A natural *musaceas* plant extract inhibits proteasome activity and induces apoptosis selectively in human tumor and transformed, but not normal and non-transformed, cells

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Abstract. Animal studies have demonstrated that a dietary polyphenol known as tannic acid (TA) exhibits anticarcinogenic activity in chemically induced cancers. Most recently, we have reported that TA and ester-bond containing green tea polyphenols are potent proteasome inhibitors *in vitro* and *in vivo*. We hypothesize that CellQuest™, a patented formula which contains high level of TA obtained from a *musaceas* (plantain) plant extract, will inhibit the tumor cell proteasome activity. Here, we report that a partially purified CellQuest fraction, S3, potently inhibits the proteasomal chymotrypsin-like activity of Jurkat T cell extracts in a concentration-dependent manner. Inhibition of the proteasome by S3 in leukemia Jurkat T, simian virus 40-transformed and prostate cancer LNCaP cells results in accumulation of ubiquitinated proteins and the natural proteasome substrate $p27^{Kip1}$, followed by induction of apoptosis. In contrast, non-transformed, immortalized human natural killer cells and normal human fibroblasts are resistant to S3-mediated proteasome inhibition and apoptosis induction. Our present study suggests that CellQuest targets and inhibits the proteasome selectively in tumor cells, which may contribute to the claimed anticancer activity.

Introduction

Over 6 million people die due to cancer each year worldwide, being the largest single cause of death in both men and women. One of the current strategies for the treatment of human cancer is to activate the cellular apoptotic death program (1,2). Currently applied radiation therapy and standard chemotherapeutic drugs kill some tumor cells through induction of apoptosis. Unfortunately, however, the majority of human cancers are resistant to these therapies (3,4). It is therefore urgent to look for novel natural or synthetic apoptosis-inducing compounds as candidate anti-tumor agents. Along this line, plant-derived compounds have great potential to be developed into anticancer drugs because of their multiple mechanisms and low side effects (5-9).

For thousands of years people have used plants and herbs as healing compounds. The active ingredients for a vast number of pharmaceutically derived medications contain components originating from phytochemical compounds. Of late, many researchers have begun to examine the unique impact of many plant extracts on cellular functions. Basic research has identified phytochemicals as agents inhibiting mutagenesis, activating apoptosis or inducing differentiation that are critical characteristics for chemoprevention. *Morinda citrifolia* (Noni), a medicinal plant used in Polynesia, has the ability to reduce carcinogen-DNA adduct formation in both cell culture and rats (10). A medicinal herb extracted from the *Hemsleya amabilis* plant inhibits tumor cell growth, colony formation, and induces apoptosis in various tumor cell lines (11). Saponins, glycosidic surfactants produced by plant cells used to solubilize membrane proteins, are also capable of decreasing tumor cell proliferation, inducing apoptosis or cell cycle arrest, or inhibiting DNA synthesis in a variety of cancer cell lines (12,13).

Tannins are plant-derived polyphenoic compounds, which can be classified into two groups: hydrolysable and condensed tannins (14-16). The hydrolysable tannins are commonly called tannic acid (TA). TA is widely found in food plants, and broadly applied to various industrial food additives (14-16). Recently, it has been shown that TA exerts cancer chemopreventative activity in various animal models (16-19). For example, TA was able to suppress skin tumor promotion induced by ultraviolet-B radiation in hairless mice (17). In addition, TA dietary intake in low doses can exert a strong

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dose-dependent chemopreventative activity against spontaneous liver tumor development in C3H male mice (18). Furthermore, it has been shown that TA increased survival rate of Balb/c mice bearing syngeneic tumors (19). Most recently, we have reported that tannic acid (20) and ester bond-containing tea polyphenols (21) potently and specifically inhibited the chymotrypsin-like activity of the proteasome *in vitro* and *in vivo*, which may contribute to the previously observed anticarcinogenic activity (16-19).

The 20S proteasome constitutes the catalytic component of the ubiquitous proteolytic machinery of the 26S proteasome (22-25). Two important functions of the proteasome in human tumor cells are to promote proliferation and to inhibit apoptosis (24,25). It has been shown that the chymotrypsin-like, but not trypsin-like, activity of the proteasome is associated with tumor cell survival (26,27). Several pro-apoptotic proteins have been identified as targets of the ubiquitin/proteasome-mediated degradation pathway, including p53 (28), $p27^{Kip1}$ (29), IκB-α (30) and *Bax* (31).

CellQuest™, a *Musaceas* plant extract, has been patented as a method of treating cancer (32). As CellQuest is a polyphenolic liquid extract from the *Musaceas genus* (such as plantain), containing high level of TA or tannin complex (5-20% dried basis) (32), we have hypothesized that CellQuest could also inhibit proteasomal activity and induce tumor cell death. Here, we report that a partially purified CellQuest supernatant fraction (S3) potently inhibits the proteasomal chymotrypsin-like activity of the Jurkat T cell extracts. Inhibition of the proteasome by S3 in leukemia Jurkat T and simian virus 40 (SV40)-transformed fibroblasts results in accumulation of ubiquitinated proteins and the natural proteasome substrate p27, followed by induction of apoptotic cell death. Importantly, we also found that immortalized, non-transformed human natural killer (NK) cells and the normal human fibroblasts were much more resistant to CellQuest S3 fraction-mediated proteasome inhibition and apoptosis induction. Finally, S3 was also able to inhibit the proteasomal activity, induce apoptotic cell death, and suppress colony formation of human prostate cancer cells. Our study suggests that inhibition of the proteasome activity by CellQuest may contribute to its previously observed anticancer activity (32).

Materials and methods

CellQuest and its supernatant preparation. CellQuest product in soup form was obtained from CellQuest, Inc., Clearwater, FL, USA. This CellQuest soup was partially purified for the use of experiments by several methods as described below. A portion of CellQuest soup was centrifuged at 1,000 x g for 30 min, followed by collection of the supernatant (named S1 fraction). Another portion of CellQuest soup was centrifuged at 48,400 x g for 30 min, followed by collection of the supernatant (named S2 fraction). Some of the S2 supernatant was filtered with a 0.2 μm filter to obtain S3 fraction. Because S3 contains similar levels of biological activities to S1 and S2 (Figs. 1 and 2), we used the S3 fraction for most of the experiments.

