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Curcumin-induced melanoma cell death is associated with mitochondrial permeability transition pore (mPTP) opening

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ABSTRACT

Here we studied the role of mitochondrial permeability transition pore (mPTP) opening in curcumin's cytotoxicity in melanoma cells. In cultured WM-115 melanoma cells, curcumin induced mitochondrial membrane potential (MPP) decrease, cyclophilin-D (CyPD)-adenine nucleotide translocator 1 (ANT-1) (two mPTP components) mitochondrial association and cytochrome C release, indicating mPTP opening. The mPTP blocker sanglifehrin A (SfA) and ANT-1 siRNA-depletion dramatically inhibited curcumin-induced cytochrome C release and WM-115 cell death. CyPD is required for curcumin-induced melanoma cell death. The CyPD inhibitor cyclosporin A (CsA) or CyPD siRNA-depletion inhibited curcumin-induced WM-115 cell death and apoptosis, while WM-115 cells with CyPD over-expression were hyper-sensitive to curcumin. Finally, we found that C6 ceramide enhanced curcumin-induced cytotoxicity probably through facilitating mPTP opening, while CsA and SfA as well as CyPD and ANT-1 siRNAs alleviated C6 ceramide's effect on curcumin in WM-115 cells. Together, these results suggest that curcumin-induced melanoma cell death is associated with mPTP opening.

1. Introduction

The incidence of malignant melanoma is increasing over the last a few decades in Caucasians and Asians, and it is one of the leading causes of cancer-related mortality around the world [1,2]. Due to the fact that malignant melanoma cells are generally less response to traditional chemo-drugs [3–5], long-term survival of metastatic melanoma patients is dismal [1,3–5], and the search for more efficient anti-melanoma agents is urgent.

Curcumin (1,7-bis(4-hydroxy-3methoxyphenyl)-1,6-heptadien-3,5-dione) is the main bioactive component of turmeric, the dietary spice made from the rhizome of Curcuma longa [6]. Groups including ours [7–9] have shown that curcumin possesses wide-range anti-tumor properties [6], probably due to its ability to induce cancer cell death and apoptosis, and to inhibit cancer-related angiogenesis [6]. Our studies [7–10] and others [6,11–13] have shown the anti-melanoma efficiency of curcumin. Several phase I and phase II clinical trials indicate that curcumin is quite safe and may exhibit therapeutic ability against melanoma [11].

The molecular mechanisms of curcumin-induced tumor cell death, however, remain to be largely ambiguous [6,14]. It has been reviewed that curcumin could inhibit tumor cells through regulating multiple signaling pathways that are important for cell proliferation, survival, apoptosis, inflammation and angiogenesis [14]. Our group has been focusing on how curcumin kills melanoma cells, and several mechanisms including MST1 (mammalian STE20-like kinase 1)-JNK (c-Jun N-Terminal Protein Kinase) activation [7,10], ceramide synthesis and reactive oxygen species (ROS) production [9,11,13] have been proposed. In the current study, we investigated the potential role of mitochondrial permeability transition pore (mPTP) opening in curcumin's effect.

The mitochondrial channel mPTP is composed of at least three proteins, including the voltage-dependent anion channel (VDAC) in the outer mitochondrial membrane (OMM), the adenine nucleotide translocator-1 (ANT-1) in the inner mitochondrial membrane (IMM), and cyclophilin-D (CyPD) in the matrix [15–17]. Under the resting condition, CyPD is sitting in the mitochondrial matrix to keep the mPTP shut [15,16,18]. However, it associates 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.

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ANT in the IMM to open the mPTP when facing critical conditions [19,20]. The consequences of mPTP opening include mitochondrial membrane potential (MMP) loss (mitochondrial depolarization), mitochondria swelling, Ca^{2+} release, and OMM rupture, and release of cell death-associated proteins (i.e. cytochrome C) to cytosol. In the current study, we found that mPTP opening might also be important for curcumin-induced melanoma cell death.

