Lansiumamide B and SB-204900 isolated from Clausena lansium inhibit histamine and TNF-α release from RBL-2H3 cells. Inflamm Res

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ORIGINAL RESEARCH PAPER

Lansiumamide B and SB-204900 isolated from *Clausena lansium* inhibit histamine and TNF-α release from RBL-2H3 cells

Takuya Matsui · Chihiro Ito · Hiroshi Furukawa · Tadashi Okada · Masataka Itoigawa

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Abstract
Aims and objective Mast cells play a central role in allergic and chronic inflammation. Extracts from *Clausena lansium* (Lour.) Skeels (Rutaceae) possess many pharmacological effects including anti-inflammatory, anti-oxidant, anti-cancer, and anti-trichomonal activities. In addition, the leaves and fruit are used in Chinese folk medicine. We have isolated and identified four known cinnamamides from this plant: lansiumamide C, lansamide I, lansiumamide B, and SB-204900. However, the biological activities of these compounds are not yet understood. The purpose of this paper is to clarify the pharmacological effects of these compounds on mast cells.
Methods We measured inflammatory molecules in A23187-stimulated rat basophilic leukemia cells (RBL-2H3) treated with these compounds using HPLC, ELISA, and immunoblotting methods. In addition, some signaling molecules were investigated by immunoblotting.
Results Lansamide I, lansiumamide B, and SB-204900 significantly decreased histamine release. Furthermore, lansiumamide B- and SB-204900-treated cells also reduced the protein and/or mRNA levels of TNF-α. SB-204900 markedly suppressed the phosphorylation of p38 MAPK.
Conclusion Our findings suggest that lansiumamide B and SB-204900 attenuate mast-cell-induced inflammation.

Keywords *Clausena lansium* · SB-204900 · Mast cell · Histamine · Pro-inflammatory cytokine

Introduction

Mast cells are ubiquitous in a diverse range of tissues and possess many chemical mediators such as histamine and pro-inflammatory cytokines [1]. These cells are also known to play a crucial role in the development of inflammatory diseases such as asthma, atopic dermatitis [2], and rheumatic synovitis [3]. Many reports indicate that mast cells play a pivotal role in the development of inflammation and the recruitment of inflammatory cells, vascular hyperpermeability, and the contraction of smooth airway muscles through diverse chemical mediators.

In the acute phase of intermediate hypersensitivity, a degranulation mechanism from activated mast cells is required for incremental increases in intracelluar Ca^{2+} concentration and activation of protein kinase C (PKC). The activation of downstream signaling molecules gives rise to pro-inflammatory cytokine production leading to the late phase reactions [4, 5]. However, the signaling pathways of degranulation and cytokine production are complicated and not fully understood.

Many natural products such as flavonoids and coumarin are known to possess anti-allergic, anti-inflammatory, and anti-cancer effects [6–8]. We have previously reported that polymethoxyflavonoids, phytoquinoids, and phenylpropanoids isolated from *Citrus* and *Illicium* plants possess

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T. Matsui · C. Ito · H. Furukawa
Faculty of Pharmacy, Meijo University, Tempaku-ku, Nagoya, Aichi, Japan

T. Matsui (✉) · T. Okada
Department of Physiology, Aichi Medical University, Yazako-karimata 1-1, Nagakute, Aichi, Japan
e-mail: tmatsui@aichi-med-u.ac.jp

M. Itoigawa
Faculty of Human Wellness, Tokai Gakuen University, Tempaku, Nagoya, Aichi, Japan

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anti-allergic activity [9, 10]. *Clausena lansium* (Lour.) Skeels belongs to the family Rutaceae and its extracts reveal antioxidant and anti-cancer activities [11]. Imperatorin, 3-formylcarbazole, clausenamide, and mafaicheenamine E have been isolated and identified from this plant [12–14]. These compounds and methanol extracts are involved in various biological activities including nootropic, antitrichomonal, anti-diabetic, and anti-cancer activities [13–15]. We have also explored the isolation and identification of four cinnamamide compounds from the same plant [16]. These four cinnamamides are not novel compounds and have already been reported by Milner et al. [17] and Lin et al. [18]. Although some of the biological effects of the extract and some of the compounds from this plant have been described, few reports have examined the pharmacological effects of the isolated compounds on mast cells. Imperatorin only inhibits caspase-1 activity and interleukin (IL)-1β production in immunoglobulin (Ig) E-stimulated mast cells and shows anti-allergic effects [19]. From our isolated compounds, we selected four cinnamamides that have similar structures to tranilast, which is a known anti-inflammatory drug. Until now, only the cytotoxic activities of these four cinnamamides have been demonstrated on a few human cancer cell lines [14] and the other biological activities are largely undefined. Therefore, we investigated whether the four cinnamamides obtained might express biological activities on mast cells.

