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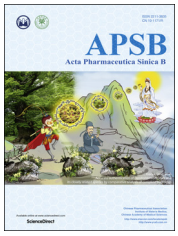

ORIGINAL ARTICLE

Mitochondrial uncoupler BAM15 inhibits artery constriction and potently activates AMPK in vascular smooth muscle cells

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KEY WORDS

BAM15;
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Smooth muscle cells;
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Metformin

Abstract Our previous studies found that mitochondrial uncouplers CCCP and niclosamide inhibited artery constriction and the mechanism involved AMPK activation in vascular smooth muscle cells. BAM15 is a novel type of mitochondrial uncoupler. The aim of the present study is to identify the vasoactivity of BAM15 and characterize the BAM15-induced AMPK activation in vascular smooth muscle cells (A10 cells). BAM15 relaxed phenylephrine (PE)-induced constricted rat mesenteric arteries with intact and denuded endothelium. Pretreatment with BAM15 inhibited PE-induced constriction of rat mesenteric arteries with intact and denuded endothelium. BAM15, CCCP, and niclosamide had the comparable IC_{50} value of vasorelaxation in PE-induced constriction of rat mesenteric arteries. BAM15 was less cytotoxic in A10 cells compared with CCCP and niclosamide. BAM15 depolarized mitochondrial membrane potential, induced mitochondrial fission, increased mitochondrial ROS production, and increased mitochondrial oxygen consumption rate in A10 cells. BAM15 potently activated AMPK in A10 cells and the efficacy of BAM15 was stronger than that of CCCP, niclosamide, and AMPK positive activators metformin and AICAR. In conclusion, BAM15 activates AMPK in vascular smooth muscle cells with higher potency than that of CCCP, niclosamide and the known AMPK activators metformin and AICAR. The present work indicates that BAM15 is a potent AMPK activator.

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1. Introduction

Mitochondria are key cellular organelles which provide energy through oxidative phosphorylation process. Normally, the nutrient oxidation is coupled with ATP production in cells *via* the electrochemical proton gradient across mitochondrial inner membrane. However, when the proton leaks back to the mitochondrial matrix through either the endogenous mitochondrial uncoupling proteins(UCPs) or the exogenous chemical uncouplers, the proton gradient decreases and the ATP production is reduced, which is referred as "mitochondrial uncoupling". Mitochondrial uncoupling is not completely harmful, "mild mitochondrial uncoupling" is reported to be protective in a variety of disorders, including obesity[1], diabetes[2], ischemia/reperfusion injury[3], Parkinson's disease[4] and aging[5]. For instance, UCP2 overexpression restored the impaired endothelium-dependent relaxation in obese diabetic mice[6], inhibited proliferation and migration of vascular smooth muscle cells induced by high glucose and Ang II[7]; in addition to overexpression of UCPs, through the mechanism of inducing mild mitochondrial uncoupling, the chemical mitochondrial uncouplers improved diabetic symptoms[2], reversed hypertriglyceridemia, fatty liver disease, and insulin resistance[8,9], and combated obesity[10] in mice.

Due to the beneficial effects of mild mitochondrial uncoupling, developing mitochondrial uncouplers to induce mild mitochondrial uncoupling is a strategy for treating not only the metabolic disorders but also the heart, vascular, and nerve injury. However, the limitation of the widely used protonophore uncouplers such as FCCP is that they induce plasma membrane depolarization, resulting in off-target effects[11]. Based on this reason, Kenwood et al.[12] developed a new mitochondrial uncoupler BAM15 which had a broad effective range and did not affect plasma membrane electrophysiology. Furthermore, they proved that BAM15 protected against kidney ischemic-reperfusion injury *in vivo*[12]. This finding brings promising hope for the development of the novel mitochondrial uncouplers.

Our previous works systemically studied the vasorelaxant effect of mitochondrial uncouplers and found that mitochondrial uncouplers inhibited artery constriction and the mechanisms involved AMPK activation in vascular smooth muscle cells[13,14]. BAM15 is a new type of mitochondrial uncoupler with different characterization from the classical uncoupler FCCP[12], which enlightens us to identify: (1) whether BAM15 shows the similar vasoactivity as the classical uncouplers? (2) whether BAM15 has the similar effect of activating AMPK as the classical uncouplers? Identification of these pharmacological properties is important for developing novel type of mitochondrial uncouplers with potential clinic use. In the present work, we compared the effects of BAM15 on artery constriction and AMPK signal in vascular smooth muscle cells with that of mitochondrial uncouplers CCCP and niclosamide. We found that BAM15 inhibited artery constriction, similarly to the effects of CCCP and niclosamide. However, BAM15 had higher potency of activating AMPK in vascular smooth muscle cells than CCCP, niclosamide and the known AMPK activators metformin and AICAR, indicating that BAM15 is a promising AMPK activator with potential use in metabolic disorders.

2. Materials and methods

2.1. Agents and animals

BAM15 (N^5,N^6-bis(2-fluorophenyl)[1,2,5]oxadiazolo[3,4-*b*]pyrazine-5,6-diamine) was purchased from Cayman chemical (USA). CCCP, acetylcholine chloride (Ach), oligomycin, rotenone and antimycin A were purchased from Sigma Aldrich Chemistry (Saint Louis, MO, USA). Niclosamide ethanolamine salt was purchased from Shanghai Rongbai biological technology Co., Ltd. (Shanghai, China). Phenylephrine (PE) was purchased from Shanghai Harvest Pharmaceutical Co., Ltd. (Shanghai, China). Mito-Tracker Green, fluo-3/AM, MitoSOX, tetramethylrhodamine methyl ester (TMRM) and hoechst were purchased from life technology (Invitrogen, Oregon, USA). Metformin HCl, AICAR, BAPTA/AM, STO609 were purchased from Selleck Chemicals (shanghai, China). AMPK, p-AMPK (Thr172) antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). Arterial smooth muscle cells (A10) were purchased from ATCC (Manassas, Virginia, USA). Adult male Sprague–Dawley rats were purchased from Charles River (Charles River Laboratory Animal, Beijing, China). All animal experiments were approved by the Institutional Animal Care and Use Committee of Harbin Medical University.

