Chronic treatment with ginsenoside Rg3 induces Akt-dependent senescence in human glioma cells

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Abstract. Therapy-induced senescence, an irreversible growth arrest, in cancer cells is regarded as a novel functional target that may improve cancer therapy. 20(S)-ginsenoside Rg3 [20(S)-Rg3], a chemical component extracted from Panax ginseng, has recently emerged as an effective anticancer medicine with evident antitumor effects and no observed toxic adverse reactions. We report here that chronic treatment with 20(S)-Rg3 in a sub-lethal concentration induced senescence-like growth arrest in human glioma cells. Glioma cells treated with 20(S)-Rg3 showed high expression of senescence-associated β-galactosidase, followed by upregulation of the CDK inhibitors p21 and p16. Moreover, reactive oxygen species (ROS) generation markedly increased in 20(S)-Rg3-treated cells compared with control cells. Consistently, co-incubation with the antioxidant N-acetyl cysteine interfered with 20(S)-Rg3-induced senescence in glioma cells. In addition, 20(S)-Rg3-induced-activation of Akt was associated with increased ROS levels, and depletion of Akt partially prevented 20(S)-Rg3-induced ROS generation and senescence induction in glioma cells. Furthermore, 20(S)-Rg3-induced senescence was partially rescued when the p53/p21 pathway was inactivated. Our data indicate that 20(S)-Rg3 induces senescence-like growth arrest in human glioma cancer through the Akt and p53/p21-dependent signaling pathways. This is the first report of a pro-senescent effect of 20(S)-Rg3 in cancer cells.

Introduction

It is widely accepted that normal somatic cells have intrinsically limited division capacity and reach a non-proliferative state called cellular senescence. Senescent cells are characterized by irreversible growth arrest, a typical gene-expression profile, hyporesponsiveness to external stimuli, increased acidic β-galactosidase activity, and a flat and enlarged morphology. Most tumors contain cell populations that have escaped the senescence barriers to proliferative potential. This capability, known as immortality, could trigger genomic instability and tumorigenesis. Therefore, cellular senescence has been proposed as a major tumor suppression mechanism (1), and therapy-induced senescence represents a novel functional target that may improve cancer therapy (2-5).

Gliomas are the most common primary brain tumor in adults, and high-grade gliomas are almost universally fatal. Despite recent advances in diagnosis and therapy, including surgical resection followed by radiation and chemotherapy, the prognosis for patients with malignant glioma remain unsatisfactory due to the high recurrence rate and relative drug resistance of high-grade glioma cells (6). Conventional anticancer drugs are associated with relatively strong toxic side-effects and drug resistance.

20(S)-ginsenoside Rg3 [20(S)-Rg3] is an effective medicinal chemical compound extracted from Panax ginseng with a $C_{42}H_{72}O_{13}$ framework and 784.3 Da molecular weight (7). 20(S)-Rg3 has been shown to be safe, and recent evidence from *in vitro* experiments and animal models have demonstrated that 20(S)-Rg3 possesses a variety of anti-mutagenic and cancer-inhibitory properties (8-12). However, the exact molecular mechanism of its antitumor effects remains unclear.

Here, we show that chronic treatment with 20(S)-Rg3 at a sub-apoptotic concentration caused senescence-like growth arrest in U87 glioma cells and that this was partially reliant on 20(S)-Rg3-induced ROS generation and induction of p53/p21. Moreover, we found that Akt plays a critical role in 20(S)-Rg3-induced ROS generation and senescence in glioma cells.

