



Petatewalide B, a novel compound from *Petasites japonicus* with anti-allergic activity



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ABSTRACT

Ethnopharmacological relevance: The giant butterbur *Petasites japonicus* is used to treat asthma and allergic diseases in traditional Korean, Japanese, and Chinese medicine.

Aim of the study: To elucidate the anti-allergic effect of *Petasites* genus, we studied effects of several compounds from *Petasites japonicus* leaves and found a novel bakkenolide-type sesquiterpine. In the present study, anti-allergic and anti-inflammatory effects of the new compound was examined using in vivo and in vitro experiments.

Materials and methods: The novel compound was isolated from *Petasites japonicus* leaves and named petatewalide B. Antigen-induced degranulation and Ca^{2+} mobilization were measured in RBL-2H3 mast cells by measuring β -hexosaminidase activity and fluorescence change of Ca^{2+} probe, fura-2. Induction of inducible nitric oxide synthase and cyclooxygenase 2 was measured by Western blotting in peritoneal macrophages. In addition, ovalbumin-induced asthma model was used for in vivo efficacy test of petatewalide B. Membrane potential was estimated by measuring fluorescence change of DiBAC in C6 glioma cells.

Results: Petatewalide B inhibited the antigen-induced degranulation of β -hexosaminidase in RBL-2H3 mast cells, but did not affect antigen-induced Ca^{2+} increase in the cells. Petatewalide B also showed inhibition of the LPS-induced induction of iNOS, but not COX-2 in mouse peritoneal macrophages. Nitric oxide production was also inhibited by petatewalide B in macrophages. In the ovalbumin-induced asthma model, petatewalide B strongly inhibited accumulations of eosinophils, macrophages, and lymphocytes in bronchoalveolar lavage fluid. Petatewalide B increased the membrane potential of C6 glioma cells in a concentration-dependent manner.

Conclusion: Petatewalide B from *Petasites* genus not only has anti-allergic and anti-inflammatory effects but also induces a transient increase of membrane potential in C6 glioma cells.

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1. Introduction

Extracts from *Petasites* genus have been shown to have anti-allergic and anti-inflammatory effects (Fiebich et al., 2005; Lee et al., 2011; Zhang et al., 2011). Lipophilic extracts of rhizomes of butterbur (*Petasites hybridus* L., Asteraceae) have been used as an anti-asthma therapy in the West (Brattstrom et al., 2010; Danesch, 2004). In Korea, Japan, and China, *Petasites japonicus* has been used

to treat asthma and allergic diseases (Lee et al., 2011, 2013b; Shimoda et al., 2006; Zhang et al., 2011). Inhibitory effects of *Petasites japonicus* extracts on mast cell degranulation and type I allergic response were reported (Shimoda et al., 2006). Identified ingredients from the *Petasites japonicus* extracts were fukinolic acid, chlorogenic acid, caffeic acid and etc. (Shimoda et al., 2006). Similarly, suppressive effect of *Petasites japonicus* extract on ovalbumin (OVA)-induced airway inflammation as an asthmatic model was reported (Lee et al., 2011). However, active constituents of leaves of *Petasites japonicus* have not been fully studied.

In the previous study, we sought to identify the active ingredients in *Petasites japonicus* leaves and to test them for anti-allergic and anti-inflammatory effects (Lee et al., 2013b). Of the compounds isolated from *Petasites japonicus*, we found that

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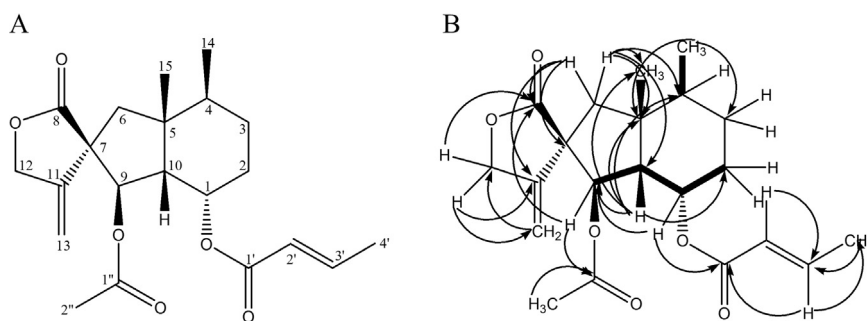


Fig. 1. Structure of petatewalide B. Structure of petatewalide B (A) and HMBC of petatewalide B (B).

bakkenolide B, a major component in its leaves exhibited significant effects in an OVA-induced asthma model (Lee et al., 2013b). In the current study, we report on isolation of a novel bakkenolide-type sesquiterpene, named petatewalide B, and its effects on various activities (Dong et al., 2010) (structure of petatewalide B is shown in Fig. 1).

2. Materials and methods

2.1. Plant material and chemicals

The leaves of *Petasites japonicus* (Siebold & Zucc.) Maxim were collected from Chungdogun, Gyungbok Province, Korea, in May 2012. The botanical identification was made by Dr. Young Whan Choi (College of Natural Resources and Bioscience, Pusan National University, Korea), and a voucher specimen (No. MW20120050) was deposited at the laboratory of the Natural Products Research Lab., College of Natural Resources and Bioscience, Pusan National University, Korea. Fura 2-AM was obtained from Calbiochem (Darmstadt, Germany) and other materials were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Extraction and isolation

The fresh leaves of *P. japonicus* (425 g) were chopped to a fine particle using an electric mixer (HMF-3100S, Hanil Electric, Seoul, Korea) and were successively extracted at room temperature with *n*-hexane, EtOAc, BuOH, and MeOH to obtain 8.477 g of hexane (MWLSH), 1.612 g of EtOAc, 3.92 g of BuOH, and 15.81 g of aqueous extract. The hexane extract (2.673 g) was evaporated in vacuo and the residue was chromatographed on a silica gel (40 μ m, Baker, NJ) column (100 $\times$ 4.0 cm²) using a step gradient 2.5%, 15%, and 25% acetone in dichloromethylene and 15%, 25%, and 50% MeOH in chloroform to obtain 62 fractions. Of these, fraction 10 (MWLSH10, 282.9 mg) was subjected to silica gel column (4 $\times$ 100 cm²) chromatography, using 2.5% methylene chloride, to give 14 fractions. MWLSH10IK (106.7 mg), a selected fraction, was subjected to LH20 sephadex gel column (3 $\times$ 100 cm²) chromatography, using 100% MeOH, to give 58.6 mg of pure compound (MWLSH10IKID, later named petatewalide B).

