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Flavones inhibit proliferation and increase mediator content in human leukemic mast cells (HMC-1)

Alexandrakis M, Letourneau R, Kempuraj D, Kandere-Grzybowska K, Huang M, Christodoulou S, Boucher W, Seretakis D, Theoharides TC. Flavones inhibit proliferation and increase mediator content in human leukemic mast cells (HMC-1).
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Abstract: *Objective*: Mast cells are involved in allergic and inflammatory reactions. These cells are also increased in the bone marrow, skin, and other organs in systemic mastocytosis. Flavonoids are naturally occurring molecules with antioxidant, cytoprotective, and anti-inflammatory activities. Some flavonoids, like quercetin, inhibit the growth of certain malignant cells in culture. Quercetin also inhibits histamine release and induces accumulation of secretory granules in rat basophilic leukemia cells. *Method*: We investigated the effect of five flavonoids: flavone, kaempferol, morin, myricetin, and quercetin at 1, 10, and 100 μM on proliferation and secretory mediator content (β-hexosaminidase, histamine, and tryptase) in human leukemic mast cells (HMC-1), the doubling time of which was about 2 d. *Results*: Flavone and kaempferol at 100 μM inhibited cell proliferation over 80% on either day 3, 4, or 5 of culture. Quercetin showed this level of inhibition only on day 5, myricetin inhibited by 50% at days 3–5, whereas morin's inhibition was $<20\%$. All flavonoids (except morin) at 100 μM increased histamine and tryptase content, but not β-hexosaminidase, equally at days 3 and 4 of culture quercetin also increased the development of secretory granules. *Conclusion*: These results indicate that certain flavonoids can inhibit HMC-1 proliferation, induce secretory granule development and the accumulation of mediators.

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Key words: mast cells; flavonoids; quercetin; β-hexosaminidase; histamine; tryptase

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Mast cells play an important role in early and late phase allergic reactions through allergen and IgE-dependent release of histamine, proteases, prostaglandins, and several multifunctional cytokines (1). Mast cell proliferation has been well documented in the bone marrow and skin of systemic mastocytosis (2), as well as in urinary bladder of interstitial cystitis patients (3). Human leukemic mast cells (HMC-1) are useful for the study of growth characteristics, synthesis of mediators, and development of secretory granules because they secrete histamine, tryptase, and many cytokines (4), but only to prolonged stimulation with phorbol esters and the cation ionophore A23187 (5). Flavonoids are low molecular weight compounds found in many plants; they exhibit antioxidant, anti-allergic activity, and anti-inflammatory activity (6). Some flavonoids, like quercetin, inhibit histamine release from rat basophils and mast cells (7–10). Quercetin also induces histamine accumulation, expression of mRNA for RMCP-II and development of secretory granules in rat basophil leukemia (RBL) cells (11). Myricetin strongly inhibits histamine and leukotriene B_4 (LTB_4) release from rat peritoneal exudate cells (12) and down-regulates nuclear factor-kappa B (NF-κB) systems that are involved in immune and inflammatory responses (13). Flavonoids such as quercetin, baicalin, and luteolin have also been reported to inhibit leukotrienes, prostaglandins D_2, and cytokines such as IL-6 and tumor necrosis factor-alpha (TNF-α) from human mast cells (10). Flavonoids have also been shown to have anti-proliferative effects on a number of transformed cells (6). In the present study, we investigated the

effect of certain flavonoids with a particular structure (flavone, kaempferol, morin, myricetin, and quercetin) on the proliferation, development of secretory granules and accumulation of granule-stored mediators β-hexosaminidase, histamine, and tryptase in HMC-1. Our results indicate that the flavones studied, except morin, inhibit HMC-1 proliferation and induce the development of secretory granules with increased accumulation of histamine and tryptase.