Materials and methods

Materials. U87 glioma cell lines were purchased from American Type Culture Collections (Manassas, VA, USA). These cell lines were cultured in Dulbecco's modified Eagle's medium (HyClone, Logan, UT, USA) supplemented with 10% fetal bovine serum and 25 U/ml penicillin/streptomycin. Anti-phospho-p53, and phospho-Akt polyclonal antibody were

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purchased from Cell Signaling Inc. (Danvers, MA, USA). Antibodies for p21, p27, β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Secondary horseradish peroxidase-conjugated anti-rabbit and anti-mouse antibodies were from Zymed Laboratories (San Francisco, CA, USA); chemiluminescent detection systems were from Pierce (Rockford, IL, USA); and Lipodin-Pro protein transfection reagent was from Abbiotec (San Diego, CA, USA). Small interfering RNA duplexes against Akt, p53, and p21 were purchased from Dharmacon Co. Transfection reagent (Oligofectamine™ 2000) was purchased from Invitrogen (Eugene, OR, USA). N-acetyl-l-cysteine and doxorubicin were obtained from Calbiochem (San Diego, CA, USA), and 2', 7'-dichlorofluorescein diacetate and MitoSOX was purchased from Invitrogen (Carlsbad, CA, USA). 20(S)-ginsenoside Rg3 were obtained from International laboratory (USA). All other biochemical reagents were from Sigma or Invitrogen.

SA β-galactosidase staining. Cellular senescence of cells was confirmed by a senescence-associated β-galactosidase activity assay as described by Dimri *et al* (13). After being grown in a semi-confluent state, senescence-associated β-galactosidase, pH 6.0, activity was examined. Cells were washed with phosphate-buffered saline (PBS) and fixed with 2% paraformaldehyde containing 0.2% glutaraldehyde in PBS for 5 min at room temperature (RT). After washing with PBS, cells were incubated with β-galactosidase reagent [1 mg/ml 5-bromo-4-chloro-3-indolyl-β-d-galactopyranoside (X-gal), 40 mm citric acid/sodium phosphate buffer, pH 6.0, 5 mm potassium ferrocyanide/potassium ferricyanide, 150 mm NaCl, 2 mm $MgCl_2$] at 37˚C.

Western blot analysis. Akt, p16, p21, p27 and p53 band were determined by western blotting. Briefly, cells were lysed with lysis buffer [50 mM Tris pH7.4, 150 mM NaCl, 1 mM EDTA pH 8.0, 1 mM protease inhibitor (Roche), 1 mM PMSF, 1 mM NaF, 1 mM sodium orthovanadate], and protein contents were determined using Bradford reagent. Equal amounts of protein (40 μg) were then separated by SDS-PAGE and transferred to nitrocellulose membranes (Schleicher & Schuell BioScience Inc). After blocking with TBS containing Tween-20 in the presence of 2.5% nonfat dry milk, the membranes were incubated with the primary antibodies at 4˚C for 16 h. Secondary antibodies were added for 1 h at RT. The antibody-antigen complexes were detected using the ECL detection system (Pierce).

Analysis of apoptosis by Annexin V staining. The amount of phosphatidylserine (PS) on cell surfaces (a measure of apoptosis) was determined using an Annexin V-FITC apoptosis detection kit (Abcam, USA), according to the manufacturer's instructions. Briefly, following H_2O_2 or staurosporine treatment, cells were harvested and washed twice with PBS. The cells were then resuspended in 0.5 ml of cold 1X binding buffer (10 mM Hepes, pH 7.4, 150 mM NaCl, 2.5 mM $CaCl_2$, 1 mM $MgCl_2$, 4% BSA), and 2.5 μl of Annexin V-FITC (fluorescein isothiocyanate) was added. Following incubation for 15 min, stained cells were analyzed by flow cytometry (Becton-Dickinson FACSorter) using CELLQuest software. Error bars represent standard deviations of the means.

RNA interference and transfection. U87 cells were plated in a 100 mm dish, transfected with 0.5 nmol of siRNA and Oligofectamine reagent in serum-free medium and incubated for 4 h at 37˚C in a CO_2 incubator. Following incubation, the cells were supplemented with growth medium containing 10% fetal bovine seerum. Cells were harvested after 72 h.

