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ORIGINAL ARTICLE

Maackiain is a novel antiallergic compound that suppresses transcriptional upregulation of the histamine H_1 receptor and interleukin-4 genes

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Abstract

Kujin contains antiallergic compounds that inhibit upregulation of histamine H_1 receptor (H1R) and interleukin (IL)-4 gene expression. However, the underlying mechanism remains unknown. We sought to identify a Kujin-derived antiallergic compound and investigate its mechanism of action. The H1R and IL-4 mRNA levels were determined by real-time quantitative RT-PCR. To investigate the effects of maackiain in vivo, toluene-2,4-diisocyanate (TDI)-sensitized rats were used as a nasal hypersensitivity animal model. We identified (−)-maackiain as the responsible component. Synthetic maackiain showed stereoselectivity for the suppression of IL-4 gene expression but not for H1R gene expression, suggesting distinct target proteins for transcriptional signaling. (−)-Maackiain inhibited of PKCδ translocation to the Golgi and phosphorylation of Tyr^{311} on PKCδ, which led to the suppression of H1R gene transcription. However, (−)-maackiain did not show any antioxidant activity or inhibition of PKCδ enzymatic activity per se. Pretreatment with maackiain alleviated nasal symptoms and suppressed TDI-induced upregulations of H1R and IL-4 gene expressions in TDI-sensitized rats. These data suggest that (−)-maackiain is a novel antiallergic compound that alleviates nasal symptoms in TDI-sensitized allergy model rats through the inhibition of H1R and IL-4 gene expression. The molecular mechanism underlying its suppressive effect for H1R gene expression is mediated by the inhibition of PKCδ activation.

Abbreviations

DNP-BSA, 2,4-dinitrophenylated bovine serum albumin; DPPH, 2,2-diphenyl-1-picrylhydrazyl; ERK, extracellular signal-regulated kinase; GPCR, G protein-coupled receptors; H1R, histamine H_1 receptor; IL, interleukin; MEK, mitogen-activated protein kinase; PARP-1, poly(ADP-ribose) porymerase-1; PKCδ, protein kinase

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C-δ; PMA, phorbol-12-myristate-13-acetate; TDI, toluene-2,4-diisocyanate; T_H1/T_H2, helper T cell type 1/2.

Introduction

Pollinosis, a seasonal allergic rhinitis caused by hypersensitivity to tree or grass pollens, affects more than 36 million people in the U.S. and about 16% of the Japanese population (Nathan et al. 1997; Baba et al. 2009). Antihistamines are a widely employed first-line treatment for nasal symptoms of pollinosis. These drugs were thought to antagonize histamine and prevent it from binding to the histamine H_1 receptor (H1R). However, nowadays, it is considered that antihistamines act as inverse agonists and antagonize histamine by blocking its binding with the H1R and suppress constitutive H1R activity (Simons 2004; Mizuguchi et al. 2012a). Recently, we reported that the H1R gene expression strongly correlated with the severity of allergic symptoms in toluene-2,4-diisocyanate (TDI)-sensitized allergy model rats and patients with pollinosis (Mizuguchi et al. 2008, 2010). Compounds that suppress upregulation of H1R gene expression can alleviate allergy symptoms (Matsushita et al. 2008; Dev et al. 2009; Shahriar et al. 2009; Nurul et al. 2011; Hattori et al. 2013). These findings strongly suggest that H1R signaling is very important for the development of pollinosis and that drugs designed for the suppression of H1R signaling will be effective for allergic diseases. Previously, we reported that histamine and phorbol-12-myristate-13-acetate (PMA) stimulation increased H1R at both mRNA and protein levels by the activation of H1R in HeLa cells endogenously expressing H1R (Das et al. 2007). Histamine- and PMA-induced upregulation of H1R gene expression was suppressed by rottlerin, a protein kinase C-δ (PKCδ)-selective inhibitor, indicating that upregulation of H1R gene expression is PKCδ dependent. Further studies showed that both histamine- and PMA-induced upregulation of H1R gene expression involved common downstream mediators of PKCδ signaling. Recently, we investigated the molecular mechanism of PMA-induced upregulation of H1R gene expression in HeLa cells and demonstrated that the PKCδ/extracellular signal-regulated kinase (ERK)/poly(ADP-ribose) polymerase-1 (PARP-1) signaling pathway was involved in PMA-induced upregulation of HIR gene expression in HeLa cells (Mizuguchi et al. 2011).

Allergic reactions are also characterized by a disruption of the helper T cell type 1/2 (T_H1/T_H2) balance toward a pronounced T_H2 profile (Holgate 1993). T_H2 cytokines, including IL-4 and IL-5, are also upregulated in TDI-sensitized rats and patients with pollinosis (Shahriar et al. 2009; Kitamura et al. 2012). We reported that the expression levels of these cytokines strongly correlated with that of H1R (Mizuguchi et al. 2008; Shahriar et al. 2009), suggesting the existence of cross talk between H1R signaling and T_H2 cytokine signaling. Consistent with this observation, we found that intranasal application of IL-4 and histamine increased H1R and IL-4 gene expression, respectively (Shahriar et al. 2009).

Kujin is the dried root of *Sophorae flavescens* AITON of the Leguminosae family. This Chinese herb has been used extensively in the treatment of allergic diseases and many other pathological conditions for many years in Asian countries. Phytochemical studies have shown that it contains quinolizidine, alkaloids, flavonoids, and triterpenoids (Chen et al. 2004; Li and Wang 2004; Piao et al. 2006; Ling et al. 2007). Kujin and its active components have been reported to possess many biologically relevant properties, exerting anti-inflammatory (Kim et al. 2002), antiasthmatic (Hoang et al. 2007), antitumor (Sun et al. 2007), and antimicrobial (Kuroyanagi et al. 1999) effects. In the previous study, we showed that Kujin extract inhibited upregulation of H1R and IL-4 gene expression in TDI-sensitized rats (Dev et al. 2009). However, so far, little work has been done to justify the usefulness of Kujin extract or to elucidate the mechanism behind its effects.

In the present study, we identified (−)-maackiain as the primary ingredient responsible for the antiallergic action of Kujin. Our data revealed that (−)-maackiain inhibits the activation of PKCδ, a key enzyme in H1R signaling, resulting in the inhibition of PKCδ translocation to the Golgi, thereby causing suppression of H1R gene transcription. Treatment with maackiain alleviated nasal symptoms and suppressed TDI-induced upregulation of H1R and IL-4 gene expression in TDI-sensitized rats. Our data suggest that (−)-maackiain is a novel antiallergic compound that alleviates nasal symptoms in TDI-sensitized allergy model rats through the inhibition of H1R and IL-4 gene expression. The molecular mechanism underlying its suppressive effect for H1R gene expression is mediated by the inhibition of PKCδ activation.