Material and methods

Material

Flavone, kaempferol, morin, myricetin, and quercetin were obtained from Sigma (St Louis, MO, USA). They were dissolved in propylene glycol, filter-sterilized and diluted in culture medium for working dilutions. HMC-1 were kindly provided by Dr J. H. Butterfield (Mayo Clinic, Rochester, MN, USA). They were grown (5) in modified Dulbecco's medium (GIBCO, Grand Island, NY, USA) containing 10% bovine calf serum (GIBCO) and 1.2 mM monothio-glycerol (Sigma) either in 25 cm^2 tissue culture plates (Corning, New York, NY, USA) or in six-well tissue culture plates (Costar, Cambridge, MA, USA) in 5% CO_2 balanced oxygen at 37°C. HMC-1 were plated at 0.2×10^6 cells per milliliter (from 3-d-old culture) and were incubated with each flavonoid at 1, 10, and 100 μM for 3, 4, or 5 d of culture.

Cell viability

HMC-1 (50 μL) with or without flavonoids were harvested from each plate ($n = 3$), centrifuged and resuspended in phosphate buffered saline (PBS). Cell viability was determined by the standard Trypan blue (0.1%) exclusion method and the viability was expressed as the percentage of cells that did not take-up trypan blue.

Cell proliferation assay

HMC-1 (0.2×10^6 cells per milliliter) incubated with or without flavonoids were counted after 3, 4, or 5 d of culture. The inhibition of proliferation was calculated by the formula $[(N_c - N_f)/N_c] \times 100\%$, where N_c is the live cell numbers in control culture and N_f is the live cell numbers in culture treated with the particular flavonoid.

β-Hexosaminidase, histamine, and tryptase content

HMC-1 were incubated with 10 or 100 μM of each flavonoid for 3, 4, or 5 d. The β-hexosaminidase was measured as follows: samples (50 μL) were mixed with 450 μL substrate (Sigma) cocktail (0.2 M Na_2HPO_4, 0.4 M citric acid 200 mg *p*-nitrophenyl-*n*-acetyl-*o*-glucosaminidine) and incubated for 2 h at 37°C. The enzymatic reaction was stopped by adding 1.5 mL of 0.2 M glycine (pH 10.7). Sample absorbance was read in a spectrophotometer at a wavelength of 405 nm and results are expressed as arbitrary units. Histamine was measured by radio immunoassay (RIA) (AMAC, Inc., Westbrook, ME, USA) and tryptase by Unicap (Kabi Pharmacia Diagnostics, Inc., Piscataway, NY, USA). The results are presented as ng/mL for histamine and tryptase.

Staining of HMC-1

HMC-1 were harvested from various culture conditions. After washing with PBS, cells were cytocentrifuged onto gelatin coated glass slides, fixed for 5 min with methanol and then air-dried. Cells were stained for 3 min with May-Grunwald (Sigma) and Giemsa (Sigma) for 10 min at room temperature (RT); they were then washed with distilled water and air-dried. An average of 50 cells from each condition were evaluated by two researchers blinded to the experimental conditions and the representative cells were photographed.

Electron microscopy of HMC-1

HMC-1 were treated with quercetin at 100 μM for 3–5 d. Adherent HMC-1 were washed at 4°C in PBS and fixed for 1 h at RT with 3% glutaraldehyde in 0.1 M cacodylate-HCl buffer (pH 7.2). The cells were then scraped and pelleted at 370 ***g*** for 10 min. They were then washed with cacodylate buffer for two times and postfixed with 1% OsO_4 in cacodylate buffer for 1 h at RT; they were again washed with cacodylate buffer and pelleted. Cells were then infiltrated with 100% propylene oxide (two times 10 min each), followed by overnight exposure to 1 : 1 mixture of propylene oxide and DMP-30. Then, the cells were embedded in outgassed Epon with DMP-30 and were placed in a 56°C oven to polymerize for 48 h. Sections (1000 Å) were stained with both uranium and lead salts and examined using a Phillips 300 transmission electron microscope (FET Philips Electron Optics CMD, Eindhoven, The Netherlands) (11). An average of 20 cells from each condition were evaluated by two researchers blinded to the experimental conditions and representative fields were photographed.

Statistical analysis

Values were calculated only for days 3 and 4 of culture, as the effect on cell proliferation on day 5

was similar, but the cell viability dropped. Results were expressed as the mean ± SD. The results were analyzed by ANOVA; significance was denoted by $P < 0.05$.