2.2. Mesenteric artery and aorta tension measurement

The methods in detail were described in our previous studies[13–16]. Briefly, The entire mesentery and thoracic aorta were removed from adult male Sprague–Dawley rats (300–350 g) and placed in a petri dish with cold physiological saline solution (PSS) aerated with gas (95% O_2 + 5% CO_2). The mesenteric arteries and thoracic aorta were dissected into 2-mm and 4-mm rings respectively. The isometric contractions of mesenteric arterial rings and thoracic aortic rings were measured by using multi wire myograph system (model 620 DMT, Danish Myo Technology, Denmark) and multi-channel myograph system (BL-420S, Chengdu Taimeng

Software Co., Ltd., China), respectively. The KPSS (60 mmol/L K^+) solution for inducing vasoconstriction was composed of (mmol/L): NaCl, 74.7; KCl, 60; $MgSO_4 \cdot 7H_2O$, 1.17; KH_2PO_4, 1.18; $NaHCO_3$, 14.9; $CaCl_2$, 1.6; D-glucose, 5.5; EDTA, 0.026.

2.3. Live and dead cell staining

The viability of A10 cells was measured by using live/dead viability/cytotoxicity assay kit as described in our previous study[17]. The numbers of live and dead cells were counted automatically with Image J software (NIH, USA).

2.4. Western blot

The protein levels were analyzed by Western blot. The methods in detail were described in our previous studies[13–15].

2.5. Measurement of cellular ATP concentration and ADP/ATP ratio

The level of ATP was measured by using the ATP bioluminescence assay kit (Beyotime, Shanghai, China). The ADP/ATP ratio was measured by use of an ADP/ATP-Lite assay kit (Vigorous Bio, Beijing, China). The methods in detail were described in our previous studies[13,14].

2.6. Measurement of mitochondrial membrane potential

The mitochondrial membrane potential was measured as described in our previous works[13,14]. Briefly, the cells were incubated in free medium containing TMRM (50 nmol/L) for 45 min and hoechst (1 μg/mL) for 12 min in dark at 37 °C, then were imaged by using confocal laser scanning microscopy (Zeiss LSM 700; Zeiss; Oberkochen, Germany). The red fluorescence serves as an indicator of the mitochondrial membrane potential and the blue fluorescence serves as an indicator of cell nucleus. Mitochondrial membrane potential was quantified as the change in fluorescence over the initial TMRM fluorescence (F/F_0).

2.7. Measurement of mitochondrial reactive oxygen species (mitoROS)

The mitoROS was detected as described in our previous works[13,14]. Cells were loaded with MitoSOX (5 μmol/L) for 20 min and Hoechst (1 μg/mL) for 12 min at 37 °C. The red fluorescence representing mitochondrial ROS was imaged by using confocal microscopy (Fluoview Fv10i, Olympus, Japan) and quantified by using Image software.

2.8. Measurement of intracellular $[Ca^{2+}]_i$

The intracellular $[Ca^{2+}]_i$ was detected as described in our previous works[13,14]. Cells were loaded with Ca^{2+}-sensitive dye Fluo-3/AM (5 μmol/L) and incubated at 37 °C for 15 min. Then, the cell nucleus was incubated with hoechst (1 μg/mL) for 12 min. The fluorescence intensity reflecting $[Ca^{2+}]_i$ was measured by confocal microscopy (Zeiss LSM 700; Zeiss; Oberkochen, Germany). The change of intracellular $[Ca^{2+}]_i$ was monitored with the relative intensity of green fluorescence.

2.9. Staining of mitochondrial morphology

The mitochondria morphology of A10 cells was observed by using a mitochondrial selective probe MitoTracker Green as described in our previous work[15].

2.10. Mitochondrial respiratory measurements

The intact whole cell respiratory function was determined by high-resolution respirometry (Oxygraph-2k; Oroboros Instruments, Innsbruck, Austria). The protocol was designed as ATP synthase inhibitor-uncoupler-inhibitor titrations to evaluate the effects of uncouplers on the maximal uncoupled respiratory capacity of the electron transfer system. A10 cells were centrifuged and suspended in culture medium, and then about 10^6 cells/cm^3 intact cells were added to the two O2k-chambers (2 cm^3). The routine respiration is followed by inhibition of ATP synthase (manual titration of oligomycin, 5 mmol/L, 1 μL) to induce the non-phosphorylating LEAK state. When respiration is stable, manual titrations of uncoupers (1 mmol/L CCCP, 1 mmol/L NEN, 1 mmol/L BAM15) were in steps of 1 μL for 10 times, 4 μL for 2 times and 10 μL for 2 or 3 times to induce maximum noncoupled flux (capacity of the election transfer system, ETS) at intervals of 120 s. Then, 1 μL rotenone (1 mmol/L) and 1 μL antimycin A (5 mmol/L) were used to get the residual respiration.

2.11. Statistical analysis

Data were expressed by mean ± SEM and analyzed by using Sigma Plot 12.5. Two-group comparisons were performed by Student's *t*-test. Multiple-group comparisons were carried out using one-way ANOVA. $P < 0.05$ was considered significant.

3. Results

3.1. BAM15 inhibits phenylephrine (PE)-induced constriction of rat mesenteric arteries

Our previous studies found that mitochondrial uncouplers inhibited artery constriction[13,14,18], and that BAM15 is a new uncoupler with different characterization from the classical uncouplers. Therefore, we examined the vasoactivity of BAM15 firstly. BAM15 relaxed PE-induced constricted rat mesenteric arteries with intact and denuded endothelium in a dose-dependent manner (Fig. 1A–F). Pretreatment with BAM15 inhibited PE-induced constriction of rat mesenteric arteries with intact and denuded endothelium (Fig. 1G–H).