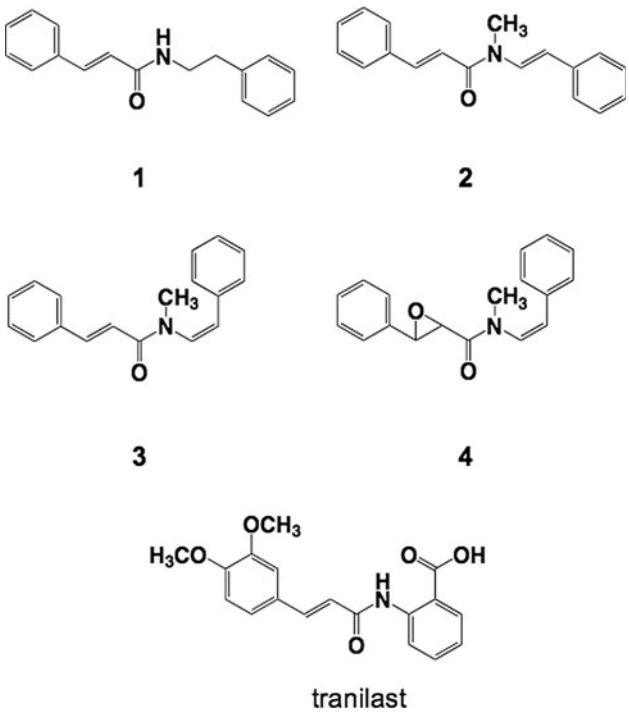


Fig. 1 Structures of compounds isolated from *Clausena lansium* (Lour.) Skeels. **1** lansiumamide C, **2** lansamide I, **3** lansiumamide B, **4** SB-204900. Tranilast is known to be an anti-inflammatory drug and its structure resembles the test compounds

Materials and methods

Plant materials

Fresh leaves of *Clausena lansium* were collected from the Nagoya City Togokusan Fruit Park in Aichi. A voucher specimen was deposited in the Faculty of Pharmacy of Meijo University. Lansiumamide C, lansamide I, lansiumamide B, and SB-204900 were isolated from *Clausena lansium* (Lour.) Skeels (Fig. 1). The purity and structures of the test compounds were confirmed by melting point and IR, UV, MS and ^{1}H-NMR spectra. Samples were dissolved in dimethyl sulfoxide (DMSO) and added to culture medium to give a final DMSO concentration of 0.1 % v/v, at which DMSO has no significant effect on the growth of the cell line tested (data not shown).

Materials

Minimum Eagle's medium (MEM), CelLytic M Cell Lysis Reagent, and anti-β-actin antibody were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). A23187 was obtained from Calbiochem (San Diego, CA, USA). Mouse COX-2 antibody was purchased from Cayman Chemical Co. (Ann Arbor, MI, USA). Antibodies to p38, phospho-p38 MAPK, p65, phospho-p65 NFκB, IκB, phospho-IκB, and PKCs were purchased from Cell Signaling Technology (Beverly, MA, USA). Western blotting detection reagents (ECL plus) were purchased from Amersham Biosciences.

Cell culture and cell viability

RBL-2H3 cells obtained from Tohoku University were grown in MEM supplemented with 10 % heat-inactivated FCS, penicillin at 100 units/mL, and streptomycin at 100 μg/mL under 5 % CO_2 at 37 °C. Cell viability was measured by MTT assay.

Histamine release assay

RBL-2H3 cells (1×10^5 cells/well) were cultured for 30 min in HEPES–Tyrode buffer containing the test compounds at defined concentrations. Cells were then stimulated with 2 μM A23187 for 30 min. Histamine contents were determined by HPLC coupled with post-column derivatization fluorometry, as described previously [10].

Cell stimulation

RBL-2H3 cells (1×10^6 cells/well) were preincubated with test compounds at the defined concentrations.

Pretreated cells were stimulated with A23187 (2 μM) and then cultured at 37 °C for 3 h (for mRNA measurement) and 8 h (for protein measurement). For signaling analysis, cells were cultured with each test compound at concentrations of 5, 25, and 50 μM at 37 °C for 30 min. Cells were then stimulated with A23187 (2 μM) at 37 °C for 15 min.

TNF-α and IL-6 release assay

Tumor necrosis factor (TNF)-α and IL-6 levels in conditioned medium were determined by using Rat TNF-α and IL-6 ELISA Kits (Quantikine, R&D Systems, Minneapolis, MN, USA), according to the manufacturer's protocols.

Immunoblotting analysis

The expression of cyclooxygenase (COX)-2 and the phosphorylated and non-phosphorylated forms of the signaling molecules (p65 NFκB, IκBα, p38 MAPK, PKC (pan) βII, PKCδ and θ) were determined by immunoblotting analysis. Briefly, the cells were lysed after stimulation in CelLytic M Cell Lysis Reagent (Sigma-Aldrich Co.) according to the manufacturer's protocol and mixed with SDS–PAGE sample buffer. The samples were then boiled for 10 min. Samples were subjected to SDS–PAGE under reducing conditions and transferred to polyvinylidene difluoride (PVDF) membranes (Immobilon-P, Millipore Co., MA, USA). PVDF membranes were incubated with 5 % skim milk or BSA for 1 h at room temperature. The membranes were incubated with the antibodies separately overnight at 4 °C and then with HRP-conjugated species-specific anti-mouse and anti-rat IgG antibodies (Cell Signaling Technology) for 1 h at room temperature. The blots were probed with the ECL Plus Western Blot Detection System, according to the manufacturer's instructions.

