Liposomal C6 Ceramide Activates Protein Phosphatase 1 to Inhibit Melanoma Cells

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Abstract

Melanoma is one common skin cancer. In the present study, the potential anti-melanoma activity by a liposomal C6 ceramide was tested *in vitro*. We showed that the liposomal C6 (ceramide) was cytotoxic and anti-proliferative against a panel of human melanoma cell lines (SK-Mel2, WM-266.4 and A-375 and WM-115). In addition, liposomal C6 induced caspase-dependent apoptotic death in the melanoma cells. Reversely, its cytotoxicity was attenuated by several caspase inhibitors. Intriguingly, liposomal C6 was non-cytotoxic to B10BR mouse melanocytes and primary human melanocytes. Molecularly, liposomal C6 activated protein phosphatase 1 (PP1) to inactivate Akt-mammalian target of rapamycin (mTOR) signaling in melanoma cells. On the other hand, PP1 shRNA knockdown or exogenous expression of constitutively activate Akt1 (CA-Akt1) restored Akt-mTOR activation and significantly attenuated liposomal C6-mediated cytotoxicity and apoptosis in melanoma cells. Our results suggest that liposomal C6 activates PP1 to inhibit melanoma cells.

1. Introduction

Melanoma is one common skin cancer [1,2,3,4,5]. It is characterized by rapid disease progression and early invasion/metastasis to other organs [6]. It is estimated that metastatic or recurrent melanoma causes over 8000 deaths each year [5]. In addition, melanoma is resistant to almost all traditional chemotherapy agents [1,2,3,4]. Currently, dacarbazine and temozolomide (TMZ) are routinely prescribed for melanoma chemotherapy. Yet, the response rate is often less 15–20% [1,2,3,4]. Therefore, it is urgent to explore novel and more potent anti-melanoma agents.

Ceramides are a family of lipid molecules that are enriched within cell membranes [7,8]. Ceramides could also function as active signaling molecules [7,8]. Among all the ceramides, the short-chain cell permeable ceramides (C2, C4, C6 and C8) have displayed promising anti-tumor activity, either alone or in combination with traditional anti-cancer agents (reviewed in [9,10,11,12]). C6 ceramide has been tested in melanoma cells, and showed decent *in vitro*

cytotoxicity [13]. Yet, the systematic use of the short-chain ceramides is extremely limited due to their poor solubility [14]. Therefore, liposome-based nanotechnology delivery systems have been developed to assist ceramide delivery *in vivo* [14,15,16,17,18]. In the current study, we investigated the potential anti-melanoma activity by a liposomal C6 ceramide [14,18]. The underlying mechanisms were also analyzed.

2. Materials and Methods

2.1. Chemicals and reagents

Liposomal C6 (ceramide), liposome ghost vehicle and free C6 (ceramide) were provided by Bo Zhang's Lab at Tianjin Medical University [19]. The caspase-3 specific inhibitor Ac-DEVD-CHO, the caspae-9 specific inhibitor Ac-LEHD-CHO and the pan caspase inhibitor Ac-VAD-CHO were purchased from Peptide Institute (Osaka, Japan). Antibodies of PP1α/β/λ were obtained from Santa Cruz Biotech (Santa Cruz, CA). All other antibodies utilized in the study were purchased form Cell Signaling Tech (Denver, MA). Cell culture reagents were provided by Calbiochem (Shanghai, China).

2.2. Cell culture

Melanoma cell lines SK-Mel2, WM-266.4, A-375 and WM-115 were obtained from American Type Culture Collection (ATCC, Manassas, VA). Melanoma cells were maintained in RPMI medium, supplemented with 10% heat-inactive fetal bovine serum (FBS), 1% penicillin–streptomycin and 4 mmol/L L-glutamine, in a CO_2 incubator at 37°C. B10BR mouse melanocytes, obtained from Shanghai Biological Institute (Shanghai, China), were cultured in Hams F12 supplement with 10 heat-inactivated calf serum (FCS, Gibco), 50 ng/mL phorbol 12-myristate 13-acetate (TPA, Sigma) and 1% penicillin–streptomycin. Primary human melanocytes from neonatal foreskin (Cascade Biologics/Life Technologies, Shanghai, China) were cultured in Medium 254 and Human Melanocyte Growth Supplement (HMGS2; Cascade Biologics/Life Technologies) and propagated for ≤15 population doublings.

