

## RESEARCH ARTICLE

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# Cytotoxicity of Simvastatin in Human Breast Cancer MCF-7 and MDA-MB-231 Cell Lines

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## Abstract

**Objective:** Statins, 3-hydroxy-3-methylglutaryl co-enzyme A (HMG-CoA) reductase inhibitors, have been shown to be effective in the treatment of cardiovascular disease. Recent reports demonstrate an anticancer effect induced by statins on lung and prostate cancer cells. The present study aimed to investigate the therapeutic potential of Simvastatin can serve as chemotherapeutic agent against human breast cancer MCF-7 and MDA-MB-231 cell lines. **Methods:** The cytotoxic effect of simvastatin against breast cancer cells were evaluated using MTT assay. The related mechanism of cell death was further determined by trypan blue staining, morphological changes observation, and drug combination index. **Results:** The results showed that simvastatin treatment substantially induced cell death in a dose-dependent and time-dependent manner on MCF-7 and MDA-MB-231 cells. Simvastatin exhibited a highly cytotoxic effect on MCF7 and MDA-MB-231 with half-maximal (50%) inhibitory concentration ( $IC_{50}$ ) 8.9  $\mu$ M and 4.5  $\mu$ M respectively. Consistently, we observed antiproliferative effect of Simvastatin was associated with apoptosis on breast cancer cell lines by determination of morphological changes. Moreover, this drug demonstrated a synergistic activity with doxorubicin on triggering cell death in MCF7 cells, but not in MDA-MB-231. **Conclusion:** Simvastatin has a potent cytotoxic effect resulting in the death of human breast cancer MCF-7 and MDA-MB-231 cell lines, demonstrating its potential as a new candidate for cancer drug.

**Keywords:** Simvastatin- cell death- breast cancer- antiproliferative- chemosensitive

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## Introduction

Breast cancer is the second most common cancer in the world and is the most common cancer in women. Based on data from Globocan, the International Agency for Research on Cancer (IARC), it was reported that in 2018 there were 2,088,849 (11.6%) new cases of breast cancer. Based on these data it is also mentioned that the number of breast cancer cases in Indonesia have the highest incidence when compared with other cancer cases, contributing up to 58,256 (16.7%) new cases (Bray et al., 2018).

Breast cancer cells consist of several subtypes. The most common breast cancer is luminal subtype breast cancer with an ER-positive type. Luminal A subtype breast cancer is characterized by ER+, PR-, and HER2- (Perou and Borresen-Dale, 2011; Ostad and Parsa, 2011). Luminal A subtype breast cancer consists of MCF-7, T47D, and SUM185 cell lines. MCF-7 is the most commonly used breast cancer cell line in researches because it has very

high hormone sensitivity due to its high ER expression (Holliday and Speirs, 2011). Other breast cancer cells are claudin-low (triple negative) subtypes that are similar to basal subtypes which are characterized by ER-, PR-, and HER2-. MDA-MB-231 is one of the claudin-low breast cancer subtype (Perou and Borresen-Dale, 2011; Ostad and Parsa, 2011).

To overcome the high incidence of breast cancer, the discovery and development of cancer treatments must continue to be attempted. Novel breast cancer drugs should have few side effects, greater therapeutic efficacy, and lower costs. The discovery of new cancer drugs is considered more effective when based on drugs that are already available. Research into possible beneficial effects of available drugs is more efficient than research on new herbal medicines because it has passed a series of standardized clinical trials. This will certainly affect the production time and research costs (Pantziarka et al., 2018).

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Statins are a class of HMG CoA reductase inhibitor drugs that have been clinically proven to treat dyslipidemia (Beckwitt et al., 2018). Currently approved statins by the FDA are atorvastatin, fluvastatin, lovastatin, pitavastatin, pravastatin, rosuvastatin, and simvastatin. Hypercholesterolemia's patients in Indonesia use statins as the first drug of choice. The most frequently prescribed statin for drug monotherapy is Simvastatin (42.8%) (Munawar et al., 2013; Putra et al., 2017), which is cheaper than other types of statins (Moon and Bogle, 2006).

