



## Research Paper

# Micellization of coenzyme Q by the fungicide caspofungin allows for safe intravenous administration to reach extreme supraphysiological concentrations

Ying Wang, Siegfried Hekimi<sup>\*</sup>

Department of Biology, McGill University, Montreal, Quebec, Canada



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## ABSTRACT

Coenzyme Q<sub>10</sub> (CoQ<sub>10</sub>; also known as ubiquinone) is a vital, redox-active membrane component that functions as obligate electron transporter in the mitochondrial respiratory chain, as cofactor in other enzymatic processes and as antioxidant. CoQ<sub>10</sub> supplementation has been widely investigated for treating a variety of acute and chronic conditions in which mitochondrial function or oxidative stress play a role. In addition, it is used as replacement therapy in patients with CoQ deficiency including inborn primary CoQ<sub>10</sub> deficiency due to mutations in CoQ<sub>10</sub>-biosynthetic genes as well as secondary CoQ<sub>10</sub> deficiency, which is frequently observed in patients with mitochondrial disease syndrome and in other conditions. However, despite many tests and some promising results, whether CoQ<sub>10</sub> treatment is beneficial in any indication has remained inconclusive. Because CoQ<sub>10</sub> is highly insoluble, it is only available in oral formulations, despite its very poor oral bioavailability. Using a novel model of CoQ-deficient cells, we screened a library of FDA-approved drugs for an activity that could increase the uptake of exogenous CoQ<sub>10</sub> by the cell. We identified the fungicide caspofungin as capable of increasing the aqueous solubility of CoQ<sub>10</sub> by several orders of magnitude. Caspofungin is a mild surfactant that solubilizes CoQ<sub>10</sub> by forming nano-micelles with unique properties favoring stability and cellular uptake. Intravenous administration of the formulation in mice achieves unprecedented increases in CoQ<sub>10</sub> plasma levels and in tissue uptake, with no observable toxicity. As it contains only two safe components (caspofungin and CoQ<sub>10</sub>), this injectable formulation presents a high potential for clinical safety and efficacy.

## 1. Introduction

Coenzyme Q (CoQ), also called ubiquinone (UQ), is a redox-active, lipid-soluble component of biological membranes. It is an obligate mobile electron carrier in the mitochondrial electron transport chain (ETC) [1,2]. In addition, CoQ has antioxidant properties that are believed to protect membrane lipids from oxidative damage [3,4]. Several other redox functions have also been described for CoQ, including in sulfide oxidation, in regulating the mitochondrial permeability transition pore, and in plasma membrane electron transport and proton translocation across lysosomal membranes [4–6].

CoQ is comprised of a redox-active benzoquinone ring linked to an isoprenoid side-chain (Fig. 1A). The length of the side-chain, indicated by a subscript, is species-specific. Humans have CoQ<sub>10</sub> and mice mostly CoQ<sub>9</sub>, with some CoQ<sub>10</sub>. The final steps of CoQ biosynthesis in the inner mitochondrial membrane begin with the assembly of the polyisoprenoid

side-chain and its attachment to the benzoquinone ring, which then undergoes a series of modifications (decarboxylation, hydroxylations and methylations) to obtain the final product [7–13]. In addition to the actual enzymes, several other proteins are necessary for CoQ biosynthesis [14–18].

Patients with mutations in genes involved in the biosynthesis of CoQ suffer from inborn primary CoQ deficiency (MIM 607426). So far, pathogenic mutations for 9 of the 11 genes currently known to participate in human CoQ biosynthesis have been identified, with an estimated 125,000 individuals affected worldwide [7,19–21]. CoQ deficiency is associated with numerous symptoms, including nephrotic syndrome, infantile encephalomyopathy, ataxia and cerebellar atrophy [7,21–47]. Like mitochondrial disease syndrome (MDS), primary CoQ deficiency is often a multi-systems disorder with heterogeneous clinical manifestations. In addition, patients with MDS and other diseases frequently suffer from secondary CoQ<sub>10</sub> deficiency [16,48–51]. A decline of CoQ<sub>10</sub> levels

<sup>\*</sup> Corresponding author.

E-mail address: [siegfried.hekimi@mcgill.ca](mailto:siegfried.hekimi@mcgill.ca) (S. Hekimi).

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is also observed during aging and in some age-related diseases (e.g., Parkinson disease) [52–54]. The mechanisms responsible for secondary deficiency remain unknown.

In addition to the possibility of treating CoQ<sub>10</sub> deficiency, supplementing CoQ<sub>10</sub> has long been put forward as a potential treatment for many conditions that have components of mitochondrial dysfunction or oxidative stress, and numerous clinical trials have been conducted with CoQ<sub>10</sub> for a variety of disorders. Examples include inborn mitochondrial disorders, heart disease, diabetes and neurodegenerative diseases [49, 55–60]. However, only oral CoQ<sub>10</sub> is currently available and, despite the sensible rationale and some positive reports, it appears to be only minimally effective [14,37,42–45,61]. In fact, although numerous oral CoQ<sub>10</sub> products are available, CoQ<sub>10</sub> supplementation is not yet FDA-approved to treat any medical condition.

One reason for the therapeutic failure of CoQ<sub>10</sub> is its poor oral bioavailability and its extreme hydrophobicity which prevents parenteral delivery. Here we report a water-soluble CoQ<sub>10</sub> formulation made by using the FDA-approved drug caspofungin (brand name Cancidas). We show that this formulation can be safely administered intravenously to animals, which raises CoQ<sub>10</sub> levels by unprecedented amounts in all tissues tested. Given that the only component of the formulation besides CoQ<sub>10</sub> is safe and approved, there should be few obstacles to its clinical development and approval for the treatment of CoQ deficiency and other diseases.

## 2. Results

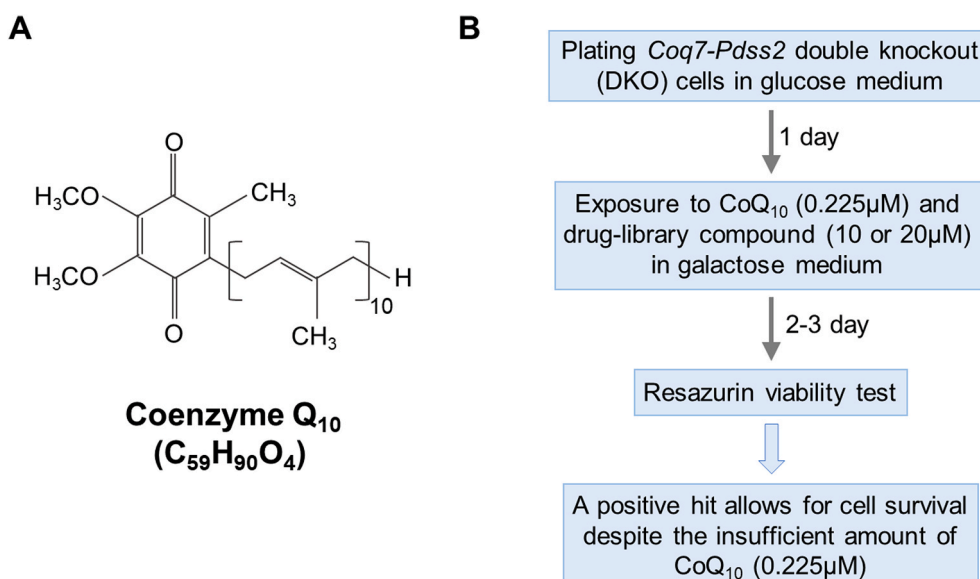
### 2.1. Identification of caspofungin (CF) in a screen for drugs that enhance uptake of exogenous CoQ

We obtained mouse cells entirely devoid of CoQ and CoQ biosynthetic intermediates by viral delivery of Cre-recombinase into mouse embryonic fibroblasts (MEFs) carrying floxed alleles of two CoQ-biosynthetic genes, *Pdss2* and *Coq7*. *Pdss2* (prenyldiphosphate synthase subunit 2) is one of two subunits of the prenyl diphosphate synthase that is responsible for the first committed step of CoQ biosynthesis, the assembly and elongation of the polyisoprenoid side chain [13]. The *Coq7* hydroxylase catalyzes the penultimate step which is the C6-hydroxylation of 5-demethoxyubiquinone (DMQ). *Coq7* null mutants, in addition to losing the ability to make CoQ, accumulate DMQ [10,11,62,63]. As detailed in Fig. S1A and B, the expression of both

