EBioMedicine xxx (2017) xxx–xxx

Contents lists available at ScienceDirect

EBioMedicine

journal homepage: www.ebiomedicine.com

Research Paper

# Antihypertrophic Effects of Small Molecules that Maintain Mitochondrial ATP Levels Under Hypoxia

Hiroaki Nagai *, Tomoko Satomi, Akiko Abiru, Kazumasa Miyamoto, Koji Nagasawa, Minoru Maruyama, Satoshi Yamamoto, Kuniko Kikuchi, Hiromitsu Fuse, Masakuni Noda, Yoshiyuki Tsujihata *

*Research, Takeda Pharmaceutical Company Limited, Fujisawa, Kanagawa 251-8555, Japan*

ARTICLE INFO

*Article history:*
Received 28 June 2017
Received in revised form 15 September 2017
Accepted 15 September 2017
Available online xxxx

*Keywords:*
Mitochondrial ATP
Cardiomyocytes
Hypertrophy
Hypoxia
Phenotypic screening

ABSTRACT

Since impaired mitochondrial ATP production in cardiomyocytes is thought to lead to heart failure, a drug that protects mitochondria and improves ATP production under disease conditions would be an attractive treatment option. In this study, we identified small-molecule drugs, including the anti-parasitic agent, ivermectin, that maintain mitochondrial ATP levels under hypoxia in cardiomyocytes. Mechanistically, transcriptomic analysis and gene silencing experiments revealed that ivermectin increased mitochondrial ATP production by inducing Cox6a2, a subunit of the mitochondrial respiratory chain. Furthermore, ivermectin inhibited the hypertrophic response of human induced pluripotent stem cell-derived cardiomyocytes. Pharmacological inhibition of importin β, one of the targets of ivermectin, exhibited protection against mitochondrial ATP decline and cardiomyocyte hypertrophy. These findings indicate that maintaining mitochondrial ATP under hypoxia may prevent hypertrophy and improve cardiac function, providing therapeutic options for mitochondrial dysfunction.


## 1. Introduction

Despite recent progress in medical therapies, heart failure remains one of the leading causes of death and healthcare burden worldwide (Ziaeian and Fonarow, 2016). Cardiomyocytes contain numerous mitochondria to support the heart's demand for adenosine triphosphate (ATP), and studies have implicated mitochondrial dysfunction in human patients and animal models of heart diseases, as demonstrated by reduced ATP levels (Beer et al., 2002), decreased enzymatic activity in the oxidative phosphorylation (OXPHOS) system (Ahuja et al., 2013), and enhanced reactive oxygen species (ROS) production (Borchi et al., 2010; Ide et al., 2000). Associations of mitochondrial dysfunction with heart diseases are also supported by previous studies, in which cardiomyopathy was observed in patients with mutations in critical mitochondrial proteins (Meyers et al., 2013). Since the heart becomes vulnerable to hypoxia in patients with heart disease and mitochondria require oxygen to synthesize ATP, hypoxia may be one of the key pathogenic factors causing impaired OXPHOS in heart diseases (Krishnan et al., 2009). Although targeting mitochondria has garnered increasing attention (Brown et al., 2017), there is no currently approved therapy that targets this organelle. Therefore, it would be valuable to identify novel therapeutic approaches for heart diseases based on mitochondrial ATP generation under pathological conditions, such as hypoxia. Although it had been challenging to directly measure mitochondrial ATP, a fluorescence resonance energy transfer (FRET)-based ATP biosensor, ATeam, has been recently reported, which made it possible to monitor spatiotemporal changes in ATP levels in a specific organelle, including mitochondria, in living cells (Imamura et al., 2009).

In this study, we identified small-molecule drugs, including the anti-parasitic drug, ivermectin, which maintain mitochondrial ATP levels under hypoxia in cardiomyocytes, through high-throughput phenotypic screens of a drug library using the aforementioned mitochondrial FRET-based ATP biosensor. We also assess the antihypertrophic effects of these mitochondrial modulators in human induced pluripotent stem cell (iPSC)-derived cardiomyocytes. Moreover, the transcriptomic analyses provide mechanistic insights into how these modulators exert their effects on mitochondrial ATP. This study provides previously unappreciated insights into the regulation of mitochondrial ATP, and has therapeutic implications for diseases involving mitochondrial dysfunction.

