JOURNAL OF CANCER PREVENTION
VOL. 21, NO. 1, MARCH 2016

http://dx.doi.org/10.15430/JCP.2016.21.1.26
pISSN 2288-3649 · eISSN 2288-3657
www.jcpjournal.org

Dehydroglyasperin D Inhibits the Proliferation of HT-29 Human Colorectal Cancer Cells Through Direct Interaction With Phosphatidylinositol 3-kinase

ORIGINAL ARTICLE

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Background: Despite recent advances in therapy, colorectal cancer still has a grim prognosis. Although licorice has been used in East Asian traditional medicine, the molecular properties of its constituents including dehydroglyasperin D (DHGA-D) remain unknown. We sought to evaluate the inhibitory effect of DHGA-D on colorectal cancer cell proliferation and identify the primary signaling molecule targeted by DHGA-D.
Methods: We evaluated anchorage-dependent and -independent cell growth in HT-29 human colorectal adenocarcinoma cells. The target protein of DHGA-D was identified by Western blot analysis with a specific antibody, and direct interaction between DHGA-D and the target protein was confirmed by kinase and pull-down assays. Cell cycle analysis by flow cytometry and further Western blot analysis was performed to identify the signaling pathway involved.
Results: DHGA-D significantly suppressed anchorage-dependent and -independent HT-29 colorectal cancer cell proliferation. DHGA-D directly suppressed phosphatidylinositol 3-kinase (PI3K) activity and subsequent Akt phosphorylation and bound to the p110 subunit of PI3K. DHGA-D also significantly induced G1 cell cycle arrest, together with the suppression of glycogen synthase kinase 3β and retinoblastoma phosphorylation and cyclin D1 expression.
Conclusions: DHGA-D has potent anticancer activity and targets PI3K in human colorectal adenocarcinoma HT-29 cells. To our knowledge, this is the first report to detail the molecular basis of DHGA-D in suppressing colorectal cancer cell growth.
(J Cancer Prev 2016;21:26-31)

Key Words: PI3K, Dehydroglyasperin D, Colorectal neoplasms, Proliferation, Cell cycle

INTRODUCTION

Colorectal cancer was the second most common form of cancer in terms of incidence rate in Korea in 2015.[1] The incidence of colorectal cancer among Korean men in 2008 was 46.9 per 100,000 people, ranking first among all Asian countries and fourth worldwide.[2] The World Cancer Research Fund announced that approximately 47% of colorectal cancer cases in the UK can be prevented by eating and drinking healthily, being physically active and maintaining a healthy body weight.[3] In addition to a balanced diet and more exercise, new bioactive agents for cancer suppression are needed.

Phosphatidylinositol 3-kinase (PI3K) is a heterodimeric signaling factor composed of a p85 regulatory subunit and a p110α catalytic subunit, which upon activation is responsible for the conversion of phosphatidylinositol-4,5-bisphosphate to phosphatidylinositol-2,4,5-trisphosphate. This leads to the recruitment and phosphorylation of Akt, and the promotion of cell growth and proliferation.[4] Activation of the PI3K/Akt pathway closely correlates with the degree of prognosis in stage II colon cancer.[5] We have recently demonstrated that PI3K is a promising anti-cancer target for natural phytochemicals.[6]

The consumption of natural phytochemicals has been linked to the prevention or delay of cancer development.[7] Compounds

Received February 15, 2016, **Revised** February 22, 2016, **Accepted** February 22, 2016

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in licorice roots exhibit various biological activities, including antioxidant, anti-inflammatory and anti-cancer effects.[8-10] Dehydroglyasperin D (DHGA-D) is a recently identified anti-obesity component in licorice, acting as a ligand for the peroxisome proliferator-activated receptor γ.[11] Although DHGA-D was recently shown to exhibit antioxidant and aldose reductase inhibitory activities,[12,13] the mechanism of action of its inhibitory effect on colon cancer cell proliferation remains unknown. Here, we report for the first time that DHGA-D is a PI3K inhibitor and suppresses anchorage-dependent and -independent human colorectal cancer cell proliferation. This appears to result from G1 cell cycle arrest via the Akt/GSK3β/cyclin D1 signaling pathway in HT-29 cells.

