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Dietary flavonoids as proteasome inhibitors and apoptosis inducers in human leukemia cells

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Abstract

It has been shown that proteasome activity is required for cancer cell survival and consumption of fruits and vegetables is associated with decreased cancer risk. Previously, we reported that grape extract could inhibit proteasome activity and induce apoptosis in tumor cells. In this study, we examined the flavonoids apigenin, quercetin, kaempferol and myricetin for their proteasome-inhibitory and apoptosis-inducing abilities in human tumor cells. We report that apigenin and quercetin are much more potent than kaempferol and myricetin at: (i) inhibiting chymotrypsin-like activity of purified 20S proteasome and of 26S proteasome in intact leukemia Jurkat T cells; (ii) accumulating putative ubiquitinated forms of two proteasome target proteins, Bax and Inhibitor of nuclear factor κβ-α in Jurkat T cells and (iii) inducing activation of caspase-3 and cleavage of poly(ADP-ribose) polymerase in Jurkat T cells. The proteasome-inhibitory abilities of these compounds correlated with their apoptosis-inducing potencies. Results from computational modeling of the potential interactions of these flavonoids to the chymotrypsin site (β5 subunit) of the proteasome were consistent with the obtained proteasome-inhibitory activities. We found that the C_4 carbon may be a site of nucleophilic attack by the OH group of N-terminal threonine of proteasomal β5 subunit and that the C_3 hydroxyl may alter the ability of these flavonoids to inhibit the proteasome. Finally, apigenin neither effectively inhibited the proteasome activity nor induced apoptosis in non-transformed human natural killer cells. Our results suggested that the proteasome may be a target of these dietary flavonoids in human tumor cells and that inhibition of the proteasome by flavonoids may be one of the mechanisms responsible for their cancer-preventive effects.

Keywords: Flavonoids; Chemoprevention; Chemotherapy; Proteasome inhibitors; Apoptosis; Computer modelling

1. Introduction

Annually, an estimated 10 million people worldwide are diagnosed with cancer and approximately 6.2 million die from the disease [1,2]. Cancer is a heterogeneous disease characterized by the growth of a malignant cell population leading to impairment of normal physiological functions [3]. Tumor cells often have multiple alterations in their apoptotic machinery and/or signaling pathways that lead to increased levels of growth and proliferation [3,4]. Overriding these mutations stimulates the apoptotic signaling pathway, leading to tumor cell death, which is a significant area of focus in anticancer drug research.

The ubiquitn-proteasome pathway plays an important role in regulating both cell cycle and apoptosis [5–8]. Proteasome is often referred to in one of two modes, the 20S particle is the catalytic core, and the 26S particle is composed of the 20S core associated with two 19S regulatory caps [8,9]. When the proteasome is analyzed in cellular extract or a whole cell system, it can be referred to as the 26S proteasome since the 19S caps are assumed present. Additionally, proteasome can be purified to contain just the 20S core particle [10]. The proteasome is an immense multi-subunit protease with at least three catalytic activities located in the 20S core: chymotrypsin-like, trypsin-like and caspase-like [9,10]. The chymotrypsin-like activity is the rate-limiting step of protein degradation [11,12]. Cleavage of substrates by the proteasomal chymotrypsin-like activity occurs on the N-terminal

Abbreviations: AMC, 7-amido-4-methyl-coumarin; BSA, bovine serum albumin; (−)-EGCG, (−)-epigallocatechin-3-gallate; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; IκB-α, Inhibitor of Nuclear Factor κB-α; PARP, poly(ADP-ribose) polymerase

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threonine (Thr) of the β5 subunit [9,13]. Moreover, binding affinities to the S_1 pocket of β5 are important for substrate specificity [14]. Recently, it has been shown that tumor cells are dependent upon the proteasome function, as proteasome inhibition leads to growth arrest in the G_1 phase of the cell cycle and/or induction of apoptosis [15,16]. However, treatment with some proteasome inhibitors in several human normal or non-transformed cell lines is not associated with induction of apoptosis [8,16,17].

Many studies report that a diet high in fruits and vegetables lowers the incidence of cancer [18–20]. We recently reported that various fruit and vegetable extracts, particularly grape extract, are capable of inhibiting the proteasome activity and that this inhibition is associated with tumor cell apoptosis [21]. Plant-derived flavonoids possess a number of physiologic effects [22]. Previously, we demonstrated that the flavonoid (−)-epigallocatechin-3-gallate [(−)-EGCG] inhibits the proteasome both in vitro and in cell culture models at concentrations comparable to those observed in the blood plasma of tea drinkers [23]. We hypothesized that some similar flavonoids found in grapes may be responsible for the proteasome-inhibitory and apoptosis-inducing activities observed previously [21].

Grapes possess a number of flavonoids, but for this study we focused on quercetin, kaempferol and myricetin as well as a similar flavonoid apigenin, found primarily in celery seed and chamomile flowers [24]. We examined the proteasome-inhibitory properties of these four flavonoids in vitro and in cultured leukemia cells. We found that these flavonoids inhibited the proteasomal chymotrypsin-like activity in a dose- and time-dependent manner both in vitro and in cultured leukemia cells. This inhibition is associated with apoptotic induction in leukemic Jurkat T cells, but not in normal, non-transformed natural killer (YT) cells. The order of potency of the four flavonoids for both inhibiting the proteasome activity and inducing tumor cell apoptosis is: apigenin > quercetin > kaempferol > myricetin. Furthermore, by using the in silico model we developed for (−)-EGCG [25], we examined whether binding affinities of the four flavonoids to the chymotrypsin-like active site of the β5 subunit of the proteasome were influenced by their chemical structures. Modifications to the structures of the flavonoids and subsequent docking analysis suggested the presence of a distinct structure-activity relationship. Specifically, deletion of the C_3 hydroxyl group from the quercetin, kaempferol and myricetin results in a binding that is nearly identical to that of apigenin, indicating that this pose may be conducive to inhibition of the chymotrypsin-like activity.

2. Materials and methods

2.1. Chemical reagents

Apigenin [4′,5,7-trihydroxyflavone], kaempferol [3,5,7-trihydroxy-2-(4-hydroxyphenyl)-4H-1-benzopyran-4-one], quercetin dihydrate [2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4H-1-benzopyran-4-one dihydrate], myricetin [3,3′,4′,5,5′,7-hexahydroxyflavone], propidium iodide, sulforhodamine 101 acid chloride, RNase A, protease inhibitor cocktail and dimethyl sulphoxide (DMSO) were purchased from Sigma–Aldrich Co. Purified 20S proteasome (*Methanosarcina thermophile*, Recombinant, *Escherichia coli*), fluorogenic proteasomal chymotrypsin peptide substrate Suc-Leu-Leu-Val-Tyr-AMC and caspase-3 specific substrate Ac-Asp-Glu-Val-Asp-AMC were obtained from Calbiochem Inc. Another fluorogenic peptide substrate Z-Gly-Gly-Leu-AMC specific for the proteasomal chymotrypsin-like activity was from BIOMOL International LP. Rabbit polyclonal antibody to Inhibitor of nuclear factor κβ-α (IκB-α), mouse monoclonal antibody to Bax (B9), rabbit polyclonal antibody to caspase 3 and goat polyclonal antibody to actin were obtained from Santa Cruz Biotechnology Inc. Mouse monoclonal antibody to human poly(ADP-ribose) polymerase (PARP) was from BIOMOL International LP and rabbit polyclonal anti-PARP cleavage site-specific antibody, fluorescein isothiocyanate (FITC) conjugate, from BioSource International Inc. Vectashield mounting medium for fluorescence with 4′,6-diamidino-2-phenylindole (DAPI) was purchased from Vector Laboratories Inc. Fetal bovine serum (FBS) was obtained from Tissue Culture Biologicals. RPMI 1640 medium, Dulbecco's modified Eagle's medium, penicillin and streptomycin were purchased from Invitrogen Co.

