Antitumor agent 25-*epi* Ritterostatin G_N1_N induces endoplasmic reticulum stress and autophagy mediated cell death in melanoma cells

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Abstract. Metastatic melanoma is the most aggressive of all skin cancers and is associated with poor prognosis owing to lack of effective treatments. 25-*epi* Ritterostatin G_N1_N is a novel antitumor agent with yet undefined mechanisms of action. We sought to delineate the antitumor mechanisms of 25-*epi* Ritterostatin G_N1_N in melanoma cells to determine the potential of this compound as a treatment for melanoma. Activation of the endoplasmic reticulum (ER) stress protein glucose-regulated protein 78 (GRP78) has been associated with increased melanoma progression, oncogenic signaling, drug resistance, and suppression of cell death. We found that 25-*epi* Ritterostatin G_N1_N induced cell death in melanoma cells at nanomolar concentrations, and this cell death was characterized by inhibition of GRP78 expression, increased expression of the ER stress marker CHOP, loss of mitochondrial membrane potential, and lipidation of the autophagy marker protein LC3B. Importantly, normal melanocytes exhibited limited sensitivity to 25-*epi* Ritterostatin G_N1_N. Subsequent *in vivo* results demonstrated that 25-*epi* Ritterostatin G_N1_N reduced melanoma growth in mouse tumor xenografts and did not affect body weight, suggesting minimal toxicity. In summary, our findings indicate that 25-*epi* Ritterostatin G_N1_N causes ER stress and massive autophagy, leading to collapse of mitochondrial membrane potential and cell death in melanoma cells, with minimal effects in normal melanocytes. Thus, 25-*epi* Ritterostatin G_N1_N is a promising anticancer agent that warrants further investigation.

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Abbreviations: PI, propidium iodide; ER, endoplasmic reticulum; GRP78, glucose regulated protein 78; PBS, phosphate buffered saline; LC3, microtubule associated light chain 3; CHOP, CCAAT/enhancer binding protein homologous protein; HSP70, heat shock protein 70; UPR, unfolded protein response; DAPK-1, death associated protein kinase; PERK, PKR-like EIF2α kinase; ATF-6, activation transcription factor-6; IRE-1, inositol requiring enzyme-1; BAX, Bcl-2 associated X; NHEM, normal human epidermal melanocytes; DMSO, dimethyl sulfoxide; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetra-zolium bromide; FACS, fluorescence activated cell sorting; Atg6, autophagy-related gene 6

Key words: anticancer agent, 25-epi Ritterostatin GN1N, melanoma, ER stress, autophagy, GRP78, mitochondrial membrane potential, therapeutic specificity

Introduction

Malignant melanoma is the most dangerous type of skin cancer, accounting for more than 75% of deaths related to skin cancer. Patients with advanced melanoma with dissemination to distant sites and visceral organs have a very poor prognosis, with a median survival time of 6 months and a 5-year survival rate of less than 5% (1). Currently, very limited treatment options are available for metastatic melanoma. Although multiple clinical trials have been initiated for novel agents to treat melanoma, these have met with limited success, thus highlighting the need for the development of new drugs and identification of novel therapeutic targets for effective cancer therapy.

The endoplasmic reticulum (ER) in eukaryotic cells is required for several critical functions, including lipid and cholesterol biosynthesis, maintenance of calcium homeostasis, and transport of nascent proteins to subcellular organelles (2). Cellular stress conditions, such as aberrant calcium signaling and accumulation of misfolded proteins could lead to ER stress and activation of a counteractive reaction called the ER stress response or unfolded protein response, which serves to restore ER functionality and homeostasis (3,4). However, prolonged induction of acute ER stress can lead to cell death (5-9). Thus,

while moderate ER stress triggers cell survival signaling, severe stress may potentiate cell death.

ER stress is regulated by two critical proteins, glucose-regulated protein 78 (GRP78) and CCAAT/enhancer binding protein homologous protein (CHOP) (10,11). GRP78 is a key member of the HSP70 protein family that functions as an ER chaperone involved in protein folding and assembly and ER-mediated stress signaling (12). Overexpression of GRP78 has been observed to cause aggressive tumor behavior and promote angiogenesis in various solid tumors (13-17). In melanoma, enhanced activation of GRP78 has been associated with poor patient survival and increased disease progression through downregulation of apoptosis and activation of unfolded protein response mechanisms (18). De Ridder *et al* found a link between humoral response to GRP78 and cancer progression in a murine model of melanoma (19). Studies have also demonstrated a distinct role of GRP78 in drug resistance; GRP78 induced doxorubicin resistance in dormant squamous carcinoma cells through inhibition of BAX activation (20). Of note, GRP78 is expressed only on the surface of cancer cells and not on the surface of normal cells, making it an important target for therapeutic intervention (17).

In contrast, prolonged expression of CHOP results in cytotoxicity (21). Incremental CHOP levels have been associated with increased apoptosis and reduced tumor growth (22,23). Furthermore, numerous studies indicate that knockdown of CHOP leads to significantly decreased drug effects in cancer cells, confirming that CHOP plays a critical role in mediating ER stress-induced cytotoxicity (24-26). Thus, ER stress can be described as a double-edged sword: moderate or chronic levels of ER stress can activate pro-survival cellular signaling pathways through GRP78, whereas severe or acute levels of ER stress can lead to cell death via activation of CHOP.