Results

Growth curve of HMC-1

The doubling time of these cells was about 2 d (day $0 = 1 \times 10^6$, day $1 = 1.1 \times 10^6$, day $2 = 1.8 \times 10^6$, day $3 = 5 \times 10^6$, day $4 = 1.8 \times 10^7$, and day $5 = 2.8 \times 10^7$), unlike the original culture generated in which the doubling time was reported to be 3 d (5) (Fig. 1).

Effect of flavonoids on HMC-1 viability

Viability was nearly 100% at 100 and 10 μM of all flavonoids at both days 3 and 4 of culture; however, on day 5 of culture, viability decreased about to 80% with 100 μM of all flavonoids except morin (results not shown). Consequently, the effect of flavonoids on the mediator content of HMC-1 was investigated for days 3 and 4 only.

Effect of flavonoids on the proliferation of HMC-1

HMC-1 were incubated with either flavone, kaempferol, morin, myricetin, or quercetin at 1, 10, or 100 μM for 3, 4, or 5 d in culture. Then the cells in each experimental condition were counted. Flavone = kaempferol = quercetin > morin decreased the proliferation of HMC-1 in a dose-dependent manner with maximal inhibition on day

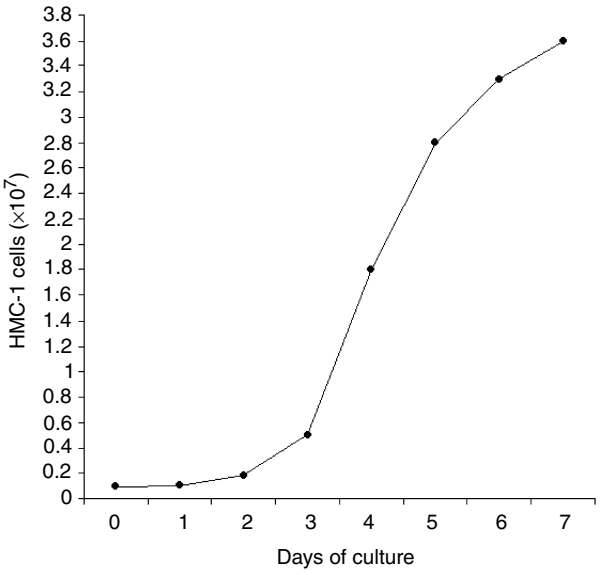

Fig. 1. Growth curve of HMC-1.

