INHIBITION OF GASTRIC H^+, K^+-ATPase BY FLAVONOIDS: A STRUCTURE-ACTIVITY STUDY

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Gastric H^+, K^+-ATPase plays a pivotal role in the final step of gastric acid secretion. Over 80 flavonoids, including flavones, flavanones, isoflavones and anthocyanidins were examined for their *in vitro* effect on gastric H^+, K^+-ATPase and some were found to be inhibitors of this enzyme. Kinetic studies showed that the inhibition of H^+, K^+-ATPase by flavonoids was competitive with respect to ATP, and non-competitive with respect to K^+. Structure–activity analysis revealed the following: (1) The inhibitory potency of flavonoids depends on the number of hydroxyl groups up to four per molecule and that above this, no marked enhancement is seen; (2) The hydroxylation pattern is an important determinant of inhibitory potency. Two adjacent hydroxyl groups (catechol-type), three adjacent hydroxyl groups (pyrogallol-type) or hydroxyl groups at C-3, C-5 and C-7 are a minimum requirement for high potency inhibition; (3) Protection of the hydroxl group(s) by glycosylation or methylation decreases potency; (4) Saturation of the C-2–C-3 double bond results in a decrease in potency; and (5) A ketone at C-4 is not essential for inhibition.

Keywords: Flavonoids; H^+, K^+-ATPase; Proton pump; Enzyme inhibitor

INTRODUCTION

Flavonoids, a group of phenolic compounds present in a wide variety of plant sources such as vegetables, fruits, herbs and tea,[1] affect the activity of enzyme systems, such as cyclic nucleotide phosphodiesterase,[2,3] lens aldose reductase,[4] protein kinase C,[5] ion-transport ATPase,[6,7]

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NADH-oxidase,[8] hyaluronidase,[9] HIV integrase,[10] arachidonate 5-lipox-idase,[11] and others. Gastric H^+, K^+-ATPase is a membrane-bound ion-transport ATPase responsible for acid secretion from the parietal cells in gastric mucosa.[12,13] This enzyme catalyses H^+ transport at the expense of ATP hydrolysis. Inhibition of the enzyme can reduce gastric acid secretion, and one of the synthetic inhibitors, omeprazole, is clinically prescribed for patients with peptic ulcer.[14,15] We previously showed that naturally occur-ring phenolic compounds, including the most common flavonoid, quercetin, are inhibitors of H^+, K^+-ATPase and that phenolic hydroxyl groups play a role in the inhibition of the enzyme.[16-24] In the present study, we carried out a more extensive survey of various types of flavonoids to examine structure–activity relationships for the inhibition of gastric H^+, K^+-ATPase so as to better understand the interactions of flavonoids with the enzyme.

MATERIALS AND METHODS

Materials

Flavonoids were obtained from Funakoshi (Tokyo, Japan). Tris(hydroxy-methyl) aminomethane (Tris), Piperazine-N,N'-bis(2-ethanesulfonic acid) (Pipes), and ATP were purchased from Sigma Chemical Co. All other chem-icals were of reagent grade. Fresh pig stomachs were obtained from a local abattoir.

Preparation of Gastric H^+, K^+-ATPase

Stomachs from freshly slaughtered pigs were flushed with tap water and mucus was removed by wiping the tissue with paper towels. The mucosal layer of the fundic region was scraped off from the underlying muscular layer and was homogenised in an ice-cold 20 mM Pipes-NaOH buffer containing 0.2 mM EDTA, 250 mM sucrose, pH 7.4, with Physcotron (Nichi-on, Tokyo, Japan). All of the following procedures were carried out at 4°C. The homogenate was centrifuged for 30 min at $18,000 \times g$. The resulting super-natant was centrifuged for 60 min at $100,000 \times g$. The pellet was resus-pended in homogenisation buffer. Gastric microsome vesicles containing H^+, K^+-ATPase were prepared by Ficoll-sucrose discontinuous density gra-dient centrifugation, as described elsewhere.[25] The membrane fraction containing vesicles was collected and lyophilsed to render vesicles freely permeable to cations, then was stored at $-80°C$ until used. Protein was determined by the method of Lowry *et al.*[26]

