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# The hederagenin saponin SMG-1 is a natural FMLP receptor inhibitor that suppresses human neutrophil activation

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ABSTRACT

The pericarp of *Sapindus mukorossi* Gaertn is traditionally used as an expectorant in Japan, China, and Taiwan. Activated neutrophils produce high concentrations of the superoxide anion ($O_2^{\bullet-}$) and elastase known to be involved in airway mucus hypersecretion. In the present study, the anti-inflammatory functions of hederagenin 3-*O*-(3,4-*O*-di-acetyl-α-L-arabinopyranoside)-(1→3)-α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranoside (SMG-1), a saponin isolated from *S. mukorossi*, and its underlying mechanisms were investigated in human neutrophils. SMG-1 potently and concentration-dependently inhibited $O_2^{\bullet-}$ generation and elastase release in *N*-Formyl-Met-Leu-Phe (FMLP)-activated human neutrophils. Furthermore, SMG-1 reduced membrane-associated $p47^{phox}$ expression in FMLP-induced intact neutrophils, but did not alter subcellular NADPH oxidase activity in reconstituted systems. SMG-1 attenuated FMLP-induced increase of cytosolic calcium concentration and phosphorylation of p38 MAPK, ERK, JNK, and AKT. However, SMG-1 displayed no effect on cellular cAMP levels and activity of adenylate cyclase and phosphodiesterase. Significantly, receptor-binding analysis showed that SMG-1 inhibited FMLP binding to its receptor in a concentration-dependent manner. In contrast, neither phorbol myristate acetate-induced $O_2^{\bullet-}$ generation and MAPKs activation nor thapsigargin-caused calcium mobilization was altered by SMG-1. Taken together, our results demonstrate that SMG-1 is a natural inhibitor of the FMLP receptor, which may have the potential to be developed into a useful new therapeutic agent for treating neutrophilic inflammatory diseases.



## 1. Introduction

Airway mucus hypersecretion is a common feature of chronic inflammatory lung diseases, including asthma, chronic obstructive pulmonary disease (COPD), and cystic fibrosis [1–3]. Activated neutrophils are recruited into the airways in acute and chronic inflammatory respiratory diseases by chemoattractants that are present in the airway [4–7]. Neutrophils play a pivotal role in the defense of the human body against infections. However, overwhelming activation of neutrophils is known to elicit tissue damage. High concentrations of reactive oxygen species (ROS) and elastase produced by activated neutrophils in the sputum of patients with airway mucus hypersecretion has been implicated in the pathogenesis of many acute and chronic pulmonary diseases including asthma, COPD, cystic fibrosis, and acute respiratory distress syndrome (ARDS) [5,8,9]. Excessive mucus production and inadequate mucus clearance in the airways causes severe coughing, airflow obstruction, airway hyperresponsiveness, and

*Abbreviations:* AC, adenylyl cyclase; Akt, protein kinase B; ARDS, acute respiratory distress syndrome; BAPTA-AM, 1,2-bis(2-aminophenoxy)ethane-N,N,N′,N′-tetra-acetic acid tetrakis(acetoxymethyl ester); cAMP, cyclic adenosine 3′,5′-monophosphate; CB, cytochalasin B; COPD, chronic obstructive pulmonary disease; ERK, extracellular regulated kinase; FMLP, *N*-Formyl-Met-Leu-Phe; GPCR, G protein-coupled receptor; H89, N-(2-((*p*-bromocinnamyl)amino)ethyl)-5-isoquinolinesulfonamide; IBMX, 3-isobutyl-1-methylxanthine; JNK, *c-Jun* N-terminal kinase; LDH, lactate dehydrogenase; MAPK, mitogen-activated protein kinase; $O_2^{\bullet-}$, superoxide anion; PDE, phosphodiesterase; PKA, protein kinase A; PKC, protein kinase C; PMA, phorbol myristate acetate; Ro318220, 3-(1-(3-(amidinothio)propyl-1H-indol-3-yl))-3-(1-methyl-1H-indol-3-yl)maleimide; ROS, reactive oxygen species; SOD, superoxide dismutase; WST-1, 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium monosodium salt.

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inflammation, and may lead to clinical deterioration and death [5,10,11]. As a result, it is important to develop drugs that inhibit mucus hypersecretion to treat these inflammatory lung diseases.

*Sapindus mukorossi* Gaertn. (Sapindaceae), also known as the soap-nut tree, is an important economic agricultural product in tropical and subtropical regions of Asia. The pericarp of *S. mukorossi* has traditionally been used as an expectorant, to relieve coughing, for detoxification, and for defervescence [12–15]. Saponins are the key bioactive compounds found in the pericarp of *S. mukorossi* [13,15–19]. The number of known natural saponins has grown rapidly in recent years. They exhibit a diverse range of pharmacological effects, including antimicrobial activity [20,21], antitumor functions [22,23], anti-platelet aggregation [17], anti-nociceptive, and anti-inflammatory effects [24,25]. A particular series of saponins was reported to have anti-inflammatory activities in *in vivo* animal models. For example, saponins exhibit inhibitory effects on Freund's complete adjuvant reagent-induced rheumatoidal arthritis in rats [24]. In addition, *Panax notoginseng* (ginseng) saponins show remarkable inhibitory effects on atherosclerosis induced by zymosan A in rats through an anti-inflammatory action and regulation of the blood lipid profile [26]. In spite of this, no record was found describing anti-neutrophilic inflammation by *S. mukorossi* in the literature.

In searching for suitable new anti-inflammatory agents from natural sources, hederagenin 3-*O*-(3,4-*O*-di-acetyl-α-L-arabinopyranoside)-(1→3)-α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranoside (SMG-1) (Fig. 1A), a saponin from *S. mukorossi* [27], was found to concentration-dependently and potently inhibit the

