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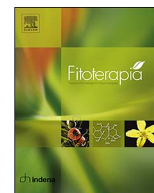

Anti-allergy activities of Kuji amber extract and kujigamberol

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ABSTRACT

Amber is fossilized tree resin and several biologically active compounds were isolated from ambers using the growth-restoring activity of the mutant yeast [*Saccharomyces cerevisiae* (*zds1*Δ *erg3*Δ *pdr1*Δ *pdr3*Δ)] involving Ca^{2+}-signal transduction. The aim of this study is to investigate the anti-allergic effect of both the methanol extract of Kuji amber (MEKA) and its main biologically active constituent, kujigamberol (15,20-dinor-5,7,9-labdatrien-18-ol) having activity against the mutant yeast. Both MEKA and kujigamberol inhibited the degranulation of RBL-2H3 cells by stimulation of thapsigargin (Tg) (IC_{50} = 15.0 μg/ml and 29.1 μM) and A23187 (IC_{50} = 19.6 μg/ml and 24.9 μM) without cytotoxicity, but not by stimulation of IgE + DNP-BSA (Ag) (IC_{50} > 50.0 μg/ml and 50.0 μM). However, both inhibited Ca^{2+}-influx in RBL-2H3 cells by all three stimulations in a dose dependent manner. Leukotriene C_4 production in RBL-2H3 cells stimulated by A23187 was also inhibited by both through the inhibition of ERK1/2 phosphorylation. In an ovalbumin-induced rhinitis model of guinea pigs, nasal administration of MEKA and kujigamberol inhibited nasal blockade in a dose-dependent manner and the effect was about 5 times potent than that of a steroid clinical drug, mometasone furoate. The growth-restoring activity of MEKA and kujigamberol against the mutant yeast is involved in the anti-allergic activities against cells and animals, and both are expected to be candidates for the development of new anti-allergy agents.

1. Introduction

Ca^{2+} is one of the important second messengers in many cell types, including yeast and immune cells [1,2]. Ca^{2+}-signal transduction is involved in many diseases and is critical for degranulation, generation of eicosanoids and production of cytokines in mast cells in response to antigen and other stimulants [3]. Type I allergy such as allergic rhinitis has been increasing especially in industrialized countries and the typical symptoms of nasal itching, sneezing, rhinorrhea and nasal congestion affect quality of life and work of patients [4]. Although current therapeutic drug such as corticosteroids for rhinitis and asthma is used in worldwide, natural products are expected to develop safe and effective therapeutic agents different from the steroids [5]. Budding yeast *Saccharomyces cerevisiae* is a simpler model organisms as eukaryote and used for drug screening [6,7].

Amber is fossilized tree resin constituted of organic polymers derived through complex maturation processes of the original plant resin and has been used mainly as ornament. Although it was used as medicine for muscle pain, headaches, skin allergies, etc. in the limited area, any biologically active compound from ambers has not been isolated until we have studied [8–11]. Our group focused on its biological activity for the first time and have already isolated several kinds of biologically active compounds involving Ca^{2+}-signal transduction inhibition from Kuji [*Araucaria* (Araucariaceae); 90–86 million years ago (Ma) (Prof. Hisao Ando, Ibaraki University, personal communication), Japan], Baltic (Pinaceae, Araucariaceae, Burseraceae; 56–34 Ma, Porland and Russia) and Dominican [*Hymenaea protera*, *Hymenaea verrucosa* (Fabaceae/Caesalpiniaceae); 45–30 Ma and 20–15 Ma, Dominican Republic] ambers [12] using a mutant *Saccharomyces cerevisiae* (*zds1*Δ *erg3*Δ *pdr1*Δ *pdr3*Δ) (YNS17 strain) [2,6,13,14]. There are three new compounds, 15,20-dinor-5,7,9-labdatrien-18-ol (kujigamberol), 13-methyl-8,11,13-podocarpatrien-19-ol (kujiol A) and 15,20-dinor-5,7,9-labdatrien-13-ol (kujigamberol B) from Kuji amber, three known compounds, agathic acid 15-monomethyl ester, dehydroabietic acid and pimaric acid from Baltic amber, and one new analogue compound, 5(10)-halimen-15-oic acid and two known compounds, 3-cleroden-15-

