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β-Lapachone attenuates mitochondrial dysfunction in MELAS cybrid cells

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ABSTRACT

Mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes (MELAS) is a mitochondrial disease caused by mutations in the mitochondrial genome. This study investigated the efficacy of β-lapachone (β-lap), a natural quinone compound, in rescuing mitochondrial dysfunction in MELAS cybrid cells. β-Lap significantly restored energy production and mitochondrial membrane potential as well as normalized the elevated ROS level in MELAS cybrid cells. Additionally, β-lap reduced lactic acidosis and restored glucose uptake in the MELAS cybrid cells. Finally, β-lap activated Sirt1 by increasing the intracellular NAD^+/NADH ratio, which was accompanied by increased mtDNA content. Two other quinone compounds (idebenone and CoQ10) that have rescued mitochondrial dysfunction in previous studies of MELAS cybrid cells had a minimal effect in the current study. Taken together, these results demonstrated that β-lap may provide a novel therapeutic modality for the treatment of MELAS.

1. Introduction

The majority of the energy required for sustained eukaryotic cell activity is produced through oxidative phosphorylation (OXPHOS) in the mitochondria. OXPHOS is carried out by a respiratory chain complex that consists of components encoded by both the nuclear and the mitochondrial genomes. Mitochondrial DNA (mtDNA) encodes 13 protein subunits of the respiratory chain complex, as well as 22 transfer RNAs (tRNA) and two ribosomal RNAs needed for mitochondrial protein synthesis [1]. Mutations in human mtDNA contribute to defects in OXPHOS and mitochondrial protein synthesis, which can eventually lead to mitochondrial diseases [2]. Mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes (MELAS) is an inherited mitochondrial disease caused by various mutations in mtDNA, the most common being the A3243G mutation that affects the mitochondrial tRNA $tRNA^{Leu(UUR)}$ [3]. The most distinctive phenotype of MELAS is the impaired OXPHOS caused by deficient mitochondrial protein synthesis and decreased respiratory chain complex activities [4]. Antioxidants, cofactors, and various vitamins are treatment options for MELAS [5], although more efficient therapies are needed.

A natural quinine-containing compound β-lapachone (3,4-dihydro-2,2-dimethyl-2H-naphthol[1,2-β]pyran-5,6-dione; [β-lap]) is a substrate of NAD(P)H:quinone oxidoreductase (NQO1). β-Lap facilitates the NQO1-dependent oxidation of NADH to NAD^+ by accepting two electrons from NADH, thereby increasing the intracellular NAD^+/NADH ratio [6,7]. Reduced β-lap is rapidly auto-oxidized in aqueous solution, but its destiny within cells is largely unknown. Idebenone, a short-chain quinone derivative and a substrate of NQO1, increased cellular redox potential and energy levels in cybrid cells obtained from a MELAS patient [8], presumably by donating its electrons directly to complex III of the respiratory chain complex after it is reduced by NQO1 at the expense of NADH oxidation, leading to restoration of ATP production. We reasoned that β-lap may similarly restore the energy production in MELAS cybrid cells. β-Lap activates Sirt1 by increasing the intracellular NAD^+ level [9]. Because Sirt1 is a key regulator of mitochondrial biogenesis and metabolism, β-lap may further improve energy metabolism in MELAS cybrid cells.

In this study, we compared the efficacies of β-lap, idebenone, and CoQ10 (a long-chain quinone and a component of the respiratory chain complex) in restoring mitochondrial dysfunction in MELAS cybrid cells. This study showed that β-lap was the most efficient at increasing energy production, reducing reactive oxygen species (ROS) and lactate generation, and restoring mitochondrial content. We suggest that β-lap may provide a better therapeutic modality for MELAS.

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2. Materials and methods

2.1. Cell culture and chemical treatment

Cybrid cell lines harboring 100% wild-type (WT) and A3243G mutation (MELAS) cells were kindly provided by Dr. Kyong Soo Park (Seoul National University, Seoul, Korea). The cells were cultured in DMEM media containing 1,000 mg/L glucose (Hyclone); supplemented with 10% FBS (Hyclone), 1% penicillin–streptomycin (Invitrogen), and 50 μg/mL uridine; and maintained at 5% CO_2 at 37 °C. β-Lap was chemically synthesized by the R&D Center, KT&G Life Sciences (Suwon, Korea). Idebenone and CoQ10 were purchased from Sigma. β-Lap, idebenone, and CoQ10 were dissolved in dimethyl sulfoxide (DMSO) at concentrations of 1 mM, 10 mM, and 100 mM, respectively, immediately before use. Cybrid cells were treated with β-lap, idebenone, or CoQ10 solution (final concentrations of 1 μM, 10 μM, and 100 μM, respectively) at 37 °C for 48 h. The same volume of DMSO was used as a control.

