

Effects of Benzyldenecyclopentanone Analogues of Curcumin on Histamine Release from Mast Cells

Agung Endro NUGROHO,^{a,b} Zullies IKAWATI,^b SARDJIMAN,^c and Kazutaka MAEYAMA^{*,a}

^aDepartment of Pharmacology, Informational Biomedicine, Ehime University Graduate School of Medicine; Shitsukawa, Toon, Ehime 791–0295, Japan; ^bDepartment of Pharmacology and Clinical Pharmacy, Faculty of Pharmacy, Gadjah Mada University; and ^cDepartment of Pharmaceutical Chemistry, Faculty of Pharmacy, Gadjah Mada University; Jogjakarta, 55281, Indonesia.

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Curcumin reportedly has anti-allergic effects and can inhibit the release of histamine from mast cells. In the present study, fourteen benzyldenecyclopentanone analogues of curcumin were studied for their effects on histamine release from rat basophilic leukemia (RBL-2H3) cells. After screening, four selected compounds: 2,5-bis(4-hydroxybenzylidene)cyclopentanone; 2,5-bis(4-hydroxy-3-methoxybenzylidene)cyclopentanone; 2,5-bis(4-hydroxy-3,5-dimethylbenzylidene) cyclopentanone; and 2,5-bis(4-hydroxy-3,5-diethylbenzylidene)cyclopentanone were studied for their concentration-dependent effects on histamine release and Ca^{2+} uptake. In RBL-2H3 cells and rat peritoneal mast cells stimulated with antigen or compound 48/80, respectively, the methoxy-hydroxy analogue was more potent than curcumin in inhibiting histamine release. In contrast, the inhibitory effects of methyl/ethyl analogues were less potent than those of curcumin. Moreover, these compounds abrogated histamine release induced by increased intracellular Ca^{2+} concentrations in response to stimulants such as thapsigargin and ionomycin. These compounds also showed potent inhibitory effects on $^{45}\text{Ca}^{2+}$ uptake in RBL-2H3 cells. The mechanism of the inhibitory effects of these curcumin analogues on histamine release appeared to be related to blockade of Ca^{2+} signaling events. These results provide useful information to guide the development of new synthetic compounds for the treatment of allergic and inflammatory diseases related to histamine or mast cells.

Key words histamine; curcumin; benzyldenecyclopentanone; mast cell; rat basophilic leukemia cell; Ca^{2+} uptake

Curcumin [1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione] is a natural yellow pigment isolated from the rhizomes of the plant *Curcuma longa* L. (turmeric). Its chemical structure is described in Fig. 1.¹⁾ The pharmacological effects of curcumin include anti-inflammatory,²⁾ antioxidant,³⁾ antiprotazoal,⁴⁾ antibacterial,⁵⁾ anticancer,⁶⁾ anti-hepatotoxic,⁷⁾ hypolipidemic,⁸⁾ and antifibrotic effects.⁹⁾ Curcumin showed no toxicity in acute toxicity studies in rats, guinea pigs, and monkeys,¹⁰⁾ as well as in humans.¹¹⁾

Several prior studies have examined the effects of curcumin on allergic diseases. An ethyl acetate extract of *Curcuma longa* L., which contains curcuminoids, exhibited a preventive effect in allergic type I and IV models.¹²⁾ Furthermore, both the ethyl acetate extract of *Curcuma longa* L. and curcumin were found to decrease histamine release from mast cells. However the ethyl acetate extract of *Curcuma longa* L. had somewhat stronger activity than that of curcumin, which may have been due to the presence of curcumin derivatives.¹³⁾ Several studies have also examined the anti-allergic activity of curcumin and curcumin-related compounds in relation to their antioxidant effects. Most of these compounds were shown to inhibit histamine release from rat basophilic leukemia (RBL-2H3) cells; however, histamine release was decreased by antioxidant compounds and by those that lacked antioxidant activity. The results suggested that the hydroxy moiety of curcumin is responsible for anti-

oxidant activity and may also contribute to the inhibition of histamine release.¹⁴⁾ In a guinea pig model of airway hyperresponsiveness, curcumin at a dose of 20 mg/kg body weight significantly inhibited ovalbumin (OVA)-induced airway constriction and airway hyperactivity.¹⁵⁾

Although curcumin has a wide range of potentially beneficial pharmacological effects, its use is limited by the compounds instability. The stability of curcumin is strongly affected by factors such as pH and exposure to light.¹⁶⁾ The kinetics of the pH-dependent degradation of curcumin in aqueous medium at various pH levels was studied by Tonnesen and Karlsen.¹⁷⁾ They found that curcumin in aqueous solution at pH less than 7 is quite stable, but with increasing pH, curcumin decomposes. The main decomposition products are ferulic acid and feruloylmethane, which rapidly undergo retro-aldol condensation to vanillin and acetone. The instability of curcumin at alkaline pH is caused by the active methylene moiety. Decomposition of curcumin can also occur as a result of light exposure, and this process is also mediated by the active methylene moiety; the degradation products include ferulic aldehyde, ferulic acid, dihydroxynaphthalene product, 4-vinylguaicol, vanillin, and vanillic acid.^{16,17)} The modification of the middle site of curcumin to compounds such as the 1,4-pentadiene-3-ones, cyclopentanone, and cyclohexanone which lack the active methylene moiety and one carbonyl moiety of curcumin may show improved stability. These compounds which still maintain the hydroxy moiety at aromatic rings show a substantial antioxidant activity.¹⁸⁾ The compounds resulting from these modification are named 1,5-diphenyl-1,4-pentadiene-3-one analogues, benzyldenecyclopentanone, and benzyldenecyclohexanone, respectively.¹⁸⁾

In the present study, we investigated the anti-allergic activ-

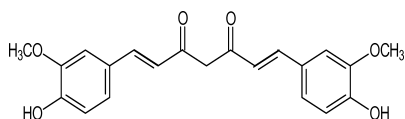


Fig. 1. Structure of Curcumin

ities of benzylidene cyclopentanone analogues of curcumin (Fig. 2). Firstly, we tested these compounds for their effects on histamine release *in vitro* using the histamine releasing agent, dinitrophenylated bovine serum albumin (DNP₂₄-BSA). The concentration-dependence of the inhibitory effects on histamine release of several selected-compounds was then examined in both RBL-2H3 cells and rat peritoneal mast cells (RPMCs) using other histamine releasing agents such as thapsigargin, ionomycin, compound 48/80 and phorbol myristate acetate (PMA).