The densities of the bands were measured by using ImageJ analysis software, and corrected to the relative density of non-stimulated cells.

Quantitation of TNF-α, IL-6 and COX-2 mRNAs

Total RNA was isolated from RBL-2H3 cells using an RNeasy Mini Kit (Qiagen K.K., Tokyo, Japan) and then reverse-transcribed to prepare cDNA using a High-capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's instructions. Quantitative real-time PCR was performed with 20 ng of cDNA with SYBR Premix EX Taq (Takara Co., Otsu, Japan) using an ABI-9500 (Applied Biosystems). PCR conditions were as follows: 10 s at 95 °C, 40 cycles at 95 °C for 5 s, and then 60 °C for 34 s, followed by standard melting curve analysis. The number of copies in each real-time PCR reaction was normalized against a housekeeping gene (*GAPDH*). TNF-α and COX-2 mRNAs coding sequences derived from GenBank were used in the Primer 3 program (http://www.frodo.wi.mit.edu/cgi-bin/primer3/primer3.cgi) to design primers for the real-time PCR assays. The following primers were used.

TNF-α: forward primer 5′-GCATGATCCGAGATGTG GAA-3′, reverse primer 5′-ACGAGCGGGAATGAGAA GAG-3′; IL-6: forward primer 5′- GTGGCTAAGGACCA AGACCA-3′, reverse primer 5′- GGTTTGCCGAGTA GACCTCA-3′; COX-2: forward primer 5′-CCCATGTCA AAACCGTGGTG-3′, reverse primer 5′-CTGTGTTTGGG GTGGGCTTC-3′; and GAPDH: forward primer 5′-TGC CACTCAGAAGACTGTGG-3′, reverse primer 5′-GGAT GCAGGGATGATGTTCT-3′.

Statistical analysis

Statistical analysis was performed by one-way analysis of variance followed by Tukey's test to compare RBL-2H3 cells in response to A23187 (positive control) using the Statistical Package for the Social Sciences (SPSS) software (version16.00; SPSS Inc. Chicago, IL). Results were expressed as mean ± SEM. P values <0.05 were considered statistically significant.

Results

Suppressive effect of four cinnamamides on histamine release

We investigated whether the four cinnamamides isolated suppressed histamine release from A23187-stimulated RBL-2H3 cells. Lansamide I, lansiumamide B, and SB-204900 significantly suppressed histamine release at concentrations of 50 μM, while lansiumamide C and tranilast did not (Fig. 2b). None of the compounds had cytotoxic effects at this concentration (Fig. 2a). The histamine release of lansiumamide B- and SB-204900-treated cells decreased by over 30 % and the activities of these two compounds showed substantially higher potency compared with lansamide I. Furthermore, these compounds showed a dose-dependent inhibitory effect at concentrations of 5, 25, and 50 μM (Fig. 2c). Comparing lansiumamide B and SB-204900, the latter possessed a slightly more potent inhibitory effect (at 50 μM, lansiumamide B 71.2 ± 2.1 % vs. SB-204900 59.1 ± 1.6 %). These findings showed that lansiumamide B and SB-204900 possess potent suppressive effects on histamine release from A23187-stimulated RBL-2H3 cells. Based on these results, we also investigated the pharmacological effects of lansiumamide B and SB-204900.

