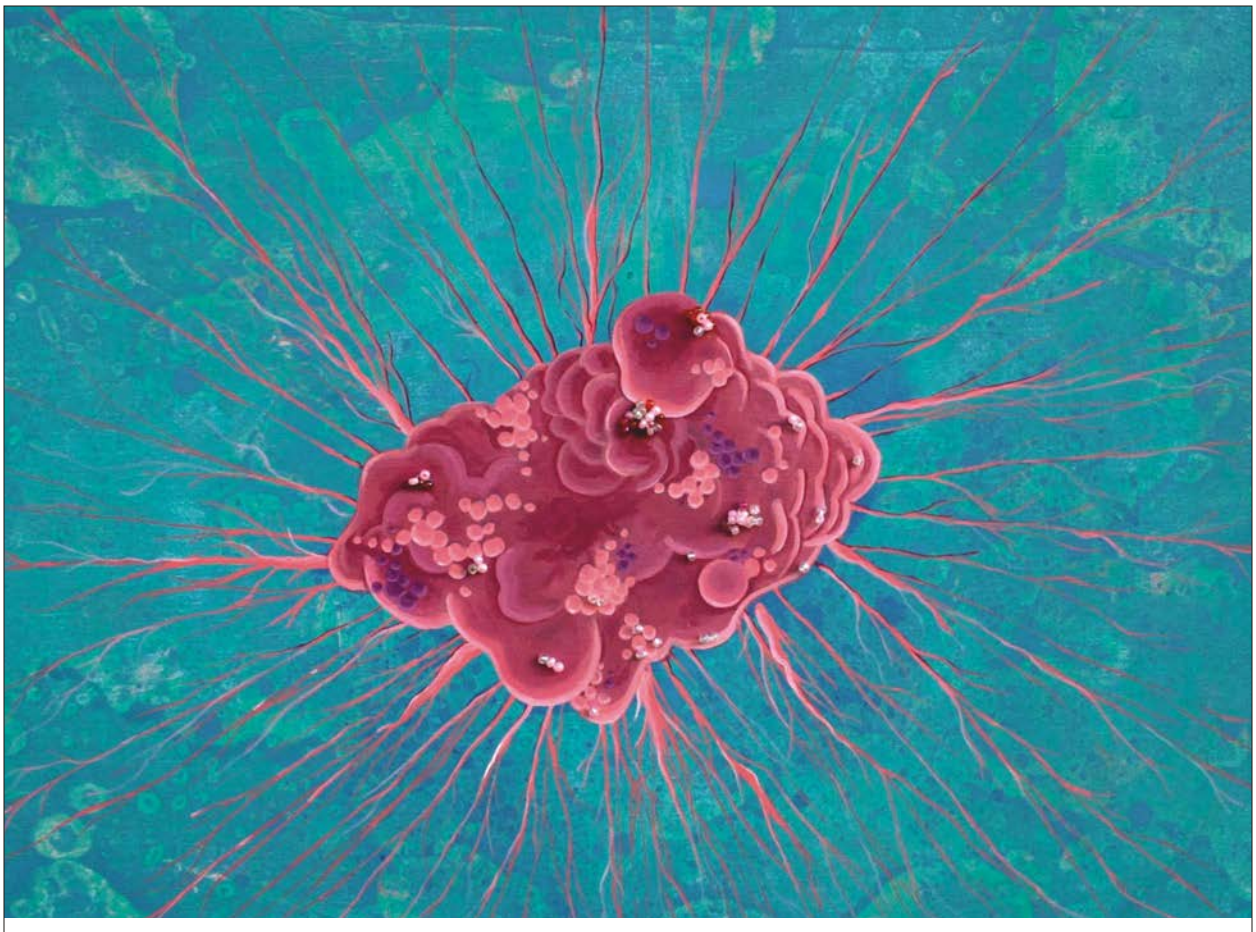

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Research Article
Mucoxin (Acetogenin) Inhibits Proliferation of T47D Breast Cancer by Suppressing Expression of Cyclin D1 Mediated by p53