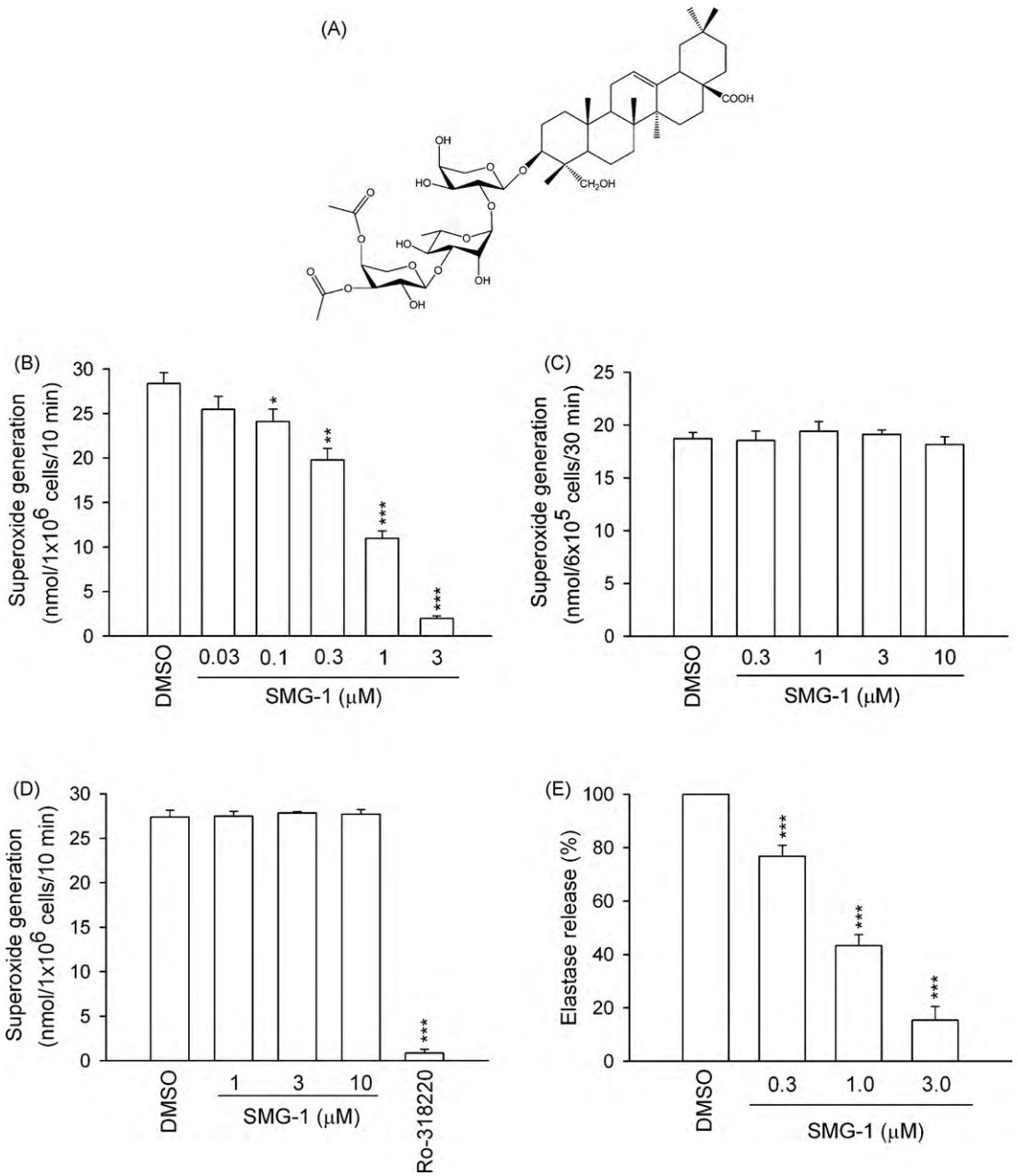


**Fig. 1.** Effects of SMG-1 on $O_2^{\bullet-}$ generation or elastase release in FMLP/CB, NAF/CB, or PMA-activated human neutrophils. (A) Chemical structure of hederagenin 3-*O*-(3,4-*O*-di-acetyl-α-L-arabinopyranoside)-(1→3)-α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranoside (SMG-1). Human neutrophils were preincubated with DMSO (control), SMG-1 (0.03–10 μM), or Ro318220 (0.1 μM) for 5 min. $O_2^{\bullet-}$ generation was induced by FMLP (100 nM)/CB (1 μg/ml) (B), NAF (20 mM)/CB (2.5 μg/ml) (C) or PMA (5 nM) (D) and measured using SOD-inhibitable cytochrome *c* reduction. (E) Elastase release was induced by FMLP/CB and measured spectrophotometrically at 405 nm. All data are expressed as the mean ± S.E.M. ($n$ = 4–7). $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ compared to the control.

generation of superoxide anion ($O_2^{\bullet-}$) and the release of elastase in *N*-Formyl-Met-Leu-Phe (FMLP)-activated human neutrophils. This study investigated the action mechanisms of SMG-1 in human neutrophils. Our data demonstrates that SMG-1 suppresses FMLP-induced respiratory burst and degranulation in human neutrophil by the attenuation of calcium ($Ca^{2+}$) mobilization, mitogen-activated protein kinases (MAPKs) phosphorylation, and protein kinase B (AKT) activation and that these effects are due to the inhibition of the FMLP receptor.

## 2. Materials and methods

### 2.1. Reagents

Powder of the pericarp of *S. mukorossi* (soap-nut) was purchased from the administrative office of Dharani Forestry and Orchards, India. SMG-1 was isolated as a pure compound (purity >97%) from *S. mukorossi* as described previously [27], and was dissolved in dimethyl sulfoxide (DMSO) to make stock solutions. H89 (N-(2-((*p*-bromocinnamyl)amino)ethyl)-5-isoquinolinesulfonamide), leupeptin, MeO-Suc-Ala-Ala-Pro-Val-p-nitroanilide, phenylmethylsulfonyl fluoride (PMSF), Ro318220 (3-(1-(3-(amidinothio)propyl-1H-indol-3-yl))-3-(1-methyl-1H-indol-3-yl)maleimide), and rolipram were obtained from Calbiochem (La Jolla, CA, USA). Fluo-3 AM and *N*-formyl-Nle-Leu-Phe-Nle-Tyr-Lys-fluorescein (FNLFNYK) were purchased from Molecular Probes (Eugene, OR, USA). 2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium monosodium salt (WST-1) was purchased from Dojindo Laboratories (Kumamoto, Japan). Ficoll-Paque Plus was purchased from GE Healthcare (Uppsala, Sweden). All other chemicals were obtained from Sigma (St. Louis, MO, USA). When drugs were dissolved in DMSO, the final concentration of DMSO in cell experiments did not exceed 0.4% and did not affect the parameters measured.

### 2.2. Extraction and isolation

The pericarp of *S. mukorossi* (soap-nut) (35.5 g) was extracted with MeOH for 72 h. The MeOH extract (28.0 g) was subjected to column chromatography over a 23.0 cm × 4.5 cm highly porous Diaion HP-20 polymer resin column and gradient elution with a gradient of $H_2O$ and 20%, 40%, 60%, 80%, and 100% aqueous MeOH. The 100% MeOH fraction was further chromatographed on a 24.0 cm × 3.0 cm Diaion HP-20 column and successively eluted with 40%, 60%, 80%, and 100% $Me_2CO$. The 40% $Me_2CO$ fraction was concentrated (4.4 g) and rechromatographed on a 23.0 cm × 2.5 cm silica gel column and eluted with a gradient of $CHCl_3$/MeOH (from 21/1 to 2/1) to give five fractions (fractions 1–5). Fraction 4 was further purified by high-performance liquid chromatography (HPLC) on a 250.0 mm × 10.0 mm silica gel column at a flow rate of 2.2 ml/min, and SMG-1 (30.5 mg) was purified by elution with $CHCl_3$/MeOH (12:1). The structure of SMG-1 was determined by mass and nuclear magnetic resonance (NMR) spectroscopic methods [27].

### 2.3. Preparation of human neutrophils

Blood was taken from healthy human donors (20–30 years old) by venipuncture, using a protocol approved by the institutional review board at Chang Gung Memorial Hospital. Neutrophils were isolated with a standard method of dextran sedimentation prior to centrifugation in a Ficoll Hypaque gradient and hypotonic lysis of erythrocytes [28]. Purified neutrophils that contained >98% viable cells, as determined by the trypan blue exclusion method, were resuspended in $Ca^{2+}$-free Hank's balanced salt solution (HBSS; Gibco, Grand Island, NY, USA) buffer at pH 7.4, and were maintained at 4 °C before use.

### 2.4. Neutrophil fractionation

Neutrophils were pretreated with 1 mM PMSF for 30 min at 4 °C, disrupted in relaxation buffer (100 mM KCl, 3 mM NaCl, 3.5 mM $MgCl_2$, 1 mM ATP, 1 mM EGTA, and 10 mM PIPES; pH 7.3) by sonication. Unbroken cells were removed by centrifugation at 300 × *g* for 5 min, and the supernatant was then centrifuged at 100,000 × *g* for 20 min at 4 °C to produce cytosolic and plasma membrane fractions.

### 2.5. Measurement of $O_2^{\bullet-}$ generation

The assay of the generation of $O_2^{\bullet-}$ was based on the superoxide dismutase (SOD)-inhibitable reduction of ferricytochrome *c* [29,30]. In brief, after supplementation with 0.5 mg/ml ferricytochrome *c* and 1 mM $Ca^{2+}$, freshly isolated neutrophils ($1 \times 10^6$ cells/ml) were equilibrated at 37 °C for 2 min and incubated with drugs for 5 min. Cells were activated with FMLP (100 nM), NAF (20 mM), or phorbol myristate acetate (PMA, 5 nM). When FMLP or NAF was used as a stimulant, 1 or 2.5 μg/ml cytochalasin B was incubated for 3 min before cell activation. $O_2^{\bullet-}$ generation by isolated neutrophil fractions was measured after the addition of 160 μM NADPH to 800 μl of relaxation buffer containing $4 \times 10^6$ cell equivalents of the membrane extract, $1.2 \times 10^7$ cell equivalents of cytosol, 2 μM GTP-γ-S, and 0.5 mg/ml ferricytochrome *c*. To facilitate the assembly of NADPH oxidase components, 100 μM sodium dodecylsulfate (SDS) and 50 μM arachidonic acid were incubated for 3 min before the addition of NADPH. Drugs were incubated for 2 min after NADPH oxidase assembly. Changes in absorbance with the reduction of ferricytochrome *c* at 550 nm were continuously monitored in a double-beam, six-cell positioner spectrophotometer with constant stirring (Hitachi U-3010, Tokyo, Japan). Calculations were based on differences in the reactions with and without SOD (100 U/ml) divided by the extinction coefficient for the reduction of ferricytochrome *c* ($\varepsilon$ = 21.1/mM/10 mm).

