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ORIGINAL ARTICLE

Quercetin reverses tamoxifen resistance in breast cancer cells

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Summary

Purpose: *To investigate the effect of quercetin on the reversal of tamoxifen resistance in breast cancer cells, and explore the underlying mechanism.*

Methods: *We established a tamoxifen-resistant breast cancer cell line (MCF-7Ca/TAM-R), and exposed it to different concentrations of quercetin (experimental group 1: 10μM, group 2: 25μM, and group 3: 50μM). Each group was further subdivided into 2 subgroups: 1) simultaneous administration of quercetin and 4-hydroxy-tamoxifen (OHT); 2) sequential administration of quercetin (12-h induction) followed by OHT. No drug exposure and OHT alone were used as controls. We determined cell survival, apoptosis, and expression of ERa (estrogen receptor a) and Her-2 (human epidermal growth factor receptor 2).*

Results: *With increasing dosage of quercetin, significant decrease in proliferation and increase in apoptosis was observed. Low concentrations of quercetin (10μM) had no effects. We found no significant difference between simultaneous and sequential mode of drug administration. Further, with increasing dosage of quercetin, we observed a gradual reduction in Her-2 expression and upregulation of ERa. Again, no difference in Her-2 and ERa protein levels between simultaneous and sequential drug administration was noticed.*

Conclusions: *Proliferation inhibition and apoptosis in MCF-7Ca/TAM-R cells increase with increasing dosage of quercetin. This suggests that quercetin can reverse tamoxifen resistance in breast cancer cells. The underlying mechanism likely involves upregulation of ERa combined with downregulation of Her-2. However, this effect is independent of whether quercetin and tamoxifen are administered simultaneously or sequentially.*

Key words: *breast cancer, endocrine therapy, quercetin, tamoxifen-resistance*

Introduction

Carcinogenesis and development of breast cancer are closely related to the levels of steroid hormones. One treatment approach for breast cancer patients who are positive for ER, involves the use of endocrine therapy. Tamoxifen is non-steroidal compound that binds competitively to ER, thereby inhibiting the growth of breast cancer cells. Because of its therapeutic properties, tamoxifen is one of the main drugs currently used as first-line endocrine therapy against breast cancer. Despite increasing reported rates of clinical benefit, some researchers found that 40% of the patients who initially responded effectively to tamoxifen administration, developed secondary resistance during prolonged course of treatment. This indicates that recurrence and metastasis could occur during the standard 5-year treatment course of tamoxifen [1,2]. This resistance to tamoxifen has serious therapeutic implications.

The first example of a reversal effect of aldose reductase inhibitors on endocrine-resistant cells through upregulation of ERα and downregulation of Her-2 was reported in the 2009 San Antonio Breast Cancer Symposium (SABCS) [3]. Quercetin is a type of aldose reductase inhibitor of the flavonoid family, and used in traditional Chinese med-

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icine. It is known to have antitumor properties. Several studies have shown that its mechanism of action is likely to involve interference with tumor cell signal transduction, inhibition of proliferation and metastasis [4-7], antiangiogenic effects [8] and reversal of multidrug resistance [9]. Other studies have shown that the antitumor effect of quercetin on breast cancer cells is possibly mediated through downregulation of Her-2 expression [9]. Therefore, quercetin seems to function through inhibition of Her-2 function.

In this study, we searched whether quercetin can reverse the tamoxifen resistance of breast cancer cells. We also looked for the possible effect of quercetin on the expression of ERα and Her-2, and the regulatory crosstalk between signaling pathways mediated by ERα and Her-2. In this study, we used the tamoxifen resistant derivative of ER-positive human breast cancer cell line MCF-7Ca (MCF-7Ca/TAM-R) to address these questions. Specifically, we assayed the effect of quercetin induction on MCF-7Ca/TAM-R cells following tamoxifen exposure. Through this study, we aimed to address the issue of secondary resistance in breast cancer patients receiving long-term tamoxifen therapy.

Methods

Materials

The human breast cancer cell line MCF-7Ca was purchased from Shanghai Cell Department of the Chinese Academy of Sciences. We procured fetal bovine serum (FBS), DMEM medium and 0.25% trypsin from Gibco (California, USA), 4-Hydroxytamoxifen (4-OH-TAM, OHT), MTT and dimethyl sulfoxide (DMSO) from Sigma (Missouri, USA), and Annexin V-FITC and PI kit from Mbchem (Shanghai, China). A flow cytometer (FACS Aria, BD, New Jersey, USA) and a microplate reader (Bio-Rad, California, USA) were also used in this study.

