Abstract. The molecular mechanisms underlying genistein-mediated reversal of chemoresistance remains unknown. In the present study, we investigated the molecular mechanisms by which genistein overcomes chemoresistance and its effect on doxorubicin-induced cytotoxicity. Consistent with previous reports, genistein combined with doxorubicin had a synergistic effect on MCF-7/Adr cells, and genistein reduced the chemoresistance of these cells. Genistein treatment increased the intracellular accumulation of doxorubicin but did not influence P-gp function. The combination of genistein and doxorubicin significantly induced cell cycle arrest and apoptosis. Genistein treatment strongly inhibited HER2/neu but not MDR-1 expression at both the mRNA and protein levels. Therefore, our results demonstrated that genistein combined with doxorubicin had a synergistic effect on MCF-7/Adr cells, and the mechanisms likely involve an increase in the intracellular accumulation of doxorubicin and suppression of HER2/neu expression.

Introduction

Breast cancer, a type of cancer originating from breast tissue, is a common cancer and the second leading cause of cancer-related mortality among women (1,2). Although there has been a sustained decline in the mortality rates in recent decades due to development of increasingly effective adjuvant medical treatments, relapse after the first 5 years is frequent and is a major contributor to breast cancer mortality (3-5). The major reason for relapse is chemoresistance acquired during the chemotherapeutic process (6-8). Therefore, overcoming acquired chemoresistance is critically important to reducing the rate of relapse and improving prognosis.

Doxorubicin, also known as Adriamycin, is an anthracycline antibiotic that functions by intercalating DNA (9). It is commonly used in the treatment of a wide range of cancers, including breast cancer, hematological malignancies, soft tissue sarcomas and many types of carcinoma (10-12). However, a long duration of doxorubicin treatment often causes cancer cell resistance to treatment and has the serious adverse effect of life-threatening heart damage (13,14). Elucidating the molecular mechanisms that underlie chemoresistance and overcoming established chemoresistance to doxorubicin are clinically relevant challenges in breast cancer treatment.

Genistein, first isolated from the dyer's broom in 1899, is a phytoestrogen and belongs to the category of isoflavones. Various studies have found that genistein has inhibitory effects on many types of cancers, including breast cancer (15,16). More importantly, several studies have shown that genistein can enhance chemotherapeutic efficacy and overcome chemoresistance in breast cancer (17-19). However, the molecular mechanisms underlying genistein-mediated reversal of chemoresistance are still largely unknown.

Materials and methods

Materials and equipment. The human breast cancer cell line MCF-7 and resistant derivative cell line MCF-7/Adr were gifted by the Department of Surgery, Wuhan Union Hospital, Hubei, China. Sensitive human breast cancer MCF-7/s cells were obtained from ATCC (USA). The MCF-7/adr cells (Michigan Cancer Foundation-7/adriamycin resistant) were kindly provided by Dr. Huang Tao (University of Science and Technology of Xiehe Hospital at Huazhong Surgery Laboratory Center, Wuhan, Hubei). The cell line is derived from the parental drug-sensitive MCF-7 cells by stepwise selection with Dox. To maintain the drug-resistant phenotype, the cells were cultured in the presence of 1 µg/ml Dox in RPMI-1640 culture medium supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen), 10 mM glutamine, and antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin). Cells were maintained at 37ºC in 5% CO2/95% air (20-22).

Genistein and rhodamine (Rh123) were obtained from Sigma-Aldrich (USA). Doxorubicin hydrochloride was purchased from Shanghai Biological Engineering Co., Ltd. (China). Oligo(dt) was synthesized by Shanghai Biological Engineering Co. Taq PCR MasterMix was purchased from Tiangen (China). Primers were synthesized by Shanghai Biological Engineering Co. Anti-human permeability glycoprotein (P-gp) monoclonal antibody was purchased from Abcam (USA). Anti-c-erbB2 [human epidermal growth factor

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receptor 2 (Her2/Neu) polyclonal antibody was purchased from Dako Corp. (USA). Anti-mouse IgG-horseradish peroxidase (HRP) and anti-rabbit IgG-HRP were purchased from Beijing Ding National Biotechnology Co. (China). A Coulter DNA PREP™ reagents kit was purchased from Beckman-Coulter (USA). An Annexin V-FITC apoptosis detection kit was purchased from Shanghai Yan-Bin Chemical Technology (China). The F-7000 fluorescence spectrometer was purchased from Hitachi (Japan), and the Hitachi UV spectrophotometer was from Eppendorf (Germany).