Autophagy is a self-digestive process that facilitates lysosomal degradation of cytoplasmic proteins and organelles as a means of maintaining cellular homeostasis and adapting to different forms of stress (27,28). Autophagy is primarily a mechanism of cell survival; however, prolonged exposure of cells to deprivation conditions such as DNA damage, oxidative stress, and starvation can lead to induction of excessive autophagy, causing depletion of cellular organelles and self-destruction (29,30). Thus, similarly to ER stress, autophagy also plays a dual role in cancer. For instance, tumors with activating mutations in Ras have been shown to employ autophagy for survival (31). Noteworthy, although nuclear p53 transactivates autophagy inducers such as DRAM1 and sestrin2, cytoplasmic p53 inhibits autophagy (32,33). Gene knockout of the autophagy regulatory protein, Beclin-1, was found to increase tumor incidence in mice with lymphoma and lung cancer (34,35). Similarly, death-associated protein kinase (DAPK-1), which has cancer metastasis suppressive properties, is activated following an accumulation of unfolded proteins in cells, leading to ER stress and initiation of autophagy through phosphorylation of Beclin-1 (36-38). Unfolded protein response, which is triggered as an ER stress response, potentially induces autophagy; binding of GRP78 to misfolded proteins leads to the release of the 3 ER membrane-associated proteins, PKR-like eIF2α kinase (PERK), activation transcription factor-6 (ATF-6), and inositol-requiring enzyme-1 (IRE-1) (39,40). Of note, although both PERK and ATF-6 promote autophagy, IRE-1 attenuates the autophagic response in cells. Furthermore, multiple recent studies have indicated that ER stress can magnify autophagy and vice versa (41-44). Hence, both ER stress and autophagy constitute valid therapeutic targets, and inhibition of either or both of these processes could lead to improved therapeutic outcomes.

25-*epi* Ritterostatin G_N1_N, an analogue of cephalostatin 1 (Fig. 1), is a potent anticancer agent with 50% inhibitory concentrations in the subnanomolar range (45). Testing of this compound in the NCI-60 cell line panel indicated that the compound is highly effective against leukemia, melanoma lung, breast, renal, colon, and prostate cancer cells (46,47). Recent work by Kanduluru *et al* outlined the synthesis of 25-*epi* Ritterostatin G_N1_N (45). However, very little is known about the mechanism of action of this novel inhibitor in cancer cells. In this study, we aimed to delineate the mechanism of antitumor activity of 25-*epi* Ritterostatin G_N1_N in melanoma cells. We found that 25-*epi* Ritterostatin G_N1_N triggered ER stress and autophagic cell death in melanoma cells and inhibited tumor growth in mouse xenografts. Importantly, 25-*epi* Ritterostatin G_N1_N was therapeutically selective toward melanoma cells, whereas, normal melanocytes were resistant to it. These findings indicate that further study is warranted of 25-*epi* Ritterostatin G_N1_N as a potential therapeutic agent for melanoma.

Materials and methods

Cell lines and reagents. A375 melanoma cells were purchased from American Type Culture Collection (Manassas, VA, USA). WM35 cells were purchased from the characterized cell line core at The University of Texas MD Anderson Cancer Center (Houston, TX, USA). WM35 PKB cells, which stably overexpress Akt, were a kind gift from Dr Jack Arbiser (Emory University School of Medicine, Atlanta, GA, USA). Normal human epidermal melanocytes were purchased from PromoCell (Heidelberg, Germany). A375 and WM35/WM35 PKB cells maintained in Dulbecco's modified Eagle's medium and RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum were grown in a cell culture incubator at 37˚C with 5% CO_2 in humidified air. Normal melanocytes were grown in M254CF medium supplemented with human melanocyte growth supplement-2 (Life Technologies, Grand Island, NY, USA) in a cell culture incubator at 37˚C with 5% CO_2. The compound 25-*epi* Ritterostatin G_N1_N was synthesized and provided by the research group of Dr Philip L. Fuchs, Purdue University (West Lafayette, IN, USA). 25-*epi* Ritterostatin G_N1_N was first dissolved in dimethyl sulfoxide and then further diluted in media to a final concentration of 0.1%. Rhodamine 123, a fluorescent dye for the detection of mitochondrial membrane potential, was purchased from Life Technologies. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetra-zolium bromide (MTT) and propidium iodide were purchased from Sigma-Aldrich (St. Louis, MO, USA), and an Annexin V-FITC kit was purchased from BD Pharmingen (San Jose, CA, USA). Antibodies were obtained from the following commercial sources: β-actin from Calbiochem (San Diego, CA, USA), GRP78 (BiP) and p62 from Santa Cruz Biotechnology (Santa Cruz, CA, USA), LC3B

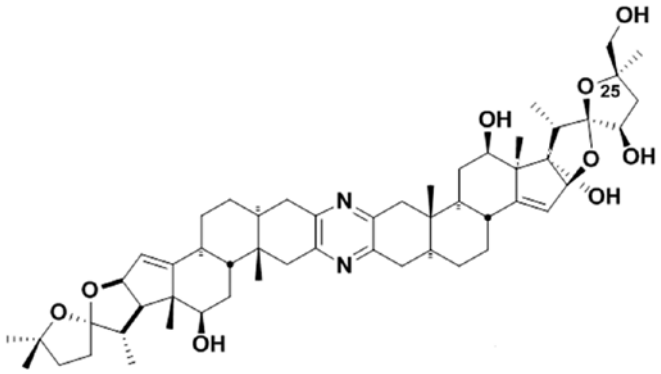

Figure 1. Structure of 25-*epi* Ritterostatin G_N1_N.

from Novus Biologicals (Littleton, CO, USA), and CHOP from Thermo Scientific (Rockford, IL, USA).

