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Structure-Activity Analysis of Flavonoids: Direct and Indirect Antioxidant, and Antiinflammatory Potencies and Toxicities

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Structure-Activity Analysis of Flavonoids: Direct and Indirect Antioxidant, and Antiinflammatory Potencies and Toxicities

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Flavonoids are secondary plant products that are well represented in healthy diets because of ingestion of fruit, vegetables, herbs, and teas. Increased consumption is correlated with decreased risks of cardiovascular disease, cancer, and other chronic diseases. Certain flavonoids confer direct antioxidant protection to cells, others induce enzymes that protect cells against oxidative and other insults ("indirect antioxidants"), and others appear to be protective by both mechanisms. Hydroxylated flavones manifest substantial direct antioxidant activity but do not effectively induce cytoprotective enzymes. Methoxylated flavones that potently induce cytoprotective enzymes were evaluated to elucidate the structural prerequisites for effective chemoprotective agents: protecting healthy cells with minimal collateral toxicity. Flavones and flavanones methoxylated at the 5-position of the A-ring were among the most potent inducers of the cytoprotective NAD(P)H:quinone-oxidoreductase 1 (NQO1) in 3 different cell lines. Other flavones were equally potent inducers, but more toxic. Flavanones contain no Michael reaction center, yet some are potent inducers of NQO1, have low cytotoxicity, and inhibit LPS-stimulated iNOS activity, which suggests a redox mechanism of action rather than the Keap1/Nrf2/ARE mechanism by which so many of the classic inducers operate. Evaluation in vivo will reveal whether differential protective advantages support their possible evaluation in a cancer prevention setting.

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INTRODUCTION

Despite advances in surgery and chemotherapy, cancer incidence continues to grow in absolute terms, mortality remains unacceptably high, and the 5-yr survival rate with many cancers has only improved slightly. Interrupting the biological mechanisms involved in carcinogenesis through the use of naturally occurring or synthetic compounds (*chemoprevention* or *chemoprotection*), which are safe for chronic, long-term use in otherwise healthy persons, would have tremendous medical and public health benefits. Chemoprotection may occur at any point along the long continuum of carcinogenesis, and many agents that target this process are under investigation. Health benefits are widely documented to be associated with higher intake of fruits and vegetables and the flavonoid-rich group of phytochemicals contained therein (1). Epidemiological studies have correlated intake of flavonoids to reduced coronary-related mortality (2,3), as well as reduced risk of cancers (4,5).

The flavonoids are a structurally diverse class of about 5000 low molecular weight polyphenolic benzo-c-pyrone derivatives, with a basic structure comprising 2 benzene rings (A and B), separated by an oxygen-containing pyran ring (C) (Fig. 1). Flavonoids can be further grouped into flavonols, flavanols, flavones, isoflavans, flavanones, and isoflavones, based on the degree of oxidation of the C-ring, the hydroxylation pattern of the A- and B-rings, and the substitution in the 3-position (Fig. 1). Most common ring substituents are hydroxyl-, methoxyl-, or sugar moieties (6). Flavonoids and their metabolites induce or inhibit phase 1 and 2 enzymes in vivo (7), but many of these flavonoids have very limited bioavailability because of high intestinal metabolism or poor absorption in vivo (8–10).

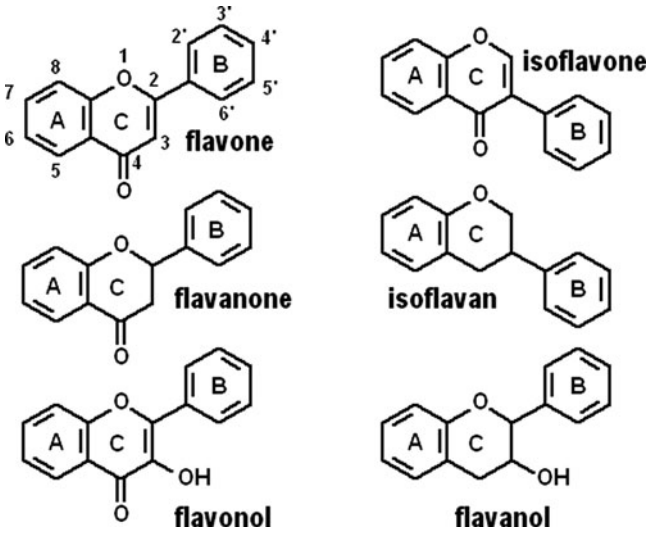

FIG. 1. Basic structure of a flavonoid and some naturally occurring flavonoids.

Recently, methoxylated flavones have emerged as a new subclass of flavonoids with unprecedented metabolic stability and thus potentially increased bioavailability (11,12). Particularly high concentrations of meth(ox)ylated flavones have been reported in *Citrus* species (13) and in a variety of plants used in various folk medicines (14), including *Arnica* species (15), neotropical nutmeg (16,17), leaves of some pepper plants (18), common field plants (19), the manuka tree in New Zealand (19), and herbs (20), but most naturally occurring flavonoids exist as glycosylated conjugates, and the aglycones are only absorbed after gastrointestinal hydrolysis.

Among their well-documented modes of action, many flavonoids inhibit cancer cell growth (7,21), down-regulate expression or activity of carcinogen-bioactivating phase 1 enzymes (8,22,23) or upregulate cytoprotective phase 2 enzymes (23,24). Induction of phase 2 enzymes (25,26) is an important step in detoxification of electrophilic and thus potentially carcinogenic xenobiotics (27). NAD(P)H:quinone-oxidoreductase 1 (NQO1) is an inducible phase 2 enzyme (24) that catalyzes the obligatory 2-electron reduction of quinones to hydroquinones thus protecting against oxidative cycling and sulfhydryl depletion. As such, the enzyme NQO1 is protective towards chemical- and radiation-induced carcinogenesis (28,29). Moreover, it has been widely used as a representative and prototypical biomarker for the coordinate induction of phase 2 enzymes, now known to occur via the Keap1-Nrf2-ARE signaling pathway (30), and inducers of which are referred to as *indirect antioxidants* (25). Many compounds that induce NQO1, including the naturally occurring polyphenols, contain Michael reaction acceptor groups (24,31), and it has been postulated that they are also essential for the NQO1 inducer activity of flavonoids (31).