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oic acid and 8-labden-15-oic acid from Dominican amber [9,15–17]. The methanol extract of Kuji amber (MEKA) and its main biologically active compound, kujigamberol have growth-restoring activity that is a different phenotype from that of 3-cleroden-15-oic acid against the mutant yeast without cytotoxicity. Furthermore, 3-cleroden-15-oic acid prevented the degranulation of rat basophilic leukemia-2H3 (RBL-2H3) cells through the inhibition of Ca^{2+}-influx stimulated by thapsigargin (Tg) or the calcium ionophore A23187, not by immunoglobulin E + dinitrophenol-bovine serum albumin [IgE + DNP-BSA (Ag)] [16].

Mast cells are a key player in type I allergy and the activation of receptors with high affinity for IgE (FcεRI) on it is to liberate β-hexosaminidase (β-HEX), a general marker of degranulation, as well as allergic mediators such as leukotrienes, histamine, cytokines and prostaglandins. RBL-2H3 cells are suitable as a model for the mast cells and have been widely used to study IgE-FcεRI interactions, related to intracellular signaling pathways in degranulation and to evaluate anti-allergic agents. After crosslinking of their IgE-bound FcεRI by allergens, or Ca^{2+}-influx by Tg or A23187, mast cells release mediators which are histamine, prostaglandin D_2 (PGD_2), the leukotrienes C_4 (LTC_4) and D_4 (LTD_4) [18,19]. The LTC_4 is a lipid mediator which is released by degranulation and causes allergy reaction such as nasal congestion [20,21].

The aim of this study is to investigate anti-allergic effect of MEKA and its main biologically active constituent, kujigamberol having activity against the mutant yeast, on cells and animals. Surprisingly, MEKA showed potent biological activities against them and the activities did not match the content of kujigamberol in MEKA. The relationship between the activity of MEKA and the quantity of kujigamberol in MEKA was also clarified and discussed.

2. Materials and methods

2.1. Chemicals and strains

Kuji amber was excavated from mines of Kuji Kohaku Co. Ltd. located in the upper part of the Tamagawa Formation of Kuji Group of Kuji city, Iwate Prefecture, northeastern Japan. Kuji amber (1029 g) was ground to a powder and extracted with MeOH. It was partitioned with EtOAc and subjected to silica gel column chromatography. Finally, kujigamberol was purified 75.7 mg as a colorless oil using HPLC [9,17]. Extracted samples of 100 mg/ml in MeOH (MEKA) were prepared for subsequent assays. HPLC analyses of MEKA and kujigamberol were carried out using the following conditions [Capcell pak 4.6 × 150 mm (Shiseido Co. Ltd., Tokyo, Japan), 75% MeOH, 1 ml/min, 220 nm]. The yeast strain used was a derivative of strain W303-1A and it was the YNS17 strain (*MATa zds1::TRP1 erg3::HIS3 pdr1::hisG URA3 hisG pdr3::hisG*) [6,9]. Difco® YPD broth and YPD agar were obtained from Becton Dickinson (Franklin Lakes, NJ, USA). An immunosuppressive drug, FK506 (tacrolimus) was kindly provided by the Fujisawa Pharmaceutical Co., Ltd. (now Astellas Pharma Inc., Tokyo, Japan). Unless otherwise stated, chemicals used were of the best commercially available grade.

2.2. Animals

Male Hartley guinea pigs for the rhinitis model were obtained from Kyudo Co. Ltd. (Tosu, Japan) at the age of 8 weeks and housed in aluminum cages in a room controlled at a temperature of 23 ± 2 °C, humidity of 61 ± 8% and with a 12 h lighting cycle (7:00–19:00). The experimental procedures employed in this study were in accordance with guidelines of the Industrial Animal Care and Use Committee at LSI Medience Co. (Kumamoto, Japan).

2.3. Growth restoring activity

Growth restoring activity was carried out using the YNS17 strain and 5 μl of each sample were added on a plate as described previously [6]. The inhibitory activity of the Ca^{2+}-signal transduction was determined by way of the strength and/or distinction of the yeast growth zone. FK506 [2.5 ng/spot, calcineurin (protein phosphatase 2B) specific inhibitor] was used as a positive control [22].