Cytotoxicity assays. The antiproliferative effect of 25-*epi* Ritterostatin G_N1_N was determined by performing the MTT assay. A375 and WM35 cells were plated in triplicate in a 96-well plate (2,000 cells per well). After overnight incubation, the cells were treated with log-scale serial diluted concentrations of 25-*epi* Ritterostatin G_N1_N and incubated at 37˚C for 72 h, followed by treatment with 50 μl of MTT reagent for 4 h. The cell media was aspirated and the formazan precipitates were dissolved in dimethyl sulfoxide. Absorbance at 570 nm was measured in a Multiskan MK3 microplate-reader (Thermo Labsystem, Franklin, MA, USA). The 50% inhibitory concentration values were then computed using GraphPad Prism (GraphPad Software, Inc., La Jolla, CA, USA).

Cell apoptosis and necrosis were measured using flow cytometry. Briefly, cells were harvested, washed in ice-cold phosphate-buffered saline (PBS), and suspended in Annexin V binding buffer. Cells were then stained with Annexin V-FITC for 15 min, washed, and stained with propidium iodide. Cell death was quantified using a BD Biosciences FACSCalibur flow cytometer (Mountain View, CA, USA) and the results were analyzed using FlowJo (TreeStar, Inc., Ashland, OR, USA).

Colony formation assay. To assess the effect of 25-*epi* Ritterostatin G_N1_N on the colony formation capacity of cells, we treated A375 and WM35 cells (5,000 cells per well) with serial diluted concentrations of 25-*epi* Ritterostatin G_N1_N for 2 weeks. At the end of this period, the cells were fixed in methanol/acetic acid (10:1) fixation solution and stained with Giemsa (Sigma-Aldrich). The stained cells were photographed and counted.

Mitochondrial membrane potential assay. Melanoma and normal melanocyte cells were stained with 0.5 μM Rhodamine 123 during the last hour of treatment with 25-*epi* Ritterostatin G_N1_N. The cells were then harvested, washed, and suspended in PBS. The mitochondrial membrane potential of the cells was measured using a FACSCalibur flow cytometer and subsequent data analysis was performed using FlowJo software.

Immunoblotting. Cellular protein extracts were separated using standard SDS-PAGE and transferred onto a nitrocellulose membrane. The membrane was probed for the indicated primary antibodies, followed by the appropriate horseradish peroxidase-conjugated secondary antibodies. The signal was developed using a supersignal-enhanced chemiluminescence kit (Thermo Scientific).

In vivo antitumor activity assay. All animal experiments were performed according to Institutional Animal Care and Use protocol at MD Anderson Cancer Center. Ten athymic nude mice were each subcutaneously inoculated with $2x10^6$ WM35 PKB cells. The mice were divided into 2 groups (5 mice each); mice in the treatment group were injected intraperitoneally with 2 mg/kg 25-*epi* Ritterostatin G_N1_N, and mice in the control group were injected with an equal volume of PBS. The mice were monitored routinely for tumor growth and body weight. Moribund animals were sacrificed according to Institutional Animal Care and Use protocol, and the time of death was recorded.

Statistical analysis. All statistical analyses were performed using GraphPad Prism version 6 (GraphPad Software, Inc.). Data are represented as means ± standard error of the mean. 95% confidence intervals were used to determine statistical significance.

Results

25-epi Ritterostatin G_N1_N inhibits growth and viability of melanoma cells at nanomolar concentrations. The effect of 25-*epi* Ritterostatin G_N1_N on the viability of A375 and other melanoma cells was determined by performing the MTT assay. Exposure to log-scale serial diluted concentrations (0-100 nM) of 25-*epi* Ritterostatin G_N1_N for 72 h resulted in concentration- and time-dependent cell death in melanoma cells, with an average 50% inhibitory concentration of 90.2 nM across four melanoma cell lines (Fig 2A and B).

To measure melanoma cell death after treatment with 25-*epi* Ritterostatin G_N1_N, we performed Annexin V/propidium iodide staining in WM35, A375, UCSD354L, and A375SM cells treated with 0.5 μM 25-*epi* Ritterostatin G_N1_N (Fig. 2C and D). Results of a follow-up colony-formation assay showed that 25-*epi* Ritterostatin G_N1_N suppressed colony formation at concentrations as low as 62.5 nM in melanoma cells (Fig. 2E and F). A375 cells, which were relatively more sensitive than WM35 cells to 25-*epi* Ritterostatin G_N1_N according to the MTT cell viability assay, were comparatively resistant to 25-*epi* Ritterostatin G_N1_N in the colony-formation assay. A concentration dependent decrease in colony formation was observed in melanoma cells at higher concentrations of 25-*epi* Ritterostatin G_N1_N. These results demonstrate that 25-*epi* Ritterostatin G_N1_N is cytotoxic and inhibits tumor growth in melanoma cells at nanomolar concentrations.