### 2.6. Measurement of elastase release

Degranulation of azurophilic granules was determined by elastase release as described previously [31,32] with some modifications. Experiments were performed using MeO-Suc-Ala-Ala-Pro-Val-p-nitroanilide as the elastase substrate. Briefly, after supplementation with MeO-Suc-Ala-Ala-Pro-Val-p-nitroanilide (100 μM), neutrophils ($1 \times 10^6$/ml) were equilibrated at 37 °C for 2 min and incubated with drugs for 5 min. Cells were activated by 100 nM FMLP and 0.5 μg/ml CB, and changes in absorbance at 405 nm were continuously monitored to assay elastase release. Results are expressed as a percent of the initial rate of elastase release in the FMLP/CB-activated, drug-free control system.

### 2.7. Lactate dehydrogenase (LDH) release

LDH release was determined by a commercially available method (Promega, Madison, WI, USA). Cytotoxicity was represented by LDH release in the cell-free medium as a percentage of the total LDH released. The total LDH released was determined by lysing cells with 0.1% Triton X-100 for 30 min at 37 °C.

### 2.8. $O_2^{\bullet-}$-scavenging activity

The $O_2^{\bullet-}$-scavenging ability of SMG-1 was determined using xanthine/xanthine oxidase in a cell-free system, based on a previously described method [33]. After 0.1 mM xanthine was added to the assay buffer (50 mM Tris (pH 7.4), 0.3 mM WST-1, and 0.02 U/ml xanthine oxidase) for 15 min at 30 °C, the absorbance

associated with the $O_2^{\bullet-}$-induced WST-1 reduction was measured at 450 nm.

### 2.9. 1,1-Diphenyl-2-picrylhydrazyl (DPPH)-scavenging activity

An ethanol solution of the stable nitrogen-centered free radical, DPPH (100 μM), was incubated with SMG-1 or α-tocopherol for 16 min at 25 °C, and the absorbance was measured at 517 nm.

### 2.10. $p47^{phox}$ membrane translocation and phosphorylation

Neutrophils were incubated with drugs for 5 min at 37 °C before being stimulated by FMLP. After 3 min, reactions were stopped by cooling to 4 °C. Cells were pelleted and resuspended in ice-cold relaxation buffer (20 mM Tris–HCl (pH 7.4), 2 mM EDTA, 2 mM EGTA, 1 mM dithiothreitol, 1 mM PMSF, and 1% dilution of Sigma protease inhibitor cocktails). Cells were then disrupted by sonication and further ultracentrifuged at 20,000 × *g* for 10 min at 4 °C to pelletize the membrane fractions. The pellet was resuspended in the ice-cold relaxation buffer containing 1% Triton X-100. Then, the preparation was re-centrifuged at 100,000 × *g* for 40 min at 4 °C. The supernatant obtained, containing solubilized membranes, was used for the analysis of $p47^{phox}$.

Enrichment and separation of phosphorylated proteins from neutrophil lysates was performed by the use of a phosphoprotein purification kit (QIAGEN), as described previously [34]. Neutrophils were incubated with drugs for 5 min at 37 °C before being stimulated with FMLP for 1.5 min. The assay was performed according to the manufacturer's instructions.

Protein concentrations were determined by the Bradford assay using BSA as the standard. Solubilized membrane fractions or enrichment of phosphorylated proteins were electrophoresed through a SDS-polyacrylamide gel and electrotransferred onto a nitrocellulose membrane (PerkinElmer, Boston, MA, USA), and blots were incubated with a mouse monoclonal anti-$p47^{phox}$ antibody (BD Transduction Laboratories, San Diego, CA, USA). A horse anti-mouse antibody conjugated to HRP was used as the secondary antibody (Vector Laboratories, Burlingame, CA, USA). The immunoreactive bands were visualized by an enhanced chemiluminescence system (ECL; Amersham Biosciences).

### 2.11. Measurement of intracellular calcium concentration ($[Ca^{2+}]_i$)

Neutrophils were loaded with 2 μM fluo-3 AM at 37 °C for 45 min. After being washed, cells were resuspended in $Ca^{2+}$-free HBSS to $3 \times 10^6$ cells/ml. The change in fluorescence was monitored using a Hitachi F-4500 spectrofluorometer in a quartz cuvette with a thermostat (37 °C) and continuous stirring. The excitation wavelength was 488 nm, and the emission wavelength was 520 nm. FMLP and thapsigargin were used to increase $[Ca^{2+}]_i$ in the presence or absence of 1 mM $Ca^{2+}$. $[Ca^{2+}]_i$ was calibrated by the fluorescence intensity as follows: $[Ca^{2+}]_i = K_d \times [(F - F_{min})/(F_{max} - F)]$; where $F$ is the observed fluorescence intensity, $F_{max}$ and $F_{min}$ were respectively obtained by the addition of 0.05% Triton X-100 and 20 mM EGTA, and $K_d$ was taken to be 400 nM.

### 2.12. Determination of cAMP concentrations

cAMP levels were assayed using an enzyme immunoassay kit (GE Healthcare, Buckinghamshire, UK). Human neutrophils were incubated with drugs for 5 min before stimulation with FMLP for another 5 min, and the reaction was terminated by adding 0.5% dodecytrimethylammonium bromide. Samples were then centrifuged at 3000 × *g* for 5 min at 4 °C. The supernatants were used as a source for the cAMP samples. The assay was performed according to the manufacturer's instructions.

### 2.13. Assay of adenylyl cyclase (AC) and phosphodiesterase (PDE) activities

Neutrophils ($5 \times 10^7$ cells/ml) were sonicated in ice-cold buffer, containing 25 mM Tris–HCl (pH 7.5), 0.25 M sucrose, 2 mM EDTA, 5 mM $MgCl_2$, 10 μM leupeptin, 100 μM PMSF, and 10 μM pepstatin. Unbroken cells were removed by centrifugation at 300 × *g* for 5 min, and then the supernatant was centrifuged at 100,000 × *g* for 40 min at 4 °C. The pellet and cytosol fraction were respectively used as sources for the AC and PDE enzymes. The reaction mixture (25 mM Tris–HCl (pH 7.5), 15 mM $MgCl_2$, 1 mM 3-isobutyl-1-methylxanthine (IBMX), 7.5 mM creatine phosphate, and 3 units of creatine phosphokinase) contained 0.5 mM dithiothreitol, 1 mM ATP, and the pellet fraction for assessing AC activity. The reaction was carried out for 20 min at 30 °C and was terminated by boiling for 3 min [35]. cAMP contents were assayed using enzyme immunoassay kits.

PDE activity was analyzed using a tritium scintillation proximity assay (SPA) system, and the assay was performed according to the manufacturer's instructions (Amersham Biosciences). Briefly, assays were performed at 30 °C for 10 min in the presence of 50 mM Tris–HCl (pH 7.5) containing 8.3 mM $MgCl_2$, 1.7 mM EGTA, and 0.3 mg/ml bovine serum albumin. Each assay was performed in a 100-μl reaction volume containing the above buffer, the neutrophil supernatant fraction, and around 0.05 μCi [$^3$H]cAMP. The reaction was terminated by the addition of 50 μl PDE SPA beads (1 mg) suspended in 18 mM zinc sulfate. Assays were performed in 96-well microtiter plates. The reaction mix was allowed to settle for 1 h before counting in a microtiter plate counter [35].

### 2.14. Immunoblotting analysis of whole-cell lysates

Neutrophils were incubated with drugs for 5 min at 37 °C before being stimulated with FMLP (0.1 μM) for 0.5 min or PMA (5 nM) for 1 min. The reaction was stopped by placing the mixture on ice, and cells were centrifuged at 4 °C. After removing the supernatants, the pellets were lysed in 150 μl buffer (50 mM HEPES (pH 7.4), 100 mM NaCl, 1 mM EDTA, 2 mM $Na_3VO_4$, 10 mM *p*-nitrophenyl phosphate, 5% β-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, 1% dilution of Sigma protease inhibitor cocktails, and 1% Triton X-100). Samples were centrifuged at 14,000 × *g* for 20 min at 4 °C to yield whole-cell lysates. Proteins derived from whole-cell lysates were separated by SDS-polyacrylamide gel electrophoresis (PAGE) using 12% polyacrylamide gels and blotted onto nitrocellulose membranes. Immunoblotting was performed using the indicated antibodies and horseradish peroxidase (HRP)-conjugated secondary anti-rabbit antibodies (Cell Signaling Technology, Beverly, MA, USA). The band was visualized by the ECL system (Amersham Biosciences).

