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ARTICLE

Inhibitory Effect of Flavonoids on 26S Proteasome Activity

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Inhibiting proteasomal degradation has been shown to induce apoptosis in tumor cells. Utilization of proteasome inhibition is therefore one approach to anticancer therapy. Some of the flavonoids can induce cell apoptosis via inhibiting proteasome 26S activity. In this study, the inhibition of 26S proteasome from pig red blood cells was analyzed on 12 flavones, 5 flavanones, and 9 isoflavones by using a proteolysis assay. Several flavonoids such as apigenin-6-hydroxy-7-*O*-β-D-glucoside, quercetin, rutin, 6-hydroxyapigenin, 5,6,4′-trihydroxy-7,3′-dimethoxyflavone, 5,6,3′,4′-tetrahydroxy-7-methoxyflavone, glycitecin, and 6,7,4′-trihydroxyisoflavone inhibited the chymotrypsin-like, caspase-like, or trypsin-like activity of 26S proteasome when Suc-LLVY-AMC, Z-LLE-AMC, and Ac-RLR-AMC were used as substrates. Three peptidase activities of flavonoids were found to be significantly correlated with one another. Flavones had significantly stronger inhibitory effects on chymotrypsin-like and caspase-like activities than flavanones and isoflavones. 5,6,3′,4′-Tetrahydroxy-7-methoxyflavone, 5,6,4′-trihydroxy-7,3′-dimethoxyflavone, and quercetin displayed a mixed type inhibition of 26S by Lineweaver−Burk plots analysis. Furthermore, 5,6,3′,4′-tetrahydroxy-7-methoxyflavone is found to have a higher inhibitory effect on 26S proteasome activities and is the only flavonoid to inhibit all three peptidase activities, whereas the inhibition of flavonoids was not affected by ubiquitin-induced stimulation of the three peptidase activities of 26S proteasome; 5,6,3′,4′-tetrahydroxy-7-methoxyflavone inhibited 75% casein degradation. These results suggest that both the 6-hydroxy and 7-methoxy positions of the flavone may play an important role in targeting 26S activity.

KEYWORDS: 5,6,3′,4′-Tetrahydroxy-7-methoxyflavone; MG132; 26S proteasome; flavonoids; 5,6,4′-trihydroxy-7,3′-dimethoxyflavone; proteasome inhibitor; ubiquitin

INTRODUCTION

Flavonoids represent one of the most prevalent classes of phenolic phytochemicals that are widely distributed in edible plants. Flavonoids are divided into anthocyanins, flavones, flavonols, flavanols, flavanones, and isoflavones. Flavonoids have been shown to have antitumor, anti-inflammation, and antioxidant activities (*1*). Natural flavonoids also been shown to modulate the function of the proteasome (*2*, *3*). However, there is no study focused on the chemical structures of flavones, flavanones, and isoflavones related to their proteasome inhibitory activities.

The ubiquitin−proteasome pathway (UPP) is an important mechanism for the degradation of the majority of intracellular protein that is required for cell proliferation and survival in eukaryotes (*4*). The regulatory role of proteasome is an energy-dependent molecular machine that not only regulates several cellular processes such as cell-cycle regulation, apoptosis, degradation of oxidized, unfolded and misfolded proteins, and antigen presentation but also is involved in drug resistance in human tumor cells (*4*).

Ubiquitin is a conserved small protein consisting of 76 amino acids of 8.6 kDa present in all eukaryotic cells. When polyubiquitin is attached to target proteins, tagged proteins are selected for destruction by cytoplasmic organelles called proteasomes (*4*). The eukaryotic 26S proteasome (M_r = 2,400,000), a multisubunit enzyme (EC 3.4.99.46), is a proteolytic cellular apparatus of the 20S core particle capped at one or both ends by the 19S regulatory particle (19S cap, PA700) (*5*, *6*). The 20S core particle, a multicatalytic protease, is a cylindrical structure composed of four stacked rings and formed by seven subunits in an α7β7β7α7 configuration. The two outer rings (called α rings) complex with the 19S regulatory particles, forming a narrow channel through which only denatured proteins can pass. The catalytic chamber is formed by the two inner β rings, each of which contain three well-characterized peptidase activities—chymotrypsin-like (ChT-L, cleaving after hydrophobic residues), trypsin-like (T-L, cleaving after basic residues), and postglutamyl peptide hydrolase-like (PGPH, caspase-like, cleaving after acidic residues) hydrolytic active sites (*6*). Proteins are degraded by the core particle in a progressive manner, generating peptides of 3−25 amino acids in length (*6*).

Proteasome inhibitors can inhibit catalytic center activity, block a large number of regulated protein degradations, induce intracellular signaling system disorders, and then create an overload situation that results in cell growth inhibition. Therefore, several groups of proteasome inhibitors, peptide aldehydes, peptide boronates, nonpeptide inhibitors, peptide vinyl sulfones, and epoxyketones, have been developed and are now widely used

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as research tools to study the role of the UPP in various cellular processes (*7*).

Two proteasome inhibitors, Bortezomib (Velcade, PS341) and NPI-0052, have been recently reported in clinical trials (*8*, *9*). Bortezomib inhibits the chymotrypsin-like activity and targets the caspase-like activity, whereas NPI-0052 inhibits the chymotrypsin-like, caspase-like, and trypsin-like activities. However, these two clinical proteasome inhibitors have their undesirable side effects. There are, however, several edible plants or traditional low-toxicity Chinese herbal medicines that target proteasome agents that are very important subjects to be studied for the pretreatment of many diseases. The beneficial health effects and low toxicity of flavonoids make them attractive to use in addition to the fact that most of them are present in many widely available foods. Flavones are naturally occurring dietary phytoestrogens in different plants and particularly rich in soybeans (*10*). Previously we have demonstrated that ginsenoside Rd may play a role in the inhibitory property of the chymotrypsin-like activity on 26S proteasome (*11*). Additionally, our recent study on the screening of new 26S proteasome inhibitors resulted in the identification of a known compound, 5,6,3′,4′-tetrahydroxy-7-methoxyflavone from *Anisomeles ovata* R. Br., as a potent 26S inhibitor. In addition to 5,6,3′,4′-tetrahydroxy-7-methoxyflavone, there are many flavone, flavanone, and isoflavone analogues from food or Chinese herbal medicine yet to be studied. This study investigated the inhibitory potentials of 12 flavones, 5 flavanones, and 9 isoflavones from different foodstuffs and edible plants for their 26S proteasome activity (**Figure 1**) using 26S proteasome isolated from pig red blood cells to study the proteasome-inhibitory abilities, the inhibition mode, and their functional kinetics in the degradation potencies of these compounds.

MATERIALS AND METHODS

Materials. MG132 and 7-amino-4-methylcoumarin (AMC) were obtained from Calbiochem (Darmstadt, Germany). Dimethyl sulfoxide (DMSO) was purchased from Sigma Chemical Co. (St. Louis, MO). Substrates Suc-Leu-Leu-Val-Tyr-AMC, Ac-Arg-Leu-Arg-AMC, and Z-Leu-Leu-Glu-AMC were purchased from Bachem (Bubendorf, Switzerland), Biomol International, L.P. (Plymouth Meeting, PA), and A. G. Scientific, Inc. (San Diego, CA), respectively. 5,6,3′,4′-Tetrahydroxy-7-methoxyflavone was purified from our laboratory. 6,7,4′-Trihydroxyisoflavone was a gift from Dr. T. S. Chang (Department of Biological Sciences and Technology, National University of Tainan, Tainan, Taiwan). Several flavonoids were provided by Dr. H. Y. Ding (Institute of Cosmetics Science, Chia Nan University of Pharmacy and Science, Tainan, Taiwan). Others were commercially available and used as received.