Assay of H^+, K^+-ATPase

The reaction mixture consisted of 40 mM Tris-HCl buffer, pH 7.4, containing 2 mM $MgCl_2$ and 10 μg membrane protein, with or without 20 mM KCl, in a total volume of 1 ml. Flavonoids were solubilised in 100% dimethyl sulfoxide and 5 or 10 μl was added to the reaction mixture before starting the reaction. The concentration of dimethyl sulfoxide in the reaction mixture was below 1% and did not affect the enzyme activity. The reaction was initiated with 2 mM ATP Tris salt, the preparation incubated for 20 min at 37°C, then was terminated by adding 1 ml ice-cold trichloroacetic acid (10%) and assayed for inorganic phosphate according to the method of Fiske and Subbarow.[27] IC_{50} values, concentrations required to inhibit the enzyme activity by 50%, were determined by extrapolation from inhibition curves using 6 concentrations in the range 0.1–300 μM.

RESULTS AND DISCUSSION

The effect of typical active flavonoids, luteolin, geraldol, quercetin-3-arabinoside and 6,7-dihydroxy flavone on gastric H^+,K^+-ATPase are shown in Figure 1. These flavonoids inhibited the enzyme in a dose-dependently manner. Kinetic studies were carried out for three different types of flavones, galangin (trihydroxy flavone), luteolin (tetrahydroxy flavone) and myricetin (hexahydroxy flavone), to elucidate the mechanism by which flavonoids inhibit H^+,K^+-ATPase. Our previous study[24] revealed that the inhibition of gastric H^+,K^+-ATPase by quercetion (pentahydroxy flavone) was competitive with respect to ATP and non-competitive with respect to K^+. These three flavonoids inhibited gastric H^+,K^+-ATPase by a similar mechanism to quercetin (Figure 2).

The effect of various types of flavonoids, including flavones, flavanones, isoflavones and anthocyanidins was examined. The IC_{50} values are shown in Tables I–VII. The parent compound, flavone, was not effective in inhibiting H^+, K^+-ATPase even at 100 μM, the maximum concentration possible due to insolubility in water (Table I). Flavanone up to 300 μM also lacked inhibitory activity. Flavones with one hydroxyl group at various positions, 3-hydroxy flavone (flavanol), 5-hydroxy flavone, 6-hydroxy flavone, 7-hydroxy flavone, 6-hydroxy flavanone, 2'-hydroxy flavanone, and 4'-hydroxy flavanone, did not exhibit inhibitory activity.

Among flavones with two hydroxyl groups, only the compounds with two adjacent hydroxyl groups (catechol orientation), 6,7-dihydroxy flavone and
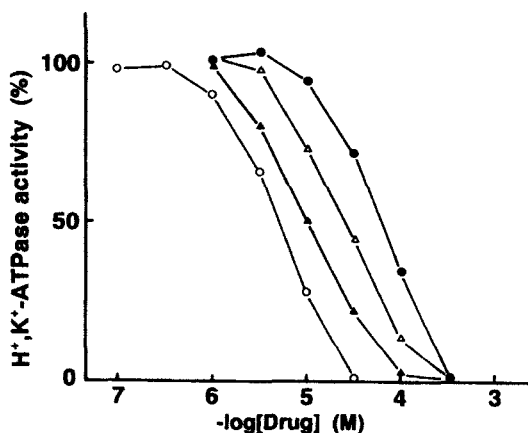
FIGURE 1   Inhibition of pig gastric H^+, K^+-ATPase by flavonoids. Microsome membranes were incubated in 40 mM Tris-HCl buffer pH 7.4, containing 2 mM $MgCl_2$, 20 mM KCl, and 2 mM ATP, for 20 min at 37°C in the presence of luteolin (○), geraldol (▲), quercetin-3-arabinoside (△), and 6,7-dihydroxy flavone (●), and inorganic phosphate was then determined. In the absence of compounds, H^+, K^+-ATPase activity was 148 μmol Pi/mg protein/h. Each point represents the mean of duplicate assays of two separate experiments.

