

The Doxorubicin-Induced G2/M Arrest in Breast Cancer Cells Modulated by Natural Compounds Naringenin and Hesperidin

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Abstract

Doxorubicin as the common drug for breast cancer has been widely proposed to use in combine with a natural compound in order to overcome its side effects such as cardiotoxicity and resistance. Previously, we reported that naringenin and hesperidin, the abundant flavanons in citrus fruit peel, increased cytotoxic and apoptosis activities of doxorubicin in doxorubicin resistant breast cancer cells (T47D and MCF-7 cells). Since doxorubicin arrests G2/M phase in most cancer cells, both flavanons are speculated to affect the similar phase in breast cancer cells. Cell cycle distributions were determined by flowcytometry using propidium iodide (PI) to stain DNA of the cells. Combination of naringenin or hesperidin with doxorubicin increased accumulation of T47D cells in G2/M phase, while in MCF-7 cells, accumulated cells in G2/M phase were decreased, accompanying with slightly increased in G1 phase. Naringenin itself had no effect on cell cycle of both cells. Whereas, hesperidin arrested G2/M and G1 phases in T47D and MCF-7 cells, respectively. The different effect of naringenin and hesperidin in T47D and MCF-7 cells is most likely caused by difference of *p53* status. In *p53* mutant, T47D cells, naringenin and hesperidin supported mechanism of doxorubicin to arrest at G2/M that to be considered via *p53*-independent pathway. Whereas, in *p53* wild-type MCF-7 cells, naringenin and hesperidin decreased G2/M arrest, suggesting that both flavanons do not utilize cell cycle arrest for their anticancer activity with doxorubicin. This study revealed that potential co-chemoterapeutic agents, naringenin and hesperidin distinctly modulated cell cycle arrest induced by doxorubicin according to the characteristic of breast cancer cells.

Keywords: *naringenin, hesperidin, doxorubicin, cell cycle, breast cancer cells.*

INTRODUCTION

According to global cancer statistics 2018 reported by International Agency for Research on Cancer, breast cancer is the most commonly diagnosed cancer in the vast majority of the countries and the leading cause of cancer death in over 100 countries among females (Bray, *et al.*, 2018). Doxorubicin is part of the anthracycline family and

is still to be a common chemotherapeutic agent for breast cancer therapy, however it causes side effects such as cardiotoxicity and resistance, resulting in poor patient prognosis and survival (Barrett-Lee,

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et al., 2009; Christowitz, *et al.*, 2019). Resistance of breast cancer toward doxorubicin is caused by internal character of the cancer or external factor/acquired factor (Wang, *et al.*, 2019). Because of these problems, many researchers propose co-chemotherapy of doxorubicin and other compounds especially natural compound to achieve more effective doxorubicin for cancer therapy.

Previous studies showed that citrus flavanons, naringenin and hesperidin, are potential compounds to be developed for co-chemotherapy with doxorubicin. Naringenin itself have cytotoxic and antiproliferative effects on several cancer cells, including gastric (Zhang, *et al.*, 2016), lung (Jin, *et al.*, 2011), breast (Kanno, *et al.*, 2005), prostate (Lim, *et al.*, 2017) and pancreas (Park, *et al.*, 2017). While, hesperidin demonstrated a cytotoxic effect on cancer cells such as leukemia (Desai, *et al.*, 2015), breast (Ye, *et al.*, 2012), liver (Yumnam, *et al.*, 2016), and kidney (Siddiqi, *et al.*, 2015). In our previous studies, the combination of naringenin or hesperidin with doxorubicin has been proved to increase the effectiveness of doxorubicin treatment on two doxorubicin resistant breast cancer cells (MCF-7 and T47D cells) through enhancement of apoptosis (Fitriasari, *et al.*, 2010; Hermawan, *et al.*, 2010; Junedi, *et al.*, 2010; Setiawati, *et al.*, 2011).

Besides apoptotic induction, cell cycle arrest is also an important mechanism in chemotherapy (Birt, *et al.*, 2003). Doxorubicin caused G2/M phase arrest on cancer cell characterized with mutated *p53* (Drummond, 2008). Whereas, naringenin induced G0/G1 and G2/M phase arrests in Hep G2 cell, and G0/G1 phase arrest in human epidermoid carcinoma A431 cells (Ahamad, *et al.*, 2014; Arul and Subramanian, 2013). Similar as naringenin, hesperidin also arrested different cell cycle phases in different cancer cells, for example G0/G1 phase in HeLa cells, and G2/M phase in human gall bladder carcinoma (Pandey, *et al.*, 2019; Wang, *et al.*, 2015).