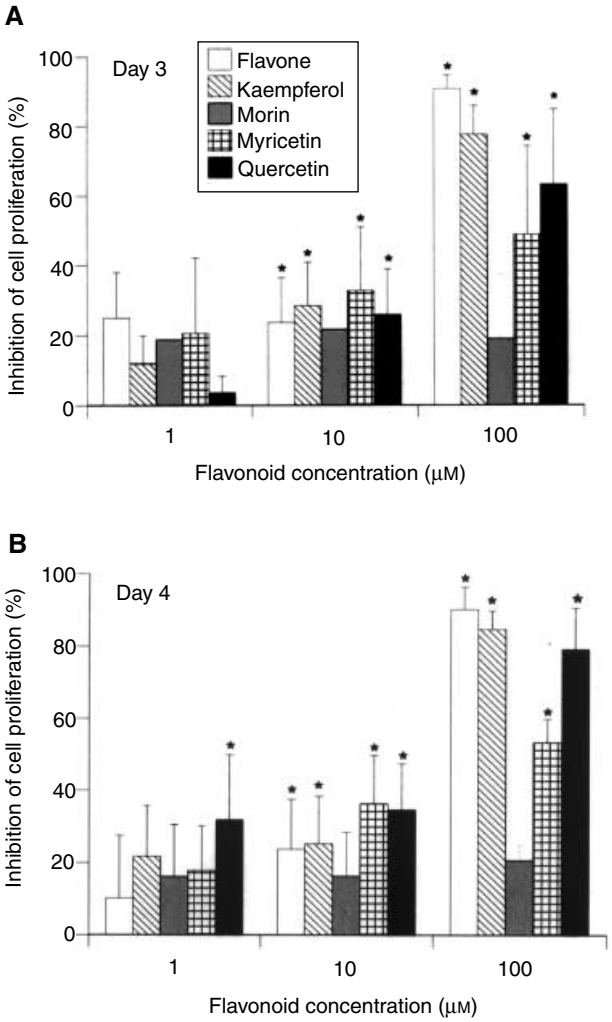

Fig. 2. Effect of flavonoids on HMC-1 proliferation. HMC-1 were incubated with flavone ($n = 5$); kaempferol ($n = 6$); myricetin ($n = 4$); quercetin ($n = 6$), and morin ($n = 3$) at 1, 10, and 100 μM for days 3 (A) and 4 (B) in culture. HMC-1 then were counted in all conditions and the percentage inhibition of proliferation was calculated. Number in parentheses indicate the number of experiments carried out. $^*P < 0.05$.

3 (Fig. 2) that remained at the same level on days 4 (Fig. 2B) and 5 (results not shown). Maximal inhibition of proliferation was obtained with flavonoid concentrations of 100 μM. There was no statistically different inhibition between 10 and 1 μM (Fig. 2). Inhibition of cell proliferation on day 4 did not differ significantly from that on day 3 (Fig. 2A,B). Morin had no effect on cell proliferation and, therefore, was not used in further experiments.

Effect of flavonoids on the morphology of HMC-1

HMC-1 were examined with May-Grunwald and Giemsa staining. Control cells from days 3 and 4

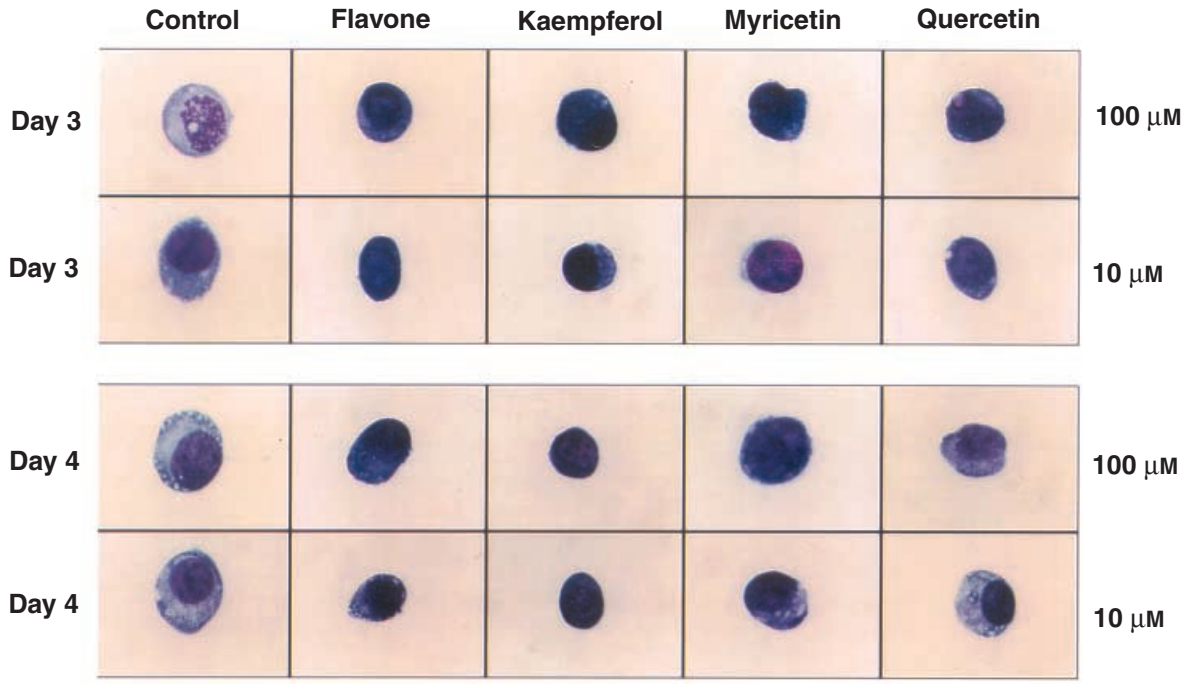

Fig. 3. Light photomicrographs of HMC-1 grown in presence of various flavonoids. HMC-1 were incubated with flavone ($n = 5$); kaempferol ($n = 6$); myricetin ($n = 4$) and quercetin ($n = 6$), at 10 or 100 μM for days 3 and 4 in culture. Micrographs are representatives of each condition. Note the density of dark purple staining because of reduction in cytoplasm and accumulation of secretory granules in the cells grown at 100 μM of flavonoids compared with control cells. Magnification 400×.

