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Research Communication

Inhibition of the Sarcoplasmic/Endoplasmic Reticulum Ca^{2+}-ATPase by Flavonoids: A Quantitative Structure-activity Relationship Study

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Summary

Flavonoids are commonly found in fruit and vegetables and have been shown to reach concentrations of several micromolars in human blood plasma. Flavonoids are also believed to have cancer chemoprotective properties. One hypothesis is that flavonoids are able to initiate apoptosis, especially in cancer cells, via a Ca^{2+}-dependent mitochondrial pathway. This pathway can be activated through an exaggerated elevation of cytosolic [Ca^{2+}], and sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPases (SERCA) play an essential role in ameliorating such changes. In this study, we demonstrate that flavonoids (especially flavones) can inhibit the activity of Ca^{2+}-ATPases isoforms SERCA1A and SERCA2B in the micromolar concentration range. Of the 25 flavonoids tested, 3,6-dihydroxyflavone (IC_{50}, 4.6 μM) and 3,3′,4′,5,7-pentahydroxyflavone (quercetin) (IC_{50}, 8.9 μM) were the most potent inhibitors. We show that polyhydroxylation of the flavones are important for inhibition, with hydroxylation at position 3 (for SERCA1A) and position 6 (for SERCA2B) being particularly relevant. © 2008 IUBMB
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Keywords flavonoid; quercetin; QSAR; Ca^{2+}-ATPase; SERCA; phytoestrogen.

INTRODUCTION

Phytoestrogens are a group of nonsteroidal plant-derived compounds, which are chemically and pharmacologically similar to the female hormone estrogen (*1*). One major group of phytoestrogens are the flavonoids (*2*), which are heterocyclic compounds, consisting of three linked rings (two of which are aromatic), designated as rings A, B, and C (Fig. 1). They have been the focus of much interest over the last few years because of their potential therapeutic uses (*3*).

Because of their abundance in fruit and vegetables, some of these flavonoids have been reported to reach levels as several micromolars in human blood plasma (*4*). The relatively high levels of flavonoids particularly in Asian diet has also been linked to comparatively low rates of diseases prevalent in western populations, including conditions such as breast, prostrate, and colon cancers, suggesting that these chemicals may have cancer protective effects (*5–8*).

One mechanism by which flavonoids are believed to be cancer chemoprotective is their ability to sensitize some cells into undergoing apoptosis, a process that has become misregulated in many cancers (*8*). In recent years, research has shown that some flavonoids can trigger apoptosis through the modulation of a number of key cellular signaling pathways that can cause increased cellular levels of caspases and decreased levels of antiapoptotic factors such as Bcl-2-type proteins (*8–11*). Although it is unclear as yet how this occurs, one pathway involves a mitochondrial-mediated process (*11, 12*). Mitochondrial-mediated apoptosis is believed to be due to the release of proapoptotic factors such as cytochrome C from the mitochondria, which then activates caspase-3. The release of these proapoptic proteins can occur via mitochondrial "Ca^{2+} overload," whereby an excessive elevation of cytosolic [Ca^{2+}] levels leads to excessive mitochondrial Ca^{2+} uptake (*13*).

The sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPases (SERCA) Ca^{2+} pumps play a central role in maintaining low levels of free cytosolic [Ca^{2+}] within cells. This Ca^{2+} pump is inhibited by a wide spectrum of hydrophobic molecules, a number of which are plant-derived natural products such as thapsigargin (*14*), curcumin (*15, 16*), and the flavonoid, quercetin (*17*). Therefore, one possible mechanism by which apoptosis is initiated by flavonoids is via Ca^{2+} pump inhibition leading to elevation of cytosolic [Ca^{2+}], and therefore initiating Ca^{2+}-dependent mitochondrial-mediated cell death. In support of this

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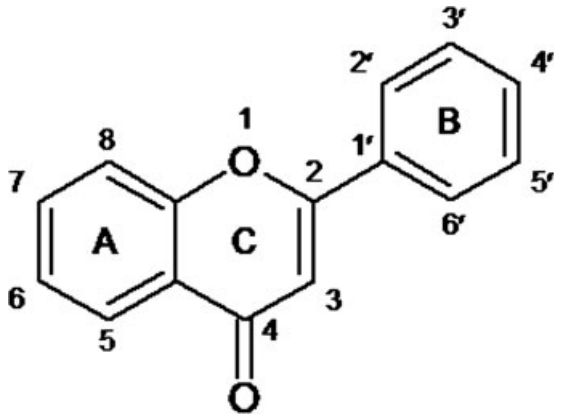