Since deregulation of cell cycle is a hallmark of the transformation of normal cells

into cancer cells, inhibitors of cell cycle have emerged as potential therapeutic drugs for the treatment of cancers-both as single-agent therapy and in combination (Bai, *et al.*, 2017). Considering the important of cell cycle modulator to control cancer cell growth, and the activity of naringenin and hesperidin itself to arrest cell cycle phases in cancer cells, we envisage that naringenin and hesperidin can support doxorubicin to arrest cell cycle in breast cancer cells. In this study, we want to pursue the better understanding of naringenin and hesperidin action to cell cycle which culminating to the synergistic cytotoxicity with doxorubicin on doxorubicin-resistant T47D cells and MCF-7 cells.

MATERIAL AND METHODS

Cell Culture and Material Treatment

The T47D cell line and MCF-7 cell line (ATCC, Manassas, VA, USA) were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco Invitrogen, NY, USA) supplemented with 10% v/v of fetal bovine serum (FBS) (Gibco) and 1% v/v of penicillin-streptomycin (Gibco) at 37°C and 5% CO₂ until 80% confluent. Cells were washed with phosphat puffer saline (PBS) and detached with 0.25% of trypsin-EDTA (Gibco, Invitrogen, Canada). Cells (1x10⁶) were seeded in each well of six-well plate and incubated for 24 h at 37°C and 5% CO₂. The 125 µM of Naringenin (MilliporeSigma, St. Louis, MO, USA), 100 µM of hesperidin (Sigma Aldrich, Steinheim, Germany), 7.5 nM or 200 nM of doxorubicin (Ebewe, PT. Ferron Par Pharmaceutical, Indonesia) and co-treatment of naringenin or hesperidin with doxorubicin were added to T47D cells and MCF-7 cells, following with incubation for 24 h at 37°C and 5% CO₂. Concentration of naringenin, hesperidin and doxorubicin for single and co-treatment were chosen based on synergistic cytotoxic activity of naringenin or hesperidin and doxorubicin in our previous experiment (Fitriasari, *et al.*, 2010; Hermawan, *et al.*, 2010; Junedi, *et al.*, 2010; Setiawati, *et al.*, 2011).

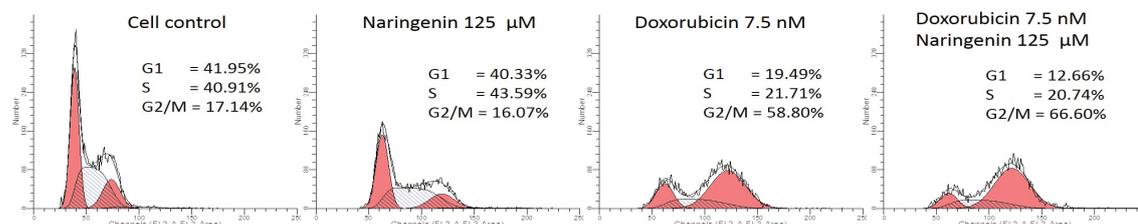


Figure 1. Effect of naringenin on cell cycle in T47D cells. Flowcytogram and number of cell cycle distribution (%) in cells without (cell control) and with naringenin, doxorubicin and combination of naringenin and doxorubicin.

Cell Cycle Analysis

After treatment, all cells were collected by trypsinizing with 100 µL of trypsin-EDTA for 3 minutes then added 1 mL of medium culture and centrifuged at 2000 rpm for 30 seconds. Cells were rinsed with cold PBS and centrifuged again at 2000 rpm for 30 seconds. Prior to analysis, propidium iodide (PI) stain solution was added to the cell pellet and resuspended for incubation at 37°C for 10 minutes in the dark. PI stain solution contained 0.1% of triton X-100 (E.Merck, Darmstadt, Germany), 20 µg/mL of RNase and 20 µg/mL of PI (Sigma-Aldrich, St. Louis, MO, USA). After staining, cells were transferred to tube for analysis with flowcytometer (BD FACS Calibur, BD Bioscience, San Jose, CA, USA). Data acquisition and analysis were performed with software Modfit LT 3.0.

RESULT

Modulation of Naringenin on T47D and MCF-7 Cells Cycles

Treatment of naringenin 125 µM to T47D cells and MCF-7 cells showed the same cell cycle distribution as the cell without treatment (Figure 1 and Figure 2). Whereas, treatment of doxorubicin 7.5 nM to T47D cells or 200 nM to MCF-7 cells as expectedly induced accumulation of cell in G2/M phase. Accumulated cells in G2/M phase was more in T47D cells after co-treatment of naringenin and doxorubicin compared to that of doxorubicin single treatment (Figure 1). On the contrary, accumulated cells in G2/M phase of MCF-7 cells after co-treatment was less than that of doxorubicin single treatment, but accumulated cell in G1 phase was slightly increased (Figure 2).