Purification of 26S Proteasome form Pig Red Blood Cells (RBCs). Fresh pig blood was collected at a local abattoir with sodium citrate as anticoagulant. Plasma and white blood cells (WBC) were removed from blood samples by centrifugation (500*g* for 10 min). RBCs were resuspended 1:1 (v/v) with cold phosphate buffer saline (PBS) and centrifuged at 3700*g* for 17 min at 4 °C. 26S proteasomes were purified from pig RBC as described previously (*12*).

Proteolysis Measurement. Proteasome activity was determined according to the methods described in our previous paper with some modifications (*11*). The peptide activity of pig 26S proteasome toward three different fluorogenic peptides was measured by incubation with 1 μL of each flavonoid or MG132 in 100 μL of reaction buffer [30 mM Tris-HCl (pH 8), 5 mM $MgCl_2$, 1 mM ATP, 0.5 mM DTT with 50 μM Suc-Leu-Leu-Val-Tyr-AMC, Ac-Arg-Leu-Arg-AMC, or Z-Leu-Leu-Glu-AMC] for 15 min at 37 °C. The final concentration of 26S proteasomes in the assays was 385 μg/mL (or 160 nM assuming a molecular mass of 2.4 MDa). Proteasome activity was assessed using synthetic peptide substrates linked to the fluorometric reporter, aminomethylcoumarin (AMC). AMC hydrolyzed from the peptides was quantified in a BioTek FL800 plate reader (Winooski, VT) using 360 nm excitation and 460 nm emission wavelengths at 37 °C for 15 min. Enzymatic activity represents mean values of triplicate independent assays and normalized for protein concentration, expressed as percent of activity in control (26S proteasome with 1 μL of DMSO).

For the effects of ubiquitin, 26S proteasomes (160 nM) were incubated with or without 7.2 μM ubiquitin for 15 min before the addition of 1 μL of flavonoid (apigenin-6-hydroxy-7-*O*-β-D-glucoside, 6-hydroxyapigenin, quercetin, 5,6,4′-trihydroxy-7,3′-dimethoxyflavone, or 5,6,3′,4′- tetrahydroxy-7-methoxyflavone) or MG132. After incubation at 37 °C, the peptide hydrolysis was measured fluorometrically.

Michaelis−Menten Kinetics of Flavonoids. One microliter of flavonoids (quercetin, 5,6,4′-trihydroxy-7,3′-dimethoxyflavone, or 5,6,3′,4′-tetrahydroxy-7-methoxyflavone) was mixed with 160 nM 26S proteasome in 100 μL of reaction buffer [30 mM Tris-HCl (pH 8), 5 mM $MgCl_2$, 1 mM ATP, 0.5 mM DTT] and substrates (Suc-LLVY-AMC or Z-LLE-AMC). The concentration of Suc-LLVY-AMC or Z-LLE-AMC was from 1 to 50 μM. The velocities of reactions were measured without or with inhibitor (10, 50, or 100 μM). The following procedures used the same protocol as described above.

Degradation of Casein by Purified 26S Proteasomes. Degradation of casein was determined according to the methods described in a previous paper with some modifications (*13*). 26S proteasomes were preincubated with inhibitors (apigenin-6-hydroxy-7-*O*-β-D-glucoside, 6-hydroxyapigenin, quercetin, 5,6,4′-trihydroxy-7,3′-dimethoxyflavone, or 5,6,3′,4′-tetrahydroxy-7-methoxyflavone) for 30 min at 37 °C to allow these slow binding inhibitors to bind to the active sites. Casein (30 μM) was then added to these pretreated 26S proteasomes and incubated at 37 °C for 2 h. The final concentration of proteasomes in the assays was 120 nM. Proteins were precipitated with 5% perchloric acid to separate from peptides. After centrifugation for 20 min at 13500*g*, the supernatant was then neutralized with KOH. The fluorescamine assay was performed at pH 6.8.

Statistical Analysis. For each proteolysis measurement, the mean of three different experiments ± standard error (SE) is presented. Student′s unpaired *t* test is used to characterize the differences between two sets of values. A standard $p < 0.05$ was set as the significance level. The data of the relationship were analyzed by Pearson′s correlation coefficient (*r*).

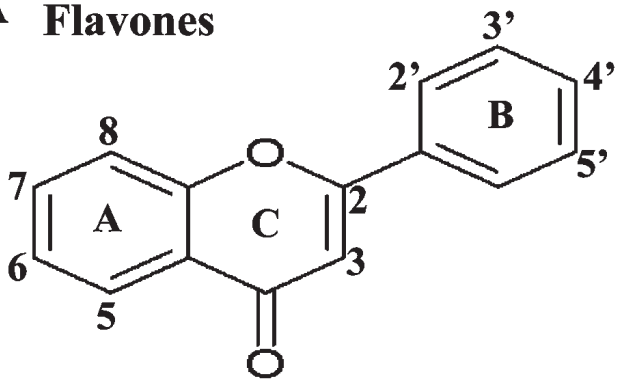
RESULTS AND DISCUSSION

Inhibitory Effects of Flavonoids on 26S Proteasome Activity. Many foods and edible plants contain flavonoids. This paper attempts to evaluate which flavonoids could more efficiently inhibit the chymotrypsin-like, trypsin-like, and caspase-like activities of 26S proteasome. The chemical structures of 12 flavones, 5 flavanones, and 9 isoflavones are shown in **Figure 1**, together with their abilities to inhibit the chymotrypsin-like, caspase-like, and trypsin-like activities of 26S proteasomes. Several flavonoids including apigenin-6-hydroxy-7-*O*-β-D-glucoside, quercetin, rutin, 6-hydroxyapigenin, 5,6,4′-trihydroxy-7,3′-dimethoxyflavone, 5,6,3′,4′-tetrahydroxy-7-methoxyflavone, glycitecin, and 6,7,4′-trihydroxyisoflavone were found to be able to inhibit the chymotrypsin-like, caspase-like, or trypsin-like activities of 26S proteasomes (**Figure 2A**).

The flavone 5,6,3′,4′-tetrahydroxy-7-methoxyflavone was found to be the strongest proteasome inhibitor compared with other flavonoids in my study. 5,6,3′,4′-Tetrahydroxy-7-methoxyflavone inhibited 90.5, 85.4, and 73.1% of the chymotrypsin-like, caspase-like, and trypsin-like activities of 26S proteasomes, respectively, with IC_{50} values of 14.0, 5.4, and 24.1 μM (**Table 1**). The IC_{50} values of apigenin-6-hydroxy-7-*O*-β-D-glucoside, quercetin, rutin, 6-hydroxyapigenin, 5,6,4′-trihydroxy-7,3′-dimethoxyflavone, 5,6,3′,4′-tetrahydroxy-7-methoxyflavone, glycitecin, and 6,7,4′-trihydroxyisoflavone are shown in **Table 1**. The other favonoids inhibited $< 20\%$ of the chymotrypsin-like activity of 26S proteasome, and there was no significant inhibition of the caspase-like and trypsin-like activities of 26S (**Figure 2A**).

