

Silver nanoparticles from *Dendropanax morbifera* Léveille inhibit cell migration, induce apoptosis, and increase generation of reactive oxygen species in A549 lung cancer cells

Verónica Castro Aceituno¹ · Sungeun Ahn¹ · Shakina Yesmin Simu² · Chao Wang¹ · Ramya Mathiyalagan² · Deok Chun Yang^{1,2}

Received: 22 January 2016 / Accepted: 9 May 2016 / Published online: 1 June 2016 / Editor: Tetsuji Okamoto
© The Society for In Vitro Biology 2016

Abstract Green synthesized silver nanoparticles have significant potential in the pharmaceutical field because of their biological functions such as antioxidant and anticancer activities. Novel silver nanoparticles synthesized from *Dendropanax morbifera* Léveille leaves (D-AgNPs) exhibit antimicrobial activity and reduce the viability of cancer cells without affecting the viability of RAW 264.7 macrophage-like cells. In this study, we evaluated the anticancer effect of D-AgNPs by measuring the levels of reactive oxygen species (ROS) production and toxicity against A549 and HepG2 cell lines. The effect of D-AgNPs on cell migration, induction of apoptosis, and modification of gene and/or protein expression of cancer-related markers was determined using A549 cells. D-AgNPs exhibited cytotoxicity in A549 and HepG2 cell at different concentrations and enhanced the production of ROS in both cell lines. An increase in cell apoptosis and a reduction in cell migration in A549 cells were also observed after D-AgNP treatment. Furthermore, the effect of D-AgNPs in A549 cells was shown to be related to modification of the EGFR/p38 MAPK pathway. Our data provide the first evidence supporting the potential of D-AgNPs as a possible anticancer agent, particularly for the treatment of non-small cell lung carcinoma.

Keywords Silver nanoparticles · Lung cancer · *Dendropanax morbifera* · EGF receptor · Apoptosis

Introduction

Cancer is one of the leading causes of mortality worldwide. Among all types of cancer, lung cancer is the major cause of cancer-related mortality (Siegel et al. 2014). Despite the variety of treatment options, the prognosis for lung cancer is dismal, with only approximately 40% of patients surviving 1 yr after diagnosis. Thus, new therapies that overcome the current obstacles to therapy such as drug resistance, side effects, and poor specificity are needed.

Nanotechnology-based therapies for cancer treatment with minimal side effects and high specificity are attracting increased interest. In particular, the application of novel metal nanoparticles synthesized from medicinal plants has gained attention because of their antimicrobial, antioxidant, and anticancer activity (Conde et al. 2012; Sur et al. 2012; Singh et al. 2015a, b). Previously, the ability of “green” silver nanoparticles to decrease cell viability and increase cell apoptosis in different cancer cells has been reported (Sankar et al. 2013; Kathiravan et al. 2014; Vasanth et al. 2014). Thus, research has focused on the selection of a natural compound with anticancer effects for the synthesis of novel silver nanoparticles to obtain a drug with high activity against cancer cells but low toxicity to normal cells.

Dendropanax morbifera Léveille, a medicinal plant used for the treatment of several diseases, including cancer, was used to synthesize a silver nanoparticle. This nanoparticle decreased the viability of A549 lung cancer cells and induced morphological changes in the nucleus but did not affect the viability of the murine macrophage cell line RAW 264.7 (paper accepted for publication), suggesting a possible anticancer

✉ Deok Chun Yang
dcyang@khu.ac.kr

¹ Department of Oriental Medicinal Biotechnology, College of Life Sciences, Kyung Hee University, Yongin 446-701, Republic of Korea

² Graduate School of Biotechnology and Ginseng Bank, College of Life Sciences, Kyung Hee University, Yongin 446-701, Republic of Korea

effect. In this study, to further investigate the anticancer effect of silver nanoparticles from *D. morbifera* Léveille (D-AgNPs), we evaluated their cytotoxicity and reactive oxygen species (ROS) generation in A549 and HepG2 cell lines. We also evaluated the ability of D-AgNPs to induce apoptosis, reduce cell migration, and modify the expression of different cancer-related markers in A549 lung cancer cells.

Materials and Methods

Nanoparticle synthesis The preparation of silver nanoparticles from *D. morbifera* Léveille was performed as described previously (paper accepted). In brief, healthy leaves of *D. morbifera* were washed with deionized water, cut into small pieces, and dried. Five grams of the dried leaves was transferred into 1 L of distilled water and boiled for 30 min. The extract was allowed to cool at room temperature, centrifuged at 5000 rpm for 10 min, and filtered through Whatman filter paper (No. 2) to give a clear filtrate. The total volume of filtrate was brought up to 100 mL, and the extract was stored at 4°C for further experiments. For preparation of silver nanoparticles, 5 mL of leaf extract was mixed with 45 mL of deionized water, and AgNO₃ solution was added to a final concentration of 1 mM. The mixture was incubated at 80°C for 1 h, and the color change of the reaction mixture was observed continuously and compared with that of the control after 10 min, 20 min, 30 min, 45 min, and 1 h. D-AgNPs were collected in the form of pellets by centrifugation at 16,000 rpm for 20 min and washed several times with deionized water to remove any unwanted substances. The final product was dried and dissolved in 1× PBS.