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Abstract

Background: Mucoxinis believed to be a promising anticancer because it is known to inhibit cell proliferation. However, given study on mucoxin still very limited, the mechanism of the substances isolated from leaf extract of *Rollinia mucosa* in regulating and eliminating cancer cells has not fully understood. This study investigated the mucoxin mechanism in affecting proliferation, expression of p53 and cyclin D1 genes in the T47D breast cancer cells. **Materials and Methods:** The cell line samples were grouped into four referred to the hour of assays undertaken after mucoxin application, namely hour 0th, 24th, 48th and 72nd. Each group was given mucoxin of six different concentrations namely 0.00 μg mL^{-1} as a control, 0.1×10^{-3}, 0.5×10^{-3}, 1×10^{-3}, 5×10^{-3} and 10×10^{-3} μg mL^{-1} with three replications. Cells proliferation assayed by flow cytometry technique using BrDU staining protocol, whereas the expression of p53 and cyclin D1 genes determined by quantitative PCR (qPCR). **Results:** Cell proliferation in each group significantly reduced by mucoxin treatment. Mucoxin enhance p53 gene expression in 48 h, while the expression of cyclin D1 supressed signifantly by mucoxin of 5×10^{-3} and 10×10^{-3} μg mL^{-1} in 48 and 72 h. Simple regression analysis showed that cell proliferation decreased with the increase of p53 expression and the suppression of cyclin D1 gene, while p53 expression positively associated to cyclin D1 expression. **Conclusion:** Mucoxin can decrease the proliferation of T47D breast cancer cells by suppressing the expression of cyclin D1 mediated by p53 gene.

Key words: Mucoxin, acetogenin, *Rollinia mucosa*, p53, cyclin-D1, T47D, breast cancer, anticancer

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Considering conventional cancer treatments cause various side effects, recently practitioners are compelled to seek alternative cancer treatment that can eliminate or minimize the side effects[1,2]. One of the alternatives medication proposed for cancer, especially breast cancer is by utilizing acetogenin, an active substances isolated from *Annona muricata* Linn.[3-5]. One type of annonaceous acetogenins is mucoxin. This bioactive substance, classified as nonclassical acetogenin was isolated from leaf extract of *Rollinia mucosa*[6].

Mucoxin, as reported by Narayan and Borhan[6] showed a high anticancer properties against the cell line MCF-7 with an ED_{50} value of 3.7×10^{-3} μg mL^{-1} compared to adriamycin ($ED_{50} = 1\times10^{-2}$ μg mL^{-1}). However, studies on mucoxinis still very limited, especially with regard to the mechanism of cancer cells elimination. Until now, most studies concerning the role of annonaceous bioactive in the field of cancer biology are using non-mucoxin acetogenin and derivatives.

One of the derivatives, bullatacin has been recognized as an acetogenin that has cytotoxic effects against multidrug-resistant human adenocarcinoma[7]. Moreover, acetogenin inhibit hypoxia-inducible factor-1 (HIF-1) activation by blocking the hypoxic induction of nuclear HIF-1α protein and block hypoxic tumor angiogenesis by pressing the hypoxic induction of HIF-1 target genes VEGF (vascular endothelial growth factors) and GLUT-1 (glucose transporter-1). Inhibition of HIF-1α activity may lead to suppress mitochondrial respiration[8] at complex I. Acetogenin is also known to inhibit the activity of deoxyribonucleic acid (DNA) and DNA to poisomerase[9]. In addition to affect mitochondrial complex I, acetogenins block the electron transport chain and stop the production of adenosinetriphosphate (ATP). 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[10]. In addition to involve in the mechanisms mentioned above, annonaceous bioactives, also reported induced apoptosis in A549 cells through mitochondrial-mediated pathway and involvement of NF-κB[11]. More recent study showed that in addition to suppress NF-κB activity, the acetogenin treatment inhibits protein kinase B (Akt) and cyclin D1 protein in human hepatocellular carcinoma[12].

Based on the facts above it is clear that the process related to cancer cannot be explained by one or two pathways only. Thus, the role of acetogenin (or mucoxin) in inhibiting angiogenesis and proliferation of cancer cells may include many factors and mechanisms. The angiogenic switch during tumorigenesis, for instance, related to the amplification of normal HIF-1 dependent responses to hypoxia via loss of p53 function[13].