2.3. Cell survival MTT assay

Standard MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Sigma) assay was performed in 96-well plates as described previously [18]. Optic density (OD) value at 570 nm of treatment group was normalized as percentage change of that of untreated control group [20].

2.4. Caspase-3/-9 activity assay

Following treatment, melanoma cells were examined for the caspase-3/-9 activity via caspase-3/-9 activity kit (Clontech Corporation, Carlsbad, CA) with the manufacturer's protocol [21]. Briefly, 200 μg of the protein lysates were incubated with 200 μM of the caspase-3 substrate DEVD-pNA or 200 μM of the caspase-9 substrate Ac-LEHD-pNA at 37°C for 2 hours. Absorbance of pNA was detected at 405 nm with a microplate reader (PerSeptive Biosystems, Framingham, MA).

2.5. Annexin V detection of apoptosis

Cell apoptosis was detected by the Annexin V Apoptosis Detection Kit (BD Biosciences, Shanghai, China). Briefly, after treatment, melanoma cells were resuspended in 1×Binding buffer, Annexin V-FITC and propidium iodide (PI) (5 μg/ml each). After 15 min incubation, cells were acquired by flow cytometry. Both early apoptotic cells (Annexin V positive, PI

negative) and late apoptotic cells (Annexin V positive, PI positive) were detected by FACScan, and subsequently analyzed by CellQuest software. Annexin V percentage was recorded.

2.6. Single-stranded DNA (ssDNA) ELISA assay of apoptosis

In the process of apoptosis, DNA denature is a characteristic marker. In the present study, denatured ssDNA was detected via a nucleosomal monoclonal antibody in an ELISA format. Detailed protocol was described in other studies [19,21,22,23]. Briefly, melanoma cells (2.5 $\times 10^4$/well) were seeded onto 96-well plates. After applied treatment, cell apoptosis was analyzed via the ssDNA ELISA kit (Chemicon, Shanghai, China) according to the attached protocol. The OD value was utilized as a quantitative indicator of cell apoptosis.

2.7. Western blots

Cells were washed and incubated in cell lysis buffer [20]. Protein samples were separated by SDS-PAGE gel and electro-transferred to PVDF membranes (Bio-Rad), followed by incubation with primary antibodies [18]. Protein bands were visualized using horseradish peroxidase (HRP)-conjugated secondary antibodies (Santa Cruz), and by the enhanced chemiluminescence (ECL) reagents [19]. The x-ray films were scanned, acquired in Adobe Photoshop, and analyzed with NIH Image J software.

2.8. Protein phosphatase activity assay

Protein phosphatase activity was determined with the [^{32}P] phosphorylase a protocol as previously described [24]. The assay was performed in a 50-μl aliquot that consisted of 50 mM Tris HCl (pH 7.4), 5 mM caffeine, 0.5 mM EGTA, 0.5 mM EDTA, 50 μM β-mercaptoethanol, and 100 ng of aprotinin (protease inhibitor) with or without 2 μg of protein lysates and 500 pmol [^{32}P] phosphorylase a [24]. The assay was initiated by adding the cell lysates and was incubated at 30°C for 5 min. Incubation was rapidly stopped by addition of 30 μl of 60% TCA and 20 μl of BSA (50 mg/ml). Tubes were held in ice for 10 min and then centrifuged at 12,000 g for 5 min. After centrifugation, ^{32}P radioactivity was counted in 80 μl of clear supernatant in 7 ml of liquid scintillation fluid. protein phosphatase activity was calculated through the same protocol as described [24]. The protein phosphatase activity of liposomal C6 treatment group was normalized to that of untreated control group.

2.9. PP1 shRNA knockdown

The pan PP1 shRNA (sc-43545-SH, Santa Cruz) and scramble control shRNA were purchased from Santa Cruz Biotech (Shanghai, China). The PP1 shRNA sequence was described in the previous study [25]. For shRNA transfection, melanoma cells were seeded at 50% confluence. The shRNA vector was introduced by Lipofectamine 2000 (Invitrogen, Carlsbad, CA), according to the manufacturer's protocol. The stable cells expressing PP1 shRNA were selected by puromycin (2.5 μg/ml) for 2–3 weeks. Western blot assay was always performed to test PP1α expression in stable cells.