Establishment of tamoxifen-resistant breast cancer cell line (MCF-7Ca/TAM-R)

The OHT impact method of high concentration and short-term exposure was used to establish the tamoxifen-resistant MCF-7Ca/TAM-R line. Briefly, MCF-7Ca cells were cultured in the logarithmic growth phase in 10 cm Petri dishes. OHT was added at a final concentration 10-6 mol/L. After changing medium every 2 to 3 days for 21 days, a stable MCF-7Ca/TAM-R cell line was established using a limiting dilution assay. The line was maintained in media containing OHT at a maintenance concentration of 10-7mol/L (mcOHT).

Grouping

The MCF-7Ca/TAM-R cells were divided into three groups: experimental group, negative control and blank control. The experimental group was further subdivided into three groups, based on the concentration of quercetin that they were subsequently exposed to: experimental group 1 (10 μM quercetin), experimental group 2 (25 M quercetin), and experimental group 3 (50 μM quercetin). Next, each of these groups was subdivided into two subgroups: group a (simultaneous administration of quercetin and OHT), and group b (sequential administration of quercetin (induced for 12 hrs, followed by OHT). In the negative control group, cells were exposed only to OHT, while in the blank control group, no drugs were added to culture. OHT was administered at a final concentration of 10^{-6} mol/L.

Determination of cell survival rate using MTT assay

Cells in all groups were cultured in 96-well plates with approx. 1×10^4 cells per well. To each well, 50μL of 1X MTT was added following the last dosage of 24, 48, and 72 hrs, and incubated at 37°C for 4 hrs. Next, 150μL DMSO were added to the culture and oscillated for 20 min for formazan crystals to dissolve completely. The optical density (OD) was measured on a microplate reader at 570 nm detection wavelength. Cell survival rates (CSRs) were calculated at different time periods following quercetin treatment using the formula: CSR % = OD value of test well / OD value of control well × 100%.

Detection of cellular apoptosis by flow cytometry

To determine the level of apoptosis, after the last dosage of 48-h, cultures in each group were digested with 0.25% trypsin to generate a single cell suspension. Next, we washed the suspension twice with precooled PBS, added 70% cold ethanol and fixed for 18 hrs at 4°C. Finally, the cell concentration was adjusted to 10^6/mL. One mL of the above cells was washed 3 times with PBS and then resuspended into one ml PI for 30 min in the dark. Ultraviolet excitation wave of 340nm wavelength and maximum emission wave with 620nm wavelength were set in flow cytometry analysis.

Evaluation of ERa and Her-2 protein expression using Western blotting

Total cellular protein from each group was extracted after the last dosage of 48 hrs, and separated on a 12% SDS-polyacrylamide gel by electrophoresis. Separated proteins were then transferred to a nitrocellulose membrane, and blocked for 2 hrs with PBST containing 5% skimmed milk. Next, the membranes were incubated with monoclonal primary antibodies against ERα and Her-2 overnight at 4°C. This was followed by a 2-h incubation with HRP-conjugated secondary antibody (goat anti-rabbit IgG-HRP) at room temperature.

Statistics

All measured data were presented as mean ±

standard deviation (±SD) and analyzed using Analysis of Variance (ANOVA). Comparison between groups was made using L-S-D method. Quantitative data were analyzed using the chi-square test and a $p<0.05$ was considered statistically significant. Data between groups were compared using the Split chi-square test. Corrective α value=0.05÷22 (split times)=0.002. In this assay, $p<0.002$ was considered as significant difference.

Results

Establishment of tamoxifen-resistant breast cancer cell line

We used the continued impact method of high OHT concentration (10^{-6}mol/L) and short-time exposure to successfully derive MCF-7Ca/TAM-R line. We observed proliferation of cells in response to OHT intervention at different concentrations, thus validating the line. In the parental MCF-7Ca line, we observed significant growth inhibition in the MTT assay at low concentrations of OHT (10^{-8}mol/L). In contrast, proliferation was not affected in the tamoxifen-resistant line. In this way, we validated the MCF-7Ca/TAM-R line.