2. Material and methods

2.1. Chemicals and reagents

Curcumin, sanglifehrin A (SfA), cyclosporine A (CsA) and mouse monoclonal anti-tubulin antibody were purchased from Sigma (St. Louis, MO). Antibodies for CyPD, Erk1/2, cytochrome C, ANT-1 and VDAC were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

2.2. Cell culture

WM-115 melanoma cells [7,9] were maintained in DMEM medium, supplemented with a 10% FBS (Sigma, St. Louis, MO), Penicillin/Streptomycin (1:100, Sigma) and 4 mmol/L L-glutamine (Sigma), in a CO_2 incubator at 37 °C .

2.3. Cell death assay

After treatment, cells were trypsinized and pelleted with cellular supernatant for 5 min at 400*g*. After pellet was resuspended in 60 μl media/well, 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 treatment, WM-115 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 20,000 cells of each sample were analyzed by flow cytometry in a FACS (Beckton Dickinson FACScan, Taibei, China). The percentage of Annexin V was recorded as apoptosis rate.

2.5. Detection of mitochondrial membrane potential (MMP)

The MMP was measured through JC-10 dye (Invitrogen) [21]. The JC-10 dye exhibits two staining spectra. In normally respiring

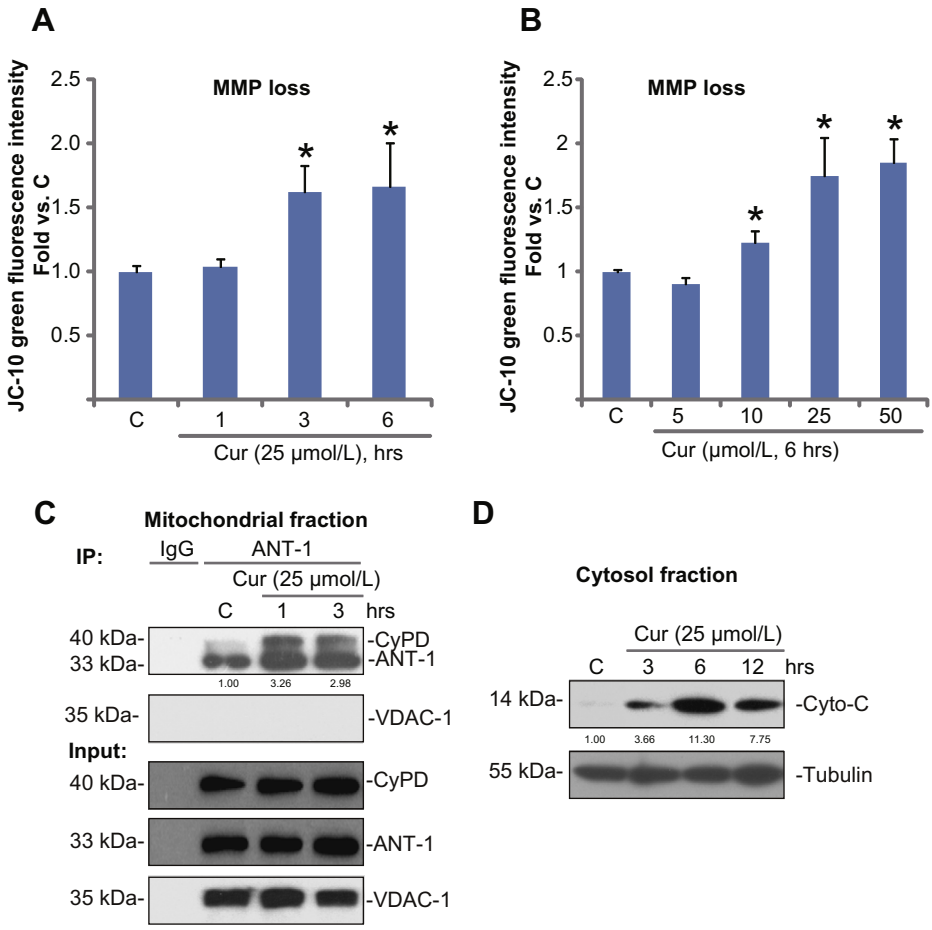

Fig. 1. Curcumin induces mPTP opening in cultured melanoma cells. The JC-10 green fluorescence intensity before and after indicated curcumin (Cur) stimulation in WM-115 cells was analyzed (A and B). The association between CyPD, ANT-1 and VDAC-1 in mitochondria before and after curcumin (Cur, 25 μM) treatment was tested by mito-IP (C). Expression of above proteins in the mitochondria was also tested (C, Input). Cytosol cytochrome C (Cyto-C) and tubulin expression before and after indicated curcumin (Cur) treatment was tested by Western blots (D). ANT-1-bound CyPD (C) and cytosol cytochrome C level (D) were quantified. Experiments in this figure were repeated three times, and similar results were obtained. $^{*}p < 0.05$ vs. control ("C") group (A and B).