2.10. Constitutively active-Akt1 (CA-Akt1) transfection and stable cells selection

The constitutively active mutant of Akt1 (CA-Akt1) cDNA sequence was provided by Dr. Teng's group at Jining Medical University [26,27]. CA-Akt1 was inserted into the pSuper-puro-GFP vector and was transfected via Lipofectamine 2000 reagent (Invitrogen), according

to the manufacturer's protocol. The stable cells were selected by puromycin (2.5 µg/ml) for 2–3 weeks. Western blot assay was performed to test CA-Akt1 in stable cells.

2.11. Statistical analysis

The values in the figures were expressed as the means ± standard deviation (SD). Statistical analysis of the data was performed by ANOVA. Values of $p < 0.05$ were considered as statistically different.

3. Results

3.1. Liposomal C6 inhibits melanoma cell survival and proliferation

Cultured WM-115 human melanoma cells were treated with the liposomal C6. MTT assay results in Fig 1A demonstrated that liposomal C6 (at 5–25 µM) dramatically inhibited WM-115 cell survival. Liposomal C6 (10 µM) exhibited a time-dependent effect, the viability OD started to decrease 48 hours after liposomal C6 treatment (Fig 1B). Meanwhile, liposomal C6
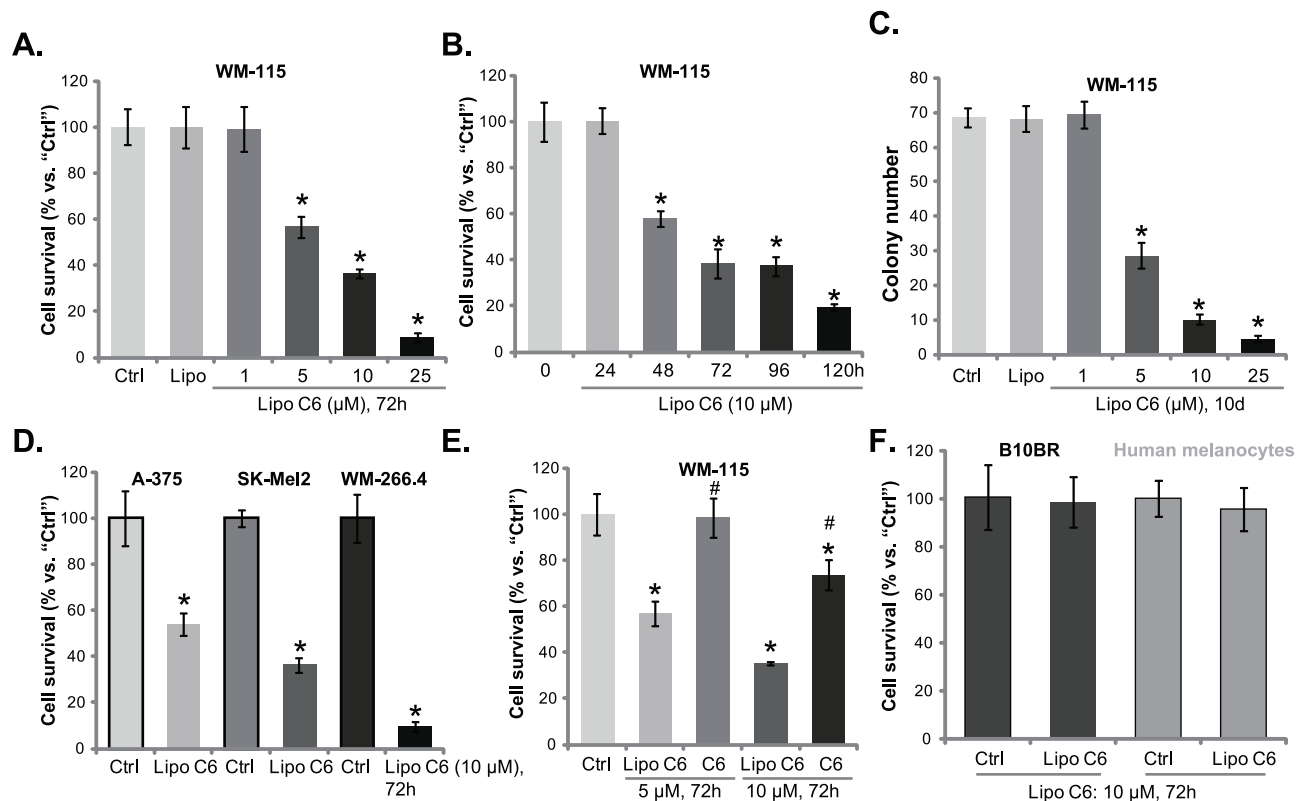