### 2.15. Receptor-binding assay

The receptor-binding assay was measured by the FACScan analysis of the binding of FNLFNYK, a fluorescent analogue of FMLP, as described previously [36] with some modifications. Neutrophils ($2 \times 10^6$/ml) were incubated with drugs for 5 min at 4 °C before being labeled by 4 nM FNLFNYK. After 30 min, cells were pelleted and resuspended in ice-cold HBSS. The binding of FNLFNYK was immediately analyzed by FACS.

### 2.16. Statistical analysis

Results are expressed as the mean ± S.E.M., and comparisons were made using Student's *t*-test. A probability of $\leq 0.05$ was considered significant.

## 3. Results

### 3.1. SMG-1 inhibits FMLP/CB-induced $O_2^{\bullet-}$ generation and elastase release in human neutrophils

To investigate whether SMG-1 reduces respiratory burst in FMLP/CB-treated human neutrophils, the amount of $O_2^{\bullet-}$ generated was measured. SMG-1 (0.03–3 μM) failed to alter basal $O_2^{\bullet-}$ generation under resting conditions, whereas it inhibited $O_2^{\bullet-}$ release in FMLP/CB-treated human neutrophils in a concentration-dependent manner with an $IC_{50}$ value of 0.64 ± 0.04 μM (Fig. 1B). In contrast, SMG-1 (0.3–10 μM) did not alter NAF/CB-induced $O_2^{\bullet-}$ production in human neutrophils (Fig. 1C). Additionally, SMG-1 (1, 3, and 10 μM) failed to affect PMA-induced $O_2^{\bullet-}$ release. Ro318220 (0.1 μM), a well-documented inhibitor of protein kinase C (PKC), was used as a positive control of PMA-caused $O_2^{\bullet-}$ generation (Fig. 1D). Culturing with SMG-1 (up to 10 μM) for 30 min did not affect cell viability, as assayed by LDH release (data not shown).

Neutrophil degranulation was also measured according to the extent of release of the primary granule-derived protease, elastase. SMG-1 (0.3, 1, and 3 μM) inhibited elastase release by human neutrophils in response to FMLP/CB in a concentration-dependent manner with an $IC_{50}$ value of 1.00 ± 0.24 μM (Fig. 1E).

### 3.2. $O_2^{\bullet-}$- and free radical-scavenging activity of SMG-1

To examine the ability of SMG-1 to scavenge $O_2^{\bullet-}$ and free radicals, the effects of SMG-1 on the cell-free xanthine/xanthine oxidase system and DPPH tests were assayed. SMG-1, at concentrations of up to 10 μM, failed to alter WST-1 or DPPH reduction. SOD and α-tocopherol were used as positive controls in the xanthine/xanthine oxidase system and DPPH assay, respectively (Fig. 2). Moreover, SMG-1 (10 μM) did not affect the removal of $O_2^{\bullet-}$ by SOD (0.5 U/ml) (Fig. 2A). These data rule out the possibility that the inhibitory effect of SMG-1 on $O_2^{\bullet-}$ release

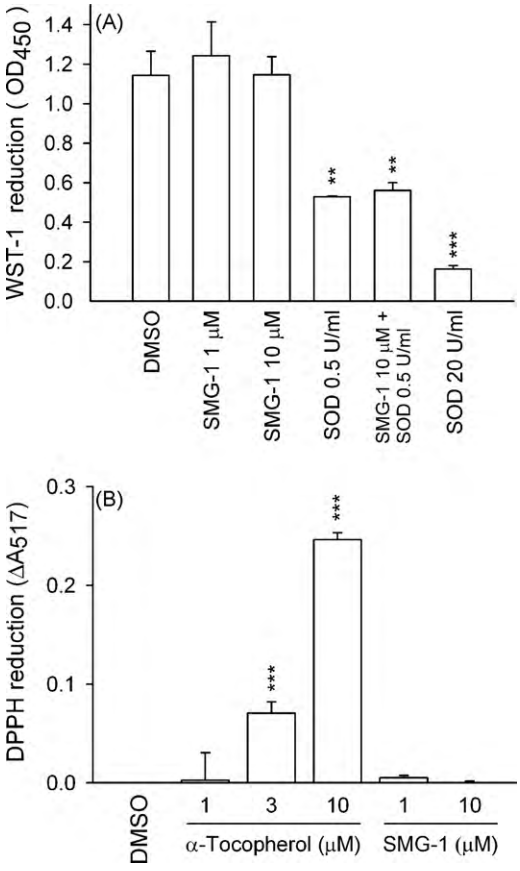


**Fig. 2.** Antioxidant effects of SMG-1 in a cell-free xanthine/xanthine oxidase system and DPPH assay. Reductions of WST-1 (A) and DPPH (B) were respectively measured spectrophotometrically at 450 and 517 nm, as described in Section 2. Data are expressed as the mean ± S.E.M. ($n = 4$). $^{**}p < 0.01$; $^{***}p < 0.001$ compared to the control.

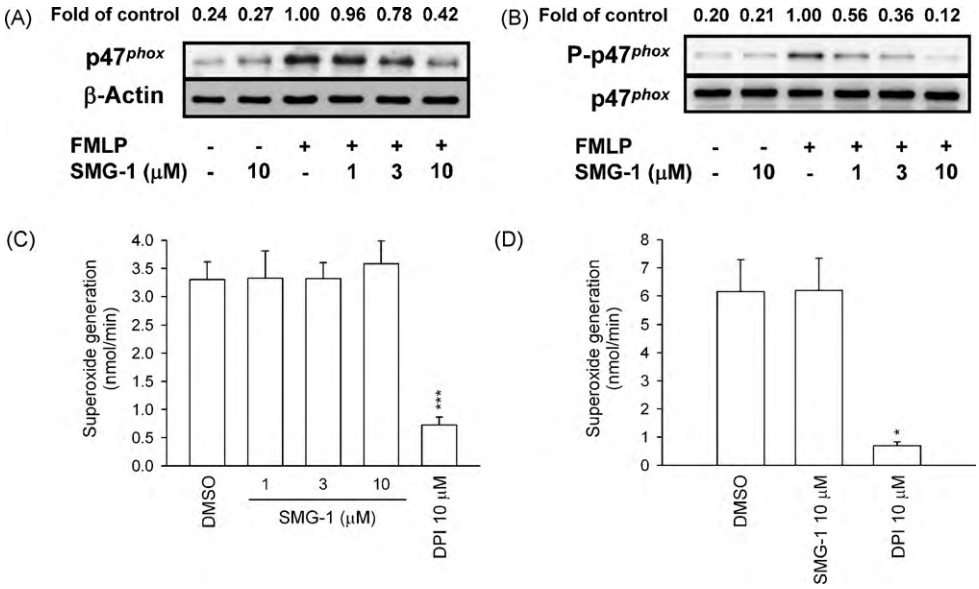


**Fig. 3.** Effects of SMG-1 on FMLP-induced $p47^{phox}$ membrane translocation and phosphorylation in intact neutrophils and $O_2^{\bullet-}$ generation in reconstituted NADPH oxidase. (A) Neutrophils were incubated with DMSO or SMG-1 (1, 3, and 10 μM) for 5 min and then stimulated with or without FMLP (1 μM) for another 3 min. The equivalent total protein was loaded according to protein concentration measurements and was also determined using an anti-β-actin antibody. Expression of $p47^{phox}$ in membrane fractions was determined by a Western blot analysis. Quantitation of the $p47^{phox}$/β-actin ratio is shown. The result shown is representative of three separate experiments. (B) Neutrophils were incubated with DMSO or SMG-1 (1, 3, and 10 μM) for 5 min and then stimulated with or without FMLP (1 μM) for another 1.5 min. Total phosphoproteins were prepared by the use of antiphosphoprotein affinity chromatography as described in Section 2. The $p47^{phox}$ proteins in total lysate ($p47^{phox}$) and in the phosphoprotein fraction (P-$p47^{phox}$) were detected by the use of an anti-$p47^{phox}$ antibody. The result shown is representative of three separate experiments. (C and D) A reactive mixture of the neutrophil cytosolic fraction and membrane fraction was preincubated with sodium dodecylsulfate (SDS, 100 μM) (C) or arachidonic acid (50 μM) (D) at 37 °C for 3 min before the addition of 160 μM NADPH. DMSO (as the control), SMG-1 (1, 3, and 10 μM), or diphenyleneiodonium (DPI, 10 μM) was incubated for 1 min before the addition of NADPH. $O_2^{\bullet-}$ generation was measured using SOD-inhibitable cytochrome *c* reduction. Data are expressed as the mean ± S.E.M. ($n = 4$). $^{*}p < 0.05$; $^{***}p < 0.001$ compared to the control.

occurs through scavenging of $O_2^{\bullet-}$ and free radicals. Additionally, SMG-1 (10 μM) did not alter uric acid formation in the xanthine/xanthine oxidase system (data not shown), suggesting SMG-1 does not inhibit the activity of xanthine oxidase.