2.3. Identification of petatewalide B

Petatewalide B (isolated as MWLSH10IKID) was obtained as a colorless sticky compound with a negative optical rotation [α]_D²⁵ -21.2 (*c*=0.03, CH₂Cl₂). Its molecular formula, C₂₁H₂₈O₆, was determined by GC-MS (*m/z* 376.19 [M]⁺). The ¹H NMR data (Table 1) for petatewalide B (MWLSH10IKID) exhibited four methyl groups at δ 0.88 (3H, d, *J*=6.5 Hz, H₃-14), 1.09 (3H, s, H₃-15), 2.00 (3H, s, -COCH₃), and 2.37 (3H, d, *J*=7.3 Hz, -COCH=CHCH₃), two olefinic protons at δ 5.60 (1H, d, *J*=10.5 Hz, H-2') and 7.02 (1H, m,

Table 1

¹H (500 MHz in CDCl₃) ¹³C NMR (125 MHz in CDCl₃), and HMBC spectroscopic data of petatewalide B.

Position	δ_C	δ_H	HMBC
1	70.32	CH 5.14, m	165.59, 80.79,
2	26.82	CH ₂ 1.82, br	
3	29.53	CH ₂ 1.66, dd, <i>J</i> =14.5/3.5 1.35, dd, <i>J</i> =13.5/ 4.0	
4	35.33	CH 1.55, m	
5	43.29	C	
6	45.79	CH ₂ 2.22, d, <i>J</i> =14.0 1.93, d, <i>J</i> =14.5	177.51, 147.80, 54.80, 43.29, 35.33, 19.56 177.51, 147.80, 54.80, 51.76, 43.29, 35.33
7	54.80	C	
8	177.51	C	
9	80.79	CH 5.73, d, <i>J</i> =11.0	177.51, 169.89, 147.80, 70.55, 54.80, 51.76
10	51.76	CH 2.73, dd, <i>J</i> =10.5/5.0	80.73, 70.32, 43.29, 35.33, 26.82, 19.56
11	147.80	C	
12	70.55	CH ₂ 4.65, s	177.51, 147.80, 108.22
13	108.22	CH ₂ 5.16, d, <i>J</i> =18.0	70.55
14	15.55	CH ₃ 0.88 d, <i>J</i> =6.5	43.29, 35.33, 29.53
15	19.56	CH ₃ 1.09, s	51.76, 45.79, 43.29, 35.33
1'	165.59	C	
2'	112.49	CH 5.60, d, <i>J</i> =10.5	152.72
3'	152.72	CH 7.02, m	165.59, 19.25
4'	19.25	CH ₃ 2.37, d, <i>J</i> =7.5	152.72
1''	169.87	C	
2''	21.19	CH ₃ 2.00, s	169.87

H-3'), two oxygenated methines at δ 5.14 (1H, m, H-1) and 5.73 (1H, d, *J*=11.0 Hz, H-9), and an exocyclic methylene groups at δ 5.16 (2H, d, *J*=18 Hz, H-13). The ¹³C NMR spectrum of petatewalide B (MWLSH10IKID), in combination with the DEPT and HMQC spectra, showed twenty one carbon signals that were classified into four methyls [δ 15.55 (C-14), 19.56 (C-15), 21.19 (C-2''), and 19.25 (C-4')], five methylenes [δ 26.82 (C-2), 29.53 (C-3), 45.79 (C-6), 70.55 (C-12), and 108.22 (C-13)], six methines [δ 70.32 (C-1), 35.33 (C-4), 80.79 (C-9), 51.76 (C-10), 112.49 (C-2'), and 152.72 (C-3')], and six quaternary carbons [δ 43.29 (C-5), 54.80 (C-7), 177.51 (C-8), 147.80 (C-11), 165.59 (C-1'), and 169.87 (C-1'')]. Close similarities of the chemical shifts from C-1 to C-15 for petatewalide B (MWLSH10IKID) with reported values for similar compounds implied that petatewalide B (MWLSH10IKID) was a bakkenolide-type sesquiterpene with acetoxy and crotonyloxy groups. The further analysis of HMQC and HMBC spectra confirmed the presence of the bakkenolide-type skeleton, the acetoxy, and the crotonyloxy groups. The HMBC correlations of H-1 to the carbonyl carbon (δ_C 165.59) of the crotonyloxy group, and H-9 to the carbonyl carbon (δ_C 169.87) of the acetoxy group, revealed that the acetoxy and the crotonyloxy groups were attached at C-9 and C-1, respectively. The positive HRTOFMS indicated a molecular ion at *m/z* 431.1503

corresponding to $[M+H+Cl+H_2O]^+$, thus indicating a molecular formula of $C_{21}H_{31}ClO_7$.

By analyzing the HSQC, HMBC, and ^1H - ^1H COSY spectra, all the proton and carbon signals were assigned unambiguously. Petatewalide B (MWLSH10IKID) was a novel compound and was named petatewalide B (Fig. 1).