Some benzylidenecyclopentanone analogues of curcumin have been reported to have antioxidant activity.¹⁸⁾ We examined the relationship between the anti-allergic activity and antioxidant activity of these compounds because previous research on curcumin indicated that these properties were positively correlated.¹⁴⁾ Other studies have qualitatively examined the structure-activity relationships of benzylidenecyclopentanone analogues of curcumin with respect to antioxidant activity.¹⁸⁾ The result of these studies may provide useful information for the development of pharmacological agents for the treatment of allergic and inflammatory diseases related to histamine or mast cells.

MATERIALS AND METHODS

Materials Curcumin or 1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione was purchased from E. Merck (Germany), and benzylidenecyclopentanone analogues of curcumin were synthesized by Sardjiman *et al.*¹⁸⁾ The chemical structures of the compounds are shown in Fig. 2. The histamine release inducers used in the study were ionomycin, thapsigargin, compound 48/80, and PMA from Sigma Chemical Co. (St. Louis, MO, U.S.A.). DNP₂₄-BSA as an antigen and monoclonal immunoglobulin (Ig) E against DNP₂₄-BSA purified from supernatant in IgE producing hybridoma, were produced in our laboratory. Eagle's minimum essential medium (MEM) and antibiotics (combination of penicillin G sodium and streptomycin sulfate) were purchased from Gibco (Grand Island, NY, U.S.A.). Fetal calf serum was obtained from JRH Biosciences (Kansas, U.S.A.). Piperazine-1,4-bis(2-ethanesulfonic acid) (PIPES) was purchased from Dojindo (Kumamoto, Japan), and *o*-phthalaldehyde was from Wako Pure Chemical Industries (Osaka, Japan).

Culture of RBL-2H3 Cells RBL-2H3 cells were cultured in MEM containing 15% fetal calf serum and antibiotics (penicillin and streptomycin) in a flask in a humidified atmosphere (5% CO₂) at 37 °C as described by Barsumian *et al.*¹⁹⁾ For the assay of histamine release, cells were seeded into 24-well culture plates at a density of 5×10^5 cells/0.4 ml per each well. The cells were incubated overnight at 37 °C. For DNP₂₄-BSA experiments, the cells were sensitized with 0.5 µg/ml of monoclonal IgE. On the second day, the medium was removed, and the cells were washed twice with 500 µl of PIPES buffer (119 mM NaCl, 5 mM KCl, 25 mM PIPES, 5.6 mM glucose, 0.4 mM MgCl₂, 1 mM CaCl₂, 40 mM NaOH, and 0.1% BSA, pH 7.2), and preincubated for 10 min at 37 °C after addition of 180 µl PIPES buffer either without (as a negative control) or with the drug. After 10 min preincubation, 20 µl of stimulant (200 ng/ml DNP₂₄-BSA, 5 µM thapsigargin, or 10 µM ionomycin) were added to each well

and the plate was incubated at 37 °C for 30 min.

Isolation of RPMCs Male Wistar rats weighing between 250–300 g and aged 3–4 months were used. The animal experiments were conducted according to the guidelines of the Animal Care Committee of the Ehime University, and all experimental protocols had been approved by this Committee. Rats were killed by decapitation and exsanguination. RPMCs were isolated by injection of 25 ml phosphate buffered saline (PBS) pH 7.4 containing 5 IU/ml heparin and 0.1% BSA into the peritoneal cavity and the abdomen was massaged for about 120 s. Afterwards, the peritoneal cavity was opened carefully, and the fluid containing mast cells were collected. The collected mast cells were centrifuged at $200 \times g$ for 5 min at room temperature and then resuspended in 2 ml PBS buffer containing 0.1% BSA. Peritoneal mast cells were separated from the other components (macrophages and lymphocytes) by layering on 4 ml of 38% BSA, and centrifuging at $800 \times g$ for 20 min at 4 °C. After the upper layer containing other components was aspirated and discarded, the remaining cell pellet was washed with 6 ml PBS buffer and resuspended in 1 ml of PIPES buffer. Mast cell preparations were about 95% pure as assessed by toluidine blue staining.

For the assay of histamine release, 120 µl of RPMCs suspension (2×10^4 cells/ml) was preincubated for 10 min at 37 °C after addition of 60 µl PIPES buffer either without (as a negative control) or with drugs at a range of concentrations (0.1–100 µM). After 10 min preincubation, 20 µl of stimulant (100 µg/ml compound 48/80, 5 µM thapsigargin, 10 µM ionomycin, or a combination of 100 nM PMA and 1 µM ionomycin) was added to each well and the plates were incubated at 37 °C for 30 min.

Assay of Histamine Release Histamine released in the medium was measured by HPLC-fluorometry as described previously.²⁰⁾ After a 30 min incubation, the plates were centrifuged at $1800 \times g$ for 5 min and 50 µl of the supernatant was mixed with 250 µl of 3% perchloric acid containing 5 mM Na₂-ethylenediaminetetraacetic acid (EDTA). After addition of 30 µl of 2 M KOH/1 M KH₂PO₄ and centrifugation at $10000 \times g$ for 15 min at 4 °C, 50 µl of the supernatant was injected directly onto a column packed with TSKgel SP-2SW cation exchanger (Tosoh, Tokyo). For measuring the total histamine content in cells, 350 µl of PIPES buffer was added to 6 wells and the cells were then sonicated. Fifty microlitres cell homogenate was used for the histamine assay described above. Histamine was eluted with 0.25 M potassium phosphate at a flow rate of 0.6 ml/min, and post-labeled with *o*-phthalaldehyde under alkaline conditions and detected using a F1080 Fluorometer (Hitachi, Tokyo) at excitation and emission wavelengths of 360 and 450 nm, respectively. The values were expressed as a percentage of net histamine release.

