Note

Effects of Hydroxy Groups in the A-Ring on the Anti-proteasome Activity of Flavone

Kasumi Nakamura,[a] Jia-Hua Yang,[b] Eiji Sato,[a] Naoyuki Miura,[a] and Yi-Xin Wu*[,a]

[a] *Department of Biochemistry, Hamamatsu University School of Medicine; 1–20–1 Handayama, Higashi-ku, Hamamatsu, Shizuoka 431–3192, Japan: and* [b] *Department of General Surgery, Putuo Hospital, Shanghai University of Traditional Chinese Medicine; Shanghai 200062, China.*

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The ubiquitin–proteasome pathway plays an important role in regulating apoptosis and the cell cycle. Recently, proteasome inhibitors have been shown to have antitumor effects and have been used in anticancer therapy for several cancers such as multiple myeloma. Although some flavones, such as apigenin, chrysin and luteolin, have a specific role in the inhibition of proteasome activity and induced apoptosis in some reports, these findings did not address all flavone types. To further investigate the proteasome-inhibitory mechanism of flavonoids, we examined the inhibitory activity of 5,6,7-trihydroxyflavone, baicalein and 5,6,7,4′-tetrahydroxyflavone, scutellarein on extracted proteasomes from mice and cancer cells. Unlike the other flavones, baicalein and scutellarein did not inhibit proteasome activity or accumulate levels of ubiquitinated proteins. These results indicate that flavones with hydroxy groups at positions 5, 6 and 7 of the A-ring lack the anti-proteasome function.

Key words ubiquitin; flavonoid; proteasome; inhibitor

The ubiquitin–proteasome pathway has an important role in regulating the cell cycle and apoptosis.[1)] This pathway involves two steps: (i) conjugation of multiple ubiquitin molecules to the target protein, and (ii) degradation of the ubiquitin-tagged protein by the 26S proteasome. The latter is a multi-subunit protease complex comprising a 20S core associated with two 19S regulatory caps. The 20S core particle is cylindrical and consists of two outer heptameric rings of α-subunits and two inner heptameric rings of β-subunits. The three main catalytic activities of proteasomes are associated with the 20S core. They are peptidylglutamyl peptide hydrolyzing (PGPH) activity (mediated by the β1 subunit), trypsin-like (T-L) activity (mediated by the β2 subunit), and chymotrypsin-like (CT-L) activity (mediated by the β5 subunit).[2)]

Flavonoids are a group of polyphenolic substances that are widely distributed in plants, fruits vegetables, tea and wine. Flavonoids reportedly have pharmacological effects that include antioxidant, antitumor, antiviral and anti-inflammatory activities.[3–8)] Recent studies have shown that certain flavonoids can inhibit proteasome activity and induce apoptosis in tumor cells, which suggests that flavonoids have potential as a new type of anticancer drugs.[9,10)] However, not all flavonoids are strong inhibitors of the proteasome. Their proteasomal inhibitiory potency is dependent upon their chemical structure. Some flavones (*e.g.*, luteolin, apigenin) are markedly more potent than other flavonoids such as flavonols (*e.g.*, kaempferol), or flavanones (*e.g.*, naringenin and eriodictyol).[11)] Flavonoid glycoside is one type of flavonoids, and some flavonoid glycosides, such as baicalin and scutellarin, reportedly affect the inhibition of proteasomes.[12)]

Flavones have been shown to have structure–activity relationships; the apoptosis-inducing potencies in tumor cells and inhibitory potencies in 20S purified proteasome were luteolin>apigenin>chrysin.[11)] Furthermore, analysis of the effects of flavonoids on the three catalytic activities of proteasomes, CT-L, T-L and PGPH activities have shown the order of inhibitory potencies to be luteolin>apigenin>chrysin in both CT-L and T-L catalytic activities.[10)] These results indicated that flavones with hydroxylated B rings are more powerful proteasome inhibitors and inducers of apoptosis in tumor cells. However these findings did not include all flavone types. In particular, the relationship between anti-proteasome activity and the hydroxy groups in the A-ring is still not clear.

Baicalein and scutellarein are major active principal flavonoids that exist in a variety of plants. They have a similarity in their chemical structures: three hydroxy groups in the A-ring, at positions 5, 6 and 7 (Fig. 1). Baicalein and scutellarein have been reported to many pharmacological effects, such as antioxidant, antitumor, anti-adipogenic, antiviral and anti-inflammatory activities.[13–16)] Whether baicalein and scutellarein can inhibit proteasome activity has not yet been reported.

