Biochemical and Biophysical Research Communications 421 (2012) 462–467

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

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

Increasing ceramides sensitizes genistein-induced melanoma cell apoptosis and growth inhibition

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ARTICLE INFO

Article history:
Received 24 March 2012
Available online 7 April 2012

Keywords:
Melanoma
Genistein
Ceramides
Apoptosis and signal transduction

ABSTRACT

The aim of the current study is to investigate the effect of ceramides on genistein-induced anti-melanoma effects *in vitro*. We found that exogenously added cell-permeable short-chain ceramides (C6) dramatically enhanced genistein-induced growth inhibition and apoptosis in cultured melanoma cells. Genistein treatment only induced a moderate intracellular ceramides accumulation in B16 melanoma cells. Two different agents including 1-phenyl-2-decanoylamino-3-morpholino-1-propanol (PDMP), a ceramide glucosylation inhibitor, and the sphingosine kinase-1 (SphK1) inhibitor II (SKI-II), a sphingosine (ceramides precursor) phosphorylation inhibitor, both facilitated genistein-induced ceramides accumulation and melanoma cell apoptosis. Co-administration of ceramide (C6) and genistein induced a significant Akt inhibition and *c*-jun-NH_2-kinase (JNK) activation, caspase-3 cleavage and cytochrome c release. Caspase-3 inhibitor z-DVED-fmk, JNK inhibitor SP 600125, or to restore Akt activation by introducing a constitutively active form of Akt (CA-Akt) diminished ceramide (C6) and genistein co-administration-induced *in vitro* anti-melanoma effect. Our study suggests that increasing cellular level of ceramides may sensitize genistein-induced anti-melanoma effects.

1. Introduction

Several biological agents have been tested as new adjuvant therapies against melanoma both *in vivo* and *in vitro* with mixed successes [1]. Among all the agents tested, genistein received major attentions. Epidemiologic studies show that consumption of genistein containing diets in Asian populations is associated with a lower incidence of certain cancers [2], Study by Li et al., suggested that dietary supplementation with genistein reduced melanoma size and lung metastasis in mice xenograft melanoma model [3]. The anti-melanoma ability of genistein is due to its ability to induce cancer cell apoptosis and growth inhibition [1], and to modulate multiple signaling pathways, including up-regulation of the cyclin-dependent kinase inhibitor (CDKi) p27(KIP1), stabilization of protein-linked DNA strand breakage, activation of p53 and others (see review in [1]).

However, the use of genistein is limited due to the fact that a relative high concentration of genistein (30–100 μM, *in vitro*) is often needed to achieve its anti-cancer effects. Our previous studies have found that exogenously added short-chain cell-permeable ceramide (C6) enhanced doxorubicin and histone deacetylase inhibitors (HDACi)-induced cancer cell cytotoxicity and apoptosis [4,5]. In the current study, we aim to sensitize genistein induced *in vitro* anti-melanoma effects by increasing the cellular level of ceramides, both exogenously and endogenously.

2. Material and methods

2.1. Chemicals and reagents

Genistein, PDMP, SKI-II, and SP 600125 were obtained from Sigma (Sigma, St. Louis, MO); Ceramides used in this study were obtained from Avanti Polar Lipids, Inc. (Alabaster, AL). Anti-AKT1, tubulin, rabbit IgG-HRP and mouse IgG-HRP antibodies were

Abbreviations: CI, combination index; PI, propidium iodide; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide. SKI-II, SphK1 inhibitor II; PDMP, 1-phenyl-2-decanoylamino-3-morpholino-1-propanol; FACS, fluorescence-activated cell sorting; mTOR, mammalian target of rapamycin; FBS, fetal bovine serum; DMEM, Dulbecco's modified Eagle medium; MAPK, mitogen activated protein kinase; JNK, *c*-jun-NH_2-kinase; SphK1, sphingosine kinase-1.

