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Structure-Requirements of Isocoumarins, Phthalides, and Stilbenes from Hydrangeae Dulcis Folium for Inhibitory Activity on Histamine Release from Rat Peritoneal Mast Cells

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Abstract—We examined the structure–activity relationships of isocoumarins, phthalides and stilbenes isolated from Hydrangeae Dulcis Folium and related compounds for the inhibition of histamine release in rat peritoneal mast cells. The activities of isocoumarins such as thunberginols A and B were more potent than those of dihydroisocoumarins such as hydrangenol and thunberginol G. The double bond at the 3-position seemed to be essential to potentiate the activity. The hydroxyl groups at the 8-, 3′- and 4′-positions of isocoumarin were essential for the activity, while the hydroxyl group at the 6-position was scarcely needed. Since the activities of benzylidenephthalides such as thunberginol F were more potent than those of hydramacrophyllols A and B, the presence of a double bond at the 3-position was needed to increase the activity. Moreover, the hydroxyl group at the 8-position was essential for the activity. On the time course study, thunberginols A, B and F completely inhibited histamine release by pretreatment at 100 μM for 1 to 15 min, whereas DSCG inhibited histamine release only following 1-min pretreatment at 1000 μM. These results suggested that the mechanisms of the inhibitory effect of thunberginols are different from that of DSCG.

Introduction

Hydrangeae Dulcis Folium, a natural medicine indigenous to Japan, is prepared from the leaves of *Hydrangea macrophylla* SERINGE var. *thunbergii* MAKINO (Saxifragaceae) via several processing steps such as crumpling, fermentation and drying. This natural medicine is listed in the Japanese Pharmacopoeia XIII and is extensively used in confectionery, drinks and food as an oral refrigerant and a sweetening. Two dihydroisocoumarins, phyllodulcin (**1**) and hydrangenol (**3**), have been isolated as the principal constituents of this natural medicine (the processed leaves), while their 8-*O*-glucosides (**2**, **4**) were isolated from the dried leaves of *Hydrangea macrophylla* var. *thunbergii*.[1]

As part of our studies of the bioactive constituents of Hydrangeae Dulcis Folium,[1,2] the methanolic extract of this natural medicine was found to show potent antiallergic, antibacterial, antioxidative, antiulcer and cholagoic activities.[3] We have reported the isolation and structural characterization of two isocoumarins [thunberginols A (**11**) and B (**12**)],[4,5] three dihydroisocoumarins [thunberginols C (**6**), D (**7**), and E (**8**)],[6] a benzylidenephthalide [thunberginol F (**17**)],[4] three dihydroisocoumarin glucosides [thunberginol G 3′-*O*-glucoside, (+)- and (−)-hydrangenol 4′-*O*-glucoside (**5**)],[6] and two phthalides [hydramacrophyllols A (**19**) and B (**20**)][7] as the antiallergic and antibacterial principles of this natural medicine.

We have developed an efficient method for transforming dihydroisocoumarin into benzylidenephthalide and isocoumarin using regiospecific lactonization reaction of 2-carboxystilbenes.[7] During the course of these synthetic studies, various isocoumarins, phthalides and stilbenes were obtained, but their antiallergic activities have not been clarified. In this paper, we describe the inhibitory activities of isocoumarins, phthalides and stilbenes from Hydrangeae Dulcis Folium or the dried leaves of *Hydrangea macrophylla* var. *thunbergii* and their synthetic derivatives on the histamine release induced by antigen-antibody reaction other than compound 48/80 and calcium ionophore A23187, which were partially reported previously.[4–7] Their structural requirements for the inhibitory activity will also be discussed.

Results and Discussion

We investigated the structure–activity relationships of the constituents isolated from Hydrangeae Dulcis

Key words: Allergies; antigens; coumarins; natural products.

