

Mdivi-1 attenuates sodium azide-induced apoptosis in H9c2 cardiac muscle cells

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Abstract. The aim of the current study was to investigate the effect of mitochondrial division inhibitor 1 (Mdivi-1) in sodium azide-induced cell death in H9c2 cardiac muscle cells. Mdivi-1 is a key inhibitor of the mitochondrial division protein dynamin-related protein 1 (Drp1). Mdivi-1 was added to H9c2 cells for 3 h, after which, the cells were treated with sodium azide for 24 h. Cell viability was measured by Cell Counting kit-8 assay. DAPI staining was used to observe nuclear morphology changes by microscopy. To further investigate the role of mitochondria in sodium azide-induced cell death, mitochondrial membrane potential ($\Delta\Psi_m$) and the cellular ATP content were determined by JC-1 staining and ATP-dependent bioluminescence assay, respectively. Reactive oxygen species (ROS) production was also assessed by use of the specific probe 2',7'-dichlorodihydrofluorescein diacetate. In addition, the expression of Drp1 and of the apoptosis-related proteins BCL2 apoptosis regulator (Bcl-2), and BCL2 associated X (Bax) was determined by western blotting. The present findings demonstrated that pretreatment with Mdivi-1 attenuated sodium azide-induced H9c2 cell death. Mdivi-1 pretreatment also inhibited the sodium azide-induced down-regulation of Bcl-2 expression and upregulation of Bax and Drp1 expression. In addition, the mitochondrion was revealed to be the target organelle of sodium azide-induced toxicity in H9c2 cells. Mdivi-1 pretreatment moderated the dissipation of $\Delta\Psi_m$, preserved the cellular ATP contents and suppressed the production of ROS. The results suggested that the mechanism of sodium azide-induced cell death in H9c2 cells may involve the mitochondria-dependent apoptotic pathway. The present results indicated that Mdivi-1 may have a cardioprotective effect against sodium azide-induced apoptosis in H9c2 cardiac muscle cells.

Introduction

Sodium azide (NaN_3), has a wide range of applications. It is used in the military setting as a substrate in explosive materials, a propulsion agent in jet aircraft and in airplane escape chutes, and in the industrial setting as an ingredient to inflate automobile airbag gas. It is also used as an insecticide, herbicide, nematocide, fungicide and bactericide in agriculture and as a potent preservative in clinical laboratories and hospitals (1-3). Sodium azide is highly toxic, similarly to cyanide poisoning, and poisoning by sodium azide poses a serious risk of death. In recent years, cases involving sodium azide poisonings were still reported in the literature, despite limited access to chemicals of this type (4-8). Various reports have demonstrated that sodium azide poisoning causes severe hypoxemia (9-11), and extensive damage in the nervous (10,12) and cardiac systems (11,13,14). A previous study demonstrated that sodium azide also causes acute kidney injury (15). It is known that aerobic organs, such as the heart, are highly sensitive to hypoxia and susceptible to injury. Previous reports have confirmed that sodium azide, a mitochondrial respiratory chain complex IV inhibitor, could induce cell death when added to cultured neonatal rat cardiac myocytes, and simulate chemical hypoxia, which was associated with the proteolysis of biochemical indicators, such as myocardial troponin I (11). This process was demonstrated to be significantly inhibited by calcium antagonists, such as nifedipine and benidipine (11,16). Inhibition of Ca^{2+} influx, and preservation of mitochondrial membrane potential ($\Delta\Psi_m$) and cellular ATP contents by benidipine were important in the protection against sodium azide-induced cardiac cell death (16). However, the exact mechanism of sodium azide-induced cardiotoxicity remains not fully understood.

Mdivi-1, a derivative of quinazolinone, is a novel mitochondrial division inhibitor (17). It is a highly efficacious small molecule serving as a selective inhibitor to suppress dynamin-related protein 1 (Drp1) self-assembly and mitochondrial fission (18-20). Drp1, a member of the dynamin family of large GTPases, which is primarily found in the cytosol, is recruited by mitochondrial fission 1 protein to translocate to the outer mitochondrial membrane and is then localized to discrete regions on the mitochondrial surface to initiate fission (21-24). Previous studies have demonstrated that the small molecule inhibitor Mdivi-1 attenuated both tubular cell apoptosis and acute kidney injury (15), and has been demonstrated to have protective

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effects by attenuating cell apoptosis in both myocardial (18,25) and cerebral ischemia/reperfusion (I/R) injury (19). In addition, Mdivi-1 has also been reported to have cardioprotective effects by ameliorating pressure overload during heart failure (26). However, the effects of Mdivi-1 on sodium azide-induced cell death in H9c2 cardiac muscle cells remain unclear.

