# Inhibition of the proteasome activity, a novel mechanism associated with the tumor cell apoptosis-inducing ability of genistein

Aslamuzzaman Kazi[a], Kenyon G. Daniel[a], David M. Smith[a],
Nagi B. Kumar[b], Q. Ping Dou[a],*

[a]*Drug Discovery Program, H. Lee Moffitt Cancer Center & Research Institute, Departments of Interdisciplinary Oncology,
Biochemistry & Molecular Biology, College of Medicine, University of South Florida, Tampa, FL 33612, USA*
[b]*Cancer Control Division, H. Lee Moffitt Cancer Center & Research Institute, Departments of Interdisciplinary Oncology,
Biochemistry & Molecular Biology, College of Medicine, University of South Florida, Tampa, FL 33612, USA*

## Abstract

Epidemiological studies have suggested that increased soy consumption is associated with reduced cancer occurrence. Genistein, a soy isoflavone, has been reported to inhibit the growth of human tumor cells although the involved molecular mechanisms are not clearly defined. Here we report that genistein inhibits the proteasomal chymotrypsin-like activity *in vitro* and *in vivo*. Computational docking studies suggest that the interaction of genistein with the proteasomal β5 subunit is responsible for inhibition of the chymotrypsin-like activity. Inhibition of the proteasome by genistein in prostate cancer LNCaP and breast cancer MCF-7 cells is associated with accumulation of ubiquitinated proteins and three known proteasome target proteins, the cyclin-dependent kinase inhibitor p27$^{Kip1}$, inhibitor of nuclear factor-κB (IκB-α), and the pro-apoptotic protein Bax. Genistein-mediated proteasome inhibition was accompanied by induction of apoptosis in these solid tumor cells. Finally, genistein induced proteasome inhibition and apoptosis selectively in simian virus 40-transformed human fibroblasts, but not in their parental normal counterpart. Our results suggest that the proteasome is a potential target of genistein in human tumor cells and that inhibition of the proteasome activity by genistein might contribute to its cancer-preventive properties.
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## 1. Introduction

Genistein is one of the simplest biosynthetic isoflavonoid compounds of the Leguminosae. It is synthesized in plants from the flavanone naringenin by a novel ring migration reaction catalyzed by the cytochrome p450 enzyme iso-flavone synthase (IFS) [1]. It is the predominant isoflavone phytoestrogen found in soy. Tofu, soy flour, soy milk, miso, soy sauce, tempeh, etc. are the major dietary sources of isoflavonoids for humans. It has been shown that 1 g of powdered soybean chips contains nearly 800 μg of daidzein and over 500 μg of genistein (primarily as glycosides), whereas 1 g of soy protein has approximately 150 μg of daidzein and 250 μg of genistein [1]. Bioavailability of dietary flavonoid components depends on relative uptake rates of conjugated and free forms, hydrolysis of glycosides by gut bacteria or gut wall enzymes, further metabolism, for example to glucuronides within the liver, and excretion rate. A recent animal study has shown that the principle meta-bolite of genistein that accumulates in the prostate is 2-(4-hydroxyphenyl)-propionic acid (2-HPPA) [2].

A growing body of epidemiological studies have shown that increased soy consumption is associated with a reduced risk of breast, colon, and prostate cancer in Asian compared with populations in the United States and Western countries [3,4]. Animal studies also found that both a soy diet and dietary genistein itself may confer a protective effect on mammary, colon, and skin tumors [5]. Different possible

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mechanisms have been suggested to be responsible for genistein-mediated antitumor activities. These include inhibition of tumor cell proliferation [6], angiogenesis [7], tumor cell invasion [8], and tumor metastasis [9], as well as its antioxidant [10] and apoptosis-inducing activities [11]. Furthermore, several potential molecular targets of genistein have been suggested in the literatures, including tyrosine kinase [12], tumor necrosis factor α [13], topoisomerase II [14], the enzymes involved in steroid metabolism (such as aromatase) [15], 17β-hydroxysteroid dehydrogenase and 5α reductase [16], c-*fos* [17], and microsomal lipid peroxidase [18]. Like other phytoestrogens, genistein also binds to the classical ER [19]. It has been shown that genistein has higher affinity to ER beta (ERβ) than ER alpha (ERα), which suggests that genistein-mediated ERβ signaling pathways may play an important role for its biological action [19]. However, the detailed molecular mechanisms and targets of genistein remain unclear.

Effects of genistein have been extensively studied in human prostate cancer cells. It has been shown that genistein is involved in cell cycle arrest caused by down-regulation of cyclin B1 and cdc25C, up-regulation of p21[Waf1] [20], apoptosis induction by activation of caspase-3 [21], NF-κβ inactivation [13], and DNA strand breakage [22]. In a recent study using human prostate cancer cell lines, it has been shown that genistein up-regulates the expression of the antioxidant enzyme glutathione peroxidase gene [23]. Although it appears that genistein elicits pleiotropic molecular effects on tumor cells including prostate cancer cells, a conclusive mechanistic pathway of genistein-mediated cancer prevention has not yet been identified.

The structural and metabolic integrity of cells is maintained by proteolysis. From yeast to man, proteasome is present in all the cells, where it plays the central role for degradation of the vast majority of intracellular proteins [24]. Typically, a protein must be targeted before it is degraded by the proteasome and this targeting process is called ubiquitination. The multi-catalytic 26S proteasome is the ubiquitous proteinase in eukaryotes responsible for the degradation of intracellular proteins [24]. The 20S proteasome, a multicatalytic complex (700 kDa), constitutes the catalytic component of the ubiquitous proteolytic machinery of the 26S proteasome [25–28]. It has been shown that the ubiquitin/proteasome-dependent protein degradation pathway plays an essential role to promote tumor cell proliferation and to protect tumor cells against apoptosis [25–28]. It has also been shown that the chymotrypsin-like, but not trypsin-like, activity of the proteasome is associated with tumor cell survival [29,30]. Cell proliferation and cell death regulators have been identified as targets of the ubiquitin/proteasome-mediated degradation pathway, including p53 [31], pRb [32], p21 [33], p27[Kip1] [34], IκB-α [35], and Bax [36]. Recently, it has been reported from our laboratory that ester bond-containing tea polyphenols, such as (−)-EGCG, potently and specifically inhib-
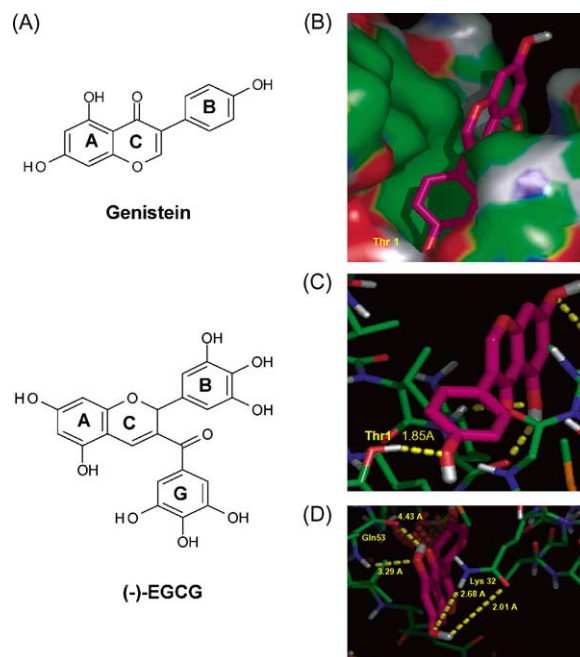


