**Puerariae radix** isoflavones and their metabolites inhibit growth and induce apoptosis in breast cancer cells

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**A B S T R A C T**

**Puerariae radix** (PR) is a popular natural herb and a traditional food in Asia, which has antithrombotic and anti-allergic properties and stimulates estrogenic activity. In the present study, we investigated the effects of the PR isoflavones puerarin, daidzein, and genistein on the growth of breast cancer cells. Our data revealed that after treatment with PR isoflavones, a dose-dependent inhibition of cell growth occurred in HS578T, MDA-MB-231, and MCF-7 cell lines. Results from cell cycle distribution and apoptosis assays revealed that PR isoflavones induced cell apoptosis through a caspase-3-dependent pathway and mediated cell cycle arrest in the G2/M phase. Furthermore, we observed that the serum metabolites of PR (daidzein sulfates/glucuronides) inhibited proliferation of the breast cancer cells at a 50% cell growth inhibition (GI50) concentration of 2.35 μM. These results indicate that the daidzein constituent of PR can be metabolized to daidzein sulfates or daidzein glucuronides that exhibit anticancer activities. The protein expression levels of the active forms of caspase-9 and Bax in breast cancer cells were significantly increased by treatment with PR metabolites. These metabolites also increased the protein expression levels of p53 and p21. We therefore suggest that PR may act as a chemopreventive and/or chemotherapeutic agent against breast cancer by reducing cell viability and inducing apoptosis.

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In this study, we attempted to elucidate the inhibitory capacities of PR isoflavones and PR metabolites on the growth of breast cancer cells.

Materials and methods

Cell culture. Human breast cancer cell lines HS578T, MDA-MB-231, and MCF-7 were purchased from American Type Culture Collection. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen), supplemented with 10% fetal bovine serum (Invitrogen) and 1% penicillin–streptomycin solution (Invitrogen) at 37 °C in a 5% CO₂ incubator.

Preparation and quantitation of serum metabolite of PR. The blood of rats withdrawn at 30 min after administration of PR decoction was centrifuged to obtain serum, which was added fourfolds of methanol, and centrifuged to remove proteins. The supernatant was evaporated to dryness below 40 °C under vacuum by using rotatory evaporator. Then the residue was dissolved with water and the isoflavone metabolites were quantitated by HPLC/UV method after hydrolysis with β-glucuronidase and sulfatase, respectively, as described previously [28].

Assay of cell proliferation. For cell proliferation assay, HS578T, MDA-MB-231, and MCF-7 cells were cultured overnight on 96-well plates. Media containing different concentrations of puerarin, daidzein, genistein (SIGMA), and serum metabolites of PR were added and incubated for 72 h. Subsequently, 20 μl of MTS was added from a stock solution (2 mg/ml) and incubated for an additional 2 h. The absorbance was read at 490 nm on a Bio-Rad model 550 microplate reader.

Cell cycle analysis. For cell cycle analysis, control or compound-treated cells (1 × 10⁶ per sample) were fixed in 70% ethanol, incubated overnight at −20 °C. The cells were washed with ice-cold PBS and subsequently suspended in staining buffer (20 μg/ml propidium iodide; 0.1% Tween 20; 0.2 mg/ml RNase A in PBS) and incubated at room temperature for a minimum duration of 30 min. Using flow cytometry (FACSort instrument and analysis software, ModFit LT), the cells were analyzed for cell cycle distribution and apoptosis.

Assay of caspase-3 activity. Caspase-3 activity was determined by using CALBIOCHEM Caspase-3 Assay Kit according to the manufacturer’s protocol. Fluorescence was measured with a multi-well plate series 4000 (PerSeptive Biosystems) at excitation and emission wavelengths of 360 and 530 nm, respectively.

Western blot. Whole cell lysates were obtained by direct dissolution of cells using an ice-cold Mammalian Protein Extraction Reagent (M-PER, PIERCE). Proteins (20 μg) were resolved by 10% SDS–PAGE and electro-transferred onto polyvinylidene difluoride (PVDF) membrane. The blot was blocked with a solution containing 5% non-fat blocking buffer (0.5 M Tris-base, 10 mM EDTA, 1.5 M NaCl, and 0.5% Tween 20) for 1 h, washed and incubated with antibodies to β-actin (NEO MARKERS, the detection of β-actin was used as an internal control in all of the data of Western blotting analysis), caspase-9 (NEO MARKERS) and p53 (NEO MARKERS) and p21 (NEO MARKERS) and Bax (NEO MARKERS). A HRP-conjugated goat anti-mouse IgG was used as secondary antibody. The immunoreactive bands were visualized using enhanced chemiluminescence.

Annexin V staining. The apoptotic effects of puerarin, daidzein, and genistein on breast cancer cells were analyzed by Annexin V staining. Breast cancer cells were placed on glass cover slips in the wells of a 6-well plate. After a 12-h cell culture, they were maintained in the same media lacking phenol red and containing charcoal-stripped PBS for 24 h and subsequently treated for 24 h with GI₅₀ puerarin, daidzein, and genistein. The cells were washed with PBS and fixed in 4% formaldehyde for 30 min, followed by staining with Annexin V (BD) at a concentration of 2 mM with incubation for 30 min. After gently washing with PBS, fluorescent nuclei were observed under a Nikon E600 fluorescence microscope. Morphology of the stained nuclei was used to determine apoptosis.

