Biochemical and Biophysical Research Communications 464 (2015) 286–291

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

ABT-737 sensitizes curcumin-induced anti-melanoma cell activity through facilitating mPTP death pathway

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ARTICLE INFO

Article history:
Received 18 June 2015
Accepted 22 June 2015
Available online 24 June 2015

Keywords:
Curcumin
Melanoma
ABT-737
Mitochondrial permeability transition pore (mPTP)
Cell death and chemo-sensitization

ABSTRACT

In the current study, we studied the potential role of ABT-737, a novel Bcl-2 inhibitor, on curcumin-induced anti-melanoma cell activity *in vitro*. The associated mechanisms were also investigated. We demonstrated that ABT-737 significantly sensitized curcumin-induced activity against melanoma cells (WM-115 and B16 lines), resulting in substantial cell death and apoptosis with co-administration. At the molecular level, curcumin and ABT-737 synergistically induced mitochondrial permeability transition pore (mPTP) opening in melanoma cells, the latter was evidenced by mitochondrial membrane potential (MPP) reduction and mitochondrial complexation between cyclophilin-D (CyPD) and adenine nucleotide translocator 1 (ANT-1). Significantly, mPTP blockers, including cyclosporin A and sanglifehrin A, remarkably inhibited curcumin and ABT-737 co-administration-induced cytotoxicity against melanoma cells. Meanwhile, siRNA-mediated knockdown of CyPD or ANT-1, the two key components of mPTP, alleviated WM-116 cell death by the co-treatment. Collectively, we show that ABT-737 sensitizes curcumin-induced anti-melanoma cell activity probably through facilitating mPTP death pathway. ABT-737 could be further investigated as a potential curcumin adjuvant in melanoma and other cancer treatment.

1. Introduction

Malignant melanoma is a major health threat in Caucasians and Asians, and it is causing dramatic cancer-related mortalities all over the world [1,2]. Even with the development of modern medicines, the overall survival of malignant melanoma has not been significantly improved [1,2]. One reason could be that malignant melanoma is among the most resistant tumors to possible all traditional chemo-agents [3–5]. Groups including ours [6–8] are thus searching for more efficient anti-melanoma agents.

Curcumin, or 1,7-bis(4-hydroxy-3methoxyphenyl)-1,6-heptadien-3,5-dione, is the main bioactive component of turmeri from the rhizome of *Curcuma longa* [9]. Studies have confirmed that curcumin exerts wide-range anti-tumor activities [9]. Curcumin is shown to inhibit cancer cell survival and proliferation, to induce cancer cell death and apoptosis, and to suppress cancer-related angiogenesis [9]. Meanwhile, we [7,8,10] and others [9,11–13] have confirmed the dramatic anti-melanoma activity of curcumin both *in vitro* and *in vivo*. Further, phase I and phase II clinical trials are also undergoing, and preliminary results have shown that curcumin is quite safe and might exhibit therapeutic ability against melanoma [11].

However, the clinical usage of curcumin is limited, in part due to the overwhelming resistance from the melanoma cells [14–16]. Overexpression and over-activity of Bcl-2, an anti-apoptotic protein, were seen in many melanomas, which are correlated with

Abbreviations: (ANT-1), Adenine nucleotide translocator 1; (CyPD), cyclophilin-D; (CsA), cyclosporin A; (mPTP), mitochondrial permeability transition pore; (MPP), mitochondrial membrane potential; (SfA), sanglifehrin A; (VDAC), voltage-dependent anion channel.

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http://dx.doi.org/10.1016/j.bbrc.2015.06.144
0006-291X/

cancer progression and poor prognosis [17]. Bcl-2 interacts with pro-apoptotic BH3 protein to inhibit cancer cell apoptosis [17]. Recent studies have developed a novel BH-3-mimetic Bcl-2 inhibitor, namely ABT-737 [18]. Its activity in melanoma cells, however, has not been fully studied. Here, we show that ABT-737 significantly sensitizes curcumin-induced anti-melanoma cell activity through facilitating mitochondrial permeability transition pore (mPTP)-mediated death pathway.