2.2. Quantification of ATP level

The intracellular ATP level was measured using the ATP colorimetric/fluorometric assay kit (Biovision). After incubation with the quinone compounds at 37 °C for 48 h, the cells were treated according to the manufacturer's instructions.

2.3. Measurement of mitochondrial membrane potential

Measurement of mitochondrial membrane potential was determined by staining with JC-1 (Sigma). The cells were plated on poly-L-lysine-coated coverslips and incubated in DMEM containing 3 μM JC-1 for 15 min at 37 °C. Immediately after incubation, fluorescent images were obtained using a Fluoview FV 1000 confocal laser-scanning microscope (Olympus). Data were analyzed using MetaMorph imaging software (Universal Imaging) to quantify the intensities of red and green fluorescence. The results were expressed as the ratio of red to green fluorescence.

2.4. Determination of intracellular lactate level

The cells were lysed in 400 μL of 6% perchloric acid (Sigma) and collected by scraping. The scraped extracts were centrifuged at 13,000 rpm for 10 min. The supernatant was neutralized by adding 1 M KOH and incubated on ice for 5 min. After one more centrifugation at 13,000 rpm for 10 min, the supernatant was analyzed using L-lactic acid assay kit (Megazyme) according to the manufacturer's instructions.

2.5. Measurement of intracellular generation of ROS

Mitochondrial superoxide generation in cybrid cells was measured using MitoSOX Red™ (Invitrogen), which is oxidized by superoxide and exhibits red fluorescence once inside the mitochondria. The cells were incubated with 1 μM MitoSOX for 15 min at 37 °C, washed twice with PBS, resuspended in 500 μL of PBS, and analyzed using flow cytometry (excitation at 510 nm and fluorescence detection at 580 nm).

2.6. Measurement of NAD^+/NADH ratio

Cells were harvested and extracted with 1 M perchloric acid or 1 M potassium hydroxide solution on ice for 15 min [10]. Extracted samples were subjected to ultrasonication with a Sonic Dismembrator (Fisher Scientific) on power setting 3 for 30 s. After centrifugation at 12,000 rpm for 10 min, the supernatant was obtained and neutralized by adding 1 M borate buffer or 1 M KH_2PO_4 on ice for 10 min. After centrifugation at 12,000 rpm for 10 min, the supernatant was filtered through a Microcon YM-3 filter (Millipore). Electrospray-ionization mass spectrometry was performed in positive ion mode using MDS Sciex API 4000 Triple Quadrupole Mass Spectrometer (Applied Biosystems) followed by chromatographic separation on an Agilent 1100 series HPLC system (Agilent technologies) equipped with an XTerra MS C18 2.1 × 150 mm, 3.5-μm column (Waters) as previously described [11].

2.7. Localization of FOXO1-GFP

The expression plasmid containing FOXO1-GFP was purchased from Addgene [12]. HeLa cells were stably transformed with the FOXO1-GFP plasmid and incubated with the quinone compounds for 1 h. The cells were washed three times with PBS and fixed with 4% paraformaldehyde (pH 7.4) in PBS for 10 min and stained briefly with Hoechst 33342. Images were obtained using a Fluoview FV 1000 confocal laser-scanning microscope equipped with 60× oil-immersion (Olympus).

2.8. Measurement of glucose uptake

Glucose uptake in cybrid cells was measured using 2-NBDG (Invitrogen), a fluorescent glucose analog. The cells were washed two times with PBS and incubated in culture medium (no glucose) containing 2-NBDG (100 μM) for 20 min. After incubation, cells were harvested with trypsin–EDTA (Invitrogen) and analyzed by flow cytometry (excitation at 465 nm and fluorescence detection at 540 nm).

