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ORIGINAL RESEARCH

Inhibition of Mitochondrial Dynamics by Mitochondrial Division Inhibitor-1 Suppresses Cell Migration and Metastatic Markers in Colorectal Cancer HCT116 Cells

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Introduction: The mitochondria are highly dynamic organelles. The mitochondrial morphology and spatial distribution within the cell is determined by fusion and fission processes of mitochondria. Several studies have used mitochondrial division inhibitor-1 (Mdivi.1) to explore the roles of mitochondrial dynamics in various pathological conditions, including diabetic cardiomyopathy, myocardial infarction, cardiac hypertrophy, Alzheimer's disease, Huntington's disease and cancers.

Purpose: The objective of the study was to investigate the role of mitochondrial dynamics in the invasiveness of HCT116 colorectal cancer cells.

Material and Methods: MTT assay was used to determine the Mdivi.1-induced toxicity in HCT116 cells. Wound healing, cell migration and colony forming assays were adopted to measure the migration and invasion activity of HCT116 cells. Furthermore, flow cytometry was used to determine the Mdivi.1-induced mitochondrial mass quantification, mitochondrial membrane potential and reactive oxygen species generation in HCT116 cells. Additionally, Western Blot analysis was used to determine the expression level of Drp1, p-Drp1, Mnf2, AMPK-α, p-AMPK-α, Cox-2, iNos and MMP9 in HCT116 cells.

Results: We found that Mdivi.1 induced toxicity and altered the morphology of HCT116 cells in concentration- and time-dependent manners. Mdivi.1 significantly increased mitochondrial mass and dissipated the mitochondrial membrane potential. Furthermore, Mdivi.1 induced reactive oxygen species (ROS) generation and mitochondrial superoxide production, leading to AMPK activation. Moreover, Mdivi.1 decreased dynamin-related protein-1 (Drp1) and phosphorylated-Drp1 expression and increased mitofusin-2 (Mfn2) expression in a concentration-dependent manner at 48 and 72 h post-treatment. Notably, Mdivi.1 induced inhibition of translocation of Drp1 from the cytosol to the outer mitochondrial membrane. Mdivi.1 significantly suppressed the invasion and migration of HCT116 cells and inhibited the formation of HCT116 cell colonies. In addition, Mdivi.1 significantly decreased the expression of metastatic markers including Cox-2, iNos, and MMP-9 in HCT116 cells.

Conclusion: Collectively, this study revealed that Mdivi.1 downregulates Drp1, upregulates Mfn2, and increases mitochondrial mass with attenuated oxidative metabolism, leading to the inhibition of cell invasion and metastasis in colorectal cancer HCT116 cells. Mitochondrial dynamics are regarded as possible drug targets for interrupting colorectal cancer cell migration and metastasis.

Plain language summary: The mitochondria are highly dynamic organelles. This study aimed to investigate the role of a mitochondrial fission inhibitor (Mdivi.1) on the viability, migration, and invasion of colorectal cancer cells (HCT116 cells), as well as the underlying mechanisms. We found that Mdivi.1 inhibited cell viability and migration via mechanisms linked to changes in mitochondrial dynamics, reactive oxygen species generation, and the downregulation of metastatic markers. This study highlighted the role of altered mitochondrial dynamics as a potential therapeutic approach for colorectal cancer treatment.

Keywords: Mdivi.1, mitochondria, HCT116, invasion, Drp1, Mfn2

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Introduction

The mitochondria, the powerhouse of the cell, are highly dynamic organelles. Unlike other subcellular organelles, in the presence of external or internal stimuli, mitochondria regulate to interchange morphology between small granules to large interconnected threads by a phenomenon known as mitochondrial dynamics.[1,2] Mitochondrial dynamics is regulated by coordination of members of dynamin family proteins in conjunction with adapter proteins and lipids leading to decidedly controlled fusion process, where two mitochondria merge together to form one mitochondrion, and fission process where one mitochondrion divides to form two mitochondria.[3,4] The fusion process of outer mitochondrial membrane is regulated by mitofusin-1 (Mfn1), mitofusin-2 (Mfn2) and inner mitochondrial membrane is regulated by optic atrophy-1 (opa1).[1–4] The fission process is regulated by cytoplasmic GTPase (dynamin-related protein-1; Drp1), dynamin-2 (Dnm2), and mitochondrial fission-1 (Fis1), and mitochondrial fission factor (Mff).[1–5] The balance between fusion and fission ensures morphology, function, abundance and spatial distribution of mitochondria within the cells to rationalize cellular microenvironments.[5] Imbalanced fusion and fission processes affect mitochondrial-dependent biological events such as reactive oxygen species (ROS) generation, oxidative phosphorylation, calcium ion storage, cell proliferation, mitophagy and apoptosis and develop pathological conditions.[6–10]

During mitochondrial fission, Drp1 is translocated to the outer mitochondrial membrane where it hydrolyzes GTP to form a polymer. Activated Drp1 then interacts with one or more fission proteins anchored on the outer mitochondrial membrane, that is, Fis1 and Mff, and facilitates mitochondrial membrane constriction.[2] Drp1 is overexpressed in several cancers and is actively involved in cancer metastasis. Drp1 is overexpressed in brain tumor-initiating cells and breast cancer cells, and is associated with mitochondrial fragmentation. Tumorigenicity of brain tumor-initiating cells is repressed by inhibition of Drp1 with Mdivi.1 in vitro and in vivo.[11] Silencing of Drp1, as well as upregulation of Mfn2, changes the morphology of the cells from fragmented to mitochondrial elongation and clusters, respectively, and suppresses the metastatic activity of various breast cancer cells. In contrast, Mfn2 silencing induces mitochondrial fragmentation resulting in higher metastatic abilities of breast cancer cells.[12] Furthermore, it is reported that Drp1 downregulation or Mfn1 upregulation decreased the migration activity of oncocytic XTC.UC1 cells.[13] Excessive mitochondrial fission has been associated with a poor prognosis in hepatocellular carcinoma (HCC) patients.[14,15] However, roles of mitochondrial dynamics on aggressiveness of colorectal cancer are largely unknown.

The mitochondrial division inhibitor 1(Mdivi.1), a derivative of quinazolinone, is a putative inhibitor of Drp1. Several studies have used Mdivi.1 to explore the roles of mitochondrial dynamics in various pathological conditions, including diabetic cardiomyopathy, myocardial infarction, cardiac hypertrophy, cancers, Alzheimer's disease, and Huntington's disease.[16–21] In various cancers, Mdivi.1 modulates the redox state of the cells, induces cytotoxicity, and decreases cancer cell proliferation through mitochondrial fission inhibition.[5,21] Mdivi.1 decreases tumorsphere formation through down-regulation of Drp1 and ROS induction in breast cancer, melanoma, and lung cancer cell lines.[21] However, the effect and underlying mechanism of Mdivi.1 on colorectal cancer aggressiveness remain unclear. Therefore, the present study aimed to explore the effect and underlying mechanism of Mdivi.1-induced disruption of mitochondrial dynamics on the proliferation, migration, and invasiveness of colorectal cancer cells using the HCT116 cell line.