Based on the above research literature, it was hypothesized that Mdivi-1, a selective inhibitor of Drp1, may prevent sodium azide-induced H9c2 cells death by improving mitochondrial function and increasing reactive oxygen species (ROS) production. Therefore, the present study assessed the effect of Mdivi-1 in sodium azide-induced H9c2 cells and its mechanism. The results revealed that inhibition of Drp1 by Mdivi-1 pretreatment prevented sodium azide-induced H9c2 cell death, suggesting that it may serve as a potential drug in the treatment of azide poisonings.

Materials and methods

Materials. Mdivi-1 was purchased from Tocris Bioscience (Bristol, UK) and dissolved in dimethyl sulfoxide (DMSO), assuring that the final concentration of DMSO was <0.01% in all experiments. Sodium azide (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) was dissolved in medium as a 1M stock solution, and then diluted to the indicated concentrations prior to use in experiments.

Cell culture. The rat embryonic ventricular myocardial H9c2 cell line (American Type Culture Collection, Manassas, VA, USA) was used in the present study. H9c2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Hyclone; GE Healthcare Life Sciences, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA). Cells were incubated at 37°C in a humidified incubator containing 95% air and 5% CO₂ and used at 70-80% of confluence. All experimental procedures and protocols were approved by Ethics Committee of Soochow University.

Cell viability assay. To assess cell viability, the Cell Counting kit (CCK-8; Dojindo Molecular Technologies, Inc., Shanghai, China) assay was used. Cells (5x10⁴/well) were seeded in 96-well plates. Following culture for 24 h, sodium azide (0.1-70 mM) was added to the cells and incubated for 24 h to make the cell injury model. For Mdivi-1 pretreatment, cells were cultured in the presence of different doses of Mdivi-1 for 3 h, prior to the sodium azide treatment and Mdivi-1 was kept in the same culture media during the 24 h sodium azide treatment. Cells without any treatment were used as control. Then, a total of 10 μ l of CCK-8 solution (Dojindo Molecular Technologies, Inc.) was added to each well and incubated for another 3 h under standard cell-culture conditions (37°C, 5% CO₂). The absorbance was determined at 450 nm wavelength (A450 nm) with a ELx808 microplate reader (BioTek Instruments, Inc., Winooski, VT, USA). Cell viability was calculated according to the mean optical density (OD) of 8 wells. The experiments were repeated at least three times.

Nuclear morphology of DAPI-stained H9c2 cells. H9c2 cells, with or without 1 μ M Mdivi-1 pretreatment for 3 h, were

incubated with 30 mM sodium azide for 24 h. In order to distinguish between programmed or non-apoptotic cell death, nuclei were stained with DAPI. Briefly, cells were washed twice with PBS and then fixed with 4% paraformaldehyde for 30 min at room temperature. Following three washes, fixed cells were stained with DAPI (1:5,000; dilution with PBS) for 5-10 min. Cells were then washed with PBS and fluorescence images were captured with a Leica DMI fluorescent microscope (Leica Microsystems GmbH, Wetzlar, Germany). The experiments were repeated at least three times.

Mitochondrial membrane potential ($\Delta\Psi_m$) measurement. $\Delta\Psi_m$ is a significant parameter of mitochondrial function. $\Delta\Psi_m$ was assessed by staining with the fluorescent probe 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazole-carbocyanide iodide (JC-1; Beyotime Institute of Biotechnology, Haimen, China). H9c2 cells were cultured in 24-well plates and either not treated (control), or exposed to sodium azide for 24 h, with or without 1 μ M Mdivi-1 pretreatment for 3 h. Subsequently, cells were stained with JC-1, according to the manufacturer's protocol. Following incubation at 37°C for 20 min, the cells were washed thrice and fresh medium without serum was added. Images were observed and captured with a fluorescent microscope: JC-1 monomer green fluorescence (excitation 490 nm, emission 525 nm) denotes the presence of low membrane potential and red J-aggregate fluorescence (excitation 525 nm, emission 590 nm) denotes the presence of high membrane potential. The positive control was treated with carbonyl cyanide m-chlorophenylhydrazone (CCCP), as provided by the kit. In order to quantify the changes of relative mitochondrial membrane potential, ratios of red/green fluorescent densities were calculated and analyzed with ImageJ v1.32 J software (National Institutes of Health, Bethesda, MD, USA).

