

# Ethacrynic Acid Inhibits Sphingosylphosphorylcholine-Induced Keratin 8 Phosphorylation and Reorganization via Transglutaminase-2 Inhibition

Hyun Jung Byun<sup>1</sup>, Kyung Jin Kang<sup>1</sup>, Mi Kyung Park<sup>1</sup>, Hye Ja Lee<sup>1</sup>, June Hee Kang<sup>1</sup>, Eun Ji Lee<sup>1</sup>, You Ri Kim<sup>1</sup>, Hyun Ji Kim<sup>1</sup>, Young Woo Kim<sup>1</sup>, Kyung Chae Jung<sup>2</sup>, Soo Youl Kim<sup>2</sup> and Chang Hoon Lee<sup>1,\*</sup>

<sup>1</sup>College of Pharmacy, Dongguk University, Seoul 100-715, <sup>2</sup>National Cancer Center, Goyang 410-769, Republic of Korea

## Abstract

Sphingosylphosphorylcholine (SPC) is significantly increased in the malicious ascites of tumor patients and induces perinuclear reorganization of keratin 8 (K8) filaments in PANC-1 cells. The reorganization contributes to the viscoelasticity of metastatic cancer cells resulting in increased migration. Recently, we reported that transglutaminase-2 (Tgase-2) is involved in SPC-induced K8 phosphorylation and reorganization. However, effects of Tgase-2 inhibitors on SPC-induced K8 phosphorylation and reorganization were not clearly studied. We found that ethacrynic acid (ECA) concentration-dependently inhibited Tgase-2. Therefore, we examined the effects of ECA on SPC-induced K8 phosphorylation and reorganization. ECA concentration-dependently suppressed the SPC-induced phosphorylation and perinuclear reorganization of K8. ECA also suppressed the SPC-induced migration and invasion. SPC induced JNK activation through Tgase-2 expression and ECA suppressed the activation and expression of JNK in PANC-1 cells. These results suggested that ECA might be useful to control Tgase-2 dependent metastasis of cancer cells such as pancreatic cancer and lung cancers.

**Key Words:** Sphingosylphosphorylcholine, Transglutaminase-2, Keratin-8 phosphorylation and reorganization, Ethacrynic acid, Migration, Invasion

## INTRODUCTION

Metastasis is the ability of cancer cells to spread from its origin to distant locations within the body and to continue its growth (Valastyan and Weinberg, 2011). The high mortality rates associated with cancer are caused by the metastatic spread of tumor cells away from the site of their origin (Park *et al.*, 2013a). In fact, metastases are the cause of 90% of cancer deaths (Steeg, 2006). Therefore, several researchers are trying to develop new anti-metastatic compounds. Recently, novel approaches have been proposed to characterize the properties of metastatic cancer cells, such as cell elasticity or mechanical properties (Beil *et al.*, 2003; Suresh, 2007). The clinical importance of viscoelasticity or cell stiffness was reported by Cross *et al.* (2007). In particular, the importance of cell elasticity or viscoelasticity in several metastatic cancer cell lines has also been reported (Beil *et al.*, 2003; Rolli *et al.*, 2010). For example, sphingosylphosphorylcholine (SPC)-

induced keratin phosphorylation and reorganization of human epithelial pancreatic cancer cells combined with the resulting changes in viscoelasticity of the cells have been suggested as a possible pathway that facilitates the migration and increased metastatic competence of pancreatic tumor cells (Beil *et al.*, 2003; Rolli *et al.*, 2010).

Keratin reorganization is achieved through phosphorylation of specific serine residues in keratin by MAP kinases, such as ERK, p38, and c-jun N-terminal kinase (JNK) (Ku *et al.*, 2002; Park *et al.*, 2011; Busch *et al.*, 2012). Several compounds such as sphingosylphosphorylcholine (SPC) and leukotriene B<sub>4</sub> induces keratin 8 (K8) phosphorylation and reorganization in PANC-1 cells (Beil *et al.*, 2003; Park *et al.*, 2011). We also showed that BLT2 and transglutaminase-2 (Tgase-2) are involved in K8 phosphorylation and reorganization (Park *et al.*, 2011; Park *et al.*, 2012).