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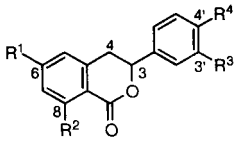
Folium and related compounds. Tables 1–4 show the IC_{50} values for inhibition of histamine release by 15-min pretreatment. Compound 48/80, widely used to analyze the mechanism of mast cell degranulation, mobilizes Ca^{2+} from intracellular Ca^{2+} storage sites followed by extracellular Ca^{2+} influx. This increase in Ca^{2+} concentration is almost entirely dependent on intracellular Ca^{2+}. Compound 48/80 acts to a pertussis toxin-sensitive GTP binding protein and activates phospholipase Cγ (PLCγ). As a result, the level of inositol-1,3,5-triphosphate (IP_3) increases followed by Ca^{2+} mobilization from the endplasmic reticulum.[8] This intracellular Ca^{2+}-dependent event progresses in less than 10 s and rapidly induces mast cell degranulation. The percentage of compound 48/80-induced histamine release was in the range of 14.6 to 34.0%. Thunberginols A (**11**), B (**12**), D (**7**), E (**8**) and F (**17**) and piceatannol (**25**) inhibited the histamine release with IC_{50} values of less than 100 μM. The inhibitory activities of isocoumarins such as **11** and **12** were more potent than those of dihydroisocoumarins, with IC_{50} values of 22 and 76 μM, respectively. The inhibitory activity of a benzilidenephthalide, **17**, was most potent among the examined compounds, and its IC_{50} value was 9.8 μM. Other than hydrangenol (**3**), **7**, **8**, **25** and lunurarin (**26**), many dihydroisocoumarins and stilbenes lacked the activity. A reference drug, DSCG, suppressed the histamine release at high concentration (37.7% inhibition at 1000 μM).

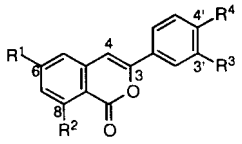
The calcium ionophore A23187 changes cell membrane permeability and induces transport of extracellular

Table 1. Inhibitory effects of dihydroisocoumarins on histamine release from rat peritoneal mast cells induced by compound 48/80, calcium ionophore A23187, and antigen–antibody reaction

Dihydroisocoumarins					IC_{50} (μM) on histamine release		
					Compound 48/80	Calcium ionophore A23187	Antigen–antibody reaction
	R^1	**R^2**	**R^3**	**R^4**			
Phyllodulcin (**1**)	H	OH	OH	OCH_3	NE[4)]	> 100 (35.0)[4)]	NE
Phyllodulcin 8-*O*-glucoside (**2**)	H	*O*-β-D-Glc	OH	OCH_3	NE[6)]	NE[6)]	NE
Hydrangenol (**3**)	H	OH	H	OH	> 100 (22.6)[4)]	NE[4)]	> 100 (16.4)
Hydrangenol 8-*O*-glucoside (**4**)	H	*O*-β-D-Glc	H	OH	NE[6)]	NE[6)]	NE
(−)-Hydrangenol 4′-*O*-glucoside (**5**)	H	OH	H	*O*-β-D-Glc	NE[6)]	NE[6)]	NE
Thunberginol C (**6**)	OH	OH	H	OH	NE[6)]	85.0[6)]	92.0
Thunberginol D (**7**)	OH	OH	OH	OH	90.0[6)]	NE[6)]	> 100 (47.0)
Thunberginol E (**8**)	OH	OH	OH	OCH_3	85.0[6)]	NE[6)]	93.0
Thunberginol G (**9**)	H	OH	OH	OH	NE[6)]	NE[6)]	89.0
Hydrangenol monomethyl ether (**10**)	H	OH	H	OCH_3	NE	NE	NE

Values in parentheses represent the % inhibition of compounds at 100 μM, and NE indicates less than 10% inhibition at 100 μM. Glc: glucopyranoside.

Table 2. Inhibitory effects of isocoumarins and disodium cromoglycate (DSCG) on histamine release from rat peritoneal mast cells induced by compound 48/80, calcium ionophore A23187, and antigen–antibody reaction

Isocoumarins and DSCG					IC_{50} (μM) on histamine release		
					Compound 48/80	Calcium ionophore A23187	Antigen–antibody reaction
	R^1	**R^2**	**R^3**	**R^4**			
Thunberginol A (**11**)	H	OH	OH	OH	22.0[4)]	9.4[4)]	64.0
Thunberginol B (**12**)	OH	OH	OH	OH	76.0[4)]	91.0[4)]	10.0
8-Deoxythunberginol A (**13**)	H	H	OH	OH	NE	NE	NE
Dehydrophyllodulcin (**14**)	H	OH	OH	OCH_3	NE[6)]	> 100 (18.3)[6)]	34.0
3′-Deoxythunberginol A (**15**)	H	OH	H	OH	NE[6)]	NE[6)]	NE
8,3′-Deoxythunberginol A (**16**)	H	H	H	OH	NE	NE	NE
DSCG					> 1000 (37.7)	> 1000 (16.7)	NE′

Values in parentheses represent the % inhibition of compounds at 100 μM or DSCG at 1000 μM, and NE and NE′ indicate less than 10% inhibition at 100 μM and at 1000 μM, respectively.