Next, I evaluated whether structural substituents of flavonoids would affect their inhibition of 26S activity. Hydroxylation at

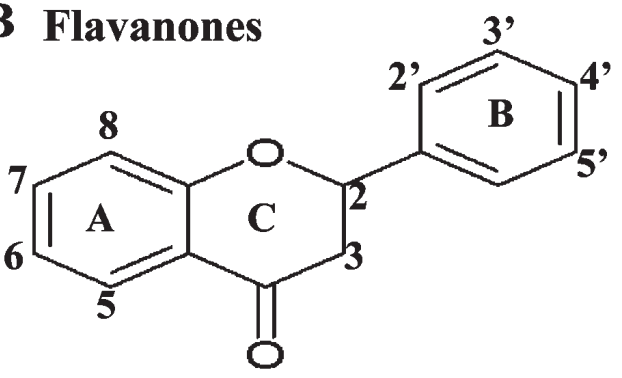
A Flavones



Flavones	3	5	6	7	8	3'	4'	5'	MW
Apigenin	-	OH	-	OH	-	-	OH	-	270.24
Apigenin-6-hydroxy-7-O-β-D-glucoside	-	OH	OH	O-Glu	-	-	OH	-	448
Cosmosiin (Apigenin-7-glucoside)	-	OH	-	O-Glu	-	-	OH	-	432
Kaempferol (3, 4', 5, 7-Tetrahydroxyflavone)	OH	OH	-	OH	-	-	OH	-	286.24
Quercetin (3, 3', 4', 5, 7-Pentahydroxyflavone)	OH	OH	-	OH	-	OH	OH		302.24
Rutin (quercetin-3-rutinoside)	O-Glu-Rha	OH	-	OH	-	-	OH	OH	610.52
6-Hydroxyapigenin	-	OH	OH	OH	-	-	OH	-	286
5, 4'- Dihydroxy-7-methoxyflavone	-	OH	-	$O\text{-}CH_3$	-	-	OH	-	284.268
5, 6, 7-Trihydroxyflavone	-	OH	OH	OH	-	-	-	-	270.24
7, 8-dihydroxyflavone	-	-	-	OH	OH	-	-	-	254.24
5, 6, 4'- Trihydroxy-7, 3'-dimethoxyflavone	-	OH	OH	$O\text{-}CH_3$	-	$O\text{-}CH_3$	OH	-	330
5, 6, 3', 4'- Tetrahydroxy-7-methoxyflavone	-	OH	OH	$O\text{-}CH_3$	-	OH	OH	-	316

*Glu: glucose; Rha: rhamnose. -: H

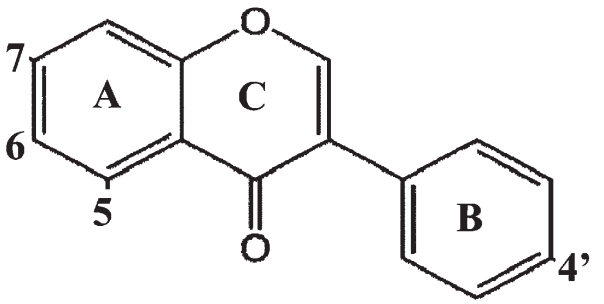
B Flavanones



Flavanones	5	7	3'	4'	MW
Eriodictyol (3', 4', 5, 7-tetrahydroxyflavanone)	OH	OH	OH	OH	288.063
Hesperidin (Hesperetin 7-rhamnoglucoside)	OH	O-Glu-Rha	OH	$O\text{-}CH_3$	610.56
Neohesperidin	OH	O-Glu-Rha	OH	$O\text{-}CH_3$	610.57
Naringin (4',5,7-Trihydroxyflavanone-7-rhamnoglucoside)	OH	O-Glu-Rha	-	OH	580.541
Poncirin	OH	O-Glu-Rha	-	$O\text{-}CH_3$	594.57

*Glu: glucose; Rha: rhamnose. -: H

C Isoflavones



Isoflavones	5	6	7	4'	MW
Daidzein	-	-	OH	OH	254.24
Genistein (5,7,4'-trihydroxyisoflavone)	OH	-	OH	OH	270.24
Genistin	OH	-	O-Glu	OH	432.41
Glycitein	-	O-CH_3	OH	OH	284.27
Formononetin (7-hydroxy-4'-methoxy isoflavone)	-	-	OH	O-CH_3	268.27
Ononin	-	-	O-Glu	O-CH_3	430.41
5, 7-Dihydroxy-4'-methoxyisoflavone	OH	-	OH	O-CH_3	284.268
4', 5-Dihydroxy-7-methoxyisoflavone	OH		O-CH3	OH	284.268
6, 7,4'-Trihydroxyisoflavone	-	OH	OH	OH	270.24

*Glu: glucose; -: H

Figure 1. Chemical structures of (**A**) 12 flavones, (**B**) 5 flavanones, and (**C**) 9 isoflavones investigated in this study.

position 6 of flavones increased the inhibitory effects on 26S activity, whereas hydroxylation at position 4′ decreased the inhibitory effects (Table 1 of the Supporting Information). Methoxylation or glycoslation at the 7- or 3′-position of flavones decreased the inhibitory effects on 26S activity (Table 1 of the Supporting Information). There were no other significant inhibitory effects on 26S activity by any other position substituents of flavanones studied (Table 2 of the Supporting Information). Hydroxylation at position 5 or 6 of isoflavones increased the inhibitory effects on 26S activity, whereas methoxylation at position 4′ decreased the inhibitory effect on 26S activity (Table 3 of the Supporting Information). I have found that all flavones showed stronger inhibitory effects on the chymotrypsin-like and caspase-like but not the trypsin-like activities of 26S than flavanones and isoflavones ($p < 0.05$) (**Figure 2B**,**C**).

Several studies have reported antitumor activity of numerous natural compounds or that they possess synergistic effects in association with chemotherapeutic drugs. Dietary flavonoids, apigenin, quercetin, kaempferol, and myricetin, have many demonstrable health-promoting actions in cancer chemoprevention by their ability to inhibit the chymotrypsin-like activity of 26S proteasome (*3*), whereas apigenin and quercetin are much more potent than kaempferol (*3*). IC_{50} values of the flavonoids for inhibition of the chymotrypsin-like activity of purified 20S proteasome and 26S proteasome in intact Jurkat T cells are around 1−12 μM (*3*). A 20 μM concentration of 5,6,3′,4′-tetrahydroxy-7-methoxyflavone significantly arrested the cell cycle of Con A-stimulated spleen cells at the G0/G1 stage (*14*). However, I have shown that the inhibitory effect of apigenin and kaempferol on 26S activity was significantly weaker than that of quercetin. Most flavonoids inhibited 26S activity, and their IC_{50} values are >50 μM (**Table 1**). These results show that minor changes in the structure of flavonoids induced major changes in the inhibitory effects. Apigenin-6-hydroxy-7-*O*-β-D-glucoside, quercetin, 6-hydroxyapigenin, 5,6,4′-trihydroxy-7,3′-dimethoxyflavone, and 5,6,3′,4′-tetrahydroxy-7-methoxyflavone show stronger proteasome inhibition (**Figure 2A** and **Table 1**), which suggests the C(3′) hydroxyl or methoxyl group may play a role in the inhibition of 26S proteasome activity (**Figures 1** and **2A**). The C(4) carbon may be a site for nucleophilic attack by the OH group of the N-terminus of threonine of the proteasomal β5 subunit. Furthermore, the C(3) hydroxyl may alter the ability of these flavonoids to inhibit the proteasome (*14*). Apigenin-6-hydroxy-7-*O*-β-D-glucoside, 5,6,4′-trihydroxy-7,3′-dimethoxyflavone, and 5,6,3′,4′-tetrahydroxy-7-methoxyflavone show low IC_{50} values of their chymotrypsin-like activity, and their structures do not have a C(3) hydroxyl group. Therefore, the C(3) hydroxyl might not be the only group these flavonids depend upon for their proteasome inhibitory activity. Our data show the inhibitory effects of chymotrypsin-like activity of flavones are stronger than those of isoflavones. This could be due to the fact that isoflavones contain a C(3) benzyl group, which may sterically hinder its protesaome inhibitory activity. Methylated dietary flavonoids did not act as proteasome inhibitors either in an in vitro system or by an in silico model (*15*). They only show an ability to weakly induce apoptosis in the systems (*15*). To understand the relationship between molecular structure and proteasome inhibitory activity, I have evaluated the relationship between hydroxyl, methoxyl, and glucosyl groups of the flavonoids and their inhibitory effects on