7,8-dihydroxy flavone exhibited inhibitory activity (Table II). Other types of dihydroxy flavones, 7,8-dimethoxy flavone, chrysin and chrysin dimethyl-ether, were ineffective, and the dihydroxy isoflavon, daidzein was also ineffective. Although the dihydroxy flavone, chrysin, was not effective, the addition of a third hydroxyl group to C-3 enhanced the inhibitory activity, and galangin was a potent inhibitor (Table III). Glycosylation of the 3-hydroxyl group of galangin (galangin-3-rutinoside) resulted in a loss of the inhibitory activity. Another type of trihydroxy flavone is baicalein, which has hydroxyl groups at C-5, C-6 and C-7 and proved to be a potent inhibitor. However, glycosylation of the 7-hydroxyl group (baicalin) reduced inhibitory activity. The third type of trihydroxy flavone is apigenin, which has no hydroxyl group at C-3 and a catechol type orientation. This flavone was not an effective inhibitor. Methylation and/or glycosylation of the hydroxyl groups of apigenin (acacetin, apiin, rhoifolin, and linarin) did not enhance the inhibitory activity. The catechol-bearing trihydroxy flavone, 7,3',4'-trihydroxy flavone, had moderate inhibitory activity, and the introduction of glucose to C-6 (homoorientin) led to a decrease in activity. The trihydroxy flavanone, naringenin, was not effective, and methylation and/or glycosylation of the hydroxyl group (sakuranetin, isosakuranetin and naringin) did not enhance the inhibitory activity. The trihydroxy iso-flavone, genistein, had weak inhibitory activity and methylation of the
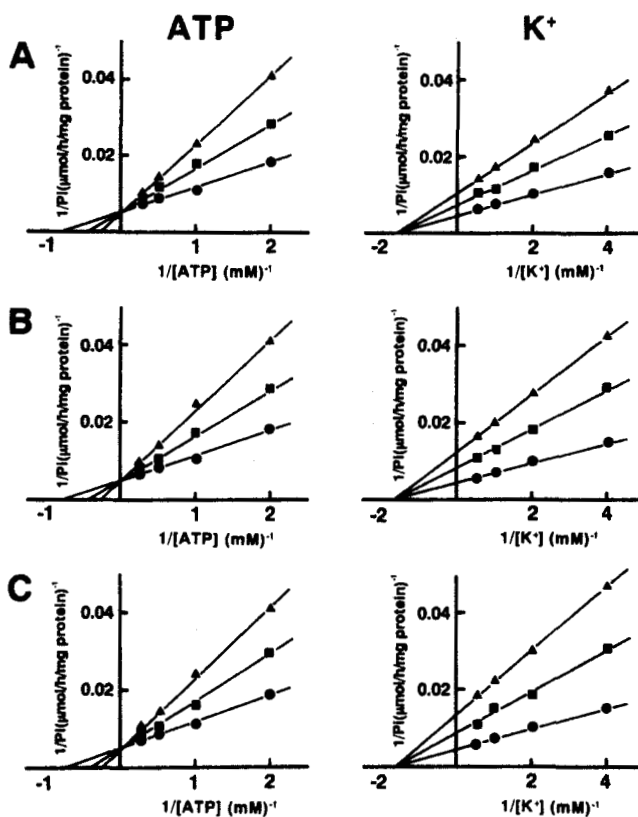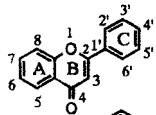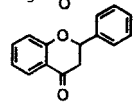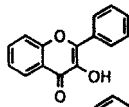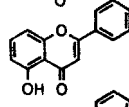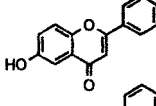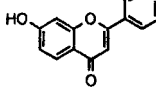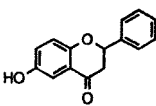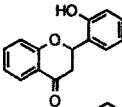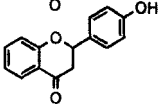
FIGURE 2 Double reciprocal plots of the rates of ATP hydrolysis by H$^+$,K$^+$-ATPase vs concentrations of ATP and the rates of ATP hydrolysis by H$^+$, K$^+$-ATPase vs concentrations of KCl in the presence of 0 (●), 1.5 μM (■) and 3 μM (▲) of (A) galangin (B) luteolin and (C) myricetin. Each point represents the mean of duplicate assays of two separate experiments.

7-hydroxy group (prunetin) resulted in a decrease in inhibition. Moreover, 6,7,4'-trihydroxy isoflavone, where the hydroxyl groups have a catecholic orientation, exhibited inhibitory activity. These results indicate that the hydroxyl group at C-3 has a role in interacting with the enzyme, and that hydroxyl groups with a catecholic orientation are also important for enzyme inhibition.