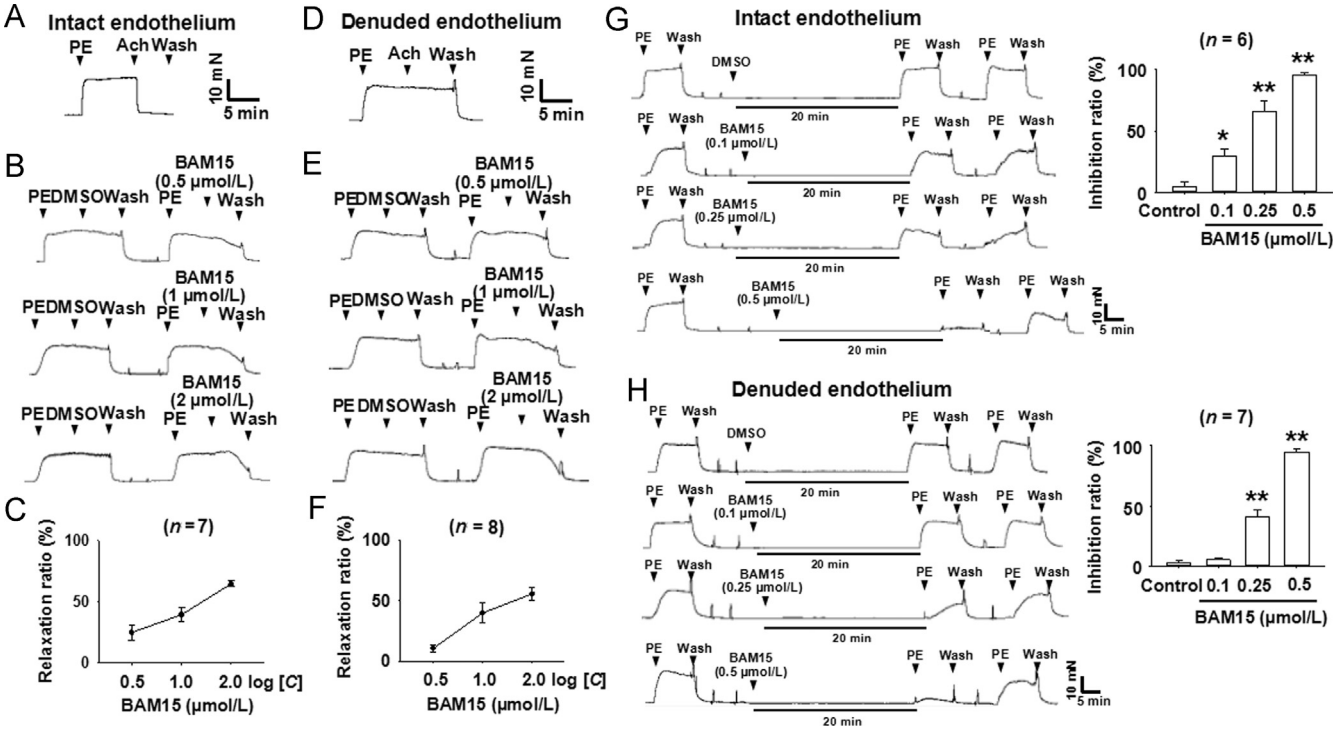

Figure 1 BAM15 inhibited phenylephrine (PE)-induced constriction of rat mesenteric arteries. (A) Acetylcholine (1 μmol/L, Ach)-induced vasorelaxation confirmed the rat mesenteric arteries with intact-endothelium. (B) and (C) BAM15 elicited dose-dependent relaxation of PE-induced constriction of rat mesenteric arteries with intact endothelium. (D) The absence of Ach (1 μmol/L)-induced vasorelaxation confirmed the rat mesenteric arteries with denuded-endothelium. (E) and (F) BAM15 elicited dose-dependent relaxation of PE-induced constriction of rat mesenteric arteries with denuded endothelium. (G) and (H) BAM15 pretreatment inhibited PE-induced vasoconstriction in rat mesenteric arteries with intact and denuded endothelium. $^{*}P<0.05$, $^{**}P<0.01$ *vs* control.

3.2. *BAM15 relaxes constricted thoracic aorta of rats*

We further examined the effect of BAM15 on PE-induced constriction of rat aorta. As shown in Fig. 2A–B, BAM15 dose-dependently relaxed PE-induced constriction of rat aorta. However, although BAM15 relaxed the rat aorta constriction induced by high K^{+} (KPSS), the KPSS-induced aorta constriction was less sensitive to BAM15 treatment than PE-induced aorta vasoconstriction (Fig. 2C).

3.3. *Comparison of vasoactivity of BAM15, CCCP and niclosamide*

Since BAM15 showed the similar vasoactivity as the classical mitochondrial uncouplers CCCP and niclosamide[13,14], we compared the efficacy of vasoactivity of BAM15, CCCP, and niclosamide (niclosamide ethanolamine salt). As shown in Fig. 3, the vasorelaxant efficacy of BAM15 was relatively lower than that of CCCP, and niclosamide, whereas BAM15, CCCP, and

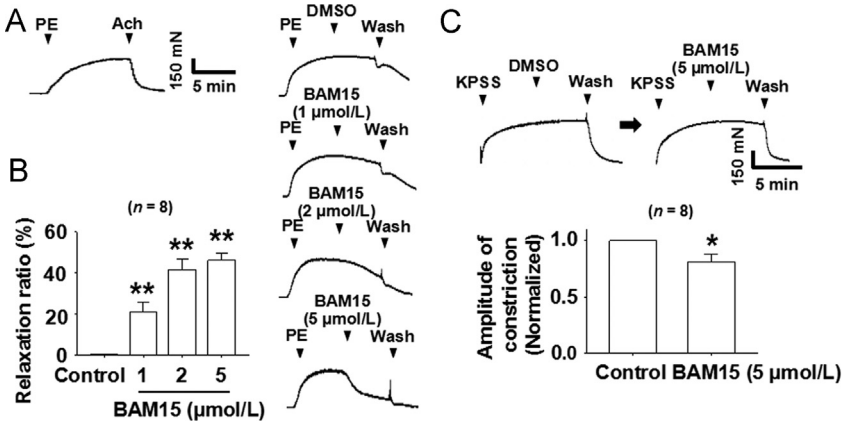

Figure 2 BAM15 relaxed vasoconstriction of rat thoracic aorta with intact endothelium. (A) and (B) BAM15 dose-dependently relaxed PE-induced vasoconstriction of rat thoracic aorta with intact endothelium. $^{**}P<0.01$ *vs* DMSO (control). (C) BAM15 (5 μmol/L) relaxed KPSS-induced vasoconstriction of rat thoracic aorta with intact endothelium. $^{*}P<0.05$ *vs* DMSO (control).