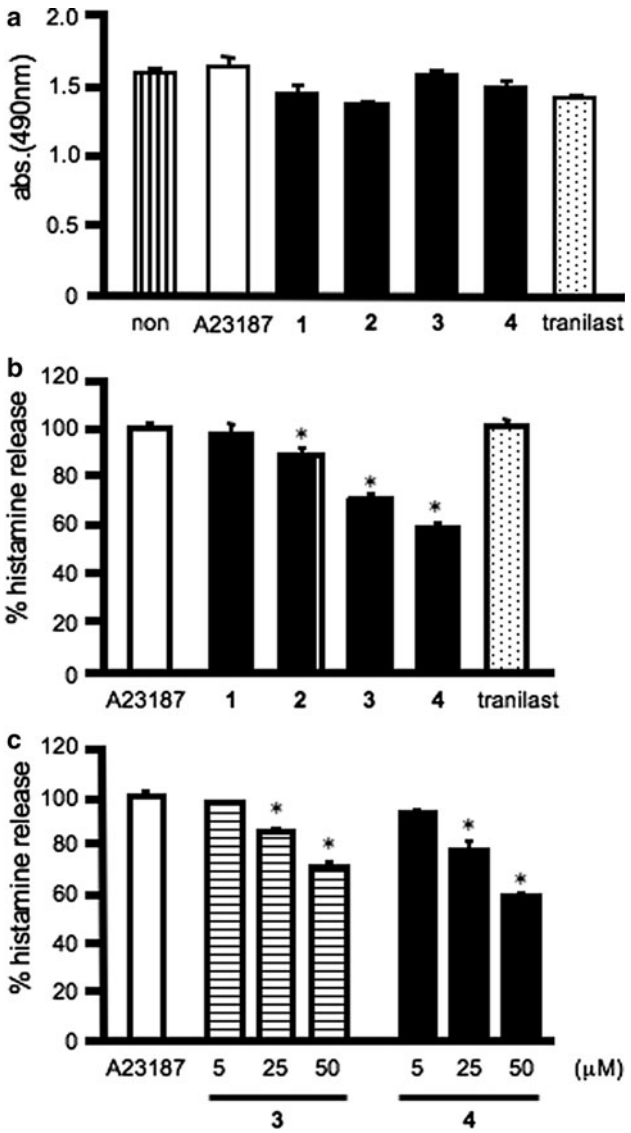


Fig. 2 The inhibitory effect of cinnamamide derivatives on histamine release from RBL-2H3 cells. RBL-2H3 cells were pretreated with each of four cinnamamides for 30 min. Cells were stimulated with A23187 (2 μM) and cultured for 30 min. **a** The four tested cinnamamides (**1**–**4**) and tranilast at 50 μM did not affect cell viability by MTT assay. **b** Comparison of the four cinnamamides (**1**–**4**) and tranilast at 50 μM in the inhibition of histamine release in response to A23187 (2 μM). *1* lansiumamide C, *2* lansamide I, *3* lansiumamide B, *4* SB-204900. **c** Dose–response analysis of lansiumamide B (**3**) and SB-204900 (**4**) at 5, 25, and 50 μM. Values represent mean ± SEM of at least three experiments. $*p < 0.05$ versus A23187 stimulation alone (*white bar*)

Inhibitory effects of lansiumamide B and SB-204900 on mRNA expression of COX-2, TNF-α, and IL-6

To explore the pharmacological effects of lansiumamide B and SB-204900 on other inflammatory molecules, we quantified the mRNA and protein levels of COX-2, TNF-α, and IL-6 by real-time PCR, immunoblotting, and ELISA methods. At 25 and 50 μM, SB-204900 significantly and dose-dependently reduced the mRNA levels of COX-2 and TNF-α in A23187-stimulated cells (Fig. 3a, b). In addition, SB-204900 at 50 μM resulted in a 53.5 % reduction of TNF-α mRNA levels compared with the positive control (A23187-stimulated cells). While IL-6 mRNA levels were reduced in a dose-dependent manner by SB-204900, the reduction was not statistically significant (Fig. 3c). Furthermore, although lansiumamide B subtly reduced COX-2, TNF-α, and IL-6 mRNA levels compared with the positive controls (Fig. 3), the results were not statistically significant.

Inhibitory effects of lansiumamide B and SB-204900 on COX-2 protein expression and TNF-α and IL-6 secretion

We analyzed the expression of each protein to investigate whether COX-2, TNF-α, and IL-6 mRNA expression reflected the level of each protein. Lansiumamide B and SB-204900 appeared to marginally inhibit the expression of COX-2 protein, but this was not statistically significant (Fig. 4a). However, the levels of TNF-α in conditioned medium from SB-204900-treated cells decreased markedly in a dose-dependent manner compared with the positive control (Fig. 4b). Similarly, levels were 47.5 % lower than that of the positive control in conditioned medium with 50 μM lansiumamide B-treated cells. At the lower concentration, SB-204900 suppressed TNF-α secretion, unlike lansiumamide B, and thus the inhibitory effect of SB-204900 appeared to be stronger than that of lansiumamide B. IL-6 protein levels in conditioned medium were not appreciably detected in any A23187-stimulated cells treated with or without the respective compounds, although an increase in mRNA expression was detected in A23187-stimulated cells (data not shown). Thus, SB-204900 seemed to possess inhibitory activities towards TNF-α secretion/production and COX-2 production in A23187-stimulated RBL-2H3 cells.

Effect of lansiumamide B and SB-204900 on the phosphorylation of signaling molecules

As previously mentioned, lansiumamide B and SB-204900 displayed suppressive effects on histamine release and on TNF-α secretion/production from A23187-stimulated cells. We analyzed the expression of some phosphorylated signaling molecules, partly to clarify the mechanisms of their pharmacological activities. It is known that PKCs may be either Ca^{2+}-dependent or independent. Firstly, we investigated the phosphorylation of PKCs that are essential for the process of degranulation. The anti-phosphorylated-PKC (pan) βII antibody, which is recognized by various PKCs, was detected in a single band at about 78 kDa (Fig. 5a).