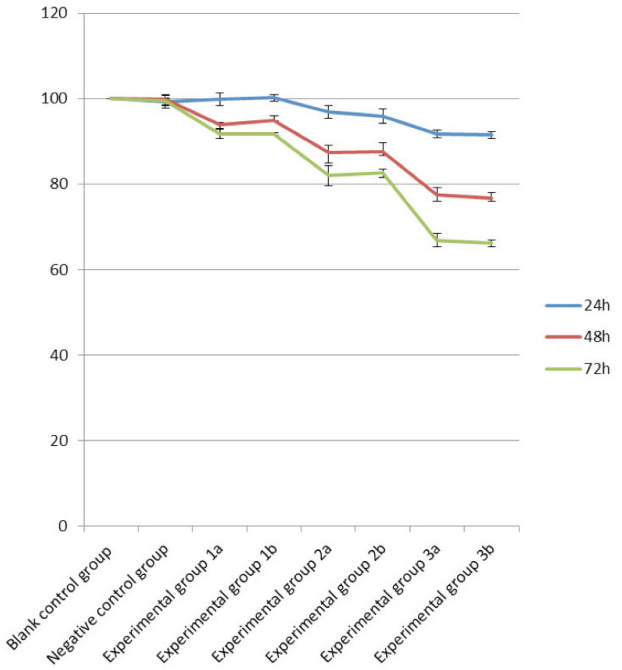

Figure 1. Cell survival curves of each experimental group at different time points after administration of quercetin and OHT.

Cell survival rate (CSR) of MCF-7Ca/TAM-R cells after quercetin administration

Optical density (OD) of proliferating cells was measured using the MTT assay, after either simultaneous or sequential administration of quercetin and OHT for 24, 48 and 72 hrs. We then calculated CSR at three time points following administration. Our results showed a significant reduction in CSR in all experimental groups ($p=0.000$), when compared to the control groups. This effect was enhanced with increasing dosage of quercetin and increasing duration of exposure. The most significant decrease in CSR was observed in the experimental group 3 after dosing for 72 hrs ($p=0.000$). However, we found no significant reduction in cell survival following either low dosage (10μM) or short-time exposure (24 hrs) of quercetin (experimental group 1 vs control groups: $p>0.05$).

Table 1. CSR (%) at different time points after quercetin administration, as measured with MTT

Hours	*Blank control*	*Negative control°*	*Experimental group 1*		*Experimental group 2*		*Experimental group 3*	
			a^{Δ}	*b*	a^{Δ}	*b*	a^{Δ}	*b*
24*	100 ±0	99.31 ±1.58	99.86 ±1.47	100.15 ±0.79	96.91 ±1.43	95.87 ±1.67	91.71 ±0.88	91.45 ±0.76
48	100 ±0	99.91 ±0.3	93.96 ±0.49	94.97 ±1.03	87.39 ±1.75	87.66 ±2.03	77.53 ±1.72▲	76.75 ±1.18▲
72	100 ±0	99.54 ±1.26	91.79 ±1.21	91.78 ±0.25	81.95 ±2.46▲	82.55 ±0.99▲	66.85 ±1.56▲	66.15 ±0.78▲

Quercetin concentration in experimental group 1= 10μM; group 2= 25μM; group 3= 50μM; a: simultaneous administration of quercetin and OHT; b: 12-h quercetin administration followed by OHT. Blank control group: no drug administered; negative control group: only OHT. Final concentration of OHT=10^{-6}mol/L. ▲$p<0.05$ (vs all other experimental groups, except a vs b within a group); $^{\Delta}p>0.05$ (a vs b); *$p>0.05$ (experimental group 1 vs experimental group 1); °$p>0.05$ (comparison between the two control groups)

