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Mitochonic Acid 5 (MA-5), a Derivative of the Plant Hormone Indole-3-Acetic Acid, Improves Survival of Fibroblasts from Patients with Mitochondrial Diseases

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Mitochondria are key organelles implicated in a variety of processes related to energy and free radical generation, the regulation of apoptosis, and various signaling pathways. Mitochondrial dysfunction increases cellular oxidative stress and depletes ATP in a variety of inherited mitochondrial diseases and also in many other metabolic and neurodegenerative diseases. Mitochondrial diseases are characterized by the dysfunction of the mitochondrial respiratory chain, caused by mutations in the genes encoded by either nuclear DNA or mitochondrial DNA. We have hypothesized that chemicals that increase the cellular ATP levels may ameliorate the mitochondrial dysfunction seen in mitochondrial diseases. To search for the potential drugs for mitochondrial diseases, we screened an in-house chemical library of indole-3-acetic-acid analogs by measuring the cellular ATP levels in Hep3B human hepatocellular carcinoma cells. We have thus identified mitochonic acid 5 (MA-5), 4-(2,4-difluorophenyl)-2-(1H-indol-3-yl)-4-oxobutanoic acid, as a potential drug for enhancing ATP production. MA-5 is a newly synthesized derivative of the plant hormone,

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indole-3-acetic acid. Importantly, MA-5 improved the survival of fibroblasts established from patients with mitochondrial diseases under the stress-induced condition, including Leigh syndrome, MELAS (myopathy encephalopathy lactic acidosis and stroke-like episodes), Leber's hereditary optic neuropathy, and Kearns-Sayre syndrome. The improved survival was associated with the increased cellular ATP levels. Moreover, MA-5 increased the survival of mitochondrial disease fibroblasts even under the inhibition of the oxidative phosphorylation or the electron transport chain. These data suggest that MA-5 could be a therapeutic drug for mitochondrial diseases that exerts its effect in a manner different from anti-oxidant therapy.

Keywords: indole-3-acetic acid; Leigh syndrome; MELAS; mitochonic acid 5 (MA-5); mitochondrial disease
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Introduction

Mitochondria are key organelles implicated in a variety of processes related to energy and free radical generation, the regulation of apoptosis and various signal pathways (Vafai and Mootha 2012). Mitochondrial dysfunction has been implicated in certain inherited mitochondrial disease phenotypes, for example MELAS (myopathy encephalopathy lactic acidosis and stroke-like episodes), MERRF (myoclonic epilepsy and ragged red fibers), Leigh syndrome (LS) and Leber's hereditary optic neuropathy (LHON) and Kearns-Sayre syndrome (Pieczenik and Neustadt 2007; Vafai and Mootha 2012). LS is a devastating neurodegenerative disease, also referred to as subacute necrotizing encephalopathy, usually emerged in infancy or early childhood. Deficiencies of the mitochondrial oxidative phosphorylation (OXPHOS) enzymes activities and several mutations of nuclear DNA or mitochondrial DNA (mtDNA) coding representative OXPHOS enzymes were reported in LS patients (Pieczenik and Neustadt 2007; Vafai and Mootha 2012). MELAS is one of the most frequent mitochondrial diseases and has a broad spectrum of manifestations, including stroke-like episodes, exercise intolerance, muscle weakness, epilepsy, dementia, migraine headaches, short stature, sensorineural hearing loss, and lactic acidosis (El-Hattab et al. 2014). LHON is characterized by bilateral, painless, subacute visual failure that develops during young adult life (Carelli et al. 2013). Kearns-Sayre syndrome (KSS) is a mitochondrial disorder characterized by onset before the age of 20 years, progressive external ophthalmoplegia, and pigmentary retinopathy. Additional clinical features of this disease include cardiac conduction defects and cerebellar ataxia (Kabunga et al. 2015). Mitochondrial diseases are clinically heterogeneous and are associated with mutations in the genes encoded by either nuclear DNA or mitochondrial DNA (Pieczenik and Neustadt 2007; Vafai and Mootha 2012). In addition, acquired mitochondrial dysfunction is also implicated in many metabolic (e.g. diabetes and atherosclerosis), neurodegenerative and neuromuscular diseases (e.g. Parkinson's and Alzheimer's diseases) (Pieczenik and Neustadt 2007; Schapira 2012).

