Silymarin increases the sensitivity of breast cancer cells to doxorubicin in doxorubicin-induced MCF-7 cells by inhibiting breast cancer resistance protein expression

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Abstract. The efflux transporter breast cancer resistance protein (BCRP) is typically overexpressed in cancer cells with reduced doxorubicin sensitivity. Silymarin is known to exert inhibitory effects against BCRP; therefore, the exposure of doxorubicin-resistant breast cancer cells to silymarin is expected to increase their sensitivity to doxorubicin. Here, we examined the effect of silymarin addition on the sensitivity of breast cancer cells to doxorubicin. MCF-7 breast cancer cells were exposed to doxorubicin for 14 days. Subsequently, the cells were treated with different concentrations of silymarin (10, 25, 50, or 100 µM) with or without doxorubicin. On days 3 and 7 following the treatment, cells were analyzed for viability and BCRP mRNA expression. MCF-7 cells exposed to doxorubicin for 14 days showed reduced sensitivity to doxorubicin, as indicated by the 9.5-fold shift in cytotoxicity concentration and the 9.7-fold increase in BCRP mRNA expression. Treatment with the combination of different concentrations of silymarin and doxorubicin significantly decreased the percent cell viability and reduced BCRP mRNA expression on days 3 and 7. The combination of doxorubicin and silymarin increased the sensitivity of MCF-7 cells to doxorubicin through the inhibition of BCRP mRNA expressions by silymarin.

1. Introduction
Resistance to chemotherapy is a substantial clinical problem that limits the effectiveness of anticancer drug treatments, accounting for treatment failure in more than 90% of patients with metastatic disease. Resistance to drugs might occur at early stages before chemotherapy (intrinsic resistance), occur during chemotherapy (extrinsic resistance), or be inducible—when cancer cells either genetically or epigenetically become resistant after a single treatment of chemotherapy over a long period [1,2]. The mechanisms underlying resistance in breast cancer cells include the active export of drugs from cells, the sequestration of drugs from cell compartments, the activation of detoxification enzymes and apoptosis suppressors, the modifications in apoptosis signaling, and the modifications in target drug expression. Additional factors, such as DNA repair defects and epigenetic regulation, are also associated with the development of drug resistance [1-3]. Among all these mechanisms, membrane
transporters play a particularly important role in the development of resistance to anticancer drugs, such as doxorubicin, in breast cancer [4].

Decrease in the uptake of drugs, including antracyclines (e.g., doxorubicin and daunorubicin), into cancer cells is caused by increase in the expression of efflux transporters, and such an increased expression of efflux transporters reportedly determines cancer resistance to antitumor agents [5]. The expression of efflux transporters, including breast cancer resistance protein (BCRP/ABCG2), P-glycoproteins (P-gp/ABCB1), and multidrug resistance protein 1 (MRP1), has been reported to increase during doxorubicin resistance [4-6]. Recently, BCRP has emerged as an important marker since it indicates cross-resistance to several structurally unrelated classes of cancer chemotherapeutic agents. Notably, the BCRP substrate spectrum includes a wide range of chemotherapeutics, including doxorubicin [7]. Previous studies have demonstrated that even at low doses and over the short-term use, doxorubicin induces a significant increase in BCRP expression [4]. Therefore, BCRP is considered a molecular target to enhance the efficacy of chemotherapy drugs [7]. Several natural isolates, particularly flavonoids, inhibit BCRP. Among these, flavonoids, such as chrysin, flavone, quercetin, genistein, silymarin, curcumin, and dibenzoylmethane, have been comprehensively studied in terms of their chemical structure and potential to inhibit BCRP overexpression in cancer cells [8]. The aim of the present study was to investigate the potential role of silymarin in enhancing the effects of doxorubicin in breast cancer cells and to study its role in modulating BCRP mRNA expression.

2. Methods

2.1 Materials and reagents
The MCF-7 breast cancer cell line was a kindly gifted by Makmal FKUI. Doxorubicin and silymarin were provided by PT Kalbe Farma (Indonesia). Dulbecco’s minimal essential medium (DMEM), fetal bovine serum (FBS), penicillin, streptomycin, Fungizone, and TrypLE Express were purchased from Gibco Ltd. (Singapore). TriPure isolation reagents were purchased from Roche Diagnostics (Singapore). MTS proliferation kits were purchased from Promega (USA), and primers used for quantitative real-time polymerase chain reaction (qRT-PCR) were purchased from First Base Ltd. (Singapore). The KAPA SYBR Fast One-Step RT-PCR Kits were purchased from KAPA Biosystems (USA).