2.9. Quantitation of mitochondrial DNA (mtDNA)

The mtDNA and nuclear DNA were isolated from cybrid cells using Blood & Cell Culture DNA mini kit (Qiagen). Relative quantity of mtDNA and nuclear DNA was assessed by quantitative real-time PCR (qRT-PCR) using a TaKaRa Thermal Cycler Dice Real Time System Single TP 815 (Takara) with SYBR Green (Takara) as fluorescent dyes. The sequences of primers were: mtDNA, 5′-AGGAC AAGAG AAATA AGGCC-3′ and 5′-TAAGA AGAGG AATTG AACCT CTGAC TGTAA-3′; D-Loop, 5′-TTCTG GCCAC AGCAC TTAAA-3′ and 5′-GGAGT GGGAG GGGAA AATAA-3′; Actin, 5′-TCACC CACAC TGTGC CCATC TACGA-3′ and 5′-CAGCG GAACC GCTCA TTGCC AATGG-3′.

2.10. Statistics

All data are reported as mean ± SD. Statistical significance was analyzed using the two-way ANOVA for multiple comparisons (Statview 5.0, SAS). $p < 0.05$ was considered statistically significant.

3. Results

3.1. β-Lap increased energy production in MELAS cybrid cells

We first investigated whether β-lap restored the reduced energy production in MELAS cybrid cells more than idebenone or CoQ10 did. β-lap was administered at a final concentration of 1 μM throughout this study, whereas idebenone and CoQ10 were used at their reported effective concentrations (10 and 100 μM, respectively) [8,13]. The ATP level was markedly reduced in MELAS cybrid cells (59% of the level in WT) after 48 h cultivation in low glucose media. Treatment with β-lap significantly increased the ATP levels in MELAS cybrid cells to 82% of the WT level. However, neither idebenone nor CoQ10 had significant effects on the ATP levels in MELAS cybrid cells under the same conditions (Fig. 1A).

Mitochondrial ATP production through respiratory chain complex is reflected by the changes in mitochondrial membrane potential (Ψm). Therefore, we measured the changes in Ψm using JC-1 dye, which loses red fluorescence when Ψm decreases. As reported in previous studies, MELAS cybrid cells had a significantly lower Ψm, as demonstrated by substantially reduced red/green JC-1 fluorescence ratio (28% of the WT level). This fluorescence ratio was significantly restored to 50% of the WT level with β-lap treatment, but not with idebenone or CoQ10 treatment (Fig. 1B).

MELAS cybrid cells generate increased amount of ROS due to impaired OXPHOS [14]. In accordance with the previous findings, the ROS levels in MELAS cybrid cells were 218% of the WT level. All of the tested compounds significantly lowered the ROS production in MELAS cybrid cells to 140–163% of the WT levels (Fig. 1C).

Taken together, these data indicate that β-lap (but not idebenone or CoQ10) significantly restored mitochondrial energy production in MELAS cybrid cells under our experimental conditions. However, all of the tested compounds were effective in reducing excessive ROS production in MELAS cybrid cells.

3.2. β-Lap rescued mitochondrial metabolism defects in MELAS cybrid cells

MELAS syndrome is characterized by lactic acidosis, which occurs due to the increased glycolysis that compensates for defects in OXPHOS [15]. As expected, lactate was 36% higher in MELAS cybrid cells than in WT cells. β-Lap markedly reduced the lactate in MELAS cybrid cells to a near-normal level. However, idebenone and CoQ10 did not significantly affect lactate in MELAS cybrid cells (Fig. 2A).

Mitochondrial dysfunction leads to impaired insulin sensitivity, as indicated by reduced glucose uptake [16]. Glucose uptake was markedly lower in MELAS cybrid cells (66% of the WT uptake), but β-lap significantly increased glucose uptake to 85% of the uptake in WT cells. Neither idebenone nor CoQ10 significantly affected glucose uptake in MELAS cybrid cells (Fig. 2B).

3.3. β-Lap increased NAD^+ metabolism in MELAS cybrid cells

Intracellular NAD^+/NADH ratio is an essential determinant of mitochondrial function and metabolism at least partially through regulation of sirtuin family proteins [17]. Direct measurement of intracellular NAD^+ and NADH levels revealed that the NAD^+/NADH ratio was significantly lower in MELAS cybrid cells (65% of WT). β-lap restored the NAD^+/NADH ratio in MELAS cybrid cells to the level of the WT cells, but the effects of idebenone and CoQ10 were negligible on the NAD^+/NADH ratio (Fig. 3A).