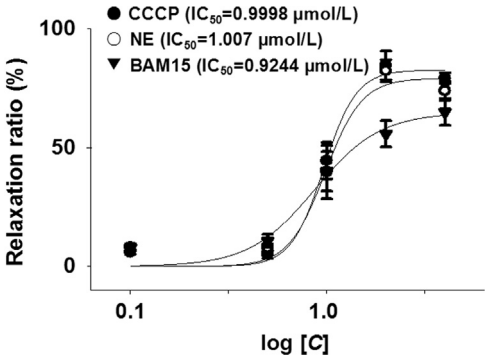

Figure 3 The comparison of vasoactivity of BAM15, CCCP and niclosamide. BAM15, CCCP and niclosamide showed equivalent vasorelaxation in rat mesenteric arteries with denuded endothelium. CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; NE, niclosamide ethanolamine salt.

niclosamide had the comparable IC_{50} value of vasorelaxation in PE-induced constriction of rat mesenteric arteries.

3.4. *Effects of BAM15, CCCP and niclosamide on cell viability, mitochondrial membrane potential, mitochondrial ROS, mitochondrial fission, and mitochondrial respiration of A10 cells*

BAM15 was reported to be less cytotoxic than FCCP in L6 and NmuLi cells[12]. We further compared the cytotoxic effect of BAM15, CCCP and niclosamide with the same concentrations (10 μmol/L) in A10 cells. As shown in Fig. 4A, the optical images showed that the cell densities in CCCP and niclosamide groups were less than that in control group, and dead cells appeared in niclosamide group; however, the cell state in BAM15 group was no significant change. By using the LIVE/DEAD® cell viability assays, we further proved that BAM15 at 10 μmol/L had no significant cytotoxicity in A10 cells, but both CCCP and niclosamide had shown significant cytotoxicity at the same concentrations (Fig. 4B).

BAM15 is a mitochondrial uncoupler, we further confirmed the mitochondrial uncoupling effect of BAM15 in A10 cells. As shown in Fig. 5A, the TMRM staining results showed that BAM15 treatment significantly depolarized the mitochondrial membrane potential in A10 cells and the analyzed data was shown in Fig. 5B. BAM15 treatment also increased mitochondrial ROS production in A10 cells (Fig. 5C–D**).** We further measured the effect of BAM15, CCCP and niclosamide on oxygen consumption rate in A10 cells by using Oxygraph-2k. As shown in Fig. 5E and F, the sequence of the potency of increasing oxygen consumption rate was niclosamide > CCCP > BAM15. However, the maximal efficacy of inducing mitochondrial uncoupling was basically equivalent for three types of mitochondrial uncouplers (Fig. 5G).

The classical mitochondrial uncouplers induced mitochondrial fission[19,20]. Since BAM15 is a new type of mitochondrial uncoupler with different characterization from the classical uncouplers, we wondered its effect on mitochondrial fission in A10 cells. As shown in Fig. 6, BAM15 at 2 μmol/L induced mitochondrial fission in A10 cells, similarly to the effects of classical uncouplers CCCP and niclosamide.

3.5. *BAM15 potently activates AMPK in A10 cells*

Our previous studies found that CCCP and niclosamide activated AMPK in A10 cells[13,14], we further examined the effect of BAM15 on AMPK in A10 cells. As shown in Fig. 7A, BAM15 treatment (from 0.25 to 5 μmol/L) for 5 min induced significant activation of AMPK in A10 cells, and the BAM15-induced AMPK activation was dose-dependent. We compared the effect of BAM15, CCCP and niclosamide on AMPK activation in A10 cells at the same concentration (1 μmol/L). CCCP and niclosamide at 1 μmol/L showed no significant effect on AMPK while BAM15 at 1 μmol/L had activated AMPK in A10 cells (Fig. 7B). We further compared the effect of the known AMPK activators metformin and AICAR with that of BAM15. Metformin and AICAR treatment (2 mmol/L) for 1 h did not affect AMPK activity, but BAM15 at 5 μmol/L significantly activated AMPK in A10 cells (Fig. 7C). We further extended the treatment time to

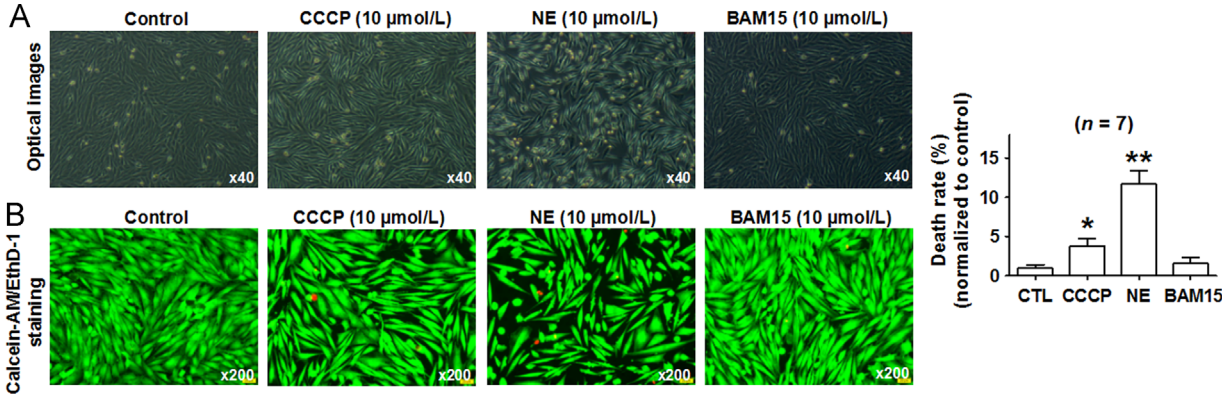

Figure 4 The effect of BAM15, CCCP and niclosamide on cell viability. (A) The optical images of A10 cells treated with BAM15, CCCP and niclosamide at 10 μmol/L for 24 h. (B) The representative images of Live and Dead staining of A10 cells treated with BAM15, CCCP and niclosamide at 10 μmol/L for 24 h, and the analyzed data. The live cells were stained in green and dead cells in red. $^{*}P<0.05$,$^{**}P<0.01$ *vs* control, $n = 7$ in each group. CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; NE, niclosamide ethanolamine salt.