Measurement of cellular ATP contents. H9c2 cells were cultured in 6-well plates, pretreated with 1 μ M Mdivi-1 for 3 h, then treated with 30 mM sodium azide for 24 h. The measurement of cellular ATP contents was performed with a firefly luciferase ATP assay kit (Beyotime Institute of Biotechnology), according to the manufacturer's instructions. In brief, cells were washed with pre-cooled PBS and lysed with ATP lysis buffer on ice. Then samples were centrifuged at 12,000 x g for 5 min in 4°C to collect the supernatant in 1.5 ml tubes and stored at -80°C until measurement. ATP contents were measured in 20 μ l of each sample (including standard) and mixed with 50 μ l of ATP detection working dilution, which was placed in advance at room temperature for 3-5 min. Luminescence (relative light units, RLU) activity was measured immediately using a luminometer (GloMax 20/20; Promega Corporation, Madison, WI, USA). In each assay, a 7-point standard curve (range, 0.1-10 μ M) for the quantification was generated. Finally, the intracellular ATP contents were expressed as nmol per mg of total protein.

Measurement of ROS production in H9c2 cells. Intracellular ROS production was assessed by using the specific probe 2',7'-dichlorofluorescein diacetate (DCFH-DA; Beyotime Institute of Biotechnology), according to the manufacturer's instructions. Intracellular ROS oxidize DCFH-DA, yielding the fluorescent compound 2',7'-dichlorofluorescein (DCF)

and measurement of the DCF fluorescence intensity is representative of the amount of ROS in the cells. H9c2 cells were cultured in 6-well plates, then exposed to 30 mM sodium azide for 24 h, with or without pretreatment with 1 μ M Mdivi-1 for 3 h. Subsequently, cells were treated with DCFH-DA (10 μ M) dissolved in serum-free DMEM (1:1,000) for 20 min at 37°C and then washed three times with serum-free DMEM. The cells were then observed for green fluorescence (excitation 488 nm, emission 525 nm) with a Leica DMI fluorescent microscope (Leica Microsystems GmbH) and analyzed with ImageJ v1.32 J software (National Institutes of Health). Fluorescence intensities of ROS were presented in arbitrary units (a.u).

Western blotting. H9c2 cells were exposed to 30 mM sodium azide for 24 h, with or without 1 μ M Mdivi-1 pretreatment for 3 h. The cultured cells were exposed to liquid nitrogen, lysed with radioimmunoprecipitation assay buffer (Beyotime Institute of Biotechnology) with protease inhibitors (1 mM PMSF; 1:100), harvested in 1.5 ml tubes by scraping, and centrifuged at 4°C at 13,362 \times g for 10 min in order to collect the supernatants. Subsequently, the proteins concentrations were determined by bicinchoninic acid assay (Pierce; Thermo Fisher Scientific, Inc.). Equal amounts of proteins (50–60 μ g) were separated by 10 and 15% SDS-PAGE and transferred to polyvinylidene fluoride membrane by a semidry electrotransferring unit (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Following blocking with TBS containing 0.1% Tween-20 (TBST) and 5% non-fat dry milk for 2 h at room temperature, the membranes were incubated with primary antibodies against Drp1 (cat. no. ABT155; dilution 1:1,000; EMD Millipore, Billerica, MA, USA), BCL2 associated X (Bax; cat. no. sc-7480; dilution, 1:200; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) and BCL2 apoptosis regulator (Bcl-2; cat. no. sc-7382; dilution, 1:200; Santa Cruz Biotechnology, Inc.) overnight at 4°C. The following day, membranes were washed and incubated with horseradish peroxidase-conjugated second antibodies (rabbit; cat. no. A0208; 1:1,000; Beyotime Institute of Biotechnology) or (mouse; cat. no. A0216; 1:1,000; Beyotime Institute of Biotechnology) at room temperature for 1 h. Finally, immunoreactivity was visualized by the enhanced chemiluminescence system (ChemiScope 5200; Clinx Science Instruments Co., Ltd., Shanghai, China) and quantitatively analyzed with ImageJ v1.32 J software (National Institutes of Health). GAPDH served as the loading control. Three independent experiments were performed.

