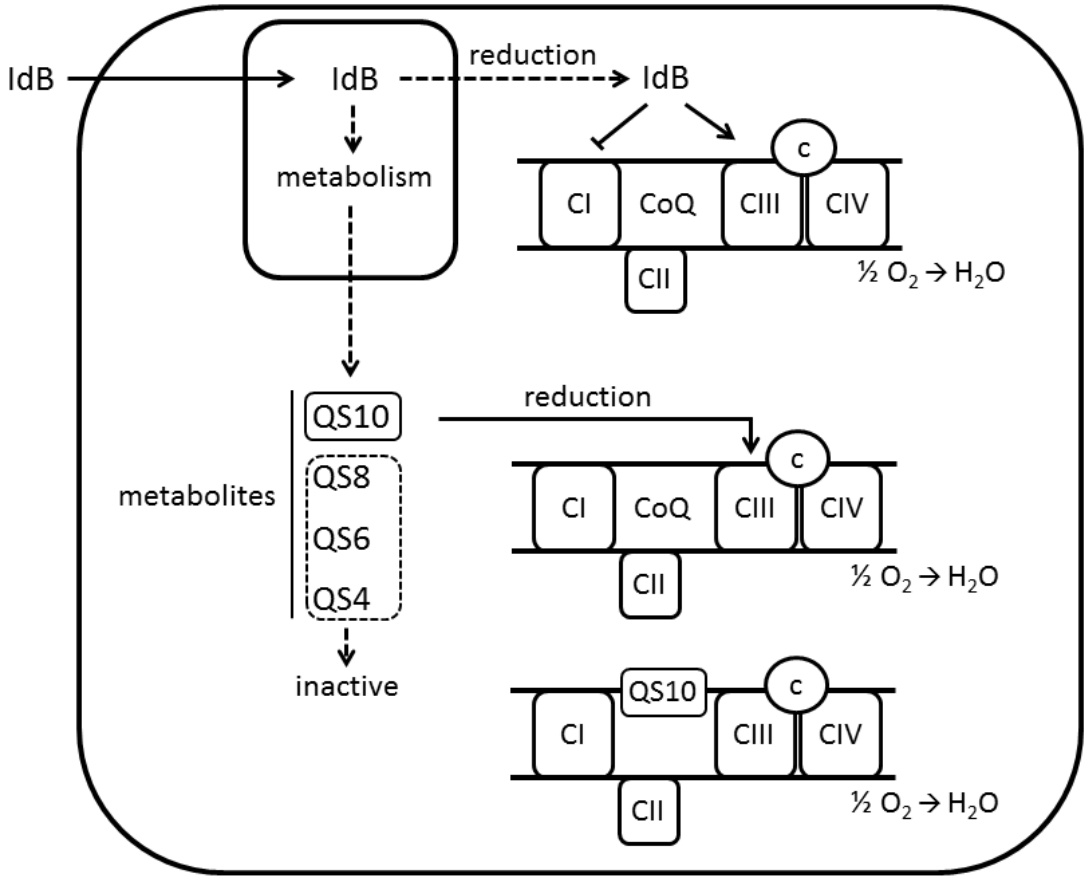


Fig. 5



Graphical abstract

Highlights

- Idebenone is a short-chain quinone used to bypass defective mitochondrial complex I
- The QS10 metabolite can partially replace endogenous coenzyme Q but also mediate electron transfer in the presence of rotenone
- QS10 but not idebenone rescues zebrafish from rotenone toxicity