Mitochondrial diseases are clinically heterogeneous and are characterized by the mitochondrial respiratory chain dysfunction. Accordingly, in mitochondrial diseases, increased oxidative stress and depletion of ATP are serious problems that can cause cell death (Schapira 2012; Vafai and Mootha 2012). Antioxidant quinones (e.g. coenzyme Q10 (CoQ10), idebenone) have been used clinically to prevent the disease progression (Avula et al. 2014; Enns 2014), but the effectiveness of such quinones has not been well established (Pfeffer et al. 2012). Therefore, an alternative effective therapeutic approach is still urgently needed.

In patients with renal failure, many uremic toxins are accumulated, such as indoxyl sulfate and *p*-cresyl sulfate (Toyohara et al. 2010). Unexpectedly, we also found the accumulation of indole-3-acetic acid (IAA), a plant hormone auxin, in uremic patients (Toyohara et al. 2010). IAA regulates growth and essential for plant's life cycle and body development, but IAA is synthesized in the mouse liver and kidney (Gordon et al. 1972) and intestinal anaerobes (Chung et al. 1975). In addition, IAA increased the growth of mouse and human fibroblasts (Abu Sinna 1983), although the precise role and mechanism has not been clarified. We are therefore interested in the bioactive properties of IAA.

Here, we screened an in-house chemical library of IAA analogs (Hayashi et al. 2012) and found that derivatives of IAA significantly increased the cellular ATP level in Hep3B human hepatocellular carcinoma cells, including mitochonic acid (MA)-5, 4-(2,4-difluorophenyl)-2-(1H-indol-3-yl)-4-oxobutanoic acid (Hayashi et al. 2012). Moreover, MA-5 improved the survival of fibroblasts established from patients with mitochondrial diseases probably increasing the ATP level independently of the membrane potential or OXPHOS complexes. Our finding changes the focus on searching drugs for mitochondrial and neurodegenerative diseases.

Materials and Methods

Human samples

The review board of Tohoku University and Kanagawa Children's Medical Center approved the study and written informed consent was obtained from all the participants.

Cell culture

Hep3B cells were purchased from ATCC and cultured in RPMI1640 with 10% fetal bovine serum (FBS), 100 units/ml penicillin and 100 μg/ml streptomycin. Fibroblasts were established from a patient with MELAS, LS, LHON or Kearns-Sayre syndrome by

Hitoshi Osaka (Tanigawa et al. 2012) and collected in Tohoku University Hospital under the approval of the Ethical Committee of Tohoku University. These mitochondrial diseases were selected from the patients under clinically treated in our hospitals.

Cell viability assay of fibroblasts from patient with mitochondrial diseases

Fibroblasts from patients with mitochondrial diseases were cultured in 1.0 g/L low-glucose Dulbecco's Modified Eagle's Medium (DMEM) with 10% FBS, 100 units/ml penicillin and 100 μg/ml streptomycin (Normal growth medium) at 37°C, 95% ambient air and 5% CO_2 and 21% oxygen for maintaining culture. In cell viability assay, low-glucose DMEM with 1% FBS and antibiotics (assay medium) was used. Mitochondrial disease fibroblasts were cultured in normal growth medium until semi-confluent and then plating at 1.5 $\times$ 10^4 cells/ 0.5 ml assay medium per each well of 48 well flat bottom culture plate in assay medium. After 24-hr incubation, L-buthionine-(*S,R*)-sulfoximine (BSO) at 100 μM or control vehicle dimethyl sulfoxide (DMSO) at 0.1% were added and cultured for another 24 hrs. After each compound was applied at indicated final concentrations, fibroblasts were cultured for 48 hrs, and cell viability was measured by Cell count Regent SF (nacalai tesque) (Enns et al. 2012). Intracellular ATP was measured with Cellno ATP assay regent (TOYO B-NET). Reactive oxygen species (ROS) were measured by OxiSelect ROS Assay Kit (Cell Biolabs). Glutathione (GSH) was measured using a GSH-Glo™ Glutathione Assay kit based on the manufacturer's instructions (Promega).