2.2. Cell culture and protein extract preparation

Human leukemia Jurkat T and non-transformed, immortalized human natural killer (YT) cells were cultured in RPMI 1640 medium supplemented with 10% FBS, 100 units/ml of penicillin, and 100 μg/ml of streptomycin. All the cell lines were maintained at 37 °C in a humidified incubator with an atmosphere of 5% CO_2. A whole cell extract was prepared as described previously [26]. Briefly, cells were harvested, washed with PBS twice, and lysed in a whole cell lysis buffer (50 mM Tris–HCl/pH 8.0, 5 mM EDTA, 150 mM NaCl, 0.5% NP-40, and 0.1% of protease inhibitor cocktail) for 30 min at 4 °C. Afterwards, the lysates were centrifuged at 14,000 × *g* for 20 min, and the supernatants were collected as whole cell extracts.

2.3. Nucleophilic susceptibility analysis

The electron density surface colored by nucleophilic susceptibility was created with the use of Quantum CAChe (Fujitsu) by performing a nuclear susceptibility analysis using the PM5 geometry and PM5 wavefunction in water. A colored "bull's-eye" with a red center denotes atoms that are highly susceptible to nucleophilic attack.

2.4. Computational binding simulation

The crystal structure of the eukaryotic yeast 20S proteasome was obtained from the Protein Database (Ref. number 1JD2) and used for all the docking studies presented here [27]. The yeast 20S proteasome is structurally very similar to the mammalian 20S proteasome, and the chymotrypsin active site between the two species is highly conserved [14,28]. The AutoDock 3.0 suite of programs, which was used for the docking calculations, uses an automated docking approach that allows ligand flexibility as described to a full extent elsewhere [29]. Autodock has been compared with various docking programs in several studies and has been found to be able to locate docking modes that are consistent with X-ray crystal structures [30,31]. Default parameters (including a distance-dependent dielectric ''constant'') were used as described in the AutoDock manual except as noted below. Dockings were performed on i386 architecture computer running the RedHat ™ Linux 9.0 operating system. The crystal structure of the 20S proteasome and the ligands were prepared for docking by following the default protocols except where noted. The energy-scoring grid was prepared by defining a 20 Å × 20 Å × 20 Å box centered on the N-terminal threonine with a space of 0.2 Å between grid points. In the search protocols, the number of genetic runs used was 100 and the number of energy evaluations was set to 5 million. AutoDock relies on an empirical scoring function, which provides approximate binding free energies. AutoDock reports a docked energy that we have referred to in this article as a ''docked free energy'' because it includes a solvation free energy term. The docked energy also includes the ligand internal energy or the intramolecular interaction energy of the ligand. In the present study, we chose to use the docked free energies because the number of rotatable bonds in our compounds is constant and because we believed that the internal energy of the ligand should not be neglected.

Dockings were chosen by fulfilling two criteria we used for resolving the docking of (−)-EGCG and related compounds to the β5 subunit [25]. Briefly, the electrophilic carbon of the C-ring of the flavonoid (Fig. 1) should lie within 4 Å of the N-terminal threonine (a distance suitable for nucleophilic attack) and the A–C double ring system (Fig. 1) should be placed into or near the hydrophobic S_1 pocket. The probability of adopting the inhibitory conformation was the number of genetic runs (out of 100) in which the molecule docked into the active site and fulfilled the above criteria.

2.5. Inhibition of purified 20S proteasome activity by flavonoids

The chymotrypsin-like activity of purified 20S proteasome was measured as follows. Briefly, 0.1 μg of purified 20S proteasome was incubated in 100 μl of assay buffer (50 mM Tris–HCl, pH 7.5) with or without different concentrations of each flavonoid and 40 μM fluorogenic peptide substrate Suc-Leu-Leu-Val-Tyr-AMC (for the proteasomal chymotrypsin-like activity) for 2 h at 37 °C. After incubation, production of hydrolyzed AMC groups was measured using a Wallac Victor3™ multilabel counter with an excitation filter of 355 nm and an emission filter of 460 nm.

2.6. Caspase-3 activity assay

Cell-free caspase-3 activities were determined by measuring the release of the AMC groups from a caspase-3 specific substrate Ac-Asp-Glu-Val-Asp-AMC. Briefly, Jurkat T cells were treated with 50 μM of each flavonoid for 12 h, followed by preparation of whole cell extracts. The cell extract (30 μg) was then incubated in 100 μl of the assay buffer (50 mM Tris–HCl, pH 7.5) along with 40 μM of caspase-3 substrate in a 96-well plate. The reaction mixture was incubated at 37 °C for 3 h and the hydrolyzed fluorescent AMC groups were quantified as described above.

2.7. Inhibition of the proteasome activity in intact tumor cells by flavonoids

To measure the inhibition of proteasome activity in living tumor cells, Jurkat T or YT cells (100 μl of 1×10^4 cells/(ml well) were cultured in 96-well plates. The next day the cells were treated by adding 1, 10 or 50 μM of each flavonoid or DMSO as control to culturing medium and incubating for 6 or 24 h, followed by 2 h additional incubation with the fluorogenic peptide substrate Z-Gly-Gly-Leu-AMC specific for the proteasomal chymotrypsin-like activity. Afterwards, production of hydrolyzed AMC groups was measured using the same plate reader and conditions mentioned above. The data were graphed and IC_{50}s determined using Microsoft™ Excel.

2.8. Western blot analysis

Jurkat T or YT cells were treated with an indicated concentration of flavonoids for indicated hours (see figure legends), followed by preparation of cell lysates. Cell lysates (40 μg) were then separated by an SDS-PAGE and electrophoretically transferred to a nitrocellulose membrane, followed by the enhanced chemiluminescence (ECL) Western blotting using specific antibodies to IκB-α, Bax, PARP, caspase 3 or actin, as described previously [16].

2.9. Immunofluorescence microscopy

Jurkat T cells were treated with or without different flavonoids for 24 h and harvested. The cells were then

