Gallic acid, a major component of *Toona sinensis* leaf extracts, contains a ROS-mediated anti-cancer activity in human prostate cancer cells

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**Article Info**

**Article history:**

Received 10 January 2009
Received in revised form 6 May 2009
Accepted 25 May 2009

**Keywords:**

Prostate cancer
*Toona sinensis*
Gallic acid
Cytotoxicity
G2/M phase

**Abstract**

Prostate cancer, the most frequently diagnosed malignancy in elderly males of the United States, has become a major health issue in Asia. Previous studies have demonstrated that leaf extracts of *Toona sinensis* Roem. contain cytotoxic activity on several cancer cells including prostate cancer cells. In this study, gallic acid is identified as the major anti-cancer compound in *T. sinensis* leaf extracts. It is cytotoxic to DU145 prostate cancer cells, through generation of reactive oxygen species (ROS) and mitochondria-mediated apoptosis, which were reversed by antioxidants catalase and N-acetylcysteine. Furthermore, gallic acid is shown to block the growth of DU145 cells at G2/M phases by activating Chk1 and Chk2 and inhibiting Cdc25C and Cdc2 activities. In addition, gallic acid has a synergistic effect with doxorubicin in suppressing the growth of DU145 cells. Taken together, our results suggest that gallic acid has the potential to be developed into an anti-prostate cancer drug and is worthy of further studies.

1. **Introduction**

Prostate cancer (PCA) is the most frequently diagnosed malignancy in elderly males and the second leading cause of cancer-related deaths in the United States [1]. It has also become a major malignancy in many Asian countries in the past two decades [2]. Scientists are putting more efforts on identifying novel botanicals that can selectively target tumor cells growth without affecting normal cells [3,4]. With regard to PCA chemoprevention, natural botanicals and dietary substances are gaining more attention and have the potential to become a major resource of the cancer chemopreventive agents [4,5].

One major biochemical change in cancer cells after treatment with anti-cancer agents is the increase in reactive oxygen species (ROS) generation which is often considered as a cancer-promoting factor [6–8]. Studies have demonstrated that high levels of ROS can cause cellular damage [9–13] and play an important role in mediating apoptosis [14,15]. Interestingly, ROS has been demonstrated to selectively kill cancer cells [8,16,17]. For instance, Hileman et al. show that ROS generated by 2-methoxyestradiol (2-ME) preferentially kill human leukemia cells without exhibiting significant cytotoxicity to normal lymphocytes [18]. Trachootham et al. demonstrate that elevated ROS levels by β-phenylethyl isothiocyanate (PEITC) can be exploited to selectively kill malignant cells. In contrast, normal cells can better tolerate oxidative insults owing to their low basal ROS output and
normal metabolic regulation [8]. This biochemical difference between normal and cancer cells may constitute a basis for modulating cellular ROS as a strategy to selectively kill cancer cells.

_T. sinensis_ Roem. (Meliaceae; _T. sinensis_), a species of arbor widely distributed in Asia, has been used as a nutritious food for a long time and the leaves cooked with eggs is a very popular vegetarian dish in Taiwan. The edible leaves were employed as an oriental medicine for treatment of enteritis, dysentery and dermatitis with no significant side effects [19]. Recently, various biological activities of _T. sinensis_ leaf extracts have been reported, including anti-cancer [20,21], anti-inflammatory [22], anti-diabetes [23,24], antioxidant [13,25], inhibiting Leydig cell steroidogenesis [26], and improving the dynamic activity of human sperm [27]. In a previous study in which the D-7000 HPLC System was applied, we reported that the specific fraction, TSL2, of the _T. sinensis_ leaf extracts contains the most significant anti-cancer activity [21, also see Materials and Methods]. In this study, we report that gallic acid (3,4,5-trihydroxybenzoic acid, GA) is the major bioactive compound isolated from TSL2 fraction of _T. sinensis_ leaf extracts and contains an anti-cancer activity, at least partly through the ROS-mediated pathway, in human prostate cancer cells.

