Cancer Chemother Pharmacol (2010) 66:999–1003
DOI 10.1007/s00280-010-1374-1

SHORT COMMUNICATION

C6 ceramide potentiates curcumin-induced cell death and apoptosis in melanoma cell lines in vitro

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Received: 29 March 2010 / Accepted: 18 May 2010 / Published online: 3 June 2010

Abstract
Purpose The majority of metastatic melanomas are resistant to diverse chemotherapeutic agents, and long-term survival for patients with melanoma who have metastatic disease is dismal. Consequently, the search for novel anti-melanoma agents is urgent. Here, we evaluate the potential effects of C6 ceramide to sensitize melanoma cell lines (B16 and WM-115 cells) to curcumin-induced cell death.
Methods MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was used to test melanoma cell viability in vitro. Hoechst 33342 fluorescence and Histone DNA ELISA was used to evaluate melanoma cell apoptosis. Apoptosis-associated proteins in melanoma cells after treatments were measured by Western blot.
Results C6 ceramide promotes curcumin-induced cell death and apoptosis in B16 and WM-115 melanoma cell lines. Curcumin itself promotes pro-apoptosis protein Caspase 3 and Caspase 9 cleavage and anti-apoptosis protein Bcl-XL and X-IAP degradation, and combination of C6 ceramide with curcumin dramatically enhances it. Caspase inhibitors largely inhibit C6-ceramide plus curcumin induced cell death and apoptosis.
Conclusion We suggest that C6 ceramide sensitizes melanoma cell to curcumin induced cell death and apoptosis in vitro, which is due to, at least in part, the augment of mitochondria apoptosis pathway. Combining C6 ceramide with traditional chemotherapy drugs such as curcumin may have potential to be used as a new therapeutic intervention against melanoma.

Keywords Curcumin · C6 ceramide · Apoptosis · Chemotherapy · Melanoma

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Introduction

Melanoma is a cutaneous tumor characterized by abnormal proliferation of melanocytes that invade the basement membrane. The majority of metastatic melanomas are resistant to diverse chemotherapeutic agents, and long-term survival for patients with melanoma who have metastatic disease is dismal [2, 3]. Consequently, the search for novel anti-melanoma agents is urgent.

Curcumin [1,7-bis(4-hydroxy-3methoxyphenyl)-1,6-heptadien-3,5-dione] is the primary bioactive component isolated from turmeric, a dietary spice made from the rhizome of Curcuma longa. Curcumin has chemotherapeutic properties in numerous experimental models and has been shown to inhibit a variety of cellular targets that promote survival and metastasis. Previous studies have shown some effects of curcumin on highly metastatic melanoma cells; however, the application of curcumin as chemotherapy drugs against melanoma is limited as a result of failure to response or chemo-resistance [4, 9, 14].

Cell-permeable ceramides (C^2 or C^6 ceramide) have shown cytotoxic effects against a variety of cancer cell lines (reviewed in [12]) including murine and human melanoma

and soft tissue sarcoma [1] as well as Jurkat leukemia and head and neck squamous cancer [10]. Recent studies, however, also indicate the potential effects of C6 ceramide to sensitize multiple cell lines to chemotherapy drugs (Taxol for example)—induced cytotoxic effects [7, 11].

Here, we investigate the potential effect of cell permeable ceramides (C6 ceramide) to augment the antitumor effect of known chemotherapeutic agent curcumin in melanoma cell lines [11]. We demonstrate that C6 ceramide, which alone has limited effects on melanoma cell death and apoptosis, radically sensitizes the melanoma cell lines to curcumin-mediated cell death and apoptosis effects in vitro.

Materials and methods

Chemicals and reagents

C6 ceramide was from Avanti (Alabaster, AB), and Paclitaxel, X-IAP, Bcl-XL, ERK1/2, goat anti-rabbit IgG-HRP and goat anti-mouse IgG-HRP antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal mouse anti-β-actin was obtained from Sigma (St. Louis, MO). Cleaved-Caspase 3 and cleaved-Caspase 9 antibody were purchased from Cell Signaling Technology (Bevery, MA).