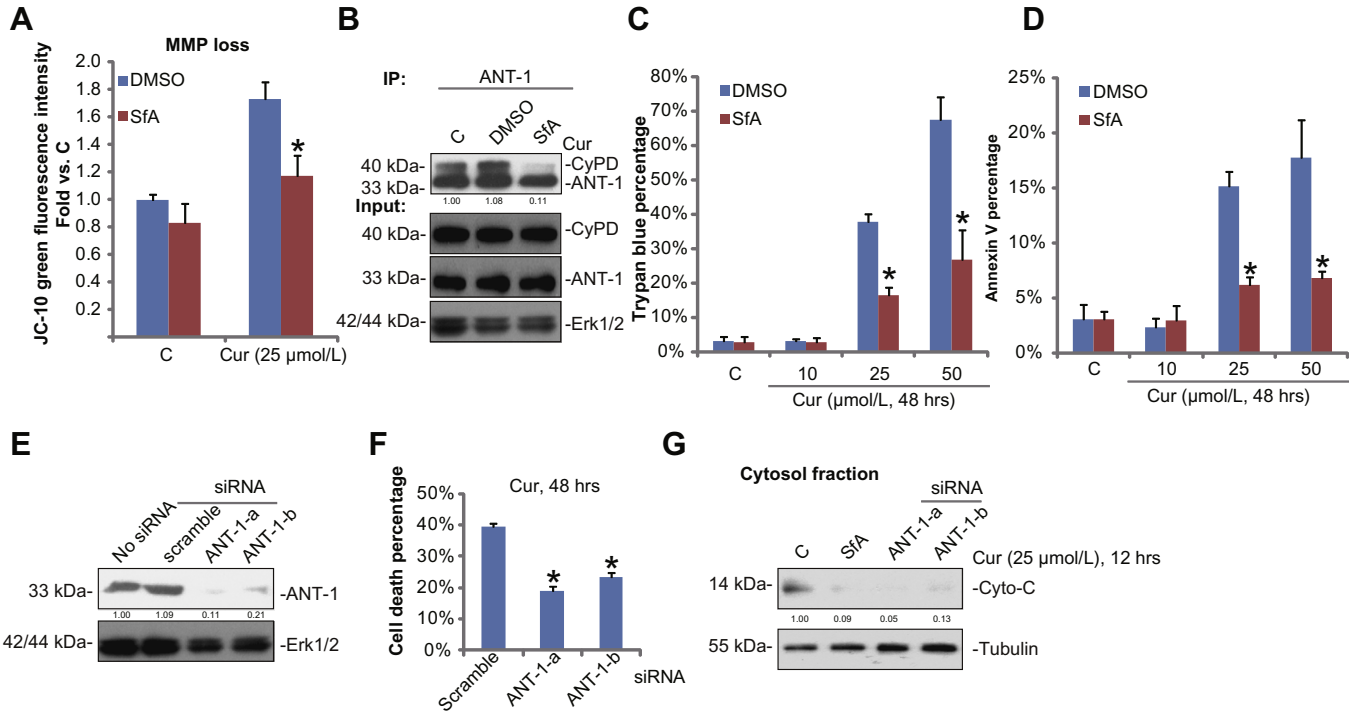

Fig. 2. Opening of mPTP is required for curcumin-induced melanoma cell death. The effect of sanglifehrin A (SfA, 2.5 μM) on curcumin (Cur, 25 μM, 3 h)-induced JC-10 green fluorescence intensity change (A) and mitochondrial CyPD-ANT-1 association (B) was shown. The effect of sanglifehrin A (SfA, 2.5 μM) on indicated curcumin (Cur, 48 h)-induced cell death and apoptosis were analyzed by trypan blue staining (C) and Annexin V FACS (D), respectively. WM-115 cells were either left untreated, or transfected with scramble siRNA, ANT-1-siRNA-a or ANT-1-siRNA-b (200 nM each, 48 h), expression of ANT-1 and Erk1/2 (the loading control) was tested (E), above cells were treated with curcumin (Cur, 25 μM, 48 h), cell death was analyzed by trypan blue staining (F). Effects of sanglifehrin A (SfA, 2.5 μM) and ANT-1 siRNAs (−a/−b) on curcumin (Cur, 25 μM, 12 h)-induced cytochrome C (Cyto-C) release were tested (G). ANT-1-bound CyPD (B), ANT-1 expression (E), and cytosol cytochrome C level (G) were quantified. Experiments in this figure were repeated three times, and similar results were obtained. *$p < 0.05$ vs. DMSO group (A, C and D). *$p < 0.05$ vs. scramble siRNA group (F). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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 loss.

2.6. Western blots

