Original Article

The effect of quercetin and doxorubicin combination in inhibiting resistance in mcf-7 cell

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Abstract:

Background*:* Doxorubicin chemotherapy may induce the development of resistance in the cancer cells. Overexpression of P-glycoprotein (P-gp) has prominent causative role in this process. Unfortunately, many inhibitory agents do not exert satisfying clinical outcome to overcome resistance. Quercetin, a polyphenolic compound, can be developed as P-gp inhibitor due to its cytotoxic properties. ***Objective*****:** To investigate the effect of quercetin and doxorubicin combination in inhibiting the development of resistance in MCF-7 cell. ***Methods*****:** Doxorubicin resistant MCF-7 cell were developed from the parent MCF-7 cell by exposing to 75 nM doxorubicin for 25 days. The inhibitory effect of resistance was evaluated *via* exposured to 75 nM doxorubicin and 750 nM quercetin combination in the same manner with resistance induction. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide) citotoxicity assay and flowcytometry assay were performed to investigate the degree of resistance and to inhibit the development of resistance. ***Results*****:** The exposure of 75 nM doxorubicin in MCF-7 cells (MCF-7 cells/dox75) exhibited sensitivity significantly decrease and upregulation of P-gp expression. The combination of 750 nM quercetin and 75 nM doxorubicin (MCF-7 cells/dox75q750) caused a significantly increase in cell sensitivity to doxorubicin ($p<0,05$) and a decrease in P-gp expression ($p> 0,05$). ***Conclusions:*** The exposure of 75 nM doxorubicin led to the development of doxorubicin-acquired resistance MCF-7 cell. The combination of quercetin and doxorubicin have potency to inhibit the development of doxorubicin-acquired resistance MCF-7 cell by increasing cell sensitivity and reducing P-gp expression level.

Keywords: MCF-7 cell line; Resistance; Quercetin; Doxorubicin; P-gp

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Introduction:

Doxorubicin is an anthracycline antibiotic which is considered as an effective chemotherapeutic agent for breast cancer, however, its usage is restricted for its cardiotoxicity and resistance.[1] There are many mechanism induce doxorubicine resistance. P-glycoprotein (P-gp) is being one of major factor which contributes it. Increased P-glycoprotein (P-gp) level contributes to the development of resistance that ultimately leads to poor therapeutic outcome.[2]

Efforts has been done to overcome resistance against chemotherapeutic agents due to P-gp upregulation by developing drug that inhibit P-gp activities, but the result has not yielded optimal results to date.[3-5] The hopes of developing P-gp modulator that could act with high degree of selectivity and low degree of toxicity has directed many investigators' research focus onto polyphenolic compounds. One of those potential compounds to restore chemotherapy resistance is quercetin. Quercetin (3,3',4',5,7-pentahydroxyflavone) is a flavonoid polyphenolic compound that has 3 rings dan 5

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hydroxyl groups.[6,7] Numerous in vitro and in vivo study using quercetin have shown not only having anti-inflammatory, antioxidative and antiviral but also having anti-cancer activities.[8-10] There is also study showed that combination of quercetin and doxorubicin increase doxorubicin effects in human breast cancer cells.[11]

Effect of quercetin on P-gp transporter to reduce chemotherapy resistance is still controversial. Quercetin was shown by one group significantly reduce doxorubicin retention in cultured rat hepatocytes.[12,13] Another group found that quercetin is a potential modulator of P-gp to restore chemotherapy resistance via inhibitation of P-gp-facilitated efflux of doxorubicin, so that enhancing doxorubicin concentration in MCF-7/ADR-RES cancer cell model.[14,15]

Apart from its controversial effect, quercetin is still considered to be effective to reduce insensitivity of neoplastic cells to chemotherapeutic agents. In this study, we have evaluated the effect of quercetin and doxorubicin combination in preventing the development of resistance by evaluating the sensitivity of MCF-7 cell and expression of P-gp in MCF-7 cell.

Materials and methods:

Chemicals. Doxorubicin injection 2 mg/mL (Ebewe) and quercetin hydrate 95% (Sigma 337951) were used in this study. Other materials used were Dulbecco's modified eagle medium (DMEM), fetal bovine serum (FBS) 10 %, penicillin-streptomycin 1% (Gibco), 1mM-glutamine, fungison 1% (Gibco), sodium bicarbonate, trypsin, aquabidest, alcohol 70%, 4(2-hydroxyethyl)-1-piperazin-ethane-sulfonic acid (HEPES), phosphate-buffered saline (PBS) (Sigma) and (3-(4,5-dimethylthiazole-2-il)-2,5-diphenyl tetra zolium bromide) (MTT). Anti-P-glycoprotein antibody (UIC2) (FITC) (cat.ab66250, Abcam, Cambridge, USA), diluted in PBS, was used in flowcytometry to assess the level of P-gp expression.