Materials and Methods

Isolation of (−)-maackiain from Kujin extract

Kujin extract was prepared as reported previously (Dev et al. 2009). In briefly, 60 g of Kujin ("Kojima Kujin M," Lot 902607; Kojima Kampo, Osaka, Japan) was added to 1 L of distilled water, boiled for 1.5 h, and filtered twice to remove insoluble materials. The extract was then concentrated and used for this study. The yield of freeze-dried extract was 20% (w/w) with respect to the dried root. To separate the crude extract into acidic, alkaline, and neutral fractions, the pH of the Kujin extracts (30 mg/mL) was first adjusted to three using 1 mol/L HCl, and then extracted three times with ethyl acetate (EtOAc) to recover the acidic fraction. Subsequently, the pH of the water-soluble fraction was adjusted to 10 with 1 mol/L NaOH and was then extracted with EtOAc to recover the alkaline fraction. Finally, pH of the water-soluble fraction was neutralized with 10% (w/v) citric acid and was designated the neutral fraction. After removal of the solvent, each fraction was tested for its ability to suppress the IgE-stimulated upregulation of IL-4 gene expression in RBL-2H3 cells. Activity was found in the acidic fraction. The acidic fraction was subjected to chromatography on a silica gel column (Silica gel 60N; Kanto Kagaku, Tokyo, Japan). Elution of the column with $CHCl_3$:MeOH (9:1) yielded 21 fractions. TLC analysis showed a common spot in two active fractions (fraction #1 and #2). These fractions were combined and rechromatography was performed on a silica gel column eluted with hexane:EtOAc (2:1). Fractions containing the common spot, which was identified using TLC analysis, were combined and their ability to suppress IL-4 gene expression in RBL-2H3 cells was confirmed. After rechromatography on a silica gel column using benzene:EtOAc (20:1), the active fraction was further purified with a Mightysil RP18 GP HPLC column (Kanto Kagaku) using 65% methanol. Finally, the pure compound was obtained by crystallization in methanol, rechromatography on the HPLC column using 55% methanol, and recrystallization in methanol. ^{1}H NMR and ^{13}C NMR spectra of the purified maackiain in $CDCl_3$ were recorded using a Bruker AV400N instrument (Hitachi High-Tech, Tokyo, Japan) with tetramethylsilane as internal standard. MS spectra were obtained with an LCT PREMIER system (Waters/Micromass, Milford, MA). IR spectra were recorded on FT/IR-6000 (JASCO, Tokyo, Japan). Optical rotations of purified and synthesized maackiain were measured using a CD-J600 spectropolarimeter (JASCO).

Synthesis of maackiain

Bis(benzonitrile)palladium(II) dichloride [$PdCl_2(PhCN)_2$] was prepared as reported previously (Doyle et al. 1960). The synthesis of (±)-3-benzylmaackiain was performed as follows. To a solution of 7-benzyloxy-2*H*-1-benzopyran (300.0 mg, 1.26 mmol, 1.0 equiv), 2-bromo-4,5-methylene dioxyphenyl (1.093 g, 5.03 mmol, 3.0 equiv) in anhydrous DMF (5 mL), suspended with potassium acetate (1.300 g, 13.2 mmol, 9.0 equiv) was added $PdCl_2(PhCN)_2$ (49.0 mg, 0.128 mmol, 0.1 equiv) in one portion. The resulting suspension was stirred at 40°C. After 24 h, addition of $PdCl_2(PhCN)_2$ (49.0 mg, 0.128 mmol, 0.1 equiv) was carried out to the resulting reaction mixture, and the same procedure was repeated after 48 h and 72 h, respectively (total amount of used $PdCl_2(PhCN)_2$ was 196.0 mg). Finally, the resulting mixture was stirred for additional 24 h (totally, 96 h). The resulting mixture was diluted with ethyl acetate (50 mL), and filtered through Florisil. The filtrate was washed with brine, dried over sodium sulfate, and concentrated *in vacuo*. The residue was purified by silica gel 60N column chromatography (dichloromethane: hexane = 1:1) to afford (±)-3-benzylmaackiain (77.1 mg, 0.206 mmol, 16% yield) as a colorless oil. m.p. 146–147°C; IR (KBr): 3446, 2924, 1619, 1541, 1506, 1474, 1434, 1377, 1329, 1269, 1146, 1038, 939, 828, 779 cm^{-1}; ^{1}H NMR (400 MHz; $CDCl_3$): δ 7.48–7.31 (m, 6H), 6.77–6.70 (m, 2H), 6.57 (d, J = 2.4, 1H), 6.45 (s, 1H), 5.93 (d, J = 10.8, 1H), 5.93 (d, J = 10.8, 1H), 5.51 (d, J = 6.8, 1H), 5.08 (s, 2H), 4.25 (dd, J = 11.2, 5.2, 1H), 3.68 (dd, J = 11.2, 11.2, 1H), 3.50 (ddd, J = 11.2, 6.8, 5.2, 1H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 160.2 (C), 156.5 (C), 154.2 (C), 148.1 (C), 141.7 (C), 136.7 (C), 131.7 (CH), 128.6 (CH × 2), 128.0 (CH), 127.4 (CH × 2), 117.9 (C), 112.7 (C), 109.8 (CH), 104.7 (CH), 102.7 (CH), 101.2 (CH_2), 93.8 (CH), 78.4 (CH), 70.0 (CH_2), 66.5 (CH_2), 40.2 (CH); HRESIMS: m/z calcd for $C_{23}H_{18}O_5Na$ $[M + Na]^+$: 397.1052, found: 397.1054. Preparation of (±)-maackiain [(±)-hydroxy-8,9-methylenedioxypterocarpan] was carried out from (±)-3-benzylmaackiain according to the previously reported method (Breytenbach et al. 1980; Tőkés et al. 1999). Separation of (−)-maackiain from (+)-maackiain was performed using a Chiral Pack IC column (0.46 ID × 25 cm, Daicel Co, Osaka, Japan) using hexane/methanol [80:20 (v/v)] as a mobile phase. The retention times of (−)-maackiain and (+)-maackiain were 73.68 min and 99.60 min, respectively.

Animal studies

Six-week-old male Brown Norway rats weighing 200–250 g (Japan SLC, Hamamatsu, Japan) were used for the present study. Rats were allowed free access to water and food and kept in a room maintained at 25 ± 2°C and 55 ± 10% humidity with a 12-h light/dark cycle. Sensitization with TDI was performed by the method described by Dev et al. (2009; Fig. 1). In brief, 10 μL of a 10%

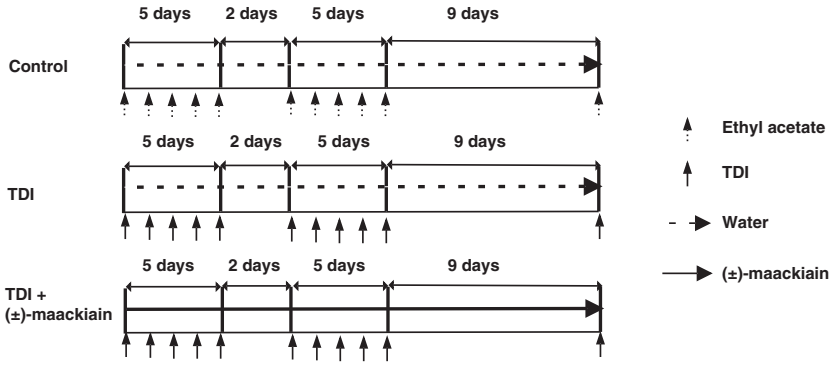


Figure 1. Experimental protocol for animal studies. Rats were sensitized with TDI for 2 weeks. The control group was sensitized with only ethyl acetate. (±)-Maackiain (5–20 mg/kg) was administered once a day for 3 weeks. (±)-Maackiain was administered 1 h before TDI application when rats were sensitized with TDI. TDI, toluene-2,4-diisocyanate.

solution of TDI in ethyl acetate (EtOAc; Wako Chemical, Tokyo, Japan) was applied bilaterally on the nasal vestibule of each rat once a day for five consecutive days. This sensitization procedure was then repeated after a 2-day interval. Nine days after the second sensitization, 10 μL of 10% TDI solution was again applied to the nasal vestibule to provoke nasal symptoms. The control group was sensitized and provoked with 10 μL of EtOAc using the same procedure. Synthetic (±)-maackiain (5–20 mg/kg) was administered orally once a day for 3 weeks and nasal symptoms were measured during the 10 min period just after TDI provocation. Symptoms included the number of sneezes and the nasal score, which included the extent of watery rhinorrhea, swelling, and redness, measured on a scale ranging from 0 to 3 (Table 1). All experimental procedures were performed in accordance with the guidelines of the Animal Research Committee of The Tokushima University.