Other materials. Fetal calf serum, propidium iodide, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), trypan blue, RNase A, and DMSO were purchased from Sigma-Aldrich (St. Louis, MO). RPMI-1640 medium, Dulbecco's modified Eagle's medium, MEM non-essential amino acids solution, MEM sodium pyruvate solution, penicillin, and streptomycin were purchased from Invitrogen (Carlsbad, CA). The fluorogenic peptide substrates Suc-Leu-Leu-Val-Tyr-AMC (for the proteasomal chymotrypsin-like activity) and Ac-Asp-Glu-Val-Asp-AMC (for the caspase-3 activity) were from Calbiochem (La Jolla, CA). Monoclonal antibody to $p27^{Kip}$ was purchased from PharMingen (San Diego, CA), monoclonal antibodies to ubiquitin and actin were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA), and a polyclonal antibody to human poly(ADP-ribose) polymerase (PARP) was from Roche Molecular Biochemicals (Indianapolis, IN).

Cell culture, cell extract preparation, and Western blot assay. Human leukemia Jurkat T and prostate cancer LNCaP cells were cultured in RPMI-1640 medium supplemented with 10% fetal calf serum, 100 units/ml of penicillin, and 100 μg/ml streptomycin. Human immortalized, non-transformed NK cells (YT line) (33) were cultured in RPMI-1640 supplemented with 1 mM MEM sodium pyruvate solution, 0.1 mM MEM non-essential amino acids solution, 10% fetal calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. Human breast cancer (MCF-7), human prostate cancer (PC-3), normal (WI-38) and SV40-transformed (VA-13) human fibroblasts were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 100 units/ml of penicillin and 100 μg/ml of streptomycin. All cells 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 homogenized in a lysis buffer [50 mM Tris-HCl (pH 8.0), 5 mM EDTA, 150 mM NaCl, 0.5% NP-40, 0.5 mM phenylmethylsulfonyl fluoride, and 0.5 mM dithiothreitol] for 30 min at 4˚C. After that, the lysates were centrifuged at 12,000 x g for 15 min at 4˚C, and the supernatants were collected as whole cell extracts. Equal amounts of protein extract (60 μg) were resolved by SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane using a Semi-Dry Transfer system (Bio-Rad, Hercules, CA). The enhanced chemiluminescence Western blot analysis was then performed using specific antibodies to the proteins of interest.

Inhibition of the proteasome activity in whole cell extracts by S1, S2 or S3 fraction. A whole cell extract (6 μg) of Jurkat T cells was incubated for 60 min at 37˚C with 20 μM of the fluorogenic peptide substrate (Suc-Leu-Leu-Val-Tyr-AMC) for the chymotrypsin-like activity of the proteasome in 100 μl of the assay buffer (50 mM Tris-HCl, pH 8.0), in the absence or presence of each supernatant of CellQuest at various dilutions. After incubation, production of hydrolyzed 7-amido-4-methylcoumarin (AMC) groups were measured using a multi-well plate reader Wallac 1420 Victor 2™ with an excitation filter of 355 nm and an emission filter of 460 nm (EG & G Wallac).

Assay for caspase-3 activity. Cell-free caspase-3 activity was determined by measuring the cleavage of AMC groups from