### 3.3. SMG-1 inhibits membrane-associated p47$^{phox}$ expression and p47$^{phox}$ phosphorylation by intact neutrophils, but does not alter subcellular NADPH oxidase activity by reconstituted systems

Stimulation of neutrophils leads to increases in their oxygen consumption through the activity of NADPH oxidase which generates $O_2^{\bullet-}$, a precursor of other ROS. The phosphorylation and translocation of the subunit of p47$^{phox}$ from the cytosol to the plasma or phagosomal membrane is a crucial step in activating NADPH oxidase [37,38]. SMG-1 (1, 3, and 10 μM) diminished the membrane-associated p47$^{phox}$ expression and p47$^{phox}$ phosphorylation caused by FMLP, and the effects of SMG-1 were also concentration dependent (Fig. 3A and B). Additionally, neutrophil membranes were isolated to assay $O_2^{\bullet-}$ production in reconstituted systems after the addition of NADPH to examine whether SMG-1 has a direct effect on the NADPH oxidase in a cell free assay. Diphenyleneiodonium (10 μM), an NADPH oxidase inhibitor, but not SMG-1 (1, 3 and 10 μM), suppressed $O_2^{\bullet-}$ generation in SDS- and arachidonic acid-reconstituted systems (Fig. 3C and D). This data indicates that SMG-1 inhibits FMLP/CB-induced $O_2^{\bullet-}$ generation by intact neutrophils but not by reconstituted NADPH oxidase.

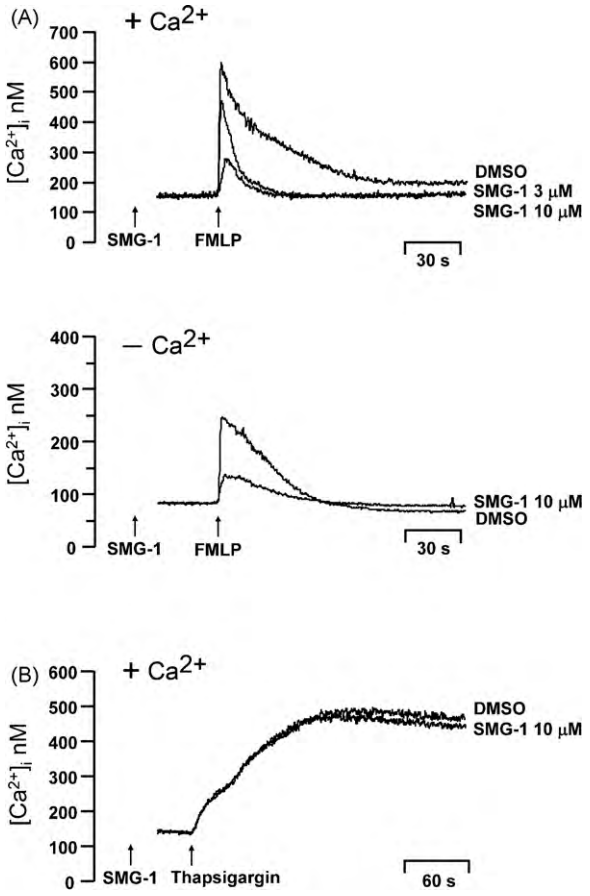


**Fig. 4.** Effect of SMG-1 on $Ca^{2+}$ mobilization in FMLP- or thapsigargin-activated human neutrophils. Fluo 3-loaded neutrophils were incubated with DMSO (as the control) or SMG-1 (1, 3, or 10 μM) for 5 min and then activated by 0.1 μM FMLP (A) or 0.1 μM thapsigargin (B) in 1 mM $Ca^{2+}$-containing or $Ca^{2+}$-free HBSS. The traces shown are from three to four different experiments.

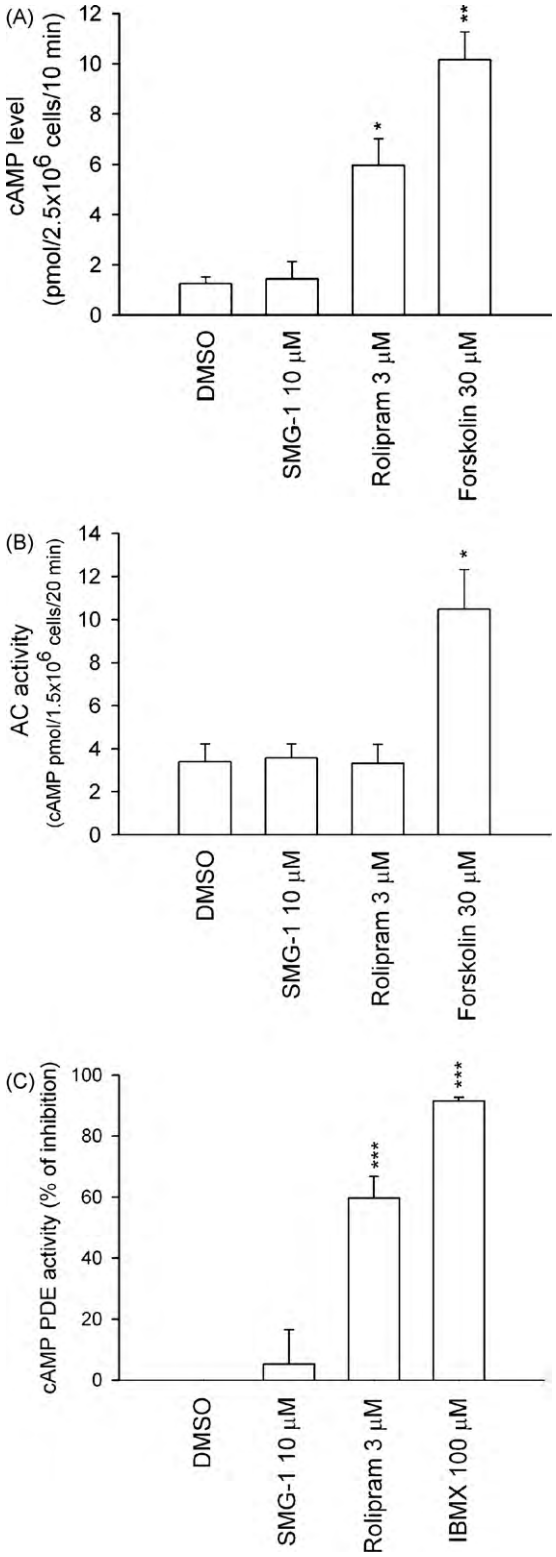


**Fig. 5.** Effects of SMG-1 on the cAMP pathway in human neutrophils. (A) Neutrophils were incubated with DMSO (as control), SMG-1 (10 μM), rolipram (3 μM), or forskolin (30 μM) for 5 min before stimulation with FMLP (0.1 μM) for another 5 min. cAMP levels were assayed using enzyme immunoassay kits. (B) Neutrophil membrane fractions were incubated with SMG-1 (10 μM), rolipram (3 μM), or forskolin (30 μM) at 30 °C for 20 min in the presence of 1 mM ATP. cAMP was assayed using enzyme immunoassay kits. (C) Neutrophil homogenates were incubated with DMSO (as control), SMG-1 (10 μM), rolipram (3 μM), or IBMX (100 μM) and then 0.05 μCi [$^3$H] cAMP was added to the reaction mixture at 30 °C for 10 min. PDE activity was measured as described in Section 2. All data are expressed as the mean ± S.E.M. ($n = 3$). $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ compared to the control.