Real-time quantitative RT-PCR

HeLa cells were cultured at 37°C under a humidified 5% CO_2, 95% air atmosphere in a minimal essential medium-α containing 8% fetal calf serum, and 1% antibiotics–antimycotics (Invitrogen, Carlsbad, CA, USA). HeLa cells cultured to 70% confluency in 6-well dishes were serum-starved for 24 h and then treated with reagents 24 h before PMA stimulation. After a 3-h treatment with PMA, the cells were harvested with 700 μL of RNAiso Plus (Takara Bio Inc. Kyoto, Japan), mixed with 140 μL of chloroform, and centrifuged at 17,400*g* for 15 min at 4°C. The aqueous phase was collected, and RNA was precipitated by the addition of isopropyl alcohol. After centrifugation at 17,400*g* for 15 min at 4°C, the resulting RNA pellet was washed with ice-cold 70% ethanol. Total RNA was resuspended in 10 μL of diethylpyrocarbonate-treated water, and 5 μg of each RNA sample was used for the reverse transcription reaction. For determination of IL-4 mRNA, RBL-2H3 cells were used instead of HeLa cells. RBL-2H3 cells were cultured at 37°C under a humidified 5% CO_2, 95% air atmosphere in a MEM containing 10% fetal calf serum and 100 IU/mL penicillin (Sigma–Aldrich) and 50 μg/mL streptomycin (Sigma–Aldrich, St, Louis, MO, USA). RBL-2H3 cells cultured 70% confluency in 6-well dished were treated with antidinitrophenol (DNP) IgE (SPE-7, 100 ng/mL; Sigma-Aldrich , St. Louis, MO, USA) 18 h before DNP-HSA (100 ng/mL) stimulation. After 2-h stimulation with DNP-HSA, the cells were harvested and total RNA was prepared. Rat nasal mucosa samples were collected in RNAlater (Applied Biosystems, Foster City, CA) 4 h after provocation. Nasal mucosa samples were homogenized using a Polytron (Model PT-K; Kinematica AG, Littau/Luzern, Switzerland) in 10 volumes of ice-cold RNAiso Plus Reagent. The homogenates were mixed with chloroform and centrifuged at 17,400*g* for 15 min at 4°C. The aqueous phase containing RNA was transferred to a new tube, and the RNA was precipitated by the addition of isopropanol and centrifugation at 17,400*g* for 15 min at 4°C. The RNA samples were reverse-transcribed to cDNA using a High Capacity cDNA Reverse Transcription kit (Applied Biosystems). TaqMan primers and the probe were designed using Primer Express (Applied Biosystems).

Table 1. Criteria for grading the severity of TDI-induced nasal responses in rats.

Nasal response	Score			
	0	1	2	3
Watery rhinorrhea	(−)	At nostril	Between 1 and 3	Drops of discharge from nose
Swelling and redness	(−)	Slightly swollen	Between 1 and 3	Strong swelling and redness

TDI, toluene-2,4-diisocyanate.

Table 2. Nucleotide sequences for primers and probes used in this study.

Primer/probe name	Sequence
Human H1R mRNA	
Sense primer	5′-CAGAGGATCAGATGTTAGGTGATAGC-3′
Antisense primer	5′-AGCGGAGCCTCTTCCAAGTAA-3′
Probe	FAM-CTTCTCTCGAACGGACTCAGATACCACC-TAMRA
Rat H1R mRNA	
Sense primer	5′-TATGTGTCCGGGCTGCACT-3′
Antisense primer	5′-CGCCATGATAAAACCCAACTG-3′
Probe	FAM-CCGAGAGCGGAAGGCAGCCA-TAMRA
Rat IL-4 mRNA	
Sense primer	5′-CAGGGTGCTTCGCAAATTTTAC-3′
Antisense primer	5′-CACCGAGAACCCCAGACTTG-3′
Probe	FAM-CCCACGTGATGTACCTCCGTGCTTG-TAMRA

H1R, histamine H_1 receptor.

Real-time PCR was conducted using a GeneAmp 7300 sequence detection system (Applied Biosystems). The sequences of the primers and TaqMan probe are listed in Table 2. To standardize the starting material, the human GAPDH gene and Rodent GAPDH Control Reagents (VIC™ Probe; Applied Biosystems) were used, and data were expressed as the ratio of H1R mRNA to GAPDH mRNA.

Antioxidant activity of (−)-maackiain

The antioxidant capacity of purified (−)-maackiain was determined using the DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging activity assay previously described by Jung et al. (Jung et al. 2005). L-Ascorbic acid was used as a positive control. In brief, various concentrations of (−)-maackiain or L-ascorbic acid in methanol were incubated with 150 μmol/L DPPH for 30 min at room temperature. Absorbance at 520 nm derived from the DPPH radical was measured. DPPH radical scavenging activity was calculated as follows:

$$\text{DPPH radical scavenging activity}\,(\%) = C - (S_t - S_b)/C \times 100,$$

where, S_t represents absorbance of sample at 520 nm; S_b, absorbance of blank (methanol solution) at 520 nm; and C, absorbance of control (DPPH in methanol only) at 520 nm.

Immunoblot analysis

HeLa cells were serum-starved for 24 h and stimulated with 100 nmol/L of PMA for 10 min in 100-mm dishes. Cells were pretreated with (−)-maackiain for 24 h before stimulation with PMA. The cells were harvested in TBS containing proteinase inhibitors (Complete Mini, Sigma-Aldrich, St. Louis, MO, USA) and phosphatase inhibitors (Phos STOP, Sigma-Aldrich, St. Louis, MO, USA), and whole cell extracts were prepared by sonication. For the immunoblot analysis, 30 μg of each protein sample was separated on a 10% SDS-PAGE gel and then transferred onto a nitrocellulose membrane (Bio-Rad, Richmond, CA, USA). The membrane was briefly rinsed in TBS containing 0.1% Tween 20 (TBS-T) and then incubated for 1 h at room temperature in TBS-T containing 5% skim milk (Difco, BD Japan, Tokyo, Japan) or 3% BSA (for detecting phosphoproteins; Sigma). The membrane was then incubated with a primary antibody [PKCδ (C-20), Santa Cruz Biotechnology (Santa Cruz Biotechnology, Santa Cruz, CA, USA); phospho-PKCδ (Tyr^{311}) and β-actin, Cell Signaling (Cell Signaling Technology Japan, Tokyo, Japan)] overnight at 4°C. Goat anti-rabbit IgG (H ± L)-HRP conjugate (Bio-Rad) was used as the secondary antibody, and proteins were visualized with an Immobilon Western Chemiluminescent HRP substrate (Merk Millipore Billerica, MA, USA).

