SPANDIDOS
PUBLICATIONS

Wogonin suppresses the LPS-enhanced invasiveness of MDA-MB-231 breast cancer cells by inhibiting the 5-LO/BLT2 cascade

JI-HYUN GO[1], JUN-DONG WEI[1], JAE-IN PARK[1], KYUNG-SEOP AHN[2] and JAE-HONG KIM[1]

[1]College of Life Sciences and Biotechnology, Korea University, Seoul 02841; [2]Natural Medicine Research Center,
Korea Research Institute of Bioscience and Biotechnology (KRIBB), Cheongju-si, Chungbuk 28116, Republic of Korea

Abstract. Wogonin, a naturally occurring bioactive monoflavonoid isolated from *Scutellariae* radix (roots of *Scutellariae baicalensis* Georgi), has known anticancer effects. However, the molecular signaling mechanism by which wogonin inhibits invasiveness in breast cancer cells remains unclear. In the present study, it was observed that wogonin exerted an inhibitory effect on the lipopolysaccharide (LPS)-enhanced invasiveness of MDA-MB-231 cells. In addition, wogonin inhibited the synthesis of interleukin-8 (IL-8) and matrix metallopeptidase-9 (MMP-9), which are critical for promoting invasiveness in MDA-MB-231 cells. Wogonin also suppressed the expression of leukotriene B4 receptor 2 (BLT2) and the synthesis of its ligand, by inhibiting 5-lipoxygenase (5-LO) in LPS-stimulated MDA-MB-231 cells. Notably, wogonin attenuated the production of IL-8 and MMP-9 by inhibiting the BLT2/extracellular signal-regulated kinase (ERK)-linked cascade. Finally, *in vivo*, LPS-driven MDA-MB-231 cell metastasis was markedly suppressed by wogonin administration. Overall, the present results suggested that wogonin inhibited the 5-LO/BLT2/ERK/IL-8/MMP-9 signaling cascade and demonstrated that this cascade may be an important target through which wogonin exerts its anticancer effects in breast cancer.

Correspondence to: Dr Jae-Hong Kim, College of Life Sciences and Biotechnology, Korea University, 5-1 Anam-dong, Sungbuk-gu, Seoul 02841, Republic of Korea
E-mail: jhongkim@korea.ac.kr

Dr Kyung-Seop Ahn, Natural Medicine Research Center, Korea Research Institute of Bioscience and Biotechnology (KRIBB), 30 Yeongudanji-ro, Ochang-eup, Cheongwon-gu, Cheongju-si, Chungbuk 28116, Republic of Korea
E-mail: ksahn@kribb.re.kr

Introduction

Leukotriene B4 receptor 2 (BLT2) is a G protein-coupled receptor for pro-inflammatory lipid mediators, such as leukotriene B4 (LTB4) and 12(S)-hydroxyeicosatetraenoic acid [12(S)-HETE] (1). In recent studies, LTB4 and its receptor, BLT2, have been demonstrated to be closely associated with tumorigenesis. Upregulated production of LTB4 and BLT2 has been previously noted in a number of human cancer cells, including pancreatic, ovarian, bladder, and prostate cancer cells, as well as lung and breast cancer cells, wherein LTB4 and BLT2 promote cancer cell proliferation, chemoresistance, invasion and metastasis (2-7).

Recent studies have demonstrated that following exposure to lipopolysaccharide (LPS) *in vitro* and *in vivo*, cancer cells become more aggressive as a result of the stimulation of the toll-like receptor 4 (TLR4)/myeloid differentiation primary response 88 (MyD88) pathway (8-10). In particular, LPS-induced TLR4 overexpression in breast cancer tissues is associated with lymph node metastasis (11,12). A previous study has demonstrated that LPS upregulates the MyD88/5-lipoxygenase (5-LO)/BLT2 cascade and that BLT2 depletion attenuates the ability of LPS to induce invasiveness and interleukin-8 (IL-8) biosynthesis in aggressive breast cancer cells. In addition, BLT2 inhibition reduces the incidence of LPS-induced metastasis in an *in vivo* breast cancer mouse model (13). On the basis of this information, pharmaceutical or natural agents that directly interfere with the production of BLT2, or antagonize its signaling functions, may be effective in attenuating breast cancer progression.

Scutellariae baicalensis Georgi is a species of herbaceous plant in the Lamiaceae family and has several specialized flavones, such as scutellarin, baicalein, oroxylin A and wogonin (14). Wogonin (5,7-dihydroxy-8-methoxyflavone), which can be found in the roots of *S. baicalensis* Georgi (*Scutellariae* radix), is one of the plant's active components. Wogonin is commonly used as a traditional medicine in East Asian countries, such as China, Japan and Korea (15), and many studies have suggested that wogonin is widely useful due to its antiviral, neuroprotective, anti-inflammatory and antitumor activities, which have been demonstrated in a

variety of *in vitro* and *in vivo* models, as well as its excellent safety profile (16-19). The potent anticancer effects of wogonin are mainly attributable to the induction of cell cycle arrest, apoptosis, and antiangiogenesis activity in various cancer cell lines (20-22). Although there are previous reports about the inhibitory effect of wogonin on the proliferation and invasion of breast cancer cells, the detailed signaling mechanisms of the anti-invasive effects of wogonin remain unclear (20,23,24). Similarly, scutellarin, a compound isolated from the aerial part of *S. baicalensis* Georgi, has been demonstrated to exert anti-invasive effects on MCF-7 breast cancer cells (25). Further studies are necessary to understand the differences in the anti-invasive signaling mechanisms of scutellarin and wogonin. In the present study, the aggressive human breast cancer cell line MDA-MB-231 was used to elucidate the potential mechanism of wogonin in breast cancer cell invasiveness.

The present results demonstrated that wogonin suppressed LPS-induced invasiveness in MDA-MB-231 cells, by downregulating the BLT2-mediated phosphorylation of extracellular signal-regulated kinase (ERK) and the subsequent production of IL-8/matrix metallopeptidase-9 (MMP-9). Additionally, treatment with wogonin suppressed the synthesis of the BLT2 ligand LTB4, by inhibiting 5-LO expression. Furthermore, wogonin significantly reduced LPS-enhanced metastasis in a breast cancer mouse model. Taken together, the present findings suggest that wogonin acts as a novel, natural, safe and effective inhibitor of the 5-LO/BLT2 cascade, and thus attenuates invasiveness and metastasis in breast cancer cells.

Materials and methods

Materials. Wogonin, whose molecular formula is $C_{16}H_{12}O_5$ (Fig. 1A), was isolated from *Scutellariae radix*. An air-dried material (60 g) was extracted with hot methanol. Following concentration, the crude extract was chromatographed on silica gel to yield a crude crystalline fraction, which was recrystallized from methanol to yield 5,7-dihydroxy-8-methoxyflavone (wogonin; 350 mg) (26). Wogonin was kindly provided by Dr Ahn (Korea Research Institute of Bioscience and Biotechnology, Daejeon, Korea). Unless otherwise indicated, samples containing wogonin of ≥98% purity were used in all experiments. Prior to experiments, wogonin was dissolved in dimethyl sulfoxide (DMSO) and diluted with medium. The final DMSO concentration did not exceed 0.1% throughout the study. Fetal bovine serum (FBS) and RPMI-1640 were obtained from Thermo Fisher Scientific, Inc. (Waltham, MA, USA), and MK886, U75302 and LY255283 were acquired from Cayman Chemical Co. (Ann Arbor, MI, USA). LPS (*Escherichia coli* serotype O55:B5), bovine serum albumin, and DMSO were acquired from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). Antibodies to 5-LO were obtained from BD Transduction Laboratories, Inc. (BD Biosciences, Franklin Lakes, NJ, USA), and antibodies to phosphorylated (p-) ERK, ERK, MMP-9 and β-actin were acquired from Cell Signaling Technology, Inc. (Danvers, MA, USA). All other chemicals were obtained from standard sources and were of molecular biological grade or higher.