Recording of cyclic voltammogram

Cyclic voltammograms were obtained for 0.5 mM CoQ10, 0.5 mM idebenone, 0.5 mM IAA and 0.5 mM MA-5 in an acetic acid/ acetonitrile mixture (4:1 by volume) containing 0.01 M sodium acetate as the supporting electrolyte, using a BASi C3 electrochemical analyzer, as previously reported (Shrader et al. 2011). Cyclic voltammograms were recorded in a glass cell using a glassy carbon disk electrode (3 mm in diameter) as the working electrode, a platinum wire as the counter electrode and an Ag/Ag^+ electrode as the reference electrode. The scan rate was 100 mV s^{-1} and all measurements were performed in air at room temperature (~ 20°C).

Statistics

The data were expressed as means ± SEM. Comparisons were made using unpaired two-tailed Student's *t*-tests, one-way ANOVA and Dunnett's test or two-way ANOVA and Tukey post hoc test, as appropriated (JMP Pro 11 software). *P* value of less than 0.05 was considered significant (**P* < 0.05, ***P* < 0.01, ****P* < 0.01).

Results

Screening for IAA derivatives that increase cellular ATP levels

IAA is a plant hormone auxin analog that regulates growth and essential for plant's life cycle and body development. IAA also increased the growth of mouse and human fibroblasts (Abu Sinna 1983). To identify the potential drugs for stimulating cell survival, we screened the in-house chemical library of IAA analogs (Hayashi et al. 2012) based on the increase of the cellular ATP contents compared with the control. As extensive metabolism and ATP synthesis are stably processed in the liver, we used Hep3B human hepatocarcinoma cells for screening the IAA analog library by measuring intracellular ATP. Among 40 analogs, 8 compounds increased cellular ATP levels by up to 3 fold compared to the control in Hep3B cells (Fig. 1A; see the chemical structures in Fig. 1B). In addition, each of compounds #1, #2, #4, #5 and #7 increased ATP levels in a dose-dependent manner (Fig. 1C). Among them, we focused on compound #5 (mitochonic acid 5; MA-5), because of its potency and suitability for large-scale production. In fact, large-scale production is difficult in some compounds.

MA-5 rescues fibroblasts from patients with mitochondrial diseases under stress-induced cell death conditions

We then analyzed the effects of MA-5 and other IAA analogs on cell survival in the presence of BSO, a glutathione synthesis inhibitor, according to the common assay method for screening drugs for mitochondrial diseases (Enns et al. 2012). BSO causes the decrease in cellular glutathione (GSH) and the increase in reactive ROS production, thereby inducing a variety of apoptotic signals (Shrader et al. 2011).

We treated patient fibroblasts with BSO and each IAA analog and examined cell viability. Although fibroblasts from a healthy individual were able to tolerate BSO treatment (data not shown), fibroblasts from the LS patient exhibited enhanced cell death within 48 hr (Fig. 2A). Under this condition, neither the indole, IAA nor 1-naphthaleneacetic acid (NAA) showed any cell-protective effect on BSO-treated LS fibroblasts (Fig. 2A). CoQ10 and α-lipoic acid are clinically used in patients with mitochondrial diseases to attempt to enhance electron transfer within the respiratory chain (Pfeffer et al. 2012), but these compounds had no effect on cell viability, as reported (Shrader et al. 2011). On the other hand, compounds #2, #4, MA-5, #21 and #35 did exert a cell-protective effect (Fig. 2A). This cell-protective effect of MA-5 was dose-dependent with an EC_{50} of 2.3 μM (Fig. 2B). In addition, MA-5 was effective in preventing cell death of BSO-treated fibroblasts derived from a patient with MELAS, LHON or Kearns-Sayre syndrome with similar EC_{50} values (Fig. 2C-E). Because the gene mutations and biochemical defects are different among these mitochondrial diseases, MA-5 may act on a pathway different from the respiratory chain.

MA-5 mediates a novel mechanism for sustentation

Quinones such as CoQ10 and idebenone are used as antioxidants to neutralize free radicals (Shrader et al. 2011). To examine the redox properties of MA-5, we recorded cyclic voltammograms. CoQ10 and idebenone exhibited reversible redox reactions, with a reduction potential of ~ −400 mV, as evidenced by an equivalent current flow in the oxidation and reduction phases (Shrader et al. 2011). On the other hand, IAA and MA-5 exhibited irreversible oxidation peaks at ~ 800 mV (Fig. 3A). Therefore, antioxidant properties like quinones cannot be expected in these IAA