In this study, we examined the inhibitory activity of flavones, including baicalein and scutellarein, on proteasomes extracted from mouse and cancer cells. Unlike other flavones, baicalein and scutellarein did not inhibit the activities of the proteasomes, nor did they accumulate ubiquitinated proteins or affect proteasome target protein. These data suggest that flavones with hydroxy groups at positions 5, 6 and 7 of the A-ring lack anti-proteasome function.

MATERIALS AND METHODS

Chemicals and Reagents Apigenin (purity≥95%), chrysin (purity>97%), baicalein (purity≥98%), and MG-132 were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). Luteolin (purity>98%) was purchased from LKT Laboratories Inc. (St. Paul, MN, U.S.A.). Scutellarein (purity≥97%) was purchased from Extrasynthese Co. (Genay, France). The fluorogenic substrates Succ-LLVY-AMC, Z-ARR-AMC, and Z-LLE-AMC (for proteasome chymotrypsin-like, trypsin-like, PGPH-like activities) were purchased from Calbiochem, Inc. (San Diego, CA, U.S.A.). Protein A-Sepharose was purchased

* To whom correspondence should be addressed. e-mail: yixinwu@hama-med.ac.jp

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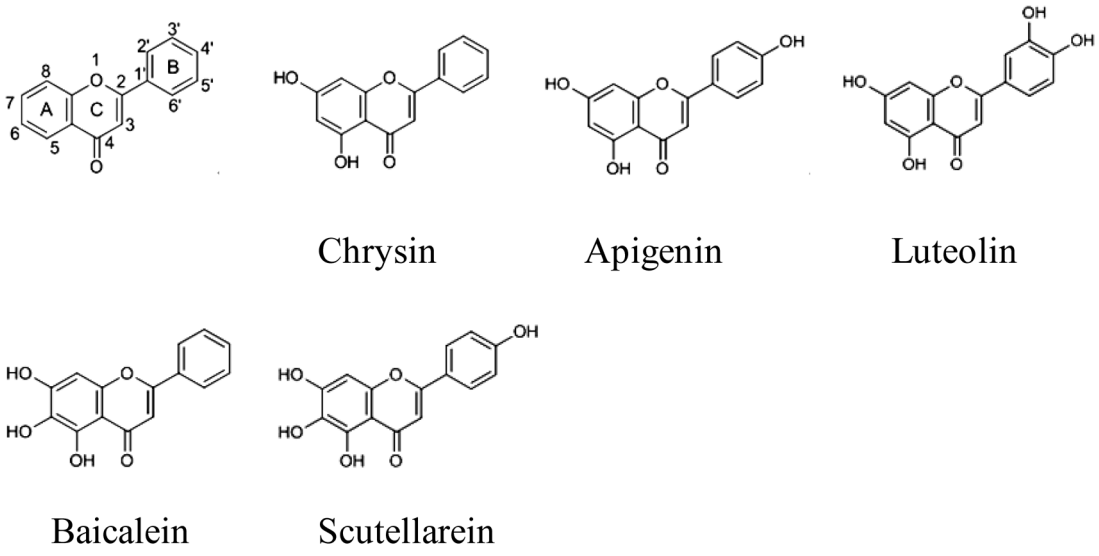

Fig. 1. Chemical Structures of Flavones

from GE Healthcare (Waukesha, WI, U.S.A.).

Cell Culture A human leukemia Jurkat T cell line was obtained from Riken Cell Bank (Tsukuba, Japan). The human colon cancer HCT-116 cell line was obtained from ATCC (U.S.A.). All cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 10 μg/mL penicillin/streptomycin. Cell cultivation was carried out at 37°C in a humidified atmosphere of 5% carbon dioxide and 95% air.