2.2 Cell culture
MCF-7 cells were cultured in DMEM supplemented with 10% FBS, 100 IU/mL penicillin, 100 µg/mL streptomycin, and 2.5 µg/mL fungizone at 5% CO₂ at 37°C.

2.3 Drug preparation
Doxorubicin, silymarin, and ritonavir were dissolved in dimethyl sulfoxide (DMSO) and subsequently diluted in DMEM to desired concentrations. Final concentrations of DMSO were less than 0.001%. DMSO (0.001%) was used as a negative control for all experiments.

2.4 Doxorubicin dose determination
To determine the appropriate dose of doxorubicin that decreased the sensitivity of the cells to drugs, MCF-7 cells were exposed to either 0.1 µM or 0.25 µM doxorubicin for 10 days. The resultant cells with decreased sensitivity to doxorubicin were labeled MCF-7/Dox.

2.5 Drug treatment
MCF-7 and MCF-7/Dox cells were divided into the following treatment groups: control (DMSO); 0.1 µM doxorubicin alone; doxorubicin in combination with 10, 25, 50, or 100 µM silymarin; doxorubicin in combination with 19 µM ritonavir (as a positive control for BCRP inhibitor); 10, 25, 50, or 100 µM silymarin alone; or 19 µM ritonavir alone. The treatment was performed in 6-well plates for 7 days.
Cell viability and BCRP mRNA expressions were analyzed on days 3 and 7. Each treatment was performed three times in duplicates.

2.6 qRT-PCR
qRT-PCR was performed with a Roquette Corbet RT-PCR machine using the KAPA SYBR Fast One-step qRT-PCR kit (KAPA Biosystems, USA). Primers used were β-actin (Forward: 3’TTCGGCTTGCAACA CACTATG5’; Reverse: 5’TCCAGACACACCCACGGATAA3’), BCRP (Forward: 3’GGCATCGTGA TGAGACTCCG5’; Reverse: 5’GCTGGAAGGTGGACAGGGA3’).

Nine samples were prepared by mixing 200 nM primers, 10 μL KAPA SYBR Green Fast qPCR master mix, 0.4 μL KAPA RT Mix (50x), 0.4 dUTP, 100 ng RNA template, and millipore sterile water to obtain a final volume of 20 μL. Then, the samples were analyzed under the following conditions: 5 min at 42 °C for cDNA synthesis, 5 min at 95 °C to inactivate reverse transcriptase, 40 cycles of 30 s each at 95 °C for denaturation, 20 s at 55 °C for annealing, and 20 s for extension at 72 °C, followed by dissociation according manufacturer’s instructions. Ct values were automatically calculated using a software. Ct data were then processed using the Livak method. BCRP was considered as the target gene and β-actin was used as a reference gene in this study.

2.7 Data analysis
Data are presented as mean ± standard deviation. Unrelated one-way ANOVA, followed by multiple comparison using the Tukey method, was used for statistical analysis (α = 0.05). All analyses were performed using the GraphPad Prism 6 software (USA).

3. Results and Discussion
After 14 days of treatment with DMSO, 0.1 μM doxorubicin, or 0.25 μM doxorubicin, the cells exhibited decreased sensitivity toward both doses (Figure 1). However, the cells treated with 0.1 μM doxorubicin exhibited increased viability over the control group (Figure 1). Therefore, the cells treated with 0.1 μM doxorubicin (MCF-7/Dox) were used for subsequent treatments with silymarin. BCRP mRNA expression (up to 9-fold over control) significantly increased following treatment with 0.1 or 0.25 μM DMSO (Figure 2).

Figure 1. MCF-7 cell viability after 14 days of treatment with DMSO, doxorubicin 0.1 μM, or doxorubicin 0.25 μM.
After 14 days of treatment with 0.1 \( \mu \text{M} \) doxorubicin, cells were divided into the following treatment groups: DMSO, doxorubicin alone, a combination of doxorubicin and silymarin or ritonavir, and silymarin or ritonavir alone. After 7 days, the effects of each treatment on cell viability (Figure 3) and BCRP mRNA expression were examined (Figure 4).

**Figure 2.** BCRP mRNA expressions in MCF-7 cells following the treatment with doxorubicin (0.1 or 0.25 \( \mu \text{M} \)) for 14 days *significantly different at \( p < 0.05 \), with one-way ANOVA followed by Tukey’s multiple comparison.