Western blots assay was performed according to the previous protocol [7,9]. For detecting proteins in the mitochondria, intact mitochondria of WM-115 cells were isolated from 2.0×10^7 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 described [10], 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-ANT-1 (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. Real-time PCR

Total RNA was extracted using Trizol protocol. 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 and glyceraldehyde phosphate dehydrogenase (GAPDH) mRNA expression. Primer sequences were as follows (CyPD: F: 5′-GAGGCACTTGTGTCCT GCTT-3′; R: 5′-CGGGTTCTTGGA ATTGGAG-3′ [22]. GAPDH: F:5′-CCTGCCAAGTATGATGACATCA-AGA-3′; R:5′-GTAGCCCAGGATGCCCTTTAGT-3′ [22]). 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 was normalized relative to that of GAPDH.

2.9. SiRNAs and transfection

Two non-overlapping siRNAs against human CyPD (CyPD siRNA-a and CyPD siRNA-b), two non-overlapping siRNAs against ANT-1 (ANT-1-siRNA-a, ANT-1-siRNA-b), and a negative control scramble siRNA were purchased from Dharmacon Research Inc. (Lafayette, CO, USA). For transfection, 3.0 μl 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 medium (no antibiotics, no FBS), cells then cultured for additional 24 h before adding 2% FBS for another 24 h. Control cells were