Statistical analysis. Statistical analysis was performed using SPSS 13.0 software (SPSS, Inc., Chicago, IL, USA). Data are presented as the mean \pm standard error of the mean and difference between groups was evaluated by one-way analysis of variance followed by Bonferroni post hoc tests. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Mdivi-1 pretreatment inhibits sodium azide-induced H9c2 cell death. In order to examine the viability of H9c2 cells following treatment with sodium azide, the CCK-8 assay was used. Cells were treated with different concentrations (0–70 mM) of sodium azide for 24 h. As illustrated in Fig. 1A,

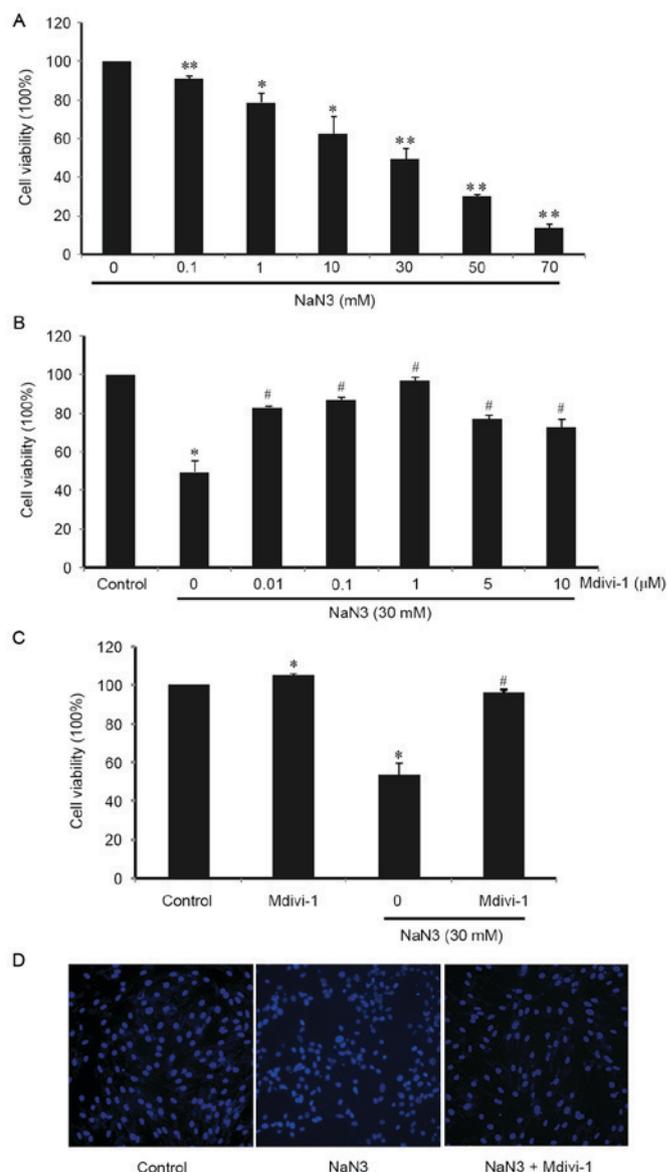


Figure 1. Effect of Mdivi-1 against NaN_3 -induced H9c2 cell death. (A) NaN_3 significantly suppressed cell viability in a dose-dependent manner. Cell viability in H9c2 cells treated with 0–70 mM NaN_3 for 24 h was assessed using the CCK-8 assay. (B) Mdivi-1 pretreatment reduced the NaN_3 -induced H9c2 cell death. Cell viability was measured by CCK-8 assay in H9c2 cells treated with 30 mM NaN_3 for 24 h, with or without pretreatment with different concentrations of Mdivi-1 for 3 h. (C) H9c2 cell viability was not affected by Mdivi-1 treatment alone. (D) Fluorescence images (magnification, $\times 200$) of H9c2 cells treated with 30 mM NaN_3 for 24 h, with or without pretreatment with Mdivi-1 for 3 h, then stained with DAPI. Data represent the mean % relative to control \pm standard error of the mean from three independent repeats. * $P < 0.05$ and ** $P < 0.01$ vs. control group; # $P < 0.05$ vs. NaN_3 group. Mdivi-1, mitochondrial division inhibitor 1; NaN_3 , sodium azide; CCK-8, Cell Counting kit-8.

cell viability was reduced following sodium-azide treatment in a dose-dependent manner. Treatment with 70 mM sodium azide resulted in ~90% of cells dying in 24 h (Fig. 1A). These results indicated that sodium azide induced cell death and cytotoxicity in a dose-dependent manner. Exposure to sodium azide at a concentration of 30 mM caused prominent cell death in H9c2 cells (by ~50%; Fig. 1A).