Fig. 1. Docking solution of genistein to the proteasomal β5 subunit. (A) Structure of genistein and (−)-EGCG. (B) Docked conformation of genistein showing the S1 pocket and the N-terminal threonine (Thr 1) of β5 subunit. (C) Distance of the B-ring hydroxyl of genistein from Thr 1. (D) Potential hydrogen bonds between genistein and backbone amino acids of the proteasome; the 'red spheres' in the image mark Thr 1. Energy of this dock is −5.15 kcal/mol.

ited the chymotrypsin-like activity of the proteasome *in vitro* ($IC_{50}$ 86–194 nM) and *in vivo* (1–10 μM) at the concentrations found in the serum of green tea drinkers [37].

Genistein consists of three rings, similar to those of A, C, and B in green tea polyphenol (−)-EGCG (Fig. 1A). Therefore, we hypothesize that genistein might be a proteasome inhibitor. Here we report that indeed genistein inhibits the chymotrypsin-like activity in purified 20S proteasome and 26S proteasome of LNCaP and MCF-7 cell extracts. Furthermore, inhibition of the proteasome by genistein in intact LNCaP and MCF-7 cells is associated with accumulation of ubiquitinated proteins and the known proteasome target proteins, including p27[Kip1], IκB-α, and Bax. Following genistein-mediated proteasome inhibition, apoptosis occurred. In addition, genistein also induced p53 protein accumulation, associated with increased levels of p53 downstream target proteins such as p21[Waf1]. Finally, the proteasome-inhibitory and apoptosis-inducing effects of genistein were observed only in SV40-transformed human fibroblasts (VA-13), but not in their parental normal lung fibroblast counterpart (WI-38).

## 2. Materials and methods

### 2.1. Materials

Highly purified Gen, Gen-G, Gly, Gly-G, Daid, Daid-G, and ADG were purchased from LC Laboratories and used

directly without additional purification. Fetal calf serum, propidium iodide, RNase A, and DMSO were purchased from Sigma–Aldrich. RPMI 1640 medium, Dulbecco's modified Eagle's medium, penicillin, and streptomycin were purchased from Invitrogen. Purified 20S proteasome (rabbit) was purchased from Boston Biochem. Fluorogenic peptide substrate Suc-Leu-Leu-Val-Tyr-AMC (for the proteasomal chymotrypsin-like activity) was obtained form Calbiochem. Monoclonal antibodies to p21[Waf1] and p27[Kip] were purchased from PharMingen; polyclonal antibodies to IκB-α, Bax (N20), ubiquitin (P4D1) and actin were obtained from Santa Cruz Biotechnology Inc. Mouse monoclonal antibody to p53 (Ab6) was obtained from Oncogene Research Products. Rabbit polyclonal antibody to human PARP was from Roche Applied Science.

## 2.2. Cell culture and extract preparation

Human 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 of streptomycin. Human breast cancer MCF-7 cells, normal (WI-38) and SV40-transformed (VA-13) human fibroblast cells were grown in Dulbecco's modified Eagle's medium containing 10% fetal calf serum, 100 units/mL of penicillin, and 100 μg/mL of streptomycin. All cells were maintained at 37° in a humidified incubator with an atmosphere of 5% $CO_2$. A whole cell extract was prepared as described previously [38]. 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°. After that, the lysates were centrifuged at 12,000 $g$ for 30 min, and the supernatants were collected as whole cell extracts.

## 2.3. Inhibition of purified 20S proteasome activity by genistein

The chymotrypsin-like activity of purified 20S proteasome was measured as follows. Briefly, 0.1 μg of purified 20S rabbit proteasome was incubated with 20 μM fluorogenic peptide substrate Suc-Leu-Leu-Val-Tyr-AMC (for the proteasomal chymotrypsin-like activity) for 30 min at 37° in 100 μL of assay buffer (50 mM Tris–HCl (pH 7.5)) with or without genistein. After incubation, production of hydrolyzed AMC groups were measured using a multi-well plate VersaFluor[TM] Fluorometer with an excitation filter of 355 nm and an emission filter of 460 nm (Bio-Rad).

## 2.4. Inhibition of proteasome activity in whole cell extracts by different soy compounds

A whole cell extract (10 μg) of LNCaP and MCF-7 cells were incubated for 60 min at 37° with 20 μM of fluorogenic peptide substrate Suc-Leu-Leu-Val-Tyr-AMC in 100 μL of the assay buffer with either genistein or another indicated isoflavone. The hydrolyzed AMCs were quantified as described above.

## 2.5. Western blot analysis

LNCaP, MCF-7, VA-13, and WI-38 cells were treated with an indicated concentration of genistein or different soy isoflavones for indicated hours (see figure legends). After that cells were harvested and lysated. Cell lysates (60 μg) were separated by an SDS–PAGE and electrophoretically transferred to a nitrocellulose membrane, followed by enhanced chemiluminescence Western blotting. The enhanced chemiluminescence (ECL) Western blot analysis was performed using specific antibodies to p27[Kip], IκB-α, Bax, p53, p21[Waf1], ubiquitin, PARP and actin, as described previously [29].

## 2.6. Flow cytometry

Cell cycle analysis based on DNA content was performed as we described previously [29]. At each time point, cells were harvested, counted, and washed twice with PBS. Cells ($5 \times 10^6$) were suspended in 0.5 mL PBS, fixed in 5 mL of 70% ethanol for over night at $-20°$, 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), ModFit LT and WinMDI V.2.8 cell cycle analysis software (Verity Software). The cell cycle distribution is shown as the percentage of cells containing $G_1$, S, $G_2$, and M DNA judged by propidium iodide staining. The apoptotic population is determined as the percentage of cells with sub-$G_1$ ($<G_1$) DNA content.