(Fig. 3) had eccentric nuclei and large cytoplasmic area that did not show any granular staining. However, HMC-1 cultured in the presence of flavone, kaempferol, myricetin, or quercetin at 10 or 100 μM had reduced cytoplasmic area, many secretory granules that often obscured the nucleus (Fig. 3).

Electron micrographs of control cells contained large nuclei, many mitochondria, and rare immature secretory granules; vacuoles without any stored electron-dense material were often observed close to the plasma membrane (Fig. 4A, black arrows). HMC-1 grown in the presence of 100 μM quercetin for 3 d (Fig. 4B) or 4 d (Fig. 4C) contained many secretory granules filled with varying amounts of electron-dense material (Fig. 4C,D, white arrows). In addition, many lipid bodies were also present in day 4 cells (Fig. 4D, asterisks). The variable extent of granule filling and different textures of the electron-dense material could be appreciated at high magnification (Fig. 4D).

Effect of flavonoids on β-hexosaminidase, histamine, and tryptase in HMC-1

HMC-1 secrete constitutively large amounts of mediators that would otherwise be stored in the secretory granules. The content of β-hexosaminidase, histamine, and tryptase was investigated in cells incubated in the presence or absence of flavonoids. Cells were incubated with 10 or 100 μM of each flavonoid for 3 and 4 d. The amount of β-hexosaminidase did not change during 3 or 4 d of culture with either flavonoid (Table 1). All the four flavonoids at 100 μM increased histamine content on days 3 and 4 of culture, whereas kaempferol and myricetin increased histamine content at 10 μM on these days (Table 1). Tryptase was increased at 10 μM on day 4 and 100 μM on days 3 and 4 of kaempferol, as well as with 100 μM quercetin on day 4. Myricetin or flavone had no effect on tryptase at any concentrations (Table 1).

Discussion

The results show that certain flavonoids with a flavone structure inhibit HMC-1 proliferation and lead to accumulation of secretory granules with variable electron granular content, indicating increased level of mediator storage. The doubling time of the HMC-1, we used, was faster (2 d) than what we had originally reported (5). This variability could be explained because of the multiple passages of these cells that have, nevertheless, been used extensively. The observed correlation between