Inhibition of the activity of deoxyribonucleic acid (DNA), for another instance, will lead p53 protein activate its downstream target genes[14]. Thus, the p53 gene should be assumed as a functional target of acetogenins. By taking into account the crucial role of p53 in the cancer regulation[15], even concluded this gene (TP53) as a key gene in human cancer.

Cyclin D1 as well as p53 is also frequently linked to various types of human cancer[16]. If annonaceous acetogenin can actually suppress NF-κB activity, the activity of cyclin D1 should also be reduced since it is known that inhibition of NF-κB causes the reduction of serum-induced cyclin D1-associated kinase activity and resulted in delayed phosphorylation of the retinoblastoma protein[17].

However, though acetogenin has been known to suppress cancer cell proliferation through various pathways, the role of annonaceous substances (especially mucoxin) in regulating expression of the key genes such as p53 and cyclin D1 in human cancer is not yet known. Therefore, this study shows what and how the mechanism of mucoxin affect proliferation, expression of p53 and cyclin-D1 genes in T47D breast cancer cells.

MATERIALS AND METHODS

Mucoxinand 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 5 mg 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 were grouped into four based on the hour of mucoxin exposure before assays done, namely hour 0, 24, 48 and 72 h. Concentration levels of mucoxinapplied in the experiment were as follows: 0, 0.1×10^{-3}, 0.5×10^{-3}, 1×10^{-3}, 5×10^{-3} and 10×10^{-3} μg mL^{-1}.

Cell culture: The cells were grown in Roswell Park Memorial Institute medium (RPMI1640) culture media supplemented with 10% Foetal Bovine Serum (FBS) Gibco™ (from Thermo

Fisher Scientific Cat. No. 26140–079) and 0.2 U mL^{-1} 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 min. Then, as much as 5×10^4 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 treatment of the cells: 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 5×10^4 cells cm^{-2} in each well. Once the cells density reach 80% confluent, the cells treated with mucoxin of different concentrations asa follows: 0 μg mL^{-1} (K), 0.1×10^{-3} μg mL^{-1} (P_1), 0.5×10^{-3} μg mL^{-1} (P_2), 1×10^{-3} μg mL^{-1} (P_3), 5×10^{-3} μg mL^{-1} (P_4) and 10×10^{-3} μg mL^{-1} (P_5). After being treated, the cells were incubated in accordance with the length of hours that have been assigned to each group, ie., 0, 24, 48 and 72 h.

Cell proliferation assays: Cells proliferation assayed by flow cytometry technique using BrDU staining protocol. The wells containing T47D cells under optimal condition stained with Bromodeoxyuridine (BrdU) 30 μM, 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.

Genes expression assays: Primers used for determining p53 expression was 5'-CTGAGGTTGGCTCTGACTGTACCACCATCC-3' as forward primer and 5'-CTCATTCAGCTCTCGGAAGCATTT GCGGTGGAC-3' as the reverse primer. While, primers used for cyclin D1 analysis was 5'-CCGTCCATGCGGAAGATC-3' as forward primer and 5'-ATGGCCAGCGGGAAGAC-3' as the reverse primer. The β-actin gene was used as a control with the forward primer 5'-TCTGGCACCACACCTTCTACAATG-3' and reverse primer 5'-AGCACAGCCTGGATAGCAACG-3'.

The RNA was extracted from the T47D cell samples using easy-spin™ (DNA free) Total RNA Extraction Kit from Intron Biotechnology. The concentration of total isolated RNA then assessed by Nano Drop 2000 Spectrophotometer from Thermo Scientific (Thermo Fisher Scientific Inc., MA, USA). Expression of p53 and cyclin D1 genes in the T47D cells were determined by quantitative PCR (qPCR) methods using RealMOD™ Green Real-time PCR Kit from Intron Biotechnology with the Cat. No. REF25109. The qPCR data, finally, analyzed using Light Cycler® software from ROCHE. Beta actin (β-actin) gene was used as the internal control (house keeping gene) while control samples was used as the calibrator genes.

Statistical analysis: Comparison of mean values between treatment was analyzed using ANOVA followed by Tukey test if the data distribution was normal and Kruskal-Wallis test if it was not. Simple linear regression analysis was carried out to test the statistical correlation significance between variables: T47D cell proliferation, p53 gene expression and cyclin D1 gene expression.