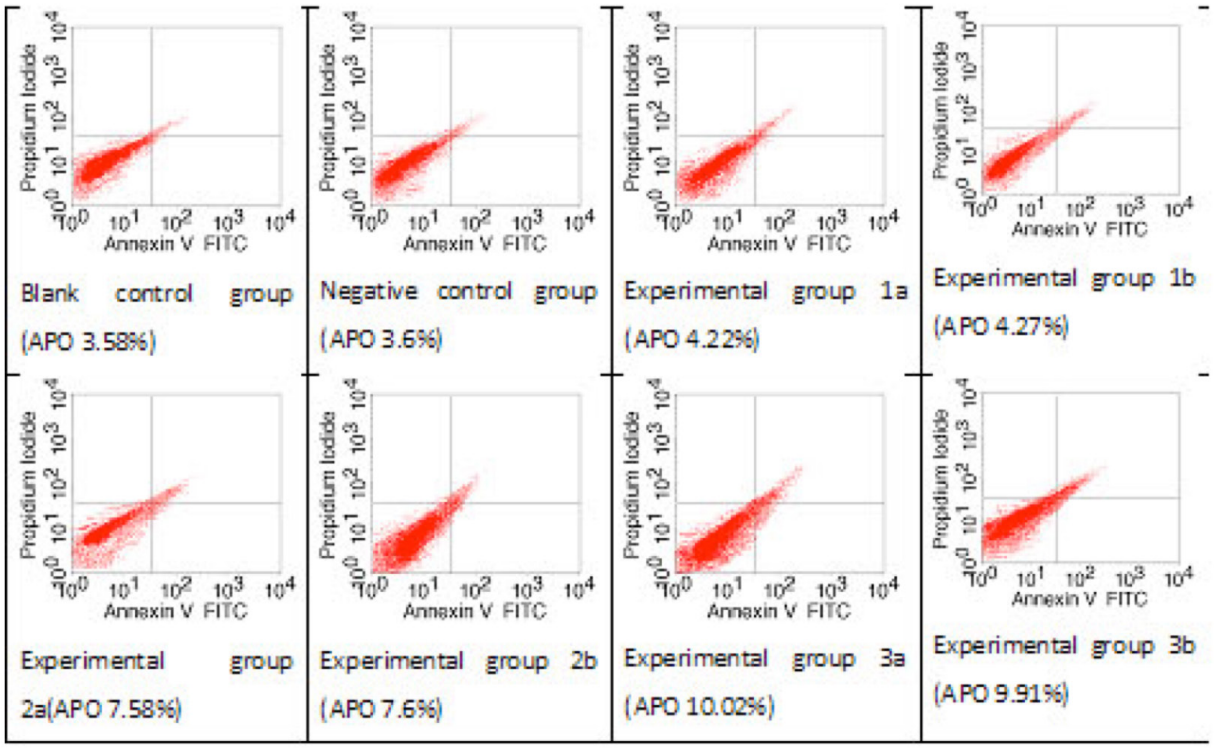

Figure 2. Apoptosis in MCF-7Ca/TAM-R cell line following quercetin administration, as detected by flow cytometry. Quercetin concentration in experimental group 1= 10 μM; group 2= 25 μM; group 3= 50 μM; a: simultaneous administration of quercetin and OHT; b: 12-h quercetin administration followed by OHT. Blank control group: no drug administered; Negative control group: only OHT. Final concentration of OHT= 10-6mol/L. APO: apoptosis rate.

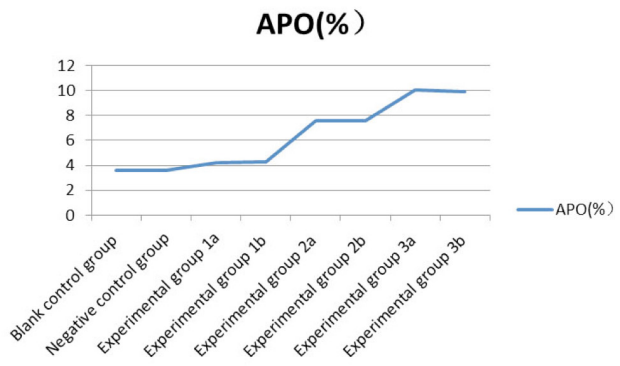

Figure 3. Apoptosis (APO) in MCF-7Ca/TAM-R cell line induced by quercetin.

In addition, there was no significant difference between the simultaneous and sequential method of administration in any experimental group and every time point assayed (p>0.05). Finally, we found no difference between the two control groups (p>0.05).

Survival curves were drawn according to the CSR values (Figure 1). Their analysis showed that while the survival curve of 24-h quercetin-exposed cells started to decline gradually, the viability of cells exposed for 72 hrs was the best amongst all experimental groups. While the survival curves of both experimental groups 2 and 3 showed a significant decrease, the latter displayed the most marked reduction. These values were similar between the simultaneous and sequential administration groups, suggesting that the sequence of drug delivery had no effect on cell survival.