Cell death caused by 25-epi Ritterostatin G_N1_N is mediated through autophagy. Further investigation of the mechanisms of action of 25-*epi* Ritterostatin G_N1_N revealed that cell death occurred as a result of autophagy. A massive collapse of the mitochondrial membrane potential was observed in melanoma cells treated with 0.5 and 1 μM 25-*epi* Ritterostatin G_N1_N

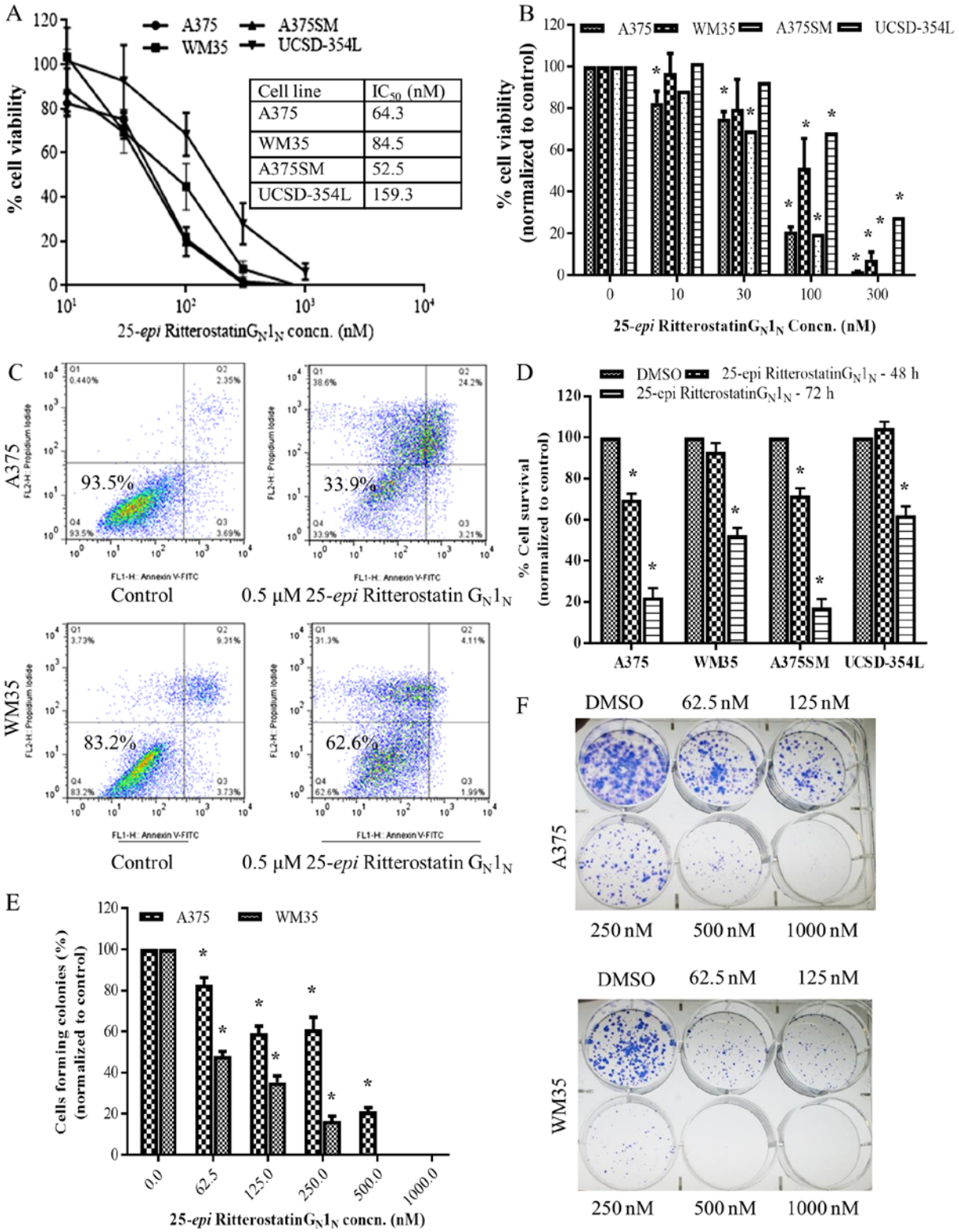

Figure 2. 25-*epi* Ritterostatin G_N1_N is cytotoxic at nanomolar concentrations in melanoma cells. (A and B) MTT assay results showing A375, WM35, A375SM, and UCSD-354L melanoma cell viability after treatment with various concentrations of 25-*epi* Ritterostatin G_N1_N for 72 h. IC_{50}, 50% inhibitory concentration. (C) Percentage of viable WM35 and A375 cells after treatment with 0.5 μM 25-*epi* Ritterostatin G_N1_N for 72 h, determined according to Annexin V-FITC/propidium iodide staining and subsequent flow cytometry analysis. (D) The bar graphs represent the percentage of viable A375, WM35, A375SM, and UCSD-354L cells (mean ± standard error) after treatment with 0.5 μM 25-*epi* Ritterostatin G_N1_N for 48 and 72 h, for 3 independent measurements, according to Annexin V-FITC/propidium iodide staining analysis. (E and F) Giemsa staining for colony formation assessment in A375 and WM35 cells treated with varying concentrations of 25-*epi* Ritterostatin G_N1_N [or dimethyl sulfoxide (DMSO) only] for 14 days. The bar graphs represent the mean (± standard error of the mean) of three independent measurements. $^*p<0.05$.

compared with untreated cells (Fig. 3A and B). This decrease in the mitochondrial membrane potential was time-dependent.