Measurement of intracellular ROS level. Cells were stained with 50 μM of DCF-DA for 30 min and then harvested. For quantitation of mitochondrial ROS, the cells were incubated with 0.2 μM MitoSOX for 30 min at 37˚C, washed with PBS, trypsinized, collected in PBS, and analyzed on a FACSCalibur. To examine the effect of N-acetylcysteine (NAC), cells were treated with 20 mM of NAC for 2 days.

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) assays. Cells were plated in 24-well tissue culture plates and allowed to attach overnight. MTT was added to each well to a final concentration of 200 μg/ml, and cells were incubated for 4 h. After removing the medium completely, the formazan product was solubilized with dimethylsulfoxide. Optical densities (OD) were measured at 490 nm. Each experiment was performed three times. Error bars represent standard deviations of the means.

Statistical analysis. Data are presented as the means ± S.D. and p-values were calculated using a Student's t-test. A value of $p<0.05$ was considered to indicate a statistically significant difference. All data presented are the representative of at least three separate experiments.

Results

Low or high doses of 20(S)-Rg3 induces either senescence or apoptosis, respectively, in glioma cells. To investigate the growth-regulatory activity of 20(S)-Rg3 in glioma cells, we treated glioma cells with various concentrations of 20(S)-Rg3. Exposure of U87 cells to 20(S)-Rg3 for 3 days resulted in a dose-dependent inhibition of cell proliferation (Fig. 1A and B). Concentrations of ≥10 μM 20(S)-Rg3 suppress the growth of U87 cells (Fig. 1B) whereas concentrations <10 μM exhibit little effect (data not shown). Next we examined the involvement of apoptosis in 20(S)-Rg3-induced growth arrest. Direct detection of apoptosis with Annexin V staining showed that apoptotic cells were not significantly increased in a sublethal dose of 20(S)-Rg3 (10 μM, 20 μM) compared with a high dose of 20(S)-Rg3 (50 μM, 100 μM) (Fig. 1C). We further examined the effects of chronic exposure of sub-apoptotic 20(S)-Rg3 on cell proliferation. Direct cell count assay indicated that the growth of U87 cells was gradually inhibited by chronic exposure of a sublethal dose of 20(S)-Rg3, which became more obvious on day 9 (Fig. 1D). 20(S)-Rg3 (20 μM) completely suppressed cell proliferation for at least nine days, while inducing only modest levels of cell death (data not shown). To test the possibility that cell growth arrest in response to 20(S)-Rg3 was caused by the induction of cellular senescence, we treated glioma cells with various sublethal concentrations of 20(S)-Rg3 and examined the expression of senescence-associated-β-galactosidase (SA-β-gal), a commonly accepted marker of senescence. As