### 3.4. Effect of SMG-1 on $[Ca^{2+}]_i$

Many cellular functions of neutrophils, such as respiratory burst and degranulation, are regulated by $Ca^{2+}$ signals [39]. FMLP-induced peak $[Ca^{2+}]_i$ values were concentration-dependently inhibited by SMG-1 (1, 3, and 10 μM) in human neutrophils in the presence or absence of extracellular $Ca^{2+}$ (1 mM) (Fig. 4A). In contrast, SMG-1 failed to change the $[Ca^{2+}]_i$ mobilization of human neutrophils caused by thapsigargin, an endoplasmic reticular $Ca^{2+}$-ATPase inhibitor (Fig. 4B).

### 3.5. Effect of SMG-1 on the cAMP pathway

Cellular cAMP concentrations are modulated either by synthesis via AC or by degradation via PDEs. To examine whether cAMP pathways are involved in the inhibitory effect of SMG-1, the cAMP concentration, AC function, and PDE activity were assayed. Rolipram (a PDE4 inhibitor, 3 μM) and forskolin (a direct AC activator, 30 μM), but not SMG-1 (10 μM), increased cAMP levels in human neutrophils (Fig. 5A). Furthermore, neither AC nor cAMP PDE activities were altered by SMG-1. Forskolin was used as a positive control for activating AC, and rolipram and IBMX were used as positive controls for inhibiting cAMP PDEs (Fig. 5B and C). Moreover, the protein kinase A (PKA) inhibitor, H89 (3 μM), restored the prostaglandin $(PG)E_1$ but not SMG-1-induced inhibition of $O_2^{\bullet-}$ production and elastase release (Fig. 6).

### 3.6. Effect of SMG-1 on phosphorylation of MAPKs and AKT

To determine whether MAPKs and AKT are involved in the inhibitory effects of SMG-1, activation of these kinases was assayed using antibodies specific for the phosphorylated, activated forms of MAPKs and AKT as determined by Western blotting. Stimulation of human neutrophils with FMLP resulted in the rapid phosphorylations of MAPKs and AKT. SMG-1 (0.3–10 μM) diminished FMLP-induced phosphorylations of ERK, JNK, p38 MAPK, and AKT in concentration-dependent manners (Fig. 7). However, SMG-1 (1 and 10 μM) did not alter PMA (5 nM)-induced phosphorylations of ERK, JNK, or p38 MAPK in human neutrophils (Fig. 8).

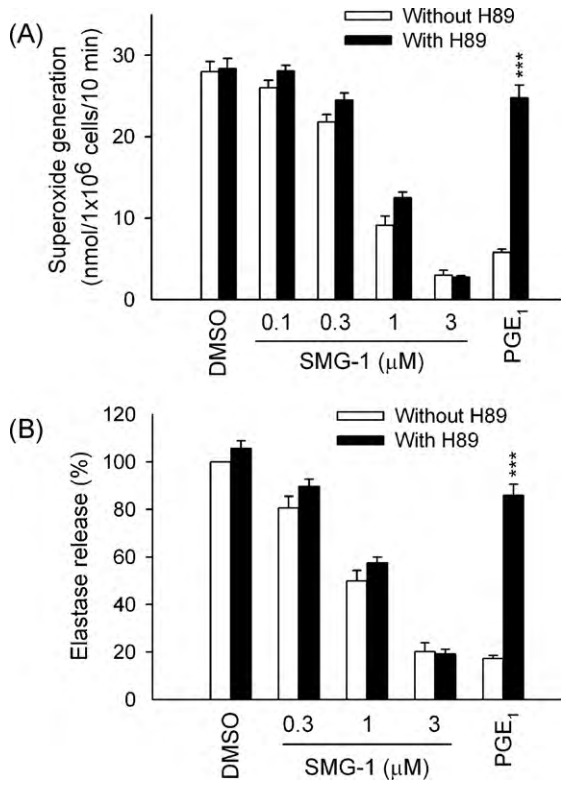


**Fig. 6.** Effects of a PKA inhibitor on the inhibition of $O_2^{\bullet-}$ generation and elastase release by SMG-1 in human neutrophils. H89 (3 μM), a PKA inhibitor, was preincubated for 5 min before the addition of SMG-1 (0.1-3 μM) or $PGE_1$ (1 μM). $O_2^{\bullet-}$ generation (A) and elastase release (B) were induced by FMLP/CB. All data are expressed as the mean ± S.E.M. ($n = 4$ or 7). $^{***}p < 0.001$ compared to the corresponding $PGE_1$.

### 3.7. SMG-1 inhibited the binding of FNLFNYK

Binding of FNLFNYK, a fluorescent analogue of FMLP [36], on the surface of neutrophils was monitored by flow cytometry. Binding of FNLFNYK to neutrophils was inhibited by FMLP (10 μM) as shown in Fig. 9. Furthermore, SMG-1 (1, 3, and 10 μM) significantly blocked the binding of FNLFNYK to the FMLP receptor in a concentration-dependent manner.

## 4. Discussion

Airway mucus hypersecretion is common in inflammatory and allergic lung diseases. Neutrophils are recruited into the lungs at the site of inflammation in response to inflammation and infection. Many airway diseases, including asthma, COPD, cystic fibrosis, and ARDS are characterized by neutrophil infiltration of the airways [9]. Therefore, suppression of neutrophils' functions using drugs is an important strategy to treat these inflammatory lung diseases. The pericarp of *S. mukorossi* has traditionally been used as an expectorant, to relieve coughing, for detoxification, and as a defervescence in Japan, China, and Taiwan [15]. However, the anti-inflammatory effect of *S. mukorossi* remains to be established. The aims of this study were to investigate the effects of SMG-1, a hederagenin saponin from this medicinal herb, on $O_2^{\bullet-}$ generation and elastase release in human neutrophils and to elucidate the signaling pathways responsible for the SMG-1-caused inhibition of neutrophilic responses. Our data suggest that the suppressive effects of SMG-1 on human neutrophil respiratory burst and degranulation are associated with inhibition of $Ca^{2+}$, JNK, p38 MAPK, and AKT signaling pathways through its blocking of the FMLP receptor.

The redox balance is particularly important in the airways because they are frequently exposed to higher oxidant burdens than other tissues. An enhanced oxidative burden was implicated in the pathogenesis of many acute and chronic pulmonary diseases including asthma, COPD, cystic fibrosis, ARDS, and lung malignancies [5,8,9]. In addition to respiratory burst, degranulation also plays a pivotal role in most neutrophil functions. Neutrophil granules contain many antimicrobial and potentially cytotoxic substances. Neutrophil elastase is a major secreted product of stimulated neutrophils and a major contributor to destruction of tissues in chronic inflammatory disease [40,41]. Neutrophil influx into airways is a predominant pathophysiologic feature of chronic inflammatory airway diseases. Neutrophil elastase exists in high concentrations in airway secretions of patients with chronic inflammatory airway diseases and induces overproduction of MUC5AC mucin, a major component of airway mucus [42]. Therefore, elastase also appears to be a target for therapy of chronic inflammatory airway diseases. SMG-1 potently suppressed FMLP-induced human neutrophil ROS formation and elastase release in concentration-dependent ways. This data supports our hypothesis that SMG-1 could act as an anti-inflammatory agent.