Table 3. Inhibitory effects of phthalides on histamine release from rat peritoneal mast cells induced by compound 48/80, calcium ionophore A23187, and antigen–antibody reaction

Phthalides				IC_{50} (μM) on histamine release		
				Compound 48/80	Calcium ionophore A23187	Antigen–antibody reaction
	C^3–C^8	R^1	R^2			
Thunberginol F (**17**)	C=C	H	OH	9.8[4)]	20.0[4)]	10.0
3′-Deoxythunberginol F (**18**)	C=C	H	H	NE	NE	NE
Hydramacrophyllol A (**19**)	C–C	β–OH	H	NT	NT	> 100 (42.2)[7)]
Hydramacrophyllol B (**20**)	C–C	α–OH	H	NT	NT	> 100 (46.8)[7)]

Values in parentheses represent the % inhibition of compounds at 100 μM. NE and NT indicate less than 10% inhibition at 100 μM and "not tested", respectively.

Table 4. Inhibitory effects of stilbenes on histamine release from rat peritoneal mast cells induced by compound 48/80, calcium ionophore A23187, and antigen–antibody reaction

Stilbenes					IC_{50} (μM) on histamine release		
					Compound 48/80	Calcium ionophore A23187	Antigen–antibody reaction
	C^7-C^8	R^1	R^2	R^3			
Hydrangeaic acid (**21**)	C=C	COOH	H	H	NE[6)]	NE[6)]	NE
3′-Hydroxyhydrangeaic acid (**22**)	C=C	COOH	H	OH	NE	> 100 (37.9)	NE
5-Hydroxyhydrangeaic acid (**23**)	C=C	COOH	OH	H	NE	> 100 (27.8)	NE
Resveratrol (**24**)	C=C	H	OH	H	NE	NE	90.7
Piceatannol (**25**)	C=C	H	OH	OH	88.5	> 100 (16.7)	82.5
Lunurarin (**26**)	C–C	H	H	H	> 100 (46.5)	75.9	> 100 (23.4)
Dihydroresveratrol (**27**)	C–C	H	OH	H	NE	NE	NE

Values in parentheses represent the % inhibition of compounds at 100 μM, NE indicates less than 10% inhibition at 100 μM.

Ca^{2+} into the cells. This dynamic Ca^{2+} influx elevates intracellular Ca^{2+} level and induces mast cell degranulation.[9] The percentage of A23187-induced histamine release was in the range of 14.7 to 25.9. Thunberginols A (**11**), B (**12**), C (**6**) and F (**17**) and lunurarin (**26**) inhibited the histamine release and their IC_{50} values were less than 100 μM. Thunberginols A (**11**) and F (**17**) strongly inhibited this histamine release as well as that induced by compound 48/80 with IC_{50} values of 9 and 20 μM, respectively. On the other hand, an isocoumarin, **12**, showed less activity than **11** and **17**. Phyllodulcin (**1**), dehydrophyllodulcin (**14**), 3′-hydoxyhydrangeaic acid (**22**), 5-hydroxyhydrangeaic acid (**23**) and piceatannol (**25**) slightly inhibited the histamine release. DSCG showed only a low level of activity (16.7% inhibition at 1000 μM).

