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Structure−Activity Relationships of Flavonoids in the Cellular Antioxidant Activity Assay

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Chemical antioxidant activity assays are used extensively to evaluate the potential bioactivity of plant foods and their phytochemical constituents, but they do not mimic the complexity of biological systems. The cellular antioxidant activity (CAA) activity assay was developed to be a more biologically relevant model to measure antioxidant activity. Structure−activity relationships of flavonoids have been determined in many chemistry antioxidant activity assays, and they vary with the protocols. The objective of this study was to determine structure−activity relationships of selected flavonoids in the CAA assay. The structures that conferred flavonoids with the most antioxidant activity in the CAA assay were a 3′,4′-*o*-dihydroxyl group in the B-ring, a 2,3-double bond combined with a 4-keto group in the C-ring, and a 3-hydroxyl group. Isoflavones had no cellular antioxidant activity. Flavanols with a galloyl moiety had higher antioxidant activity than those without, and a B-ring 3′,4′,5′-trihydroxyl group further improved their efficacy. ORAC values for flavonoids were not related to their CAA values. Knowledge of structure−activity relationships in the CAA assay may be helpful in assessing potential in vivo antioxidant activity of flavonoids.

KEYWORDS: Flavonoids; antioxidant; antioxidant activity; cancer; free radicals

INTRODUCTION

Cancer is the second leading cause of death in the United States (*1*). Cancer is a disease in which abnormally high proliferation of mutated cells occurs. Oxidative stress may be the most important factor causing oxidative DNA damage that can eventually lead to mutations if left unrepaired (*2*). Consumption of fruits and vegetables has been linked to reduced risk of cancer in several epidemiological studies (*3, 4*). The dietary phytochemicals in fruits and vegetables are likely responsible for decreased cancer risk by reducing oxidative stress and modulating signal transduction pathways involved in cell proliferation and survival (*5, 6*).

The flavonoids are a class of widely distributed phytochemicals with antioxidant and biological activity. They have structures consisting of two aromatic rings linked by three carbons in an oxygenated heterocycle (**Figure 1**). Differences in the structure of the heterocycle, or C-ring, classify them as flavonols, flavones, flavanols (catechins), flavanones, anthocyanidins, or isoflavonoids (isoflavones) (*6*). Flavonols are characterized by a 2,3-double bond, a 4-keto group, and a 3-hydroxyl group in the C-ring. Flavones lack the 3-hydroxyl moiety, and flavanones have a saturated C-ring. The 2,3-double bond and 4-keto group are absent from flavanols or catechins. The B-ring of isoflavones is linked to C-3 of the C-ring, instead of C-2, as it is for the other flavonoid subclasses. Flavonoids, as constituents of plant foods, have been implicated in the reduction of cancer risk. In the Zutphen Elderly Study, flavonoid intake from fruits and vegetables was inversely associated with all-cause cancer risk and cancer of the alimentary and respiratory tract (*7*). Lung cancer risk has also been inversely associated with flavonoid (*8*) and quercetin intake (*9*). Although not definitive, many other epidemiological studies have shown a trend for decreased cancer risk with higher flavonoid consumption, and these studies have been reviewed recently (*10, 11*).

Concepts related to the presence of antioxidants in foods and their potential health benefits to humans are becoming recognized (*6*). The cellular antioxidant activity (CAA) assay was recently introduced by our laboratory to measure the antioxidant activity of antioxidants, foods, and dietary supplements in hepatocellular carcinoma (HepG2) cells (*12*). It was developed in response to the need for a cell culture model to assess potential in vivo antioxidant capacities (*13*) that would be more biologically relevant than the chemistry antioxidant activity assays in common usage. Chemical antioxidant activity assays, such as the oxygen radical absorbance capacity (ORAC) (*14*), Trolox equivalent antioxidant capacity (TEAC) (*15*), total radical-scavenging antioxidant parameter (TRAP) (*16, 17*), 2,2-diphenylpicrylhydrazyl (DPPH) free radical (*18*), ferric reducing/antioxidant power (FRAP) (*19*), total oxyradical scavenging capacity (TOSC) (*20*), and peroxyl scavenging capacity (PSC) (*21*),

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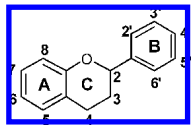

Figure 1. Generic structure of flavonoids.

are simple methods that measure antioxidant activity in controlled systems, but are not reflective of biological activity because they do not account for cell uptake, partitioning of antioxidants between aqueous and lipid phases, or phase I and phase II metabolism. The CAA assay measures the inhibition of peroxyl radical-induced oxidation of dichlorofluorescin by antioxidants in cell culture. Therefore, it is a more biologically relevant method than the popular chemical antioxidant activity assays and is an important tool to study the potential bioactivity of foods, antioxidants, and dietary supplements.

It was proposed by Bors et al. (*22*) that three structural moieties are important for antioxidant and radical-scavenging activity by flavonoids: (1) an *o*-dihydroxyl group in the B-ring; (2) a 2,3-double bond combined with a 4-oxo group in the C-ring; and (3) hydroxyl groups at positions C-3 and C-5. The structure−activity relationships for flavonoids have been investigated in many chemical antioxidant activity assays (*23*−*29*), and the required structural features for high activity are often those proposed by Bors et al. (*22*), but not always. Thus, structure−activity relationships depend on the protocol employed, and it is necessary to define them to predict activity under the investigative set of conditions. The ability of the CAA assay to measure the antioxidant activity of phenolic compounds and fruit extracts in cell culture was reported recently (*12*), but the molecular structures that dictate efficacy in the CAA assay have yet to be characterized.