Cell lines, development of resistant subline. The MCF-7 cell, which is a model cell line for human mammary carcinoma, was used as parental cell line. The cells were obtained from *Laboratorium Penelitian dan Pengujian Terpadu* (LPPT) Universitas Gadjah Mada (UGM), Yogjakarta. MCF-7 cells were maintained as an attached type monolayer culture in DMEM medium supplemented with 10% FBS, 1% penicillin-streptomycin, and 1 mM L-glutamine. Resistant cells were developed by the *in vitro* culture of sensitive parental cells in the presence of doxorubicin for as long as 25 days. The IC_{50} value from parental MCF-7 cells was used to established the most ideal concentration in development of doxorubicin-resistant MCF-7 cell. The development of doxorubicin-resistant MCF-7 cell was done by giving doxorubicin concentration below the IC_{50} value of parental MCF-7 cell, and the cells were then incubated 24 hours, twice a week for a period of 25 days. The evaluation of cell sensitivity and level of P-gp expression were performed to investigate the resistance developed in MCF-7. Evaluation of cell sensitivity was determined using MTT cytotoxicity assay. The degrees of resistancy were calculated by comparing IC_{50} value of resistant cell line to IC_{50} value of sensitive parent MCF-7 cell and by comparing the level of expression P-gp in resistant cell to parental MCF-7 cell line. The level of P-gp expression was measured *via* FACSCalibur flowcytometer (BD Biosciences).

Assay for inhibition of resistance development in MCF-7 cell. To know whether the combination of doxorubicin and quercetin can inhibit the development of resistance, MCF-7 cells were treated with doxorubicin-quercetin combination in the same manner with resistance development. The cells were incubated 24 hours with doxorubicin-quercetin combination twice a week for as long as 25 days. Inhibition of resistance development was determined with MTT cytotoxicity assay and P-gp expression level. The analysis of inhibition degree was established by comparing mean viability values, IC_{50} value, and level of P-gp expression in the cells that had been treated with doxorubicin-quercetin combination with resistant cells. The IC_{50} value and level of P-gp expression in cells that had been treated with doxorubicin-quercetin combination were expected to be lower than those of resistant cells.

Assay for cell sensitivity. Parental cells, resistant cells and cells that had been treated with doxorubicin-quercetin combination were tested in 96-well microtiter plates. Cells were seeded into each well (1×10^4) with the exception of medium controls-containing wells. The plates were incubated at 37°C for 24 h. Then, cells were incubated with increasing concentration of doxorubicin in 5% CO_2 at 37°C for 24 h. The cells were incubated again for further 4 h with MTT-containing medium (0,5 mg/mL). The reaction stopped with 100 µL 10% SDS solution in 0,1 N HCl solution was added to each well. The plates were further incubated at 37°C overnight to allow the dissolution of formazan crystals

that were produced by mitochondrial enzymes in the viable cells. The viability of cells was determined by measuring the optical density of the chromogenic product at 595 nm with a Bio Rad 680 XR ELISA Reader. The viability of cells and IC_{50} value were determined for each cell line.

Assay of P-gp expression. This assay was slightly modified from the procedure of Boyerinas (2012).[16] In order to evaluate the expression of P-gp, parental cells, resistant cells and cells that had been exposed to doxorubicin-quercetin combination were collected respectively through trypsinization, and the concentration of each cells was adjusted to $6x10^5$-$1x10^6$ cells/test. Cells were suspended in 100 µL warm UIC2 binding buffer (PBS, 1%BSA) at the concentration of 10^6 per 100 µL. Tubes were incubated at 37° C for 10 minutes. FITC-labeled UIC2 (10 µL) was added to each sample and tubes were incubated for another 30 minutes. The 1 mL cold UIC2 binding buffer was added to each tube and cells were centrifuged at 2000 rpm for 5 minutes and washed twice in cold UIC2 binding buffer. Cells were then resuspended in 150 µL cold UIC2 binding buffer and the FITC fluorescence was read using a FACSCalibur flowcytometer (BD Biosciences).

This study was done after obtaining ethical approval.

Results:

Development of resistant subline. The investigation of doxorubicin concentration to develop resistant cells had been done by using several concentration below the IC_{50} value of parental MCF-7 cell. In this study, 75 nM doxorubicin concentration was the only concentration that could make MCF-7 cells viable until the end of the study. MCF-7 cells that had been incubated 24 h every 2 times a week for 25 days with 75 nM doxorubicin was designated as MCF-7/dox75 cells. After incubated 24 hours twice a week for as long as 25 days, MCF-7/dox75 cells were then tested to prove whether the cells become less sensitive to doxorubicin.