2. Material and methods

2.1. Chemicals and reagents

ABT-737 were obtained from Selleck (Shanghai, China). Cyclosporine A (CsA) and sanglifehrin A (SfA) were obtained from Sigma (Sigma, St. Louis, MO). Antibodies against adenine nucleotide translocator 1 (ANT-1); cyclophilin-D (CyPD), voltage-dependent anion channel (VDAC) and β-tubulin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

2.2. Cell culture

WM-115 and B16 melanoma cells [8,10] were maintained in DMEM medium, supplemented with a 10% FBS (Gibco, Shanghai, China), Penicillin/Streptomycin (1:100, Gibco) and 4 mmol/L L-glutamine (Gibco), in a CO_2 incubator at 37 °C.

2.3. Cell death assay by trypan blue staining

After the treatment, cells were trypsinized and pelleted with cellular supernatant for 5 min at 400 g. After pellet was resuspended, the cell death percentage was determined by counting cells using a hemocytometer after addition of trypan blue, which stained the cytoplasm of dead cells. Cell death percentage (%) = the number of trypan blue stained cells/the number of total cells (× 100%).

2.4. Apoptosis assay

After the treatment, cells were washed with cold PBS and incubated with 0.5 ml of Binding Buffer (10 mM HEPES, pH 7.4, 150 mM NaCl, 2.5 mM $CaCl_2$, 1 mM $MgCl_2$, and 4% BSA), containing 3 μg/mL Annexin V-FITC for 10 min. Cells were then washed with cold PBS and resuspended. A total of 10,000 cells of each sample were analyzed by flow cytometry in a FACS (Beckton Dickinson FACScan, Shanghai, China). The percentage of Annexin V was recorded as apoptosis ratio.

2.5. Detection of mitochondrial membrane potential (MMP)

As previously reported [6], the MMP was measured through JC-10 dye (Invitrogen, Shanghai, China). The JC-10 dye exhibits two staining spectra. In normally respiring cells, the dye forms aggregates in the mitochondrial membrane, exhibiting orange fluorescence. When the membrane potential is lost, monomeric JC-10 forms in the cytosol, exhibiting green fluorescence. WM-115 cells were stained with 5 μg/mL of JC-10 for 10 min at 37 °C. Cells were then washed twice with PBS, and resuspended in fresh culture medium and read immediately on a microplate reader with an excitation filter of 485 nm. The green fluorescence intensity was recorded as the indicator of MMP reduction [6].

2.6. Western blots

Western blots assay was performed according to the previous protocol [6,8,10]. For detecting proteins in the mitochondria, intact mitochondria of WM-115 cells were isolated from 1.0×10^7 WM-115 cells using the "Mitochondria Isolation Kit for Cultured Cells" (Pierce, Rockford, IL). The band intensity was quantified through ImageJ software. The intensity of each indicated band was normalized to the intensity of the corresponding load control. The value was expressed as fold change vs. control group, which labeled as "1.00".

2.7. Mitochondrial immunoprecipitation (mito-IP)

As reported [6], 500 μg of cell lysates from mitochondrial fractions were pre-cleared with 20 μL of protein A/G PLUS-agarose (Santa Cruz) for 1 h. The supernatant was then rotated overnight with 2 μg of anti-CyPD (Santa Cruz Biotech). Next, the lysates were centrifuged for 5 min at 4 °C in a micro-centrifuge to remove nonspecific aggregates. The protein A/G PLUS-agarose (35 μL) was then added to the supernatants for 4 h at 4 °C. Pellets were washed six times with PBS, resuspended in lysis buffer, and then assayed by Western blots.

2.8. SiRNAs and transfection

As previously reported [6], two non-overlapping siRNAs against human CyPD (CyPD siRNA-a and CyPD siRNA-b) [6], two non-overlapping siRNAs against ANT-1 (ANT-1-siRNA-a, ANT-1-siRNA-b) [6], and one negative control scramble siRNA were all purchased from Dharmacon Research Inc. (Lafayette, CO, USA). For transfection, 3.0 μL of PLUS™ Reagent (Invitrogen, Carlsbad, CA) was diluted in 90 μL of RNA dilution water (Santa Cruz, CA) for 5 min at room temperature. Then, 0.2 nmol of target-siRNA was added to PLUS™ Reagent and left for 5 min at room temperature. To this was added 4.0 μL of Lipofectamine (Invitrogen) and incubation for another 30 min. Finally, the complex was added to the well containing 1.0 ml of culture medium (no antibiotics, no FBS), cells then cultured for additional 24 h before adding 2% FBS for another 24 h. Control cells were transfected with the same amount of scramble siRNA (200 nM). The efficiency of siRNA was determined by Western blots.