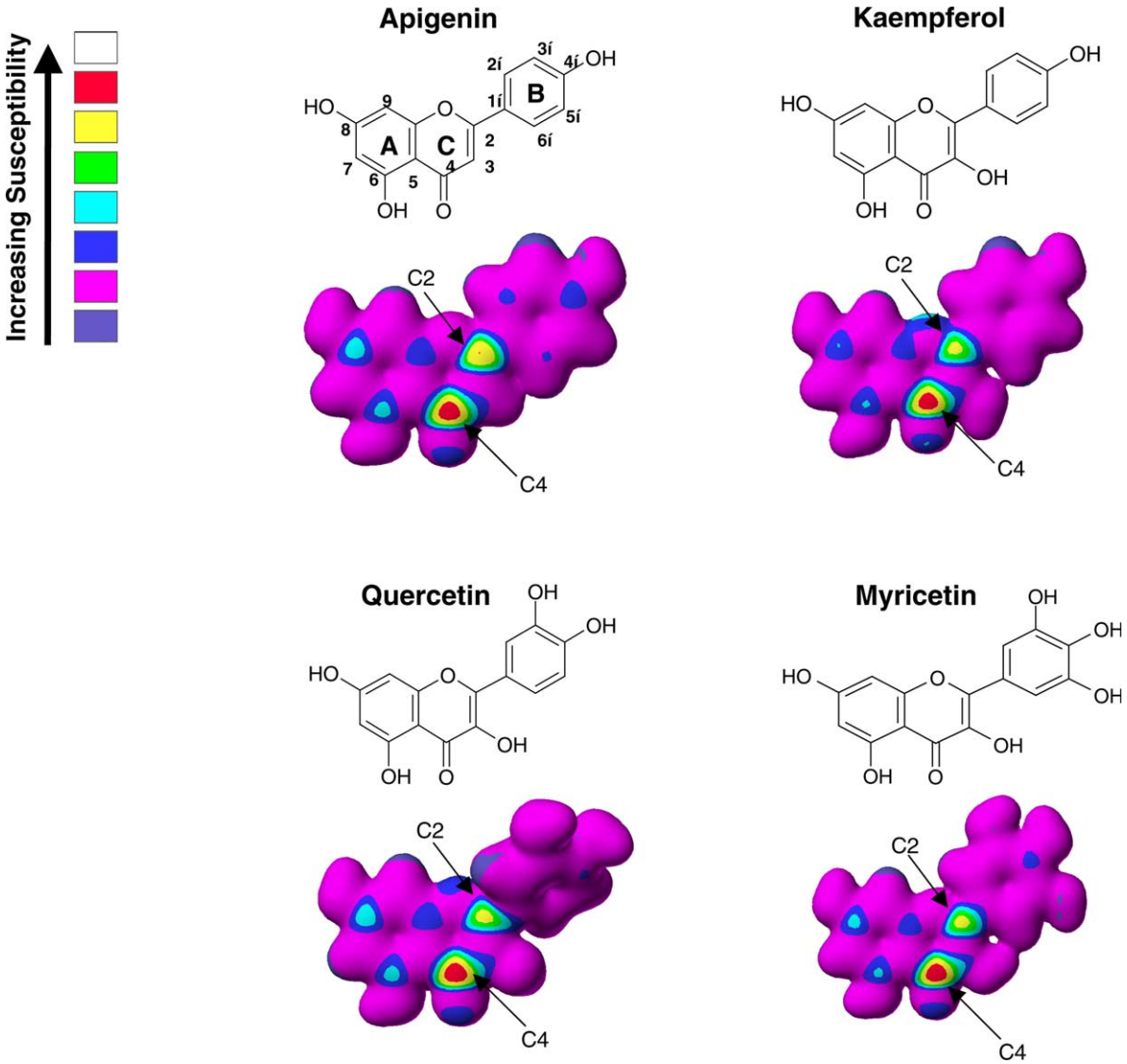

Fig. 1. Chemical structures and nucleophilic susceptibility of the four flavonoids. Each of the flavonoids was analyzed using the CAChe software system for nucleophilic susceptibility. In every case a highly susceptible site was found at the C_4 carbon that could be nucleophilically attacked by the OH group of N-Thr of proteasomal β5 subunit.

washed three times in PBS and fixed in 70% ethanol for 1 h. After three washes in PBS, the cells were permeabilized in 0.1% Triton -X-100 containing sulforhodamine for a final concentration of 5 μg/ml for 15 min at room temperature and washed three times again. Cells were blocked in 1% bovine serum albumin (BSA) in phosphate buffered saline (PBS) for 20 min and then the PARP p85-FITC antibody was added to the blocking solution for 1:100 dilution and incubated for 30 min at 4 °C in the dark with mild shaking. After three additional washes, the cell suspension was transferred to microscope slides with a drop of Vectorshield mounting medium with 4′,6-diamidino-2-phenylindole (DAPI). The cells were visualized and digital photographs were taken with Zeiss Axiovision microscope (Carl Zeiss Microscope Inc., Hallbergmoons, Germany).

3. Results

3.1. Potencies of apigenin, kaempferol, quercetin and myricetin to inhibiting the chymotrypsin-like activity of purified 20S proteasome

Previously, we reported that grape extracts induce apoptosis in tumor cells, associated with inhibition of proteasome activity [21]. To further investigate the involved active grape components, we chose three dietary flavonoids commonly found in grapes, kaempferol, quercetin and myricetin [32] for the current study (Fig. 1). As a comparison, a structurally related natural flavonoid apigenin (Fig. 1), found primarily in celery seed and chamomile flowers [24], was also used.

We first performed a cell-free proteasome activity assay in the presence of each of these four flavonoids at different

concentrations. The chymotrypsin-like activity of purified 20S proteasome was inhibited by all of the flavonoids with different potencies (Table 1; in 20S). Apigenin was found to be the most potent inhibitor with an IC_{50} value of 1.8 μM. Interestingly, kaempferol, which contains an extra –OH at 3-position compared to apigenin (Fig. 1), was six-fold less potent with an IC_{50} value of 10.5 μM, suggesting that the C_3 hydroxyl group interferes the proteasome-inhibitory function of these flavonoids. Although both quercetin and myricetin have a C_3 hydroxyl group (Fig. 1), quercetin was a more potent proteasome inhibitor than myricetin (IC_{50} values 3.5 μM versus 10.0 μM; Table 1, in 20S). We noticed that quercetin has two hydroxyl groups on its B-ring, while myricetin has three and kaempferol has only one (Fig. 1). It is possible that the two hydroxyls of quercetin in the *para* and *meta* positions at B-ring may allow the C_3 hydroxyl group to be removed more easily (also see Fig. 2).

Table 1
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[a]

Flavonoids	IC_{50} (μM; in 20S)	IC_{50} (μM; in 26S)
Apigenin	1.8 (±0.03)	1 (±0.02)
Kaempferol	10.5 (±0.15)	11 (±0.31)
Quercetin	3.5 (±0.05)	2 (±0.09)
Myricetin	10.0 (±0.17)	12 (±0.17)

[a] Inhibition of the chymotrypsin-like activity of purified 20S proteasome and 26S proteasome in intact Jurkat T cells was measured as described under "Section 2". The experiment was repeated three times with the similar results. The data are presented as mean values ± S.D.