2.4. Animals

Six-week old male Balb/c (19–22 g) mice were purchased from Daehan Biolink (DBL; Seoul, Korea), housed in a Laboratory Animal facility in Pusan National University, and provided unrestricted amounts of food and water. The animal protocol used in this study was reviewed and approved by the Pusan National University–Institutional Animal Care Committee (PNU–IACUC) on their ethical procedures and scientific care, and it has been approved (Approval Number ED-PNU2012-0068).

2.5. Cell culture

Rat RBL-2H3 mast cells and rat C6 glioma cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA), cultured at 37 °C in a 5% CO₂ humidified incubator, and maintained in high glucose DMEM containing 10% (v/v) heat-inactivated fetal bovine serum, 100 units/mL penicillin, 50 µg/mL streptomycin, 2 mM glutamine, and 1 mM sodium pyruvate (Lee et al., 2013b).

2.6. Measurement of degranulation

Degranulation was estimated by measuring β -hexosaminidase release according to the method announced by Dearman et al. (2005). RBL-2H3 cells (2×10^5 cells/well in 24-well plates) were sensitized with 0.5 µg/ml of monoclonal anti-dinitrophenyl specific mouse IgE (DNP-IgE, D8406, Sigma-Aldrich) overnight at 37 °C in a 5% CO₂ incubator. To remove DNP-IgE before stimulation, cells were washed twice with PIPES buffer (pH 7.2), containing 25 mM PIPES, 110 mM NaCl, 5 mM KCl, 5.6 mM glucose, 0.4 mM MgCl₂, 0.1% BSA, and 1 mM CaCl₂. Then, the cells were incubated in 500 µl PIPES buffer. Different concentrations of petatewalide B were then added to each well and incubated at 37 °C for 30 min. Human dinitrophenyl albumin (10 µg/ml; DNP-hAb, A6661 Sigma-Aldrich) was then added, and cells were incubated for another 30 min at 37 °C to induce degranulation. Aliquots (50 µl) of medium were then transferred to a 96-well microplate and incubated for 60 min with 50 µl of 1 mM 4-nitrophenyl N-acetyl- β -D-glucosaminide (N9376, Sigma-Aldrich) in 0.1 M citrate buffer (pH 4.5). Cells were lysed with an equal volume of 0.5% Triton X-100 at 37 °C for 1 h. Total β -hexosaminidase activity in the cell lysates was also measured. The reaction was terminated by adding 250 µl of 0.05 M sodium carbonate buffer (pH 10.0; 0.05 M Na₂CO₃ / 0.05 M NaHCO₃). Absorbance (OD) at 410 nm was measured using a microplate reader. Release (%) was calculated by the ratio of released β -hexosaminidase activity in the media to total β -hexosaminidase activity in the cells. In “spontaneous”, neither DNP-HSA nor sample was added to cells to confirm the spontaneous β -hexosaminidase release from cells in conditions without sample. In “total”, cells were lysated and the total amount of β -hexosaminidase contained in the cells were measured. In “sample”, both DNP-HSA and samples were treated to cells to confirm the β -hexosaminidase release from cells (Lee et al., 2013b).

ratio of β -hexosaminidase release (%) =

$$\left(\frac{\text{OD control or OD sample} - \text{OD spontaneous}}{\text{OD total}} \right) \times 100$$

2.7. Measurement of intracellular Ca^{2+} concentrations

Cells were trypsin-digested, allowed to sediment, incubated in complete medium containing monoclonal anti-dinitrophenyl specific mouse IgE (0.5 µg/ml, DNP-IgE, D8406, Sigma-Aldrich) at 37 °C for 2 h, resuspended in Hepes-buffered medium (HBM), containing 20 mM Hepes (pH 7.4), 103 mM NaCl, 4.8 mM KCl, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄, 0.5 mM CaCl₂, 25 mM NaHCO₃, 15 mM glucose, 2.5 mM probenecid, and 0.1% bovine serum albumin (fatty acid free), and then incubated for 40 min with 5 µM of fura 2-AM. Intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$) were estimated by measuring changes in fura-2 fluorescence at an emission wavelength of 510 nm and two excitation wavelengths (340 nm and 380 nm) every 0.1 s using a F4500 fluorescence spectrophotometer (Hitachi, Japan). The ratios of fluorescence intensities ($\lambda_{340}/\lambda_{380}$) at these two wavelengths were used as a surrogate of $[Ca^{2+}]_i$, as previously described (Lee et al., 2015; Park et al., 2014).

2.8. Isolation and culture of mouse peritoneal macrophages

Mouse peritoneal macrophages were isolated from peritoneal cavity of a 3% thioglycol-treated C57BL/6 mouse and cultured at 37 °C in a 5% CO₂ humidified incubator, as previously described (Michaud et al., 2010). The isolated macrophages were maintained in RPMI1640 containing 10% (v/v) heat-inactivated fetal bovine serum, 100 units/mL penicillin, 50 µg/mL streptomycin, 2 mM glutamine, and 1 mM sodium pyruvate for 24 h and then incubated in 0.5% FBS-containing media for 18 h. Samples for proteins were made after 24 h treatment of 100 ng/ml LPS (Sigma-Aldrich, L2630). Petatewalide B was added 1 h before LPS treatment (Lee et al., 2013b).

2.9. Induction of asthma in BALB/c mice and cell counting

Mice were divided into three groups ($n=5$), that is, a phosphate-buffered saline (PBS)-injected control group, an OVA-injected asthma group, and petatewalide B-treated asthma group (1 mg/kg). Asthma was induced by the intraperitoneal injection of OVA (Sigma-Aldrich, A5503) and aluminum hydroxide (Sigma-Aldrich, 329186) at 1st day and 14th day. From 28th day, mice were exposed to nebulized OVA for 20 min for three consecutive days (Aoki et al., 2010). Two days later (32th day), bronchoalveolar lavage fluids (BALF) were collected from the lungs (Lee et al., 2013b). Cells in BALF attached to slide glass using a Cellspin (5 min, 500 rpm, Hanil Electric). Cells were fixed in methanol for 30 s. The slides were processed in May-Grunwald solution (Sigma-Aldrich, 32856) for 5 min, and subsequently in Giemsa solution (Sigma-Aldrich, 32884) for 12 min. After rinsing, the slides were covered, and then, immune cells were counted. Five animals for each group were analyzed ($n=5$).

