RESEARCH ARTICLE

in vitro Modulation of P-glycoprotein, MRP-1 and BCRP Expression by Mangiferin in Doxorubicin-Treated MCF-7 Cells

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Abstract

The multidrug resistance phenotype is one of the major problems in development of cancer cell resistance to chemotherapy. Some natural compounds from medicinal plants have demonstrated promising capacity in enhancing anticancer effects in drug resistant cancer cells. We aimed to investigate whether mangiferin might have an ability to re-sensitize MCF-7 breast cancer cells previously treated with short-term doxorubicin *in vitro*, through the modulation of efflux transporters, P-glycoprotein (P-gp), MRP1 and BCRP. We exposed MCF-7 breast cancer cells pretreated with doxorubicin for 10 days to mangiferin (10, 25 or 50 μM) for 96 hours. Afterwards, we evaluated influence on cell viability and level of mRNA expression of P-gp, MRP1 and BCRP. Doxorubicin given in combination with mangiferin at low concentrations (10 and 25 μM) failed to give significant reduction in cell viability, while at the highest concentrations, the combination significantly reduced cell viability. The mRNA expression analysis of P-gp, MRP1 and BCRP showed that mangiferin had inhibitory effects on P-gp but no effects on MRP1 and BCRP. In conclusion, we suggest that mangiferin at high concentrations can be used as chemosensitizer for doxorubicin therapy. This effect might be attributed by inhibitory effects of mangiferin on P-glycoprotein expression.

Keywords: Doxorubicin - mangiferin - P-glycoprotein - MRP1 - BCRP.

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Introduction

Doxorubicin is an effective chemotherapeutic agents, which has been used extensively for treatment in various cancer, including breast cancer (Smith et al., 2006; Ghebeh et al., 2010; Andreopoulou and Sparano, 2013). Unfortunately, the incidence of cancer cell resistance to doxorubicin is quite common, which leads to unsuccessful treatments in many patients (Smith et al., 2006). In a phase 3 clinical trial, the result showed that the time to progression in breast cancer patients treated with pegylated doxorubicin were 7-9 months (Sparano et al., 2009).

One of the cause of the cause of the doxorubicin resistance is a phenomenon known as multidrug resistance MDR (Choi, 2005; Wind and Holen, 2011). The most common MDR is due to the increased efflux pumps in the cell membrane including P-glycoprotein, multidrug resistance associated protein-1 (MRP1) and breast cancer resistance protein (Choi, 2005). Doxorubicin was known as substrate as well as inducers of the major efflux transporters in breast cancer cells, P-glycoprotein, MRP1 and BCRP (Chien and Moasser, 2008; Choi, 2005).

Recently, several studies have documented the ability of natural compounds to increase the sensitivity of cancer cells to anticancer drugs by inhibiting efflux transporters (Nabekura, 2010). One of the promising natural compounds with anticancer activity and possible chemosensitizing activity is mangiferin (Zhang et al., 2013).

Mangiferin is natural polyphenol with C-glycosylxanthone structure, which can be found in many plant species, among which is the mango tree (*Mangifera indica*) (Matkowski et al., 2013). Several studies indicated that *Mangifera indica* exerts various pharmacological effects such as antibacterial, antifungal, anthelmintic, antiparasitic, antitumor, antispasmodic, antipyretic, antidiarrheal, antiallergic and immunomodulation (Shah et al., 2010). Aside from its anticancer activities, previous study in HK-2 proximal tubule cell line and Caco-2 cell line, showed that phyto-drug prepared from the stem bark of *Mangifera indica* L. inhibited the multidrug transporter, P-glycoprotein (Chieli et al., 2010).

In this study we aim to investigate whether mangiferin have the ability to re-sensitize breast cancer cells previously treated with short-term doxorubicin *in vitro*, through the modulation of efflux transporters, P-gp, MRP1 and BCRP.

Materials and Methods

Materials

MCF-7 cell line, a model cell line for human mammary

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carcinoma was used. The cells were kind gift from the Laboratory of the Agency for the Assessment and Application Technology (BPPT), Serpong, Indonesia. Doxorubicin, verapamil, nelfinavir and dimethylsulphoxide were purchased from Sigma-Aldrich (Singapore). Mangiferin was purchased from Plamed Science Technology Company, Xi-an, China. Dubelco Minimal Essential Medium (DMEM), fetal bovine serum (FBS), Penicillin/Streptomycin and fungizone were obtained from Gibco Ltd (Singapore). Tripure isolation reagents and LightCycler RNA Master SYBR Green I kit were purchased from Roche Diagnostics (Singapore). Primers used were purchased from 1st BASE Ltd, Singapore.