2.9. Real-time PCR

Total RNA was extracted using Trizol reagent (Invitrogen, Shanghai, China) based on the protocol attached. Total RNA was reverse-transcribed using the reverse transcriptase (Promega, Madison, WI, USA). The cDNA derived from 0.5 μg of total RNA was amplified by real-time polymerase chain reaction (PCR). The SYBR Green PCR kit (Applied Biosystems, Foster City, CA) was used to detect the CyPD, ANT-1 and glyceraldehyde phosphate dehydrogenase (GAPDH) mRNA expression. Primer sequences were reported early [6]. The PCR was performed in triplicate and was conducted using a Real-Time PCR Detection System (7500; ABI, Carlsbad, CA, USA). The PCR data were analyzed. mRNA expression of CyPD or ANT-1 was normalized to that of GAPDH.

2.10. Statistical analysis

The values in the figures were expressed as the means ± standard deviation (SD). Statistical analysis of the data between the control and treated groups was performed by ANOVA. Values of $\boldsymbol{p} < 0.05$ were considered as statistically different.

3. Results

3.1. ABT-737 sensitizes curcumin-induced anti-melanoma cell activity in vitro

The current study was set to understand the potential role of ABT-737 on curcumin-induced activity against melanoma cells. WM-115 melanoma cells were treated with applied concentrations of curcumin, or plus ABT-737 (100 nM) co-administration. Results demonstrated that ABT-737 remarkably enhanced curcumin-induced anti-survival (Fig. 1A), and pro-death (Fig. 1B) activities against WM-115 melanoma cells. Meanwhile, curcumin-activated apoptosis, tested by Annexin V FACS assay, was also significantly increased with ABT-737 co-treatment (Fig. 1C). Note that ABT-737 alone at test concentration (100 nM) only induced moderate cell death and apoptosis (less than 10–20%) in WM-115 cells (Fig. 1A–C). The similar curcumin-sensitization effect by ABT-737 was observed in B16 melanoma cells (Fig. 1D). Collectively, these results demonstrate that ABT-737 enhances curcumin-induced activity against melanoma cells *in vitro*.

3.2. Curcumin and ABT-737 synergistically induce mPTP opening

Our previous study demonstrates that curcumin induces mPTP opening, which mediates subsequent melanoma cell death and apoptosis [6]. Next, we studied the role of ABT-737 on the process. MMP was measured through JC-10 dye assay [6]. When the membrane potential is lost, monomeric JC-10 will form in the cytosol, exhibiting green fluorescence [6]. Thus, JC-10 green fluorescence intensity could be examined as an indicator of MMP reduction [6]. Results in Fig. 2A showed that curcumin and ABT-737 synergistically induced mPTP opening, resulting in a substantial MMP reduction. The combined activity was more potent than either agent alone (Fig. 2A). Meanwhile, curcumin and ABT-737 also synergistically induced CyPD and ANT-1 mitochondrial association, known as the initial step of mPTP opening (Fig. 2B) [6,19]. Again, the combined activity was remarkably potent than either single agent alone (Fig. 2B). Note that the expression of major mPTP components (CyPD, VDAC and ANT-1) was not affected after curcumin and/or ABT-737 treatment (Fig. 2B, input). As expected, the mPTP blockers sanglifehrin A (SfA) [20] and CsA [20,21] almost completed blocked co-administration-induced MMP reduction in WM-115 cells (Fig. 2A). Significantly, curcumin and ABT-737 co-treatment-induced WM-115 (Fig. 2C) and B15 (Fig. 2E) cell viability reduction and cell death (Fig. 2D) were dramatically inhibited by the two mPTP inhibitors. SfA and CsA alone was non-cytotoxic to above melanoma cells (Fig. 2C and D, and not shown data). These results suggest that curcumin and ABT-737 synergistically induce mPTP opening to possibly mediate melanoma cell death.