2. Materials and methods

2.1. Isolation of gallic acid from _T. sinensis_ leaf extracts

The leaves used in this preparation were obtained from _T. sinensis_ Roem. grown in Tuku (Yunlin County, Taiwan) and were picked and washed briskly with water. To investigate the potential effect against cancer cells, five different extraction fractions (TSL1, TSL2, TSL3, TSL4 and TSL5) were collected and recorded using a D-7000 HPLC System (Hitachi Ltd., Japan). In this study, we report that gallic acid (3,4,5-trihydroxybenzoic acid, GA) is the major bioactive compound isolated from TSL2 fraction of _T. sinensis_ leaf extracts and contains an anti-cancer activity, at least partly through the ROS-mediated pathway, in human prostate cancer cells.

2.2. Drugs and chemicals

DMEM-F12, DMEM and RPMI 1640 obtained from Gibco (Invitrogen Corporation, CA, USA). Fetal bovine serum and penicillin/streptomycin/amphotericin B were obtained from Biological Industries, Haemek, Israel. 2′,7′-Dichlorodihydrofluorescein diacetate (H₂DCFDA) and rhodamine 123 were obtained from Molecular Probes ( Eugene, OR, USA). Tetrazolium salt (XTT) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Antibodies recognizing Chk1, p-Chk1(Ser345), Chk2, p-Chk2(Ser516), Cdc2, p-Cdc2(Tyr15), Cdc25C, p-Cdc25C(Ser216), Cyclin B1 and p-Cyclin B1(Ser147) were purchased from Cell Signaling Technology (Beverly, MA, USA). Primary antibodies against poly-(ADP) ribose polymerase (PARP), caspase-9, caspase-3, Bcl-xl, Bax and cytochrome _c_ were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Monoclonal antibody against _ß_-actin was obtained from Abcam (Cambridge, MA, USA). DMSO, catalase (CAT), N-acetylcycteine (NAC), propidium iodide, and all other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

2.3. Cell cultures

Cancer cell lines of SKOV3, HeLa, RL95-2, HepJ5, DU145, PC-3 and LNCap were purchased from ATCC (Manassas, VA, USA). DU145 and PC-3 cells were grown in DMEM supplemented with 10% fetal bovine serum and penicillin/streptomycin/amphotericin B. LNCap cells were grown in RPMI 1640 and the other cell lines were grown in DMEM-F12, all supplemented with 10% fetal bovine serum and penicillin/streptomycin/amphotericin B.

2.4. XTT cell proliferation assay

Cells were plated out at a density of 8000 cells/well in 96-well microtiter plates. After treating with the extraction fractions for 48 or 72 h, the cytotoxicity of TSL2 and GA were determined by XTT colorimetric assay (Roche Molecular Biochemicals). Briefly, the culture medium was removed, and 100 µl of fresh culture medium with a pre-formulated 50 µl XTT mixed reagent were added. The culture plate was incubated at 37°C for 4 h. The optical density was measured at 490 nm with reference wavelength at 650 nm by using an ELISA reader. The 50% inhibitory concentration (IC₅₀) was calculated.

2.5. Cellular morphology analysis

DU145 cells grown on 6 cm dishes were treated with GA at different dosages of 0, 25, 50 and 100 µg/mL for 24 and 48 h. The morphological changes were observed under an inverted microscope (Nikon TS100).

2.6. Subcellular fractionation for detection of cytochrome _c_ release

DU145 cells were treated with 100 µg/mL GA for designated periods. The cells were then washed with cold PBS and suspended in 500 µl of buffer A (20 mM Heps-KOH, pH 7.4, 250 mM sucrose, 10 mM KCl, 1 mM MgCl₂, 1.5 mM

2.7. Western blot analysis

DU145 cells were treated with 100 µg/mL GA for designated periods. The cells were then washed with cold PBS and suspended in 500 µl of buffer A (20 mM Heps-KOH, pH 7.4, 250 mM sucrose, 10 mM KCl, 1 mM MgCl₂, 1.5 mM
sodium EDTA, 1.5 mM sodium EGTA, 1 mM dithiothreitol, cocktail protease inhibitor). The lysates were homogenized by pipetting up and down 50 strokes and centrifuged at 200g for 10 min at 4 °C. The supernatant was centrifuged further at 16,000g for 30 min to obtain the cytosolic fraction. The pellet was again lysed with 100 μl buffer B (50 mM Hepes, pH 7.4, 1% Nonidet P40, 10% glycerol, 1 mM EDTA, 2 mM dithiothreitol, cocktail protease inhibitor) and centrifuged at 16,000g for 30 min at 4 °C to obtain the mitochondrial fraction. The cytochrome c was detected from cytosolic and mitochondrial aliquots by Western blotting.