The structure–function relationships of closely related flavonoids are therefore of special interest, and, for example, the position of a substituent aromatic ring on the flavone nucleus may dramatically affect both the inducer potency (32) and its direct antioxidant activity (29). Notably missing from the cancer prevention literature, are some of the most potent naturally occurring flavonoid inhibitors of cell proliferation (e.g., 5,7-dimethoxyflavone (33), which was anticipated to be a potent NQO1-inducer due to the juxtaposition of its electron-withdrawing methoxyl-groups in relation to the oxygen at position 4 of ring C). Dinkova-Kostova and colleagues demonstrated that the presence of unsubstituted hydroxyl groups at specific positions of the aromatic ring(s) of some families of plant polyphenols significantly increased the NQO1 inducer potency (31). We expected that these structural requirements would also apply to flavonoids, with hydroxylated or methoxylated flavones modifying NQO1-induction according to their electron withdrawing potencies. Furthermore, certain mono- or dimethoxylated flavones, in contrast to their nonmethylated analogues, have potent biological activity and antiproliferative effects selective for cancer cells, but low cytotoxic or cytostatic activity in normal cells (33).

In this study we examined a range of flavonoids selected based on both their expected range of phase 2 enzyme induction potency and their enhanced capacity for cellular uptake. They are thus of special interest as potential chemoprevention agents or lead compounds for synthesis. In addition to examining the indirect antioxidant capacity of these flavonoids by measuring their ability to induce NQO1 in murine hepatoma cells, we measured their ability to inhibit lipopolysaccharide (LPS)-dependent induction of iNOS (inducible nitric oxide synthase) in mouse macrophages, their cytotoxic effects in both a human cancer and noncancer cell line, their direct antioxidant radical quenching potency by the quenching of $ABTS^{\bullet+}$ (2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid)), and the uptake kinetics of a representative subset.

MATERIALS AND METHODS

Chemicals

Flavonoids (>97% purity) were purchased from Indofine Chemical Co. (Somerville, NJ) and Sigma-Aldrich Chemical Co. (St. Louis, MO). Dimethylsulfoxide (DMSO), and other solvents were HPLC grade and obtained from J.T. Baker (Phillipsburg, NJ). 3-[4,5-Dimethylthiazolyl-2-yl]-2,5-diphenyltetrazolium bromide (MTT), N-(1-naphthyl) ethylenediamine dihydrochloride, sulfanilamide, bovine serum albumin (BSA), 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). Bicinchoninic acid (BCA) protein assay kit was obtained from Thermo Fisher Scientific LSR (Rockford, IL). Cell culture medium and serum were from Gibco-BRL (Grand Island, NY). All other chemicals were of analytical grade.

Cell Culture

Cells were obtained from the American Tissue Culture Collection (Manassas, VA). Hepa 1c1c7 murine hepatoma cells,

which are highly inducible for NQO1 (34), were cultured as previously described at 37°C and 5% CO_2 in α-minimum essential medium supplemented with 10% heat- and charcoal-treated fetal bovine serum (FBS) (31,32). Human colon carcinoma (Caco-2) cells and normal human bronchial epithelial BEAS-2B cells were cultured as described previously (22,35,36). RAW264.7 cells were cultured as described (37). Caco-2 cells have been used extensively in the study of flavonoid transport and metabolism and are considered a good model of human intestinal cell function (38). BEAS-2B cells in culture have typical epithelial cell morphology (39), and although they are immortalized through Adenovirus SV40 hybrid virus, they were derived from normal human bronchial epithelial cells (40). BEAS-2B cells have been used extensively in inflammation (41) and carcinogenesis studies (22,33,42,43), and, as a noncancerous cell line, are thought to be a good model of normal bronchial epithelial cells (36,44,45) in the study of initiation of carcinogenesis in the human lung.

"Prochaska" NQO1 Assay (Indirect Antioxidant Activity)

Hepa 1c1c7 cells were seeded into 96-well tissue culture plates for 24 h (10,000 cells per well) and exposed to serial dilutions (0–200 μM) of selected flavonoids in the cell culture medium for 48 h. Vehicle dimethyl sulfoxide (DMSO, 0.1% final volume) was used as a control in all experiments. Using the Prochaska microtiter plate bioassay (34), the potency of selected flavonoids to induce the phase 2 enzyme NQO1 was determined. The concentration required to double the specific enzymatic activity of NQO1 [E.C. 1.6.99.2] (the "CD") was used as a measure of inducer potency. Replicate samples of cell lysates were pipetted to fresh 96-well plates for determination of protein content using the BCA method (46).

Chemoprotective Index

The Chemoprotective Index (CI) for each flavonoid was calculated by dividing the cell viability (IC_{50}) concentration by the CD concentration obtained in the Prochaska NQO1 induction assay described above. This quotient provides an approximate index of the inducer potency relative to cytotoxicity – higher values suggesting potentially more desirable agents (47).

Cellular Uptake Studies

Hepa 1c1c7 cells were seeded at 500,000 cells per well in 3 ml medium in 6-well (3.5 cm diameter) tissue culture plates for 24 h. Uptake was then monitored over the next 48 h following addition of 10 μM final concentration of each of the flavonoids delivered in DMSO (0.1% final concentration). Wells containing flavonoid but no cells served as blanks; DMSO was added to control wells containing cells only. Culture medium was sampled at time points up to 48 h and specific flavonoid concentration was determined and compared to amounts remaining in blank wells. Flavonoid concentrations were determined by HPLC with a Waters Symmetry C18, 5 μm, 3.9 $\times$ 150 mm column and an isocratic mobile phase consisting of 60:40 methanol:water with 0.3% TFA; 0.9 ml/min flow rate (48), on a Waters 2690 Alliance separations module using a 2996 PDA detector tuned to the peak UV absorption wavelength for each of the compounds.