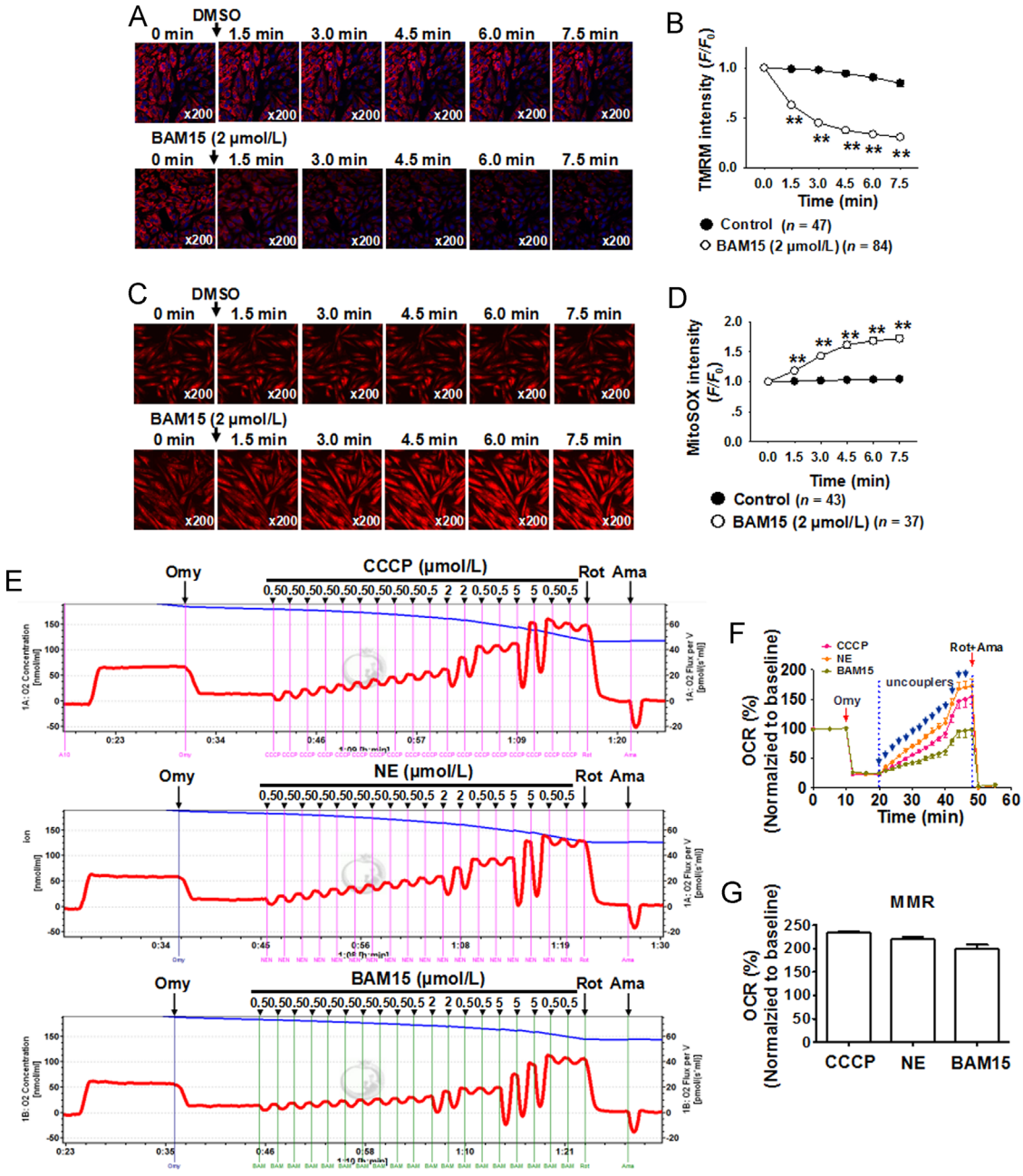

Figure 5 BAM15 induced mitochondrial uncoupling in A10 cells. (A) and (B) TMRM staining images showed that BAM15 (2 μmol/L) treatment depolarized mitochondrial membrane potential and the summarized data. $^{**}P<0.01$ *vs* control. (C) and (D) BAM15 (2 μmol/L) treatment increased mitochondrial ROS production in A10 cells. $^{**}P<0.01$ *vs* control. (E) and (G) Representative profiles and summarized data for oxygen consumption in A10 cells treated with BAM15, CCCP and niclosamide ($n = 6$). Omy, oligomycin (2.5 μmol/L); Rot, rotenone (0.5 μmol/L); Ama, antimycin A (2.5 μmol/L). CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; NE, niclosamide ethanolamine salt. OCR, oxygen consumption rate; MMR: maximal mitochondrial respiration.

6 h, metformin still could not activate AMPK; AICAR-induced AMPK activation could be detected, but the AICAR-induced AMPK activation was markedly less than that of BAM15 at 5 μmol/L (Fig. 7D). The above results suggested that BAM15 was a potent AMPK activator.