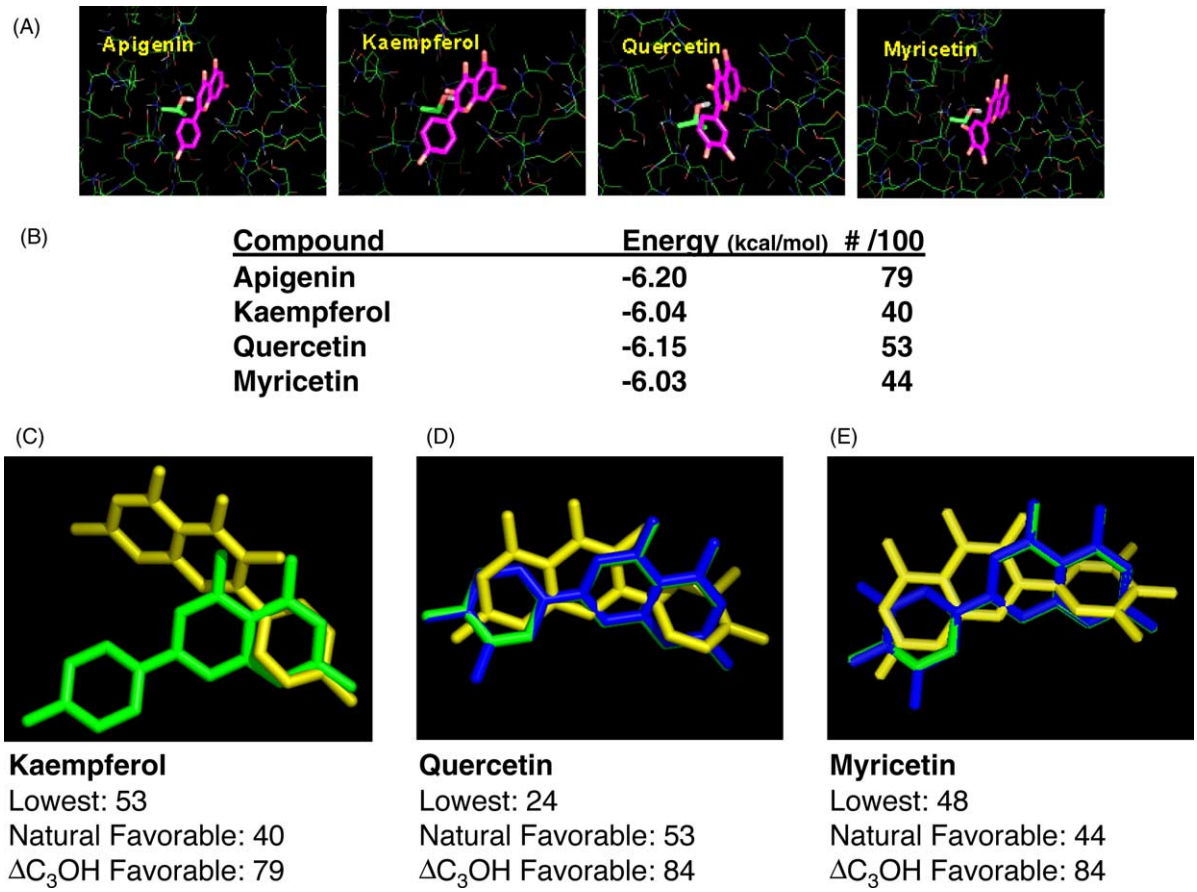

Fig. 2. Comparison of docking poses for natural flavovoids and "ΔC_3OH" flavovoids. Docking poses favorable for inhibition proteasome chymotrypsin-like activity and probabilities of assuming this pose. (A) Each of the flavonoids was docked to the β5 subunit of the proteasome. The compounds are shown in pink while the hydroxyl group of the N-terminal threonine of the β5 subunit of the proteasome is shown in green. One-hundred poses were found and clustered by energies. (B) The energy of the inhibitory pose and the number of runs (out of 100) that adopted the inhibitory pose. Apigenin and quercetin were found to have the most favorable energy and the highest number of poses in the cluster. (C–E) Each of the compounds was examined for the number of members adopting the lowest energy pose, the inhibitory favorable pose, and the pose adopted when the C_3 hydroxyl is removed (ΔC_3OH). (C) Since ΔC_3OH kaempferol is apigenin, these two compounds were compared directly. The lowest energy pose of kaempferol (yellow) contained 53 out of 100 poses. For kaempferol, the number of poses in the inhibitory favorable pose is 40 out of 100. Apigenin (green), which is ΔC_3OH kaempferol, adopts this pose 79 times in 100 runs. (D) The lowest energy pose of quercetin (yellow) was compared to ΔC_3OH quercetin (blue) and apigenin (green). Out of 100 poses, 24 adopt the lowest energy pose, 53 poses of the natural compound adopt the favorable inhibitory pose, and 84 poses of the ΔC_3OH quercetin adopt the favorable inhibitory pose. (E) The lowest energy pose of myricetin (yellow) was compared to ΔC_3OH myricetin (blue) and apigenin (green). Out of 100 poses, 48 adopt the lowest energy pose, 44 poses of the natural compound adopt the favorable inhibitory pose, and 84 poses of the ΔC_3OH myricetin adopt the favorable inhibitory pose. In each case, removal of the C_3 hydroxyl results in a dramatic pose shift and dramatic increase in the number of members that adopt a favorable docking pose. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

3.2. Docking studies show that apigenin and quercetin are the most likely to adopt an inhibitory pose with superior energy in the β5-subunit

Each of the four flavonoids was then examined for sites of nucleophilic susceptibility. Analysis revealed that all of them possessed a single site at C_4 with similar strength (Fig. 1), suggesting that this site could be attacked, and subsequently covalently bound, by the OH group of N-Thr of proteaosmal β5 subunit [25]. To further investigate the chemical nature of these four flavonoids to inhibit the chymotrypsin-like activity of the proteasome, each was docked to the active site of the proteasome β5-subunit, which is responsible for the chymotrypsin-like activity [25]. Autodock arranges its results by energy and clusters of solutions that adopt the same pose (see Section 2 for details). The results for apigenin showed that 79 poses (the largest cluster out of 100 poses) adopted a conformation favorable for nucleophilic attack on C_4 with energy of −6.20 kcal/mol (Fig. 2A and B). In comparison, kaempferol adopted this pose 40 times out of 100 with energy of −6.04 kcal/mol (Fig. 2A and B). Quercetin adopted this pose 53 times out of 100 with energy of −6.15 kcal/mol (Fig. 2A and B), while myricetin adopted this pose 44 times out of 100 with energy of −6.03 kcal/mol (Fig. 2A and B). The order of the docking energy is therefore: apigenin < quercetin < kaempferol, myricetin. The lower the docking energy is and the larger the cluster is, the greater the inhibitory potency is predicted [25]. Indeed, the docking data (Fig. 2B) are consistent with the order of the potencies of these four flavonoids to inhibit the chymotrypsin-like activity of purified 20S proteasome (Table 1; in 20S).

Of interest was the dramatic increase in the probability of apigenin adopting this pose, which fulfilled the criteria for a proteasome-inhibitory pose as compared to the other three flavonoids (from 40–53% to 79%; Fig. 2B). One of the key differences between apigenin and the other three flavonoids is the absence of a hydroxyl group at the C_3 position (Fig. 1), suggesting that removing this group increases the probability of favorable poses with superior energy in the β5-subunit. This hypothesis is supported by a previous report suggesting the C_3 position may play a role in the biological activity of these flavonoids [33]. We then examined the lowest energy poses of kaempferol, quercetin and myricetin. At its lowest energy pose, kaempferol is elevated by 90° from the active site of β5-subunit (Fig. 2C; yellow versus green). The probability of kaempferol adopting the lowest energy pose is 53%, as opposed to 40% for adopting the proteasome-inhibitory pose (Fig. 2C). However, removal of the C_3 hydroxyl (making kaempferol structurally identical to apigenin) results in the compound adopting the proteasome-inhibitory pose by 79% (Fig. 2C, green). Kaempferol seems to possess a nearly equivalent probability to adopt its lowest energy pose by 53% (Fig. 2C, yellow) or the inhibitory pose by 40% (Fig. 2C, green) as compared to apigenin, which strongly favors the inhibitory pose (79%; Fig. 2C, green). This may contribute to the reduced inhibitory nature of kaempferol.

Quercetin, although it does not rise up from the active site, undergoes a similar change when the C_3 hydroxyl is removed (Fig. 2D). The lowest energy pose of quercetin (Fig. 2D, yellow) is rotated 180° compared to apigenin. When the C_3 hydroxyl is removed, quercetin adopts a pose almost exactly the same as apigenin (Fig. 2D, blue versus green). Statistically, quercetin adopts its lowest energy pose 24% of the time and the favorable pose 53% of the time. Removal of the C_3 hydroxyl raises this to 84%. The addition of hydroxyl groups on the B-ring may contribute to quercetin's lowest energy pose resting in the active site, as compared to kaempferol (Fig. 2D versus C). Additionally, the capability of quercetin to adopt a favorable docking pose (53%), as compared to the lowest energy pose (24%), may contribute to its inhibitory nature.

Similarly, myricetin docks in its lowest energy pose 180° rotated, as compared to apigenin (Fig. 2E, yellow versus green). As with quercetin, the addition of hydroxyls on the B-ring may contribute to myricetin's position in the active site as opposed to raised in the manner of kaempferol. However, different from quercetin but similar to kaempferol, myricetin adopts its lowest energy pose 48% of the time and the favorable pose 44% (Fig. 2E, yellow versus blue). When the C_3 hydroxyl is removed, the probability of adopting the favorable pose rises to 84%.