MATERIALS AND METHODS

1. Reagents

RPMI 1640 medium, basal medium eagle (BME), gentamicin, and L-glutamine were purchased from Invitrogen (Carlsbad, CA, USA). Antibodies against p-Akt (S473), p-PTEN (S380), PTEN, p-ERK1/2 (T202/Y204), ERK, p-$p90^{RSK}$ (T359/S363), $p90^{RSK}$, p-JNK1/2 (T183/Y185), JNK1/2, p-GSK3β (S9), GSK3β, cyclin D1, CDK4, cyclin E, p-CDK2 (Y15), CDK2, and p-RB (S780) were obtained from Cell Signaling Biotechnology (Beverly, MA, USA). Antibodies against p85 and p110 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The antibody against β-actin was obtained from Sigma-Aldrich (St. Louis, MO, USA) and the active PI3K protein was obtained from EMD Millipore (Billerica, MA, USA), CNBr-Sepharose 4B, glutathione-Sepharose 4B, [γ-^{32}P] ATP and the chemiluminescence detection kit were purchased from Amersham Pharmacia Biotech (Piscataway, NJ, USA), and the protein assay kit was from Bio-Rad Laboratories (Hercules, CA, USA).

2. Cell culture

HT-29 human colon cancer cells were cultured in monolayers at 37°C in a 5% CO_2 incubator in McCoy's 5A medium containing penicillin (100 units/mL), streptomycin (100 μg/mL), L-glutamine (2 mM), and 10% FBS (Gemini Bio-Products, Calabasas, CA, USA).

3. Anchorage-independent growth assay

HT-29 cells (8×10^3 cells/mL) were suspended and exposed to DHGA-D (5 or 10 μM) in 1 mL of 0.33% BME agar containing 10% FBS or in 3.5 mL of 0.5% BME agar containing 10% FBS. The cultures were maintained at 37°C in a 5% CO_2 incubator for 1 week before colonies were counted under a microscope using Image-Pro Plus software (V.4) (Media Cybernetics, Silver Spring, MD, USA).

4. MTS assay

Cells (1×10^3 cells/well) were seeded in 96-well plates, incubated for 24 hours, and then treated with the indicated doses of DHGA-D. After incubation for 1, 2, or 3 days, 20 μL of CellTiter 96 AQueous One Solution (Promega, Medison, WI, USA) was added and the cells were incubated for 1 hour at 37°C in a 5% CO_2 incubator. Absorbance was measured at 492 nm.

5. Western blot assays

Cells (1.5×10^6) were cultured in 100 mm dishes for 24 hours before treatment with DHGA-D (5 and 10 μM) for 18 hours. The cells were then harvested and disrupted with lysis buffer before protein concentration was measured using a dye-binding protein assay kit as described in the manufacturer's manual. Protein lysates (40 μg) were subjected to 10% SDS PAGE and transferred to polyvinylidene difluoride membranes (EMD Millipore). The membranes were incubated with the specific primary antibodies at 4°C overnight. Protein bands were visualized by a chemiluminescence detection kit after hybridization with a horseradish peroxidase-conjugated secondary antibody.

6. Phosphatidylinositol 3-kinase assay

Active PI3K (100 ng) was incubated with DHGA-D for 10 minutes at 30°C. The mixture was then incubated with 20 μL of 0.5 mg/mL phosphatidylinositol (Avanti Polar Lipids) for 5 minutes at room temperature, followed by incubation with reaction buffer (100 mmol/L HEPES, pH 7.6, 50 mmol/L $MgCl_2$, and 250 μmol/L ATP containing 10 μCi of [γ-^{32}P]ATP) for an additional 10 minutes at 30°C. The reaction was stopped by the addition of 15 μL of 4 N HCl and 130 μL of chloroform/methanol (1:1). After vortexing, 30 μL of the lower chloroform phase was spotted onto a 1% potassium oxalate-coated silica gel plate that had been previously activated for 1 hour at 110°C. The resulting ^{32}P-labeled phosphatidylinositol-3-phosphate was separated by thin-layer chromatography, and the radiolabeled spots were visualized by autoradiography.