The MCF-7/dox75 cells showed decreased sensitivity to doxorubicin. The decreased sensitivity was marked by the increase in the percentage of viable cell and IC_{50} value (as shown in Table 1 and Table 2). The IC_{50} value of these cells was almost 10 folds higher than that of the parental MCF-7 cell line (20,06 ± 0,31 versus 2,689±0,05).

Flowcytometric analysis on P-gp expression demonstrated that the level of P-gp expression in MCF-7/dox75 cells was significantly higher than parental MCF-7 cell (24,145 ±4,01versus 3,33±0,42) (Figure 1). The results proved that doxorubicin in the concentration of 75 nM could induce resistant MCF-7 cell line, by triggering the increase of P-gp expression level.

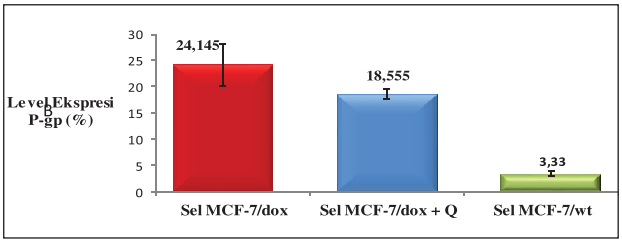
Combination of doxorubicin and quercetin increased sensitivity and decreased P-gp expression in MCF-7 cell lines. After incubated 24 h with the combination of 75 nM doxorubicin and 750 nM quercetin twice a week for as long as 25 days, cells showed significantly increased sensitivity to doxorubicin treatment (as shown in Table 1). The percentage of viable MCF-7/dox75q750 cells was lower than MCF-7/dox75 cells. According to the IC_{50} value in Table 2, MCF-7/dox75q750 cells was almost 2-3 folds more sensitive to doxorubicin than MCF-7/dox75 cells. The effects of doxorubicin-quercetin combination in MCF-7 cells were showed as not significantly decreasing the percentage of P-gp expression and mean fluorescences intensity (MFI) (p>0,05) (Figure 2).

Table 1. The percentage of parental MCF-7 cell viability, MCF-7/dox75 cell and MCF-7/dox75q750 cells by using MTT assay

Doxorubicin concentrations (µg/mL)	Viable cells (mean ± SE)*					
	Parental MCF-7 cell		MCF-7/dox75cell		MCF-7/dox75q750 cells	
	1	2	1	2	1	2
1,5625	60,72±12,15	52,31±3,70	65,78±2,70	63,43±4,13	67,97±0,39	58,59±3,10
3,125	40,76±0,59	44,63±3,19	68,89±0,65	58,99±6,61	68,58±10,68	81,64±1,99
6,25	39,94±1,43	38,33±6,68	66,8±0,82	67,47±5.52	53,17±6,48	65,37±7,31
12,5	23,60±0,93	26,99±0,31	61,17±4,34	62,03±0,69	46,45±0,99	51,81±1,11
25	12,96±0,84	14,15±0,31	49,13±0,08	57,28±1,91	42,42±0,89	29,02±7,31
50	8,61±4,73	2,06±0,51	37,99±2,78	28,09±4,89	29,09±0,39	5,42±0
100	3,55±1,27	0,79±0,72	24,54±0,81	17,20±1,97	13,57±0,99	-5,96±0,89

*data were derived from two independent experiments

Table 2. IC_{50} values (Mean±SE) of doxorubicin in parental MCF-7 cell, MCF-7/dox 75 cell and MCF-7/dox75q750 cell.



Cell Lines	IC_{50} values (µg/mL)*
Parental MCF-7	2,69 ± 0,05
Sel MCF-7/dox75	20,06 ± 0,31
Sel MCF-7/dox75q750	8,44 ± 0,29

*SE values were derived from the IC_{50} values of two independent experiments. The data were statistically analyzed using independent t-test with p<0,05.

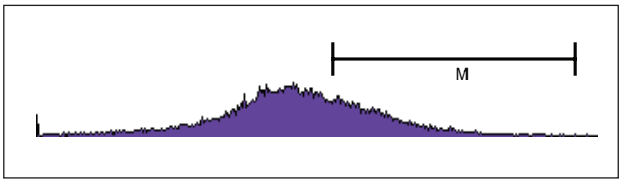


Figure 1. Expression of Pgp in MCF-7/dox75 cells, MCF-7/dox75q750 cells and parental MCF-7 cell lines by using flowcytometry assay.