Cytotoxicity studies Evaluation of cell toxicity was performed by 3, 4, 5-dimethylthiazol-2-yl)-2,5-diphenyletrazolium bromide (MTT) assay. A549 and HepG2 cells were exposed to silver nanoparticles from *D. morbifera* Léveille leaves for 48 h. After treatment, 20 µL of MTT assay solution (5 mg/mL) was added to each well and incubated for 3 h. The medium was replaced by 100 µL of DMSO, and the amount of formazan formed by viable cells after incubation for 30 min was measured using a multi-mode plate reader (BioTek Instrument, Winooski, VT) at a test wavelength of 570 nm with a reference wavelength of 630 nm (Ahn et al. 2015a, b).

Cell lines and cell culture The human lung cancer cell line A549 and hepatocellular carcinoma cell line HepG2 were purchased from the Korean Cell Line Bank (Seoul, South Korea). A549 cells were grown in RPMI 1640 culture medium (GenDEPOT Inc., Barker, TX), and HepG2 cells were grown in DMEM media (Welgene, Gyeongsangbuk-do, South Korea), both of which were supplemented with 10%

fetal bovine serum (FBS) and 1% penicillin G and streptomycin (Gibco-BRL, Gaithersburg, MD), at a temperature of 37°C in a humidified incubator with a 5% CO₂ atmosphere.

Measurement of intracellular ROS The DCF-DA method was used to detect the levels of intracellular ROS. A549 and HepG2 cells were seeded in 96-well plates at $1.0\text{--}1.2 \times 10^4$ cells per well. The day after plating, the cells were treated with different concentrations of silver nanoparticles from *D. morbifera* Léveille leaves for 48 h. After addition of 25 mM DCF-DA solution and incubation for 30 min, the fluorescence of 2',7'-dichlorofluorescein was measured using a microplate reader (BioTek Instrument, Winooski, VT) with an excitation wavelength of 485 nm and emission wavelength of 535 nm (Ahn et al. 2015a, b).

Wound healing assay A549 cells were seeded in a 12-well plate (2.5×10^5 cells/well) and incubated in a humidified atmosphere at 5% CO₂ and 37°C. After the cells reached complete confluence, the medium was replaced with serum-free growth medium for 24 h. The cell monolayers were scratched using a 10-µL plastic pipette tip, and each well was washed twice with 1× PBS to remove dead or floating cells. The cells were stimulated with epidermal growth factor (EGF) at 20 ng/mL, followed by treatment with silver nanoparticles from *D. morbifera* Léveille leaves (D-AgNPs) at 10 µg/mL according to the following treatment schedule: 2% FBS, 2% FBS + EGF, 2% FBS + EGF + D-AgNPs. To quantify the wound healing, two randomly selected areas were photographed under an optical microscope (Eclipse ME600L; Nikon Instruments, Melville, NJ), and the images were analyzed by the T-scratch program (Gebaeck 2009). The motility of the treated cells was determined relative to that of the EGF vehicle control.

Hoechst nuclear staining A549 cells were exposed to silver nanoparticles from *D. morbifera* Léveille leaves (D-AgNPs) for 48 h. Cells were washed twice with 1× PBS, fixed with 3.7% (v/v) formaldehyde for 5 min at room temperature, and washed twice with PBS. To dye the nucleus, the cells were stained with Hoechst 33258 solution (2 µg/mL) for 30 min in dark conditions at room temperature. Nuclear morphologies of the Hoechst-positive cells were observed and photographed under a fluorescence microscope (×400, Optinity, Korean Labtech, South Korea) for further analysis.

Quantitation of apoptosis by HT TiterTACS™ A549 cells were plated in 96-well plates and incubated for 24 h before exposure to silver nanoparticles from *D. morbifera* Léveille for 48 h. DNA damage was evaluated and quantified with a colorimetric apoptosis detection kit (Titer TACS, R&D Systems, Minneapolis, MN) following the manufacturer's instructions. Briefly, the cells were fixed and labeled

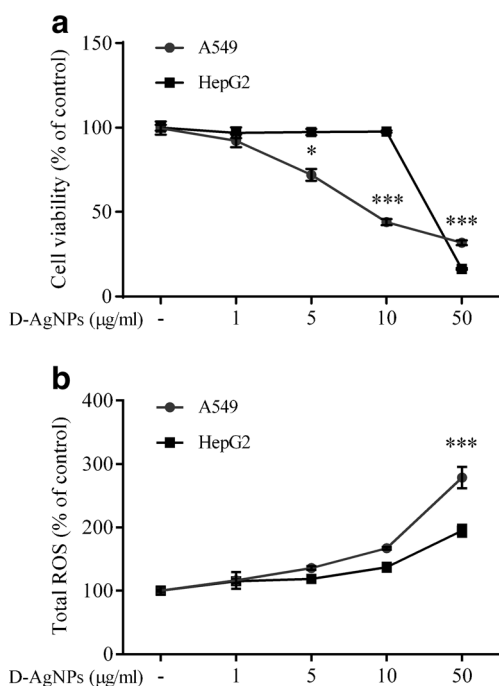


Figure 1 Effect of D-AgNPs on cell viability and ROS generation in A549 and HepG2 cell lines. (a) Cell viability was measured after 48 h of D-AgNP treatment by MTT assay. (b) ROS generation was determined by DCFH-DA fluorescence assay. Data are representative of three independent experiments. * $p < 0.05$ and *** $p < 0.001$ vs. control. D-AgNPs silver nanoparticles from *Dendropanax moribifera* Léveille, ROS reactive oxygen species.

according to the protocol prior to colorimetric analysis. Cells were incubated with TACS-Sapphire™ substrate, and the colorimetric reaction was stopped with 5% phosphoric acid after

1 h. Absorbance was measured at 450 nm using a microplate reader (BioTek Instrument, Winooski, VT).