*Abbreviations:* ATP, adenosine triphosphate; BNP, brain natriuretic peptide; CsA, cyclosporin A; ET-1, endothelin 1; FCCP, carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone; FPKM, fragments per kilobase of transcript per million mapped reads; FRET, fluorescence resonance energy transfer; HIF, hypoxia inducible factor; iPSC, induced pluripotent stem cell; mPTP, mitochondrial permeability transition pore; OXPHOS, oxidative phosphorylation; ROS, reactive oxygen species; UPR, unfolded protein response.

* Corresponding authors.
*E-mail addresses:* hiroaki.nagai@takeda.com (H. Nagai), yoshiyuki.tsujihata@takeda.com (Y. Tsujihata).

http://dx.doi.org/10.1016/j.ebiom.2017.09.022

## 2. Materials and Methods

### 2.1. Chemicals and Compounds

The Prestwick Chemical Library was purchased from PerkinElmer. Dimethyl sulfoxide (DMSO), 2-deoxy-D-glucose (2-DG), diazoxide, cyclosporin A (CsA), *N*-acetyl cysteine (NAC), IPA-3, bifonazole, verapamil, tunicamycin, and thapsigargin were purchased from Wako. Carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP), deferoxamine mesylate, importazole, ivermectin, and oligomycin A were purchased from Sigma-Aldrich. Mito-TEMPO was purchased from Enzo Life Sciences. Compound 968 was purchased from Calbiochem. Dexrazoxane was purchased from TCI Chemicals. Cariporide was purchased from Cayman Chemical. Nifuroxazide was purchased from MP Biomedicals. 2-Methylthioadenosine triphosphate (2-meSATP) was purchased from Tocris. Azoramide was purchased from Ark Pharm, Inc.

### 2.2. Plasmids and siRNA

The plasmid vectors expressing ATeam (AT1.03 and mitAT1.03-YEMK in pcDNA3.1) (Imamura et al., 2009) were obtained under license to Osaka University. Control siRNA (ON-TARGETplus siRNA-SMARTpool, Non-targeting pool, D-001810-10-05) and Cox6a2 siRNA (ON-TARGETplus siRNA-SMARTpool, L-043169-01-0005) were purchased from Dharmacon/Thermo Fisher Scientific.

### 2.3. Cell Culture

HL-1 cardiomyocytes, obtained from William Claycomb (Louisiana State University, New Orleans, LA, USA), were cultured as previously described (Claycomb et al., 1998). In brief, Claycomb medium (Sigma-Aldrich), supplemented with 2 mM L-glutamine, 100 U/mL penicillin/streptomycin, 100 μM norepinephrine in 30 mM L-ascorbic acid, and 10% fetal bovine serum (FBS), was replaced every other day. The culture flasks and plates were precoated with a solution of 0.02% (weight/volume) gelatin containing 5 μg/mL fibronectin (Sigma-Aldrich). HeLa cells (ECACC, 93021013) were purchased from DS Pharma Biomedical, and cultured in DMEM (Gibco) containing 10% FBS and 100 U/mL penicillin/streptomycin. Human iPSC-derived cardiomyocytes (CMC-100-010-001) were purchased from Cellular Dynamics International (CDI) and cultured in maintenance medium (CDI, CMM-100-120-001) according to the manufacturer's instructions. Cells were maintained at 37 °C in a humidified 5% $CO_2$ incubator.

### 2.4. Generation of HL-1 Cardiomyocytes Stably Expressing ATeam

HL-1 cardiomyocytes were reverse-transfected with plasmids expressing either mitAT1.03-YEMK (mito-ATeam) for mitochondrial ATP measurement or AT1.03 (cyto-ATeam) for cytosolic ATP measurement using Lipofectamine LTX (Invitrogen) in Opti-MEM (Gibco), according to the manufacturer's instructions. Four days after transfection, selection using 0.25 mg/mL geneticin (Gibco) was started. Populations of cells expressing ATeam at moderate levels were then selected using FACS Aria II cell sorter (Becton Dickinson) based on YFP fluorescence. The enriched ATeam stable cells were cultured as described above in the presence of geneticin.