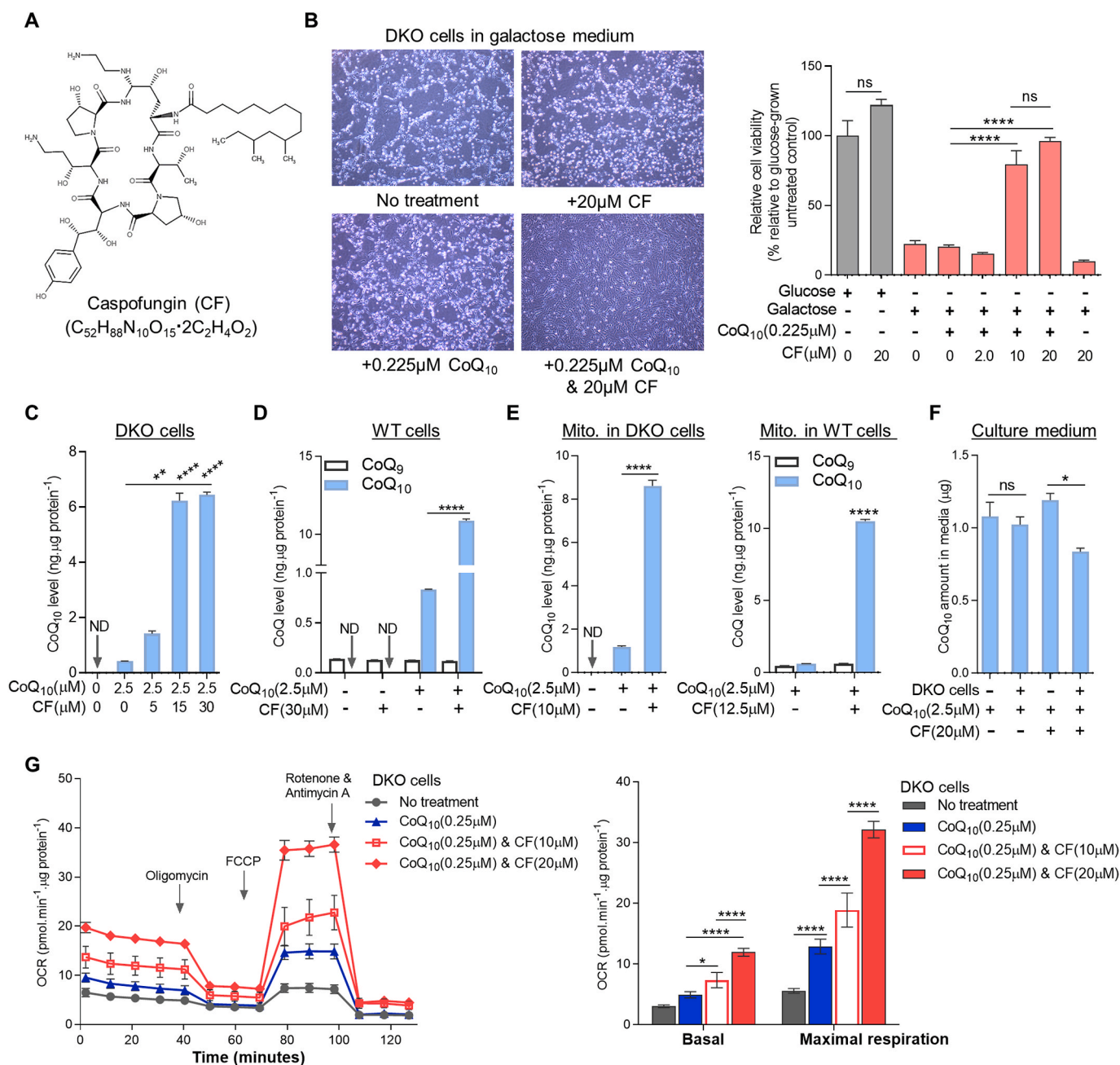
genes was lost in the *Pdss2<sup>fl/fl</sup>;Coq7<sup>fl/fl</sup>* MEFs after infection with the Cre-bearing retrovirus, with CoQ and DMQ becoming undetectable, as expected. Below we refer to the cells with the excised genes as double knockout (DKO) cells, with the *Pdss2<sup>fl/fl</sup>;Coq7<sup>fl/fl</sup>* MEFs infected with an empty viral vector serving as controls. DKO cells show no growth impairment under standard growth conditions in a medium containing high levels of glucose. However, they display a severely impaired oxygen consumption rate (OCR) and a virtually complete loss of spare respiratory capacity (Fig. S1C). These cells die within 2 days after transfer from a glucose-rich medium to a glucose free, galactose-containing, medium. The replacement of glucose with galactose forces cells to rely more heavily on aerobic respiration for energy production. A dose-dependent rescue of survival in galactose medium was observed when the medium was supplemented with CoQ<sub>10</sub> (Fig. S1D).

We used the DKO cells to screen a library of FDA-approved drugs (Selleckchem) for compounds that can boost the cellular uptake of exogenous CoQ. The screen design is shown in Fig. 1B. Briefly, we screened for compounds that can rescue the death of DKO cells in a glucose free, but galactose-containing medium (referred to as galactose medium below) supplemented with a very small amount of CoQ<sub>10</sub> (an amount that is insufficient by itself to restore the viability of DKO cells in this medium). The screen could therefore identify compounds that can boost the uptake or the functional efficiency of the minimal amount of added CoQ<sub>10</sub>. We tested the 1018 compounds of the library at two concentrations (10 and 20 μM). Only one compound, caspofungin acetate (CF) (Fig. 2A), was able to rescue DKO cell viability in presence of the minimal amount of CoQ<sub>10</sub> (Fig. 2B). CF is an antifungal drug that inhibits fungal cell wall synthesis and has no known target in mammalian cells [64]. Addition of CoQ<sub>10</sub> at 0.225 μM or CF alone did not affect survival of DKO cells in galactose medium, but inclusion of both completely rescued viability (Fig. 2B and Fig. S2).

To examine the effect of CF on CoQ uptake into cells, we used higher concentrations of CoQ<sub>10</sub> (2.5 μM) than to examine viability. As shown in Fig. 2C and D, in comparison to treatment with CoQ<sub>10</sub> alone, there was a much greater, dose-dependent, increase of total cellular CoQ<sub>10</sub> in both DKO and *Pdss2<sup>fl/fl</sup>;Coq7<sup>fl/fl</sup>* MEFs (referred to as WT cells below) when CF was added in combination with CoQ<sub>10</sub>. In WT cells the presence of CF increased CoQ<sub>10</sub> uptake >13-fold, resulting in >78-fold more CoQ<sub>10</sub> than endogenous CoQ<sub>9</sub> (Fig. 2D). As part of its biological function, CoQ can exist in both reduced and oxidized states. However, reduced CoQ is inherently unstable and readily converts to oxidized CoQ when exposed



**Fig. 1.** Screen design for compounds that enhance cellular uptake of exogenous CoQ<sub>10</sub>. (A) Structure of CoQ<sub>10</sub>. (B) We screened for the ability of compounds to rescue the survival of DKO cells in galactose medium supplemented with an amount of CoQ<sub>10</sub> (0.225 μM) that is insufficient to allow for survival by itself.



**Fig. 2. Caspofungin (CF) increases cellular uptake of exogenous CoQ.** (A) Chemical structure of CF, a semi-synthetic lipopeptide composed of a cyclic hexapeptide *N*-linked to an acyl fatty acid side-chain. (B) Viability of DKO cells after 2 days of treatment under various conditions. Cell viability was measured by the resazurin viability assay ( $n = 8$ ). See Fig. S2 for cell viability data measured by crystal violet staining. (C–D) Changes of CoQ<sub>10</sub> levels in DKO or WT cells treated with CoQ<sub>10</sub> and/or CF for 3 days ( $n = 3$ ). (E) Mitochondrial CoQ concentrations in DKO or WT cells after 3-day treatment with either CoQ<sub>10</sub> and/or CF ( $n = 3$ ). Mito. means mitochondria. (F) Total CoQ<sub>10</sub> amount in culture media (10 ml per 10 cm dish) after 2 days of incubation under different conditions ( $n = 3$ ). In b–f, data shown are mean  $\pm$  SEM. One-way ANOVA followed by Tukey’s post hoc test or Student’s *t*-test was used to compare CoQ<sub>9</sub> or CoQ<sub>10</sub> levels between groups. (G) Comparison of the effects of CoQ<sub>10</sub> treatment only and co-treatment of CoQ<sub>10</sub> and CF on mitochondrial respiration of DKO cells. Representative oxygen consumption rate (OCR) traces are shown on the left. Bar graph shows summary data. Error bars represent standard deviations (SD) ( $n = 5$ ). The data were subjected to two-way ANOVA followed by Tukey’s multiple comparison test. ND: not detectable. ns: not significant. \* $p < 0.05$ , \*\* $p < 0.05$ , and \*\*\*\* $p < 0.0001$ . (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

to air and light during the extraction process [65]. Therefore, the amount of absorbance at 275 nm at which we measure CoQ, and which corresponds to oxidized CoQ, measures the total CoQ concentration (See Materials and Methods).