The objective of the study was to investigate the structure−activity relationships of flavonoids in the CAA assay. The influence of flavonoid lipophilicity was evaluated, and CAA values were compared to ORAC values. We hypothesize that, due to the additional complexity introduced by cell uptake, metabolism, and membrane partitioning, chemical antioxidant activities cannot predict antioxidant behaviors in the CAA assay or in vivo.

MATERIALS AND METHODS

Chemicals. 2′,7′-Dichlorofluorescin diacetate (DCFH-DA), fluorescein disodium salt, apigenin, (+)-catechin hydrate, chrysin, daidzein, (−)-epicatechin, (−)-epicatechin gallate (ECG), (−)-epigallocatechin (EGC), (−)-epigallocatechin gallate (EGCG), galangin, genistein, 6-hydroxy-2,5,7,8-tetramethylchoman-2-carboxylic acid (Trolox), kaempferol, luteolin, morin hydrate, naringenin, quercetin dihydrate, rutin hydrate, and taxifolin were purchased from Sigma-Aldrich, Inc. (St. Louis, MO). Myricetin and quercetin-3-β-D-glucoside (Q-3-G) were obtained from Fluka Chemical Corp. (Milwaukee, WI). Dimethyl sulfoxide was obtained from Fisher Scientific (Pittsburgh, PA), and 2,2′-azobis(2-amidinopropane) dihydrochloride (ABAP) was purchased from Wako Chemicals USA, Inc. (Richmond, VA). Methanol was bought from Mallinckrodt Baker, Inc. (Phillipsburg, NJ). The HepG2 cells were obtained from the American Type Culture Collection (ATCC) (Rockville, MD). Williams' Medium E (WME) and Hanks' Balanced Salt Solution (HBSS) were purchased from Gibco Life Technologies (Grand Island, NY). Fetal bovine serum (FBS) was obtained from Atlanta Biologicals (Lawrenceville, GA).

Preparation of Solutions. A 200 mmol/L stock solution of DCFH-DA in methanol was prepared, aliquoted, and stored at −20 °C. A 200 mmol/L ABAP stock solution in water was prepared, and aliquots were stored at −40 °C. Working flavonoid solutions were prepared in dimethyl sulfoxide before further dilution in treatment medium (WME with 2 mM L-glutamine and 10 mmol/L Hepes). Final treatment solutions for cellular antioxidant activity assay contained 0.5% dimethyl sulfoxide, and solutions for cytotoxicity experiments contained 1% dimethyl sulfoxide.

Cell Culture. HepG2 cells were grown in growth medium (WME supplemented with 5% FBS, 10 mmol/L Hepes, 2 mmol/L L-glutamine, 5 μg/mL insulin, 0.05 μg/mL hydrocortisone, 50 units/mL penicillin, 50 μg/mL streptomycin, and 100 μg/mL gentamycin) and were maintained at 37 °C and 5% CO_2 as described previously (*12*). Cells used in this study were between passages 12 and 32.

Cytotoxicity. The cytotoxicity of flavonoids toward HepG2 cells was measured, as described previously (*12, 30*). Briefly, HepG2 cells were seeded at 4×10^4/well on a 96-well plate in 100 μL growth medium and incubated for 24 h at 37 °C. The medium was removed, and the cells were washed with PBS. Flavonoids in 100 μL growth medium were applied to the cells, and the plates were incubated at 37 °C for 24 h. The medium was removed, and the cells were washed with PBS before a volume of 50 μL/well methylene blue staining solution (98% HBSS, 0.67% glutaraldehyde, 0.6% methylene blue) was applied to each well, and the plate was incubated at 37 °C for 1 h. The dye was removed, and the plate was immersed in fresh deionized water until the water was clear. The water was tapped out of the wells, and the plate was allowed to air-dry briefly before 100 μL of elution solution (49% PBS, 50% ethanol, 1% acetic acid) was added to each well. The microplate was placed on a benchtop shaker for 20 min to allow uniform elution. The absorbance was read at 570 nm with blank subtraction using the MRX II Dynex spectrophotometer (Dynex Inc., Chantilly, VA). The median cytotoxic concentration (CC_{50}) was calculated for each flavonoid.

Cellular Antioxidant Activity (CAA) of Flavonoids. The CAA assay protocol was described previously (*12*). Briefly, HepG2 cells were seeded at a density of 6×10^4/well on a 96-well black, clear-bottom microplate in 100 μL of growth medium/well. Twenty-four hours after seeding, the growth medium was removed, and the wells were washed with PBS. Wells were treated in triplicate for 1 h with 100 μL of treatment medium containing tested flavonoid plus 25 μmol/L DCFH-DA. When a PBS wash was utilized, wells were washed with 100 μL of PBS. Then 600 μmol/L ABAP was applied to the cells in 100 μL of HBSS, and the 96-well microplate was placed into a Fluoroskan Ascent FL plate reader (ThermoLabsystems, Franklin, MA) at 37 °C. Emission at 538 nm was measured after excitation at 485 nm every 5 min for 1 h.