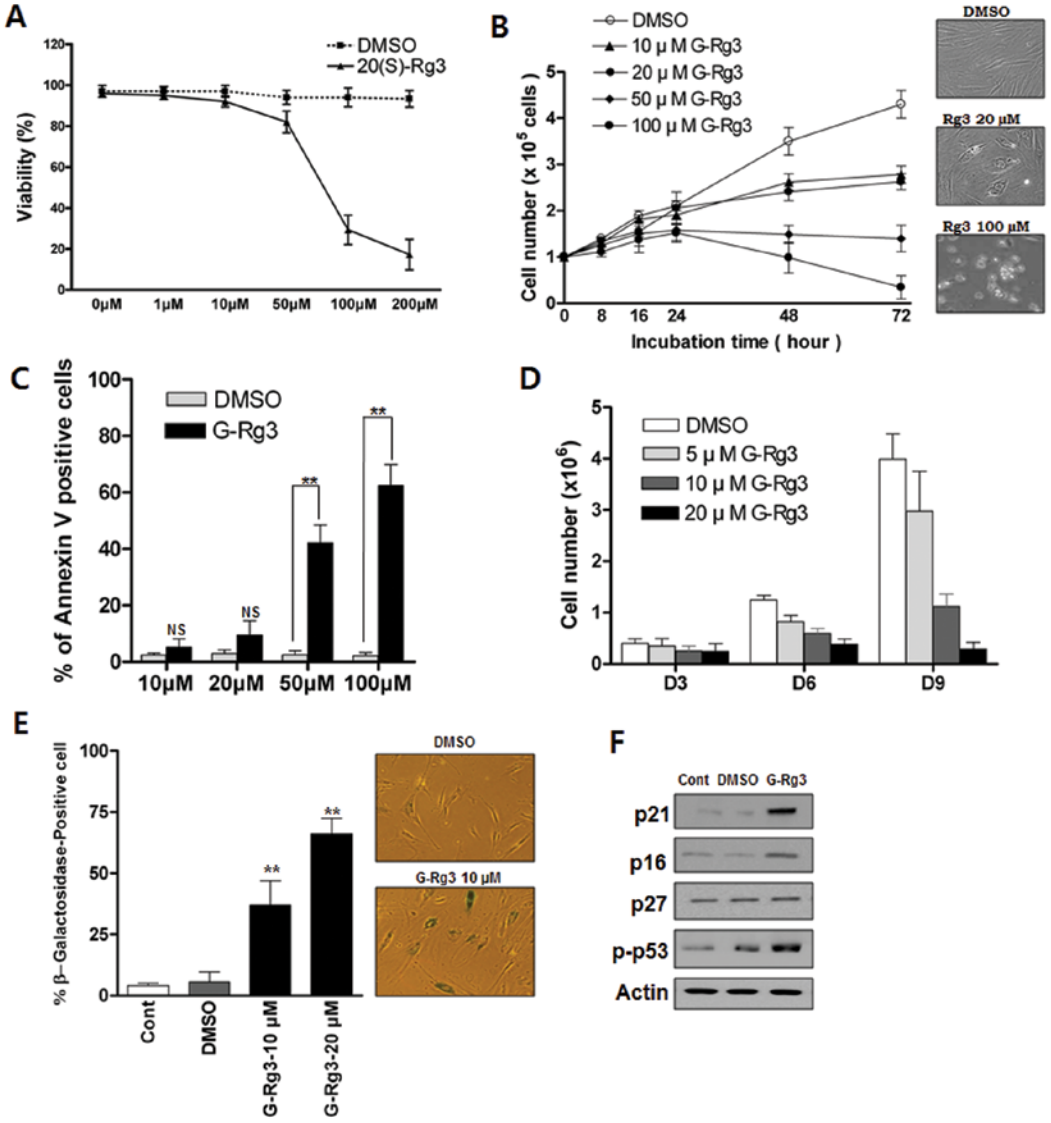

Figure 1. Chronic treatment of 20(S)-Rg3 in U87 cells induces cell senescence. (A) U87 cells were treated for 72 h with the indicated concentrations of 20(S)-Rg3 and the relative cell viability was determined by measuring the absorbance or optical density at 490 nm (OD490) in the MTT staining assay. Error bars represent the SE from three independent experiments. (B) U87 cells were treated with the indicated concentrations of 20(S)-Rg3. Cell numbers were counted at the indicated time points. Data are the mean ± SEM of three independent experiments. (C) U87 cells were treated with the indicated concentrations of 20(S)-Rg3. Cells were stained with Annexin V-FITC. Annexin V determined by flow cytometry. Error bars represent the SE from three independent experiments. A double asterisk (**) denotes P<0.01 in Student's t-test. (D) U87 cells were treated with the indicated concentrations of 20(S)-Rg3. Cell numbers were counted at the indicated time points. Data are the mean ± SEM of three independent experiments. (E) U87 cells were treated with empty control, DMSO, 10 μM and 20 μM 20(S)-Rg3 for 9 days. Cells were analyzed for the senescence-associated β-galactosidase activity and stained cells were counted. Data are the mean ± SEM of three independent experiments. (F) U87 cells were treated with empty control, DMSO, or 20 μM 20(S)-Rg3. After 9 days, cells were collected and the expression levels of p21, p16, p27 and p-p53 were analyzed by western blotting. β-actin was used as a loading control.

