Delivery of the reduced form of vitamin K$_2$(20) to NIH/3T3 cells partially protects against rotenone induced cell death

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Mitochondria generate energy through the action of the electron transport chain (ETC) and ATP synthase. Mitochondrial malfunction can lead to various disorders, including neurodegenerative diseases. Several reports have shown that menaquinone-4 (MK-4, vitamin K$_2$(20)), a safe drug for osteoporosis, may improve mitochondrial function. Here, we hypothesized that the efficient delivery of menahydroquinone-4 (MKH), an active form of MK-4, could exert a supporting effect. We verified the effects of MKH delivery on mitochondrial dysfunction by using MK-4 and MKH ester derivatives in NIH/3T3 mouse fibroblast cells treated with mitochondrial inhibitors. MK-4 and MKH derivatives suppressed cell death, the decline in mitochondrial membrane potential (MMP), excessive reactive oxygen species (ROS) production, and a decrease in intrinsic coenzyme Q$_9$ (CoQ$_9$) induced by rotenone (ROT, complex I inhibitor). MK-4 and MKH derivatives delivered MKH to NIH/3T3 cells, acting as an effective MKH prodrug, proving that the delivered MKH may reflect the mitigation effects on ROT-induced mitochondrial dysfunction. MKH prodrugs are also effective against 3-nitropropionic acid (3-NP, complex II inhibitor) and carbonyl cyanide-m-chlorophenylhydrazone (CCCP, uncoupler)-induced cell death. In conclusion, MKH delivery may mitigate mitochondrial dysfunction by maintaining MMP, ROS, and CoQ$_9$, indicating that MKH prodrugs may be good candidates for treating mitochondrial disorders.

Mitochondria are integral for normal cell functioning as they generate the ATP required to maintain vital cell function in the respiratory chain behaving as “powerhouses of the cell.” Mitochondrial quality control and dysfunction are also implicated in the production of reactive oxygen species (ROS), regulation of cell death, and etiology of neurological disorders such as Huntington’s disease, Parkinson’s disease, and Alzheimer’s disease. Therefore, focusing on mitochondrial function is necessary to elucidate the etiology of diseases for which there are still no effective treatment strategies and advance effective drug development.

Mitochondria are organelles with a double-membrane structure containing five complexes in the inner membrane. In complexes I–IV (electron transport chain: ETC), protons are pumped into the intermembrane space during electron transport. The concentration gradient of protons creates a mitochondrial membrane potential (MMP), which is used by complex V (ATP synthase) (Fig. 1). During the process of ATP production, the leaked electrons from the ETC generates ROS, while a reduced form of coenzyme Q (CoQ), known as a representative antioxidant in the ETC, protects against mitochondrial damage caused by ROS as a part of homeostasis mechanism. However, the production of excess ROS over the defense system in problematic situations causes oxidative stress and decreases membrane potential, resulting in an inability to produce energy and mitochondrial dysfunction. Rotenone (ROT), a major complex I inhibitor, interferes with the conversion of CoQ to reduced CoQ (CoQH$_2$) by binding to NADH dehydrogenase, which is part of complex I. As a result, electrons flow back, and ROS are generated, leading to cell death. Therefore, ROT has been widely used in experimental models to explore drug effectiveness against mitochondrial dysfunction. 

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Menaquinone-4 (MK-4, vitamin K2(20)) has been clinically used as a therapeutic agent for osteoporosis in Japan, and its long-term safety has been confirmed. It has been reported that MK-4 suppresses neuronal cell death mediated by ROT-induced microglial activation. However, a recent study showed that vitamin K is not a substitute for CoQ10. Other studies have reported that MK-4 can act as a mitochondrial electron transporter. Thus, whether MK-4 is effective in mitochondrial dysfunction remains controversial. MK-4 cannot act as a cofactor for γ-glutamyl carboxylase (GGCX) for the post-translational modification of vitamin K-dependent proteins (VKDP) until MK-4 is reduced to menaquinone-4 (MKH)18. MKH is stoichiometrically converted to MK-4 epoxide (MKO) when it acts as a cofactor for GGCX (Fig. 2). Since MKH has a strong reduction capability, MKH but not MK-4 functions as a ROS scavenger. As a result, it would exert a protective effect against mitochondrial ROS. It has also been revealed that MKH is supplied not only by the reduction of exogenous MK-4 but also by biosynthesizing vitamin K1 by UBIAD119 and has been reported to be highly distributed in the brain in the form of MK-420. Therefore, if the efficacy of MK-4 is proven, it is speculated that our study will contribute to the development of safe and effective drugs for intractable diseases such as neurodegenerative disorders. However, MK-4 may not be well delivered to cells.

