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Anti-proliferative and Apoptotic Effects of Mucoxin (Acetogenin) in T47D Breast Cancer Cells

INTRODUCTION

In Indonesia the incidence of breast cancer was high, about 37 per 100,000 people, compared to other Asian countries where the average is only 20 per 100,000 people[1]. Considering most people with cancer ended with the death[2], the high incidence of breast cancer in Indonesia is a serious threat for the country. However, given the conventional cancer treatments causing various side effects, recently practitioners are compelled to seek alternative cancer treatment that can eliminate or minimize the side effects. Among the various alternative cancer drugs that is believed to be safe by people with cancer, especially in Indonesia, is plant-derived substances[3-4].

One of the alternatives medication proposed for cancer, especially breast cancer, is by utilizing acetogenin, an active substances isolated from Annonaceae plant family, Annona muricata Linn[5-8]. Among the various annonaceous acetogenin derivatives, mucoxin is the latest and claimed the most powerful to eradicate cancer cells because the only type acetogenin containing a hydroxylated trisubstituted tetrahydrofuran (THF) ring[9]. Unfortunately, though mucoxin claimed as a promising cancer drug, has manufactured and marketed on line all over the world, including Indonesia, a thorough study of the role of the bioactive materials on cellular level is very rare.

Scientific reports about the biological properties of mucoxin that can be accessed via the

internet is still limited to the antitumor and cytotox activities. For instance, it was found that mucoxin is a highly potent and specific antitumor agent against MCF-7 (breast carcinoma) cell lines (ED50 = 3.7 x $10^{(-3)}$µg/mL compared to adriamycin, ED50 = 1.0 x $10^{(-2)}$µg/mL) and A-549 lung cancer[9-10]. In addition, mucoxin appeared to have significant activity against a number of human cancer cell lines such as epidermoid carcinoma, K562 and HL-60[11].

Lack of scientific information about mucoxin role in regulating and suppressing cancer cells, especially breast cancer, certainly need to be enriched and improved through in-depth study. To confirm the anticancer effects, mainly the antiproliferative and apoptotic, of mucoxin, a non-classical acetogenin purified from plant extract of Rollinia mucosa against cancer cells, T47D breast cancer cells were used as the target.

METHOD

The mucoxin and cell lines

Bioactive substances tested in this study is mucoxin (acetogenin) ID AG E 32919 and CAS No. 183195995 obtained from Angene International Limited. The product package contains 5mg of pure mucoxin in powder form. Whereas human breast cancer cell line used in this study was T47D (ATCC® HTB133™) obtained from American Type Culture Collection (Manassas, VA 20108 USA) with a lot number 61062006.

Experimental design

A randomized block design, six concentrations of treatment and three replications is the experimental design applied in this research. The T47D cell lines was divided into four groups based on the hour of mucoxin exposure until assays done, namely hour 0th, 24th, 48th, and 72nd. Concentration levels of mucoxin applied in the experiment were as follows: 0ng/ml, 0.1ng/ml, 0.5ng/ml, 1ng/ml, 5ng/ml and 10ng/ml. Each treatment dose was made in three replicates.

Cell culture

The cells were grown in Roswell Park Memorial Institute medium (RPMI 1640) culture media supplemented with 10% Foetal Bovine Serum (FBS) Gibco™ (from Thermo Fisher Scientific Cat. No. 26140 079) and 0.2 units/ml bovine insulin (from Sigma Aldrich Cat. No. I5500 and CAS RN 11070 73 8) at 37°C in 5% CO_2. Thawing process performed in waterbath at 37°C for 2-4 minutes. Then, as much as 5x10^4 cells/cm^2 was taken into T-flask and incubated at 37°C in CO_2 5%. When cells density reached 80% confluent, trypsinization done using 0.25% Trypsin + 0.53 mM EDTA solution and then subcultured into new culture vessels, also at 37°C in CO_2 5%. After two times passaging the T47D cells ready to be treated.