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http://dx.doi.org/10.1016/j.bbrc.2012.04.012

purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-p-Akt (Ser473), p-Akt (Thr308), p-S6 (Ser 235/236) and cleaved-Caspase 3 antibodies were obtained from Cell Signaling Technology (Bevery, MA).

2.2. Cell culture

Melanoma cell lines B16, WM451 and MeWo were maintained in a DMEM medium (Sigma, St. Louis, MO), supplemented with a 10% FBS (Invitrogen, Carlsbad, CA), 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.

2.3. Live cell counting by trypan blue staining

The number of viable melanoma cells (trypan blue positive) after indicated treatment/s was counted, and the percentage (%) of viable cells was calculated by the number of the trypan blue stained cells of the treatment group divided by that of the untreated control group.

2.4. Clonogenicity assay

B16 cells (4×10^4) were suspended in 1 ml of DMEM containing 1% agar (Sigma, St. Louis, MO), ten percent (10%) FBS and indicated agents. The cell suspension was then added on top of a pre-solidified 1% agar in a 100 mm culture dish. The medium was replaced every three days. After 12 days of incubation, colonies were photographed at 4×. Colonies larger than 50 μm in diameter were quantified for both size and number using Image J Software. Each experiment was repeated three times, in triplicates.

2.5. Cell viability assay (MTT dye assay)

As reported before [5], melanoma cell viability after indicated treatment/s was measured by the 3-[4,5-dimethylthylthiazol-2-yl]-2,5 diphenyltetrazolium bromide (MTT) method. Twenty micro-liters (20 μl) of MTT tetrazolium salt (Sigma, St. Louis, MO), dissolved in PBS at a concentration of 5 mg/ml was added to melanoma cells with indicated treatment/s and incubated in CO_2 incubator for additional 4 h. The medium was then aspirated from each well before 150 μl of DMSO (Sigma, St. Louis, MO) was added to dissolve formazan crystals, the absorbance of each well was obtained using a Dynatech MR5000 (Nanjing, China) plate reader at a test wavelength of 490 nm with a reference wavelength of 630 nm.

2.6. Analysis of apoptosis by propidium iodide (PI) fluorescence-activated cell sorting (FACS)

For FACS analysis, melanoma cells with indicated treatment/s were stained by fluorescein isothiocyanate-conjugated the fluorescent dye PI. Apoptosis percentage was calculated as the number of PI positive cells divided by the total number of cells.

2.7. Caspase-3 activity assay

Before making the protein extract, floating cells were collected and combined with cells growing on the dish and washed two times with cold PBS (Same for other procedures). The melanoma cells were lysed in 2× caspase lysis buffer (25 mM HEPES-NaOH, pH 7.4, 10% sucrose, 0.1% CHAPS, 2 mM EDTA, 5 mM dithiothreitol). Cell lysates were spun for 4 min in a microcentrifuge, and a protein assay was performed on the supernatant. Fifty micro-grams (50 μg) of total protein was mixed with 2× caspase assay buffer (25 mM HEPES-NaOH, pH 7.4, 5 mM DTT) and a 100 μM concentration of one of the following caspase fluorogenic substrates: Ac-DEVD-AFC for caspase-3. After incubation at 37 °C for 4 h, the fluorometric detection of cleaved AFC product was performed on a CytoFluor Multi-Well Plate Reader Series 4000 (PerSeptive Biosystems) using a 400-nm excitation filter and a 530-nm emission filter. For preparation of the AFC calibration curve, 80 μM free AFC was diluted in the caspase assay buffer without substrate to give 1.6, 3.2, and 4.8 μM of free AFC, and fluorescence was measured on the fluorometer, the caspase-3 activity in the treatment group was expressed as fold changes vs. the untreated control.

2.8. Quantification of apoptosis by ELISA

As reported before [4,5],the cell apoptosis ELISA Detection Kit (Roche, Palo Alto, CA) was used to quantify apoptosis in melanoma cells after indicated treatment/s. Briefly, 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 (Nanjing, China) at 405 nM.