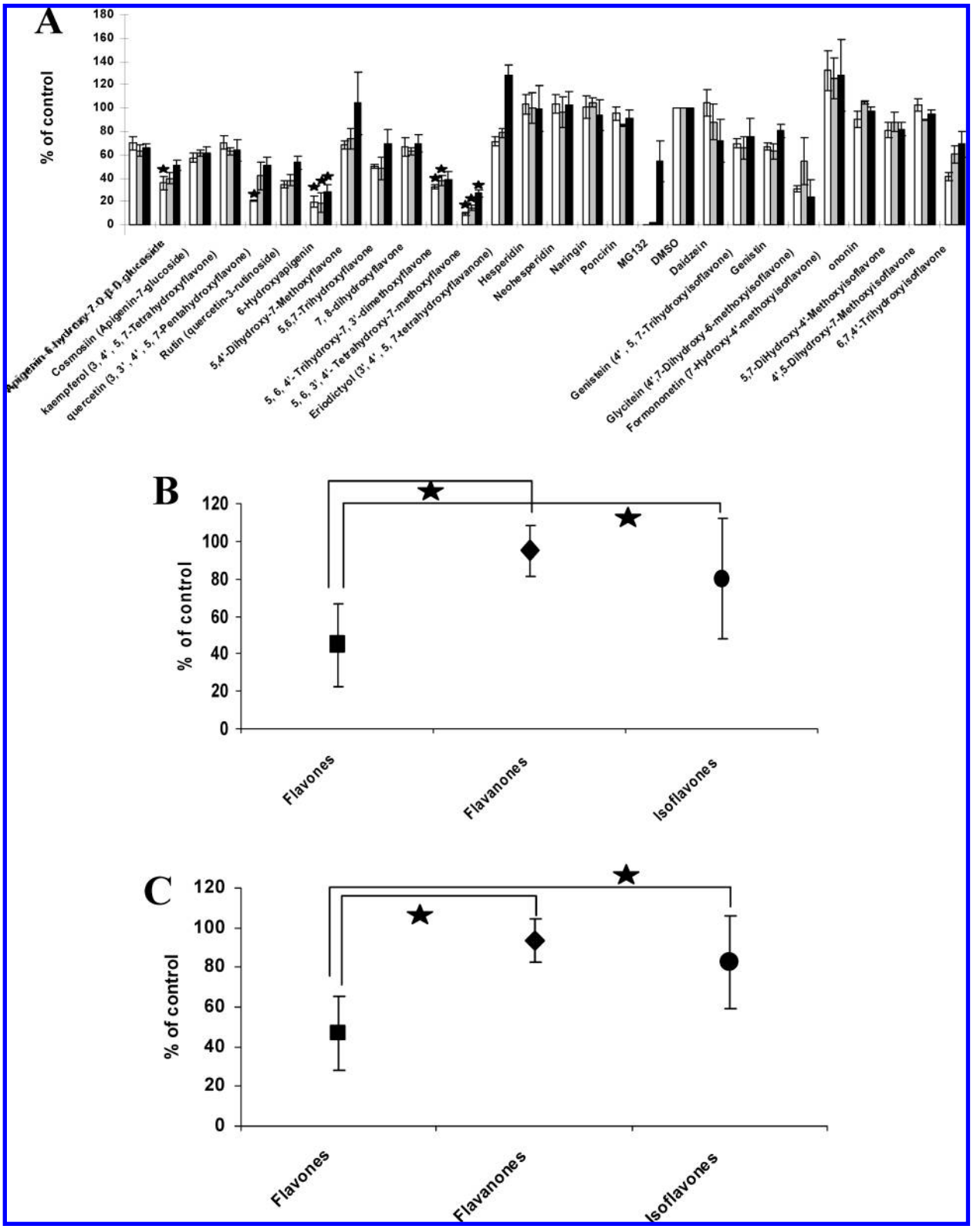


Figure 2. (**A**) Comparison of percentage of chymotrypsin-like (white bars), caspase-like (gray bars), and trypsin-like activities (black bars) of 26S proteasome inhibited by 100 μM concentrations of 12 flavones, 5 flavanones, 9 isoflavones, and MG132 (26S proteasome with DMSO = 100%). Data are reported as mean $\pm$ SEM. Each sample is the mean of three determinations. The star (★) indicates values where difference from control value (DMSO) was statistically significant at $P < 0.01$. Comparison of all samples (100 μM) is on chymotrypsin-like (**B**) and caspase-like activity (**C**) of 26S proteasomes. Flavones ($n = 12$, ■), flavanones ($n = 5$, ♦), and isoflavones ($n = 9$, ●) represent the mean $\pm$ SE. The star (★) indicates values of flavanones and isoflavones where difference from the value of flavones was statistically significant at $P < 0.05$. The significance of the differences between flavones, flavanones, and isoflavones was determined by unpaired Student's *t* test (★, $p < 0.05$).

proteasomes. I have demonstrated that 5,6,4′-trihydroxy-7,3′-dimethoxyflavone with a 3′-methoxyl group had low proteasome inhibitory activity when compared to 5,6,3′,4′-tetrahydroxy-7-methoxyflavone (**Figure 2A** and Table 1 of the Supporting

Information). Apigenin-6-hydroxy-7-*O*-β-D-glucoside with 7-glucoside has low proteasome inhibitory activity when compared to 6-hydroxyapigenin (**Figure 2A** and Table 1 of the Supporting Information). Overall, flavones that have more than three hydroxyl groups such as apigenin-6-hydroxy-7-*O*-β-D-glucoside, quercetin, rutin, 6-hydroxyapigenin, 5,6,4′-trihydroxy-7,3′-dimethoxyflavone, and 5,6,3′,4′-tetrahydroxy-7-methoxyflavone show stronger 26S proteasome inhibitory activity (**Table 2**). Therefore, the number of hydroxyl groups may play an important role in the inhibitory effects on 26S activity by flavones, whereas methoxyl and glucosyl groups could hinder the flavone for nucleophilic attack and thus alter the ability of these flavonoids to inhibit the proteasome.

Table 1. IC_{50} Values of the Investigated Compounds on 26S Proteasome Activity

	IC_{50} (μM)		
flavonoids and isoflavonoids	chymotrypsin-like	caspase-like	trypsin-like
apigenin-6-hydroxy-7-*O*-β-D-glucoside	27.8	64.9	
quercetin (3,3′,4′,5,7-pentahydroxyflavone)	38.5	71.8	79.9
rutin	66.0	57.0	
6-hydroxyapigenin	61.7	16.1	54.7
5,6,4′-trihydroxy-7,3′-dimethoxyflavone	28.9	40.7	
5,6,3′,4′-tetrahydroxy-7-methoxyflavone	14.0	5.4	24.0
glycitecin	138.4	99.4	17.1
6,7,4′-trihydroxyisoflavone	113.1		

Table 2. Inhibitor Mode of Investigated Compounds on 26S Proteasome Activity

		Michaelis constants K_i (μM)		
test sample	inhibition mode	chymotrypsin-like	caspase-like	trypsin-like
5,6,3′,4′-tetrahydroxy-7-methoxyflavone	mixed	11.2	15.8	—[a]
5,6,4′-trihydroxy-7,3′-dimethylflavone	mixed	32.7	44.4	—
quercetin	mixed	60.6	116.3	—

[a] Unable to establish.