2.10. Western blotting

Macrophages were harvested and resuspended in lysis buffer. Concentrations of proteins were determined using the BCA protein assay (Thermo Scientific, Rockford, IL, USA). Equal amounts of proteins were resolved by 10% SDS-polyacrylamide gel electrophoresis and electrophoretically transferred to nitrocellulose membranes, which were blocked in Tris-buffered saline containing 0.1% Tween 20 (TBS-T) and 5% skim milk, and then incubated with specific rabbit antibodies recognizing cyclooxygenase 2 (COX-2) and inducible nitric oxide synthase (iNOS) (Cell Signaling Technology, Danvers, MA, USA). Anti-rabbit horseradish-linked IgG was used as the secondary antibody. Signals were developed using an enhanced

chemiluminescence system (Advanst, USA) (Kang et al., 2013).

2.11. Nitrite measurement

Production of NO was estimated by measuring the amount of nitrite, a stable metabolite of NO, using Griess reagent, as previously described (Lee et al., 2013a). Cells were pretreated with different concentrations of petatewalide B for 1 h and then stimulated with LPS (100 ng/ml) for 24 h. Nitrite concentrations in media were determined using the Griess Reagent System (Promega, Madison, WI, USA).

2.12. Measurement of membrane potential

C6 glioma cells were sedimented, resuspended with a HEPES-buffered medium consisting of 20 mM of HEPES (pH 7.4), 103 mM NaCl, 4.8 mM KCl, 1.2 mM KH_2PO_4 , 1.2 mM MgSO_4 , 0.5 mM CaCl_2 , 25 mM NaHCO_3 , 15 mM glucose and 0.1% bovine serum albumin (fatty acid free), and then incubated for 30 min with 5 μM of DiBAC₄(3). Fluorescence emissions at 530 nm wavelength from excitation wavelength (488 nm) were measured every 0.1 s using an F4500 fluorescence spectrophotometer (Hitachi, Japan). Membrane potential was estimated by measuring fluorescence change of DiBAC-loaded cells (Lee and Im, 2006; Lee et al., 2007).

2.13. Cell viability assay

C6 glioma cells were seeded in a 96-well plate at a density of

1×10^4 cells per well and cultured for 24 h at 37 °C in a 5% CO_2 humidified incubator. The cells were incubated with petatewalide B for 48 h. Cytotoxicity was examined using an EZ-Cytox cell viability assay kit (ITSBIO, Seoul, Korea). Cell viability was determined by measuring the absorbance at 450 nm using an EZ read 400 microplate reader (Biochrom; Miami, FL, USA). Optical density (OD) of untreated cells was defined as 100% (Park et al., 2014).

2.14. Statistics

The results are expressed as the means $\pm$ SEs of the numbers of indicated determinations. Statistical significances of the differences were determined by analysis of variance (ANOVA) with turkey's post hoc, and statistical significance was accepted for p values < 0.05 . Analyses were performed using GraphPad Prism software (GraphPad Software, Inc., La Jolla, CA, USA).

3. Results

3.1. Petatewalide B inhibited antigen-induced degranulation in RBL-2H3 mast cells

Degranulation of histamine, leukotrienes, and prostaglandins from antigen-exposed mast cells is a key step in allergic response. Therefore, we utilized rat RBL-2H3 mast cells to determine whether petatewalide B inhibits antigen-induced degranulation. Degranulation was measured by assessment of β -hexosaminidase