Luteolin, a flavone with four hydroxyl groups at C-5, C-7, C-3' and C-4', was a potent inhibitor (Table IV). This flavone has a catechol orientation in ring C. Methylation of the 3'- or 4'-hydroxyl group (chrysoeriol and diosmetin) showed a marked decrease in inhibitory activity. Glucosylation of the 7-hydroxyl group (luteolin-7-glucoside) decreased as did glucosylation
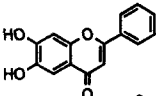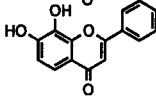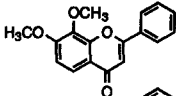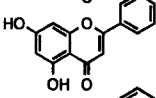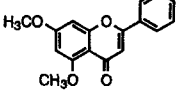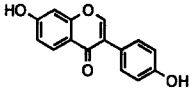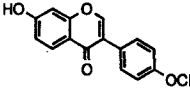
TABLE I   Effect of flavonoids with or without one hydroxy group on H^+, K^+-ATPase

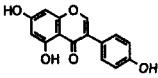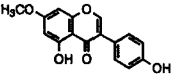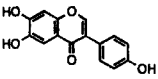| Structure	Name	Inhibition IC_{50} (μM)
	flavone	> 100
	flavanone	> 300
	3-hydroxy flavone (flavonol)	> 100
	5-hydroxy flavone	> 100
	6-hydroxy flavone	> 100
	7-hydroxy flavone	> 100
	6-hydroxy flavanone	> 100
	2'-hydroxy flavanone	> 100
	4'-hydroxy flavanone	> 100

IC_{50} values were calculated from concentration–activity curves of six concentrations and expressed as the mean of two measurements. The variation between the replicates was in the range of 2–11% of the mean. Substrate concentration (ATP) was 2 mM.

of the 7- and 3'-hydroxyl groups (luteolin-7,3',-diglucoside) as well as intro-duction of glucose to C-8 (orientine). Introduction of a C-6-methoxy group (6-methoxyluteolin) did not decrease inhibitory activity. Scutellarein with four hydroxyl groups at C-5, C-6, C-7 and C-4' (pyrogallol-type) was a moderate inhibitor but less potent than luteolin. Another type of flavone

TABLE II   Effect of dihydroxy flavonoids on H^+, K^+-ATPase

Structure	Name	Inhibition IC$_{50}$ (μM)
	6,7-dihydroxy flavone	63
	7,8-dihydroxy flavone	52
	7,8-dimethoxy flavone	> 100
	chrysin	> 100
	chrysin dimethylether	> 100
	daidzein	> 100
	pratol	> 100

IC$_{50}$ values were calculated from concentration–activity curves of six concentrations and expressed as the mean of two measurements. The variation between the replicates was in the range of 2–11% of the mean. Substrate concentration (ATP) was 2 mM.

with four hydroxyl groups, 7,8,3',4'-tertahydroxy flavone, which has two pairs of catechol oriented hydroxyl groups, was seen to be more potent than luteolin. The introduction of a fourth hydroxyl group to C-2' (datiscetin) of galangin decreased inhibitory activity, while the introduction of a hydroxyl group to C-4' (kaempferol) retained activity. Kaempferol has no catechol-oriented hydroxyl group. Methylation of the 4'-hydroxy group (kaempferide) enhanced inhibitory activity, an event which may be related to the increase in hydrophobicity. Elimination of the 3-hydroxyl group at kaempferol (apigenin) resulted in a loss of potency thereby suggesting the importance of the 3-hydroxyl group. Fisetin is a flavonol with four hydroxyl groups, and has a catechol-oriented hydroxyl group in ring C like luteolin. Fisetin was also a potent inhibitor similar to luteolin. The inhibitory activity

TABLE III   Effect of trihydroxy flavonoids on H^+, K^+-ATPase

Structure	Name	Inhibition IC_{50} (μM)
	galangin	6.3
	galangin-3-rutinoside	> 100
	baicalein	5.5
	baicalein-7-glucoside (baicalin)	140
	apigenin	> 100
	vitexin	> 100
	acacetin	> 100
	apiin	> 100
	rhoifolin	> 100
	linarin	> 100
	7,3',4'-trihydroxy flavone	42
	homoorientin	210
	naringenin	> 100
	naringin	> 100
	sakuranetin	> 100
	isosakuranetin	> 100

TABLE III (*Continued*)

Structure	Name	Inhibition IC$_{50}$ (μM)
	genistein	100
	prunetin	> 100
	6,7,4'-trihydroxy isoflavone	10

IC$_{50}$ values were calculated from concentration–activity curves of six concentrations and expressed as the mean of two measurements. The variation between the replicates was in the range of 2–11% of the mean. Substrate concentration (ATP) was 2 mM.

was decreased by methylation of the 3'-hydroxyl group of fisetin (geraldol). Additional methylation of the 4'-hydroxyl group (fisetin 3',4'-dimethoxyl-ether) further decreased the activity.