Mucoxin treatments

The mucoxin preparation was made by diluting the powder of mucoxin in 1 ml of 0.1% DMSO. The stock solution is then diluted further in accordance with the needs of the treatment concentrations (six levels). After subcultured for two times the cells were diluted with RPMI and seeded in 24-wells plate with a cells density of 5x10^4 cells/cm^2 in each well. Once the cells density reach 80% confluent, the cells treated with mucoxin of different concentrations as follows: 0ng/ml (K), 0.1ng/ml (P_1), 0.5ng/ml (P_2), 1ng/ml (P_3), 5ng/ml (P_4) and 10ng/ml (P_5). After being treated, the cells were incubated in accordance with the length of hours that have been assigned to each group, i.e. 0, 24, 48, and 72 hours.

Cell Proliferation Assay

Cells proliferation assayed by flow cytometry (FCM) technique using BrDU staining protocol. The wells containing T47D cells under optimal condition stained with Bromodeoxyuridine (BrdU) 30 iM, incubated, washed with PBS, and then trypsinized. After the incubation, cells were harvested and washed by adding flow cytometry staining buffer and diluted until in each tube containing 10^5 10^8 cells. After being washed twice with flow cytometry staining buffer, anti-BrdU fluorochrome-conjugated antibody was added. Cell quantity in the samples then determined using flow cytometry at wavelength 530 nm.

Apoptosis Assay

Apoptotic effect of mucoxin was identified by flow cytometry technique using FITC Annexin V Apoptosis Detection Kit with PI from Biolegend. The T47D cells washed with cold BioLegend's Cell Staining Buffer and resuspended in Annexin V Binding Buffer at a concentration of about 1.0 x 10^6

cells/ml. After FITC Annexin V (5 µl) and Propidium Iodide Solution (10 µl) being added, the suspension then incubated in the dark at room temperature for 15 minutes. Lastly, after the cell lines in each tube diluted with 400 µl of Annexin V Binding Buffer, the suspension analyzed by flow cytometry machine at wavelength 530 nm.

Statistical Analysis

Comparison of mean values of quantitative data between treatments (mucoxin dose) and between group of exposure time was analyzed using ANOVA followed by LSD test.

RESULTS

Effect of Mucoxin on Proliferation

Quantification results of the flow cytometric (FCM) of the effects of mucoxin application on the proliferation of T47D cells of each exposure hour group are presented in Table 1. To determine whether the exposure hour has an effect on the proliferation, one way ANOVA also applied for comparing the average value of proliferation between the groups in which the results are shown in Table 2.

Based on the ANOVA and post hoc test shown in Table 1 and Table 2 it can be assumed that the mucoxin doses as well as the exposure hour have effects on T47D cell proliferation. In all exposure groups, the mucoxin treatment significantly reduced the percentage of cell proliferation. The p-values of the comparative mean value of proliferation between treatment dose in exposure hour 0, 24, 48 and 72 respectively are 0.007, 0.009, 0.006, 0.006. The sharpest decline (>50%) occurred in the group of 48-hour exposure by the mucoxin dose of 5ng/ml (P4) and 10ng/ml (P5).

Effect of Mucoxin on Apoptosis

Gating strategy for quantification of the T47D cell apoptosis after mucoxin treatment in each hour group is ilustrated in Figure 1. The quantitative results from the FCM graph readings of each treatment group, the ANOVA results followed by LSD test against mean values of the effect of the mucoxin doses on the apoptosis of T47D cells of each exposure hour group are presented in Table 3. For comparing the average value of apoptotic cells between the exposure hour groups, the one way ANOVA has also applied and result in the data shown in Table 4.

Referring the data in Table 3 and Table 4, it was also clear that both mucoxin doses and exposure hour have effects on T47D cell apoptosis. In all exposure groups, the mucoxin treatment significantly increase the percentage of apoptotic cells. The p-values of the comparative mean value of apoptosis between treatment dose in exposure hour 0, 24, 48 and 72 respectively are 0.008, 0.012, 0.005, 0.005. However, the high increases (>50%)

Table 1: Effect of mucoxin application on the proliferation of T47D cells of each exposure hour group