The rates of net histamine release and spontaneous release were calculated according to the following equation:

$$\text{Net release (\%)} = \frac{(\text{histamine content in the supernatant of cells stimulated} - \text{histamine content in the supernatant of unstimulated cells})}{(\text{total histamine content} - \text{histamine content in the supernatant of unstimulated cells})} \times 100.$$

$$\text{Spontaneous release (\%)} = \frac{(\text{histamine content in the supernatant of unstimulated cells})}{(\text{total histamine content})}$$

×100.

The percent inhibition of histamine release was calculated according to the following equation.

Inhibition of histamine release (%) = [(histamine release in the absence of the test compound – histamine release in the presence of the test compound) / histamine release in the absence of the test compound] × 100.

Uptake of $^{45}\text{Ca}^{2+}$ This experiment was conducted using RBL-2H3 cells in a 24 well-plate.²¹⁾ After overnight incubation at 37 °C, the cells were washed twice with 500 μl of PIPES buffer and then preincubated for 10 min at 37 °C in 180 μl PIPES buffer either without (as a negative control) or with the drug. After preincubation, 20 μl of PIPES buffer containing 5 $\mu\text{Ci}/\text{ml}$ of $^{45}\text{Ca}^{2+}$ and 5 μM of thapsigargin as Ca^{2+} uptake stimulant was added into each well, and the plate was incubated at 37 °C for 15 min. After this time, the reaction was stopped by washing with ice-cold Ca^{2+} -free buffer containing 100 μM La^{3+} . The cells were lysed with 0.3 ml of 0.1% Triton X, and 100 μl of the solution was combined with 10 ml of scintillation cocktail for radioactivity counting. The values were expressed as the percentage of maximum uptake in the absence of inhibitor compounds.

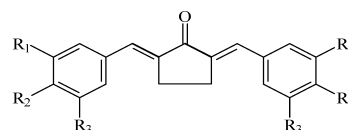
Statistical Analysis All data were expressed as mean $\pm$ S.E.M. One-way analysis of variance (ANOVA) followed by the least significant difference (LSD) test was used for statistical analyses. *p*-values less than 0.05 were considered significant.

RESULTS

Screening of the Inhibitory Effects of Histamine Release All test compounds, illustrated in Fig. 2, were benzylidenecyclopentanone analogues of curcumin. These compounds were screened for their effects on histamine release from RBL-2H3 cells. Additional studies were then performed to determine the concentration-dependency of their effects on RBL-2H3 cells and RPMCs treated with various inducers of histamine release. Because curcumin (the parent compound) was previously shown to have anti-allergic ef-

fects *in vitro* and *in vivo*,^{12–15,22–23)} we compared the inhibitory effects of benzylidenecyclopentanone analogues of curcumin on histamine release in RBL-2H3 cells with those of curcumin itself. In this study, DNP₂₄-BSA was used as an antigen to stimulate histamine release from IgE-sensitized RBL-2H3 cells. Compounds with highly potent inhibitory effects on histamine release with minimal effects on spontaneous histamine release were used for additional *in vitro* anti-allergic experiments.

The effects of fourteen benzylidenecyclopentanone analogues of curcumin (a concentration of 100 μM) on histamine



No.	R1	R2	R3	Chemical name	MW
1.	H	H	H	2,5-Dibenzylidenecyclopentanone	260.12
2.	H	OH	H	2,5-Bis(4-hydroxybenzylidene)cyclopentanone	292.11
3.	H	OCH ₃	H	2,5-Bis(4-methoxybenzylidene)cyclopentanone	320.14
4.	H	CH ₃	H	2,5-Bis(4-methylbenzylidene)cyclopentanone	288.15
5.	H	C ₄ H ₉	H	2,5-Bis(4-tert butylbenzylidene)cyclopentanone	372.25
6.	H	N(CH ₃) ₂	H	2,5-Bis(4-dimethylaminobenzylidene)cyclopentanone	346.21
7.	OCH ₃	OH	H	2,5-Bis(4-hydroxy-3-methoxybenzylidene)cyclopentanone	352.13
8.	H	Cl	H	2,5-Bis(4-chlorobenzylidene)cyclopentanone	328.04
9.	Cl	H	H	2,5-Bis(2-chlorobenzylidene)cyclopentanone	328.04
10.	Cl	Cl	H	2,5-Bis(3,4-dichlorobenzylidene)cyclopentanone	395.96
11.	CH ₃	OH	CH ₃	2,5-Bis(4-hydroxy-3,5-dimethylbenzylidene)cyclopentanone	348.17
12.	C ₂ H ₅	OH	C ₂ H ₅	2,5-Bis(4-hydroxy-3,5-diethylbenzylidene)cyclopentanone	404.24
13.	<i>i</i> -C ₃ H ₇	OH	<i>i</i> -C ₃ H ₇	2,5-Bis(4-hydroxy-3,5-diisopropylbenzylidene)cyclopentanone	460.20
14.	<i>i</i> -C ₄ H ₉	OH	<i>i</i> -C ₄ H ₉	2,5-Bis(4-hydroxy-3,5-ditert.butylbenzylidene)cyclopentanone	516.36

Fig. 2. Structure, Chemical Name and Molecular Weight of Benzylidenecyclopentanone Analogues of Curcumin