Tgase-2 is a multifunctional protein. In addition to catalyzing Ca<sup>2+</sup>-dependent transamidation reactions, it can bind

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## \*Corresponding Author

E-mail: uatheone@dongguk.edu  
Tel: +82-31-961-5213, Fax: +82-31-961-5206

and hydrolyze GTP/GDP (Mhaouty-Kodja, 2004). The selective expression of Tgase-2 in chemoresistant and metastatic cancer cells, such as pancreatic, lung and ovarian tumors makes it a promising therapeutic target (Verma *et al.*, 2006; Chhabra *et al.*, 2009; Park *et al.*, 2013b). However, studies on Tgase-2 inhibitors modulating metastasis of cancers were not enough. We screened inhibitory effects of some compounds on Tgase-2.

Ethacrynic acid (ECA) is a diuretic that inhibits cellular ion flux that leads to an increase in intracellular Na concentrations (Fig. 1A) (Vivas and Chiaraviglio, 1989; Li and El-Mallakh, 2004). ECA is used rarely as a diuretic because other potent agents have been introduced. Nevertheless, ECA still has a place in the modern practice of medicine (Wall *et al.*, 2003; Han *et al.*, 2005). ECA showed new pharmacological activities independent of diuretic activity. For example, ECA suppressed the all-retinoic acid-induced monocyte chemoattractant protein-1 production and is known to reduce the retinoid-induced ear edema in mice (Kim *et al.*, 2010).

In this study, we found that ECA inhibited Tgase-2 and confirmed the involvement of Tgase-2 in SPC-induced K8 phosphorylation and reorganization. Our finding suggested the possibility that ECA might be used as antimetastatic drugs.

## MATERIALS AND METHODS

### Material

D-erythro SPC was obtained from Matreya (Pleasant Gap, PA, USA). The phosphospecific antibody to detect K8 Ser431 was purchased from Abcam (Cambridge, UK). The anti-Tgase-2 antibody was supplied by Labvision Corporation-NeoMarkers (Thermo scientific, Fremont, CA, USA). Peroxidase-labeled secondary antibodies and lentiviral shRNA were acquired from Santa Cruz Biotechnology (Santa Cruz, CA,

USA). Alexa Fluor 594 goat anti-mouse antibody was obtained from Molecular Probes, Inc. (Eugene, OR, USA).

### Cell culture

The human pancreatic carcinoma cell line, PANC-1 (ATCC CRL 1469), was obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with L-glutamine (2 mM), penicillin-streptomycin (10,000 IU/ml and 10,000 µg/ml, respectively), and sodium pyruvate (1 mM). The PANC-1 cells were maintained in medium containing 10% (v/v) fetal calf serum (FCS). The cells were incubated at 37°C in a humidified atmosphere containing 10% CO<sub>2</sub>. The cells were washed twice in serum-free DMEM and incubated in serum-free DMEM 18 hours before the respective experiments.

### In vitro Tgase-2 inhibition assay

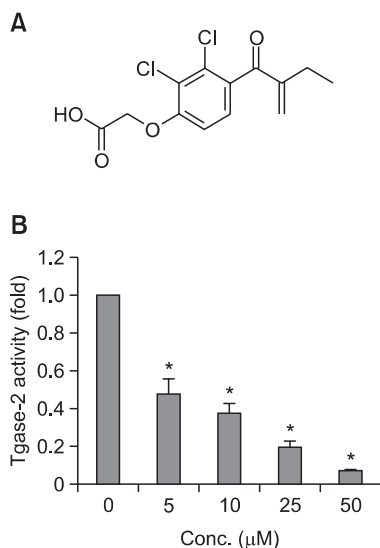
The inhibitory effect of each compound was determined by measuring the incorporation of [1,4-<sup>14</sup>C] putrescine into succinylated casein (Park *et al.*, 2013a). Following 10 min of pre-incubation of 2.5 milliunits (mU) of Tgase-2 from the guinea pig liver with each concentration of chemicals in 0.1 ml of reaction buffer solution without 10 mM CaCl<sub>2</sub>, we added 0.4 ml of substrate solution containing 5 mg of succinylated casein and 100 nCi of [1,4-<sup>14</sup>C] putrescine. After further incubation at 37°C for 1 h, the reaction was terminated by the addition of 4 ml of cold (4°C) 7.5% (w/v) TCA. TCA-insoluble precipitates were collected in GF/A glass fiber filters (Millipore Co.), washed with cold 5% (w/v) TCA, dried and assessed for incorporation of radiolabel using a scintillation counter (Beckman Coulter Co.). The resultant data represent the means of three independent experiments.