Figure 1. Structure of the parent (flavone) molecule shown with its ring letter designations and atom numbers. In flavanones, the 2=3 bond is saturated, whereas in isoflavonoids, the B ring is attached to the C ring at C3 instead of C2.

view, a recent study showed that the flavonoid baicalein induced apoptosis in SCC-4 cells via a Ca^{2+}-dependent mitochondrial pathway (*18*). Furthermore, we have also shown that various environmental pollutants can induce apoptosis in mammalian cells by Ca^{2+}-ATPase inhibition leading to mitochondrial depolarisation and activation of caspases (*19, 20*).

In this study, we investigate the inhibition of SERCA Ca^{2+} pumps (isoforms type 1A found in skeletal muscle and isoforms 2B found ubiquitously in most mammalian cells) by a wide range of flavonoids, some of which are commonly found within the human diet to ascertain their structure-activity relationship.

EXPERIMENTAL PROCEDURES

Flavonoids (purity ≥97%) were purchased from Alexis Biochemicals (Switzerland), Sigma/Aldrich (USA), Lancaster Synthesis (UK), and Apin Chemicals (UK). They were dissolved in dimethylsulfoxide (DMSO) to give a stock solution of 20 mM. The flavonoid solutions were added to the assays such that the solvent never exceeded more than 1.5% (v/v) of the assay volume, which had no effect on activity. All other reagents were of analytical grade.

Sarcoplasmic reticulum (SR) was prepared from skeletal muscle of rabbit as described in (*21*), which contains ~80% SERCA1A protein. Porcine brain microsomes, which contain predominantly SERCA2B (~5% of the total protein), were prepared as described in (*22*).

The effect of flavonoids on skeletal muscle Ca^{2+}-ATPase (SERCA1A) activity was investigated at pH 7.2, employing a coupled enzyme assay as previously described by Michelangeli and Munkonge (*21*). Typically, 15 μg/mL of SR Ca^{2+}-ATPase was added to a buffer containing 40 mM HEPES/KOH (pH 7.2, 25°C), 5 mM $MgSO_4$, 0.42 mM phosphoenolpyruvate, 0.15 mM NADH, 8.0 U pyruvate kinase, 20 U lactate dehydrogenase, 1.01 mM EGTA, and 2.1 mM ATP. In all experiments, unless otherwise stated, optimal free Ca^{2+} concentration of 6 μM was used.

Ca^{2+}-ATPase activities in porcine brain microsomes were performed using the phosphate liberation assay as described by Longland et al. (*23*). Briefly, 50 μg of microsomal membranes were resuspended in 1 mL of buffer containing 45 mM HEPES/KOH (pH 7.0), 6 mM $MgCl_2$, 2 mM NaN_3, 0.25 M sucrose, and 12.5 μg/mL of A23187 ionophore. EGTA (1 mM) and $CaCl_2$ were also added to give a free [Ca^{2+}] of 3 μM. Assays were preincubated at 37°C for 10 min with the flavonoids prior to activation with ATP (6 mM). The reaction was stopped after 40 min by the addition of 0.25 mL 6.5% (w/v) trichloroacetic acid. The assays were put on ice for 10 min prior to centrifugation for 10 min at 20,000*g*. Supernatant (0.5 mL) was added to 1.5 mL buffer containing 11.25% (v/v) acetic acid, 0.25% (w/v) copper sulfate, and 0.2 M sodium acetate. Ammonium molybdate (0.25 mL, 5% w/v) was then added and mixed thoroughly. ELAN solution (0.25 mL) was added (consisting of 2% (w/v) *p*-methyl-aminophenol sulfate and 5% (w/v) sodium sulfite). The color intensity was measured after 10 min at 870 nm absorbance. Controls were also performed in the absence of added Ca^{2+} to calculate the rate of Ca^{2+}-independent ATP hydrolysis.