Cell culture. The human breast cancer cell line MDA-MB-231 was obtained from the Korean Cell Line Bank (Seoul, Korea). The MDA-MB-231 cells were maintained in RPMI-1640 containing 10% heat-inactivated FBS and antibiotic-antimycotic solution (Thermo Fisher Scientific, Inc.) at 37°C in a humidified 5% CO_2 atmosphere.

Cell viability. Cell viability was assessed by cell counting assays. Briefly, $5x10^4$ cells per well were plated in a 12-well plate and incubated in complete culture medium prior to drug exposure. After 24 or 48 h of treatment, aliquots were removed, and viable cells were counted in triplicate by trypan blue (Sigma-Aldrich; Merck KGaA) exclusion. Based on the results of the cytotoxicity assay, a nontoxic concentration of wogonin was selected to use on the MDA-MB-231 cells in subsequent experiments.

Semiquantitative reverse transcription-polymerase chain reaction (RT-PCR) analysis. Total RNA was extracted from the cells with Easy-Blue (Intron, Sungnam, Korea), according to the manufacturer's instructions. A portion (2 µg) of the RNA was subjected to reverse transcription with M-MLV reverse transcriptase (Beams Bio, Gyunggi, Korea), and then, semiquantitative PCR analysis was performed with a PCR PreMix kit (Intron, Sungnam, Korea) and specific forward and reverse primers (Genotech, Daejeon, Korea), according to the manufacturer's instructions. The sequences of the primers used for PCR amplification are listed in Table I. The specificity of all primers was confirmed by sequencing the PCR products. All data were normalized to corresponding data for GAPDH. The thermocycling conditions were as follows: Initial denaturation at 95°C for 5 min, followed by amplification (number of cycles, temperature and duration per step for each gene are listed in Table I), and a final extension phase at 72°C for 10 min. The reaction products were separated by electrophoresis on a 1.5% agarose gel, stained with EtBr, and then visualized by a ChemiDoc™ XRS+ System (Bio-Rad Laboratories, Hercules, CA, USA).

Western blot analysis. The cells were washed with ice-cold PBS, scraped into a lysis buffer [20 mM Tris-HCl, pH 7.5; 150 mM NaCl; 0.5% Nonidet P-40; 5 mM EDTA; 1% Triton X-100; and protease inhibitors (100 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 2 µg/ml leupeptin, and 2 µg/ml aprotinin)], maintained at 4°C and then heated at 95°C for 5 min. Protein concentrations were determined using the Bradford method. A total of 50 µg protein was loaded into each lane, and then, the proteins were separated by 8% SDS-PAGE prior to being transferred electrophoretically to a polyvinylidene fluoride membrane at 100 V for 60 min. The membrane was exposed to TBS containing 0.05% Tween-20 and 5% dried nonfat milk for 1 h and then incubated with primary antibodies against 5-LO (cat. no. 610695; 1:1,000; BD Transduction Laboratories Inc.; BD Biosciences), MMP-9 (cat. no. CST 3852), p-ERK (cat. no. 9101S), total ERK (cat. no. 9102) and β-actin (cat. no. 4970S) (1:1,000; all from Cell Signaling Technology, Inc.). Secondary antibodies were anti-rabbit (cat. no. 7074S; 1:2,000) or anti-mouse antibodies (cat. no. 7076S, 1:2,000) both from Cell Signaling Technology, Inc., and then, the proteins were visualized using enhanced chemiluminescence reagent (Amersham; GE Healthcare, Chicago, IL, USA), according to the manufacturer's recommendations.

RNA interference (RNAi). BLT2-specific (5'-CCACGCAGU CAACCUUCUG-3') (27) and pre-designed control (scrambled)

Table I. Sequences of primers used in the present study.

Author, year	Gene	Primer		Sequence (5'-3')	Denaturation/annealing/extension Temperature (°C)	Time per step (sec)	Total cycles	Amplicon size (bp)	(Refs.)
Park and Kim, 2013	GAPDH	Forward		CTGCACCACCAACTGCTTAGC	95/58/72	20/15/15	21	337	(13)
		Reverse		CTTCACCACCTTCTTGATGTC					
	BLT1	Forward		TATGTCTGCGGAGTCAGCATGTACGC	95/67/72	30/30/30	31	346	
		Reverse		CCTGTAGCCGACGCCCTATGTCCG					
	BLT2	Forward		AGCCTGGAGACTCTGACCGCTTTCG		20/20/15	30	380	
		Reverse		GACGTAGCACCGGGTTGACGCTA					
Kim et al, 2012	IL-8	Forward		ATGACTTCCAAGCTGGCCGTGGCT	95/61/72	20/20/20	20	292	(5)
		Reverse		TCTCAGCCCTCTTCAAAAACTTCTC					
Kim et al, 2010	MMP-9	Forward		CACTGTCCACCCCTCAGAGC	95/62/72	20/20/20	31	273	(29)
		Reverse		GCCACTTGTCGGCGATAAGG					

BLT, leukotriene B4 receptor; IL-8, interleukin-8; MMP-9, matrix metallopeptidase-9.

small interfering (si) RNAs (cat. no. SN-1003) were obtained from Bioneer (Daejeon, Korea). The siRNAs were introduced into the cells by transfection in Opti-MEM (cat. no. 31985070; Thermo Fisher Scientific, Inc.) for the indicated times using Oligofectamine (cat. no. 12252-011; Thermo Fisher Scientific, Inc.).

Invasion assay. The invasive potential of MDA-MB-231 cells was assessed using BioCoat Matrigel Invasion Chambers (BD Biosciences), as described (5,13). Cells (3.5x10⁴) were harvested with RPMI-1640 supplemented with 0.5% FBS and seeded in rehydrated Matrigel inserts containing the same media. RPMI-1640 supplemented with 5% FBS, which served as a chemoattractant, was added to the lower chamber. MDA-MB-231 cells were incubated at 37°C for approximately 24 h, after which the cells on the upper surface of each filter were removed, and the remaining cells were fixed in methanol and stained with hematoxylin-eosin (H&E). The cells in 10 randomly selected high-power (x40) fields were then counted with a CKX41 microscope (Olympus Corporation, Tokyo, Japan) equipped with a DP71 digital camera (Olympus Corporation). Each sample was assayed in triplicate.

IL-8 and LTB4 measurements. Conditioned media were immediately frozen and lyophilized. ELISA kits for human IL-8 and LTB4 were obtained from BD Biosciences (cat. no. 550799) and Enzo Life Sciences (cat. no. ADI-900-068; Farmingdale, NY, USA), respectively. Each assay procedure was conducted according to the manufacturer's instructions for each kit. The concentrations of the mediators were determined at 450 nm for IL-8 and 405 nm for LTB4, using an Epoch microplate spectrophotometer (BioTek Instruments, Winooski, VT, USA).

In vivo metastasis assay. This study was approved by the Ethics Committee of Korea University, and all experimental animals used herein were handled according to the guidelines approved by the Institutional Animal Care and Use Committee of Korea University. All animals were maintained under a 12-h light/dark cycle and housed at a density of 5 mice per static polycarbonate microisolator cage on disposable bedding. Wire-lid food hoppers within the cages were filled to capacity with rodent chow, and water was supplied by a bottle. For the spontaneous metastasis assays, cultured MDA-MB-231 cells were treated with LY255283 (10 μM), wogonin (20 μM) or DMSO, prior to being treated with LPS (1 μg/ml) for 24 h, as described previously (13). Then, six-week-old female nude (BALB/C) mice (Daehan Biolink, Chungbuk, Korea) were injected unilaterally in the fourth right mammary fat pad with cultured MDA-MB-231 (3.5x10⁶) cells in 100 μl of PBS; the cells were injected subcutaneously at the base of the nipple. Wogonin (20 mg/kg), LY255283 (2.5 mg/kg) or DMSO was injected intraperitoneally three times at 5-day intervals beginning immediately following cell implantation. The animals were sacrificed 14 weeks post-cell implantation, and the number of metastatic nodules on the surface of the small bowel was determined.