Materials and Methods

Materials

HCT116 colorectal cancer cells were obtained from American Type Culture Collection (Manassas, VA, USA). DMEM/Ham's F-12 media, penicillin and streptomycin were purchased from Gibco (Thermo Fisher Scientific). Mdivi.1 was purchased from Santa Cruz biotechnologies. Inc. (Waltham, MA). MitoTracker Deep Red was purchased from Invitrogen (Carlsbad, MA USA). TMRM (tetramethylrhodamine methyl ester) was purchased from Abcam (USA). DMSO and Crystal violet were purchased from Sigma-Aldrich (St. Louis, MO, USA). MTT was purchased from Bio Basic Inc. (Markham, Canada). Antibodies against Drp, p-Drp, Mfn2, AMPK-α, p-PAMK-α, Cox-2, iNos, MMP9 and β-actin were from Cell Signaling Technology (Danvers, MA). All other chemicals used were of scientific standard and from commercial sources.

Cell Culture

HCT116 colorectal cancer cells were cultured in 1:1 Dulbecco's modified Eagle's medium (DMEM) and Ham's F-12 medium containing FBS (10%), penicillin (100 U/mL), and streptomycin (100 mg/mL) in humidified atmosphere of 5% CO_2 / 95% O_2 at 37 °C.

Cell Viability Assays

The toxicity of Mdivi.1 against HCT116 cells was evaluated using MTT assays, as reported previously.[22] Cells were seeded in 96-well cell culture plates (5000 cells/well) and grown for 24 h. Cells were incubated with Mdivi.1 for 24, 48, 72, and 96 h followed by incubation with MTT reagent (10 µL of 5 mg/mL) for 4 h in the dark at 37 °C. Subsequently, 150 µL of DMSO was added to dissolve formazan crystals, and absorbance at 570 nm was detected using the Synergy™ Neo2 Multi-Mode microplate reader (BioTek Instruments, Winooski, VT, USA). The data were presented as percentage of cell viability compared to control.

Observation of Morphological Changes

HCT116 cells were incubated with vehicle (DMSO) or Mdivi.1 at selected concentrations for 48 h at 37 °C. Morphology of cells was observed with images being captured by a bright-field microscope (Zeiss, AX10, USA).

Wound Healing Assay

HCT116 cells were grown in 12-well plates until 80–90% confluency was reached. Scratching was done to create a wound using a sterile 200 µL pipette tip. The floating cells were removed by washing with serum-free DMEM/F12 medium. The cells were treated with Mdivi.1 or vehicle with 10% FBS for 24 h at 37 °C. Following treatment, the medium was changed to 1% FBS and the cells were grown for another 24 h. Finally, the cell migration was recorded under a microscope (Zeiss, AX10, USA) with images being captured at 0, 24, and 48 h for analysis. The data were presented as colony formation rate over percentage of control.

Cell Migration Assay

The migration activity was determined by growing the HCT116 cells in Transwell chambers (8-µm pores; Corning Costar, Corning, NY, USA). HCT116 cells were seeded and grown in 12 well cell culture plates. The cells were incubated with vehicle or Mdivi.1 for 16 h in DMEM/F12 medium supplemented with 10% FBS. Following treatment, the cells were trypsinized and 5000 cells in 200 µL of DMEM/F12 medium supplemented with 1% FBS were seeded in the upper transwell chamber for 24 h and 48 h, and 600 µL DMEM/F12 medium with 10% FBS was added in the lower chamber. Subsequently, non-migrated cells in the upper chambers were removed by a cotton swab and washed with D-PBS. The penetrated cells were then fixed with paraformaldehyde (4%), stained with crystal violet, and counted in five different areas under a microscope (Zeiss, AX10, USA). The data were analyzed as the percentage of migrated cells over control.

Colony Forming Assay

HCT116 cells were seeded and cultured in 6-well plates. The cells were treated with different concentrations of Mdivi.1 or vehicle for 16 h in DMEM/F12 supplemented with 10% FBS. Cells were then trypsinized, seeded into 6-well plate (500 cells/well), and grown in DMEM/F12 medium supplemented with 1% FBS for 10 days or until colony formation. The colonies were washed with D-PBS, fixed with paraformaldehyde (PFA; 4%) for 20 min at room temperature, and stained with crystal violet for 15 min. The colonies were washed with D-PBS to remove extra stain and photographed. Subsequently, to determine the rate of proliferation, crystal violet stain was dissolved in 1 mL of methanol, and the absorbance (595 nm) was recorded by using a Synergy™ Neo2 Multi-Mode microplate reader (BioTek Instruments, Winooski, VT, USA). The data were analyzed as the percentage of colony formation rate over control.

Analysis of Mitochondrial Mass Quantification

The mitochondrial mass was analyzed using flow cytometry. Briefly, HCT116 cells were seeded and cultured in 6-well plates for 24 h, followed by incubation with Mdivi.1 for 48 and 72 h. After treatment, the cells were washed, trypsinized, and incubated with MitoTracker Deep Red (10 nM) for 20 min in dark. Subsequently, the cells were washed, resuspended, and analyzed by flow cytometry (BD AccuriTM C6 Plus, BD Biosciences, Becton-Dickinson). The data were analyzed as fold changes with respect to the vehicle.

Analysis of Mitochondrial Membrane Potential

Mitochondrial membrane potential (MMP) was analyzed using the TMRM assays kit (Abcam, Waltham, MA, USA). Briefly, HCT116 cells were seeded and cultured in 6-well plates for 24 h, followed by incubation with Mdivi.1 for 48 and 72 h. The cells were then washed, trypsinized, and incubated with TMRM (400 nM) for 20 min in dark. Cells were washed, resuspended, and analyzed by flow cytometry (BD AccuriTM C6 Plus, BD Biosciences, Becton-Dickinson). MMP was determined as the ratio TMRM to mitochondrial mass in HCT116 cells, using MitoTracker Deep Red. The data were analyzed as the mean MMP referenced to the control.

Determination of Reactive Oxygen Species

ROS generation was analyzed using dichloro fluorescein diacetate assay (DCFDA) (Abcam, Waltham, MA, USA). Briefly, HCT116 cells were treated with Mdivi.1 for 48 and 72 h. After that, the cells were washed, trypsinized, and incubated with DCFDA (20 μM) for 30 min in dark. Dichlorofluorescein fluorescence distribution signals were detected using flow cytometry (BD AccuriTM C6 Plus, BD Biosciences, Becton-Dickinson). The data are presented as the mean fluorescence of DCF compared with control.