Subcellular localization of PKCδ

To determine the subcellular localization of PKCδ, HeLa cells were plated onto 35-mm glass-bottomed dishes (Asahi Techno Glass, Chiba, Japan). HeLa cells were serum-starved for 24 h. The cells were then stimulated with 100 nmol/L PMA for 5 min. The cells were treated with the (−)-maackiain (30 μmol/L) for 24 h before PMA stimulation. After stimulation, the cells were washed once with PBS and fixed with ice-cold methanol, and the PBS was then replaced. The subcellular localization of the PKCδ was determined with anti-PKCδ antibody as the primary antibody and Cy3-conjugated donkey anti-rabbit IgG as the secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) using a confocal laser microscope (LSM510; Carl Zeiss, Oberkochen, Germany). Localization of the Golgi was determined with anti-58K Golgi marker Protein antibody (as the primary antibody, Abcam, Tokyo, Japan) and DyLight488-conjugated donkey anti-rabbit IgG (as the secondary antibody, Jackson ImmunoResearch).

PKCδ kinase assay

The PKCδ kinase assay was performed using the PKCδ kinase enzyme system (Promega, Madison, WI, USA) and ADP-Glo kinase assay kit (Promega), according to the manufacturer's instructions. In brief, recombinant PKCδ (3 ng) and substrates (50 μmol/L ATP and 0.2 mg/mL CREBtide) were incubated with or without

various concentrations of (−)-maackiain or staurosporine, a PKC inhibitor, for 20 min at 25°C. The reaction was stopped by the addition of the ADP-Glo Reagent solution, and the luminescence derived from the ADP formed was measured using an Infinite M200 microplate reader (Tecan Japan, Kanagawa, Japan).

Statistical analysis

The results are shown as mean ± SEM. The data from the experiments using TDI-sensitized rats are shown as mean ± SD. Statistical analyses were performed using unpaired *t*-tests or ANOVA with Dunnett's multiple comparison test using the GraphPad Prism software (GraphPad Software Inc., La Jolla, CA). $P < 0.05$ was considered statistically significant.

Results

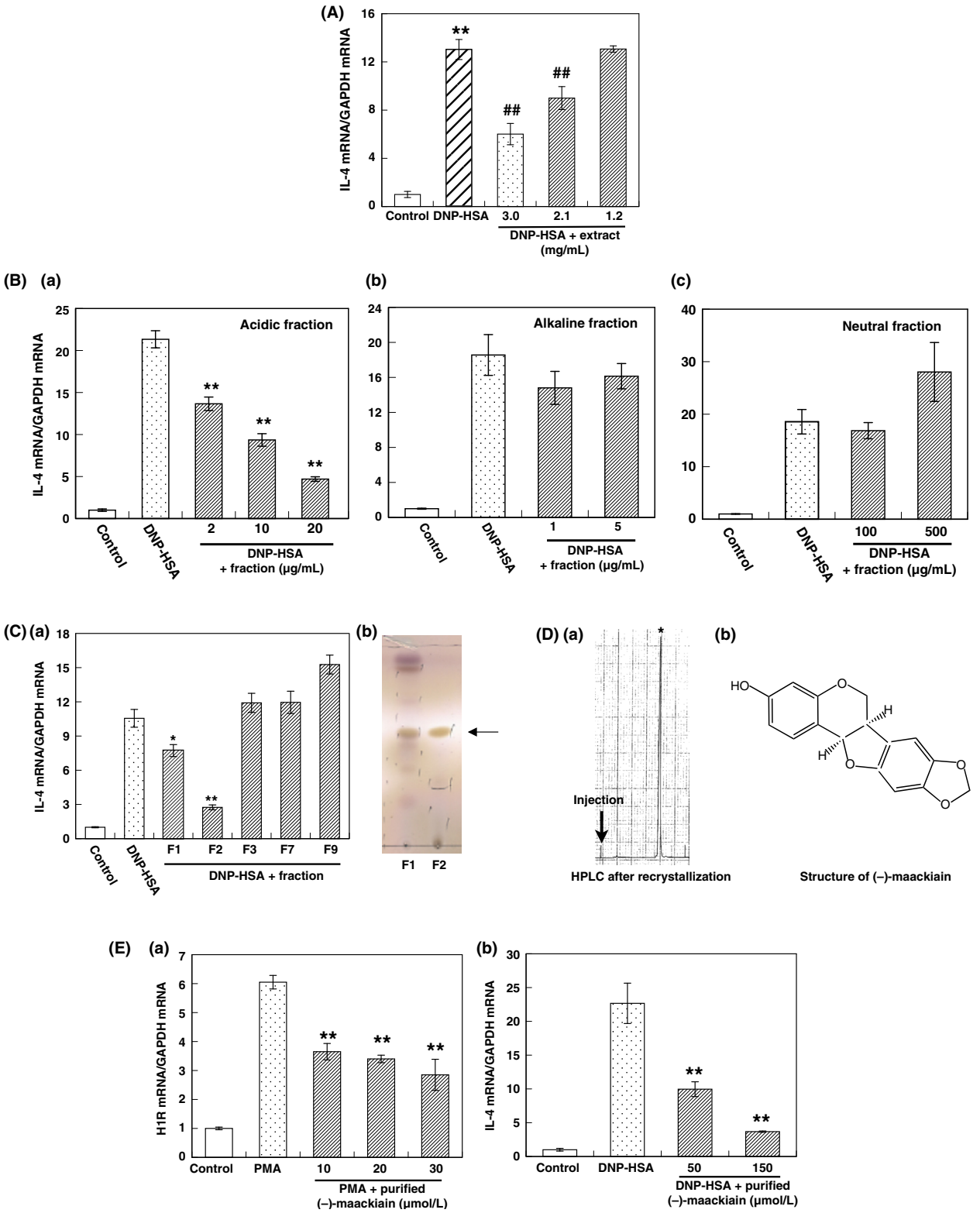
Identification of (−)-maackiain as an anti-allergic compound in Kujin

We have previously reported that Kujin extract alleviates nasal symptoms and suppresses TDI-induced upregulation of H1R and IL-4 gene expression in the nasal mucosa of TDI-sensitized rats (Dev et al. 2009). To identify the active compounds responsible for this effect, we investigated the effect of Kujin extract on stimuli-induced upregulation of H1R and IL-4 gene expression in cell systems. Kujin extract inhibited stimuli-induced H1R (Nurul et al. 2011) and IL-4 mRNA elevations (Fig. 2A). Therefore, we tried to purify the active compounds from Kujin extract by partitioning the fractions and assessing the suppressive activity of each fraction on the IgE-stimulated upregulation of IL-4 gene expression in RBL-2H3 cells. Kujin extract was partitioned into acidic, basic, and neutral fractions (Fig. 2B), and the active (acidic) fraction was further purified using silica gel column chromatography (Fig. 2C). Pure compound was obtained by rechromatography using silica gel, followed by reversed-phase HPLC and crystallization (Fig. 2D). The purified compound was identified as maackiain by structure determination using a combination of several 2D NMR spectroscopic techniques and mass spectrometry. Determination of the specific rotation of the purified maackiain ($[\alpha]^{16}_{D} = -176.4°$) revealed that (−)-maackiain (Fig. 2D) is the active compound. Purified (−)-maackiain suppressed upregulation of H1R and IL-4 gene expression (Fig. 2E).