To determine the role of Mdivi-1 in sodium-azide-treated H9c2 cells, cells were pretreated with various concentrations (0.01–10 μ M) of Mdivi-1 for 3 h and then exposed to 30 mM

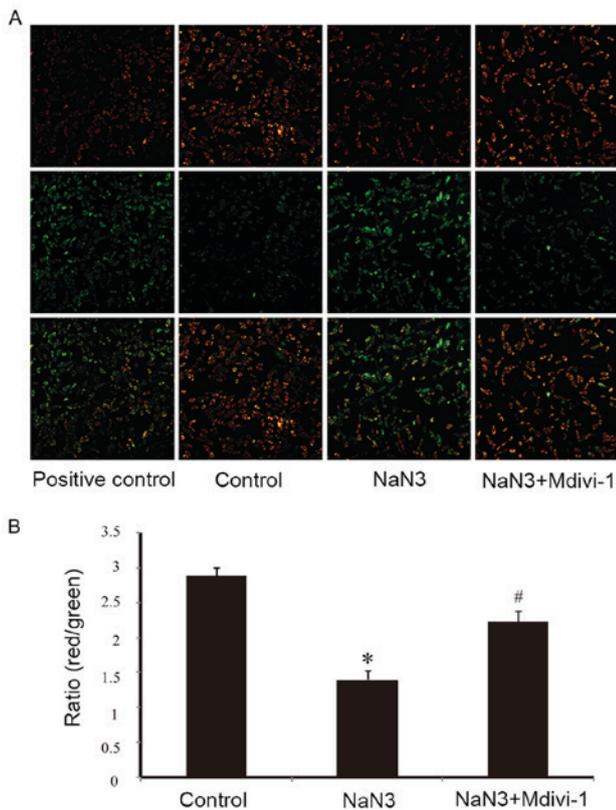


Figure 2. Mdivi-1 moderates the NaN_3 -induced dissipation of $\Delta\Psi_m$ in H9c2 cells. H9c2 cells were treated with 30 mM NaN_3 for 24 h, with or without pretreatment of 1 μM Mdivi-1 for 3 h. $\Delta\Psi_m$ was evaluated by JC-1 staining. (A) Representative fluorescence microscopy images (magnification, $\times 100$). JC-1 aggregates (red) indicate intact $\Delta\Psi_m$, while JC-1 monomers (green) indicate dissipation of $\Delta\Psi_m$. H9c2 cells treated with 10 μM CCCP were used as positive control, and untreated cells were used as control. (B) Ratio of red fluorescence to green fluorescence was quantified to indicate the changes of $\Delta\Psi_m$. * $P < 0.05$ vs. control group; # $P < 0.05$ vs. NaN_3 group. Mdivi-1, mitochondrial division inhibitor 1; NaN_3 , sodium azide; $\Delta\Psi_m$, mitochondrial membrane potential; CCCP, carbonyl cyanide m-chlorophenylhydrazone.

sodium azide for 24 h. Using the CCK-8 assay as mentioned above, the viability of cells was evaluated (Fig. 1B). The results indicated that there was a dose-dependent response when treating with different dose (0.01 to 10 μM) of Mdivi-1. Pretreatment with Mdivi-1 resulted in an increase of cell viability compared with sodium azide-treated cells alone (Fig. 1B). The optimal concentration of Mdivi-1 to prevent sodium azide-induced H9c2 cells death was 1 μM (Fig. 1B). Furthermore, it was also confirmed that cell viability was unaffected by Mdivi-1 treatment alone (Fig. 1C).

In addition, H9c2 cells were stained with DAPI and observed by fluorescence microscopy. In sodium azide-treated H9c2 cells, atypical morphology of apoptosis was detected and Mdivi-1 pretreatment exhibited an obvious ameliorative effect (Fig. 1D), supporting the above results from the CCK-8 assays. These findings suggest that Mdivi-1 protected against sodium azide-induced H9c2 cell death.

Mdivi-1 pretreatment moderates the sodium azide-induced dissipation of $\Delta\Psi_m$ in H9c2 cells. As illustrated in Fig. 2A by JC-1 staining, exposure of H9c2 cells to sodium azide (30 mM) for 24 h resulted in dissipation of $\Delta\Psi_m$, as observed by an increase in green fluorescence compared to normal