Gene expression studies A549 cells were seeded at a density of 1×10^6 cells/well in a six-well plate. After incubation for 24 h, the cells were subjected to serum starvation for 8 h followed by 48-h treatment with or without silver nanoparticles from *Dendropanax moribifera* Léveille at 25 µg/mL in the presence or absence of EGF stimulation. RNA was isolated using TRIzol reagent (Sigma, St. Louis, MO) as described previously (Ahn et al. 2015a, b). First-strand complementary DNA (cDNA) was synthesized using a cDNA synthesis kit (Thermo Scientific, EU, Lithuania) according to the manufacturer's instructions. One microliter of the cDNA product was used as the template for quantitative real-time polymerase chain reaction (RT-PCR) amplification using a R-Corbett Rotor-Gene Model 6000 (Corbett Research, Sidney, Australia) with a SYBR Green qPCR Super Mix UDG kit (Invitrogen, Carlsbad, CA). RT-PCR was carried out using the following primers: Bcl-2, 5'-AAT GGG CAG CCG TTA GGA AA-3' (forward) and 5'-GCG CCC AAT ACG ACC AAA TC-3' (reverse); Bax, 5'-GGC CCT TTT GCT TCA GGG TT-3' (forward) and 5'-GGA AAA AGA CCT CTC GGG GG-3' (reverse); EGFR 5'-CCA ACC AAG CTC TCT TGA GG-3' (forward) and 5'-GCTTTCGGAGATGTTGCTTC-3' (reverse); ELK1 5'-TGA GCT GTA GGG AAA CGC AG-3' (forward) and 5'-CAG GGG TAC CTG TGT GTA GC-3' (reverse); JNK, 5'-CGG TGA GGA ACT ACG TGG AG-3' (forward) and 5'-ACC

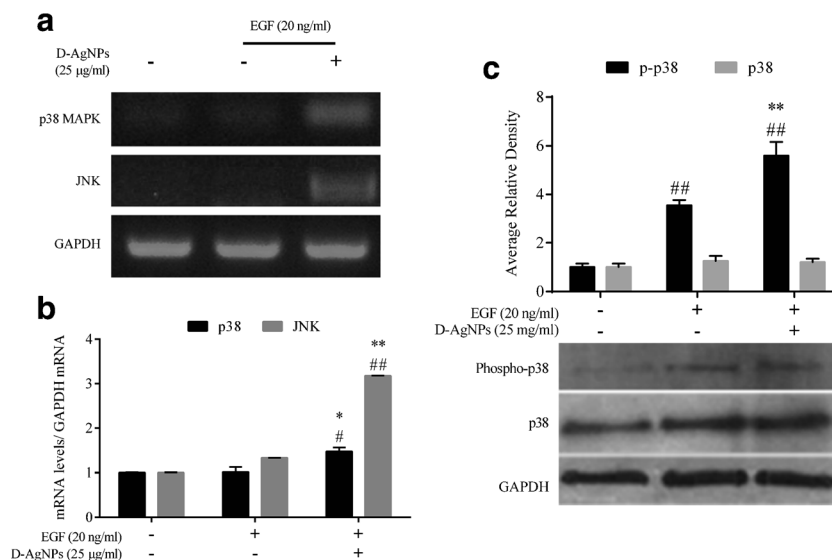


Figure 2 Effect of D-AgNPs on the p38 MAPK pathway in A549 lung cancer cells. Cells were starved for 20 h before treatment and EGF external stimulation. After 48 h of treatment, RNA and protein were isolated from whole cells. (a) mRNA expression levels were evaluated by RT-PCR and visualized by gel electrophoresis. (b) mRNA expression levels were quantified using qRT-PCR and normalized to GAPDH