Based on its characteristics, statins are divided into lipophilic and hydrophilic types. Simvastatin itself is a type of lipophilic statin (Campbell et al., 2006; Feldt et al., 2015; Desai et al., 2015; Beckwitt et al., 2018). It has been reported (Fong, 2014), based on its hepatoselectivity, lipophilic statins tend to have higher exposure to non-liver tissue, whereas hydrophilic statins tend to be more hepatoselective. This occurs because lipophilic statins can be transported passively and non-selectively by diffusion into liver or non-liver tissue.

Statins have a major effect on reducing cholesterol concentrations (Hu et al., 2012; Cardwell et al., 2015). In addition, statins can also inhibit the growth of cancer cells through antiproliferative effects and cell death (Chan et al., 2003; Al-Husein et al., 2012; Gazzero et al., 2012; Wang et al., 2016; Wang et al., 2018). Colorectal cancer shows a non-significant apoptotic effect on statin drugs (Bardou et al., 2010). The administration of Simvastatin to lung and prostate cancer cells cause a significant apoptotic effect (Wang et al., 2018; Goc et al., 2012).

Based on the description above, Simvastatin has been shown to have a significant effect on apoptosis of lung and prostate cancer cells (Wang et al., 2018; Goc et al., 2012). Researchers suspect this is due to the inhibitory effect of proliferation and direct cell death due to the administration of Simvastatin (Beckwitt et al., 2018; Cardwell et al., 2015). Based on the description above, the author wants to evaluate the cytotoxic effects of Simvastatin and its effect on MCF-7 and MDA-MB-231 breast cancer cell lines' death.

## Materials and Methods

### *Chemicals and reagents*

RPMI 1640 medium (cat No. 11875093), fetal bovine serum (FBS) (cat No. 10270106) were purchased from Sigma-Aldrich, USA. L-glutamine (cat No. 25030164) and penicillin streptomycin (cat No. 15140122) were purchased from Thermo Fisher, USA. Simvastatin (cat No. S6196-5MG, Sigma Aldrich, USA) diluted with dimethylsulfoxide (DMSO, cat No. D8418, Sigma Aldrich, USA) at a concentration of 1 mM. 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) (cat No. M2128), were purchased from Sigma-Aldrich, USA. Doxorubicin was provided by Pharmacy of Hasan Sadikin General Hospital, Bandung, Indonesia. All the other chemicals were of analytical grade purchased from Merck, USA.

### *Cell Culture*

The human breast cancer cells MCF-7 and

MDA-MB-231 cell lines were used to evaluate cytotoxic activity of Simvastatin. The MCF-7 cells were derived from Dr. Ahmad Faried and Prof. Hiroyuki Kuwano (Gunma University, Japan) which was obtained from American Type Culture Collection (ATCC, Manassas, Virginia). MDA-MB-231 cells were from Dr. Thordur Oskarsson (DKFZ, Germany). All cell lines were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine and 1% penicillin/streptomycin in a humidified cell culture incubator that contained 5% CO<sub>2</sub>, 21% O<sub>2</sub>, 37°C. All experiments were conducted triplicate and from 3 different experiments at the Laboratory of Cell Culture and Cytogenetics, Faculty of Medicine, Universitas Padjadjaran, Indonesia.

### *Cytotoxicity Assay (MTT Assay)*

Serial dilutions of Simvastatin were added into each of 96-well plates, then the cells were seeded at a density of  $1 \times 10^4$  cells/well a day before treated with simvastatin concentrations of 0.5, 1, 3, 5, 10, 20, 50, 75, 100, 125, and 150  $\mu$ M and then incubated for 24, 48 and 72 hours. After incubation, medium was removed, cytotoxic evaluation of Simvastatin against MCF-7 and MDA-MB-231 cancer cell lines were analyzed using MTT assay according to the protocol in previous studies (Rezano et al, 2013). The soluble formazan product was quantified using a micro-titer plate reader (Thermo Scientific- Multiscan EX, Singapore) at 550 nm and percentage of cell death was calculated using the following formula:

$$\% \text{ cell death} = (1 - (\text{average absorbance of triplicate drug wells}) / (\text{average absorbance of control wells})) \times 100\%$$