2.7. Neutral comet assay for detection of DNA double-strand breaks (DSBs)

DSBs were determined by neutral comet single-cell gel electrophoresis (Trevigen, Gaithersburg, MD, USA) all according to the instruction. Briefly, DU145 cells were treated with GA (100 μg/mL) at indicated times as previously described. Cell were combined with 1% low melting point agarose at a ratio of 1:10 (v/v) and immediately pipetted 75 μl onto Cometslide at 4 °C and placed in darkness for 10 min. Slides were immersed in ice-cold lysis solution (Trevigen) for 30–60 min in 50 ml of 1X TBE (90 mM Tris–HCl, 90 mM boric acid, and 2 mM EDTA, pH 8.0). Finally, transferred slides from 1X TBE buffer were placed in a horizontal electrophoresis apparatus at 20 V for 10 min and stained with 1:10,000 SYBR Green I (Trevigen).

The comet length was measured from the trailing edge of the nucleus to the leading edge of the tail. This length was indicative of the extent of DNA damage. The analysis software TriTek Comet Image was used to calculate the measurement for each slide. Calculations were averaged per replicate.

2.8. Assessment of mitochondrial membrane potential

The levels of mitochondrial membrane potential (ΔΨm) were determined according to methods described previously [28]. Briefly, after GA treatment, DU145 cells were loaded with 10 μM rhodamine 123 and incubated at 37 °C for 30 min in the dark. Cells were then harvested, washed and resuspended in PBS and analyzed immediately using flow cytometry assay with the excitation wavelength at 488 nm and the emission wavelength at 525 nm.

2.9. Detection of intracellular ROS accumulation

Intracellular ROS accumulation was monitored using H$_2$DCFDA, which is a specific probe for the presence of hydrogen peroxide [29]. After treatments with GA, cells were loaded with 20 μM H$_2$DCFDA and incubated at 37 °C for 30 min in the dark. Cells were then collected, washed and resuspended in PBS and analyzed immediately using flow cytometry with the excitation and emission wavelengths of 490 and 530 nm, respectively.

2.10. Measurement of cell cycle/DNA content

Cell cycle and sub-G1 distribution were determined by FACS analysis. DU145 cells at a concentration of $2 \times 10^5$ were seeded onto 6 cm dishes and treated with GA for
designated periods. The cells were collected and fixed overnight in 70% ethanol at -20 °C. The following day, 1 mL of propidium iodide solution (50 μg/mL) was added and incubated in darkness for 30 min at room temperature. The relative DNA content of these cells was analyzed by FACSscan cytometry (Becton Dickinson, San Jose, CA, USA) based on red fluorescence. Quantitation of the fraction of each cell cycle stage was performed with ModFit LT for Mac 3.0 software (Becton Dickinson).

2.11. Immunoblotting analysis

The detailed procedures for immunoblotting analysis were followed according to Chang et al. (2006). In brief, 100 μg of protein lysate per sample was denatured in 4X SDS–PAGE sample buffer and subjected to SDS–PAGE on tris–glycine gel as needed. The separated proteins were transferred onto nitrocellulose membrane followed by blocking with 5% non-fat milk in TBS-T. Membranes were incubated with specific primary antibodies, washed with Tris-buffered saline with Tween 20 (TBS-T), then incubated with peroxidase-conjugated secondary antibodies. Finally, the signals were detected by enhanced chemiluminescence detection kit (Amersham, NJ, USA).

2.12. Statistics

Data are presented as means ± SD and comparisons were made using Student’s t test. A probability of 0.05 or less was considered statistically significant.

3. Results

3.1. Purification of gallic acid from the TSL2 fraction of T. sinensis leaf extracts that contains the most significant anti-cancer activity

In our previous study, TSL2 fraction of T. sinensis leaf extracts contains the most significant anti-cancer activity [21]. In this study, we determined to identify the pure compounds with anti-cancer activity from TSL fraction (please see Section 2 for the detailed procedures). Gallic acid, the natural phenolic component purified from the TSL2 extracts, was identified and collected for further studies (Fig. 1).