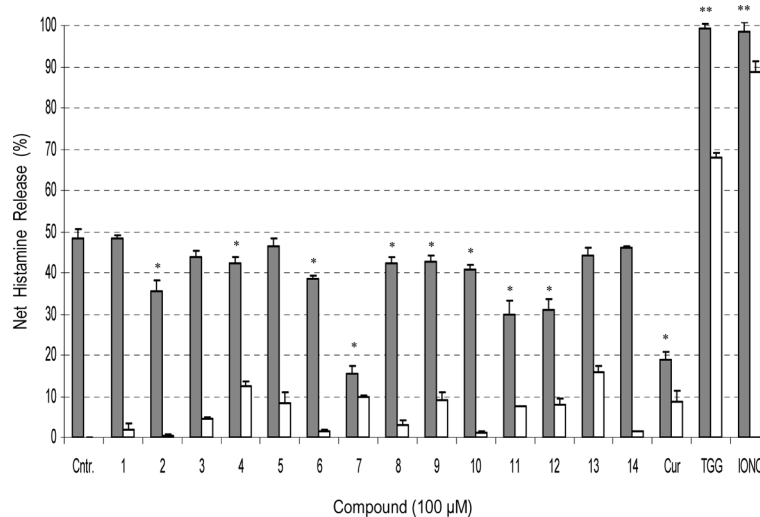


Fig. 3. Effect of Benzylidenecyclopentanone Analogues of Curcumin (100 μM) on Histamine Release from RBL-2H3 Cells in the Presence (Solid Bar) or Absence (Open Bar) of DNP-BSA 20 ng/ml as an Antigen to Stimulate Histamine Release

Data represent mean $\pm$ S.E.M. and are 3 independent experiments. Compounds 1–14 refer to Fig. 2. * Significant difference ($p < 0.05$) compared to the negative control value. ** The compound increased histamine release significantly ($p < 0.05$). Cur = curcumin, TGG = thapsigargin 0.5 μM , Iono = ionomycin 1 μM .

release were examined (Fig. 3). Four compounds that inhibited histamine release by more than 25% and that had minimal effects on spontaneous histamine release were selected for the next experiments. These compounds were: 2,5-bis(4-hydroxybenzylidene)cyclopentanone (compound **2**); 2,5-bis(4-hydroxy-3-methoxybenzylidene)cyclopentanone (compound **7**); 2,5-bis(4-hydroxy-3,5-dimethylbenzylidene)cyclopentanone (compound **11**); and 2,5-bis(4-hydroxy-3,5-diethylbenzylidene)cyclopentanone (compound **12**).

Concentration-Dependent Inhibitory Effects on Histamine Release from RBL-2H3 Cells Curcumin and four selected compounds were studied for their concentration-dependent inhibitory effects on histamine release from RBL-2H3 cells induced by DNP₂₄-BSA, thapsigargin or the ionophore ionomycin. The concentrations used in these experiments were 0.1, 0.3, 1, 3, 10, 30 and 100 μ M, and the IC₅₀ values of their inhibitory effects were used to compare potencies (Fig. 4).

DNP₂₄-BSA stimulated histamine release from RBL-2H3 cells by $52.1 \pm 0.5\%$ ($n=3$). In this study, all four compounds showed gradual inhibitory effects on histamine release from RBL-2H3 cells. At higher concentrations (3 μ M or greater), the inhibitory effects of curcumin and compound **7** increased progressively, whereas the effects of compounds **11** and **12** increase more gradually. Compound **2**, which has a hydroxy moiety on the aromatic ring, showed less potent inhibitory effects than those of the other compounds. Curcumin and compound **7** had the most potent inhibitory effects on histamine release (more than 50%), and the effect of compound **7** was more potent than that of curcumin.

Thapsigargin (0.5 μ M) and ionomycin (1 μ M), which act on Ca²⁺ influx and intracellular calcium pathways, stimulated histamine release by $74.3 \pm 7.1\%$ ($n=3$); and $78.8 \pm 9.1\%$ ($n=3$), respectively. In these experiments, curcumin and its analogues, compounds **7**, **11**, and **12** potently inhibited histamine release, whereas compound **2** showed only moderate inhibitory effects. Moreover, curcumin and compounds **7** and **11** at the highest dose (100 μ M) strongly inhibited histamine release. These effects of curcumin and its analogues were presumed to be related to intracellular Ca²⁺ signaling events in RBL-2H3 cells. Based on IC₅₀ values in experiments with RBL-2H3 cells, the potency order was compound **7** > curcumin > **11** > **12** > **2** (Table 1).

Concentration-Dependent Inhibitory Effects on Histamine Release from RPMCs The histamine release stimulants for RPMCs in this study were compound 48/80, thapsigargin, ionomycin and PMA (Fig. 5). Compound 48/80, acting directly on G proteins in mast cells increased histamine

