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Amurensin G induces autophagy and attenuates cellular toxicities in a rotenone model of Parkinson's disease

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ABSTRACT

Although Parkinson's disease is a common neurodegenerative disorder its cause is still unknown. Recently, several reports showed that inducers of autophagy attenuate cellular toxicities in Parkinson's disease models. In this report we screened HEK293 cells that stably express GFP-LC3, a marker of autophagy, for autophagy inducers and identified amurensin G, a compound isolated from the wild grape (*Vitis amurensis*). Amurensin G treatment induced punctate cytoplasmic expression of GFP-LC3 and increased the expression level of endogenous LC3-II. Incubation of human dopaminergic SH-SY5Y cells with amurensin G attenuated the cellular toxicities of rotenone in a model of Parkinson's disease. Amurensin G inhibited rotenone-induced apoptosis and interfered with rotenone-induced G2/M cell cycle arrest. In addition, knockdown of beclin1, a regulator of autophagy, abolished the effect of amurensin G. These data collectively indicate that amurensin G attenuates cellular toxicities through the induction of autophagy.

1. Introduction

Autophagy involves the degradation and recycling of intracellular components of eukaryotic cells and plays a role in the degradation of long-lived proteins and organelles [1]. Targeted intracellular components are enclosed in an autophagosome, which fuses with a lysosome for degradation or recycling of its contents. Autophagy is activated by various stimuli such as nutrient depletion, the accumulation of damaged organelles, and infection with cytoplasmic pathogens. Autophagy can be monitored by microscopic and biochemical methods, and the microtuble-associated protein light chain 3 (LC3) protein is a useful autophagy marker because it associates with the isolation membrane and spherical autophagosomes. The cellular localization of LC3 can be easily visualized using a recombinant protein fused with green fluorescent protein (GFP) [2]. Punctuate GFP-LC3 spots in the cytoplasm reveal autophagosome formation, and the number of GFP-LC3 spots represents the level of autophagy induction. Western blot analysis with anti-LC3 antibody has also been developed to monitor autophagy. Cells contain two forms of LC3. Conjugation of LC3-I with the phosphatidyl ethanolamine (PE) generates LC3-II, which tightly associates with the autophagosome membrane [2]. The level of LC3-II represents the current autophagic activity, which can be easily monitored since the migration of LC3-II (18 kDa) is faster than that of LC3-I (16 kDa) [3].

Parkinson's disease (PD) is a common neurodegenerative disorder that is characterized by a selective and progressive loss of dopaminergic neurons and the presence of Lewy body inclusions in neurons of the substantia nigra [4]. The molecular mechanism of PD is not clearly understood; however, reduced activity of complex I of the electron transport chain in mitochondria has been implicated in PD pathogenesis [5]. The mitochondrial complex I inhibitor rotenone has been shown to injure nigral dopaminergic neurons and to mimic the biochemical lesions of PD in vivo and in vitro [5,6]. Rotenone produces reactive oxygen species and later induces α-synuclein aggregates, a major component of Lewy body inclusions [7,8]. It is reported that α-synuclein aggregates can be targeted into the autophagic vesicle and degraded by autophagy [9], therefore autophagy enhancers have been considered for potential therapeutic use and several reports have shown that enhancing autophagy alleviates Parkinson's disease [7,10–12]. For example, rapamycin, an inhibitor of mTOR (mammalian target of rapamycin) kinase, activates autophagy, thereby inhibiting rotenone-induced apoptosis [9,13]. These results collectively suggest that autophagy activators have potential applications in the prevention and treatment of Parkinson's disease.

Vitis amurensis, a wild grape, is widely distributed in East Asia and its fruit is used for juice and wine [14]. The root and stem of *V. amurensis* have been used as traditional medicines to treat cancer and pain [15]. Recently, oligostilbenes with anti-inflammatory