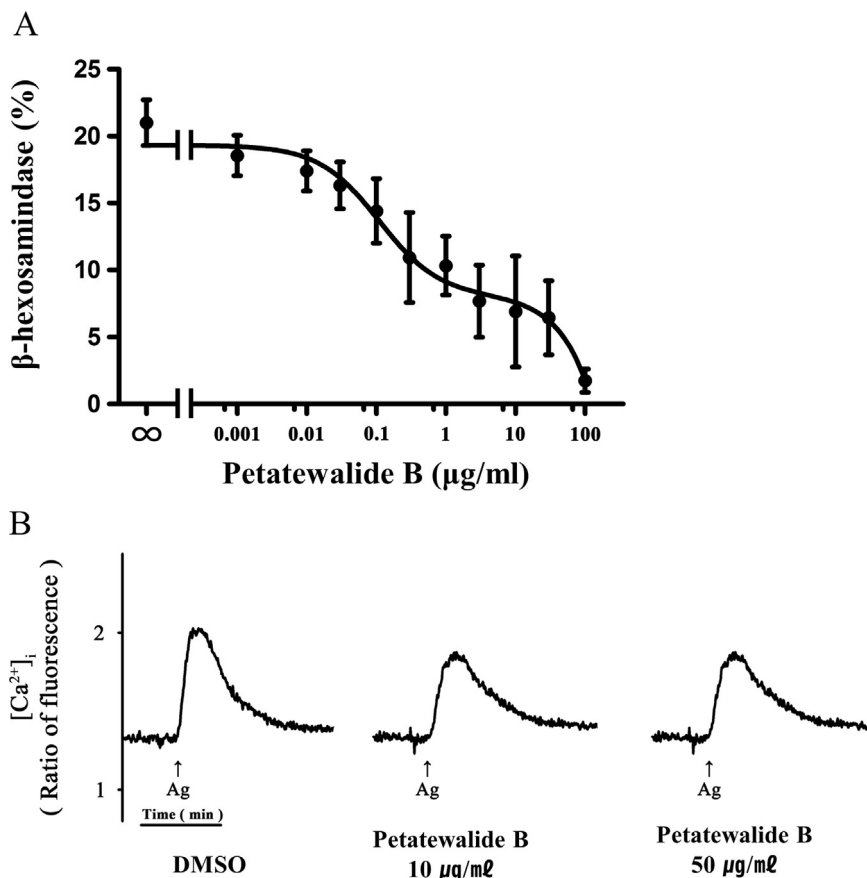


Fig. 2. Effects of petatewalide B on antigen-induced release of β -hexosaminidase and intracellular increase of Ca^{2+} in RBL-2H3 mast cells. (A) RBL-2H3 cells sensitized overnight with anti DNP-IgE were treated with different concentrations of petatewalide B, and, 30 min later, challenged with DNP human serum albumin. Antigen-induced degranulation was determined by measuring the amount of released β -hexosaminidase activity (A). (B) RBL-2H3 cells were sensitized overnight with anti DNP-IgE, loaded with Fura-2 AM for 45 min, and washed twice with PBS. The cells were then pre-incubated with petatewalide B for 10 min and challenged with DNP human serum albumin. Arrows indicate when the antigen was added. The data shown are representative of three independent experiments.

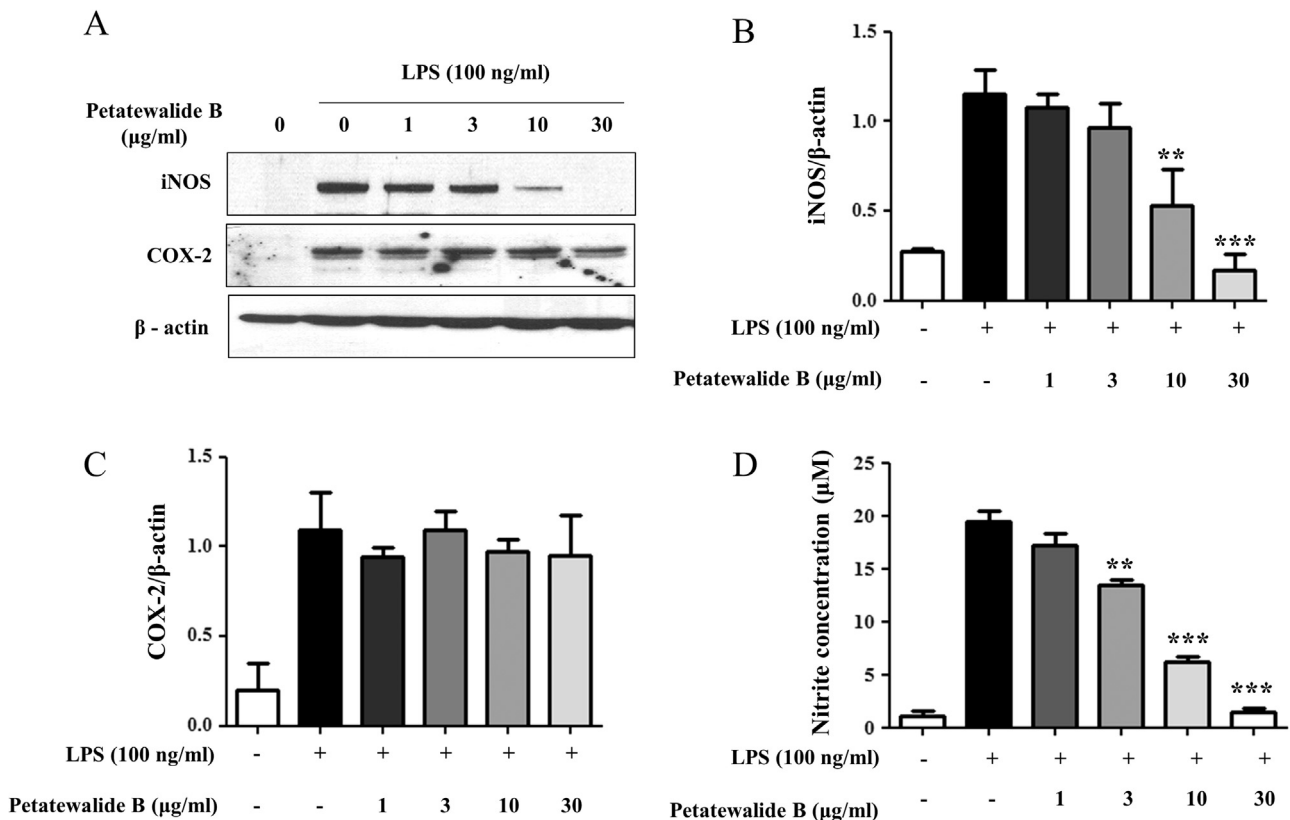


Fig. 3. Inhibitory effects of petatewalide B on iNOS expression and NO production in mouse peritoneal macrophages. Mouse peritoneal macrophages were pretreated with petatewalide B and, 1 h later, stimulated with LPS, to induce the expression of iNOS and COX-2. Results are representative of three independent experiments that yielded similar results (A). Protein levels were quantitated using ratios to β -actin protein levels (B for iNOS and C for COX-2). Values are expressed as the mean $\pm$ SE ($n=3$). Statistical significance: ** $P < 0.01$, *** $P < 0.001$ vs. LPS-treated group. (D) Mouse peritoneal macrophages were treated with petatewalide B and, 1 h later, stimulated with 100 ng/ml LPS. LPS-induced production of nitrite was measured. Results are expressed as the mean $\pm$ SE of three independent experiments in triplicate. Statistical significance: ** $p < 0.01$, *** $p < 0.001$ vs. LPS-treated group.

activity in media after antigen exposure, as previously described (Dearman et al., 2005). Antigen-induced release of β -hexosaminidase was inhibited by petatewalide B in a concentration-dependent manner (Fig. 2A). Degranulation is evoked by the elevation of intracellular Ca^{2+} after antigen-exposure in mast cells (Choi et al., 2012). Next, intracellular Ca^{2+} increase caused by antigen exposure was measured to determine whether petatewalide B inhibited the increase (Lee et al., 2005). However, petatewalide B did not affect antigen-induced $[\text{Ca}^{2+}]_i$ increase, similar to responses by bakkenolide B in RBL-2H3 cells (Lee et al., 2013b) (Fig. 2B).