We further measured CoQ levels in mitochondria and observed that there was >7–17 times more CoQ<sub>10</sub> uptake in cells co-treated with CF and CoQ<sub>10</sub> relative to cells treated with the same amount of CoQ<sub>10</sub> alone (Fig. 2E). Consistent with this, we found that after incubation of DKO

cells with CF and CoQ<sub>10</sub>, the amount of CoQ<sub>10</sub> in the culture media was decreased, further pointing to a dramatic and active CoQ<sub>10</sub> uptake by the cells (Fig. 2F). Of note, no treatment had any effect on the level of endogenous CoQ (CoQ<sub>9</sub> in mouse cells) (Fig. 2D and E). In order to confirm the intra-mitochondrial functionality of the exogenous CoQ<sub>10</sub> after uptake, we measured effects on mitochondrial respiration. After 2 days of treatment with 0.25  $\mu$ M of CoQ<sub>10</sub>, DKO cells showed no change of basal respiration rate, but a small increase of mitochondrial

respiratory capacity (assessed by addition of the uncoupler FCCP). However, a much larger dose-dependent increase was observed for both basal and maximal respiration after 2 days of treatment with CF (10 or 20  $\mu\text{M}$ ) and CoQ<sub>10</sub> (0.25  $\mu\text{M}$ ) simultaneously (Fig. 2G). CF by itself had no effect, as shown in Fig. S3.

## 2.2. Caspofungin increases the water solubility of CoQ<sub>10</sub>

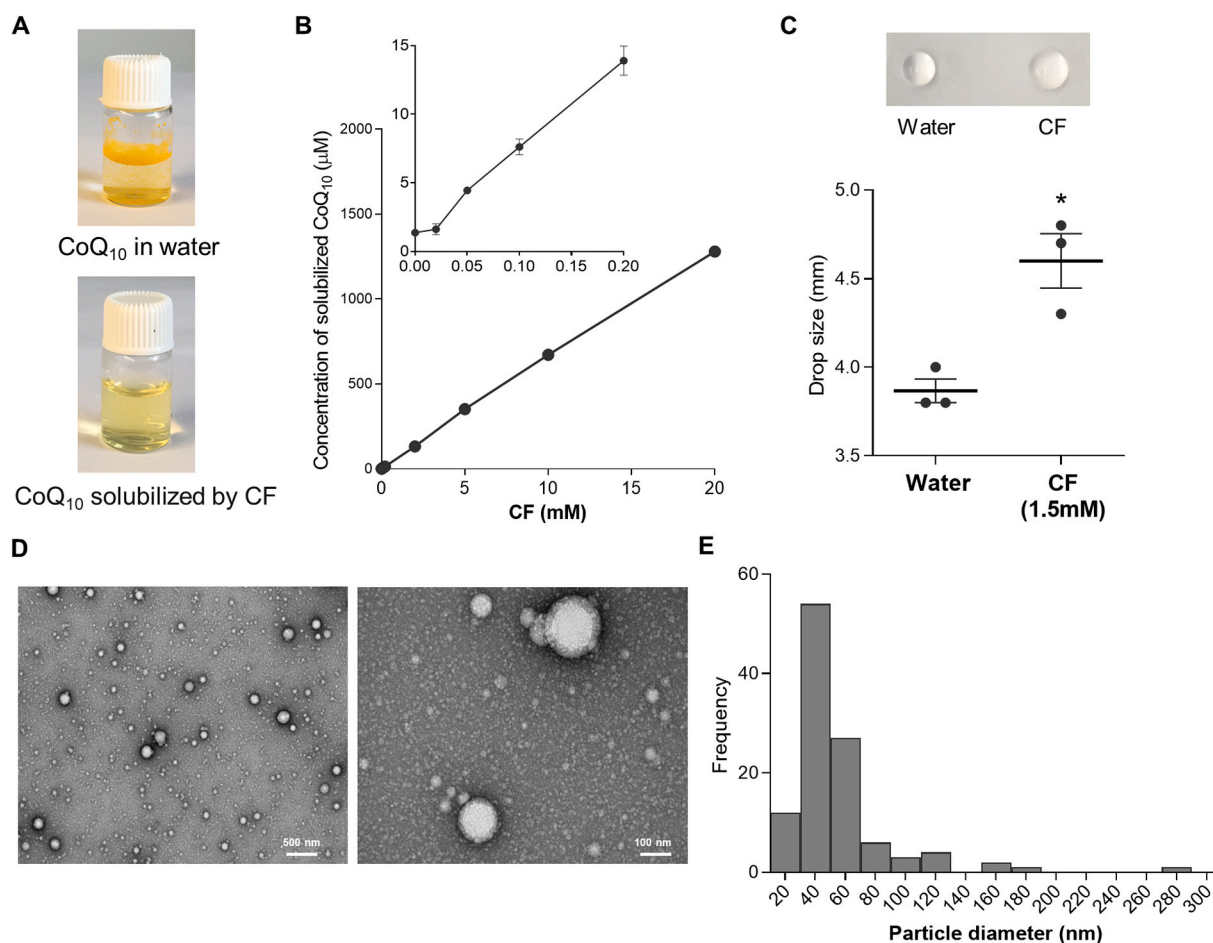
The solubility of CoQ<sub>10</sub> in water is extremely low. Fig. 3A shows that the yellow powder that is pure CoQ<sub>10</sub> cannot dissolve in water. As CF is water-soluble, we added CoQ<sub>10</sub> powder into a CF water solution and mixed by sonicating for ~2 min. The mixture was then filtered through a 0.22  $\mu\text{m}$  sterile filter to remove undissolved material and to sterilize the solution. This resulted in a clear yellow solution with 2–5 mM of CoQ<sub>10</sub> (Fig. S4). The aqueous solubility of CoQ<sub>10</sub> by itself is reported to be around 0.7 ng/ml [66]. Therefore, a CoQ<sub>10</sub> concentration of >2 mM indicates an  $>2.4 \times 10^6$  fold increase of solubility. Fig. 3B shows that the solubilization of CoQ<sub>10</sub> by CF follows a linear dose-dependency. In the drop-collapse test, which is commonly used for rapid determination of wetting properties of a surfactant [67], we observed that CF is surface-active (Fig. 3C). Transmission electron microscopy (TEM) of the

CF/CoQ<sub>10</sub> mix revealed sharp-contrast particles with spherical morphology that have a wide size distribution with a mode of 40 nm (Fig. 3D and E). In contrast, CF by itself produces very few and smaller particles (Fig. S5). Taken together these observations indicate that CF acts as a surfactant that solubilizes CoQ<sub>10</sub> by micellization.