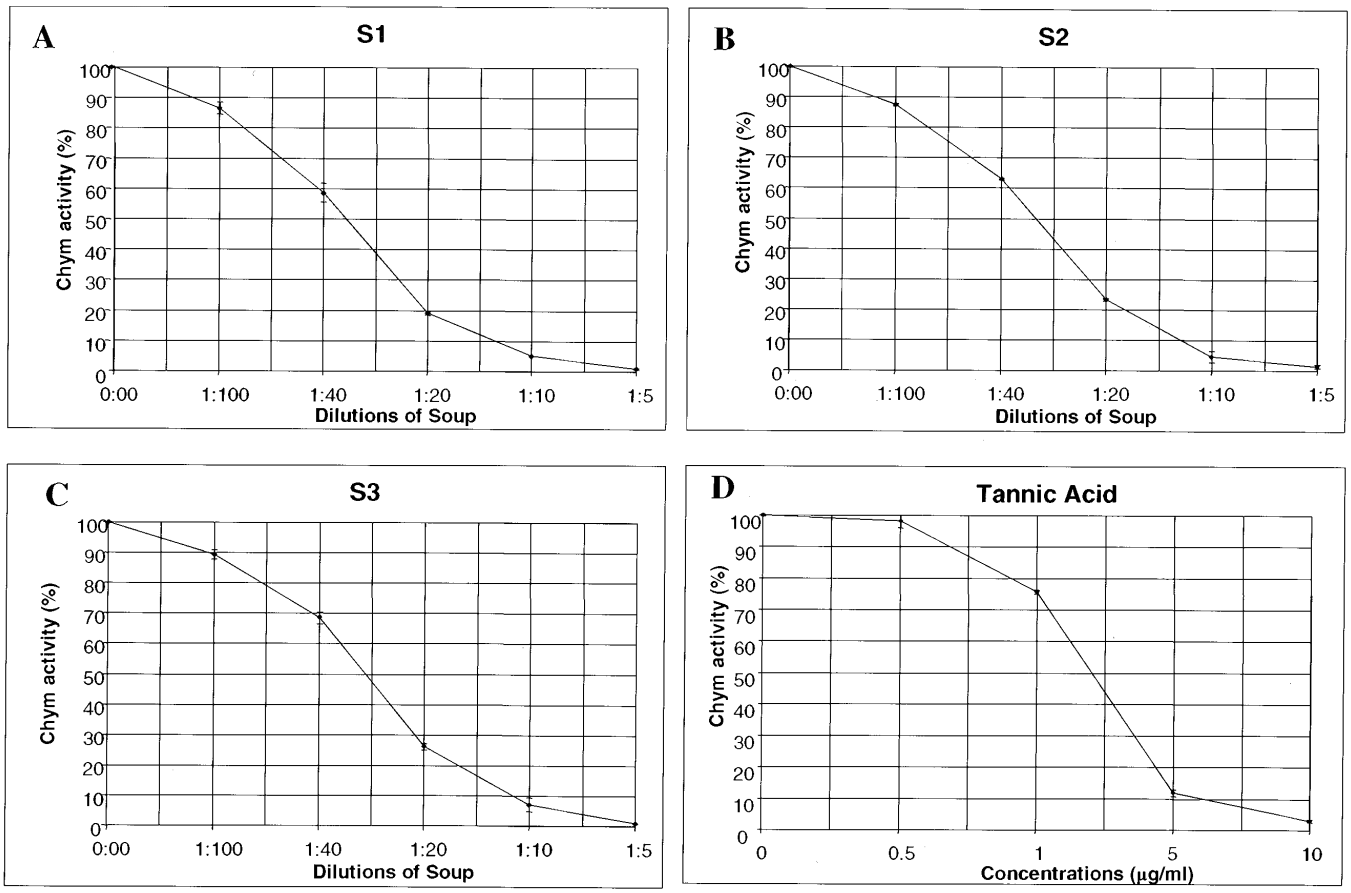

Figure 1. Inhibition of the proteasome activity in Jurkat cell extracts by CellQuest S1, S2 and S3 fractions. A Jurkat cell extract was incubated for 1 h with a fluorogenic peptide substrate for the proteasomal chymotrypsin-like (Chym) activity, in the absence or presence of each CellQuest supernatant at indicated dilutions or tannic acid at different concentrations, followed by measurement of free AMC groups as described in Materials and methods. Values are mean triplicates and error bars denote standard deviations.

a caspase-3 substrate (Ac-Asp-Glu-Val-Asp-AMC). Briefly, a prepared protein extract (30 µg) was incubated in a buffer containing 50 mM Tris/pH 7.5 along with the caspase substrate at 40 µM in a 96-well plate. The reaction mixture was incubated at 37˚C for 1 h. After incubation, the liberated florescent AMC groups were quantified as described above.

Trypan blue assay. The trypan blue dye exclusion assay was performed by mixing 20 µl of cell suspension with 20 µl of 0.4% trypan blue dye before injecting into a hemocytometer and counting. The number of cells that absorbed the dye and those that exclude the dye were counted, from which the percentage of nonviable cell number to total cell number was calculated.

Morphological assessment of prostate cancer cells. To assess morphological changes of cells, prostate cancer LNCaP cells grown in 6 cm-tissue culture dishes were treated with S3 fraction at different dilutions for the indicated hours, followed by observing them under a phase-contrast microscope (Leica; Wetzlar, Germany) and taking photographs (x100). Apoptotic cells were identified by their distinct morphological changes (34).

Flow cytometry. Cell cycle analysis based on DNA content was performed as we described previously (26). At each time point, cells were harvested, counted, and washed twice with PBS. Cells ($5x10^6$) were suspended in 0.5 ml PBS, fixed in 5 ml of 70% ethanol overnight at -20˚C, centrifuged, resuspended again in 1 ml of propidium iodide staining solution (50 µg propidium iodide, 100 units RNase A and 1 mg glucose/ml PBS), and incubated at room temperature for 30 min. The cells were then analyzed with FACScan (Becton-Dickinson Immunocytometry, CA), ModFit LT and WinMDI V.2.8 cell cycle analysis software (Verity Software; Topsham, ME). The cell cycle distribution is shown as the percentage of cells containing G_1, S, G_2, and M phase DNA judged by propidium iodide staining. The cell death population is determined as the percentage of cells with sub-G_1 ($<G_1$) DNA content.

Soft agar assay. The soft agar assay was performed as described previously (35) with a few modifications. In brief, in a 6-well plate, a bottom feeder layer (0.6% agar) was prepared with DMEM media containing 10% fetal bovine serum, 100 units/ml penicillin, and 100 µg/ml streptomycin. A top layer (0.3% agar) was prepared with DMEM and the same media as described above but containing $2x10^4$ prostate cancer LNCaP cells and indicated dilutions of CellQuest S3 fraction or H_2O as a control. Plates were incubated at 37˚C in 5% CO_2 in a humidified incubator for two weeks. MTT (1 mg/ml) was added to each well and incubated overnight to