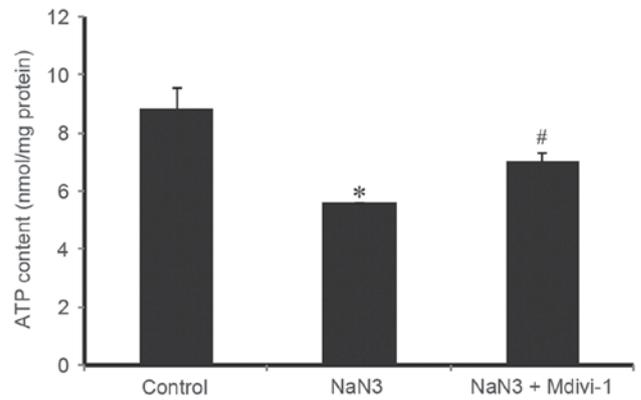


Figure 3. Effect of Mdivi-1 on NaN_3 -induced cellular ATP contents. H9c2 cells were treated with NaN_3 for 24 h, with or without pretreatment of 1 μM Mdivi-1 for 3 h and the contents of ATP were assessed. Data are expressed as means \pm standard error of the mean from three independent repeats. * $P < 0.05$ vs. control group; # $P < 0.05$ vs. NaN_3 group. Mdivi-1, mitochondrial division inhibitor 1; NaN_3 , sodium azide.

mitochondria that exhibit red fluorescence. Pretreatment with Mdivi-1 (1 μM) was able to moderate the decline of $\Delta\Psi_m$ indicating the protective effects of Mdivi-1 (Fig. 2A). CCCP, which can induce mitochondrial membrane potential dissipation, was used as a positive control (Fig. 2A). The ratio of red/green fluorescence was used to quantify the effects of sodium azide and Mdivi-1 on $\Delta\Psi_m$. As presented in Fig. 2B, the ratio of red to green fluorescence was significantly decreased following sodium azide treatment, while pretreatment with Mdivi-1 partially reversed this effect and resulted in an increase in the ratio in H9c2 cells. These results indicated that Mdivi-1 improved sodium azide-induced mitochondrial dysfunction.

Mdivi-1 pretreatment reverses the downregulation of sodium azide-induced mitochondrial ATP energy production. To evaluate whether Mdivi-1 could affect the ATP contents in H9c2 cells exposed to sodium azide, ATP contents in treated cells were quantitatively determined by a specific ATP assay kit. As illustrated in Fig. 3, treatment with 30 mM sodium azide for 24 h resulted in a significant decrease in the cellular ATP contents compared with control, while pretreatment with 1 μM Mdivi-1 reversed this effect. These results suggested that Mdivi-1 could reverse the sodium azide-induced downregulation of mitochondrial ATP energy production.

Mdivi-1 pretreatment inhibits sodium azide-induced accumulation of mitochondrial ROS. It is well-established that mitochondria are the main source of cellular ROS, and ROS have an important role in apoptosis activation. Therefore, the role of ROS in sodium azide-induced H9c2 cell death and the implication of Mdivi-1 on this process was examined. As presented in Fig. 4, compared with control cells, the DCF fluorescence intensity increased significantly in cells following sodium azide (30 mM) treatment for 24 h, indicating that sodium azide resulted in mitochondrial ROS production. Mdivi-1 (1 μM) pretreatment markedly inhibited the accumulation of ROS (Fig. 4A and B). These results suggested that sodium azide-induced apoptosis was associated with oxidative stress and indicated that Mdivi-1 may inhibit apoptosis by