Dietary modulation of signaling cascades is a potential chemopreventive mechanism controlling cellular growth, proliferation, and differentiation. Genistein, daidzein, glycitein, and equol from soybean have a prophylactic effect on prostate cancer via ERK1/2 activation (*16*, *17*). Genistein also has an antiproliferative property on mitogen-stimulated cell growth of in vitro cultured human breast cancer cells and therefore is a candidate for study in the prevention of breast cancer (*18*). Additionally, the presented data show that glycitein but not genistein had high inhibitory with low IC_{50} on caspase-like 26S activity (**Figure 2A** and **Table 1**).

Relationships among Chymotrypsin-like, Caspase-like, and Trypsin-like Activities of 26S Proteasome and Flavonoids. To date there is no information addressing the relationship among molecular weight and the chymotrypsin-like, caspase-like, and trypsin-like activities of 26S proteasome and flavonoids. Our data showed that there is no discernible relationship between the molecular weight of flavonoids and the inhibitory effect of 100 μM flavonoids on 26S activity (**Figure 3A**). However, chymotrypsin-like activity increased when caspase-like or trypsin-like activity was significantly increased in the cross-section studies. The inhibitory effect was strongly correlated with each activity as analyzed by Pearson's correlation coefficients (r) of 0.95, 0.83, and 0.86 ($p < 0.001$) (**Figure 3B**−**D**).

I investigated the relationship among three peptidase activities of flavonoids. The inhibitory effect of each activity was strongly correlated with another activity. The primary structure

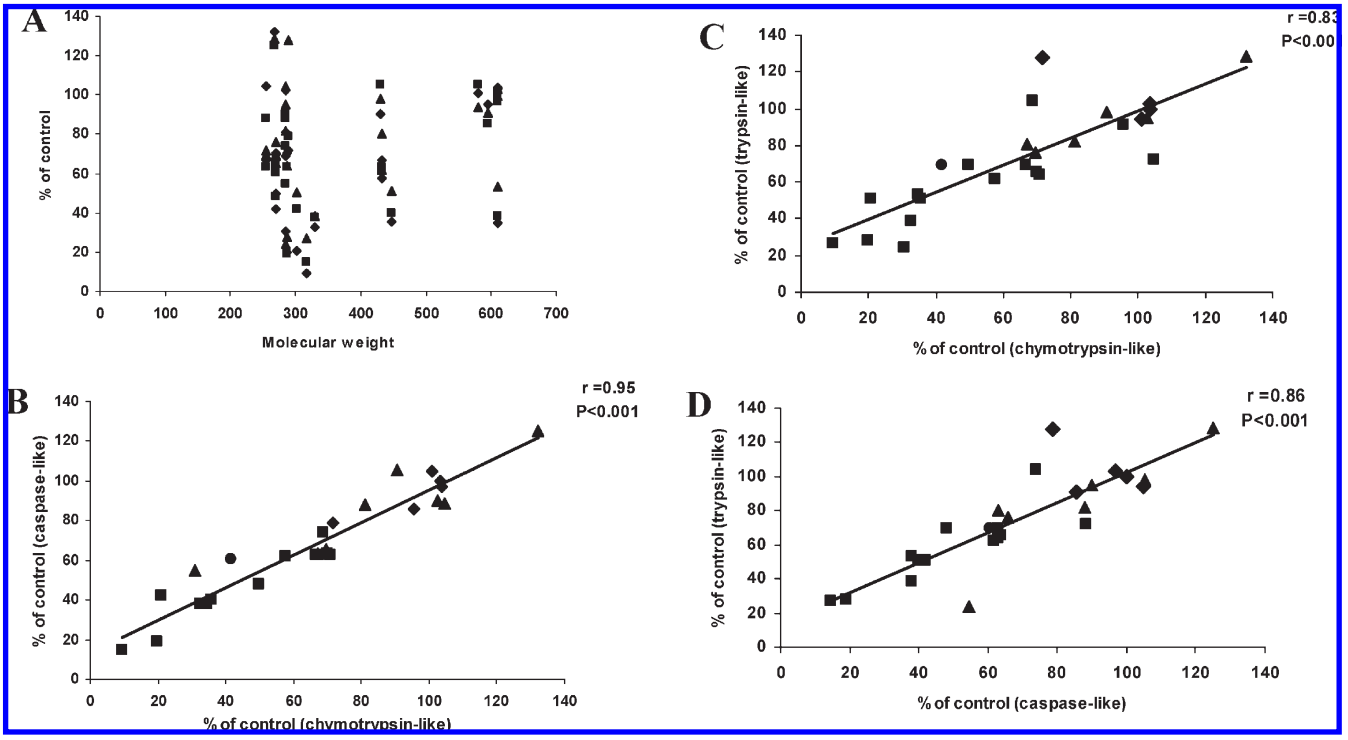


Figure 3. (**A**) Relationship between the molecular weight of 100 μM flavonoids and their inhibitory effects on chymotrypsin-like (▲), caspase-like (■), and trypsin-like activities (●) of 26S. Relationship between (**B**) chymotrypsin-like and caspase-like, (**C**) chymotrypsin-like and trypsin-like, and (**D**) caspase-like and trypsin-like activities of 26S is affected by 100 μM flavonoids: flavones (■); flavanones (◆); isoflavones (▲). Abbreviations: r, Pearson's coefficient.