Quantification of Cellular Antioxidant Activity (CAA). After blank subtraction and subtraction of initial fluorescence values, the area under the curve for fluorescence versus time was integrated to calculate the CAA value at each flavonoid concentration as

$$\text{CAA unit} = 1 - \left(\int \text{SA} / \int \text{CA}\right) \quad (1)$$

where $\int$SA is the integrated area under the sample fluorescence versus time curve and $\int$CA is the integrated area from the control curve. The median effective dose (EC_{50}) was determined for the flavonoids from the median effect plot of $\log(f_a/f_u)$ versus log (dose), where f_a is the fraction affected (CAA unit) and f_u is the fraction unaffected (1 − CAA unit) by the treatment. The EC_{50} values were stated as mean ± SD for triplicate sets of data obtained from the same experiment. EC_{50} values were converted to CAA values, expressed as micromoles of quercetin equivalents (QE) per 100 μmol of flavonoid, using the mean EC_{50} value for quercetin from five separate experiments. Experimental variation for the assay was discussed previously (*12*).

Measurement of Oxygen Radical-Scavenging Capacity (ORAC). The peroxyl radical scavenging efficacy of flavonoids was measured using the ORAC assay (*31*). Briefly, 20 μL of blank, Trolox standard, or flavonoid in 75 mmol/L potassium phosphate buffer, pH 7.4 (working buffer), was added to triplicate wells in a black, clear-bottom, 96-well microplate. Each of the solutions contained 0.2% dimethyl sulfoxide. The triplicate samples were distributed throughout the microplate and were not placed side-by-side, to avoid any effects on readings due to location. In addition, no outside wells were used, as use of those wells results in greater variation. A volume of 200 μL of 0.96 μmol/L fluorescein in working buffer

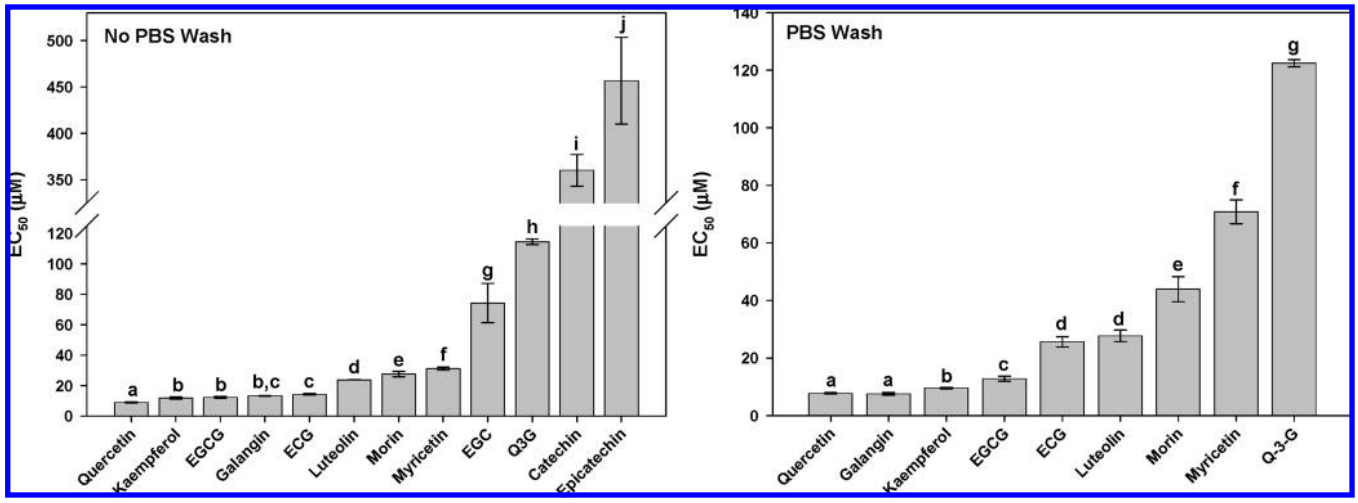

Figure 2. EC_{50} values for selected flavonoids in the CAA assay (mean ± SD, $n = 3$). In each graph, bars having no letters in common are significantly different ($p < 0.05$).

Table 1. EC_{50} and CAA Values for Flavonoids in the CAA Assay (Mean ± SD, $n = 3$)