## 2.7. Molecular modeling and docking studies

The crystal structure of the eukaryotic yeast 20S proteasome was obtained from the Protein Data Base [39], and used for the docking studies. 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 [40]. The Autodock Tools suite of programs (Autotors, Autogrid, and Autodock) employs an automated docking approach allowing ligand flexibility described to a full extent elsewhere [41]. Autodock has been found to produce docked conformations that predict X-ray crystal structures with bound ligands [42,43]. Default parameters were used as described in the Autodock manual except as noted below. The Autodock software was run on an i386 architecture computer operating with the Redhat Linux 6.0[TM] operating system. The crystal structure of the 20S proteasome and genistein were prepared for

docking by following the default protocols except were noted. A $20\,\text{Å} \times 20\,\text{Å} \times 20\,\text{Å}$ box centered on the β5 catalytic N-terminal threonine served as the scoring grid and genistein was limited to this search space during docking. The number of docking runs was set to 100. For the Lamarckian genetic algorithm [41], the maximum number of energy evaluations were changed to 5 million. The default parameters were kept for mutation, crossover, and elitism. The pseudo-Solis and Wets local search method was used. Autodock arranged dockings by cluster ranks of docking conformations and free energy from the lowest free energy conformation to the highest. The selected docking for genistein was the cluster rank that provided the greatest number of members and not just the lowest free energy dock. In the case of genistein 60 out of 100 dockings finished in the same conformation and this docking was chosen as the most likely binding mode for genistein and analyzed. Structural output from Autodock was visualized using PyMOL [44] on a computer running the Windows98[TM] operating system.

## 3. Results

### 3.1. Computational modeling of genistein binding to the chymotrypsin site of 20S proteasome

The proteasomal β5 subunit is responsible for its chymotrypsin-like activity (cleavage after hydrophobic residues), which depends on the presence of the N-terminal Thr (Thr 1) residue [25]. In addition, an S1 pocket of the β5 subunit defined by the hydrophobic residues plays an important role in the substrate specificity and proteasome inhibitor binding [45,46].

Most recently, we have established, for the first time, a computational molecular model that shows how an ester bond-containing green tea polyphenol such as (–)-EGCG binds and targets the chymotrypsin site of the proteasome β5 subunit in a conformation suitable for a nucleophilic attack.[1] Similar to (−)-EGCG, genistein also consists of three rings, A, C, and B and phenolic hydroxyl groups (Fig. 1A), suggesting that genistein might be a proteasome inhibitor. But different from (−)-EGCG, genistein lacks the gallate group (Fig. 1A), which allowed us to predict that genistein would be less potent than (−)-EGCG.

To test this hypothesis, we first docked genistein to the yeast 20S proteasome. We found that in 60 out of 100 runs with 5-million energy evaluations, genistein docks primarily in the S1 pocket of the active site of the proteasome β5 subunit. Figure 1B shows this conformation as an example.

Our computational docking data also shows that the hydroxyl group of the B-ring of genistein lies in close proximity to Thr 1 with a distance of 1.85 Å; this interaction may sterically block Thr 1 (Fig. 1C). As a comparison, the ester bond-carbon of (−)-EGCG is situated at 3.18 Å from Thr 1 that is suitable for a nucleophilic attack.[1] Since genistein lacks a group that can undergo a nucleophilic attack, unlike the ester bond-carbon of (−)-EGCG, it is unlikely that genistein could covalently modify Thr 1 of β5 subunit of the proteasome.

Both genistein and (−)-EGCG place their A–C rings into the S1 pocket[1] (Fig. 1B). However, different from (−)-EGCG, the C-ring of genistein has a double-bonded oxygen which was placed into the hydrophobic S1 pocket (Fig. 1A and B). The placement of this oxygen into the pocket shifts the rings upwards from the pocket slightly. Furthermore, the presence of the oxygen in this hydrophobic environment may reduce the stability of genistein to remain in this position. By contrast, (−)-EGCG lacks this doubled-bonded oxygen and would not suffer a loss of binding stability[1] (Fig. 1A).

There are four potential hydrogen bonds that could be formed within the complex of genistein and the proteasomal β5 subunit (Fig. 1D). The backbone hydrogen on the nitrogen of Gln 53 and the backbone oxygen of Gln 53 appear to be at the appropriate distance (3.29 and 4.43 Å, respectively) to hydrogen bond with the first hydroxyl group of A-ring of genistein (Fig. 1A and D). Likewise, the backbone hydrogen of the nitrogen of Lys 32 and its oxygen appear to be at the proper distance (2.01 and 2.68 Å, respectively) to hydrogen bond with the ortho-hydroxyl group of A-ring (Fig. 1D). This contrasts with the eight potential hydrogen bonds that could form within the complex of (−)-EGCG and the proteasome β5 subunit.[1]

Lastly, the docked free energy of binding for genistein to the β5 subunit is –5.15 kcal/mol, higher than that of (−)-EGCG[1] (−10.52 kcal/mol). The higher predicted free binding energy for genistein suggests that binding of genistein to β5 subunit is less stable (though still spontaneous) than that of (−)-EGCG.

Therefore, when compared to (−)-EGCG, it is apparent that genistein lacks covalent modification of Thr 1 and the extensive hydrogen-bond stabilization. In addition, there is potential disruption of genistein binding in the S1 pocket and a greater free energy of binding than (−)-EGCG possesses[1] (Fig. 1). These docking studies suggest that genistein is a weaker proteasome inhibitor than the green tea polyphenol (−)-EGCG.