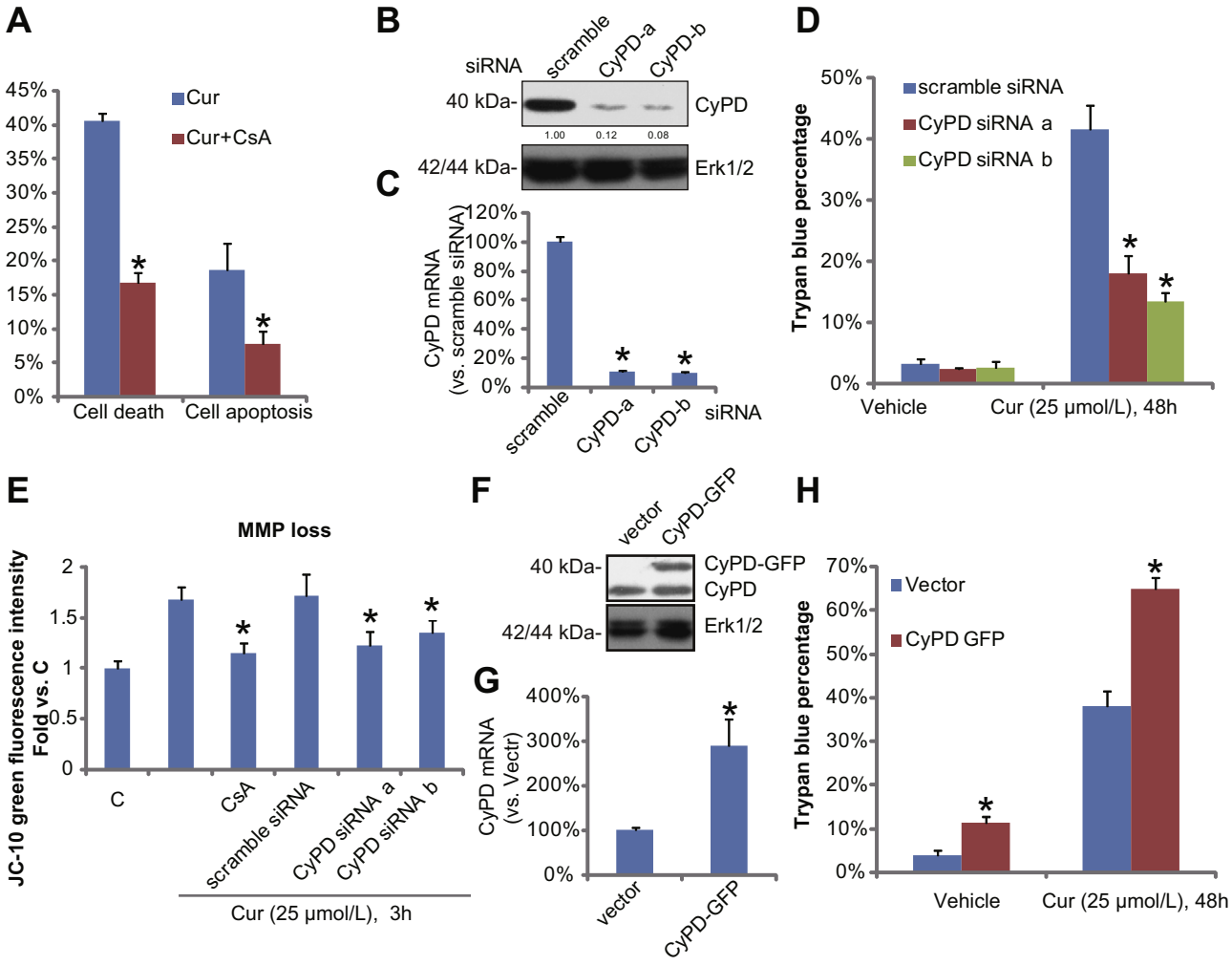

Fig. 3. Involvement of mitochondrial protein CyPD in curcumin-induced melanoma cell death. The effect of CsA (0.5 μM) on curcumin (Cur, 25 μM, 48 h)-induced cell death and apoptosis in WM-115 cells was detected by trypan blue staining and Annexin V FACS (A). The protein and mRNA expression of CyPD in WM-115 cells transfected with scramble or CyPD siRNAs (−a/−b, 200 nM each, 48 h) were shown in (B) and (C), respectively. Above cells were also treated with curcumin (Cur, 25 μM, 48 h), trypan blue staining was utilized to test cell death (D). The JC-10 green fluorescence intensity in curcumin (Cur, 25 μM, 3 h) stimulated WM-115 cells, co-treated with or without CsA (0.5 μM) or CyPD siRNAs was tested (E). The protein and mRNA expressions of CyPD in stable WM-115 cells transfected with empty vector or CyPD-GFP were shown (F and G). CyPD expression (B) was quantified. Above WM-115 cells were treated with curcumin (Cur, 25 μM, 48 h), cell death was analyzed (H). Experiments in this figure were repeated three times, and similar results were obtained. $^*p < 0.05$ vs. curcumin only group (A and E). $^*p < 0.05$ vs. scramble siRNA group (C and D). $^*p < 0.05$ vs. vector group (G, H). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

transfected with the same amount of scramble siRNA (200 nmol/L). The efficiency of siRNA was determined by Western blot and/or RT-PCR.

2.10. Cyp-D vector and transfection

The CyPD plasmid (pSuper-puromycin-GFP-CyPD) and the empty vector (pSuper-puromycin-GFP) were gifts from Dr. Bi [23]. Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA) was used to transfect Cyp-D plasmid or the empty vector (2 μg/well) into cells according the manufacturer's protocol. Stable clones were selected: the cell culture medium was replaced with fresh puromycin (0.25 μg/ml)-containing medium every 2–3 days, until resistant colonies can be seen (3 weeks). The CyPD expression was always detected by Western blots in the resistant colonies.