The flavanone eriodictyol, which has catechol-oriented hydroxyl groups in ring C, was slightly weaker than the corresponding flavone, luteolin (Table IV). Methylation of the 3'- or 4'-hydroxyl group (homoeriodictyol and hesperetin) resulted in a marked decrease in activity. Glycosylation of the 7-hydroxyl group (eriodictyol-7-glucoside and neocriocitrin) also reduced inhibitory activity. Dihydrofisetin is another type of flavanone with four hydroxyl groups. The inhibitory activity of dihydrofisetin was about 20 times weaker than that of the corresponding flavone, fisetin. A similar decrease in inhibitory activity was observed between quercetin and taxifolin (Table V). In the case of eriodictyol, the decrease in potency by saturation of the double bond between c-2 and C-3 was slight, as noted above. Thus, conversion of the C-2 and C-3 double bond to a single bond decreased the activity.

As previously reported,[24] quercetin is one of the most potent inhibitors, and has catechol-oriented hydroxyl groups in ring C (Table V). Glycosylation of the 3-hydroxyl group decreased the inhibitory activity. In particular, the introduction of a disaccharide residue resulted in a marked decrease in activity (avicularin, quercitrin, hyperoside, isoquercetin compared with rutin and paltatoside). The inhibitory activity of rhamnetin was as potent as that of quercetin, suggesting that the 7-hydroxyl group is not essential for the inhibition. In the case of quercetin, methylation of the 3'- or 4'-hydroxyl group (isorhamnetin and tamarixetin) led to some decrease in activity. Methylation of the 3' or 4'-hydroxyl group causes a loss of the catechol-oriented hydroxyl groups. However as demonstrated from the finding that

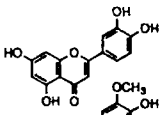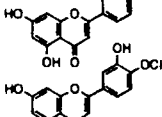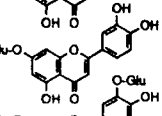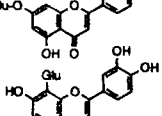TABLE IV   Effect of tetrahydroxy flavonoids on H^+, K^+-ATPase
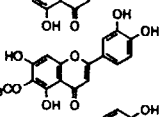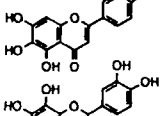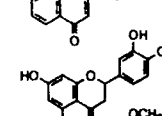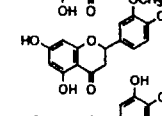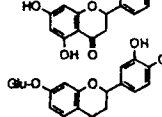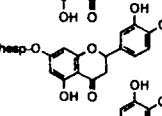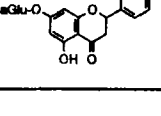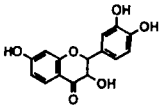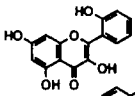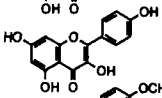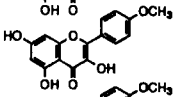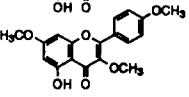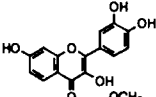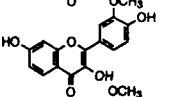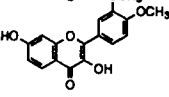
Structure	Name	Inhibition IC_{50} (μM)
	luteolin	5.2
	chrysoeriol	> 100
	diosmetin	> 300
	luteolin-7-glucoside	30
	luteolin-7,3'-diglucoside	> 100
	luteolin-8-C-glucoside (orientine)	160
	6-methoxyluteolin	6.0
	scutellarein	47
	7,8,3',4'-tetrahydroxy flavone	2.1
	eriodictyol	6.9
	homoerpodictyol	> 100
	hesperetin	> 100
	eriodictyol-7-glucoside	> 300
	neoeriocitrin	> 100
	hesperidin	> 100

TABLE IV (*Continued*)

Structure	Name	Inhibition IC$_{50}$ (μM)
	dihydrofisetin (fustin)	90
	datiscetin	29
	kaempferol	7.4
	kaempferide	2.9
	kaempferol-3,7,4'-trimethylether	> 100
	fisetin	4.2
	geraldol	10
	fisetin-3',4'-dimethylether	> 100