3.3. CyPD is required for curcumin and ABT-737 co-administration-induced melanoma cell death

To further support the role of mPTP in co-administration-induced cytotoxic effect in melanoma cells, siRNA method was applied to knockdown CyPD, the key component of mPTP [6,22,23]. We applied two non-overlapping siRNAs against CyPD [6]. Western blot and RT-PCR results confirmed that both siRNAs showed excellent efficiency, resulting in over 85% of CyPD downregulation in WM-115 cells (Fig. 3A and B). As expected, curcumin and ABT-

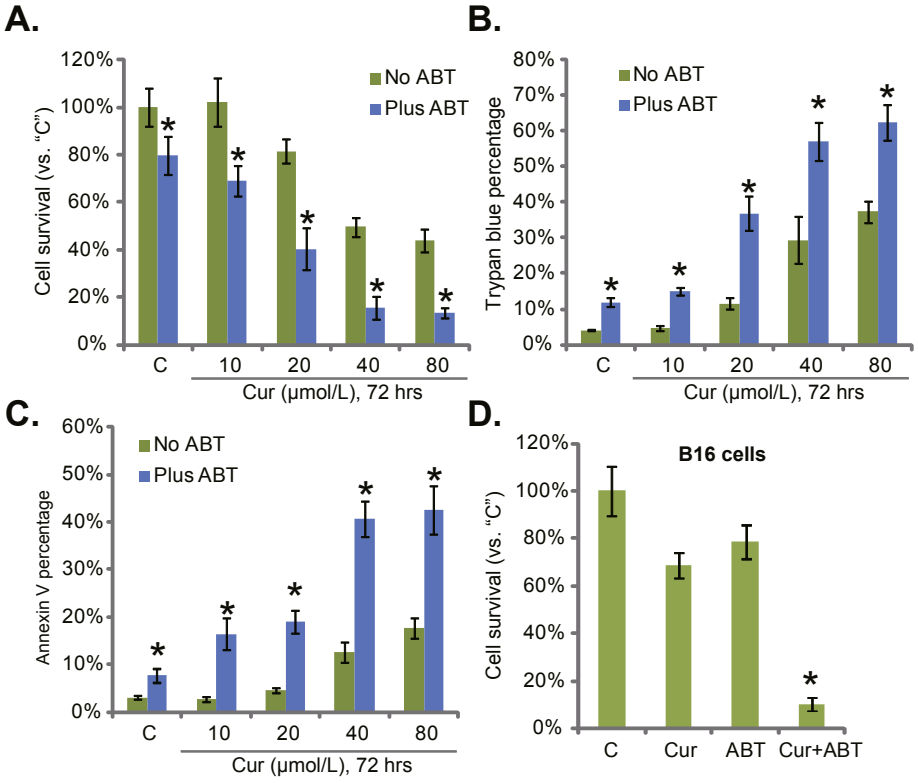

Fig. 1. ABT-737 sensitizes curcumin-induced activity against melanoma cells. WM-115 melanoma cells were treated with applied concentrations of curcumin (Cur, 0–80 μM), or plus ABT-737 (ABT, 100 nM), cells were further cultured for 72 h, cell viability, cell death and apoptosis were tested by MTT assay (**A**), trypan blue staining assay (**B**) and Annexin V FACS assay (**C**), respectively. B16 melanoma cells were treated with curcumin (Cur, 20 μM), ABT-737 (ABT, 100 nM), or both for 72 h, cell survival was tested by MTT assay (**D**). Experiments in this figure were repeated three times, and similar results were obtained. For each assay, n = 5 (Same for all figures). "C" stands for medium control group (Same for the all following figures). *$p < 0.05$ vs. "Cur" only group.