## 2.3. In vitro cellular uptake of CF/CoQ<sub>10</sub> micelles

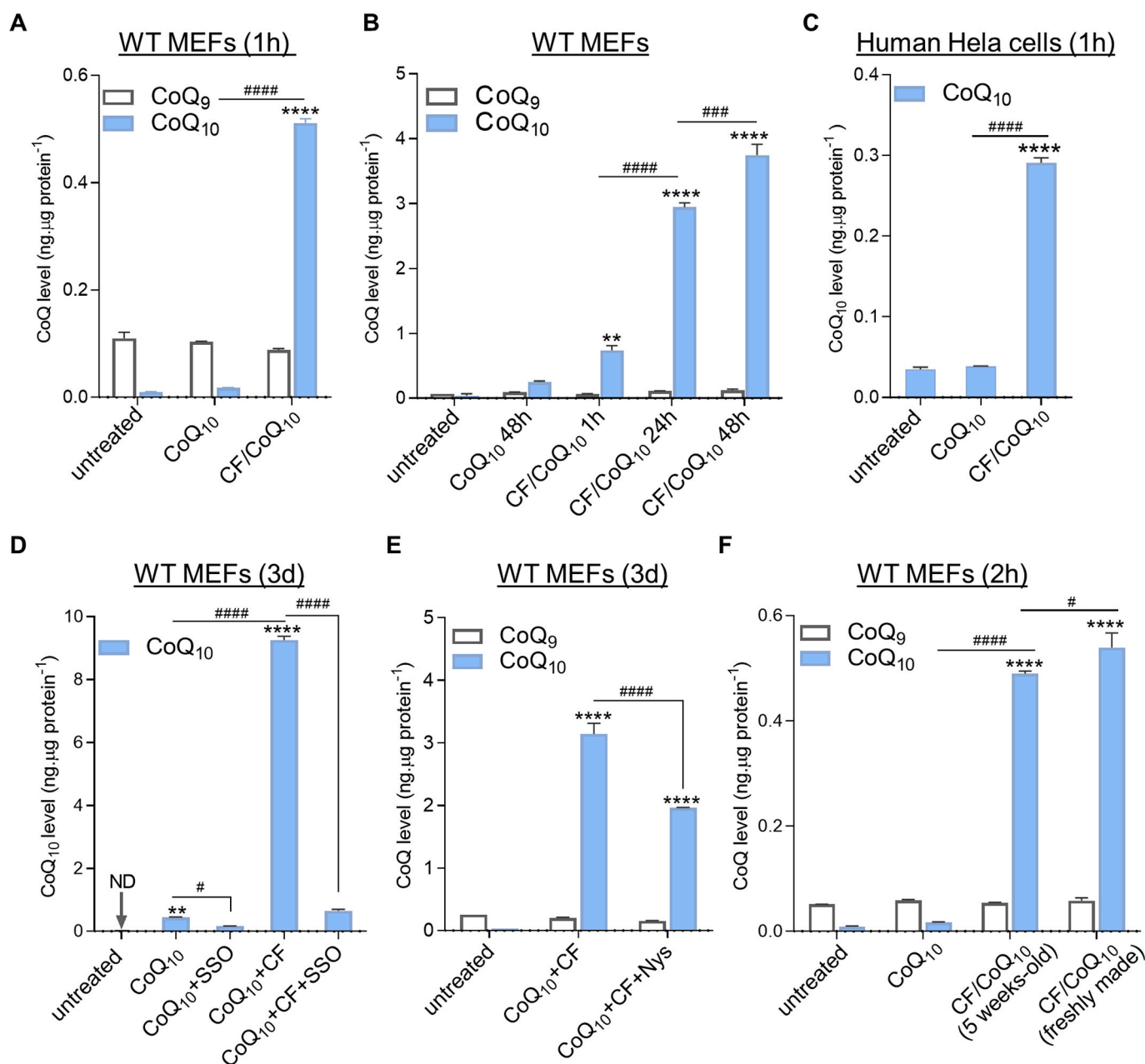
We tested the capacity of CF/CoQ<sub>10</sub> micelles to deliver CoQ<sub>10</sub> into cells and mitochondria. The liquid formulations of CF/CoQ<sub>10</sub> was prepared as described above. To treat cells with CoQ<sub>10</sub> alone, we used a 0.5 mM CoQ<sub>10</sub> stock in ethanol. Fig. 4A shows that 1-h after addition of CF/CoQ<sub>10</sub>, there was already a dramatic increase of CoQ<sub>10</sub> in WT cells, while there was essentially no uptake of free CoQ<sub>10</sub> at the same dose. Furthermore, the total CoQ<sub>10</sub> loading increased with time (Fig. 4B). Quick uptake was also observed in human HeLa cells (Fig. 4C).

We sought to establish the nature of the molecular mechanism that allows for uptake of CoQ<sub>10</sub> via CF/CoQ<sub>10</sub> micelles. The class B scavenger receptor CD36 is a ubiquitous membrane glycoprotein that has been implicated in CoQ uptake by brown adipose tissue in mice [68]. We tested the effect of sulfosuccinimidyl oleate (SSO) which binds



**Fig. 3. CF increases the water solubility of CoQ<sub>10</sub>.** (A) Pictures of CoQ<sub>10</sub> in aqueous solution alone or solubilized by CF. The final concentration of CF and CoQ<sub>10</sub> in the clear yellow solution is 4 mM and 2.2 mg/mL (2.5 mM), respectively. (B) Concentration of solubilized CoQ<sub>10</sub> in aqueous CF solutions of various concentrations. The smaller graph on the upper left zooms over the region where CF concentrations are lower than 0.2 mM. Each point represents the average and SEM of 2 samples (some error bars are not visible due to their small size). In A, the CF/CoQ<sub>10</sub> water solution was prepared by sonication-aided mixing followed by filtration with 0.22  $\mu\text{m}$  filters to remove undissolved material. In B, to better control the mixing force and time, CF and CoQ<sub>10</sub> were mixed by overnight shaking at 220 rpm and insoluble particles were removed by centrifugation prior to quantification of CoQ<sub>10</sub> concentrations. (C) Drop collapse test result. Photos of typical samples are shown, and the graph below presents the average drop size of 3 independent measures. \* $p < 0.05$ , unpaired student's *t*-test. (D) Negative-staining TEM images of CF/CoQ<sub>10</sub> nanoparticles at two different magnifications. (E), Particle size distribution histogram determined from TEM images of a clear mixture of CF and CoQ<sub>10</sub>. See Fig. S5 for TEM images of nanoparticles in a CF-only solution. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)





**Fig. 4. Rapid cellular uptake of CF/CoQ<sub>10</sub> micelles and CoQ accumulation over time.** (A) CoQ levels in WT MEFs after 1-h treatment with free CoQ<sub>10</sub> or micellar CF/CoQ<sub>10</sub> (n = 2). (B) Quantification of time-dependent cellular uptake of CF/CoQ<sub>10</sub> micelles (n = 3). (C) Changes of CoQ<sub>10</sub> levels in human HeLa cells after 1-h treatment with micellar CF/CoQ<sub>10</sub> or free CoQ<sub>10</sub> (n = 2). (D-E) Effects of sulfosuccinimidyl oleate (SSO) or nystatin (Nys) on uptake of CF/CoQ<sub>10</sub> micelles. The dosage for SSO and Nys was 75 μM and 40 μg/mL (43.2 μM), respectively. CoQ<sub>10</sub> levels at the end of 3-day treatment are shown. n = 3. (F) Comparison of CoQ-delivery efficacy between 5-weeks old and freshly made CF/CoQ<sub>10</sub> micelles. WT MEFs were used, and CoQ extraction was carried out after 2-h treatment. For all micelle treatment groups, the micellar solutions of CF/CoQ<sub>10</sub> used to treat cells were prepared by sonication-aided mixing (see Materials and Methods) of 4 mM of CF and excess CoQ<sub>10</sub> powder. CoQ<sub>10</sub> concentration in the solutions varied between 2 mM and 5 mM because they were prepared at different times and sometimes made with different batches of materials. However, the final concentration of CoQ<sub>10</sub> in the treatment media was always 6 μM. Data are mean ± SEM, and one-way ANOVA followed by Tukey's multi-comparisons was used to compare CoQ<sub>9</sub> or CoQ<sub>10</sub> levels between groups. ND: not detected. \*\*p < 0.01 and \*\*\*\*p < 0.0001 (vs. untreated cells). #p < 0.05, ###p < 0.001, and ####p < 0.0001 (comparison of CoQ<sub>10</sub> levels between treatment groups). No difference in CoQ<sub>9</sub> levels was found between any groups.

irreversibly and specifically to the hydrophobic cavity of CD36 [69]. We observed an inhibitory effect of SSO on the small amount of uptake of free CoQ<sub>10</sub> and a very dramatic effect on the large uptake of CF/CoQ<sub>10</sub> micelles (Fig. 4D). Although the inhibition of CoQ<sub>10</sub> uptake by SSO is dramatic, it is not complete (Fig. 4D). We therefore also tested nystatin, which disrupts caveolae-dependent endocytosis [70]. The moderate effect that was observed could imply a small role for caveolae but could also result from toxicity or an indirect effect on CD36 (Fig. 4E). Finally,