Proteasome Extraction 26S Proteasomes were prepared from the liver of mice as described by Momose *et al.*[17] Briefly, livers from female mice (ICR) were homogenized in 3 volumes of 50 mM Tris–HCl buffer (pH 7.5) containing 1 mM dithiothreitol (DTT), 2 mM ATP and 0.25 M sucrose. The homogenate was centrifuged at 70000×***g*** for 1 h in a Beckman 60Ti rotor. The resulting supernatant was recentrifuged at 85000×***g*** for 5 h. The precipitate was suspended in a suitable volume of 25 mM Tris–HCl buffer (pH 8) containing 1 mM DTT, 2 mM ATP and 20% (v/v) glycerol, and then was centrifuged at 13000 rpm for 30 min at 4°C. The supernatant obtained was stored at −80°C and used as proteasomes. Proteasome extraction from cells was carried out as described previously.[18] Briefly, cells (1×10^7) were harvested, resuspended in 1 mL ATP/DTT lysis buffer [10 mM Tris–HCl (pH 8), 5 mM ATP, 0.5 mM DTT, 5 mM $MgCl_2$], and incubated on ice for 10 min. This was followed by sonication for 15 s. Lysates were centrifuged at 500×***g*** for 10 min at 4°C. The resulting supernatant containing proteasomes was stable at −80°C for ≥1 month with the addition of 20% (v/v) glycerol. Protein concentrations of proteasome extractions from mice and cells were measured using a bicinchoninic acid (BCA) protein assay reagent (Pierce, Rockford, IL, U.S.A.) with bovine serum albumin as a standard.

Antibodies Rabbit polyclonal antibody against inhibitor of nuclear factor κB-alpha (IκB-α), goat polyclonal antibody against actin, and p27, a mouse monoclonal antibody against Bax, were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, U.S.A.). Secondary antibodies were purchased from Jackson Immunoresearch Laboratories Inc. (West Grove, PA, U.S.A.).

Enzyme Assay The fluorogenic substrates Succ-LLVY-AMC, Z-ARR-AMC, and Z-LLE-AMC were used to measure chymotrypsin-like (CT-L), trypsin-like (T-L), and PGPH proteasome activities. Assays were carried out in 50 μg, 50 mM ethylenediaminetetraacetic acid (EDTA) and 50 μM fluorogenic substrates in a total volume of 200 μL of ATP/DTT lysis buffer at 37°C. The fluorescent rate was determined using a Synergy HT (Bio-TEK Instruments Inc., Winooski, VT, U.S.A.) at an excitation wavelength of 395 nm and emission wavelength of 460 nm.

Immunoprecipitation Cultured cells were treated with flavones. At the end of the incubation period, cells were lysed in lysis buffer. Equal amounts of protein from cell lysates were incubated with 5 μL of rabbit polyclonal IκB-α antibody for 2 h at 4°C. Protein A-Sepharose was then added for one hour. Immune complexes were washed with lysis buffer, eluted by boiling in sample buffer and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western immunoblotting.

Electrophoresis and Western Blotting SDS-PAGE was performed according to Laemmli (1970). Cultured cells treated with flavones were prepared for whole cell extracts. An equal amount of protein extract from each sample was then separated by SDS-PAGE. After electrophoresis, the gels were transferred to nitrocellulose filters for immunoblotting. The membranes were blocked with 5% dry skim milk in TBST buffer [20 mM Tris–HCl (pH 7.5), 150 mM NaCl, and 0.05% Tween-20] for 1 h. After washing in TBST buffer, the membranes were probed with primary antibodies in 1% skim milk/TBST buffer for one hour at room temperature. The primary antibody reactions were detected with peroxidase-conjugated goat anti-rabbit or anti-mouse immunoglobulin G (IgG) in 1% skim milk/TBST buffer, and developed using an enhanced chemiluminescence Western blotting kit (ECL, GE Healthcare) according to the manufacturer's specifications.

Statistical Analysis Statistical analyses were performed with Microsoft Excel software. Student's *t*-test was applied for independent analysis to evaluate differences between the treatment group and control group.