Western Blot Analysis

Proteins extracted for Western blot analysis were prepared as described previously.[22] Briefly, HCT116 cells were treated with Mdivi.1 for the indicated time intervals. Cells were kept on ice and lysed with RIPA cell lysis reagent containing 1% PhosSTOP and protease inhibitors (Roche, Mannheim, Germany). Amount of protein in lysates were determined using the Bradford reagent using the Lowry method. The protein extracts (20 μg) were resolved using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis before transferred to a nitrocellulose membrane. The membrane was blocked with 5% (w/v) nonfat milk and incubated overnight at 4 °C with antibodies against Drp1 (1:1000), p-Drp1 (1:1000), Mnf2 (1:1000), AMPK-α (1:1000), p-AMPK-α (1:1000), Cox-2 (1:1000), iNos (1:1000), MMP-9 (1:1000) or β-actin (1:1000). After washing with TBST, horseradish peroxidase-conjugated goat anti-rabbit secondary antibodies were added to the blots and incubated for an hour at room temperature. The signals were determined using an ECL plus chemiluminescence kit (Bio-Rad ChemiDocTM Imaging System, CA, USA).

Statistics

Data are presented as mean ± S.D. from at least three separate experiments and statistically compared with the untreated vehicle group and/or within treated groups using a repeated measures analysis of variance (ANOVA) followed by Tukey's post hoc test, using GraphPad Prism software. Differences were considered statistically significant at $p < 0.05$. Columns that do not share the same superscript letters (a,b,c,d,e,f) are considered statistically significant.

Results

Effect of Mdivi.1 on HCT116 Cell Viability

The growth inhibitory effect of Mdivi.1 on HCT116 cells was evaluated using the MTT assay. Cells were treated with different concentrations of Mdivi.1 at different time points, followed by MTT assay. The data showed that Mdivi.1 inhibited the growth of HCT116 cells in a concentration- and time-dependent manner (Figure 1A). Furthermore, the cells were treated with different concentrations of Mdivi.1 for 48 h to observe morphological changes. The data showed that Mdivi.1 induced morphological changes, including loss of cellular shape and geometry, and reduced the number of cells in a concentration-dependent manner

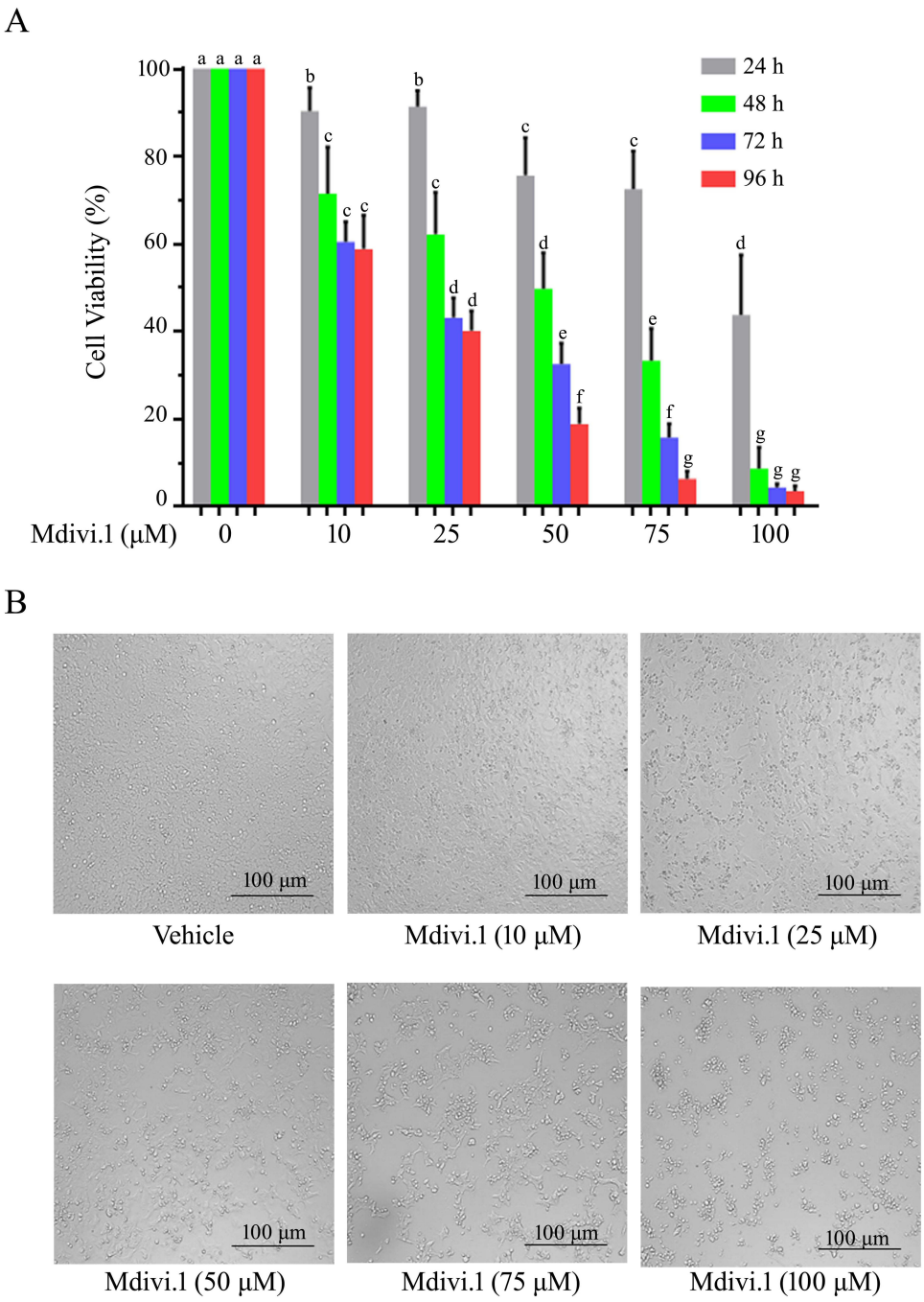

Figure 1 Mdivi.1-induced reduced cell viability in HCT116 cells. (**A**) Cells were treated with indicated concentrations of Mdivi.1 followed by MTT assay. Mdivi.1 induced toxicity in concentration-dependent and time-dependent manners. The data were analyzed and presented as Mdivi.1-induced reduced cell viability over percentage (%) of control. Columns not sharing the same superscript letters (a,b,c,d,e,f,g) differ significantly ($p < 0.05$, one-way ANOVA; Tukey's post hoc test). (**B**) Mdivi.1 induced loss of cellular geometry and inhibited the proliferation of cells.

(Figure 1B). Mdivi.1 induced cells death at high concentrations. For further mechanistic studies, 48 h and 72 h Mdivi.1-incubation time points were selected.