### 3.2. Inhibition of chymotrypsin-like activity of purified 20S proteasome by genistein

To test whether genistein inhibits the proteasomal activity, we performed a cell-free proteasome activity assay with purified 20S rabbit proteasome in the absence or

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[1] Smith DM, Daniel KG, Wang Z, Guida WC, Chan TK, Dou QP. Development of tea polyphenol proteasome inhibitors: applications to rational drug design, 2003 (accepted with minor revision).
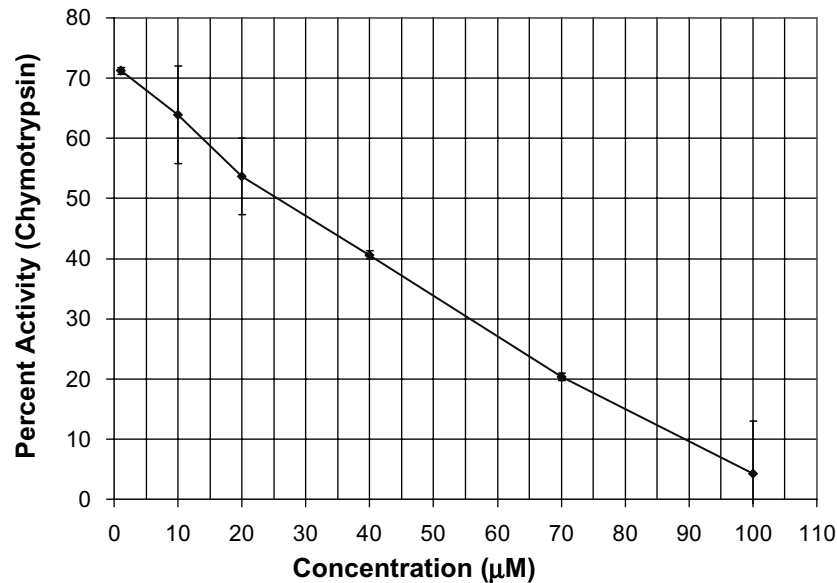
Fig. 2. Inhibition of purified 20S proteasome activity *in vitro* by genistein. 0.1 μg of purified rabbit 20S proteasome was incubated with 20 μM flurogenic peptide substrate (for the chymotrypsin-like activity) with genistein at various concentrations, as described in Section 2. Concentration-dependent inhibition of purified 20S proteasome by genistein is plotted. All values are means of independent triplicate experiments. Error bars denote standard deviations.

presence of genistein at various concentrations. The result in Fig. 2 demonstrates that genistein inhibits the chymotrypsin-like activity of purified 20S proteasome in a concentration-dependent manner: ∼30% inhibition at 1 μM. The $IC_{50}$ value was found to be 26 μM (Fig. 2).

### 3.3. Inhibition of the proteasomal chymotrypsin-like activity by genistein in solid tumor cell extracts

We then tested if genistein could inhibit the 26S-proteasome activity in tumor cell extracts. Protein extracts were prepared from exponentially growing human prostate cancer LNCaP and breast cancer MCF-7 cells, and used in the cell-free proteasome activity assay. We found that genistein also inhibited the proteasomal chymotrypsin-like activity in these tumor cell extracts in a concentration-dependent manner: ∼50% inhibition at 70 and 65 μM, respectively (Fig. 3A). The concentrations of genistein needed to inhibit the proteasome activity in LNCaP and MCF-7 cell extracts (Fig. 3A) were higher than were needed for the inhibition of purified 20S proteasome (Fig. 2), suggesting the binding of genistein to cellular proteins or degradation of genistein by cellular enzymes (see Section 4). These results are consistent with our previous study where higher concentrations of (−)-EGCG are necessary for inhibition of the proteasome activity in cell extracts than the purified 20S proteasome [37]. Furthermore, when different soy isoflavones were tested for the proteasome-inhibitory activity in LNCaP cell extract, genistein was found to be the most potent, followed by 6″-*O*-acetyldaidzin, genistin, glycitein, and daidzen (Fig. 3B). Under the same experimental conditions, the tripeptidyl proteasome inhibitor LLnL and (−)-EGCG were found to be more potent than genistein (Fig. 3B).
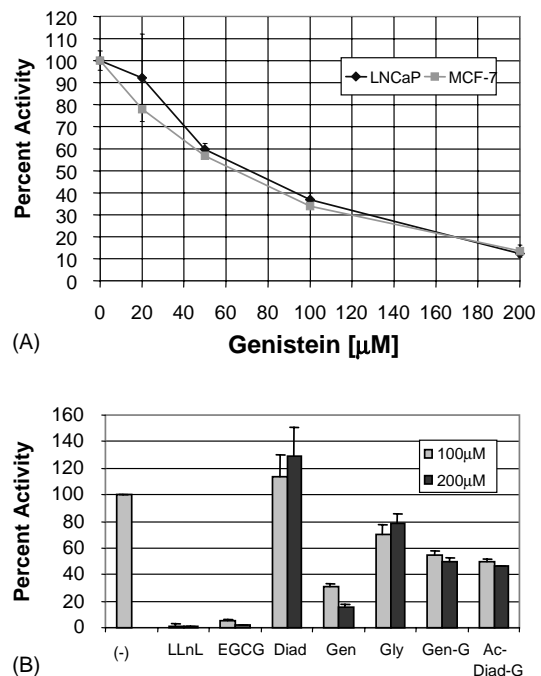


(A)



(B)

Fig. 3. Inhibition of proteasomal chymotrypsin-like activity by isoflavones in solid tumor cell extracts. (A) LNCaP and MCF-7 cell extracts (10 μg) were incubated for 1 hr at 37° with 20 μM fluorogenic peptide substrate for the proteasomal chymotrypsin-like activity, in the absence or presence of genistein at indicated concentrations, followed by measurement of free AMC groups. The "0" indicates without addition of genistein. (B) LNCaP cell extract (10 μg) was incubated for 1 hr at 37° with 20 μM fluorogenic peptide substrate for the proteasomal chymotrypsin-like activity, in the absence or presence of different soy compounds at 100 or 200 μM, followed by measurement of free AMC groups. The (−) symbol indicates without addition of any soy compound. LLnL and (−)-EGCG were used as positive controls. All values are means of independent triplicate experiments. Error bars denote standard deviations.

### 3.4. Inhibition of the proteasomal activity by genistein in intact prostate and breast cancer cells

We then tested whether genistein could inhibit tumor cellular proteasome activity. Both kinetics and genistein concentration-dependent experiments were performed using prostate cancer LNCaP cells, followed by measuring levels of p27$^{Kip1}$, IκB-α and Bax, three well-known target proteins of the proteasome [34–37]. Treatment of LNCaP cells at 100 μM of genistein increased accumulation of p27$^{Kip1}$ in a time-dependent manner: by 2-, 2-, and 4-fold at 12, 24, and 48 hr treatment, respectively, compared to untreated (0 hr) control cells (Fig. 4A). Genistein treatment also induced expression of IκB-α by 7-, 8-, and 6-fold at 8, 12, and 24 hr, respectively (Fig. 4A). Like p27 and IκB-α, Bax levels were also increased by up to 4-fold after 4–24 hr treatment (Fig. 4A). Inhibition of proteasome activity by genistein should also increase the level of polyubiquitinated proteins, because most of the proteasome-mediated protein degradation pathways require ubiquitination [25–28]. Indeed, when the LNCaP cell lysates were immunoblotted with an antibody to ubiquitin, increased levels of ubiquitinated proteins were detected in a time-dependent manner (Fig. 4A). The highest level of ubiquitinated proteins was detected at 48 hr (Fig. 4A).