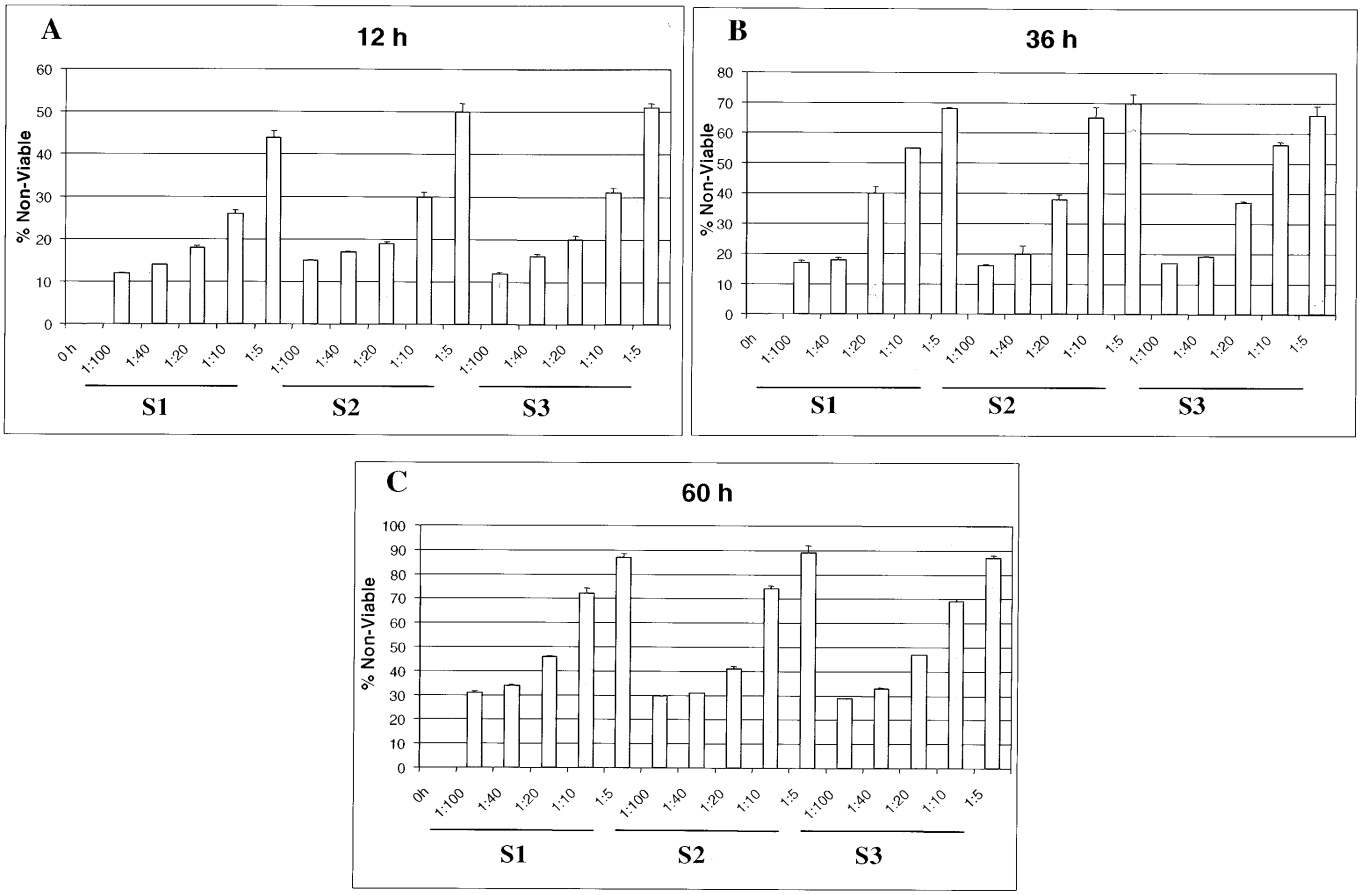

Figure 2. Induction of the Jurkat T cell death by S1, S2 and S3. Jurkat T cells were incubated for 12, 36 or 60 h with each CellQuest supernatant, followed by trypan blue incorporation assay to measure cell death rates. Standard deviations are shown with error bars from a mean of at least three independent experiments.

allow complete formation of purple formazan crystals. The plates were then scanned and photographed, and the number of colonies was quantified by Quantity one v.4.0.3 software (Bio-Rad, Hercules, CA).

Results

Partially purified CellQuest fractions inhibit the proteasomal chymotrypsin-like activity under cell-free conditions. We tested the hypothesis that CellQuest containing high level (5-20%) of TA (32) inhibits the chymotrypsin-like activity of the proteasome, associated with induction of apoptosis (20). To do so, we first prepared three CellQuest supernatant fractions, S1, S2 and S3 (Materials and methods), and determined their potencies to inhibiting the chymotrypsin-like activity of the 26S proteasome using a Jurkat T cell extract (Fig. 1). In this experiment, all the S1, S2 and S3 fractions inhibited the proteasomal chymotryptic activity in a concentration-dependent manner with similar potencies: 10-15% inhibition at 1:100 dilution, 30-40% inhibition at 1:40 dilution, and 95% inhibition at 1:10 dilution (Fig. 1A-C). As a control, a commercial TA (Sigma) also inhibited the proteasomal chymotrypsin-like activity in a concentration-dependent fashion (Fig. 1D). Therefore, CellQuest contains a proteasome-inhibitory activity.

Partially purified CellQuest fractions induce Jurkat T cell death. CellQuest is believed to have anti-tumor activity (32). We then investigated whether the three partially CellQuest fractions could induce tumor cell death that might be responsible for, or contribute to, its claimed anti-tumor activity (32). Jurkat T cells were incubated with S1, S2 or S3 at various concentrations for 12, 36 or 60 h, followed by trypan blue incorporation assay. We found that all the three CellQuest fractions induced Jurkat cell death in both concentration- and time-dependent manner (Fig. 2). For example, at 36 h, S1, S2 and S3 fractions at 1:100, 1:20 and 1:5 dilutions killed ~20, ~40 and ~70% of Jurkat T cells, respectively (Fig. 2B). In addition, these three preparations at 1:10 dilution induced cell death by up to 35, 65, and 75% after treatment for 12, 36 and 60 h, respectively (Fig. 2). The similar potency of S1, S2 and S3 suggests that the cell death-inducing factor present in CellQuest soup is water-soluble and is relatively small in size (Fig. 2), associated with the proteasome-inhibitory activity (Fig. 1). Here after, only S3 fraction was used for further experiments of this study.

S3 fraction inhibits the proteasomal activity and induces apoptosis preferentially in Jurkat T and SV40-transformed over normal and non-transformed cells. We then determined whether S3 fraction was able to inhibit the proteasomal activity in intact Jurkat T cells (Fig. 3, lanes 1-9). Jurkat cells