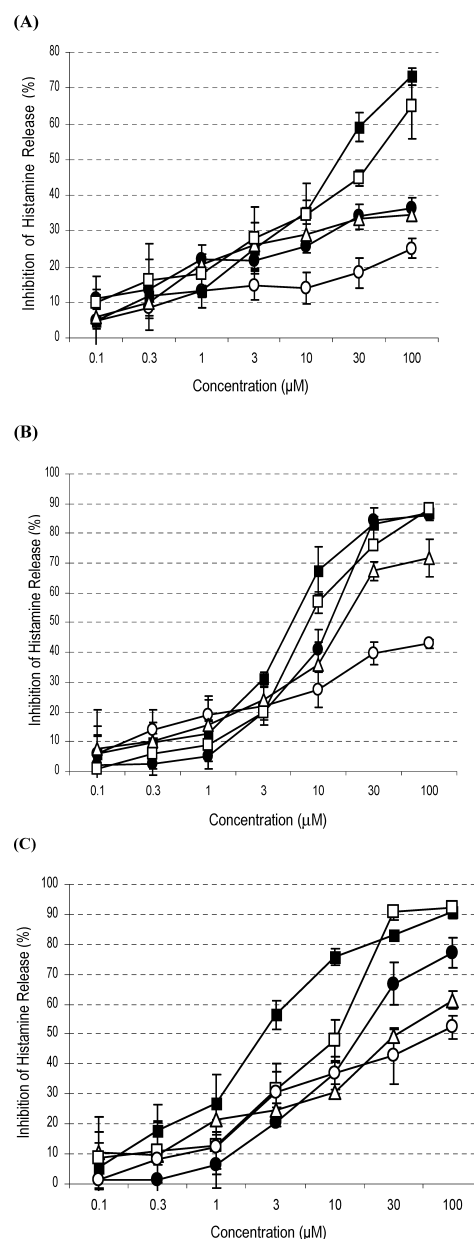


Fig. 4. Concentration-Dependence of Benzylidenecyclopentanone Analogues of Curcumin on Histamine Release from RBL-2H3 Cells Induced by DNP-BSA 20 ng/ml (A), Thapsigargin 0.5 μ M (B), or Ionomycin 1 μ M (C)

The data were representative of 3 independent experiments. $\circ$ =compound **2**; $\blacksquare$ =compound **7**; $\bullet$ =compound **11**; $\triangle$ =compound **12**; and $\square$ =curcumin.

Table 1. The Mean of IC₅₀ of Benzylidenecyclopentanone Analogues of Curcumin on the Histamine Release from RBL-2H3 Cells with Several Histamine Release Inducers

Compound	IC ₅₀ values (μ M)		
	DNP-BSA	Thapsigargin	Ionomycin
2,5-Bis(4-hydroxybenzylidene)cyclopentanone (compound 2)	>100	>100	82.55
2,5-Bis(4-hydroxy-3-methoxybenzylidene)cyclopentanone (compound 7)	22.38	6.64	2.56
2,5-Bis(4-hydroxy-3,5-dimethylbenzylidene)cyclopentanone (compound 11)	>100	14.21	16.11
2,5-Bis(4-hydroxy-3,5-diethylbenzylidene)cyclopentanone (compound 12)	>100	18.91	33.56
Curcumin	48.28	8.71	11.00

DNP-BSA 20 ng/ml; thapsigargin 0.5 μ M; ionomycin 1 μ M.

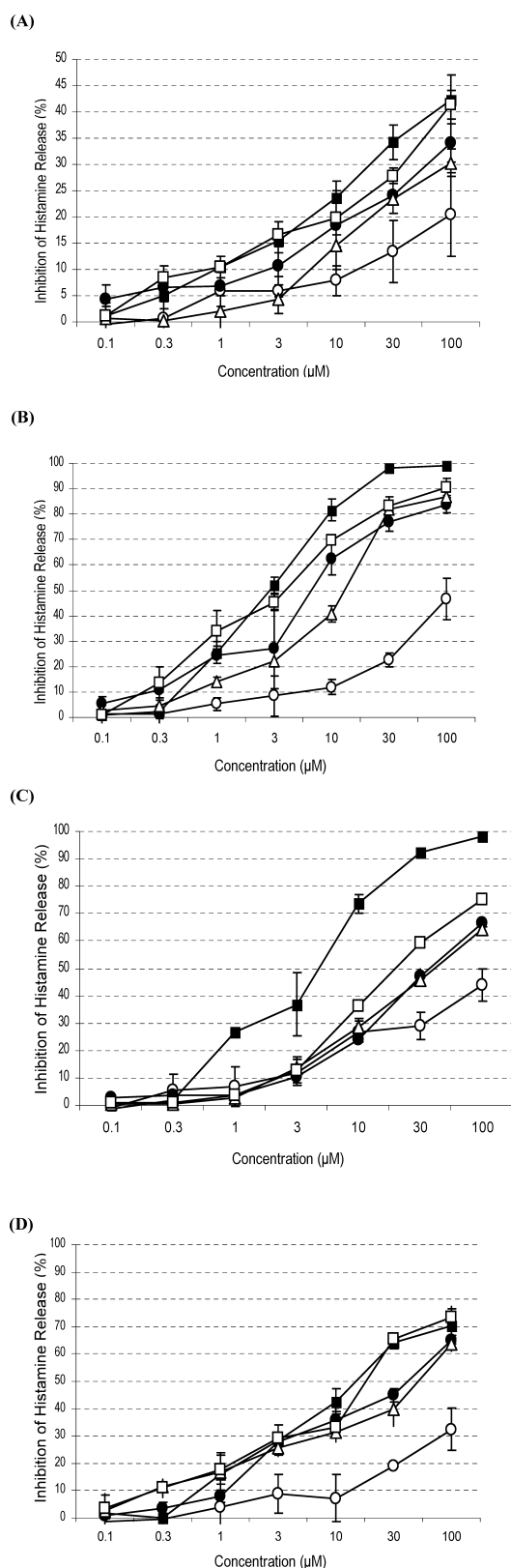


Fig. 5. Concentration-Dependence of Benzylidenecyclopentanone Analogues of Curcumin on Histamine Release from Rat Peritoneal Mast Cells Induced by Compound 48/80 $10 \mu\text{g/ml}$ (A), Thapsigargin $0.5 \mu\text{M}$ (B), Ionomycin $1 \mu\text{M}$ (C), or Phorbol Myristate Acetate 10 nM –Ionomycin $0.1 \mu\text{M}$ (D).

The data were representative of 3 independent experiments. ○=compound 2; ■=compound 7; ●=compound 11; △=compound 12; and □=curcumin.

release from RPMCs by $78.5 \pm 1.1\%$ ($n=3$) of the total cellular content of histamine. Curcumin and its four selected analogues mildly inhibited ($<50\%$) histamine release induced by compound 48/80.