shown in Fig. 1E, ~67% of U87 cells after chronic 20(S)-Rg3 treatment at a 20 μM concentration were stained positively compared with only ~5% cells with positive staining in the DMSO control group (Fig. 1E). Cells that were positive for SA-β-gal showed an enlarged and flattened morphology that was consistent with cellular senescence (Fig. 1E). These findings indicated that 20(S)-Rg3 could induce senescence in addition to apoptosis. An immunoblot analysis showed that the level of p53 and cyclin-dependent kinase inhibitor p21CIP were markedly increased in 20(S)-Rg3-treated cells (Fig. 1F). However, there was no increase in the expression of p27Kip1 and only a moderate elevation of p16INK4 in 20(S)-Rg3-treated U87 cells (Fig. 1F).

20(S)-Rg3 triggers senescence by an increase of mitochondrial ROS level. As reactive oxygen species (ROS) are well-known inducers of senescence, we evaluated whether treatment of glioma cells with 20(S)-Rg3 increases oxidative stress. ROS levels, as assessed by dichlorofluorescein (DCF), were increased after 20(S)-Rg3 treatment of U87 glioma cells (Fig. 2A). As mitochondrial ROS are the major cellular source of ROS, we also examined the fluorescence of MitoSOX Red as a mitochondrial superoxide indicator. Fluorescence intensity of MitoSOX Red was significantly increased in U87 cells, consistent with increased intracellular ROS levels (Fig. 2B). To address the role of ROS in senescence induced by 20(S)-Rg3, we treated U87 cells with the ROS scavenger N-acetyl-l-