AMPK is mainly activated by the cellular AMP/ATP ratio increase or Ca^{2+}/calmodulin-dependent protein kinase kinase (CaMKKβ) signal[21]. Our previous works found that the classical uncouplers CCCP and niclosamide increase intracellular $[Ca^{2+}]_i$ concentration in A10 cells[13,14]. Therefore, we speculated that

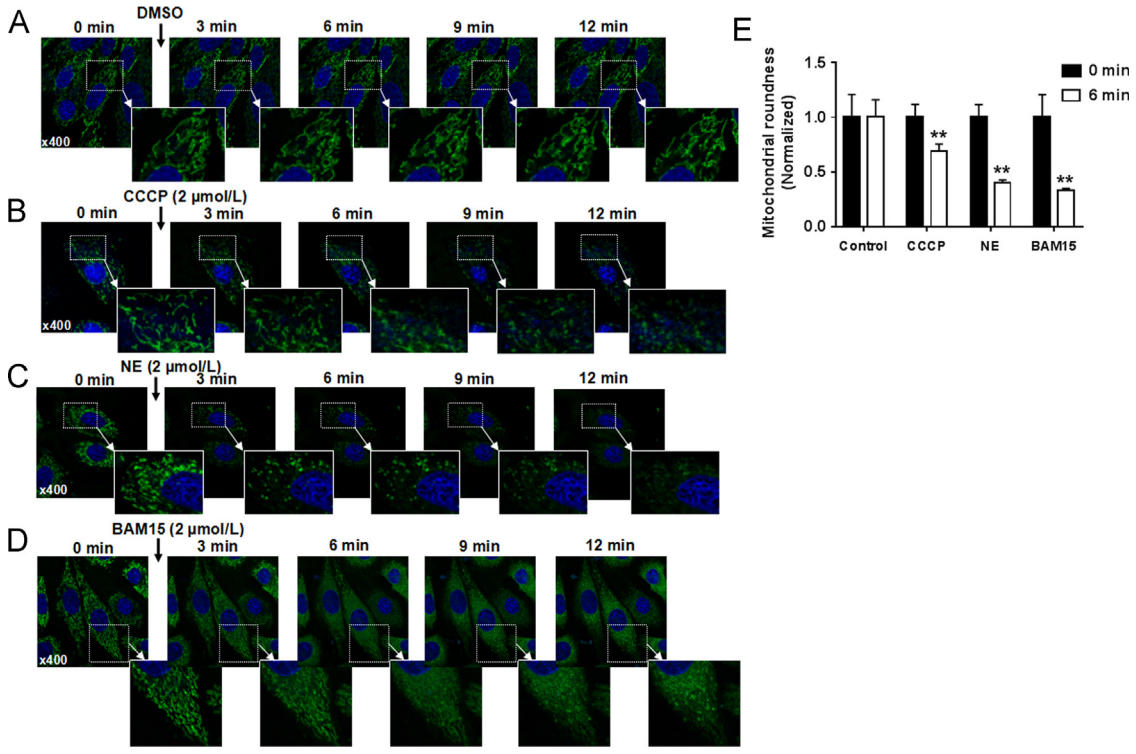

Figure 6 BAM15, CCCP, and niclosamide induced mitochondrial fission in A10 cells. (A)–(D) The framed areas were enlarged for clear exhibition. CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; NE, niclosamide ethanolamine salt. (E) Mitochondrial roundness was analyzed by using Image-Pro Plus software and normalized to the mean of mitochondrial roundness at 0 min. Roundness = Perimeter^2/(4πArea). $^{**}P<0.01$ *vs* 0 min, $n \geq 46$ in each group.

BAM15 might activate AMPK in A10 cells through increasing intracellular $[Ca^{2+}]_i$. However, we did not detect the BAM15-induced significant increase of intracellular $[Ca^{2+}]_i$ in A10 cells (Fig. 8A); the intracellular calcium chelator BAPTA/AM and the selective CaMKK inhibitor STO-609 did not inhibit BAM15-induced AMPK activation in A10 cells (Fig. 8B–C), indicating that BAM15-induced AMPK activation was not through Ca^{2+}/CaMKK pathway. We further examined the effect of BAM15 on cellular ATP level and ADP/ATP ratio in A10 cells, results showed that BAM15 treatment for 5 min reduced ATP production and increased ADP/ATP ratio (Fig. 8D), indicating that BAM15-induced AMPK activation might be through reducing ATP level and increasing ADP/ATP ratio.

4. Discussion

BAM15 is a novel mitochondrial uncoupler identified recently, the most important characterization of which is that it does not depolarize the plasma membrane[12]. Here, we found that BAM15 showed similar vasoactivity as the classical mitochondrial uncouplers CCCP and niclosamide[13,14]. Especially, we proved that BAM15 is a potent AMPK activator, the potency of which was higher than that of CCCP, niclosamide and the known AMPK activators metformin and AICAR; more importantly, it is less cytotoxic. In view of AMPK as the therapeutic target in multiple diseases[22–24], the present work indicates that BAM15 could be developed as a novel drug with AMPK as target.

Although the most important finding of the present study was that BAM15 was a potent AMPK activator; however, the first purpose of the present study was to compare the vasoactivity of the three uncouplers BAM15, CCCP and niclosamide. We had found that mitochondrial uncouplers CCCP and niclosamide induced vasorelaxation[13,14]. Since BAM15 is a new type of mitochondrial uncoupler with different characterization from the classical uncouplers, we compared the vasoactivity of the three uncouplers firstly. The three uncouplers had the comparable IC_{50} value of vasorelaxation in PE-induced constriction of rat mesenteric arteries but the vasorelaxant efficacy of BAM15 was relatively lower than that of CCCP, and niclosamide. Our previous work showed that AMPK activation was involved in CCCP- and niclosamide-induced vasorelaxation[13,14]. Next, we compared the effect of BAM15, CCCP, and niclosamide on AMPK signal in A10 cells. Surprisingly, we found that BAM15 strongly activated AMPK in A10 cells, and the potency was significantly higher than that of CCCP, niclosamide and the known AMPK activators metformin and AICAR. Therefore, the mechanism of uncoupler-induced AMPK activation and vasorelaxation remained to be clarified.