	no PBS wash		PBS wash		cytotoxicity
flavonoid	EC_{50} (μmol/L)	CAA (μmol of QE/100 μmol)	EC_{50} (μmol/L)	CAA (μmol of QE/100 μmol)	CC_{50} (μM)
quercetin	8.93 ± 0.44	99.1 ± 4.8	7.71 ± 0.26	105.7 ± 3.7	>100
kaempferol	11.9 ± 0.8	74.6 ± 4.8	9.57 ± 0.27	85.1 ± 2.4	>100
EGCG	12.3 ± .7	72.1 ± 3.8	12.8 ± 0.9	63.8 ± 4.4	>100
galangin	13.3 ± .2	66.3 ± 1.1	7.56 ± 0.46	107.8 ± 6.4	>80
ECG	14.2 ± 0.4	62.1 ± 1.9	25.6 ± 1.8	31.9 ± 2.2	>200
luteolin	23.8 ± .0	37.1 ± 0.0	27.7 ± 2.0	29.5 ± 2.1	>80
morin	27.6 ± 1.8	32.1 ± 2.1	43.9 ± 4.3	18.6 ± 1.8	>200
myricetin	31.1 ± 1.0	28.4 ± 0.9	70.7 ± 4.2	11.5 ± 0.7	>200
EGC	74.2 ± 12.9	12.1 ± 2.2	>100		>200
Q-3-G	115 ± 2	7.7 ± 0.1	122 ± 1	6.6 ± 0.1	>200
catechin	360 ± 17	2.5 ± 0.1	>800		>1000
epicatechin	457 ± 47	1.9 ± 0.2	>800		>1000
taxifolin	>200		>200		>200
genistein	no activity		no activity		>100
daidzein	no activity		no activity		>100
apigenin	no activity		no activity		>80
naringenin	no activity		no activity		>200
chrysin	no activity		no activity		>200
rutin	no activity		no activity		>200

was added to each well and incubated at 37 °C for 20 min, with intermittent shaking, before the addition of 20 μL of freshly prepared 119 mmol/L ABAP in working buffer using a 12-channel pipetter. The microplate was immediately inserted into a Fluoroskan Ascent FL plate reader (ThermoLabsystems) at 37 °C. The decay of fluorescence at 538 nm was measured with excitation at 485 nm every 4.5 min for 2.5 h. The areas under the fluorescence versus time curve for the samples minus the area under the curve for the blank were calculated and compared to a standard curve of the areas under the curve for 6.25, 12.5, 25, and 50 μmol/L Trolox standards minus the area under the curve for blank. ORAC values were expressed as mean micromoles of Trolox equivalents (TE) per micromole of flavonoid ± SD for at least three separate experiments.

Octanol−Water Partition Coefficients (*P*). Log *P* values for the selected flavonoids were estimated using the log *P* add-on for ChemSketch 10.0 (Advanced Chemistry Development, Inc., Toronto, ON, Canada).

Statistical Analyses. All results are presented as mean ± SD, and statistical analyses were performed using Minitab 15 (Minitab Inc., State College, PA). Differences between means were detected by ANOVA, followed by multiple comparisons using Fisher's least significant difference test. ANOVA was performed on log-transformed EC_{50} values for CAA because the assumptions of normally distributed residuals and equal variances were not met by the untransformed data. Correlations were determined using linear regression. Results were considered to be significant when $p < 0.05$.

RESULTS AND DISCUSSION

Efficacy of Selected Flavonoids in the CAA Assay. Nineteen flavonoids were evaluated for their cellular antioxidant activities using the CAA assay. The EC_{50} values for those with quantifiable activity are depicted in **Figure 2**, and the EC_{50} values and their corresponding CAA values for each flavonoid tested are listed in **Table 1**. When the cells were not washed with PBS between flavonoid and ABAP treatments (no PBS wash protocol), quercetin had the highest activity, or lowest EC_{50} value ($p < 0.05$), followed by kaempferol, EGCG, and galangin, which had similar EC_{50} values ($p > 0.05$). The efficacies of the remaining flavonoids in the no PBS wash protocol were in the following order: ECG > luteolin > morin > myricetin > EGC > Q-3-G > catechin > epicatechin; taxifolin had low, but unquantifiable, activity.

When the HepG2 cells were washed with PBS between flavonoid and ABAP treatments (PBS wash protocol), the order of efficacy was quercetin = galangin > kaempferol > EGCG > ECG = luteolin > morin > myricetin > Q-3-G.

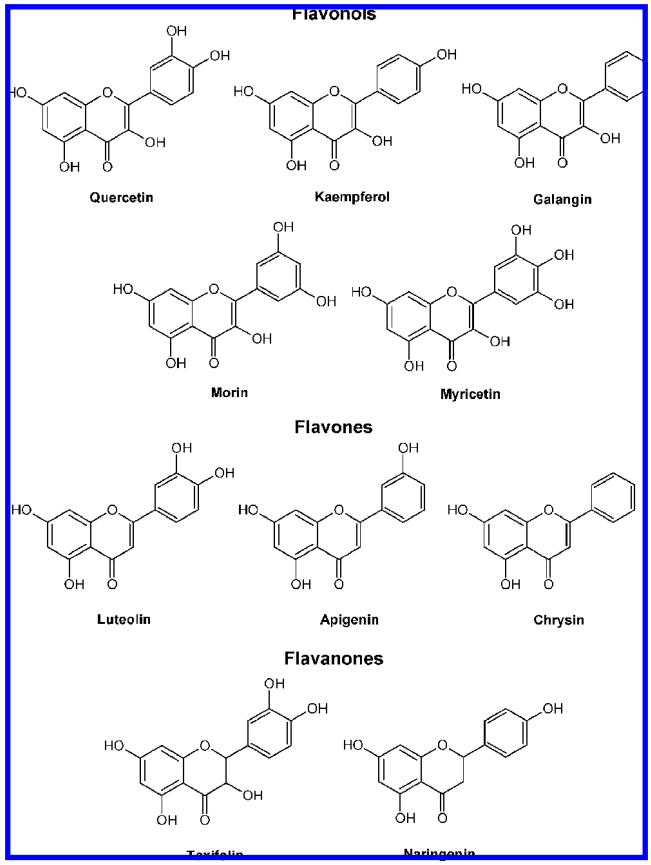

Figure 3. Structures of flavonoids showing differences in B-ring hydroxylation within subclasses.