Fig 1. Liposomal C6 inhibits melanoma cell survival and proliferation. Cultured melanoma cell lines (WM-115, SK-Mel2, WM-266.4 and A-375), B10BR mouse melanocytes or primary human melanocytes were either left untreated ("Ctrl"), treated with applied concentrations of liposomal C6 ceramide ("Lipo C6", A-F), liposomal ghost vehicle ("Lipo", for A, C), or free C6 ceramide ("C6", for E), cells were further cultured for applied time, cell survival was tested by MTT assay (A, B, D-F), and cell proliferation was evaluated by colony formation assay (C, for WM-115 cells). Experiments were repeated four times, and similar results were obtained. Data were presented as mean ± SD. * p<0.05 vs. "Ctrl" group. # p<0.05 vs. "Lipo C6" only group (E).

treatment dose-dependently inhibited the number of WM-115 colonies (Fig 1C), suggesting its anti-proliferative activity. Note that liposomal ghost ("Lipo") showed almost no effect on melanoma cell survival nor proliferation (Fig 1A and 1C).

The potential effect of liposomal C6 on other melanoma cell lines was also analyzed. Three other established melanoma cell lines (SK-Mel2, WM-266.4 and A-375) were cultured and treated with liposomal C6 (10 μM). MTT assay results in Fig 1D showed that liposomal C6 was cytotoxic against all these melanoma cell lines. To compare the efficiency between regular free C6 and liposomal C6, WM-115 cells were treated with same concentration (10 μM) of free C6 or liposomal C6, MTT results showed that liposome-packed C6 was significantly more potent than free C6 in suppressing WM-115 cells (Fig 1E). Same results were also obtained in other tested melanoma cell lines (Data not shown). The potential activity of liposomal C6 on normal melanocytes (non-cancerous cells) was tested. MTT results in Fig 1F showed that liposomal C6 (10 μM) failed to inhibit survival of B10BR mouse melanocytes and primary human melanocytes, implying its selective cytotoxicity to cancer cells. Collectively, these results indicate that liposomal C6 exerts cytotoxic and anti-proliferative activity against cultured human melanoma cells.

3.2. Liposomal C6 activates apoptosis in melanoma cells

Next, we studied the potential effect of liposomal C6 on cell apoptosis. WM-115 cells were treated with indicated concentration of liposomal C6. Results in Fig 2A and 2B showed that liposomal C6 dose-dependently increased activity of caspse-3 and caspase-9 in WM-115 cells. In addition, liposomal C6 (5–25 μM) significantly increased Annexin V percentage (Fig 2C) and ssDNA ELISA OD (Fig 2D). All these results indicated apoptosis activation by liposomal C6 in WM-115 cells (Fig 2A–2D). To study the role of apoptosis in liposomal C6-induced melanoma cytotoxicity, three caspase-based apoptosis inhibitors were applied. Results showed that the caspase-3 specific inhibitor Ac-DEVD-CHO, the caspse-9 specific inhibitor Ac-LEHD-CHO and the pan caspase inhibitor Ac-VAD-CHO dramatically inhibited liposomal C6 (10 μM)-induced WM-115 cell viability reduction (Fig 2E). ssDNA apoptosis ELISA results in Fig 2F confirmed significant apoptosis activation in three other melanoma cell lines after liposomal C6 (10 μM) treatment. Once again, liposomal C6 was more potent than free C6 in inducing apoptosis in WM-115 cells (Fig 2G). Notably, ssDNA ELISA assay results in Fig 2H demonstrated that liposomal C6 failed to induce significant apoptosis in B10BR mouse melanocytes and primary human melanocytes. These results against confirmed its selective activity in cancerous cells. Collectively, liposomal C6 induces caspase-dependent apoptotic death in melanoma cells.