2.11. 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 a Student's t test. Values of $p < 0.05$ were considered as statistically different.

3. Results

3.1. Curcumin induces mPTP opening in cultured melanoma cells

We first tested the status mitochondrial membrane potential (MMP) before and after curcumin treatment in WM-115 melanoma cells. MMP was measured through JC-10 dye as reported [21]. When the membrane potential is lost, monomeric JC-10 will form in the cytosol, exhibiting green fluorescence. Thus, JC-10 green fluorescence intensity could be examined as an indicator of MMP loss. As demonstrated, the JC-10 green fluorescence intensity was significantly increased after curcumin treatment in WM-115 cells, indicating MMP loss (Fig. 1A and B). The effect of curcumin on MMP was both time- and dose- dependent (Fig. 1A and B). Meanwhile, curcumin induced CyPD and ANT-1 mitochondrial association, known as the initial step of mPTP opening (Fig. 1C) [19]. Note that the expression of major mPTP components (CyPD, VDAC and ANT-1) was not changed after curcumin stimulation

(Input) (Fig. 1C). We didn't see a direct association between ANT-1 and VDAC in our system regardless of curcumin stimulation (Fig. 1C). One of the main consequences of mPTP opening is cytochrome C release from mitochondria [24,25], we found that the level of cytosol cytochrome C increased dramatically after curcumin stimulation (Fig. 1D). These results together suggest that curcumin induces mPTP opening in cultured melanoma cells.

3.2. Opening of mPTP is required for curcumin-induced melanoma cell death

In the present study, we wanted to know the possible role of mPTP opening (see Fig. 1) in curcumin-induced cytotoxicity. Sanglifehrin A (SfA), the mPTP blocker [26] was applied here. Results in Fig. 2A showed that SfA suppressed curcumin-induced MPP loss. Further, the mito-IP data confirmed the inhibitory effect of SfA on curcumin-induced mitochondrial CyPD-ANT-1 association (Fig. 2B). These results indicated that SfA inhibited curcumin-induced mPTP opening in WM-115 cells. Significantly, SfA significantly inhibited curcumin-induced WM-115 cell death (Fig. 2C) and apoptosis (Fig. 2D), indicating that mPTP opening might be required for curcumin-induced cytotoxicity in WM-115 cells. To further support this hypothesis, data showed that WM-115 cells with ANT-1 siRNA-knockdown (Fig. 2E) were protected from curcumin (Fig. 2F), note that we utilized two non-overlapping siRNAs (−a/−b) to knock-down ANT-1 expression (Fig. 2E). We found that curcumin-induced cytochrome C release was inhibited by SfA or ANT-1-siRNAs (Fig. 2G). Together, these results suggest that opening of mPTP is required for curcumin-induced cytochrome C release, and WM-115 melanoma cell death/apoptosis.

3.3. Involvement of mitochondrial protein CyPD in curcumin-induced melanoma cell death

In light of above results, we predicted that CyPD, the key component of mPTP [27,28], should also be required for curcumin-induced melanoma cell death. Our data showed that CsA, the CyPD inhibitor [26,29], dramatically inhibited cell death and apoptosis by curcumin in WM-115 cells (Fig. 3A). Further, siRNA-knocking down of CyPD dramatically inhibited curcumin-induced increase of trypan blue staining (Fig. 3B–D). Once again, we applied two non-overlapping siRNAs against CyPD (Fig. 3B and C). Meanwhile, data in Fig. 3E demonstrated that CsA and CyPD siRNAs inhibited curcumin-induced MPP loss, indicating the role of CyPD in curcumin-induced mPTP opening. On the other hand, WM-115 cells with CyPD over-expression (Fig. 3F and G) were hypersensitive to curcumin-induced cell death (Fig. 3H). Together, these results confirmed the important role of CyPD for curcumin-induced melanoma cell death.