7. Pull-down assays

DHGA-D-Sepharose 4B beads were prepared according to the manufacturer's instructions (Amersharm Pharmacia Biotech) as described in previous study.[14] Cellular supernatant fractions (500 μg) and active PI3K proteins (200 ng) were incubated with DHGA-D–Sepharose 4B (or Sepharose 4B alone as a control) beads in reaction buffer.[14] After incubation with gentle rocking

overnight at 4°C, the beads were washed 5 times with washing buffer,[14] and proteins bound to the beads were analyzed by immunoblotting with an anti-PI3K p110 subunit antibody.

8. Cell cycle analysis

Cells (7×10^4) were seeded in 60 mm dishes and cultured for 24 hours before treatment for 48 hours with the indicated concentrations of DHGA-D. The cells were harvested by trypsinization, fixed with ethanol, stained with propidium iodide, and then analyzed for cell cycle phase by flow cytometry.

9. Statistical analysis

Data are expressed as mean ± SD, and Student's *t*-test was used to perform statistical analysis for single comparisons. A probability value of $P < 0.05$ was used as the criterion for statistical significance.

RESULTS

1. Dehydroglyasperin D inhibits anchorage-dependent and -independent HT29 cell growth

Unregulated cell growth is one of the hallmarks of cancer,[15] and we first investigated the effect of DHGA-D on anchorage-dependent and -independent growth of HT-29 colorectal cancer cells. DHGA-D strongly suppressed both anchorage-dependent and -independent HT29 cell growth (Fig. 1).

2. Dehydroglyasperin D inhibits phosphorylation of Akt, but not mitogen-activated protein kinases or PTEN in HT-29 cells

The PI3K/Akt signaling pathway plays a major role in the regulation of cell proliferation and survival[16] and mitogen-activated protein kinases (MAPKs) are also known to regulate cell proliferation.[17] To identify the major signaling molecule targeted by DHGA-D, we determined the effect of DHGA-D on PI3K signaling pathway factors and MAPK family members. Western blot analysis showed that DHGA-D suppressed Akt phosphorylation without affecting phosphorylation of PTEN or expression of the p85 and p110 subunits of PI3K (Fig. 2A). Additionally, DHGA-D did not appear to affect the expression or phosphorylation of ERK, p90RSK, or JNK1/2 (Fig. 2B).

3. Dehydroglyasperin D inhibits phosphatidylinositol 3-kinase activity by directly binding to p110 subunit of phosphatidylinositol 3-kinase

Because DHGA-D appeared to singularly suppress Akt, a major substrate of PI3K, without affecting PI3K expression or PTEN phosphorylation, we hypothesized that DHGA-D may directly bind with PI3K. Kinase assays with active PI3K revealed that DHGA-D significantly suppressed PI3K kinase activity. Pull-down assays further confirmed that DHGA-D was able to physically