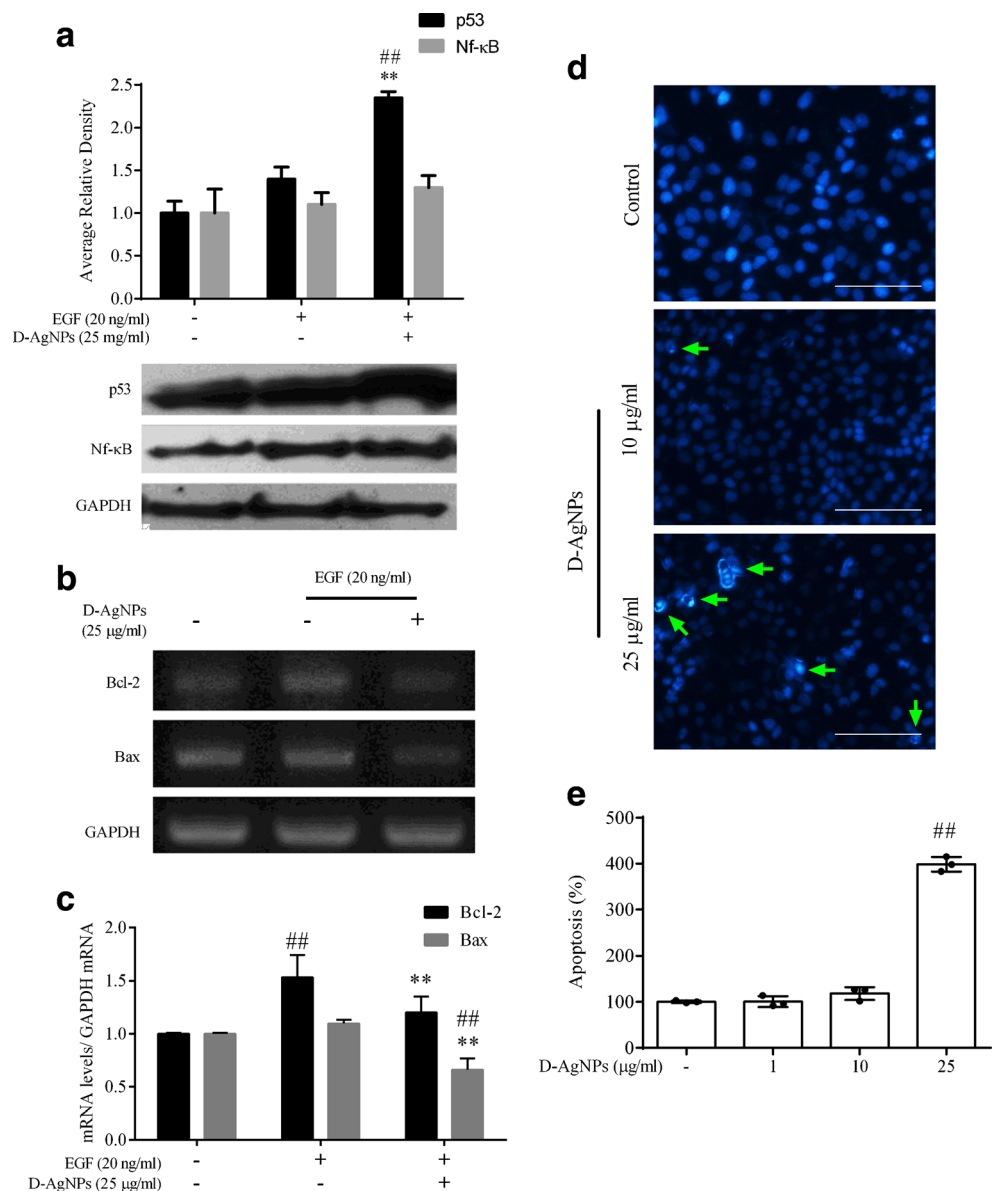
expression. (c) Protein expression levels of phospho-p38 and p38 by western blot were analyzed by ImageJ software. Data are representative of three independent experiments. ## $p < 0.05$ and ### $p < 0.01$ vs. control. * $p < 0.05$ and ** $p < 0.01$ vs. EGF-control. D-AgNPs silver nanoparticles from *Dendropanax moribifera* Léveille, GAPDH glyceraldehyde 3-phosphate dehydrogenase.

ATC GCT CTC AAC CCT TG-3' (reverse); p38 MAPK, 5'-CGA CTT GCT GGA GAA GAT GC-3' (forward) and 5'-TCC ATC TCT TCT TGG TCA AGG-3' (reverse); GAPDH, 5'-AAT GGG CAG CCG TTA GGA AA-3' (forward) and 5'-GCG CCC AAT ACG ACC AAA TC-3' (reverse). Amplifications were performed at an initial temperature of 95°C for 10 min, followed for 40 cycles of 95°C for 10 s, 60°C for 15 s, and 72°C for 20 s. The relative gene expression levels were normalized to the amount of *GADPH* mRNA using the delta cycle threshold (Ct) method (Livak and Schmittgen 2001).

Western blot studies A549 cells were seeded in 100-mm dish culture plates at 5×10^6 cells/dish. After 24-h incubation, the cells were subjected to serum starvation for 20 h followed by 48-h treatment in the presence or absence of silver

nanoparticles from *D. morbifera* Lévelle at different concentrations. Cells were stimulated with EGF (20 ng/mL) for 30 min prior to protein isolation. After stimulation, the cells were rinsed twice with ice-cold PBS and total proteins were solubilized with 2× sodium dodecyl sulfate (SDS) loading buffer (100 mM Tris-Cl (pH 6.8), 4% (w/v) SDS, 0.2% (w/v) bromophenol blue, 20% glycerol and 200 mM β -mercaptoethanol) for 5 min at room temperature (RT). The protein was denatured at 95°C for 10 min and stored at -20°C (Dubon and Park 2015). For immunoblotting, proteins of total cell lysates were resolved in 10% SDS-polyacrylamide gel electrophoresis at 120 V and transferred to PVDF membrane (ATTO Corporation, Tokyo, Japan) at 100 V for 2 h. The membranes were blocked at RT for 1 h in 5% skim milk and then incubated with specific antibodies against phospho-p38, p38, phospho-EGF Receptor (Tyr1068), EGF receptor,

Figure 3 D-AgNPs induce apoptosis in A549 lung cancer cells. (a) Western blot assay for p53 and NF- κ B proteins. Density analysis was performed by ImageJ software. (b) Gene expression levels were evaluated by RT-PCR and visualized by gel electrophoresis. (c) mRNA expression levels were quantified using qRT-PCR and normalized to GAPDH expression. (d) Hoechst 33258 staining in A549 cells. Apoptotic cells are indicated with arrows. Scale bar, 10 μ m. (e) The percentage of apoptotic cells was determined by HT TiterTACS™ assay. Data are representative of three independent experiments. # $p < 0.05$ and ## $p < 0.01$ vs. control. * $p < 0.05$ and ** $p < 0.01$ vs. EGF-control. D-AgNPs silver nanoparticles from *Dendropanax morbifera* Lévelle, GAPDH glyceraldehyde 3-phosphate dehydrogenase.