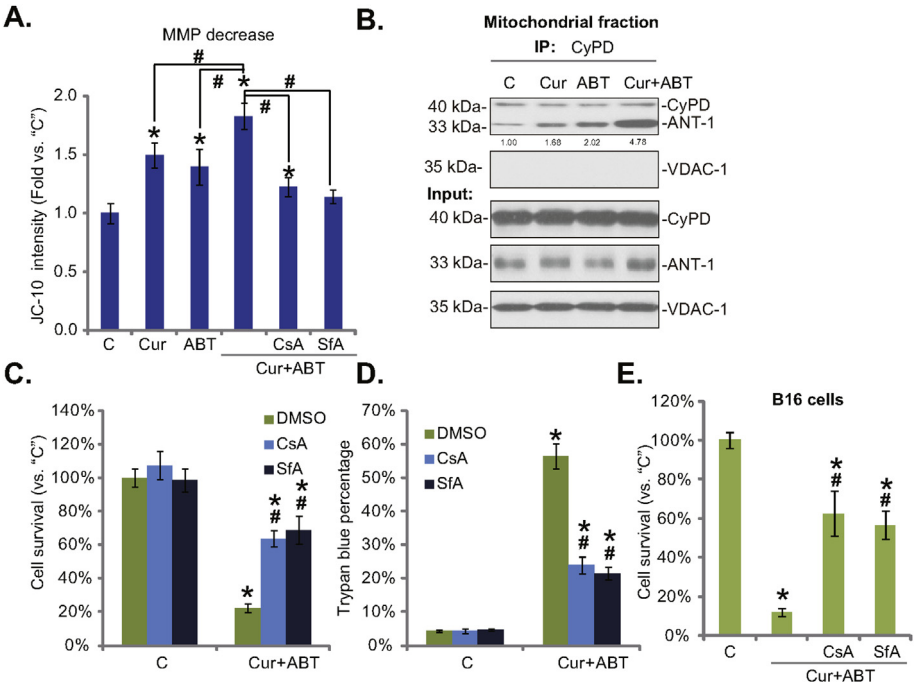

Fig. 2. Curcumin and ABT-737 synergistically induce mPTP opening. WM-115 melanoma cells were stimulated with curcumin (Cur, 20 μM) and/or ABT-737 (ABT, 100 nM) for 3 h, MMP reduction was analyzed by JC-10 dye assay (**A**), mitochondrial ANT-1 and Cyp-D association was examined by Mito-IP (**B**). WM-115 or B16 melanoma cells, pretreated with sanglifehrin A (SfA, 2.5 μM) or cyclosporin A (CsA, 0.5 μM), were stimulated with curcumin (Cur, 20 μM) plus ABT-737 (ABT, 100 nM), MMP reduction was tested 3 h after co-administration (**A**), cell survival was tested by MTT assay (**C** and **E**, 72 h after co-treatment), and cell death was tested by trypan blue staining assay (**D**, 72 h after co-treatment). CyPD-associated ANT-1 expression was quantified (**B**). Experiments in this figure were repeated three times, and similar results were obtained. $^{*}p < 0.05$ vs. "C" group. $^{\#}p < 0.05$ vs. "ABT + Cur" group.

737 co-administration-induced MMP reduction was largely inhibited in CyPD-silenced WM-115 cells (Fig. 3C). As a consequence, co-administration-induced WM-115 cell death was alleviated in CyPD-depleted cells (Fig. 3D and E). Similar results were also obtained in B16 melanoma cells (Data not shown). These results, together with the above data, indicate that mitochondrial protein CyPD, probably through complexation with VDAC, is involved in curcumin and ABT-737 co-administration-induced activity against melanoma cells.

3.4. SiRNA-knockdown of ANT-1 inhibits curcumin and ABT-737 co-administration-induced melanoma cell death

We again utilized siRNA method to silence ANT-1, another key component of mPTP. As showed in Fig. 4A–B, ANT-1 protein and mRNA expressions of were significantly downregulated by targeted ANT-1 siRNAs (ANT-1-siRNA-a, ANT-1-siRNA-b) in WM-115 cells [6]. We again applied two non-overlapping siRNAs against ANT-1 [6]. Importantly, ANT-1 silencing remarkably alleviated co-administration-induced MMP reduction (Fig. 4C). As a result, curcumin and ABT-737-induced WM-115 cell death was also inhibited (Fig. 4D and E). Above results were also seen in B16 melanoma cells (Data not shown). These results once again confirm the role of ANT-1 and mPTP in mediating curcumin and ABT-737 co-administration-induced cytotoxicity in melanoma cells.

4. Discussions

In the past decades, the incidence of malignant melanoma has been rising in both Eastern and Western countries [24]. Its prognosis and mortality rate are not even close to be satisfactory [24–28]. Currently, there has been no effective chemo-agents for the this devastating disease [24]. One important challenge clinically is its incredible resistance to almost all traditional chemotherapy [25–28]. Groups including ours [7,8,10] have shown that curcumin could possibly overcome the chemo-resistance by inducing melanoma cell death and apoptosis [5].