3.2. Petatewalide B inhibited induction of iNOS and production of NO in peritoneal macrophages

Inflammatory responses are also involved in allergic responses (Wills-Karp, 2004). Accordingly, we measured the effect of petatewalide B on expression of iNOS and COX-2 in mouse peritoneal macrophages (Lee et al., 2013b). LPS induced the expression of iNOS and COX-2 at the protein level in macrophages (Fig. 3A). Petatewalide B inhibited induction of iNOS in a concentration-dependent manner (Fig. 3A and B) but not COX-2 (Fig. 3A and C). To confirm the inhibitory effect of petatewalide B on induction of iNOS, nitric oxide (NO), the enzyme product was measured. As shown in Fig. 3D, LPS (100 ng/ml) induced the production of NO, and treatment with petatewalide B resulted in significant concentration-dependent inhibition of NO production (Fig. 3D), consistent with the inhibitory effects of petatewalide B on protein expression of iNOS (Fig. 3A and B).

3.3. Petatewalide B inhibited accumulation of eosinophils, macrophages, and lymphocytes in BALF of OVA-induced asthma model mice

The anti-allergic and anti-inflammatory effects of petatewalide B observed in RBL-2H3 cells and in peritoneal macrophages in vitro were further confirmed in OVA-induced asthma model mice in vivo. In RBL2H3 cells, effective concentrations of petatewalide B were 1–10 $\mu\text{g/ml}$ for degranulation inhibition (Fig. 2). Effective concentration of petatewalide B in peritoneal macrophages was 10–30 $\mu\text{g/ml}$ in vitro (Fig. 3). Considering a whole body of mice as a compartment, 1 mg/kg intraperitoneal injection would be 20 $\mu\text{g}/20\text{ g}$, that is 1 $\mu\text{g/mL}$. Therefore, we used a dose of 1 mg/kg for the in vivo OVA-induced asthma model.

The numbers and population of the cells in BALF were analyzed. Total cell numbers in BALF were increased as 230% in the OVA-induced asthma group compared to PBS control group (Fig. 4A). The increment of total cells in OVA group was inhibited by petatewalide B treatment about 60% (Fig. 4A). Further analyses of cell populations in BALF supported this observation (Fig. 4B). In particular, eosinophil numbers were increased as 494% in the BALF of the OVA group compared to PBS control group. This increment of eosinophils was inhibited in the OVA+petatewalide B group about 59% (Fig. 4B). Furthermore, BALF macrophage and lymphocyte counts in the OVA group were 153% and 270% increased, respectively compared to PBS group (Fig. 4B). In the OVA+petatewalide B group, the increased counts of macrophages and lymphocytes were inhibited about 58% and 87%, respectively (Fig. 4B).