NF- κ B, p53, and GAPDH overnight at 4°C. The blots were then washed five times with TBS-T, followed by incubation with goat anti-mouse or anti-rabbit IgG secondary antibody for 2 h at RT. Immunolabeling was visualized by enhanced chemiluminescence detection (Millipore Corporation, Billerica, MA). Band densities were measured using ImageJ software (Collins 2007).

Statistical analysis Statistical analysis was performed using GraphPad 6.04 software (La Jolla, CA). Results are expressed as mean \pm SEM. The statistical significance of differences between values was evaluated by one-way ANOVA. Differences were considered significant at $p \leq 0.05$.

Results

D-AgNPs induce cell toxicity and ROS generation by p38 phosphorylation We examined the ability of silver nanoparticles from *D. moribifera* L  veille (D-AgNPs) to reduce cell viability and trigger ROS generation in A549 and Hep2 cell lines. A549 cells treated with D-AgNPs showed decreased cell viability at 5 μ g/mL. For the HepG2 cell line, the D-AgNPs reduced cell viability up to 50 μ g/mL (Fig. 1a). Furthermore, the generation of ROS was increased in A549 and HepG2 cell lines from 5 μ g/mL but was significant at 50 μ g/mL. A lower concentration of D-AgNPs (1 μ g/mL) did not generate an

increase in ROS levels in the cells (Fig. 1b). Next, the activation of p38 MAPK by D-AgNPs and the relationship between this activation and the generation of intracellular ROS in A549 lung cancer cells was evaluated. We found that mRNA expression of p38 MAPK and JNK was enhanced at 25 μ g/mL D-AgNPs (Fig. 2a, b). Also, our results showed that D-AgNPs at 25 μ g/mL increased the phosphorylation of p38 MAPK but did not significantly affect the expression of p38 (Fig. 2c).

D-AgNPs induce apoptosis We determined the effect of silver nanoparticles from *D. moribifera* L  veille (D-AgNPs) on apoptosis in the A549 cell line. First, we evaluated the expression of p53 and NF- κ B proteins by western blot analysis. We found that expression levels of p53 were enhanced by D-AgNPs at 25 μ g/mL whereas the expression of NF- κ B was not affected (Fig. 3a). Next, we determined the mRNA expression levels of Bcl-2 and Bax, well-known apoptotic markers. Our results showed a decrease in the expression of Bcl-2 and Bax genes after treatment with 25 μ g/mL D-AgNPs (Fig. 3b, c). Additionally, the nuclei of A549 cells were stained by Hoechst 33258 dye and quantification of the apoptotic effect of D-AgNPs was performed by HT TiterTACSTM. Our data showed an induction of apoptosis through changes in the nucleus and also through the percentage of apoptotic cells induced by D-AgNPs, which was higher at 25 μ g/mL than at 10 μ g/mL (Fig. 3d, e).

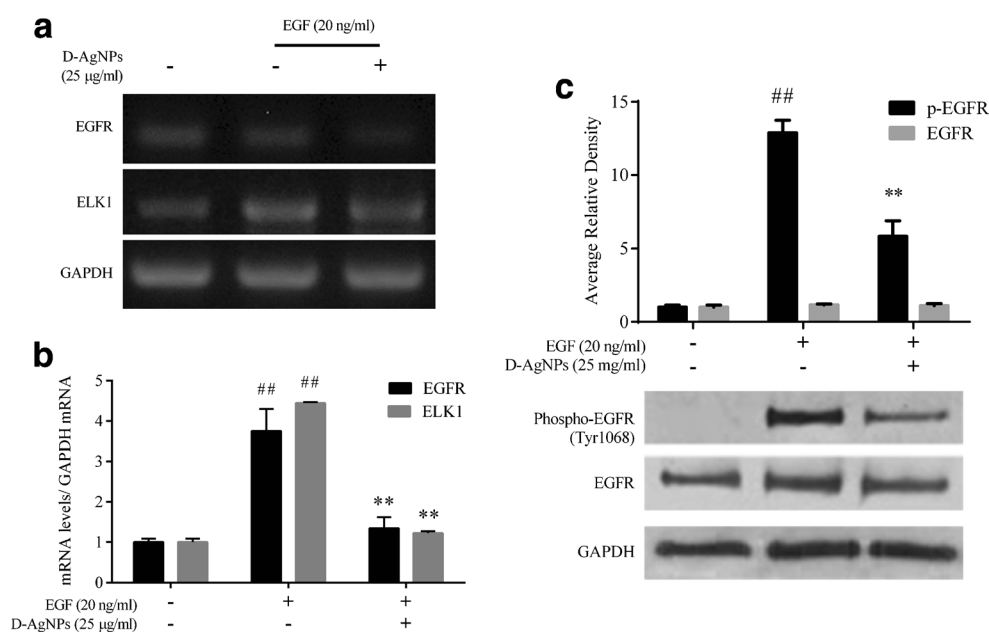


Figure 4 Inhibition of EGFR-induced phosphorylation by D-AgNPs. A549 cells were starved for 20 h and then treated with D-AgNPs at 25 μ g/mL for 48 h. (a) Expression levels were evaluated by RT-PCR and visualized by gel electrophoresis. (b) mRNA expression levels were quantified using qRT-PCR and normalized to GAPDH expression. (c) Protein expression levels of phospho-EGFR and EGFR were measured

by western blotting and analyzed by ImageJ software. The figure shows a representative result of three experiments. ^{##} $p < 0.01$ vs. control. ^{**} $p < 0.01$ vs. EGF-control. *EGF* epithelial growth factor, *EGFR* epithelial growth factor receptor, *D-AgNPs* silver nanoparticles from *Dendropanax moribifera* L  veille, *GAPDH* glyceraldehyde 3-phosphate dehydrogenase.