In our laboratory, we synthesized two MKH ester derivatives, MKH 1,4-bis-N,N-dimethylglycinate (MKH-DMG) and MKH 1,4-bis-hemi-succinate (MKH-SUC), which overcome the shortcomings of MK-4. In the present study, we aimed to verify whether MKH delivered by MKH prodrugs affected ROT-induced mitochondrial dysfunction and cell death. We have previously reported that MKH-DMG and MKH-SUC can act as effective MKH prodrugs in vitro and in vivo. We have also shown that their MKH delivery via intracellular hydrolysis activation is independent of the reductive activation pathway for MK-4. In this study, we evaluated the influence of MKH prodrugs on cell death, decrease in MMP, and intracellular CoQ levels following exposure to mitochondrial inhibitors, mainly ROT, in NIH/3T3 cells.

Results
MK-4 and MKH derivatives mitigate ROT-induced cell death. To verify whether MK-4 and MKH derivatives affect ROT (complex I inhibitor)-induced cell death in NIH/3T3 cells, cell viability was assessed using the CellTitre-Blue® (CTB, G8080, Promega Japan, Tokyo, Japan) reagent. As preliminary tests, the cytotoxicity of ROT, MK-4, or MKH derivatives to NIH/3T3 cells was evaluated. ROT treatment (0.01, 0.1, 1, and 10 µM for 24 h) induced a dose-dependent decrease in cell survival rate. The maximum reduction rate in cell viability was approximately 40% of the vehicle group (0.1% dimethylsulfoxide: DMSO) at the highest dose in the 0.01–10 µM dose range (Supplementary Fig. S1). In the absence of ROT, treatment with MK-4 and MKH derivatives for 24 h did not affect cell viability at concentrations of up to 3 µM (Supplementary Fig. S1). Based on the results of the preliminary cytotoxicity tests, the concentrations of the drug treatments were determined as follows: ROT, 10 µM; MK-4 and MKH derivatives, 0.03, 0.3, and 3 µM each. As shown in Fig. 3, the cell viability in the groups treated with MK-4 and MKH derivatives at 0.3 and 3 µM was higher than that with ROT only, while 0.03 µM MK-4 and MKH derivatives did not affect ROT-induced cell death. At 0.3 µM the effects of MK-4 and MKH-SUC treatments reached a plateau, while that of MKH-DMG treatments peaked at 3 µM (Fig. 3). These results showed that the MK-4 and MKH derivatives mitigated ROT-induced cell death.

MK-4 and MKH derivatives alleviate ROT-induced dysfunction of mitochondria. To observe the mitochondrial state before ROT-induced cell death occurs, MMP was evaluated by JC-1 staining. The changes
Figure 2. Schematic illustration of the vitamin K cycle and concept of the menahydroquinone-4 delivery system. MKH, menahydroquinone-4; MK-4, menaquinone-4; MKO, menaquinone-4 epoxide; VK, vitamin K; VKDP, vitamin K-dependent protein; GGCX, γ-glutamyl carboxylase.

Figure 3. Influence of MK-4 and MKH derivatives on ROT-induced cell death. The NIH/3T3 cells were treated with 0.03–3 μM MK-4, MKH-DMG, or MKH-SUC for 24 h in the presence of 10 μM ROT. Cell viability was determined using the CTB assay. ***p < 0.001 versus vehicle group (0.1% DMSO + 0.1% ethanol); ###p < 0.001 versus ROT only group (Tukey’s test). Mean ± SD (n = 3).
in fluorescence from red to green of JC-1 showed a reduction in MMP. Treatment with 10 µM ROT for 6 h decreased the red fluorescence intensity compared to that in the vehicle group (0.1% DMSO + 0.1% ethanol), as shown in Fig. 4a. The red/green fluorescence intensity ratio was reduced by ROT, as shown in Fig. 4b, indicating mitochondrial dysfunction. Treatment with all three MK-4 and MKH derivatives alleviated the ROT-induced MMP reduction, as shown in Fig. 4a,b. These data indicated that MK-4 and MKH derivatives protected NIH/3T3 cells from ROT-induced mitochondrial damage.