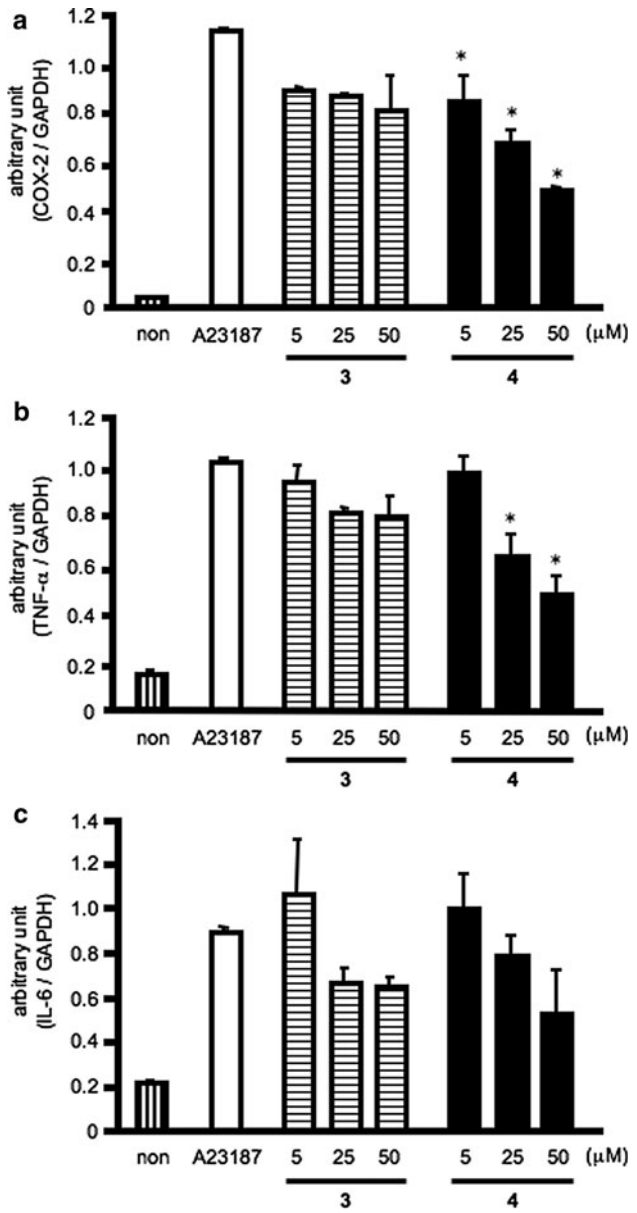


Fig. 3 Effects of lansiumamide B and SB-204900 on the expression of mRNA pro-inflammatory molecules. RBL-2H3 cells were pretreated with lansiumamide B (**3**) and SB-204900 (**4**) at 5, 25, and 50 μM for 30 min. Cells were stimulated with A23187 (2 μM) and cultured for 3 h. The mRNA levels of COX-2 (**a**), TNF-α (**b**) and IL-6 (**c**) were measured by real-time PCR. Values represent mean ± SEM of at least three experiments. *$p < 0.05$ versus A23187 stimulation alone (*white bar*)

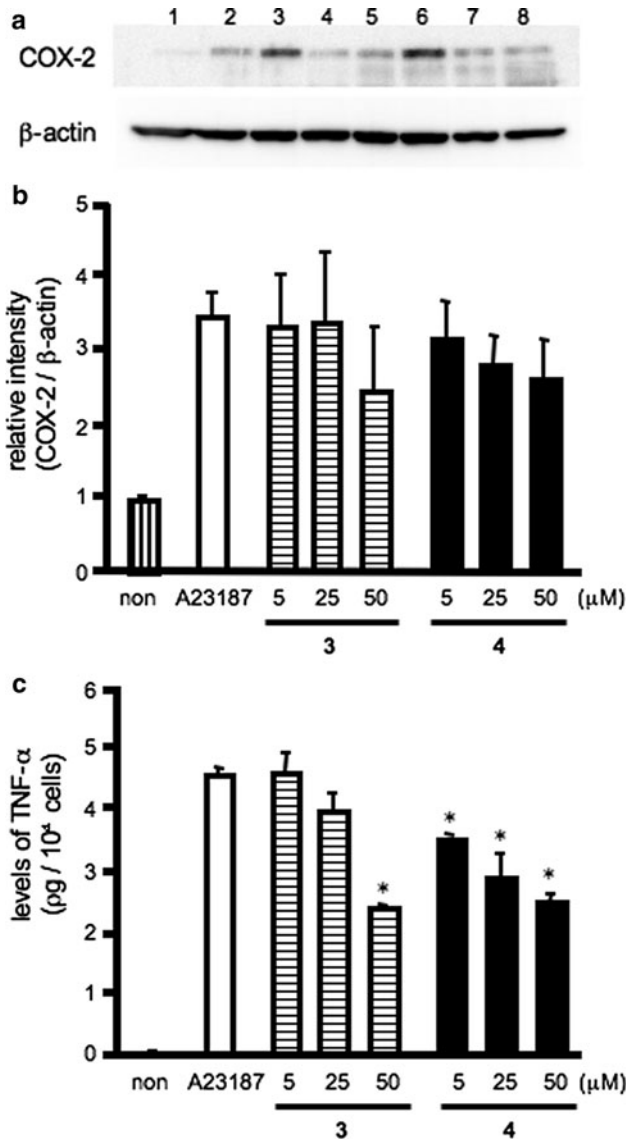
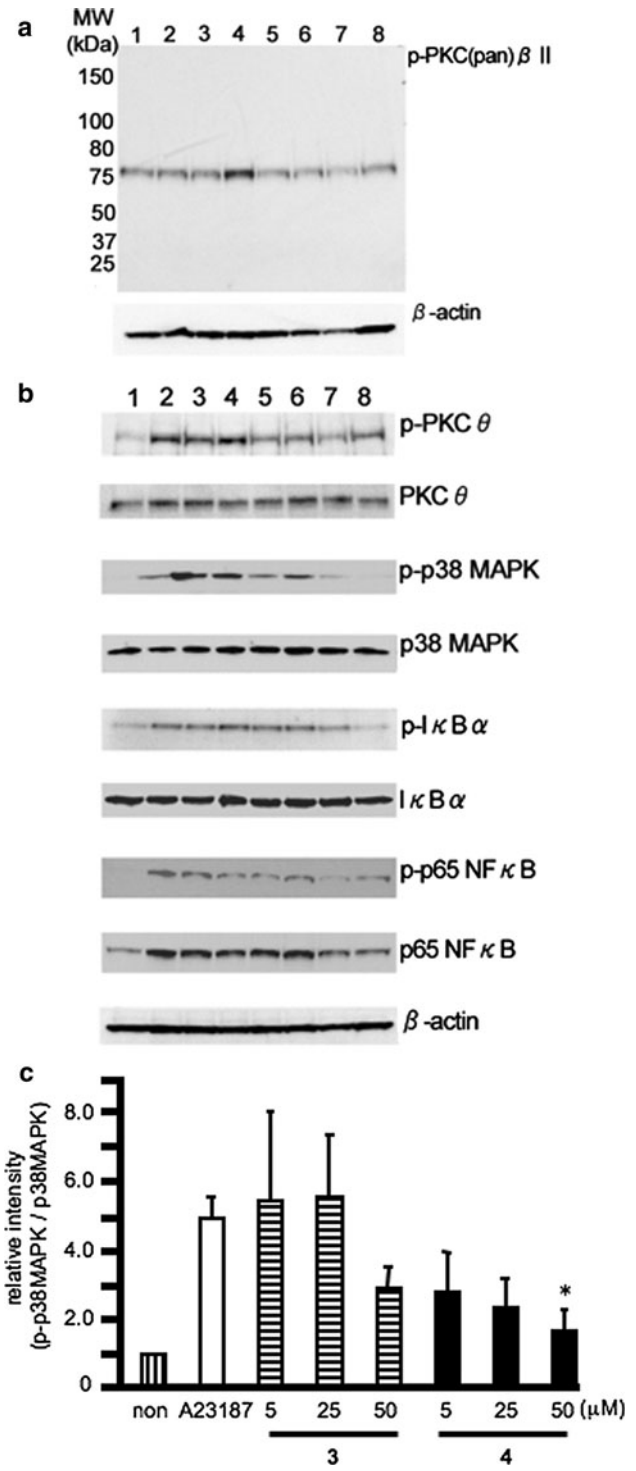