### Western blot

The PANC-1 cells were harvested and lysed in 50 mM Tris-Cl (pH 7.5), 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 2 mM EDTA, sterile solution and protease inhibitors (Gendepot, Barker, TX, USA) (Park *et al.*, 2011). The protein concentrations of the supernatants were determined using Coomassie Plus (Pierce Biotechnology Inc., Rockford, IL, USA), as recommended by the manufacturer. The protein lysates were loaded onto a precast 4% to 12% polyacrylamide gradient gel (Invitrogen, Carlsbad, CA, USA). The proteins were separated by SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Pall, Pensacola, FL, USA). The membranes were blocked in 5% non fat milk and probed with the appropriate primary antibodies such as anti-Tgase-2, keratin-8, and JNK. After incubation with the primary antibody, the membranes were washed with TBS+0.1% Tween 20 and incubated with the appropriate peroxidase-conjugated secondary antibodies followed by development with a chemiluminescence substrate (Pierce Biotechnology Inc., Rockford, IL, USA) and exposure to X-ray film (Kodak, Rochester, NY, USA).

### Confocal microscopy

PANC-1 cells were grown on coverslips and fixed 24 hours later with fresh 4% paraformaldehyde, pH 7.0, for 10 min at room temperature. Fixed cells were permeabilized with a 10 min wash in 0.1% Triton X-100 at room temperature followed by several washes in PBS with 3% bovine serum albumin (PBS/BSA). The phosphospecific antibody detecting K8



**Fig. 1.** ECA induced Tgase-2 expression in Panc1 cells. (A) The structure of ethacrynic acid (B) Expression level of Tgase-2 in PANC-1 cell stimulated with the indicated concentration of ECA for 30 min. \* $p < 0.05$  was considered statistically significant.

Ser431 (Abcam, Cambridge, MA, USA) primary antibody was incubated with coverslips overnight at 4°C (Park *et al.*, 2011). Excess antibody was removed with four washes in PBS/BSA. Species-specific second antibodies conjugated to goat anti-rabbit IgG antibody (Alexa Fluor 488, 1:500 Molecular Probes) or goat anti-mouse IgG antibodies (Alexa Fluor 594, 1:500, Molecular Probes) were then reacted with the coverslips for 1 hour at room temperature followed with four washes in PBS/BSA. The final samples were mounted onto slides and visualized using a Zeiss Axiophot confocal microscope.

**Migration**

Migration of PANC-1 cells through 8-µm size-limited pores was assessed in response to SPC according to Park’s report (Park *et al.*, 2011). PANC-1 cells (5×10<sup>4</sup> cells per well) were treated with the indicated concentrations of SPC for 1 hour. PANC-1 cells plated in the upper chamber were allowed to migrate for 5 hours to establish the temporal kinetics of migration. The transwell membranes were then fixed and stained with Diff-Quik® staining kit (Kobe, Japan). Membranes were removed from transwells, and cells on the under surface of the millipore membrane were counted under a light microscope (average of 5 semi-random non-overlapping fields at 200× magnification). All treatments were performed in triplicate wells.

**Invasion assay using transwell plates**

Cell invasion was studied using matrigel-coated (0.5 lg/ml) transwell inserts, as described previously (Park *et al.*, 2013a). Trypsinised cells were suspended in serum-free medium, and 2×10<sup>5</sup> cells were added to the upper chamber of the transwell inserts. Medium with 10% serum was added to the lower chamber. After a 16 h incubation with PANC-1 cells, the non-migrated cells on the upper surface of the membrane were removed, and the cells on the lower surface were stained using the Hema 3 staining system (Fisher Scientific, Houston, TX, USA), photographed (200× magnification) and counted in 10 randomly selected fields. All experiments were repeated at least three times with two replicates each.