2.9. Western blots

As reported early [4,5], B16 cells with indicated treatments were lysed with lysis buffer (200 mM NaCl (pH 7.4), 1% Triton X-100, 10% glycerol, 0.3 mM EDTA, 0.2 mM Na_3VO_4, and protease inhibitor cocktails (Roche Diagnostics, Indianapolis, IN). Aliquots of 40 μg of protein from each sample were separated by 10–12.5% SDS–PAGE and transferred onto a PVDF membrane (Millipore, Bedford, MA). After blocking with 10% instant nonfat dry milk for 1 h, membranes were incubated with specific antibody overnight at 4 °C, followed by incubation with corresponding secondary antibody for 1 h at room temperature. Antibody binding was detected with the enhanced chemiluminescence (ECL) detection system (Amersham Biosciences, Piscataway, NJ).

2.10. Analysis of cellular ceramides levels

The cellular ceramides level was analyzed by the methods mentioned early [5], and it was expressed as fold changes vs. the untreated control group.

2.11. Generation of constitutively active Akt1 (CA-Akt) transfected stable B16 cells

Similar to previously reported [5], a plasmid encoding a constitutively active Akt1 cDNA (Plasmid 16244) was obtained from addgene (Cambridge, MA). B16 melanoma cells were seeded in a 6-well plate with 60–70% of confluence. Two microgram (2 μg) of CA-Akt plasmid or vector control was added to 90 μl of RNA dilution water (Santa Cruz, CA) containing 3.5 μl of PLUS™ Reagent (Invitrogen, Carlsbad, CA), 3.6 μl of Lipofectamine (Invitrogen, Carlsbad, CA) was then added to the complex. After a 30 min of incubation, the transfection complex was formed and added to wells containing 2 ml of medium. Successfully transfected cells were selected by puromycin (2.5 μg/ml). Western blots were performed to test transfection efficiency.

2.12. Statistical analysis

Individual experiment was analyzed separately (no pooling of samples was used). In each experiment, a minimum of three wells/dishes of each treatment was used. Each experiment was repeated a minimum of three times. In each experiment, the mean value of the repetitions was calculated and this value was used

in the statistical analysis. All data were normalized to control values of each assay and are presented as mean ± standard deviation (SD). Data were analyzed by one-way ANOVA followed by a Scheffe's *f*-test by using SPSS software. $P < 0.05$ is considered statistically significant. CalcuSyn software was utilized to calculate combination index (CI), CI < 1 was considered as synergism.

3. Results

3.1. Exogenously added short-chain ceramides sensitize genistein-induced anti-melanoma effects in vitro

To test whether increasing the ceramides level can enhance genistein-induced anti-melanoma effect *in vitro*, a cell-permeable short-chain ceramide (C6) was directly added to B16 melanoma cells. Three independent assays including trypan blue stained cells counting, "MTT" cell viability assay and "clonogenicity" assay were used to test B16 cell growth after genistein and/or C6 ceramide treatments. Genistein or C6 ceramide as a single agent showed a moderate anti-growth effect in B16 cells, co-administration of the two caused a significant cell growth inhibition (Fig. 1A–C). MTT results in Fig. 1B showed a 40.2% loss of B16 cell viability after 4 days of genistein (50 μM) treatment and 11.5% loss by C6 ceramide (5 μg/ml or 12.72 μM) treatment, combination of the two caused a synergistic 77.9% loss of cell viability, combination index (CI) < 1. Clonogenicity assay results in Fig. 1C showed that the number of clone with size more than 50 μm was down to 19.7% (or a 81.3% loss) of the untreated control group after genistein (50 μM) and C6 ceramide (5 μg/ml) co-administration, compared to a 31.1% loss by genistein treatment alone, or a 18.7% loss after the C6 ceramide treatment alone. Similar results were also seen in two other melanoma cell lines WM-451 and MeWo (Fig. 1E–F). Short-chain C2 and C4 ceramides also enhanced genistein-induced B16 melanoma cell viability loss, while long-chain ceramide C14 had no effect (Fig. 1F). C6 ceramide, among all the short-chain ceramides tested, demonstrated the most significant effect (Fig. 1F).