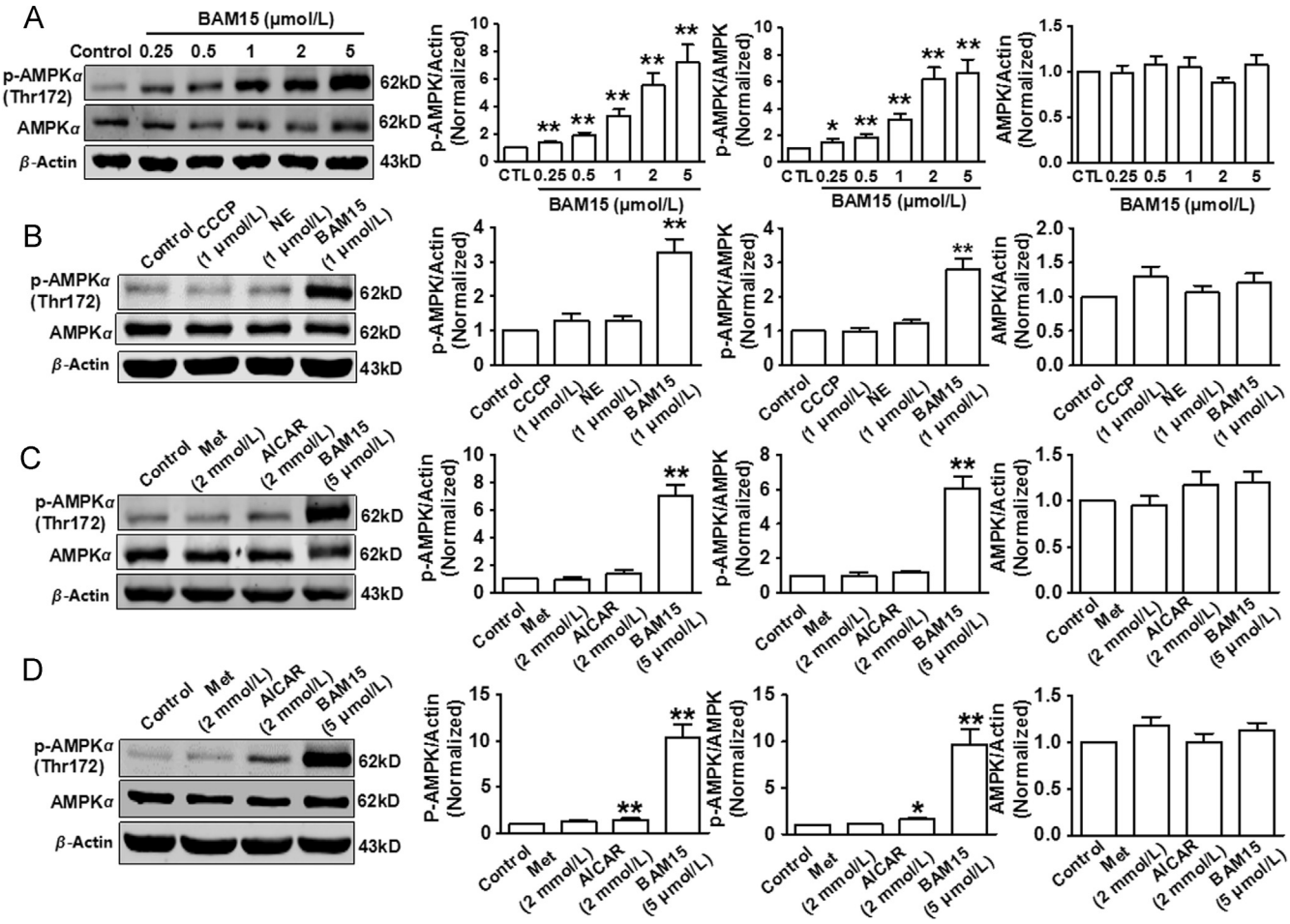

Figure 7 BAM15 activated AMPK in A10 cells. (A) BAM15 treatment for 5 min induced AMPK activation in A10 cells in a dose-dependent manner. $^{*}P<0.05$, $^{**}P<0.01$ *vs* control, $n=9$ in each group. (B) BAM15 but not CCCP and niclosamide treatment for 5 min at 1 μmol/L activated AMPK in A10 cells. $^{**}P<0.01$ *vs* control, $n = 11$ in each group. CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; NE, niclosamide ethanolamine salt. (C) BAM15 (5 μmol/L) but not metformin (2 mmol/L) and AICAR (2 mmol/L) treatment for 1 h activated AMPK in A10 cells. $^{**}P<0.01$ *vs* control, $n=6$ in each group. Met, metformin. (D) BAM15 (5 μmol/L) treatment for 6 h significantly activated AMPK compared with AICAR (2 mmol/L) and metformin (2 mmol/L). $^{*}P<0.05$, $^{**}P<0.01$ *vs* control, $n=7$ in each group. Met, metformin.

Metformin is the first-line drug for type 2 diabetes and is being proved to be therapeutic for multiple cardiovascular diseases and cancer. Metformin-induced activation of the energy-sensor AMPK mainly contributes its pharmacological actions, and the mechanisms of metformin-induced AMPK activation have been intensively studied. It was reported that metformin inhibited complex I of the electron transport chain, preventing mitochondrial ATP production, thus, increasing cytoplasmic AMP/ATP ratio and activating AMPK[25]. On the other hand, some studies showed that metformin treatment efficiently activated AMPK without disrupting energy state[26], so a lysosomal mechanism of AMPK activation was further proposed[27]. AICAR is another positive AMPK activator. AICAR is a nucleoside that is phosphorylated to the nucleotide 5-amino-4-imidazolecarboxamide riboside 5′-monophosphate (ZMP), which does not perturb the cellular contents of ATP, ADP or AMP, but mimics the effects of AMP on the AMPK activation[28]. Metformin and AICAR activate AMPK in many cell types and tissues to exert their therapeutic actions. For instance, they activated AMPK in MCF-7 and A549 cancer cells to inhibit tumor growth[29], activated AMPK in THP-1 monocytes to inhibit monocyte-to-macrophage differentiation[30], and activated AMPK in human pulmonary arterial smooth muscle cells to inhibit cell proliferation[31]. However, Metformin and AICAR had to be used at the mmol/L level in the *in vitro* experiments in these studies[29–32]. In the present study, we compared the effect of BAM15, metformin and AICAR on AMPK signal in A10 cells in the same experimental conditions. We found that the potency of AMPK activation by BAM15 was thousand times more than that of metformin and AICAR. More importantly, BAM15 at the concentrations activating AMPK showed no cytotoxic effect, indicating that BAM15 would be a new AMPK activator with potential clinical significance.