**Statistical analysis**

The data are expressed as the mean ± S.E.M. of at least three in-dependent experiments performed in triplicate. A *p* value <0.05 was considered significant.

**RESULTS**

**ECA inhibits transglutaminase-2**

We have shown that Tgase-2 is involved in SPC-induced K8 phosphorylation and reorganization by JNK activation leading to migration of metastatic pancreatic cancer cells (Park *et al.*, 2011). Therefore, Tgase-2 inhibitor may be effective for metastasis treatment. To obtain a Tgase-2 inhibitor, we firstly screened a single compound library comprised of used drugs and natural extracts. We found that ECA has a concentration-dependent Tgase-2 inhibitory effect (Fig. 1B).

**ECA suppressed the SPC-induced K8 phosphorylation and reorganization in PANC-1 cells**

In previous report, cystamine (CTM), a well-known Tgase inhibitor, suppressed the SPC-induced K8 phosphorylation

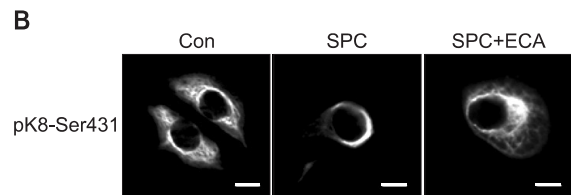
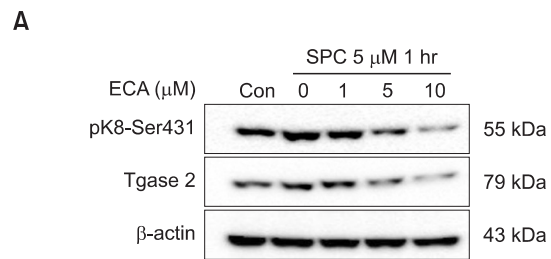
and reorganization (Park *et al.*, 2011). So we examined whether ECA, a newly found Tgase-2 inhibitor, could suppress the SPC-induced K8 phosphorylation and reorganization. SPC induced phosphorylation of serine 431 of K8 and ECA concentration dependently inhibited the SPC-induced K8 phosphorylation (Fig. 2A). SPC also induced ring like perinuclear reorganization of K8 in PANC-1 cells and Tgase-2 is involved in this event. ECA inhibited the SPC-induced perinuclear reorganization of K8 (Fig. 2B).

**ECA suppressed the SPC-induced migration and invasion of PANC-1 cells**

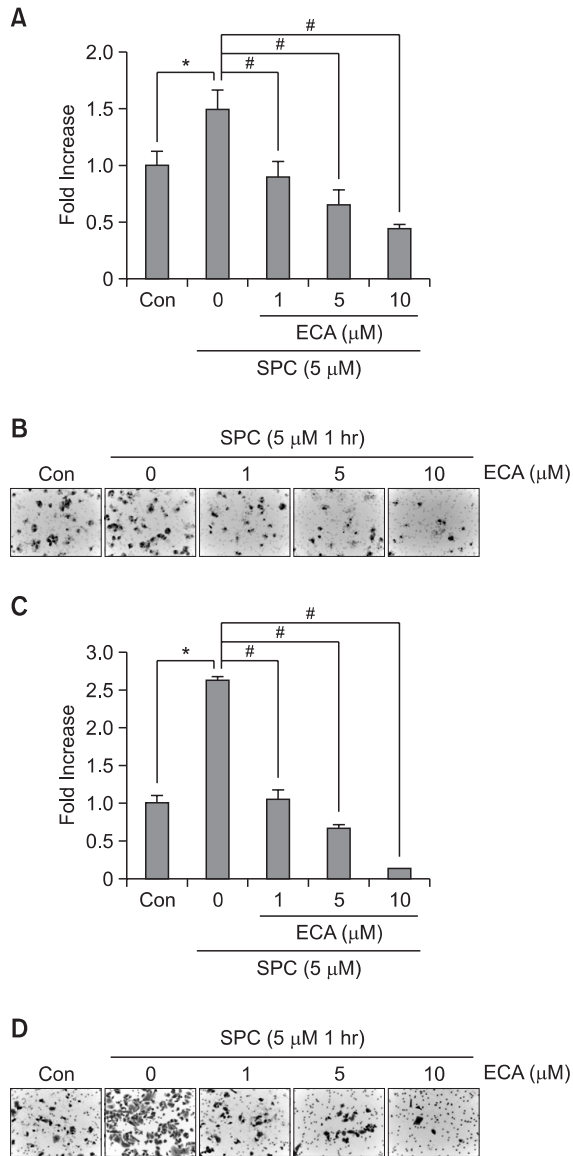
The expected final outcome of SPC-induced reorganization of the keratin network in PANC-1 cells is increased migratory properties (Beil *et al.*, 2003). Therefore, in previous report, we demonstrated that Tgase-2 is involved in the SPC-induced migration of PANC-1 cells by CTM and gene silencing (Park *et al.*, 2011). SPC treatment induced the increased migration and invasion of PANC-1 cells (Fig. 3). ECA concentration-dependently inhibited the SPC-induced migration and invasion of PANC-1 cells (Fig. 3). No cytotoxic effects of ECA were observed in our experimental setting of migration and invasion.

