Batatasin I, a Naturally Occurring Phenanthrene Derivative, Isolated from Tuberous Roots of Dioscorea batatas Suppresses Eicosanoids Generation and Degranulation in Bone Marrow Derived-Mast Cells

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To find anti-inflammatory compounds from the tuberous roots of Dioscorea batatas, we isolated 6-hydroxy-2,4,7-trimethoxyphenanthrene (batatasin I) from the dichloromethane (CH2Cl2) fraction of this plant. Batatasin I inhibited both the generation of prostaglandin D2 (PGD2), leukotriene C4 (LTC4), and degranulation reaction in mouse bone marrow-derived mast cells (BMMCs). This compound inhibited cyclooxygenase-2 (COX-2) dependent PGD2 generation in a dose dependent manner, with IC50 values of 1.78 μM. Western blotting probed with specific anti-COX-2 antibodies showed that the decrease in the quantity of the PGD2 generation was accompanied by a decrease in the COX-2 protein level. In addition, this compound also inhibited the production of 5-lipoxygenase (5-LOX) dependent LTC4 in a dose dependent manner (IC50 1.56 μM). Batatasin I also inhibited the mast cell degranulation reaction (IC50 6.7 μM) in BMMCs. This result indicates that batatasin I could be developed as an anti-inflammatory agent through further investigation.

Key words Dioscorea batatas; batatasin I; prostaglandin D2; leukotriene C4; bone marrow-derived mast cell; inflammation

Mast cells (MCs) are involved in the pathophysiology of inflammation and immediate-type allergic reactions. MCs are activated by allergen-specific immunoglobulin E (IgE) binding, with its high-affinity IgE receptor (FceRI) or Stem Cell Factor (SCF, also known as c kit-ligand, KL) to c-Kit, MCs release various preformed chemical mediators and newly synthesized inflammatory mediators. During the early phase of a reaction, MCs release histamine, tryptase, prostaglandin D2 (PGD2), leukotriene C4 (LTC4), platelet-activating factor, chemokine, tumor necrosis factor and various other interleukins.1—3) The enzymes responsible for PG synthesis, cyclooxygenases (COXs), are now known to exist as two isoforms, COX-1 and COX-2.4,4) COX-1 is constitutively expressed in majority of the cells and tissues and is the major isoenzyme seen in gastrointestinal tissue, whereas COX-2 is an inducible isozyme, the expression of which is elevated in response to inflammatory cytokines and endotoxin.5,6) The discovery of COX-2 led to the new concept that selective inhibition of COX-2 activity explains both the therapeutic benefits and the side effects associated with the use of non-steroidal anti-inflammatory drugs (NSAIDs), whereas inhibition of COX-1 is responsible for their gastric and renal side effects as well as for their anti-thrombogenic activity. However, the role of COX-1 in inflammation7 and sudden cardiac arrest and other dangerous side effects associated with selective COX-2 inhibitors cannot be excluded.8,9) LTC4, another class of inflammatory lipid mediators, are synthesized from arachidonic acid by the action of 5-lipoxygenase (5-LOX) in mast cells, eosinophils, and monocytes.10) The development of dual inhibitors that can simultaneously inhibit COX-2 and 5-LOX might synergistically inhibit inflammation and reduce the undesirable side effects that are commonly associated with non steroidal anti-inflammatory drugs (NSAIDs).11)

The rhizome of Dioscorea (D.) batatas is widely distributed in East Asia, including Korea, China, Japan and Taiwan. Plants of the genus D. batatas have long been used in oriental folk medicine, and D. batatas has been cultivated as a health food in Korea. Phytochemical constituents of D. batatas such as polysaccharide,12) storage protein,12,13) mannose-binding lectin,14) steroidal saponin,15,16) and phenanthrene derivatives17—19) Different scientists have reported different biological activities of D. batatas such as anti-oxidative,21,22) cholesterol-lowering,23) anti-cholinesterase activity,24) anti-inflammatory activity,25) growth hormone-releasing activity,25) antifungal activity of phenanthrene compounds17—19) and immune cell-stimulating activity.26)

Previously, our group reported anti-inflammatory activity of D. batatas in LPS stimulated RAW264.7 cells.27) In the course of isolating the active compound(s) from this plant 6-hydroxy-2,4,7-trimethoxyphenanthrene (batatasin I) was isolated from the dichloromethane (CH2Cl2) fraction of the tuberous roots of this plant and its anti-inflammatory activity was evaluated. Even though, the antifungal activity of batatasin I had been reported elsewhere,18,19) the exact mechanisms of the anti-inflammatory effects of batatasin I have not been reported till date. Therefore, for the first time, we have investigated anti-inflammatory activity of batatasin I on the production of eicosanoid generation as well as degranulation in SCF-treated BMMCs.