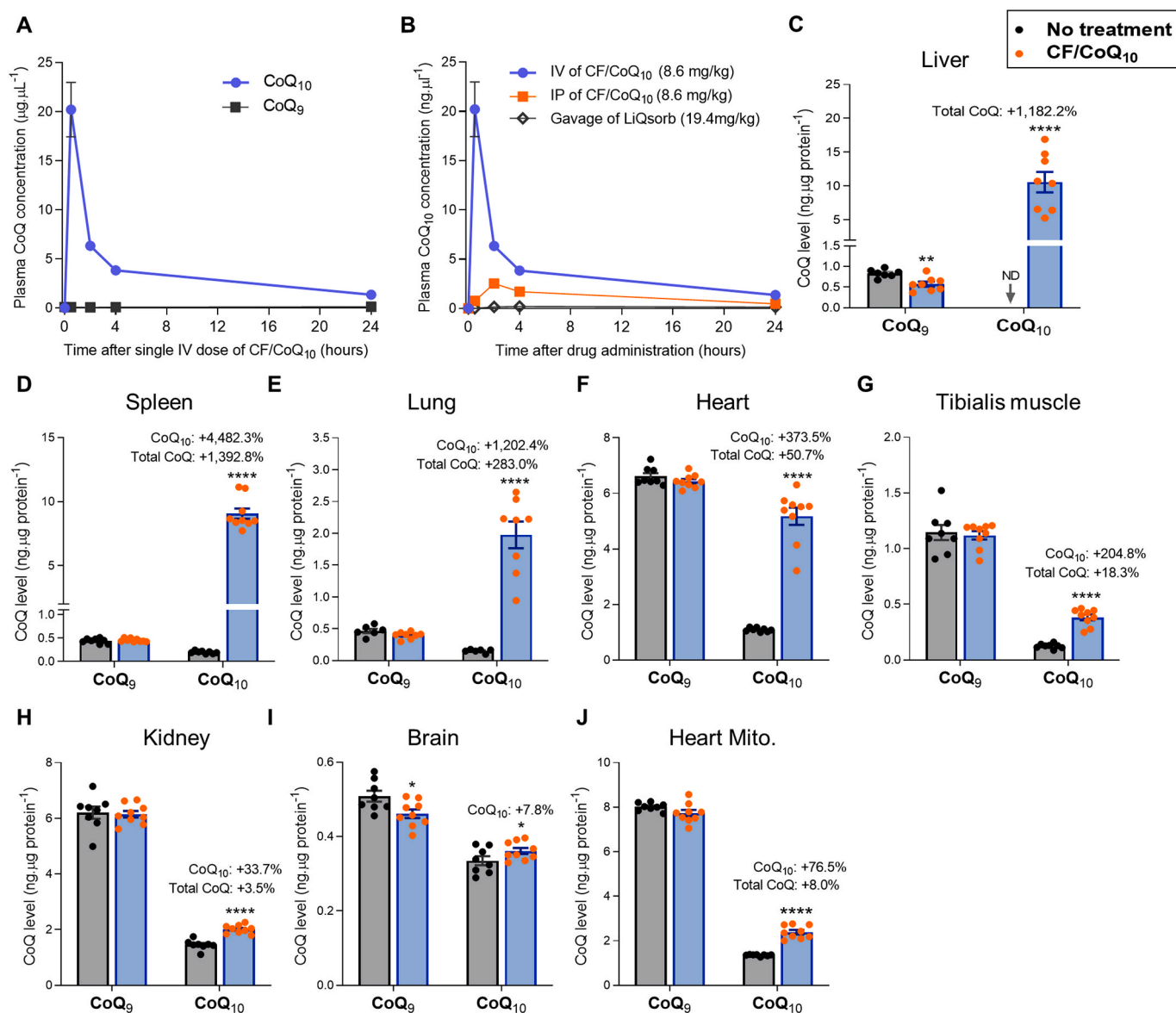
the storage stability of CF/CoQ<sub>10</sub> micelles was studied by comparing the efficiency of CoQ<sub>10</sub> delivery by micellar CF/CoQ<sub>10</sub> stored under different conditions (Fig. 4F and Supplementary Results). The observation of efficient CoQ<sub>10</sub> delivery by CF/CoQ<sub>10</sub> after storage under various conditions, suggesting high physicochemical stability of the micelles.

#### 2.4. In vivo administration of CF/CoQ<sub>10</sub> micelles by intravenous (IV) injection

We injected wild-type mice through the tail vein with micellar CF/CoQ<sub>10</sub> and followed the plasma CoQ concentrations after a single dose of 8.6 mg/kg body weight (BW) of CoQ<sub>10</sub> (Fig. 5A and Fig. S10). The treatment created a dramatic supraphysiological peak of CoQ<sub>10</sub> concentration, followed by fast clearance. At the dose used, the CoQ<sub>10</sub> plasma concentration reached  $20.23 \pm 6.22$   $\mu\text{g}/\text{ml}$  after 30 min, which is > 160 times higher than that of CoQ<sub>9</sub>, the major endogenous CoQ species in mice. A rapid decline in concentration, representing a distribution phase, was observed for the first 4 h after dosing. However, notably, 24 h after dosing, the plasma concentration of CoQ<sub>10</sub> was  $1.37 \pm 0.29$   $\mu\text{g}/\text{mL}$ , that is, still about 10 times higher than that CoQ<sub>9</sub> ( $0.14 \pm 0.01$   $\mu\text{g}/\text{mL}$ ). We also administered CF/CoQ<sub>10</sub> by intraperitoneal (IP) injection for comparison and observed a much lower plasma peak level

of CoQ<sub>10</sub> (Fig. 5B). For a direct comparison of CF/CoQ<sub>10</sub> treatments with an available oral CoQ product, we administered LiQsorb (Tishcon) by oral gavage. LiQsorb is a liposomal formulation of CoQ<sub>10</sub> that we administered at 19.4 mg/kg BW, which is approximately equivalent to the highest safe daily dose for humans (1200 mg/day) [71], and about 1.6 times higher than the IV and IP dose of CF/CoQ<sub>10</sub>. Compared to IV CF/CoQ<sub>10</sub>, the plasma CoQ<sub>10</sub> level increase after the one-time gavage of LiQsorb was excessively small (Fig. 5B).

CoQ<sub>10</sub> tissue uptake was studied by performing single daily injections of CF/CoQ<sub>10</sub> for 10 consecutive days and scoring CoQ 2 h after the last injection. Fig. 5, C-J present the findings in female C57BL/6J mice at a dosage of 12.0 mg/kg BW/day of CoQ<sub>10</sub>. The most dramatic increase of CoQ<sub>10</sub> concentrations was seen in the liver, spleen and lung, with a 3.8- to 14.9-fold increase of total CoQ (CoQ<sub>9</sub>+CoQ<sub>10</sub>) (Fig. 5, C-E). The heart showed a >4.7-fold increase of CoQ<sub>10</sub> and a ~50.7% increase in total CoQ (Fig. 5F). The CoQ<sub>10</sub> increase in skeletal muscle was



**Fig. 5. Plasma and tissue CoQ levels after intravenous administration of CF/CoQ<sub>10</sub>.** (A) Plasma CoQ concentrations versus time after a single tail vein injection of CF/CoQ<sub>10</sub> (8.6 mg of CoQ<sub>10</sub> per kg BW). (B) Plasma CoQ<sub>10</sub> concentrations after a single administration of CF/CoQ<sub>10</sub> (via the tail vein or by IP injection) in comparison to one-time gavage with LiQsorb. n = 4–6 mice per group. (C–J) CoQ concentrations in various tissues or isolated heart mitochondria 2 h after 10 daily injections of CF/CoQ<sub>10</sub> (at a dosage of 12.0 mg of CoQ<sub>10</sub> per kg BW/day). Data are mean  $\pm$  SEM (n = 7–10/group). When not shown, error bars are smaller than the symbol size. \*p < 0.05 and \*\*\*\*p < 0.0001 (Student's *t*-test). The labels above CoQ<sub>10</sub> bars indicate the percentage increase of CoQ<sub>10</sub> and total CoQ levels (CoQ<sub>9</sub>+CoQ<sub>10</sub>) in the treated mice compared to no-treatment control. 10-wk-old female C57BL/6J mice were used in the experiment. ND: not detectable.

~3.0-fold which resulted in ~18.3% higher total CoQ (Fig. 5G). The kidney also showed significant uptake but to a lesser degree: ~33.7% increase of CoQ<sub>10</sub> and ~3.5% increase in total CoQ (Fig. 5H). The smallest (but significant) elevation of CoQ<sub>10</sub> level (~7.8%) was observed in the brain, where the level of endogenous CoQ<sub>9</sub> was lowered and therefore total CoQ levels remained unchanged (Fig. 5I). The low uptake of CoQ<sub>10</sub> might be due to the blood-brain barrier. The low CoQ<sub>9</sub> levels, for which we have no explanation, were not seen with a different mouse strain (see below). For the heart, we extended our analysis to purified mitochondria and observed a ~76.5% increase in the level of CoQ<sub>10</sub> and a ~8.0% increase in total CoQ (Fig. 5J).