Statistical analysis. The data are representative of three independent experiments, and the results are presented as the mean ± standard deviation. Comparisons between groups were performed with one-way analysis of variance, followed by
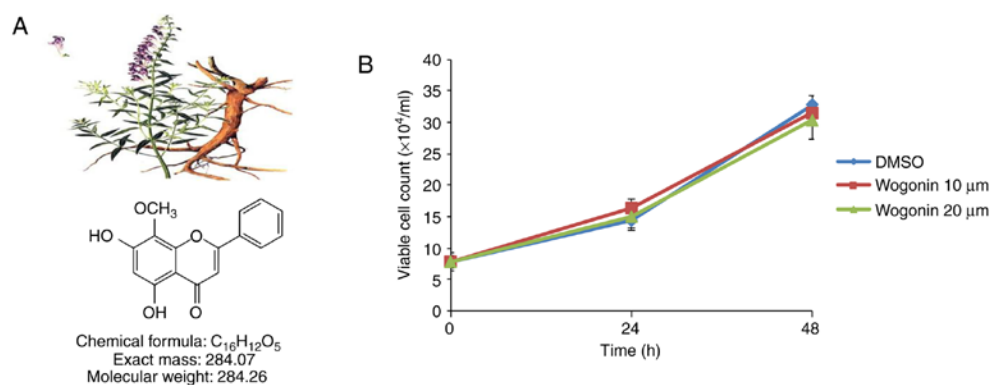
Figure 1. Effect of wogonin on MDA-MB-231 cell viability. (A) The plant *Scutellariae baicalensis* Georgi and the chemical structure of the active compound 5,7-dihydroxy-8-methoxyflavone (known as wogonin). (B) MDA-MB-231 cells were incubated in the presence or absence of 0-20 μM wogonin, and cell viability was determined using a cell counting assay. Data are presented as the mean $\pm$ standard deviation of three independent experiments.
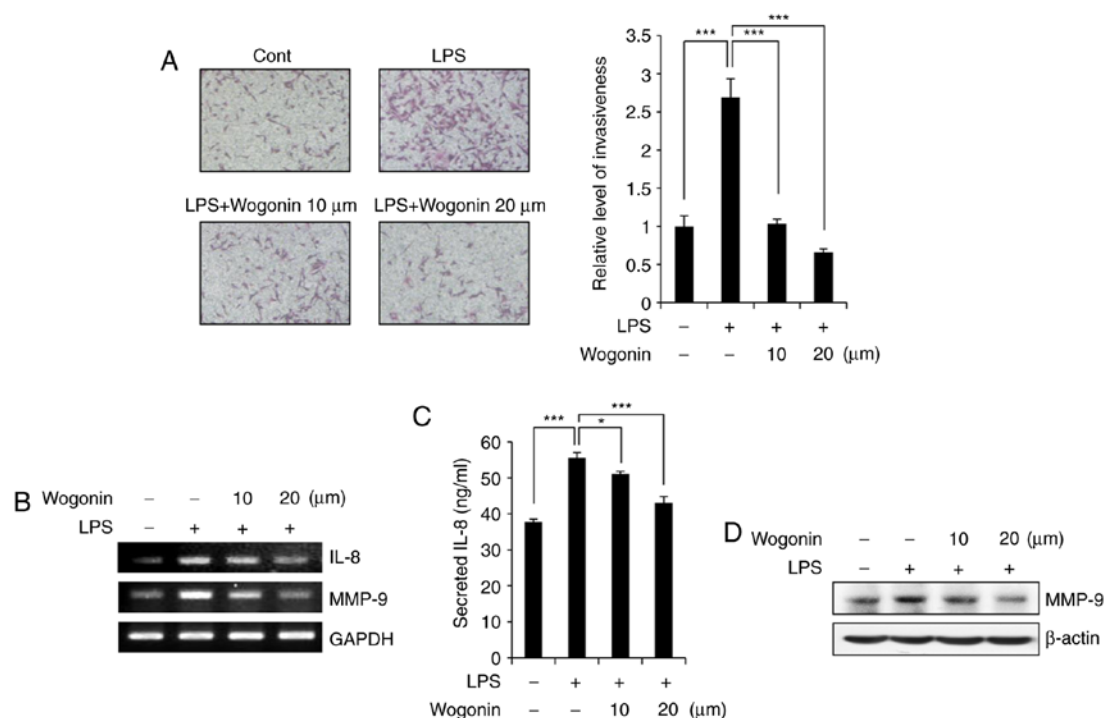

Figure 2. Wogonin inhibits the invasiveness of MDA-MB-231 breast cancer cells by downregulating IL-8 and MMP-9 expression. (A) MDA-MB-231 cells were incubated with wogonin (10 or 20 μM) and then treated with or without LPS (1 μg/ml), prior to Transwell invasion assays. Representative fields of invading cells stained with H&E and quantification are shown. (B) Cells were incubated with wogonin (10 or 20 μM) for 1 h, and then, they were incubated with or without LPS (1 μg/ml) for 24 h. The cells were subsequently assayed for IL-8 and MMP-9 mRNA expression by semiquantitative RT-PCR. (C) The levels of secreted IL-8 in the cell supernatants were measured by ELISA. (D) Cell lysates were subjected to western blot analysis with antibodies to MMP-9 and β-actin (loading control). The semiquantitative RT-PCR images are representative of three independent experiments. Quantitative data are presented as the mean $\pm$ standard deviation of three independent experiments. *P<0.05 and ***P<0.005, with comparisons indicated by lines. IL-8, interleukin-8; MMP-9, matrix metallopeptidase-9; LPS, lipopolysaccharide; RT-PCR, reverse transcription-polymerase chain reaction.

Tukey's post-hoc test. SPSS software (IBM SPSS Statistics for Windows, version 21.0; IBM Corp., Armonk, NY, USA) was used for statistical analysis. P<0.05 was considered to indicate a statistically significant difference.

Results

Wogonin inhibits the invasiveness of MDA-MB-231 breast cancer cells by downregulating IL-8 and MMP-9 expression. Before studying the anti-invasive activity of wogonin, the effect of wogonin on cell viability was assessed. MDA-MB-231 cells