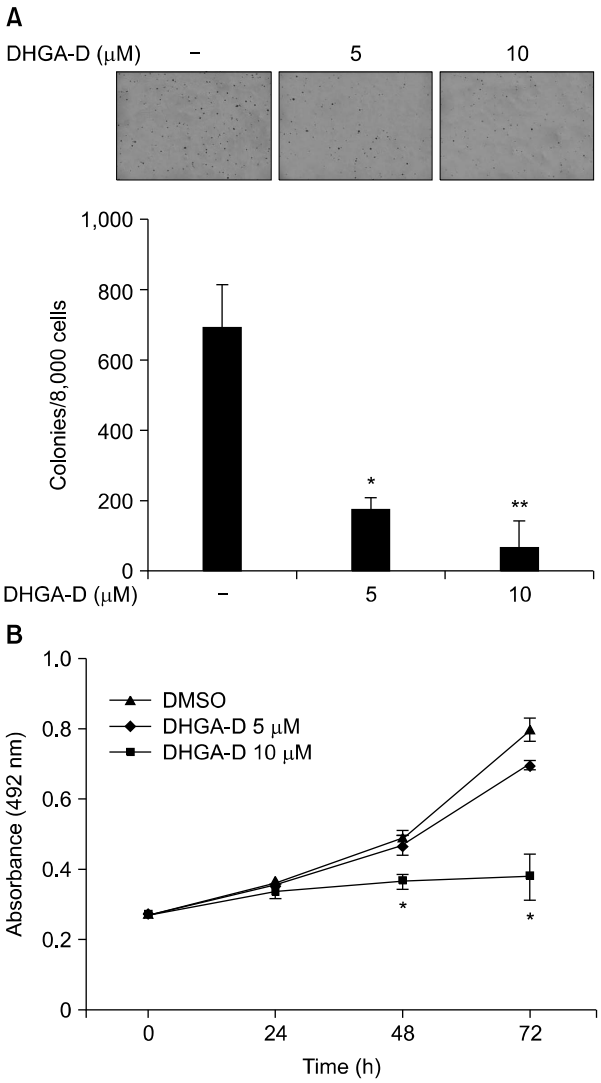

Figure 1. Dehydroglyasperin D (DHGA-D) inhibits anchorage-dependent and -independent growth of HT-29 cells. (A) DHGA-D inhibits anchorage-independent HT-29 cell growth. HT-29 cells were treated as described in the Materials and Methods and colonies were counted 10 days later. Data are shown as mean ± SD of the colony numbers as determined from three separate experiments (magnification: ×40). (B) DHGA-D inhibits anchorage-dependent HT-29 cell growth. Cell viability was measured as described in the Materials and Methods. Data are represented as mean ± SD as determined from three independent experiments. The asterisks (*and **) indicate a significant difference ($P < 0.05$ and $P < 0.01$, respectively) between the treatment groups and the vehicle control. DMSO, dimethyl sulfoxide.

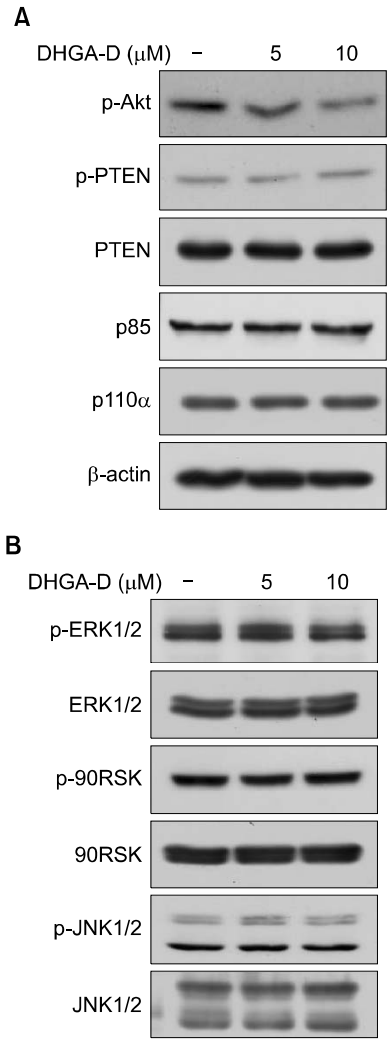

Figure 2. Dehydroglyasperin D (DHGA-D) inhibits phosphorylation of Akt in HT-29 cells. (A) DHGA-D inhibits Akt phosphorylation, but not phosphorylation of PTEN or expression of the p85 and p110 subunits of phosphatidylinositol 3-kinase in HT-29 cells. (B) DHGA-D does not affect phosphorylation of ERK1/2, RSK, or JNK1/2 in HT-29 cells. Western blot analysis was performed as described in the Materials and Methods using the indicated antibody.