Although curcumin has displayed dramatic activity in melanoma as well as in many other cancers, a high concentration of curcumin (20–100 μM) is often needed to efficiently kill cancer cells *in vitro* [8,10], which significantly limits its potential clinical use [14,15]. Thus, our group has been focusing on the curcumin-sensitization strategies. For example, we showed that ceramide production is important for curcumin-mediated melanoma cell apoptosis. Meanwhile 1-phenyl-2-decanoylamino-3-morpholino-1-propanol (PDMP), the glycosphingolipid biosynthesis inhibitor, enhances curcumin-induced ceramide production and anti-melanoma activity against melanoma cells [8]. Further, we demonstrated that with the help from a short-chain ceramide (C6), low concentrations of curcumin (1–10 μM) could be extremely cytotoxic to melanoma cells [6]. We suggest that curcumin-sensitization effect by C6 ceramide is probably due to its ability to facilitate mPTP-mediated mitochondrial death pathway [6].

It is well-known that mitochondria play a vital role in dictating both apoptotic and necrotic cell death [29–31]. Under stress conditions, mitochondria exert its pro-death function through regulating mPTP [29–31]. mPTP opening will cause mitochondrial swelling, outer membrane bleach and pro-death proteins (*i.e.* cytochrome C) release, eventually leading to cell death and apoptosis [32]. It is well-accepted that mPTP is composed of at least three major proteins, ANT on the mitochondrial inner membrane, VDAC on the mitochondrial outer membrane, and CyPD in the mitochondrial matrix [32]. Inhibition, mutation of knockdown of ANT-1 and other components of mPTP could alleviate cell death and apoptosis by a number of stimuli [33–36]. In the current study, we showed that curcumin and ABT-737 synergistically induced mPTP

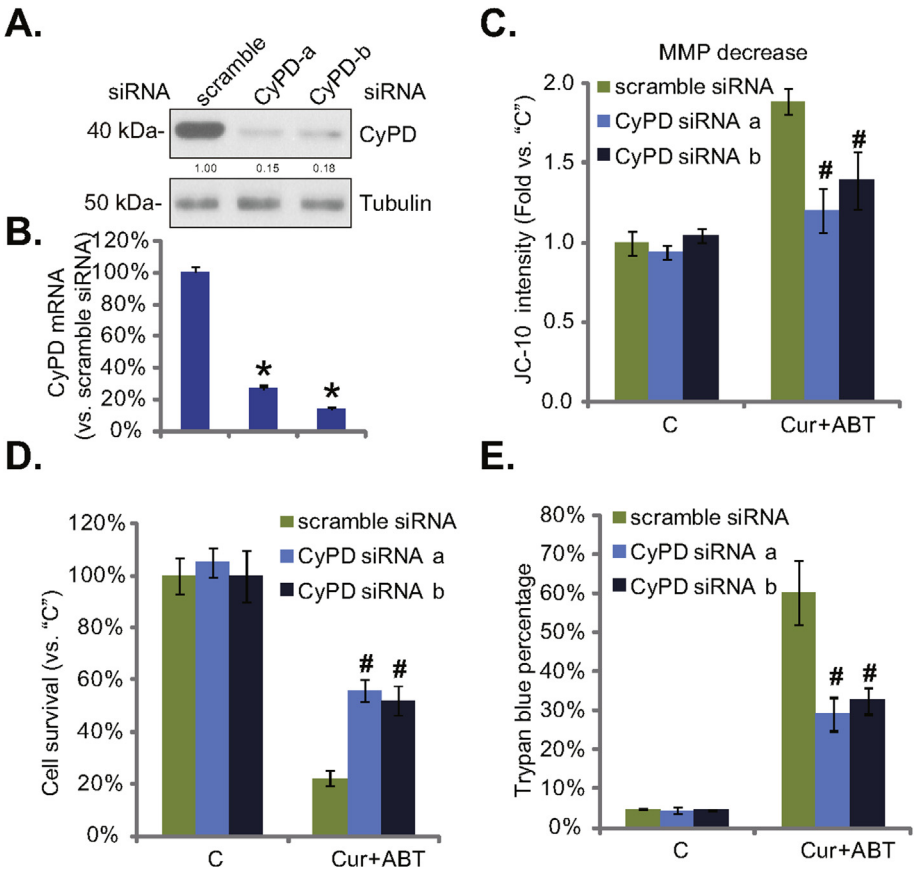

Fig. 3. CyPD is required for curcumin and ABT-737 co-administration-induced melanoma cell death. The protein and mRNA expression of CyPD in WM-115 cells transfected with scramble or CyPD siRNAs (-a/-b, 200 nM each, 48 h) were shown in (**A**) and (**B**), respectively. Above cells were also treated with curcumin (Cur, 20 μM) plus ABT-737 (ABT, 100 nM), MMP reduction was analyzed by JC-10 day assay after 3 h (**C**), cell survival and cell death were tested by MTT assay (**D**) and trypan blue staining assay (**E**) after 72 h, respectively. CyPD protein expression (vs. Tubulin) was quantified (**A**). Experiments in this figure were repeated three times, and similar results were obtained. *p < 0.05 vs. scramble siRNA group (**B**). #p < 0.05 vs. "ABT + Cur" group (**C**–**E**).