When LNCaP cells were treated with genistein at different concentrations, we also observed a concentration-dependent accumulation of p27$^{Kip1}$ (data not shown) and IκB-α (Fig. 4B). Genistein at 50, 100, and 200 μM increased IκB-α levels by 12–16-fold, compared to the control cells (Fig. 4B). Treatment of breast cancer MCF-7 cells for 48 hr with 100 μM of genistein also increased expression of p27$^{Kip1}$ (by 13-fold) and IκB-α as well as a putative ubiquitinated form of IκB-α (indicated by an arrow) in comparison to vehicle (DMSO)-treated cells
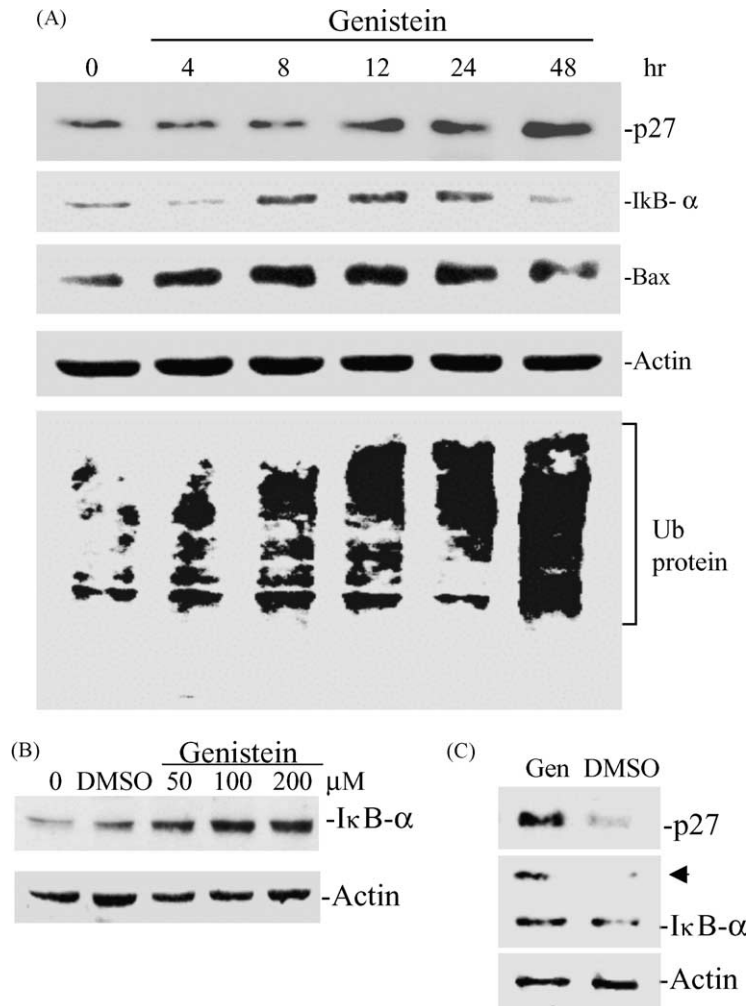


Fig. 4. Accumulation of p27, IκB-α, Bax, and ubiquitinated proteins by genistein. Exponentially growing LNCaP cells (0 hr) were treated with 100 μM of genistein for the indicated hours (A) or indicated concentrations for 24 hr (B), or MCF-7 cells treated with 100 μM of genistein for 48 hr (C). The solvent DMSO was used as a negative control. After each treatment, Western blot assay was performed using specific antibodies to p27, IκB-α, Bax, ubiquitin or actin. Molecular masses of IκB-α, Bax, and actin are 40, 21 and 43 kDa, respectively. The bands indicated in (A) are ubiquitin-containing proteins (Ub protein). Data are representatives of at least three independent experiments.

(Fig. 4C). These data support the conclusion that genistein inhibits the proteasome activity in intact solid tumor cells.

### 3.5. Induction of apoptotic cell death in prostate cancer cells by genistein

Treatment of LNCaP cells with 100 μM of genistein showed apoptosis-specific cleavage of PARP at 8 hr, which then further increased after 12–24 hr, but decreased slightly at 48 hr (Fig. 5A). The decreased apoptosis at 48 hr was probably due to the decreased cellular concentrations of genistein by metabolism and/or tumor cell recovery. In addition, when LNCaP cells were treated with various concentrations of genistein for 24 hr, a concentration-dependent induction of PARP cleavage was observed (Fig. 5B).

To further examine the tumor cell-killing effects of genistein, we performed flow cytometry analysis using LNCaP cells treated with 100 μM genistein for up to 48 hr (Fig. 6). Again, we observed a time-dependent increase in the cell population containing sub-$G_1$ DNA content ($<G_1$), a measurement of cell death-associated DNA degradation: 4% increase at 12 hr, 29% at 24 hr, and 37% at 48 hr (Fig. 6). No significant changes were observed in the cell cycle distribution before 24 hr (Fig. 6).

Therefore, genistein-induced cell death was time- and concentration-dependent.