Cell culture

MCF-7 cell line was cultured in DMEM supplemented with 10% heat-inactivated fetal bovine serum, 100 IU/mL penicillin, 100 μg/mL streptomycin and 1% Fungizone. Medium was routinely changed everyday. The cells were sub-cultured when reaching 90% confluence. All the cell culture plates were purchased from NUNC Thermo Fisher Scientific.

Treatment of doxorubicin-treated MCF-7 with mangiferin

MCF-7 cells were grown in a medium containing doxorubicin 0.1 μM for 10 days. The medium was changed every day. Briefly, 100,000 of the MCF-7 cells that were grown in medium containing doxorubicin 0.1 μM were seeded in a 6-well culture dish. Mangiferin 10 or 25 or 50 μM were administered to the culture dish every 24-hour in combination with doxorubicin μM up to 96-hour. Verapamil 50 μM was used as positive control for P-gp and MRP1 inhibitor, while nelfinavir 15 μM was used as positive control for BCRP inhibitor. Mangiferin, verapamil and nelfinavir were dissolved in DMSO, then were diluted in serum free medium, with final concentrations <0.01%. Medium and treatments were changed everyday. The cells were harvested after 96-hour drug administrations. Afterwards, the cells were analyzed for cell viability using trypan blue exclusion method. RNA were isolated and quantified for mRNA expressions using qRT-PCR. All treatments were done in 4 separate experiments.

RNA isolation

Total RNA was isolated using Tripure Isolation Reagents (Roche) according to the manufacturer's protocol. Quantity and purity of the RNA were determined by measuring 260/280 absorbance using NanoDrop spectrophotometer. RNA was then subjected to quantitative real-time reverse transcription polymerase chain reactions (qRT-PCR)

qRT-PCR

The mRNA expressions of the following drug transporters are quantified: P-glycoprotein, MRP1 (Multidrug resistance protein 1) and BCRP (Breast Cancer Resistance Protein).

Quantitative real-time reverse transcription quantitative polymerase chain reaction (qRT-PCR) was performed using LightCycler RNA Master SYBR Green I kit (Roche) and the LightCycler 2.0 Instrument (Roche, USA), according to the manufacturer's instruction. Primers for transporters are as described previously (Farabegoli et al., 2010): P-glycoprotein, P-gp F: CCCATCATTGCAATAGCAGG; P-gp R:GTTCAAACTTCTGCTGGTCA; BCRP, BCRP F:TTCGGCTTGCAACAACTATG; BCRP R:TCCAGACACACCACGGATAA; MRP1 F:ATGTCACGTGGAATACCAGC MRP1 R: GAAGACTGAACTCCCTTCCT. ß-actin was used as housekeeping gene. The sequence of the primers were: ß-actin F:GGCATCGTGATGGACTCCG; ß-actin R: GCTGGAAGGTGGACAGCGA. Annealing temperature were 58°C for Pgp and MRP1; 60°C for MRP1, and 55°C for BCRP. The relative changes in mRNA transporter expression levels were calculated using Livak method (Livak and Schmittgen, 2001).

Data analysis

The data were presented in the form of means ± standard deviation (SD). Graphs were created using GraphPad Prism software. Statistical significance was calculated using One-Way ANOVA followed by post hoc test, with $p<0.05$ were considered significant.

Results

Our result showed that after 10-day incubation of MCF-7 cells with doxorubicin, the cells had already shown decreased sensitivity towards doxorubicin as given in the increased percentage of viable cells over control (Figure 1).

After 96-hour of drug administration to MCF-7 cells pretreated with doxorubicin, it was shown that the addition of mangiferin to doxorubicin tends to increase the sensitivity of the cells to doxorubicin. Lower concentrations of mangiferin (10 and 25 μM) did not cause significant reduction in MCF-7 cell count, while the higher concentrations of mangiferin 50 μM. Treatment of the cells with verapamil (positive control for P-gp and MRP inhibitor) and nelfinavir (positive control for BCRP) also resulted in a significant reduction of percentage of viable cells (Figure 2).