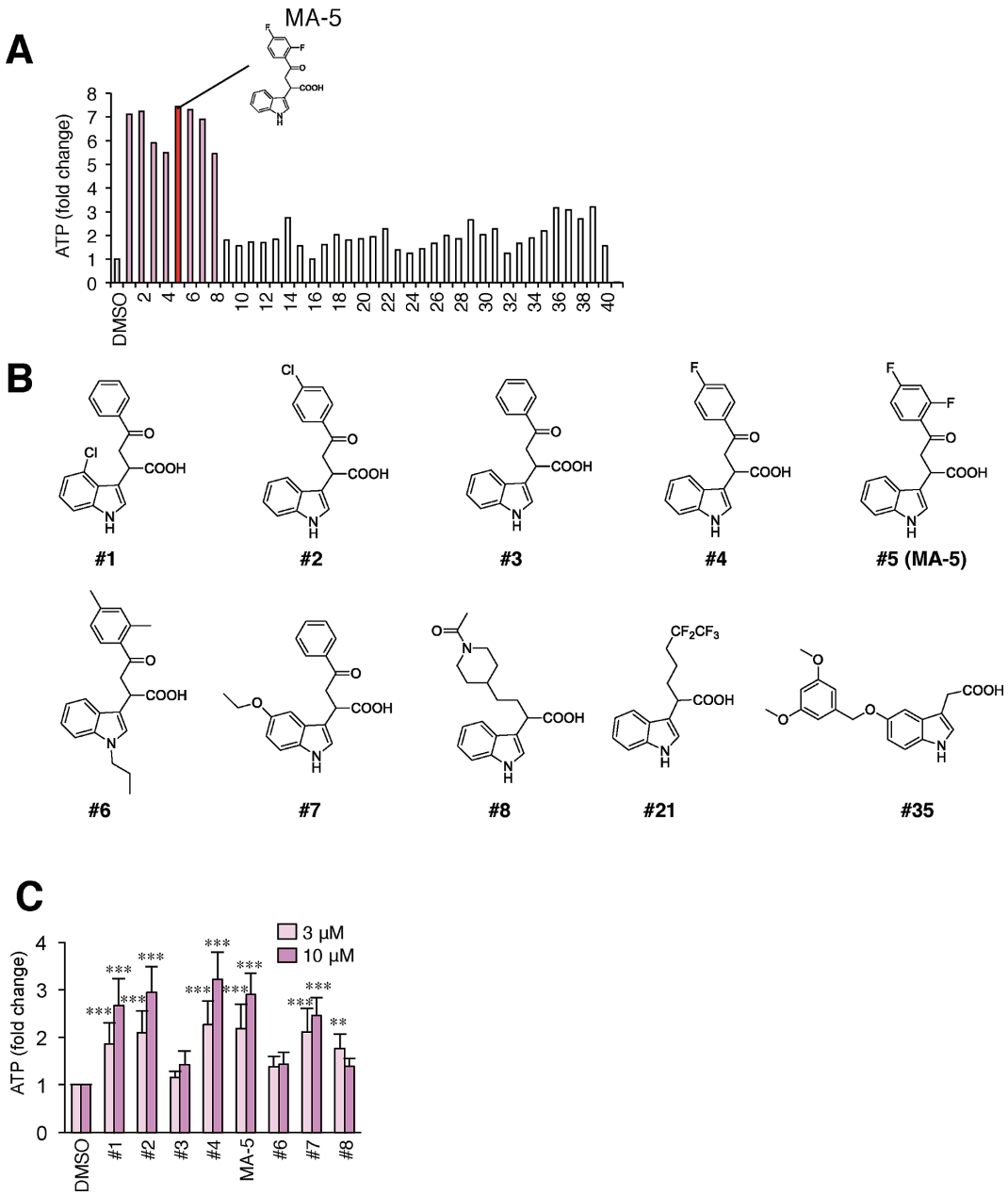

Fig. 1. MA-5 increases intracellular ATP contents in Hep3B cells.
(A) Intracellular ATP contents were measured in Hep3B cells after 24-h incubation with each of 40 IAA analogs at 3 μM (n = 1). (B) Chemical structures of #1-8, #21 and #35. (C) Intracellular ATP contents were measured in Hep3B cells after 24-h incubation with each of compounds #1- #8 at 3 or 10 μM (n = 4). $^{**}P < 0.01$, $^{***}P < 0.001$, one-way ANOVA and Dunnett's test vs. DMSO.

derivatives.