Cell Toxicity Assay (MTT)

The most potent flavonoids in the NQO1-activity assay were subsequently tested for cell toxicity using the MTT assay (49) in Hepa 1c1c7, Caco-2, and BEAS-2B cells. Cells were seeded in 96-well tissue culture plates at 10,000 cells/well and incubated with selected flavonoids for 48 h. Cells were then rinsed in DPBS and incubated with the tetrazolium salt MTT (10 μL of 5 mg/ml added to 100 μL DPBS) for 2 h at 37°C. DMSO (200 μL) was then added to each well, mixed to solubilize the formazan, and optical density determined spectrophotometrically by measuring absorbance at 570/630 nm using a Spectramax Plus microplate reader (Molecular Devices, Sunnyvale, CA).

Inhibition of iNOS Induction

The capacity to inhibit LPS-induced iNOS induction was evaluated in murine macrophage-like RAW264.7 cells by the Griess reaction (50). Briefly, cells were cultured at 37°C in a humidified 5% CO_2 atmosphere in Dulbecco's Minimum Essential Medium containing 10% heat-inactivated FBS. Murine RAW264.7 cells were plated in 96-well tissue culture plates (20,000 cells per well) and grown for 24 h before exposure to serial dilutions of test compounds in the presence of 10 ng/ml LPS. Cells were incubated for an additional 48 h. Wells treated with LPS, but without flavonoid, served as controls. Nitrite concentration in culture supernatant was measured as an indicator of iNOS induction (NO production) in the cells. Briefly, 100 μL of each supernatant fraction were mixed with the same volume of Griess reagent [1% sulfanilamide in 5% phosphoric acid and 0.1% N-(1-naphthyl)ethylenediamine dihydrochloride in distilled water], and $Abs_{550\ nm}$ was plotted against a standard curve of sodium nitrite (37,50).

Direct Antioxidant Assessment ($ABTS^{\bullet+}$)

Direct antioxidant activity was measured with the $ABTS^{\bullet+}$ radical cation decolorization assay (25). Briefly, 50 μl of the test compound were serially diluted in ethanol across all wells of columns 2 through 12 in a 96-well microtiter plate, starting with a stock concentration of 90 μM, which was diluted into 300 μL total volume for a final concentration of 15 μM. Column 1 contained only 50 μL ethanol and served as the blank; the bottom half of the plate, rows E–H, served as sample controls and received only 250 μL ethanol per well; the top half of the plate, rows A–D, received 250 μL of a 124 μM ethanolic solution of $ABTS^{\bullet+}$. The plates were read immediately in a microtiter plate reader (Spectramax Plus, Molecular Devices, Sunnyvale, CA) at 734 nm continuously for 5 min and the 2-min measurement was used for comparative analyses. Each experiment contained 1 plate of Trolox, a synthetic vitamin E analog, which was used to construct a standard curve (not shown) to calculate Trolox Equivalents (TE; defined as the concentration of Trolox providing an oxygen radical cation decolorization rate equivalent to 1 μM of the test compound).

Statistical Analyses

Results were expressed as means of at least 3 separate wells per treatment. Standard errors of the mean were calculated. Because all standard errors of the mean values were very small and ranged between 1% and 7% of the arithmetic mean, error bars were not included in either tables or figures, for the purpose of clarity. Trend analyses (nptrend and ranksum) and Spearman correlation analyses were performed using Stata, version 11.2, (StataCorp, College Station, TX).

RESULTS

Cytoprotective Enzyme Induction

Twenty-nine flavones and 8 flavanones (Figs. 1 and 2) were evaluated for their potential to induce the representative phase 2 enzyme NQO1 in Hepa 1c1c7 cells (see Table 1), with special focus on a series of methoxylated compounds (no. 10–26,32–36) and their hydroxylated analogues (no. 2–5,27,30–33).The structural isomers α- and β-naphthoflavone (no. 28,29) were included in some comparisons because of their extremely low- and high-potency, respectively, which bracket the range of potencies of the hydroxylated and methoxylated compounds evaluated (Table 1). Potent inducers have CD values in the low micromolar range or below. The hydroxylated flavone quercetin (no. 27) had a CD of 1.8 μM, and 3′,4′-dihydroxyflavone (no. 5), a catechol, had comparable potency (CD = 2.3 μM), and increased the specific activity of NQO1 to a maximum 12-fold at 50 μM. Among the methoxylated flavones (no. 10–26), 5-methoxyflavone (no. 11), 5,3′-dimethoxyflavone (no. 21), 5,4′-dimethoxyflavone (no. 20), and 5,7-dimethoxyflavone (no. 19), were extremely potent inducers of NQO1 in murine Hepa 1c1c7 cells (CD $\leq$ 0.2 μM). Other flavones (e.g., 5-hydroxy,7-methoxyflavone) (no. 7), 3-methoxyflavone (no. 10), 3,6-dimethoxyflavone (no. 18), and 4′-methoxyflavone (no. 14), also demonstrated good NQO1 induction potency with CD-values ranging from 0.3 to 8 μM. Higher degrees of methoxylation (e.g., trimethoxyl-) or substitutions at positions other than the 5, 7, 3′, and 4′ carbons of flavones did not result in superior NQO1 inducer potencies.