**Figure 3.** Percent viability of MCF-7/Dox cells following treatment with doxorubicin, doxorubicin + ritonavir, or doxorubicin + silymarin for 3 or 7 days (over control) **significantly different at \( p < 0.001 \) vs Dox 0.1 (Day 3) and ## significantly different at \( p < 0.001 \) vs Dox 0.1 (Day 7), with one-way ANOVA followed by Tukey’s multiple comparison.

Dox 0.1 = 0.1 \( \mu \text{M} \) doxorubicin; Dox 0.1 + Rito 19 = 0.1 \( \mu \text{M} \) doxorubicin + 19 \( \mu \text{M} \) ritonavir; Dox 0.1 + Syl 10 = 0.1 \( \mu \text{M} \) doxorubicin + 10 \( \mu \text{M} \) silymarin; Dox 0.1 + Syl 25 = 0.1 \( \mu \text{M} \) doxorubicin + 25 \( \mu \text{M} \) silymarin; Dox 0.1 + Syl 50 = 0.1 \( \mu \text{M} \) doxorubicin + 50 \( \mu \text{M} \) silymarin; Dox 0.1 + Syl 100 = 0.1 \( \mu \text{M} \) doxorubicin + 100 \( \mu \text{M} \) silymarin.
Figure 4. BCRP mRNA expressions in MCF-7/Dox cells following treated with doxorubicin, doxorubicin + ritonavir, or doxorubicin + silymarin for 3 or 7 days

**significantly different at p < 0.001 vs Dox 0.1 (Day 3) and ##significantly different at p < 0.001 vs Dox 0.1 (Day 7), with one-way ANOVA followed by Tukey’s multiple comparison.

Dox 0.1 = 0.1 μM doxorubicin; Dox 0.1 + Rito 19 = 0.1 μM doxorubicin + 19 μM ritonavir; Dox 0.1 + Syl 10 = 0.1 μM doxorubicin + 10 μM silymarin; Dox 0.1 + Syl 25 = 0.1 μM doxorubicin + 25 μM silymarin; Dox 0.1 + Syl 50 = 0.1 μM doxorubicin + 50 μM silymarin; Dox 0.1 + Syl 100 = 0.1 μM doxorubicin + 100 μM silymarin.

The viability of cells treated with doxorubicin and silymarin as well as of those treated with doxorubicin and ritonavir significantly decreased at all doses (Figure 3). A dose-dependent effect of silymarin on cell viability was observed, with the strongest effect observed at the highest dose of 0.1 μM doxorubicin and 100 μM silymarin. The time of exposure did not affect the viability, except for that in the 0.1 μM doxorubicin and 100 μM silymarin group.

BCRP mRNA expression in MCF-7 decreased following cells treatment with the combination of doxorubicin and silymarin/ritonavir compared with that following treatment with doxorubicin alone (Figure 4). Doxorubicin in combination with 10 μM silymarin exhibited the weakest suppression of BCRP mRNA expression, while the remaining three concentrations did not exhibit any significant differences. No clear association was observed between the time of exposure and BCRP mRNA expression.

Furthermore, after 14 days of treatment with doxorubicin 0.1 μM, the effects of each drug alone on cell viability (Figure 5) and BCRP mRNA expression (Figure 6) for 7 days were assessed: DMSO, doxorubicin alone, and silymarin or ritonavir alone. Silymarin alone was not potent enough to induce a significant decrease in cell viability when administered for 3 days only. However, when the time of exposure was extended to 7 days, treatment with 50 and 100 μM of silymarin significantly decreased in cell viability compared with treatment with doxorubicin alone (Figure 5).

BCRP mRNA expression significantly decreased at all silymarin doses investigated (Figure 6). Increasing the time of exposure tended to increase the suppressive effect of silymarin on BCRP mRNA expression (Figure 6).
Figure 5. Percent viability of MCF-7/Dox cells treated with doxorubicin, ritonavir, or silymarin for 3 or 7 days (over control)

*significantly different at p < 0.05, with one-way ANOVA followed by Tukey’s multiple comparison.
Dox 0.1 = 0.1 μM doxorubicin; Rito 19 = 19 μM ritonavir; Syl 10 = 10 μM silymarin; Syl 25 = 25 μM silymarin; Syl 50 = 50 μM silymarin; Syl 100 = 100 μM silymarin.