Sirt1 is a member of sirtuin family proteins, which are NAD^+-dependent class III group of histone deacetylases [18]. NAD^+ is a strictly required cofactor for Sirt1, and β-lap may increase Sirt1 activity by increasing cellular NAD^+ levels [19]. We monitored the activation of Sirt1 by examining the nuclear translocation of FOXO1, which is a substrate of Sirt1. When deacetylated by Sirt1, FOXO1 moves into the nucleus and activates the downstream genes [12]. HeLa cells transfected with a FOXO1-GFP expression plasmid were treated and observed under a fluorescent microscope. β-Lap caused nuclear translocation of FOXO1, but idebenone and CoQ10 did not (Fig. 3B). These data suggest that β-lap activates Sirt1 activity by elevating the intracellular NAD^+/NADH ratio.

3.4. β-Lap increased mitochondrial components encoded by mtDNA

We next investigated the effects of β-lap on the mitochondrial components encoded by mtDNA. The mtDNA content was

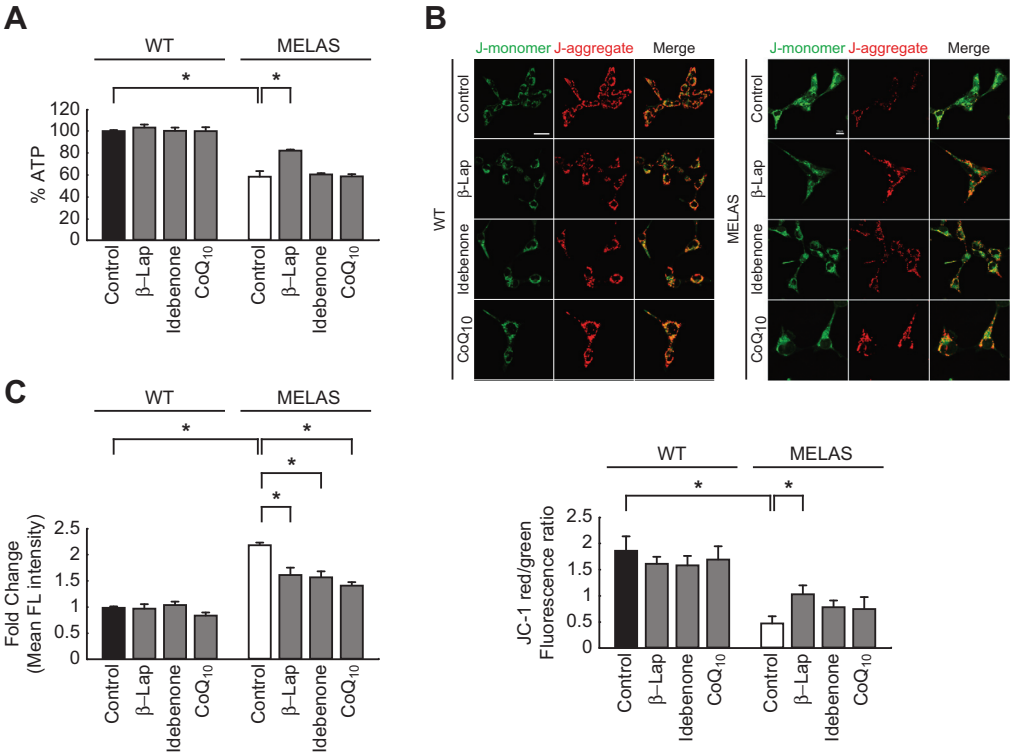

Fig. 1. Effects of β-lap, idebenone, and CoQ10 on mitochondrial energy production. WT and MELAS cybrid cells were treated with β-lap (1 μM), idebenone (10 μM), or CoQ10 (100 μM) for 48 h. (A) ATP levels were measured and the values relative to WT cells were plotted. Data are shown as the mean ± SD ($n = 3$). $^*p < 0.05$. (B) Mitochondrial membrane potential (Ψm) was evaluated using JC-1 dye. The ratios of red to green fluorescence were quantified using MetaMorph imaging software and plotted. Scale bar, 20 μm. Data are shown as the mean ± SD ($n = 100$). $^*p < 0.05$. (C) ROS levels were measured by flow cytometry using MitoSOX™. Data are shown as the mean ± SD ($n = 3$). $^*p < 0.05$.