RESULTS

Mucoxin effects on cell proliferation: Effects of mucoxin treatment on T47D breast cancer cells are presented in Table 1. Based on the data it is clear that mucoxin treatments (P1-P5) effect on the cell proliferation compared to control (K) in all cell groups (all values of $p<0.05$). However, high inhibitions occurred in the cell group 48 h by concentration treatment of 1×10^{-3} μg mL^{-1} (P3), 5×10^{-3} μg mL^{-1} (P4) and 10×10^{-3} μg mL^{-1} (P5).

Mucoxin effects on p53 gene expression: Effects of mucoxin treatment on p53 gene expression in T47D cells after mucoxin treatment are presented in Table 2. Compared to control (K), mucoxin treatments (P1-P5) effect on the p53 gene expression in all cell groups (all values of $p<0.05$) and the high effect of mucoxin occurred in cell group 48 h.

Based on the data on Table 2 it can be inferred that mucoxin able to promote, stabilize and activate expression of p53 gene in T47 cell lines by exposure for 0, 24 and 48 h. The inference was supported by highly significant difference ($p<0.001$) between mean values of p53 expression.

Mucoxin effects on cyclin-D1 gene expression: The expression profile of cyclin-D1 gene in T47D breast cancer treated with mucoxin and the statistical analysis results are presented in Table 3. In all cell groups, the mucoxin treatments (P1-P5) compared to control (K) effect on the cyclin D1 gene expression (all values of $p<0.05$). However, a

Table 1: Mean proliferation (%) of T47D breast cancer after giving mucoxin

Cell groups (h)	Treatments*	Mean (%)	SD	p
0	K	96.403	0.320	0.007
	P1	95.883	0.223	
	P2	95.457	0.210	
	P3	94.233	0.180	
	P4	94.160	0.104	
	P5	93.900	0.144	
24	K	95.507	0.927	0.009
	P1	95.180	0.885	
	P2	94.417	0.280	
	P3	93.837	0.532	
	P4	89.823	1.242	
	P5	81.177	1.661	
48	K	96.133	0.610	0.006
	P1	80.907	0.310	
	P2	65.370	1.532	
	P3	57.940	0.955	
	P4	45.860	0.366	
	P5	45.427	0.386	
72	K	95.383	0.716	0.006
	P1	87.897	0.120	
	P2	78.470	1.779	
	P3	75.593	0.932	
	P4	74.876	0.140	
	P5	73.810	0.519	

*Concentration of mucoxin, K: 0 μg mL^{-1}, P_1: 0.1×10^{-3} μg mL^{-1}, P_2: 0.5×10^{-3} μg m^{-1}, P_3: 1×10^{-3} μg m^{-1}, P_4: 5×10^{-3} μg mL^{-1} and P_5: 10×10^{-3} μg mL^{-1}

Table 2: Expression of p53 gene (number of copies) in T47D cells treated with mucoxin

Cell groups (h)	Treatments*	Mean (copies)	SD	p
0	K	336,568	31,194	0.008
	P1	221,907	14,928	
	P2	272,000	15,426	
	P3	3206,000	89,867	
	P4	465,167	51,726	
	P5	313,000	51,730	
24	K	257,400	5,797	0.0001
	P1	291,840	7,516	
	P2	311,773	10,817	
	P3	371,230	15,758	
	P4	433,200	29,371	
	P5	287,300	10,850	
48	K	318,010	55,911	0.005
	P1	465,507	10,657	
	P2	861,670	6,760	
	P3	935,133	56,241	
	P4	1148,367	21,034	
	P5	742,700	16,488	
72	K	263,100	35,851	0.0001
	P1	478,500	17,022	
	P2	319,000	7,550	
	P3	436,333	39,678	
	P4	332,000	65,092	
	P5	111,370	10,070	

*Concentration of mucoxin, K: 0 μg mL^{-1}, P_1: 0.1×10^{-3} μg mL^{-1}, P_2: 0.5×10^{-3} μg m^{-1}, P_3: 1×10^{-3} μg m^{-1}, P_4: 5×10^{-3} μg mL^{-1} and P_5: 10×10^{-3} μg mL^{-1}

consistent inhibitory effect of the mucoxin treatments on the expression of cyclin D1 is seen in cell groups 48 and 72 h.