The docking results support the argument that the C_3 hydroxyl group interferes with the binding of the flavonoids to the active site of the β5-subunit and that removing this moiety would increase the binding affinity and inhibitory potency of flavonoids. Likewise, the addition of hydroxyls on the B-ring seems to alter the ability of these compounds to adopt a proteasome-inhibitory pose. In the presence of the C_3 hydroxyl, a single *para* substitution (kaempferol; Fig. 1) dramatically reduces the likelihood of this compound to adopt the inhibitory pose. However, a second *meta* substitution (quercetin; Fig. 1) restores the likelihood of the compound adopting the inhibitory pose. A third substitution in the *meta* position (myricetin; Fig. 1) again disrupts the binding and reduces the probability of the compound to adopt the inhibitory pose. Therefore, the C_3 hydroxyl group appears to be the most significant group, in these compounds, in directing the docking pose. However, additional hydroxyls on the B-ring seem to more subtly alter probabilities of the binding poses. These docking results correlate well to the relative inhibitory potencies of these compounds to a purified proteasome (Fig. 2 versus Table 1, in 20S).

3.3. Apigenin and quercetin are more potent proteasome inhibitors in intact Jurkat T cells than kaempferol and myricetin

To determine whether these flavonoids could also inhibit the activity of 26S proteasome in living tumor cells, human

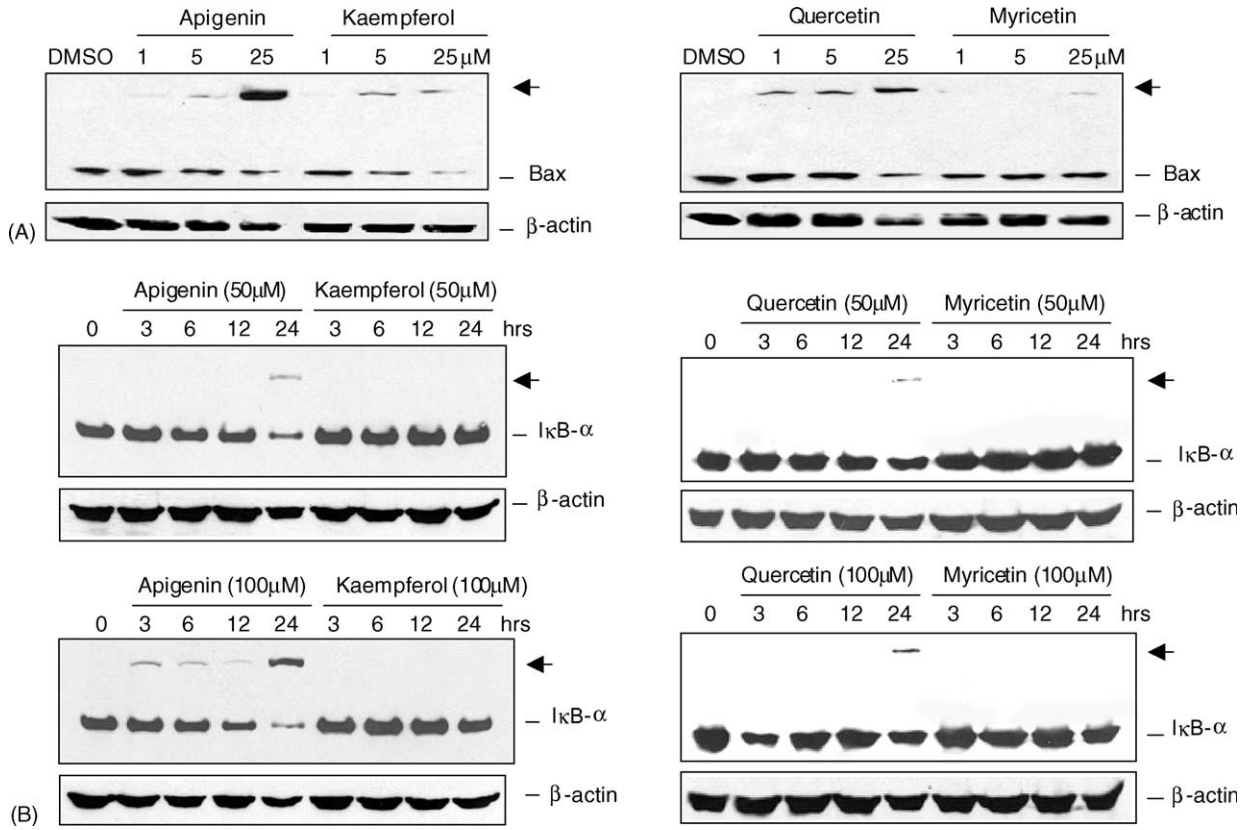

Fig. 3. Accumulation of putative ubiquitinated forms of Bax and IκB-α by the flavonoids. (A) Jurkat T cells were treated with solvent or indicated concentrations of four different flavonoids for 24 h, followed by a Western blot assay using antibodies to Bax and actin. Molecular masses of Bax and actin are 23 and 43 kDa, respectively. The band of 55 kDa, indicated by arrows, is putative ubiquitinated Bax (15). (B) Western blot analysis of the time- and concentration-dependent action of flavonoids in Jurkat T cells. After treatment of Jurkat T cells by the flavonoids in indicated concentrations and hours, the cell extracts were analyzed by Western blot using specific anti-IκB-α antibody. Molecular mass of IκB-α is 37 kDa. The band of 56 kDa, recognized by specific anti-IκB-α antibody, is putative ubiquitinated IκB-α (23) and indicated by arrows. The experiment was repeated three times with the similar results.

leukemia Jurkat T cells were treated with each of these four flavonoids at various concentrations, followed by an additional incubation with a fluorogenic proteasome peptide substrate specifically for the proteasomal chymotrypsin-like activity. Afterwards, cells were measured for levels of hydrolyzed AMC groups. The results from this cell culture study (Table 1; in 26S) were consistent with the data generated with purified 20S proteasome (Table 1; in 20S) and from computational modeling (Fig. 2). Apigenin potently inhibited the proteasomal chymotrypsin-like activity in intact Jurkat cells in a concentration-dependent manner with an IC_{50} of 1 μM (Table 1; in 26S). Quercetin was slightly less potent than apigenin with an IC_{50} of 2 μM (Table 1; in 26S). In contrast, kaempferol and myricetin were much less potent than apigenin with IC_{50}s of 11 and 12 μM, respectively (Table 1; in 26S).

Having shown that the flavonoids inhibit the proteasomal chymotrypsin-like activity in a cell-free system and in intact tumor cells (Table 1), we then determined whether the flavonoids could have an effect on proteasome target proteins, such as Bax [15] and IκB-α [34], in intact tumor cells. Previously by performing a coupled immunoprecipitation and Western blotting assay, we identified a ubiquitinated form of Bax with molecular mass 55 kDa (p55) [15]. Jurkat T cells were treated for 24 h with apigenin, kaempferol, quercetin or myricetin at 1, 5 or 25 μM, followed by Western blotting using a Bax-specific antibody. We observed that a band of p55, similar to the previously reported ubiquitinated Bax [15], was accumulated to a much higher level by apigenin than kaempferol at 25 μM (indicated by an arrow, Fig. 3A). In addition, quercetin treatment also increased the levels of p55 in a dose-dependent manner while myricetin had much less effect under identical conditions (Fig. 3A).