### 3.6. Effects of other isoflavones in prostate cancer cells

We then treated LNCaP cells with each of seven different isoflavones at 50 μM for 24 hr, using the vehicle DMSO as a negative control. The cell lysates were immunoblotted with antibodies to p27$^{Kip1}$, Bax and PARP. Increased levels of p27$^{Kip1}$ were also observed with daidzein (by 14-fold), daidzin (by 10-fold), and glycitin (7-fold), in addition to genistein (6-fold) (Fig. 5C). In this experiment, vehicle (DMSO) had little effect (Fig. 5C). Note that accumulation of p27$^{Kip1}$ by isoflavones is probably a transient process (see Fig. 4) and the peak levels of p27$^{Kip1}$ induced by different isoflavones might therefore be found at different times. In comparison to other isoflavones, genistein showed higher induction (by 12-fold) of Bax accumulation at 36 hr (Fig. 5C). Consistent with this, the highest levels of p85/PARP cleavage fragment were observed with genistein, followed by daidzein and glycitein (Fig. 5C). These results are consistent with the previous reports that genistein is the most potent soy isoflavone [7,47].
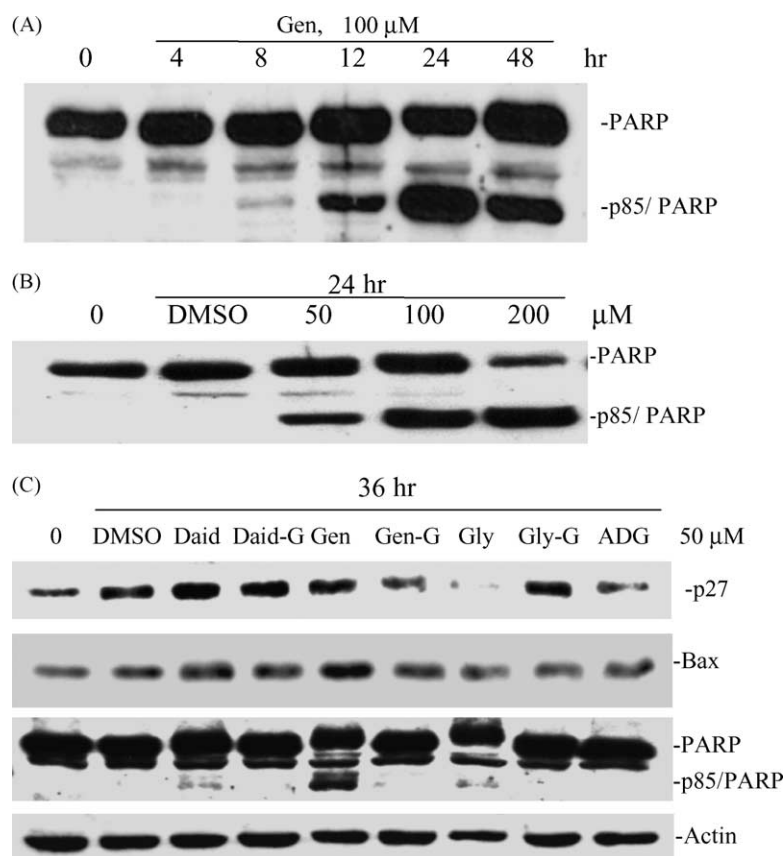


Fig. 5. Time- and concentration-dependent induction of apoptosis by genistein in LNCaP cells. Exponentially growing LNCaP cells (0 hr) were treated with 100 μM of genistein for the indicated hours (A) or indicated concentrations for 24 hr (B), or treated with 50 μM of different soy compounds for 36 hr (C), followed by Western blot assay using specific PARP, p27, Bax, or actin antibodies. Molecular mass of intact PARP is 116 kDa and a cleaved PARP fragment is 85 kDa. Data are representatives of at least three independent experiments.
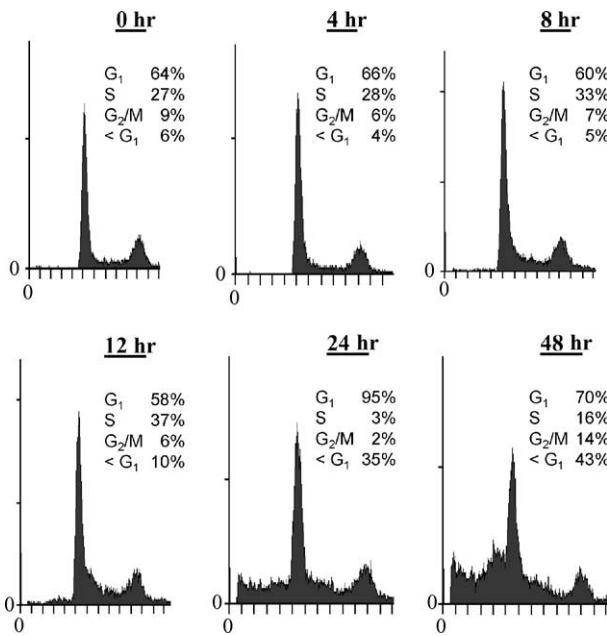
Fig. 6. Measurement of sub-$G_1$ (<$G_1$) cell population after genistein treatment. Exponentially growing LNCaP cells (0 hr) were treated with 100 μM of genistein for the indicated hours, followed by flow cytometry analysis. The cell cycle distribution was measured as the percentage of cells that contain $G_1$, S, $G_2$, and M DNA (cells in $G_1$, S, $G_2$, and M = 100%) and cell death-associated DNA degradation is measured by the increase in the percentage of cell population with <$G_1$ DNA content (<$G_1$). Similar results were observed in three independent experiments.
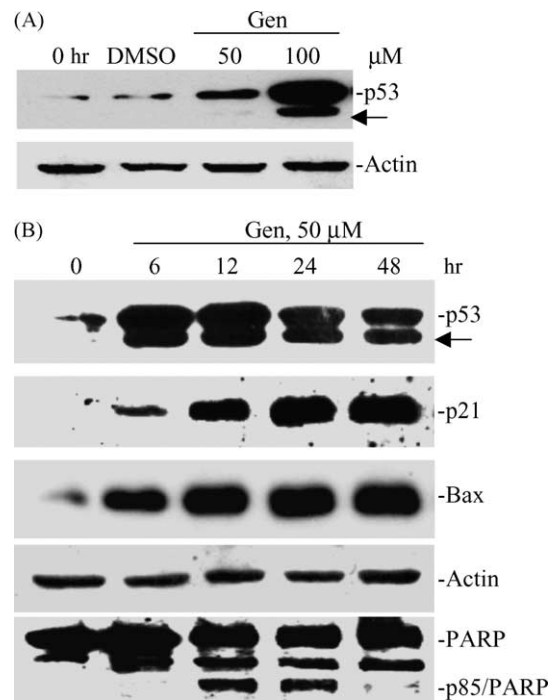


Fig. 7. Accumulation of p53 and its down stream target proteins by genistein prior to induction of apoptosis. LNCaP cells were treated with 50 or 100 μM of genistein for 24 hr with DMSO as a control (A), or with 50 μM of genistein for the indicated hours (B), followed by Western blot assay using specific antibodies to p53, p21, Bax, PARP and actin. A cleaved form of p53, with molecular mass 48 kDa, is indicated by an arrow. Data are representatives of at least three independent experiments.