The formation of $O_2^{\bullet-}$, a precursor of other ROS, by NADPH oxidase is directly or indirectly linked to damage to or destruction of surrounding tissues. NADPH oxidase is a multicomponent enzyme, including cytosolic- and membrane-bound proteins, and remains unassembled in resting cells. Membrane components

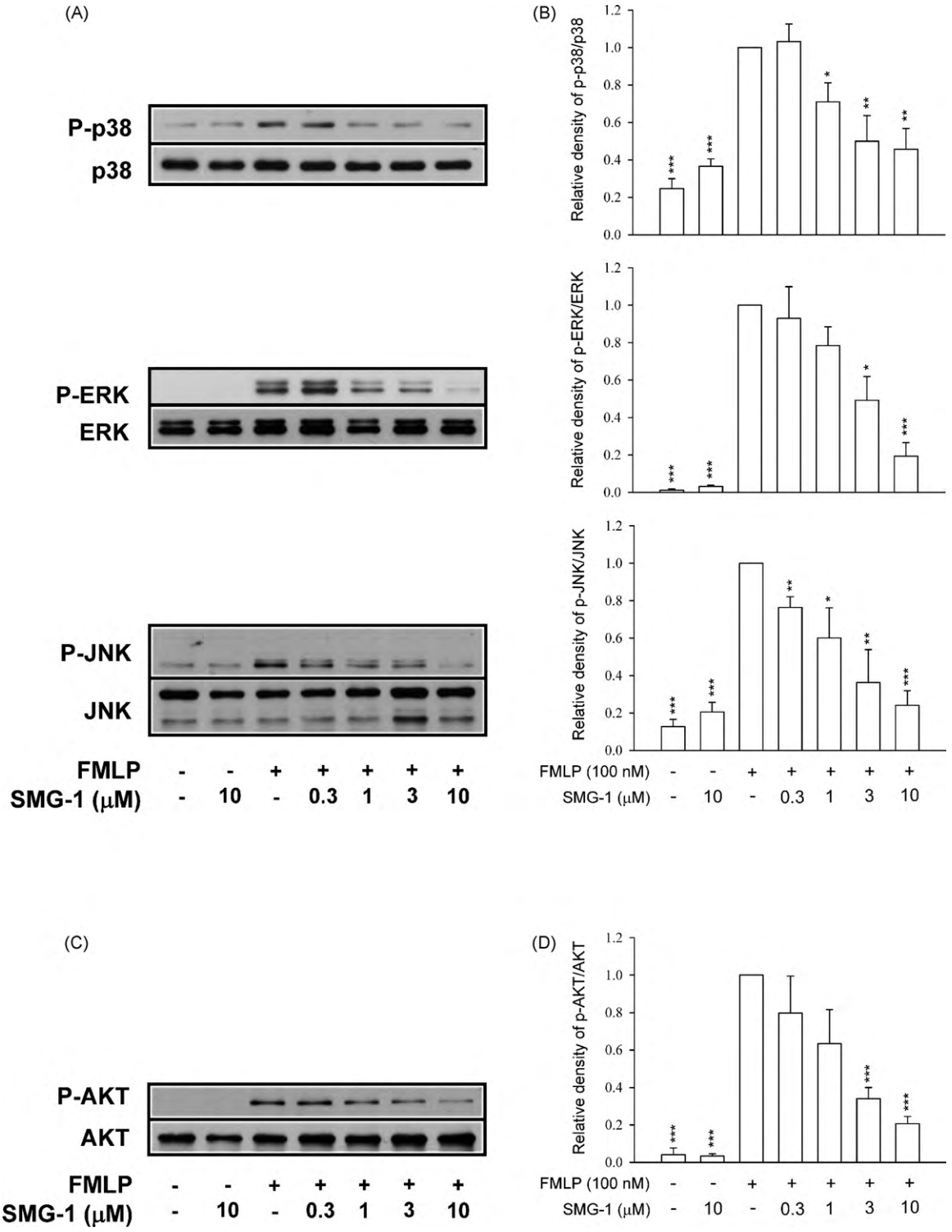


**Fig. 7.** Effects of SMG-1 on phosphorylation of MAPKs and AKT in FMLP-activated human neutrophils. Human neutrophils were incubated with DMSO or SMG-1 (0.3–10 μM) for 5 min before stimulation with FMLP (0.1 μM) for 0.5 min at 37 °C. Phosphorylations of p38, p42/44, JNK (A) and AKT (C) were analyzed by an immunoblot analysis using antibodies against the phosphorylated form and the total of each protein as described in Section 2. (B and D) Bands from (A) and (C) were analyzed by densitometer. Quantitation of the p-MAPK/MAPK and p-AKT/AKT ratios is shown. Representative images from one of three or four experiments are shown.

include a stable, heterodimeric flavocytochrome $b_{558}$ composed of two subunits, gp91$^{phox}$ and p22$^{phox}$. Cytosolic components include four soluble factors, p67$^{phox}$, p47$^{phox}$, p40$^{phox}$, and Rac, a small G-protein. Upon cell stimulation by soluble inflammatory mediators, such as FMLF, cytosolic components are translocated to the plasma or phagosomal membrane, where NADPH oxidase is assembled [38,43]. The formation of $O_2^{\bullet-}$ in neutrophils can be inhibited by modulating cellular signaling pathways, and also by directly scavenging $O_2^{\bullet-}$. SMG-1 inhibited $O_2^{\bullet-}$ generation by FMLP-activated intact neutrophils but not by a cell-free xanthine/xanthine oxidase system or by SDS- or arachidonic acid-reconstituted NADPH oxidase systems, suggesting that SMG-1 does not inhibit $O_2^{\bullet-}$ release through either scavenging $O_2^{\bullet-}$ formation or inhibiting NADPH oxidase activity. Indeed, the ability

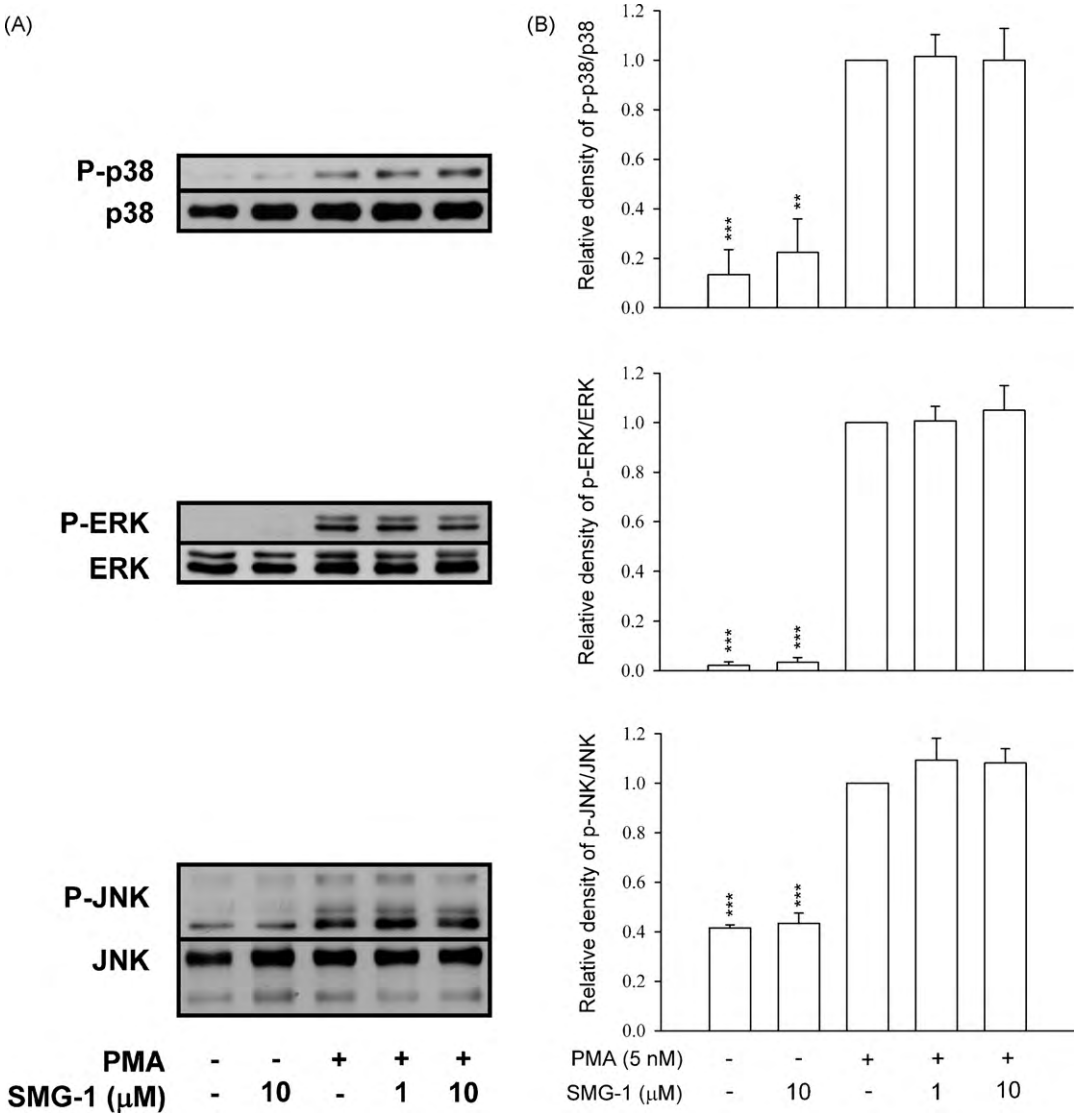


**Fig. 8.** Effects of SMG-1 on the phosphorylation of MAPKs in PMA-activated human neutrophils. (A) Human neutrophils were incubated with DMSO or SMG-1 (1 and 10 μM) for 5 min before stimulation with PMA (5 nM) for 1 min at 37 °C. Phosphorylations of p38, p42/44, and JNK were analyzed by an immunoblot analysis using antibodies against the phosphorylated forms and the total of each protein as described in Section 2. (B) Bands from (A) were analyzed by densitometer. Quantitation of the p-MAPK/MAPK ratios is shown. Representative images from one of three experiments are shown.

of SMG-1 to diminish membrane-associated $p47^{phox}$ in FMLP-activated human neutrophils demonstrates that SMG-1 exerts its inhibitory influence upstream of NADPH oxidase.