Previously we have also reported that the green tea polyphenol proteasome inhibitor (−)-EGCG was able to accumulate a candidate ubiquitinated IκB-α of 56 kDa (p56) [23]. Jurkat T cells were then treated with various concentrations of each of these four flavonoids for different hours, followed by measuring levels of IκB-α. Levels of a p56 band, detectable by the specific antibody to IκB-α, significantly increased with treatment by apigenin and quercetin in both dose- and time-dependent manner (indicated by an arrow, Fig. 3B). In contrast, the p56 band was not seen in cells treated with kaempferol or myricetin under identical conditions (Fig. 3B). Therefore, apigenin and quercetin are more potent proteasome inhibitors than kaempferol and myricetin in intact Jurkat T cells, which

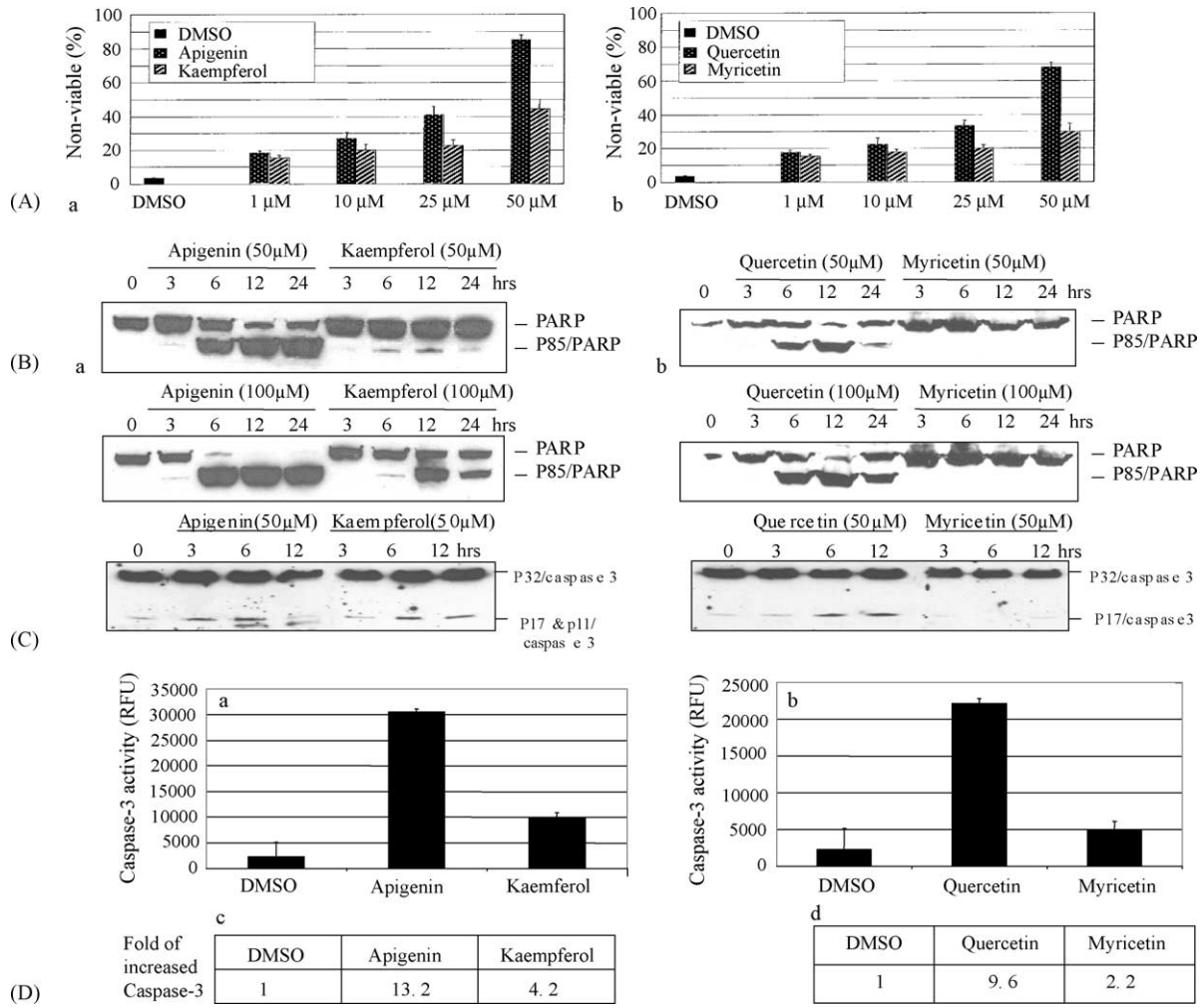

Fig. 4. The flavonoids induce cell death and apoptosis. (A) Jurkat T cells were treated with the solvent (DMSO) and indicated concentrations of apigenin and kaempferol (a) or quercetin and myricetin (b) for 24 h, followed by trypan blue dye exclusion assay. The percentage of non-viable Jurkat T cells is shown. The data represented are as the mean number of dead cells over total cell population ± S.D. (B) Cleavage of the apoptotic marker PARP was induced by the flavonoids in time- and dose-dependent manner. Jurkat T cells were treated with the DMSO or varying concentrations of apigenin and kaempferol (a) or quercetin and myricetin (b) for indicated hours. Intact PARP (p116) and PARP cleavage fragments (p85) are presented. (C) Cleavage of caspase 3 was induced in Jurkat T cells by 50 μM of four flavonoids, respectively, for different time points. Full-length of caspase 3 (p32) and cleaved caspase 3 (p17 and p11) are indicated. (D) The caspase-3 activity was significantly increased by the flavonoids. Jurkat T cells were treated with 50 μM of four different flavonoids followed by the caspase-3 activity assay (see Section 2). The results were represented as relative fluorescence units (RFU) (a and b) and increased fold (c and d). The experiment was repeated three times with the similar results.

was consistent with the proteasome-inhibitory potencies in 20S and 26S proteasome (Table 1) as well as the docking energies and probabilities (Fig. 2) of these flavonoids. The nature of these putative ubiquitinated Bax and IκB-α proteins induced by the flavonoids will be confirmed by a coupled immunoprecipitation and Western blotting assay and by using cells transfected with a haemagglutinin (HA)-tagged uquiquitin in the near future.

3.4. Apigenin and quercetin are more potent inducers of cell death and apoptosis than kaempferol and myricetin

It has been shown that inhibition of the proteasomal chymotrypsin-like activity is associated with induction of tumor apoptotic cell death [16,35]. We then investigated the cell death-inducing potencies of these four flavonoids. Jurkat T cells were treated with 1, 10, 25 or 50 μM of apigenin, kaempferol, quercetin or myricetin for 24 h, and then analyzed with the Trypan blue dye exclusion assay to determine the extent of cell death (Fig. 4A). A dose-dependent cell death was observed when each of these flavonoids was used. At 50 μM treatment, apigenin and kaempferol resulted in 80% and 45%, respectively, non-viable cells (Fig. 4A, panel a), and quercetin and myricetin resulted in 70% and 30%, respectively, non-viable cells (Fig. 4A, panel b). These results suggest that the order of potency for induction of cell death is: apigenin > quercetin > kaempferol > myricetin.

To confirm that these flavonoids induce apoptotic cell death, we compared their apoptosis-inducing activities by measuring levels of PARP cleavage and caspase-3 activity

in Jurkat T cells. Both apigenin and quercetin at 50 μM induced apoptosis-specific PARP cleavage at as early as 6 h (Fig. 4B). In contrast, very low levels of the cleaved PARP p85 were detected in cells treated with 50 μM of kaempferol, and no PARP cleavage was found after treatment with 50 μM myricetin for even 24 h (Fig. 4B). When concentrations of these four flavonoids were increased to 100 μM, a dose-dependent PARP cleavage was observed (Fig. 4B). Importantly, PARP cleavage induced by apigenin occurred after induction of putative ubiquitinated IκB-α (i.e., 100 μM for 3 h; Fig. 4B versus Fig. 3B). Comparing the four flavonoids in the PARP cleavage assay, apigenin was more potent than quercetin than kaempferol and than myricetin (Fig. 4B). Myricetin is the weakest flavonoid of this set. There was no PARP cleavage induced by myricetin at even 100 μM (Fig. 4B).