3.3. Liposomal C6 activates protein phosphatase, and inhibits Akt-mTOR signaling in melanoma cells

Previous studies have shown that short-chain ceramides could activate the protein phosphatase 1 (PP1) [28,29] and de-phosphorylates Akt to exert cytotoxic or anti-proliferative activity [30]. We thus analyzed protein phosphatase activity in liposomal C6-treated melanoma cells using the method described [24]. Results demonstrated that liposomal C6 dose-dependently increased protein phosphatase activity in both WM-115 (Fig 3A) and A-375 melanoma cells (Fig 3B). As a result, Akt activation was largely inhibited (Fig 3C and 3E). In addition, pP70S6K1, the indicator of mammalian targeted of rapamycin (mTOR) activation, was also inhibited (Fig 3C and 3E). Akt and P70S6K1 phosphorylations in WM-115 and A375 cells were quantified (Fig 3D and 3F). Since, Akt-mTOR activation plays a vital role in melanoma cell survival and
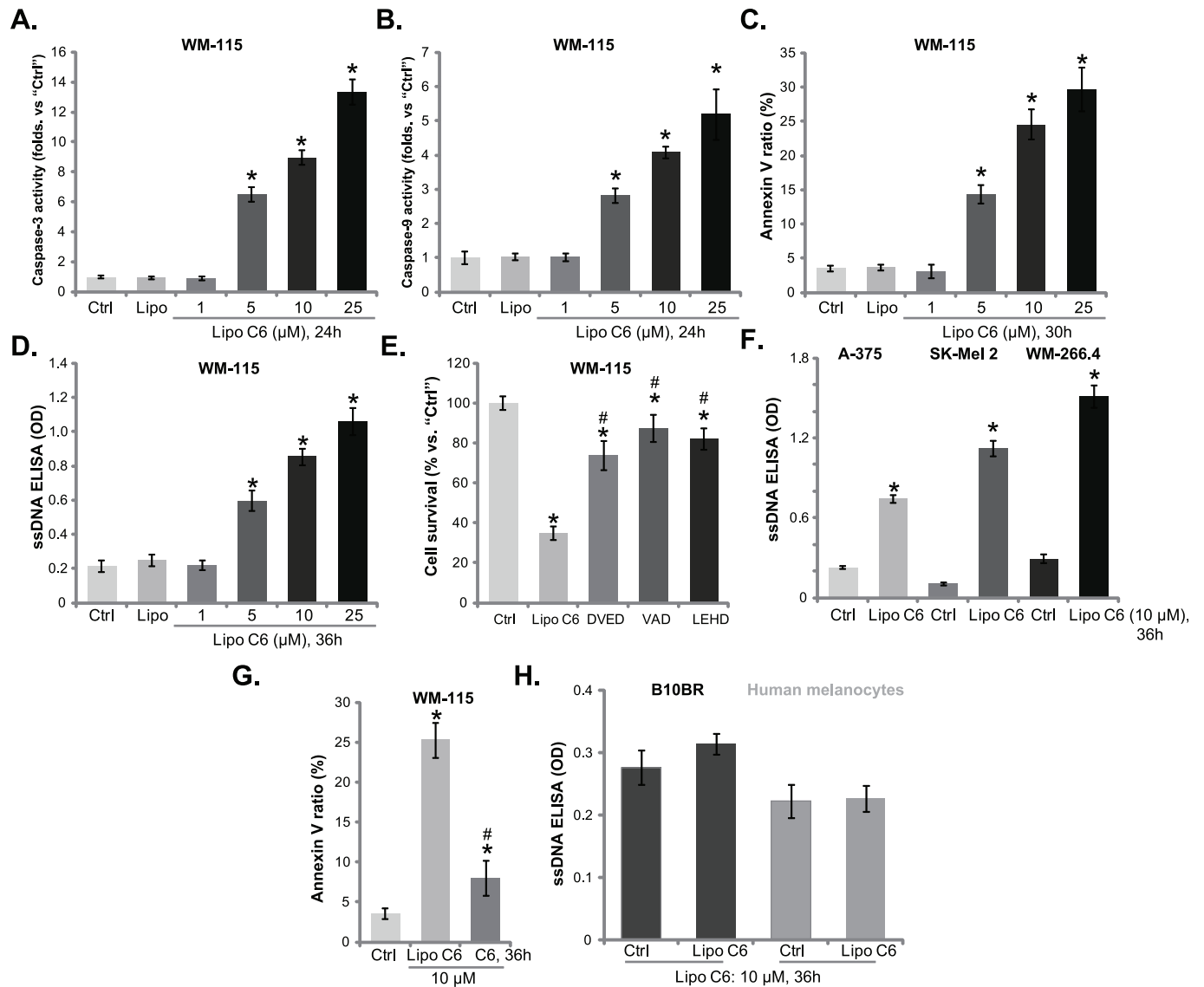
Fig 2. Liposomal C6 activates apoptosis in melanoma cells. Melanoma cell lines (WM-115, SK-Mel2, WM-266.4 and A-375), B10BR mouse melanocytes or primary human melanocytes were either left untreated ("Ctrl"), treated with applied concentration of liposomal C6 ceramide ("Lipo C6", A-D, F-H), liposomal ghost vehicle ("Lipo", for A-D), or free C6 ceramide ("C6", for G), cells were further cultured for applied time, cell apoptosis was analyzed by listed assays (A-D, F-H). WM-115 cells, pre-treated with Ac-DVED-CHO ("DVED", the caspase-3 inhibitor), Ac-LEHD-CHO ("LEHD", the caspase-9 inhibitor) or Ac-VAD-CHO ("VAD", the pan-caspase inhibitor) (50 μM each, 1 hour), were treated with liposomal C6 (10 μM), cells were further cultured for 72 hours, cell survival (E, MTT assay) was analyzed. Experiments were repeated four times, and similar results were obtained. Data were presented as mean ± SD. * $p < 0.05$ vs. "Ctrl" group. # $p < 0.05$ vs. "Lipo C6" only group (E and G).