3.2. Cytotoxicity of T. sinensis extracts on various cancer cell lines

To investigate the potential effect against cancer cells, there were five different extraction fractions from the leaves of T. sinensis (TSL1, TSL2, TSL3, TSL4 and TSL5), which were dissolved in PBS. The fraction TSL2 had the most potent cytotoxic effect on cancer cells (data not shown). The results prompted us to further explore the cytotoxicity of TSL2 towards various malignant cell lines. The potency of TSL2 on cell proliferation was tested by XTT assays. After treating TSL2 for 72 h, DU145, a prostate cancer line, possessed the most pronounced anti-proliferation activity with an IC50 value of 17.5 μg/ml (Table 1).

3.3. Effect of GA on inhibition of cell growth in prostate cancer cells (PCA cells)

The PCA cells viability correlated with GA were determined by XTT proliferation assays as shown in Table 2. GA inhibited cell proliferation in all prostate cancer cell lines especially in DU145. The IC50 values of GA were 15.6 ± 2.1 μg/mL. Therefore, DU145 cells were treated with GA at various concentrations of 0, 25, 50 and 100 μg/mL for 24 and 48 h. Phase-contrast micrographs revealed that GA induced cell shrinkage and apoptotic vacuoles display in a dose- and time-dependent manner (Fig. 2).

Table 1

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Cell line</th>
<th>IC50 (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ovarian cancer</td>
<td>SKOV3</td>
<td>28</td>
</tr>
<tr>
<td>Cervical cancer</td>
<td>Hela</td>
<td>&gt;100</td>
</tr>
<tr>
<td>Endometrial cancer</td>
<td>RL95-2</td>
<td>&gt;100</td>
</tr>
<tr>
<td>Liver cancer</td>
<td>Hepg5</td>
<td>30</td>
</tr>
<tr>
<td>Prostate cancer</td>
<td>DU145</td>
<td>17.5</td>
</tr>
</tbody>
</table>

Cells were treated with TSL2 for 72 h and then determined by XTT proliferation assays. Results are expressed as means ± SD of three different experiments.

Table 2

<table>
<thead>
<tr>
<th>Cell line</th>
<th>IC50 (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DU145</td>
<td>15.6 ± 2.1</td>
</tr>
<tr>
<td>LNCaP</td>
<td>20.7 ± 1.1</td>
</tr>
<tr>
<td>PC-3</td>
<td>16.1 ± 3.5</td>
</tr>
</tbody>
</table>

Cells were treated with varying concentrations of GA for 48 h and then determined by XTT proliferation assays. Results are expressed as means ± SD of three different experiments.

3.4. Effect of GA on induction of DNA double-strand breaks in DU145

The nuclear DNA integrity was examined by using the comet assays under neutral electrophoresis condition so that DNA double-strand breaks could be detected. DNA strand breaks in DU145 cells were analyzed after exposure to 100 μg/mL GA for 0, 1, 4, 8, 12, and 24 h. The images of representative nuclei after electrophoresis showed a control group with normal cell nucleus (left panel) and a GA-treated group with increasing degrees of DNA migration (right panel) (Fig. 3A). Previous studies have recommended that tail length (TL) or tail moment (TM) could serve as a quantitative parameter for analysis [30–32]. TM has also been reported as an optimal parameter for assessment of DNA breakage [31,33]. According to Yasuhara et al. [34], a cell containing more than 50% of the total DNA in the comet tail was defined as an apoptotic cell. GA caused DNA breakage and led to an increased TM, indicating that cells were at their apoptotic stages, in a time-dependent manner (Fig. 3B).

3.5. Effects of GA on the expression of pro-apoptotic and anti-apoptotic proteins in DU145 cells

The effects of GA on the expression of the pro-apoptotic protein Bax and the anti-apoptotic protein Bcl-xl in DU145 cells were then determined. The level of Bcl-xl was significantly reduced, while that of Bax was significantly increased after 12 h or 24 h of treatment (Fig. 4). We also investigated whether caspase activation was involved in GA-induced apoptotic death of DU145 cells. The levels of the cleaved caspase-3 were increased over time, while those of full-length caspase-9, caspase-3, and PARP were decreased. These data indicated that GA inactivates anti-apoptotic protein, but activates pro-apoptotic proteins in a time-dependent manner (Fig. 4).