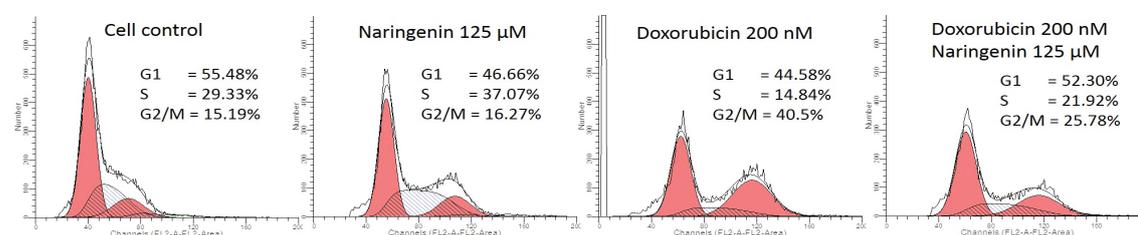


Figure 2. Effect of naringenin on cell cycle in MCF-7 cells. Flowcytogram and number of cell cycle distribution (%) in cells without (cell control) and with naringenin, doxorubicin and combination of naringenin and doxorubicin.

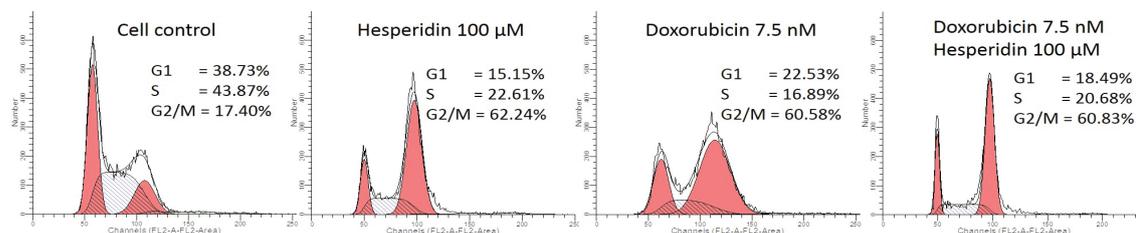


Figure 3. Effect of hesperidin on cell cycle in T47D cells. Flowcytogram and numbers of cell cycle distribution (%) in cells without (cell control) and with hesperidin, doxorubicin and combination of hesperidin and doxorubicin.

Modulation of Hesperidin on T47D and MCF-7 Cells Cycles

Hesperidin 100 µM in T47D cells induced cell accumulation in G2/M phase (Figure 3) similar as doxorubicin single treatment, whereas in MCF-7 cells, it induced cells accumulation in G1 phase (Figure 4). Combination of hesperidin and doxorubicin in T47D cells accumulated cells in G2/M phase with percentage of cells 60.83%, similar as that of doxorubicin single treatment 60.58% (Figure 3). Looking at the flowcytometry graph in Figure 3, combination of hesperidin and doxorubicin in T47D cells showed similar profile as hesperidin single treatment than doxorubicin single treatment, indicating the high effect of hesperidin to modulate cell cycle in the presence of doxorubicin. In MCF-7, combination of hesperidin and doxorubicin decreased the accumulated cells in G2/M phase and slightly increased in G1 phase (Figure 4).

DISCUSSION

In the T47D cells, eventhough naringenin did not induced any cell cycle arrest, it increased cell accumulation in G2/M phase which induced by doxorubicin, whereas in MCF-7, its combination with doxorubicin reduced the accumulated cells in G2/M phase, but slightly increased in G1 phase, compared to that of doxorubicin single treatment (Table 1). The different effect of naringenin to the co-treatment with doxorubicin in T47D and MCF-7 is possibly because of the different *p53* in both cells. The wild-type *p53* in MCF-7 was able to undergo the process of G1 phase arrest, oppositely the mutant *p53* in T47D could not. High *p53* level triggers expression of CDK interacting protein/kinase inhibitory protein such as *p21* and *p27*, which leads to G1 phase arrest (Zohny, et al., 2019). Recent study have shown that induction of G1 phase arrest by naringenin is positively