To confirm the apoptosis-specific PARP cleavage, we also performed an immunostaining assay with a specific FITC-conjugated antibody to the cleaved p85 PARP fragment. The results from Jurkat T cells treated with various flavonoids have shown again that apigenin and quercetin induced more PARP cleavage than kaempferol than myricetin (Fig. 5A). Quantitation of these results demonstrate that the order of potency to produce the cleaved PARP fragment is: apigenin > quercetin > kaempferol > myricetin (Fig. 5C).

Caspase-3 is an essential effector caspase, responsible for cleaving PARP in many cell systems [36]. We then measured capases-3 activity levels in Jurkat T cells treated with these four flavonoids. The fold of increased caspase-3 activity is: apigenin 13.2 > quercetin 9.6 > kaempferol 4.2 > myricetin 2.2 (Fig. 4D), consistent with the levels of PARP cleavage (Figs. 4B and 5C). Finally, cleavage of caspase-3 into active fragments p17 and p11, which is responsible for caspase-3 activation [37], was also measured by Western blotting (Fig. 4C). The order of levels of caspase-3 p17/p11 fragments generated by these four flavonoids were: apigenin > quercetin, kaempferol > myricetin (Fig. 4C). Therefore, the order of potency of these flavonoids to inhibit the proteasome (Table 1 and Fig. 3) correlates well with their abilities to induce tumor cell apoptosis (Figs. 4 and 5). These results support the functional significance of inhibition of tumor cellular proteasome activity by flavonoids. Our study is also consistent with previous reports that overexpression of Bax and IκB-α causes tumor cell apoptosis [38–40].

3.5. Non-transformed human natural killer cells are more resistant to apigenin treatment than leukemia cells

Thus far, we have shown that flavonoids such as apigenin can inhibit the proteasome activity and induce tumor cell apoptosis. However, whether apigenin could affect human normal or non-transformed cells was unknown. To determine whether apigenin was able to induce apoptosis preferentially in tumor/transformed versus normal/non-transformed cells, we treated both human leukemic Jurkat

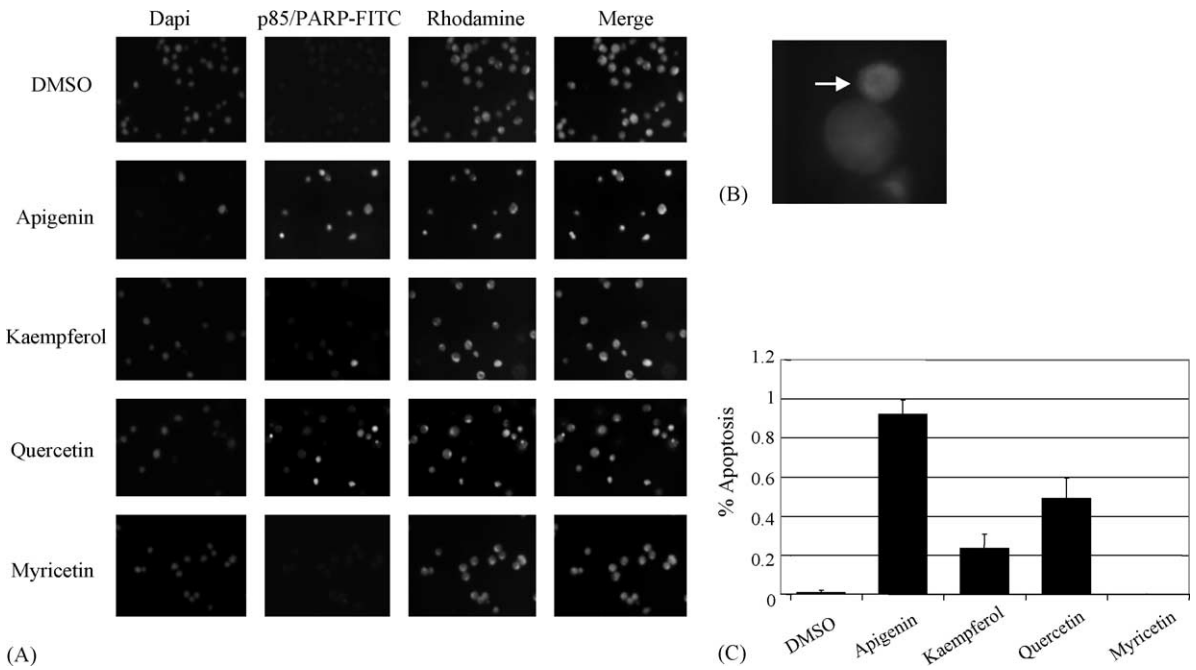

Fig. 5. Immunostaining assay showed that apigenin was more potent inducer of apoptosis than quercetin, kaempferol and myricetin. Jurkat T cells were treated with DMSO or 50 μM of four different flavonoids for 24 h and harvested followed by incubation with specific antibody to the p85 cleaved PARP fragment conjugated to FITC, nuclear marker DAPI, cytosol marker Rhodamine, and photographed (A). The merged data shows apoptosis in treated Jurkat T cells (A). An enlarged image of the merged data from apigenin treatment is represented (B). A schematic representation of the percentage of apoptotic cells indicates that apigenin has the most potent effect on Jurkat T cells (C). Magnification was 100× (A) or 400× (B). Data shown are representative scanned wells from triplicate experiments.

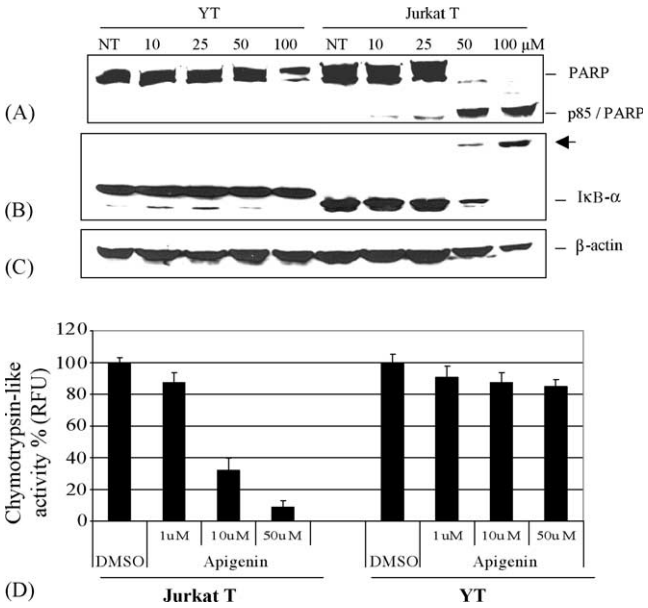

Fig. 6. The flavonoid apigenin induces apoptosis and inhibits the proteasomal activity selectively in tumor cells but not in non-transformed cells. Human leukemic Jurkat T and non-transformed, immortalized human NK cells (YT) were treated with apigenin at indicated concentrations. (A–C) Twenty-four hour treatment, followed by Western blot analysis using specific antibodies to PARP (A), IκB-α (B) and actin (C). The band of 56 kDa, indicated by an arrow in B, is a putative ubiquintinated IκB-α protein (23). (D) Six-hour treatment, followed by a 2 h additional incubation with the fluorogenic peptide substrate Z-Gly-Gly-Leu-AMC specific for the proteasomal chymotrypsin-like activity. Afterwards, production of hydrolyzed AMC groups was measured using a Wallac Victor3^{TM} multilabel counter with an excitation filter of 355 nm and an emission filter of 460 nm. Triplicates of each time point wee performed.