Effect of synthetic maackiain on upregulation of H1R and IL-4 gene expression in cultured cells and nasal symptoms in TDI-sensitized rats

The synthesis of racemic maackiain has already been reported using $HgCl_2$ as a catalyst (Breytenbach et al. 1980). To eliminate the possibility of Hg contamination in the final product, we established a new method for the synthesis of (−)-maackiain that does not use $HgCl_2$. In our method, the Heck reaction was used for the preparation of (±)-3-Benzyloxy-8,9-methylenedioxypterocarpan [(±)-3-Benzyloxymaackiain] from 2-Bromo-4,5-methylenedioxyphenol and 7-Benzyloxy-2H-1-benzopyran. After racemic maackiain synthesis (Fig. 3), we separated (−)-maackiain from (+)-maackiain using a chiral column (Fig. 4A) and investigated the effect of these compounds on the stimuli-

Figure 2. Identification of (−)-maackiain as an antiallergic compound in Kujin. (A) Effect of Kujin extract on IgE/antigen-stimulated IL-4 mRNA expression in RBL-2H3 cells. RBL-2H3 cells were treated with 100 ng/mL anti-DNP IgE for 18 h and then stimulated with 100 ng/mL DNP-HSA for 2 h. Kujin extract was treated 24 h before DNP-HSA stimulation. After stimulation, total RNA was isolated, and the IL-4 mRNA levels were determined by real-time quantitative RT-PCR. Data are presented as the mean ± SEM ($n = 3$). **, $P < 0.01$ versus control; ##, $P < 0.01$ versus DNP-BSA; one-way ANOVA and Dunnett's multiple comparison test. (B) Effect of the fractions resulting from pH partition on IgE/antigen-stimulated upregulation of IL-4 mRNA expression in RBL-2H3 cells. Acid fraction (a), alkaline fraction (b), and neutral fraction (c) were treated 24 h before DNP-HSA stimulation. Data are presented as the mean ± SEM ($n = 3$). **, $P < 0.01$ versus DNP-HSA; one-way ANOVA and Dunnett's multiple comparison test. (C) Effect of the fractions obtained from silica gel column chromatography on IgE/antigen-stimulated upregulation of IL-4 mRNA expression in RBL-2H3 cells (a), Fractions (20 μg/mL for F1, F3, F7, and F9; 50 μg/mL for F2) were treated 24 h before DNP-HSA stimulation. Data are presented as the mean ± SEM ($n = 3$). **, $P < 0.01$ and *, $P < 0.05$ versus DNP-HSA; unpaired *t*-tests. (b), TLC analysis of the active fractions indicates existence of the common compound in F1 and F2 (arrow). (D) Elution profile from HPLC of the purified active compound (a), and the structure of (−)-maackiain (b). (E) Effect of purified (−)-maackiain on PMA-induced H1R mRNA elevation in HeLa cells (a) and IgE/antigen-stimulated IL-4 mRNA elevation in RBL-2H3 cells (b). (a), HeLa cells were serum-starved for 24 h, and stimulated with 100 nmol/L PMA for 3 h. Purified (−)-maackiain was treated 24 h before PMA stimulation. After stimulation, total RNA was isolated, and the H1R mRNA levels were determined by real-time quantitative RT-PCR. In (b), RBL-2H3 cells were treated with 100 ng/mL anti-DNP-HSA IgE for 18 h and then stimulated with 100 ng/mL DNP-HSA for 2 h. Purified (−)-maackiain was treated 24 h before DNP-HSA stimulation. After stimulation, total RNA was isolated, and the IL-4 mRNA levels were determined by real-time quantitative RT-PCR. Data are presented as the mean ± SEM ($n = 3$). **, $P < 0.05$ versus PMA or DNP-HSA; one-way ANOVA and Dunnett's multiple comparison test. H1R, histamine H_1 receptor; PMA, phorbol-12-myristate-13-acetate.



induced upregulation of H1R and IL-4 gene expression. As shown in Figure 4B, both (+)- and (−)-maackiain suppressed H1R mRNA elevation, whereas only (−)-maackiain inhibited IL-4 mRNA elevation, suggesting distinct target molecules for the H1R and IL-4 signaling pathways. Next, we investigated the effect of synthetic maackiain on nasal