**Cells and culture.** Cells were cultured in RPMI-1640 culture medium with 10% fetal calf serum at 37°C with 5% CO₂. Cells were trypsinized with 0.25% trypsin and passed every 2 or 3 days. MCF-7/Adr cells were maintained in medium supplemented with doxorubicin (1.0 µg/ml) for two weeks. Cells were diluted from 5x10⁴ to 5x10⁵/ml for use in the experiments.

**Preparation of genistein.** The purity of genistein was greater than 98%. Twenty-five milligrams of genistein was dissolved in 1 ml dimethylsulfoxide (DMSO) and stored at 4°C. Twenty-five milligrams of doxorubicin was dissolved in 10 ml saline and stored at 20°C. For experiments, doxorubicin was added to measure proliferation. Q-value was calculated by the formula of Guinness (17-19). IC₅₀ and the reversal fold were also calculated. The reversal fold = IC₅₀ (chemotherapy drugs)/IC₅₀ (genistein).

**MTT assay.** MCF-7/Adr cells in the logarithmic growth phase were seeded in 96-well culture plates. To each well, we added 30 µmol/l genistein and 0.07, 0.7, 7 or 70 µM doxorubicin, in a final volume of 200 µl (n=8 for each concentration). Wells with medium only (no cells) were used to measure the background, and seeded wells given untreated medium served as the controls. Forty-eight hours after treatment, MTT was added to measure proliferation. Q-value was calculated by the formula of Guinness (17-19). IC₅₀ and the reversal fold were also calculated. The reversal fold = IC₅₀ (chemotherapy drugs)/IC₅₀ (genistein).

**Fluorospectrophotometry.** Quantification of doxorubicin concentrations was carried out using a standard curve. The standard curve was established using standard solutions of different doxorubicin concentrations (0, 0.01, 0.05, 0.25, 1.25, 6.25 and 31.25 µM). Cell culture medium was added to yield final genistein concentrations of 7, 15, 30, 60 and 120 µmol/l, and 0.7, 7 and 70 µM doxorubicin. After experimental culture, the supernatants were collected and centrifuged, and fluorescence intensity was measured. The concentration of doxorubicin in the supernatant was calculated according to the standard curve.

**Analyses of cell cycle and apoptosis by flow cytometry.** MCF-7/Adr cells in the logarithmic growth phase were seeded in 24-well plates. The cells were divided into following groups: control group (culture medium), doxorubicin groups (10 or 100 µM), genistein groups (30 or 60 µmol/l), low-dose combination group (1 µM doxorubicin + 30 µmol/l genistein), and high-dose combination group (10 µM doxorubicin + 60 µmol/l genistein). The final concentration of DMSO was 0.1%. Then cells were harvested and washed twice with cold PBS.

**RT-PCR.** MCF-7/Adr cells in the logarithmic growth phase were seeded in 6-well plates and divided into the following groups: control, doxorubicin (1 or 10 µM), genistein (15, 30 or 60 µmol/l). After 48 h of treatment, cells were harvested and total-RNA was extracted. The A260:A280 ratio was ≥1.8. RT-PCR was performed according to the two-step RT-PCR amplification kit instructions. The primers were as follows: mdr-1 forward, 5'-CCCATCATTGCAATAGCAGG-3' and reverse, 5'-GTTCAAACACTTCTGCTCCTCA-3'; Her2/neu forward, 5'-GCCCTCATCCACATACCCCAC-3' and reverse, 5'-CATTTCCTCCAGCCTACTCT-3'; β-actin forward, 5'-AGCAGGACATCCCCAAATT-3' and reverse, 5'-GGG CACGAAGGCTCATCATT-3'. The PCR product of mdr-1 is 157 bp; the PCR product of Her2/neu is 220 bp; and the PCR product of control β-actin is 200 bp. PCR conditions were as follows: 94°C denaturation for 30 sec, 57°C refolding for 30 sec, and 72°C extension for 30 sec, for a total of 30 cycles.