To demonstrate reproducibility and possible effects of genetic background, we repeated the 10 injections experiments in a mixed C57BL/6J × 129/Sv background. Our findings were essentially indistinguishable from those made with the pure C57BL/6J background (Fig. S7 A-H). In this background we also scored CoQ in tissues and in heart mitochondria after a single dose injection (8.6 mg/kg body weight of CoQ<sub>10</sub>), and observed elevated CoQ<sub>10</sub> levels in the liver, spleen, lung, heart, and heart mitochondria, but not in other tissues (Fig. S7, I-O). In the tissues in which we observed CoQ<sub>10</sub> uptake after a single injection, the elevation of CoQ<sub>10</sub> was much lower than the elevation in the same tissues after 10 injections. This indicates that repeated injections allow for tissue accumulation of CoQ<sub>10</sub>. On the other hand, the plasma concentrations achieved after 1 or 10 daily injections were very similar (Fig. S8A). This suggests that there is rapid clearance of CF/CoQ<sub>10</sub> micelles from circulation and distribution into tissues. All animal experiments described above were with females. We therefore also tested male C57BL/6J mice with the 10 injections protocol but examined only blood, heart, liver and heart mitochondria for CoQ levels. As with females there was a dramatic elevation of plasma CoQ<sub>10</sub> concentration, a ~3.3-fold increase of CoQ<sub>10</sub> level in the heart, and a ~9.7-fold increase of total CoQ in the liver (Figs. S9A-C). In the heart mitochondria, we observed a ~63.2% increase of CoQ<sub>10</sub> level and a ~8.3% higher level of total CoQ (Fig. S9D).

Intriguingly, in experiments with both females and males, and in both genetic backgrounds, we found that the dramatic rise of CoQ<sub>10</sub> concentration in the plasma resulting from CF/CoQ<sub>10</sub> injection was accompanied by a reduction of plasma CoQ<sub>9</sub> levels (Figs. S8, S9A, and S10). In addition, we observed a small decrease of CoQ<sub>9</sub> in the liver, but in no other tissues, after 10 injections of CF/CoQ<sub>10</sub> in C57BL/6J mice (both male and female) (Fig. 5C and Fig. S9C), but not in the mixed C57BL/6J × 129/Sv background (Fig. S7A).

For a direct comparison with 10 daily injections of CF/CoQ<sub>10</sub>, we administrated LiQsorb by oral gavage daily for 10 days. As shown above, we observed a much smaller increase of plasma CoQ<sub>10</sub> level after one-time gavage of LiQsorb compared to one-time IV injection of CF/CoQ<sub>10</sub>. In fact, we observed a CoQ<sub>10</sub> peak concentration of  $0.20 \pm 0.04 \text{ ng } \mu\text{l}^{-1}$ , 4 h after LiQsorb feeding (Fig. S11A). This is about 100 times less than that detected after a single dose of intravenous CF/CoQ<sub>10</sub> ( $20.22 \pm 6.22 \text{ ng } \mu\text{l}^{-1}$ , 0.5 h after dosing). At tissue level, we found a small uptake only in the liver and spleen after oral feeding of LiQsorb for 10 days (Fig. S11, B and C), and this is a >100-fold lesser uptake compared to IV dosing of CF/CoQ<sub>10</sub> in these organs (Fig. 5C and D). Other tissues examined (heart, kidney, muscle, lung and brain) showed no detectable increase in CoQ<sub>10</sub> after LiQsorb gavage (Fig. S11, D-H). As with CF/CoQ<sub>10</sub> IV treatment, LiQsorb feeding also resulted in a decrease in the level of CoQ<sub>9</sub> in the plasma and liver with no effect in other tissues (Fig. S11, B-I). In the blood, CoQ is thought to be carried in liver-derived lipoproteins which may serve to deliver dietary CoQ into other tissues [72]. Thus, low liver CoQ<sub>9</sub> could result in low serum CoQ<sub>9</sub>. However, how CoQ<sub>10</sub> dosing results in low liver CoQ<sub>9</sub> remains to be investigated.

We sought to demonstrate phenotypic rescue of CoQ deficiency in mice by parenteral delivery of CF/CoQ<sub>10</sub>. We previously showed that complete loss of *Coq7* by induced conditional knockout (KO) in young adults, led to progressive but rapid loss of CoQ, severe ETC dysfunction, and death after ~9 months [73]. Oral CoQ<sub>10</sub> supplementation had no

meaningful effect on the mutant phenotypes [73]. Unfortunately, the KO animals become rapidly too sick to allow for reliable IV treatment. We therefore turned to intraperitoneal (IP) injection to determine if parenteral treatment with micellar CF/CoQ<sub>10</sub> could alleviate their phenotype. We started the IP CF/CoQ<sub>10</sub> treatment (~8.6 mg/kg BW/day) at 4 months following the induction of the *Coq7* KO. At that time, the condition of the animals had already deteriorated significantly but with no mortality [73]. As shown in Fig. 6A, we found that the treated *Coq7* KO mice lived significantly longer than saline-treated KO mice. Blood lactate is a systemic measure of mitochondrial dysfunction. In a separate experiment we therefore scored blood lactate after IP treatment starting ~2 weeks after KO induction. We measured blood lactate after 2 months of treatment and observed significantly lower levels in the treated animals (Fig. 6B). As shown above, single CF/CoQ<sub>10</sub> IP injections are sufficient to increase plasma CoQ<sub>10</sub> level, but to a much lesser degree than by IV. Furthermore, although IP injections resulted in significant CoQ<sub>10</sub> uptake in the liver there was no measurable uptake in the heart after 5 daily injections (see Supplementary Results). Taken together, the positive effects of IP treatment on lactate levels and survival, despite comparatively poor effect on plasma and organ CoQ<sub>10</sub> levels, suggest that profound phenotypic rescue could be obtained by IV CF/CoQ<sub>10</sub>.

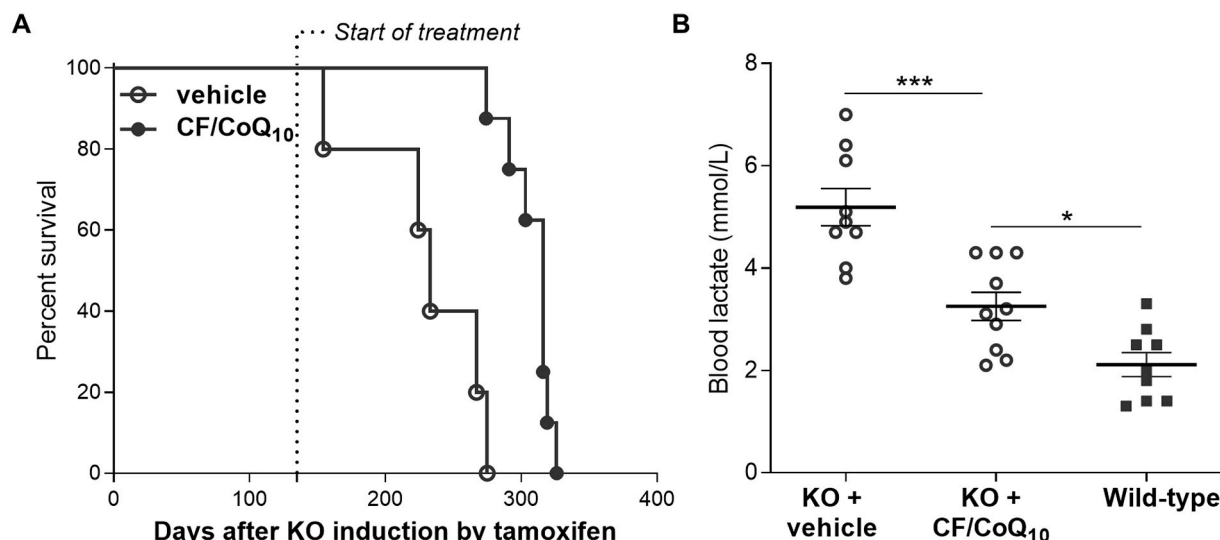
### 2.5. Effects of caspofungin analogs on CoQ<sub>10</sub> solubilization and uptake

CF is the first echinocandin antifungal agent to gain FDA-approval for clinical use. It is a semisynthetic water-soluble lipopeptide composed of a cyclic hexapeptide, of which all six amino acids are hydroxylated, and a branched C<sub>14</sub> fatty acid side-chain (Fig. 2A) [74,75]. There are two other semi-synthetic echinocandin derivatives, anidulafungin (AFG) and micafungin (MFG), which are also approved anti-fungal agents for candidiasis. Structurally, they differ from CF in a number of ways but particularly at the side chains: MFG has an aromatic side-chain (3,5-diphenyl-substituted isoxazole ring side chain) and the side chain of AFG is an alkoxytriphenyl (terphenyl) side-chain [74-76] (Fig. S14). AFG is practically insoluble in water whereas MFG is water soluble. We found that both compounds did not produce drop collapse (Fig. S15A). However, mixing CoQ<sub>10</sub> with MFG by sonication resulted in an increase of CoQ<sub>10</sub> aqueous solubility, while it was ineffective with AFG (Fig. S15B). Both showed no effect on uptake of CoQ<sub>10</sub> from the medium (Fig. S15C), and consistently failed to enhance the rescue of DKO cells in galactose medium supplemented with the minimal, ineffective, amount of CoQ<sub>10</sub> (0.225 μM) (Fig. S15D).