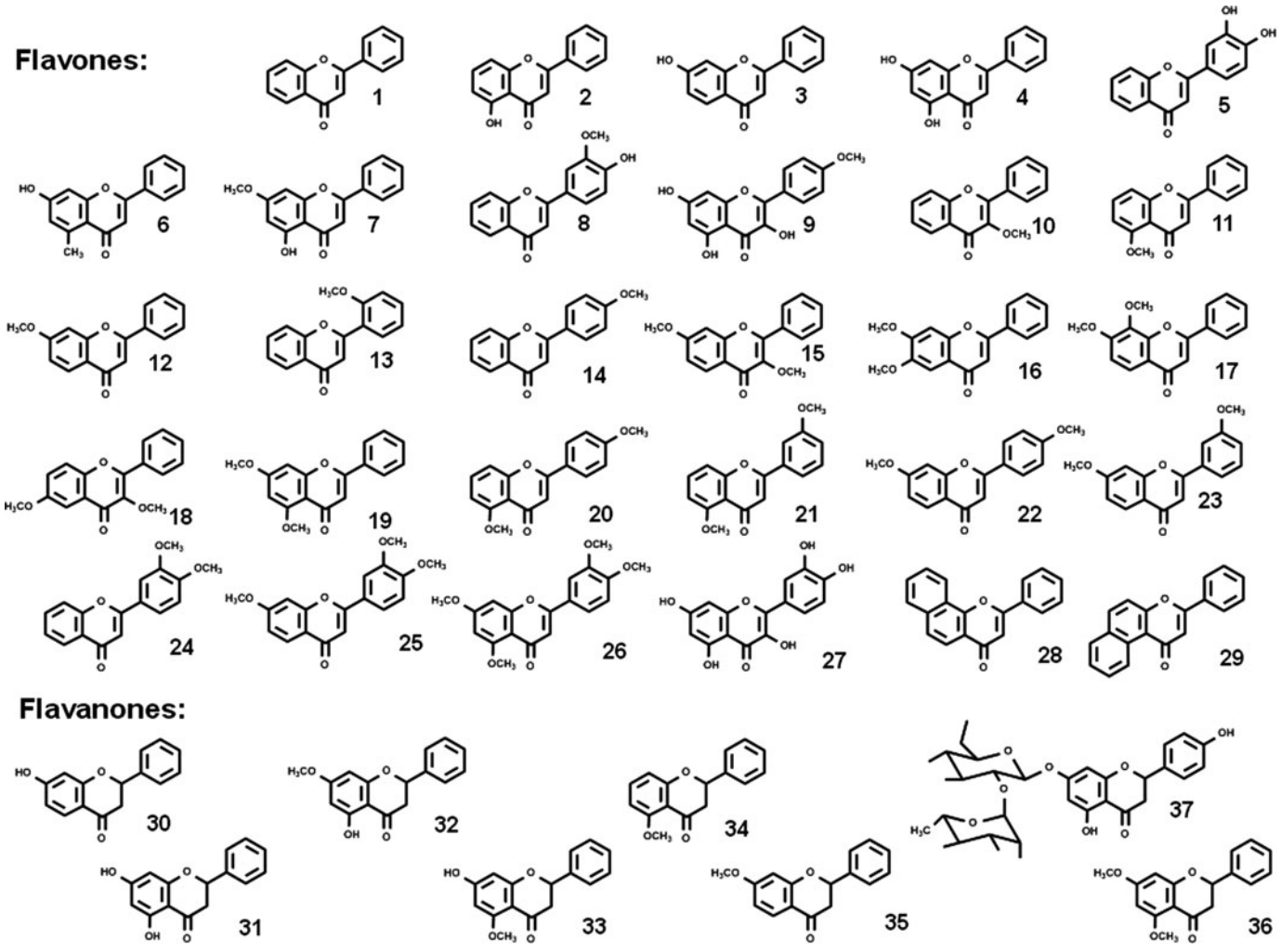

FIG. 2. Structures of selected flavones and flavanones; compound numbers are referred to in text and tables.

TABLE 1
Induction of NQOI specific activity in Hepa1c1c7 cells by flavones and flavanones

No.[a]	Flavonoid	Common name	NQO1		
			CD (μM)[b]	CI[c]	CI rank[d]
	Flavone				
1	flavone		1.9	21	13
2	5-hydroxyflavone	primuletin	>100.0	<1.4	31
3	7-hydroxyflavone		Inactive		
4	5,7-dihydroxyflavone	chrysin	15.0	5.1	26
5	3′,4′-dihydroxyflavone	4-hydroxyflavonol	2.3	6.1	24
6	7-hydroxy,5-methylflavone		20.0	10.9	16
7	5-hydroxy,7-methoxyflavone	tectochrysin	0.3	567	4
8	4′-hydroxy,3′-methoxyflavone		50.0	2.0	30
9	3,5,7-trihydroxy,4′methoxyflavone	kaempferide	6.25	8.7	17
10	3-methoxyflavone		0.32	248	8
11	5-methoxyflavone		0.03	1090	2
12	7-methoxyflavone		9.0	5.6	25
13	2′-methoxyflavone		25.0	2.1	29
14	4′-methoxyflavone		8.0	14.0	14
15	3,7-dimethoxyflavone		37.0	3.5	28
16	6,7-dimethoxyflavone		13.0	7.5	22
17	7,8-dimethoxyflavone		Inactive		
18	3,6-dimethoxyflavone		2.0	49.4	10
19	5,7-dimethoxyflavone	chrysin dimethyl ether	0.2	435	6
20	5,4′-dimethoxyflavone		0.085	728	3
21	5,3′-dimethoxyflavone		0.06	1652	1
22	7,4′-dimethoxyflavone		31.0	6.2	23
23	7,3′-dimethoxyflavone		7.5	13.7	15
24	3′,4′-dimethoxyflavone		18.0	26.7	11
25	7,3′,4′-trimethoxyflavone		Inactive		
26	5,7,3′,4′-tetramethoxyflavone	luteolin tetramethyl ether	12.5	24.2	12
27	3,5,7,3′,4′-pentahydroxyflavone	quercetin	1.8	8.5	19
28	7,8-benzoflavone	α-naphthoflavone	500		
29	5,6-benzoflavone	β-naphthoflavone	0.015		
	Flavanone				
30	7-hydroxyflavanone		30	8.6	18
31	5,7-dihydroxyflavanone	pinocembrin	70	3.9	27
32	5-hydroxy,7-methoxyflavanone	pinostrobin	0.3	319	7
33	5-methoxy,7-hydroxyflavanone	alpinetin	15	7.7	21
34	5-methoxyflavanone		0.15	527	5
35	7-methoxyflavanone		15	8.0	20
36	5,7-dimethoxyflavanone		0.7	176	9
37	4′,5,6-trihydroxyflavanone,7-rhamnoglucoside	naringin	Inactive		

[a]No. = Compound numbers are referred to in the text, figures, and other tables. [b]CD = Concentration required to double the specific activity of NQO1. [c]Chemoprotective Index (CI) = IC_{50}/CD. [d]CI rank = relative CI ranking with 1 being the highest (CI = 1652) and 31 the lowest (CI = <1.4).