## 3.7. Induction of p53 by genistein in prostate cancer LNCaP cells

Prostate cancer LNCaP cells express wild-type p53 gene [48] and genistein was shown to induce p53-dependent apoptosis [49–51]. We also investigated whether genistein could affect p53 levels under our experimental conditions. Consistent with previous reports [49–51], when LNCaP cells were treated with 50 or 100 μM of genistein for 24 hr, levels of p53 significantly increased by 11.5- or 25-fold, respectively, in comparison to the untreated control cells (Fig. 7A). In addition, genistein treatment also greatly increased expression of another form of p53 with faster mobility (~48 kDa; Fig. 7A), which has been demonstrated to be a calpain-cleaved fragment of p53 with increased activity [52]. In a kinetic experiment, treatment of LNCaP cells with 50 μM of genistein for 6 hr significantly increased levels of p53 (by 25-fold) and its cleaved form (Fig. 7B). Induction of both wild-type (12-fold) and cleaved p53 were also observed at 12 hr, which were decreased at 24 and 48 hr (to ~4-fold; Fig. 7B). The genistein-induced p53 is probably functional since p21 and Bax, two p53 target gene products [53,54], were also significantly increased in a time-dependent manner (Fig. 7B). Levels of p21 increased by 18-, 56-, 87-, and 73-fold, respectively, after 6, 12, 24 or 48 hr treatment (Fig. 7B). In addition, expression of Bax protein was increased by up to 24-fold

after genistein treatment (Fig. 7B). It appears that Bax accumulation by genistein is due to activation of p53-mediated transcription (Fig. 7B) and/or inhibition of proteasome-mediated degradation (Figs. 4 and 5). Importantly, apoptosis-specific PARP cleavage was observed at as early as 12 hr, which occurred after induction of p53, p21 and Bax (Fig. 7B), consistent with the previous reports [49–51] that induction of p53 is involved in genistein-mediated prostate cancer cell apoptosis.

## 3.8. Normal human WI-38 fibroblasts are more resistant to genistein-induced proteasome inhibition and apoptosis than their SV40-transformed counterpart

Previously, we have shown that proteasome inhibitors selectively induce apoptosis in tumor and transformed human cells [29,37]. To investigate whether genistein has any differential effects on transformed over normal human cells, the normal human fibroblast cell line WI-38 and its SV40-transformed derivative (VA-13) were treated with 100 μM genistein for up to 48 hr, followed by the measurement of proteasome inhibition and apoptosis induction.

Similar to LNCaP prostate cancer cells (Fig. 4A), the treatment of the transformed VA-13 cells with genistein increased the level of ubiquitinated proteins (Fig. 8A), indicating inhibition of the proteasomal activity. The increase in ubiquitinated proteins was transient and peaked
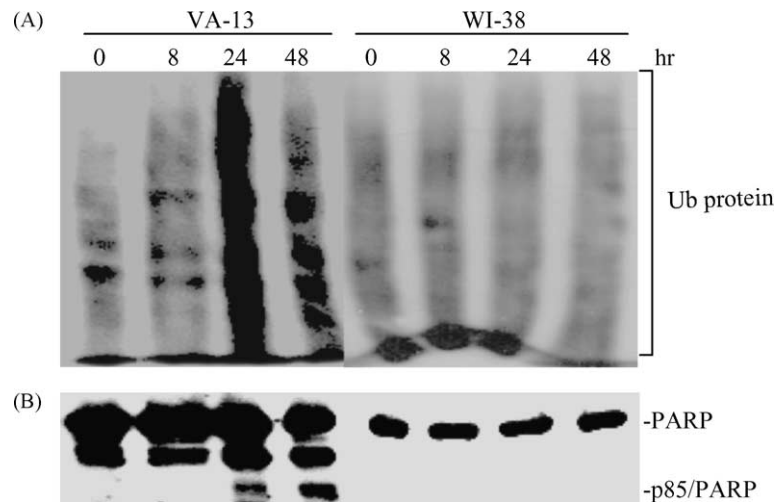
Fig. 8. Preferable accumulation of ubiquitinated proteins and PARP cleavage by genistein in the transformed fibroblasts *over* the normal human fibroblasts. Exponentially growing normal (WI-38) and SV40-transformed (VA-13) human fibroblasts (0 hr) were treated with 100 μM of genistein for the indicated hours, followed by Western blot assay using specific antibodies to ubiquitin and PARP. Data are representatives of at least three independent experiments.

at 24 hr (Fig. 8A). Associated with proteasome inhibition, apoptosis occurred in VA-13 cells after genistein treatment. The cleaved PARP fragment p85 was first detected at 24 hr and its level was further increased after 48 hr treatment (Fig. 8B). In contrast, genistein treatment of normal WI-38 cells for up to 48 hr did not increase levels of ubiquitinated proteins and failed to induce apoptosis (Fig. 8A and B). These data suggest that genistein could inhibit the proteasome activity and induce cell death selectively in the transformed *over* the normal human fibroblasts.

## 4. Discussion

To date, the molecular mechanism for the genistein's cancer-preventive effects is poorly understood. In this study, we report that genistein was able to inhibit the proteasomal activity both *in vitro* and *in vivo* (Figs. 2–4). Among different soy compounds genistein was the most potent inhibitor of the proteasomal chymotrypsin-like activity (Fig. 3B). Inhibition of proteasome activity by genistein in LNCaP and MCF-7 cells was associated with increased levels of p27, IκB-α, Bax, and ubiquitinated proteins, accompanied by induction of apoptotic cell death (Figs. 4–6). We also reported that genistein selectively accumulated ubiquitinated proteins and induced apoptosis in SV40-transformed over normal human fibroblasts (Fig. 8). Our results suggest that the proteasome is a potential target of genistein in human tumor cells and that inhibition of the proteasome activity by genistein might contribute to its cancer-preventive properties.

Recently, we reported that ester bond-containing tea polyphenols, such as (−)-EGCG, inhibited the chymotrypsin-like activity of the proteasome [37]. Due to structural similarities of genistein to (−)-EGCG (Fig. 1A), we hypothesized that genistein might have proteasome-inhibitory activity. Both computational docking and *in vitro* proteasome activity studies confirmed that genistein is a proteasome inhibitor, with potency weaker than green tea polyphenol (−)-EGCG[1] (Figs. 1–3). The $IC_{50}$ value of (−)-EGCG to a purified 20S proteasome is found to be in a range of 0.1–0.2 μM [37], which were similar to the concentrations found in the serum of green tea drinker [55]. We also found that genistein at 1 μM could inhibit ∼30% of the chymotrypsin-like activity of purified 20S proteasome (Fig. 2). It has been reported that plasma levels of genistein are in a range of 0.5–2.5 μM and the concentrations of genistein also vary in different tissues and organs [56,57]. It is, therefore, possible that a partial inhibition of the proteasome activity by genistein at a physiological concentration might contribute its reported cancer-preventative effects [3–5].