Interconnection between variables: Results of simple linear regression analysis of the strength of the relationship between the experimental variables are presented in Table 4. Cell proliferation decreased with the accretion of p53 ($R^2 = 0.06$; $p = 0.037$) and the reduction of cyclin D1 ($R^2 = 0.182$; $p = 0.0001$), while the expression of p53 gene positively related to the expression of cyclin D1 ($R^2 = 0.063$; $p = 0.033$).

Table 3: Expression of cyclinD1gene (number of copies) in T47D cells treated with mucoxin

Cell groups (h)	Treatments*	Mean (copies)	SD	p
0	K	50,430	19,659	0.0001
	P1	60,207	2,108	
	P2	152,767	5,755	
	P3	121,997	5,503	
	P4	102,073	3,069	
	P5	88,487	3,445	
24	K	71,900	2,506	0.0001
	P1	59,123	1,558	
	P2	88,707	1,421	
	P3	47,157	1,659	
	P4	68,343	1,498	
	P5	73,867	5,515	
48	K	54,717	3,082	0.0001
	P1	60,067	2,829	
	P2	67,327	2,111	
	P3	61,440	2,787	
	P4	43,540	1,640	
	P5	32,267	3,595	
72	K	86,167	2,902	0.0060
	P1	98,947	1,485	
	P2	92,547	1,004	
	P3	87,827	1,487	
	P4	45,123	5,204	
	P5	26,533	2,359	

*Concentration of mucoxin: K: 0 μg mL^{-1}, P_1: 0.1×10^{-3} μg mL^{-1}, P_2: 0.5×10^{-3} μg m^{-1}, P_3: 1×10^{-3} μg m^{-1}, P_4: 5×10^{-3} μg mL^{-1} and P_5: 10×10^{-3} μg mL

Table 4: Results of simple regression analysis between research variables

Independent variables	Dependent variables	Constant	Slope	F	p	R^2
p53 expression	T47D proliferation	86.446	-0.006	4.502	0.0370	0.060
cyclin D1 expression	T47D proliferation	66.268	0.231	15.591	0.0001	0.182
p53 expression	cyclin D1 expression	66.049	0.012	4.708	0.0330	0.063

Refers to the data in Table 4, it can be inferred that the increase of stabilization and activation of the p53 gene was relating to the inhibition of T47D cell proliferation. Given the coefficient of determination (R^2) is 0.06, so that the p53 gene expression accounted for 6% of the cell proliferation inhibition. In this contex the inhibitory effects of mucoxin on the proliferation of T47D cells most probably related to suppression of cyclin-D1 gene. The $R^2 = 0.063$ between p53 and cyclin-D1 gene means that 6.3% of the expression of cyclin-D1 can be accounted for by the expression of the p53 gene.

DISCUSSION

Based on the data presented above it was revealed that mucoxin treatments inhibit T47D cell proliferation, enhance p53 gene expression and suppress the expression of cyclin-D1 gene. Furthermore, by referring to the regression analysis data these three dependent variables significantly interrelated to each other (all values of $p<0.05$).

The interconnection between p53, cyclin-D1 and proliferation of T47D cells clearly seen from the difference of expression time (hours) of the genes and the proliferation. In this study p53 was expressed earlier than cycline-D1. In addition, p53 is expressed in the first 24 h of mucoxin exposure while, T47D cells starting to proliferate at 48 h. Thus it can be assumed that mucoxin promote p53 expression which then suppress cyclin-D1 gene expression. Although, the effect was relatively low (6.3%), but the effects of p53 on the cyclin-D1 at the protein level would be significant enough.

Current findings consistent with the existing theory and some previous reports indicated that the increase in stabilization and activation of p53 plays a key role both in the process of proliferation and apoptosis by acting as a transcriptional regulatory protein of certain genes[18,19].

Genes known to be activated by p53 for its transcription are WAFI/CIP1/p21, GADD45, 14-3-3, Bax, Bak, Puma and Noxa. The 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[20].

