Antiallergic Potential on RBL-2H3 Cells of Some Phenolic Constituents of *Zingiber officinale* (Ginger)

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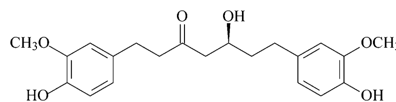
Received September 1, 2008

In the present study on five pure phenolic compounds (**1**−**5**) isolated from the rhizomes of *Zingiber officinale* (ginger) and investigated for their antiallergic potency, rat basophilic leukemia (RBL-2H3) cells were incubated with these compounds and the release of β-hexosaminidase was measured kinetically. The data obtained suggest that ginger rhizomes harbor potent compounds capable of inhibiting allergic reactions and may be useful for the treatment and prevention of allergic diseases.

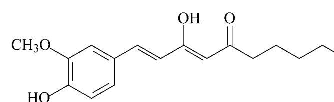
Ginger, the powdered rhizomes of the herb *Zingiber officinale* Roscoe (Zingiberaceae), is used widely as a spice throughout the world. In Chinese medicine, ginger has been used traditionally as a treatment for rheumatism, nervous diseases, gingivitis, toothache, asthma, stroke, constipation, and diabetes.[1] Its extracts have been reported to have potential anti-inflammatory,[2] antioxidative,[3−5] antithrombosis,[6] and cancer chemopreventive activities[7] and to be effective in reducing the symptoms of arthritis in humans.[8] In addition, phytochemicals isolated from ginger species have been documented for the treatment of chemotherapy-associated nausea,[9] the suppression of platelet aggregation,[10] and the inhibition of COX-2 and nitric oxide synthase.[11] Since inflammation is a partial manifestation of the allergic response, this prompted us to investigate the possible antiallergic effects that ginger constituents might possess.

The physiological consequences of type I allergic diseases are mainly highlighted by immunoglobulin E (IgE)-mediated activation of mast cells or basophils.[12,13] Mast cells are distributed widely throughout mucosal and connective tissues. They are considered the pivotal cell type in defending against parasites and bacteria in innate immunity, but, on the other hand, they are also the major effector cells of IgE-mediated allergic inflammation.[14−16] Allergen-specific IgE is directly involved in the initiation of the allergic cascade by binding to the high-affinity receptors for IgE (FcεRI) expressed on the surface of either mast cells or basophils. Re-exposure to the same allergen will cause the cross-linking of FcεRI-bound IgE molecules and trigger downstream signaling events, finally resulting in the elevation of intracellular calcium levels for mast cells to degranulate various inflammatory mediators.[17]

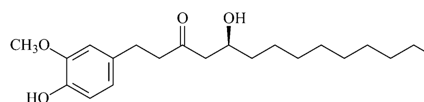
RBL-2H3 cells are tumor analogues of mucosal mast cells and express high levels of FcεRI on their surface. These cells have been used extensively as a model for the study of mast cell degranulation through the antigen-induced aggregation of FcεRI.[18] Among the various inflammatory mediators produced by mast cells, β-hexosaminidase is stored in the secretory granules of the cells and is released by exocytosis when mast cells are immunologically activated, such as during the cross-linking of FcεRIs.[19] Therefore, measurements of inhibitory capacity on released β-hexosaminidase have been used commonly as a reliable parameter to predict possible antiallergic activities of either natural or synthetic compounds. As the possible antiallergic effects of *Z. officinale* constituents remain undefined, we report in the current study the isolation of five active known compounds (**1**−**5**) from ginger rhizomes and the investigation of their potential antiallergic ability using the degranulation model of RBL-2H3 cells. We show herein that antigen-induced activation of RBL-2H3 cells could be inhibited by some of these compounds.