**ECA suppressed the SPC-induced JNK activation and expression**

Tgase-2 is involved in SPC-induced K8 phosphorylation via JNK activation (Park *et al.*, 2011). So, we examined whether ECA suppressed the Tgase-2-dependent JNK activation. SPC treatment increased the phosphorylation of JNK and ECA treatment suppressed the phosphorylation and expression of JNK (Fig. 4A).



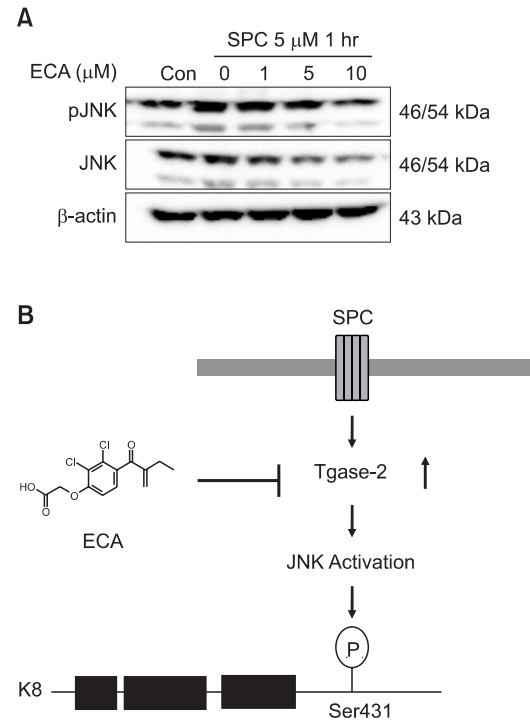
**Fig. 2.** ECA suppressed the SPC-induced K8 phosphorylation and reorganization. (A) Effect of ECA on SPC-induced K8 phosphorylation. The PANC-1 cells were treated with various amounts of ECA and with or without SPC (5 µM). (B) Confocal microscopic examination of the effect of ECA on SPC-induced K8 phosphorylation of PANC-1 cells. PANC-1 cell were treated with ECA (5 µM) 30 min and SPC (5 µM) for 1 hr. Immunostaining was performed using K8-Ser431 (green).



**Fig. 3.** ECA suppressed the SPC-induced migration and invasion. (A) Effect of ECA on SPC-induced migration of PANC-1 cell. (B) Microscopic images showing the effect of ECA on SPC-induced migration of PANC-1 cell. (C) Effect of ethacrynic acid on SPC-induced invasion of PANC-1 cell. (D) Microscopic images showing the effect of ethacrynic acid on SPC-induced invasion of PANC-1 cell. In (A), (B), (C) and (D), PANC-1 cell ( $5 \times 10^4$  cell per well) were treated with or without SPC ( $5 \mu\text{M}$ ) and various concentration of ECA. \* $p$  or # $p < 0.05$  was considered statistically significant.