Increase of intracellular Ca^{2+} signal contributes to mitochondrial fission[15,33–35], we speculated that the classical mitochondrial

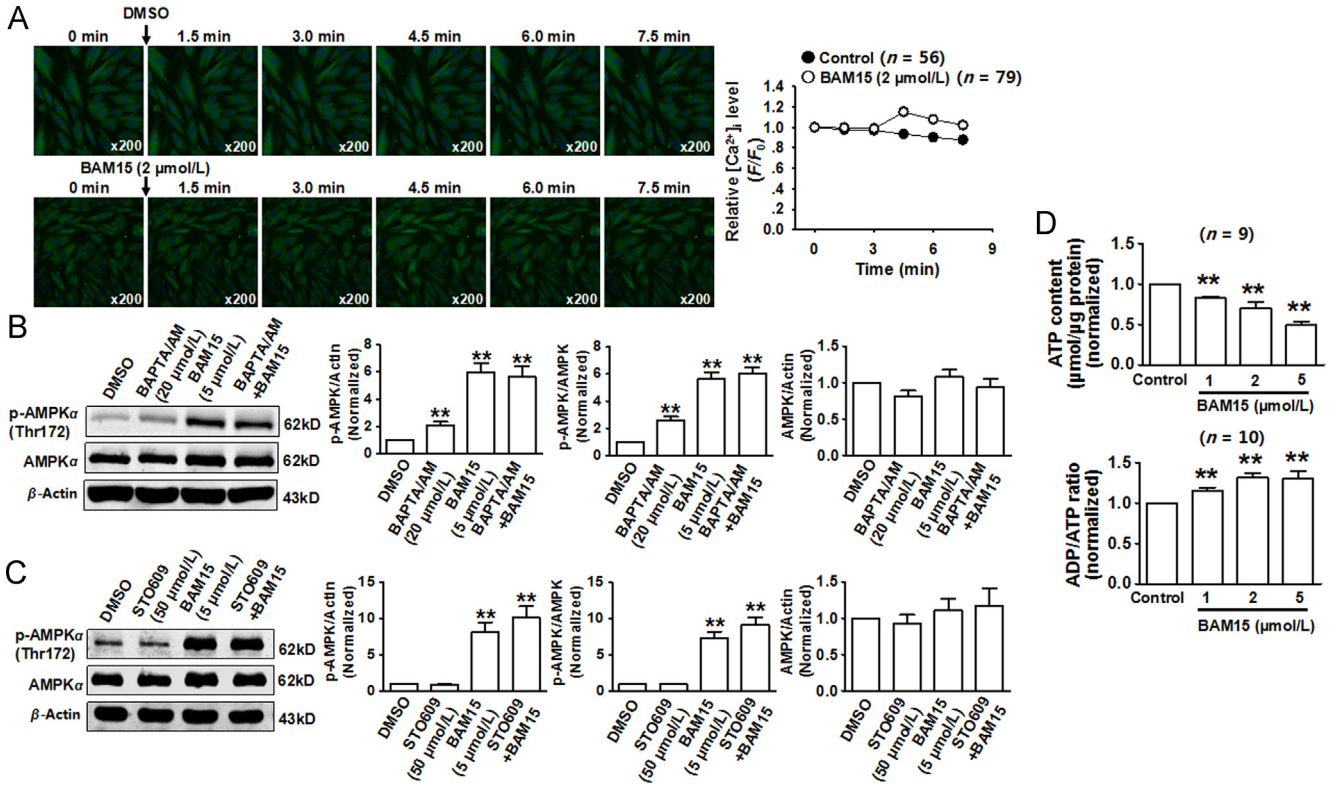

Figure 8 BAM15-induced AMPK activation in A10 cells were through reducing ATP production but not Ca^{2+}/CaMKK pathway activation. (A) The representative time-lapse images and the summarized data showed that BAM15 (2 μmol/L) did not significantly affect cytosolic $[Ca^{2+}]_i$ in A10 cells. (B) and (C) BAPTA/AM and STO609 pretreatments did not inhibit BAM15-induced AMPK activation in A10 cells. A10 cells were treated with BAM15 (5 μmol/L, 5min) after BAPTA-AM (20 μmol/L, 1h) and STO-609 (50 μmol/L, 1 h) pretreatments. $n=9$ in (B) and $n=10$ in (C). $^{**}P<0.01$ *vs* DMSO (control). (D) BAM15 treatment for 5 min reduced ATP production and increased ADP/ATP ratio in A10 cells. $^{**}P<0.01$ *vs* control.

uncouplers might stimulate mitochondrial fission through plasma membrane depolarization-induced $[Ca^{2+}]_i$ increase. Our previous works showed that the classical uncouplers CCCP and niclosamide increased $[Ca^{2+}]_i$ in A10 cells[13,14], and the present data showed that they induced mitochondrial fission in A10 cells. BAM15 did not depolarize the plasma membrane and we found that it did not significantly affect $[Ca^{2+}]_i$ in A10 cells indeed. However, BAM15 induced mitochondrial fission in A10 cells, indicating that mitochondrial uncoupler-induced mitochondrial fission might be not through the increase of $[Ca^{2+}]_i$. Previous studies reported that CCCP induced mitochondrial fission in various cell types including HeLa cells, MDCK cells and fibroblasts[36,37], and several mechanisms might be involved, including inducing dephosphorylation of Drp1 at S637[38], activating mitochondrial permeability transition[39], and stimulating mitochondrial fusion protein OPA1 to be cleaved by the inducible protease OMA1[40–42]. BAM15 might share the similar mechanisms of inducing mitochondrial fission as CCCP.

BAM15 has the general properties of mitochondrial uncouplers, including inducing mitochondrial membrane potential depolarization, increasing mitochondrial oxygen consumption rate, reducing ATP production, activating AMPK; however, compared with the classical uncouplers, BAM15 is significantly less cytotoxic, as evidenced by other[12] and our present data. These results indicate that the mitochondrial uncoupling effect of mitochondrial uncouplers could be separated from their cytotoxic effect, for instance, BAM15 strongly activates AMPK but is less cytotoxic. Therefore, it is promising to develop mitochondrial uncouplers as AMPK activator with clinical application.

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