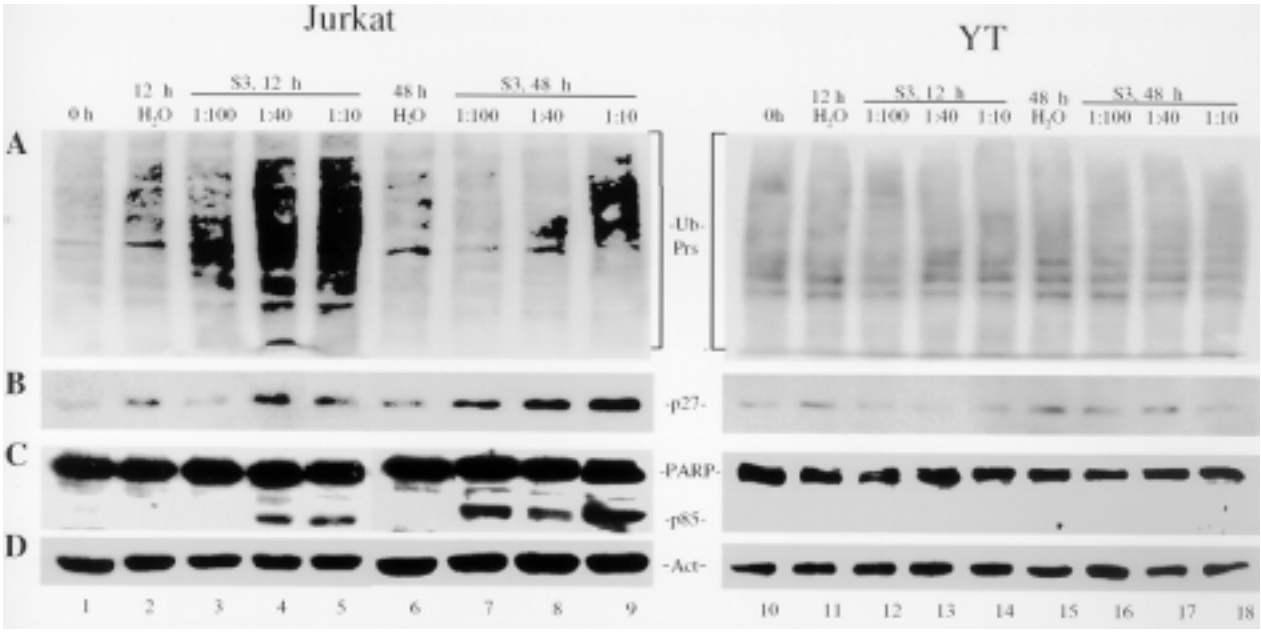

Figure 3. Selective induction of apoptosis by S3 fraction in leukemic Jurkat T over non-transformed, immortalized NK cells. Jurkat T and NK (YT) cells were treated with H_2O (control) and different dilutions of S3 fraction for the indicated hours, followed by a Western blot assay using specific antibodies to ubiquitin (A), p27 (B), PARP (C), and actin (D). Untreated control cells are indicated as 0 h. The intact PARP (116 kDa) and a PARP cleavage fragment (p85) are indicated. Actin was used as a loading control. Similar results were observed in three independent experiments.

were treated with S3 at different dilutions or the vehicle (H_2O) for either 12 or 48 h, followed by measuring levels of ubiquitinated proteins and the proteasome target protein p27. After 12-h treatment, an increase in levels of ubiquitinated proteins was observed in a S3 concentration-dependent manner, which was decreased after 48-h treatment (Fig. 3A, lanes 2-5 vs. 6-9 vs. 1). In addition, p27 expression was significantly increased, again in a concentration-dependent fashion, after 48 h (Fig. 3B, lanes 6-9 vs. 1).

To determine whether S3-induced cell death (Fig. 2) is due to apoptosis, we measured cleavage of PARP in the Jurkat cells treated with S3 fraction. After 12-h treatment, PARP cleavage was observed in cells treated with S3 at 1:40 and 1:10, but not 1:100, dilutions (Fig. 3C, lanes 1-5). However, after 48-h treatment, PARP cleavage was found in cells treated with S3 at all the dilutions (Fig. 3C, lanes 6-9). Taken together, these results indicate that CellQuest S3 fraction contains both proteasome-inhibitory and apoptosis-inducing activities.

We then determined effects of S3 on immortalized, non-transformed human NK cells (YT). Impressively, under the same conditions, S3 at even the highest concentration (1:10 dilution) for the longest incubation (48 h) was unable to accumulate either ubiquitinated proteins or p27 (Fig. 3, A and B, lanes 10-18). Associated with that, S3 also failed to induce apoptosis-specific PARP cleavage in the non-transformed NK cells (Fig. 3C, lanes 10-18).

To further investigate the tumor-selective effects of CellQuest S3 fraction, normal, non-immortalized human fibroblast cell line WI-38 and its SV40-transformed derivative (VA-13) were tested. Again, S3 treatment of transformed VA-13 cells for 12 h showed concentration-dependent accumulation of ubiquitinated proteins, compared to the control water-treated cells (Fig. 4A, lanes 1-4). In addition,

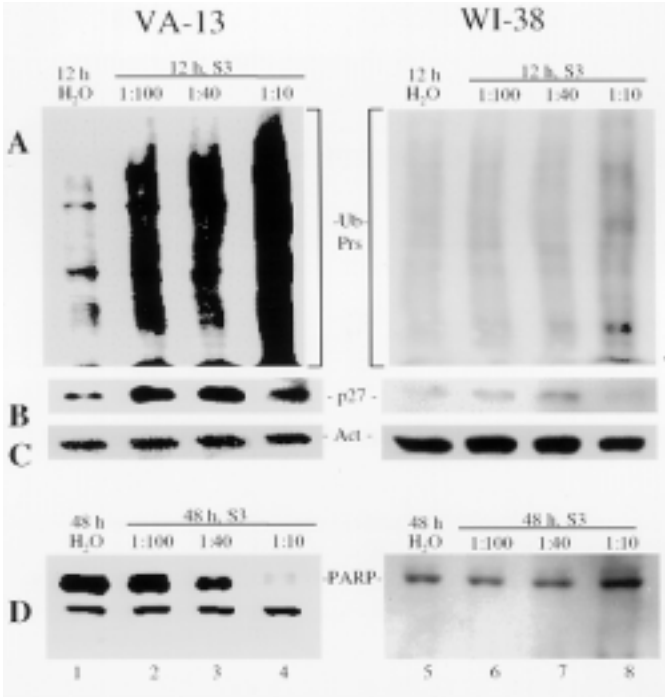

Figure 4. Preferential accumulation of ubiquitinated proteins and p27 and decreased levels of PARP protein by S3 in the transformed fibroblasts over the normal human fibroblasts. Normal (WI-38) and SV40-transformed (VA-13) human fibroblasts were treated with H_2O (control) and different dilutions of S3 fraction for the indicated hours followed by Western blot assay using specific antibodies to ubiquitin (A), p27 (B), actin (C), and PARP (D). Similar results were observed in three independent experiments.