All activity measurements were measured over a range of flavonoid concentrations, each concentration repeated 3–5 times. The mean activity value for each concentration ± SD was then plotted using Fig P (Biosoft, UK), and the IC_{50} values were determined by graphical extrapolation.

RESULTS AND DISCUSSION

Table 1 lists the flavonoids that show inhibition of the skeletal muscle SERCA1A Ca^{2+}-ATPase activity. This table also shows their structures and their IC_{50} values for inhibition (*i.e.*, concentration required to cause 50% inhibition). In this study, 25 flavonoids (19 flavones, 4 isoflavones, and 2 flavonones) were investigated on their effects on SERCA1A activity. Of the flavonoids that caused inhibition of activity, their IC_{50} values ranged from 6.1 (for 3,6-dihydroxyflavone) to 237 μM (for Hesperetin) (Table 1). Table 2 lists the flavonoids tested that caused no inhibition of SERCA1A activity. For this study, we designated flavonoids to be inactive if they showed less than 20% inhibition of activity at a concentration of 300 μM. It is unlikely that the inhibition by these flavonoids is due to indirect effects caused by their antioxidant properties, as in previous studies Ca^{2+}-ATPase inhibition by quercetin (a potent flavonoid antioxidant) could be reversed by removing it from the ATPase (*17*).

Analysis of the results presented in Tables 1 and 2 identifies a number of key features that may be important in determining the potency of Ca^{2+}-ATPase inhibition for these flavonoids. The most striking feature that deduced here was that isoflavones and flavanones showed little or no inhibition of Ca^{2+} ATPase activity when compared with the flavones. It can also be deduced that the variability in potency is due to the positioning

Table 1
IC_{50} values for flavonoid inhibition of SERCA1A

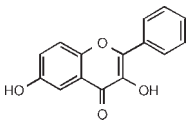
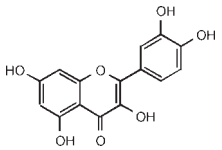
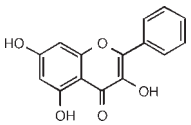
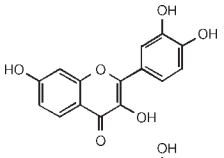
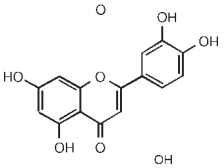
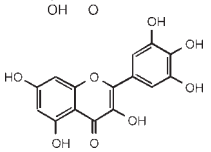
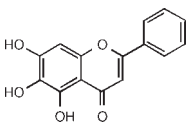
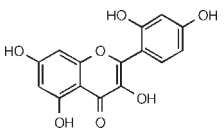
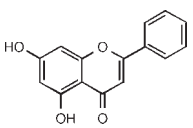
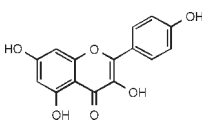
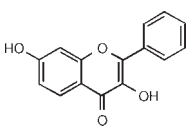
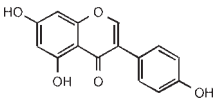
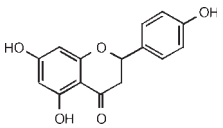
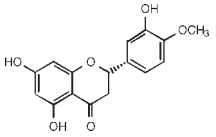
Flavonoid	Structure	IC_{50} (μM)
Flavones		
3,6,-Dihydroxyflavone		6.1 ± 0.4
3,3′,4′,5,7-Pentahydroxyflavone (Quercetin)		8.9 ± 0.2
3,5,7-Trihydroxyflavone (Galangin)		8.9 ± 0.5
3,3′,4′,7-Tetrahydroxyflavone (Fisetin)		10.9 ± 0.7
3′,4′,5,7-Tetrahydroxyflavone (Luteolin)		13.6 ± 0.6
3,3′,4′,5,5′,7-Hexahydroxyflavone (Myricetin)		14.6 ± 1.3
5,6,7-Trihydroxyflavone (Baicalein)		20.3 ± 0.7
2′,3,4′,5,7-Pentahydroxyflavone (Morin)		27.1 ± 2.1
5,7-Dihydroxyflavone (Chrysin)		33.5 ± 1.5
3,4′,5,7-Tetrahydroxyflavone (Kaempferol)		53.9 ± 1.5
3,7-Dihydroxyflavone		102 ± 2