The expressions of P-gp, MRP1 and BCRP were measured. The result showed that the addition of mangiferin to doxorubicin slightly reduced the expression of P-gp mRNA expressions at lower concentrations (10

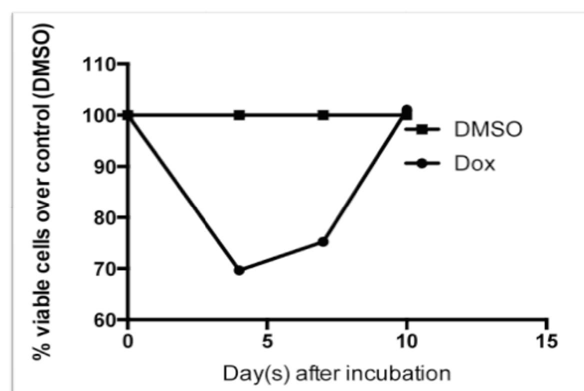

Figure 1. Percentage of viable cells after incubation of MCF-7 cells with doxorubicin or DMSO

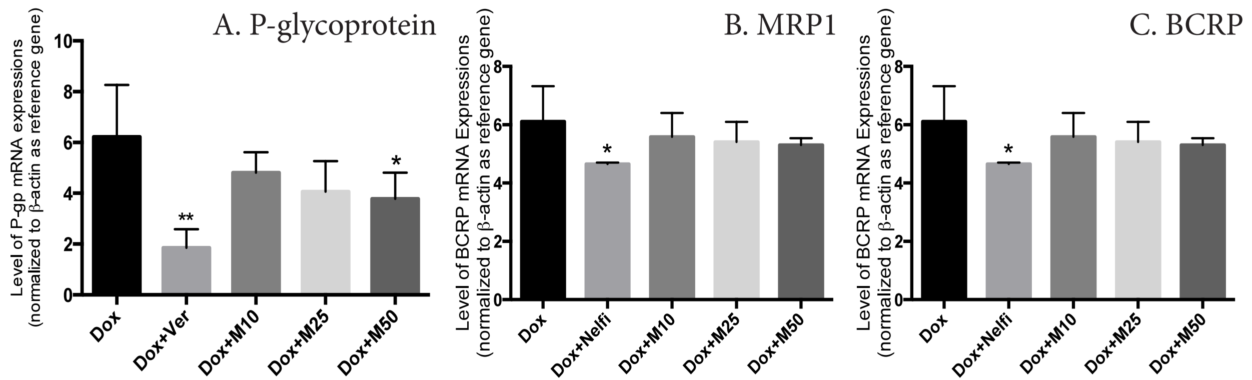

Figure 3. The expressions of A) P-glycoprotein mRNA; B) MRP1 mRNA; C) BCRP mRNA after 96-hour (4-day) treatment of MCF-7 cells with doxorubicin alone or combination of doxorubicin+mangiferin 10 μM/mangiferin 25 μM/mangiferin 50 μM/verapamil 50 μM/nelfinavir 15 μM. Results were shown as mean±SD (N=4). Dox=doxorubicin 0.1 μM; M10=mangiferin 10 μM, M25=mangiferin 25 μM; M50=mangiferin 50 μM; ver=verapamil 50 μM; nelfi=nelfinavir 15 μM. (*) significant difference versus doxorubicin at p<0.05; (**) significant difference versus doxorubicin at p<0.001

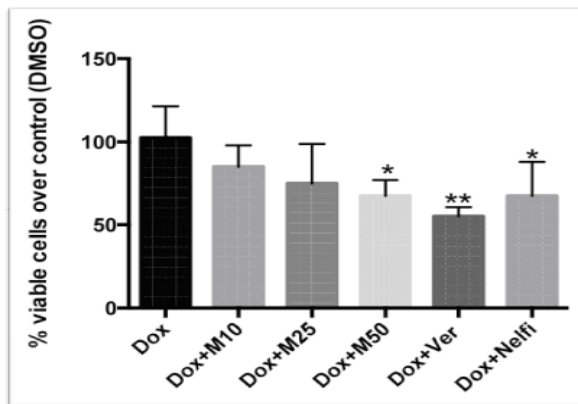

Figure 2. Percentage of viable cells *vs* control (DMSO) after 96-hour treatment with doxorubicin 0.1 μM, doxorubicin with Mangiferin 10 μM/Mangiferin 25 μM/Mangiferin 50 μM/Verapamil 50 μM/Nelfinavir 15 μM. Results were shown as mean ± SD (N=4).Dox=doxorubicin 0.1 μM; M10=mangiferin 10 μM, M25=mangiferin 25 μM; M50=mangiferin 50 μM; ver=verapamil 50 μM; nelfi=nelfinavir 15 μM. (*) significant difference versus doxorubicin at p<0.05; (**) significant difference versus doxorubicin at p<0.001.

and 25 μM), while at the highest concentrations (50 μM) showed a significant reduction of Pgp mRNA expressions (Figure 3).