IC$_{50}$ values were calculated from concentration–activity curves of six concentrations and expressed as the mean of two measurements. The variation between the replicates was in the range of 2–11% of the mean. Substrate concentration (ATP) was 2 mM.

galangin was as potent as an inhibitor isorhamnetin and tamarixetin, maintenance of the inhibitory activity in these two flavones is attributed to their hydroxyl groups at C-3, C-5 and C-7. Morin has five hydroxyl groups at C-3, C-5, C-7, C-2' and C-4' but was less potent than quercetin. Another type of flavone with five hydroxyl groups, robinetin, which has adjacent trihydroxyl groups (pyrogallol-type), but lacks a hydroxyl group at C-5, was the most potent inhibitor. Ombuin, which has no catechol-oriented hydroxyl group in ring C and 3- and 5-hydroxyl groups, was not effective. The multi-methoxy containing compounds 5,7,3'-trihydroxy-3,4'-dimethoxy flavone, retusin, eupatorin, eupatorin-5-methylether, sinensietn and tangeretin were also ineffective (Table V)

The hexahydroxy flavones, myricetin and quercetagetin, were potent inhibitors (Table VI). However, introduction of the sixth hydroxyl group to pentahydroxy flavones did not enhance the inhibitory activity. The inhibitory activity of myricetin and quercetagetin were a little weaker than the pentahydroxy flavones, quercetin and robinetin. Glycosylation of the 3-hydroxyl group (myricitrin) resulted in a decrease in activity.

The anthocyanidins, pelargonidin, cyanidin and delphinidin, which correspond to kaempferol, quercetin and myricetin, respectively, in terms of the positions of the hydroxyl groups, were also potent inhibitors of gastric H^+, K^+-ATPase (Table VII). These anthocyanidins were as active as the flavonoids or even more so. We have reported elsewhere that catechins are inhibitors of gastric H^+, K^+-ATPase.[22] These observations show that a

TABLE V   Effect of pentahydroxy flavonoids on H^+, K^+-ATPase
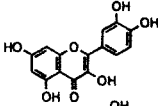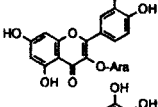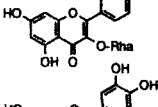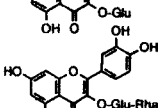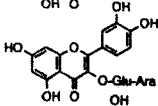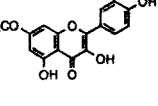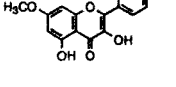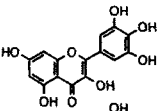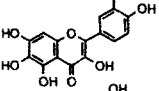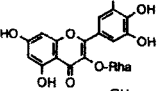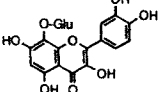
Structure	Name	Inhibition IC_{50} (µM)
	quercetin	3.4
	quercetin-3-arabinoside (avicularin)	26
	quercetin-3-rhamnoside (quercitrin)	17
	quercetin-3-galactoside (hyperoside)	20
	quercetin-3-glucoside (isoquercetin)	9.5
	quercetin-3-gluco-rhamnoside (rutin)	100
	quercetin-3-gluco-arabinoside (peltatoside)	> 100
	rhamnetin	2.7

TABLE V (*Continued*)

Structure	Name	Inhibition IC_{50} (μM)
	isorhamnetin	5.8
	tamarixetin	6.8
	morin	20
	robinetin	1.5
	ombuin	> 100
	5,7,3'-trihydroxy-3,4'-dimethoxy flavone	> 100
	retusin	> 100
	eupatorin	> 100
	eupatorin-5-methylether	> 100
	sinensetin	> 100
	tangeretin	> 100
	taxifolin	44
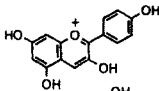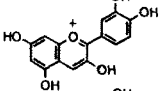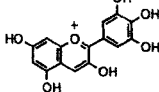
IC_{50} values were calculated from concentration–activity curves of six concentrations and expressed as the mean of two measurements. The variation between the replicates was in the range of 2–11% of the mean. Substrate concentration (ATP) was 2 mM.
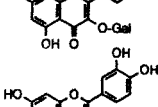
TABLE VI   Effect of hexahydroxy flavonoids on H^+, K^+-ATPase

Structure	Name	Inhibition IC_{50} (μM)
	myricetin	4.8
	quercetagetin	4.0
	myricitrin	40
	gossypin	8.3

IC_{50} values were calculated from concentration–activity curves of six concentrations and expressed as the mean of two measurements. The variation between the replicates was in the range of 2–11% of the mean. Substrate concentration (ATP) was 2 mM.