To confirm the role of autophagy in mediating cytotoxicity, we treated A375 cells with 25-*epi* Ritterostatin

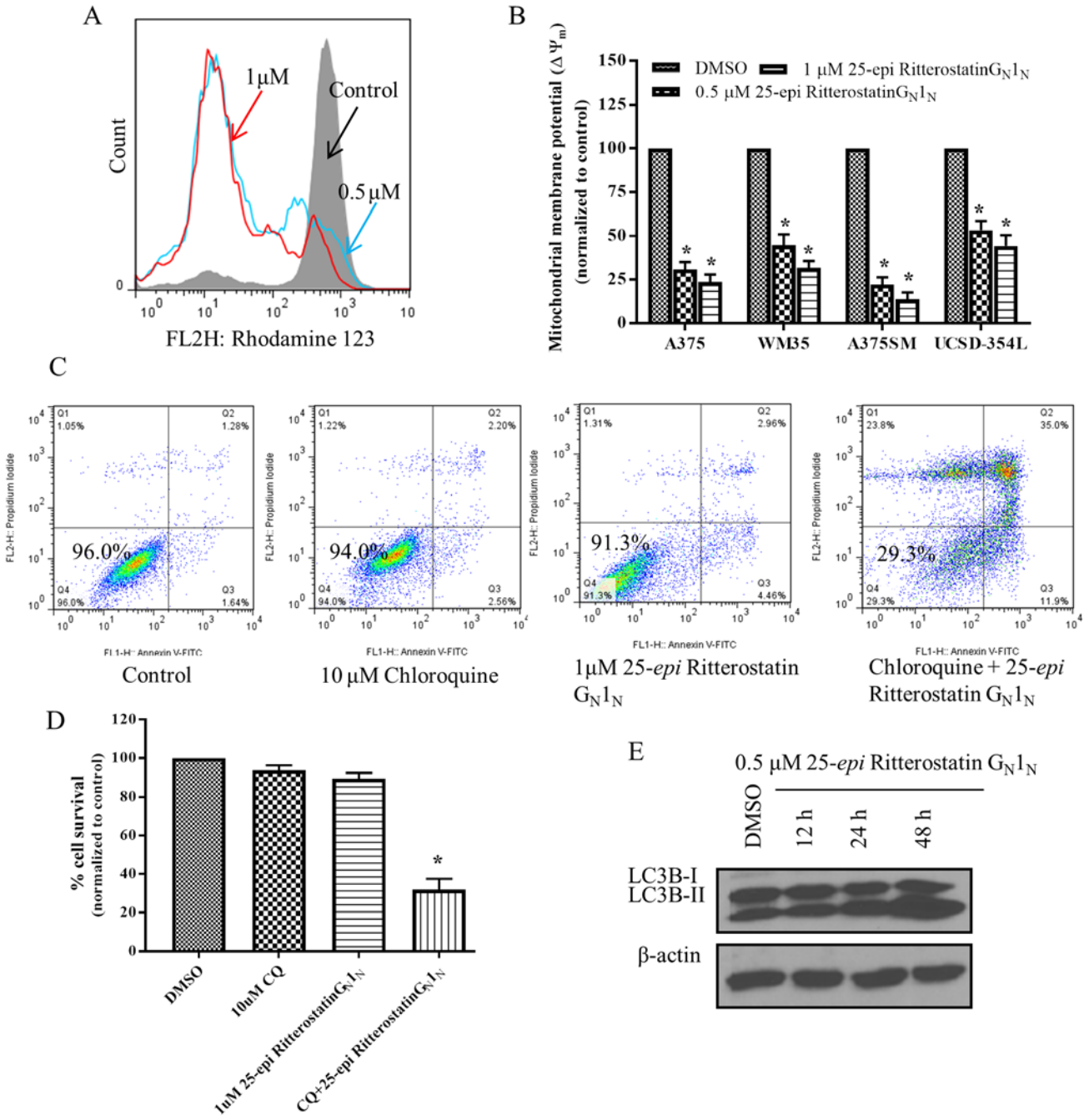

Figure 3. 25-*epi* Ritterostatin G_N1_N triggers autophagic cell death in melanoma cells. (A and B) Rhodamine 123 staining results indicate the mitochondrial membrane potential changes in A375, WM35, A375SM, and UCSD-354L cells treated with 0.5 or 1 μM 25-*epi* Ritterostatin G_N1_N for 48 h. The bar graphs represent the mean (± standard error of the mean) of 3 independent measurements. (C) Percentage of viable A375 cells at 24 h after treatment with 10 μM chloroquine alone, 1 μM 25-*epi* Ritterostatin G_N1_N alone, or 10 μM chloroquine followed by 1 μM 25-*epi* Ritterostatin G_N1_N, according to Annexin V-FITC/propidium iodide staining and flow cytometry analysis. (D) The bar graphs represent the mean (± standard error of the mean) of three independent measurements for Annexin V-FITC/propidium iodide stained A375SM cells treated with 1 μM 25-*epi* Ritterostatin G_N1_N ± 10 μM chloroquine for 24 h. (E) Western blot results show expression of the autophagic proteins LC3B-I and LC3B-II in A375 cells treated with 0.5 μM 25-*epi* Ritterostatin G_N1_N for the indicated time points. DMSO, dimethyl sulfoxide (control). *p<0.05.

G_N1_N in combination with chloroquine. Chloroquine is a lysosomotrophic agent known to promote rapid cell death in autophagic cells by inhibiting lysosome acidification and degradation of autophagosomes, thus sensitizing the cells to drug action (48,49). For this experiment, 1 μM 25-*epi* Ritterostatin G_N1_N was added to A375 cells pretreated with 10 μM chloroquine for 1 h. A significant increase in cell death was observed in cells pretreated with chloroquine compared with cells treated with 25-*epi* Ritterostatin G_N1_N alone (Fig. 3C and D). Furthermore, cell death was rapid in the cells pretreated with chloroquine; only 29.3% of cells were viable at 24 h after treatment (Fig. 3C). However, the viability of A375 cells treated with 25-*epi* Ritterostatin G_N1_N or chloroquine alone was very similar to that of the control cells. Western blot analysis of A375 cells treated with 0.5 μM 25-*epi* Ritterostatin G_N1_N showed time-dependent