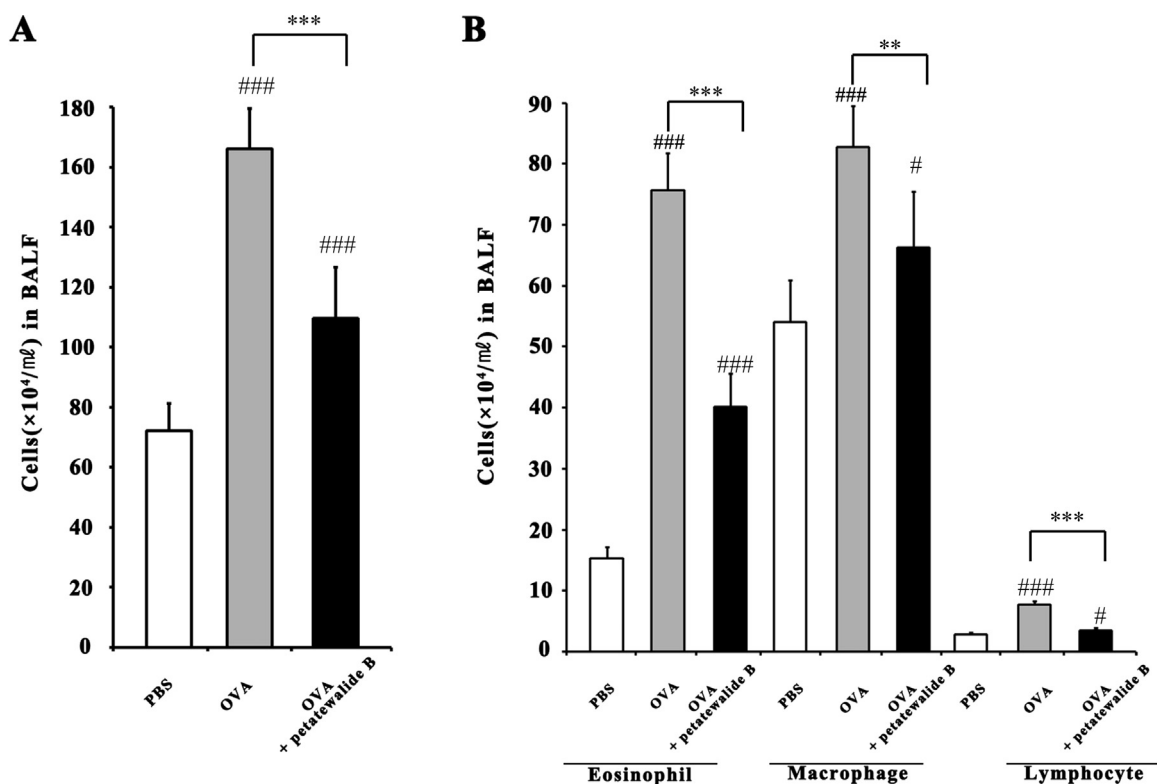


Fig. 4. Effect of petatewalide B on immune cells accumulation in BALF of OVA-induced asthmatic mice. Five mice were used for each group. Mice were sensitized with OVA twice by intraperitoneal injection (days 1st and 14th) and were later challenged with nebulized OVA on days 28th, 29th, and 30th. Petatewalide B was administrated by intraperitoneal injection (1 mg/kg) on these days 30 min before OVA challenge. Cells in BALF were stained using the May-Grunwald staining and counted. (A) Total cell counts in the BALF of PBS control mice, OVA-induced asthmatic mice (OVA), and petatewalide B-treated asthma mice (OVA+petatewalide B). (B) Cells counts of eosinophils, macrophages, and lymphocytes in the BALF of these three groups. The values shown are means $\pm$ SEs ($n=5$). Statistical significance: # $p < 0.05$ and ### $p < 0.001$ vs. PBS controls. ** $p < 0.01$, and *** $p < 0.001$ vs. the OVA-treated group.

3.4. Petatewalide B increased membrane potential in C6 glioma cells

Modulation of membrane potential plays important roles in neuronal cells and smooth muscle cells. Previously, modulation of membrane potential by natural compounds has been investigated in glioma cells (Choi et al., 2008; Lee et al., 2006). Thus, in this study, we measured membrane potential in C6 glioma cells and tested the effect of petatewalide B on the resting membrane potential. As shown in Fig. 5, petatewalide B increased membrane potential in a concentration-dependent manner (Fig. 5A and B). This response was specific to petatewalide B, because s-petasin did not induce such an increase in membrane potential in the cells (Fig. 5C). The increase of membrane potential by petatewalide B was transient. The highest increase observed with 100 μ g/ml petatewalide B also returned to the baseline after 20 min (data not shown). We also measured cytotoxicity of petatewalide B in C6 glioma cells, which showed slight cytotoxicity of approximately 20% (Fig. 5D). However, this cytotoxicity appears not to be correlated with increase of membrane potential, because concentration-response curves are not superimposed between two results (Fig. 5B and D). Increase of membrane potential by 100 μ M petatewalide B was more than 5 folds that of 50 μ M (Fig. 5B). However, there was no such increase in cytotoxicity between two concentrations (Fig. 5D).

4. Discussion

The genus *Petasites* has been reported to have anti-allergic and anti-inflammatory effects (Fiebich et al., 2005; Lee et al., 2011; Zhang et al., 2011). Many studies on action mechanism and active

constituents for the effects of *Petasites* genus have elucidated L-type Ca^{2+} channel blockage by petasin and isopetasin (Bickel et al., 1994; Fiebich et al., 2005; Resnati et al., 2002; Thomet et al., 2001; Wang et al., 2010) and inhibition of leukotriene B_4 and cysteinyl leukotriene production in eosinophils and neutrophils (Thomet et al., 2001). Similarly, inhibition of cysteinyl leukotriene biosynthesis was shown with *Petasites hybridus* extracts in isolated peritoneal macrophages (Bickel et al., 1994) and isopetasin and oxopetasin esters were found to inhibit the biosynthesis of cysteinyl leukotrienes (Bickel et al., 1994).

Inhibitory effects of *Petasites japonicus* extracts on mast cell degranulation and type I anti-allergic response were reported (Shimoda et al., 2006). Similarly, suppressive effect of *Petasites japonicus* extract on OVA-induced airway inflammation as an asthmatic model was reported (Lee et al., 2011). However, active constituents of leaves of *Petasites japonicus* have not been fully studied. In the previous study, we reported on the anti-allergic and anti-inflammatory effects of bakkenolide B in *Petasites japonicus* leaves (Lee et al., 2013b). Bakkenolide B inhibited antigen-induced degranulation in RBL-2H3 mast cells and induction of inducible NOS and COX-2 in mouse peritoneal macrophages (Lee et al., 2013b). In the current study, we isolated a new bakkenolide-type constituent from *Petasites japonicus* leaves and named it petatewalide B, because its structure is similar to that of petatewalide A (Dong et al., 2010). Petatewalide A was initially isolated from *Petasites tatewakianus* and its weak cytotoxicity to HeLa, MCF-7, and LLC cell lines was reported (Dong et al., 2010). However, study of the effect of petatewalide A on anti-allergic and anti-inflammatory effects has not been conducted. In the current study, we found that petatewalide B has inhibitory effects on mast cell degranulation and on LPS induction of iNOS in macrophages. Because