MK-4 and MKH derivatives suppress ROT-induced ROS. ROT is believed to induce ROS in the mitochondria by blocking complex I in the respiratory chain. Therefore, to confirm the influence of MK-4 and MKH derivatives on ROT-induced ROS production, intracellular ROS levels were measured using 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) reagent. The intracellular ROS levels were standardized by the number of living cells, as indicated in Fig. 5. The ROS level in the ROT-only group was 1.7-fold higher than that in the vehicle group. In contrast, the levels of all three MK-4 and MKH derivatives were lower than those of the ROT-only group, indicating similar levels to that of the vehicle group. These data indicated that MK-4 and MKH derivatives maintained intracellular ROS levels in the normal state, even in the presence of ROT.

MK-4 and MKH derivatives suppress ROT-induced heme oxygenase-1 expression. Heme oxygenase-1 (HO-1) is induced by such as ROS and inflammatory cytokines. To evaluate the effect of ROT-induced ROS on the amount of HO-1 expression, real-time PCR and western blotting were performed. HO-1 gene expression increased eightfold in response to ROT compared to vehicle treatment (Fig. 6a). In contrast, the HO-1 gene expression levels of MK-4 and MKH derivatives were 2.7- to 4.0-fold lower than that of ROT (Fig. 6a). HO-1 protein expression was clearly increased by ROT, and the MK-4 and MKH derivatives suppressed ROT-induced HO-1 expression (Fig. 6b). The whole blot image can be found in Supplementary Figure S2. These data show that MK-4 and MKH derivatives suppress ROT-induced HO-1 gene and protein levels, supporting their suppressive effect on ROT-induced ROS.

MK-4 and MKH derivatives deliver MKH to NIH/3T3 and suppress ROT-induced coenzyme Q9 decrease. To confirm MKH delivery by MK-4 and MKH derivatives and to investigate the effect of ROT-induced mitochondrial damage on the endogenous coenzyme \( Q_9 \) (\( \text{CoQ}_9 \))/reduced coenzyme \( Q_9 \) (\( \text{CoQ}_9 \text{H}_2 \)) balance, we measured intracellular MK-4, MKO, \( \text{CoQ}_9 \), and \( \text{CoQ}_9 \text{H}_2 \) levels using LC–MS/MS. MKH cannot be measured directly because it is easily oxidized to MK-4 in presence of air. The MKO level reflects the amount of MKH delivered due to the conversion of MKH to MKO, which acts as a cofactor for the post-translational modification of VKDP by GGCX. In the vehicle group, neither MK-4 nor MKO was detected. Intracellular MKO was observed in the drug-treated groups, indicating that both the MK-4 and MKH derivatives functioned...
as MKH prodrugs. After 6 and 24 h of drug treatment, intracellular MKO levels were higher than intracellular MK-4 levels at every time point. The maximum intracellular drug level of MK-4 was approximately 0.05 nmol/mg protein, and that of MKO was approximately 0.4 nmol/mg protein (Fig. 7a,b). These results suggested that the delivered MKH was efficiently utilized in the VKDP production process. The delivery of MKH by MK-4 and MKH derivatives was in the order MKH-SUC > MK-4 > MKH-DMG 6 h after drug treatment (Fig. 7a). After 24 h of treatment, the order of MKH delivery was MKH-DMG > MK-4 ≈ MKH-SUC (Fig. 7b). These results indicated that the ester hydrolysis of MKH-SUC in cells is fast, whereas that of MKH-DMG is steady.