Mdivi.1 Increases Mitochondrial Mass and Decreases Mitochondrial Membrane Potential in HCT116 Cells

Since Mdivi.1 inhibits the fission of mitochondria and inhibition of mitochondrial fission could increase the mitochondrial mass in treated cells, we determined the effect of Mdivi.1 on mitochondrial mass quantification to evaluate the

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effect of Mdivi.1 on mitochondrial dynamics in HCT116 cells. HCT116 cells were treated with the indicated concentrations of Mdivi.1 for 48 h and 72 h. At 10 μM, Mdivi.1 did not induce any change in mitochondrial mass compared with that in the vehicle group. However, Mdivi.1 significantly increased mitochondrial mass at 25 μM, with the maximum effect observed at 50 μM for 72 h (Figure 2A). Changes in mitochondrial mass induced by Mdivi.1 treatment are known to affect the mitochondrial membrane potential. Thereafter, we determined the effect of Mdivi.1 on mitochondrial membrane potential in Mdivi.1-treated HCT116 cells at different time points. The data showed that Mdivi.1 treatment at concentrations of 10 μM for 48 and 72 h and concentrations of 25 μM for 48 h did not significantly reduce the mitochondrial membrane potential (Figure 2B). However, Mdivi.1 treatment with 25 μM Mdivi.1 for 72 h and 50 μM Mdivi.1 for 48 and 72 h significantly reduced the mitochondrial membrane potential in HCT116 cells. Notably, decreased mitochondrial membrane potential was in line with increased mitochondrial mass (Figure 2A and B). Taken together, these data suggest that Mdivi.1 induced inhibition mitochondrial fission, leading to a decreased mitochondrial membrane potential in HCT116 cells.

Mdivi.1 Increases Reactive Oxygen Species (ROS) Generation and Mitochondrial Superoxide Production in HCT116 Cells

Growing evidences suggest that Mdivi.1 inhibits mitochondrial fission, which could impact other mitochondrial events and alter the redox state of the cells. Therefore, we measured ROS levels using the DCFDA assay. The cells were treated with different concentrations of Mdivi.1 for 48 and 72 h. The results showed that Mdivi.1 increased ROS levels in a concentration-dependent manner and induced changes in the redox state of the cells as compared to the vehicle group, with tert-butyl hydroperoxide (TBHP) being used as a positive control (Figure 2C). Interestingly, the ROS levels after 72 h of incubation were lower than those after 48 h of incubation with Mdivi.1 (Figure 2C). However, the increase in mitochondrial mass was greater after 72 h than that after 48 h of incubation with Mdivi.1 (Figure 2A). Notably, increased ROS levels were not associated with an increase in mitochondrial mass. Furthermore, we measured mitochondrial superoxide production in Mdivi.1-treated HCT 116 cells by MitoSOX staining. Mitochondrial superoxide levels were higher in Mdivi.1-treated cells than in vehicle-treated cells (Figure 2D). However, mitochondrial superoxide levels after treatment with Mdivi.1 at 50 μM Mdivi for 72 h were slightly lower than those after 48 h. These data suggest that Mdivi.1 induced ROS generation and mitochondrial superoxide production in HCT116 cells.

Mdivi.1 Activates AMPK in HCT116 Cells

Mdivi.1 inhibits the oxygen consumption rate in cancer stem cells, resulting in the depletion of ATP-linked respiration.[21] ATP deficiency stimulates AMP-activated protein kinase (AMPK) activation. Therefore, we further evaluated the effect of Mdivi.1 treatment on APMK activation using Western blot analysis. We found that Mdivi.1 at concentrations of 25 μM and 50 μM increased AMPK-α phosphorylation, as indicated by the increased phosphorylated AMPK (p-AMPK) to AMPK ratios at 48 h and 72 h post-treatment, indicating AMPK activation (Figure 3A and B).

Mdivi.1 Modulates Mitochondrial Dynamics in HCT116 Cells

The mitochondria are highly dynamic organelles. There is a balance between mitochondrial fusion (Mfn1, Mfn2, Opa1) and fission (Drp1, Dnm2, Fis1) proteins, which determines the morphology, function, abundance, and spatial distribution of mitochondria.[5] Therefore, in the Mdivi.1 treated cells, we measured the expression of Drp1 and Mfn2, which are involved in mitochondrial fission and fusion, respectively. Mdivi.1 decreased the expression of Drp1 and phosphorylated Drp1 (p-Drp1) in a concentration-dependent manner at 48 h and 72 h (Figure 3C and D). Mdivi.1 increased the protein content of Mnf2 at 48 and 72 h. Surprisingly, Mfn2 expression levels decreased following Mdivi.1 treatment at 50 μM for 72 h. Activated Drp1 is translocated to the outer mitochondrial membrane where it hydrolyzes GTP to form a polymer. Activated Drp1 then interacts with one or more of fission protein anchored on the outer mitochondrial membrane ie Fis1 and Mff and facilitates mitochondrial membrane constriction.[23] Subsequently, we determined the expression of Drp1 in cytosol and mitochondrial extracts of cells treated with Mdivi.1 for 48 h and 72 h. The data showed that Drp1 expression levels in the cytosolic fraction of Mdivi.1-treated cells remained unchanged at 48 h (Figure 3E) but decreased at