Fig. 4 Effects of lansiumamide B and SB-204900 on protein levels of COX-2 and TNF-α. RBL-2H3 cells were pretreated with lansiumamide B (**3**) and SB-204900 (**4**) at 5, 25, and 50 μM for 30 min. Cells were stimulated with A23187 (2 μM) and cultured for 8 h. COX-2 (**a**, **b**) in cell lysate and TNF-α (**c**) in conditioned medium were measured by immunoblotting and ELISA. Lanes in the immunoblot images of COX-2 (**a**) show untreated cells (non-vehicle dimethyl sulfoxide, *lane 1*), A23187-stimulated cells (*lane 2*), lansiumamide B (**3**)-treated cells (5, 25, and 50 μM) + A23187 (*lanes 3*, *4*, and *5*) and cells treated with SB-204900 (**4**) (5, 25, and 50 μM) + A23187 (*lanes 6*, *7*, and *8*). The band densities of COX-2 protein were measured by ImageJ software. The relative intensities (**b**) were corrected by the band densities of β-actin. Values represent mean ± SEM of at least three experiments. *$p < 0.05$ versus A23187 stimulation alone (*white bar*)

We predicted that this single band was PKCδ or θ. Furthermore, we attempted to confirm the band by using anti-phosphorylated-PKCδ and θ antibodies. The phosphorylated form of PKCθ was detected but not that of PKCδ (Fig. 5a, b). Although the relative intensities of phosphorylated PKCθ on lansiumamide B at 5 μM and SB-204900 at 5 and 25 μM were slightly reduced compared with the positive controls, but these changes were not statistically significant (Fig. 5b). Subsequently, we determined the phosphorylation of p38 MAPK, IκBα, and p65 NFκB, which are known to participate in pro-inflammatory cytokine production (Fig. 5b, c). The relative intensity of phospho-p38 MAPK in cells treated with SB-204900 at 5,



◀ **Fig. 5** Effects of lansiumamide B and SB-204900 on the expression of signaling molecules. RBL-2H3 cells were pretreated with each of lansiumamide B (**3**) and SB-204900 (**4**) at 5, 25, and 50 μM for 30 min. Cells were stimulated with A23187 (2 μM) and cultured for 15 min. Cells were then subjected to immunoblotting analysis by using anti-phosphorylated-PKC (pan) βII (**a**), anti-phosphorylated (*p*-) and non-phosphorylated antibodies of PKCθ, p38 MAPK, IκB, and p65 NFκB (**b**). Lanes in immunoblotting images (**a, b**) show untreated cells (non-vehicle dimethyl sulfoxide, *lane 1*), A23187-stimulated cells (*lane 2*), lansiumamide B (**3**)-treated cells (5, 25, and 50 μM) + A23187 (*lanes 3*, *4*, and *5*) and cells treated with SB-204900 (**4**) (5, 25 and 50 μM) + A23187 (*lanes 6*, *7*, and *8*). The relative intensity of phosphorylated p38 MAPK (**c**) was corrected by non-phosphorylated forms. Values represent means ± SEM of at least three experiments. $*p < 0.05$ versus A23187 stimulation alone (*white bar*)