Cellular ROS are scavenged by the thioredoxin system and glutathione (GSH) (Che et al. 2014). Idebenone inhibits ROS accumulation and prevents H_2O_2-induced cytotoxicity (Lim et al. 2008). On the other hand, our direct monitoring of the ROS levels revealed that MA-5 had no effect on the cellular ROS levels under basal and H_2O_2-treated conditions (Fig. 3B). In addition, MA-5 did not affect the cellular GSH levels in the presence or absence of BSO (Fig. 3C). These data suggest that the cell-protective mechanism of MA-5 is different from those of the cellular antioxidant quinones.

Effects of signal inhibitors on MA-5 function

It was reported that erythropoietin modulates ATP level via GATA and HIF transcriptional pathways (Imagawa et al. 2003). Aryl hydrocarbon receptor (AhR)-mediated ATP modulation was also postulated (Jiang et al. 2011). However, a GATA inhibitor, AhR inhibitor and HIF inhibitor had no effect on MA-5-mediated cell protection (Fig. 4A). In plants, nitric oxide (NO) and cGMP are messengers in the IAA-induced root growth and development

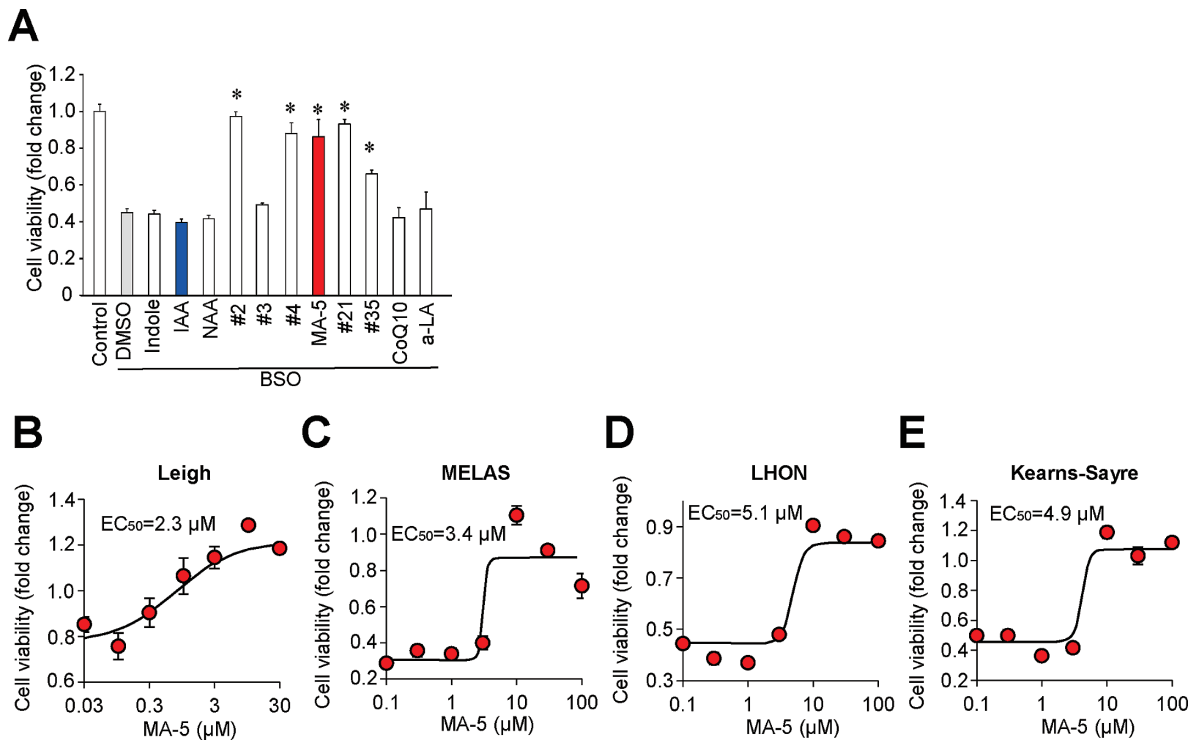

Fig. 2. Effects of MA-5 on the survival of fibroblasts established from mitochondrial diseases.
(A) The viability of BSO-treated LS fibroblasts that were also exposed to each compound at 10 μM (n = 8), including indole, IAA, 1-naphthaleneacetic acid (NAA), compound #2, #3, #4, MA-5 (#5), #21, #35, CoQ10 and α-lipoic acid (α-LA). *P < 0.05 vs. DMSO/BSO. MA-5 improved the viability of BSO-treated fibroblasts from LS (B), MELAS (C), LHON (D), and Kearns-Sayre syndrome (E). The ranges of MA-5 concentrations were 0.03-30 μM for B and 0.01-100 μM for C-E (n = 8).