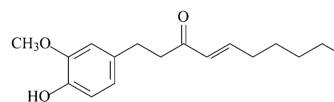
hexahydrocurcumin (**1**)



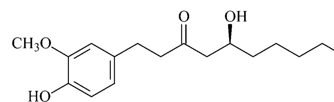
6-dehydrogingerdione (**2**)



10-gingerol (**3**)



6-shogaol (**4**)



6-gingerol (**5**)

Table 1 summarizes the inhibitory effects (IC_{50}) of the five phenolic ginger compounds (**1**−**5**) used on the growth of RBL-2H3 cells. Most compounds exhibited little or undetectable inhibition on the viability of RBL-2H3 cells under the experimental conditions used, except for 6-dehydrogingerdione (**2**), with weak cytotoxicity (IC_{50} 38.5 μM). On the other hand, a higher degree of

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10.1021/np800555y CCC: $40.75 © 2009 American Chemical Society and American Society of Pharmacognosy
Published on Web 03/09/2009

Table 1. IC_{50} Values on the Viability of RBL-2H3 Rat Basophilic Leukemia Cells after 12 h Treatment with Compounds **1**−**5**

compound	RBL-2H3 cytotoxicity (IC_{50}, μM)
hexahydrocurcumin (**1**)	152.3
6-dehydrogingerdione (**2**)	38.5
10-gingerol (**3**)	111.7
6-shogaol (**4**)	90.4
6-gingerol (**5**)	185.1

inhibition on cell viability was observed only at higher concentrations for the remaining compounds used in the assays [(hexahydrocurcumin (**1**) and 6-gingerol (**5**) at 200 μM, 10-gingerol (**3**) and 6-shogaol (**4**) at above 120 μM, respectively)]. These results indicate that, at physiologically relevant concentrations, these ginger constituents showed either insignificant or no cytotoxic effects on cell proliferation.

Degranulation is a characteristic feature of activated mast cells or basophils upon the stimulation of cross-linking antigens.[20] To assess the possible modulatory effects of constituents **1**−**5** from *Z. officinale* on the activation of RBL-2H3 cells, these five compounds were added at various concentrations and incubated with the cells at different time periods before a combination of mIgE-DNP and DNP-BSA was added to trigger the activation of the cells. The inhibitory capacity of each compound was evaluated as the release of β-hexosaminidase in the culture medium as compared to that from untreated cells. As shown in Figure 1, compounds **2**−**5** exhibited similar inhibitory capacities and kinetics, in that they showed a moderate initial inhibition (60−70%) at the lowest concentration (1 μM) and they all reached their highest degree of inhibition at higher concentrations (e.g., 100 and 200 μM). Among all of the compounds tested, hexahydrocurcumin (**1**) demonstrated the most potent inhibitory capacity. The inhibitory capacity of hexahydrocurcumin (**1**) was also evident in a dose- and time-dependent manner to reach almost full inhibition when used at 200 μM for the RBL-2H3 cells. It is also worth mentioning that all compounds but 6-shogaol (**4**) exhibited a high degree of inhibition (>90%) at all concentrations tested when longer incubation times (24−48 h) were employed, suggesting that the prolonged stimulation of RBL-2H3 cells with lower concentrations of these ginger constituents might be sufficient to elicit inhibitory effects and impede the degranulation process of RBL-2H3 cells.

The present study demonstrates that selected phenolic constituents from ginger rhizomes exhibit high potency in inhibiting the release of inflammatory mediators from RBL-2H3 cells. Additionally, when used at physiological concentrations, these compounds did not cause significant reduction in cell viability, indicating they possibly influence signaling pathways leading to the reduced degranulation of RBL-2H3 cells. Therefore, further investigation on the molecular mechanim(s) by which these ginger compounds elicit their inhibitory effects on allergic responses is warranted.