In the CoQ9H2 measurement, a part of the CoQ9H2 was oxidized to CoQ9 during the extraction process. Although oxidation could not be completely prevented, extraction was carried out promptly and simultaneously for all samples. The intracellular CoQ9 level in the ROT-treated group at 6 h decreased compared to that in the vehicle group and reached 10% of the vehicle group after 24 h. Simultaneous treatment with MK-4 or MKH derivatives significantly suppressed the ROT-induced decrease in intracellular CoQ9 level, which was about 70–90% of the vehicle group after 24 h (Fig. 7c,d). On the other hand, the amount of CoQ9H2 detected was approximately 5–10% of that of CoQ9. ROT treatment markedly reduced CoQ9H2 after 6 h, but the MK-4 and MKH derivatives slightly affected the low levels of CoQ9H2 (Fig. 7e,f).

**Figure 5.** Influence of MK-4 and MKH derivatives on ROT-induced ROS. The NIH/3T3 cells were treated with 3 µM MK-4, MKH-DMG, or MKH-SUC for 6 h in the presence of 10 µM ROT. Intracellular ROS levels were determined by DCFH-DA staining. The relative luminescence unit (RLU) obtained using the DCFH-DA reagent was standardized by RLU from the CTB assay as the living cell number. ***p < 0.001 versus vehicle group; ###p < 0.001 versus ROT only group (Tukey’s test). Mean ± SD (n = 3).

**Figure 6.** Influence of MK-4 and MKH derivatives on HO-1 induced by ROT. The NIH/3T3 cells were treated with 3 µM MK-4, MKH-DMG, or MKH-SUC for 6 h in the presence of 10 µM ROT. (a) HO-1 mRNA level obtained by qPCR. (b) HO-1 protein was detected by western blotting. ***p < 0.001 versus vehicle group; ###p < 0.001 versus ROT only group (Tukey’s test). Data are presented as mean ± SD (n = 3).
MK-4 and MKH derivatives protect against 3-NP- and CCCP-induced cell death but not against AA and OA. To elucidate the protective effects of MK-4 and MKH derivatives on other respiratory chain complex inhibitors; 3-nitropropionic acid (3-NP, complex II inhibitor), antimycin A (AA, complex III inhibitor), oligomycin A (OA, complex V inhibitor), and carbonyl cyanide m-chlorophenyl hydrazone (CCCP, uncoupler) were used. Cell viability was measured using CTB reagent, and the concentrations of all inhibitors used in the experiment were determined by preliminary experiments (Supplementary Fig. S3). Each of the inhibitors,
3-NP, AA, OA and CCCP, reduced the cell viability to 50%, 80%, 20%, and 20% of the vehicle group, respectively (0.1% ethanol) (Fig. 8). The MK-4 and MKH derivatives effectively mitigated the cytotoxicity of 3-NP and CCCP in a dose-dependent manner (Fig. 8a,d), whereas they did not affect AA- and OA-induced cell death (Fig. 8b,c).

Discussion
For vitamin K to exert pharmacological effects in post-translational modification of VKDP, antioxidation, and ROS scavenging, its naphthoquinone scaffold must be converted to naphthohydroquinone (a two-electron reduced form). It has been revealed that MKH (hydroquinone form of MK-4) is supplied not only by the reduction of exogenous MK-4 but also by biosynthesizing from vitamin K1 by UBIAD119, and it has been reported that MKH is highly distributed in the brain in the form of MK-4 20. To test the hypothesis of whether MKH is effective in mitochondrial dysfunction, which may correspond to various pathologies, such as Alzheimer’s disease and Parkinson’s disease, we investigated the effects of MK-4 and MKH derivatives as prodrugs of MKH on mitochondrial inhibitor-induced cytotoxicity in vitro. Vos et al. reported that MK-4 is responsible for ETC in Drosophila experiments16; however, Cerqua et al. showed that MK-4 is not a substitute for CoQ10 in mammalian cells15. Thus, whether MKH contributes to electron transport remains controversial.

NIH/3T3 cells are derived from mouse fibroblasts and are widely used for assessing basic biological effects such as cytotoxicity and elucidation of molecular mechanisms25,26. We employed NIH/3T3 cells to evaluate the essential effects of MKH derivatives on mitochondria. In his study, MK-4 and MKH derivatives showed a partial suppressive effect on NIH/3T3 cell death and mitochondrial dysfunction induced by mitochondrial inhibitors of complex I, II (ROT, 3-NP) and uncoupler (CCCP). Moreover, MK-4 and MKH derivatives acted as MKH prodrugs in NIH/3T3 cells and suppressed excessive ROS production and loss of endogenous CoQ9 induced by ROT. Based on these results, it is speculated that MKH is a key player in mitigating mitochondrial dysfunction induced by mitochondrial inhibitors.