EGC, catechin, epicatechin, and taxifolin had low activity at the concentrations tested. Genistein, daidzein, apigenin, naringenin, chrysin, and rutin had no activity in either protocol.

Structure−Activity Relationships of Selected Flavonoids in CAA Assay. The molecular structures of flavonoids that dictate their efficacies in the cellular antioxidant activity were investigated. The generic structure of flavonoids is illustrated in **Figure 1**. Flavonoids undergo extensive phase I and phase II metabolism within enterocytes upon absorption and other tissues after transport (*32*), which will ultimately affect their bioactivities. The incorporation of cellular metabolism into the assay is one of the features that make the CAA an improvement over chemistry antioxidant activity assays. HepG2 cells were used because the results are similar to those obtained from intestine-like Caco-2 cells, but with much less variation (unpublished data). Because flavonoid metabolism will influence efficacy, a flavonoid structure−activity examination was warranted as a first step toward characterizing the CAA assay and as a comparison of the CAA assay to chemistry antioxidant activity assays. The three structural features proposed to be essential for flavonoid antioxidant activity by Bors et al. (*22*)—a B-ring *o*-dihydroxy group, a 2,3-double bond combined with a 4-oxo group in the C-ring, and a 3-hydroxyl group—were examined, as well as quercetin glycosylation and structures particular to isoflavones and flavanols.

*B-Ring Hydroxylation of Flavonols, Flavones, and Flavanones (**Figure 3**).* The number and positioning of the B-ring hydroxyl groups in flavonoids were important to cellular antioxidant activity. **Figure 3** shows the hydroxylation patterns of tested flavonoids from the flavonol, flavone, and flavanone subclasses. Of the flavonols tested in the no PBS wash protocol, quercetin, which has a 3′,4′-*o*-dihydroxyl group, had the highest activity ($p < 0.05$) with an EC_{50} of 8.93 ± 0.44 μmol/L (**Table 1**). Kaempferol and galangin had similar efficacies, despite the lack of B-ring hydroxyl groups on galangin, and had only slightly higher EC_{50} values, or slightly lower activities, than quercetin ($p < 0.05$). Morin, which has two B-ring hydroxyl groups in the meta configuration, had much lower activity (EC_{50} = 27.6 ± 1.8 μmol/L; $p < 0.05$) than quercetin. The presence of a *m*-diphenolic moiety in the B-ring reduced activity compared to the ortho configuration in the TEAC assay, as well (*25*). Myricetin has an extra 5′-hydroxyl group compared to quercetin and had even lower activity than morin (EC_{50} = 31.1 ± 1.0 μM). Of those tested, luteolin was the only flavone with activity in the CAA assay. The flavanone, taxifolin, had low but unquantifiable activity, whereas naringenin had none.

In the PBS wash protocol, the flavonols with lowest EC_{50} values for cellular antioxidant activity were quercetin (7.71 ± 0.26 μmol/L) and galangin (7.56 ± 0.46 μmol/L), followed closely by kaempferol. Morin, possessing 3′,5′-*m*-hydroxylation, had a higher EC_{50} value (43.9 ± 0.43 μmol/L; $p < 0.05$), and the addition of another hydroxyl group in myricetin further decreased activity. Again, only the flavone and flavanone with B-ring catechol groups had cellular antioxidant activity.

These results indicate that a 3′,4′-*o*-dihydroxyl group is an indicator of substantial antioxidant activity for flavonoids in the CAA assay, especially for those not belonging to the flavonol subclass. This is consistent with previous reports that a B-ring catechol group is essential for high antioxidant activity in many different systems (*23, 25−29, 33*). The higher antioxidant activity of flavonoids with an *o*-dihydroxyl group in the B-ring has been attributed to their greater radical stability through increased electron delocalization (*22*) and intramolecular hydrogen bonding between the 3′- and 4′-hydroxyls (*34*). An additional 5′-hydroxyl group in the B-ring, as seen in myricetin, has been shown to decrease antioxidant activity in other assays, which may be due to a pro-oxidant effect introduced by the pyrogallol group (*27*).

*Presence of 2,3-Double Bond, 4-Keto Group, and 3-Hydroxyl Moiety (**Figure 4**).* The EC_{50} values for flavonoids in the no PBS wash and PBS wash protocols showed similar trends (**Figure 2**). For flavonoids with a B-ring catechol group (quercetin, luteolin, taxifolin, and catechin), the loss of any of the C-ring functional groups, the 2,3-double bond, 4-keto group, or 3-hydroxyl group, tended to result in a reduction of activity. When the 2,3-double bond conjugated to the 4-keto group was present, the absence of the 3-hydroxyl group, as in luteolin, moderately increased the EC_{50} value and decreased cellular antioxidant activity. Removal of the 3-hydroxyl group from a flavonol introduces an approximately 20° twist of the B-ring relative to the A- and C-rings, and nonplanar molecules cannot delocalize electrons across the molecule effectively and have less scavenging activity (*34*). In addition, hydrogen bonding between the 4-keto and 3-hydroxyl or 5-hydroxyl groups stabilizes flavonoid radicals (*34*). The 4-keto group, along with the 5-hydroxyl moiety, is the most important site for the chelation of transition metal ions, which can catalyze oxidative chain reactions (*35*).