25, and 50 μM was reduced to 43.6, 52.2, and 66.1 %, respectively, in a dose-dependent manner compared with the positive controls (Fig. 5c). At 50 μM, SB-204900 significantly suppressed the phosphorylation of p38 MAPK, while lansiumamide B at 50 μM resulted in a 41.8 % reduction. Lansiumamide B and SB-204900 had virtually no effect on the phosphorylation of p65 NFκB and IκBα, even though the relative intensity of their signaling molecules in SB-204900-treated cells marginally decreased compared with the positive controls. Thus, SB-204900 showed, at least in part, inhibitory effects on the phosphorylation of p38 MAPK.

Discussion

Many natural products such as flavonoids and curcumin are reported to show anti-allergic and anti-inflammatory activities on mast cells in in vivo and in vitro studies [7, 20–22]. We have previously shown that phytoquinoids, phenylpropanoids, and polymethoxyflavonoids inhibit chemical mediators from A23187-stimulated RBL-2H3 cells [9, 10]. Some components such as (−)-clausenamide, imperatorin, and 3-formylcarbazole, isolated and purified from *Clausena lansium*, have already been the subjects of biological activity assays and show anti-cancer and anti-trichomonal activities [12, 13, 15]. However, the biological activities of the four cinnamamides in the present study which were isolated and identified from the plant are poorly understood.

Lansiumamide C did not inhibit histamine release from A23187-stimulated RBL-2H3 cells, similarly to tranilast, while lansamide I showed modest inhibition. In addition, lansiumamide B and SB-204900 significantly and dose-dependently attenuated the release of histamine from A23187-stimulated cells. These findings suggest that lansamide I, lansiumamide B, and SB-204900 have an ability to suppress histamine release from activated RBL-2H3 cells. However, this study has not clearly defined a correlation between the inhibitory effects and the structural features of the compounds. Lansamide I, lansiumamide B, and SB-204900 each possess the common structure of *N*-methylamide. Moreover, lansiumamide B and SB-204900, which showed potent inhibitory effects on histamine release, have *cis*-olefinic protons unlike lansiumamide C, lansamide I, and tranilast. Based on these findings,

we predict that the inhibitory effects require *N*-methyl-amide, and that *cis*-olefinic protons enhance this action.

We focused on lansiumamide B and SB-204900 in terms of their inhibitory effects on histamine release. Furthermore, we investigated whether the compounds have inhibitory effects on COX-2 and the pro-inflammatory cytokines that are commonly known to be expressed from mast cells after the acute phase in intermediate allergy.

Mast cells, as well as other inflammatory cells such as macrophages, also produce prostaglandin D_2 (PGD_2) through the induction of COX-2 [1]. Lansiumamide B and SB-204900 significantly decreased mRNA levels, while protein expression was marginally attenuated. Although the reduction of COX-2 mRNA in lansiumamide B- and SB-204900-treated cells did not fully reflect the expression of the proteins, it is possible that these compounds inhibit COX-2 production at the transcription stage.

In the pro-inflammatory cytokine assay, TNF-α levels in conditioned medium with lansiumamide B- or SB-204900-treated cells showed a dose-dependent decrease compared with RBL-2H3 cells stimulated with A23187 alone. SB-204900 also reduced the expression of TNF-α mRNA, while lansiumamide B showed little effect. It is well known that both pre-stored and de novo synthesized TNF-α exists in mast cells [23]. To date, it is known that the activation of mast cells results in marked degranulation, the secretion of granule contents either by rapid exocytosis associated with enhanced intracellular Ca^{2+} levels, or by piecemeal degranulation, which is a lesser known mechanism [3, 24]. Our preliminary experiments showed that the alteration in TNF-α mRNA expression of cells treated with 50 μM lansiumamide B cultured for 5 h after stimulation was the same as that of cells cultured for 3 h (data not shown). Lansiumamide B seemed to have an effect on TNF-α secretion but not on its production. Thus, we considered the possibility that lansiumamide B and SB-204900 may affect at least two inhibitory sites, which bring about a reduction in TNF-α levels. Lansiumamide B and SB-204900 may inhibit the degranulation pathways or, alternatively, SB-204900 may inhibit the transcription stage. Unfortunately, the inhibitory effects of these compounds on IL-6 secretion from A23187-stimulated RBL-2H3 cells are not clear since we could not detect IL-6 in the conditioned medium. However, the results of the IL-6 mRNA assay leave open the possibility that SB-204900 has an inhibitory effect on IL-6 production in A23187-stimulated RBL-2H3 cells. Although the inhibitory effects of lansiumamide B and SB-204900 on other inflammatory cytokines is not clear, our results raise the possibility that the compounds may suppress TNF-α secretion and/or production from mast cells. Furthermore, the inhibitory effects of SB-204900 on histamine and TNF-α were marginally stronger than that of lansiumamide B. It is likely that the presence of the trans-epoxide in SB-204900 contributed to this difference in pharmacological activity. Maneerat et al. [14] recently reported that lansiumamide B and SB-204900 are weakly cytotoxic in some human cancer cell lines. However, until now the pharmacological effects of the compounds have not been clarified. RBL-2H3 cells possess twin properties of mast cells and basophils [25, 26], and thus we conducted a preliminary examination of the inhibitory effects of these compounds on TNF-α secretion from another mast cell line. Lansiumamide B and SB-204900 reduced TNF-α levels in a dose-dependent manner in conditioned medium from A23187-stimulated P-815 and HMC-1 cells, which are known mouse and human mast cell lines, respectively (data not shown). Although we could not exclude that these compounds have inhibitory effects on basophils, our findings demonstrated the potent inhibitory effects that they have on histamine and TNF-α release from activated mast cells, which adds to their novel pharmacological effects.