## DISCUSSION

Metastatic cancer cells are reported to have unique mechanical characteristics, such as soft stiffness and elasticity (Cross *et al.*, 2007). Keratins are one of the main intermediate filaments that control the mechanical characteristics of cells (Bordeleau *et al.*, 2008). This study focused on ECA, Tgase-2 inhibitor modulating the SPC-induced keratin phosphorylation and reorganization in PANC-1 cells that controls the viscoelasticity and migratory properties of cancer cells.



**Fig. 4.** Ethacrynic acid suppressed the SPC-induced JNK activation. (A) Effect of ECA on SPC-induced JNK activation in PANC-1 cell. The PANC-1 cell treated with or without SPC ( $5 \mu\text{M}$ ) and various concentration of ECA. (B) Proposed effects of ECA on SPC-induced K8 phosphorylation via JNK activation.

MAP kinase is involved in keratin reorganization through the phosphorylation of keratin (Ku *et al.*, 2002; Park *et al.*, 2011; Busch *et al.*, 2012), but there are few studies on the other proteins affecting keratin reorganization, except plectin (Cheng *et al.*, 2008). Recently, we reported that Tgase-2 is involved in SPC-induced keratin reorganization via JNK activation (Park *et al.*, 2011). Tgase-2 mediates the metastasis and chemoresistance of several cancer cells and is a new and interesting target (Kim, 2011). However, effective Tgase-2 inhibitors are not yet available to clinical application although several approaches revealed promising Tgase-2 inhibitors (Lai *et al.*, 2008; Lee *et al.*, 2013; Park *et al.*, 2013a). So, we examined inhibitory effects of some drugs on Tgase-2 since drug can be easily applicable to cancer treatment. We found that ECA concentration-dependently inhibited the Tgase-2 (Fig. 1B). The inhibitory mechanism of ECA against Tgase-2 is not clear but the molecular structure of ECA contains an exo-methylene group conjugated to a carbonyl group (Fig. 1A). This electrophilic "eneone" moiety can alkylate thiol groups in proteins or glutathione via a Michael-type addition reaction (Han *et al.*, 2005). Interestingly, one of key residues of Tgase-2 is cysteine residue at 277<sup>th</sup> amino acid (Lee *et al.*, 1993). Thus, ECA might modify critical thiol residues in 277<sup>th</sup> Tgase-2.

The results showed that ECA suppressed the phosphorylation of K8 and perinuclear keratin reorganization (Fig. 2). These observations confirmed that Tgase-2 is involved in SPC-induced K8 phosphorylation and perinuclear reorganization of K8 (Park *et al.*, 2011).

SPC-induced keratin phosphorylation and reorganization



led to increased migration of PANC-1 cells and Tgase-2 inhibition by ECA suppressed the SPC-induced migration and invasion (Fig. 3). ECA is known to have diverse effects such as glutathione-S-transferase inhibition and thiol-adduct formation. So these diverse effects also might be involved in inhibition of migration and invasion. However, to our knowledge, we could not find reports about suppressing the migration of cancer cells via GST inhibition. However, thiol-adduct formation of ECA might contribute to inhibition of Tgase-2 since Tgase-2 has cysteine residue at 277th in active site. In previous paper, we showed that SPC induced migration of PANC-1 cells via Tgase-2 expression (Park *et al.*, 2011). Therefore, ECA might suppress the SPC-induced migration by inhibition of Tgase-2.

Tgase-2 is involved in SPC-induced JNK activation and ECA, Tgase-2 inhibitor, suppressed the JNK activation in PANC-1 cells (Fig. 4A). Especially, ECA also suppressed the JNK expression (Fig. 4A). These results suggested that ECA inhibited JNK expression via Tgase-2 inhibition.

Our findings confirmed the role of ECA as a Tgase-2 inhibitor in the suppression of SPC-induced K8 phosphorylation and reorganization of PANC-1 cells via JNK (Fig. 4B). Therefore, ECA might be helpful in modulating the Tgase-2 involved metastasis of cancer cells such as pancreatic cancers, and lung cancers.

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