D-AgNPs downregulate expression of EGF receptor To elucidate the possible anticancer mechanism of silver nanoparticles from *D. morbifera* Léveille (D-AgNPs) in A549 lung cancer cells, we analyzed EGFR expression after overstimulation with external EGF (20 ng/mL). First, we confirmed whether mRNA expression levels of EGFR were affected by the presence of D-AgNPs. Compared with the EGF-control group, cells treated with D-AgNPs (25 µg/mL) for 48 h showed significantly decreased mRNA expression of *EGFR* and *ELK1* (Fig. 4a, b). We also determined the protein expression of phospho-EGF receptors and EGF receptors by western blotting and found that 48-h treatment of D-AgNPs (25 µg/mL) reduced the amount of phospho-EGF receptor, but did not affect the expression of EGF receptor (Fig. 4c).

D-AgNPs decrease cell migration Since EGFR overexpression is related to cell migration, a wound healing assay was performed to observe the effect of silver nanoparticles from *D. morbifera* Léveille on cell migration. The level of wound healing was measured by the average decrease in distance between the edges of the wounds at different time points in the presence or absence of EGF (20 ng/mL) stimulation. The wound healing assay revealed that A549 cells that were not treated with D-AgNPs effectively healed the wounds, with a decrease to ~90% of the original distance after 24 h. However, treatment with 25 µg/mL D-AgNPs significantly repressed wound healing in A549 cells (Fig. 5).

Discussion

In the present study, we found that silver nanoparticles from *D. morbifera* Léveille (D-AgNPs) were more toxic toward A549 cells than HepG2 cells (Fig. 1a). Therefore, subsequent studies on the effect of D-AgNPs in cancer cells were performed in the A549 cell line. We found that the anticancer effect might be linked to the production of ROS, modification in the expression of apoptosis-related proteins, and reduction of cell migration.

Intracellular ROS production is involved in the apoptosis signal transduction pathway (Green and Reed 1998). Also, previous studies suggested that increased oxidative stress is related to the apoptotic response induced by several anticancer agents (Luo et al. 2010). In our study, we found that the levels of ROS production were significantly enhanced by D-AgNPs at 50 µg/mL compared to controls over a period of 48 h (Fig. 1b). We also observed that D-AgNPs at 25 µg/mL induced activation of the phospho-38 MAPK pathway (Fig. 2). It was previously suggested that ROS acted as upstream regulators, resulting in the activation of p38 MAPK (Peverelli et al. 2010). Besides, many lines of evidence suggest that members of the MAPK family, including p38 MAPK, play crucial roles in cell growth, survival, and death (Peverelli et al.

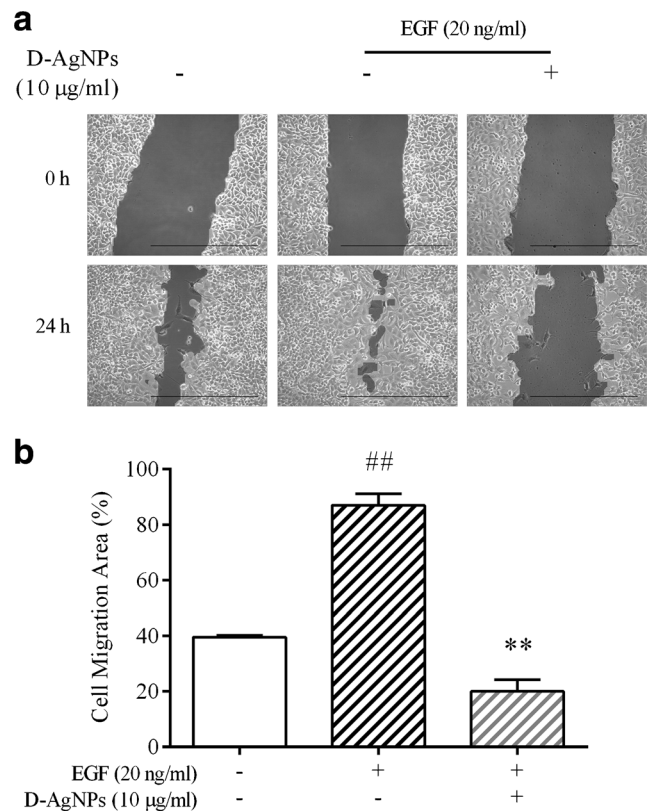


Figure 5 D-AgNPs reduces EGF-enhanced migration of A549 lung cancer cells. (a) Effect of D-AgNPs on wound healing assay. (b) Analysis of migration ratio (%) within 24 h in wound healing assay. Data are shown as mean \pm SEM ($n = 3$). ^{##} $p < 0.01$ vs. control; ^{**} $p < 0.01$ vs. EGF-control. EGF epidermal growth factor, D-AgNPs silver nanoparticles from *Dendropanax morbifera* Léveille.