Treatments	Hours of Exposure			
	0	24	48	72
K (0 ng/ml)	96.40±0.32^{a}	95.51±0.93^{a}	96.13±0.61^{a}	95.38±0.72^{a}
P1 (0.1 ng/ml)	95.88±0.22^{b}	95.18±0.89^{a}	80.91±0.31^{b}	87.90±0.12^{b}
P2 (0.5 ng/ml)	95.46±0.21^{c}	94.42±0.28^{ab}	65.37±1.53^{c}	78.47±1.78^{c}
P3 (1 ng/ml)	94.23±0.18^{d}	93.84±0.53^{b}	57.94±0.96^{d}	75.59±0.93^{d}
P4 (5 ng/ml)	94.16±0.10^{de}	89.82±1.24^{c}	45.86±0.37^{e}	74.88±0.14^{de}
P5 (10 ng/ml)	93.90±0.14^{e}	81.18±1.66^{d}	45.43±0.39^{e}	73.81±0.52^{e}
ANOVA (P-value)	0.007	0.009	0.006	0.006

Values are the mean ± SD percentage of proliferated cells (n=3); numbers in the same column that shared the same superscript was not statistically different at α=0.05 based on LSD test

Table 2: Effect of exposure hour on the proliferation of T47D cells given mucoxin of six different concentration

Treatments	Hours of Exposure				ANOVA
	0	24	48	72	(P-value)
K (0 ng/ml)	96.40±0.32^{a}	95.51±0.93^{a}	96.13±0.61^{a}	95.38±0.72^{a}	0.272
P1 (0.1 ng/ml)	95.88±0.22^{a}	95.18±0.89^{a}	80.91±0.31^{c}	87.90±0.12^{b}	0.000
P2 (0.5 ng/ml)	95.46±0.21^{a}	94.42±0.28^{a}	65.37±1.53^{c}	78.47±1.78^{b}	0.000
P3 (1 ng/ml)	94.23±0.18^{a}	93.84±0.53^{a}	57.94±0.96^{c}	75.59±0.93^{b}	0.000
P4 (5 ng/ml)	94.16±0.10^{a}	89.82±1.24^{b}	45.86±0.37^{d}	74.88±0.14^{c}	0.000
P5 (10 ng/ml)	93.90±0.14^{a}	81.18±1.66^{b}	45.43±0.39^{d}	73.81±0.52^{c}	0.000

Values are the mean ± SD percentage of proliferated cells (n=3); numbers in the same line that shared the same superscript was not statistically different at α=0.05 based on LSD test

Table 3: Effect of mucoxin application on the apoptosis of T47D cells of each exposure hour group

Treatments	Hours of Exposure			
	0	24	48	72
K (0 ng/ml)	6.81±1.74^{a}	7.22±1.97^{a}	5.68±0.37^{a}	10.03±0.23^{a}
P1 (0.1 ng/ml)	9.63±0.32^{bc}	7.70±1.44^{a}	9.66±0.31^{b}	15.76±0.62^{b}
P2 (0.5 ng/ml)	7.47±1.12^{a}	10.55±0.08^{b}	17.14±0.40^{c}	26.05±0.35^{c}
P3 (1 ng/ml)	10.78±0.49^{c}	10.19±0.36^{b}	20.94±1.02^{d}	39.80±1.21^{d}
P4 (5 ng/ml)	10.02±0.03^{c}	10.94±0.42^{b}	32.47±1.52^{e}	58.50±0.75^{e}
P5 (10 ng/ml)	10.86±0.68^{c}	11.23±0.66^{b}	52.71±1.09^{f}	75.73±2.53^{f}
ANOVA (P-value)	0.008	0.012	0.005	0.005

Values are the mean ± SD percentage of apoptotic cells (n=3); numbers in the same column that shared the same superscript was not statistically different at α=0.05 based on LSD test

Table 4: Effect exposure hour on the apoptosis of T47D cells given mucoxin of six different concentration

Treatments	Hours of Exposure				ANOVA
	0	24	48	72	(P-value)
K (0 ng/ml)	6.81±1.74^{a}	7.22±1.97^{a}	5.68±0.37^{a}	10.03±0.23^{b}	0.0210
P1 (0.1 ng/ml)	9.63±0.32^{b}	7.70±1.44^{a}	9.66±0.31^{b}	15.76±0.62^{c}	0.0001
P2 (0.5 ng/ml)	7.47±1.12^{a}	10.55±0.08^{b}	17.14±0.40^{c}	26.05±0.35^{d}	0.0000
P3 (1 ng/ml)	10.78±0.49^{a}	10.19±0.36^{a}	20.94±1.02^{b}	39.80±1.21^{c}	0.0000
P4 (5 ng/ml)	10.02±0.03^{a}	10.94±0.42^{a}	32.47±1.52^{b}	58.50±0.75^{c}	0.0000
P5 (10 ng/ml)	10.86±0.68^{a}	11.23±0.66^{a}	52.71±1.09^{b}	75.73±2.53^{c}	0.0000