3.2. C6 ceramide sensitizes genistein-induced melanoma cell apoptosis

Using three independent apoptosis assays including FACS sorting PI stained cells (Fig. 2A), Histone DNA-ELISA assay (Fig. 2B) and caspase-3 activity assay (Fig. 2C), we demonstrated a significant pro-apoptosis outcome by C6 ceramide and genistein co-administration in B16 melanoma cells. The percentage of PI positive B16 cells increased to 50.8% after the co-administration, compared to 10.1% of the C6 ceramides only treatment group, and 21.7% of the genistein only treatment group (Fig. 2A, CI < 1). 50 μM of genistein caused 24.8 folds increase of caspase-3 activity, 5 μg/ml of C6 ceramide resulted in 12.4 fold increase of caspase-3 activity, combination of the two led to a 65.0 fold increase of caspase-3 activity (Fig. 2C). Notably, caspase-3 inhibitor z-VAD-fmk (ZVAD) significantly reduced C6 ceramide plus genistein co-administration-induced B16 cell viability loss, suggesting that caspase-3 activation and apoptosis are responsible for growth inhibition.

3.3. PDMP and SKI-II facilitates genistein-induced intracellular ceramides production, melanoma cytotoxicity and apoptosis

Next we aimed to enhance the intracellular ceramides level by regulating ceramides metabolism pathways. First, B16 melanoma cells were treated with 1-phenyl-2-decanoylamino-3-morpholino-1-propanol (PDMP) to increase levels of ceramides and the precursor sphingolipid by promoting their synthesis and inhibiting