TABLE VII   Effect of anthocyanidins on H^+, K^+-ATPase

Structure	Name	Inhibition IC_{50} (μM)
	pelargonidin	7.0
	cyanidin	1.0
	delphinidin	2.9

IC_{50} values were calculated from concentration–activity curves of six concentrations and expressed as the mean of two measurements. The variation between the replicates was in the range of 2–11% of the mean. Substrate concentration (ATP) was 2 mM.

ketone at C-4 is not essential for H^+, K^+-ATPase inhibition. The ketone group at C-4 was found to be important for maintaining inhibitory activity in most enzyme systems,[4,5,8–10,29] a prominent difference between gastric H^+, K^+-ATPase and others.

The importance of hydroxyl groups in inhibiting H^+, K^+-ATPase has been noted in various naturally occurring phenolic compounds including catechins[22] and stilbenes.[20] In the case of salvianolic acid A[17] and cassigarol A,[19] protection of the hydroxyl groups by acetylation resulted in a loss of inhibition of enzyme activity and acid secretion. The present study also suggests that hydroxyl groups play a role which enables flavonoids to exert H^+, K^+-ATPase inhibition, and that the existence of hydroxyl groups at some positions are required for effective inhibition. The existence of at least two adjacent hydroxyl groups (catechol-type) is important for enzyme inhibition. A similar result has been noted for stilbene derivatives.[20] Although the parent compound, stilbene itself, is not an effective inhibitor, introduction of the hydroxyl group(s) markedly potentiates inhibitory activity. In the current study, three hydroxyl groups at the required position of ring A, like galangin and baicalein, exhibited nearly maximum inhibition. The inhibitory activity was enhanced by an increase in the number of hydroxyl groups up to four, above which no marked enhancement was observed. Since an increase in the number of hydroxyl groups decreases the hydrophobicity of the molecule, effective interaction between flavonoids and the enzyme may be impaired. Glycosylation and methylation of the hydroxyl group(s) decreased the inhibitory activity, except for that of kaempferide. The presence of the C-2 and C-3 double bond is also important for the inhibition of H^+, K^+-ATPase. Ferriola et al. noted the importance of the C-2 and C-3 double bond of flavonoids for the inhibition of protein kinase C, and postulated a structural model for an active inhibitor, which includes a planar benzopyrone ring system with a 7-hydroxy and a coplanar 2-(3′,4′-dihydroxy)-phenyl ring. The opening of ring B of flavones produces chalcones. We have shown that chalcone inhibited gastric H^+, K^+-ATPase with an IC_{50} value of 48 μM, in the same experimental system.[8] Thus, the structure of ring B is variable. Among other enzyme systems, the influence of the opening of ring B on inhibitory potency is small for lens aldose reductase[4] and succinoxidase,[29] while it is profound[5] protein kinase C.

Flavonoids have been shown to inhibit ATP-dependent enzymes such as kinase and ion-transporting ATPase. Our previous kinetic studies revealed that quercetin[24] and other phenolic compounds[16–22,28] inhibited gastric H^+, K^+-ATPase, competitively with respect to ATP. Three potent inhibitors with different numbers of hydroxyl groups, galangin, luteolin and myricetin behaved as competitive inhibitors with respect to ATP, in a similar manner to quercetin (Figure 2) so that inhibition of H^+, K^+-ATPase by other active flavonoids can be reasonably assumed to be similar. Competitive inhibition

with respect to ATP by flavonoids has been noted for protein kinase C,[5] Ca^{2+}, Mg^{2+}-ATPase[7] and phosphatidylinositol-3-kinase.[30] Thus, some instances of ATP-dependent enzyme inhibition are related to competition for the binding of ATP.