Experimental Section

General Experimental Procedures. Optical rotations were measured with a JASCO DIP-370 digital polarimeter. UV spectra were obtained in MeCN using a JASCO V-530 spectrophotometer. The IR spectra were measured on a Hitachi 260-30 spectrophotometer. ^{1}H (400 MHz, using $CDCl_3$ as solvent for measurement), ^{13}C (100 MHz), DEPT, HETCOR, COSY, NOESY, and HMBC NMR spectra were obtained on a Unity Plus Varian NMR spectrometer. LRFABMS and LREIMS were obtained with a JEOL JMS-SX/SX 102A mass spectrometer or a Quattro GC-MS spectrometer with a direct inlet system. Silica gel 60 (Merck, 230−400 mesh) was used for column chromatography. Precoated silica gel plates (Merck, Kieselgel 60 F-254, 0.20 mm and Merck, Kieselgel 60 F-254, 0.50 mm) were used for analytical and preparative TLC, respectively. Spots were detected by spraying with 50% H_2SO_4 and then heating on a hot plate.

Plant Material. The roots of *Z. officinale* (ginger) were purchased from a local market in Kaohsiung, Taiwan, in July 2006, and were identified by Dr. Yen-Ray Hsui of the Division of Silviculture, Taiwan Forestry Research Institute, Taipei, Taiwan. A voucher specimen (Hsui-Zo-1) was deposited at Fooyin University.

Extraction and Isolation. The rhizomes (25.6 kg) of *Z. officinale* were chipped, air-dried, and extracted repeatedly with $CHCl_3$ (50 L × 4) at room temperature. The combined $CHCl_3$ extracts (896.5 g) were then evaporated further and separated into 20 fractions by column chromatography on silica gel (3.8 kg, 70−230 mesh) with gradients of *n*-hexane−$CHCl_3$. Fr. 8 (81.2 g), eluted with $CHCl_3$−MeOH (60:1), was next subjected to silica gel CC ($CHCl_3$−MeOH mixtures) and yielded 6-dehydrogingerdione (**2**, 163 mg). Fr. 10 (86.9 g), eluted with $CHCl_3$−MeOH (50:1), was next repeatedly subjected to silica gel CC ($CHCl_3$−MeOH mixtures) and yielded 6-shogaol (**4**, 328 mg). Fr. 11 (78.4 g), eluted with $CHCl_3$−MeOH (40:1), was subjected to silica gel column chromatography and yielded pure 10-gingerol (**3**, 197 mg). Fr. 12 (105.8 g), eluted with $CHCl_3$−MeOH (40:1), was subjected to silica gel CC ($CHCl_3$−MeOH mixtures) and yielded pure 6-gingerol (**5**, 311 mg). Fr. 15 (117.1 g), eluted with $CHCl_3$−MeOH (30:1), was subjected to silica gel CC ($CHCl_3$−MeOH mixtures) and yielded pure hexahydrocurcumin (**1**, 251 mg). These compounds were identified by spectroscopic data analysis and comparison with literature values.[21,22]

Chemicals and Reagents. Delbecco's modified minimal medium (DMEM) powder, [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (MTT), *p*-nitrophenyl-*N*-acetyl-D-glucosaminide (p-NAG), and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, MO). Dinitrophenyl-conjugated bovine serum albumin (DNP-BSA) was purchased from Pierce (Rockford, IL). All other chemicals and reagents were purchased at the highest purity and quality possible. Mouse anti-DNP IgE (mIgE-DNP) antibody was a generous gift from Dr. Daniel H. Conrad (Virginia Commonwealth University, Richmond, VA).

Cells Cultures. The mucosal mast cell-derived rat basophilic leukemia (RBL-2H3) cell line[23] was purchased from the American Type Culture Collection and grown in DMEM with 10% FBS and 100 U/mL penicillin plus 100 μg/mL streptomycin. The cells were maintained in 75 cm^2 culture dishes at 37 °C in a humidified chamber with 5% CO_2 in air and were routinely passed at a ratio of 1:2 at least every 4 days until the day of the experiment. Prior to this, cells were washed and replenished with fresh culture medium frequently.