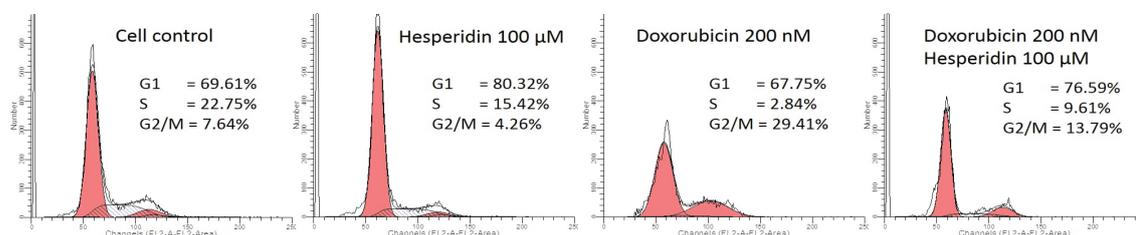


Figure 4. Effect of hesperidin on cell cycle in MCF-7 cells. Flowcytogram and numbers of cell cycle distribution (%) in cells without (cell control) and with hesperidin, doxorubicin and combination of hesperidin and doxorubicin.

Tabel 1. Summary of cell cycle arrest in T47D cells and MCF-7 cells upon single and co-treatment of doxorubicin, naringenin and hesperidin.

	T47D	MCF-7
Doxorubicin	G2/M	G2/M
Naringenin	No cell cycle arrest	No cell cycle arrest
Naringenin-Doxorubicin	G2/M	G2/M, little high G1
Hesperidin	G2/M	G1
Hesperidin-Doxorubicin	G2/M	G2/M, little high G1

correlated with high expression of *p53* (Hermawan, *et al.*, 2021). Based on the consideration of mutant *p53*, naringenin in T47D cells positively supports G2/M arrest induced by doxorubicin possibly via *p53* independent pathway. Whereas, in MCF-7 cells, naringenin regulates cell cycle different from the pathway of doxorubicin-induced G2/M arrest presumably via *p53* dependent pathway.

Single treatment of hesperidin and its co-treatment with doxorubicin triggered G2/M phase arrest in T47D. Whereas, in MCF-7, single treatment of hesperidin triggered G1 phase arrest, and its co-treatment with doxorubicin decreased G2/M phase arrest and slightly increased cell accumulation in G1 phase compared to that of doxorubicin single treatment (Table 1). The recent study showed that expression of *p53* in MCF-7 was upregulated by administration of hesperidin (Hermawan, *et al.*, 2020). Therefore, it is reasonable that hesperidin single treatment in MCF-7 induced G1 phase because of high *p53* expression. While activity of hesperidin to G2/M arrest in T47D cells is most probably through *p53* independent. Even though hesperidin itself has effect on cell cycle of T47D cells and MCF-7 cells, co-treatment with doxorubicin showed the similar result as co-treatment of naringenin and doxorubicin. The summary in Table 1 clearly display that the modulation of naringenin and hesperidin to doxorubicin-induced G2/M arrest is correlated to the character of breast cancer cells. Further researches are needed to uncover the mechanism of naringenin and hesperidin in combination with doxorubicin to modulate cell cycle phases in breast cancer cell with different type and expression of *p53*.

Considering our present and previous studies (Fitriasari, *et al.*, 2010; Hermawan, *et al.*, 2010; Junedi, *et al.*, 2010; Setiawati, *et al.*, 2011) in breast cancer cells which possessed mutant *p53*, application of naringenin or hesperidin for co-treatment with doxorubicin increases cytotoxic activity of doxorubicin through G2/M cell cycle arrest and apoptosis. Meanwhile, in breast cancer cells with wild-type *p53*, the synergistic effect of cytotoxicity in co-treatment is through apoptosis but not G2/M cell cycle arrest. Naringenin and hesperidin as co-chemotherapeutic agent differently modulated cell cycle arrest caused by doxorubicin depend on the characteristic of breast cancer cells. Therefore, it is important to identify the abnormal genes or proteins and its expression level in breast cancer cells before deciding chemotherapeutic agent and its combination materials.

CONCLUSION

The activity of naringenin and hesperidin to modulate G2/M phase arrest induced by doxorubicin is depend on the characteristic of breast cancer cells especially *p53* status. Co-treatment of naringenin or hesperidin and doxorubicin in *p53* mutant T47D cells exaggerates G2/M phase arrest, while in *p53* wild-type MCF-7 cells, naringenin or hesperidin reduces G2/M phase arrest and slightly increase accumulation in G1 phase. It is recommended to identify the abnormality and expression of *p53* in breast cancer cells in order to decide co-chemotherapeutic agent which positively modulated G2/M arrest induced by doxorubicin.

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