Figure 6. BCRP mRNA expressions in MCF-7/Dox cells following treatment with doxorubicin, ritonavir, silymarin for 3 or 7 days

**significantly different at p < 0.001 vs Dox 0.1 (Day 3) and ***significantly different at p < 0.001 vs Dox 0.1 (Day 7), with one-way ANOVA followed by Tukey’s multiple comparison.
Dox 0.1 = 0.1 μM doxorubicin; Rito 19 = 19 μM ritonavir; Syl 10 = 10 μM silymarin; Syl 25 = 25 μM silymarin; Syl 50 = 50 μM silymarin; Syl 100 = 100 μM silymarin.

Doxorubicin is an important chemotherapeutic agent used in the treatment of solid tumors, including breast cancer. However, its effectiveness is limited due to the short duration required for the development of resistance [10]. Our results are in agreement with those of many previous studies demonstrating that the sensitivity of cancer cells can decrease within a very short period of 14 days, which is accompanied by an approximately 10-fold increase in BCRP mRNA expression.
Using lower doses of doxorubicin (14 and 21 nM), Calcagno et al. have demonstrated that both BCRP mRNA and protein levels increased within a short period of only 10 days. The authors have concluded that altered BCRP expression might be an early phenotype that arises during the development of doxorubicin resistance [4].

Longer periods of resistance to doxorubicin have been observed in several other previous studies. Nurulita et al. have observed the viability of MCF-7 cells following exposure to doxorubicin at different dosages using the immunofluorescence method and western blotting analysis. They have reported that cancer cell resistance was induced after exposure to doxorubicin at 100 nM for 5 weeks, which was followed by an increase in P-glycoprotein expression [11]. Conversely, Lukyanova et al. have used doxorubicin at 0.1–32 μg/mL for 2 months to induce resistance in cells, which altered cell morphology and increased cell adhesion, thus showing that MCF-7 cells resistant to doxorubicin change in ultrastructure [12]. These resistant cells appear to have a larger cell size as well as an increased number of microtubules compared with their parent cells.

Our results demonstrated that silymarin is a potent BCRP inhibitor; however, silymarin did not exert a notable effect on cell viability when administered alone. Thus, silymarin appears to be a good candidate chemosensitizer for doxorubicin. The addition of silymarin to doxorubicin resulted in decreased cell viability and BCRP mRNA expression. In another study, Tamaki et al. have demonstrated that silymarin could weakly inhibit BCRP [8]. However, they have used only a single dose of silymarin, whereas we used repeated doses of silymarin in the present study. Thus, our study confirms that repeated doses of silymarin are required to inhibit BCRP expression. Increasing treatment duration from 3 to 6 days did not increase the effects of silymarin, but this maintained the effects of silymarin on cell viability and BCRP suppression. In this study, we used ritonavir as a positive control for BCRP inhibition [13,14] on the basis of the study by Gupta et al. wherein they have tested the effects of ritonavir in combination with doxorubicin and have proposed ritonavir as a candidate for BCRP inhibition [14]. While we found that silymarin alone at low doses is not capable of suppressing cell viability, several studies have previously described the anticancer potential of silymarin [15-17]. Agarwal and colleagues in their research have demonstrated that silymarin (silybin) showed synergistic effects as an anticancer drug when combined with chemotherapeutic drugs, such as doxorubicin, cisplatin, or carboplatin, in MCF-7 and MDA-MB468 cells [15]. Thus, it is likely that treatment with doxorubicin at 0.1–10 μg/ml and silymarin (silybin) at 0.1 μM facilitates apoptosis.

Silybin and its derivatives are also known as P-gp inhibitors [18]. A study by Colombo et al. has shown that the combination of silymarin with doxorubicin or paclitaxel showed antiproliferative effects on both sensitive (LoVo) and resistant (LoVo/DX) colon cancer cells. At low doses, silymarin exerted synergistic effects with doxorubicin and paclitaxel on LoVo cells, while at high doses, these effects applied to both cell types [19]. Taken together, these results suggest that silymarin may be beneficial for the treatment of colon cancer in combination with other chemotherapeutic drugs [19].

4. Conclusion
In conclusion, silymarin in combination with doxorubicin can increase the sensitivity of breast cancer cells to doxorubicin. This outcome is attributed to the inhibitory effect of silymarin on the expression of BCRP. We posit that silymarin is useful as a chemosensitizer in doxorubicin therapy.

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