RESEARCH ARTICLE

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Tylophorine Abrogates G2/M Arrest Induced by Doxorubicine and Promotes Increased Apoptosis in T47D Breast Cancer Cells

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Abstract

Background: The effects of tylophorine, a natural alkaloid found in *Tylophora indica*, administered as a single compound or in combination with doxorubicin on cell cycling and apoptosis were assessed in T47D breast cancer cells, selected as a model system for breast cancer. **Methods:** Cell cycle distribution and apoptosis were examined by flow cytometry. Caspase 3 and 9 expression was determined by immunocytochemistry.**Result:** We found that tylophorine did not significantly influence the cell cycle distribution of T47D cells. However, the alkaloid did prevent accumulation of cells in the G2/M phase. In addition, tylophorine increased the number of apoptotic cells. Expression of proapoptotic proteins (caspases 3 and 9) was up-regulated upon administration of tyloporine alone or in combination with doxorubicin. **Conclusions:** Tylophorine alone or in combination with doxorubicin induced apoptosis in T47D breast cancer cells through modulation of the cell cycle and affecting the expression of caspases 3 and 9.

Keywords: Tylophorine- cell cycle- apoptosis- caspase 3 and 9- T47D

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Introduction

Cancer is a pathological condition characterize by abnormal, quick, and uncontrollable cell growing. Cancer cells grown infinitely and invade surrounding tissues (Nafrialdi and Sulistia, 2007). Based on global statistical data of cancer, breast cancer is the most deadly cancer in (Parkin et al., 2002).

Many efforts have been done in the treatment of breast cancer such as chemotherapy, radiotherapy, hormonal, until surgery. The most common attempt is chemotherapy because this method is the ultimate theraphy to treat cancer cells the metastasis stage (Hanahan, 2000). The most common chemotherapeutic agents widely used in the medication of breast cancer are doxorubicin, cisplatin and 5-fluorouracil. However; these chemotherapeutic agents have some limitations as they lead to new other problems such as cancer cell resistance and the presence of toxic effects on normal cells (Fimognari et al., 2006).

One attempt to overcome this problem is by utilizing natural chemotherapeutic agents, especially from plant-derivate compounds. One of the plant-derived compounds is thylophorin, this phenanthroindolizidine alkaloid was found in *Tylophora indica* (Saraswati et al., 2013) and also presents in other Ficus family including *Ficus septica* (Wu et al., 2003).

Tylophorine is reported to have anti-cancer activity by affecting the cell cycle profile of liver cancer cells (HepG2), gastric cancer cells (NUGC-3) and nasopharyngeal cancer cells (HONE-1) (Wu et al., 2009). It also, demonstrated cytotoxic effect on nasopharyngeal (HONE-1) and gastric (NUGC-3) cancer cells (Damu et al., 2005).;In this study we further investigated the anti-cancer effect of tylophorin on breast cancer cells T47D by evaluating the influence on apoptosis process.

Materials and Methods

Tylophorine was obtained from Toronto Research Chemical Inc (catalog number T898200). This compound was dissolved in DMSO (Dimethyl Sulfoxide), prepared into aliquots, and stored at -20 ^{o}C. Primary monoclonal antibodies for to antigen caspase 3 (catalog number 9662) and caspase 9 (catalog number 9502). Doxorubicin (Ebewe) was obtained from PT Ferron Par Pharmaceutical (Cikarang, Indonesia).

Cell lines and culture conditions

T47D (Human ductal breast epithelial tumor cell line) was obtained from Laboratory of Parasitology, Faculty of Medicine, Universitas Gadjah Mada Indonesia. The cells were cultured in DMEM (Dulbecco's Modified

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Eagle's Medium) (Gibco) with 10% Fetal Bovine Serum (Gibco), 2% Sodium bicarbonate (Gibco) and HEPES (4-2(2-hydroxyethyl)-1-piperazineethanesulfonic acid) (Invitrogen). The cell lines were maintained at 37°C in a humidified incubator containing 5% CO_2.

Cell cycle analysis and Apoptotic assay

Induction of apoptosis was performed by incubating the test substances within 24 hours into a 6-well plate with $5x10^5$ cells/sinks. Observation was done with flow cytometry using Annexin V as a reagent. At the end of the incubation time, the medium was collected and the cell were harvested with trypsin (200 µL / well). The cells suspension were centrifuged at 2,000 rpm for 5 minutes and the cells pellet was washed with the addition of PBS and centrifuged at 2,000 rpm for 5 minutes. The supernatant was removed and the cells pellet was collected for analysis. The cells pellet was used for the cell cycle assay and apoptosis assay. For the cell-cycle assay, the cells was resuspended in 25 µL PI (50x) + 1 µl RNase + 0.5 µl Triton-X + PBS ad 500 µl. Where as for the apoptosis assay the cells was resuspended in 600 µL buffer, 12 µL PI, and 12 µL Annexin-V. The cell suspension was homogenized and incubated for 5 minutes at room temperature. The cells were then transferred to flowcyto-tube for analysis in a flowsitometer.