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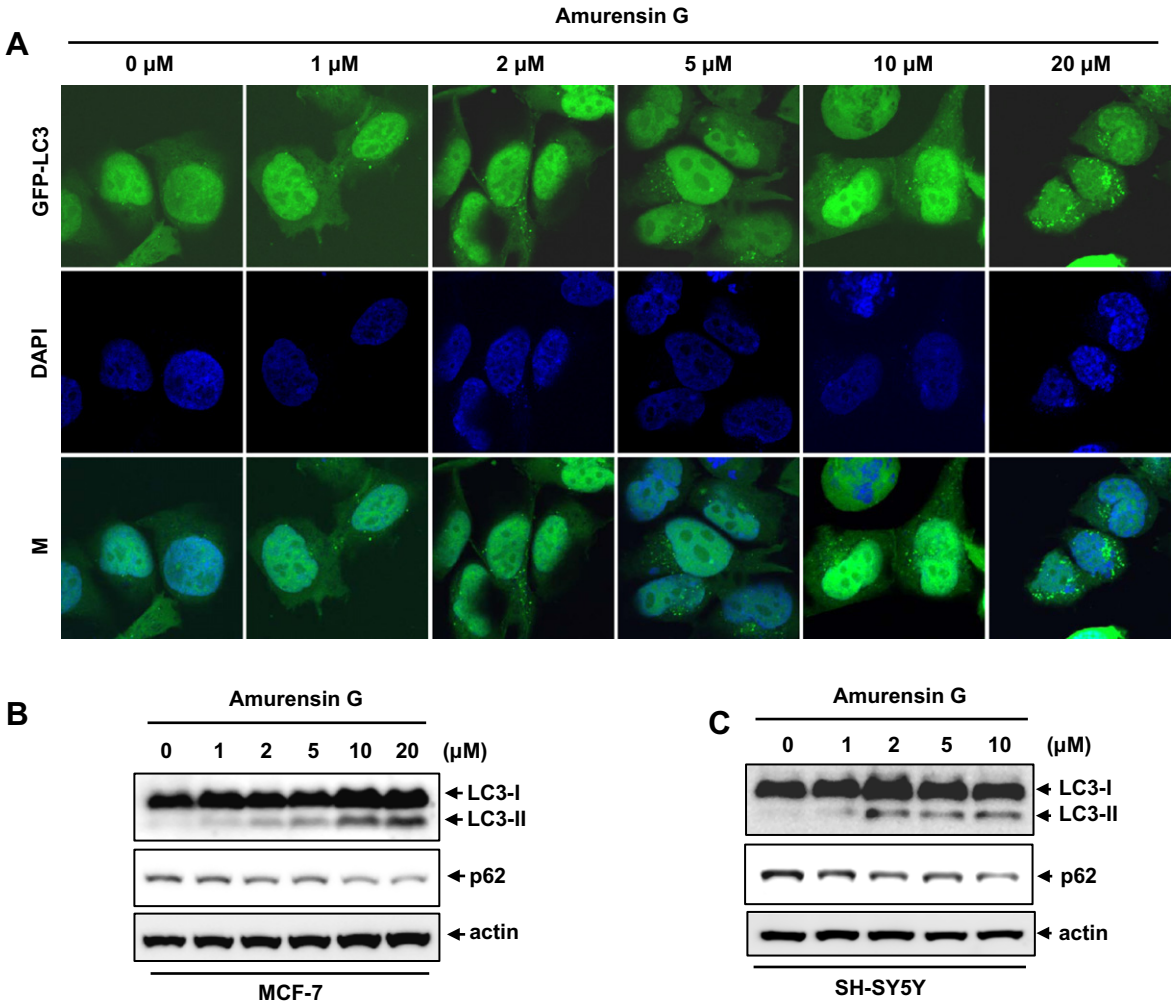

Fig. 1. Amurensin G induced autophagy. (A) HEK293 cells stably expressing GFP-LC3 (GFP-LC3 cells) were treated with the indicated concentrations (0, 1, 2, 5, 10, and 20 μM) of amurensin G for 24 h. Cells were fixed and examined by confocal microscopy (bars = 10 μm). (B) MCF7 breast cancer cells were treated with amurensin G for 24 h and the expression of autophagosome markers was examined by Western blotting. The level of LC3-II was increased and the level of p62 was decreased in cells treated with amurensin G. (C) Human dopaminergic SH-SY5Y neuronal cells were treated with amurensin G for 24 h. Cell lysates were subjected to Western blot analysis with the indicated antibodies.

activity were isolated from the root of *V. amurensis* and their structures shown to contain resveratrol oligomers [16]. One of these resveratrol oligomers, amurensin G, has been reported to have anti-cancer effects including induction of apoptosis, down-regulation of the multidrug resistance gene, and inhibition of angiogenesis [17–19].