Cell culture

Melanoma cell lines B16 and WM-115 (Purchased from Shanghai Maisha Biological Technology Co., Ltd) were maintained in a DMEM medium (Sigma, St. Louis, MO), supplemented with a 10% FBS (Sigma, St. Louis, MO), Penicillin/Streptomycin (1:100, Sigma, St. Louis, MO) and 4 mM L-glutamine (Sigma, St. Louis, MO), in a CO_2 incubator at 37°C.

Western blot

Western blot was measured as described in [6].

Cell viability assay (MTT dye assay)

Cell viability was measured by MTT method as described [6].

Assessment of the percentage of apoptotic cells

To detect apoptotic cells, cells were stained with DNA-binding dye Hoechst 33342 (Sigma, St. Louis, MO). Cells with indicated treatment were fixed with 4% formaldehyde in phosphate buffered saline (PBS) for 10 min at 4°C and then washed with PBS. Cells were incubated for 15 min with 5 μg/ml of Hoechst 33342 (Sigma, St. Louis, MO) to stain the nuclei. After washing with PBS, the apoptotic cells were observed under a confocal fluorescence microscope (Leica TCS SMD FCS, Leica, Germany). Cells exhibiting condensed chromatin and fragmented nuclei (Hoechst 33342 stain, Blue) were scored as apoptotic cells. For each Hoechst experiment, at least 200 cells in 10 random scope fields were counted for apoptotic rate.

Quantification of apoptosis by ELISA

The Cell Apoptosis ELISA Detection Kit (Roche, Palo Alto, CA) was also used to detect apoptosis. After indicated treatments, the cytoplasmic histone/DNA fragments from cells were extracted and bound to immobilized anti-histone antibody. Subsequently, the peroxidase-conjugated anti-DNA antibody was used for the detection of immobilized histone/DNA fragments. After addition of substrate for peroxidase, the spectrophotometric absorbance of the samples was determined by using Dynatech MR5000 plate reader at 405 nM.

Statistical analysis

The values in the figures are expressed as the means ± standard deviation (SD). Statistical analysis of the data between the control and treated groups was performed by a student t test. Values of $P < 0.05$ were considered as statistically significant.

Results

C6 ceramide enhances curcumin-induced melanoma cell death and apoptosis in vitro

First, we tested the potential effects of C6 ceramide on curcumin-induced cell death by using MTT assay (Melanoma cell death is indicated as reduced cell viability). As shown in Fig. 1a and b, C6 ceramide significantly enhanced curcumin induced cell death in B16 cells. Notably, when B16 cells were cultured 10% FBS, either C6 ceramide or curcumin alone had little effects on tumor cell death; however, combination of these two drugs largely induced B16 (Fig. 1c). Similar results were also seen in WM-115 cells (Data not shown). We also measured the possible effects of C6 ceramide on other chemotherapy drugs. C6 ceramide largely enhanced Taxol-induced cell death in B16 cells (Fig. 1d). However, the chemo-sensitization effects of C6 ceramide appear chemotherapy drugs dependent, since C6 ceramide had almost no effects on Cis-platin-induced cell death in B16 cells (Fig. 1e). Similar results were also seen in WM-115 cells (Data not shown). Next, we tested the effects of C6 ceramide on curcumin-induced melanoma cell apoptosis. C6 ceramide largely enhanced curcumin-induced cell apoptosis in both B16