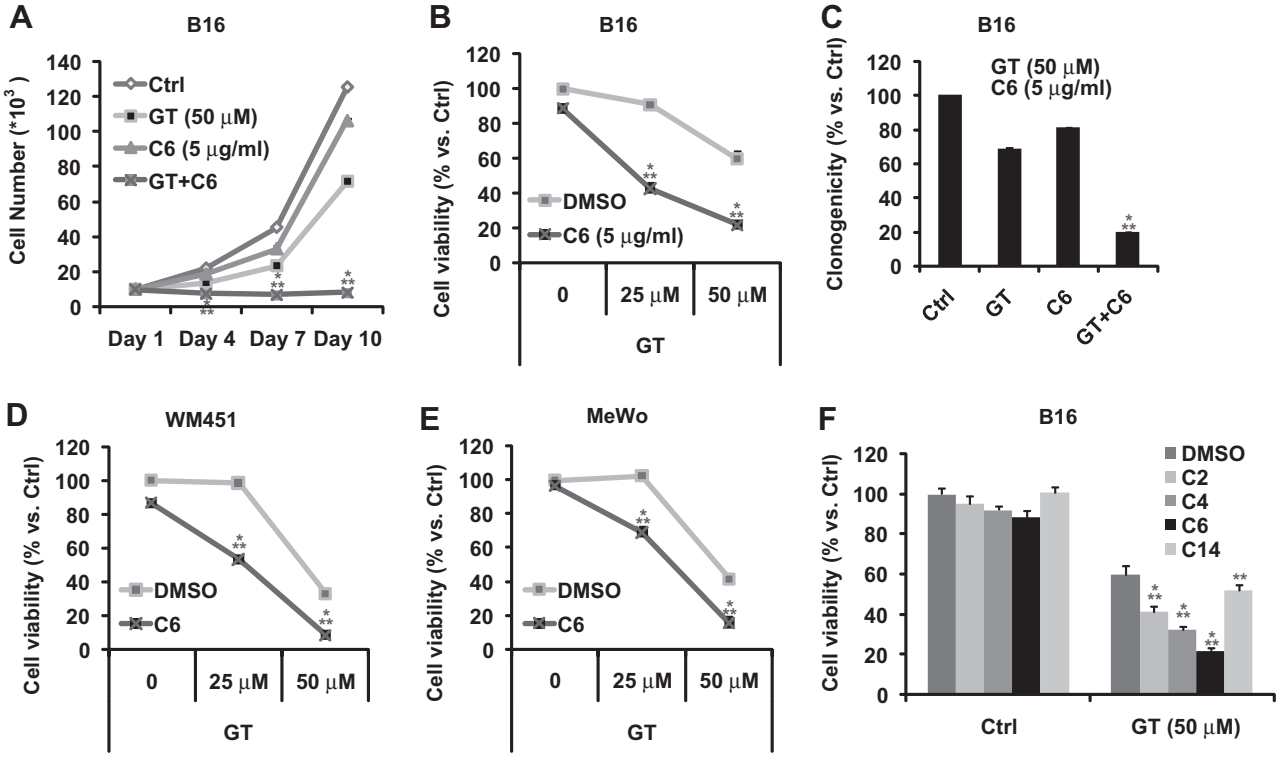

Fig. 1. Exogenously added short-chain ceramides sensitize genistein-induced anti-melanoma effects *in vitro*. Melanoma cell lines B16, WM-451 and MeWo were either left untreated (Ctrl) or exposed to genistein (GT, 25-50 μM), indicated ceramides (12.72 μM) or a combination of both agents; cell growth was analyzed by counting trypan blue stained cells (A), "MTT" cell viability assay (B, D–F) or clonogenicity assay (C). The values in the figures are expressed as the means ± standard deviation (SD) (Same for the rest of the figures). All experiments were repeated three times and similar results were obtained (Same for the rest of figures). $^{*}P < 0.05$ vs. the genistein treatment group; $^{**}P < 0.05$ vs. the C6 ceramides treatment group. Statistical significance was analyzed by ANOVA (Same for the rest of the figures).

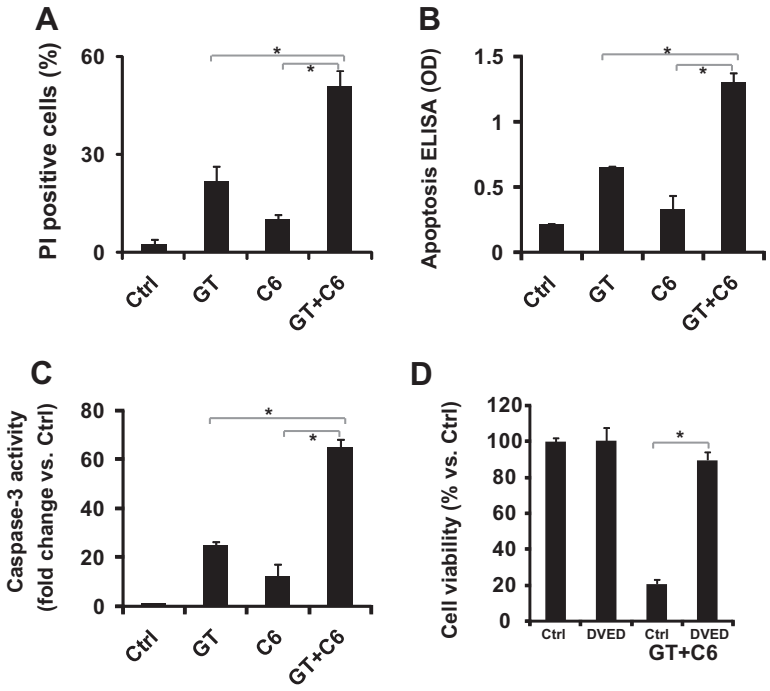

Fig. 2. C6 ceramide sensitizes genistein-induced melanoma cell apoptosis. B16 melanoma cells were either left untreated (Ctrl) or exposed to 50 μM of genistein (GT), 5 μg/ml of C6 ceramide (C6) or a combination of both (GT + C6), cell apoptosis was determined by FACS sorting PI stained cells (A, 36 h after treatment/s), Histone-DNA ELISA (B, 32 h after treatment/s) or caspase-3 activity assay (C, 24 h of treatment/s). B16 melanoma cells were either left untreated (Ctrl) or exposed to genistein (GT, 50 μM) plus C6 ceramide (5 μg/ml) (GT + C6), with or without z-VAD-fmk (ZVAD 60 μM) for 96 h, cell viability was analyzed by MTT assay (D). $^{*}P < 0.05$.