The cyclic nucleotide, cAMP, is an important second messenger with a variety of physiological and pathophysiological manifestations. Recently, our team and others demonstrated that the elevation of intracellular cAMP levels can suppress several FMLP-induced neutrophil functions, including respiratory burst and degranulation [35,44,45]. cAMP is formed from ATP by the action of the enzyme, AC, and is degraded by cAMP PDEs. Our results showed that $PGE_1$, which activates the Gαs protein to stimulate AC of neutrophils, inhibited $O_2^{\bullet-}$ production and elastase release in FMLP-induced human neutrophils. In contrast, SMG-1 failed to elevate the concentrations of cAMP or alter the activities of AC or cAMP PDE. Furthermore, pretreatment with H89, a PKA inhibitor, did not restore the SMG-1-induced inhibition of $O_2^{\bullet-}$ production or elastase release. These results indicate that the cAMP-dependent pathway does not mediate the inhibition of respiratory burst or degranulation by SMG-1.

The intracellular signaling mechanisms responsible for NADPH oxidase activation and degranulation in neutrophils are very complex and remain elusive. The bacterial peptide, FMLP, activates neutrophils by binding to the GPCR on the membrane. Stimulation of GPCR induces the $Ca^{2+}$ signal via activation of phospholipase C, which hydrolyzes phosphatidylinositol 4,5-bisphosphate into inositol triphosphate and diacylglycerol, resulting in an increase in $[Ca^{2+}]_i$ and activation of PKC, respectively [46,47]. Significant inhibition of FMLP-induced increase in $[Ca^{2+}]_i$ mobilization by SMG-1 was observed in human neutrophils. However, SMG-1 did not change the $[Ca^{2+}]_i$ concentration of human neutrophils caused by thapsigargin. These results indicate that SMG-1 specifically inhibits $Ca^{2+}$ mobilization caused by FMLP.

In addition to the increase in $[Ca^{2+}]_i$, stimulation of human neutrophils by FMLP resulted in the rapid phosphorylation of several proteins, including MAPKs and AKT. Activation of these signal transduction pathways is known to be responsible for various physiological responses [48]. It is well known that the phosphatidylinositol-3-kinase (PI3K) pathway plays an important role in neutrophil activation in response to agonists that trigger GPCRs [49–51]. Upon activation of PI3K, AKT is recruited to the plasma membrane, where it undergoes phosphorylation and activation. The MAPK family of signaling cascades consists of

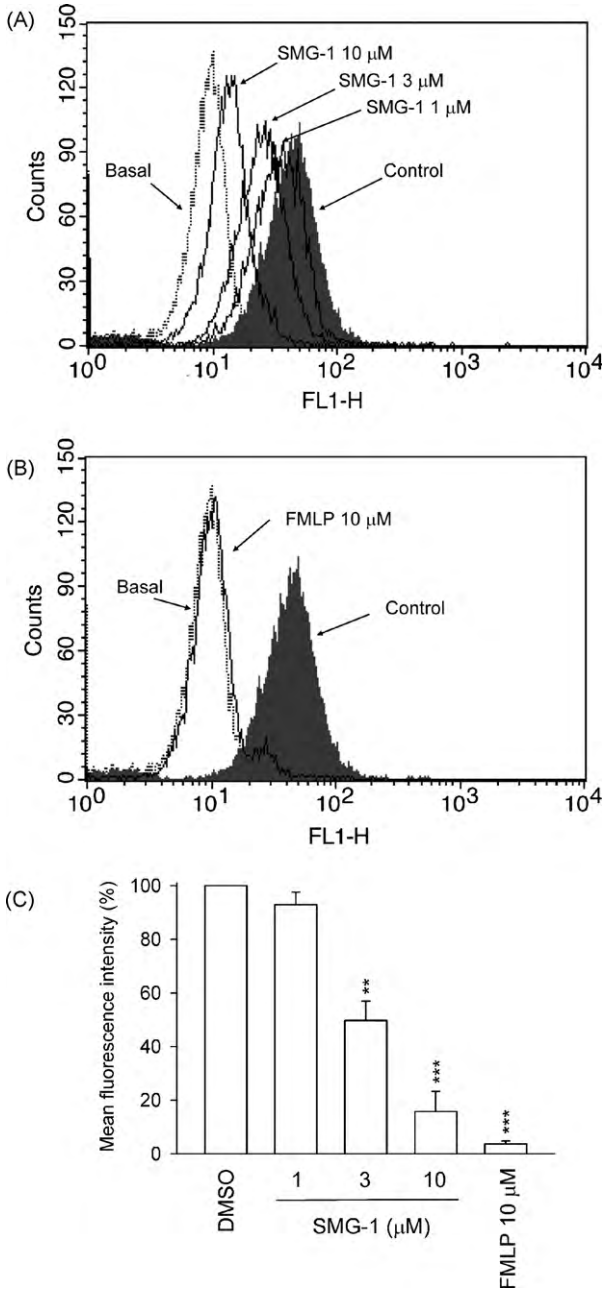


**Fig. 9.** Effects of SMG-1 on FNLFNYK binding to FMLP receptor in human neutrophils. Neutrophils ($2 \times 10^6$/ml) were incubated with SMG-1 (1, 3, and 10 μM) (A) or FMLP (10 μM) (B) for 5 min at 4 °C before being labeled by 4 nM FNLFNYK (4 nM) for 30 min. The fluorescence intensity of FNLFNYK was monitored using flow cytometry. (C) Mean fluorescence intensities are showed as the mean ± S.E.M. ($n = 4$). $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ compared to the control.

ERK, p38 kinase, JNK, ERK3/4, and the big MAPK1. The present study showed that the FMLP-induced phosphorylations of ERK, JNK, p38 MAPK, and AKT were diminished by SMG-1 in concentration-dependent ways. In contrast, PMA-activated release of $O_2^{\bullet-}$ and phosphorylation of MAPKs by neutrophils were not inhibited by SMG-1, suggesting that it might not inhibit PKC.

Based on these observations, we show that SMG-1 appears to interfere with the multiple FMLP-stimulated signaling pathways. Moreover, SMG-1 failed to alter human neutrophil $O_2^{\bullet-}$ production caused by NAF, a direct activator of G-protein. We therefore postulate that SMG-1 is a natural inhibitor of the FMLP receptor. Our data confirmed that SMG-1 blocked the binding of FNLFNYK to the FMLP receptor in a concentration-dependent manner. In summary, this study shows that SMG-1, a hederagenin saponin from the herbal medicine *S. mukorossi*, inhibits human neutrophil proinflammatory responses, including respiratory burst and degranulation. The suppressive effects of SMG-1 are associated with inhibition of the $Ca^{2+}$, JNK, p38 MAPK, and AKT signaling pathways through its blockade of the FMLP receptor. Our data revealed an active ingredient as well as the novel mechanism-mediated anti-inflammatory properties of *S. mukorossi* in human neutrophils. SMG-1 does not show structural similarity to any known inhibitor of FMLP receptor, thus providing a new chemical skeleton for the development of useful new therapeutic agents for the treatment of neutrophilic inflammatory diseases.

## Acknowledgements

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