3.6. Effect of GA on cell cycle distribution in DU145 cells

Deregulated cell cycle progression in cancer cells is an effective strategy to halt tumor growth. Effects of GA on cell cycle progression in DU145 cells were then examined. DU145 cells were treated with GA at a dosage of 0, 25, 50, and 100 μg/mL for 24 h or 48 h and FACS analysis was performed. The DNA histogram showed that GA increased the population of cells at the G2/M phase in a dose-dependent manner (Fig. 5A). The G2/M population increased significantly from 18.2 ± 1.1% to 31.4 ± 3.8%, while the G1 population significantly decreased from 46.6 ± 2.0% to 35.8 ± 4.9% after 24 h of treatment. Similar results were observed at 48 h after GA treatment. The percentage of S phase cells was not significantly affected by GA treatment. These results indicated that GA arrested DU145 cells at G2/M phase.
We further assessed the effects of GA on the expression of cell cycle–regulating proteins by immunoblotting analysis. Exposure of DU145 cells to GA at 100 μg/mL resulted in down-regulated expression of cyclin B1, Cdc2, and Cdc25C (Fig. 5B). In contrast, an increase in levels of p-Cdc2(Tyr15), p-Cdc25C(Ser216), p-Chk1(Ser345), and p-Chk2(Ser516) expressions was also observed upon GA treatment. Alteration of the expression levels and phosphorylation status of the above mentioned G2/M-related proteins upon GA treatment led to the enrichment of DU145 cells at G2/M phase.

3.7. Effects of GA on induction of mitochondrial apoptosis pathway and cytochrome c release

To determine whether GA induced apoptosis by triggering the mitochondrial apoptotic pathway, we measured the changes of mitochondrial membrane potential (ΔΨm) and the release of cytochrome c from mitochondria into cytosol. In Fig. 6A, upon 100 μg/mL GA treatment,
the fluorescent intensity, an indicator for mitochondrial membrane potential, was reduced from 84.9 ± 5.8 to 22.4 ± 10.6 after 24 h of treatment, consistent with the results that DU145 cell apoptosis occurred 24 h after treatment with GA (Figs. 2–4). We further demonstrated the release of cytochrome c from mitochondria to cytosol in a time-dependent manner (Fig. 6B). This was accompanied by a decrease in mitochondrial cytochrome c levels. These results suggest that GA triggered cell apoptosis is through induction of mitochondrial apoptotic signaling pathways. Although the levels of cytochrome c in cytosol were increased within 12 h, a sudden decrease was observed at 24 h. We therefore speculated that after 24 h of exposure to GA this may partially induce apoptosis. Nonetheless, this needs to be further clarified.

3.8. Effect of GA on ROS generation in DU145

ROS is known to be an important element in the induction of apoptosis, it is therefore interesting to know whether GA stimulates ROS generation in DU145 cells. In this study, we used H$_2$DCFDA-derived fluorochrome as an indicator of peroxides and superoxide accumulation. After treated with GA, a time-dependent increase of ROS production was observed within 12 h after GA treatment (Fig. 7A). To further address the possibility that ROS acted as initiators in GA-induced DU145 apoptosis, cells were pretreated with antioxidants, CAT or NAC, for 4 h, followed by treatment with 100 μg/mL GA for another 24 h. The fluorescence intensity showed that both CAT and NAC reversed GA-induced ROS accumulation. Quantitative analysis showed that CAT and NAC significantly reduced GA-induced ROS levels from 180.3 ± 10.9 to 127.3 ± 17.8 and 154.0 ± 10.5, respectively (Fig. 7B). In addition, pretreatment of cells with CAT or NAC significantly protected the cells from GA-induced apoptosis, with the percentage of apoptotic cells decreasing from 18% to 3% and 8%, respectively (Fig. 7D). Taken together, these results revealed that GA induced ROS generation, and resulted in ΔΨm loss and apoptosis.