Table 1
(Continued)

Flavonoid	Structure	IC_{50} (μM)
Isoflavones and flavanones		
4′,5,7-Trihydroxyisoflavone (Genistein)		121 ± 6
4′,5,7-Trihydroxyflavanone (Naringenin)		185 ± 5
4′-Methoxy-3′,5,7-trihydroxyflavanone (Hesperetin)		237 ± 4

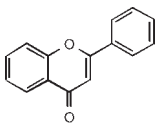
of the hydroxyl groups on the three rings, A, B, or C, rather than the degree of hydroxylation (*i.e.*, number of hydroxyl groups on the rings of the flavonoid backbone). This can be observed from the fact that the three most potent flavonoids; 3,6-dihydroxyflavone, galangin, and quercetin, contain 2, 3 and 5- hydroxyl groups, respectively. However, at least two hydroxyl groups appear to be required, because no inhibition was observed with either flavone or any of the monohydroxylated flavones tested. This appears to be different to that found for the effects of flavonoids on the enhancement of antioxidant activity (*24*), where the degree of hydroxylation was an important factor. In addition, the attachment of functional groups other than hydroxyl groups (*i.e.*, methoxy groups found in hesperetin and formononetin and sugar residues found in rutin) also appears to be detrimental to flavonoid inhibition of SERCA1A.

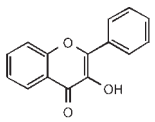
When comparing the hydroxylation positions on the flavonoids with their potency for Ca^{2+}-ATPase inhibition, it can also be deduced that:

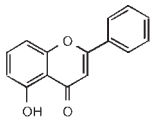
1 The fused A and C rings needs to be polyhydroxylated, with a hydroxyl group at position 3 on ring C appearing to be particularly important. The observation that hydroxylation at position 3 on ring C appears important may be due to the fact that hydroxylation at this position of the flavone hinders the rotation of the B ring, with respect to A and C ring, therefore adopting a "twisted" rather than planar conformation (*25*). Such a conformation could therefore be more favorable for flavonoid binding to SERCA1A.
2 A hydroxyl group at position 6 on the A ring may also assist in helping to increase potency, because 3,6-dihydroxyflavone is considerably more potent than 3,7-dihydroxyflavone (IC_{50} values 6.1 and 102 μM, respectively). However, hydroxylation of position 8 on ring A may be unfavorable because both chrysin (5,7-dihydroxyflavone) and luteolin (3′,4′,5,7-tetrahydroxyflavone) are more potent inhibitors than 7,8-dihydroxy- and 3′,4′,7,8-tetrahydroxy-flavones, respectively.
3 Although hydroxylation of the B ring does not appear to be necessary for the flavonoids to act as inhibitors, hydroxyl groups on this ring do appear to be able to exert subtle effects on potency. The addition of a 4′-hydroxyl group to galangin (producing kaempferol) increases the IC_{50} 6-fold and a similar effect is seen between chrysin and apigenin. However, the addition of a second hydroxyl group at the 2′ position (morin) halves or at the 3′ position (quercetin) completely abolishes this effect. If the 4′-hydroxyl group is flanked by hydroxyl groups at both the 3′ and 5′ positions (myricetin), this also appears to virtually abolish the increase in IC_{50}.

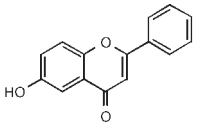
In this study, we also investigated the effects of a limited number of flavonoids on the inhibition of Ca^{2+}-ATPase activity in porcine brain microsomes, which contains mainly isoform SERCA2B (Table 3). From the data given in Table 3, it can be seen that 3,6-dihydroxyflavone is also the most potent inhibitor for SERCA2B. The only isoflavone tested, genistein, showed no inhibition in SERCA2B, whereas for SERCA1A, it inhibited only weakly (IC_{50} =121 μM). Also unlike SERCA1A, in SERCA2B, the hydroxyl group at position 3 on ring C appears to be relatively unimportant because quercetin and luteolin, which differ in the presence of the 3-hydroxyl group, have similar IC_{50} values. However, the hydroxyl group at position 6 on ring A may be more important because baicalein (5,6,7-trihydoxyflavone) is considerably more potent than galangin (3,5,7-trihydroxyflavone).