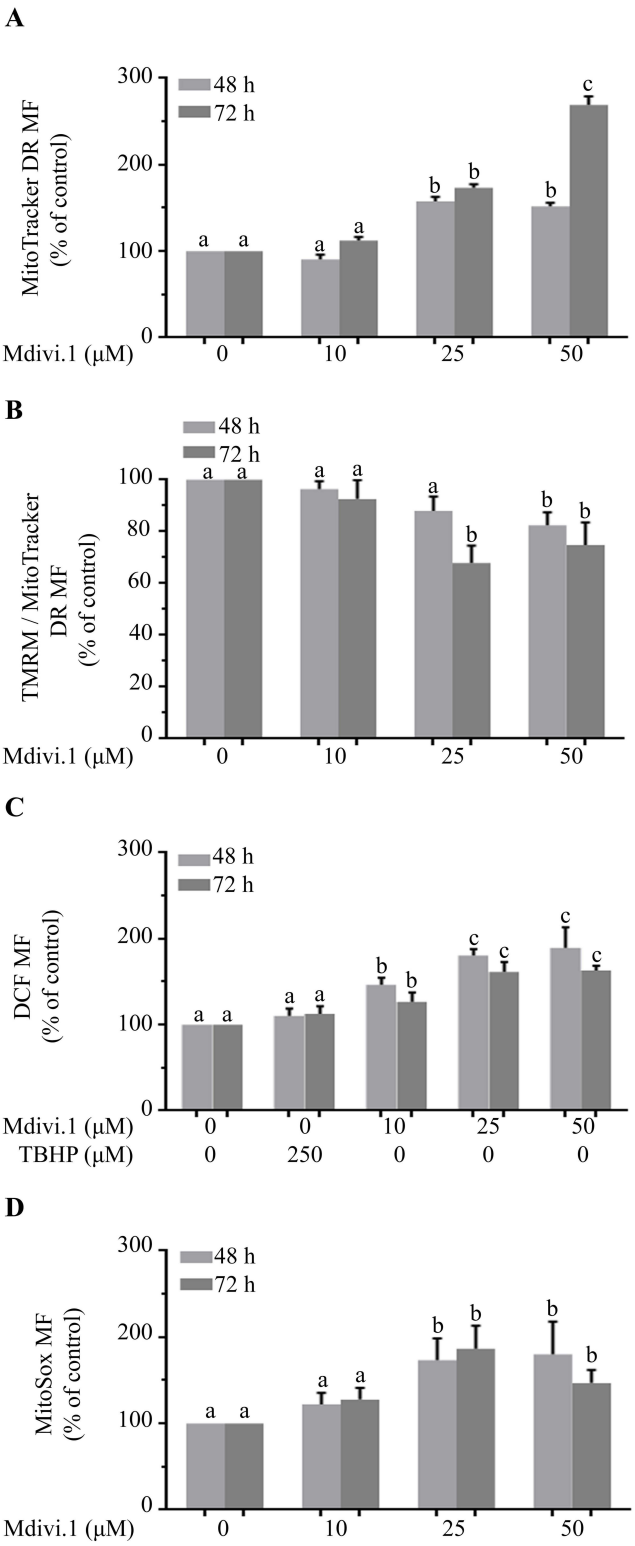

Figure 2 Effect of Mdivi.1 on mitochondrial processes. (**A**) HCT116 cells were incubated with indicated doses of Mdivi.1 for different time points followed by mitochondrial mass index measurement. Mdivi.1 increased mitochondrial mass index. The data were analyzed and presented as MitoTracker deep red mean fluorescence (MitoTracker DR MF) over percentage (%) of control. (**B**) Mdivi.1 induced loss of mitochondrial membrane potential. The data were analyzed and presented as ratio tetramethylrhodamine methyl ester (TMRM) fluorescence to mitochondrial mass index (TMRM/MitoTracker DR MF) over percentage (%) of control. (**C**) Mdivi.1 increased total ROS level in HCT116 cells. The data were analyzed and presented as dichloro fluorescein mean fluorescence (DCF MF) over percentage (%) of control. (**D**) Mdivi.1 induced mitochondrial superoxide production in HCT116 cells. The data were analyzed and presented as MitoSox mean fluorescence (MitoSox MF) over percentage (%) of control. (**A–D**) Columns not sharing the same superscript letters (a,b,c) differ significantly ($p < 0.05$, one-way ANOVA; Tukey's post hoc test).

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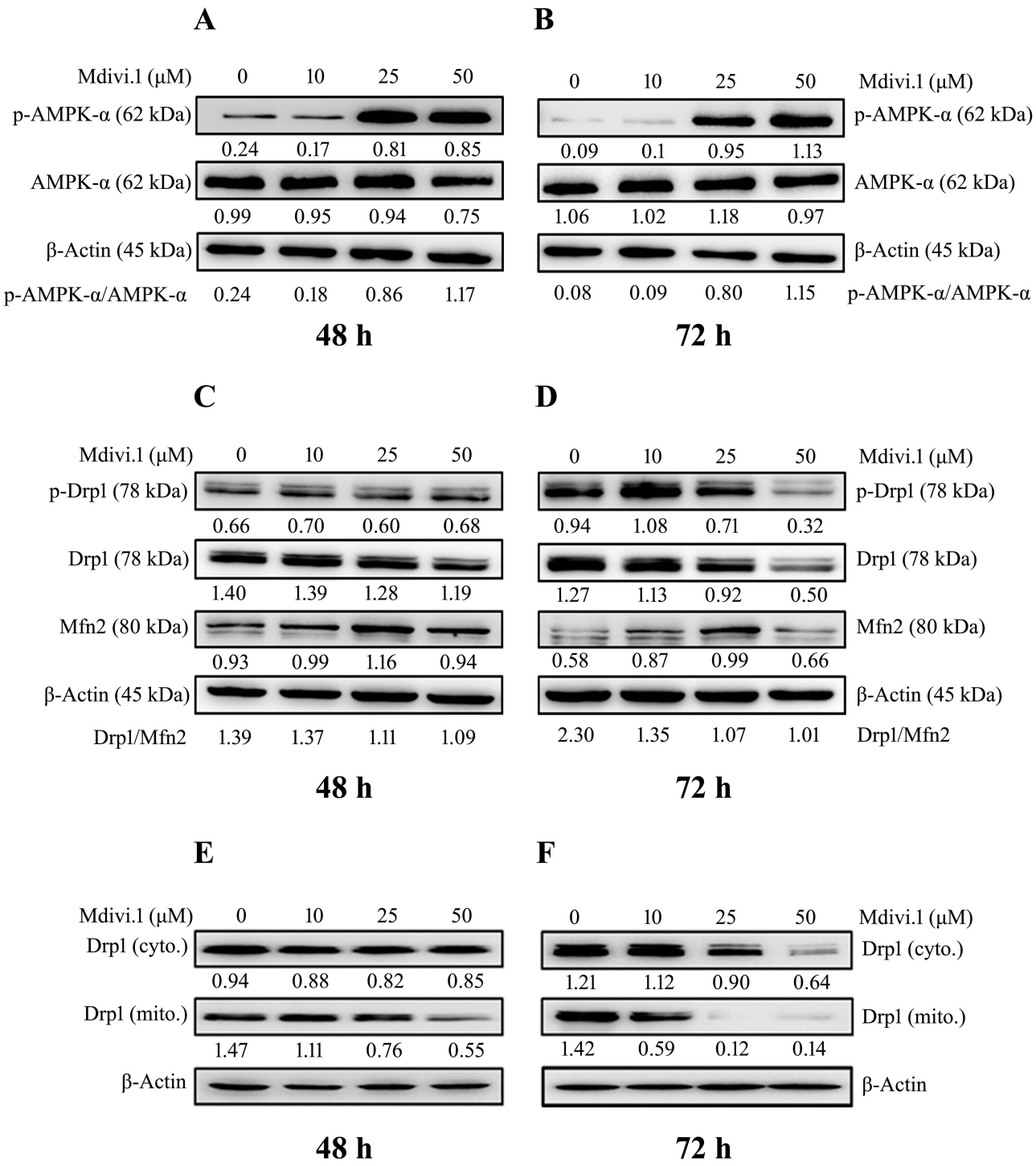

Figure 3 Mdivi.1 impaired mitochondrial dynamics. (**A** and **B**) Mdivi.1 induced activation of AMPK-α through its phosphorylation and increased the ratio of p-AMPK-α/AMPK-α at higher dose-incubation at 48 h and 72 h. (**C** and **D**) Mdivi.1 decreased the expression of Drp1 and pDrp1 and increased the expression of Mfn2 in HCT116 cells. (**E** and **F**) Mdivi.1 modulated the expression of Drp1 in mitochondrial and cytosolic fractions. Numbers below represent the densitometric analyses of each protein marker.

72 h compared to those in the vehicle-treated group (Figure 3F). Furthermore, Drp1 expression levels in the mitochondrial fraction were significantly reduced in a concentration- and time-dependent manner, indicating the Mdivi.1 inhibition of Drp1 translocation from the cytosol to mitochondria (Figure 3E and F).