their metabolism [6–9]. With the same purpose, sphingosine kinase-1(SphK1) inhibitor II (SKI-II) was also used here. SphK1 is the master kinase that regulates the balance between ceramides and sphingosine-1-phosphate (S1P). Increased expression and/or activity of SphK1 lead to increased pro-survival S1P level and less ceramides to favor cancer progression, while inhibitors of SphK1 not only inhibit S1P production, but also increase ceramides level, pushing cancer cells toward apoptosis [10,11]. Results in Fig. 3A demonstrated that genistein only moderately increased intracellular ceramides level in B16 cells. Both PDMP and SKI-II enhanced genistein-induced ceramides accumulation (Fig. 3A), cell viability loss (cytotoxicity, Fig. 3B) and the percentage of PI positive cells (apoptosis, Fig. 3C).

3.4. C6 ceramide and genistein co-administration-induced anti-melanoma effects involves JNK activation and Akt inhibition

We then focused the changes of signaling events after C6 ceramide and genistein co-administration in melanoma cells. Western blots results in Fig. 4A showed that co-administration of C6 ceramide and genistein caused a significant decrease in Akt activation (Akt and its downstream S6 phosphorylation) together with significant increases of JNK activation (JNK1/2 phosphorylation), caspase-3 cleavages and cytochrome c release. JNK inhibitor SP600125 (Fig. 4B–C) and a constitutively active form of Akt (CA-Akt) (Fig. 4D–F) inhibited C6 ceramide and genistein-induced cell viability loss and apoptosis, arguing that both JNK activation and Akt inhibition are involved in the process.

4. Discussion

Under a number of stress conditions, ceramides production is a well-known mediator of apoptosis [12,13]. However, tumor cells are capable of removing excess ceramides through diverse metabolic pathways [14,15]. Previous studies have identified a number of agents that facilitate tumor cell apoptosis through enhancing intracellular ceramides accumulation. These agents can either

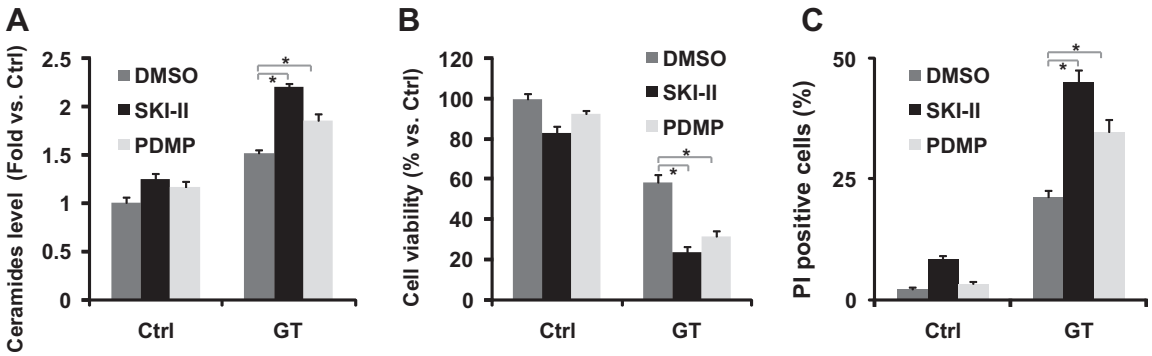

Fig. 3. PDMP and SKI-II facilitates genistein-induced intracellular ceramides production, melanoma cytotoxicity and apoptosis. B16 melanoma cells were pre-treated with PDMP (10 μM) or SKI-II (5 μM) for 1 h, cells were then exposed to 50 μM of genistein (GT), after 24 h, cellular ceramides level was analyzed, and was expressed as fold changes vs. untreated control (A). B16 cells viability after 96 h of indicated treatment/s was analyzed by MTT assay (B). The percentage of PI positive cells was analyzed by FACS sorting 36 h after treatment/s (C). $^{*}P < 0.05$.