In this report, we established an assay system using HEK293 cells stably expressing the autophagosomal marker GFP-LC3. We screened for autophagy activity among 100 single plant compounds and found that amurensin G is a potent autophagy inducer. Furthermore, amurensin G inhibited rotenone-induced apoptosis and attenuated neurotoxicity in a rotenone model of Parkinson's disease.

2. Materials and methods

2.1. Plant materials

Amurensin G is prepared from the stem of *V. amurensis* as described previously [18]. Amurensin G. Brown amorphous powder; mp 263–264 °C; [a]25 + 28°(*c* 0.1, MeOH); UV (MeOH) l_{max} (log *e*) 213 (3.80), 217 (3.95), 224 (4.00), 282 (4.32) nm; IR (film) v_{max} 3320, 1610, 1510, 1180, 1040, 840 cm^{-1}; ^{1}H and ^{13}C NMR were in accordance with reported data; EI-MS *m*/*z* 681 $[M + H]^+$ (Calc. for $C_{42}H_{32}O_9$).

2.2. Cell culture and cell proliferation assay

HEK293, MCF7, and SH-SY5Y cells were grown in DMEM medium (Welgene, Korea) supplemented with 10% fetal bovine serum (Gibco, Grand Island, NY, USA). A HEK293 stable cell line expressing GFP-LC3 was generated as described previously [23] and the GFP-LC3 plasmid was provided by Yoshimori [20]. Cell proliferation was measured using the [4,5-dimethylthiazol-2-yl]-2,5-diphenyltrazolium bromide (MTT) assay. Briefly, cells were seeded in a 24-well plate and pretreated with amurensin G for 12 h. Cells were treated with rotenone and cell proliferation was examined using the MTT assay.

2.3. Annexin V staining and cell cycle analysis

Apoptosis induction was examined by annexin V staining. FITC-conjugated annexin V (annexin V-FTIC) was purchased from KOMA Biotechnology (Seoul, Korea). SH-SY5Y cells were detached from the plate and incubated with annexin V-FITC for 10 min. The stained cells were analyzed in a FACSCalibur (Beckton–Dickinson,