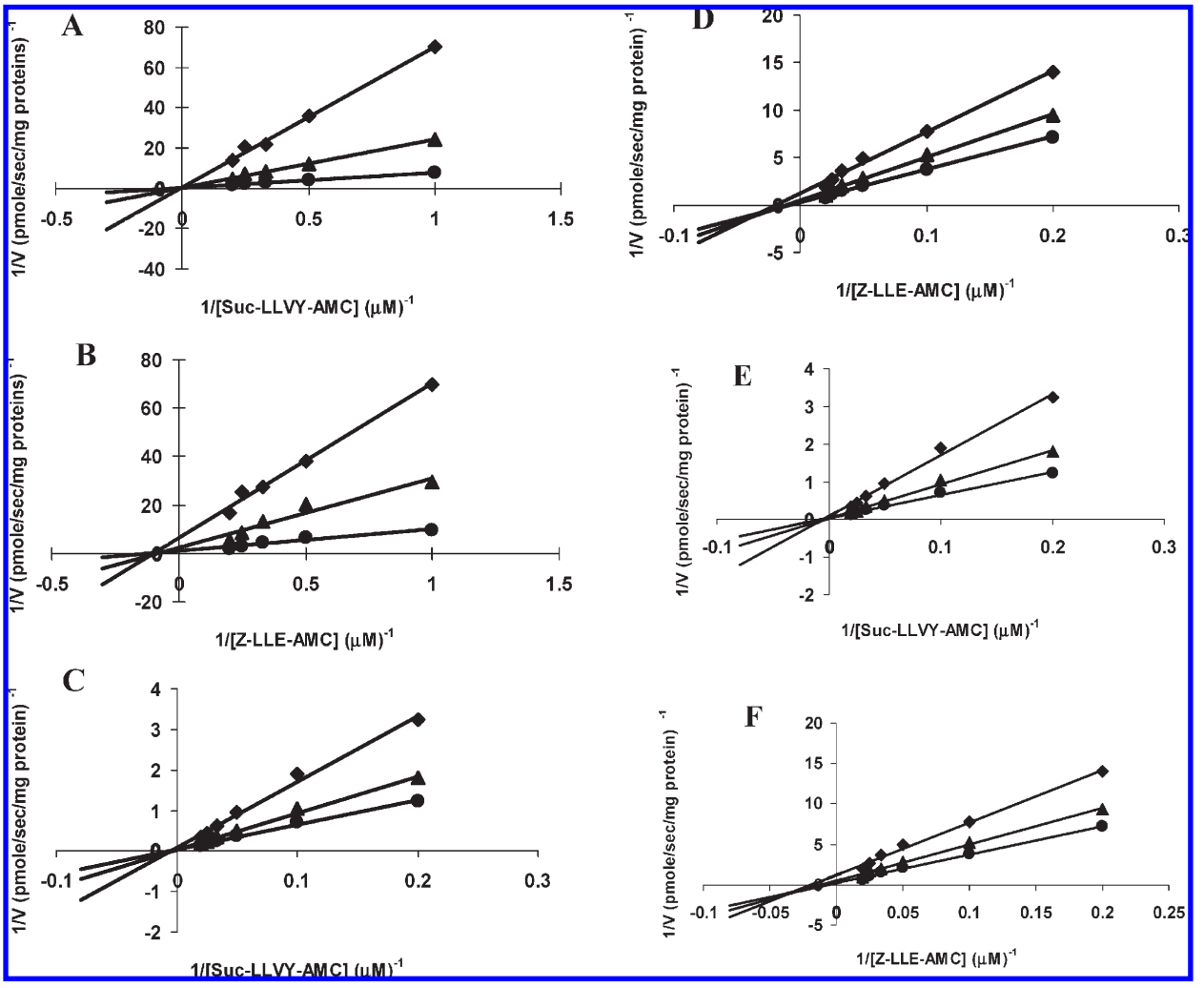


Figure 4. Lineweaver−Burk plots of 5,6,3′,4′-tetrahydroxy-7-methoxyflavone (**A**, **B**) 5,6,4′-trihydroxy-7,3′-dimethoxyflavone (**C**, **D**) [0 (◆), 50 μM (▲), 100 μM (●)] and quercetin (**E**, **F**) [0 (◆), 10 μM (▲), 100 μM (●)] effected on 26S proteasome activity when the concentration of the substrate Suc-LLVY-AMC or Z-LLE-AMC was 0.5, 1, 2, 3, 4, or 5 μM (**A**−**D**) or 5, 10, 20, 30, 40, or 50 μM (**E**, **F**). Each point represents the mean of three independent determinations.

of flavonoids might be the factor determining relationship among the inhibitory effects of chymotrypsin-like, caspase-like, and trypsin-like activities of 26S proteasomes. The flavones with low IC_{50} on 26S proteasomes were selected for study in the following experiments.

Michaelis−Menten Kinetics of Flavonoids on Chymotrypsin-like and Caspase-like Activities. To further investigate the inhibition kinetics, these three flavnoids, 5,6,3′,4′-tetrahydroxy-7-methoxyflavone, 5,6,4′-trihydroxy-7, 3′-dimethoxyflavone, and quercetin, were analyzed by using the Lineweaver−Burk method (**Figure 4**). The experiment employed was the same protocol as described under Materials and Methods. The concentration of Suc-LLVY-AMC or Z-LLE-AMC was from 1 to 50 μM. The velocities of reactions were measured without or with inhibitor (10, 50, or 100 μM). The Lineweaver−Burk plot is shown in Figure. **4**. The Michaelis−Menten kinetics of chymotrypsin activity showed a V_{max} = 4.69 pmol of AMC s^{-1} (mg of proteins)$^{-1}$ and K_m (Suc-LLVY-AMC) = 35.36 μM and caspase activity with a V_{max} = 1.12 pmol of AMC s^{-1} (mg of proteins)$^{-1}$ and K_m (Z-LLE-AMC) = 10.35 μM. The K_i values of 26S chymotrypsin and caspase activities in the presence of these three flavonoids at 10, 50, or 100 μM are shown in **Table 2**. All flavonoids show the mixed type inhibition.

The inhibition kinetics analyzed by Lineweaver−Burk plots indicates that three flavonoids are inhibitors affecting multiple targets as mixed type inhibitors. During the experiment, the flavonoid 5,6,3′,4′-tetrahydroxy-7-methoxyflavone was tested at a concentration of 100 μM, which exceeded its K_i value for the chymotryptic activity (11.23 μM) and caspase-like activity (15.8 μM) by 8.8- and 6.3-fold, respectively. The flavonoid 5,6,3′,4′-tetrahydroxy-7-methoxyflavone displayed a mixed type noncompetitive kinetics in inhibiting chymotrypsin-like or caspase-like activity when its substrate was Suc-LLVY-AMC or Z-LLE-AMC, with KI′ < KI. This suggested that the kinetic rate of enzyme−5,6,3′,4′-tetrahydroxy-7-methoxyflavone association is faster than that of enzyme−substrate−5,6,3′,4′-tetrahydroxy-7-methoxyflavone association. The efficiency of flavonoid inhibition of chymotrypsin-like and caspase-like activity may depend on their chemical structures, chiefly the 3′ OH group. The degradable fragments of proteins are affected by the association of chymotrypsin-like or caspase-like enzyme with 5,6,3′,4′-tetrahydroxy-7-methoxyflavone. The other two peptidase activities are not affected.

Inhibition by Flavonoids on Three Peptidase Activities That Are Stimulated by Ubiquitin. Binding of model polyUb substrates to the 19S regulator of mammalian and yeast 26S proteasomes enhances the peptidase activities of the 20S proteasome about 2-fold in a process requiring ATP hydrolysis (*19*, *20*). Ubiquitin is present in all eukaryotic cells by current knowledge. When ubiquitin was added into the assay mixture to mimic the cell in vivo, it stimulated the 26S proteasome activity to about 1.2-fold