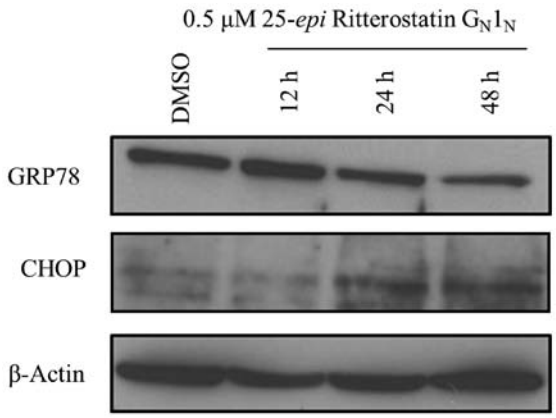

Figure 4. 25-*epi* Ritterostatin G_N1_N inhibits GRP78 expression in melanoma cells. Immunoblotting results showing GRP78 and CHOP expression in A375 cells treated with 0.5 μM 25-*epi* Ritterostatin G_N1_N for 12, 24, or 48 h are shown. DMSO, dimethyl sulfoxide (control).

lipidation of the microtubule-associated autophagy marker protein, LC3B and increased turnover of its phosphotidyl-ethanolamine-conjugated, faster-migrating isoform, LC3B-II (Fig. 3E). The presence of LC3 protein in autophagosomes and increased conversion of LC3-I to LC3-II has been regarded as a key indicator of autophagy (50,51).

Taken together, the increased expression of LC3B-II, collapse of mitochondrial membrane potential, and rapid apoptotic cell death after pretreatment with chloroquine indicate that 25-*epi* Ritterostatin G_N1_N exerts its antitumor effect in melanoma through induction of autophagy.

25-epi Ritterostatin G_N1_N induces endoplasmic reticulum (ER) stress in melanoma cells. Several studies have indicated that ER stress can lead to activation of autophagy in cells (41,42). Glucose-regulated protein 78 (GRP78) is thought to be a major sensor of ER stress and is associated with melanoma progression and increased drug resistance in melanoma and other cancers (18,52-54). Since our findings indicated that autophagy was activated by 25-*epi* Ritterostatin G_N1_N in melanoma cells, we investigated the effect of 25-*epi* Ritterostatin G_N1_N on GRP78 expression. 25-*epi* Ritterostatin G_N1_N triggered a time-dependent decrease in GRP78 expression in A375 cells. (Fig. 4). Noteworthy, an initial activation of GRP78 expression was observed at 12 h after treatment with 25-*epi* Ritterostatin G_N1_N, before decreasing at later time points. This early GRP78 activation might be a result of its initial efforts to contain and neutralize ER stress.

Simultaneously, we observed time-dependent upregulated expression of CHOP, the ER stress marker protein known to mediate cytotoxicity, in cells treated with 25-*epi* Ritterostatin G_N1_N. Taken together, these findings suggest that treatment with 25-*epi* Ritterostatin G_N1_N triggered ER stress, leading to autophagy and cell death.

25-epi Ritterostatin G_N1_N exhibits minimal toxicity in normal melanocytes. To determine whether the effects of 25-*epi* Ritterostatin G_N1_N were specific to melanoma cells, we also treated normal melanocytes with 0.5 and 1 μM 25-*epi* Ritterostatin G_N1_N and performed Annexin V/propidium iodide staining to analyze cell viability (Fig. 5A). We found that 96.6% of normal melanocytes (normalized to control)