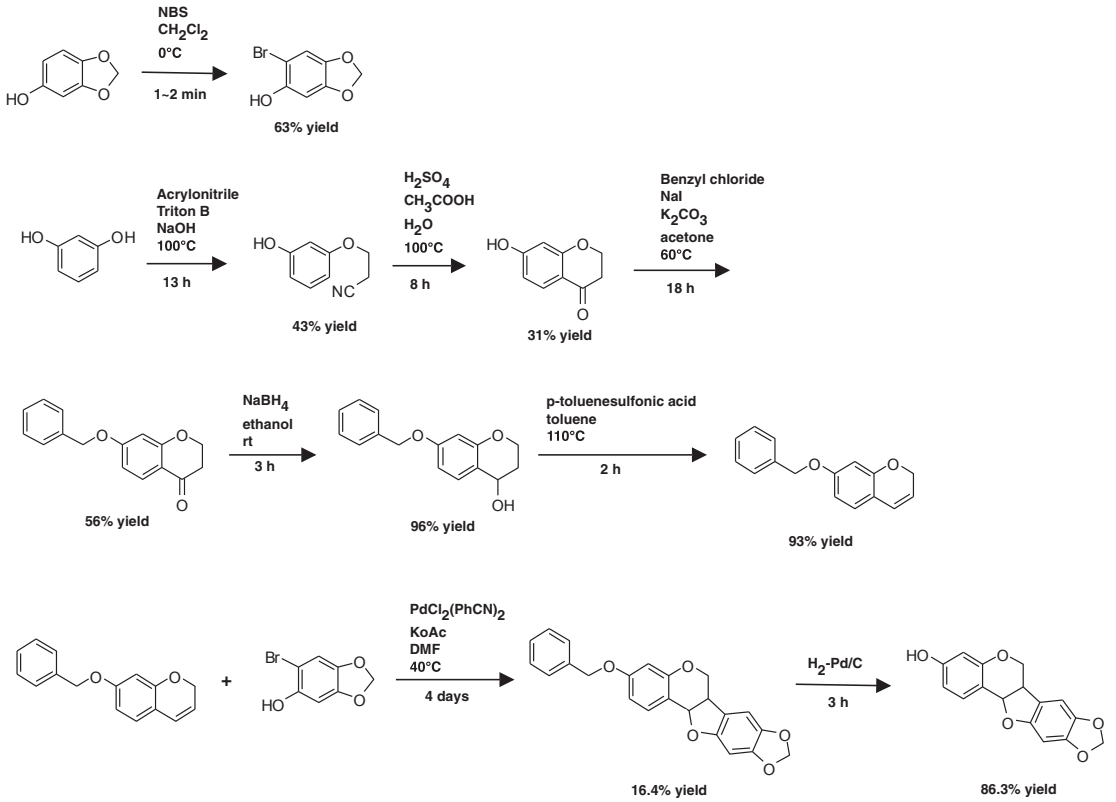


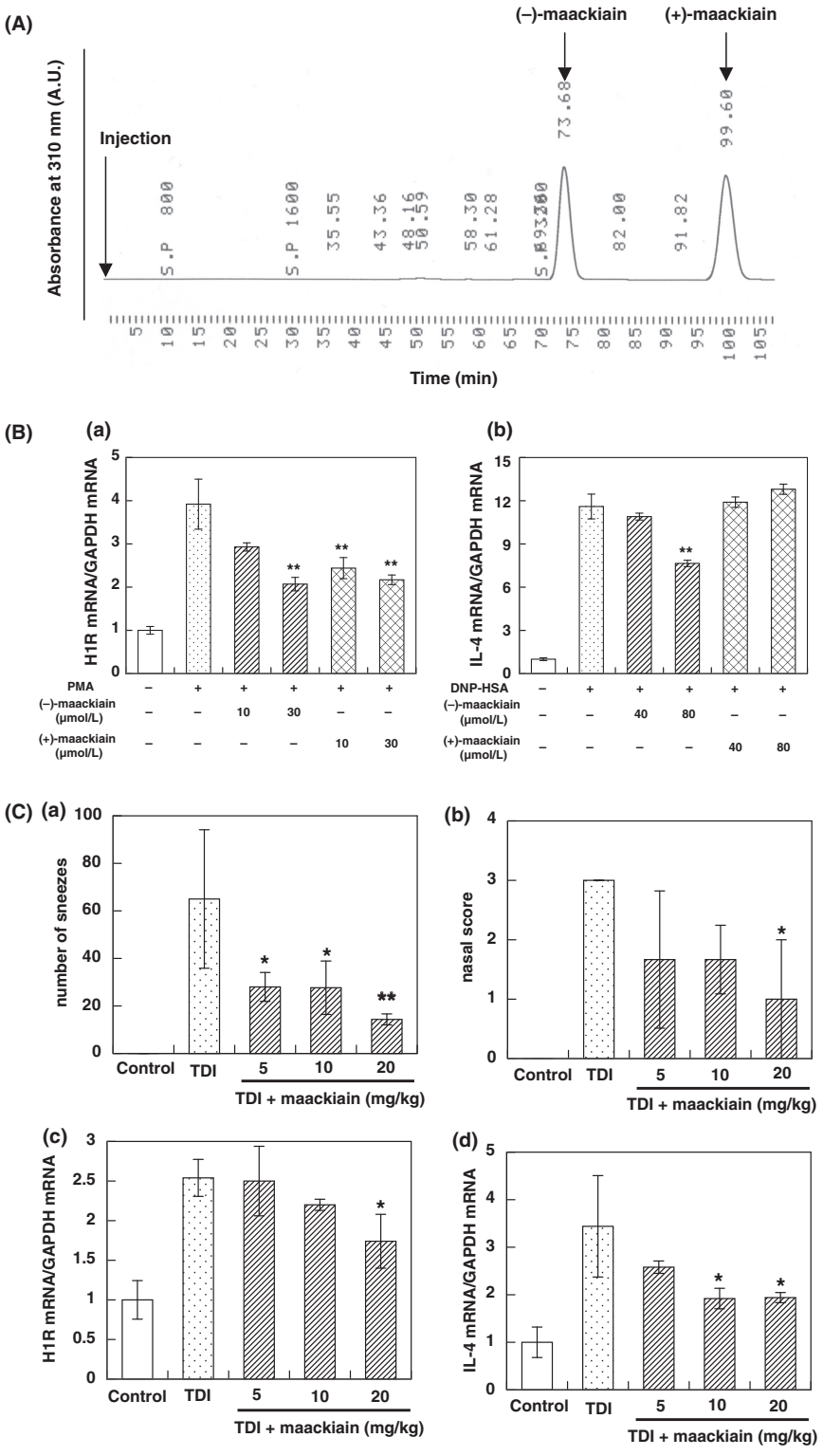
Figure 3. Synthetic pathway of (±)-maackiain.

symptoms and upregulation of allergic disease-sensitive gene expression in TDI-sensitized rats. Application of TDI causes nasal symptoms such as sneezing and watery rhinorrhea. Repeated application of TDI also increased H1R and IL-4 mRNA expression in the nasal mucosa of TDI-sensitized rats (Mizuguchi et al. 2008). Oral treatment with racemic maackiain (5–20 mg/kg) for 3 weeks alleviated nasal symptoms and suppressed upregulation of H1R and IL-4 gene expression in TDI-sensitized rats (Fig. 4C).

Molecular mechanism of suppressive effect of (−)-maackiain on H1R gene expression in HeLa cells

Pharmacological activity of many flavonoids has been explained by their antioxidant activities. Therefore, we investigated whether the antiallergic effect of (−)-maackiain is due to its antioxidant activity using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay,

Figure 4. Characterization of synthesized (−)-maackiain. (A) Separation of (−)-maackiain from (+)-maackiain by Chiral column chromatography. (B) Effect of synthesized (−)-maackiain and (+)-maackiain on PMA-induced upregulation of H1R mRNA expression in HeLa cells (a) and on IgE/antigen-stimulated upregulation of IL-4 mRNA expression in RBL-2H3 cells (b) (a), HeLa cells were serum-starved for 24 h, and stimulated with 100 nmol/L PMA for 3 h. Synthesized (−)- or (+)-maackiain was treated 24 h before PMA stimulation. After stimulation, total RNA was isolated, and the H1R mRNA levels were determined by real-time quantitative RT-PCR. (b), RBL-2H3 cells were treated with 100 ng/mL anti-DNP IgE for 18 h and then stimulated with 100 ng/mL DNP-HSA for 2 h. Synthesized (−)- or (+)-maackiain was treated 24 h before DNP-HSA stimulation. After stimulation, total RNA was isolated, and the IL-4 mRNA levels were determined by real-time quantitative RT-PCR. Data are presented as the mean ± SEM ($n = 3$). **, $P < 0.01$ versus PMA or DNP-HSA; one-way ANOVA and Dunnett's multiple comparison test. (C) Effect of (±)-maackiain on TDI-induced nasal symptoms and up-regulations of H1R and IL-4 gene expressions in the nasal mucosa of TDI-sensitized rats. Rats were sensitized and provoked as described in Materials and Methods. (a), The number of sneezes were counted for 10 min just after TDI-provocation. (b), Degrees of swelling and redness and watery rhinorrhea were scores according to the criteria listed in Table 1 on a scale ranging from 0 to 3. (c, d), Rats were sacrificed 4 h after TDI provocation and nasal mucosa was collected. Total RNA was isolated from the nasal mucosa as described in Materials and Methods, and H1R and IL-4 mRNAs were determined by real-time quantitative RT-PCR. Data are presented as the mean ± SD ($n = 3$). **, $P < 0.01$ and *, $P < 0.05$ versus TDI; one-way ANOVA and Dunnett's multiple comparison test. H1R, histamine H_1 receptor; H1R, histamine H_1 receptor, PMA, phorbol-12-myristate-13-acetate; TDI, toluene-2,4-diisocyanate.