MATERIALS AND METHODS

Plant Material The dried tuberous roots (9.5 kg) were extracted with methanol (MeOH) under reflux 3 times and the filtrate was concentrated under reduced pressure to give a MeOH extract (631 g). The MeOH extract was suspended in

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water and partitioned with CH₂Cl₂. A part of the CH₂Cl₂ extract was separated by silica gel (Merck 7734) column chromatography with n-hexane : ethylacetate (10 : 0—3 : 7) giving D₁—D₆ fractions. 6-Hydroxy-2,4,7-trimethoxyphenanthrene was obtained from fraction D₆. Its chemical structure was established as batatasin I by comparison of ¹H- and ¹³C-NMR data with those reported previously.¹⁹ Batatasin I used in this study showed a single spot on TLC and the purity of this compound is above 99.5% based on HPLC analysis. Batatasin I was prepared by dissolving in dimethyl sulfoxide (DMSO) and final concentrations of DMSO were adjusted to 0.1% (v/v) in culture media. Control with DMSO alone was run in all cases.

**Preparation and Activation of BMMCs** BMMCs isolated from male Balb/cJ mice (Sam Taco, Inc., Seoul) were cultured in 20% PWM-SCM (pokeweed mitogen-spleen cell conditioned medium) as a source of interleukin-3 (IL-3). After 3 weeks, >98% of the cells was found to be BMMCs checked by the previously described procedure.⁵

**Determination of Prostaglandin D₂ (PGD₂)** For measuring inhibitory activity on COX-2 by MeOH, CH₂Cl₂ fraction, batatasin I and licofelone (Toronto Research Chemicals Inc., North York, ON, Canada). BMMCs suspended at a cell density of 5 x 10⁶ cells/ml in enriched medium were preincubated with aspirin (10 µg/ml) for 2 h in order to irreversibly inactivate preexisting COX-1. After washing, BMMCs were activated with SCF (100 ng/ml, STEMCELL Technologies Inc., Vancouver, BC, Canada), interleukin (IL)-10 (100 U/ml) and lipopolysaccharide (LPS, 100 ng/ml) at 37 °C for 8 h with or without MeOH, CH₂Cl₂ fraction and batatasin I previously dissolved in DMSO. All reactions were stopped by centrifugation at 120 g for 4 °C for 5 min. The supernatant and cell pellet were immediately frozen in liquid N₂ and stored at −80 °C for further analysis. Concentrations of PGD₂ in the supernatant were measured using PGD₂ assay kit (Cayman Chemical, Ann Arbor, MI, U.S.A.) according to manufacturer’s instruction. In this assay conditions, COX-2-dependent phases of PGD₂ generation reached approximately 5.5 ng/10⁶ cells. All data was the arithmetic mean of triplicate determinations.

**Determination of Leukotriene C₄ (LTC₄)** BMMCs suspended in enriched medium at a cell density of 1 x 10⁶ cells/ml were pretreated with MeOH, CH₂Cl₂ fraction, batatasin I and licofelone for 15 min at 37 °C and stimulated with SCF (100 ng/ml). After 20 min of stimulation, the supernatants were isolated for further analysis by EIA. LTC₄ was determined using an enzyme immunoassay kit (Cayman Chemical, Ann Arbor, MI, U.S.A.) according to manufacturer’s instruction. Under the conditions employed, the amount of LTC₄ reached approximately 23 ng/10⁶ cells. All data was the arithmetic mean of triplicate determinations.

**Assay of β-Hexosaminidase Release** β-Hexosaminidase, a marker of mast cell degranulation, was quantified by spectrophotometric analysis of the hydrolysis of substrate (p-nitrophenyl-2-acetamido-2-deoxy-β-D-glucopyranoside, Sigma-Aldrich). Briefly, 25 µl of cell-free supernatants were mixed with 50 µl of substrate in 0.1 M citrate, pH 4.5. After incubation for 1 h at 37 °C, 175 µl of stop solution (0.2 M NaOH—glycine) was added to stop the reaction, and absorbance was measured at 405 nm. Values were expressed as percentage release relative to total β-Hex in the cells. All data was the arithmetic mean of triplicate determinations.