Results

PR isoflavones induced growth inhibition in breast cancer cells

As the basis for further characterization of isoflavone-induced cellular response in HS578T, MDA-MB-231, and MCF-7 cells, we first examined cell proliferation/growth inhibition in the three cell lines following incubation with puerarin, daidzein, and genistein using the MTT assay. A significant inhibition of cell viability was
observed upon treatment with various concentrations of puerarin, daidzein, and genistein (Fig. 1) in a dose-dependent manner. The 50% cell growth inhibition concentrations (GI50) for the three different types of breast cancer cell are listed in Table 1.

Effects of PR isoflavones on cell cycle progression and apoptosis induction in breast cancer cells

We investigated the effects of puerarin, daidzein, and genistein on the cell cycle distribution in breast cancer cells. Treatment of HS578T, MDA-MB-231, and MCF-7 cell lines with puerarin, daidzein, and genistein resulted in a significant accumulation of cells in the G2–M phase and induced apoptosis when compared with the vehicle controls (Table 2). Annexin V analysis was employed in order to evaluate the early stages of apoptosis. We observed that the fluorescence in the drug-treated group increased in comparison with that observed in the control, which indicated that the isoflavones inhibited the tumor cells via the induction of apoptosis (Fig. 2).

Cell death induced by PR isoflavones is mediated by a caspase-3-dependent pathway

Caspase-3 is a key member of the caspase family, a group of cysteine proteases that mediate apoptotic execution [29]. The induction of apoptosis in the breast cancer cell lines by isoflavones was confirmed by detection of increased caspase-3 activity in the isoflavone-treated HS578T and MDA-MB-231 cells (Fig. 3).

Serum metabolites of PR induced apoptosis in the MCF-7 cell line

A pharmacokinetic investigation of isoflavones in PR revealed the virtual absence of the parent forms of puerarin, daidzin, and daidzein in the bloodstream. However, the sulfates and glucuronides of daidzein were found to be the major metabolites present in the sample [28]. Therefore, in order to mimic the in vivo situation, we prepared the serum metabolites of PR from rats to investigate their effect on the MCF-7 cell line.

MCF-7 cells were treated with the serum metabolites of PR for 72 h, and it was observed that daidzein sulfates/glucuronides inhibited proliferation of the breast cancer cells a GI50 at 2.35 μM of daidzein sulfates/glucuronides. This result demonstrated a markedly more potent growth-inhibitory effect of the serum metabolites of PR on the MCF-7 cell line than the other isoflavones tested.

Table 1
Chemical structures and GI50 values of puerarin, daidzein, and genistein on HS578T, MDA-MB-231, and MCF-7 breast cancer cells.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Structure</th>
<th>Cells</th>
<th>GI50 (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Puerarin</td>
<td><img src="image" alt="Puerarin Structure" /></td>
<td>HS578T</td>
<td>46</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MDA-MB-231</td>
<td>71</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MCF-7</td>
<td>69</td>
</tr>
<tr>
<td>Daidzein</td>
<td><img src="image" alt="Daidzein Structure" /></td>
<td>HS578T</td>
<td>43</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MDA-MB-231</td>
<td>70</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MCF-7</td>
<td>68</td>
</tr>
<tr>
<td>Genistein</td>
<td><img src="image" alt="Genistein Structure" /></td>
<td>HS578T</td>
<td>29</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MDA-MB-231</td>
<td>52</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MCF-7</td>
<td>69</td>
</tr>
</tbody>
</table>

Table 2
Cell cycle distribution of breast cancer cells treated with PR isoflavones and PR metabolites.