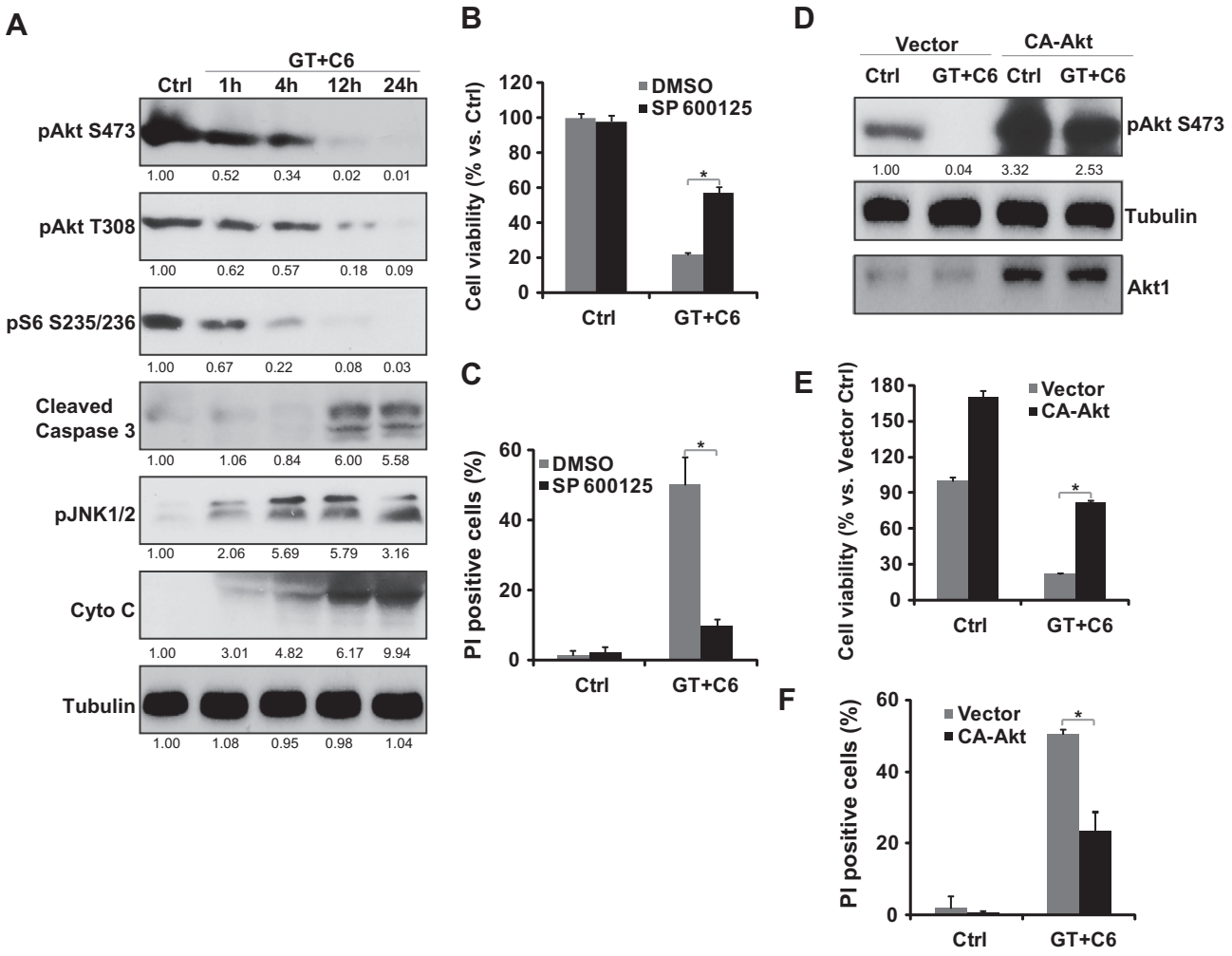

Fig. 4. C6 ceramide and genistein co-administration-induced anti-melanoma effects involves JNK activation and Akt inhibition. B16 melanoma cells were either left untreated (Ctrl) or exposed to 50 μM of genistein plus 5 μg/ml of C6 ceramide (GT + C6) for indicated time points, p-Akt, pS6, p-JNK, cleaved caspase-3, cytosol cytochrome c and tubulin were tested by Western blots, and results were quantified by Image J software after normalization to tubulin. B16 cells were pretreated with JNK inhibitor (SP600125, 10 μM, 1 h pretreatment), followed by 50 μM of genistein plus 5 μg/ml of C6 ceramide (GT + C6) treatments, cell viability was analyzed by MTT assay 96 h after treatments (B), the percentage of apoptotic cells was measured by FACS sorting PI stained cells (C). Empty vector and CA-Akt transfected stable B16 cells were exposed to 50 μM of genistein plus 5 μg/ml of C6 ceramide (GT + C6), Western blots were used to test Akt phosphorylation and expression after 12 h of treatments (D). B16 cells viability after 96 h of GT + C6 treatments was analyzed by MTT assay (E) and cell apoptosis was analyzed by FACS sorting PI stained cells (F). $^{*}P < 0.05$.

promote ceramides synthesis and/or inhibit ceramides metabolically clearance [7,16–18]. In the current study, we found that genistein treatment only caused a moderate increase of intracellular ceramides level in B16 melanoma cells, which may not be enough to result in a significant cell apoptosis due to metabolic removal. Co-administration of ceramides metabolism inhibitors PDMP [7,9] or SKI-II [11,19,20], facilitated ceramides accumulation and significantly enhanced genistein-induced melanoma cell apoptosis and growth inhibition. Our results are consistent with previous studies which demonstrate that PDMP potentiates Taxol [8], Vincristine [8] and curcumin [7] induced cytotoxicity/apoptosis by increasing ceramides accumulation. SKI-II is also known to increase ceramides accumulation, and cell apoptosis (See review in [11]).