S3 treatment of VA-13 cells also increased levels of p27 protein (Fig. 4B, lanes 1-4). Associated with inhibition of the proteasome activity by S3 in VA-13 cells, the levels of intact PARP were decreased after 48-h treatment in an S3

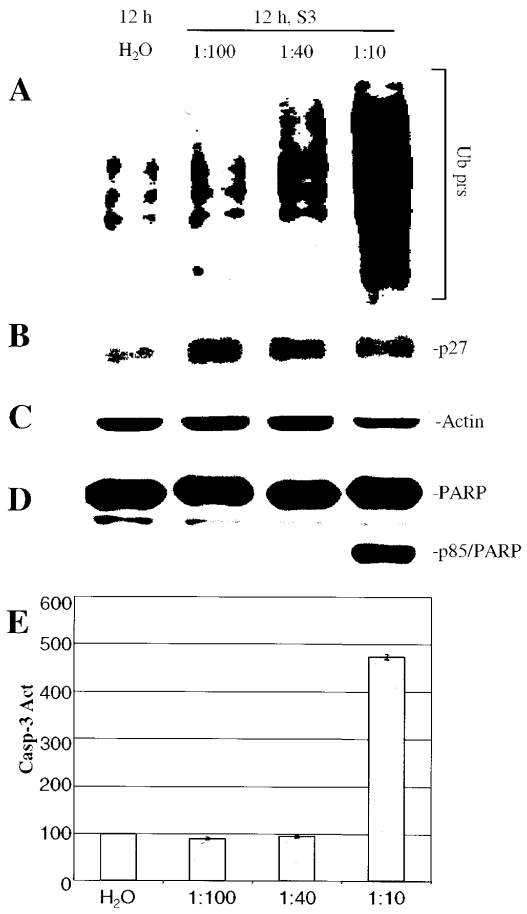

Figure 5. Proteasome inhibition and apoptosis induction by S3 fraction in human prostate cancer LNCaP cells. LNCaP cells were treated with H_2O and different dilutions of S3 for the indicated hours, followed by a Western blot (A-D) and cell-free caspase-3 (Caps-3) activity assay (E). Standard deviations are given with error bars from a mean of at least three different experiments in E. Similar results were observed in multiple independent experiments.

concentration-dependent manner (although no p85 were observed in this experiment; Fig. 4D, lanes 1-4), suggesting that cell death had occurred. In contrast, the treatment of normal WI-38 cells with different dilutions of S3 only slightly increased levels of ubiquitinated proteins and p27, and did not cause any decrease in the level of intact PARP protein (which instead increased) (Fig. 4, lanes 5-8). These data confirm that CellQuest S3 fraction could inhibit the proteasome activity and induce cell death preferentially in human tumor and transformed over the normal and non-transformed cells.

Proteasome inhibition and apoptosis induction by S3 in intact human prostate cancer LNCaP cells. To investigate effects of CellQuest S3 fraction on human solid tumor cell lines, prostate cancer LNCaP cells were treated with S3 at various dilutions for 12 h, followed by measurement of proteasome inhibition and apoptosis. Significant increase in levels of ubiquitinated proteins and p27 protein were again observed in these cells treated with S3, but not with the control (H_2O) (Fig. 5A-B), consistent with the blockage of ubiquitin/proteasome degradation pathway by S3 in intact LNCaP cells. In the same experiment, apoptosis occurred in LNCaP cells treated with S3 at 1:10, but not other dilutions. Apoptosis was measured by both cleavage of PARP (Fig. 5D) and induction of cell-free caspase-3 activity (by 5-fold) (Fig. 5E).

To further examine the solid tumor cell-killing effects of S3 fraction, we performed flow cytometry analysis using LNCaP cells treated with S3 at different concentrations for 12 or 48 h (Fig. 6). Again, we observed a time- and concentration-dependent increase in the cell population containing sub-G_1 DNA content ($<G_1$), a measurement of cell death-associated DNA degradation. After 12-h treatment in cells exposed to S3 at 1:40 and at 1:10 dilution, sub-G_1 DNA content was increased by 4 and 18%, respectively (Fig. 6). Cells containing sub-G_1 DNA content were further increased by 69% after treatment of S3 at 1:10 dilution for 48 h (Fig. 6).

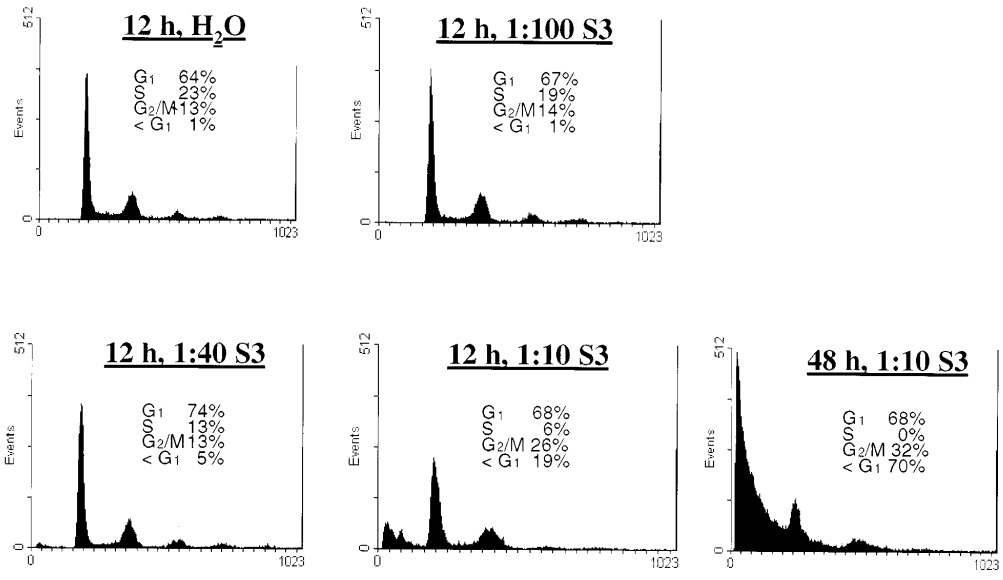

Figure 6. S3 fraction induces DNA fragmentation in prostate cancer LNCaP cells. The LNCaP cells were treated with H_2O (control) and different dilutions of S3 for the indicated hours, followed by flow cytometry analysis. Cell death-associated DNA degradation is measured by the increase in the percentage of the cell population with $<G_1$ DNA content. Similar results were observed in three independent experiments.