**Preparation of Nuclear and Cytosolic Extracts** Cell pellets were resuspended in wash buffer containing 10 mM N-(2-hydroxyethyl)piperazine-N’-2-ethanesulfonic acid (HEPES) buffer (pH 8.0), 1.5 mM MgCl₂, 10 mM KCl, 1 mM phenylmethylsulfonyl fluoride and 1 mM protease inhibitor cocktail (La Jolla, CA, U.S.A.), and then lysed in lysis buffer A containing wash buffer with 0.1% NP40 by incubating on ice for 10 min. Lysates were then centrifuged at 10000 g for 5 min. Supernatants were removed and stored at −70 °C as a cytosolic fraction. Nuclear pellets were washed with wash buffer and resuspended in buffer B containing 20 mM HEPES (pH 8.0), 25% glycerol, 420 mM NaCl, 1.5 mM MgCl₂, and 0.2 mM ethylenediaminetetraacetic acid (EDTA), and the protease inhibitor cocktail. This suspension was incubated for 30 min at 4 °C followed by centrifugation at 10000 g.

**Sodium Dodecyl Sulfate (SDS) Polyacrylamide Gel Electrophoresis/Immunoblot Analysis** After activation with SCF, IL-10 and LPS, BMMCs (1x10⁶ cells/ml) were washed once with 10 mM phosphate buffer (pH 7.4) containing 150 mM NaCl (phosphate buffer saline (PBS)) and lysed in PBS containing 0.1% SDS and 10 mM β-mercaptoethanol. The lysate (1 x 10⁶ cells equivalent) was applied to 10% SDS-polyacrylamide gels. After running the gel, the protein bands were blotted onto nitrocellulose membranes (Schleicher and Schuell, Dassel, Germany) using a semi-dry blotter (MilliBlot-SDE system, Millipore, Bedford, MA, U.S.A.) according to the manufacturer’s instructions. Membranes were then washed once with 10 mM Tris—buffered saline (TBS, pH 7.2) containing 0.1% tween-20 (TBS-T), and then blocked for 1 h in TBS-T containing 3% skim milk. After washing the membranes with TBS-T, an antibody directed against COX-2, phospho-cPLA₂, phospho-PLCγ1 and 5-LOX were added at a dilution of 1 : 3000—5000 in TBS-T. After incubation for 2 h followed by washing three times, membranes were treated for 1 h with horseradish peroxide-conjugated goat anti-rabbit IgG (Zymed, South San Francisco, CA, U.S.A.) (diluted to 1 : 7000) in TBS-T. The protein bands were visualized using an enhanced chemiluminesence (ECL) system (Amersham Corp., Newark, NJ, U.S.A.).

**Statistical Analysis** All values are represented as arithmetic means ± S.D. One-way analysis of variance (ANOVA) was utilized to determine the statistical significance. Linear regression analysis was conducted to calculate the IC₅₀ value.

**RESULTS AND DISCUSSION**

In our ongoing search for anti-inflammatory compounds from the *D. batatas*, we found that the CH₃Cl₁ fraction (100 µg/ml) strongly inhibited COX-2 dependent PGD₂ (99.6%) in SCF/LPS/IL-10 treated BMMCs, 5-LOX dependent LTC₄ generation (58.9%) and degranulation reaction (85.3%) in SCF-treated BMMCs. In addition, the inhibition of PGD₂ generation was accompanied by the inhibition of COX-2 protein expression (Fig. 1). This result led us to search active compound(s) from the CH₃Cl₁ fraction of the *D. batatas*. Repeated normal-phase silica gel column chromatographies of the CH₃Cl₁ fraction lead to the isolation of 6-hydroxy-2,4,7-trimethoxyphenanthrene (batatasin I) (Fig. 2). We first examined the cytotoxic effect of batatasin I on BMMCs using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
over time. Immediate PGD2 generation, defined as occurring following activation of BMMCs. 32,33) Following activation of BMMCs that PGD2 is the major prostanoid secreted from the activated batatasin I on PGD 2 production was examined to determine the direct effects are mediated by COX-2 protein expression. As shown in Fig. 3B, COX-2 protein was not detected in unstimulated BMMCs, whereas cytokine combination stimulated BMMCs induce the expression of detectable COX-2 protein and COX-2 protein expression was inhibited in a dose-dependent manner by batatasin I. The inhibitory effect of the highest concentration of batatasin I was comparable to that of licofelone (COX-2/5-LOX dual inhibitor) which was used as a positive control. 34) These results clearly demonstrated that decrease in the PGD2 generation was accompanied by a decrease in the COX-2 protein level.