Increased apoptosis of MCF-7Ca/TAM-R cells induced by quercetin

Next, we measured the level of apoptosis in all experimental groups following a 48-h exposure, using flow cytometry. We found significant apoptosis in all groups but to different degrees (Figures 2 and 3, p=0.000). Specifically, with increasing dosage of quercetin, the level of apoptosis was also increased. The most significant effect was observed in the 50μM dosage group (experimental group 3, p=0.000). We observed no effect in the low quercetin dosage (10μM) group (experimental group 1 vs control group: p>0.002). In addition, there was no significant difference in cell death between simultaneous and sequential methods of administration (p>0.002). The two control groups

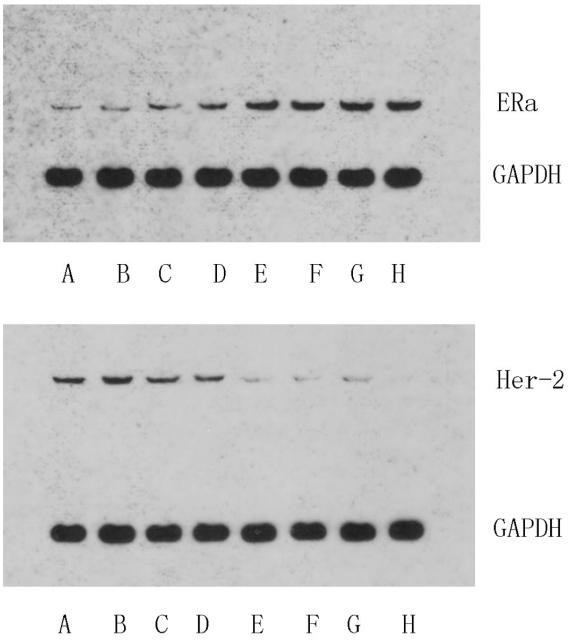

Figure 4. Expression of ERα and HER2 protein detected by Western blotting. **A:** Blank control group (no drug); **B:** Negative control group (only OHT); **C:** Simultaneous administration of 10μM quercetin and OHT; **D:** 10μM quercetin induction for 12 hours followed by OHT; **E:** Simultaneous administration of 25μM quercetin and OHT; **F:** 25μM quercetin induction for 12 hours followed by OHT; **G:** Simultaneous administration of 50μM quercetin and OHT; **H:** 50μM quercetin induction for 12 hours followed by OHT; Final concentration of OHT= 10-6mol/L.

were also statistically similar (p>0.002).

Changes in ERa and Her-2 expression in MCF-7Ca/TAM-R cells in response to quercetin

We estimated ERα and Her-2 protein expression by Western blotting (Figure 4). We detected bands at 66kD (corresponding to ERα), 185kD (Her-2) and 37kD (internal control GAPDH) (Figure 4). With increasing dosage of quercetin, gradual downregulation in Her-2 expression and upregulation in ERα was observed. In all experimental groups, there was no significant difference between subgroups subjected to simultaneous vs sequential methods of administration of quercetin and tamoxifen. By downregulating Her-2 expression, quercetin could block the regulatory crosstalk between signaling pathways mediated by ERα and Her-2. Moreover, quercetin also seemed to enhance ERα expression. Altogether, our data suggest that tamoxifen resistance in MCF-7Ca/TAM-R cells was reversed by exposure to quercetin.

Discussion

The most common treatment strategy for breast cancer involves surgery in combination with a secondary treatment approach such as chemotherapy, radiotherapy, endocrine therapy or molecular targeted therapy. Endocrine therapy is often preferred because it is believed to reduce the risk of recurrence and metastasis in patients who are ER-positive. Tamoxifen is a non-steroidal receptor antagonist which inhibits the growth of tumor cell by competitively binding to the ER. Following long-term successful clinical use, tamoxifen has now been established as an important component of endocrine therapy against breast cancer.

The rate of clinical success following tamoxifen administration has also been steadily increasing. However, few researchers have noted that tamoxifen has no therapeutic effect in almost 50% of breast cancer patients that are positive for hormone receptors. Meanwhile, secondary resistance has also been shown to occur as a consequence of a prolonged course of tamoxifen treatment in 40% of the patients who responded effectively to the treatment in its initial phase [1,2]. This therapeutic failure is compounded by the fact that drugs belonging to the family of selective estrogen receptor modulators (SERMs) tend to promote tumor growth when patients develop resistance to tamoxifen [10]. Such acquired resistance owing to long-term medication limits the efficacy and applicability of tamoxifen in breast cancer patients.