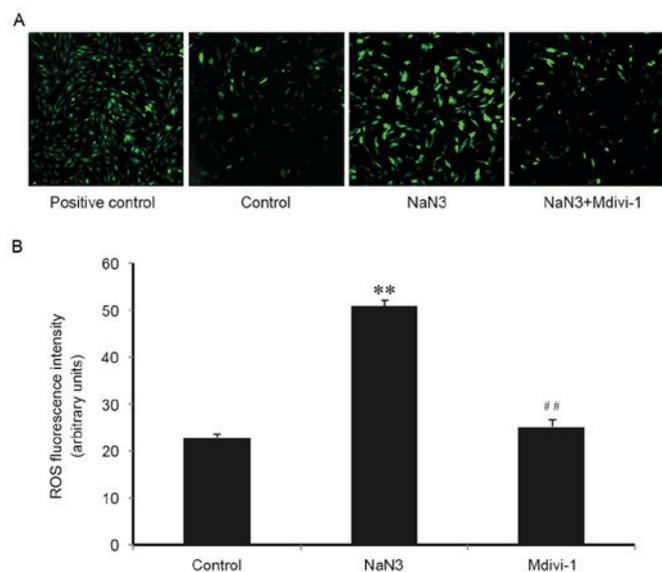


Figure 4. Mdivi-1 inhibits the NaN_3 -induced accumulation of ROS in H9c2 cells. H9c2 cells were treated with NaN_3 for 24 h, with or without pretreatment of $1 \mu\text{M}$ Mdivi-1 for 3 h. Cells were then incubated with the ROS probe 2',7'-dichlorofluorescein diacetate for 20 min and fluorescence was evaluated by microscopy. H9c2 cells treated with $10 \mu\text{M}$ CCCP were used as the positive control and untreated cells were used as the control. (A) Representative images (magnification, $\times 100$) and (B) quantification of fluorescence intensity are presented. Data are expressed as means \pm standard error of the mean from three independent repeats. ** $P < 0.01$ vs. control group; ## $P < 0.01$ vs. NaN_3 group. Mdivi-1, mitochondrial division inhibitor 1; NaN_3 , sodium azide; ROS, reactive oxygen species; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone.

alleviating ROS accumulation and protecting against oxidative stress-induced cell injury.

Mdivi-1 pretreatment inhibits expression of Drp1 and apoptosis-related proteins. The present study further explored the protein expression levels of Drp1 and apoptosis-related proteins in the treated H9c2 cells. As presented in Fig. 5A, Drp1 and the proapoptotic protein Bax were expressed at relatively low levels in the control untreated cells. Protein expression levels of Drp1 and Bax were significantly increased following treatment with sodium azide (Fig. 5). Pretreatment with Mdivi-1 ($1 \mu\text{M}$) for 3 h resulted in a significant decrease in both Drp1 and Bax expression compared with sodium-azide treated cells alone (Fig. 5). By contrast, expression of the antiapoptotic protein Bcl-2 was decreased in the sodium azide group compared with the untreated control group, while Mdivi-1 pretreatment prevented the Bcl-2 expression decrease (Fig. 5). These results indicated that Mdivi-1 exhibited an antiapoptotic effect.

Discussion

The present study explored the mechanism underlying the mitochondria-dependent apoptosis in sodium azide-induced cardiotoxicity with an *in vitro* model of the H9c2 myocardial cell line. Notably, the findings provided the first experimental evidence that Mdivi-1, a mitochondrial division inhibitor, had protective effects against sodium azide-induced cell death by apoptosis. Pretreatment with Mdivi-1 inhibited the sodium azide-induced upregulation of Drp1 expression, and attenuated H9c2 cell death. In addition, Mdivi-1 pretreatment

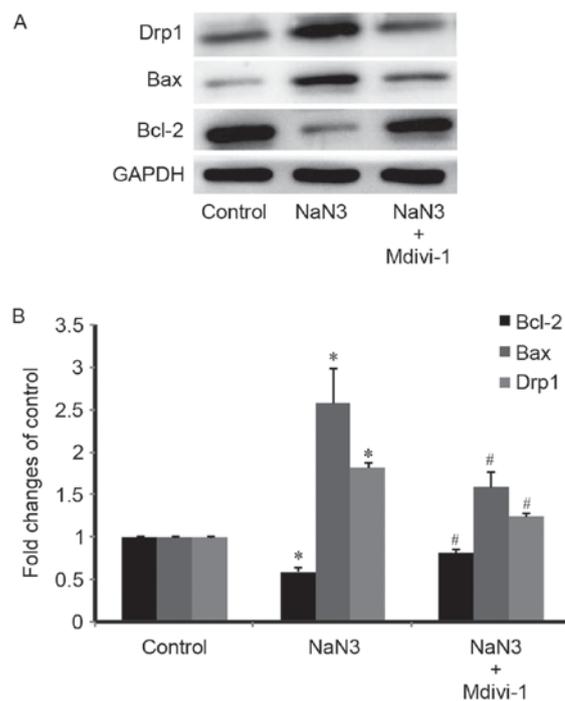


Figure 5. Effect of Mdivi-1 on Drp1, Bcl-2 and Bax protein expression. H9c2 cells were treated with NaN_3 for 24 h, with or without pretreatment of $1 \mu\text{M}$ Mdivi-1 for 3 h, and then analyzed by western blotting. (A) Representative images and (B) quantification of protein signals as a fold change relative to control are presented. * $P < 0.05$ vs. control group; # $P < 0.05$ vs. NaN_3 group. Mdivi-1, mitochondrial division inhibitor 1; NaN_3 , sodium azide; Drp1, dynamin-related protein 1; Bcl-2, BCL2 apoptosis regulator; Bax, BCL2 associated X.

inhibited the apoptosis of H9c2 cells by modulating Bax and Bcl-2 expression. In addition, Mdivi-1 pretreatment improved the sodium azide-induced mitochondrial dysfunction by inhibiting mitochondrial membrane potential dissipation, improving mitochondrial ATP energy production, alleviating the overproduction of ROS and protecting against oxidative stress-induced cell injury.