We found that exogenously added cell-permeable short-chain ceramides (C2, C4 and C6) sensitized genistein induced anti-melanoma effect *in vitro*, which further confirms our hypothesis. The results here are consistent with our previous findings where we found C6 ceramide significantly enhanced cytotoxicity and apoptosis by the known chemotherapeutics including doxorubicin and histone deacetylase inhibitor (HDACi) [4,5].

The serine–threonine kinase Akt, which is downstream signal of phosphatidylinositol-3-kinase (PI3K), controls cancer cell progression [21], and is frequently over-expressed and/or over-activated in melanoma and many other tumors, as such, it is involved in melanoma progression and chemo-resistance [22], and Akt signaling represents potentially important therapeutic and/or chemo-preventive targets in melanoma [22]. Studies have confirmed that ceramides activate serine/threonine phosphatases including protein phosphatase 1 (PP1) and Protein phosphatase 2A (PP2A) to dephosphorylate Akt [23–25]. We found here that co-administration of C6 ceramide and genistein also caused a significant Akt dephosphorylation, and to restore Akt activation by introducing a CA-Akt diminished co-administration-induced melanoma cell apoptosis and cell viability loss, suggesting that Akt inhibition is involved in the process.

Studies have shown that ceramides-induced cytotoxicity and cell apoptosis involve activation of JNK [7,8,26], here we also observed that co-administration of C6 ceramide and genistein induced a JNK activation in melanoma cells. Further, SP 600125, the JNK inhibitor, inhibited cell apoptosis and growth inhibition

by the C6 ceramide plus genistein co-administration, suggesting that JNK activation may contribute the process.

Acknowledgments

This research was supported in part by Grants from the National Natural Science Foundation of China (810701297 and 81101188), Postdoctoral Science Foundation of China (No. 20100470649).

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