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proliferation [31], our results suggest that liposomal C6 activates protein phosphatase to inhibit Akt-mTOR signaling and melanoma cell proliferation.

3.4. Activation of PP1 is required for liposomal C6-induced anti-melanoma cell activity

To further support a role of PP1-Akt signaling in liposomal C6-induced anti-melanoma cell activity, we utilized shRNA method to knockdown PP1, and stable WM-115 cells expressing PP1 shRNA were selected. Western blot results in Fig 4A showed that the pan PP1 shRNA
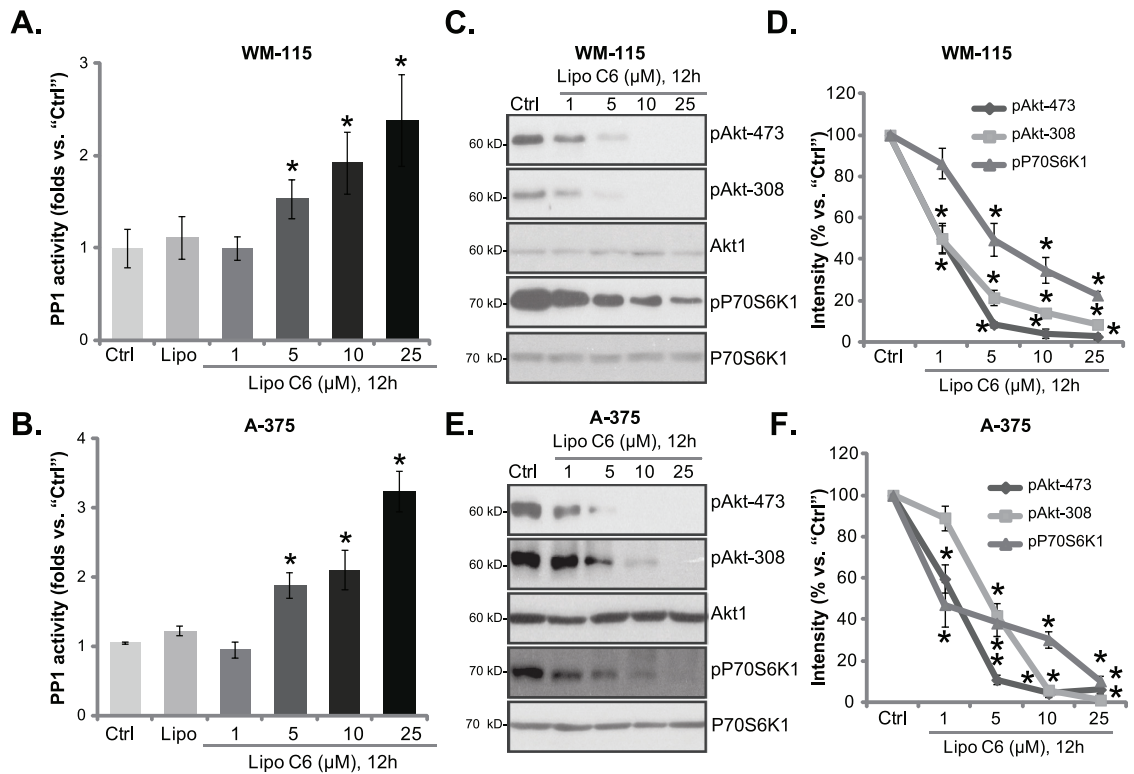
Fig 3. Liposomal C6 activates protein phosphatase, and inhibits Akt-mTOR signaling in melanoma cells. Cultured melanoma cell lines (WM-115 and A-375) were either left untreated ("Ctrl"), treated with applied concentration of liposomal C6 ceramide ("Lipo C6") or liposomal ghost ("Lipo"), cells were further cultured for applied time, protein phosphatase activity (A and B) and expression of listed kinases (C and E) were tested. Akt and P70S6K1 phosphorylations were quantified (D and F). Experiments were repeated three times, and similar results were obtained. Data were presented as mean ± SD. * $p < 0.05$ vs. "Ctrl" group.