The 2,3-double bond combined with the 4-keto group delocalizes electrons from the B-ring (*22*), and the loss of one or both characteristics dramatically reduced cellular antioxidant activity. This is demonstrated when the EC_{50} values of quercetin to taxifolin and catechin and that of kaempferol to naringenin are compared. Similar trends were seen in the TEAC assay (*25*); however, C-ring unsaturation did not affect antioxidant activity

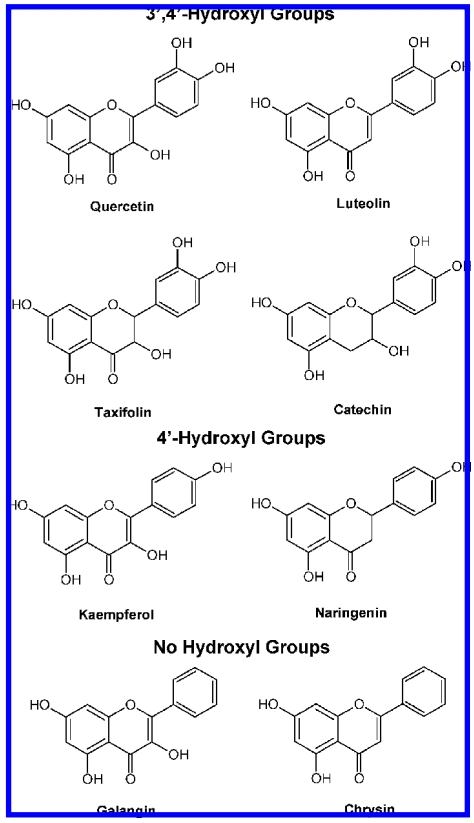

Figure 4. Flavonoids with similar B-ring hydroxylation patterns and different C-ring structural features.

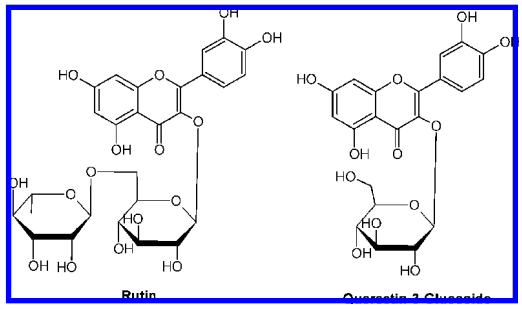

Figure 5. Quercetin glycoside structures.

in the ORAC assay (*23*). The 2,3-double bond may be more important for cellular antioxidant activity than the 3-hydroxyl group because a greater increase in EC_{50} values accompanied the loss of that structural feature; compared to quercetin, luteolin maintained higher activity than taxifolin. In the absence of B-ring hydroxylation, the 3-hydroxyl group was important to antioxidant activity, as shown by the low EC_{50} value for galangin and the absence of activity by chrysin.

*Glycosylation (**Figure 5**).* The 3-glycosylation of quercetin dramatically affected its antioxidant activity in the CAA assay, and also the type of esterified sugar was important. Q-3-G maintained a low amount of activity, whereas rutin had none. It is interesting to note that rutin had the highest antioxidant activity in the ORAC assay (**Table 2**). This will be discussed further below. The much higher EC_{50} values of Q-3-G and rutin compared to luteolin, which has the same base structure, could possibly be explained by the greater twist of the B-ring compared to the A- and C-rings introduced by glycosylation, as the torsion angle for rutin is almost 30°, whereas the torsion angle for luteolin is only around 20° (*34*).

*Isoflavones (**Figure 6**).* The isoflavones genistein and daidzein had no activity in the CAA assay (**Table 1**). Genistein and daidzein were effective reducers of the $ATBS^{\bullet+}$ cation in the TEAC assay, but performed poorly at reducing the Fe(III) complex in the FRAP assay, quenching galvinoxyl radicals, and inhibiting microsomal lipid peroxidation (*36*). The experiments showed that isoflavones are poor hydrogen donors and have activities only at levels beyond which are achievable in vivo. Guo et al. (*37*) also reported limited antioxidant activity of isoflavones against a variety of oxidants and free radicals, and genistein had low efficacy against ABAP-induced oxidation of liposomes (*23*). In agreement, another earlier study found genistein to be a poor antioxidant in liposome and micelle systems oxidized using ABAP (*38*), although it was an effective antioxidant in assays where hydrogen peroxide or iron was involved in the oxidation, despite not being a good metal chelator. The isoflavone metabolite, equol, which is identical to daidzein except for having a saturated C-ring, had much higher antioxidant activity against Fe(II)-, Fe(III)-, and ABAP-induced oxidation of liposomes compared to genistein and daidzein (*39*). The absence of a 2,3-double bond could be a major determinant of isoflavone antioxidant activity. It is not surprising, therefore, that genistein and daidzein did not have activity in the CAA assay, which involves ABAP-induced oxidation of a cell membrane. Further research is needed to determine if isoflavones with no 2,3-double bond have cellular antioxidant activity.