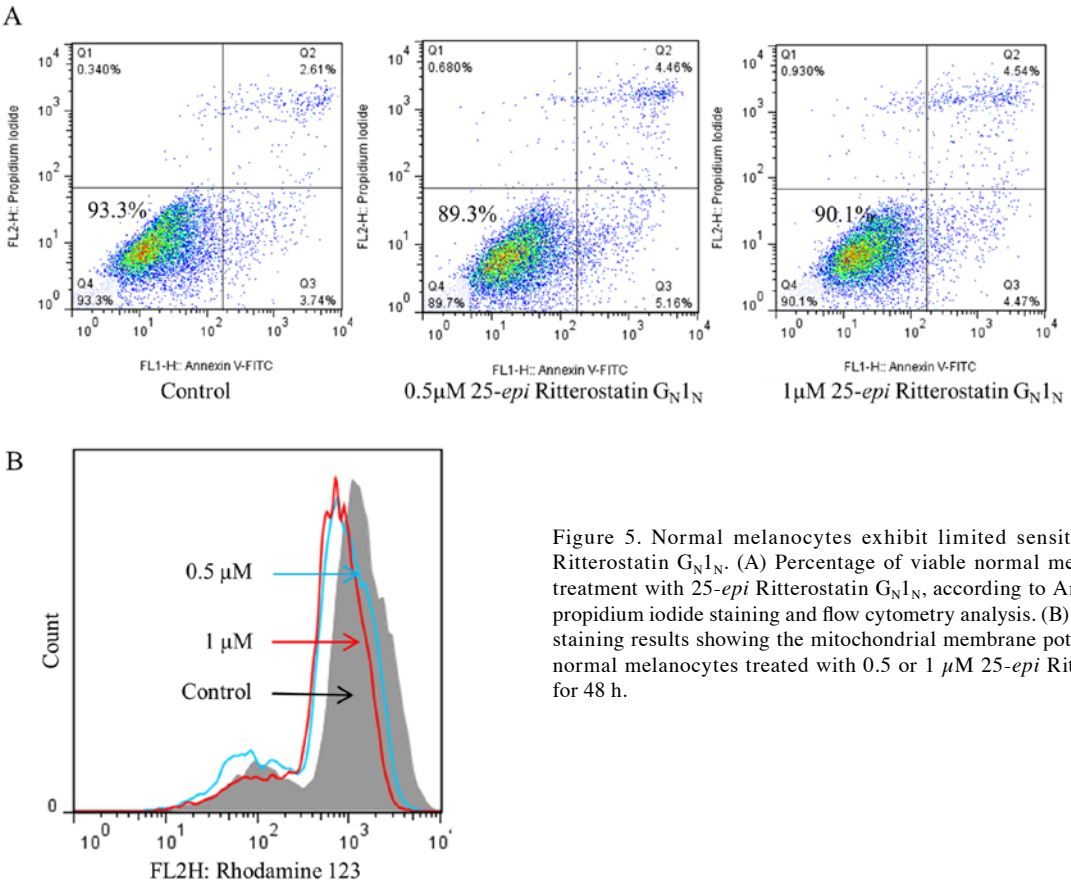

Figure 5. Normal melanocytes exhibit limited sensitivity to 25-*epi* Ritterostatin G_N1_N. (A) Percentage of viable normal melanocytes after treatment with 25-*epi* Ritterostatin G_N1_N, according to Annexin V-FITC/propidium iodide staining and flow cytometry analysis. (B) Rhodamine 123 staining results showing the mitochondrial membrane potential change in normal melanocytes treated with 0.5 or 1 μM 25-*epi* Ritterostatin G_N1_N for 48 h.

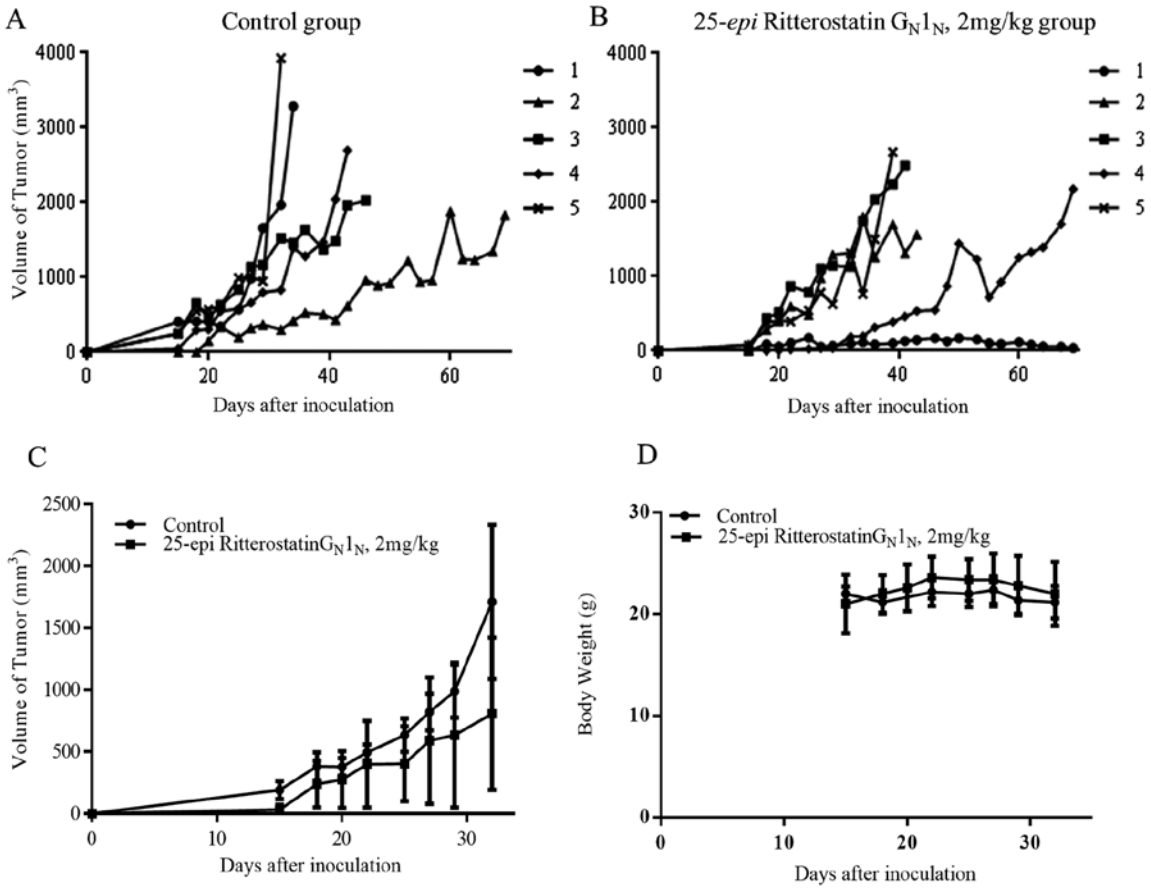

Figure 6. 25-*epi* Ritterostatin G_N1_N exhibits a therapeutic effect *in vivo*. (A) Individual tumor growth curves in the control group (5 mice injected with WM35 PKB cells and treated with phosphate-buffered saline only). (B) Individual tumor growth curves in the treatment group (5 mice injected with WM35 PKB cells and treated with 2 mg/kg 25-*epi* Ritterostatin G_N1_N 3 times per week). (C) Mean tumor growth in the control and treatment groups. (D) Mean body weight in the control and treatment groups. $^*p<0.05$.

were viable after treatment with 1μM 25-*epi* Ritterostatin G_N1_N, compared with 36% of A375 cells treated with 0.5μM 25-*epi* Ritterostatin G_N1_N. Additionally, biochemical analysis for changes in mitochondrial membrane potential indicated that the normal melanocytes were resistant to the effect of 25-*epi* Ritterostatin G_N1_N on mitochondrial membrane integrity (Fig. 5B). Subsequent immunoblotting analysis revealed no changes in the lipidation expression profile of the autophagy marker protein LC3B (data not shown). These findings indicate that the antitumor activity of 25-*epi* Ritterostatin G_N1_N is specific to melanoma cells, with limited toxicity in normal melanocytes.