were treated with wogonin at the indicated concentrations (10 or 20 μM). The viability of MDA-MB-231 cells was not significantly affected in 24 or 48 h of treatment with wogonin at concentrations up to 20 μM (Fig. 1B), suggesting that wogonin exhibits no cytotoxicity at these doses in MDA-MB-231 cells. To determine whether wogonin has any effect on the LPS-enhanced invasive potential of MDA-MB-231 cells, Matrigel-coated Transwell chambers were used. Similar to previous studies (13), MDA-MB-231 cell invasiveness was increased in the LPS-treated group compared with the control group (Fig. 2A). However, wogonin significantly
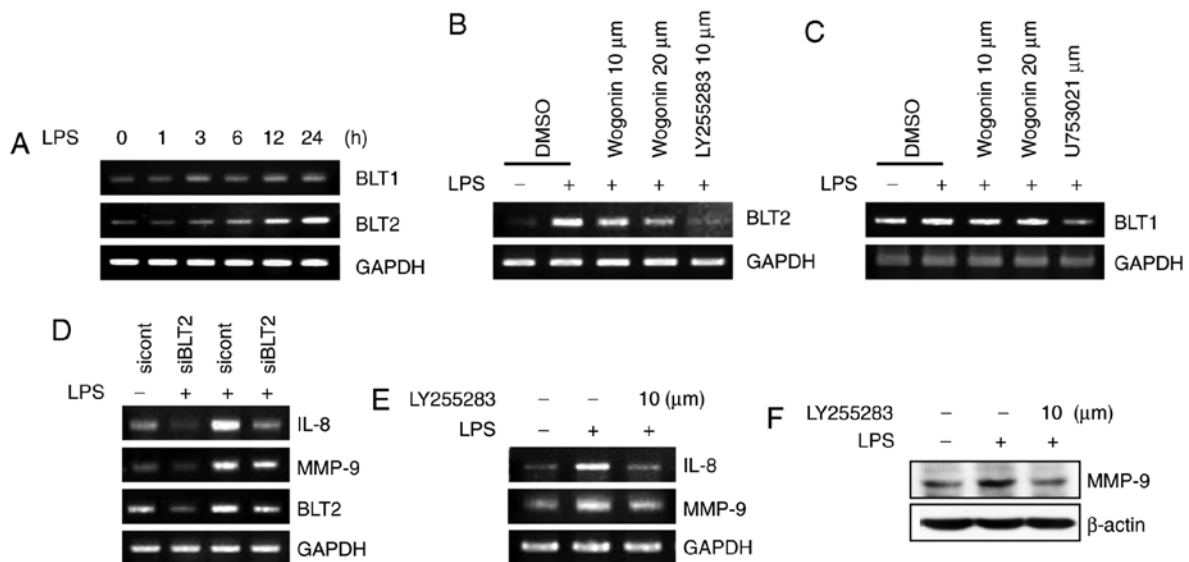
Figure 3. Wogonin inhibits BLT2 upregulation in LPS-stimulated MDA-MB-231 cells. (A) Cells were treated with LPS (1 µg/ml) for the indicated times (0, 1, 3, 6, 12 and 24 h), and then, the cell lysates were assayed for BLT1 and BLT2 mRNA expression by semiquantitative RT-PCR. (B) Cells were incubated with wogonin (10 or 20 µM) or LY255283 (10 µM) for 1 h, and then, they were stimulated with LPS for 24 h. The cells were subsequently assayed for BLT2 and (C) BLT1 mRNA expression by semiquantitative RT-PCR. (D) Cells were transfected with BLT2 (siBLT2) or control (siCont) siRNA for 24 h and then treated with LPS for 24 h. IL-8, MMP-9 and BLT2 mRNA expression was subsequently assayed by semiquantitative RT-PCR. (E) Cells were incubated with LY255283 (10 µM) for 1 h and then stimulated with LPS for 24 h. The cell lysates were assayed by semiquantitative RT-PCR, or (F) by western blot analysis. BLT, leukotriene B4 receptor; LPS, lipopolysaccharide; RT-PCR, reverse transcription-polymerase chain reaction; si, small interfering; IL-8, interleukin-8; MMP-9, matrix metallopeptidase-9.

suppressed LPS-induced invasiveness in a dose-dependent manner. Specifically, treatment with wogonin at 10 and 20 µM inhibited cell invasion by ~98% compared with the control (Fig. 2A). Previous studies have demonstrated that IL-8 and MMP-9 expression is required for breast cancer cell invasiveness and metastasis (5,13,28). To understand the signaling mechanism by which wogonin suppressed the LPS-induced invasiveness of the MDA-MB-231 cells, the mRNA expression levels of IL-8 and MMP-9 were examined by RT-PCR (Fig. 2B). IL-8 and MMP-9 expression was indeed markedly increased by LPS treatment, and 20 µM wogonin clearly suppressed LPS-induced IL-8 and MMP-9 overexpression, in a dose-dependent manner (Fig. 2B). This effect was also confirmed at the protein level, by ELISA for IL-8 (Fig. 2C) and by western blotting for MMP-9 (Fig. 2D). Taken together, these results suggest that wogonin attenuated cell invasiveness by downregulating the expression of IL-8 and MMP-9 in LPS-stimulated MDA-MB-231 cells.

Wogonin inhibits BLT2 upregulation in LPS-stimulated MDA-MB-231 cells. Next, the results demonstrated that LPS stimulation resulted in upregulated BLT2 mRNA levels in MDA-MB-231 breast cancer cells (Fig. 3A), consistent with previous reports (13). Semiquantitative RT-PCR revealed that LPS markedly increased BLT2 mRNA levels in a time-dependent manner, but had little impact on BLT1 expression (Fig. 3A). Thus, the hypothesis that wogonin may suppress BLT2 expression was examined. Wogonin clearly suppressed LPS-induced BLT2 mRNA expression in a dose-dependent manner (Fig. 3B), but did not have an effect on BLT1 expression (Fig. 3C). Previous reports suggested that the BLT2 cascade contributes to the production of IL-8 and MMP-9 (3,13,29). Furthermore, BLT2 depletion using siRNA has been reported to attenuate

the LPS-induced invasive activity of MDA-MB-231 cells (13). In agreement with these results, BLT2 depletion using siRNA, or the selective BLT2 antagonist LY255283, clearly attenuated LPS-induced IL-8 and MMP-9 mRNA and protein expression in MDA-MB-231 cells (Fig. 3D-F); however, the BLT1 antagonist U75302 did not exert such effects (data not shown). These results suggest that BLT2 may be the effector for the wogonin anti-invasion activity.

Wogonin suppresses the synthesis of the BLT2 ligand in LPS-stimulated MDA-MB-231 cells. Recent studies have suggested that 5-LO expression was increased in response to LPS stimulation in MDA-MB-231 cells (13,30). Thus, whether wogonin affects the expression of 5-LO and its metabolite, LTB4, was examined in LPS-treated MDA-MB-231 cells. 5-LO expression was markedly inhibited by wogonin treatment in LPS-stimulated MDA-MB-231 cells (Fig. 4A). In addition, wogonin significantly inhibited LPS-induced production of the 5-LO metabolite LTB4 (Fig. 4B). Under the same experimental conditions, LPS-induced IL-8 and MMP-9 expression was suppressed by pretreatment with the 5-LO-activating protein (FLAP) inhibitor, MK886 (Fig. 4C-E). Taken together, these results suggest that wogonin inhibits 5-LO and thus attenuates the production of its metabolite, LTB4, and the subsequent synthesis of IL-8 and MMP-9.