It should be noted that the 5,7-dihydroxyflavone (no. 4) (CD = 15) is about a fourfold more potent inducer than its flavanone congener (no. 31) (CD = 70), and the same holds true for the 5,7-dimethoxyflavone (no. 19) (CD = 0.2) and its flavanone congener (no. 36) (CD = 0.7). However, both dimethoxylated compounds are potent inducers.

We selected 8 flavanones (flavanones lack a double bond in the 2–3 position on ring C and thus do not contain a potential

Michael reaction center) to compare to their flavone structural analogs. Only 5-hydroxy,7-methoxyflavanone (no. 32) had comparable potency (see Table 1) to its flavone analogue (both CDs = 0.3 μM). Both flavones and flavanones with methoxyl-groups at the 5-position of the A-ring had greater NQO1 inducer potency than those with methoxyl groups at the 7 position. CD values were 0.03 and 9.0 μM for the 5-methoxy- (no. 11) and 7–methoxy- (no. 12) flavone, respectively, and 0.15 and 15 μM for their flavanone congeners (no. 34,35), respectively. The NQO1 inducer potency of the 5-methoxylated flavanone (no. 34) (CD = 0.15 μM) was dramatically attenuated when a hydroxyl group occupied the 7-position on the A-ring (CD = 15 μM for 5-methoxy,7-hydroxyflavanone) (no. 33), whereas hydroxylation of 7-methoxylated flavonoids [which are not as active in their monomethoxylated state; CD = 9 and 15 μM for the flavone (no. 12) and flavanone (no. 35), respectively], resulted in a dramatic enhancement of activity [CD = 0.3 μM for both 5-hydroxy,7-methoxyflavone (no. 7) and flavanone (no. 32)].

The CI (chemoprotective index) is the ratio of cell viability (data not shown; IC_{50} value obtained from the protein assay and used as a proxy for adherent viable cells) to the CD. It provides a helpful measure of the in vitro chemoprotective potential of a compound by highlighting the range or window of concentration between NQO1 inducer potency and toxicity. Flavonoids with a CI > 100 (Table 1) included 4 out of 5 flavones and 2 of 3 flavanones with a methoxyl-group at position 5 of the A-ring. Notably, the tetramethoxyflavone (no. 26) was not in this category, and 5,3′-dimethoxyflavone (no. 21) and 5-methoxyflavone (no. 11) had the highest chemoprotective indices (1652 and 1090, respectively), giving robust NQO1 induction at 60 and 30 nanomolar concentrations, respectively (Table 1). Trends for changes in chemoprotective index follow those outlined in the previous paragraph for CD. For ease of comparison, we have also provided a relative ranking of CIs in the Tables, whereby a ranking of "1" is associated with the highest CI and a ranking of "31" is associated with the lowest CI.

Cellular Uptake Kinetics

A small group of flavonoids with radically contrasting CDs and structures was evaluated in a cell culture uptake assay. All of the tested flavonoids were completely taken up within 48 h, with half-lives for uptake ($t_{1/2}$) ranging from 4 to 24 h. At the extremes of potency were β- and α-naphthoflavone which had CD values of 0.015 and 500 μM, respectively, which had virtually identical uptake AUC's (138 and 143 μM·h, respectively), and $t_{1/2}$ values of 12 and 14 h, respectively. Pinocembrin, quercetin, pinostrobin, alpinetin, and 5,7-dimethoxyflavanone had AUC's of 43, 77, 88, 128, and 237 μM·h, respectively (Table 2). There was absolutely no correlation between uptake and NQO1 induction potency over this greater than 3000-fold difference in potencies (P for trend $z = 0.69$, $p > 0.490$; linear regression coefficient $r^2 = 0.0000$, $t = 1.31$, $P = 0.237$). Differences in cellular uptake rates are therefore very unlikely to account for the large differences in inducer potencies among these compounds.

Cancer vs. Noncancer Derived Cells: Toxicity upon Exposure to Flavonoids

The toxicity/tolerance of 12 of the most potent NQO1-inducing flavonoids were further compared in a panel of cell lines: (a) human colon carcinoma (Caco-2) cells, (b) human transformed normal bronchial epithelial (BEAS-2B) cells, and (c) murine hepatoma Hepa 1c1c7 cells. The MTT cell toxicity/cell viability assay quantifies mitochondrial dehydrogenase activity, thus indirectly assessing the number of metabolically active cells (51). IC_{50} values were determined for each of the 12 compounds in each cell line (Table 3). Cell viability upon flavonoid exposure varied among cell lines (see Table 3): Normal human bronchial epithelial BEAS-2B cells were less

TABLE 2
Flavonoid uptake by Hepa1c1c7 cells after 48 h

No.	Flavonoid	Uptake		CI rank[c]
		$t_{1/2}$ (h)[a]	AUC_{48}[b]	
	Flavone			
27	3,5,7,3′,4′-pentahydroxyflavone	7	77	19
28	7,8-benzoflavone	14	143	Unranked
29	5,6-benzoflavone	12	138	Unranked
	Flavanone			
31	5,7-dihydroxyflavanone	4	43	27
32	5-hydroxy,7-methoxyflavanone	9	88	7
33	5-methoxy,7-hydroxyflavanone	12	128	21
36	5,7-dimethoxyflavanone	24	237	9

[a]$t_{1/2}$ = time required for uptake of 50% (10 μM starting conc). [b]AUC_{48} = area under the curve; 48 h exposure to 10 μM flavonoids. [c]Chemoprotective Index (CI) rank = relative CI ranking with 1 being the highest (CI = 1652) and 31 the lowest (CI = <1.4).