2010). Our data indicate that p38 MAPK might play an important role in D-AgNP-induced cytotoxicity in A549 cells.

Apoptosis is an ordered cellular suicidal mechanism that regulates normal physiological processes and plays a crucial role in maintaining normal homeostasis (Hail et al. 2006). We previously showed that D-AgNPs induced morphological changes in A549 lung cancer cells (paper accepted for publication). In view of the fact that cell apoptosis lead to modifications in cell morphology (Fig. 3d), we evaluated the expression of p53 and NF- κ B at protein levels, as well as the mRNA expression of Bcl-2 and Bax, to determine the possible mechanisms involved in the induction of apoptosis by D-AgNPs in A549 cells. D-AgNPs enhanced the expression of p53 but did not affect the expression of NF- κ B in the presence of external EGF stimulation (Fig. 3a). Previous studies found that basal levels of p53 present in the control cells sufficed to inhibit EGF-stimulated proliferation. Also, there were no significant differences in the expression levels of p53 between non-stimulated and EGF-stimulated cells (Godar et al. 2008). Since inhibition of cell proliferation, induction of apoptosis, and senescence are thought to be the major biological output of the p53 pathway in response to various types of cell

physiologic stresses (Levine et al. 2006), activation of p53 in A549 cells might be the mechanism involved in the induction of cell apoptosis by D-AgNP treatment. Also, the mRNA levels of Bcl-2 and Bax were decreased in A549 cells after treatment with D-AgNPs at 25 $\mu\text{g}/\text{mL}$ (Fig. 3b, c). Previous studies reported that the Bcl-2 and Bax genes can independently regulate a common apoptotic pathway (Chao and Korsmeyer 1998). Also, some studies indicated that the decreased expression levels of Bax in the presence of anticancer drugs are associated with mutation of the *TP53* encoding p53 (Perego et al. 1996). Mutation of the *TP53* gene was previously reported in A549 cells (Supino et al. 2001). Thus, we suggest that the independent regulation of Bax and Bcl-2 might be also involved in the induction of morphological changes of the nucleus and the induction of apoptosis by D-AgNPs (Fig. 3d, e). Taken together, our data suggest that activation of p53 and inactivation of Bax and Bcl-2 mediate the induction of apoptosis by D-AgNPs through multiple pathways in A549 lung cancer cells. In vivo experiments are required to fully elucidate the mechanism by which D-AgNPs induce apoptosis in A549 cells.

Mutation and overexpression of epithelial growth factor receptors (EGFRs) has been linked with drug resistance, cell survival, and migration of various cancer cells (Lurje and Lenz 2009). Thus, we investigated whether D-AgNPs modified the phosphorylation of EGFR as well as cell migration. Since overexpression of EGFR was previously detected after external stimulation with EGF in A549 cells (Lauand et al. 2013), we used EGF stimulation at 20 ng/mL in this study. D-AgNPs at 25 $\mu\text{g}/\text{mL}$ reduced the mRNA levels of *EGFR* and *ELK1* genes (Fig. 4a). Also, phosphorylation of EGFR protein was reduced by D-AgNPs after 48 h of treatment (Fig. 4b). Next, to determine the effect of D-AgNPs on cell migration after EGFR overexpression, A549 cells were treated with D-AgNPs in the presence of external EGF stimulation. The results of the wound healing assay demonstrated that D-AgNPs at 10 $\mu\text{g}/\text{mL}$ significantly decreased the migration of A549 cells compared with EGF-control cells (Fig. 4). Taken together, these results indicate that D-AgNPs reduced cell migration and also suggested that this effect might be linked to a reduction in EGFR expression in A549 lung cancer cells.

Conclusion

In this study, we showed that silver nanoparticles from *D. morbifera* Léveille (D-AgNPs) reduced cell viability and induced ROS generation in two different cancer cell lines. Furthermore, we found that ROS generation induced by D-AgNPs might be related to modification of the p38 MAPK pathway. D-AgNPs at a concentration of 25 $\mu\text{g}/\text{mL}$ also reduced expression of phospho-EGFR and decreased mRNA expression of *EGFR* and *ELK1*. Additionally, D-AgNPs

reduced cell migration at a concentration of 10 $\mu\text{g}/\text{mL}$ after enhancement of the basal migration of A549 cells using external EGF. Finally, D-AgNPs at 25 $\mu\text{g}/\text{mL}$ increased the expression of p53 protein and decreased the mRNA levels of Bcl-2 and Bax, molecules related to apoptosis induction, and increased the percentage of apoptotic A549 cells. These results indicate that D-AgNPs exhibit anticancer effects in A549 lung cancer cells in vitro.

Acknowledgments This work was supported by the Korea Institute of Planning and Evaluation for Technology in Food, Agriculture, Forestry and Fisheries, Republic of Korea (313038-03-2-SB010).