Wogonin attenuates ERK phosphorylation and thus inhibits IL-8/MMP-9 production in LPS-stimulated MDA-MB-231 cells. Previous reports have demonstrated that ERK lies downstream of BLT2 and upstream of MMP-9 in MDA-MB-231 cells (24,30). Exposure to LPS increased ERK phosphorylation in a time-dependent manner (Fig. 5A). By contrast, LPS-induced ERK phosphorylation was markedly inhibited
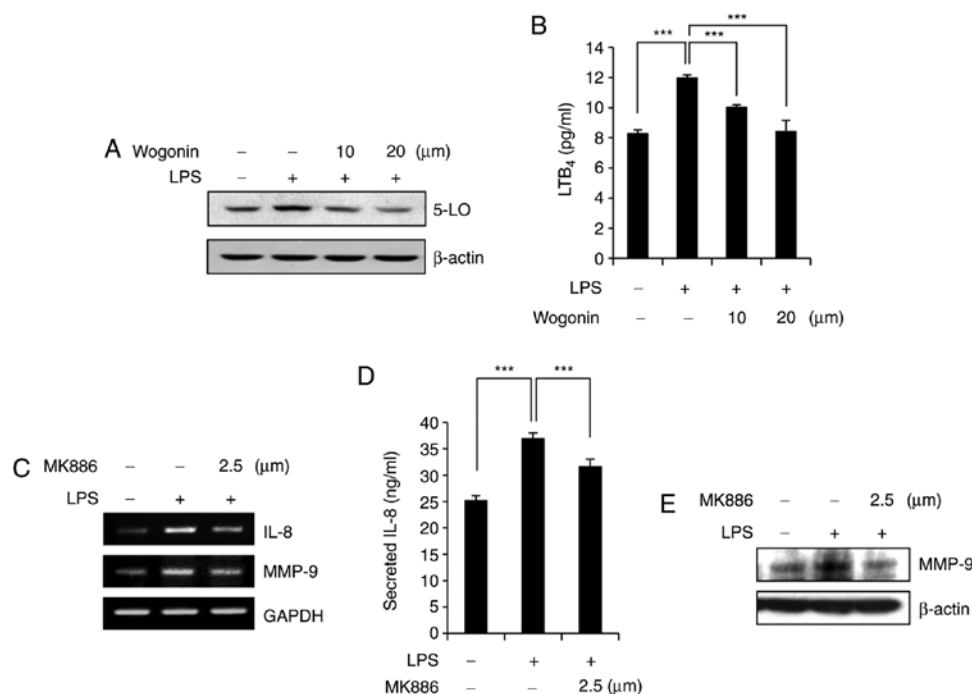
Figure 4. Wogonin suppresses the synthesis of the BLT2 ligand in LPS-stimulated MDA-MB-231 cells. (A) Cells were incubated with wogonin (10 or 20 μM) for 1 h and then stimulated with LPS (1 μg/ml). After 24 h, the cell lysates were subjected to western blot analysis with antibodies to 5-LO and β-actin (loading control). (B) LTB4 levels in the culture supernatants were measured by ELISA. (C) Cells were treated with MK886 (2.5 μM) for 1 h and then stimulated with LPS for 24 h, after which IL-8 and MMP-9 mRNA expression was assayed by semiquantitative RT-PCR. (D) IL-8 levels in the culture supernatants were analyzed by ELISA. (E) Total cell lysates were analyzed by western blotting to determine the protein expression of MMP-9 and β-actin (loading control). Quantitative data are presented as the mean ± standard deviation of three independent experiments. ***P<0.005, with comparisons indicated by lines. BLT, leukotriene B4 receptor; LPS, lipopolysaccharide; 5-LO, 5-lipoxygenase; LTB4, leukotriene B4; IL-8, interleukin-8; MMP-9, matrix metallopeptidase-9; RT-PCR, reverse transcription-polymerase chain reaction.
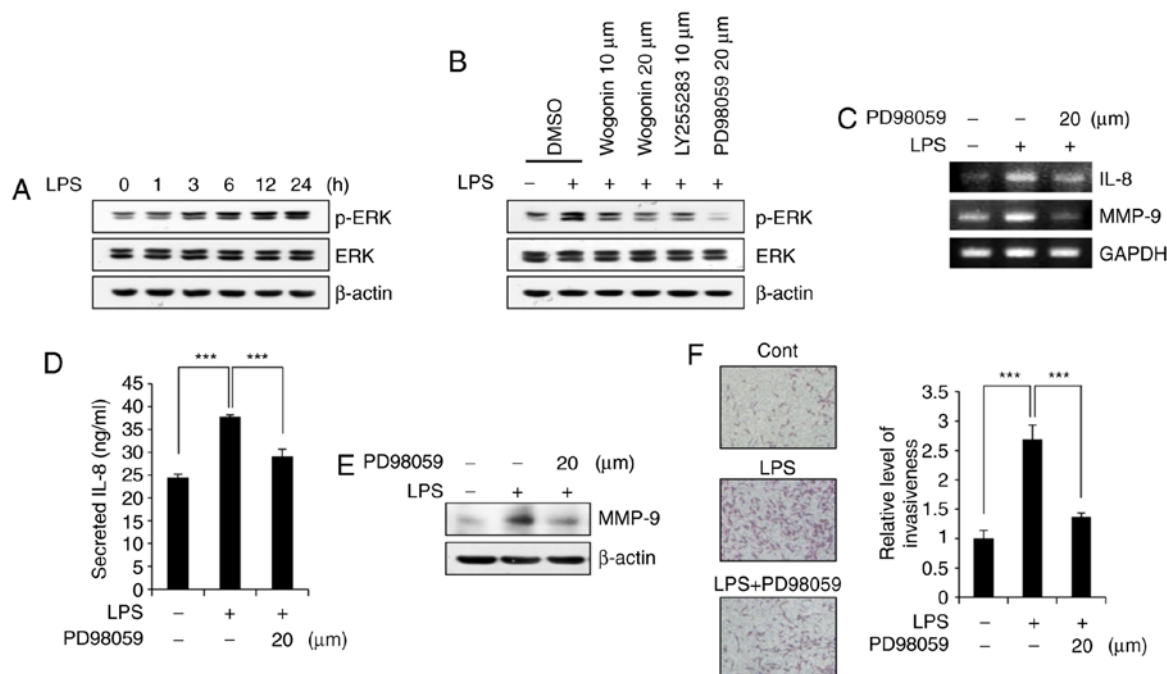