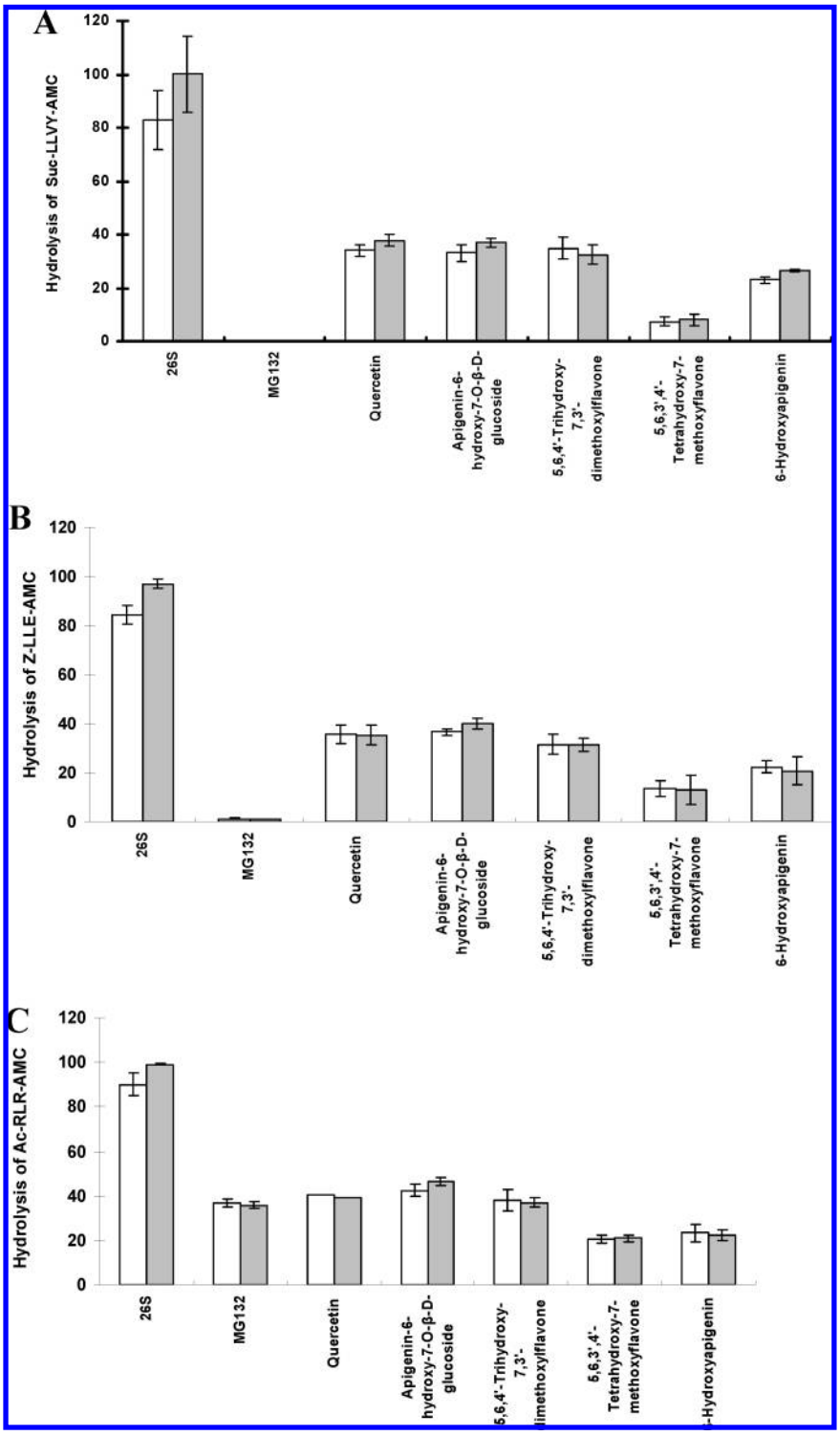


Figure 5. Inhibition of ubiquitin-induced stimulation of proteasome activity by flavonoids. 26S proteasomes were preincubated without (white bars) or with (gray bars) ubiquitin and then treated with 100 μM inhibitors (MG132, apigenin-6-hydroxy-7-*O*-β-D-glucoside, 6-hydroxyapigenin, quercetin, 5,6,4′-trihydroxy-7,3′-dimethoxyflavone, or 5,6,3′,4′- tetrahydroxy-7-methoxyflavone). Chymotrypsin-like (**A**), caspase-like (**B**), and trypsin-like (**C**) activities were assayed. Proteasome activities are arbitrary values. Values are the means ($\pm$SD) from three independent experiments.

for all three peptidase activities. To test whether the flavonoids' inhibitory effects were influenced by ubiquitin-induced 26S proteasome activity, 160 nM 26S proteasomes isolated from pig erythrocytes were preincubated with or without 7.2 μM ubiquitin and the reaction products were detected by peptide assay. I noted that stimulation induced by ubiquitin on the chymotrypsin-like, caspase-like, and trypsin-like activities could be blocked by treatment of the 26S proteasome with 100 μM flavonoids (**Figure 5**). I have found that the inhibitory effects induced by ubiquitin on the chymotrypsin-like activity of 26S proteasome were increased about 1.1-fold by apigenin-6-hydroxy-7-*O*-β-D-glucoside, quercetin, 6-hydroxyapigenin, and 5,6,3′,4′-tetrahydroxy-7-methoxyflavone (**Figure 5**), whereas 5,6,4′-trihydroxy-7,3′-dimethoxyflavone decreased the ubiquitin-induced stimulation of the chymotrypsin-like activity of 26S proteasome (**Figure 5A**). Similar results were seen on the stimulation induced

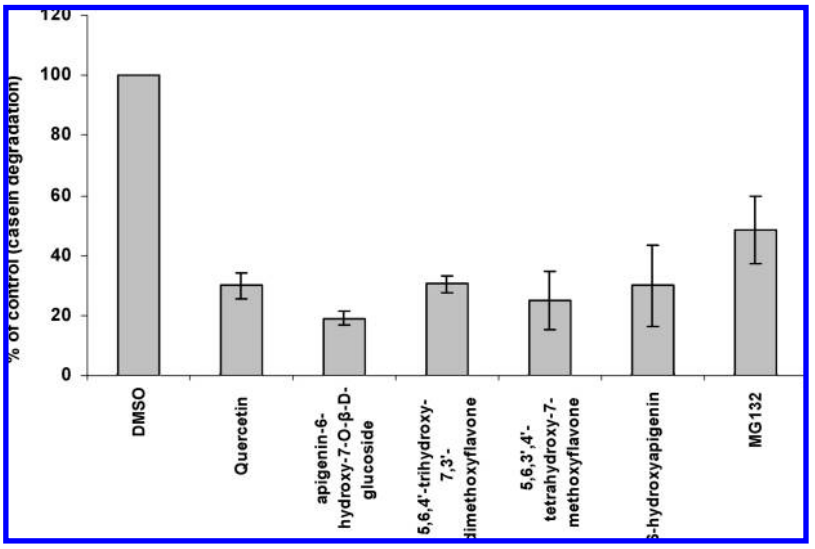


Figure 6. Degradation of casein by purified pig 26S proteasome. Reactions contained 120 nM 26S proteasome and 1.2 μM casein in the presence of DMSO (control) or 100 μM inhibitors (MG132, 6-hydroxyapigenin, apigenin-6-hydroxy-7-*O*-β-D-glucoside, quercetin, 5,6,4′-trihydroxy-7,3′-dimethoxyflavone, or 5,6,3′,4′-tetrahydroxy-7-methoxyflavone). Proteolysis is expressed as the percentage of casein degraded. Each measurement was carried out using at least three independent experiments.

by ubiquitin on the caspase-like and trypsin-like activities of 26S proteasome inhibited by flavonoids (**Figure 5B**,**C**).

These findings agree with previously published data that preincubation of ubiquitin with 26S stimulated all three peptidase activities (*19*, *20*). Thus, ubiquitin may bind to 19S to minimally open the narrow channel and hence allow peptide substrate to access the active site of 20S proteasome. Interestingly, there is no effect detected on the inhibitory of flavonoids by ubiquitin-induced stimulation of proteasome activity.

Inhibitory Effects of Flavonoids on the Degradation of Casein. The 26S proteasome has the capacity to degrade certain native disordered and nonubiquitinated proteins, such as casein. Protein degradation in vitro and in vivo is reduced significantly only when either the trypsin-like or caspase-like sites are inhibited, together with the chymotrypsin-like sites (*13*). It is noted that apigenin-6-hydroxy-7-*O*-β-D-glucoside, 6-hydroxyapigenin, quercetin, 5,6,4′-trihydroxy-7,3′-dimethoxyflavone, and 5,6,3′,4′-tetrahydroxy-7-methoxyflavone all displayed low IC_{50} values of chymotrypsin-like activity of 26S proteasome. I have examined the degradation of those compounds on casein by the 26S proteasome. The inhibition of casein degradation was found to be apigenin-6-hydroxy-7-*O*-β-D-glucoside > 5,6,3′,4′-tetrahydroxy-7-methoxyflavone > 6-hydroxyapigenin $\cong$ quercetin > 5,6,4′-trihydroxy-7,3′-dimethoxyflavone > MG132 (**Figure 6**). However, the degradation enzymatic velocities of these five flavonoids are dissimilar to the kinetics of fluorogenic peptide hydrolysis. Proteasomes usually cut on average every eighth peptide bond in a polypeptide (*21*). A polypeptide usually is hydrolyzed on average every eighth peptide bond (*21*) by determining the appearance of new amino groups with fluorescamine (*22*). The data showed the ability to degrade casein could be due to chymotrypsin-like activity of 26S proteasome. Our results indicate that blocking both the caspase- and trypsin-like sites will render a 45% decrease in casein hydrolysis (*13*).