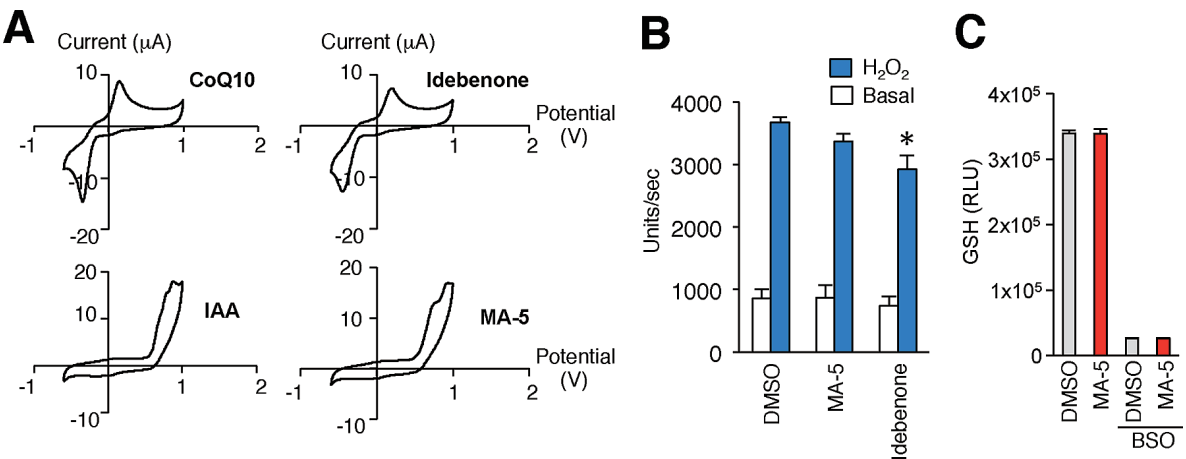

Fig. 3. Effects of MA-5 on mitochondrial membrane potential and ROS generation.
(A) Cyclic voltammograms of CoQ10, idebenone, IAA, and MA-5 at 0.5 mM. (B) ROS formation within fibroblasts from a normal volunteer with or without 1 mM H_2O_2 treatment, administrated with 0.1% DMSO, MA-5 or idebenone at 10 μM (n = 3). *P < 0.05, unpaired two-tailed Student's t-test vs. 1 mM H_2O_2 treated samples with 0.1% DMSO. (C) GSH was measured with or without BSO treatment following administration of 0.1% DMSO or MA-5 (10 μM) in fibroblasts from LS patient (n = 4).

(Pagnussat et al. 2003). To further evaluate these systems, we examined the effects of NO scavengers as well as inhibitors of NO synthase, guanylate cyclase, phosphodiesterase (PDE) 5, protein kinase G (PKG) and an ATP-sensitive K^+ channel. Most of inhibitors of NO and cGMP signal cascades did not block the cell-protective effect of MA-5 even in the presence of BSO (Fig. 4B). Among them, only LY83583 (a guanylate cyclase inhibitor) showed cell protective effects on BSO-treated LS fibroblasts comparable with MA-5 (Fig. 4B), the implication of which remains