Cell Viability Assay. A methylthiazoleterazolium (MTT) assay was used to measure cell viability to test if the compounds used in this study could induce cell death. The method is based upon the ability of a mitochondrial dehydrogenase enzyme from viable cells to cleave the tetrazolium rings of the pale yellow MTT and form impermeable crystals of a dark blue formazan, thus resulting in accumulation within healthy cells. Briefly, RBL-2H3 cells were seeded in a 6 cm dish. After reaching 70−80% confluence, cells were either left untreated (as positive control) or treated with different concentrations of the test compounds. Three hours before the end of incubation, a stock MTT solution (5 mg/mL, dissolved in PBS) was diluted 1:10 in culture medium and added to a culture dish, followed by a 2 h incubation at 37 °C. At the end of the incubation, the medium was removed and replaced with 1 mL of DMSO to dissolve the formazan crystals. After addition of DMSO, the culture dishes were gently shaken for 20 min in the dark to ensure maximal dissolution of formazan crystals. Aliquots of each sample were added to a 96-well plate and read at 600 nm on a multiwall scanning spectrophotometer. The degree of cell viability by the test compounds was calculated as the percentage OD (600 nm) of the control value. All experiments were repeated at least three times. In consideration of the possible antiproliferative effects of DMSO, cultures were added with maximal 0.5% DMSO and used as positive controls, which was found not to affect the growth of the RBL-2H3 cells.

Measurement of Degranulation. The degree of antigen-induced degranulation in RBL-2H3 cells was determined by a β-hexosaminidase activity assay as previously described.[24] Briefly, RBL-2H3 cells were dispensed into a 96-well plate at a density of 1×10^5 cells per well and incubated at 37 °C for 4−6 h to allow their complete plate adherence. Subsequently, test compounds were added at different final concentrations, and incubation was allowed to continue for various time periods before the addition of mIgE-DNP. For the positive control, cells were incubated with mIgE-DNP alone without the addition of any test compound. After another 16 h incubation, IgE-sensitized RBL-2H3 cells were washed twice in prewarmed Tyrode's buffer (135 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2$, 1.0 mM $MgCl_2$, 5.6 mM glucose,