There has been no previous research revealed the mechanism of mucoxin in suppressing proliferation and increasing apoptosis by activate and stabilize p53. However, in Taiwan, by studying the annonacin cytotoxicity, Yuan *et al.*[21] suggested that annonacin can arest the cell cycle at S1 phase in prostate cancer cells depended on caspase 3 and Bax. Moreover, Rachmawati *et al.*[22] also reported that activation and stabilization of p53 in cervical cancer cells plays a key role in apoptosis.

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[23]. 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[24]. In this study the stress signals, most likely and should be, originating from mucoxin.

The activated p53 gene will be role as a protein regulator which triggering a variety of biological responses mainly on the process of cell proliferation and cell apoptosis[19]. When p53 being active, one or more phosphorylation of serine residues at the N-terminus or C-terminus will take place which then bind to the enhancer elements of the downstream targets of p53 Ou *et al.*[25]. The downstream targets of p53 will respod the bonding by activating its self or not. Activation of the downstream targets determined by the cell types, the damage levels and any other parameters that have not yet identified[26]. Until now there are hundreds types of p53 target genes with various cellular function and mechanism.

In general, there are three main pathways of p53 downstream activation i.e. arresting the cell cycle when DNA damage occurs, repairing the damaged DNA and controlling the apoptosis when the damage can not be repaired anymore[27]. The arestting effects of p53 on cell cycle can be explain as follows. The activation of p53 in cell cycle would increase endogenous regulation of $p21^{waf1/Cip1}$ mRNA and its protein. The $p21^{waf1/Cip1}$ then bind cyclin-CDK complex[28]. Excessive expression of $p21^{waf1/Cip1}$ arrests the cell cycle at the G1 phase (resting phase) by blocking the cyclin E-CDK2 complex. The excessive expression of $p21^{waf1/Cip1}$ can also stop E2F from inducing expression of gene needed by cells for entering S phase[26].

Another target gene of p53 which lead to the inhibition of cell proliferation is 14-3-3δ GADD45, a protein involved in the G2 phase regulation. GADD45 bind CDC2 that would prevent cyclin B-2CDC from being a complex so that the kinase activities are inhibited. Meanwhile, 14-3-3δ protein would transfer the cyclin B-CDC2 complex from nucleus to cyclin-D1 complex which is separated from the target protein. Thus, excessive expression of 14-3-3δ would induce cell division arrested in G2 phase[23].

Regulation of cell cycle arrest in G1 and G2 phase by p53 will give cells additional time to repair gene-level damages before entering the critical phase of DNA synthesis and mitosis. p53 known to involved indirectly in the process of NER through P48 gene transactivation important for the repair efficiency of a particular DNA damage[29].

Moreover, p53 also known to take a part in the BER process by controling the stability of the DNA polymerase β-AP-DNA complex, the early stage of the DNA damage repair in the BER process[30]. Furthermore, p53 also involved in DNA repair processes through the Nucleotide Excision Repair (NER) and the Base Excision Repair (BER)[27].

Allegedly, mucoxin application triggering or causing stabilization of the p53 gene resulting in the enhancement of p53 gene expression. Mucoxin might arest the cell cycle at the G1. This is because, in the cell cycle, external interference such as the provision of anti-cancer mucoxin would be repaired completely before entering the S, G2 and M phase. In the G1 phase there were cyclin D1 and CDKs which when joined together could activate transcriptional genes, so it can be presumed that mucoxin would suppress the expression of cyclin D1 when the expression of p53 increased. The arrest of cell cycle by the decrease of cyclin D1 may allow cells to repair the DNA damages. When the damage can not be repaired, the cell cycle can not enter the S phase.

CONCLUSION

In summary, mucoxin reduced proliferation of T47D breast cancer cell by suppressing expression of cyclin-D1 gene mediated by the increase expression of p53 gene. Thus, mucoxin deserved classified as a promising anticancer agent.

SIGNIFICANCE STATEMENTS

The significance of this research findings are:

- Fulfill the lack of scientific data on the biological properties of mucoxin which has been manufactured and marketed worldwide online

- Inform cancer therapy practitioners and patients that mucoxin indeed inhibit the proliferation of breast cancer cells
- Provides a scientific overview of the mechanism of mucoxin affecting expression of gene responsible for regulating proliferation of cancer cells

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