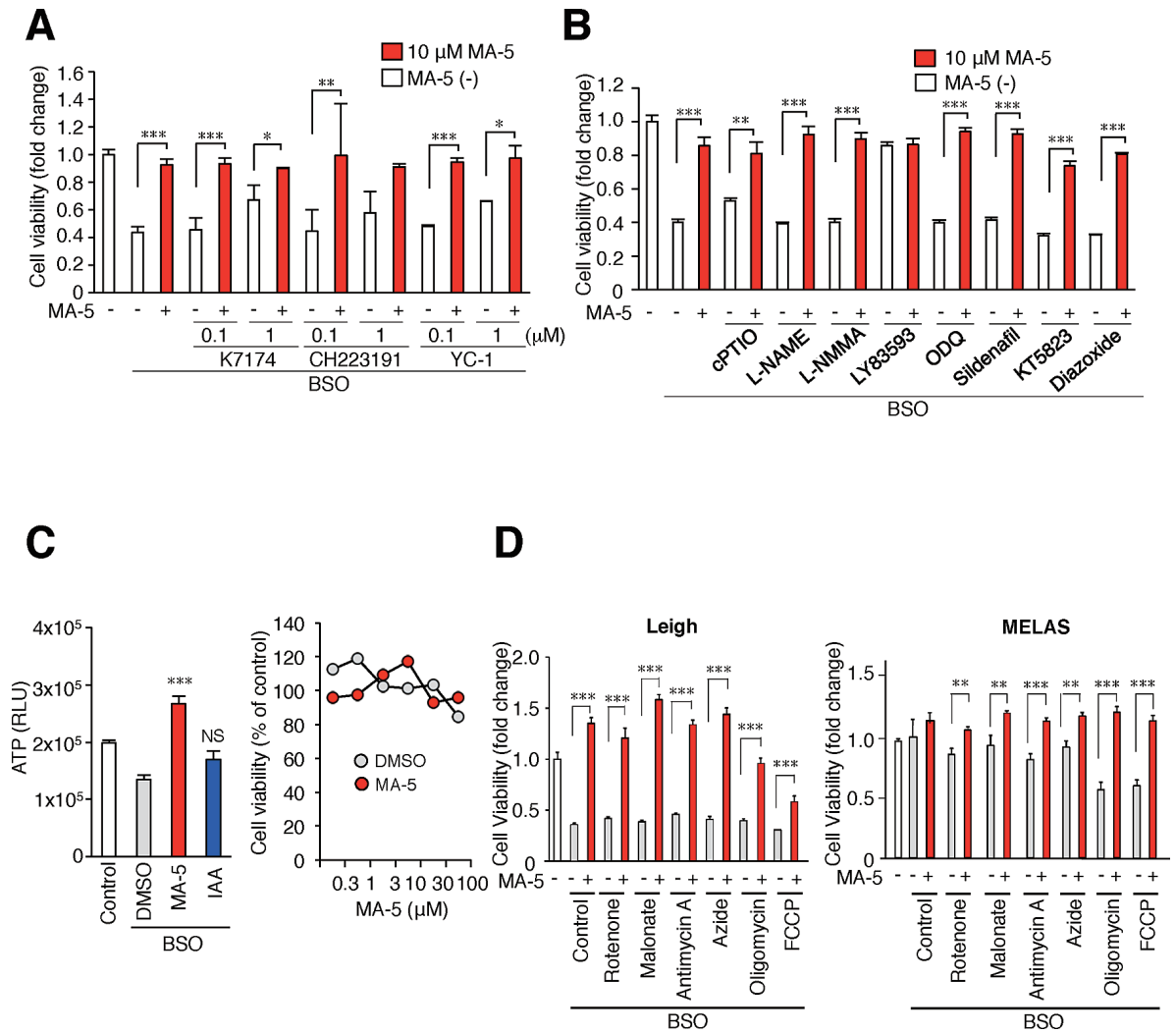

Fig. 4. Effects of MA-5 on mitochondrial respiration.
(A) Viability of fibroblasts from the LS patient after BSO treatment. K7174 (GATA inhibitor), CH233191 (AhR inhibitor) and YC-1 (HIF inhibitor) alone or with MA-5 were examined (n = 3). $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, unpaired two-tailed Student's *t*-test vs. respective control. (B) Viability of fibroblasts from the LS patient after BSO treatment. MA-5 was added with carboxy-PTIO (cPTIO, NO scavenger); Nω-Nitro-L-arginine methyl ester hydrochloride (L-NAME, NO synthase inhibitor); NG-Methyl-L-arginine (L-NMMA, NO synthase inhibitor); LY83583 [guanylate cyclase (GC) inhibitor]; 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ, GC inhibitor); sildenafil (PDE5 selective inhibitor); KT5823 (PKG inhibitor); or diazoxide (selective ATP-sensitive K^+ channel activator) (n = 3) . $**P < 0.01$, $***P < 0.001$, unpaired two-tailed Student's *t*-test vs. respective control. (C) Intracellular ATP contents were measured in BSO-treated LS fibroblasts exposed to 0.1% DMSO, 10 μM MA5 or 10 μM IAA (left, n = 8). Control indicated the value in untreated LS fibroblasts. NS, not significant. $***P< 0.001$, one-way ANOVA and Dunnett's test vs. DMSO. Cell toxicity assay of MA-5 (0.01-100 μM) in LS fibroblasts (right, n = 8). (D) Cell viability assay with malonate (1 mM), antimycin A (1 μM), azide (1 μM), oligomycin (1 μM) or FCCP (1 μM) in the presence or absence of MA-5 (10 μM) in BSO-treated LS fibroblasts (left) and BSO-treated MELAS fibroblasts (right) (n = 8). $**P < 0.01$, $***P < 0.001$, unpaired two-tailed Student's *t*-test vs. respective control.