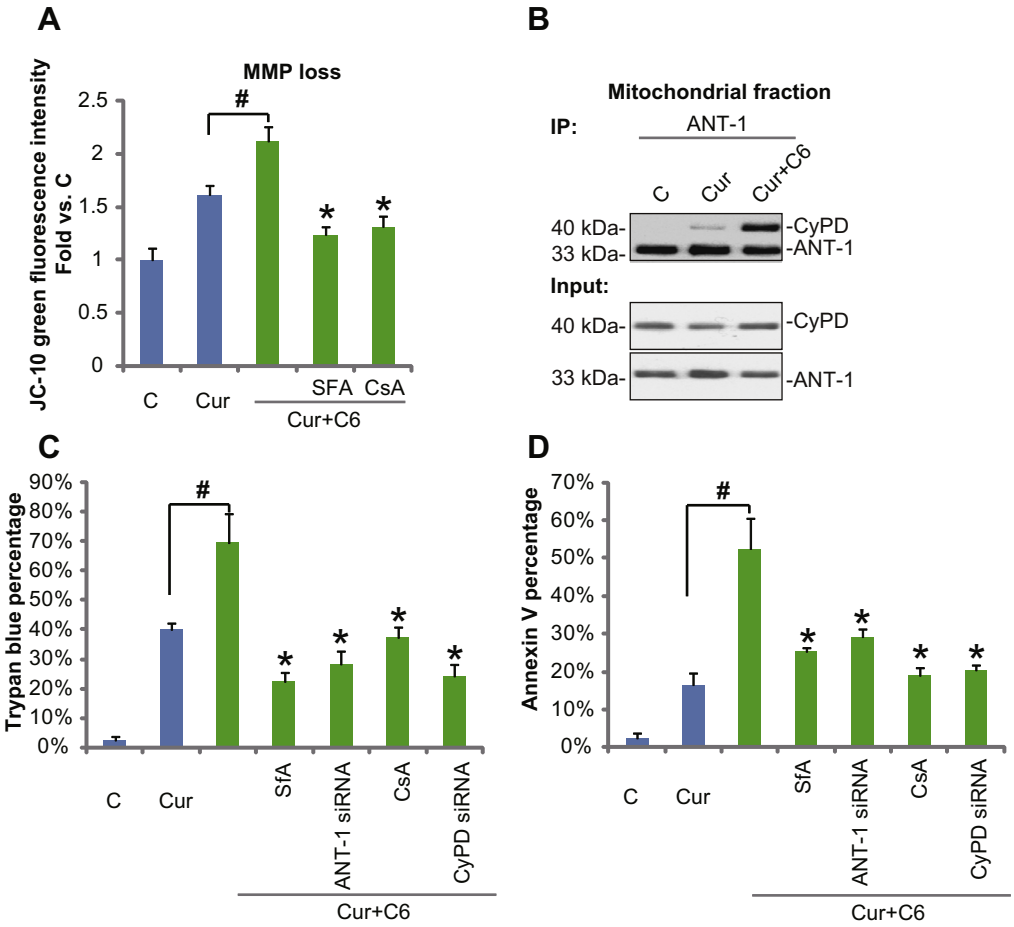

Fig. 4. C6 ceramide enhanced curcumin-induced cytotoxicity through facilitating mPTP opening . JC-10 green fluorescence intensity of WM-115 cells treated 3 h with curcumin (Cur, 25 μM), curcumin + C6 ceramide (5 μg/ml) (Cur + C6), Cur + C6 + SfA (2.5 μM) or Cur + C6 + CsA (0.5 μM) was shown (A). The mitochondrial CyPD-ANT-1 association ("IP") and expression ("input") in WM-115 cells treated 3 h with curcumin (Cur, 25 μM) or with C6 ceramide (5 μg/ml) (Cur + C6) were shown (B). WM-115 cells were treated for 48 h with curcumin (Cur, 25 μM), curcumin + C6 ceramide (5 μg/ml) (Cur + C6), Cur + C6 + SfA (2.5 μM), Cur + C6 + ANT-1-siRNA-a (see above), Cur + C6 + CsA (0.5 μM), Cur + C6 + CyPD-siRNA-a (see above), cell death and apoptosis were examined by trypan blue staining (C) and Annexin V FACS (D), respectively. Experiments in this figure were repeated three times, and similar results were obtained. $^{*}p < 0.05$ vs. Cur + C6 group (A, C and D). $^{\#}p < 0.05$ (A, C and D). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.4. C6 ceramide enhanced curcumin-induced cytotoxicity through facilitating mPTP opening