The effect and delivery of MKH-DMG were slower and milder than those of MK-4 and MKH-SUC (Figs. 3, 7a,b). Our previously published reports show a correlation between intracellular MKH delivery and efficacy, consistent with our results23,24. If MKH delivery is important for its suppressive effect on mitochondrial dysfunction,
it can be expected that the efficacy of MKH-DMG at low therapeutic concentrations will be lower than those of MK-4 and MKH-SUC. Therefore, it seems reasonable that the suppressive effects of MKH-DMG on the increase in cell death induced by mitochondrial inhibitors were lower than those of MK-4 and MKH-SUC (Figs. 3, 8a,d). It can be expected that ROS quenching or electron transport in complex I instead of CoQ. Menke et al. reported that ROT-exposed cells to stress (Fig. 6), and we speculate that it is because of ROS. MK-4 and MKH derivatives significantly suppressed the decrease in MMP, increase in ROS production and increase in HO-1 expression (Figs. 4, 5, 6). Therefore, we hypothesize that MK-4 and MKH derivatives relieved stress on cells by suppressing ROS overproduction, resulting in reduced MMP depletion and cell death.

CoQ₉ (oxidized form) and CoQ₉H₂ (two-electron reduced form) are electron transporters in the mitochondria, and CoQ₉ is more abundant than CoQ₁₀ in rodents. NADH dehydrogenase in complex I activate the ETC by reducing CoQ₉ → CoQ₉H₂. The MK-4 and MKH derivatives maintained endogenous CoQ, even in the presence of ROT (Fig. 7c,d). The ratio of CoQ₉H₂/CoQ₉ in NIH/3T3 cells in the vehicle group was low (Fig. 7c,e). To our knowledge, although no data indicating the ratio in NIH/3T3 cells have been found, low ratios have been observed in certain tissues, excluding the liver, adipose, and plasma. Previous reports have demonstrated that ROT and MKH dehydrogenase inhibitor, increases the ratio of CoQ₉H₂/CoQ₉ within a short period of drug treatment. In the present study, ROT treatment decreased not only CoQ₉H₂ (Fig. 7c,f) but also CoQ₉, particularly at 24 h. In conclusion, that the absolute amount of CoQ₉ was lost due to ROT-induced mitochondrial collapse. Remarkably, CoQ₉H₂ remained low despite the high CoQ₉ levels maintained by MK-4 and MKH derivatives (Fig. 7d,f). This result suggests that ROT strongly blocks the reduction of CoQ₉ to CoQ₉H₂, independent of normal CoQ₉ levels.

The following chemicals were obtained commercially: rotenone (R8875), indole-3-acetic acid (IAA), 3-nitropropionic acid (N5636), and oligomycin A (AG-CN2-0517) from AdipoGen Life Sciences, Inc. (San Diego, CA, US); carbonyl cyanide m-chlorophenylhydrazone (CCCP; 034-16993) from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan); and coenzyme Q₉ (CoQ₉) from Cayman Chemical (Ann Arbor, MI, USA). The following antibodies were used: heme oxygenase 1 (HO-1) from Cell Signaling Technology (Danvers, MA, USA) and monoclonal mouse anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) from Sigma-Aldrich.
Cell culture. The mouse fibroblast-like cell line NIH/3T3 (RCB2767) was obtained from the RIKEN BioResource Research Center (Ibaraki, Japan). Cells were maintained in Dulbecco’s Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F-12; Thermo Fisher Scientific, Waltham, MA, USA) containing 10% fetal bovine serum (FBS; HyClone Standard Fetal Bovine Serum Collected and Processed in USA, Cytiva, Tokyo, Japan) and 1% penicillin/streptomycin (Thermo Fisher Scientific) at 37 °C under humidified 5% CO₂ atmosphere.