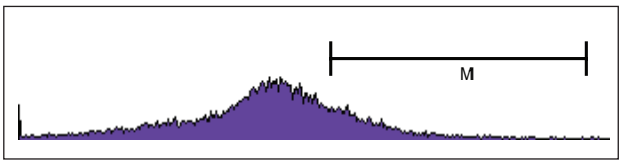


Figure 2. P-gp expression in MCF-7/dox75 cells based on MFI. MFI, *mean fluorescence intensity.*

Discussion:

In this study, MCF-7 cells were used as a model of resistance development in cancer cells due to exposure against 75 nM doxorubicin (concentration below IC_{50} of sensitive parental cell lines) for as long as 25 days. Exposure to doxorubicin reduce the sensitivity of the cancer cells, marked by the increase of IC_{50} value to almost 10 folds (20,06 ± 0,31) relative to parental MCF-7 cells with IC_{50} value for as much as 2,689±0,05. Exposure to 75 nM doxorubicin was shown as having enhancing effect on the level of P-gp expression. This effect was statistically-significant in 95% confidence interval (p<0,05). These observations consistent with the finding of Kars *et al*.[17] They have shown that anticancer drugs, such as doxorubicin, cause the development of acquired doxorubicin-resistance MCF-7 cell line. Doxorubicin is a substrate of P-gp. Exposure to doxorubicin will increase *MDR-1* gene transcription in MCF-7 cells, thus upregulating the level of P-gp expression.[18,19] The change in P-gp expression and activity as a drug efflux pump will affect the intracellular accumulation of the drug. This accumulative process is a complex process involving the uptake, retention, distribution, and the efflux of drug from the cell. The upregulation of P-gp will increase drug efflux, thus reducing intracellular drug availability that ultimately leads to diminished drug efficacy.[19,20]

Increasing cancer cells sensitivity to chemotherapeutic drugs and inhibiting P-gp expression are ideal modalities in preventing resistance against chemotherapy due to MDR-1 gene activation and upregulation of P-gp expression. Some natural compound-derived P-gp inhibitors have been developed to overcome the development of resistance. Those inhibitors are expected to increase the uptake of chemotherapeutic agents by cancer cells, thus enhancing their clinical efficacies.[19,21]

Flavonoid may act as modulatory agent in inhibiting P-gp expression and activity. Alteration of cell membrane permeability, downregulation of P-gp expression, and specific binding to H region of P-gp protein (this binding is inhibitory in nature that directly affect drug and another substrates by P-gp, and inhibit ATPase reactivity) are some mechanisms exhibited by flavonoid as P-gp modulator.[22-25] Quercetin is considered to be effective in restoring sensitivity of doxorubicin-resistant tumour cells, moreover combination quercetin and doxorubicin exhibits synergistic inhibitory effect against cancer cell growth.[26] This flavonoid improves cancer cell sensitivity to doxorubicin treatment and inhibits the expression of proteins encoded in *MDR1* gene such as P-gp, MRP and BCRP.[14,27,28]

Another research done on FM3A/M cells showed that quercetin could enhance the sensitivity of cancer cells to 3 folds.[29] In this study, it was found that combination quercetin and doxorubicin was known as an enhancer of the cell sensitivity to doxorubicin. This effect is clearly supported by the findings that IC_{50} in MCF-7/dox75q750 cells was significantly lower than the IC_{50} value observed in MCF-7/dox75 cells (8,44 ± 0,292 versus 20,06 ± 0,218).

This study also found that the level of P-gp expression in MCF-7/dox75q750 was lower than that of MCF-7/dox75 cells. Unfortunately, the effect of quercetin in

downregulating P-gp expression was not statistically-significant (p>0,05). There are some explanations why the enhanced sensitivity to doxorubicin was not followed by significant downregulation of P-gp expression in MCF-7 cells. Quercetin may exhibit those effects via another mechanism of action. (1) The modulatory effect of quercetin on resistance is not only facilitated via P-gp pathway, but also via glutathione enzyme pathway. It was found that Quercetin is a xenobiotic that competitively inhibits GSH and GST P1-1 enzymatic activities via reversible covalent binding to Cys17 residue.[30] (2) According to Borska et al.[15] who investigated quercetin effects on the expression and function of P-gp, the combination of doxorubicin and quercetin had been significantly reduce the percentage of viable MCF-7 cell and not significantly downregulated P-gp expression. Even though it was not significantly downregulated P-gp expression, it might be affect transport of doxorubicin. Lack of P-gp expression might be caused by increased doxorubicin concentration in the cell, which further induced apoptosis.

Conclusions:

In can be concluded that the development of resistance to doxorubicin in MCF-7 cell can be induced by 75 nM doxorubicin treatment for as long as 25 days. The combination of 75 nM doxorubicin and 750 nM quercetin for as long as 25 days in MCF-7 cell reduces the development of resistance to doxorubicin by enhancing sensitivity to doxorubicin and reduce P-gp expression in MCF-7 cells.

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Conflict of interest: non declared

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