TABLE 3
Effect of flavonoids on viability of 3 cell lines (Hepa1c1c7, Caco2, and BEAS-2B)

No.	Flavonoid	IC_{50} (μM)[a]			CI rank[b]
		Hepa1c1c7	Caco2	BEAS-2B	
5	3′,4′-dihydroxyflavone	∼25	25–50	25–50	24
7	5-hydroxy,7-methoxyflavone	>200	>200	>200	4
9	3,5,7-trihydroxy,4′methoxyflavone	∼50	25–50	>200	17
10	3-methoxyflavone	∼22	20–100	>100	8
11	5-methoxyflavone	∼13	25–50	∼25	2
12	7-methoxyflavone	50–100	50–100	>100	25
14	4′-methoxyflavone	50–100	>200	>200	14
18	3,6-dimethoxyflavone	50–100	50–100	50–100	10
19	5,7-dimethoxyflavone	50–100	50–100	>100	6
20	5,4′-dimethoxyflavone	25–50	50–100	>50	3
21	5,3′-dimethoxyflavone	∼50	50–100	>50	1
	Flavanone				
32	5-hydroxy,7-methoxyflavanone	100–200	100–200	>200	7

[a]IC_{50} in MTT assay of cell viability. [b]Chemoprotective Index (CI) rank = relative CI ranking with 1 being the highest (CI = 1652) and 31 the lowest (CI = <1.4).

susceptible to toxicity, and Caco-2 cells were more sensitive than either BEAS-2B or Hepa 1c1c7 cells, however the same trends were observed in all 3 cell lines examined.

The flavonoids with the lowest IC_{50} values across the 3 cell lines evaluated were 3′,4′-dihydroxyflavone (no. 5) and 5-methoxyflavone (no. 11) (viability assays in Fig. 3A). The flavonoids with the highest IC_{50} values were 5-hydroxy,7-methoxyflavanone (no. 32) (viability assays in Fig. 3B) and 4′-methoxyflavone (no. 14), closely followed by 5,7-dimethoxyflavone (no. 19) (viability assays in Fig. 3C), 7-methoxyflavone (no. 12) and 5-hydroxy,7-methoxyflavone (no. 7). Improved tolerance of the 5-methoxy flavonoids was observed when these molecules contained a second methoxyl group at positions 3′- or 4′- (B-ring), or 7- (A-ring). These data compare well to assessment of toxicity by proxy, monitoring cellular protein levels in adhered cells in the NQO1 bioassay by the BCA method (data not shown).

Potent Inhibition of iNOS-Induction by Flavonoids

We have previously established the very close correlation between potency of Keap1-Nrf2-ARE mediated induction of phase 2 cytoprotective enzymes and the suppression of LPS-stimulated iNOS induction spanning more than 6 orders of magnitude of concentration and 7 different structural classes of compounds (37). It has now been established that there is much cross-talk between the Nrf2 (cytoprotective) and NF-κB (pro-inflammatory) signaling pathways and that their respective target proteins can even regulate the activity of each other (reviewed in Ref. 52). We thus evaluated a subset of the flavonoids in Table 4 for their ability to inhibit LPS-induced iNOS induction in murine macrophage-like RAW264.7 cells. The strongest inhibitors were 5-methoxyflavanone, 5,3′-dimethoxyflavone, and 5,7-dimethoxyflavone (Table 4). Congruent with our data on NQO1 induction, 5-methoxylation facilitated the inhibition of iNOS induction, whereas 5-hydroxylation did not.

Direct Antioxidant Assessment ($ABTS^{\bullet+}$) of Trolox Equivalent Antioxidant Capacity

Of the 37 flavonoids tested, only 4 had significant direct antioxidant capacity as measured by their equivalence to the Vitamin E analog, Trolox. Data are reported as TE or Trolox Equivalents. The compounds with activity were 3′4′-dihydroxyflavone (0.9 μM), 3,5,7-trihydroxy-4′-methoxyflavone (1.2 μM), 7-hydroxy,5-methylflavone (0.25 μM), and 4′-hydroxy,3′-methoxyflavone (0.2 μM). Gallic acid (3,4,5-trihydroxybenzoic acid) and caffeic acid (3,4-dihydroxycinnamic acid) were used as positive controls and had Trolox equivalent antioxidant capacity values of 2.3 μM and 1.2 μM, respectively.

DISCUSSION

Flavonoids have long been known to be antioxidants and more recently have attracted considerable interest as cancer chemoprotective candidates (24,25,32,33,53,54). Animals and humans are exposed to a large variety of flavonoids, through ingestion and dermal contact. In particular, they are exposed to flavones such as apigenin and luteolin; flavanones such as hesperetin and naringenin; flavonols such as quercetin, kaempferol, and myricetin; isoflavones such as daidzein and genistein; flavanols such as catechin, epigallocatechin gallate, and proanthocyanidins; and anthocyanins such as cyanidin and delphinidin. Various combinations of these compounds are found in abundance in many fruits, vegetables, herbs, spices, and plant-based