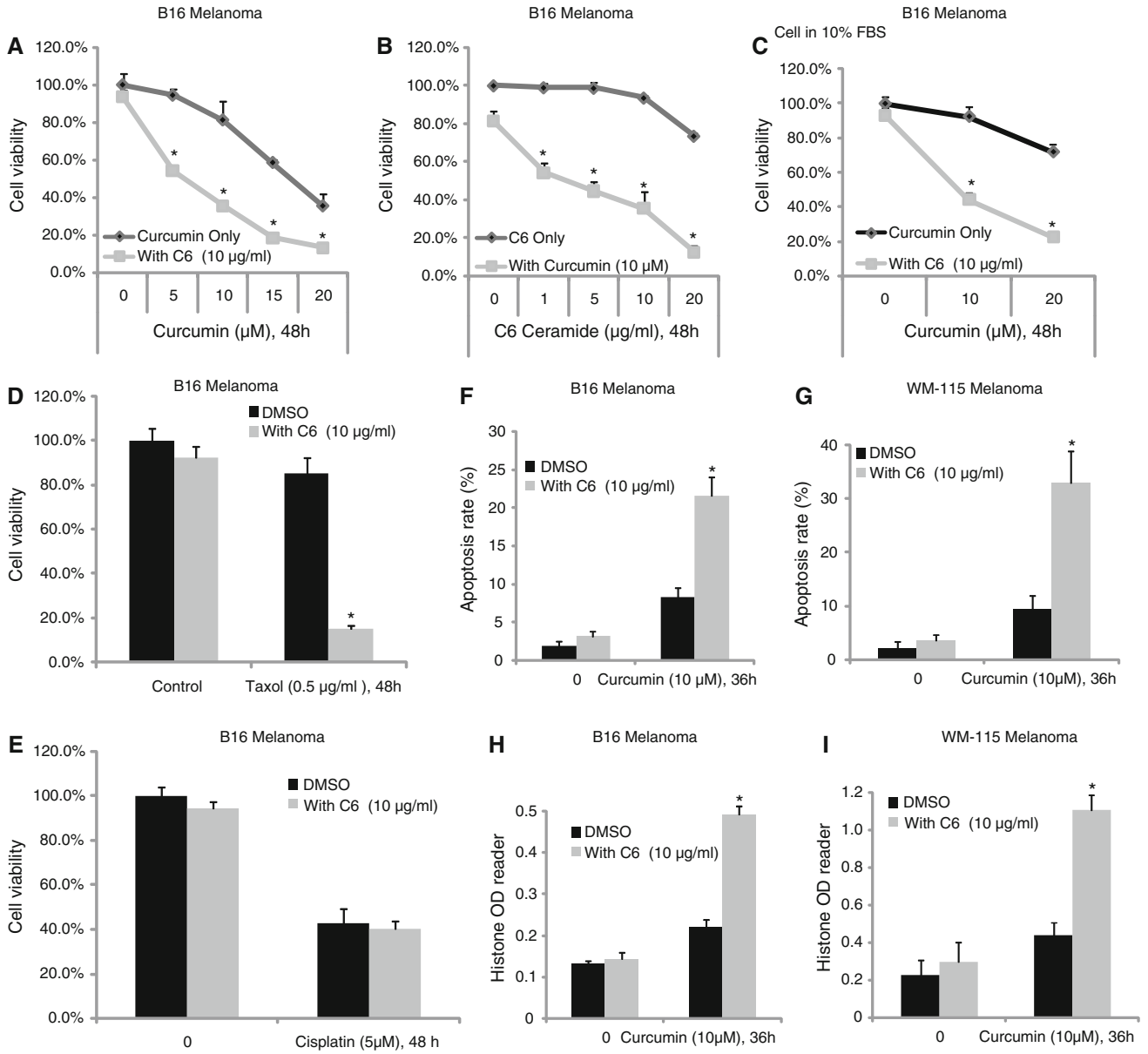

Fig. 1 C6 ceramide enhances curcumin- and Taxol-induced melanoma cell death in vitro. Melanoma cell lines B16 (**a**–**c**) were treated with combination of curcumin (at a different concentration) and C6 ceramide (at a different concentration) in the presence or absence of 10% FBS for 48 h, and cell viability was detected by MTT assay. B16 cells were treated with C6 ceramide (10 μg/ml) with Taxol (0.5 μg/ml) or Cis-platin (5 μM) for 48 h, and cell viability was detected by MTT assay (**d**, **e**). The apoptosis of B16 and WM-115 cells with indicated treatment (Control, 10 μM of curcumin, 10 μg/ml of C6 Ceramide and curcumin plus C6 ceramide) for 36 h was detected by Hoechst 33342 assay (**f**, **g**) and Histone DNA apoptosis ELISA assay (**h**, **i**). The values in the figures are expressed as the means ± SD. $^*P < 0.05$ vs. group without C6 ceramide treatment. All experiments were repeated at least three times, and similar results were obtained

(Fig. 1f, h) and WM-115 (Fig. 1g, i) cells as evidenced by Hoechst 33342 assay and Histone DNA ELISA assay.