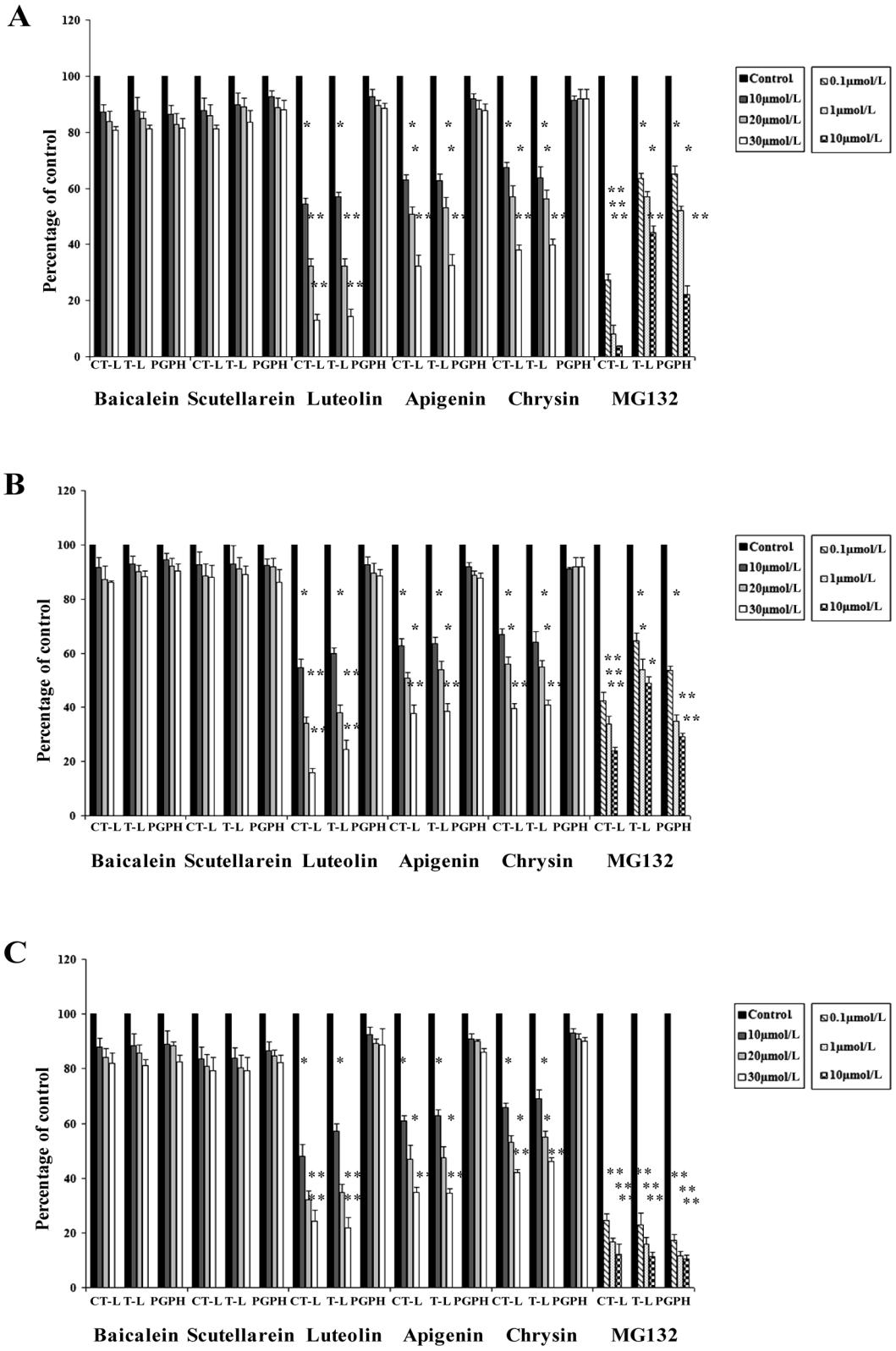

Fig. 2. Effects of Luteolin, Apigenin, Chrysin, Baicalein and Scutellarein on the Three Individual Activities (CT-L, T-L and PGPH) of Extracted Proteasome from the Liver of Mice and Cultured Cells

Proteasome extracts were prepared from the liver of mice (A), HCT-116 cells (B), and Jurkat T cells (C). Extracts were assayed for the individual catalytic activities of the proteasome after treatment with different concentrations (Control, 10 μM, 20 μM, and 30 μM) of luteolin, apigenin, chrysin, baicalein and scutellarein and (0.1 μM, 1 μM, 10 μM) of MG132 (A). p Values were calculated as indicated ($^{**}p<0.001$; $^{*}p<0.01$ as compared with respective control). Columns show mean values of five independent experiments; bars: S.D.