2.4. Cell culture and cytotoxicity

RBL-2H3 cells (ATCC CRL-2256, Manasass, VA, USA) were maintained in DMEM supplemented with 10% heat-inactivated FBS (Biowest SAS, Nuaillé, France) and antibiotics [penicillin (50 units/ml)-streptomycin (50 μg/ml), Gibco, Thermo Fisher Scientific Inc., Waltham, MA, USA] [23,24]. Cell viability was determined using the MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide] assay (Dojindo Lab., Kumamoto, Japan). RBL-2H3 cells were plated in triplicate at a concentration of 3×10^4 cells/well in a 96-well plate and incubated for overnight prior to treatment with various concentrations of MEKA or kujigamberol for 48 h. After 10 μl of MTT (5 mg/ml) was added to each well and it was incubated for 4 h at 37 °C. Isopropanol (100 μl in 0.04 N HCl) was added to dissolve the MTT formazan and the absorbance was measured at 560 nm with a microplate reader (Infinite F200 PRO, Tecan, Männedorf, Switzerland).

2.5. Measurement of the degranulation (β-HEX assay)

RBL-2H3 cells were grown overnight in 96-well plates (3×10^4 cells/well), 100 μl DMEM was added and then sensitized with 0.5 μg/ml IgE (Yamasa Co., Tokyo, Japan) for 2 h. After washing, cells were incubated in 98 μl of Tyrode buffer (137 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, 5.6 mM D-glucose, 20 mM HEPES, 1 mg/ml BSA, pH 7.3) and 2 μl of each sample for 30 min, then stimulated with 10 μl DNP-BSA (1.0 μg/ml, Merck Millipore Co., Billerica, MA, US) as an antigen for 10 min. RBL-2H3 cells were also stimulated with 10 μl Tg (2 μM, Sigma-Aldrich Co., St. Louis, MO, USA) for 20 min or 10 μl A23187 (20 μM, Sigma-Aldrich Co.) for 30 min without the sensitization by IgE. The supernatant (30 μl) was then transferred to a 96-well plate and incubated with 70 μl substrate [1.3 mg/ml, PNAG (4-nitrophenyl-*N*-acetyl-β-D-glucosaminide), Sigma-Aldrich Co.] in 0.1 M citrate buffer (pH 4.5) at 37 °C for 90 min. The reaction was stopped by adding 100 μl of stop solution (0.4 M glycine-NaOH). The absorbance was measured at 405 nm by a microplate reader. The inhibition (%) of β-HEX release by the sample was calculated using the following equation, and IC_{50} values were determined graphically. Inhibition (%) = $[1 - (T - N)/(C - N)] \times 100\%$: Control (C) was DNP-BSA (+), test sample (−); test (T) was DNP-BSA (+), test sample (+); test sample (+); and normal (N) was DNP-BSA (−), test sample (−). Quercetin was used as a reference compound (IC_{50} = 5.7 μM) [16,23,24].

2.6. Measurement of Ca^{2+} mobilization

RBL-2H3 cells were grown overnight in 96-well plates (3×10^4 cells/well), 100 μl DMEM was added and then sensitized with 0.5 μg/ml IgE (Yamasa Co., Tokyo, Japan) for 2 h. After washing, cells were incubated in 100 μl the loading buffer [Fluo 4-AM (2.5 μM, Dojindo Lab.) in Tyrode buffer with 1.25 mM probenecid, Wako] for 30 min at 37 °C. After discarding the loading buffer, cells were incubated in 98 μl of Tyrode buffer with 1.25 mM probenecid and 2 μl of each sample in MeOH for 30 min. Fluo 4-AM loaded RBL-2H3 cells were excited at 485 nm, and the fluorescence emission was observed at 535 nm using a microplate reader. Each 10 μl DNP-BSA (1.0 μg/ml), Tg (2 μM) or A23187 (20 μM) was added to each well at 60 s and the fluorescence measured for 1000 s [16,23,24].

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