Immunocytochemistry

T47D cells were seeded at $5x10^5$ cells/well on coverslips in 24-well plate until 80% confluent (24 h incubation). The medium was then replaced by fresh medium containing tylophorine, doxorubicin or their combination. The cells was then incubated in a humidified incubator (37°C and 5% CO_2) O_2 for 24 h. After incubation, the medium was discharded and the cells were washed with PBS and then fixed with cold methanol for 10 min at -4°C. Afterward, the cells were washed with PBS and blocked in hydrogen peroxide blocking solution for 10 min at room temperature. The cells were incubated with primary antibody of caspase 3 and 9 for 1 h at room temperature. The cells

were washed three times with PBS, then incubated with secondary antibody for 10 min. After washing with PBS, the cells were incubated in 3,3 diaminobenzidine solution

for 10 min and then washed with aquadest. After this step, the cells were counterstained with Mayer-Haematoxylin for 3 min. After incubation, the coverslips were taken and the stained cells were washed with aquadest, and then immersed with xylol and alcohol. The expression of caspase 3 and 9 proteins were observed using a light microscope (Nikon, Japan) and photographed using a digital camera (Canon, Japan). Positive and negative expressions of protein were represented by a dark brown and purple color in a the cells cytoplasm, respectively.

Results

In the previous study, we demonstrated that tylophorine and doxorubicin exerted cytotoxic effect in T47D cells with the IC50 of 113 and 0.13 µM. Respectively (Sundhani, 2015). Thus we used these IC50 values in this present study.

Cell cycle modulation

The amount of DNA contained in the cell is used to determine the distribution of cells including G0/G1, S and G2/M phases. Cell cycle profiles of T47D cells upon the treatment was analysed using flowcytometer and illustrated in (Figure 1). Most of the T47D cells accumulated in the G2-M phase indicating that. The treatment of tylophorine, and doxorubicin alone or combination can modulate the cell cycle and lead to the accumulation in certain phases. The cells cycle was shifted to G2-M after treatment with tylophorine 28.8 µM and 56.5 µM with the percentage of 29,72%, and 29,83%, respectively. After treatment with a doxorubicin the G2-M phase reached 46,43%. Whereas the combination doxorubicin and tylophorine showed cell cycle arrest in G2-M phase as much as 28,06% and 30,71% for tylophorine 28.8 µM and 56.5 µM respectively

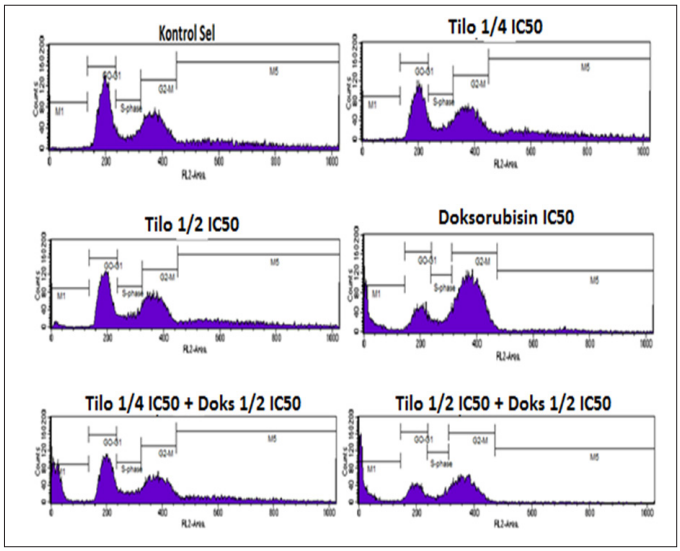

Figure 1. Detection of Cell Cycle Distribution Using Flowcytometry. 5 x 105 cells were seeded in 6-well plates and incubated for 24 hours in medium without or with treatment. Flowcytometric detection was done using PI reagent after treatment with tylophorine, doxorubicin, and their combination.