The signal transmission pathway via antigen-antibody reaction is initiated by IgE cross-linking by a specific antigen. The aggregation of high affinity IgE receptors (FcεRI) induces phosphorylation of the β and γ subunits of FcεRI[10] followed by activation of tyrosine kinases such as Lyn and Syk.[11,12] These tyrosine kinases phosphorylate phospholipase Cγ (PLCγ).[13] Subsequently, PLCγ hydrolyzes membrane phospholipids and produces diacyl glycerol (DG) and IP_3. The elevation of IP_3 leads to Ca^{2+} mobilization from intracellular Ca^{2+} storage sites[14] followed by an influx of extracellular Ca^{2+}.[15] The rise in intracellular Ca^{2+} level causes mast cell degranulation and production of metabolic arachidonate mediators. The percentage of IgE-mediated histamine release was in the range of 15.0 to 32.4%. Thunberginols A (**11**), B (**12**), C (**6**), E (**8**), F (**17**), and G (**9**), dehydrophyllodulcin (**14**), resveratrol (**24**) and piceatannol (**25**) showed inhibitory activities at less than 100 μM. The inhibitory activity of **11** on antigen-induced histamine release was less than those of compound 48/80 or calcium ionophore A23187 (IC_{50} value:

64 μM). On the other hand, **12** showed more potent activity than compound 48/80 or the calcium ionophore A23187 (IC_{50} value: 10 μM). Thunberginol F (**17**) also showed potent activity with an IC_{50} value of 10 μM. Thunberginol D (**7**), hydrangenol (**3**), hydramacrophyllols A and B (**19** and **20**) and lunurarin (**26**) slightly inhibited histamine release. DSCG showed no activity under these conditions.

In comparison of the inhibitory effects of active isocoumarins (**11**, **12**) or their 3-dehydro compounds (**9**, **7**) on histamine release induced by various stimuli, the activities of isocoumarins seemed to be more potent than those of dihydroisocoumarins. The presence of a double bond at the 3-position contributes to fixation of the 3-phenyl group to the lactone moiety, so that isocoumarins preserve the plane structure. On the other hand, as the 3-phenyl group of a dihydroisocoumarin is not fixed to the lactone moiety, its position was suggested to be crooked or rotated relative to the lactone moiety. The plane structure of the isocoumarin was presumed to contribute to the expression and enhancement of the activity.

Thunberginol A (**11**) more potently inhibited histamine release induced by non-physiological stimulants than by antigen-antibody reaction. The effect of **11** on stimulation by the calcium ionophore A23187 was more potent than on that by compound 48/80. In contrast, **12** showed more potent activity on histamine release via antigen-antibody reaction than those mediated by non-physiological stimulants. The effects of **12** on stimulation by non-physiological stimulants were similar. These observations suggested that **11** mainly inhibits the Ca^{2+} increase induced by non-physiological Ca^{2+} mobilizers, especially extracellular Ca^{2+} influx, and lower pathways leading to histamine release. On the other hand, **12** seemed to suppress the intracellular Ca^{2+} increase and other signal transduction pathways initiated by antigen-antibody reaction such as tyrosine phosphorylation.

Kimura et al. reported the inhibitory effects of 4-acyl isocoumarins such as oosponol (4-hydroxymethylketone-8-hydroxyisocoumarin), metabolic products isolated from *Oospora astringens* (air-borne fungi originated from house dust), on compound 48/80-induced histamine release.[16] They also reported the structure–activity relationships which an acyl group at the 4-position was important for inhibition of histamine release.

From the results of our investigation of isocoumarins and dihydroisocoumarins, the 8-hydroxyl group appears to be essential for the activity. That is, hydrangenol (**3**) and thunberginol A (**11**) suppressed histamine release, while hydrangenol 8-*O*-glucoside (**4**) and 8-deoxythunberginol A (**13**) lacked activity. The reason why the hydroxyl group at the 8-position is essential for the activity is unclear. However, it may be important for the proton of the 8-hydroxyl group to form a hydrogen bond to an adjacent carbonyl group at the 1-position.

The 4′-hydroxyl group seemed to be essential because **3** showed inhibitory activity, while (−)-hydrangenol 4′-*O*-glucoside (**5**) and hydrangenol monomethyl ether (**10**) did not. Comparison of phyllodulcin (**1**), thunberginol E (**8**) and **10** indicated that the 3′-hydroxyl group recovered the activity of the inactive compound with the 4′-methoxyl group. Moreover, the profile of the activity of dehydrophyllodulcin (**14**) was subtly different from that of thunberginol A (**11**). The mechanisms of action of these compounds may be different.

The 3′-hydroxyl group in 3-phenyl isocoumarins with the 8-hydroxyl group seemed to be essential, because **11** suppressed histamine release, while 3′-deoxythunberginol A (**15**) did not.