In conclusion, apigenin-6-hydroxy-7-*O*-β-D-glucoside, 6-hydroxyapigenin, quercetin, 5,6,4′-trihydroxy-7,3′-dimethoxyflavone, and 5,6,3′,4′-tetrahydroxy-7-methoxyflavone show low IC_{50} values of chymotrypsin-like activity. A recent study showed that the dietary flavonoid quercetin reduced bortezomib-induced apoptosis through formation of cyclic boronate esters between the catechol group of quercetin and the boric acid group of bortezomib (*23*). Apigenin-6-hydroxy-7-*O*-β-D-glucoside and 5,6,4′-trihydroxy-7,3′-dimethoxyflavone without a catechol group may be potential drugs to combine with bortezomib to inhibit 26S proteasomes. 5,6,3′,4′-Tetrahydroxy-7-methoxyflavone is shown to have higher inhibitory of 26S activity by peptide assay and kinetic mode, whereas apigenin-6-hydroxy-7-*O*-β-D-glucoside is the best to inhibit casein degradation. 5,6,4′-Trihydroxy-7,3′-dimethoxyflavone is the most useful inhibitor by ubiquitin-stimulated 26S activity. It appears that the 7- and 3′-positions of flavones could be regulatory sites for the inhibitory activity of 26S proteasome. These results may be useful in the evaluation of the relationship between chemical structure and biological activity of flavonoids and could be useful for the development of potential novel drugs against a number of diseases that involve the ubiquitin−proteasome pathway.

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There is no conflict of interest for this work.

Supporting Information Available: Tables 1−3. This material is available free of charge via the Internet at http://pubs.acs.org.

LITERATURE CITED

(1) Bonfili, L.; Cecarini, V.; Amici, M.; Cuccioloni, M.; Angeletti, M.; Keller, J. N.; Eleuteri, A. M. Natural polyphenols as proteasome modulators and their role as anti-cancer compounds. *FEBS J.* **2008**, *275*, 5512–5526.

(2) Chen, D.; Chen, M. S.; Cui, Q. C.; Yang, H.; Dou, Q.,P. Structure−proteasome-inhibitory activity relationships of dietary flavonoids in human cancer cells. *Front. Biosci.* **2007**, *12*, 1935–1945.

(3) Chen, D.; Daniel, K. G.; Chen, M. S.; Kuhn, D. J.; Landis-Piwowar, K. R.; Dou, Q. P. Dietary flavonoids as proteasome inhibitors and apoptosis inducers in human leukemia cells. *Biochem. Pharmacol.* **2005**, *69*, 1421–1432.

(4) Glickman, M. H.; Ciechanover, A. The ubiquitin−proteasome proteolytic pathway: destruction for the sake of construction. *Physiol. Rev.* **2002**, *82*, 373–428.

(5) Peters, J. M.; Franke, W. W.; Kleinschmidt, J. A. Distinct 19 and 20 S subcomplexes of the 26 S proteasome and their distribution in the nucleus and the cytoplasm. *J. Biol. Chem.* **1994**, *269*, 7709–1778.

(6) Groll, M.; Koguchi, Y.; Huber, R.; Kohno, J. Crystal structure of the 20 S proteasome:TMC-95A complex: a non-covalent proteasome inhibitor. *J. Mol. Biol.* **2001**, *311*, 543–548.
(7) Kisselev, A. F.; Goldberg, A. L. Proteasome inhibitors: from research tools to drug candidates. *Chem. Biol.* **2001**, *8*, 739–758.
(8) Adams, J. The development of proteasome inhibitors as anticancer drugs. *Cancer Cell.* **2004**, *5*, 417–421.
(9) Chauhan, D.; Singh, A.; Brahmandam, M.; Podar, K.; Hideshima, T.; Richardson, P.; Munshi, N.; Palladino, M. A.; Anderson, K. C. Combination of proteasome inhibitors bortezomib and NPI-0052 trigger in vivo synergistic cytotoxicity in multiple myeloma. *Blood* **2008**, *111*, 1654–1664.
(10) Nufer, K. R.; Ismail, B.; Hayes, K. D. The effects of processing and extraction conditions on content, profile, and stability of isoflavones in a soymilk system. *J. Agric. Food Chem.* **2009**, *57*, 1213–1218.
(11) Chang, T. L.; Ding, H. Y.; Kao, Y. W. The role of ginsenoside Rd in inhibiting of 26S proteasome activity. *J. Agric. Food Chem.* **2008**, *56*, 12011–12015.
(12) Liu, C. W.; Li, X.; Thompson, D.; Wooding, K.; Chang, T. L.; Tang, Z.; Yu, H.; Thomas, P. J.; DeMartino, G. N. ATP binding and ATP hydrolysis play distinct roles in the function of 26S proteasome. *Mol. Cell* **2006**, *24*, 39–50.
(13) Kisselev, A. F.; Callard, A.; Goldberg, A. L. Importance of the different proteolytic sites of the proteasome and the efficacy of inhibitors varies with the protein substrate. *J. Biol. Chem.* **2006**, *281*, 8582–8590.
(14) Rao, Y. K.; Fang, S. H.; Hsieh, S. C.; Yeh, T. H.; Tzeng, Y. M. The constituents of *Anisomeles indica* and their anti-inflammatory activities. *J. Ethnopharmacol.* **2009**, *121*, 292–296.
(15) Landis-Piwowar, K. R.; Milacic, V.; Dou, Q. P. Relationship between the methylation status of dietary flavonoids and their growth-inhibitory and apoptosis-inducing activities in human cancer cells. *J. Cell. Biochem.* **2008**, *105*, 514–523.
(16) Teas, J.; Hurley, T. G.; Hebert, J. R.; Franke, A. A.; Sepkovic, D. W.; Kurzer, M. S. Dietary seaweed modifies estrogen and phytoestrogen metabolism in healthy postmenopausal women. *J. Nutr.* **2009**, *139*, 939–944.
(17) Clubbs, E. A.; Bomser, J. A. Glycitein activates extracellular signal-regulated kinase via vascular endothelial growth factor receptor signaling in nontumorigenic (RWPE-1) prostate epithelial cells. *J. Nutr. Biochem.* **2007**, *18*, 525–532.
(18) Barnes, S. The chemopreventive properties of soy isoflavonoids in animal models of breast cancer. *Breast Cancer Res. Treat.* **1997**, *46*, 169–179.
(19) Bech-Otschir, D.; Helfrich, A.; Enenkel, C.; Consiglieri, G.; Seeger, M.; Holzhütter, H. G.; Dahlmann, B.; Kloetzel, P. M. Polyubiquitin substrates allosterically activate their own degradation by the 26S proteasome. *Nat. Struct. Mol. Biol.* **2009**, *16*, 219–225.
(20) Li, X.; Demartino, G. N. Variably modulated gating of the 26S proteasome by ATP and polyubiquitin. *Biochem. J.* **2009**, *421*, 397–404.
(21) Kisselev, A. F.; Akopian, T, N.; Woo, K. M.; Goldberg, A. L. The sizes of peptides generated from protein by mammalian 26 and 20 S proteasomes. Implications for understanding the degradative mechanism and antigen presentation. *J. Biol. Chem.* **1999**, *274*, 3363–3371.
(22) Akopian, T. N.; Kisselev, A. F.; Goldberg, A. L. Processive degradation of proteins and other catalytic properties of the proteasome from *Thermoplasma acidophilum*. *J. Biol. Chem.* **1997**, *272*, 1791–1798.
(23) Liu, F. T.; Agrawal, S. G.; Movasaghi, Z.; Wyatt, P. B.; Rehman, I. U.; Gribben, J. G.; Newland, A. C.; Jia, L. Dietary flavonoids inhibit the anticancer effects of the proteasome inhibitor bortezomib. *Blood* **2008**, *112*, 3835–3846.

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