Table 1. Cell Cycle Distribution of T47D Cells after Treatment

Fase	Control	Doxorubicin 0.13 µM	Tylophorine 28.8 µM	Tylophorine 56.5 µM	Tylophorine 28.8 µM + Doxorubicin	Tylophorine 56.5 µM + Doxorubicin
Sub G0	4.84	14.67	1.61	2.56	15.07	19.9
G0-G1	29.07	19.82	27.91	29.84	28.49	25.52
S	13.29	10.27	10.88	10.27	10.8	13.78
G2-M	26.91	46.43	29.72	29.83	28.06	30.71

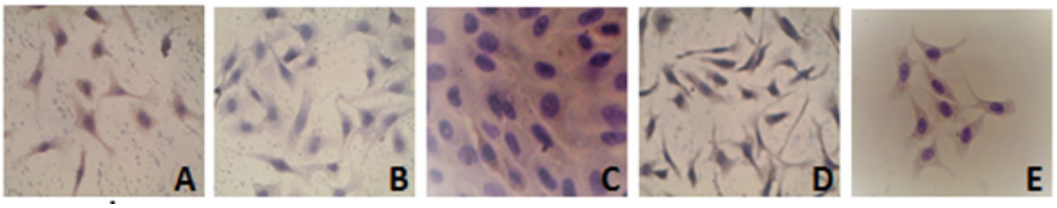

Figure 2. Expression of Caspase 3 on T47D Cells (400x). A, Control cells with caspase 3 antibodies; B, Control CELLS without caspase 3 antibodies; C, Tylophorine 113 µM; D, Doxorubicin 0.13 µM; E, Combination of 113 µM and 0.13 µM. Expression of Caspase is characterized by the brown color of the cell cytoplasm.

Table 2. Percentage of Apoptotic T47D Cells

Phase	Control (%)	Tylophorine 28.8 µM (%)	Tylophorine 56.5 µM (%)	Doxorubicin 0.13 µM (%)	Tylophorine 28.8 µM + Doxorubicin (%)	Tylophorine 56.5 µM + Doxorubicin (%)
Apoptosis	4.59	6.23	7.93	9.96	52.83	53.76
Necrosis	1.69	1.98	2.47	5.21	13.6	10.11

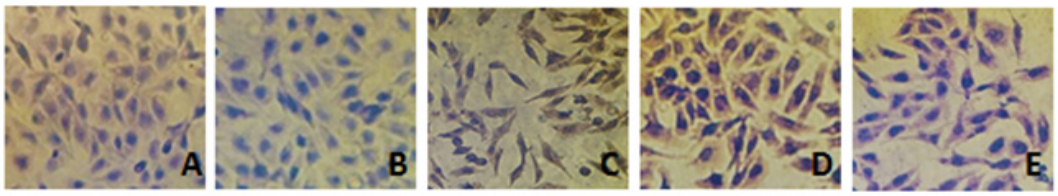

Figure 3. Expression of Caspase 9 on T47D Cells (400x). A, Control cells with caspase 9 antibodies; B, Control cells without caspase 9 antibodies; C, Tylophorine 113 µM; D, Doxorubicin 0.13 µM; E, Combination of 113 µM and 0.13 µM. Expression of Caspase is characterized by the brown color of the cell cytoplasm.

(Table 1).

Induction of Apoptosis

The percentage of apoptotic cells (early and late apoptosis) was also calculated by flowcytometer. Table 2 showed the result of the apoptosis assay. The percentage of apoptotic cells in the control group was lower than that of tylophorine, doxorubicin, and the combination thereof. This suggested that tylophorine at 28.8 µM and 56.5 µM was able to induce apoptosis in T47D cells 6.23% and 7.93% respectively.

Expression of Caspase 3 and 9

Evaluation of Caspase 3 and 9 was performed using immunocytochemical method. Positive expression of Caspase 3 and 9 are characterized by brown color of the cell cytoplasm. Observed under the microscope the cells without antibodies showed no brown color in the cytoplasm suggesting that. The expression of Caspase 3 and 9 proteins was negative (Figure.2B, 3B). Addition with antibodies was able to visualize Caspase 3 and 9 expressions indicated by brown cytoplasm. A single treatment of tylophorine or doxorubicin increased the expression of both Caspase 3 and 9 compared to control cells. The combination of tylophorine and doxorubicin was also increased the level expression of the protein compared to control cells.