Table 2. ORAC and Log *P* Values for Selected Flavonoids

flavonoid	ORAC[a,b] (μmol of TE/μmol)	lipophilicity (log *P*)
quercetin	8.04 ± 2.37 cde	2.07
kaempferol	7.19 ± 1.29 def	2.05
EGCG	4.55 ± 0.40 fgh	2.08
galangin	2.63 ± 1.31 gh	2.83
ECG	7.71 ± 1.57 cde	2.67
luteolin	8.55 ± 0.85 cde	2.4
morin	6.12 ± 1.95 efg	2.62
myricetin	4.55 ± 0.50 fgh	2.11
EGC	3.11 ± 0.73 cde	ND[c]
Q-3-G	8.11 ± 1.86 cde	1.75
catechin	12.4 ± 4.0 ab	0.49
epicatechin	9.14 ± 1.31 cd	0.49
taxifolin	9.74 ± 1.20 bcd	1.82
genistein	13.4 ± 2.8 a	2.96
daidzein	8.52 ± 2.11 cde	2.78
apigenin	10.7 ± 1.5 bc	3.04
naringenin	9.23 ± 2.79 bcd	2.42
chrysin	3.79 ± 0.67 gh	2.88
rutin	13.7 ± 1.7 a	1.76

[a] Mean ± SD, $n \geq 3$. [b] Values with no letter in common are significantly different ($p < 0.05$). [c] No data available.

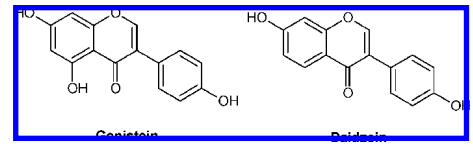

Figure 6. Isoflavone structures.

*Flavanols (Catechins) (**Figure 7**).* Catechin and epicatechin had low activity (EC_{50} = 360 ± 17 and 457 ± 47 μM, respectively) in the no PBS wash protocol and very low, unquantifiable, activity in the PBS wash protocol. In both protocols, the presence of a galloyl group in the flavanols EGCG and ECG imparted them with very high activity, and low EC_{50} values, in the CAA assay compared to catechin, epicatechin, and EGC. An additional B-ring hydroxyl group at the 5′-position gave EGC a much lower EC_{50} value than catechin and epicatechin in the no PBS wash protocol and slightly increased the activity of EGCG over ECG in both methods (**Figure 2**).

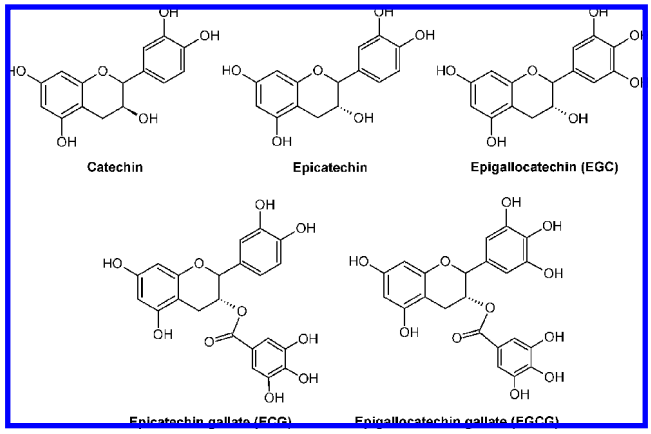

Figure 7. Structures of flavanols (catechins).

This trend is similar to that for the scavenging activities of flavanols against ABAP-generated radicals in phosphate buffer (*40*) and in the TEAC assay (*25*).

Correlations between CAA and Lipophilicity. Log *P* (octanol−water partitioning coefficient) values were estimated using computer software (**Table 2**) and compared to the EC_{50} values for CAA. Some of the most lipophilic compounds, apigenin, genistein, chrysin, and daidzein, had no cellular antioxidant activity, and so were not included in the correlation analysis. Many of the more hydrophilic flavonoids, such as epicatechin, catechin, Q-3-G, rutin, and taxifolin, also had low or no cellular antioxidant activity. The EC_{50} values obtained from the PBS wash CAA method were not related to the log *P* values of the flavonoids ($R^2 = 0.232$, $p > 0.05$, $n = 9$), but the EC_{50} values for the cellular antioxidant activity of flavonoids in the no PBS wash protocol were significantly negatively correlated to their lipophilicity ($R^2 = 0.864$, $p < 0.001$, $n = 11$). The antioxidant activity values from the no PBS wash protocol may have been more reflective of the interactions of the flavonoids with the cell membrane, as the PBS wash likely removed flavonoids with weak interactions, leaving only those that were taken up by the cells, deeply embedded in the lipid bilayer, or tightly bound to the cell membranes to scavenge peroxyl radicals.