Values are the mean ± SD percentage of apoptotic cells (n=3); numbers in the same line that shared the same superscript was not statistically different at α=0.05 based on LSD test

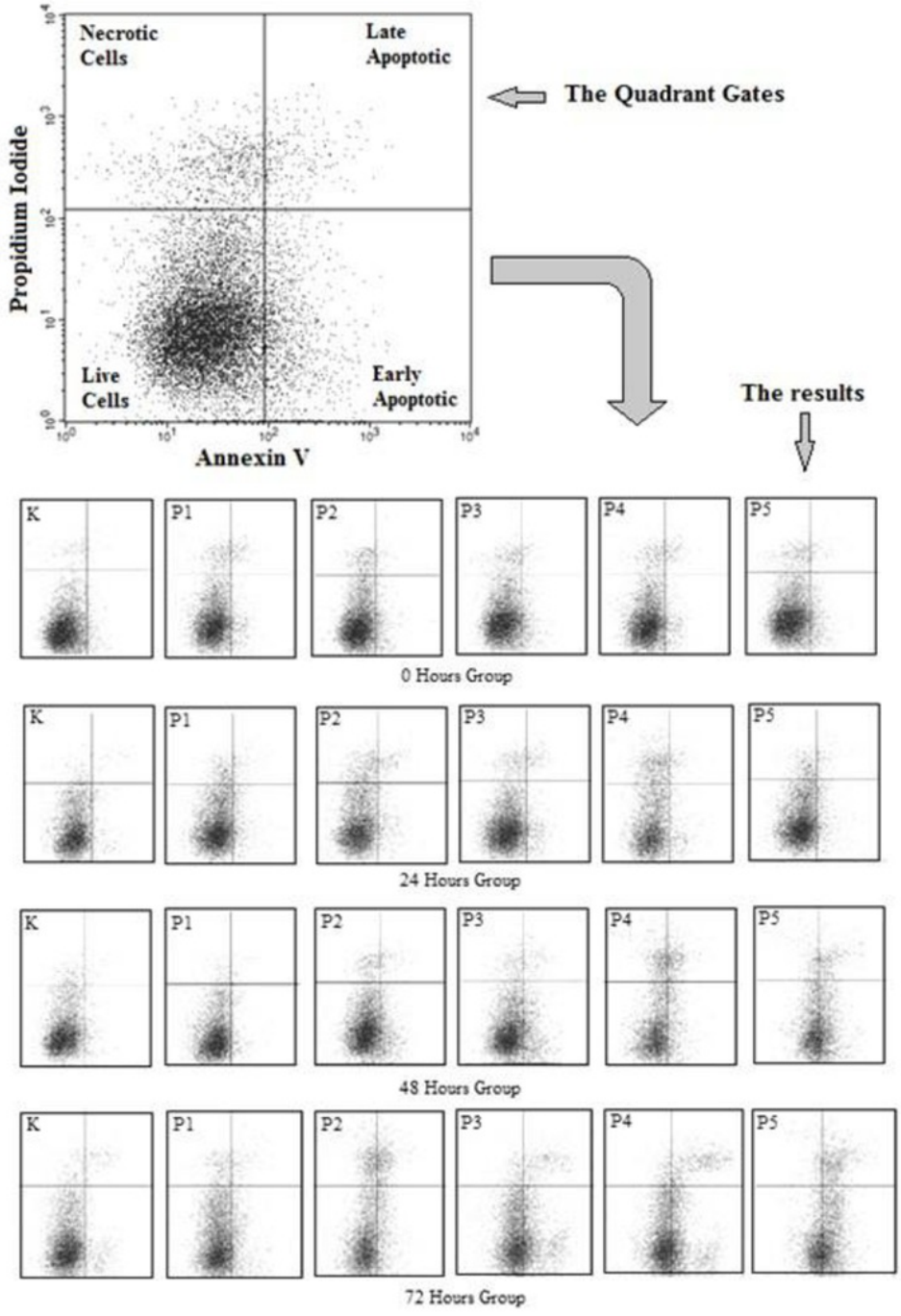

Fig. 2: Gating strategy for apoptotic assay of T47D cell lines by flow cytometri technique using Annexin V and Propidium Iodide

occurred in the group of 48-hour exposure by mucoxin dose of 10ng/ml (P4) and 72-hour eposure by mucoxin level of 5ng/ml (P4) and 10ng/ml (P5).