significantly downregulated PP1α/β/λ expression in stable WM-115 cells. Consequently, liposomal C6-induced Akt-mTOR inhibition was almost reversed (Fig 4A, also see quantification results in the panel). Significantly, protein phosphatase activity increase by liposome C6 was compromised in PP1-silenced WM-115 cells (Fig 4B). Meanwhile, liposomal C6-induced cytotoxicity (Fig 4C) and apoptosis (Fig 4D) were attenuated in PP1 shRNA-expressing WM-115 cells. These results suggest that liposomal C6 activates PP1 to inhibit Akt-mTOR, causing melanoma cell growth inhibition and apoptosis.

We next introduced a constitutively-active (CA) Akt1 [13] to WM-115 cells. Western blot results in Fig 4A confirmed CA-Akt1 expression (high Akt1 expression, "white star") in WM-115 cells. CA-Akt1 restored Akt-mTOR activation in liposomal C6-treated WM-115 cells (Fig 4A). More importantly, CA-Akt1-expressing WW-115 cells were resistant to liposomal C6, presenting with significantly reduced cell death (Fig 4C) and apoptosis (Fig 4D). Together, these results indicate that PP1-Akt signaling is required for liposomal C6-induced anti-melanoma cell activity *in vitro*.

4. Discussion

Despite the promising anti-cancer activity by the short-chain ceramides [7,10,32], the process of developing these compounds as active pharmaceutical agents has been hampered due to their insolubility [14]. Therefore, liposome-based nanotechnology delivery systems have been
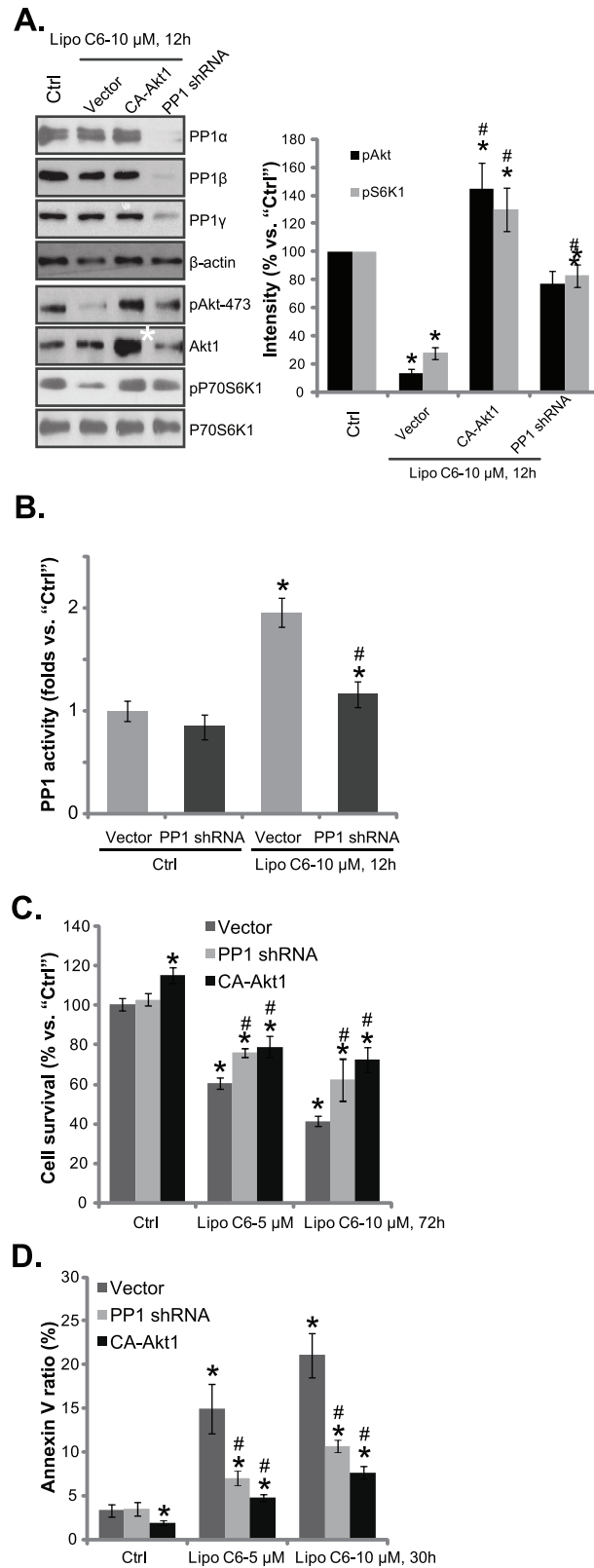
Fig 4. Activation of PP1 is required for liposomal C6-induced anti-melanoma cell activity *in vitro*.
Stable WM-115 cells expressing the pan PP1 shRNA, constitutively-activate mutant Akt1 ("CA-Akt1"), or
empty vector ("pSuper-puro") were treated with or without applied concentration of liposomal C6 ceramide