3.9. Effect of GA on cell cycle-specific apoptosis in DU145 cells

Since differential sensitivity to apoptosis is linked to a distinct phase of the cell cycle [35,36], we analyzed which cell cycle stage DU145 cells were most sensitive to GA. DU145 cells were enriched at G0/G1, S, or G2/M phases by pretreatment with the specific cell cycle inhibitors, mimosine, thymidine, or nocodazole (Fig. 8A). The specific phase-enriched cells were then treated with GA for 24 h. Interestingly, GA had the most significant cytotoxicity on nocodazole-induced G2/M-enriched DU145 cells (Fig. 8B).

3.10. Synergistic cytotoxicity of GA and doxorubicin (Dox) on DU145 cells

To determine whether GA has synergistic effect with other anti-cancer drugs, DU145 cells were treated with varying concentrations of GA alone or in combination with doxorubicin (Dox) [37,38]. Our result
showed that the combination of GA and Dox had the most significant inhibitory effect on DU145 cell proliferation as determined by XTT assay (Fig. 9).

4. Discussion

Gallic acid, a polyhydroxyphenolic compound, is one of the major bioactive compounds isolated and purified from T. sinensis. It is widely distributed in various plants and fruits, such as gallnuts, sumac, oak bark, green tea, apple-peels, grapes, strawberries, pineapples, bananas, lemons and in red and white wine [39–41]. Various pharmacological activities of GA have been reported, including anti-cancer [22] and antioxidant [14]. Studies have also demonstrated that GA selectively induces cancer cell death by apoptosis; however, GA shows no cytotoxicity against normal cells [42–44]. Furthermore, GA is considered a helpful phytochemical for cancer chemoprevention [45]. In this study, we found that GA inhibited cell growth of human PCA cell lines, including DU145, PC-3, and LNCaP. Highly significant cytotoxicity was shown in DU145 cells (Table 2).

In this study, we investigated the molecular mechanism by which GA induced PCA cells to undergo apoptosis. As shown in Table 2 and Fig. 2, GA was both an inhibitor of cell proliferation and an inducer of apoptosis, in dose- and time-dependent manners. In addition, GA induced a time-dependent DNA double-strand breaks, presented by “comet tail” in neutral comet assay, in DU145 cells (Fig. 3) [46,47]. These results are consistent with the previous reports that GA induces apoptosis by activating a pre-existing apoptotic pathway [34]. Indeed, GA induced the activation of PARP, caspase-9, and caspase-3, which preceded the onset of apoptosis (Fig. 4). GA also resulted in an increase in pro-apoptotic Bax expression and a decrease in anti-apoptotic Bcl-xL expression. Fig. 4 shows a change in the ratio of Bax to Bcl-xL contributing to the apoptosis-promotion activity of GA. Furthermore, we observed that GA blocked cancer cell proliferation by arresting cells at G2/M phase (Fig. 5A), by down-regulating activities of cyclin B1, Cdc2 and Cdc25C (Fig. 5B), which were required for G2/M transition [48,49].

Cell cycle checkpoint controls ensure chromosomal integrity through the cell cycle progression. Kinases Chk1 and Chk2 were activated at G2-phase checkpoint by damaged or unreplicated DNA [50,51]. Chk1 and Chk2 inactivate Cdc25C through its phosphorylation [52,53]. Cdc25C was a protein phosphatase responsible for dephosphorylating and activating Cdc2, a crucial step in regulating the entry of all eukaryotic cells into the M phase of the cell.
In this study, we observed that GA, through the phosphorylation and activation of Chk1 and Chk2 kinases, induced Ser216 phosphorylation and inactivation of Cdc25C phosphatase. This was unable to dephosphorylate Tyr15 of Cdc2 and therefore kept Cdc2 at inactive status as well as arrested cells at G2/M phase (Fig. 5) [56].