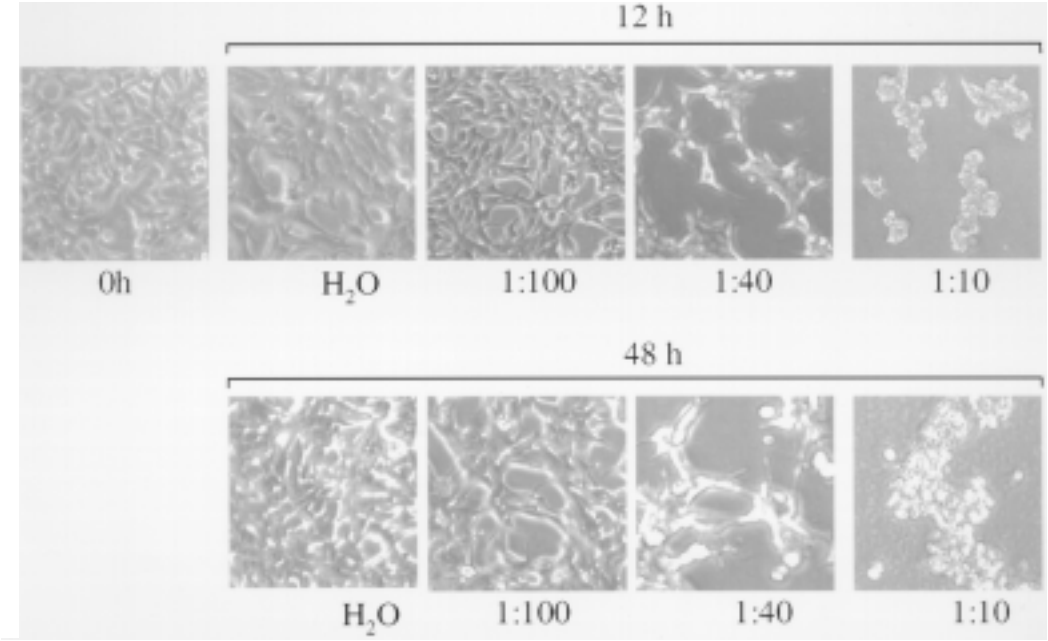

Figure 7. Morphological changes of the human prostate cancer cell LNCaP treated with S3 fraction. LNCaP cells were treated with H_2O (control) and different dilutions of S3 for the indicated hours to observe the morphological changes of cells. Untreated control cells are indicated as 0 h. Photographs are under a phase contrast microscope (x100).

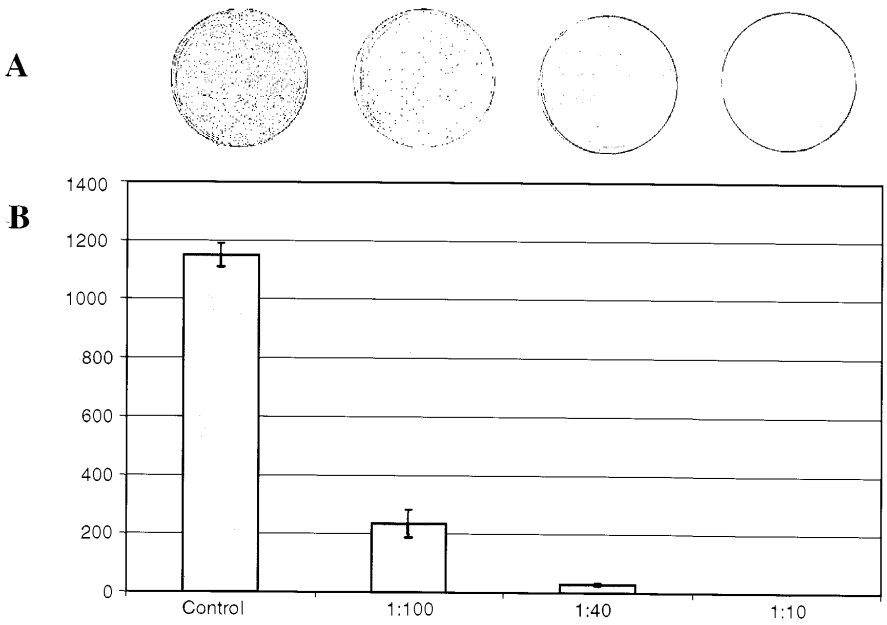

Figure 8. S3 inhibits soft agar colony formation. Prostate cancer LNCaP cells were plated in soft agar with H_2O (control) or different dilutions of S3 fraction as described in the Materials and methods. Cells were then cultured for 14 days without addition of new soup. A representative plate from each treatment was scanned and selected for presentation (A). Colonies were quantified with an automated counter and presented as mean values from triplicate independent experiments (B). Error bars denote standard deviations.

Inhibition of the proteasome activity and induction of apoptotic death were also observed in human prostate cancer cell line PC-3 and breast cancer cell line MCF-7 after S3 treatment (unpublished data). These results are consistent with the conclusion that inhibition of the proteasome activity by CellQuest S3 fraction in intact solid tumor cells is associated with induction of apoptosis.

In addition to measuring molecular changes in LNCaP cells treated with S3 (Fig. 5), we also observed striking changes in their cellular morphology (Fig. 7). At the start of treatment, LNCaP cells were elongated but became spherical with time increasing (Fig. 7). This morphological change appeared within 12 h with 1:40 and 1:10 dilutions of S3, and the number of cells displaying such changes increased with increased time of incubation (Fig. 7). At a lower dilution (1:100), a little cellular disintegration observed at 12 h and it became prominent at 48 h (Fig. 7). Along with the appearance of spherical cells, disintegrated cells and cells with condensed

nuclear chromatin appeared in time- and dilution-dependent fashion. The spherical morphology preceded the appearance of apoptotic cells (Fig. 7). In the same experiment, H_2O was used as a negative control, and the control cells did not exhibit any morphological changes at either time points (Fig. 7).