with L-ascorbic acid as a positive control. (−)-Maackiain at concentrations up to 200 μmol/L showed no DPPH scavenging activity per se (Fig. 5A). This result suggests that the mechanism of the suppressive effect of maackiain on nasal symptoms and H1R and IL-4 gene expressions was not due an antioxidant activity but proceeded

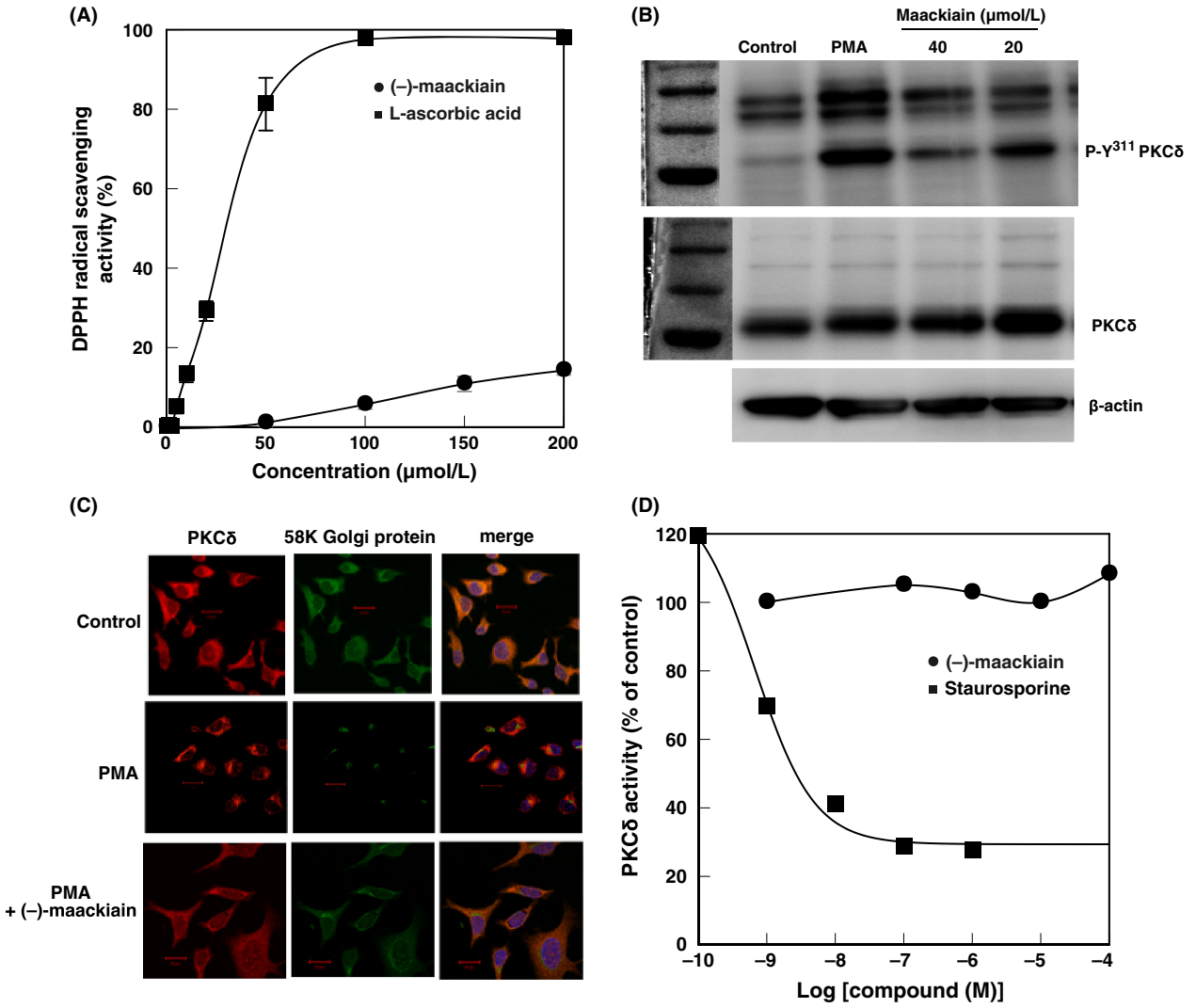


Figure 5. Antiallergic activity of (−)-maackiain is not due the antioxidant activity. (A) DPPH radical scavenging assay. Various concentrations of (−)-maackiain (●) or L-ascorbic acid (■) in methanol were incubated with 150 μmol/L DPPH for 30 min at room temperature. Absorbance at 520 nm derived from the DPPH radical was measured. DPPH radical scavenging activity was calculated as described in Materials and Methods. (B) Effect of (−)-maackiain on the PMA-induced phosphorylation of Tyr^{311} on PKCδ. HeLa cells were serum-starved for 24 h and treated with 100 nmol/L PMA for 10 min. (−)-Maackiain was treated 24 h before stimulation with PMA. After stimulation, total cell lysate was prepared and subjected to immunoblot analysis. (C) Effect of (−)-maackiain on translocation of PKCδ in response to PMA stimulation. HeLa cells were serum-starved for 24 h and treated with 100 nmol/L PMA for 5 min. The cells were treated with 30 μmol/L (−)-maackiain 24 h before PMA stimulation. The subcellular localization of PKCδ was determined using a confocal laser microscope. Scale bars = 20 μm. (D) Effect of (−)-maackiain on PKCδ kinase enzymatic activity. Recombinant PKCδ (3 ng) and substrate (50 μmol/L ATP and 0.2 mg/mL CREBtide) were incubated with or without various concentrations of (−)-maackiain (●) or staurosporine (■) for 20 min at 25°C. The reaction was stopped by the addition of the ADP-Glo Reagent solution, and the luminescence derived from the ADP formed was measured using an Infinite M200 microplate reader. DPPH, 2,2-diphenyl-1-picrylhydrazyl; PMA, phorbol-12-myristate-13-acetate.

through an unknown mechanism. Previously, we demonstrated that the PKCδ/ERK/PARP-1 pathway is involved in the histamine- and PMA-induced upregulation of H1R expression level in HeLa cells (Mizuguchi et al. 2011) and that the translocation of PKCδ to the Golgi and phosphorylation of Tyr^{311} on PKCδ are crucial steps in this signaling pathway. Therefore, we investigated the effect of (−)-maackiain on PMA-induced translocation and phosphorylation of PKCδ. Pretreatment with (−)-maackiain inhibited PMA-induced phosphorylation of Tyr^{311} on PKCδ, and its translocation to the Golgi (Fig. 5B and C). However, (−)-maackiain did not inhibit PKCδ kinase activity (Fig. 5D). These data suggest that inhibition of PKCδ activation and not the inhibition of

PKCδ enzymatic activity is the mechanism of (−)-maackiain action.