**Statistical methods.** SPSS 17.0 software was used for variance and regression analyses and pairwise comparisons Q-test. Data are presented as means ± standard deviation (SD). Comparison between groups was performed via t-test. P<0.05 was considered to indicate a statistically significant difference.

**Results**

MCF-7/Adr cells are resistant to doxorubicin-induced cell death. It is known that MCF-7/Adr cells are resistant to doxorubicin once the cell line is established. To confirm that MCF-7/Adr cells do have this biological feature, we compared...
the sensitivity of MCF-7/S and MCF-7/Adr cells. As shown in Fig. 1, doxorubicin inhibited the cell proliferation of MCF-7/S cells in a dose- and time-dependent manner; however, doxorubicin had no inhibitory effect on MCF-7/Adr cells. In MCF-7/Adr cells, the IC50 for a 48-h treatment was 117.15 µM (4.75±0.04 to 556.21±51.35 µM), whereas the IC50 for a 72-h treatment was 54.42 µM (8.95±0.08 to 486.90±45.33 µM).

Taken together, our results confirmed that the MCF-7/Adr cells were resistant to doxorubicin.

Genistein enhances the cytotoxic effect of doxorubicin in MCF-7/Adr cells. Several studies have shown that genistein can enhance the cytotoxic effects of doxorubicin and reduce the chemoresistance of tumor cells (17-19). To test this observation, we treated MCF-7/Adr cells with genistein (30 µmol/l) along with increasing doses of doxorubicin. We found that genistein had a synergistic effect with doxorubicin at all tested doses (Q>1.15; Table I). The inhibitory effect of doxorubicin and genistein was significantly greater than that of the control and doxorubicin alone (P<0.01; Table I).

Genistein enhances the cytotoxic effect of doxorubicin in MCF-7/Adr cells. Several studies have shown that genistein can enhance the cytotoxic effects of doxorubicin and reduce the chemoresistance of tumor cells (17-19). To test this observation, we treated MCF-7/Adr cells with genistein (30 µmol/l) along with increasing doses of doxorubicin. We found that genistein had a synergistic effect with doxorubicin at all tested doses (Q>1.15; Table I). The inhibitory effect of doxorubicin and genistein was significantly greater than that of the control and doxorubicin alone (P<0.01; Table I).

Genistein increases the intracellular accumulation of doxorubicin.Our results revealed that genistein enhanced the cytotoxic effect of doxorubicin. To better understand the molecular mechanism of this observation, we investigated whether genistein increases the intracellular accumulation of doxorubicin in MCF-7/Adr cells. The intracellular accumulation of doxorubicin was estimated by the formula y = 152.55 + 99.496x, R²=0.954. As presented in Table II, genistein significantly increased the intracellular accumulation of doxorubicin (P<0.01). In the MCF-7/Adr cells, increased concentration of doxorubicin alone did not significantly upregulate the intracellular accumulation of doxorubicin, and the maximal intracellular loading of doxorubicin was extremely...
limited in the doxorubicin alone group (Table II). However, genistein treatment significantly increased the intracellular accumulation of doxorubicin in a dose-dependent manner. More importantly, the maximal loading of doxorubicin was greatly increased with genistein treatment (Table II).

Genistein has no effect on the intracellular accumulation or excretion of Rh123. To further understand the mechanism underlying doxorubicin accumulation in MCF-7/Adr cells, we tested the effect of genistein on the intracellular accumulation of Rh123, a fluorescent substrate of P-gp. Rh123 is transported

Table III. Effect of genistein on doxorubicin-induced MCF-7/Adr cell cycle arrest.

<table>
<thead>
<tr>
<th>Group</th>
<th>48 h</th>
<th>72 h</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>G&lt;sub&gt;1&lt;/sub&gt;</td>
<td>S</td>
</tr>
<tr>
<td>Control</td>
<td>3</td>
<td>60.9</td>
</tr>
<tr>
<td>30 µmol/l Gen</td>
<td>3</td>
<td>43.8</td>
</tr>
<tr>
<td>60 µmol/l Gen</td>
<td>3</td>
<td>34.4</td>
</tr>
<tr>
<td>30 µmol/l Gen + 1 µM Dox</td>
<td>3</td>
<td>58.1</td>
</tr>
<tr>
<td>60 µmol/l Gen + 10 µM Dox</td>
<td>3</td>
<td>32.3</td>
</tr>
</tbody>
</table>

<sup>a</sup>P<0.01 vs. control; <sup>b</sup>p<0.05 vs. 48 h; <sup>c</sup>p<0.05 vs. 30 µmol/l Gen + 1 µM Dox. Dox, doxorubicin; Gen, genistein.