C6 ceramide enhances curcumin-induced mitochondria apoptosis pathway

Mitochondria apoptosis pathways were detected in curcumin plus C6 ceramide–treated melanoma cell lines in vitro. As shown in Fig. 2c and d, C6 ceramide treatment largely enhanced pro-apoptosis protein Caspase 3 and 9 cleavage (activation) in both B16 and WM-115 cells, while the expression of anti-apoptosis proteins including Bcl-XL and X-IAP was down-regulated (Fig. 2a and b). These results indicate that C6 ceramide facilitates melanoma cells to curcumin-induced mitochondria apoptosis pathway. We next investigated the possible effects of Caspase inhibitors on curcumin plus C6 ceramide–induced melanoma cell death and apoptosis. As shown in Fig. 2e–h, general Caspase

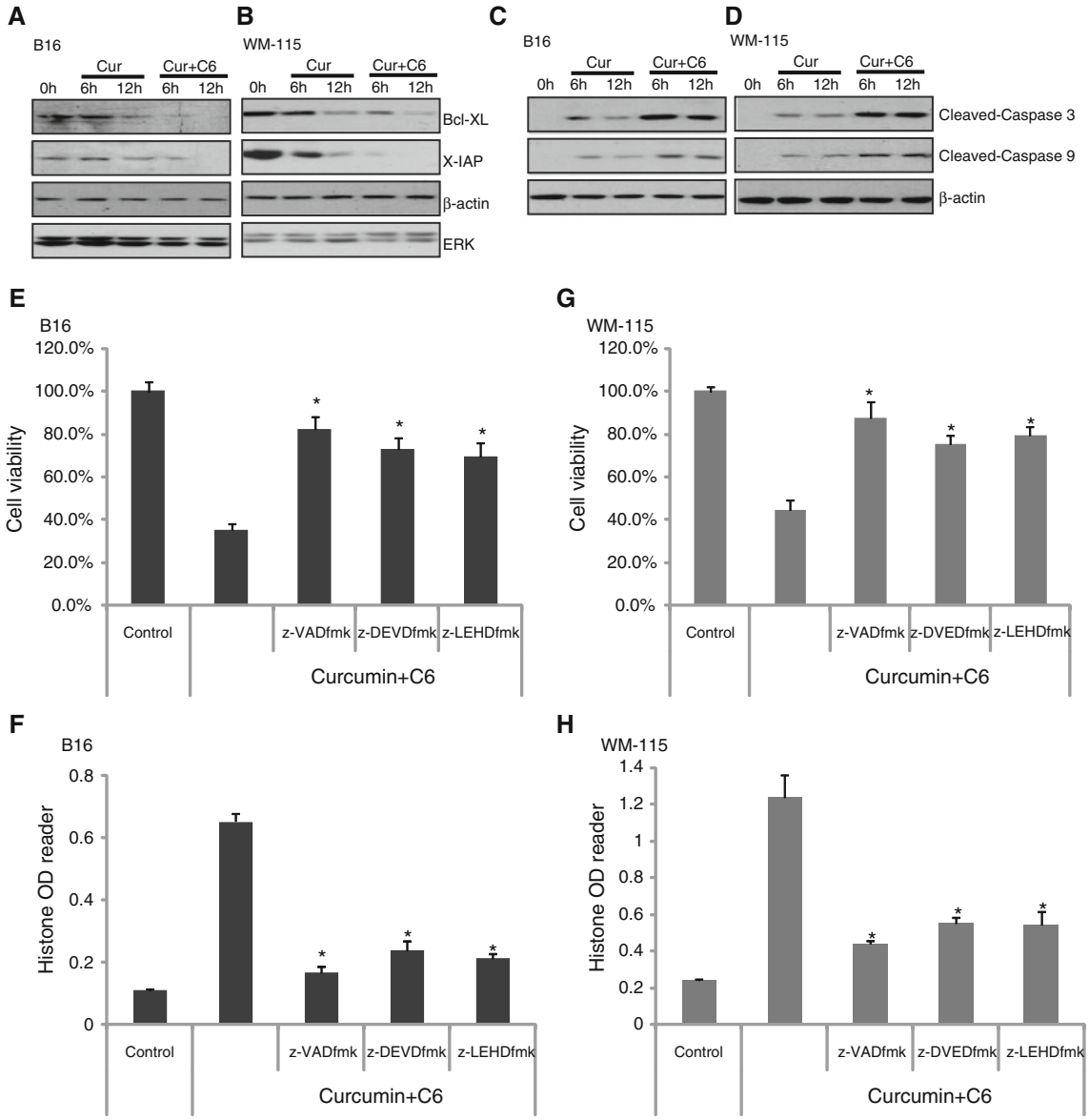

Fig. 2 C6 ceramide enhances curcumin-induced mitochondria apoptosis pathway. B16 and WM-115 cells were treated with 10 μM of curcumin with or without 10 μg/ml of C6 ceramide for 6 and 12 h, and the expression levels of Bcl-XL, X-IAP, cleaved-Caspase 3, cleaved-Caspase 9, *β*-actin and ERK1/2 were detected by Western Blot (**a**–**d**). B16 (**e**, **f**) and WM-115 (**g**, **h**) cells were pretreated with general Caspase inhibitor z-VADfmk (50 μM), Caspase-3-specific inhibitor Z-DEVDfmk (40 μM) and Caspase-9-specific inhibitor z-LEHDfmk (40 μM) for 1 h, followed by curcumin (10 μM) plus C6 ceramide (10 μg/ml), cell viability was detected by MTT assay (**e**, **g**) 48 h after treatments and cell apoptosis were detected by Histone DNA ELISA assay 36 h after treatment (**f**, **h**). The values in the figures are expressed as the means ± SD. **P* < 0.05 vs. C6+ curcumin treatment. All experiments were repeated at least three times, and similar results were obtained

inhibitor z-VADfmk, Caspase-3 specific inhibitor Z-DEVDfmk and Caspase-9 specific inhibitor z-LEHDfmk largely inhibited curcumin plus C6 ceramide–induced cell death and apoptosis in both cells.

Discussion

In this study, we show that C6 ceramide sensitizes melanoma cell lines to curcumin- and Taxol-induced cell death (Fig. 1). Such an effect was not observed, however, for Cisplatin (Fig. 1e), indicating chemo-drug-dependent sensitization for C6 ceramide. C6 ceramide enhances curcumin-induced melanoma cell apoptosis (Figs. 1f–i, 2). Inhibition of mitochondria apoptosis pathway effectively represses curcumin plus C6 ceramide–induced cytotoxicity in melanoma cell lines (Fig. 2).