A dose-response curve was plotted into four parametric logistic models by Sigmaplot ver.12 to enable the calculation of half-maximal (50%) inhibitory concentration (IC<sub>50</sub>) that kills MCF7 or MDA-MB-231 cells. The American National Cancer Institute guidelines set the IC<sub>50</sub> cut off value for evaluating drug cytotoxic activity if less than 20  $\mu$ M after 48 and 72 hours incubation (Abdel-Hameed et al., 2012)

### *Cell Death Percentage*

Determination of membrane integrity via trypan blue dye exclusion test was used to evaluate cell death and chemosensitivity against simvastatin and doxorubicin. Half-maximal inhibitory concentration of Simvastatin from MTT assay was used to set concentration course for cell death analysis. Cells were seeded on 6-well plates at density  $5 \times 10^5$  cells/well prior treatment. Afterward, cells were treated with simvastatin (Sim) concentrations of 1, 2, 5, 9, 30, 50  $\mu$ M; 9  $\mu$ M Simvastatin + 5  $\mu$ M Doxorubicin (Sim9 and Dox5); 9  $\mu$ M Simvastatin + 10  $\mu$ M Doxorubicin (Sim9 and Dox10) for MCF-7 cell line and 1, 2, 5, 9, 15, 30, 50  $\mu$ M Simvastatin; 5  $\mu$ M Simvastatin + 5  $\mu$ M Doxorubicin (Sim5 and Dox5); 5  $\mu$ M Simvastatin + 10  $\mu$ M Doxorubicin (Sim5 and Dox10) for MDA-MB-231 cell line Both cell lines were then incubated for 48 hours and then the percentage of cell viability and cell death were calculated using the formula below:

Cell Viability (%)=(Viable Cell Count)/(Total Cell)  
×100%

Cell Death (%)=(Dead Cell Count)/(Total Cell)  
×100%

#### Morphological Analysis

Observation of morphological changes of apoptotic cells after Simvastatin treatment was conducted as Moongkarndi et al., (2004) with slight modification. Using an inverted phase-contrast microscope (100X), cells changes such as cell shrinking, membrane blebbing, and ballooning were observed in predicting the apoptotic mechanism for cell death. Digital images were captured with camera connected with a computer and Toupview Software (version x64, 3.7.7892).

#### Combination Index (CI)

Cells were treated with different concentration of Simvastatin alone or in combination with Doxorubicin followed by the cytotoxic assay. As Chou (2010) reported in his previous work, Combination Index was analyzed with cell death percentage using Compusyn software.

#### Statistical Analysis

Statistical analysis was performed using SigmaPlot version 14.0 (SYSTAT Software Inc., USA), Compusyn, and IBM SPSS Statistics version 20 (SPSS Inc., USA). Analyzed data were obtained from three replications in two different experiments and presented as mean and standard deviation (mean ± standard deviation). The cytotoxic effects of MCF-7 and MDA-MB-231 cell death from Simvastatin were analyzed by the four parametric logistic regression methods using SigmaPlot version 14.0. One-way ANOVA was conducted to determine the statistical significance (P) of each difference between the control group and the treatment group. A P value of <0.05 indicates a statistically significant difference.

## Results

*The Simvastatin has cytotoxic activity in MCF-7 and MDA-MB-231 cell lines*

The percentage of cell death after treatment of cell lines with Simvastatin and Doxorubicin were listed in Table 1. From these results, a calculation is performed to find the IC<sub>25</sub>, IC<sub>50</sub>, and IC<sub>75</sub> cytotoxicity values of Simvastatin as presented in Table 2 and Figure 1.