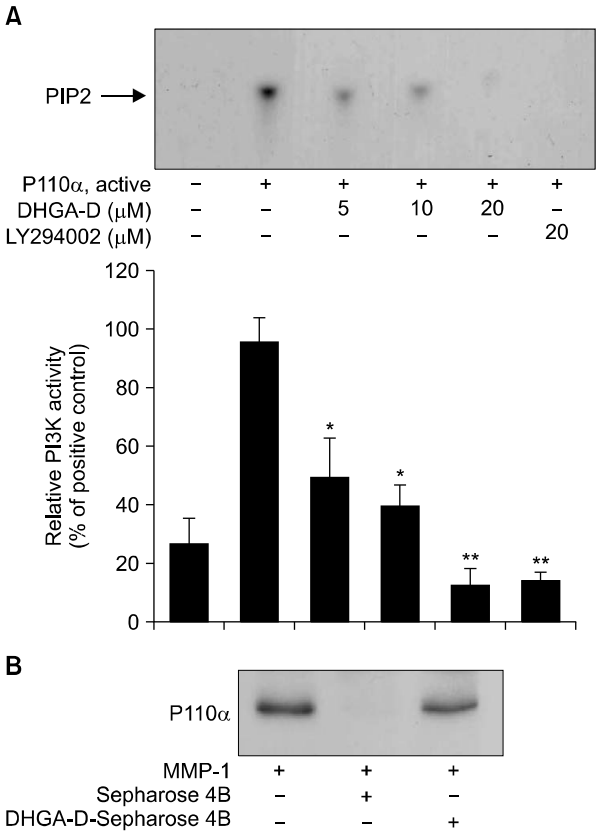

Figure 3. Dehydroglyasperin D (DHGA-D) directly inhibits phosphatidylinositol 3-kinase (PI3K) activity by binding to PI3K. (A) DHGA-D directly inhibits PI3K activity. Kinase assays were performed with active PI3K as described in the Materials and Methods. Data are representative of 3 independent experiments. The asterisks (* and **) indicate a significant difference ($P < 0.05$ and $P < 0.01$, respectively) between treatment groups and the vehicle control, or LY294002 as the positive control. (B) DHGA-D binds to the p110 subunit of PI3K. The pull-down assay was performed as described in the Materials and Methods.

bind to the p110 subunit of PI3K (Fig. 3B).

4. Dehydroglyasperin D induces cell cycle arrest of HT-29 cells at G1 phase and inhibits phosphorylation of glycogen synthase kinase 3β and retinoblastoma and expression of cyclin D1

We determined whether the inhibition of HT-29 cell growth was associated with cell cycle arrest. DHGA-D significantly induced G1 cell cycle arrest (Fig. 4A). To determine the mechanism responsible for arrest at the G1 phase, we examined the phosphorylation of GSK3β and expression of cyclin D1 and CDK4, which are signaling factors involved in the transition from G1 to S phase. Treatment of DHGA-D significantly suppressed phosphorylation of GSK3β and retinoblastoma (RB) and expression of cyclin D1, but not CDK4, CDK2, and cyclin E (Fig. 4B).

DISCUSSION

Colorectal cancer remains the third most common cancer type in men worldwide and the second most common cancer in women in Asia.[18] In Korea, the incidence of colorectal cancer continues to increase.[2] The risk of colorectal cancer is closely linked to diet and other lifestyle factors. Multiple lines of epidemiological evidence indicate that a higher consumption of fruits and vegetables correlates strongly with a lower risk of