### 2.5. Animals

The care and use of the animals and the experimental protocols used in this study were approved by the Experimental Animal Care and Use Committee of Takeda Pharmaceutical Company Limited. All experiments were performed in accordance with the guidelines and regulations of Takeda Pharmaceutical Company Limited (Shonan Research Center IACUC Guidelines). C57BL/6J mice were purchased from CLEA Japan, Inc. All mice used in this study were maintained in pathogen-free barrier animal facilities.

### 2.6. Generation of Mito-ATeam Knock-in Mice

The targeting vector pROSA26-mitAT1.03-YEMK was constructed, in which a CAG-loxP-Neo cassette-loxP-mitAT1.03-YEMK unit was inserted into the ROSA26 locus by the Red/ET recombination system (Gene Bridges) to modify the bacterial artificial chromosome with the mouse ROSA26 gene (Advanced Geno Techs Co., RP23-184A7), as shown in Fig. 3a. pROSA26-mitAT1.03-YEMK was electroporated into embryonic stem cells (ESCs) derived from C57BL/6J mice. Clones with the inserted allele were screened by genomic PCR after G418 selection. The Neo cassette was removed by transient expression of pCAG-Cre plasmids in recombinant ESCs. Chimeric mice were generated by the tetraploid complementation method described previously (Yamamoto et al., 2013). The chimeric mice were crossed with C57BL/6J mice to obtain mice heterozygous for the mutant allele. Heterozygous mice were intercrossed to obtain animals homozygous for the mutant allele and wild-type littermates.

### 2.7. Isolation of Primary Neonatal Cardiomyocytes

Neonatal murine cardiomyocytes were isolated from mito-ATeam knock-in mice and wild-type littermates at 1 day of age and maintained as described previously (Sreejit et al., 2008). Isolated primary cardiomyocytes were cultured for up to 5 days for subsequent studies.

### 2.8. Imaging

All confocal images were acquired with a high-content screening system, Cell Voyager 7000 (CV7000; Yokogawa), which was equipped with a confocal scanner unit, live-cell incubator (maintained at 37 °C and 5% $CO_2$) with a customized lid for hypoxia experiments, and built-in liquid handler. Hypoxic gas (1% $O_2$, 5% $CO_2$, and 94% air) was supplied using a gas mixer (Tokken, Inc.).

#### 2.8.1. Live-Cell Imaging of ATP in HL-1 Cardiomyocytes

ATeam stable cells were seeded at a density of 10,000 cells per well into 96-well imaging plates and treated with compounds typically for 24 h. DMSO (0.1%) was used as a negative control. ATeam biosensors were excited at 445 nm, and emission was recorded at 480/17 nm for CFP and 543/22 nm for YFP at ×20 (0.75 NA UPLSAPO objective, 37 °C) or ×60 (1.2 NA UPLSAPO objective, 37 °C) magnification. Images were acquired before and after adding 10 μM oligomycin A, or under normoxia (21% $O_2$) followed by hypoxia (1% $O_2$) and reoxygenation (21% $O_2$) for the indicated times at 5- to 10-min intervals. Image analysis was performed using CV7000 analysis software (Yokogawa); a region of interest (ROI) was defined based on CFP fluorescence intensity; fluorescence intensity of YFP (as well as that of CFP) in the ROI was quantified. The YFP/CFP emission ratio (FRET ratio) per ROI was then calculated by dividing YFP fluorescence intensity by CFP fluorescence intensity using TIBCO Spotfire (TIBCO). Ratiometric FRET images were obtained using ImageJ software (National Institutes of Health).

#### 2.8.2. Live-Cell Imaging of Mitochondrial ATP in Primary Cardiomyocytes

Neonatal cardiomyocytes were seeded at a density of 200,000 cells per well into 96-well imaging plates and treated with compounds for 24 h in Dulbecco's modified Eagle's medium (DMEM)/F-12 (Gibco) supplemented with 0.2% bovine serum albumin (BSA) and 100 U/mL penicillin/streptomycin. Live-cell imaging experiments and image analysis were performed as described above.

Please cite this article as: Nagai, H., et al., Antihypertrophic Effects of Small Molecules that Maintain Mitochondrial ATP Levels Under Hypoxia, EBioMedicine (2017), http://dx.doi.org/10.1016/j.ebiom.2017.09.022