When used in human prostate or breast cancer cell extracts, the $IC_{50}$ value of genistein to inhibit the proteasomal chymotrypsin-like activity increased (Fig. 3A), suggesting binding of genistein to cellular proteins and/or enzymatic degradation of genistein. Consistently, 50–100 μM of genistein was able to inhibit the proteasome activity in intact tumor cells (Fig. 4). These results are consistent with the previous studies, where higher concentrations of other proteasome inhibitors, even specific ones, are needed to inhibit the cellular proteasome. For example, the $IC_{50}$ value of the specific proteasome inhibitor *clasto*-lactacystin-β-lactone was in a range of 0.1–0.6 μM to inhibit the chymotrypsin-like activity of purified 20S proteasome [37,58]. However, only 20% of the proteasomal chymotrypsin-like activity was inhibited by 10 μM of β-lactone when used in intact Jurkat T cells [37]. Also, we have shown that the $IC_{50}$ value of (−)-EGCG was 0.1–0.2 μM to inhibit a purified 20S proteasomal chymotrypsin-like activity. However, 1–10 μM

of (−)-EGCG was needed to inhibit the proteasome activity in living Jurkat T cells [37].

The concentrations of genistein (50–200 μM) we used in prostate and breast cancer cells are similar to those other researcher used in various cell culture systems. Constantinou *et al.* [59] have reported that genistein at 100–300 μM induces apoptosis in MCF-7 cells within 2 days. In another study using MCF-7 cells, Xu and Loo [50] have reported that genistein at 50 μM can induce apoptosis with prolonged incubation. Zhou *et al.* [60] reported an $IC_{50}$ value of genistein was >50 μM in human prostate cancer cell lines LNCaP, DU-145, and PC-3, although *in vitro* growth of vascular endothelial cells was inhibited by genistein at lower concentrations. Ono-zawa *et al.* [47] showed that 40 μM ($IC_{50}$) genistein inhib-ited the growth of LNCaP most effectively and that genistein at physiologic concentrations may inhibit pros-tate tumor growth through a combination of subtle direct effects on tumor neovasculature. It is possible that genis-tein can be bound by many cellular proteins and/or degraded by cellular enzymes, which might be responsible for the high concentrations reported. It remains to be answered by researchers in the field why genistein at greater than plasma concentrations is needed for *in vitro* and *in vivo* treatment.

The accumulation of proteasome inhibitor target pro-teins p27 and IκB-α in LNCaP and MCF-7 cells (Fig. 4) was due to inhibition of the proteasome activity by genis-tein, which is supported by the following evidence. First, as discussed above, genistein is a proteasome inhibitor *in vitro* (Figs. 2 and 3), which was also supported by the computational model of genistein binding to the protea-somal β5 subunit (Fig. 1B–D). In addition, genistein significantly increased levels of ubiquitinated proteins in LNCaP and VA-13 cells (Figs. 4A and 8A). Further-more, accumulation of both p27 and IκB-α proteins was observed in both time- and concentration-dependent man-ner (Fig. 4B and data not shown). Bax has been shown to be a target of the proteasome [36] and also a transcriptional target of p53 [54]. The molecular mechanisms for accu-mulation of Bax by genistein (Figs. 4 and 7) probably involve both inhibition of Bax degradation and induction of p53-mediated Bax transcription. Further studies will distinguish these two possibilities. Finally, p53 is also regulated by ubiquitin/proteasome-mediated degradation pathway [31]. Whether the observed significant induction of p53 protein by genistein treatment (Fig. 7) [49–51] is due to inhibition of the proteasome activity will be inves-tigated in the near future.

The following arguments are consistent with the idea that inhibition of the proteasome activity by genistein is functional in solid tumor cells. First, when LNCaP cells were treated with 100 μM of genistein, accumulation of ubiqitinated proteins and Bax occurs at 4 hr and levels of IκB-α and p27 protein were increased at 8 and 12 hr, respectively (Fig. 4A), while apoptotic cell death occurs

at 12 hr (Figs. 5A and 6). Second, both accumulation of IκB-α protein (Fig. 4B) and PARP cleavage (Fig. 5B) were genistein concentration-dependent. Therefore, accumula-tion of Bax and IκB-α by genistein prior to apoptosis is consistent with the fact that Bax and IκB-α act as cell death promoters [35,36].

Consistent with other reports that genistein is the most potent compound among the soy isoflavones [13,47], we also found that genistein was the most potent one among all the tested isoflavones to induce Bax accumulation and PARP cleavage (Fig. 5C). However, daidzein, daidzin, genistin and glycitin, in addition to genistein, were able to accumulate p27 protein (Fig. 5C). These results suggest that accumulation of Bax and IκB-α is associated with apoptosis induction while p27 accumulation is probably associated with $G_1$ arrest [37,61].

Previously, genistein was shown to be able to induce p53-dependent apoptosis [49–51]. We also found that genistein treatment induced accumulation of p53 protein at as early as 4 hr (Fig. 7B and data not shown). In addition, it appears that genistein treatment also activates a calpain enzyme that cleaves p53 into a p48 fragment (Fig. 7) which has increased activity [52]. Induction of p53 and its cleaved form was time- and concentration-dependent (Fig. 7). Both p53 and the cleaved p48 fragment should be functional because expression of p21 and Bax, two well-known p53 downstream targets [53,54] were also increased in the same experiments (Fig. 7). Our studies are consistent with the previous studies about p53-dependent induction of apop-tosis by genistein [49–51].

One of the important criteria for potential anticancer drugs is the ability to selectively kill tumor cells, but not normal cells. Our results suggest that genistein is able to selectively increase ubiquitinated proteins and induce apoptosis in SV40-transformed, but not in the parental normal, human fibroblasts (Fig. 8). The mechanism for failure of normal human cells to respond to genistein remains unknown, which is clearly important for under-standing the cancer-preventative effects of genistein.

In conclusion, our results suggest that proteasome inhibi-tion is a novel mechanism that might be responsible for, or at least contribute to, the apoptosis-inducing ability of genis-tein and its previously observed anticarcinogenic activity [3–5]. This study further provides evidence that dietary soy phytochemical-containing soybean products have the poten-tial to be developed into chemopreventive agents.

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