Discussion

In the present study, we showed that (−)-maackiain is the prominent component responsible for the antiallergic activity of Kujin. Maackiain is a pterocarpan that is widely distributed in leguminous plants (Shibata and Nishikawa 1963). Bioactivities of maackiain, including anticancer and antimicrobial effects, have been reported (Honda and Tabata 1982; Aratanechemuge et al. 2004). (−)-Maackiain also inhibits aryl hydrocarbon hydroxylase (Gelboin et al. 1981). However, the antiallergic activity of maackiain had not yet been reported. We demonstrated that pretreatment with synthetic racemic maackiain for 3 weeks significantly suppressed TDI-induced nasal symptoms and upregulation of H1R and IL-4 gene expression in TDI-sensitized rats (Fig. 4C). To our knowledge, this is the first report to show the antiallergic activity of maackiain. As shown in Figure 4C, 5 mg/kg of racemic maackiain treatment showed nearly maximal alleviation of sneezing and nasal scores although suppression of H1R and IL-4 gene expressions were suppressed moderately. We previously reported that treatment with histamine up-regulated IL-4 and IL-5 gene expression and IL-4 treatment stimulated H1R gene expression in normal (i.e., non-TDI-sensitized) rats (Shahriar et al. 2009). We also reported that the expression of H1R gene is strongly correlated with those of IL-5 (Kitamura et al. 2012), IL-9, IL-13, and CD40L (H. Mizuguchi, H. Ogishi, and H. Fukui, unpubl. data). These data suggest the existence of the cross talk between the H1R signaling and IL-4 signaling. This is perhaps the reason why strong suppression of nasal symptoms was observed even if suppression of mRNA expression in each protein is moderate.

Pharmacological activity of many flavonoids has been explained by their antioxidant activities. However, (−)-maackiain did not show any DPPH scavenging activity per se (Fig. 5A). Furthermore, studies using synthetic (−)-maackiain and (+)-maackiain also revealed that maackiain showed stereoselectivity for IL-4 gene suppression but not for H1R gene expression (Fig. 4B), suggesting the existence of distinct target proteins for these transcriptional signaling. We did not obtained clear dose-dependency of maackain, especially experiments with HeLa cells. Over 30 μmol/L of (−)-maackiain showed cell toxicity in HeLa cells. On the other hand, (−)-maackiain did not show any cell toxicity up to 150 μmol/L in RBL-2H3 cells. This cell-dependent toxicity failed to use suitable concentration of (−)-maackiain to obtain the clear dose-dependency.

H1R gene expression is highly correlated with the severity of allergic symptoms, and compounds that suppress H1R gene expression alleviate allergic symptoms (Das et al. 2007; Mizuguchi et al. 2011; Kitamura et al. 2012; Hattori et al. 2013). T_H2 cytokines are also suggested to play important roles in the pathogenesis of allergic inflammation (Holgate 1993). We have demonstrated the cross talk between H1R signaling and T_H2 cytokine signaling in patients with pollinosis (Mizuguchi et al. 2008; Shahriar et al. 2009) and have shown that suppression of H1R signaling could inhibit T_H2 cytokine signaling. Therefore, we consider suppression of H1R signaling to be crucial for the treatment of allergic diseases. Recently, we have reported that PKCδ/ERK/PARP-1 pathway is involved in the PMA-induced upregulation of H1R expression level in HeLa cells (Mizuguchi et al. 2011). And the translocation of PKCδ to the Golgi and phosphorylation of Tyr^{311} on PKCδ are crucial steps in this signaling pathway. Pretreatment with (−)-maackiain inhibited PMA-induced phosphorylation of Tyr^{311} on PKCδ, and its translocation to the Golgi (Fig. 5B and C). However, (−)-maackiain did not inhibit PKCδ kinase activity (Fig. 5D). These data suggest that the inhibition of PKCδ activation is the mechanism of (−)-maackiain action for suppression of H1R gene expression. We identified additional antiallergic compounds, including epigallocatechin-3-O-gallate (EGCG) and quercetin that suppress the upregulation of H1R gene expression in HeLa cells and TDI-sensitized rats (Matsushita et al. 2008; Hattori et al. 2013). These compounds also inhibit the phosphorylation of Tyr^{311} on PKCδ (Mizuguchi et al. 2012b; Hattori et al. 2013). These findings suggest that inhibition of PKCδ activation could be a novel therapeutic strategy for allergic diseases. So far, we have no direct evidence that activation of PKCδ upregulates H1R gene expression in allergic nasal mucosa and suppression of its activation is target to maackiain treatment. However, as described above, pretreatment with compounds that inhibit PKCδ activation suppressed nasal symptoms. These findings support the possibility that the activation of PKCδ in the nasal mucosa is also involved in the nasal symptoms in allergy model rats. Recently, it is demonstrated that H1R immunoreactivity was observed in epithelial cells and vascular endothelial cells in human nasal mucosa (Shirasaki et al. 2012). H1R mRNA was increased in human nasal epithelial cells after stimulation with diesel exhaust particles that are known to cause chronic airway diseases, and histamine stimulation induced production of granulocyte macrophage-colony-stimulating factor (GM- CSF) and IL-8 in human nasal epithelial cells (Terada et al. 1999). In addition, involvement of H1R-PKC-ERK signaling pathway in eliciting GM- CSF and IL-8 production from bronchial epithelial cells stimulated

by histamine was reported (Matsubara et al. 2006). Involvement of PKCδ was also reported by Masaki et al. (2011). These findings suggest that epithelial cells are one of the candidates responsible for histamine-induced upregulation of H1R gene expression. As HeLa cells are the cell line derived from cervical cancer cells arising in epithelial cells, we believe that HeLa cells can be the model to analyze molecular mechanism of histamine-induced upregulation of H1R gene expression in the nasal mucosa although HeLa cells are not representative for typical target cells involved in allergic reactions. Furthermore, very recently, we found that Heat shock protein 90 (Hsp90) is one of the target proteins for (−)-maackiain and disruption of the interaction between PKCδ and Hsp90 is the mechanism of suppression of PKCδ activation by (−)-maackiain (Y. Nariai, H. Mizuguchi, T. Ogasawara, H. Nagai, Y. Sasaki, Y. Okamoto, Y. Yoshimura, Y. Kitamura, H. Nemoto, N. Takeda, and H. Fukui, unpubl. data). This finding combined with the data that (−)-maaackiain does not inhibit PKCδ enzymatic activity suggests that enzymatic activity of PKCδ is not directly related to H1R gene upregulation.

In summary, we have shown that (−)-maackiain is a novel antiallergic compound that alleviates nasal symptoms in TDI-sensitized allergy model rats through the inhibition of H1R and IL-4 gene expression. The molecular mechanism underlying its suppressive effect for the upregulation of H1R gene expression is the inhibition of PKCδ activation. Our data also suggest that PKCδ is a novel target protein for allergic diseases and compounds that suppress PKCδ activation may improve allergic symptoms or prevent development of allergic diseases, contributing to their clinical application for allergic diseases.

Author Contributions

Mizuguchi designed the project and wrote the manuscript. Nariai, Kato, Nakano, and Kanayama performed the experimental work. Nariai, Kashiwada, Nemoto, Kawazoe, and Kitamura analyzed the data and participated in the data interpretation. Takaishi and Takeda supervised the research and wrote the manuscript. Fukui conceived the project, supervised the research, and wrote the manuscript.

Disclosure

The authors declare no financial conflicts of interest.

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