Previous studies suggest that mitochondria are highly dynamic organelles that continually undergo fusion and fission, which have been implicated in a variety of biological processes, including cell apoptosis, autophagy, division, embryonic development and metabolism (27,28). Changes in mitochondrial dynamics, which can affect cardioprotection, vascular smooth cell proliferation, myocardial I/R and heart failure, have an important role in maintaining their function in cardiovascular health and disease (29-31). Mdivi-1, a novel mitochondrial division inhibitor, reduces apoptotic cell death and has cardioprotective capacity to block apoptotic cell death against I/R injury (25). In addition, inhibition of Drp1 by Mdivi-1 attenuates cerebral ischemic injury via inhibition of the mitochondria-dependent apoptotic pathway following cardiac arrest (32). Therefore, in the present study, Mdivi-1 was used in order to explore the mechanism in sodium azide-induced apoptosis in terms of mitochondria function and oxidative stress.

As the main regulators of energy production and apoptosis in the cells, mitochondria have key roles in cell function, whose structural, biochemical, or functional abnormality can lead to cell injury (33,34). It is known that this organelle

is not only the major site of ATP production, but also serve an important role in apoptosis (35). To explore the impact of sodium azide on mitochondria in the present study, the changes of mitochondrial membrane potential ($\Delta\Psi_m$) were first explored in H9c2 cells treated with sodium azide, with the hypothesis that these changes likely also affect the energy production. JC-1 staining was used to evaluate changes in $\Delta\Psi_m$. The results demonstrated a decline in mitochondrial membrane potential following sodium azide treatment, but this decline was reversed by Mdivi-1 treatment. These data indicated that the sodium azide-induced dissipation of $\Delta\Psi_m$ in mitochondria was moderated by Mdivi-1. Then, the ATP contents were also quantitatively determined. The results demonstrated that the cellular ATP contents in the sodium azide-treated cells were decreased compared with the control cells, suggesting that mitochondrial function was hindered by sodium azide treatment. The present results demonstrated that Mdivi-1 pretreatment had a protective effect in this sodium azide-induced mitochondrial dysfunction.

Furthermore, mitochondria are a major source of ROS in myocytes. Increasing evidence has suggested that ROS overload is associated with the pathogenesis of cardiovascular diseases, such as myocardial infarction and heart failure (36). A previous study has demonstrated that ROS is important in apoptosis of myocytes (37). However, whether ROS has a role in apoptosis of sodium azide-treated H9c2 cells remained unclear. In the present study, sodium azide treatment was demonstrated to result in an increase of mitochondrial ROS production in H9c2 cardiomyocytes. Notably, Mdivi-1 pretreatment significantly inhibited the accumulation of ROS.

Previous studies have suggested that sodium azide could induce cell apoptosis in neonatal rat cardiac myocytes (38,39). To identify the molecular mechanism of apoptosis in the sodium azide-treated H9c2 cells, the expression levels of the Bcl-2 family proteins were examined in the present study. This family of proteins, consisting of both proapoptotic and anti-apoptotic members, includes Bax, Bcl-2 and BCL2 extra-large. Bcl-2 is an important cellular protein, which prevents the release of proapoptotic factors, such as cytochrome c, from the mitochondria into the cytosol, and thus prevents apoptotic cell death (40). By contrast, Bax, as a proapoptotic factor, causes the collapse of the mitochondrial membrane potential and subsequent increase in mitochondrial permeability, triggers the caspase cascade and finally leads to apoptosis (41,42). In the present study, the results demonstrated that sodium azide-induced H9c2 cell apoptosis was associated with decrease of Bcl-2 and increase of Bax expression. Of note, pretreatment with Mdivi-1 attenuated the sodium azide-induced upregulation of Bax and downregulation of Bcl-2.

In conclusion, the present study indicates that the mechanism of sodium azide-induced cardiotoxicity may involve the mitochondria-dependent apoptotic pathway. Mdivi-1 was demonstrated to have a protective effect on the sodium azide-induced H9c2 cell damage, suggesting that it may serve as a therapeutic agent in the treatment of sodium azide-induced cardiotoxicity. However, the present study was limited as Mdivi-1 was only used as a pretreatment, and therefore, only a preventative, and not a therapeutic, effect has been demonstrated. Thus, further studies are required to confirm whether Mdivi-1 may also act as a therapeutic agent.

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