Mdivi.1 Inhibits HCT116 Cells Migration

The effect of Mdivi.1 on cell migration was evaluated using wound closure, colony formation, and transwell chamber assays. The wound closure assay showed that cells in the vehicle-treated group migrated to close the scratched wound and Mdivi.1 significantly suppressed the migration of cells toward the scratched wounds in a concentration-dependent manner at 24 and 48 h (Figure 4A and B). Furthermore, data from colony-forming assays showed that Mdivi.1 treatment significantly inhibited colony formation in a concentration-dependent manner (Figure 5A and B). The data from the Transwell chamber assay further demonstrated that Mdivi.1 inhibited the migration of HCT116 cells in a concentration-dependent manner (Figure 5C). These data demonstrated the anti-metastatic potential of Mdivi.1 in HCT116 cells.

Effect of Mdivi.1 on Metastatic Markers

Mdivi.1 induced down-regulation of Drp1, which is associated with anti-metastatic activity in various cancer cells.[3,5,21] To test whether other metastatic markers including Cox-2, iNos, and MMP-9 may contribute to the anti-metastatic activity of Mdivi.1, we analyzed the expression of Cox-2, iNos, and MMP-9 in HCT116 cells after incubation with various concentrations of Mdivi.1 for 48 and 72 h. The data showed that Mdivi.1 significantly decreased the expression of Cox-2, iNos, and MMP-9 in a concentration-dependent manner at 48 and 72 h (Figure 6A), suggesting that Mdivi.1 is a potent anti-metastatic agent.

Discussion

Several studies have shown that Mdivi.1 inhibits the invasive and metastatic activities of cancer cells by targeting mitochondrial dynamics.[5,21,24] Mdivi.1 dissipates mitochondrial membrane potential and shakes the redox state of cancer cells.[5,21] It is well established that increased mitochondrial fission leads to fragmented mitochondria or decreased mitochondrial mass with impaired oxidative phosphorylation.[5,21,25] Mitochondrial dynamics do not merely embody static metabolic phenotypes, but rather dynamic processes that modulate mitochondrial mass, oxidative potential, and energy budgets in response to internal or external stimuli and metabolic demands of the cells.[26,27] Therefore, modulation of mitochondrial dynamics may represent an attractive target for inducing toxicity in cancer cells. The present study demonstrated the toxicity of Mdivi.1 in HCT116 colorectal cancer cells with altered cell morphology. Furthermore, Mdivi.1-treated HCT116 cells showed elevated mitochondrial mass with dissipated mitochondrial membrane potential.

The effect of Drp1 on oxidative potential is cell-type specific. Drp1 knockdown increases oxygen consumption rate and ATP production in Ras-transformed MEFs and SK-MEL-28 cells.[28] Contrary to the former notion, Drp1 knockdown decreases the maximal oxygen consumption rate in HRas-transformed HEK-TtH cells and in the T387 BTICs.[11,29] Inhibition of mitochondrial fission results in an impaired oxidative state of MCF7 cells and renal NRK-49F cells.[21,25] Subsequent evidence is in line with our study where Mdivi.1 increased overall ROS levels and mitochondrial superoxide levels in the cells.[5,21,25] This study shows that Mdivi.1 increased mitochondrial mass and induces reactive oxygen species generation and mitochondrial superoxide production, which negatively impacts mitochondrial metabolism. It is well established that reactive oxygen species generation and mitochondrial superoxide production put mitochondria under stress conditions, leading to a decreased oxygen consumption rate. Likewise, Mdivi.1 inhibits the oxygen consumption rate in cancer stem cells, resulting in depletion of ATP-linked respiration.[21] ATP deficiency stimulates AMP-activated protein kinase (AMPK) activation. Our data agree with this notion, where AMPK-α was activated through its phosphorylation at higher concentrations, indicating that Mdivi.1 treatment put mitochondrial activation under extreme oxidative stress conditions and attenuated ATP production, leading to the activation of AMPK-α.

Mitochondrial fission processes are initiated by internal or external stimuli depending on the conditions of the cells, leading to the activation of cytoplasmic Drp1, which is then translocated to the outer mitochondrial membrane.[2] Several studies have indicated that Mdivi.1 decreases the expression level of Drp1 and increases Mfn2 expression in multiple cancer cell lines.[5,11,30] Our results are in line with previous evidence and indicate that Mdivi.1 inhibits the total expression level of Drp1 and p-Drp1 and increases the expression level of Mfn2 in HCT116 cells. Furthermore, Mdivi.1 treatment decreased the expression levels of Drp1 and p-Drp1 in the mitochondrial fractions, indicating the inhibition of translocation of activated Drp1 from the cytosol to the mitochondria. Literature evidences showed the tumor