Mitochondria are particularly affected in the early apoptotic process and are thought to act as central coordinators of cell death [57,58]. Mitochondrial dysfunction induces the opening of the mitochondrial permeability transition pore (PTP), dissipation of ΔΨm, and release of apoptogenic proteins (cytochrome c and AIF) [58,59]. However, there is also accumulating evidence that ΔΨm loss is a late event in the apoptotic process. In this study, we demonstrated that GA disrupted the function of mitochondria in the early apoptotic process in a time-dependent manner (Fig. 6A). GA led to the release of cytochrome c from mitochondria into cytosol (Fig. 6B). Pro-apoptotic protein Bax and anti-apoptotic protein Bcl-xL form conducting channels in the outer mitochondrial membrane, and control the mitochondrial cytochrome c release [60]. Many anti-cancer agents or apoptotic stimuli trigger cytochrome c release through either down-regulation of Bcl-2/Bcl-xL and/or up-regulation of Bax. Upon GA treatment, down-regulation of Bcl-xL and up-regulation of Bax were observed (Fig. 4). Since cytochrome c is released in death receptor-mediated apoptosis is followed by caspase activation [61]. Our results demonstrated that GA, through the reduction of ΔΨm, induced cytochrome c release and caspase activation (Fig. 10).

Mitochondria are a source of ROS during apoptosis [62] and the reduced ΔΨm leads to the increased generation of ROS and apoptosis [63]. Enhancement of ROS production has long been associated with the apoptotic response induced by anti-cancer agents [8,64]. Our results showed...
an increasing ROS production upon GA treatment (Fig. 7A) and we speculated that intracellular generation of ROS could be a critical factor in GA-induced apoptosis. To verify this speculation, GA-treated cells were pretreated with antioxidants CAT or NAC. The results showed that both CAT, a \( \text{H}_{2}\text{O}_{2} \)-scavenging enzyme, and NAC, a scavenger of oxygen-free radicals, caused a significant inhibition on GA-generated ROS production (Fig. 7B). Although the effects of CAT and NAC on GA-induced ROS generation were not completely blocked, the involvement of ROS system in these responses showed a statistically significant difference, that suggesting other pathways may be involved. Nonetheless, more studies are necessary to clarify the role of GA in mediating PCA cell apoptosis. We also had found a significant recovery of \( \Delta \Psi \text{m} \) (Fig. 7C), and markedly reversed GA-induced apoptosis was shown (Fig. 7D). We speculate that GA induces ROS generation through a series of steps and that the effects are accumulative.

Since differential sensitivity to apoptosis is linked to distinct phases of the cell cycle [35,36,65], we further analyzed the cytotoxic effect of GA on cells enriched at G0/G1, S or G2/M phases by treating the cells with mimosine, thymidine or nocodazole (Fig. 8A). Interestingly, cells enriched at G2/M phase were more susceptible to GA treatment (Fig. 8B), indicating a cell cycle-specific cytotoxicity of GA. Our result extends these earlier observations by showing for the first time that the effect of GA on the cell cycle can be exploited for cancer therapy to render tumor cells more susceptible for apoptosis. Clinically, resistance to apoptosis is a major cause of primary or acquired nonresponsiveness of cancer cells leading to treatment failure. Therefore, in terms of a clinical perspective, a combined sensitizer (gallic acid)/inducer (cytotoxic drugs, nocodazole) strategy may be a novel approach to enhance the efficacy of anti-cancer therapy in human prostate cancers [65].

Since cancer development involves many aspects of the cell, treatment with a single agent is rarely effective [66]. Two or more cellular processes are usually targeted in therapy. Therefore, combination therapy is now a prominent approach in cancer chemotherapy. Many advantages of this approach There are many advantages of this approach including targeting more than one critical molecular process, delivering lower dose agents with lower toxicity and increasing patient tolerance. Currently there is growing interest in the use of combination chemotherapy allowing the delivery of lower drug dosages each with different modes of activity [66–68]. GA has been shown to sensitize a variety of human cancer cell lines for apoptosis induced by different anti-cancer drugs [13,22,43,44]. In this study, our data showing that GA has synergistic effects with doxorubicin in suppressing the growth of DU145 prostate cancer cells (Fig. 9). This suggests that GA in combination with the commercially used chemotherapeutic agents (Dox) has the potential to be developed as a better chemotherapeutic agent for treatment of PCA and to decrease the side effects caused by high doses of currently used chemotherapeutic agents.

To the best of our knowledge, this is the first study to show that GA, a natural plant compound, inhibited the growth of DU145 human PCA cells by activating preexist-
Phellinus linteus