Figure 5. Wogonin attenuates ERK phosphorylation in LPS-stimulated MDA-MB-231 cells. (A) Cells were treated with LPS (1 μg/ml) for the indicated times (0, 1, 3, 6, 12 and 24 h), and then p-ERK and ERK protein levels were measured by western blotting. (B) Cells were incubated with wogonin (10 or 20 μM), LY255283 (10 μM), PD98059 (20 μM) or DMSO for 1 h and then stimulated with LPS for 24 h. The cell lysates were then subjected to western blot analysis for ERK activation. (C) Cells were incubated with PD98059 (20 μM) for 1 h, and then, they were incubated with or without LPS (1 μg/ml) for 1 h. The cells were assayed for IL-8 and MMP-9 mRNA tarpression by semiquantitative RT-PCR. (D) The levels of secreted IL-8 in the cell supernatants were measured by ELISA. (E) The cell lysates were analyzed by western blotting to determine MMP-9 expression. β-actin was used as a loading control. (F) Cells were incubated with PD98059 (20 μM) or DMSO and then treated with or without LPS (1 μg/ml), prior to Transwell invasion assays. Representative fields of invading cells stained with H&E and quantification are shown. Quantitative data are presented as the mean ± standard deviation of three independent experiments. ***P<0.005, with comparisons indicated by lines. ERK, extracellular signal-regulated kinase; LPS, lipopolysaccharide; p-, phosphorylated; IL-8, interleukin-8; MMP-9, matrix metallopeptidase-9; RT-PCR, reverse transcription-polymerase chain reaction.
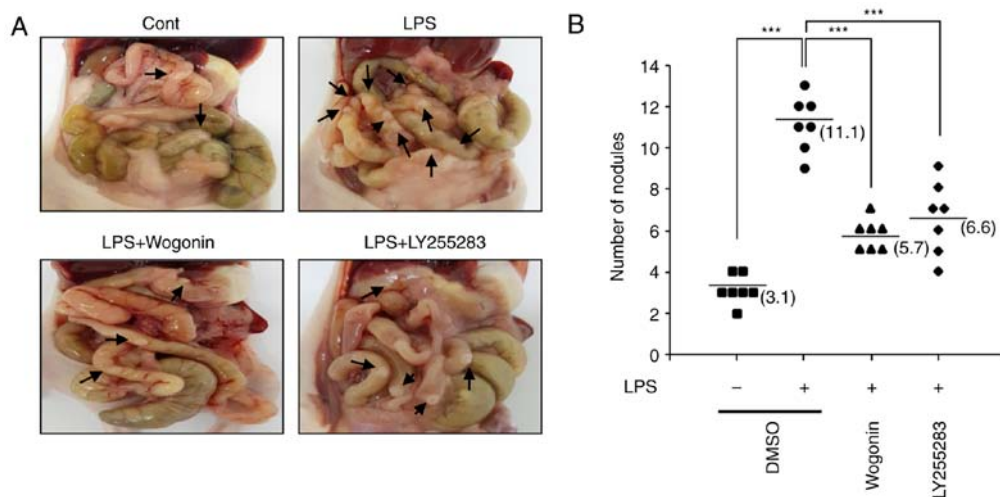
Figure 6. Wogonin significantly reduces LPS-induced metastasis in an orthotopic breast cancer model. MDA-MB-231 cells were incubated with wogonin (20 μM), LY255283 (10 μM) or DMSO for 1 h and then treated with or without LPS (1 μg/ml) for 24 h, prior to being implanted in the mammary fat pads of nude mice. Then, the animals were injected intraperitoneally with wogonin (20 mg/kg), LY255283 (2.5 mg/kg) or DMSO three times at 5-day intervals. At 14 weeks post-implantation, the small bowel was examined for metastatic nodules. (A) Representative images of metastatic modules (arrows) per experimental group. (B) Quantification of *in vivo* metastasis assay. Data were obtained from seven mice per experimental group. ***P<0.005, with comparisons indicated by lines. LPS, lipopolysaccharide.
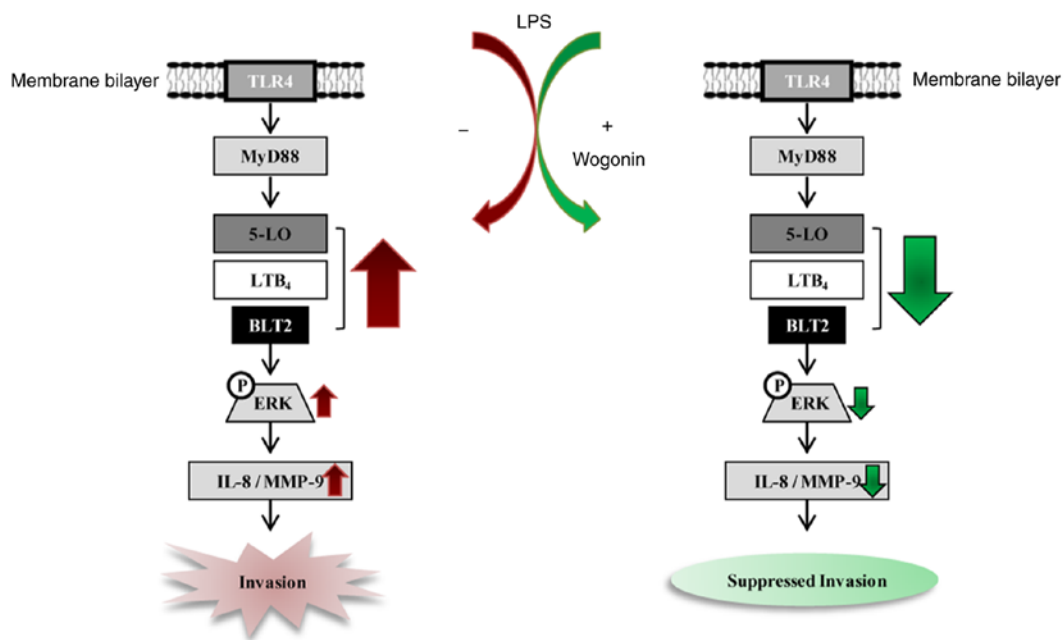

Figure 7. Schematic representation of the anti-invasive effects of wogonin in LPS-stimulated MDA-MB-231 cells, via the 5-LO/BLT2 pathway. TLR4, toll-like receptor 4; MyD88, myeloid differentiation primary response 88; 5-LO, 5-lipoxygenase; LTB4, leukotriene B4; BLT2, leukotriene B4 receptor 2; ERK, extracellular signal-regulated kinase; IL-8, interleukin-8; MMP-9, matrix metallopeptidase-9.

by wogonin in a dose-dependent manner (Fig. 5B). Treatment with PD98059, an ERK inhibitor, suppressed LPS-stimulated IL-8 and MMP-9 expression at both the transcript (Fig. 5C) and protein levels (Fig. 5D and E) and inhibited LPS-stimulated invasiveness (Fig. 5F). These data suggest that wogonin inhibits IL-8/MMP-9 production in LPS-stimulated MDA-MB-231 cells, most likely through ERK activation.

Wogonin significantly reduces LPS-induced metastasis in an orthotopic breast cancer model. Previously, BLT2 inhibition has been demonstrated to significantly reduce LPS-induced metastasis in an orthotopic breast cancer model (13). Thus,

the effect of wogonin on breast cancer metastasis induced by LPS exposure was investigated *in vivo*. To study the effect of wogonin on the LPS-driven metastasis of MDA-MB-231 cells *in vivo*, the mouse orthotopic injection tumor metastasis model was used. MDA-MB-231 cells were pretreated with wogonin (20 μM) or DMSO, and then, they were treated with LPS (1 μg/ml) for 24 h prior to being implanted into the mammary fat pads of mice. At 14 weeks post-implantation, metastatic nodules were counted on the small bowel of the mice. The number of nodules in the small bowel was significantly reduced by wogonin, compared with the mice treated with LPS alone (Fig. 6A and B). Taken together, these results indicate that

wogonin exerted an inhibitory effect on LPS-induced metastasis *in vivo*.

Discussion

The present study demonstrated that wogonin suppressed the LPS-enhanced invasiveness and metastasis of the breast cancer cell line MDA-MB-231 *in vitro* and *in vivo*. Additionally, the results demonstrated that wogonin inhibited the 5-LO/BLT2/ERK/IL-8/MMP-9 cascade. These findings suggest that this cascade may be the target through which wogonin exerts its anticancer effects in breast cancer.

Breast cancer progression involves many steps, including tumor growth, cancer cell invasion, and cancer cell dissemination throughout the body (31). Cancer invasion has a central role in metastasis and is the main cause of death in millions of breast cancer patients (32). In particular, MDA-MB-231 cells are highly aggressive and invasive, and the options for the treatment of tumors comprising of such invasive cells are limited. Thus, identifying and controlling the molecular mechanisms that regulate the tumor cell invasion process is key for the development of therapeutic interventions to prevent tumor metastasis and reduce mortality in breast cancer patients.

Wogonin has been demonstrated to inhibit the development of malignancies and has attracted increasing attention as a promising anticancer compound. Wogonin has been previously reported to effectively inhibit the mobility and invasiveness of various solid tumors, including breast cancer (24), osteosarcoma (33), hepatoma and melanoma (34,35). Although the anti-invasive and antimetastatic effects of wogonin have been proven, the molecular mechanisms of its effects are not fully understood. In addition, its main target metabolite in cancer, especially breast cancer, remains unclear. Previous reports have suggested that wogonin inhibits the effects of eicosanoids generated by phospholipase A2 activation and lipoxygenase-induced fatty acid oxidation. Wogonin significantly inhibits the release of histamine and LTB4 in rat peritoneal exudate cells (36). Additionally, in rat macrophages, wogonin attenuates the biosynthesis of LTB4 and 5-hydroxyeicosatetraenoic acid (5-HETE) (37). However, there are no reports on the inhibitory effects of wogonin on leukotrienes produced by lipoxygenases, such as 5-LO, in cancer cells. Thus, the present study is the first to demonstrate that wogonin targets the 5-LO/BLT2 signaling pathway in cancer cells and thus inhibits invasiveness. Similar to our observation, 12-LO inhibitor baicalein has been reported to inhibit the invasion of MDA-MB-231 cells through downregulating various signaling pathways, including MAPK, Wnt/β-catenin or BLT2 (13,38,39). However, under our experimental conditions, wogonin was demonstrated to significantly inhibit the expression levels of 5-LO, but not 12-LO (data not shown). Therefore, it can be speculated that 5-LO, not 12-LO, may be an important target for the action of wogonin.