("Lipo C6"), expression of listed proteins was tested by Western blots (A); Relative protein phosphatase activity was shown (B); Cell survival (C), and cell apoptosis (D) were tested by MTT assay and Annexin V assay, respectively. Experiments were repeated three times, and similar results were obtained. Data were presented as mean ± SD. *$p<0.05$ vs. untreated "Ctrl" group. # $p<0.05$ vs. "Lipo C6" of Vector group.

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developed to assist ceramide delivery *in vivo* [19,33,34,35]. It has been shown that system delivery of liposomal C6 could offer rapid tissue distribution without causing apparent toxicities [15]. In addition, liposomal C6 showed a selective response to cancerous cells [15,18,19]. Recent studies have also concluded that experimental mice were well-tolerated to the liposomal C6 systematic administration [18,19]. In the current study, our *in vitro* studies showed that liposomal C6 (ceramide) exerted potent anti-proliferative and pro-apoptotic activities against a panel of human melanoma cell lines (SK-Mel2, WM-266.4, A-375 and WM-115). Its efficiency was better than free C6 ceramide. Intriguingly, liposomal C6 was non-cytotoxic to B10BR mouse melanocytes and primary human melanocytes.

At the molecular level, we showed that shRNA knockdown of PP1 or introduction of CA-Akt1 alleviated liposomal C6-mediated anti-melanoma activity. These results indicate that PP1-mediated Akt-mTOR inactivation mediated, at least in part, liposomal C6's cytotoxicity in melanoma cells. However, it should be noted that PP1 shRNA or CA-Akt1 didn't completely block liposomal C6' cytotoxicity, indicating that other mechanisms besides the PP1-Akt signaling may also contribute to its actions. As a matter of fact, studies have identified other signaling mechanisms by (liposomal) C6 in various cancer cells, including JNK activation [36], AMP activated protein kinase (AMPK) activation [18,37,38,39,40], growth factor receptor degradation [40] and many others. A recent study by Zhang et al., showed that acute treatment (30 min) of liposomal C6 inhibited melanoma cell migration via phosphorylation of PI3K and PKCζ [41]. Reversely, knockdown or pharmacological inhibition of PKCζ or PI3K restored cancer cell migration following liposomal C6 treatment [41]. It will be interesting to test these signalings in liposomal C6-treated melanoma cells as well.

5. Conclusions

Metastatic and recurrent melanoma is still a great challenge to treat [6,42,43]. Stage III melanoma patients are often treated adjuvantly with interferon (IFN)-α, yet its response is far from satisfactory. The metastatic melanoma patients (stage IV) have a median survival of 6–10 months even with current treatments, and the 5-year survival is less than 5% [6,42,43]. Therefore, alternative treatment agents are urgently needed [6,42,43]. Our results show that liposomal C6 potently inhibits melanoma cells *in vitro*. Therefore, the liposomal C6 could be further studied for possible treatment of melanoma.

Acknowledgments

Author Contributions

Conceived and designed the experiments: FJ KJ JY XH.

Performed the experiments: FJ KJ SH QB ZS JY XH.

Analyzed the data: FJ KJ SH JY XH.

Contributed reagents/materials/analysis tools: FJ KJ JY XH.

Wrote the paper: FJ KJ SH QB ZS JY XH.

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