*The Simvastatin induces cell death in MCF-7 and MDA-MB-231 cell lines*

An incubation time of 48 hours was chosen for this study because it is considered the most effective incubation time for simvastatin. These cells have gone through its adaptation phase, namely from the lag phase to the log phase which has more stable cell proliferation. Data obtained from Table 3, Table 4, Figure 2, and Figure 3 showed half-maximal inhibitory concentration of Simvastatin can kill >50% of MCF-7 and MDA-MB-231 cell lines. The effect of Simvastatin on the increase in both cell death can be proven by the similarity of effects between the treatment of Simvastatin and Doxorubicin, which were a conventional chemotherapy.

*The Simvastatin alters cell morphology of MCF-7 and MDA-MB-231 cell lines*

Upon Simvastatin treatment, morphological observations were also made using an inverted microscope and the results of cell changes were presented in Figure 4 (MCF-7) and Figure 6 (MDA-MB-231). The observations revealed signs of cells undergoing apoptosis such as cell shrinking, cell rounding, and cell detaching after Simvastatin treatment.

*Doxorubicin and Simvastatin combination can increase lysed cell morphology*

When the treatment combination with Doxorubicin given, induce lysed cell morphology are more prominent. Morphological observations were made using an inverted

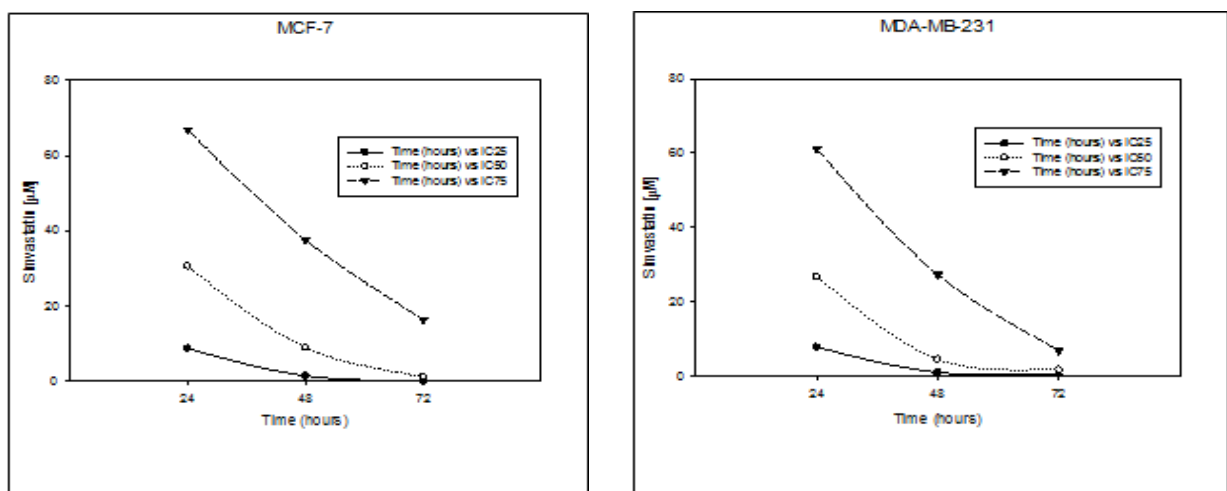


Figure 1. Graph of Time Relationships versus IC<sub>25</sub>, IC<sub>50</sub>, IC<sub>75</sub> Simvastatin concentrations. Data were presented as mean and SD from triplicate data. Drug curves were created and IC<sub>50</sub> of each cell lines were analyzed using four parametric logistic model by Sigmaplot ver.14. The graph shows the cell response to the time of administration of Simvastatin with concentrations of IC<sub>25</sub>, IC<sub>50</sub>, IC<sub>75</sub> in the MCF-7 and MDA-MB-231 cell lines.