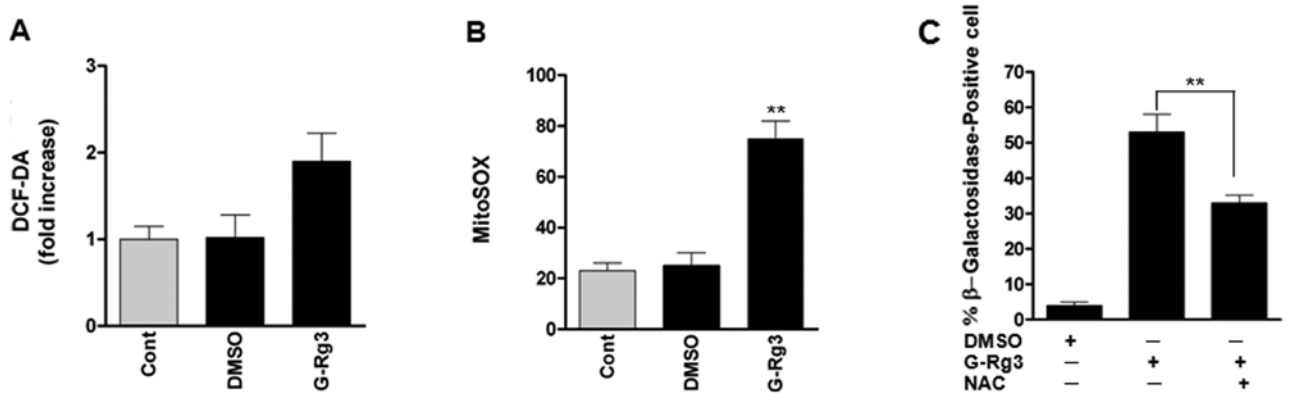

Figure 2. 20(S)-Rg3-induced senescence largely dependent of ROS. (A) U87 cells were treated with empty control, DMSO, and 20 μM 20(S)-Rg3 for 9 days. Cells were stained with dichlorofluorescein diacetate (DCF-DA), fixed and immediately analyzed by FACS. (B) U87 cells were treated with empty control, DMSO and 20 μM 20(S)-Rg3 for 9 days. Cells were used for flow cytometric analysis of mitochondrial ROS levels using MitoSOX. A double asterisk (**) denotes P<0.01 compared with control cells (ANOVA, Dunnett was used as post-test). (C) U87 cells were treated with DMSO and 20 μM 20(S)-Rg3 alone, or pretreated with 20 mM NAC for 16 h. Cells were analyzed for the senescence-associated β-galactosidase activity and stained cells were counted. A double asterisk (**) denotes P<0.01 in Student's t-test. The data are the mean ± SEM of three independent experiments.

cysteine before 20(S)-Rg3 exposure. NAC treatment reduced percentages of SA-β-gal-positive cells (Fig. 2C). These data suggest that elevated ROS levels contribute to senescence induction by 20(S)-Rg3.

Activation of the Akt contributes to senescence induction by 20(S)-Rg3 and is associated with elevation of ROS. It has been reported that PI3K/Akt signaling cascades are frequently deregulated in glioma and Akt activation is involved in glioma cell senescence (14,15). To examine whether Akt activity is involved in 20(S)-Rg3-induced senescent glioma cells, we measured phospho-Akt levels. U87 cells treated with 20 μM 20(S)-Rg3 were characterized by increases in phospho-Akt (Fig. 3A). To address whether the increase in levels of phospho-Akt is important for the induction of senescence in 20(S)-Rg3-treated human glioma cells, we employed si-RNA against Akt (Fig. 3B). As shown in Fig. 3C, depletion of Akt significantly decreased the SA-β-gal activity compared with control siRNA-infected cells. As ROS generation has been shown to be involved in the Akt in glioma cells (16), we next examined whether the increased ROS levels caused by 20(S)-Rg3 occurred as a result of activation of Akt or was a secondary effect of this drug. Transfection of Akt siRNA reduced ROS generation by approximately 32% (Fig. 3D). These data strongly suggest that AKT may play an important role in regulating cellular ROS status and senescence in glioma cells.

Role of the p53/p21 pathway in the senescence of human glioma cells treated with 20(S)-Rg3. The p53/p21 signaling pathway is a major senescence-triggering pathway in response to various stresses. As shown in Fig. 1F, the treatment of U87 cells with 20(S)-Rg3 significantly increased the expression levels of p53 and p21. Therefore, we examined whether 20(S)-Rg3-mediated senescence requires signaling of the p53/p21 pathway in U87 cells. We transfected U87 cells with p53 and/or p21 si-RNA and looked for senescent phenotypes. As shown in Fig. 4B, p53 siRNA transfected cells showed a significant decrease in SA-β-Gal activity. Cells transfected with p21 siRNA or co-transfected p53 siRNA showed similar results with p53 depleted cells (Fig. 4B). These data suggest that the p53/p21 plays an essential role in the maintenance of senescence triggered by 20(S)-Rg3.

Discussion

The induction of apoptosis was previously defined as the mechanism of 20(S)-Rg3 action for the growth arrest of various tumor cells (9,10,12). In agreement with these reports, our data showed that a high concentration of 20(S)-Rg3 could induce apoptosis in glioma cells. Here, we showed that 20(S)-Rg3 also induces cellular senescence after prolonged treatment at sub-lethal drug concentrations.

Cellular senescence, along with mitotic catastrophe and apoptosis, has been proposed as one of the mechanisms mediating the antitumor effects of chemotherapies (2-5). Tumor cells were considered to have lost the ability to senesce, as cellular senescence would provide an important barrier to tumorigenesis (17,18). Previous data have shown that cancer cell senescence can be induced by treatment with chemotherapeutic drugs, radiation, genetic manipulation, or other agents (19). The induction of senescence by chemotherapeutic drugs was dose-dependent and correlated with the growth arrest observed in various cells (2,20-22). In contrast to cell death, however, senescence leaves tumor cells alive and physiologically active (3). In human tumor cell lines treated *in vitro* and *in vivo* with differentiating agents, terminal proliferative arrest with minimal toxicity to normal cells has been observed (1,23). Therefore, understanding the mechanism of premature senescence is crucial for the development of safer and more effective cancer treatment strategies.