The mechanism underlying endocrine resistance is complex. Currently available studies on the effects of long-term tamoxifen administration have mostly focused on abnormal activation of growth factor signaling pathways and downregulation or loss of ERα [11-13]. Molecular regulatory crosstalk between Her-2 and ERα induces phosphorylation of ERα by activating a mitogen-activated protein kinase (MAPK). Research from Hurtado laboratory [14] showed that growth factors and estradiol (estrogen 2, E2) synergistically promote proliferation of ER-positive breast cancer cells. Estrogen receptors are composed of conservative amino acid sequences around Ser118 which provide the phosphorylation site that can be recognized by MAPK. Studies have indicated that Her-2 may activate MAPK to induce phosphorylation of ERα, leading to the growth and expansion of tumor cells [15]. Moreover, inhibitors of Her-2 and MAPK were shown to enhance sensitivity to tamoxifen [16]. In another study,

Sheng and Zhu demonstrated that growth factors can activate ERα mediated signaling pathways by phosphorylating MAPK *in vivo*. This in turn contributes to cell proliferation [17]. This result drew keen interest of researchers. These findings are important because they suggest that breast cancer patients who overexpress Her-2 might not be sensitive to tamoxifen therapy, or are more prone to developing tamoxifen resistance. This hypothesis was confirmed in the study by James et al. [18].

Another major cause of resistance to endocrine therapy is downregulation or loss of ERα expression [11,13] . Brodie et al. showed that the expression of ERα in resistant tumor cells is only 50% of that seen in healthy control cells [15]. In addition, Vesuna et al. reported that the transcription factor Twist can induce promoter methylation resulting in downregulation of ER in the human breast cancer cell line MCF-7, which is then followed by resistance to tamoxifen and fulvestrant (SERM drug family) [13]. These findings raised the question of whether increase in ERα expression could reverse tamoxifen resistance. In this regard, Zhang et al. found that elemene, extracted from *Radix curcumae* of the zingiberaceae family of plants can induce an increase in ERα expression, and return sensitivity to tamoxifen therapy in MCF-7Ca/TAM-R cells [12].

Aldose reductase inhibitors are known to upregulate ERα and downregulate Her-2 expression, to reverse the resistance to endocrine drugs in breast cancer cells [3]. Quercetin is an aldose reductase inhibitor of the flavonoid family that has been used in Chinese herbal medicine. It can downregulate Her-2 expression and inhibit proliferation of breast cancer cells [9]. Furthermore, quercetin can also function as a metabolic antagonist by inhibiting glucose uptake and metabolism in breast cancer cells through ER-independent signaling [19]. In the present study, we investigated the sensitivity of MCF-7Ca/TAM-R cells to tamoxifen treatment in response to quercetin induction. Our results show that tamoxifen can decrease proliferation and enhance apoptosis in the MCF-7Ca/TAM-R cell line. We further showed that this effect increases with increasing dosage of quercetin. This suggests that quercetin has the ability to enhance the sensitivity to tamoxifen and thus improve its therapeutic properties. Additional experiments revealed a possible molecular mechanism underlying the function of quercetin. In MCF-7Ca/TAM-R cells treated with quercetin, we observed significant reduction in Her-2 expression, which could interrupt the regulatory crosstalk between ERα and Her-2 mediated signaling. This could lead to the upregulation of ERα which was also detected in these cells. In addition, both sequential as well as simultaneous administration of quercetin and tamoxifen produced similar changes in Her-2 and ERα expression. Altogether, our results strongly suggest that quercetin can reverse tamoxifen resistance in a breast cancer cell line.

Quercetin is widely found in flowers, leaves and fruits of a variety of plants, and hence can be easily extracted and purified. In addition to easy availability, it is safe and inexpensive. Our findings not only provide the theoretical basis for subsequent studies on endocrine resistance in breast cancer, but also partially resolve the issue of secondary resistance arising from long-term drug administration. With low side-effects and excellent potency ratio, quercetin is a promising drug for clinical use as a reversal agent of secondary resistance to tamoxifen in breast cancer patients. It is important to note that while this study only explored the *in vitro* cellular and molecular effects of quercetin on a tamoxifen-resistant breast cancer cell line, the physiological mechanism of tamoxifen resistance in patients could be much more complex. The efficacy and safety of quercetin *in vivo* needs to be further demonstrated.

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