T cells and immortalized, non-transformed natural killer cells (YT cell line) [41] with apigenin at various concentrations for 24 h (Fig. 6). Indeed, apigenin at 10–25 μM induced apoptosis-specific PARP cleavage in Jurkat T cells (Fig. 6A), whose levels were further increased when 50–100 μM of apigenin was used (Fig. 6A). In contrast, no PARP cleavage was detectable in the YT cells after treatment with apigenin at even 100 μM (Fig. 6A). We also examined the levels of the proteasome target protein IκB-α in both Jurkat T and YT cell lines treated by apigenin. The data show that accumulation of the putative ubiquitinated form of IκB-α (p56) was observed in Jurkat T cells not in YT cells (Fig. 6B), suggesting that apigenin might fail to inhibit the proteasome activity in non-transformed YT cells, resulting in lack of apoptosis.

To confirm the differential effects of apigenin on the proteasoma activity of Jurkat T versus YT cells, both cell lines were treated with apigenin at 1, 10 or 50 μM for 6 h, followed by a 2 h additional incubation with a fluorogenic peptide substrate specific for the proteasomal chymotrypsin-like activity. Afterwards, production of hydrolyzed AMC groups was measured (Fig. 6D). In Jurkat T cells, treatment with apigenin caused a concentration-dependent inhibition of the proteasomal chymotrypsin-like activity with >90% inhibition at 50 μM (Fig. 6D). In sharp contrast, the proteasomal chymotrypsin-like activity in YT cells was decreased by only ∼15% with apigenin at the highest concentration used (Fig. 6D). Therefore, the proteasome activity in non-transformed YT cells is not effectively inhibited by apigenin, which may be responsible for lack of apoptosis in these cells (Fig. 6A).

4. Discussion

Cancer is a disease, where the treatment can be as debilitating as the disease. Therefore, prevention could be considered as important as treatment in cancer. Diet can play a vital role in cancer prevention. Studies have shown that a diet high in fruits and vegetables is associated with a reduced risk of cancer [18–20]. Likewise, proteasome inhibition has been developed as a chemotherapeutic strategy. It has been shown that inhibition of the proteasome activity is associated with induction of apoptosis in tumor, but not normal cells [8,42,43]. Previously we reported that tea flavonoids with an ester bond, such as (−)-EGCG, possess proteasome-inhibitory properties [23]. Since the flavonoids presented here are structurally similar to (−)-EGCG, we hypothesized that these compounds may be proteasome inhibitors and that may contribute to their cancer-preventative properties [21].

We have previously reported computational modeling studies that examine those properties contributing to the ability of (−)-EGCG to bind and inhibit the β5-subunit of the proteasome [25]. These properties include: a site susceptible to nucleophilic attack, a binding pose that placed that site near the hydroxyl group of the N-terminal threonine, and a binding pose that places the double ring system in or near the S_1 pocket [25]. In the current study, computational electron density analysis determined that each of the flavonoids possesses a site susceptible for nucleophilic attack at the C_4 position by the OH of β5 N-terminal threonine (Fig. 1). Next, we docked the four flavonoids to the β5-subunit of the proteasome and found that these compounds can adopt a pose suitable for nucleophilic attack on the C_4 position by the OH group of N-Thr of β5 subunit. Interestingly, apigenin had a much greater probability of adopting the inhibitory pose (Fig. 2), leading us to suspect that the absence of the C_3 hydroxyl group may have a significant role in the binding poses adopted by apigenin and its capacity for proteasome inhibition. This was supported by removing the C_3 hydroxyl group from the structures of quercetin, kaempferol and myricetin, which resulted in dramatic increases in the probabilities of these compounds to adopt poses that fulfill the two criteria used to identify potential proteasome inhibition (Fig. 2C–E). These data suggest that the C_3 hydroxyl may contribute to the binding mode of these compounds in the active site. This is in agreement with a previous study that suggested the C_3 hydroxyl might be important for biological functions [33]. The computational model suggested

that the order of proteasome-inhibitory potency would be: apigenin ≥ quercetin > kaempferol ≥ myricetin. Consistent with the prediction from the docking results, our preliminary data showed that both chrysin and luteolin, two analogs of apigenin without the C_3 hydroxyl (chrysin with no B-ring OH while luteolin with two B-ring OH groups), are inhibitors of purified 20S proteasome and inducers of apoptosis, with potency comparable to that of apigenin (data not shown).

The computational model was verified through biological evaluation of all four flavonoids. First, the four flavonoids were tested for their ability to inhibit the chymotrypsin-like activity of purified 20S proteasome. It was found that both apigenin and quercetin could potently inhibit the proteasome chymotrypsin-like activity, whereas kaempferol and myricetin were less potent (IC_{50} values of 1.8 μM and 3.5 μM versus 10.5 μM and 10 μM; Table 1, in 20S). The ability of these compounds to inhibit the proteasome chymotrypsin-like activity was further verified in intact Jurkat T cells. The results supported the prediction of the computer simulation: apigenin and quercetin were similar to each other in potency (IC_{50} of 1 μM and 2 μM; Table 1, in 26S). Likewise, kaempferol and myricetin were inferior inhibitors but shared similar potency (IC_{50} of 11 μM and 12 μM; Table 1, in 26S).

To further verify that these flavonoids can inhibit the proteasome, we measured the accumulation of proteasome target proteins. Apigenin and quercetin were the most potent inhibitors showing accumulation of putative poly-ubiquitinated Bax and IκB-α in a dose- and time-dependent manner (Fig. 3). Treatment with kaempferol or myricetin resulted in much less accumulation of these putative ubiquinated forms of these proteins (Fig. 3), further supporting that these two compounds are not potent proteasome inhibitors.

Of particular interest in cancer prevention and treatment is the preferential induction of apoptosis in tumor cells rather than normal cells. Apigenin was the strongest inducer of apoptosis in leukemia Jurkat T cells, followed by quercetin, kaempferol and myricetin, as first measured by PARP cleavage (Figs. 4 and 5). Capase-3 activity revealed a similar comparison with apigenin and quercetin inducing a 13- and 9-fold increase, respectively, in comparison to 4- and 2-fold increases by kaempferol and myricetin (Fig. 4D). When non-transformed natural killer YT cells are treated with 100 μM apigenin, there is no induction of apoptosis, as compared to human leukemia Jurkat T cells (Fig. 6A). Consistently, we found no evidence that apigenin was capable of proteasome inhibition owing to the lack of accumulation of proteasome target proteins (Fig. 6B). Indeed, apigenin at the highest concentration tested inhibited only 15% of the proteasomal chymotryptic activity in YT cells, in contrast to a >90% inhibition in Jurkat T cells (Fig. 6D). Consistent with the idea about failure of apigenin to inhibit the cellular proteasome activity in non-transformed YT cells, we have also found that some tea polyphenol proteasome inhibitors were able to inhibit the proteasomal chymotrypsin-like activity in SV40-transformed, but not normal, WI-38 cells [44], and that the soy isoflavone genistein was able to accumulate ubiquitinated proteins in the transformed, but not normal cells [45]. These data support the argument that apigenin and quercetin might have the potential to be developed into cancer-preventative agents that function through the mechanism of proteasome inhibition selectively in cancer *over* normal cells.

In conclusion, proteasome inhibition may contribute to the cancer-preventative effects of apigenin and quercetin. Computer modeling shows that the C_4 carbon may serve as a site of nucleophilic attack by N-Thr of proteaosmal β5 subunit and that the presence of the C_3 hydroxyl may affect the ability of these flavonoids to bind to the chymotrypsin active site of the proteasome. Removal of this hydroxyl appears to dramatically improve the capability of the flavonoid to bind to the proteasome as is demonstrated by apigenin. Proteasome inhibition appears to be the cause of apoptosis induction in Jurkat T cells. The results here not only provide impetus for further study of dietary flavonoids as cancer-preventative agents but also help describe some of the key structural characteristics of these compounds in fulfilling that role.

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