Gain-of-function mutations in the PI3K/Akt signaling pathway are frequently found in human glioblastomas (14). In mammalian cells, the activation of Akt has been reported to induce proliferation and survival, thereby promoting tumorigenesis (24-26). However, previous studies demonstrated that the constitutive activation of Akt promotes senescence-like arrest of cell growth through the regulation of intracellular ROS levels (15,16,27,28). Thus, Akt-induced growth arrest may be another antitumorigenic mechanism, similar to

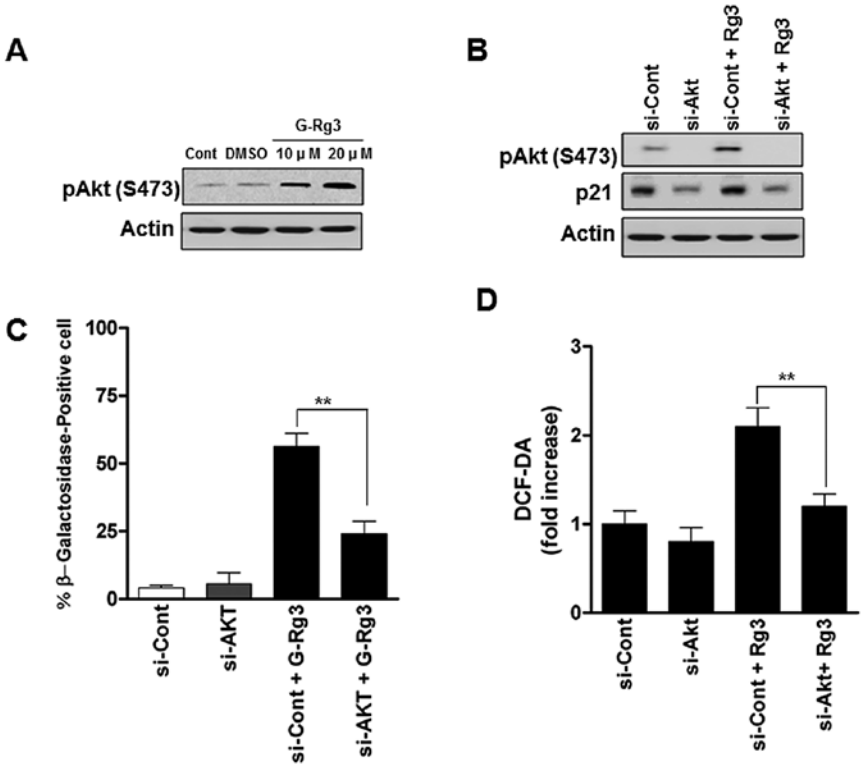

Figure 3. Akt is essential for the induction of 20(S)-Rg3-induced glioma cell senescence. (A) U87 cells were treated with empty control, DMSO, 10 μM and 20 μM 20(S)-Rg3 for 9 days. Cells were collected and the expression levels of pAkt (S473) were analyzed by western blotting. β-actin was used as a loading control. (B) U87 cells transfected with 200 nM siRNA for 72 h. U87 cells transfected with 200 nM siRNA every 3 days for 12 days. Three days after the first transfection, cells were treated with 20 μM 20(S)-Rg3 for 9 days. Cells were collected and the expression levels of pAkt, p21 were analyzed by western blotting. β-actin was used as a loading control. (C) U87 cells transfected with 200 nM siRNA every 3 days for 12 days. Three days after the first transfection, cells were treated with 20 μM 20(S)-Rg3 for 9 days then cells were used for β-galactosidase analysis and stained cells were counted. (D) U87 cells transfected with 200 nM siRNA against Akt every 3 days for 9 days, followed by the addition of 20 μM 20(S)-Rg3 for 9 days. The cells were then stained with dichlorofluorescein diacetate (DCF-DA), fixed and immediately analyzed by FACS. The control scrambled siRNA was labeled as 'Cont'. A double asterisk (**) denotes P<0.01 in Student's t-test. The data are the mean ± SEM of three independent experiments.