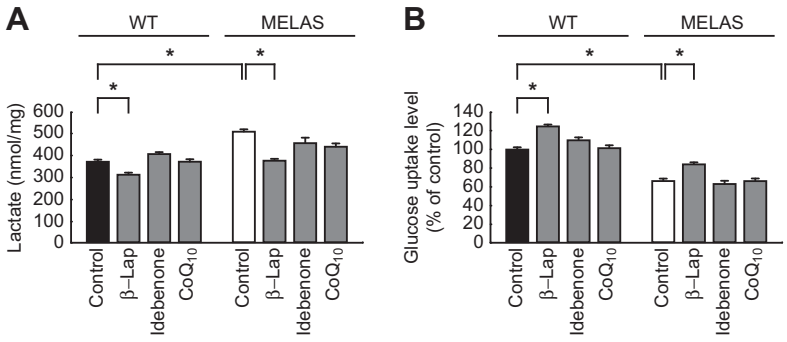

Fig. 2. Effects of β-lap, idebenone, and CoQ10 on mitochondrial metabolism. WT and MELAS cybrid cells were treated with β-lap (1 μM), idebenone (10 μM), or CoQ10 (100 μM) for 48 h. (A) Lactate contents were measured and plotted. Data are shown as the mean ± SD ($n = 3$). $^*p < 0.05$. (B) Glucose uptake levels were measured and plotted. Data are shown as the mean ± SD ($n = 3$). $^*p < 0.05$.

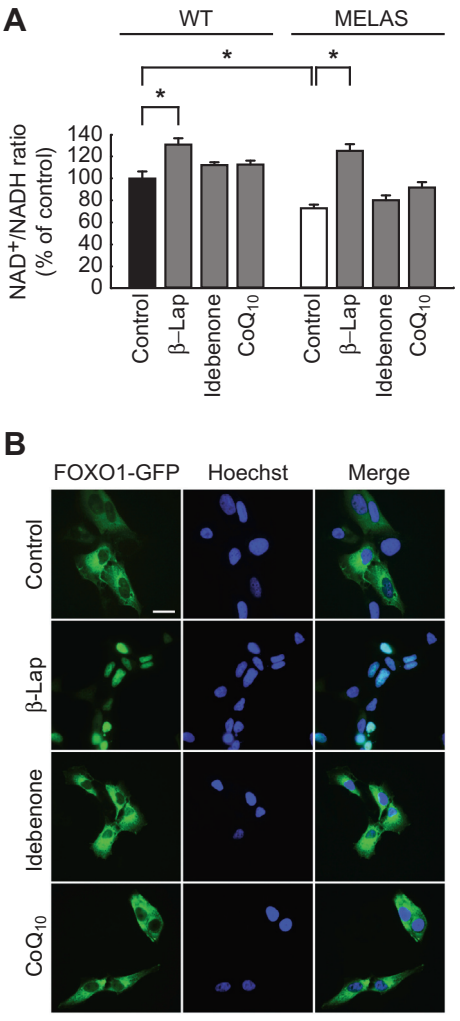

Fig. 3. Effects of β-lap, idebenone, and CoQ10 on NAD^+ metabolism. (A) WT and MELAS cybrid cells were treated with β-lap (1 μM), idebenone (10 μM), or CoQ10 (100 μM) for 48 h. NAD^+ and NADH were measured and the ratios were plotted. Data are shown as the mean ± SD ($n = 3$). $^*p < 0.05$. (B) HeLa cells containing FOXO1-GFP expression plasmids were treated with β-lap (1 μM), idebenone (10 μM), or CoQ10 (100 μM) for 1 h and observed under fluorescent microscope. Note that the GFP fluorescence was co-localized with nuclear staining only when the cells were treated with β-lap. Scale bar, 20 μm.

measured by performing PCR with mtDNA-specific and D-loop-specific primers. Both primers produced similar results and showed that the mtDNA content was reduced by about 25% in MELAS cybrid cells and restored to the level of the WT cells with β-lap, but not with either idebenone or CoQ10 (Fig. 4).