opening, evidenced by MMP decrease and mitochondrial CyPD-ANT-1 association, which mediated subsequent melanoma cell death. mPTP blockers (CsA and SfA) as well as siRNA knockdown of CyPD or ANT-1 dramatically inhibited co-administration-exerted cytotoxicity against melanoma cells. Thus, mPTP opening might be the key signaling mechanism for the synergism.

It should be noted that blockage of mPTP opening, through mPTP blockers (CsA and SfA) or CyPD/ANT-1 siRNA knockdown, only alleviated but not abolished the synergistic activity against melanoma cells by the co-administration. It is possible that the interfering methods (siRNA and pharmacological method) applied here were incomplete against mPTP. What is more likely, however, is that other cell apoptosis/death pathways could also be involved the activity by the co-treatment [14]. These other signalings pathways likely work separately or together with mPTP pathway to promote melanoma cell death and apoptosis [14]. The hypothesis

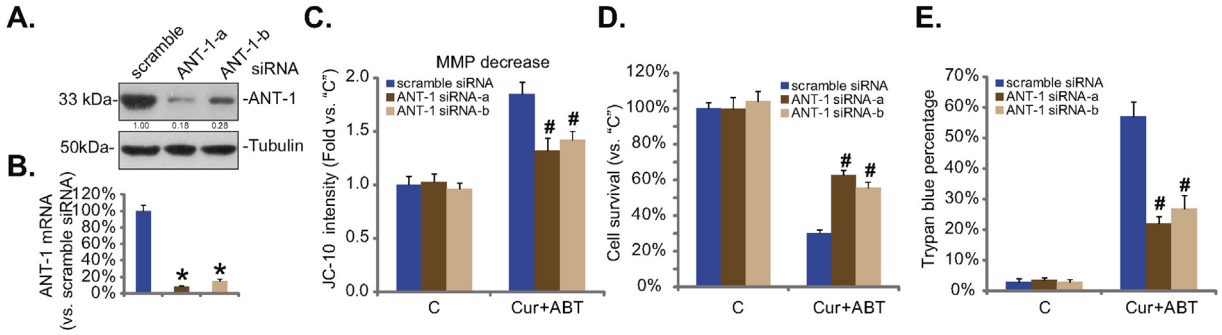

Fig. 4. Silencing of ANT-1 inhibits curcumin and ABT-737 co-administration-induced melanoma cell death. WM-115 cells were transfected with scramble siRNA, ANT-1-siRNA-a, ANT-1-siRNA-b (200 nM each, 48 h), protein expression of ANT-1 and Tubulin (the loading control) was tested (**A**), ANT-1 mRNA was also examined (**B**). Above cells were treated with curcumin (Cur, 20 μM) plus ABT-737 (ABT, 100 nM), MMP reduction was analyzed by JC-10 day assay after 3 h (**C**), cell survival and cell death were tested by MTT assay (**D**) and trypan blue staining assay (**E**) after 72 h, respectively. ANT-1 protein expression (vs. Tubulin) was quantified (**A**). Experiments in this figure were repeated three times, and similar results were obtained. *p < 0.05 vs. scramble siRNA group (**B**). #p < 0.05 vs. "ABT + Cur" group (**C**–**E**).

needs further investigations. Future studies will also be needed to test the synergism and the associated signaling changes using *in vivo* models. Together, we demonstrate that ABT-737 sensitizes curcumin-induced anti-melanoma cell activity probably through facilitating mPTP death pathway. ABT-737 could be further investigated as a potential curcumin adjuvant in melanoma and other cancer treatment.

Conflict of interests

No conflict of interests were stated by all authors.

Acknowledgments

This work is supported by funding from Shandong Health and Family Planning Commission (2014WS0275).

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2015.06.144.

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