Figure 3. Effect of genistein on doxorubicin-induced cell cycle arrest analyzed by flow cytometry. The y-axis represents cell number. (A) Control, (B) 30 µmol/l genistein (48 h), (C) 60 µmol/l genistein (48 h), (D) 60 µmol/l genistein (72 h), (E) 30 µmol/l genistein + 1 µM doxorubicin (48 h), (F) 30 µmol/l genistein + 1 µM doxorubicin (72 h), (G) 60 µmol/l genistein + 10 µM doxorubicin (48 h) and (H) 60 µmol/l genistein + 10 µM doxorubicin (72 h).
by P-gp and therefore can be used as a measure of drug extrusion by the plasma membrane (17-19). The intracellular accumulation assay showed that genistein had no effect on the intracellular accumulation of Rh123 (Fig. 2). Assays of Rh123 excretion by MCF7/S and MCF7/Adr cells also showed that genistein had no effect on the excretion of Rh123 (Fig. 2). These results suggest that genistein specifically affects the intracellular accumulation of doxorubicin but not that of Rh123.

Genistein increases doxorubicin-induced cell cycle arrest. Doxorubicin intercalates into DNA and induces cell cycle arrest. We tested whether genistein increases doxorubicin-induced cell cycle arrest. Compared to the control, both genistein and doxorubicin significantly induced MCF-7/Adr cell cycle arrest at the G2/M phase (P<0.01; Table III and Fig. 3). Genistein treatment at 60 µmol/l for 48 h and 72 h resulted in decreased percentages of cells in the G1 and G2/M phases but increased the percentages of cells in the S phase. The effect of combined genistein and doxorubicin treatment on cell cycle arrest was much stronger than that of either genistein or doxorubicin alone (Table III and Fig. 3).

Genistein enhances doxorubicin-induced apoptosis. We tested whether genistein enhances doxorubicin-induced apoptosis. Doxorubicin alone did not cause obvious apoptosis in the MCF-7/Adr cells. However, when treated with genistein alone or in combination with doxorubicin, MCF-7/Adr cells became apoptotic (P<0.01; Table IV and Fig. 4). The genistein and doxorubicin combination group showed the highest percentage of apoptotic cells (Table IV and Fig. 4).

Genistein suppresses Her2/neu mRNA expression but not mdr-1 mRNA expression. To better understand the molecular mechanism of genistein's effects, we tested the influence of genistein on the expression of mdr-1 and Her2/neu mRNA. Genistein and doxorubicin alone or in combination had no effect on mdr-1 mRNA expression (P>0.05; Fig. 5A). However, genistein downregulated Her2/neu mRNA expression in a dose-dependent manner (P<0.01; Fig. 5A). The combination of genistein and doxorubicin also significantly suppressed Her2/neu mRNA expression (P<0.01; Fig. 5A).

### Table IV. Effect of genistein on doxorubicin-induced apoptosis.

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Annexin (+) PI (-)</th>
<th>Annexin (+) PI (+)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>3</td>
<td>5.92±0.22</td>
<td>0.24±0.02</td>
</tr>
<tr>
<td>Dox (10 µM)</td>
<td>3</td>
<td>13.8±0.85</td>
<td>0.02±0.01</td>
</tr>
<tr>
<td>Gen (µmol/l)</td>
<td>3</td>
<td>26.0±0.99</td>
<td>0.11±0.01</td>
</tr>
<tr>
<td>Gen (60 µmol/l) + Dox (10 µM)</td>
<td>3</td>
<td>35.8±1.57&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>0.57±0.04</td>
</tr>
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</table>

<sup>aP<0.01 vs. control; bP<0.05 vs. Dox or Gen. Dox, doxorubicin; Gen, genistein.</sup>

*Figure 4. The effect of genistein on doxorubicin-induced apoptosis. (A) Control, (B) 10 µM doxorubicin, (C) 60 µmol/l genistein, (D) 60 µmol/l genistein + 10 µM doxorubicin.*
Genistein suppresses c-erbB2 but not P-gp expression.