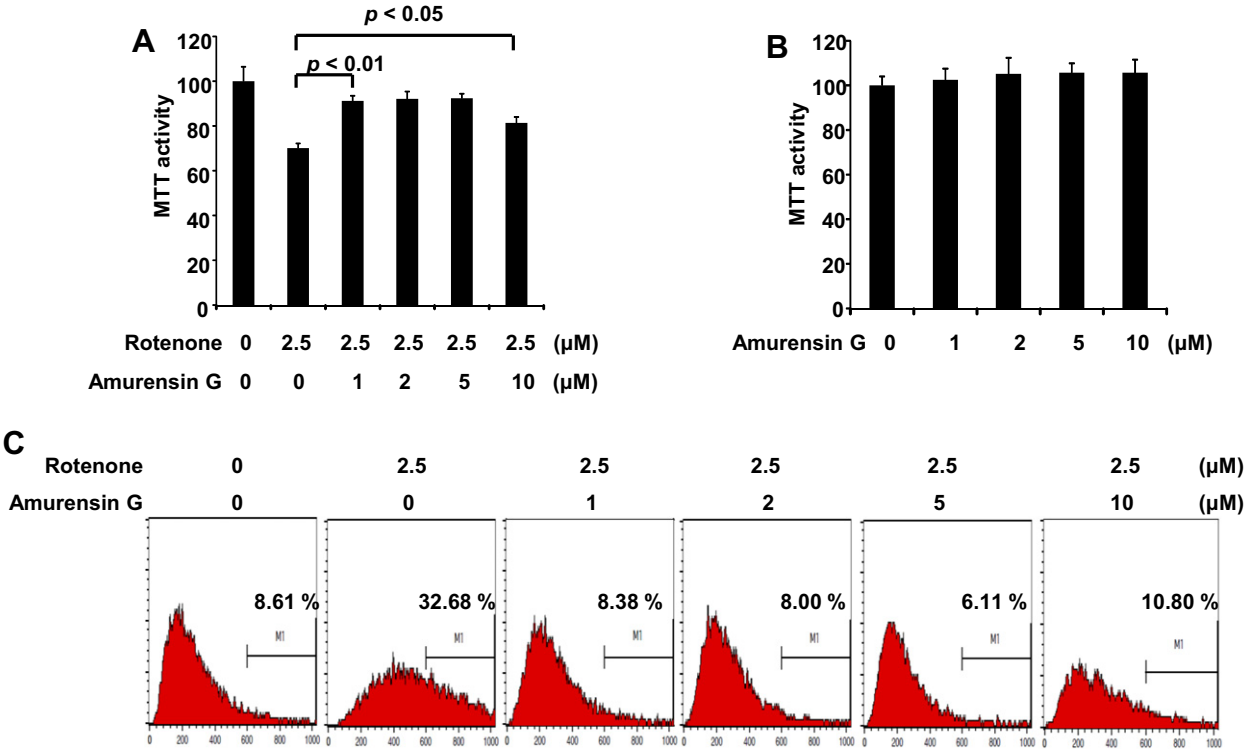

Fig. 2. Amurensin G inhibited rotenone-induced apoptosis. (A) SH-SY5Y cells were incubated with the indicated concentration of amurensin G for 24 h, and cells were treated with rotenone for 12 h. Cell viability was determined by MTT assay. The MTT assay was performed in triplicate and the standard deviation is shown. (B) Treatment with amurensin G alone is not cytotoxic. SH-SY5Y cells were incubated with the indicated concentrations of amurensin G for 24 h and cell viability was determined by MTT assay. (C) Amurensin G reduced the induction of apoptosis. SH-SY5Y cells were treated sequentially with amurensin G for 24 h and rotenone for 12 h, stained with annexin V-FITC, and analyzed by flow cytometry (10,000 cells/sample).

Mountain View, CA, USA). For cell cycle analysis, SH-SY5Y cells were washed with phosphate buffered saline (PBS) and fixed with 70% ethanol. After centrifugation, cells were washed and resuspended in PBS containing 50 μg/ml propidium iodide (PI) and 10 mg/ml RNase A (Sigma, St. Louis, MO, USA). Cells were analyzed in a FACSCalibur. At least 10,000 cells per sample were analyzed.

2.4. Western blotting

The cells were harvested and lysed in lysis buffer (150 mM NaCl, 50 mM HEPES [pH 8.0], 0.5% NP-40) containing a protease inhibitor cocktail (Roche, Mannheim, Germany). For protein immunoblot analysis, polypeptides in whole cell lysates were resolved by SDS–PAGE and transferred to nitrocellulose membrane filters. Detection was conducted with a 1:2000 or 1:5000 dilution of primary antibody using an enhanced chemiluminescence (ECL) system. The images were acquired using Chemidoc-it 410 imaging system (UVP, Upland, CA). To detect insoluble ubiquitinated protein, cells were lysed in lysis buffer and the insoluble pellet was lysed with buffer containing 8 M urea. Cell lysates were boiled and subject to Western blotting. The antibodies for LC3 and p62 were purchased from Sigma, the antibody for beclin-1 from Cell Signaling Technology (Danvers, MA, USA), and the antibody for α-synuclein from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

2.5. Immunofluorescence and confocal microscopy

GFP-LC3 cells were grown on sterilized glass coverslips. After drug treatment, cells were fixed with 4% paraformaldehyde and stained with DAPI solution. For immunostaining, cells were blocked with 10% goat serum in PBS and stained with a 1:500 dilution of primary antibody in PBS and then reacted with 1:1000 dilution of Alexa 488-conjugated secondary antibody (Invitrogen, Carlsbad, CA, USA). Finally, slides were washed three times with PBS and mounted in mounting medium (Vector, Burlingame, CA, USA). Antibody for α-synuclein was purchased from Cell Signaling Technology (Beverly, MA, USA). Images were captured with a Carl Zeiss LSM710 confocal microscope (Oberkochem, Germany).

2.6. RNA interference of beclin-1

Small interfering RNA (siRNA) was purchased from ST. PHARM (Seoul, Korea). The nucleotide sequence of beclin-1 siRNA #1 was 5′-GGA CAA CAA GUU UGA CCAU-3′ and that of beclin-1 siRNA #2 was 5′-CAG GAA CUC ACA GCU CCAU-3′. Transfection of siRNA into SH-SY5Y cells was conducted using Lipofectamine RNAiMAX reagent (Invitrogen) in accordance with the manufacturer.