Thapsigargin and ionomycin increased histamine release from RPMCs by $62.3 \pm 0.4\%$ ($n=3$); and $65.3 \pm 0.3\%$ ($n=3$), respectively. Curcumin and compounds 7, 11, and 12 showed inhibitory effects ($>50\%$) on histamine release. Moreover compound 7 strongly almost completely inhibited histamine release from RPMCs induced by either thapsigargin or ionomycin, $99.2 \pm 0.2\%$ ($n=3$) and $98.1 \pm 0.8\%$ ($n=3$), respectively.

The synergistic activation of diacylglycerol (DAG) and cytosolic Ca resulted in the degradation in RPMCs. In the preliminary experiment, we checked the dose response effects of the combination of PMA, a protein kinase C (PKC) activator, and ionomycin on histamine release in RPMCs. Histamine release induced by each 10 nM PMA and $0.1 \mu\text{M}$ of ionomycin itself, was $13.5 \pm 0.5\%$ ($n=3$) and $9.6 \pm 1.0\%$ ($n=3$), respectively, but by the combination of these was $61.5 \pm 0.6\%$ ($n=3$). We therefore chose these doses for the stimulation with the combination of PMA and ionomycin to get their synergistic action. The combination of PMA (10 nM) and low-dose ionomycin ($0.1 \mu\text{M}$) increased histamine release by $88.9 \pm 1.4\%$ ($n=3$) in RPMCs (shown in Fig. 5D). Curcumin and its selected analogues all inhibited PMA and ionomycin-induced histamine release, with compound 7 showing the highest potency. The potency order in all RPMC experiments was: compound 7 > curcumin > 11 > 12 > 2 (Table 2).

Inhibition of $^{45}\text{Ca}^{2+}$ Influx The effect of curcumin and its analogues on intracellular Ca^{2+} signaling was investigated by direct measurement of radiolabelled Ca^{2+} uptake in RBL-2H3 cells after stimulation with thapsigargin (Fig. 6). Curcumin and its analogues (compounds 7, 11 and 12) inhibited intracellular $^{45}\text{Ca}^{2+}$ incorporation dose-dependently. The potency order of their inhibitory effects on $^{45}\text{Ca}^{2+}$ influx was: compound 11 > 7 > curcumin > 12 > 2 (Table 3). Intracellular accumulation of $^{45}\text{Ca}^{2+}$ was almost completely inhibited at $100 \mu\text{M}$, the highest concentration tested.

DISCUSSION

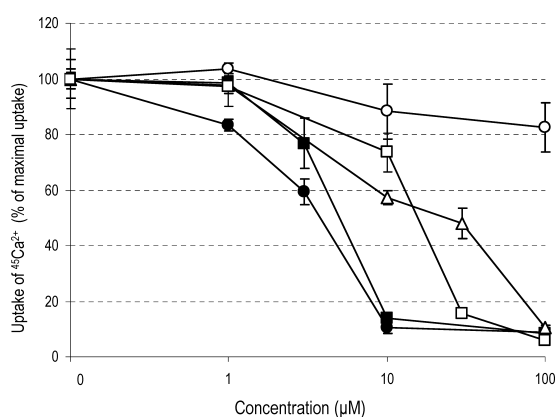
Benzylidenecyclopentanone analogues of curcumin have been synthesized in an attempt to improve upon the therapeutic properties of the parent compound. Sardjiman *et al.* synthesized a series of benzylidenecyclopentanone analogues of curcumin in order to improve upon the stability of curcumin.¹⁸⁾ Curcumin is stable at $\text{pH} < 7$, but readily decomposes at higher pH. Curcumin is also affected by light, which can cause it to decompose to ferulic aldehyde, ferulic acid, dihydroxynaphthalene product, 4-vinylguaiacol, vanillin and vanillic acid.^{16,17)} Cyclopentanone analogues of curcumin (Fig. 2) are thought to be more stable than the parent compound.

In the present study, curcumin and its analogues were first screened for their effects on histamine release in RBL-2H3 cells induced with DNP_{24} -BSA as an antigen. Furthermore, the concentration dependency of curcumin and four selected analogues were studied in: (1) RBL-2H3 cells treated with DNP_{24} -BSA, thapsigargin and ionomycin to induce his-

Table 2. The Mean of IC₅₀ of Benzyldenecyclopentanone Analogues of Curcumin on the Histamine Release from Rat Peritoneal Mast Cells with Several Histamine Release Inducers

Compound	IC ₅₀ values (μM)			
	C 48/80 ^{a)}	Thapsigargin	Ionomycin	PMA-IONO
2,5-Bis(4-hydroxybenzylidene)cyclopentanone (compound 2)	>100	>100	>100	>100
2,5-Bis(4-hydroxy-3-methoxybenzylidene)cyclopentanone (compound 7)	21.93	2.85	5.52	16.90
2,5-Bis(4-hydroxy-3,5-dimethylbenzylidene)cyclopentanone (compound 11)	71.62	7.56	40.12	47.11
2,5-Bis(4-hydroxy-3,5-diethylbenzylidene)cyclopentanone (compound 12)	96.82	14.51	46.29	60.21
Curcumin	41.18	4.39	21.94	20.36

a) IC₃₀ was used as a parameter instead of IC₅₀ value since the 50% inhibitory effect was not reached until the highest experimental concentration. C48/80=compound 48/80 10 μg/ml; thapsigargin 0.5 μM; ionomycin 1 μM; PMA-IONO=phorbol myristate acetate 10 nM-ionomycin 0.1 μM.

Fig. 6. Inhibition of ⁴⁵Ca²⁺ Uptake by Benzyldenecyclopentanone Analogues of Curcumin in Thapsigargin(0.5 μM)-Stimulated RBL-2H3 Cells

The data were representative of 3–5 independent experiments. ○=compound 2; ■=compound 7; ●=compound 11; △=compound 12; and □=curcumin.