On the other hand, the 6-hydroxyl group did not seem to be important for the activity based on comparison between **1** and **8**, **3** and **6**, **7** and **9**, and **11** and **12**. However, as the profile of the activity of **11** on various types of histamine release reactions was different from that of **12**, the presence of the 6-hydroxyl group may contribute slightly to inhibition of signal transmission on different types of histamine release.

From the results of inhibitory activities of phthalides, only thunberginol F (**17**), which conforms to the plane structure due to the presence of a double bond at the 3-position, potently inhibited histamine release. The activities of hydramacrophyllols (**19**, **20**), in which the 8-phenyl groups do not conform to the plane structure against phthalides rings, showed less activity. 3′-Deoxythunberginol F (**18**) lacking the 3′-hydroxyl group showed no activity. These results suggested that the plane structure and the 3′-hydroxyl group are essential for the activity similarly to the case of isocoumarins.

In the case of stilbenes, piceatannol (**25**) potently inhibited the histamine release induced by antigen–antibody reaction. Tsuruga et al. reported that **25** inhibited histamine release from rat peritoneal mast cells induced by compound 48/80 and concanavalin A.[17] Our observations for **25** using antigen–antibody reaction agreed with this report, although the structure–activity relationships were not clarified.

The time courses of the effects of the active compounds, thunberginols A (**11**), B (**12**) and F (**17**), and DSCG

Table 5. Time course study of thunberginol A (**11**), B (**12**), F (**17**) and disodium cromoglycate (DSCG) on histamine release from rat peritoneal mast cells induced by antigen–antibody reaction

		% Inhibition of histamine release Preincubation time (min)		
	Conc. (μM)	1	5	15
Thunberginol A (**11**)	100	97.2	100.1	125.3
Thunberginol B (**12**)	100	122.3	74.7	87.4
Thunberginol F (**17**)	100	105.8	102.4	91.3
DSCG	1000	95.6	17.1	−1.4

Values represent the means of 3 to 4 experiments.

were examined (Table 5). Thunberginols A (**11**), B (**12**) and F (**17**) potently inhibited the histamine release by 1- to 15-min pretreatment at the concentration of 100 μM. DSCG showed an inhibitory effect after only 1-min pretreatment at the concentration of 1000 μM, and no inhibitory effects were observed following by 5- or 15-min pretreatment. These results suggested that the inhibitory mechanism of thunberginols is different from that of DSCG.

Conclusion

The structural requirements of constituents of Hydrangeae Dulcis Folium and related compounds for inhibitory activity on mast cell degranulation were clarified as follows. The activities of isocoumarins such as thunberginols A (**11**) and B (**12**) were more potent than those of dihydroisocoumarins such as hydrangenol (**3**) or thunberginol G (**9**). The double bond at the 3-position seemed to be essential for the activity. The hydroxyl groups at the 8-, 3′- and 4′- positions of isocoumarin were essential for the activity, while the hydroxyl group at the 6-position was scarcely needed. Since the activity of a benzylidenephthalide, thunberginol F (**17**), was more potent than those of hydramacrophyllols (**19**, **20**), the existence of a double bond at the 3-position appeared to be necessary to increase the activity. Moreover, the hydroxyl group at the 8-position was essential for the activity. The inhibitory mechanism of thunberginols A (**11**), B (**12**) and F (**17**) seemed to be different from that of DSCG.

Experimental

Animals

Rats were purchased from Kiwa Laboratory Animals Co., Ltd. (Wakayama, Japan) and housed in an air-conditioned room at 23 ± 2°C for more than 3 days. Standard laboratory chow (MF, Oriental Yeast Co., Ltd.) and tap water were given freely.

Materials

Anti-dinitrophenyl (Anti-DNP) IgE was purchased from Seikagaku Co., Ltd., and divided into small volumes and stocked at −20°C. Dinitrophenylated bovine serum albumin (DNP-BSA) was prepared from bovine serum albumin fraction V (Sigma) and sodium dinitrobenzenesulfonate (Tokyo Kasei Organic Chemicals Co., Ltd.) according to the method of Tada et al.[18] Disodium cromoglycate (DSCG) was obtained from Biomol Res. Lab. Inc. The mast cell medium (MCM) consisted of 150 mM NaCl, 2.7 mM KCl, 0.9 mM $CaCl_2$, 5.6 mM glucose, and 5 mM HEPES in distilled water, and pH was adjusted to 7.4 by addition of 3N NaOH. The test samples were isolated or synthesized according to previous reports.[1–7] For histamine release test, each compound was diluted in DMSO and dispersed with MCM. The final concentration of DMSO was 0.1%.