3. Results

3.1. Amurensin G induces autophagy

Recent data indicate that the activation of autophagy alleviates Parkinson's disease. We screened for novel autophagy inducing compounds using HEK293 cells that stably express GFP-LC3 (GFP-LC3 cells) [3]. When autophagy is induced, the cytoplasmic punctate staining of GFP-LC3 is distinctive from staining in the control (data not shown). We aimed to treat GFP-LC3 cells with up to 100 different compounds isolated from plants and examine GFP-LC3 staining using fluorescent microscopy. In the middle of screening we found that autophagy was induced by amurensin G, which is isolated from wild grape (*V. amurensis*). We next treated GFP-LC3 cells with various concentrations (0, 1, 2, 5, 10, and 20 μM) of amurensin

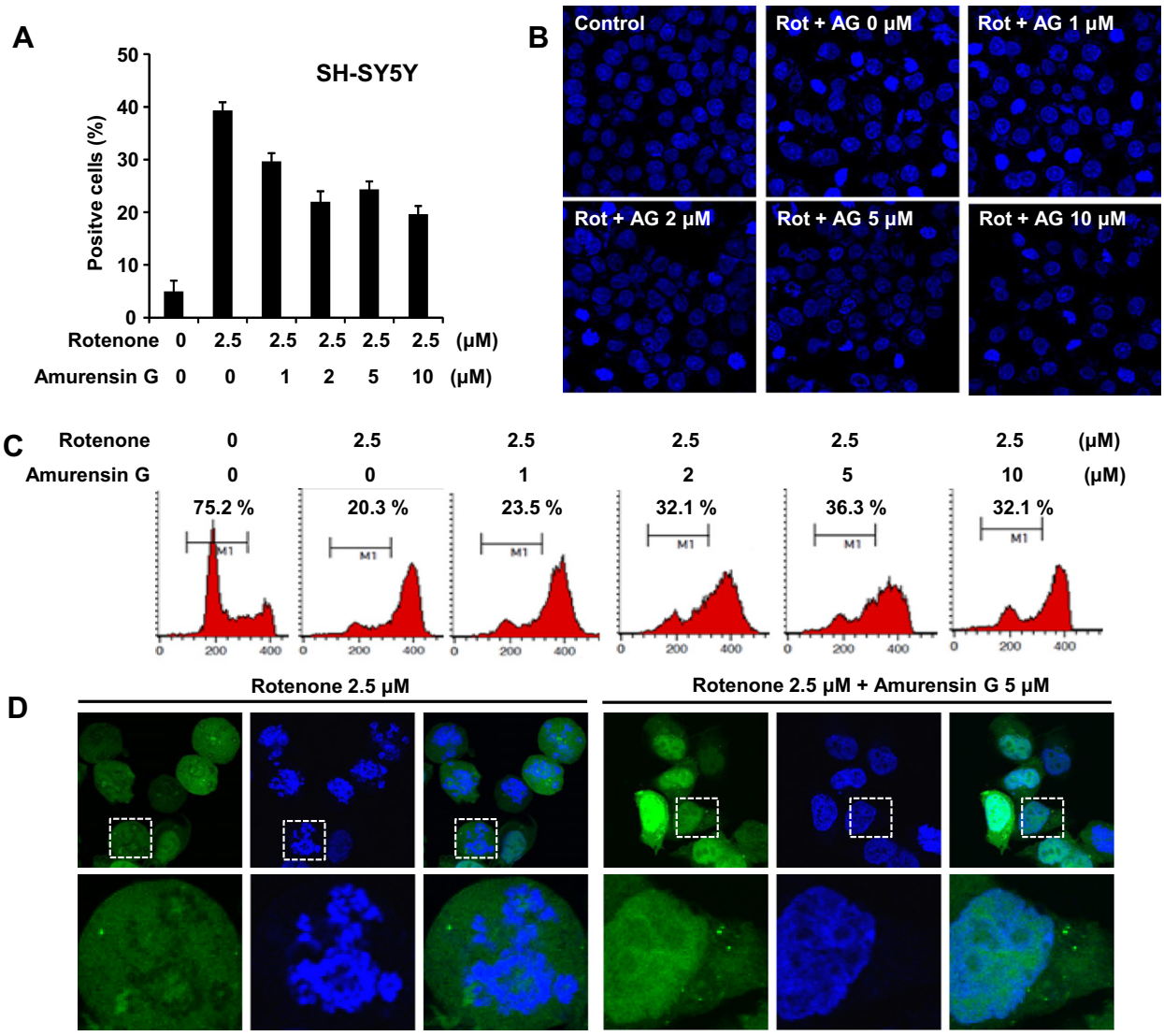

Fig. 3. Amurensin G inhibits rotenone-induced G2/M cell cycle arrest. (A and B) Amurensin G (AG) reduced nuclear condensation induced by rotenone (Rot). SH-SY5Y cells were treated sequentially with amurensin G for 24 h and rotenone for 12 h and stained with DAPI. (C) SH-SY5Y cells were incubated with the indicated concentration of amurensin G for 24 h. After rotenone treatment for 12 h, cells were stained with propidium iodide and analyzed by flow cytometry. The percentage values in the figure indicate the proportion of cells in G1 or S phase. (D) GFP-LC3 cells were treated sequentially with amurensin G for 24 h and rotenone for 12 h. Amurensin G reduced the rotenone-induced condensation of chromosomes.

G and examined the cytoplasmic pattern of GFP-LC3 protein staining. Punctate cytoplasmic GFP-LC3 staining was evident at a concentration of 1 μM amurensin G and an increased concentration of amurensin G resulted in more distinctive GFP-LC3 spots in the cytoplasm, suggesting that amurensin G induces autophagy in a dose-dependent manner (Fig. 1A). To confirm our results, we also tested the induction of