Discussion

In this study, we reported the anti-cancer potential of tylophorine, a natural product derived from medicinal plants. This study is a part of the effort to develop a new anti-cancer agents either as an extract, fraction, single compound or combination of an active natural compound with an established drugs. To improve the pharmacological profile (Febriansah et al., 2014; Nugroho et al., 2014a; Sunarwidhi et al., 2014). Interestingly in line with the growing use of medical plants, the development of anti-hypertensive, anti-allergy, anti cancer and anti-diabetic drugs either as a single agent or combination is increasing (Harwoko et al., 2014; Nugroho et al., 2011a; Nugroho et al., 2011b; Nugroho et al., 2013b).

In this study, tylophorine a plant-derived alkaloid was

evaluated for its apoptotic effect and its effect on cell cycle distribution in T47D breast cancer cells. Evaluation of cell cycle distribution and apoptosis were performed using flowcytometry, and caspase 3 and 9 protein expression was determined by immunocytochemical method. Tylophorine and doxorubicin as have been evaluated for its cytotoxic effect in our previous study. IC50 values of tylophorine and doxorubicin were 113 and 0.13 μM respectively (Sundhani, 2015).

The treatment of tylophorine or doxorubicin, and a combination of both compounds in T47D cells exhibited accumulation of cells in the G2-M phase within 24-hour incubation period. The highest cells' accumulation in the G2-M phase occurred after the treatment with doxorubicin alone (Table 1). This indicating that the drug inhibits cell cycle and causes death in T47D cancer cells. This effect might occur-by linking the DNA, generating free radicals, interacting with cellular membranes and inhibiting topoisomerase II which play a role in DNA repair (Husein, 2007). Treatment of tylophorine induced a higher G1 phase accumulation in T47D cells than that of doxorubicin. Interestingly combination of tylophorine and doxorubicin shifted the cells' accumulation from G2-M to G1 phase. The facts suggest that tylophorine can improve doxorubicin effect by changing the inhibition of cell cycle phase G2/M to phase G1.

Treatment of the cells at with tylophorine was performed with two concentrations of 28.8 μM and 56.5 μM exhibited apoptotic effect, even though these IC50 values were lower than this of doxorubicin, combination of tylophorine with doxorubicin was able to increase significantly the apoptotic effect in comparison to the single administration (Table 2). This indicated that there is a synergistic effect of tylophorine and doxorubicin when these compounds are combined. In the previous study, combination of doxorubicin of *Ficus septica* alkaloid fraction which might contain tylophorine enhanced cytotoxic effects of doxorubicin synergistically through inducing apoptosis by increasing the expression of cleaved Poly (ADP-ribose) polymerase (cPARP) (Nugroho, 2012).

The mechanism of apoptotic effect of the compounds used in this study is most likely influenced by the p53 gene. It is related to the characteristics of T47D cells that are p53 gene mutations that function in apoptotic events. In addition to the characteristics of T47D cells, it also expresses caspase 3 wildt ype, caspase 7 wild type, and ER/RR positive and sensitive to doxorubicin (Schafer et al., 2000). Phenantroindolisidine alkaloids including tylophorine have been shown to induce apoptosis by inhibiting extrinsic pathways by strengthening the action of TNF-α tumors that induce caspase 8 and poly (ADP-ribose) polymerase (PARP) cutting (Min et al., 2010). Tylophorine plays an important role in the indication of apoptosis of cancer cells through activation of caspase 3 and induces cytochrome c release which then will be bound by Apop-1 (apoptosis activating factor), which will form apoptosome. Apoptosom will activate caspase 9, and then the caspase 9 will activate caspase 3 that plays a role in apoptosis event (Ganguly et al., 2002). It indicates that tylophorine may cause apoptosis by increasing the expression of caspase 3 and 9 proteins in breast cancer cells.

Evaluation of caspase 3 and 9 expressions was done using immunocytochemical method. From the observations under the microscope treatment of the cells with tylophorine, doxorubicin or the combination increased the expressions of caspase 3 and 9 characterized by brown colour on the cytoplasm. This finding is in line with previous study by Vakkala et al., (1999) showing that the level of caspase 3 was increased by treatment of various chemotherapy agents. Caspase 3 is expressed in 90% of breast cancer cells. Caspase 3 expression is a signal transduction pathway in the apoptotic process at the downstream level. The initiation of caspase 9 activates caspase 3 which acts as the executor of a series of downstream cascades of proteolytic activity (apoptosis) in the form of digestion of protein structures in the cytoplasm and the destruction of DNA chromosomes (Rahmawati et al., 2010).

In conclusion, in this study we demonstrated that tylophorine alone or combination with doxorubicin induced apoptosis in T47D breast cancer cells through modulation of cell cycle and up regulating the expression of caspase 3 and 9 enzymes.

Competing interest

The authors declare that there is no conflict of interest related to this publication.

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