References

[1] Harborne, J.B. (Ed.) (1988) *The Flavonoids: Advances in Research Since 1980.* London: Chapman and Hall.
[2] Ferrell, J.E., Chang Sing, P.D.G., Leow, G., King, R., Mansour, J.M. and Mansour, T.E. (1979) *Mol. Pharmacol.* **16**, 556.
[3] Kuppusamy, U.R. and Das, N.P. (1992) *Biochem. Pharmacol.* **44**, 1307.
[4] Varma, S.D. and Kinoshita, J.H., (1976) *Biochem. Pharmacol.* **25**, 2502.
[5] Ferriola, P.C., Cody, V. and Middleton, E. (1989) *Biochem. Pharmacol.* **38**, 1617.
[6] Kuriki, Y. and Racker, E. (1976) *Biochemistry* **15**, 4951.
[7] Shoshan, V. and MacLennan, D.H. (1981) *J. Biol. Chem.* **256**, 887.
[8] Hodnick, W.F., Bohmont, C.W., Capps, C. and Pardini, R.S. (1987) *Biochem. Pharmacol.* **36**, 2873.
[9] Kuppusamy, U.R., Khoo, H.E. and Das, N.P. (1990) *Biochem. Pharmacol.* **40**, 397.
[10] Fesen, M.R., Pommier, Y., Leteurtre, F., Hiroguchi, S., Yung, J. and Kohn, K.W., (1994) *Biochem. Pharmacol.* **48**, 595.
[11] Yoshimoto, T., Furukawa, M., Yamamoto, S., Horie, T. and Watanabe-Kohno, S. (1983) *Biochem. Biophys. Res. Commun.* **116**, 612.
[12] Sachs G, Chang., Rabon, E., Schackmann, R., Lewin, M. and Saccomani, G., (1976) *J. Biol. Chem.* **251**, 7690.
[13] Forte, J.G., Machen, T. and Obrink, K.J. (1980) *Annu. Rev. Physol.* **42**, 111.
[14] Lorentzen, P., Jackson, R., Wallmark, B. and Sachs, G. (1987) *Biochim. Biophys. Acta* **897**, 41.
[15] McTavish, D., Buckley, M.M.T. and Heel, R.C. (1991) *Drugs* **42**, 138.
[16] Murakami, S., Kijima, H., Isobe, Y., Muramatsu, M., Aihara, H., Baba, K. and Kozawa, M. (1989) *J. Pharm. Pharmacol.* **42**, 723.
[17] Murakami, S., Kijima, H., Isobe, Y., Muramatsu, M., Aihara, H., Otomo, S., Li, L. and Ai, C. (1990) *Planta Med.* **56**, 360.
[18] Murakami, S., Isobe, Y., Kijima, H., Nagai, H., Muramatsu, M. and Otomo, S. (1991) *Planta Med.* **57**, 305.
[19] Murakami, S., Arai, I., Muramatsu, M., Otomo, S., Baba, K., Kido, T. and Kozawa, M. (1992) *Biochem. Pharmacol.* **44**, 33.
[20] Murakami, S., Arai, I., Muramatsu, M., Otomo, S., Baba, K., Kido, T. and Kozawa, M. (1992) *Biochem. Pharmacol.* **44**, 1947.
[21] Murakami, S., Muramatsu, M. and Otomo, S., (1992) *J. Nat. Prod* **55**, 513.
[22] Murakami, S., Muramatsu, M. and Otomo, S., (1992) *J. Pharm. Pharmacol.* **44**, 926.
[23] Murakami, S., Arai, I., Muramatsu, M., Otomo, S., Baba, K. and Kozawa, M. (1992) *Pharm. Pharmacol. Lett.* **2**, 108.
[24] Murakami, S., Muramatsu, M. and Otomo, S. (1992) *J. Enz. Inhibit.* **5**, 293.
[25] Saccomani, G., Stewart, H.B., Shaw, D., Lewin, M. and Sachs, G. (1977) *Biochim. Biophys. Acta* **465**, 311.
[26] Lowry, O.H., Rosebrough, N.J., Farr, A.L and Randall. R.J. (1951) *J. Biol. Chem.* **193**, 265.
[27] Fiske, C.H. and Subbarow, Y. (1925) *J. Biol. Chem.* **66**, 375.
[28] Murakami, S., Muramatsu, M., Aihara, H. and Otomo, S. (1991) *Biochem. Pharmacol.* **42**, 1447.
[29] Hodnick, W.F., Kung, F.S., Roettger, W.J., Bohmont, C.W. and Pardini, R.S. (1986) *Biochem. Pharmacol.* **35**, 2345.
[30] Matter, W.F., Brown, R.F. and Vlahos, C.J., (1992) *Biochem. Biophys. Res. Commun.* **186**, 624