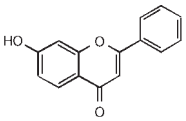
Table 2
Flavonoids which show little or no inhibition

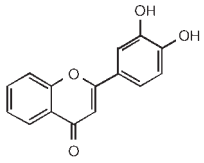

Flavone

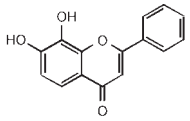

3-Hydroxyflavone

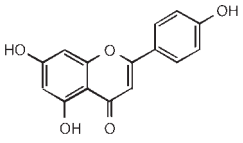

5-Hydroxyflavone
(Primuletin)

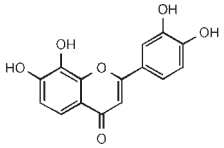

6-Hydroxyflavone

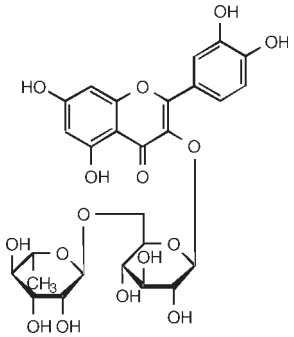

7-Hydroxyflavone

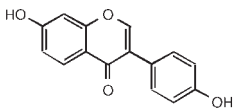

3′,4′-Dihydroxyflavone

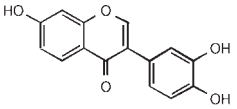

7,8-Dihydroxyflavone


4′,5,7-Trihydroxyflavone
(Apigenin)


3′,4′,7,8-
Tetrahydroxyflavone


(Rutin)


4′,7-Dihydroxyisoflavone
(Daidzein)


3′,4′,7-
Trihydroxyisoflavone

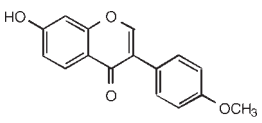

4′-Methoxy-7-
hydroxyisoflavone
(Formononetin)

Table 3
IC_{50} values for inhibition of SERCA2B in brain microsomes

Flavonoid	IC_{50} (μM)
3,6-Dihydroxyflavone	4.6 ± 0.5
5,6,7-Trihydroxyflavone (Baicalein)	22.5 ± 7.7
3,3′,4′,5,7-Pentahydroxyflavone (Quercetin)	24.9 ± 7.3
3′,4′,5,7-Tetrahydroxyflavone (Luteolin)	30.0 ± 2.3
3,3′,4′5,5′,7-Hexahydroxyflavone (Myricetin)	52.0 ± 6.5
3,5,7-Trihydroxyflavone (Galangin)	72.6 ± 10.9
2′3,4′,5,7-Pentahydroxyflavone (Morin)	86.0 ± 2.8
4′,5,7-Trihydroxyisoflavone (Genistein)	No inhibition

Several studies on the effects of flavonoids on estrogen receptor (ER) binding and sulfotranferase inhibition have been undertaken to assess their structure-activity relationship (*26, 27*). In both cases, the structural requirements for either ER binding or sulfotransferase inhibition were completely different to the structural requirements for SERCA inhibition. These findings, therefore, indicate that the flavonoid binding domains on each of the proteins is highly distinct, and if as is likely, flavonoids have multifunctional effects on cells, mixtures of flavonoids are likely to be more efficacious than adding one flavonoid alone.

In summary, some naturally occurring flavonoids are able to inhibit intracellular SERCA Ca^{2+} pumps in the micromolar concentration range, a range that has been shown to exist in human blood plasma. As such, it may be likely that these flavonoids could potentially act upon dysfunctional/transformed cells to instigate their demise through a Ca^{2+}-mediated process. Several recent studies to support of this view have shown that a number of flavonoids can cause both an increase in intracellular [Ca^{2+}] levels and induce cell death in several different types of cancer cells (*18, 28*). Finally, ongoing studies in our laboratory are underway to investigate whether a correlation exists between potency of flavonoid inhibition of Ca^{2+} pumps and their ability to induce cell death.

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