autophagy by amurensin G in MCF7 cells, a breast cancer cell line that has commonly been used to test autophagy, by examining expression of the autophagy marker proteins LC3 and p62 (Fig. 1B). The level of LC3-II was increased and the level of p62 was decreased in a dose-dependent manner by treatment with amurensin G. These results collectively indicate that amurensin G can activate autophagy.

3.2. Amurensin G protects SH-SY5Y cells against rotenone-induced death

Because amurensin G induces autophagy in both GFP-LC3 cells and MCF7 cells, we examined the protective effect of amurensin G against a cell model of Parkinson's disease in which human dopaminergic SH-SY5Y neuronal cells were exposed to the mitochondrial complex I inhibitor rotenone. First, we examined whether amurensin G induces autophagy in SH-SY5Y cells. As expected, treatment with amurensin G resulted in the induction of GFP-LC3 in SH-SY5Y cells (Fig. 1C). Next, we examined the protective role of amurensin G against rotenone. SH-SY5Y cells were pretreated with amurensin G for 24 h and then treated with rotenone for 12 h. Cell proliferation was measured using the MTT assay. Rotenone treatment alone decreased cell survival, whereas amurensin G pretreatment enhanced the survival of rotenone-treated cells (Fig. 2A). However, a higher concentration (10 μM) of amurensin G was not as effective as lower concentrations (1–5 μM). In addition, a single treatment with amurensin G did not decrease cell proliferation and did not result in cell death (Fig. 2B). These results suggest that amurensin G protects SH-SY5Y cells from rotenone toxicity.

Next, we examined whether amurensin G interferes with rotenone-induced apoptosis using an annexin V assay. SH-SY5Y cells were pretreated with amurensin G before rotenone treatment. Cells were then stained with annexin V and analyzed by flow cytometry. Rotenone treatment increased the level of annexin V