Octanol−water partitioning coefficients are a measure of lipophilicity and are commonly used to predict the distribution and fate of toxins and pharmaceuticals in the body and chemicals in the environment (*41*). Glycosylation and hydroxylation both decrease the lipophilicity of flavonoids, and sugar esterification is the greater modulator (*42*). The lipophilicity of flavonoids may play a role in their accessibility to free radicals, so membrane partitioning is thought to be important in dictating their antioxidant activity (*43, 44*). Flavonoids with very high or very low lipophilicity had low antioxidant activities against Fe(III)-induced lipid peroxidation of mouse liver microsomes (*45*), and that may also be the case in the CAA assay. As was observed in the ABAP-induced oxidation of linolenic acid in micelles (*26*), flavonoids with higher log *P* values tended to have greater antioxidant activity, but structural features dictated the activities of compounds with similar lipophilicities.

Correlations between CAA and ORAC Values. The ORAC assay measures the ability of antioxidants to scavenge peroxyl radicals generated by ABAP and delay the decay in fluorescence of the fluorescein probe. The ORAC values for selected flavonoids are listed in **Table 2**. Rutin, genistein, and catechin had the highest activities in the ORAC assay (13.7 ± 1.7, 13.4 ± 2.8, and 12.4 ± 4.0 μmol of TE/μmol, respectively; $p <$ 0.05), followed by apigenin, taxifolin, and naringenin, which were not significantly different from catechin ($p > 0.05$). Galangin, EGC, chrysin, myricetin, EGCG, and morin had the lowest antioxidant activities in the ORAC assay (2.63 ± 1.31, 3.11 ± 0.73, 3.79 ± 0.67, 4.55 ± 0.50, 4.55 ± 0.40, and 6.12 ± 1.95 μmol of TE/μmol, respectively; $p < 0.05$). The antioxidant activity ranking of tested flavonoids in the ORAC assay was different from the results reported by Cao et al. (*24*) and Ou et al. (*46*), but was more in agreement with the ranking reported by Aaby et al. (*47*). Our ORAC values of tested flavonoids also tended to be higher than the values presented in those studies. Variations in results can likely be explained by differences in protocols. Cao et al. (*24*) and Aaby et al. (*47*) both used β-phycoerythrin, not fluorescein, as a probe. The Prior group later showed that the values obtained from using a fluorescein probe tended to be higher compared to those from using β-phycoerythrin and that the compounds with the highest activities using one probe did not always have the highest activities with the other (*46*). In addition, the reagent concentrations, solvents used to dissolve the flavonoids, pH of the buffers, and reaction times differed. The degree of hydroxylation has been cited as the biggest determinant of antioxidant activity in the ORAC assay (*23, 24*); however, our ORAC data do not support that idea.

To attempt to explain the CAA of flavonoids by their radical-scavenging abilities in a simple system, their CAA and ORAC values were compared. There was no significant association between flavonoid ORAC and CAA values ($R^2 = 0.214$, $p > 0.05$, $n = 12$ for the no PBS wash protocol; $R^2 = 0.080$, $p > 0.05$, $n = 9$ for the PBS wash protocol). This was also found when the relationship between ORAC and the prevention of oxidative stress in HepG2 cells for broccoli extracts was examined (*48*). In fact, many of the flavonoids with the highest ORAC values had no CAA (rutin, genistein, apigenin, and naringenin) or low CAA (catechin, taxifolin, and epicatechin). Conversely, galangin and EGCG were among the flavonoids with the lowest ORAC values, but they exhibited high activity in the CAA assay. Quercetin, kaempferol, and luteolin, which had high cellular antioxidant activity, were only moderately effective in the ORAC assay compared to the other tested flavonoids. The lack of correlation between the CAA and ORAC assays is likely due to the biological components of the CAA assay; because it monitors oxidative stress in cells, not a test tube, it accounts for some aspects of cell uptake, distribution, and metabolism of antioxidant compounds.

Concluding Remarks. The structural features that confer flavonoids with high cellular antioxidant activity in the CAA assay include a 3′,4′-*o*-dihydroxyl group, a 2,3-double bond in conjugation with a 4-keto moiety, and a 3-hydroxyl group. Galloylated flavanols were more efficacious than flavanols without gallate conjugation, and a B-ring pyrogallol group also improved activity. Glycosylation of quercetin attenuated or abolished activity. Genistein and daidzein completely lacked cellular antioxidant activity, and the structural requirement for isoflavone activity must be studied further. ORAC values for flavonoids were not correlated to CAA values. The CAA assay may be more predictive of peroxyl radical scavenging activity in biological systems than the chemical antioxidant activity assays. Research should be done to compare results from the CAA assay to in vivo biomarkers of antioxidants and oxidative stress to determine if CAA is indicative of biological activity.

ABBREVIATIONS USED

ABAP, 2,2′-azobis(2-amidinopropane) dihydrochloride; CAA, cellular antioxidant activity; DCFH-DA, 2′,7′-dichlorofluorescin

diacetate; DPPH, 2,2-diphenylpicrylhydrazyl; ECG, (−)-epicatechin gallate; EGC,(−)-epigallocatechin; EGCG, (−)-epigallocatechin gallate; FRAP, ferric reducing/antioxidant power; ORAC, oxygen radical absorbance capacity; Q-3-G, quercetin-3-β-D-glucoside; QE, quercetin equivalents; TE, Trolox equivalents; TEAC, Trolox equivalent antioxidant capacity; Trolox, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid.

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