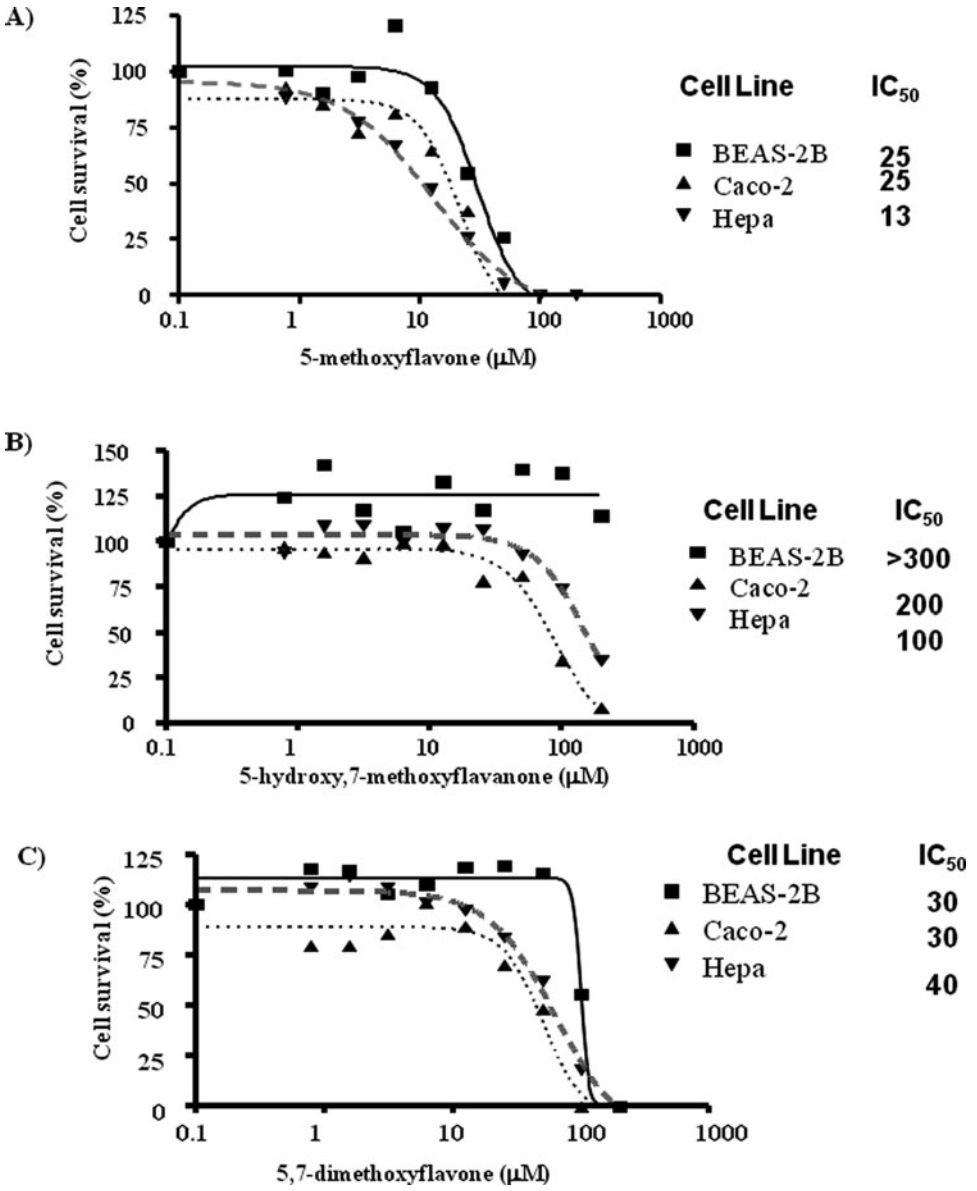

FIG. 3. MTT assays of (A) 5-methoxyflavone, (B) 5-hydroxy,7-methoxyflavanone (pinostrobin), and (C) 5,7-dimethoxyflavone, in 3 cell lines.

drinks and foods, and they have now been widely introduced into lotions, creams, cosmetics and other commercial products for topical application. Estimated dietary flavonoid intake can reach 50–800 mg per day (1), and the proliferation of fortified foods and dietary supplements can be expected to further increase the flavonoid intake in affluent populations.

Mechanisms of flavonoid action are not always obvious, and epidemiological studies do not always reach similar conclusions (1). It has become clear that in humans and in animal models of conditions involving pathological inflammation, cardiovascular disease, and cancer, many of these flavonoids and other polyphenols and/or their metabolites exhibit a variety of effects with implications for prevention or treatment. Importantly, flavonoids have been shown to inhibit many enzymes involved in the carcinogenesis process, including lipoxygenases (7,23), cyclooxygenases (23,55), xanthine oxidase (23), mitochondrial succinoxidase (23), NADH oxidase (23), phospholipase A2 (7,23), topoisomerases (23,56), and protein kinases (7,23). Some flavonoids have been shown to induce cytoprotective enzymes and thus have an indirect antioxidant effect (32). The ultimate result may be concentration-, organ-, and/or cell-specific. Therefore, for comparison purposes, both cancer cells (Hepa 1c1c7 and Caco-2) and immortalized normal (BEAS-2B) cells were used in this study. Both the intestinal carcinoma (Caco-2) cells (models for the intestinal epithelial barrier), and normal transformed human bronchial epithelial (BEAS-2B) cells are considered reasonable proxies for human epithelium, and they represent tissues that are readily and repeatedly exposed to

TABLE 4
Flavonoid inhibition of LPS-stimulated iNOS induction in RAW 264.7 cells

No.	Flavonoid	Inhibition		CI rank[c]
		IC_{50} (μM)[a]	Max. (%)[b]	
4	5,7-dihydroxyflavone	>20	40	26
11	5-methoxyflavone	5	80	2
12	7-methoxyflavone	~5	<50	25
19	5,7-dimethoxyflavone	2.5	90	6
20	5,4′-dimethoxyflavone	5	89	3
21	5,3′-dimethoxyflavone	2.5–5.0	73	1
	Flavanone			
31	5,7-dihydroxyflavanone	stimul[d]	stimul	27
32	5-hydroxy,7-methoxyflavanone	>20	20	7
34	5-methoxyflavanone	~1.2	94	5
36	5,7-dimethoxyflavanone	~5	90	9

[a]Flavonoid concentration required for 50% reduction of iNOS induction. [b]Maximum flavonoid inhibition of iNOS induction. [c]Chemoprotective Index (CI) rank = relative CI ranking with 1 being the highest (CI = 1652) and 31 the lowest (CI = <1.4). d: Stimulated (did not inhibit) LPS-stimulated iNOS induction.

dietary flavonoids. Murine hepatoma (Hepa 1c1c7) cells on the other hand have a highly inducible NQO1which can be upregulated many-fold in specific activity. This system has a long history in the discovery of new cancer preventive candidates (34).

To investigate whether flavonoids can act both as direct antioxidants and indirect antioxidants (inducers of cellular detoxification phase 2 enzymes), we first examined the NQO1 induction potency of 37 structurally related flavonoids in murine hepatoma cells. 5,7-dimethoxyflavone (no. 19) has been shown previously to be selectively inhibitory to cancer cells compared to immortalized but noncancer-derived normal human bronchial epithelium (BEAS-2B) cells (33), and it served as our reference compound. This flavone was about twice as cytotoxic in mouse hepatoma cells as it was in BEAS-2B cells, suggesting either organ, tissue, or cancer tissue specificity. However, in general, we observed less toxicity to flavonoids in BEAS-2B cells than in either of the two cancer cell lines evaluated (Hepa 1c1c7 and Caco-2). Thus, the antiproliferative effects of methoxylated flavones appears to be selective for cancer cells as has been described previously using other cell lines (33).