Table 3. The Mean of IC₅₀ of Benzyldenecyclopentanone Analogues on ⁴⁵Ca²⁺ Uptake in Thapsigargin(0.5 μM)-Stimulated RBL-2H3 Cells

Compound	IC ₅₀ values (μM)
2,5-Bis(4-hydroxybenzylidene)cyclopentanone (compound 2)	>100
2,5-Bis(4-hydroxy-3-methoxybenzylidene)cyclopentanone (compound 7)	5.98
2,5-Bis(4-hydroxy-3,5-dimethylbenzylidene)cyclopentanone (compound 11)	4.35
2,5-Bis(4-hydroxy-3,5-diethylbenzylidene)cyclopentanone (compound 12)	25.71
Curcumin	18.11

mine release, and (2) RPMCs treated with compound 48/80, thapsigargin, ionomycin and PMA as histamine inducers. The results of this screening study provided information for the development of new pharmacological agents to treat allergic and inflammatory diseases.

In the present study, curcumin and some benzyldenecyclopentanone analogues inhibited DNP₂₄-BSA-induced histamine release from RBL-2H3 cells, suggesting that these compounds might alter the effect of DNP₂₄-BSA on RBL-2H3 cells by affecting its interaction with IgE on the cell surface or by altering intracellular signal transduction involved in degranulation. In the present study, compound 2,5-dibenzylidenecyclopentanone, a curcumin analogue without moi-

eties on the aromatic rings (see Fig. 2), did not influence histamine release from RBL-2H3 cells. Analogues with moieties attached at the *para* or/and *meta* position of the aromatic ring have been shown with varying potency, to inhibit histamine release. In the present study, compounds with hydroxy moieties at the *para* position inhibited histamine release from RBL-2H3 cells, while compounds without hydroxy moieties had weak effects on histamine release. These results indicate the importance of the *para* hydroxy moieties at the aromatic rings for anti-histamine releasing potency.

Four benzyldenecyclopentanone analogues of curcumin were shown to have the most potent inhibitory effects on histamine release from RBL-2H3 cells induced by DNP₂₄-BSA; in order of potency these were 2,5-bis(4-hydroxy-3-methoxybenzylidene)cyclopentanone>2,5-bis(4-hydroxy-3,5-dimethylbenzylidene)cyclopentanone>2,5-bis(4-hydroxy-3,5-diethylbenzylidene)cyclopentanone>2,5-bis(4-hydroxybenzylidene)cyclopentanone. All these potent compounds have hydroxy moieties at the *para* position, while all compounds without hydroxy moieties showed weak effects on histamine release. These results indicated that hydroxy moieties had an important role in the inhibitory effect on histamine release. Previous study reported that the hydroxy moiety of curcumin plays an important role in its anti-allergic effect because it influences antioxidant properties.¹⁴⁾ Most hydroxybenzylidenecyclopentanone analogues of curcumin showed antioxidative effect by inhibiting radical reaction-mediated lipid peroxidation.¹⁸⁾ However, diisopropyl- and ditert-butylbenzylidenecyclopentanone (compounds 13 and 14, respectively) only weakly inhibited histamine release even though these compounds have hydroxy moieties at the *para* position (see Fig. 2). This finding showed positive relationship with previous study¹⁸⁾ that the antioxidative effects of diisopropyl- and ditert-butylbenzylidenecyclopentanone were less potent than those of dimethyl- and diethylbenzylidene cyclopentanone (compounds 11 and 12). These results indicate that increasing bulkiness adjacent to the *para* hydroxy groups has a negative influence on anti-allergy activity. Since the hydroxy moiety of curcumin has been suggested to play an important role in its anti-allergic effect, the steric factor of diisopropyl- and ditert-butyl is more prominent than the stabilization factor and prefers to hinder the action of hydroxy moiety.

Compound 48/80 is known to activate mast cell secretory processes by increasing the rate of GTPγS binding to G-proteins (Go/Gi mixture).^{24,25)} In turn, the activation of G-proteins can trigger intracellular signaling events such as activa-

tion of phospholipase C (PLC), PKC and Ca^{2+} signaling, which ultimately results in the release of histamine from these cells. Compound 48/80 also stimulated histamine release from RPMCs in both the presence and absence of extracellular calcium.²⁶⁾ The present study demonstrates that curcumin and some of its benzyldenecyclopentanone analogues can inhibit histamine release from RPMCs induced by compound 48/80, although the inhibition did not exceed 50%. These findings suggest that curcumin and its analogues might inhibit G-protein-mediated signaling events.

Thapsigargin, a sesquiterpene lactone isolated from the plant *Thapsia garganica*, is a stimulant for histamine release from RPMCs. Its target is the ATP-dependent Ca^{2+} pump in the endoplasmic reticulum, and it can increase the concentration of cytosolic free calcium ion.^{21,27,28)} Ca^{2+} release from intracellular store plays a major role in the opening of cell membrane Ca^{2+} channels to cause Ca^{2+} influx in mast cells.²⁶⁾ Ionomycin, a selective Ca^{2+} ionophore, also induces histamine release from mast cells by increasing in intracellular Ca^{2+} concentration, both through the release from intracellular Ca^{2+} pools (endoplasmic reticulum) and *via* Ca^{2+} influx.²⁹⁾ In our study, curcumin and some of its benzyldenecyclopentanone analogues inhibited histamine release from RBL-2H3 cells and RPMCs induced by either thapsigargin or ionomycin. Hydroxymethoxybenzyldenecyclopentanone inhibited histamine release by 90% and close to 100% in RBL-2H3 cells and RPMCs, respectively. These data were supported by the finding that curcumin and its analogues potently suppressed intracellular $^{45}\text{Ca}^{2+}$ uptake (Fig. 6). Moreover, hydroxymethoxybenzyldenecyclopentanone suppressed strongly intracellular $^{45}\text{Ca}^{2+}$ uptake by more than 90%. These indicate that curcumin analogues alter or block the Ca^{2+} signaling in mast cells. The potent inhibitory effects of curcumin and its analogues on Ca^{2+} uptake may underlie the inhibitory effect of these compounds on histamine release from mast cells. Our results parallel those of a previous *in vitro* anti-inflammatory study which also showed that curcumin inhibits Ca^{2+} influx.³⁰⁾