C6 ceramide promotes curcumin-induced Caspase 3 and Caspase 9 cleavage or activation while down-regulates anti-apoptosis protein Bcl-XL and X-IAP (Fig. 2) in both

cells. Further, general Caspase inhibitor z-VADfmk as well as Caspase-3-specific inhibitor Z-DEVDfmk and Caspase-9-specific inhibitor z-LEHDfmk largely inhibit curcumin plus C6 ceramide–induced cell death and apoptosis (Fig. 2). However, it is noticed that Caspase inhibitors inhibit, but not reverse, C6+ curcumin–induced cell death and apoptosis (Fig. 2e–h), which suggests that Caspase-independent apoptosis pathway may also been involved, which needs further investigation. Based on these data, we suggest that mitochondria apoptosis pathway might be the major pathway mediating the curcumin plus C6 ceramide–induced melanoma cell death.

Cutaneous malignant melanoma is a severe and life-threatening skin cancer. The incidence of melanoma is increasing at an alarming rate among Caucasian populations [8, 13]. Currently, there is no effective treatment for metastatic melanoma [8, 13]. One of the challenges in melanoma treatment is its resistance to chemotherapy. Melanoma cells, particularly those with mutant p53, are strongly resistant to conventional chemotherapy. Previous studies have shown that curcumin may overcome the chemoresistance by inducing apoptosis in a p53-independent manner [5]. However, a relative high dose of curcumin (30–100 μM) has to be used to kill different melanoma cell lines [5]. In the present study, we found that a relatively low dose of curcumin (1–10 μM) can reach the same or even stronger effects on melanoma cell death/apoptosis in the presence of C6 ceramide (Fig. 1). C6 ceramide dramatically sensitizes the melanoma cell lines to curcumin-induced cell death and apoptosis in vitro. This research has provided a new mechanism of C6+ curcumin action in melanoma cells and should further establish its future use as a valid chemo-preventive and chemo-therapeutic agent against melanoma.

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