We examined the expression of P-gp and c-erbB2 proteins. Genistein and doxorubicin alone or in combination had no effect on the expression of P-gp (P>0.05; Fig. 5B). However, genistein downregulated the expression of c-erbB2 (P<0.01; Fig. 5B).

Discussion

It is well recognized that chemotherapy can significantly improve the prognosis of breast cancer patients; however, the development of multi-drug resistance is the main cause of failure of chemotherapy for long therapeutic durations (23-25). Therefore, reversing and overcoming chemoresistance are urgent and clinically relevant issues that should be resolved by basic research. P-gp-mediated drug resistance is considered a main mechanism by which cancer cells avoid the effects of chemotherapeutic drugs (26,27). Overexpression of the Her2/neu oncogene has been observed in approximately 30% of breast cancer patients and is believed to contribute to the failure of chemotherapy (28). Genistein not only inhibits the growth of many types of cancers, but also has a similar structure to drugs that can reverse chemoresistance (29,30). This suggests that genistein may be able to reverse chemoresistance, and indeed, several reports have shown that genistein reverses chemoresistance (29,30). However, the molecular mechanisms of genistein's action are not fully understood. Several mechanisms have been proposed: i) genistein enhances apoptosis by direct inhibition of CYP24 enzyme activity (31); ii) genistein upregulates expression of cell cycle regulators P21WAF1/CIP and Bax (32); and iii) genistein induces apoptosis via upregulation of PTEN and Bax and enhancement of Bcl-2 and Bcl-XL expression (33). It has been reported that genistein enhances the cytotoxic effect of gefitinib in T790M non-small cell lung cancer cells by inhibiting p-EGFR, p-Akt and p-mTOR expression and promoting caspase-3 and PARP activity (34). Consistent with previous reports, we also found that genistein significantly improved chemotherapeutic efficacy.

Genistein derived from soybeans and other food sources is less expensive than conventional chemotherapeutic drugs. This is significant as most cancer patients are under tremendous economic pressure due to the expensive cost of chemotherapeutic drugs.

Doxorubicin is a first-line chemotherapeutic drug in many cancer types, including breast cancer (35). Therefore, elucidating the molecular mechanisms of chemoresistance and overcoming such resistance are critically important in the clinic. MCF-7/Adr is a well established doxorubicin-resistant cell line and has been widely used to study chemoresistance (36,37). Using MCF-7/Adr cells as a model, we found that genistein greatly increased the intracellular accumulation of doxorubicin, leading to doxorubicin-induced cell death. The intracellular accumulation of doxorubicin was not dependent on P-gp; we did not observe a functional change in P-Gp protein using Rhl23, a lipophilic cationic fluorescent dye that can specifically bind to P-gp. This was further confirmed by our RT-PCR and western blot data, which revealed that genistein did not influence the expression of P-gp. These results suggest that genistein increases the intracellular accumulation of doxorubicin by a P-gp-independent mechanism. However, the exact molecular mechanism requires further investigation.

Her2 (also known as Neu) is a member of the epidermal growth factor receptor (EGFR/ErbB) family (38). Thirty percent of breast cancer patients show amplification or overexpression of the Her2/neu gene, and overproduction of this gene contributes to chemoresistance. Overexpression of Her2/neu results in activation of downstream oncogenic pathways, such as the Ras/MAPK and PI3K/Akt pathways. Genistein inhibits receptor tyrosine kinase (RTK) activation and subsequently blocks Her2/neu/PI3K/Akt-mediated chemoresistance (39). Seo et al. reported that genistein and quercetin inhibit the growth of MCF-7 human breast cancer cells and MCF-7/Her2 vascular endothelial cell proliferation by inhibition of NF-xB activation (40). Choi and Kim showed that soy isoflavone aglucones and genistein exhibit anticancer effects by affecting ERα and c-erbB2 receptor expression (41).

The results presented here along with those of previous studies demonstrate that genistein at concentrations of 20-30 µM or greater can significantly inhibit tumor growth. This concentration is far below the IC50 value for cultured bone marrow stromal progenitor cells (CFU-F) and granulocyte-macrophage progenitor cells (CFU-GM). This suggests that the drug's toxic effects on the bone marrow are very minimal. Genistein may be a promising multidrug-resistance reversal agent in the clinical treatment of breast cancer.
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References