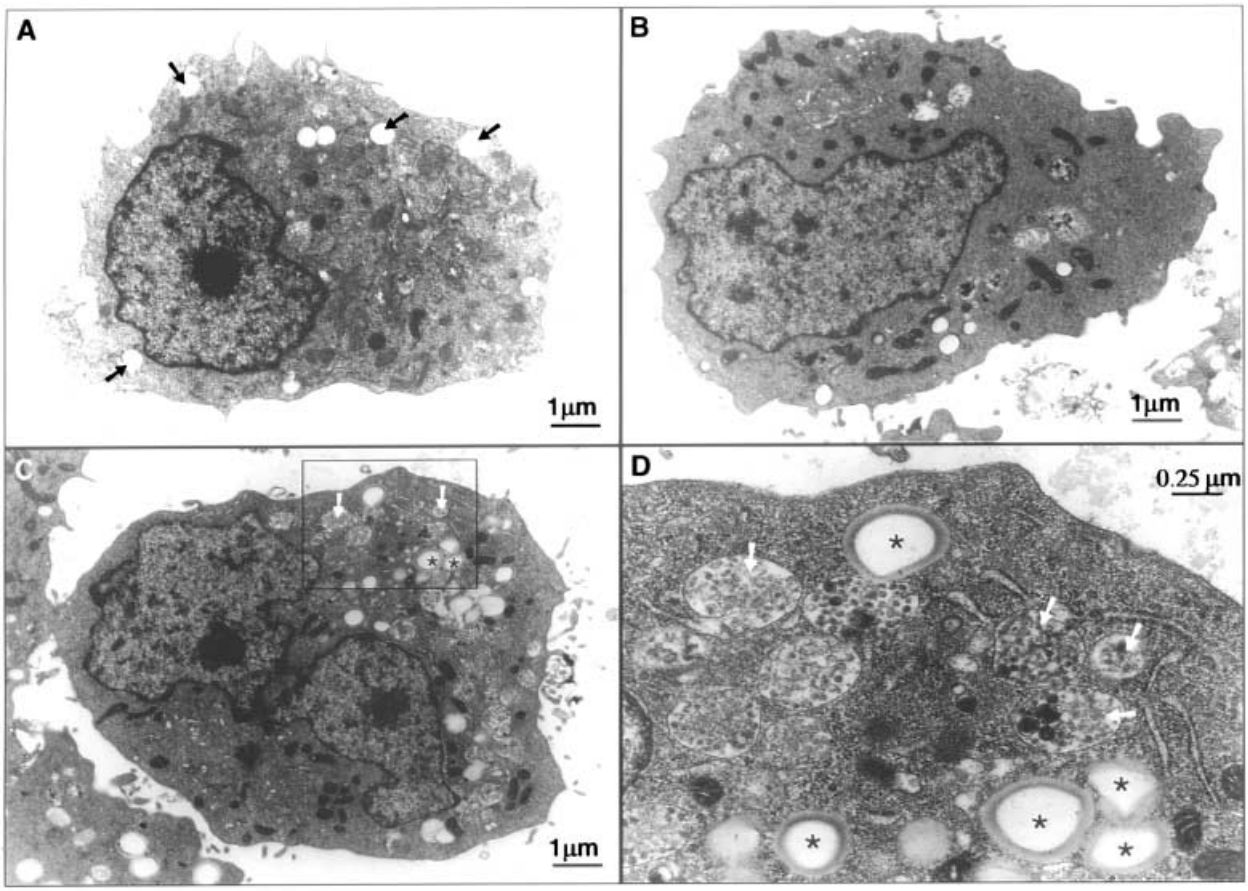

Fig. 4. Electron micrographs of HMC-1. (A) A 3-d-old cell grown in the absence of any flavonoid. Note large nucleus and abundance of mitochondria, with hardly any secretory granules, but many empty cytoplasmic vacuoles (black arrows). (B) 3-d-old and (C) 4-d-old cells grown in the presence of 100 μM quercetin show numerous secretory granules filled with fine electron-dense material (white arrows). (D) Higher magnification of a portion of the cell shown in 4C (rectangular area) show the different stages of 'maturation' of the secretory granules (white arrows), as evidenced by the appearance of the electron-dense material found therein; many lipid bodies (asterisks) are also noted.

Table 1. Effect of flavonoids on intracellular β-hexosamininidase, histamine, and tryptase in HMC-1

	β-Hexosaminidase (arbitrary units)		Histamine (ng/cell)		Tryptase (ng/cell)	
Groups	Day 3	Day 4	Day 3	Day 4	Day 3	Day 4
Control	0.7 ± 0.2	1.0 ± 0.3	4.2 ± 0.1	3.6 ± 0.7	2.1 ± 0.5	1.4 ± 0.6
Flavone 10 μm	1.1 ± 0.8	1.4 ± 0.6	6.2 ± 2.2	7.2 ± 3.7	3.6 ± 1.1	3.7 ± 2.2
Favone 100 μm	2.3 ± 1.8	2.3 ± 2.0	22.8 ± 0.4*	24.3 ± 22.2*	7.8 ± 5.6*	10.3 ± 11.3
Kaempferol 10 μm	1.1 ± 0.7	1.9 ± 0.8	7.2 ± 2.6	9.2 ± 1.5*	5.7 ± 1.9	4.2 ± 1.5
Kaempferol 100 μm	0.9 ± 0.7	1.4 ± 0.6	13.6 ± 8.0*	13.5 ± 2.6*	10.8 ± 4.4*	8.8 ± 2.1*
Myricetin 10 μm	1.0 ± 0.6	2.0 ± 1.0	6.6 ± 2.2	14.2 ± 1.6*	3.1 ± 1.5	3.1 ± 1.9
Myricetin 100 μm	1.6 ± 0.8	2.6 ± 0.5	25.4 ± 14.1*	8.1 ± 1.6*	3.8 ± 2.3	3.8 ± 1.2
Quercetin 10 μm	0.9 ± 0.4	1.2 ± 0.4	6.4 ± 1.2	5.3 ± 1.1	3.7 ± 1.8	2.4 ± 0.2
Quercetin 100 μm	0.9 ± 0.5*	1.3 ± 0.5	7.1 ± 3.4*	7.6 ± 2*	4.4 ± 3.1	7.8 ± 3.9*