Table 1. Percentage of MCF-7 and MDA-MB-231 Cell Death after Treated with Simvastatin and Doxorubicin for 24, 48, and 72 hours on MTT Assay

Drug	Dose (µM)	Cell Death (%)						P-value
		MCF-7			MDA-MB-231			
		24 hours	48 hours	72 hours	24 hours	48 hours	72 hours	
Simvastatin	Control	0	0	0	0	0	0	< 0.001
	0.5	18.2	14.3	41.8	8.3	22.6	25.6	
	1	15.3	18.7	46.3	11.6	31.4	41.3	
	3	15.3	31.9	65	17.3	45.2	65.5	
	5	25.1	40.4	66.1	18.4	54.3	72.3	
	10	27.5	42.3	68.7	31.6	58.3	78.9	
	20	31.7	51.1	69.8	39.8	73.4	81.6	
	50	64.8	77.4	85.6	60.8	74.8	94.4	
	75	85.3	81.9	96	92.9	90.6	97.4	
	100	96.1	85.1	99.3	98.4	90.8	99.3	
	125	101.9	88.7	99.9	100.6	91.6	99.4	
	150	102.8	90.2	99.7	101.8	95.6	100.3	
	Doxorubicin	5	59	48.4	55.6	51.2	60.3	
10		61.4	50.4	58.8	56.3	63.2	70.3	

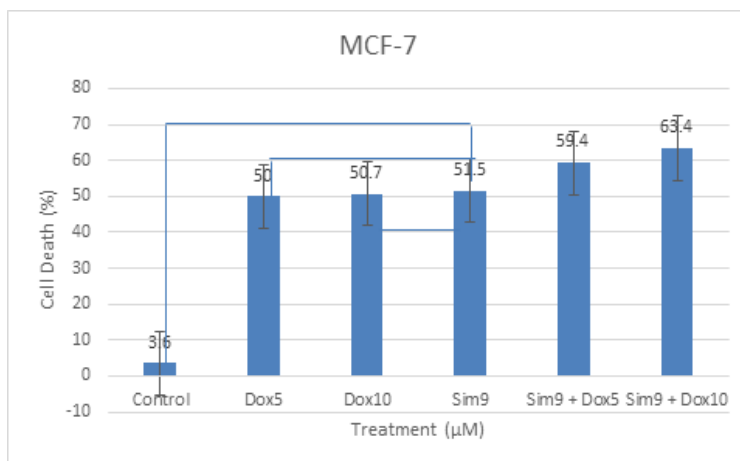


Figure 2. Cell Death Percentage with Various Concentration of Treatments and Its Significance Values. A significant effect of Simvastatin on MCF-7 breast cancer cell lines was demonstrated.

microscope and the results of cell changes were presented in Figure 5 (MCF-7) and Figure 7 (MDA-MB-231).

*Simvastatin induces synergistic cell death with Doxorubicin in MCF-7*

Our data showed a promising cytotoxic effect of Simvastatin by inducing cell death. Considering that cancer cells were activating multiple pathways for escaping from cell death. It is important to target cancer cells with conventional chemotherapy. Here,

we evaluated combination effect of Simvastatin with Doxorubicin showed synergistic effect against MCF-7 with Combination Index were 0.34 for Sim9 and Dox5; and 0.21 for Sim9 and Dox10. The data suggested that Simvastatin can increase chemosensitivity of Doxorubicin in MCF-7 cells. However, Combination Index were not identical (CI>1) in MDA-MB-231 cells. The administration of drug dosages and the effects of combination treatment are presented in Figure 8 and Figure 9.

Table 2. IC<sub>25</sub>, IC<sub>50</sub>, and IC<sub>75</sub> values in MCF-7 and MDA-MB-231 Breast Cancer Cells Treated with Simvastatin for 24, 48, and 72 hours. Data obtained from three data replications in two different experiments (P<0.001).