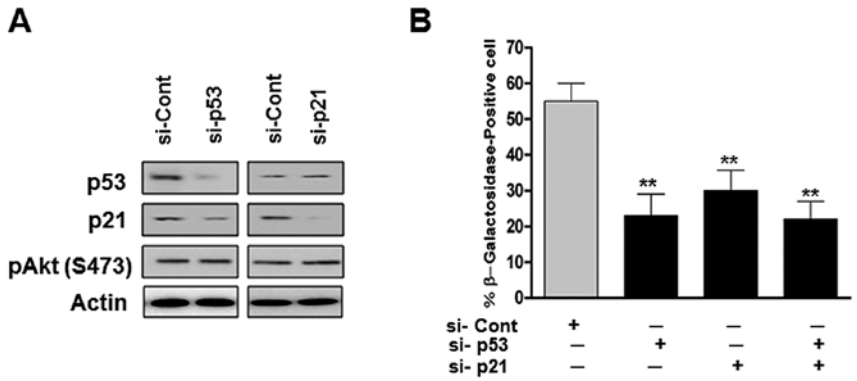

Figure 4. Inactivating the p53/p21 pathway partially protects against 20(S)-Rg3-induced senescence. (A) U87 cells were transfected with si-p53, si-p21 or the control siRNA. After 72 h, cells were collected and the expression levels of p53, p21 and pAkt wre analyzed by western blotting. β-actin was used as a loading control. (B) U87 cells were transfected with 200 nM siRNA every 3 days for 12 days. Three days after the first transfection, cells were treated with 20 μM 20(S)-Rg3 for 9 days then cells were used for β-galactosidase analysis and stained cells were counted. The control scrambled siRNA was labeled as 'Cont'. A double asterisk (**) denotes P<0.01 compared with control cells (ANOVA, Dunnett was used as post-test). The data are the mean ± SEM of three independent experiments.

Ras-induced senescence (29,30). In this study, we revealed that 20(S)-Rg3-induced senescence is associated with Akt activation, and depletion of Akt partially prevented Rg3-induced ROS generation and senescence induction in glioma cells. Elevated intracellular ROS levels have been widely accepted as triggers of cellular senescence (31-33). Senescent cells increase ROS generation (34,35), and premature cellular senescence can be readily induced by sub-lethal doses of pro-oxidants (36,37). In the present study, ROS levels were elevated during 20(S)-Rg3-induced senescence, and the ROS

scavenger NAC reverted 20(S)-Rg3-induced senescent phenotypes. However, decreases in intracellular ROS levels by NAC did not affect 20(S)-Rg3-induced Akt activation (data not shown). These data suggest that 20(S)-Rg3 induces senescence by a mechanism largely dependent on Akt/ROS signaling. Furthermore, 20(S)-Rg3-induced arrest was accompanied by substantial increases in p53 and p21, and the depletion of p53 or p21 prevented 20(S)-Rg3-induced premature senescence in U87 cells. Thus, senescence may occur in a manner dependent on the p53/p21 pathway. However, further studies are required to determine the exact mechanism involved.

In summary, our study presented a new anticancer mechanism of 20(S)-Rg3 action by inducing senescence. Chronic treatment with 20(S)-Rg3 at a sub-apoptotic concentration elicits ROS generation via Akt activation and p53/p21-dependent senescence-like growth arrest in glioma cells. Our study provides useful insight for the future development of 20(S)-Rg3 as a novel class of anticancer agents.

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