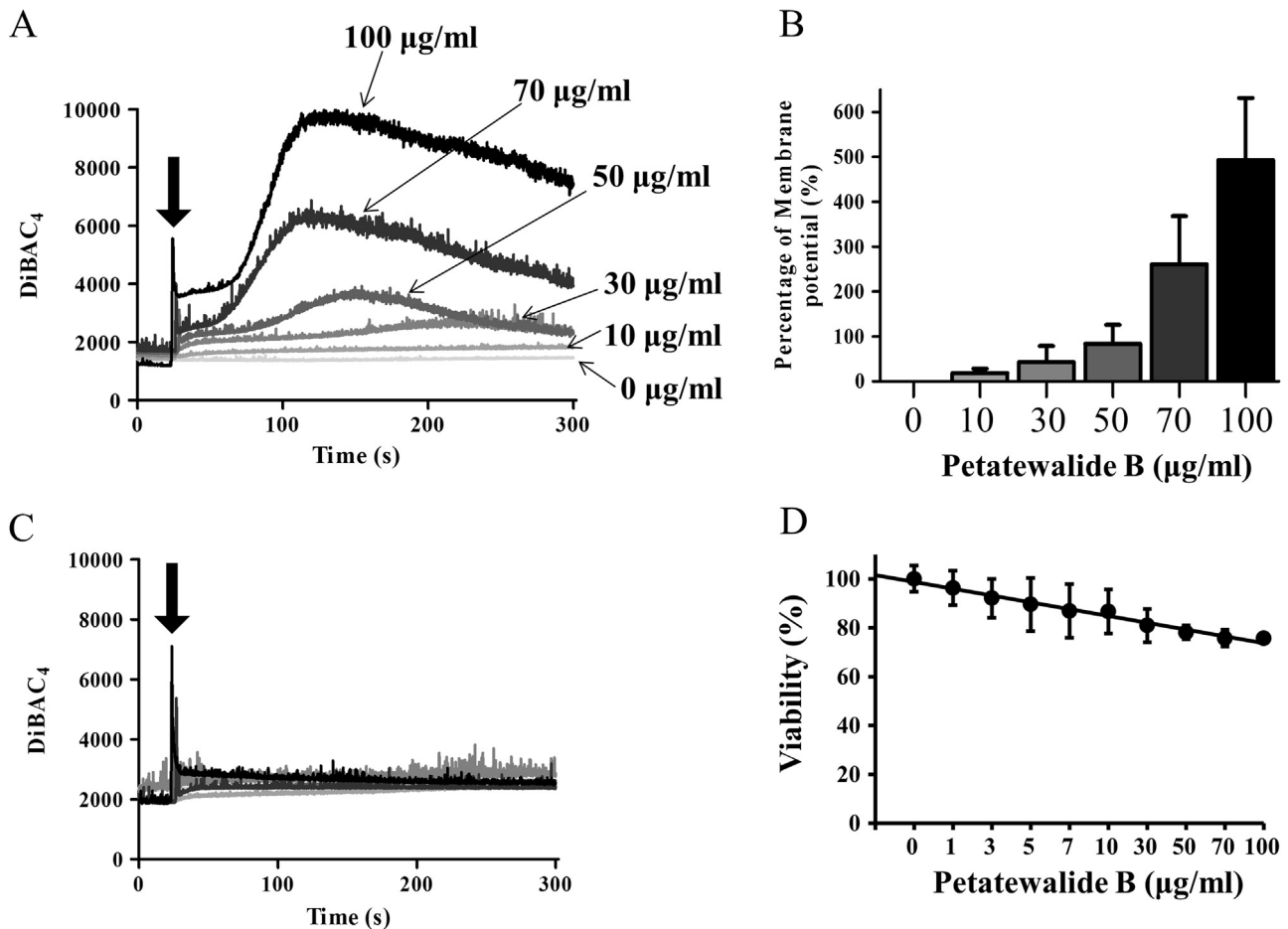


Fig. 5. Petatewalide B increased membrane potential in C6 glioma cells. C6 glioma cells loaded with DiBAC4 were treated with petatewalide B. Arrows indicate when petatewalide B was added (A). In A, traces are representative of three independent experiments with different concentrations of petatewalide B. In B, the increases by petatewalide B compared to baseline fluorescence are shown as mean $\pm$ SE ($n=3$). In C, traces are representative of three independent experiments with different concentrations of s-petasin. In D, cytotoxicity of petatewalide B was shown. Data are two independent experiments in triplicate.

petatewalide B did not inhibit increase in $[Ca^{2+}]_i$ by antigen treatment in mast cells, petatewalide B-induced inhibition of degranulation seems not to be mediated via the inhibition of Ca^{2+} channel or Ca^{2+} increase in mast cells. Further in vivo experiments with petatewalide B showed strong inhibition on OVA-induced asthma. Therefore, anti-allergic effects of *Petasites japonicus* extracts on type I anti-allergic response and on OVA-induced airway inflammation could be explained with pure compounds such as petatewalide B and bakkenolide B (Lee et al., 2011, 2013b; Shimoda et al., 2006). In addition, anti-allergic effect of a total bakkenolide fraction from *Petasites tricholobus* on OVA-sensitized allergic rhinitis in Wistar rats can now be explained in part with pure bakkenolides, that is petatewalide B and bakkenolide B (Lee et al., 2013b; Zhang et al., 2011). The action mechanism might be inhibition of degranulation in mast cells and suppression of iNOS in macrophages.

The genus *Petasites* (Astraceae) has been reported to be a rich source of sesquiterpene lactones (Dong et al., 2010; Liao et al., 1997; Wang et al., 2009; Wu et al., 1999; Zhang et al., 2011); for example, 32 bakkenolides were isolated from *Petasites formosanus* (Wu et al., 1999). Bakkenolide-type sesquiterpenes isolated from this genus have been reported to exhibit anti-platelet aggregation activity (Liao et al., 1997). In addition, bakkenolide B from *Valeriana jatamansi* was found to have a weak neuroprotective effect against MPP⁺-induced neuronal cell death in dopaminergic neuroblastoma SH-SY5Y cells (Xu et al., 2011). We also found membrane potential increasing effect of petatewalide B in C6 glioma

cells. To increase the membrane potential, movement of positive or negative ions such as Ca^{2+} influx, K^+ efflux, or blockage of Cl^- influx should be modulated by petatewalide B. Further studies are necessary in order to determine the exact mechanism of membrane potential increase by petatewalide B.

In summary, we isolated and identified a novel bakkenolide-type sesquiterpene from *Petasites japonicus* leaves, named petatewalide B. Petatewalide B showed not only anti-allergic and anti-inflammatory responses in mast cells and macrophages, but also increase of membrane potential in C6 glioma; further investigation on petatewalide B is necessary.

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