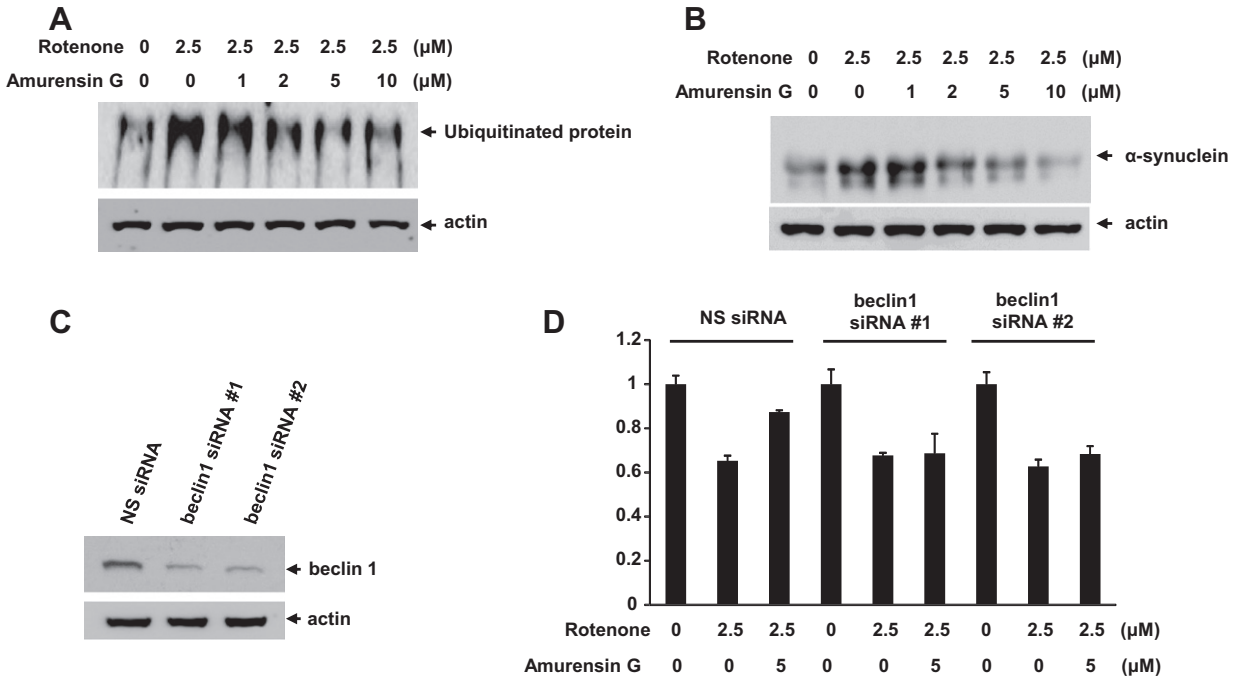

Fig. 4. Amurensin G attenuates rotenone-induced neurotoxicity (A and B) SH-SY5Y cells were incubated with amurensin G for 24 h and treated with rotenone for 12 h. Insoluble cellular fractions were lysed directly with 2× SDS buffer and equal amounts of cell lysate were subjected to Western blot analysis with anti-ubiquitin antibody (A) or anti-α-synuclein antibody (B). (C) Knockdown of beclin 1 abrogated the effect of amurensin G. SH-SY5Y cells were transfected with non-specific (NS) siRNA or siRNAs against beclin 1 for 48 h. Equal amounts of cell lysate were subject to Western blot analysis with the indicated antibodies. (D) Knockdown of beclin 1 reduced the protective effect of amurensin G. SH-SY5Y cells were transfected with siRNA and treated with rotenone in the presence or absence of amurensin G. Cell viability was measured by MTT assay.

staining, whereas pretreatment with amurensin G treatment reduced the level of annexin V staining (Fig. 2C). Amurensin G effectively inhibited apoptosis at concentrations from 1 to 5 μM, in agreement with results of the MTT assay.

3.3. Amurensin G inhibits rotenone-induced cell cycle arrest

Rotenone is reported to induce DNA condensation, a feature of apoptosis, in SH-SY5Y cells [21]. SH-SY5Y cells were pretreated with amurensin G and then incubated with rotenone and fixed for DAPI staining. Rotenone treatment induced up to 40% apoptotic cells with enhanced DAPI staining and amurensin G treatment decreased the number of apoptotic cells (Fig. 3A and B).

Higher concentrations of rotenone (1–5 μM) were reported to induce cell cycle arrest at the G2/M stage of the cell cycle [22]. To determine whether amurensin G affects rotenone-induced cell cycle arrest, SH-SY5Y cells were pretreated with amurensin G before rotenone treatment and examined by flow cytometry. Treatment with rotenone alone induced cell cycle arrest at G2/M, as reported previously, and amurensin G pretreatment decreased the level of G2/M arrest in a dose-dependent manner (Fig. 3C). The rescue of cell cycle arrest peaked at a concentration of 5 μM amurensin G.

In addition, rotenone treatment of HEK293 cells stably expressing GFP-LC3 resulted in condensed DNA and apoptotic nuclei, and amurensin G interfered with the formation of apoptotic nuclei (Fig. 3D). These results collectively indicate the amurensin G interferes with rotenone-mediated cell cycle arrest and cellular cytotoxicity.