In mast cells antigen stimulation leads to the activation of phospholipase C (PLC) γ 1 and one product of DAG causes the activation of PKC and another product, inositol 1,4,5-trisphosphate causes the movement of Ca^{2+} from the endoplasmic reticulum, which triggers store-operated Ca^{2+} entry through specialized Ca^{2+} release-activated Ca channels.³¹⁾ PMA alone elicited no histamine release in RBL-2H3 cells but with a suboptimal concentration of Ca ionophore caused the substantial histamine release.³²⁾ In RPMCs, PMA alone also caused a few amount of histamine release but the combination with Ca ionophore induced the high amount of histamine release.^{33,34)} Degranulation induced by antigen stimulation is involved in the synergistic activation of PKC and the increase in intracellular Ca concentration. The Ca influx causes the translocation of PKC from cytosol to the membrane and enhances its activity.^{35,36)} The fusion proteins synaptosome-associated protein (SNAP)-23, SNAP25 and syntaxin4 are target for PKC-mediated phosphorylation, suggesting that PKC might play a role in the terminal granule mobilization and membrane fusion events associated with the degranulation process.³⁷⁾ With respect to PKC, there are eleven isozymes of PKC which are classified in three families, the classical PKCs (α , β I, β II and γ), the novel PKCs

(δ , ϵ , θ and η) and the atypical PKCs (ζ , ι/λ) according to the dependence of both DAG and Ca^{2+} , only DAG and neither DAG nor Ca^{2+} , respectively. In mast cells α , β I, β II, δ , ϵ and θ PKCs contribute to signaling cascade leading to degranulation and generation of arachidonic acid metabolites mediators and cytokines.³⁷⁾ However, definite roles of PKC isozymes remain uncertain in mast cells because of conflicting reports due to heterogeneity of mast cells such as RBL-2H3 cells, mouse bone marrow derived mast cells and peritoneal mast cells and use of non-selective PKC modulators.³⁸⁾ In this study the combination of 10 nM PMA and low concentration of ionomycin (0.1 μM) was used to get the synergistic action in RPMCs. In RPMCs the involvement of PKCs, especially the classical PKCs, in degranulation is important. Curcumin and its analogues effectively inhibited histamine release from RPMCs induced by PMA and ionomycin in combination like compound 48/80, which suggests that these compounds may alter the interaction between PKC and intracellular Ca^{2+} during degranulation processes.

Finally, the hydroxybenzyldenecyclopentanone analogues of curcumin showed inhibitory effect on histamine release from both RBL-2H3 cells and RPMCs induced by several histamine stimulants such as DNP₂₄-BSA, thapsigargin, ionomycin, compound 48/80 and PMA. These indicated that hydroxybenzyldenecyclopentanone analogues of curcumin inhibit histamine release by altering some intracellular signaling events in mast cells. These curcumin analogues have most potent inhibitory effects on histamine release induced by Ca^{2+} stimulants, *i.e.*, thapsigargin and ionomycin, showing that their inhibitory effect on histamine release is closely related to Ca^{2+} signaling events in mast cells. Since the curcumin analogues suppressed strongly intracellular $^{45}\text{Ca}^{2+}$ uptake, the inhibitory effect on Ca^{2+} entry from extracellular space might be a major role in their inhibitory effect on histamine release from mast cells, although it is not excluded that these analogues may directly inhibit the movement of Ca^{2+} from intracellular Ca stores. The direct targets of these analogues should be clarified for the final conclusion.

The addition of moieties (*i.e.*, methoxy, methyl or ethyl) at the *meta* position of its aromatic rings enhanced the inhibitory effects on the histamine release. The inhibitory effects of methyl- and ethyl hydroxybenzyldenecyclopentanone were less potent than that of curcumin, but the methoxy analogues were slightly more potent. Since antioxidative of curcumin analogues are related to their anti-allergic effect, the stabilization of hydroxy moiety at aromatic ring has an important role on the antioxidative activity.¹⁸⁾ The addition of moieties (*i.e.*, methoxy, methyl or ethyl) is suggested enhancing the mesomeric stabilization of hydroxy moiety at aromatic ring. Methoxy moiety at the *meta* position has an influence to support electrons to the aromatic ring *via* resonance with the cloud of electrons surrounding the hydroxy moiety, causing a high reactivity especially for radical species.

The present results support those of a previous study which showed that the biological activity of 2,5-bis(4-hydroxy-3-methoxybenzylidene)cyclopentanone was better than that of curcumin.¹⁸⁾ The antioxidant and anti-cyclooxygenase activities of this compound are 2- and 7-times higher, and the anti-inflammatory activity 5-times higher than those of curcumin at a dose of 20 mg/kg, *p.o.* Moreover, the hy-

droxyl radical scavenging activity of this compound was also higher than that of curcumin. Free radicals derived from metabolites of unsaturated fatty acids participate in the induction of histamine release.^{39,40} Another previous study reported that the inhibitory effect of curcumin on histamine release was closely related to its antioxidant properties.¹⁴ The structure rigidity and stability of this compound may be responsible for the more potent effects as compared with curcumin, although both compounds have the same moieties at the aromatic ring. Cyclopentanone analogues that lack the active methylene moiety and one carbonyl moiety of curcumin and which include the hydroxy moiety, which is responsible for antioxidant activity, may have greater stability and rigidity than the parent compound.¹⁸

In conclusion, the hydroxymethoxybenzylidenecyclopentanone analogue of curcumin inhibits histamine release potently by altering some intracellular signaling events in mast cells, especially by blocking Ca^{2+} uptake into mast cells, and will be a good candidate for the anti-allergic and anti-inflammatory drug.

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