RESULTS

Different from Other Flavones, Baicalein and Scutellarein Did Not Inhibit the Activity of Extracted Proteasomes To study the effects of flavones on proteasome activity, proteasomes extracted from mouse liver and cancer cells were treated with various concentrations of apigenin, baicalein, chrysin, luteolin and scutellarein. The three individual activities (CT-L, T-L and PGPH) of the proteasomes were measured by monitoring release of the fluorophore aminomethylcoumarin (AMC) from peptide substrates specific for each activity (Succ-LLVY-AMC for CT-L, Z-ARR-AMC for T-L, and Z-LLE-AMC for PGPH). Proteasome extracts prepared from mouse liver and cultured cells were incubated with various concentrations (5, 15 or 30 μmol/L) of flavones. Luteolin, apigenin and chrysin inhibited both CT-L and T-L catalytic activities in all the extracted proteasomes in a dose-dependent manner and acted weakly on PGPH catalytic activity. However neither baicalein nor scutellarein significantly affected the three individual activities of the proteasomes (Fig. 2). Table 1 shows the values of the half-maximal inhibitory concentration (IC_{50}) of flavones toward the three individual activities of the extracted proteasomes. However, neither baicalein nor scutellarein significantly affected the three individual activities (see Fig. 2); the IC_{50} values were>500 μmol/L (Table 1). We then compared the action of flavones with the well-known proteasome inhibitor MG-132. MG-132 significantly affected the three individual activities (see Fig. 2); the IC_{50} values were 1.2 to 2 μM in HCT-116 cells, 0.3 to 1 μM in Jurkat T cells, and 0.2 to 1.2 μM in mice (see Table 1). These data showed that, unlike other flavones, baicalein and scutellarein did not inhibit the activity of extracted proteasome.

Unlike Other Flavones, Baicalein and Scutellarein Do Not Affect the Accumulation of Ubiquitinated Proteins To further confirm the anti-proteasome effect of different flavones, we determined whether these flavones had an effect on the accumulation of proteasome target proteins in tumor cells.

Ubiquitinated IκB-α protein is reportedly recognized and degraded by proteasomes leading to the release of NF-κB, which is then translocated to the nucleus. When proteasome activity is inhibited, ubiquitinated IκB-α protein stays bound to nuclear factor-kappa B (NF-κB), which prevents its translocation to the nucleus.[19] Therefore, accumulation of ubiquitinated IκB-α protein after treatment with flavones could confirm proteasomal inhibition. To confirm proteasomal inhibition caused by flavones, Jurkat T cells were treated for 24 h with each flavone at 15 μM, and ubiquitinated IκB-α protein was determined by Western blot analysis (Fig. 3A). Chen *et al.* reported a ubiquitinated form of IκB-α protein with a molecular weight of 56 kDa.[11] The level of this p56 band was determined by the specific antibody against IκB-α after treatment with luteolin, apigenin or chrysin. As a comparison, treatment of Jurkat T cells with MG132 had a similar effect on the accumulation of ubiquitinated IκB-α protein. However when the cells were treated with baicalein or scutellarein, no increase in the level of the p56 band was observed. To confirm

Table 1. IC_{50} (μM) Values for Flavones toward Inhibition of CT-L, T-L and PGPH Catalytic Activities in Extracted Proteasome

	Mouse liver			HCT-116			Jurkat T		
	CT-L	T-L	PGPH	CT-L	T-L	PGPH	CT-L	T-L	PGPH
Apigenin	17.4±1.4	17.7±1.0	>1000	18.5±1.8	19.8±0.5	>1000	16.5±1.7	17.0±1.1	>1000
Chrysin	21.5±1.1	21.3±0.7	>1000	21.6±0.2	21.5±1.1	>1000	21.6±0.8	25.2±1.8	>1000
Luteolin	11.6±0.4	12.1±0.5	>1000	11.8±0.9	13.5±0.9	>1000	9.2±1.1	12.4±0.3	>1000
Baicalein	>500	>500	>500	>500	>500	>500	>500	>500	>500
Scutellarein	>500	>500	>500	>500	>500	>500	>500	>500	>500
MG-132	<0.1	3.4±0.7	0.6±0.15	<0.01	6.1±2.9	0.13±0.05	<0.01	<0.01	<0.01