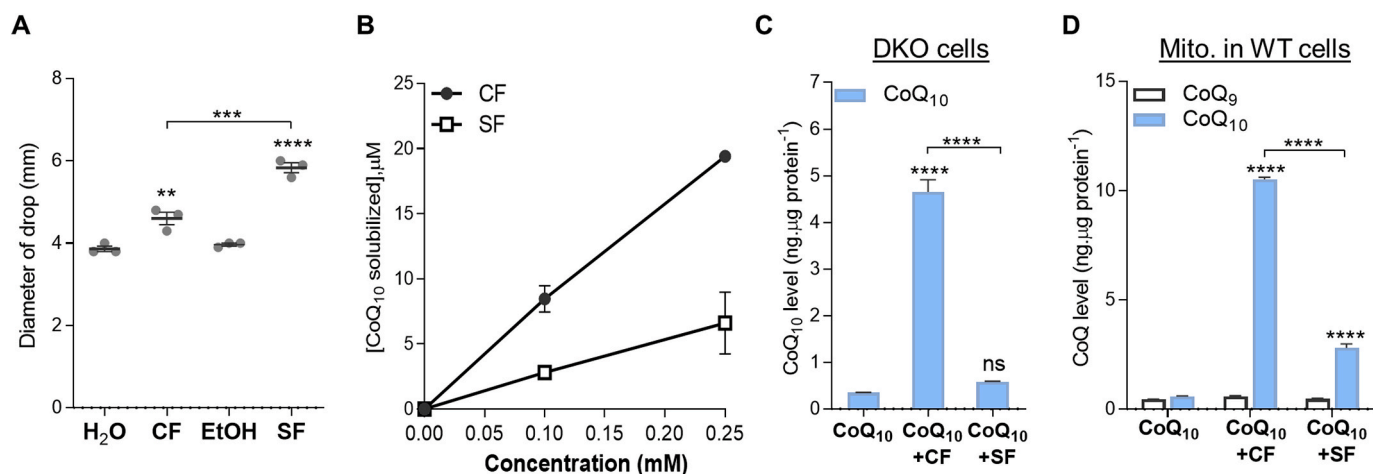
We also tested two other commercially available lipopeptides, daptomycin and surfactin. Daptomycin, which is highly soluble in water and contains a 10-carbon carboxylic acid chain (Fig. S14), showed only a small effect on CoQ<sub>10</sub> uptake (Supplementary Results). Interestingly, although surfactin is a much stronger surfactant than CF (Fig. 7A), it is substantially poorer at solubilizing CoQ<sub>10</sub> (Fig. 7B). We speculate that this is attributable to its very poor aqueous solubility. Although surfactin could enhance CoQ<sub>10</sub> uptake, the effect was much smaller for both cells and mitochondria compared to CF (Fig. 7, C and D). Thus, it appears that CF possesses a unique set of properties by which the micelles it forms with CoQ<sub>10</sub> are stable, have a high CoQ<sub>10</sub> content, can be efficiently taken up by cells, and can release their CoQ<sub>10</sub> once in the cells. We also tested the ability of a dozen other well-known surfactants to solubilize and deliver CoQ<sub>10</sub>, including block copolymers. None compares advantageously with CF. The findings are presented in Supplementary Results.

## 3. Discussion

Despite only moderate surfactant activity (Fig. 3C), CF solubilizes CoQ<sub>10</sub> by forming micellar nanoparticles (Fig. 3D). The CF/CoQ<sub>10</sub> micelles we observed were spherical and mostly smaller than 200 nm in diameter, with a mode of 40 nm (Fig. 3E). A nanoparticle size range of



**Fig. 6.** Effects of intraperitoneal CF/CoQ<sub>10</sub> treatment on *Coq7* KO mice. (A) Kaplan-Meier survival curves comparing CF/CoQ<sub>10</sub>-treated *Coq7* KO mice to vehicle (saline)-treated mutant mice. Administration of CF/CoQ<sub>10</sub> treatment was conducted by intraperitoneal (IP) injection and started at ~ 4 months after induction of the *Coq7* KO ( $n = 5$  for vehicle treatment and  $n = 8$  for CF/CoQ<sub>10</sub> treatment).  $p < 0.001$  by log-rank test. (B) IP administration of CF/CoQ<sub>10</sub> decreases blood lactate concentration in *Coq7* KO mice. IP injection of micellar CF/CoQ<sub>10</sub> started ~2 weeks after completion of induction of *Coq7* KO (by IP injections of tamoxifen) and was performed every day until the mice died. The blood lactate level was measured after 2 months of CF/CoQ<sub>10</sub> treatment. Means  $\pm$  SEM are shown ( $n = 9-10$ ). \* $p < 0.05$  and \*\*\*\* $p < 0.0001$  (one-way ANOVA with a Tukey's multi-comparison test).



**Fig. 7.** Effects of surfactin on CoQ<sub>10</sub> solubilization and uptake. (A) Drop collapse test of surfactin (SF). CF and SF were tested at the same concentration of 1.5 mM \*\* $p < 0.01$ , \*\*\*\* $p < 0.0001$  vs water or ethanol (EtOH) control for CF and SF, respectively; \*\*\* $p < 0.001$  comparing CF and SF (one-way ANOVA and Tukey's multi-comparison test). (B) Aqueous CoQ<sub>10</sub> solubilization as a function of CF or SF concentrations. (C-D) CoQ<sub>10</sub> levels in DKO cells or mitochondrial CoQ levels in WT cells after 3 days of co-treatment of CoQ<sub>10</sub> and CF or SF. The final concentrations of the test compounds in cell medium were: CoQ<sub>10</sub>: 2.5  $\mu$ M, CF: 12.5  $\mu$ M, SF: 12.5  $\mu$ M. Statistical analysis was by one-way ANOVA followed by Tukey's post-hoc test. ns: not significant. \*\*\*\* $p < 0.0001$ . All data are presented as mean  $\pm$  SEM ( $n = 3$ ). Some error bars are concealed by the symbols.

20–200 nm is preferred for IV delivery, as it allows for long circulation times and sufficient extravasation and tissue uptake [77–79]. A critical micelle concentration (CMC) in the low millimolar range is considered desirable in a drug carrier as it indicates a good stability following dilution in the blood after IV administration [80]. The CMC of CF/CoQ<sub>10</sub> micelles is close to 50  $\mu$ M (Fig. 3B). Therefore, CF/CoQ<sub>10</sub> are unlikely to dissociate rapidly due to high dilution upon IV injection.

In fact, our CF/CoQ<sub>10</sub> formulation can be safely administered to mice by IV injection and CoQ<sub>10</sub> reaches all the tissues examined. Following intravenous CF/CoQ<sub>10</sub> at 8.6 mg/kg BW of CoQ<sub>10</sub>, the highest plasma CoQ<sub>10</sub> concentration detected was >160 times higher than the normal level of endogenous CoQ<sub>9</sub> (CoQ<sub>10</sub> was undetectable in our measurements of plasma CoQ from untreated mice) (Fig. 5A and Fig. S10). To the best of our knowledge, the greatest increase in CoQ<sub>10</sub> plasma level

reported upon long-term oral administration was never more than 10 times that of CoQ<sub>9</sub> [81–84]. Note that although CoQ<sub>9</sub> levels were slightly reduced by administration of CF/CoQ<sub>10</sub> (figs. S8, S9A and S10), the effect on CoQ<sub>9</sub> levels were negligible with respect to the total elevation of CoQ (CoQ<sub>10</sub>+CoQ<sub>9</sub>).