Incubation Period (hour)	MCF-7			MDA-MB-231		
	IC <sub>25</sub>	IC <sub>50</sub>	IC <sub>75</sub>	IC <sub>25</sub>	IC <sub>50</sub>	IC <sub>75</sub>
24	8.7 µM	30.6 µM	66.9 µM	7.8 µM	26.7 µM	61.2 µM
48	1.4 µM	8.9 µM	37.6 µM	1.0 µM	4.5 µM	27.3 µM
72	0.0 µM	1.1 µM	16.3 µM	0.4 µM	1.7 µM	6.8 µM



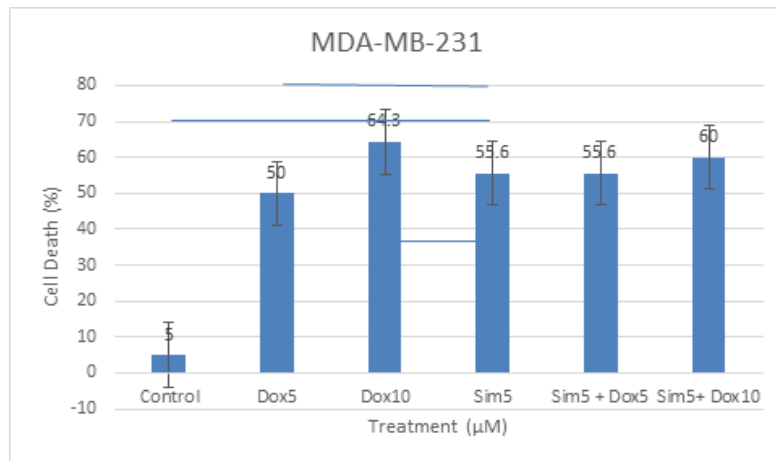


Figure 3. Cell Death Percentage with Various Concentration of Treatments and Its Significance Values. A significant effect of Simvastatin on MDA-MB-231 breast cancer cell lines was demonstrated.

## Discussion

The use of statins as anticancer agents becomes an emerging interest due to their pleiotropic effects on various cells and tissues. Accumulating evidences suggests the beneficial effects of statins extend beyond their cholesterol-lowering properties. Preclinical data have shown antiproliferative, proapoptotic, anti-invasive and radiosensitizing properties of statins. In cancer patients, the efficacy of statins as anticancer agents have been evaluated both in monotherapy and in combination therapy. However, there are very limited studies on

Simvastatin for its anticancer activities. The effect of simvastatin in bladder cancer cell line reveals reduction of cell viability in a dose- and time- dependent manner but not induces apoptosis (Wang et al., 2016). Similarly, simvastatin treatment in human leukemic natural killer cell line demonstrates cell proliferation inhibition and cell cycle arrest at G2/M (Crosbie et al, 2013). Previous report from Ghavami et al., (2009), Simvastatin activates novel apoptotic pathway in primary human lung mesenchymal cells.

Here our data shows a promising anti-tumor activity of Simvastatin in breast cancer cells. The results