25-epi Ritterostatin G_N1_N triggers inhibition of tumor growth in vivo. Akt overexpression has been found to lead to rapid tumor growth, increased angiogenesis, and a more pronounced glycolytic mechanism in parental WM35 cells (55). Therefore, we used WM35 cells with activated Akt expression (WM35 PKB cells) as a study model to assess the antitumor activity of 25-*epi* Ritterostatin G_N1_N. WM35 PKB cells ($2x10^6$) were injected subcutaneously into female athymic nude mice, followed by randomizing the mice into two groups of five each. The treatment group received 2 mg/kg 25-*epi* Ritterostatin G_N1_N 3 times per week via intraperitoneal injection, and the control group received PBS. Tumor volume and mouse body weight were recorded. Our results showed that 25-*epi* Ritterostatin G_N1_N substantially suppressed melanoma tumor growth in the mouse xenografts (Fig. 6A-C). In addition, we found that body weight did not differ substantially between the control (Fig. 6D) and the treatment groups, suggesting that 25-*epi* Ritterostatin G_N1_N does not cause any toxic effects in mice.

Discussion

Malignant melanoma is one of the most aggressive forms of cancer, with increasing incidence rates. Although multiple clinical trials have been initiated with novel agents to treat melanoma, very few have been successful, thus highlighting the need to identify potential therapeutic agents through mechanistic studies. Our results demonstrate that 25-*epi* Ritterostatin G_N1_N exhibits antitumor activity in melanoma cells by inducing autophagy and enhanced ER stress, with minimal toxic effects in normal melanocytes, suggesting that further study of 25-*epi* Ritterostatin G_N1_N as a potential treatment for melanoma is necessary.

The chaperone protein GRP78 (also called BiP) acts as the primary sensor of ER stress due to accumulation of unfolded proteins in the ER lumen, which occurs as a result of oxidative stress and cellular toxicity induced by calcium ionophores and other agents (56). During ER stress, GRP78 binds to these unfolded proteins, leading to the release of resident ER

transmembrane proteins such as PERK, ATF6, and IRE1, which then activate downstream signaling cascades promoting global repression of protein synthesis and subsequent restoration of protein function (57-60). GRP78 also supports cell survival by preventing caspase-7 activation and stabilization of mitochondrial function (61,62). In melanoma, GRP78 has been linked to tumor progression and drug resistance (18). Our results showed that 25-*epi* Ritterostatin G_N1_N inhibits GRP78 and activates CHOP expression in a time-dependent fashion, leading to ER stress-mediated cell death.

Autophagy is a cellular self-digestive process that facilitates degradation of long-lived proteins and damaged organelles in cells to release energy and nutrients during stress and starvation conditions. Numerous studies have indicated that autophagy has a tumor-suppressive role in cancer (35). Work by Yue *et al* demonstrated that activation of Beclin-1, the mammalian orthologue for the yeast autophagy-related gene Atg6, inhibited *in vitro* tumor cell proliferation and *in vivo* tumor growth in mice (34), and also Sivridis *et al* investigated the expression of two autophagy-related proteins, Beclin-1 and LC3A, in 79 specimens of nodular cutaneous melanoma (63). Their results confirmed those from other researchers demonstrating that downregulation of autophagic capacity is related to human carcinogenesis (64,65). During autophagy, the light chain protein LC3B is localized to autophagosomes and is therefore considered a hallmark for autophagy. Our results demonstrate that the cytotoxic effect of 25-*epi* Ritterostatin G_N1_N is mediated by autophagy. In addition to increased lipidation of LC3B, we observed a collapse of the mitochondrial membrane potential. Importantly, the combination of 25-*epi* Ritterostatin G_N1_N with the lysosomotrophic agent chloroquine resulted in rapid cell death. Moreover, this induction of autophagy and loss of mitochondrial membrane potential by 25-*epi* Ritterostatin G_N1_N was not observed in normal melanocytes, indicating that 25-*epi* Ritterostatin G_N1_N has therapeutic selectivity to melanoma cells.

To the best of our knowledge, the current study is the first to characterize the *in vitro* and *in vivo* antitumor effects of 25-*epi* Ritterostatin G_N1_N in melanoma. We found that 25-*epi* Ritterostatin G_N1_N inhibits melanoma cell growth by activation of ER stress and autophagy. We also found that intraperitoneal injection of 2 mg/kg 25-*epi* Ritterostatin G_N1_N 3 times per week substantially reduced the growth of melanoma in mice. Importantly, we observed no significant change in body weight between mice treated with 25-*epi* Ritterostatin G_N1_N at this dosage and control mice. These findings indicate that 25-*epi* Ritterostatin G_N1_N could be a promising treatment for melanoma. Nevertheless, additional *in vivo* and clinical studies need to be conducted to ascertain the effectiveness of this compound in inhibiting melanoma growth in humans.

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