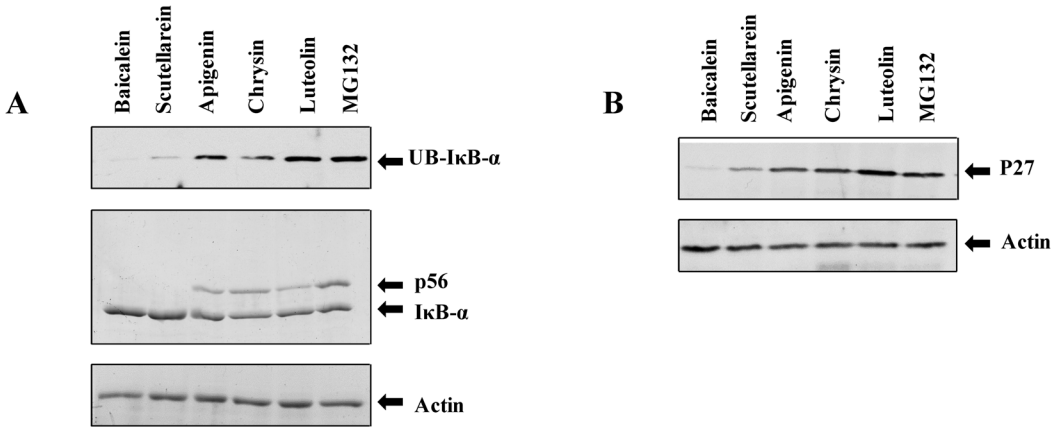

Fig. 3. Effects of Luteolin, Apigenin, Chrysin, Baicalein and Scutellarein on Accumulation of Ubiquitinated Proteins and Proteasome Target Proteins in Jurkat T Cells

Jurkat T cells treated with 15 μM of luteolin, apigenin, chrysin, baicalein, scutellarein and 0.1 μM of MG132 for 24 h. The proteins of cell extracts were analyzed with SDS-PAGE and Western blot with IκB-α antibody (middle panel of A) and actin (lower panel of A). Extracted proteins were incubated with rabbit polyclonal IκB-α antibody and Protein A sepharose, and then the immuno complexes were analyzed by immunoblotting with anti-ubiquitin (upper panel of A). The proteins of cell extracts were analyzed with SDS-PAGE and Western blot with p27 antibody (upper panel of B) and actin (lower panel of B).

that p56 is ubiquitinated from IκB-α protein, we performed an immunoprecipitation-Western blot analysis using protein extracts prepared from cells. Briefly, Jurkat T cells were treated with each flavone (at 15 μM) and MG132 (at 1 μM) for 24 h, and then the cells were lysed in lysis buffer. Equal amounts of extracted protein were incubated with rabbit polyclonal IκB-α antibody and protein A-sepharose, and the immuno complexes were analyzed by immunoblotting. Levels of ubiquitinated IκB-α were determined by specific antibody against ubiquitin. The results showed treatment with luteolin, apigenin, chrysin and MG132 resulted in levels of ubiquitinated IκB-α band that were significantly higher than for treatment with baicalein or scutellarein. We also investigated ubiquitinated IκB-α protein accumulation in HCT-116 cells, and found the ubiquitinated p56 band increased when the cells were treated with luteolin, apigenin, chrysin, but not when treated with baicalein or scutellarein (data not shown).

Baicalein and Scutellarein Did Not Affect Accumulation of p27 The cyclin-dependent kinase inhibitor $p27^{Kipl}$ (p27) is reported to be the target of proteasome, and inhibition of the cellular proteasome is associated with accumulation of p27.[20] If flavones inhibit proteasome activity, we would expect to see an increase in levels of p27. To investigate this possibility, Jurkat T cells were treated with flavones for $\leq$24 h, and accumulation of p27 was determined by Western blot analysis (Fig. 3B). When treated with luteolin, apigenin, chrysin and MG132, the level of the p27 band was significantly increased compared to when treated with baicalein or scutellarein. In addition, we investigated the accumulation of p27 in HCT-116 cells. The level of p27 band was increased when the cells were treated with luteolin, apigenin, chrysin but did not increase when treated with baicalein or scutellarein (data not shown).

These data suggest that unlike other flavones, baicalein and scutellarein are not involved in the ubiquitin/proteasome-mediated degradation pathway.