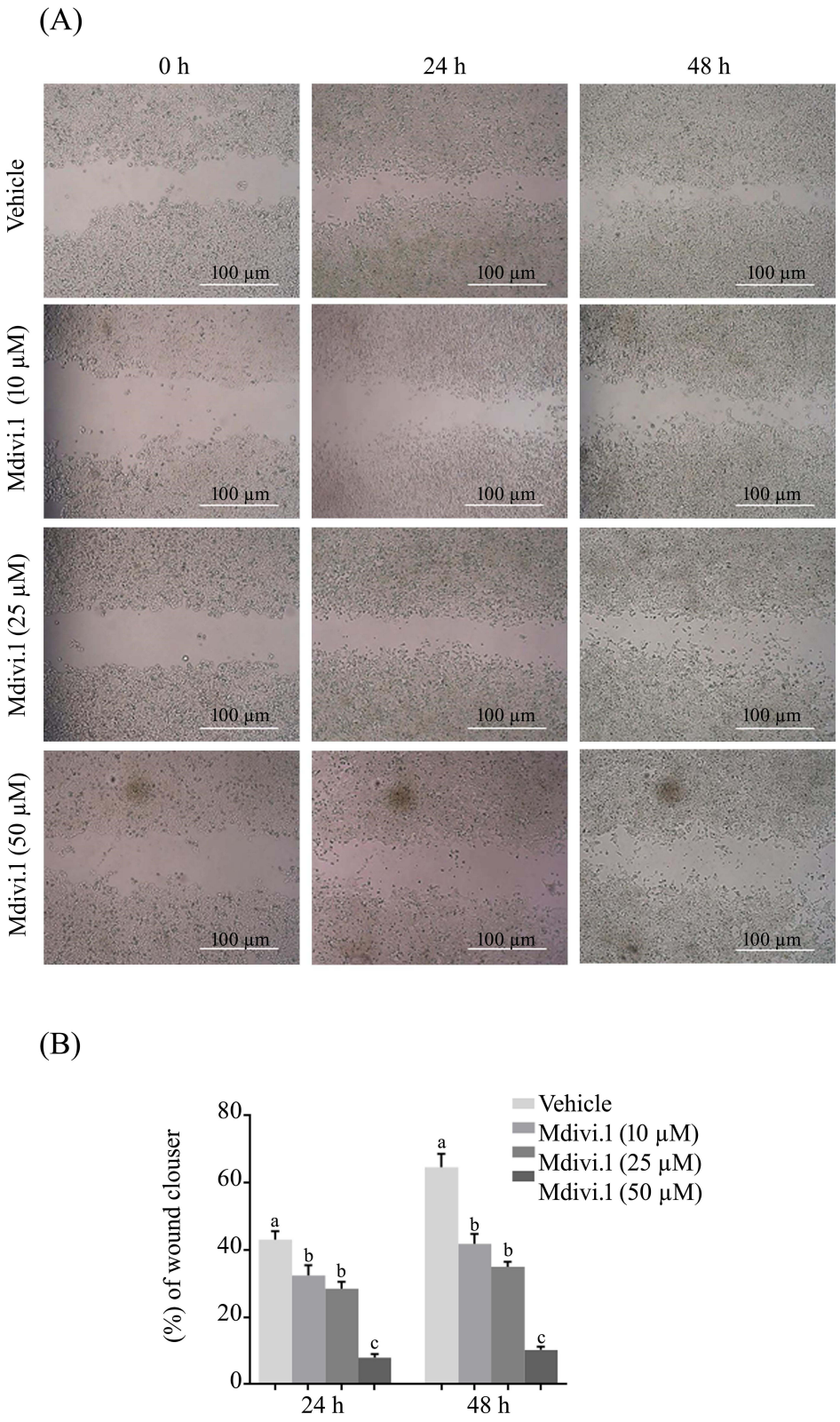

Figure 4 Effect of Mdivi.1 on wound closure in HCT116 cells. (**A**) The cells were grown until 80–90% confluent followed by wound induction through scratching with sterile pipette tip. The cells were treated with Mdivi.1 or vehicle with 10% FBS for 24 h at 37 °C. Following treatment, the medium was changed to 1% FBS and wound closure activity was observed. Mdivi.1 inhibited wound closure at indicated concentrations and time points. (**B**) The data were analyzed by ImageJ software and presented as percentage (%) of wound closure. Columns not sharing the same superscript letters (a,b,c) differ significantly ($p < 0.05$, one-way ANOVA; Tukey's post hoc test).

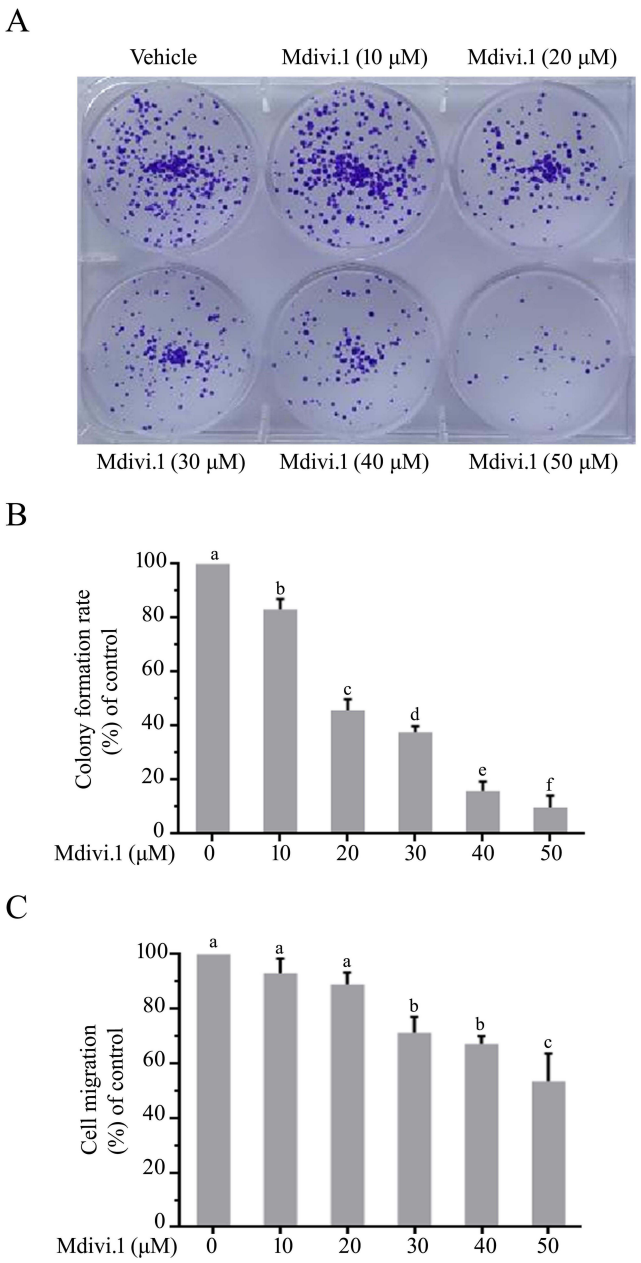

Figure 5 Anti-migration activity of Mdivi.1 in HCT116 cells. (**A**) The cells-treated with indicated concentrations of Mdivi.1 for 16 h were trypsinized, seeded (500 cells/well), and grown in DMEM/F12 medium supplemented with 1% FBS for 10 days or until colony formation. The colonies were washed with D-PBS, fixed with paraformaldehyde and stained with crystal violet for 15 min. The colonies were washed to remove extra stain and photographed. Mdivi.1 treatment inhibited the formation of colonies in a concentration-dependent manner in HCT 116 cells. (**B**) Crystal violet stain absorbed by the colonies was dissolved in methanol followed by absorbance at 595 nm. The data were analyzed and presented as colony formation rate over percentage (%) of control. (**C**) Mdivi.1 inhibited the migration of HCT116 cells in a concentration-dependent manner as determined by transwell chamber. The data were analyzed and presented as cell migration rate over percentage (%) of control. Columns not sharing the same superscript letters (a,b,c,d,e,f) differ significantly ($p < 0.05$, one-way ANOVA; Tukey's post hoc test).

cell-selective activity of Mdivi.1 leading to enhance the sensitivity of A2780 and cisplatin-resistant A2780 human ovarian cancer cells to death receptor ligands including TRAIL, FAS and TNF-α. However, Mdivi.1 does not induce cytotoxicity in non-transformed normal human fibroblast NHDF cell.[31] Furthermore, pre-incubation of Mdivi.1 partially restores mitochondrial morphology by protecting the outer mitochondrial membrane and retains a round or oval geometry of mitochondria in Glutamate-insulted cortical neurons. Glutamate-induced loss of mitochondrial membrane potential is mitigated by Mdivi.1 pretreatment in cortical neuron.[32] Kim et al reported the reversal of Palmitate-induced ROS generation, -mitochondrial superoxide production and -loss of mitochondrial membrane potential with pre-incubation of