Histamine release from rat peritoneal mast cells induced by compound 48/80, calcium ionophore A23187, and antigen–antibody reaction

Histamine release test was performed according to a slight modification of the method reported previously.[4–7]

Collection of rat peritoneal exudate cells

Male Wistar rats weighing 300 to 400 g were sacrificed, and 10 mL of cooled MCM was injected into the abdominal cavity. After gentle massage of their belly for 2 min, the cavity was opened and the exudate fluid was removed to a plastic tube using a plastic pipette. The abdominal cavity was repeatedly washed with 10 mL of MCM. The exudate fluid was centrifuged (120×*g*, 4°C, 10 min) and washed 3 times with MCM. The peritoneal exudate cells were suspended in 3 mL of MCM. After staining cells with 0.5% toluidine blue in 0.1M citrate buffer (pH 4.8), the number of mast cells was counted under a microscope. The viability of peritoneal cells was determined by the trypan blue exclusion test.

Purification of rat peritoneal mast cells and histamine releaser-induced histamine release

Mast cells were purified from peritoneal exudate cells by the Percoll density gradient separation method.[19] Percoll fluid (Pharmacia) and decuple concentrated HAM F-10 medium (Gibco) were mixed at a ratio of 9:1, and 7 mL of the fluid was decanted into a polycarbonate centrifuge tube. The tube was centrifuged (17000×*g*, 4°C, 20 min) using an angle rotor. One mL of peritoneal exudate cells suspension was applied gently on top of the Percoll density gradient, followed by centrifugation (200×*g*, 4°C, 20 min) in a swing rotor. After removal of the erythrocyte and monocyte layers near the surface of the gradient fluid, the mast cell fraction was collected. The mast cell fraction was washed 3 times with MCM (200×*g*, 4°C, 10 min), and suspended in 3 mL of MCM. The number of mast cells was counted after toluidine blue staining, and the viability of cells was checked by the trypan blue exclusion test. The mast cell suspension (10^4 cells/1.8 mL) in a plastic tube was preincubated at 37°C for 10 min, and 200 μL of the test sample diluted in MCM was added. After incubation for 15 min, 222 μL of compound 48/80 (5 μg/mL) or calcium ionophore A23187 (10 μg/mL) was added, and incubation was continued for 10 min. The plastic tube was cooled in ice water for more than 10 min and centrifuged (120×*g*) for 10 min at 4°C. The released histamine in supernatant was determined by the *o*-phthaldialdehyde fluorescence method using HPLC.[20]

Sensitization of mast cells with anti-DNP IgE and antigen–antibody reaction induced-histamine release from mast cells

One mL of anti-DNP IgE (PCA titer: ×1000, diluted in MCM) was added to 3 mL of peritoneal exudate cell suspension in a plastic tube, and incubated at 37°C for 1 h. After sensitization, the tube was cooled in ice water, and the peritoneal exudate cells were washed 3 times

with 5 mL of MCM. The mast cells in peritoneal exudate cell suspension were adjusted to 10^4 cells/1.62 mL, and incubated at 37°C for 10 min. The test sample diluted with 180 μL of MCM was added and incubated for 15 min. One- and 5-min pretreatments with **11**, **12**, **17** and DSCG were also performed for time course study. Antigen–antibody reaction was initiated by simultaneous addition of 200 μL of phosphatidyl L-serine (1 mg/mL) and 222 μL of DNP-BSA (1 mg/mL). After 20 min incubation, the tube was cooled in ice water for more than 10 min and centrifuged (120×*g*) for 10 min at 4°C. The released histamine in the supernatant was determined by the method described previously. The inhibition (%) was calculated according to the following equation.

$$\text{Inhibition}\,(\%) = \left[1 - \frac{(\text{Histamine released by reaction} - \text{Spontaneously released histamine})}{(\text{Total histamine in mast cells} - \text{Spontaneously released histamine})}\right] \times 100$$

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