Activators of the Keap1-Nrf2-ARE inducer system via the reactive cysteines of Keap1 function by (a) adding to Michael reaction groups, which we consider further in subsequent paragraphs; (b) reacting with Keap1 cysteine residues; and (c) oxidative cycling, generating reactive oxygen species that oxidize SH- groups (57). The formation of reactive oxygen species from phenols (flavonoids with hydroxylation on the A or B ring) might thus be expected from various compounds used in this study. Also, either 1,2- or 1,4-diphenols would create catechols or hydroquinones, respectively, and by 1- and 2-electron oxidative cycling these would lead to radical formation [we have tested only the former (no. 51), and not the latter]. Indeed, 3′,4′-dihydroxyflavone is a potent NQO1 inducer but is also cytotoxic. Recent work suggested that the ability of hydroxylated flavonoids to induce the transcription of a human NQO1-ARE-controlled luciferase construct in Hepa 1c1c7 cells was correlated to their redox properties (58). However, these investigators point to the need for a free hydroxyl at C-3 (C-ring), which is not supported by our observations on the potency of flavanones. Our earlier demonstration that some flavonoids are bifunctional inducers (24,32) leaves open the possibility of AhR/XRE mediated regulation of Nrf2. This mechanism has been recently reviewed (52), but the experiments presented here do not permit evaluation of this possibility.

A number of the 29 tested flavones and 3 of the 8 tested flavanones induced NQO1 substantially. Neither hydroxylations nor methoxylations on the B-ring had a substantial effect, and in fact single methoxyl substitutions resulted in 4-fold and 13-fold reduced NQO1 induction potency (no. 14,13), respectively, and greater cytotoxicity than the parent flavone (no. 1) (see Table 1). Previous work has shown that B-ring hydroxylation is not essential for NQO1 inducer activity of flavones, but that the presence of a hydroxyl group on the 3 position (C-ring) may affect the potency of induction (59). Our results suggest that in fact the opposite may be true since the 3-methoxyflavone (no. 10) was almost an order of magnitude more potent than its parent flavone (no. 1) and CI was likewise 10-fold higher. Substitutions at C-7 (A-ring) also had a pronounced NQO1-inducing effect, which was muted by additional methoxylation at C-6 or C-3′. Similarly, the induction potential of the 3-methoxyflavone (no. 10) (CD = 0.32 μM), was muted by a second methoxylation at the 6-position (no. 18) (CD = 2.0 μM), and even more dramatically by a second methoxylation at the 7-position (no. 15) (CD = 37 μM). A single methoxylation at the 5-position resulted in better NQO1 inducer potency than either methoxylation or hydroxylation at any other position, for both flavones (no. 11) and

flavanones (no. 34) (CD = 0.03 and 0.15 μM, respectively), and it also resulted in greatest inhibition of LPS-induced iNOS activity in macrophages. NQO1 inducer potency of 5-methoxyflavone was comparable to that of the 4′-bromoflavone, the most potent synthetic flavone reported to date, although the synthetic flavone was even less toxic than the methoxylated flavone (60). Antiinflammatory (inhibition of LPS-stimulated iNOS induction) and phase 2 induction potencies are reasonably congruent (Table 4), as has been shown previously for triterpenoid analogues (37,61) and many other structurally diverse NQO1 inducers (37)

The most potent NQO1 enzyme induction was observed in flavones with a methoxyl-group at C-5. 5,3′-dimethoxyflavone (no. 21*)* had the highest CI value (CI = 1652), with NQO1 induction occurring at nanomolar concentrations, and much lower cytotoxicity than its monomethoxylated analogue 5-methoxyflavone (no. 11) (Table 1). Other 5-methoxyflavones and flavanones were also potent inducers with low toxicity, and thus had some of the highest chemoprotective indices observed. Importantly, the fact that the flavanones are quite active as NQO1 inducers indicates that the C2-C3 double bond, and consequently the Michael acceptor functionality, is not required for inducer activity in this class of compounds. This finding is in contrast to our earlier views.

These observations are interesting because many previous works on flavonoid mode of action have assumed a priori that a hydroxyl group substituent is required for any biological activity. This is clearly the case for direct antioxidant effects (see Ref. 62) and as we have confirmed. Each of the few flavonoids that had direct antioxidant activity had hydroxyl substitutions, but these compounds also had very low CIs (Table 1). Interestingly, none of the methoxylated flavones or flavanones that were potent NQO1 inducers had any direct antioxidant activity. It is clear that to obtain indirect antioxidant activity (e.g., induction of the phase 2 response), methoxylation at specific positions is required. Other recent findings also point to the advantages of methoxylated flavonoids from the perspective of uptake and bioavailability (33). Compounds with mixed functionality, or with hydroxyl groups or with methoxyl groups at positions other than the 5-carbon can also serve as strong inducers; however, this goes hand-in-hand with greater toxicity.

In conclusion, some structural generalizations can be drawn from evaluating the potency of 37 flavonoids to induce the cytoprotective phase 2 response by measuring NQO1 induction in a murine hepatoma cell line. The presence of methoxyl substituents at C-5 results in the most potent NQO1 induction, and produces only limited toxicity in a panel of cancer cell lines, and little to no toxicity in a human normal bronchial epithelial cell line. These differences appear not to be related to differential cellular uptake (structural benzoflavone isomers had nearly identical uptake kinetics, despite huge differences in inducer potencies). We cannot rule out metabolic interconversions following uptake, as being in part responsible for differential activity, although we view this as unlikely. Inhibition of LPS-stimulated iNOS production was less clearly correlated with structure, despite the fact that antiinflammatory activity and phase 2 inducer activity are probably equally important contributors to the protective effects of flavonoids that have been reported in many biological models and share many common molecular targets. The direct antioxidant activity of many of these compounds suggests that redox mechanisms may be more important in the induction of Keap1/Nrf2 by flavonoids. Based on our results, these methoxylated dietary compounds, and in particular the 5-methoxyflavones, are attractive for further evaluation of their roles in preventing carcinogenesis.

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