Cell viability assay. Cell viability was assessed using the CellTiter-Blue® (CTB, Promega) by the following cell viability assay method. NIH/3T3 cells were plated at a density of 1.0 × 10⁵ cells per well in 96-well black plates and allowed to attach for 24 h. Then, drug media, including 10 μM of ROT and 0.03–3 μM of MK-4, MKH-DMG, or MKH-SUC at the different concentrations indicated in the related result section, were exposed for 24 h. Fluorescence signals as cell viability were measured using a microplate reader (Infinite M200 PRO, Tecan, Kanagawa, Japan) according to the manufacturer’s instructions.

Measurement of the mitochondrial membrane potential. MMP in cells was assessed using the JC-1 MitoMP Detection Kit (Dojindo Lab, Kumamoto, Japan) according to the manufacturer’s instructions. JC-1 accumulated in mitochondria forms red fluorescent aggregates at high membrane potentials, whereas JC-1 monomers show green fluorescence, indicating low membrane potentials. For the analysis of MMP, NIH/3T3 cells were cultured in a collagen-I-coated 96-well black plate at a density of 1.0 × 10⁴ cells/well or in a collagen-I-coated glass dish (IWAKI, AGC TECHNO GLASS Co. Ltd, Shizuoka, Japan) at a density of 2.5 × 10⁵ cells/dish. The cells were allowed to adhere for 24 h. The cells were then treated with MK-4, MKH-DMG, or MKH-SUC in the presence of ROT for 6 h. Then, the cells were stained with JC-1 (1 mg/mL in DMSO) and incubated at 37 °C for 30 min in the dark. The cells were washed twice with serum-free media, visualized under a fluorescence microscope (BZ-X810, KEYENCE, Osaka, Japan), and quantified using a microplate reader (Infinite M200 PRO). The obtained ROS levels were standardized by cell viability using the CTB reagent, as described in the Cell Viability Assay section.

Intracellular ROS measurement. Intracellular ROS levels were examined using DCFH-DA (Thermo Fisher Scientific). The DCFH-DA probe is rapidly oxidized by ROS and converted into fluorescent 2′,7′-dichlorofluorescin (DCF). The DCF fluorescence intensity is proportional to the ROS level in the cytoplasm. Briefly, NIH/3T3 cells were seeded at a density of 1.0 × 10⁵ cells/well in a collagen-I-coated 96-well black plate and allowed to adhere for 24 h. The cells were treated with MK-4, MKH-DMG, or MKH-SUC in the presence of ROT for 6 h. The cells were washed once with serum-free media and incubated in 100 μL of 10 μM DCFH-DA for 30 min in the dark at 37 °C. After incubation, intracellular fluorescence was measured at excitation and emission wavelengths of 485 and 530 nm, respectively, using a microplate reader (Infinite M200 PRO). The obtained ROS levels were standardized by cell viability using the CTB reagent, as described in the Cell Viability Assay section.

Real-time quantitative PCR. Total RNA was extracted from cultured cells using a High Pure RNA Isolation Kit (Roche Diagnostics K.K., Tokyo, Japan). cDNA was reverse-transcribed using the ReverTra Ace-a-reverse transcription kit (Toyobo, Shanghai, China), and quantification was performed using LightCycler® 480 SYBR Green I Master (Roche Diagnostics K.K.). The sequences of the primers used were as follows: heme oxygenase-1 (HO-1), forward primer: 5′-CAGTCGGAGATGACACCTGAG-3′; reverse primer: 5′-GTGTTTCCTCTGTCAGCATGCACC-3′; beta-2 microglobulin (B2M), forward primer: 5′-ACGAGACAGTTTCAGATGTTCG-3′; reverse primer: 5′-GGTTCTTTCTGGTCTGGTCT-3′. B2M was used as an internal control.