Inhibition of prostate cancer cell colony formation by S3 in soft agar assay. So far, we have demonstrated that CellQuest S3 fraction can inhibit the proteasome *in vitro* and *in vivo* and that inhibition of cellular proteasome activity is associated with apoptosis in tumor and transformed, but not normal or non-transformed cells (Figs. 1-7). We then wanted to test the effects of S3 fraction in soft agar assay, an analysis that mimics tumor growth in tissue. Prostate cancer LNCaP cells were plated in soft agar (Materials and methods) along with different dilutions of S3 or H_2O, followed by a 14-day incubation to allow for colony formation (Fig. 8). The H_2O-treated plates allowed for the development of ~1,150 colonies, which was inhibited by S3 in a concentration-dependent manner (Fig. 8). S3 at dilution 1:100, 1:40 and 1:10 inhibited formation of colonies by 80, 97 and 100%, respectively (Fig. 8). These data demonstrate that CellQuest S3 fraction can inhibit prostate cancer cell growth and colony formation in a semi-*in situ* assay.

Discussion

Plants consumed by humans contain thousands of polyphenolic compounds. The effect of dietary polyphenols has recently drawn attention of the medical scientific community. An extract from the *Musaceas* (plantain/banana) plant, named CellQuest and formulated by CellQuest, Inc., contains high level of TA (32). Previously, we reported that TA was a potent proteasomal chymotrypsin-like inhibitor and an apoptosis inducer in tumor cells (20).

In this study, we investigated the effects of CellQuest on proteasome and tumor cell sensitivity. We found that fractions S1, S2 and S3, although prepared in different ways (Materials and methods), had similar potency in inhibiting the proteasomal chymotrypsin-like activity *in vitro* (Fig. 1). The amount of a fraction (~1:30 dilution) required to inhibit 50% the proteasomal chymotrypasin-like activity was equivalent to 11.5 µg/ml TA, based on their absorbance at 280 nM (unpublished data). However, the same inhibitory effect was observed with only ~2 µg/ml purified TA. These results suggest that CellQuest contains different forms of TA and/or different compounds that have proteasome-inhibitory activity.

In addition, CellQuest S3 fraction was also able to inhibit the proteasome activity in intact tumor or transformed cells, as shown by increased levels of ubiquitinated proteins and the natural proteasome target p27 (Figs. 3-5). The following arguments are consistent with the idea that inhibition of the proteasome activity by CellQuest is functional in human tumor or transformed cells. First, when Jurkat T, VA-13 or LNCaP cells were treated with different dilutions of S3, accumulation of ubiquitinated proteins at 12 h and p27 at 48 h was concentration-dependent (Figs. 3-5). Decreased ubiquitinated protein levels at 48 h were probably due to increased activities of deubiquitination enzymes. We also noted that the accumulation of p27 at 12 h was not concentration-dependent (Figs. 3-5), which was probably due to the fact that TA transiently inhibits the proteasomal activity. Second, PARP cleavage was time- and concentration-dependent (Figs. 3-5). Third, caspase 3 activation was concentration-dependent (Fig. 5E). Lastly, cell death was time- and concentration-dependent (Figs. 2, 6 and 7). Inhibition of tumor cellular proteasome by CellQuest occurs prior to apoptosis, consistent with the idea that the proteasome is required for tumor cell survival.

Pronounced morphological changes correlated with induction of apoptosis by different dilutions of S3 (Fig. 7). LNCaP cells became spherical and irregularly shaped. It has been shown that apigenin, a flavone, can induce G_2/M phase arrest and morphological elongation and arborization of neutrites in rat neuronal cells (36). Genistein, a natural isoflavonoid phytoestrogen, also blocks the cell cycle at the G_2/M transition and induces morphological changes (37). Genistein-treated LNCaP cells developed denderite-like structure that became progressively elongated over time (37). In addition to the appearance of these spherical and irregularly-shaped cells, LNCaP cells exhibited apoptotic characteristics, namely chromatin condensation, nuclear fragmentation, and cell shrinkage, after longer incubation and/or with higher concentrations of S3 (Fig. 7).

One of the important criteria for potential anticancer drugs is the ability to selectively kill tumor, but not normal cells. In this study we found that CellQuest S3 inhibited the proteasomal activity and induced apoptosis selectively in human Jurkat cells, but not non-transformed, immortalized human NK cells (YT) in a time- and concentration-dependent manner (Fig. 3). In addition, S3 was able to inhibit the proteasomal activity and induce cell death preferentially in SV40-transformed (VA-13), but not the normal parental, human fibroblasts (WI-38) (Fig. 4). This study is consistent with our previous studies that showed that green tea polyphenols can selectively inhibit tumor cell growth in human tumor and transformed cells (21).

Malignant transformation of a cell leads to tumor formation and metastasis. In order for tumor development, cancer cells have to form a node *in vivo* as the first step. The desired effect of any anticancer drug is to inhibit tumor growth and formation *in situ*. Soft agar colony formation is an assay that has been developed to mimic tumor cellular growth in tissue. We hypothesized that the S3 that induce cell death should be able to inhibit colony formation in soft agar assay. Indeed, when LNCaP prostate cancer cells were cultured in the presence of S3, inhibition of colony growth and formation was observed in dilution-dependent manner, as compared to control cells. Higher dilutions of S3 can completely inhibit colony formation (Fig. 8). This study is consistent with other studies demonstrating that plant extract or purified plant polyphenols have anticancer activity and inhibit colony formation (11,38).

In conclusion, our study suggests that the anticancer effect of CellQuest (32) may be due to inhibition of proteasomal activity and consequent induction of apoptosis. In addition, it appears that human tumor and transformed cells are much more sensitive to CellQuest-mediated proteasome inhibition and apoptosis induction than normal and non-

transformed cells. This selectivity may also contribute to the antitumor activity of CellQuest (32). It should be noted that as a plant extract, CellQuest contains a variety of compounds that may act on different pathways of tumor cell growth and survival. Inhibition of the proteasome activity may be only one of the pathways mediated by CellQuest. Our study provides further evidence that plant phytochemical-containing polyphenolic compounds might be used as potential chemo-prevention agents.

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