<table>
<thead>
<tr>
<th>Cells</th>
<th>Treatments</th>
<th>Control (%)</th>
<th>Puerarin (%)</th>
<th>Daidzein (%)</th>
<th>Genistein (%)</th>
<th>PR metabolites (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HS578T</td>
<td>Sub-G1</td>
<td>0.2</td>
<td>12.67</td>
<td>5.3</td>
<td>10.48</td>
<td>3.5</td>
</tr>
<tr>
<td></td>
<td>G1</td>
<td>69</td>
<td>61</td>
<td>62</td>
<td>46</td>
<td>51</td>
</tr>
<tr>
<td></td>
<td>S</td>
<td>12.8</td>
<td>3.33</td>
<td>7.7</td>
<td>18.52</td>
<td>18.5</td>
</tr>
<tr>
<td></td>
<td>G2/M</td>
<td>18</td>
<td>23</td>
<td>25</td>
<td>25</td>
<td>27</td>
</tr>
<tr>
<td>MDA-MB-231</td>
<td>Sub-G1</td>
<td>0.26</td>
<td>8.3</td>
<td>8.3</td>
<td>11.94</td>
<td>7.37</td>
</tr>
<tr>
<td></td>
<td>G1</td>
<td>58</td>
<td>54</td>
<td>56</td>
<td>48</td>
<td>59</td>
</tr>
<tr>
<td></td>
<td>S</td>
<td>25.74</td>
<td>17.7</td>
<td>14.7</td>
<td>23.06</td>
<td>12.63</td>
</tr>
<tr>
<td></td>
<td>G2/M</td>
<td>16</td>
<td>20</td>
<td>21</td>
<td>17</td>
<td>21</td>
</tr>
<tr>
<td>MCF-7</td>
<td>Sub-G1</td>
<td>1.7</td>
<td>4.9</td>
<td>4.2</td>
<td>4.9</td>
<td>5.5</td>
</tr>
<tr>
<td></td>
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<td>59</td>
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<td>4.8</td>
<td>29.1</td>
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<tr>
<td></td>
<td>G2/M</td>
<td>17.4</td>
<td>34</td>
<td>36</td>
<td>34</td>
<td>14</td>
</tr>
</tbody>
</table>
The phenotypic characteristics of the MCF-7 cells treated with serum PR metabolites were evaluated by microscopic inspection of their general morphology. We observed that the MCF-7 cells formed apoptotic bodies and subsequently floated after treatment with 2.35 μM of serum PR metabolites for 72 h (Fig. 4A). Moreover, early apoptosis was detected after treatment with PR metabolites for 24 h, and this treatment also induced cell cycle arrest at the G2–M phase in MCF-7 cells (Fig. 4B; Table 2).

Serum metabolites of PR increased the expression of apoptotic proteins

Changes in the expression of various breast cancer cell apoptotic proteins were analyzed by Western blotting. We observed that the expressions of caspase-9, p53, p21, and Bax were all significantly increased after the treatment with serum metabolites of PR for 24 h (Fig. 4C). On the basis of the above results, it is suggested that the serum PR metabolite-induced MCF-7 cell death can be attributed to changes in the expression levels of various apoptotic proteins.

Discussion

In this study, we investigated the molecular mechanisms underlying the effects of the serum metabolites of PR and related isoflavones on the human breast cancer cell lines HS578T, MDA-MB-231, and MCF-7. After 72 h of incubation, puerarin, daidzein, and genistein significantly reduced the viability of breast cancer cells compared with the vehicle control in a dose-dependent manner.

Puerarin, daidzein, and genistein were able to prevent the entry of HS578T, MDA-MB-231, and MCF-7 cells into the G0/G1 phase of the cell cycle and induced apoptosis in these cells, thereby suggesting that the observed growth-inhibitory effect of various isoflavones on HS578T, MDA-MB-231, and MCF-7 cells was mediated through modulation of cell cycle progression and induction of apoptosis in these cells.

Caspase-3 is a critical component of the cell death machinery. Apoptosis induction was further confirmed by determining caspase-3 protein expression in the cells. We demonstrated that puerarin, daidzein, and genistein induced apoptosis of HS578T, MDA-MB-231, and MCF-7 cells through a caspase-3-mediated pathway. The serum metabolites of PR also activated caspase-3 in HS578T cells (data not shown), which indicated that PR metabolites may induce the same pathway as puerarin, daidzein, and genistein.

We investigated the in vitro effect of the serum metabolites of PR against the breast cancer cell line MCF-7. The PR metabolites...
inhibited the growth of breast cancer cells with a $G_{50}$ value of 2.35 μM, which was considerably lower than that of puerarin, daidzein, and genistein. On the basis of data from an in vivo animal study, a serum concentration of 2.35 μM is considered to be achievable [28]. The serum metabolites of PR inhibited cell proliferation via an up-regulation of p21/Waf1, p53, caspase-9, and Bax, as revealed by western blotting. Furthermore, early apoptosis in MCF-7 cells was detected by Annexin V staining.

Breast cancer is the most common malignancy in women, and it is highly curable if diagnosed at an early stage. It is now well established that adjuvant systemic therapy improves survival in patients with early stage breast cancer, and current treatment options include chemotherapy (e.g., anthrocyclines and taxanes) and hormone therapy (e.g., tamoxifen and aromatase inhibitors). On the basis of the results of this study, PR appears to be a potential candidate for the treatment of breast cancer. In conclusion, the serum metabolites of PR exhibited potent anticancer activity on MCF-7 cells, implying that PR is a promising chemopreventive or chemotherapeutic agent.

Acknowledgments

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References


Fig. 4. Apoptosis was induced by serum metabolites of PR in breast cancer cells and was associated with the expression of apoptosis-related proteins. (A) Microscopic observations revealed that the MCF-7 cells treated with PR metabolites for 72 h had the highest number of apoptotic bodies. (B) MDA-MB-231, and MCF-7 cells treated with PR metabolites for 24 h revealed early apoptosis when stained with Annexin-V-fluos. (C) MCF-7 cell apoptosis induced by PR metabolites was associated with the expression of caspase-9, p53, p21, and Bax. The blot was later stripped and reprobed with β-actin antibody to ensure equal loading of the cell extracts.