Our previous studies have shown that a cell permeable short-chain ceramide (C6 ceramide) sensitized curcumin-induced cytotoxicity in melanoma cells (also see data in Fig. 4C and D) [7,9]. Here, we tested whether mPTP opening played a role. Results in Fig. 4A demonstrated that C6 ceramide enhanced curcumin-induced MMP loss in cultured WM-115 melanoma cells. The CyPD inhibitor CsA and the mPTP inhibitor SfA dramatically inhibited mPTP loss by curcumin plus C6 ceramide co-administration (Fig. 4A). Meanwhile, C6 ceramide facilitated mitochondrial CyPD-ANT-1 association by curcumin (Fig. 4B). Significantly, SfA and CsA as well as CyPD/ANT-1 siRNA depletions alleviated curcumin plus C6 ceramide-induced cell death and apoptosis in WM-115 cells (Fig. 4C and D). Together these data indicated that C6 ceramide enhanced curcumin-induced melanoma cell death and apoptosis probably through facilitating mPTP opening.

4. Discussion

The incidence of malignant melanoma is increasing at an alarming rate worldwide [30]. Currently, there is no effective treatment for the metastatic melanoma [30]. One of the challenges in melanoma treatment is its incredible resistance to the traditional chemotherapy. Previous studies have shown that curcumin may overcome the chemo-resistance by inducing apoptosis in a p53-independent manner [5], however the underlying mechanisms are not fully understood [14]. In the present study, we found that curcumin-induced melanoma cell death was associated with mPTP opening, which was confirmed by MMP loss, mitochondrial CyPD-ANT-1 association and cytochrome C release with curcumin treatment. Thus, mPTP opening might be the key signaling hub for curcumin-induced death of melanoma cells.

CsA is known bind to CyPD and to block CyPD's peptidyl-prolyl*cis-trans*isomerase (PPIase) activity, thus shutting down the mPTP [26,31]. SfA, generally known as mPTP blocker, binds to CyPD at a different site from CsA, but more potently inhibits the PPIase activity of CyPD [26]. In the current study, we found that both CsA and SfA inhibited curcumin-induced MMP loss and melanoma cell death, suggesting that curcumin-induced cytotoxicity might require mPTP opening. To further support this theory, we found that WM-115 cells with CyPD/ANT-depletion were resistant to curcumin. Reversely, cells with CyPD over-expression were hyper-sensitive to curcumin-induced death. These results together suggest that mPTP opening is required for curcumin-induced melanoma cell death.

When using alone, a relative high dose of curcumin (30–100 μM) has to be used to efficiently kill melanoma cells. Our previous studies have found that a relatively low dose of curcumin (1–10 μM) could reach much stronger efficiency on melanoma cell death/apoptosis in the presence of C6 ceramide [7,9]. Here we found that the chemo-sensitization effect of C6 ceramide is probably due to its ability to promote mPTP opening. C6 ceramide facilitated curcumin-induced mPTP opening and following melanoma cell death. While CsA, SfA as well as CyPD/ANT-1 depletions significantly inhibited C6 ceramide's role on curcumin in WM-115 cells. Thus, we might discover the key mechanism of C6 ceramide in sensitizing curcumin's cytotoxicity.

We should point out that all the inhibitors (CsA and SfA) or siRNAs (CyPD and ANT-1) used in this study only inhibited, but not totally abolished curcumin-induced cytotoxicity. This could possibly due to these inhibitors or siRNAs didn't block mPTP opening. However, it is more likely that other signalings besides mPTP opening could also contribute to the curcumin-induced melanoma cell death. These pathways might work together, or independently, with mPTP signaling to cause melanoma cell death, which need further characterizations. Further studies will also be needed to explore the upstream signalings and downstream targets for mPTP opening in curcumin treated cells.

Conflict of interests

No conflict of interests were stated by all authors.

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