5-LO controls another key pathway in arachidonic acid metabolism; the major products of the pathway include LTB4 and the cysteinyl leukotrienes, which are potent pro-inflammatory lipid mediators involved in chronic inflammatory diseases and cancer (40). 5-LO is expressed in many cancer cells and participates in angiogenesis, invasiveness, and cellular proliferation (41). The present results demonstrated that 5-LO and, thus, the downstream BLT2/ERK/IL-8/MMP-9 cascade are potential targets of wogonin. In clinical studies, IL-8 and MMP-9 are overexpressed in breast tumor tissues compared with normal tissues, and the expression of these factors is correlated with high invasion potential (42,43). Consistent with these findings, we previously reported that the expression of IL-8 promoted MDA-MB-231 cell invasiveness and metastasis through the activation of the BLT2 signaling pathway (13). Previous reports have also revealed that wogonin suppresses IL-8 and MMP-9 expression in tumor cells. For example, wogonin attenuated IL-8 expression in LPS-induced colorectal adenocarcinoma cells (44) and inhibited breast cancer cell invasion and metastasis *in vitro,* by suppressing phorbol-12-myristate-13-acetate (PMA)-induced MMP-9 expression (24).

The current results demonstrated that ERK lies downstream of the BLT2 cascade in MDA-MB-231 cells (30). ERK activation is critical for IL-8 and MMP-9 expression and the promotion of the degradation of the basement membrane and the infiltration of surrounding tissues for the facilitation of breast tumor metastasis (45-47). In the present study, it was demonstrated that wogonin significantly attenuated the activation of ERK in LPS-stimulated MDA-MB-231 cells, which indicated that the antimetastatic effect of wogonin in breast cancer may be dependent on BLT2 expression. Additionally, wogonin remarkably suppressed LPS-enhanced metastasis to the small bowel, suggesting that wogonin inhibited BLT2-induced breast cancer metastasis. These findings provide a preliminary basis for the development of wogonin-based therapeutic herbal medicines for the treatment of metastatic breast cancer.

In summary, the present study demonstrated that wogonin suppressed the ability of LPS to stimulate the invasiveness and metastasis of MDA-MB-231 cells. Furthermore, the results revealed that the molecular mechanism responsible for the effects of wogonin may be associated with the 5-LO/BLT2/ERK/IL-8/MMP-9 cascade (Fig. 7). Thus, the 5-LO-/BLT2 axis is a novel pathway through which wogonin may exert its anti-invasive actions in LPS-stimulated MDA-MB-231 cells.

Availability of data and materials

The analyzed datasets generated during the study are available from the corresponding author on reasonable request.

Authors' contributions

JHG designed the study, performed the experiments, analyzed data, and was a major contributor in writing the manuscript. JDW contributed to the interpretation of the results. JIP conducted the animal study. KSA provided the wogonin and critically revised the manuscript for intellectually important content. JHK provided critical feedback and contributed to the design of the present study, supervised the study and wrote the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All experiments involving animals were approved by the Ethics Committee of Korea University, and performed according to the guidelines approved by the Institutional Animal Care and Use Committee of Korea University.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Funk CD: Prostaglandins and leukotrienes: Advances in eicosanoid biology. Science 294: 1871-1875, 2001.
2. Hennig R, Osman T, Esposito I, Giese N, Rao SM, Ding XZ, Tong WG, Buchler MW, Yokomizo T, Friess H and Adrian TE: BLT2 is expressed in PanINs, IPMNs, pancreatic cancer and stimulates tumour cell proliferation. Br J Cancer 99: 1064-1073, 2008.
3. Kim EY, Seo JM, Kim C, Lee JE, Lee KM and Kim JH: BLT2 promotes the invasion and metastasis of aggressive bladder cancer cells through a reactive oxygen species-linked pathway. Free Radic Biol Med 49: 1072-1081, 2010.
4. Park J, Park SY and Kim JH: Leukotriene B4 receptor 2 contributes to chemoresistance of SK-OV-3 ovarian cancer cells through activation of signal transducer and activator of transcription-3-linked cascade. Biochim Biophys Acta 1863: 236-243, 2016.
5. Kim H, Choi JA, Park GS and Kim JH: BLT2 up-regulates interleukin-8 production and promotes the invasiveness of breast cancer cells. PLoS One 7: e49186, 2012.
6. Seo JM, Park S and Kim JH: Leukotriene B4 receptor 2 promotes invasiveness and metastasis of ovarian cancer cells through signal transducer and activator of transcription 3 (STAT3)-dependent up-regulation of matrix metalloproteinase-2. J Biol Chem 287: 13840-13849, 2012.
7. Lee JW and Kim JH: Activation of the leukotriene B4 receptor 2-reactive oxygen species (BLT2-ROS) cascade following detachment confers anoikis resistance in prostate cancer cells. J Biol Chem 288: 30054-30063, 2013.
8. Harmey JH, Bucana CD, Lu W, Byrne AM, McDonnell S, Lynch C, Bouchier-Hayes D and Dong Z: Lipopolysaccharide-induced metastatic growth is associated with increased angiogenesis, vascular permeability and tumor cell invasion. Int J Cancer 101: 415-422, 2002.
9. Szajnik M, Szczepanski MJ, Czystowska M, Elishaev E, Mandapathil M, Nowak-Markwitz E, Spaczynski M and Whiteside TL: TLR4 signaling induced by lipopolysaccharide or paclitaxel regulates tumor survival and chemoresistance in ovarian cancer. Oncogene 28: 4353-4363, 2009.
10. Chen X, Zhao F, Zhang H, Zhu Y, Wu K and Tan G: Significance of TLR4/MyD88 expression in breast cancer. Int J Clin Exp Pathol 8: 7034-7039, 2015.
11. Yang H, Wang B, Wang T, Xu L, He C, Wen H, Yan J, Su H and Zhu X: Toll-like receptor 4 prompts human breast cancer cells invasiveness via lipopolysaccharide stimulation and is over-expressed in patients with lymph node metastasis. PLoS One 9: e109980, 2014.
12. Ahmed A, Wang JH and Redmond HP: Silencing of TLR4 increases tumor progression and lung metastasis in a murine model of breast cancer. Ann Surg Oncol 20 (Suppl 3): S389-S396, 2013.
13. Park GS and Kim JH: Myeloid differentiation primary response gene 88-leukotriene B4 receptor 2 cascade mediates lipopolysaccharide-potentiated invasiveness of breast cancer cells. Oncotarget 6: 5749-5759, 2015.
14. Li HB and Chen F: Isolation and purification of baicalein, wogonin and oroxylin A from the medicinal plant *Scutellaria baicalensis* by high-speed counter-current chromatography. J Chromatogr A 1074: 107-110, 2005.
15. Brekhman II, Grinevitch MA and Kyu KB: Oriental medicine: A computerized study of complex recipes and their components: Herbs most frequently used in traditional Japanese and Korean medicine. Am J Chin Med 9: 134-143, 1981.
16. Guo Q, Zhao L, You Q, Yang Y, Gu H, Song G, Lu N and Xin J: Anti-hepatitis B virus activity of wogonin in vitro and in vivo. Antiviral Res 74: 16-24, 2007.
17. Khan NM, Haseeb A, Ansari MY, Devarapalli P, Haynie S and Haqqi TM: Wogonin, a plant derived small molecule, exerts potent anti-inflammatory and chondroprotective effects through the activation of ROS/ERK/Nrf2 signaling pathways in human Osteoarthritis chondrocytes. Free Radic Biol Med 106: 288-301, 2017.
18. Xu Y, Yang B, Hu Y, Lu L, Lu X, Wang J, Xu F, Yu S, Huang J and Liang X: Wogonin prevents TLR4-NF-kappaB-medicated neuro-inflammation and improves retinal ganglion cells survival in retina after optic nerve crush. Oncotarget 7: 72503-72517, 2016.
19. Huynh DL, Sharma N, Kumar Singh A, Singh Sodhi S, Zhang JJ, Mongre RK, Ghosh M, Kim N, Ho Park Y and Kee Jeong D: Anti-tumor activity of wogonin, an extract from *Scutellaria baicalensis*, through regulating different signaling pathways. Chin J Nat Med 15: 15-40, 2017.
20. Huang KF, Zhang GD, Huang YQ and Diao Y: Wogonin induces apoptosis and down-regulates survivin in human breast cancer MCF-7 cells by modulating PI3K-AKT pathway. Int Immunopharmacol 12: 334-341, 2012.
21. Lin CM, Chen YH, Ong JR, Ma HP, Shyu KG and Bai KJ: Functional role of wogonin in anti-angiogenesis. Am J Chin Med 40: 415-427, 2012.
22. Yang H, Hui H, Wang Q, Li H, Zhao K, Zhou Y, Zhu Y, Wang X, You Q, Guo Q and Lu N: Wogonin induces cell cycle arrest and erythroid differentiation in imatinib-resistant K562 cells and primary CML cells. Oncotarget 5: 8188-8201, 2014.
23. Ma X, Xie KP, Shang F, Huo HN, Wang LM and Xie MJ: Wogonin inhibits IGF-1-stimulated cell growth and estrogen receptor alpha expression in breast adenocarcinoma cell and angiogenesis of chick chorioallantoic membrane. Sheng Li Xue Bao 64: 207-212, 2012.
24. Chen P, Lu N, Ling Y, Chen Y, Hui H, Lu Z, Song X, Li Z, You Q and Guo Q: Inhibitory effects of wogonin on the invasion of human breast carcinoma cells by downregulating the expression and activity of matrix metalloproteinase-9. Toxicology 282: 122-128, 2011.
25. Hou L, Chen L and Fang L: Scutellarin inhibits proliferation, invasion, and tumorigenicity in human breast cancer cells by regulating HIPPO-YAP signaling pathway. Med Sci Monit 23: 5130-5138, 2017.
26. Harrison LJ, Sia GL and Sim KY: 5,7-Dihydroxy-8-methoxyflavone from *Tetracera indica*. Planta Med 60: 493-494, 1994.
27. Choi JA, Lee JW, Kim H, Kim EY, Seo JM, Ko J and Kim JH: Pro-survival of estrogen receptor-negative breast cancer cells is regulated by a BLT2-reactive oxygen species-linked signaling pathway. Carcinogenesis 31: 543-551, 2010.
28. Mehner C, Hockla A, Miller E, Ran S, Radisky DC and Radisky ES: Tumor cell-produced matrix metalloproteinase-9 (MMP-9) drives malignant progression and metastasis of basal-like triple negative breast cancer. Oncotarget 5: 2736-2749, 2014.
29. Kim EY, Seo JM, Cho KJ and Kim JH: Ras-induced invasion and metastasis are regulated by a leukotriene B4 receptor BLT2-linked pathway. Oncogene 29: 1167-1178, 2010.
30. Park GS and Kim JH: LPS up-regulates ICAM-1 expression in breast cancer cells by stimulating a MyD88-BLT2-ERK-linked cascade, which promotes adhesion to monocytes. Mol Cells 38: 821-828, 2015.