3.4. Amurensin G attenuates rotenone-induced neurotoxicity

A major feature of Parkinson's disease is an increase in insoluble ubiquitinated proteins and α-synuclein protein [13]. As rotenone is reported to induce accumulation of ubiquitinated proteins, we examined whether amurensin G interferes with the accumulation of ubiquitinated proteins in SH-SY5Y cells by immunoblotting with anti-ubiquitin antibody. Rotenone treatment led to accumulation of high molecular weight insoluble ubiquitinated protein, which was reduced by pretreatment with amurensin G (Fig. 4B). Next, we examined the effect of amurensin G on the level of α-synuclein. Rotenone treatment increased the level of α-synuclein, and pretreatment with amurensin G repressed the induction of α-synuclein (Fig. 4B). These results collectively indicate that amurensin G impairs rotenone-induced cellular toxicity by reducing the level of ubiquitinated protein and α-synuclein.

3.5. The inhibitory effect of amurensin G requires autophagy induction

Because autophagy inducers interfere with rotenone-induced cell death, we investigated whether the protective effect of amurensin G requires autophagy induction. To test this, we silenced the expression of beclin 1, an important regulator of autophagy, by transfection with two different beclin 1-specific siRNAs. Western blot analysis with anti-beclin 1 antibody showed that beclin 1 expression was efficiently silenced by the siRNAs (Fig. 4C). Next, we examined whether amurensin G protected against rotenone-induced cell death in the absence of beclin 1. Amurensin G did not inhibit rotenone-induced cell death in SH-SY5Y cells transfected with beclin 1 siRNA (Fig. 4D), indicating that the suppression of rotenone-induced cell death by amurensin G involves autophagy.

4. Discussion

Autophagy is a protein degradation pathway that is often induced in response to damaged organelles and intracellular infection. The pathology of Parkinson's disease is closely related with the accumulation of aberrant proteins such as α-synuclein, a major component of Lewy body inclusions. In this report, we used the

chemical rotenone to simulate Parkinson's disease in a cellular model and observed an elevated level of insoluble ubiquitinated proteins and α-synuclein following rotenone treatment. Recent reports showed that autophagy reduced the accumulation of pathologic protein in several neurodegenerative diseases including Alzheimer's disease, Huntington's disease, and Parkinson's disease [23]. Rapamycin, a typical autophagy inducer, can alleviate these neurodegenerative diseases by eliminating the protein aggregates. Therefore autophagy inducers are potentially useful in the treatment of diseases caused by protein accumulation.

To find novel autophagy inducers we initiated a screen of natural products extracted from plants. We treated HEK293 cells stably expressing GFP-LC3 with each product and selected those that induced the formation of GFP-LC3 spots in the cytoplasm. Because transient over-expression of GFP-LC3 often results in false-positive results, we used a GFP-LC3 stable cell line to reduce the background. After extensive screening, we isolated several compounds that induced autophagy and amurensin G was one of these compounds. Amurensin G treatment resulted in cytoplasmic LC3 foci in HEK293 cells and changes in the expression of autophagy markers, specifically increased expression of LC-II and decreased expression of p62. These results collectively indicate that amurensin G is a novel autophagy inducer. Next, we examined whether amurensin G affects cellular toxicity in a rotenone model of Parkinson's disease. Amurensin G treatment inhibited rotenone-induced apoptosis and decreased the level of α-synuclein. These results indicate that amurensin G is a candidate compound for the treatment of Parkinson's disease, and further research is required to test its activity in vivo.

Recent reports showed that amurensin G is potentially useful in the treatment of cancer. It has been reported that amurensin G suppresses the expression of the multidrug resistance (MDR) gene and also sensitizes TRAIL-resistant human leukemic cells [14,19]. In this study we showed that amurensin G treatment itself does not affect cell viability; however, when combined with other treatments the induction of autophagy by amurensin G may contribute to cell death. As excessive autophagy can contribute to cell death, further study will be required to reveal the role of amurensin G-mediated autophagy induction in cancer cell death. Similarly, amurensin G is reported to protect against β amyloid-mediated neurotoxicity, a cellular model of Alzheimer's disease [14]. It is possible that amurensin G inhibits β amyloid-induced cell death by modulating the level of autophagy. It will also be exciting to examine whether amurensin G is active in the treatment of Huntington's disease.

Acknowledgments

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