unknown, because ODQ, another guanylate cyclase inhibitor, did not increase cell viability. Therefore, MA-5 may react with a novel target to enhance ATP production and to protect cells.

MA-5 acts on mitochondria without affecting the electron transport chain or OXPHOS

To investigate the cell-protective mechanism of MA-5, we measured the intracellular ATP level in fibroblasts from the LS patient. Under the oxidative condition with BSO treatment, the reduced ATP level was obviously increased by MA-5, but not by IAA (Fig. 4C, left). The elevated ATP level was unrelated to the improved cell survival, since no effect of MA-5 on the cell viability was observed at doses up to 100 μM (Fig. 4C, right).

We next evaluated whether the cell-protecting effect of MA-5 is dependent on mitochondrial OXPHOS, using the mitochondrial electron transport chain (ETC) inhibitors, rotenone (complex I), malonate (complex II), antimycin A (complex III), azide (complex IV) and oligomycin (complex

V) as well as a mitochondrial uncoupler, carbonyl cyanide-p- trifluoromethoxyphenylhydrazone (FCCP). We examined the effect of each inhibitor on the cell survival of LS and MELAS fibroblasts (Fig. 4D). In BSO-treated LS fibroblasts, cell viability was significantly decreased and the addition of an ECT inhibitor or FCCP did not further change the cell survival. In MELAS fibroblasts, the effect of BSO was relatively weak compared to that of LS fibroblasts but an ECT inhibitor or FCCP clearly decreased cell viability. Under these different conditions, MA-5 completely rescued cell viability (Fig. 4D).

Discussion

In this study, we have shown that the plant hormone IAA analogs act in human cells to increase the cellular ATP level. Among them, MA-5 improves survival of fibroblasts from patients with mitochondrial diseases probably by facilitating ATP production. The principal theory of ATP production is based on the chemiosmotic theory that the movement of protons across an electrochemical potential difference could mainly provide the energy to produce ATP (Mitchell 1961). However, MA-5 works even after the administration of membrane ionophore FCCP that cancels the mitochondrial membrane proton gradient, or oligomycin that inhibits Fo-ATP synthase.

Our data indicated that MA-5 does not exert any evident influence on mitochondrial OXPHOS or membrane potential, even though OXPHOS is responsible for more than 90% of cellular ATP production (Pieczenik and Neustadt 2007). One of the possibilities of MA-5 acting mechanisms is the effect on the oligomerization of ATP synthase. The oligomerization of ATP synthase is essential for the maintenance of cristae junctions (Habersetzer et al. 2013). In addition, the oligomerization of ATP synthase does increase the local pH gradient and membrane potential and optimizes ATP synthesis without changing whole mitochondrial membrane potential (Strauss et al. 2008).

Another possibility is attributed to its structure. Because indoles have some structural homology to the adenosine group of ATP (Coskuner and Murray 2014), our data further predict that MA-5 directly binds to a novel unveiled ATP-binding site within certain enzymes and directly exert its functions. In addition, we found that MA-5 exerted its potency even under treatment of oligomycin that inhibits Fo subunit of ATPase (Fig. 4D). Recently, Uchihashi et al. (2011) reported that the $\alpha 3\beta 3$ subcomplex (referred to as F1) is suffices as the motor upon ATP hydrolysis. In the isolated $\alpha 3\beta 3$ stator ring of ATPase, the three β subunits cyclically propagate conformational states in the counterclockwise direction without the γ-subunit (Uchihashi et al. 2011). Further binding and mutation experiments are necessary to reveal these mechanisms of MA-5 on ATP production.

In conclusion, MA-5 provides an alternative therapeutic strategy for treating patients with mitochondrial diseases and various metabolic diseases.

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Conflict of Interest

The authors declare no conflict of interest.

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