In all the tissues examined, including the liver, kidney, heart, skeletal muscle, spleen, lung and brain, we observed a significant increase of CoQ<sub>10</sub> levels after 10 daily IV doses of 8.6–12 mg of CoQ<sub>10</sub>/kg of BW (Fig. 5, C–I and fig. S7, A–G). Uptake in all these organs was cumulative (10 injections produce much more uptake than one-time injection). In the liver, spleen, lung, heart and skeletal muscle, from 18.3% to 1392% higher levels of total CoQ were observed. Further studies are needed to address the mechanism behind the differences in uptake between tissues. Remarkably, an increase in the levels of CoQ<sub>10</sub> and total CoQ was



found in heart mitochondria after only a single IV dosing (Fig. 5G, Fig. S7, H and O, and Fig. S9D). In addition to patient studies suggesting general secondary CoQ deficiency in mitochondrial disorders [48,49,85], a recent animal study demonstrated that mitochondrial dysfunction in the heart leads to low CoQ biosynthesis [51]. Furthermore, cardiac manifestations are prevalent in mitochondrial disease patients [86]. The quick uptake of exogenous CoQ<sub>10</sub> in heart mitochondria provided by CF/CoQ<sub>10</sub> treatment is thus particularly encouraging.

Both CF and CoQ<sub>10</sub> are already used in the clinic and have very favorable safety profiles [53,87]. We did not observe any adverse effects on the mice during the 10-day treatments at ~12.0 mg/kg and ~19.4 mg/kg for CoQ<sub>10</sub> and CF, respectively (Fig. S18). Previous studies with mice showed that oral CoQ<sub>10</sub> supplementation in wild-type animals can result in some uptake in the liver, ovaries and brown adipose tissue but perhaps not in other tissues despite sporadic positive reports [68,73,83,88–90]. In the present study, we directly compared oral feeding with LiQsorb at a ~1.6 times higher dose than that used with IV CF/CoQ<sub>10</sub> and observed only low plasma concentrations (Fig. 5B and Fig. S11A). At tissue level, LiQsorb gavage only increased CoQ<sub>10</sub> in the liver and spleen with a much weaker effect than with IV CF/CoQ<sub>10</sub> even in these organs (Fig. 5C and D, and Fig. S11, B and C). Taken together, our results demonstrate a promise of high delivery and excellent tolerance for intravenous CF/CoQ<sub>10</sub>.

The potential range of uses of CF/CoQ<sub>10</sub> to treat CoQ deficiencies and other diseases remains to be established. How each tissue in an organism lacking sufficient CoQ will react to effective CoQ supplementation is not clear. Some tissue might be refractory to correction of the damage sustained through the deficiency, while in others CoQ might show a disproportional beneficial effect, as has been repeatedly suggested for the kidney [73,91,92]. It is encouraging that treatment of *Coq7* KO mice with CF/CoQ<sub>10</sub> led to increased survival despite the need to use the much less efficient IP route (Fig. 6A).

For use in patients, the CF/CoQ<sub>10</sub> formulation can very likely be further developed and improved. Methods exist to improve micelle drug loading capacity and optimal micelle shapes and sizes can be engineered. Finally, structure-function relationship studies could lead to discovery of CF analogs with further enhanced CoQ<sub>10</sub> solubilization and delivery properties. However, the potential toxicity of a new chemical entity is necessarily unknown, in contrast to CF which is known to be safe.

## 4. Materials and Methods

### 4.1. Cell culture and compound screening

*Pdss2<sup>fl/fl</sup>;Coq7<sup>fl/fl</sup>* MEFs were isolated from E12.5 embryos and cultured in standard DMEM supplemented with 10% (v/v) FBS and 1% (v/v) antibiotic-antimycotic (referred to as glucose medium). To excise the floxed genes, *Pdss2<sup>fl/fl</sup>;Coq7<sup>fl/fl</sup>* MEFs were infected with a pBabe-Puro-Cre retrovirus as previously described [63]. The resulting cells lacking the expression of both *Pdss2* and *Coq7* genes were referred to as DKO cells. *Pdss2<sup>fl/fl</sup>;Coq7<sup>fl/fl</sup>* MEFs infected with empty pBabe-puro retrovirus were used as wild-type control. For use in compound screening, DKO cells were seeded in 96-well plates in glucose medium for 12–24 h. After washing once with phosphate-buffered saline (PBS), medium was switched to DMEM deprived of glucose but supplemented with 10 mM galactose, 10% dialyzed FBS, and 1% antibiotic-antimycotic (referred to as galactose medium). The compounds of the Selleckchem FDA-approved drug library were added at 2 different concentrations (10 and 20 μM) along with or without 0.225 μM CoQ<sub>10</sub>. Cell viability was determined after 2–3 days of culture in galactose medium by resazurin assay.

### 4.2. Preparation of CF/CoQ<sub>10</sub> micelle solution for cell treatments and animal tests

An excess of CoQ<sub>10</sub> powder were added to 4 mM of CF solution in water followed by mixing by sonication for 2–3 min (QSonica XL-2000 sonicator at the power setting of 8–9). The sonication mixture was then passed through a 0.22-μm-pore-size sterile filter to remove undissolved material and to sterilize it for use in cell treatments or animal tests. Filtrate concentration of CoQ<sub>10</sub> was determined from absorbance at 275 nm using a molar extinction coefficient of 14.2 mM<sup>-1</sup>cm<sup>-1</sup>. The filtrate (CF/CoQ<sub>10</sub>) was kept at 4 °C before use.

### 4.3. Electron microscopy

A droplet of CF or CF/CoQ<sub>10</sub> solution was directly deposited onto an electron microscope grid (carbon-coated copper grid) and the samples were allowed to dry for 2 min under ambient conditions. Then, a droplet of 1% uranyl acetate was added on to grids and left standing for 45 s. Observations were performed on a Tecnai 12 BioTwin Transmission Electron Microscope (FEI Electron Optics), operated at 120 kV, using a 40-μm objective aperture. Images were digitized with the use of an AMT XR80C (8 megapixel) charge-coupled device camera and Image Capture Engine Software (version 601).

### 4.4. Mice

C57BL/6 mice were obtained from Charles River Laboratories. *Pdss2<sup>fl/fl</sup>* mice were kindly provided by Dr. David L. Gasser (University of Pennsylvania) [13]. All procedures were approved by McGill University's Animal Care Committee and were in accordance with the ethical guidelines of the Canadian Council on Animal Care. All intravenous injections of CF/CoQ<sub>10</sub> solution via the tail vein were made in a volume of 0.1 ml, which corresponds to 8.6–12.0 mg/kg body weight (BW) per dose. LiQsorb (Tishcon) treatment was given by oral gavage at dose of 19.4 mg/kg BW daily for 10 days.

### 4.5. CoQ detection by high performance liquid chromatography (HPLC)

CoQ quantification was carried out by HPLC, as previously described with some modifications [25,63,93]. An Agilent 1260 Infinity LC system equipped with a quaternary pump (G7111A) and a variable wavelength detector (G7114A) was used. Cells and mouse tissues were thoroughly homogenized in a radioimmunoprecipitation buffer (20 mM Tris-HCl, pH 7.5, 1% NP-40, 0.5% deoxycholate, 10 mM EDTA, 150 mM NaCl) before CoQ extraction. Culture medium (500 μL) and mouse plasma (100 μL) samples were directly used for CoQ extraction with ethanol and hexane (v/v 2/5). Chromatography was carried out on a reverse-phase C18 column (2.1 × 50 mm, 1.8 μm, Agilent) with 70% methanol and 30% ethanol as the mobile phase at a flow rate of 0.3 mL/min. The detector was set at 275 nm. CoQ<sub>9</sub> and CoQ<sub>10</sub> peaks in extracts were identified and quantified using pure CoQ standards. CoQ concentrations in cells or mouse tissues were normalized to the amount of protein measured with BCA assay (Thermo Scientific). The whole procedure was carried out under normal conditions with exposure to air and light. Given that CoQ in the reduced state is highly unstable and is readily oxidized in the presence of oxygen and light, the detection at 275 nm (maximal absorbance of oxidized CoQ), should measure total CoQ.

### 4.6. Statistical analysis

Quantitative data were analyzed by GraphPad Prism Version 8.4.2 (GraphPad Software, Inc.). Comparisons between untreated and treated mice were performed using an unpaired two-tailed Student's *t*-test unless otherwise specified. For multiple comparisons, one-way ANOVA followed by an appropriate post-hoc comparison test was performed. Kaplan-Meier survival data was analyzed by the log rank test. For all

analyses, the level of statistical significance is set at  $p < 0.05$ .

### Declaration of competing interest

The authors have no conflicts of interest to declare.

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### Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.redox.2020.101680>.

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