Mangiferin at all concentrations did not seem to influence the expressions of MRP1 and BCRP mRNA expressions.

Discussion

In this study, we exposed breast cancer cells (MCF-7) doxorubicin for 10 days with a relatively low dose. Many other studies that were aimed at reversing the tumor cell resistance to doxorubicin or other anthracycline were using resistant cell lines developed in a long period. Kars et al. (Kars et al., 2006) used MCF-7 breast cancer cells that were exposed to doxorubicin for over a year. While this model is ideal to understand the mechanism of cancer cells resistance to drugs, but this kind of model did not mimic *in vivo* situation.

Our results showed that even in a short period of administration, after 10 days, doxorubicin had failed to suppress the cancer cell growth. Several other studies also showed similar results as ours. Smith et al. established doxorubicin-resistant cell line from the surviving population of MDA-MB-231 breast cancer cells after treatment with doxorubicin for 24-hour (Smith et al., 2006), while Calcagno et al. managed to develop doxorubicin-resistant cell line from MCF-7, IGROV-1 and S-1 clones after exposing doxorubicin for 10 days (Calcagno et al., 2008).

After 10-day administration with low doxorubicin concentrations, we added mangiferin or positive controls (verapamil or nelfinavir) to the surviving MCF-7 cells. Mangiferin at lower concentrations had failed to reduce the percentage of viable cells, while mangiferin at the highest dose and both positive controls (verapamil and nelfinavir) showed significant effects in the reduction of viable cells.

The significant effect of verapamil in suppressing cell growth is in accordance to several other studies. Donmez et al. (2011) reported verapamil had synergistic effect with doxorubicin in MCF-7 cells with established resistant to doxorubicin, while Zhang et al. showed that verapamil might be used to reverse the resistance of MCF-7 cells to adriamycin (Zhang et al., 2007). Verapamil is a specific first generation of P-gp efflux pump, but it also showed inhibitory effects on MRP1 (Wong et al., 2009). In our study, the inhibitory effects of verapamil to the expressions of P-gp and MRP1 were shown to be very strong.

Nelfinavir also had significant effect in reducing the percentage of cell growth. This effect might be due to its inhibitory effect to the BCRP mRNA expression, which were shown to be strong. A study by Bruning et al. reported that tamoxifen and nelfinavir also had synergistic anticancer effects that are independent to the estrogen receptor status of the cells (Bruning et al., 2010).

Mangiferin, on the other hand, only showed significant result on the highest dose. At the lower concentrations, mangiferin did not suppress the expressions of P-glycoprotein enough to cause an evident reduction in percentage of viable cells. Our result also showed that mangiferin did not influence the expressions of MRP1 and BCRP mRNA expressions at all. On the other hand, at higher concentrations, while mangiferin did not possess

any effect on the expressions of MRP1 and BCRP, the inhibitory effects towards P-glycoprotein were enough to re-sensitize the cells to doxorubicin. Our results are in agreement to the previous study done by Chieli et al. that showed that phytodrug from *Mangifera indica* stem bark decreased the P-gp expressions in a concentration and time-dependent increase (Chieli et al., 2009).

Li et al. (2013) proved mangiferin as low as 12.5 μM had significant anticancer effects on cells with estrogen-positive and negative type cells, through the regulation of matrix metalloproteinases, epithelial to mesenchymal transition, and ß-catenin pathway (Li et al., 2013), while Du Plessis-Stoman showed that mangiferin-mediated down-regulation of NFϰB showed potential for chemotherapeutic agent-mediated cell death (du Plessis-Stoman et al., 2011). But apparently, those signaling regulations are incapable to reverse the sensitivity of cells to doxorubicin.

Doxorubicin treatments showed increase in level of mRNA expressions of P-gp, MRP1 and BCRP, but our results showed that mangiferin only modulates the mRNA expressions of P-gp and not MRP1 or BCRP.

Based on our result, we believe that mangiferin might be a promising candidate agent to reverse doxorubicin-resistant breast cancer cells. But further studies on the concentrations and time needed for the reversal are needed to explore this potential strategy.

In conclusion, mangiferin at high concentrations can be suggested as a chemosensitizer for doxorubicin therapy. This effect is attributed by the inhibitory effect of mangiferin on P-glycoprotein expressions.

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