DISCUSSION

Proteasome inhibitors have recently emerged as a new type of antitumor therapy. Their clinical efficacy has been shown in multiple myeloma and non-Hodgkin lymphoma. Bortezomib (PS-341, Velcade), is the first proteasome inhibitor approved by the Food and Drug Administration for the treatment of patients with multiple myeloma and non-Hodgkin lymphoma.[21] Although the data from the bortezomib trials showed significant clinical benefit, some toxicity was observed. The most common side effects include nausea, fatigue, and diarrhea, but thrombocytopenia, peripheral neuropathy, neutropenia, lymphopenia and hyponatremia were also observed.[22] Therefore, there is a need to search for other proteasome inhibitors with fewer or non-toxic side effects.

Recent studies have shown that some flavonoids could inhibit proteasome activity and induce apoptosis in tumor cells, suggesting that flavonoids could have potential as a new type of anticancer drugs.[9,10] However, not all flavonoids are strong proteasome inhibitors. For example, some flavones are markedly more potent than other flavonoids such as flavonols or flavanones. To identify safe and more effective anti-proteasome drugs, the association between the structure of flavonoids and anti-proteasome activity must be clarified. In this study, we focused on the relationship between flavone structure and inhibition of proteasome activity. When we examined the inhibitory activity of various flavones on the extracted proteasomes from mice and cancer cells, we found that, unlike other flavones, baicalein and scutellarein did not inhibit proteasome activity. In addition, treatment with baicalein and scutellarein did not lead to the accumulation of ubiquitinated proteins or affect proteasome target protein p27.

Previous studies have reported that flavones play a specific role in inhibition of both CT-L and T-L proteasome catalytic activities. In this study, we found that luteolin, apigenin and chrysin inhibited both CT-L and T-L catalytic activities in proteasome extracted from mouse liver and cultured cells in a dose-dependent manner. In contrast, baicalein and scutellarein did not inhibit the activity of extracted proteasome.

Baicalein and scutellarein apparently have antitumor and anti-inflammatory activities. Recent studies have showed that baicalein and scutellarein can induce apoptosis of cancer cells.[23,24] The mechanism of apoptosis induced by baicalein and scutellarein has been widely reported. Baicalein and scutellarein effectively up-regulated the expression of mitochondrial Bax and caspase-3, down-regulated the expression of Bcl-2, and decreased the mitochondrial transmembrane potential ($\Delta\Psi$m).[23–25] These data suggest that the mechanism by which baicalein and scutellarein induce apoptosis involves the mitochondrial signaling pathway. It has been reported that luteolin, apigenin and chrysin in addition to inducing apoptosis through the ubiquitin–proteasome pathway, also can induce apoptosis through the mitochondrial signaling pathway.[26–28] In this study we found that, unlike with luteolin, apigenin and chrysin, baicalein and scutellarein did not inhibit the activity of proteasomes. However all the flavones in this study significantly enhanced caspase-3 activity in all treated cells (data not shown). These data suggest that the apoptotic pathways of baicalein and scutellarein might not be related to the ubiquitin–proteasome pathway. The results suggest that different flavone compounds might differ in their apoptotic pathway involvement, depending on their chemical structure.

Baicalin and scutellarin are flavonoid glycosides, *i.e.*, baicalein 7-*O*-β-glucuronide and scutellarein 7-*O*-β-glucuronide. Recent studies showed that baicalin and scutellarein affect the inhibition of proteasomes, specifically inhibiting the CT-L activity.[12] Combined with the results of this study, these data suggest that flavones with 5,6,7-hydroxy groups of the A-ring linked by β-glucuronide at position 7 in the A ring, will exhibit anti-proteasome activity.

Apigenin, baicalein, chrysin, luteolin and scutellarein are all flavones, but have different chemical structures. Both baicalein and scutellarein have three hydroxy groups at adjacent positions in the A-ring, *i.e.*, hydroxy groups at positions 5, 6 and 7, while luteolin, apigenin and chrysin have only two hydroxy groups at positions 5 and 7 in the A-ring. Our data suggest that flavones with hydroxy groups at positions 5, 6 and 7 of the A-ring lack anti-proteasome function. In addition, scutellarein has a hydroxylated B-ring. Our data showed that the lack of anti-proteasome function of scutellarein is very similar to that of baicalein, which means the hydroxy group in the B-ring is not an important factor in term of the lack of anti-proteasome function. These results will be beneficial to the further development of proteasome inhibitors for clinical application.

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Conflict of Interest The authors declare no conflict of interest.

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