Western blot analysis. NIH/3T3 cells were seeded at a density of 4.0 × 10⁵ cells/dish in a collagen-I coated 60 mm dish and treated with 10 μM ROT with or without 3 μM MK-4, MKH-DMG, or MKH-SUC for 6 h. Cultured cells were washed with ice-cold PBS and lysed in RIPA buffer (0.5% NP-40, 0.25% sodium deoxycholate, 0.05% SDS, 150 mM NaCl, and 50 mM HEPES, pH 7.4) containing a protease inhibitor cocktail (Nacalai Tesque, Kyoto, Japan) on ice. A plastic cell scraper was used to scrape the adherent cells. Cell lysates were then clarified by centrifugation at 20,600 × g at 4 °C for 15 min, and the supernatant was collected. The total protein concentration was determined using the Pierce ™ BCA protein assay kit (Thermo Fisher Scientific). Proteins (every 10 μg) were separated by SDSPAGE using Super Sep ™ Ace 15% 13-well gels (FUJIFILM Wako) and transferred onto PVDF membranes (Bio-Rad, Hercules, CA, USA). The membrane was blocked using Blocking One solution (Nacalai Tesque) and incubated with anti-HO-1 (1:1,000) and anti-GAPDH antibodies (1:10,000), respectively, at 25 °C for 1 h. After washing, the membranes were treated with the appropriate secondary antibodies and visualized using Immunostar LD (FUJIFILM Wako). Protein expression was analyzed using the ImageJ software (version 1.53 k).

Determination of intracellular MKO, MK-4, CoQ₉, and CoQ₉H₂. NIH/3T3 cells were seeded at 3.0 × 10⁵ cells/well in 6-well plates and allowed to attach for 24 h. Next, the cells were cultured in a medium with or without 3 μM MK-4, MKH-DMG, or MKH-SUC in the presence of 10 μM ROT. The medium was then removed, and the cells were washed twice with PBS. The cells were collected in 1 mL PBS and sonicated on ice. Cell homogenates were combined with an equal volume of ethanol and three times the volume of n-hexane, vortexed for 2 min, and centrifuged at 1750 × g for 10 min. The organic layer was evaporated using N₂ gas. The residue was reconstituted with 200 μL of ethanol and analyzed using LC–MS/MS, as described below. The protein concentration in the cell homogenate was determined using a BCA protein assay kit (Thermo Fisher Scientific).
LC–MS/MS. LC–MS/MS was performed using an LC–MS-8060 liquid chromatography-mass spectrometer (Shimadzu, Kyoto, Japan) and a Shimadzu UFLC System (Shimadzu, Kyoto, Japan). Separations were performed on a Shim-pack XR-C8 column (ϕ 2.2 μm, 3 × 75 mm, Shimadzu) using a mobile phase comprising 10 mM ammonium acetate and 0.1% acetic acid in methanol (pump A) and ethanol (pump B) under gradient elution, at a flow rate of 0.2 mL/min. A binary gradient was established as follows: (A) 10 mM ammonium acetate and 0.1% acetic acid in methanol and (B) 10 mM ammonium acetate and 0.1% acetic acid in ethanol; 30% B at 0 min, 30% B at 2.0 min, 70% B at 10.01 min, 70% B at 12.00 min. The column temperature was maintained at 40 °C. The mass spectrometer was equipped with an electrospray ionizer and operated in the positive ion mode. Identification and quantitation were performed under the MS/MS-multiple reaction monitoring (MRM) mode, using the following transition ions: m/z 461.0 → 81.0 for the [M + H]⁺ MKO adduct; m/z 445.0 → 187.0, for the [M + H]⁺ MK-4 adduct; m/z 796.2 → 197.0, for the [M + NH₄]⁺ CoQ₉H₂ adduct; and m/z 445.0 → 187.0. Retention times were as follows: MKO, 3.5 min; MK-4, 3.9 min; CoQ₉H₂, 5.5 min; and CoQ₉, 7.4 min.

Statistical analysis. Comparisons among groups were performed by one-way ANOVA with Tukey’s test, and the analyses were carried out using GraphPad Prism 6 (GraphPad Software, San Diego, CA, USA); results with p < 0.05 were considered significant.

Data availability
The datasets generated during and/or analyzed during the current study are available from the corresponding author upon reasonable request.

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Author contributions
J.T. designed this research. E.T. performed the experiments with assistance from S.S., K.T., D.W., A.Y., M.K., and H.Y. Moreover, K.M., S.S., S.G., and E.T. analyzed the data, E.T. wrote the manuscript, K.M., S.S., S.G, and K.K. helped with review and editing, Y.K., K.I., J.T., and K.M. oversaw the research. All the authors have approved the final version of the manuscript.

Competing interests
The authors declare no competing interests.

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