References

- Ahn S, Siddiqi MH, Aceituno VC, Simu SY, Zhang J, Perez ZEJ, Kim Y-J, Yang D-C (2015a) Ginsenoside Rg5: Rk1 attenuates TNF- α /IFN- γ -induced production of thymus- and activation-regulated chemokine (TARC/CCL17) and LPS-induced NO production via down-regulation of NF- κ B/p38 MAPK/STAT1 signaling in human keratinocytes and macrophages. *In Vitro Cell Dev Biol Anim* 1:1–9
- Ahn S, Siddiqi MH, Noh H-Y, Kim Y-J, Kim Y-J, Jin C-G, Yang D-C (2015b) Anti-inflammatory activity of ginsenosides in LPS-stimulated RAW 264.7 cells. *Sci Bull* 60:773–784
- Chao DT, Korsmeyer SJ (1998) BCL-2 family: regulators of cell death. *Annu Rev Immunol* 16:395–419
- Collins TJ (2007) ImageJ for microscopy. *BioTechniques* 43:25–30
- Conde J, Doria G, Baptista P (2012) Noble metal nanoparticles applications in cancer. *J Drug Deliv* 2012:751075. doi:10.1155/2012/751075
- Dubon MJ, Park KS (2015) Substance P enhances the proliferation and migration potential of murine bone marrow-derived mesenchymal stem cell-like cell lines. *Exp Ther Med* 9:1185–1191
- Gebaek T (2009) TScratch: a novel and simple software tool for automated analysis of monolayer wound healing assays (vol 46, pg 265, 2009). *BioTechniques* 46(6):383–383
- Godar S, Ince TA, Bell GW, Feldser D, Donaher JL, Bergh J, Liu A, Miu K, Watnick RS, Reinhardt F (2008) Growth-inhibitory and tumor-suppressive functions of p53 depend on its repression of CD44 expression. *Cell* 134:62–73
- Green DR, Reed JC (1998) Mitochondria and apoptosis. *Science* 281:1309
- Hail N Jr, Carter B, Konopleva M, Andreeff M (2006) Apoptosis effector mechanisms: a requiem performed in different keys. *Apoptosis* 11: 889–904
- Kathiravan V, Ravi S, Ashokkumar S (2014) Synthesis of silver nanoparticles from *Melia dubia* leaf extract and their in vitro anticancer activity. *Spectrochim Acta A Mol Biomol Spectrosc* 130:116–121
- Lauand C, Rezende-Teixeira P, Cortez BA, Niero E, Machado-Santelli GM (2013) Independent of ErbB1 gene copy number, EGF stimulates migration but is not associated with cell proliferation in non-small cell lung cancer. *Cancer Cell Int* 13:32
- Levine A, Hu W, Feng Z (2006) The P53 pathway: what questions remain to be explored? *Cell Death Differ* 13:1027–1036
- Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2- $\Delta\Delta\text{CT}$ method. *Methods* 25:402–408
- Luo M, Liu X, Zu Y, Fu Y, Zhang S, Yao L, Efferth T (2010) Cajanol, a novel anticancer agent from *Pigeonpea* [*Cajanus cajan* (L.) Millsp.] roots, induces apoptosis in human breast cancer cells through a ROS-mediated mitochondrial pathway. *Chem Biol Interact* 188: 151–160

- Lurje G, Lenz H-J (2009) EGFR signaling and drug discovery. *Oncology* 77:400–410
- Perego P, Giarola M, Righetti SC, Supino R, Caserini C, Delia D, Pierotti MA, Miyashita T, Reed JC, Zunino F (1996) Association between cisplatin resistance and mutation of p53 gene and reduced bax expression in ovarian carcinoma cell systems. *Cancer Res* 56:556–562
- Peverelli E, Olgiati L, Locatelli M, Magni P, Fustini MF, Frank G, Mantovani G, Beck-Peccoz P, Spada A, Lania A (2010) The dopamine–somatostatin chimeric compound BIM-23A760 exerts anti-proliferative and cytotoxic effects in human non-functioning pituitary tumors by activating ERK1/2 and p38 pathways. *Cancer Lett* 288:170–176
- Sankar R, Karthik A, Prabu A, Karthik S, Shivashangari KS, Ravikumar V (2013) *Origanum vulgare* mediated biosynthesis of silver nanoparticles for its antibacterial and anticancer activity. *Colloids Surf B: Biointerfaces* 108:80–84
- Siegel R, Ma J, Zou Z, Jemal A (2014) Cancer statistics, 2014. *CA Cancer J Clin* 64:9–29
- Singh P, Kim YJ, Wang C, Mathiyalagan R, El-Agamy Farh M, Yang DC (2015a) Biogenic silver and gold nanoparticles synthesized using red ginseng root extract, and their applications. *Artif cells Nanomed Biotechnol* 1–6. doi:10.3109/21691401.2015.1008514
- Singh P, Kim YJ, Yang DC (2015b) A strategic approach for rapid synthesis of gold and silver nanoparticles by *Panax ginseng* leaves. *Artif Cells Nanomed Biotechnol* 1–9. doi:10.3109/21691401.2015.1115410
- Supino R, Perego P, Gatti L, Caserini C, Leonetti C, Colantuono M, Zucco V, Carenini N, Zupi G, Zunino F (2001) A role for c-myc in DNA damage-induced apoptosis in a human TP53-mutant small-cell lung cancer cell line. *Eur J Cancer* 37:2247–2256
- Sur I, Altunbek M, Kahraman M, Culha M (2012) The influence of the surface chemistry of silver nanoparticles on cell death. *Nanotechnology* 23:375102
- Vasanth K, Ilango K, MohanKumar R, Agrawal A, Dubey GP (2014) Anticancer activity of *Moringa oleifera* mediated silver nanoparticles on human cervical carcinoma cells by apoptosis induction. *Colloids Surf B: Biointerfaces* 117:354–359