Although we consider that these compounds inhibit downstream signaling that involves the release and/or production of chemical mediators following increments in cytoplasmic Ca^{2+} levels, the phosphorylation of few signaling molecules has been analyzed. Ozawa and co-workers reported that RBL-2H3 expressed Ca^{2+}-dependent (α, β) and Ca^{2+}-independent (δ, ε, ζ) isomers of PKC, and PKCβ and δ participated in mast cell degranulation [27–29]. Additionally, some reports have shown that downstream signaling after activation of PKC leads to cytokine production in mast cells [5, 30, 31]. This study could not detect the phosphorylation of PKCδ in positive controls, despite the anti-phosphorylated-PKC (pan) βII antibody detected in a single band at 78 kDa in all cells. While the phosphorylated form of PKCθ was constitutively expressed on non-stimulated cells, its expression was enhanced by A23187. Until now, there has been little information on the role of PKCθ in mast cells; however, Liu et al. [32] reported that it induced IL-3 gene transcription and degranulation in antigen-stimulated RBL-2H3 cells via ERK activation. The inhibitory effects of flavonols such as quercetin and kaempferol on the release of pro-inflammatory cytokines have been shown to be involved in the inhibition of phosphorylated PKCθ in human mast cells [33, 34]. Lansiumamide B and SB-204900 at low concentrations showed marginal effects on PKCθ, while the relative intensity of phosphorylated forms in the treated cells was not statistically significant compared with positive controls. Thus, the mechanism by which these compounds have an effect on the signaling pathways via PKCθ has yet to be determined.

Some reports show that the activation of mast cells initiated by FcεRI or Toll-like receptor leads to the phosphorylation of NFκB and p38 MAPK [4, 30, 35]. The phosphorylated forms are known to enhance the production

of pro-inflammatory cytokines and prostaglandins. We investigated the expression of p38 MAPK, IκBα, and p65 NFκB and the results show that lansiumamide B and SB-204900 significantly inhibited the phosphorylation of p38 MAPK. However, these compounds did not affect the expression of phosphorylated IκBα and p65 NFκB, suggesting that they inhibit the activation of other transcriptional factors.

To date, the p38 MAPK pathway is known to control a variety of biological functions such as the cell cycle, inflammation, and apoptosis [36, 37]. Some reports on the expression of inflammation molecules indicate that the p38 MAPK pathway induces COX-2 in a variety of inflammatory cells including mast cells, macrophages, and NIH-3T3 fibroblasts [38–40]. Gibbs and colleagues reported that the phosphorylation of p38 MAPK is expressed in antigens or A23187-stimulated human basophils and its expression is linked to the secretion of histamine, leukotriene C4, IL-4, and IL-13 [41]. Besides COX-2 expression, another previous report showed that the phosphorylation of p38 MAPK and JNK induced TNF-α production in rat peritoneal mast cells stimulated with substance P [42]. The relationship between the activation of p38 MAPK and the chemical mediators in A23187-stimulated RBL-2H3 cells has still not been fully elucidated. In our preliminary experiments using 10 μM SB202190, a commercial p38 MAPK inhibitor, we found a 32 % reduction in TNF-α levels in conditioned medium and a 26 % reduction in COX-2 protein expression in A23187-stimulated RBL-2H3 cells, but no effects on histamine release. Our preliminary data lends support to some of the above-mentioned reports, which show that the p38 MAPK pathway is concerned in TNF-α secretion and COX-2 protein expression. Although there is no evidence that lansiumamide B and SB-204900 can directly suppress the phosphorylation of p38 MAPK, the present study might indicate that SB-204900 mainly inhibits the p38 MAPK pathway, leading to a reduction in TNF-α and COX-2.

In conclusion, this study is the first report to show that lansiumamide B and SB-204900 possess some novel pharmacological actions, including the inhibition of histamine release, IL-6 and COX-2 production, and TNF-α secretion/production in A23187-stimulated RBL-2H3 cells. These pharmacological actions might be attributable to suppression of the phosphorylation of p38 MAPK. Elucidation of the precise pharmacological mechanisms remains problematic; however, it appears that lansiumamide B and SB-204900 might be useful in the treatment of allergy and/or inflammation. Since one report shows that the bioactivity of natural products in in vitro assays is not necessarily reflected in in vivo assays [43], we would like to examine the in vivo effects of lansiumamide B and SB-204900 in a future study.

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