HMC-1 were incubated with flavone, kaempferol, myricetin, quercetin and morin at 10 or 100 μm for day 3 or 4 in culture ($n = 4$–7). The cells were centrifuged and the β-hexosamininidase, histamine and tryptase content were measured in the cells. Results are expressed as mean ± SD.
*Significant ($P < 0.05$) as compared with their respective controls, except for these two.

proliferation arrest and increased mediator content suggests that the main mechanism for mediator accumulation could be slow cell division with continuous amine/protein synthesis. The HMC-1 content of histamine and tryptase was increased by all flavonoids except morin. Although both rat and human mast cells contain β-hexosaminidase (21), this enzyme was not affected, possibly due to its storage in a compartment other than secretory granules such as in lysosomes. Quercetin had been

reported to inhibit stimulated histamine release from various mast cells and basophils (7–10). Quercetin also inhibited IgE-mediated release of histamine, LTC_4 and PGD_2 from human cultured mast cells (9). Various flavonoids, again except for morin, had previously also been shown to inhibit lymphocyte secretion of interleukin-2 (14).

Plant polyphenols and flavonoids had previously been reported to inhibit proliferation of two human lymphoid tissue-derived cell lines (14). Moreover, quercetin inhibited proliferation of human acute myeloid and lymphoid leukemic cells without affecting normal hematopoiesis (15). These findings are in accordance with previous reports showing that quercetin could block proliferation of HL-60 leukemia cells by inducing an accumulation of the cells in the G_2/M phase of the cell cycle (16) and could inhibit progression of human gastric cancer cells from G_1 to the S phase of growth (17). The growth inhibitory effect of quercetin on a human lymphoblastoid cell line was shown to be through the action on a type II estrogen-binding site, an effect not shared by hesperidin, which does not bind to such sites (18). The latter action may explain quercetin's ability to inhibit the growth of estrogen sensitive cells, such as human breast cancer cells in culture (19). Quercetin's action on HMC-1 may be related, as human mast cells were shown to express cytoplasmic estrogen receptors (20).

The ultrastructural observations indicate that quercetin induces accumulation of secretory granules with variable electron-dense content. However, we could not make any direct correlation between the increase in histamine or tryptase content and appearance of secretory granules. Granule maturation, as judged by histochemistry, was reported to not necessarily correspond to the full complement of mature granule content in bone marrow derived mast cells cultured with recombinant c-*kit* ligand (24). Moreover, some of the mast cell mediators may be released differentially without exocytosis (25, 26). These findings are important as mastocytoma cells do not normally accumulate secretory granules even at stages expressing increased membrane receptors for IgE (27).

The results indicate that the structural requirements of flavonoids with respect to the inhibition of both proliferation and accumulation of mediators in HMC-1 are similar to those previously reported for RBL cells (22). For instance, flavone, quercetin, and kaempferol (but not morin), induced accumulation of secretory granules in RBL cells (22) and quercetin increased the content of RMCP-II (11). The structural requirements included keto and hydroxyl groups in the middle rings and a unique hydroxylation pattern of the B-ring. Morin's lack of inhibitory activity may be due to one additional hydroxyl group in the 2′ position of the B-ring (6), not shared by the other flavonoids tested; such a group could result in some steric interference (10) of a binding site. Morin could not inhibit proliferation also of a human lymphoblastoid cell line (18) or mitogen-induced lymphocyte proliferation (23).

The reported inhibitory activity of flavonoids could be due to their reported ability to inhibit various enzymatic systems *in vitro* (6,10), such as protein kinase C (PKC) translocation in human cultured mast cells (9), as well as to suppress NF-κB activation (13).

Unique flavonoids that are flavones with hydroxylation at positions 3′, 4′, or 5′ have the potential to be used as therapeutic agents (6, 28).

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