LTs are key lipid mediator that are significantly involved in immunoregulation and elevated in a variety of diseases, including asthma, inflammation and various allergic conditions. When activated by diverse stimuli, the mast cells release arachidonic acid through phospholipase A2 (PLA2), and rapidly generate both PGD2 and the parent molecule of 5-LOX/LTC4 synthase in mast cells, and LTC4 is a family of the cysteinyl leukotrienes (LTC4, LTD4 and LTE4). 6) LTC4 is metabolites of arachidonic acid derived from action of 5-LOX/LTC4 synthase in mast cells, and LTC4 is a family of eicosanoid lipid mediators have been implicated in inflammation, proliferation and allergic conditions like asthma. 35—38) The development of a specific inhibitor for 5-LOX would not only be useful as a tool in investigating the regulatory mechanism behind LT biosynthesis, it can also be utilized for therapeutic purposes. 39) Thus, we investigated the effects of batatasin I on 5-LOX dependent LTC4 generation in BMMC. Figure 4 shows that the BMMC stimulated with SCF for 15 min produced approximately 23 ng/106 cells, and batatasin I strongly inhibited this LTC4 biosynthesis in a dose-dependent manner with an IC50 value of approximately 1.56 μM. The inhibitory effect of 5 μM batatasin I was comparable to that of licofelone (COX-2/5-LOX dual inhibitor) which was used as a positive control. 34) These results clearly demonstrated that decrease in the PGD2 generation was accompanied by a decrease in the COX-2 protein level.
Translocation of 5-LOX and phospho-cPLA2α to the nuclear membrane are required for the generation of LTC₄. To investigate the mechanism of batatasin I on the LTC₄ generation, we examined whether batatasin I affects phosphorylation of cPLA2α and translocation of both phosphorylated cPLA2α and 5-LOX to the nuclear membrane. As shown in Figs. 5A and B, batatasin I dose-dependently inhibited not only phosphorylation of cPLA2α but also translocation of phosphorylated cPLA2α (nuclear-p-cPLA2α) and 5-LOX (nuclear-5-LOX). This result showed that inhibition of LTC₄ generation by batatasin I occurred through the attenuation of translocation of phosphorylated cPLA2α and 5-LOX. COX-2/5-LOX dual inhibitors might synergistically inhibit inflammation and avoid some of the disadvantages of selective COX-2 inhibitors and spares the gastrointestinal mucosa. This result clearly demonstrates that batatasin I has COX-2/5-LOX dual inhibitory activity and might be a good candidate for developing COXs/5-LOX dual inhibitors.

When mast cells are activated by various stimuli, the release of histamine bears a close parallel to that of β-hexosaminidase (β-Hex), which is one of degranulation markers. Therefore, the inhibitory activity of batatasin I on the degranulation reaction in the BMMCs was examined. As shown in Fig. 6, batatasin I inhibited the dose-dependent inhibition of β-Hex release with an IC₅₀ value of 2.7 μM. Since intracellular calcium mobilization was a necessary signal for degranulation of mast cells, we examined the effect of batatasin I on the phosphorylation of phospholipase Cγ1 (PLCγ1). PLCγ1 phosphorylation activates generation of inositol-3 phosphate, which triggers subsequent activation of calcium signals. As demonstrated in Fig. 6, batatasin I significantly inhibited the tyrosine phosphorylation of PLCγ1 in a dose-dependent manner.

Previously other groups reported that D. batatas induced occupational asthma and rhinitis caused, as confirmed by specific inhalation challenge tests. However, the results of this study strongly suggest that batatasin I showed anti-inflammatory effects via the inhibition of both eicosanoid generation and degranulation reaction in activated mast cells. SCF has been shown to induce histamine release of mature connective tissue-type mast cells as well as to induce direct mast cell chemotaxis. In addition, SCF induce airway hyperreactivity in allergic and normal mice. Further, SCF
is an important role in the pathophysiology of asthma and inhibition of SCF attenuates chronic allergen-induced asthma.\(^{37,48}\) Taken together, the present study suggests batatasin I represents a potential therapeutic approach in the treatment of an airway allergic-inflammatory diseases.

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**REFERENCES**