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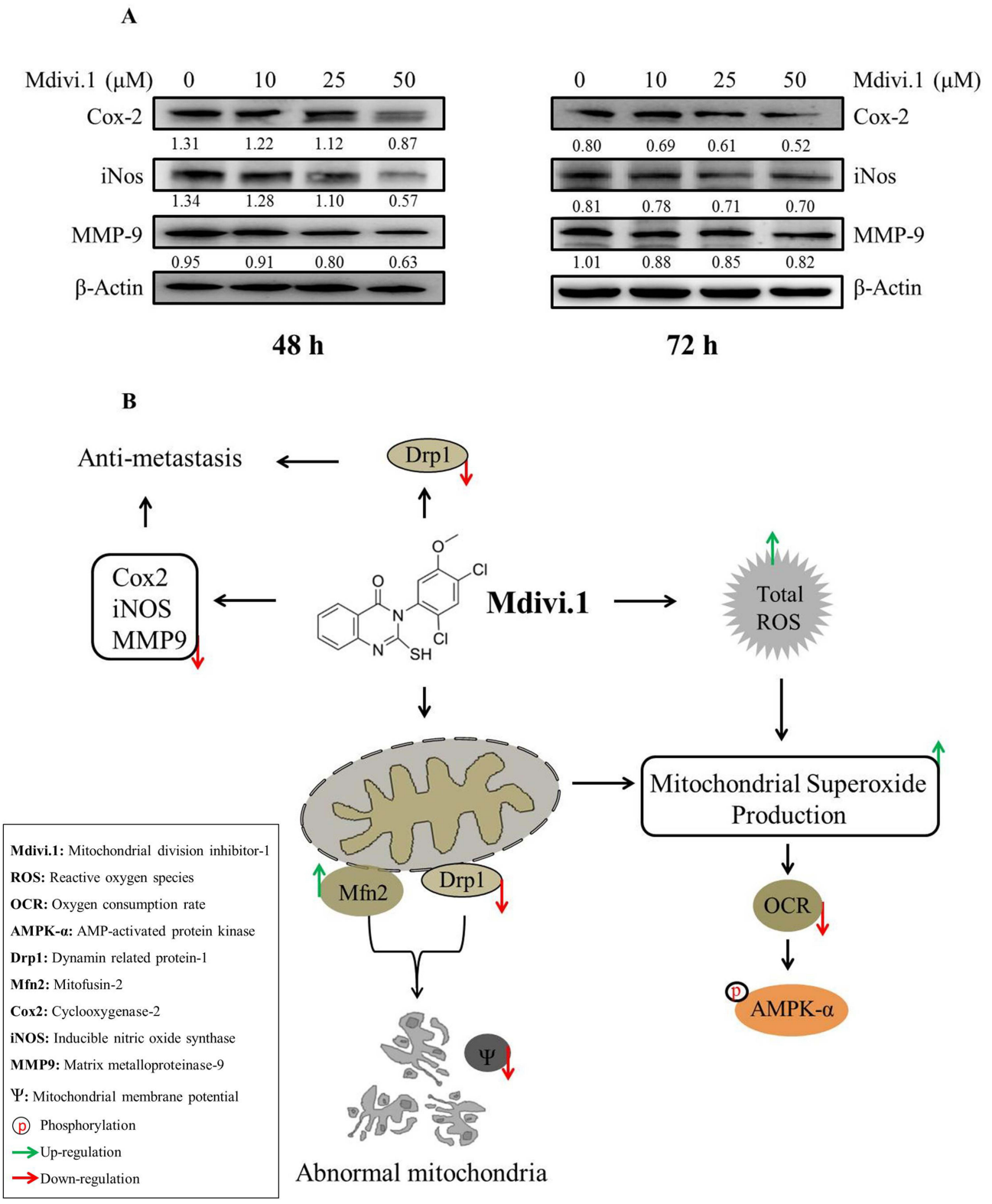

Figure 6 Mdivi.1 down-regulated metastatic markers. (**A**) Mdivi.1 decreased the expression of Cox-2, iNos and MMP9 in HCT116 cells. Numbers below represent the densitometric analyses of each protein marker. (**B**) Schematic representation of possible mode of action of Mdivi.1 on mitochondrial dynamics in HCT116 cells.

Mdivi.1 in normal hippocampal neural stem cells (NSCs).[33] Additionally, Mdivi.1 decreases palmitate-induced Drp1 expression level and inhibits the translocation of Drp1 from cytoplasm to mitochondria in hippocampal NSCs. Mdivi-1 changes the expression of the Bcl-2 family proteins, inhibits the release of cytochrome c and caspase-3 activation, thereby enhancing the survival of hippocampal NSCs exposed to palmitate.[33] The promising tumor cell-selective activity develops Mdivi.1 a lead compound to selectively kill cancer cells by disrupting mitochondrial dynamics.

Overexpression of Drp1 is associated with cancer cell invasion and metastasis, and maintains stem cell properties.[11,30,34] Excessive mitochondrial fission has been associated with poor prognosis in patients with hepatocellular carcinoma (HCC) patients.[35–37] Mitochondrial localization, trafficking, and redistribution toward the periphery of the cells are required for cell motility, invasion, and migration. Downregulation of Drp1 by silencing and upregulation of Mfn2 changes the morphology of the mitochondria from fragmented to mitochondrial elongation and clusters, respectively, inhibits the movement of mitochondria, and restricts them to the central region of the cells, which suppresses the metastatic activity of various breast cancer cells. In contrast, Mfn2 silencing induced mitochondrial fragmentation, resulting in higher metastatic abilities of breast cancer cells.[12] Furthermore, cell migration and invasion activity of oncocytic XTC.UC1 cells were decreased by the downregulation of Drp1 and upregulation of Mfn2.[13] Our results are in agreement with the previous observation as Mdivi.1 induced anti-metastatic activity and inhibits the invasion and migration of HCT116 cells in a concentration-dependent manner.

It is well established that excessive mitochondrial fission is associated with a poor prognosis of cancers, and overexpression of Drp1 enhances cancer stem cell characteristics and induces cancer invasion and metastasis.[21,23] We found that Mdivi.1 significantly decreased the expression of Cox-2, iNos, and MMP-9, known metastatic markers, in a concentration-dependent manner. Further studies are needed to explore whether Mdivi.1-induced anti-metastatic activity and changes in metastatic markers are Drp1-dependent. A schematic representation of Mdivi.1-induced anti-metastatic activity through modulation of mitochondrial dynamics and metastatic markers is shown in Figure 6B.

Conclusion

In conclusion, this study demonstrated that in HCT116 cells, Mdivi.1 inhibits mitochondrial fission, leading to the accumulation of dysfunctional mitochondria with dissipated mitochondrial membrane potential, ROS generation, and AMPK activation. Furthermore, Mdivi.1 inhibits the migration and metastatic markers in HCT116 colorectal cancer cells. Targeting mitochondrial dynamics is a promising approach for the treatment of colorectal cancer.

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Disclosure

The authors report no conflicts of interest in this work.

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