4. Discussion

Many mitochondrial diseases, which are characterized by mitochondrial dysfunction, are caused by mutations in the mtDNA as well as in the nuclear genome. The mtDNA encodes a small portion of the respiratory chain complex subunits and some of the RNA components of the mitochondrial translational apparatus, whereas the nuclear genome encodes the majority of the protein subunits in the respiratory chain complex. Mutations in either the mtDNA or the nuclear genome disrupt respiratory chain function, particularly in tissues that have a high energy demand such as the muscle and neural tissues [20]. Manifestations of mitochondrial diseases include epilepsy, intellectual disability, skeletal and cardiac myopathy, sensorineural hearing loss, endocrine diseases, and renal impairment [21]. MELAS is a mitochondrial disease characterized by seizures, stroke-like episodes, and lactic acidosis [22], and at least 30 mtDNA mutations have been associated with MELAS [4].

Treatment of mitochondrial diseases is largely restricted to cofactor supplementation [23]. CoQ10, an essential component of respiratory chain complex, is widely used for MELAS therapy because of its critical role in energy metabolism, and has been shown to effectively reverse respiratory defects in MELAS fibroblast and cybrid cells [24]. Idebenone is a short-chain quinone compound and a substrate of NQO1. Through the enzymatic reaction of NQO1, idebenone accepts two electrons at the expense of NADH oxidation and directly donates the electrons to the respiratory chain, leading to the continued generation of ATP through OXPHOS [9].

The quinone-containing compound β-lap has a number of pharmacological effects [25,26]. NQO1 mediates the reduction of β-lap using NADH as an electron source [6]. The reduced form of β-lap is unstable and is rapidly re-oxidized to its original form in aqueous solutions, and this futile cycle is presumed to increase the NAD^+/NADH ratio [27]. β-Lap has been shown to prevent obesity-related metabolic phenotypes in mice [28] and age-related decline of muscle and brain functions [29] through regulation of NAD^+ metabolism.

Sirt1, activated by NAD^+ as a cofactor, is associated with the regulation of lifespan, metabolism, and cellular survival [30,31]. Previous studies show that Sirt1 improves mitochondrial function through AMPK and Sirt1 activation in several animal models of metabolic diseases [9,32], and activation of Sirt1 promotes mitochondrial biogenesis and protects against metabolic dysregulation [33]. We previously showed that β-lap increases Sirt1 activity through elevation of intracellular NAD^+/NADH ratio in neuronal

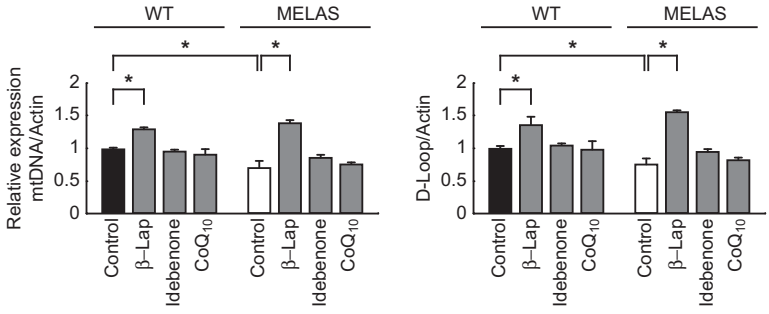

Fig. 4. Effects of β-lap, idebenone, and CoQ10 on mtDNA expression. WT and MELAS cybrid cells were treated with β-lap (1 μM), idebenone (10 μM), or CoQ10 (100 μM) for 48 h. The contents of mtDNA were determined by PCR with mtDNA- and D-loop-specific primers. Data are shown as the mean ± SD ($n = 3$). $^{*}p < 0.05$.

cells [19]. In this study, we found that β-lap activated Sirt1 and increased mtDNA content whereas idebenone and CoQ10 did not, suggesting that the beneficial effects of β-lap in MELAS cybrid cells are attributable at least in part to the β-lap-mediated activation of Sirt1. However, we did not investigate this mechanism in detail in the present study.

In previous studies, both idebenone and CoQ10 restored metabolic dysfunction in MELAS fibroblast and cybrid cells. However, these compounds did not show the same effects in this study, possibly due to (1) different experimental conditions and (2) different cybrid cells between the studies. Acute (1 h) but not long-term treatment (48 h) with idebenone restored deficits in energy production of MELAS cybrid cells. The MELAS cybrid cells using tissues from individual patients and were thus unique in their genetic background. Comparative studies of these compounds should be performed in different types of MELAS cybrid cells to clarify the effect of β-lap on the disease. Nevertheless, this study suggests that β-lap is an effective therapeutic target for treatment of MELAS syndrome.

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