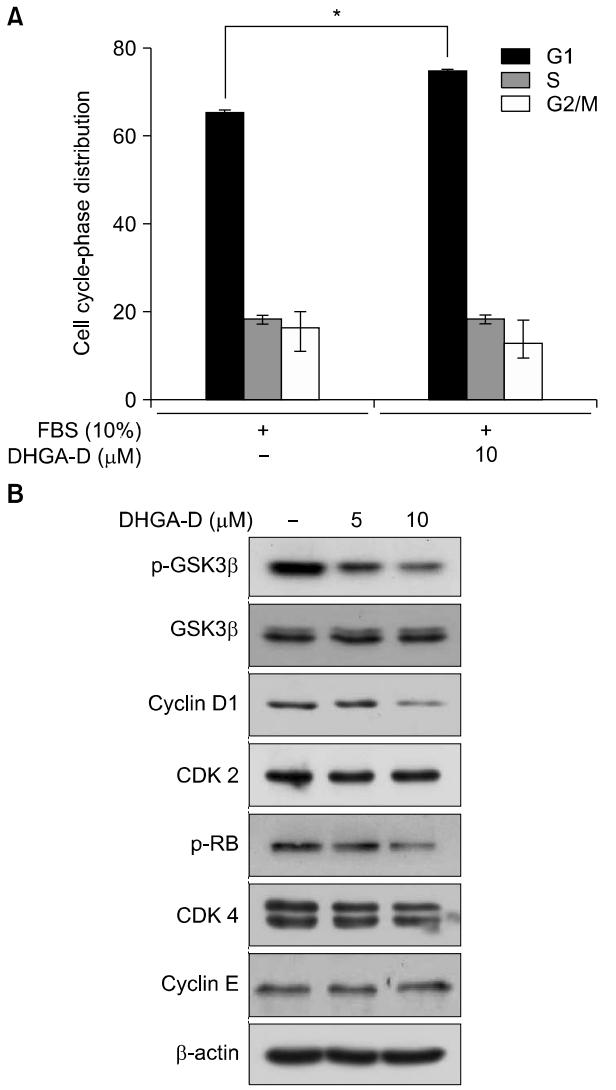

Figure 4. Dehydroglyasperin D (DHGA-D) induces G1 cell cycle arrest and inhibits phosphorylation of GSK3β and cyclin D1 expression in HT-29 cells. (A) DHGA-D induces G1 cell cycle arrest. Cells were cultured in the presence or absence of DHGA-D (10 μM) for 48 hours. Cell cycle analysis was performed by flow cytometry. Data are shown as means ± SD. The asterisks (*) indicate a significant difference ($P < 0.05$) between treatment groups and the vehicle control. (B) DHGA-D inhibits the GSK3β/cyclin D1 signaling pathway in HT-29 cells. Western blot analysis was performed as described in the Materials and Methods using the indicated antibody.

various cancers.[7,19] As such, both the National Cancer Institute and National Institutes of Health recommend the higher consumption of natural and unprocessed foods, including vegetables and fruits.[7,19] Licorice has long been used as a traditional medicine in East Asian cultures and a number of studies have uncovered its biological functions including antioxidant, anti-inflammatory and anti- cancer effects.[8-10] Additionally, compounds present in licorice have been recently identified as effective bioactive agents.[9,10,13,20] In our recent study, DHGA-D exhibited chemopreventive properties with anti-inflammatory effects and directly suppressed mixed-lineage kinase activity.[21] However, its biological functions and underlying molecular mechanisms in colon cancer have not been previously investigated.

We confirmed that DHGA-D significantly suppressed anchorage-dependent and -independent HT-29 cell growth. PI3K/Akt signaling and MAPK family members are known to be key regulators of cell proliferation.[16,17] Interestingly, DHGA-D only suppressed Akt phosphorylation without having an observable effect on ERK phosphorylation. Furthermore, DHGA-D did not appear to affect expression of the PI3K subunits, p85 and p110, or PTEN. Based on these observations, we hypothesized that DHGA-D might interact with PI3K directly and interfere its activity. Kinase assays with active PI3K and pull-down assays confirmed that DHGA-D directly suppressed PI3K activity by binding to PI3K. Although much remains to be elucidated concerning how natural phytochemicals elicit anti-cancer effects, recent studies suggest that at least some phytochemicals can act as small molecule inhibitors of oncogenic enzymes.[22] Our results showing that PI3K is a molecular target of DHGA-D and is related to colon cancer growth support the notion that the physical inhibition of oncogenic enzymes is a more likely explanation for the potent bioactivity of some phytochemicals. Accumulating evidence suggests that the PI3K/Akt pathway is a promising target for the control of colorectal cancer.[23,24]

Our cell cycle analysis suggests that G1 arrest could be a major mechanism responsible for inhibition of HT-29 cell proliferation by DHGA-D. Between the quiescence phase (G0) and the G1 phase of the cell cycle, cyclin D1 acts as a mitogenic signal sensor and is expressed as a delayed-early response to myriad mitogenic signals, which forces cells to enter the proliferative cycle from G0 phase.[25] Activation of Akt by PI3K negatively regulates GSK3β and consequently increases progression of the cell cycle from G0 to G1 and the promotion of cyclin D1 stability.[24] Our recent study also showed that inhibition of PI3K down-regulates GSK3β phosphorylation and cyclin D1 expression and finally induces G1 cell cycle arrest in melanoma.[6] Therefore, these accumulated lines of evidence support the hypothesis that DHGA-D induces G1 phase arrest by suppressing the PI3K/Akt/ GSK3β/cyclin D1 signaling cascade in HT-29 cells.

In summary, DHGA-D inhibits anchorage-dependent and -independent growth of human colon cancer cells. This inhibition is mediated primarily through attenuation of the

PI3K/Akt/GSK3β/cyclin D1 signaling pathway by direct physical inhibition of PI3K. Taken together, DHGA-D is a promising inhibitor of PI3K and has potential as an antineoplastic agent to suppress the growth of colon cancer cells.

ACKNOWLEDGMENTS

This study was supported by a grant from Korea Food Research Institute (to S.K.J.) and Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education (2014R1A1A2054979 to C.H.J.).

CONFLICTS OF INTEREST

No potential conflicts of interest were disclosed.

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