DISCUSSION

Based on the data presented above it was revealed that the application of mucoxin presumably inhibit proliferation and increase apoptosis of cell T47D. Due to a lack of data describing the biological properties of mucoxin in cancer cells, so the best approach to explain the effect is by reference to a similar substance derived from plants of the same family, Annonaceae, i.e. acetogenins.

The anti proliferative effect of annonaceous acetogenin has revealed by previous studies. Reference[12], by using acetogenin bullatacin, suggested that the annonaceous acetogenin is cytotoxic against multidrug-resistant human mammary adenocarcinoma cells. Another study suggested that a mono tetra hydrofuran acetogenin, annonacin, arrest cancer cells at the G1 phase and causes cytotoxicity[13]. Such effects may be due to inhibition properties of acetogenin on the activity of deoxyribonucleic acid (DNA) and DNA topoisomerase[14]. Moreover, the substances also affect mitochondrial complex I, block the electron transport chain and stop the production of adenosinetriphosphate (ATP). In addition, this substance also activates adenosine monophosphate-activated protein kinase (AMPK) and inhibits the signaling pathway of the mammalian target of rapamycin complex 1 (mTORC1) in colon cancer cells[15].

Current findings also confirm the apoptotic effects of Annona muricata leaves ethyl acetate extract (AMEAE) against lung cancer A549 cells. The bioactives substance induced apoptosis through mitochondrial-mediated pathway and involvement of NF-κB. AMEAE effectivley reduce the activation of NF-κB signaling pathway by suppressing the induced translocation of NF-κB from cytoplasm to nucleus[16].

More recent study showed that in addition to suppress NF-êB activity, the acetogenin treatment inhibits protein kinase B (Akt) and cyclin D1 protein in human hepatocellular carcinoma[17]. Cyclin D1 is a potein frequently linked to various types of human cancer[18]. If annonaceous acetogenin can actually suppress NF-κB activity, the activity of cyclin D1 would also be reduced since it is known that inhibition of NF-kB causes the reduction of serum-induced cyclin D1-associated kinase activity and resulted in delayed phosphorylation of the retinoblastoma protein[19].

The other genetic factors frequently interconnected with NF-kB and/or cyclin-D1 is protein p53. The p53 gene that encode p53 protein is a tumor suppressor. As a tumor suppressor, p53 plays a very important role to prevent excessive cell proliferation and maintain genomic integrity[20]. This gene will be activated by the cells in response to the internal or external stress signals. Stress signals can be either DNA damage due to viral infection, radiation as well as chemotherapy drugs, hypoxia, excessive expression of oncogene, nutritional deficiencies or ribosomal dysfunction. The stress signals could induce various upstream mediators such as 14ARF and Mdm2 that make p53 stable and active[21]. In this study the stress signals, most likely and should be, originating from mucoxin.

Genes known to be activated by p53 for its transcription are WAFI/CIP1/p21, GADD45, 14-3-3, Bax, Bak, Puma, and Noxa. WAFI/CIP1/p21 is a gene that encodes a protein CDK inhibitor which will cause hypo-phosphorylated of Rb so that E2F inactive. The GADD45 gene that encode GADD45 protein function in arresting cell cycle by enhancing p21 performance as the CDK inhibitor. Protein p43, the product of 14-3-3 gene, acts as a negative regulator that arrest cell cycle at the G2/M phase. The protein Bax and Bak, on other hand, are the propoptotic protein that directly increase the permeability of mitochondria. Whereas Puma and Noxa are genes that encode proteins BH3 which also play a role in the intrinsic pathway for apoptosis[22].

CONCLUSION

Mucoxin is proven to have anti-proliferative and pro-apoptotic properties against the T47D

breast cancer cells and, thus, mucoxin deserved classified as a promising anticancer agent.

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Conlict of Interest

The authors declare no conflicts of interest.

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