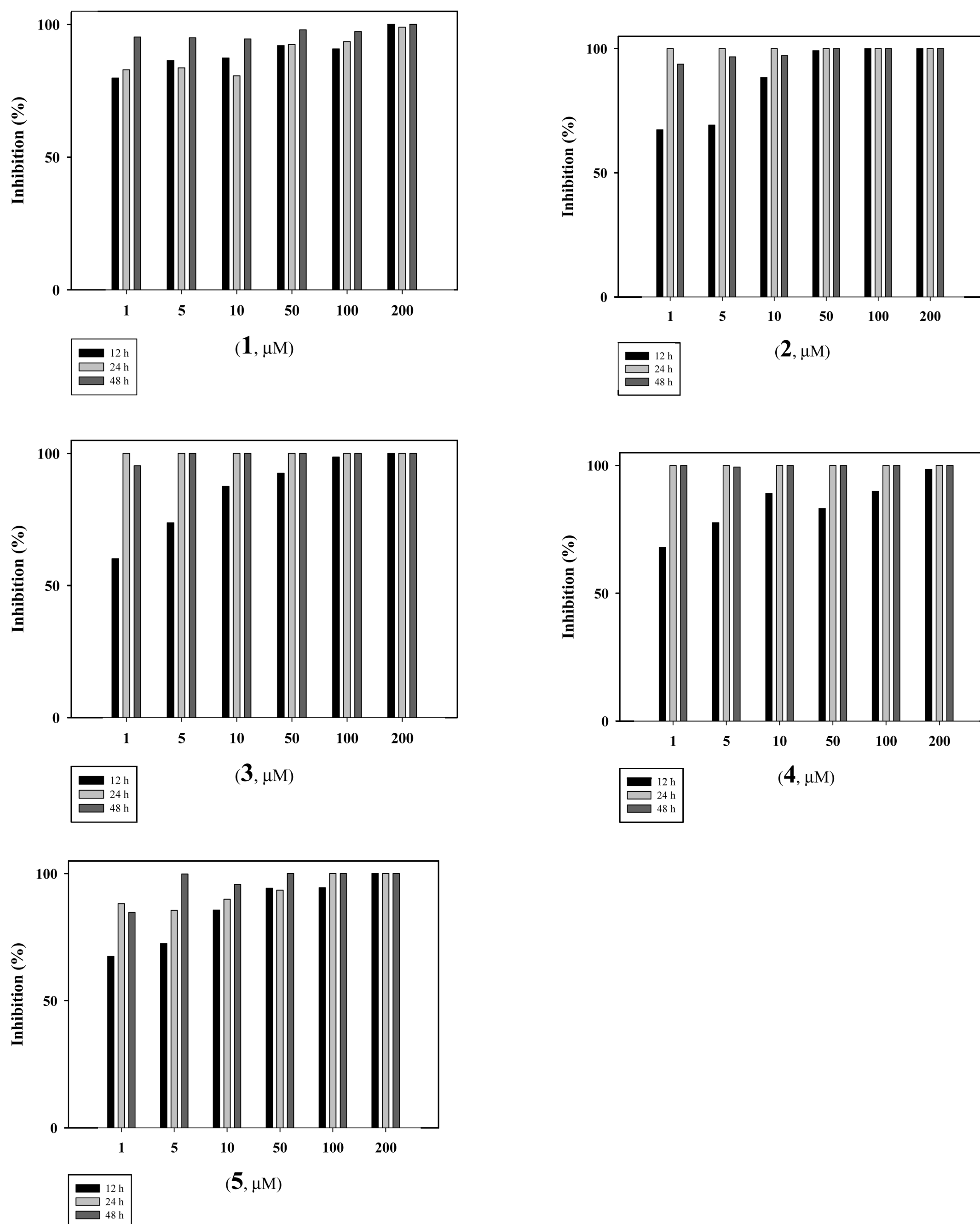

Figure 1. Time-course inhibitory effects of different concentrations of ginger bioactive compounds on β-hexosaminidase release from antigen-stimulated RBL-2H3 cells.

20 mM HEPES, and 1 mg/mL BSA at pH 7.4) and stimulated by adding the cross-linking antigen DNP-BSA diluted in Tyrode's buffer at 1 μg/mL at 37 °C for 1 h. The reaction was stopped by cooling the plate in an ice bath for 15 min. As controls for the measurement of the total amount and spontaneous release of β-hexosaminidase, unstimulated cells were either lysed with 1% Triton X-100 solution or left untreated (without the addition of antigen), respectively. Aliquots of supernatants collected from the control and experimental wells were transferred into a 96-well microplate and incubated with an equal volume of 1 μM p-NAG prepared in 0.1 M citrate buffer (pH 4.5) as substrate for at 37 °C for 1 h. The reaction was quenched by the addition of 150 μL stop buffer (0.1 M Na_2/$NaHCO_3$, pH 10.0) followed by the measurement of the absorbance at 405 nm on a microplate reader. The inhibition percentage of β-hexosaminidase release from RBL-2H3 cells was calculated using the following equation:

$$\text{Inhibition } (\%) = \left[1 - \frac{(\text{OD}_{\text{sample}} - \text{OD}_{\text{spontaneous}})}{(\text{OD}_{\text{total}} - \text{OD}_{\text{spontaneous}})}\right] \times 100$$

Statistical Analysis. All results are expressed as the mean values. Statistical comparisons were carried out using the Student's *t*-test for paired values.

Acknowledgment. This investigation was supported by a grant from the National Science Council of the Republic of China (NSC) (NSC96-2320-B-037-038-MY2) to B.-H.C., and by grants from NSC (NSC97-2221-E-037-002) and the Center of Excellence for Environmental Medicine (KMU-EM-97-2.1.b) to H.-M.W. The authors also thank M.-Y. J. Wang for English editing.

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NP800555Y