31. Truong D, Puleo J, Llave A, Mouneimne G, Kamm RD and Nikkhah M: Breast cancer cell invasion into a three dimensional tumor-stroma microenvironment. Sci Rep 6: 34094, 2016.

32. Jemal A, Ward E and Thun MJ: Recent trends in breast cancer incidence rates by age and tumor characteristics among U.S. women. Breast Cancer Res 9: R28, 2007.

33. Huynh DL, Kwon T, Zhang JJ, Sharma N, Gera M, Ghosh M, Kim N, Kim Cho S, Lee DS, Park YH and Jeong DK: Wogonin suppresses stem cell-like traits of CD133 positive osteosarcoma cell via inhibiting matrix metalloproteinase-9 expression. BMC Complement Altern Med 17: 304, 2017.

34. Liu X, Tian S, Liu M, Jian L and Zhao L: Wogonin inhibits the proliferation and invasion, and induces the apoptosis of HepG2 and Bel7402 HCC cells through NF-kappaB/Bcl-2, EGFR and EGFR downstream ERK/AKT signaling. Int J Mol Med 38: 1250-1256, 2016.

35. Zhao K, Wei L, Hui H, Dai Q, You QD, Guo QL and Lu N: Wogonin suppresses melanoma cell B16-F10 invasion and migration by inhibiting Ras-medicated pathways. PLoS One 9: e106458, 2014.

36. Lim BO: Effects of wogonin, wogonoside, and 3,5,7,2',6'-penta hydroxyflavone on chemical mediator production in peritoneal exduate cells and immunoglobulin E of rat mesenteric lymph node lymphocytes. J Ethnopharmacol 84: 23-29, 2003.

37. Zeng H, Dou S, Zhao J, Fan S, Yuan X, Zhu S, Li L, Zhang W and Liu R: The inhibitory activities of the components of Huang-Lian-Jie-Du-Tang (HLJDT) on eicosanoid generation via lipoxygenase pathway. J Ethnopharmacol 135: 561-568, 2011.

38. Wang L, Ling Y, Chen Y, Li CL, Feng F, You QD, Lu N and Guo QL: Flavonoid baicalein suppresses adhesion, migration and invasion of MDA-MB-231 human breast cancer cells. Cancer Lett 297: 42-48, 2010.

39. Ma X, Yan W, Dai Z, Gao X, Ma Y, Xu Q, Jiang J and Zhang S: Baicalein suppresses metastasis of breast cancer cells by inhibiting EMT via downregulation of SATB1 and Wnt/β-catenin pathway. Drug Des Devel Ther 10: 1419-1441, 2016.

40. Peters-Golden M and Henderson WR Jr.: Leukotrienes. N Engl J Med 357: 1841-1854, 2007.

41. Gautam S, Roy S, Ansari MN, Saeedan AS, Saraf SA and Kaithwas G: DuCLOX-2/5 inhibition: A promising target for cancer chemoprevention. Breast Cancer 24: 180-190, 2017.

42. Green AR, Green VL, White MC and Speirs V: Expression of cytokine messenger RNA in normal and neoplastic human breast tissue: Identification of interleukin-8 as a potential regulatory factor in breast tumours. Int J Cancer 72: 937-941, 1997.

43. Merdad A, Karim S, Schulten HJ, Dallol A, Buhmeida A, Al-Thubaity F, Gari MA, Chaudhary AG, Abuzenadah AM and Al-Qahtani MH: Expression of matrix metalloproteinases (MMPs) in primary human breast cancer: MMP-9 as a potential biomarker for cancer invasion and metastasis. Anticancer Res 34: 1355-1366, 2014.

44. Wang W, Xia T and Yu X: Wogonin suppresses inflammatory response and maintains intestinal barrier function via TLR4-MyD88-TAK1-mediated NF-kappaB pathway in vitro. Inflamm Res 64: 423-431, 2015.

45. Karroum A, Mirshahi P, Benabbou N, Faussat AM, Soria J, Therwath A, Mirshahi M and Hatmi M: Matrix metalloproteinase-9 is required for tubular network formation and migration of resistant breast cancer cells MCF-7 through PKC and ERK1/2 signalling pathways. Cancer Lett 295: 242-251, 2010.

46. Kim S, Jeon M, Lee JE and Nam SJ: MEK activity controls IL-8 expression in tamoxifen-resistant MCF-7 breast cancer cells. Oncol Rep 35: 2398-2404, 2016.

47. Jung YS and Lee SO: Apomorphine suppresses TNF-α-induced MMP-9 expression and cell invasion through inhibition of ERK/AP-1 signaling pathway in MCF-7 cells. Biochem Biophys Res Commun 487: 903-909, 2017.