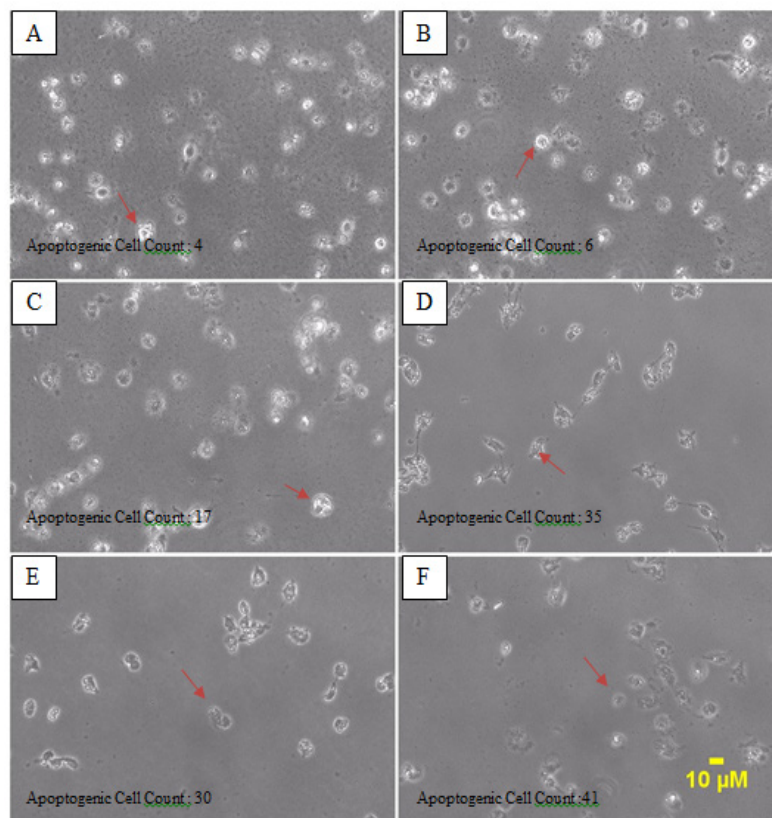


Figure 4. Morphological Studies by Inverted Microscope at Actual Magnification 100 $\times$  on MCF-7. At concentration of Simvastatin (A) 1 $\mu\text{M}$ , (B) 2 $\mu\text{M}$  and (C) 5 $\mu\text{M}$ , intact MCF-7 breast cancer cell morphology were seen. At concentration of (D) 9 $\mu\text{M}$ , (E) 30 $\mu\text{M}$ , and (F) 50 $\mu\text{M}$ , lysed MCF-7 breast cancer cell morphology were seen.

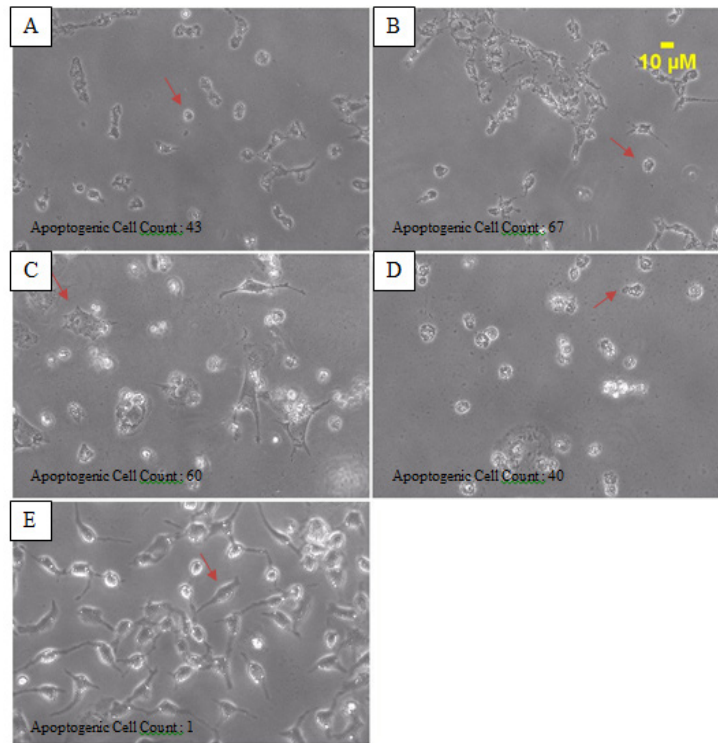


Figure 5. Morphological Studies by Inverted Microscope at Actual Magnification 100× on MCF-7. At a concentration of Simvastatin (A) 9μM Simvastatin + 5μM Doxorubicin, (B) 9μM Simvastatin + 10μM Doxorubicin, (C) 5μM Doxorubicin (positive control), and (D) 10μM Doxorubicin (positive control), lysed MCF-7 breast cancer cell morphology was seen. (E) Negative control of MCF-7 breast cancer cell lines were 80-90% confluent after incubation for 48 hours.

showed simvastatin has a cytotoxic effect on MCF-7 and MDA-MB-231 breast cancer cell lines. The results of this study proved that Simvastatin has a cytotoxic

effect on MCF-7 and MDA-MB-231 breast cancer cell lines. Treatment of Simvastatin against these cell lines caused significant cell death ( $P < 0.001$ ) when compared

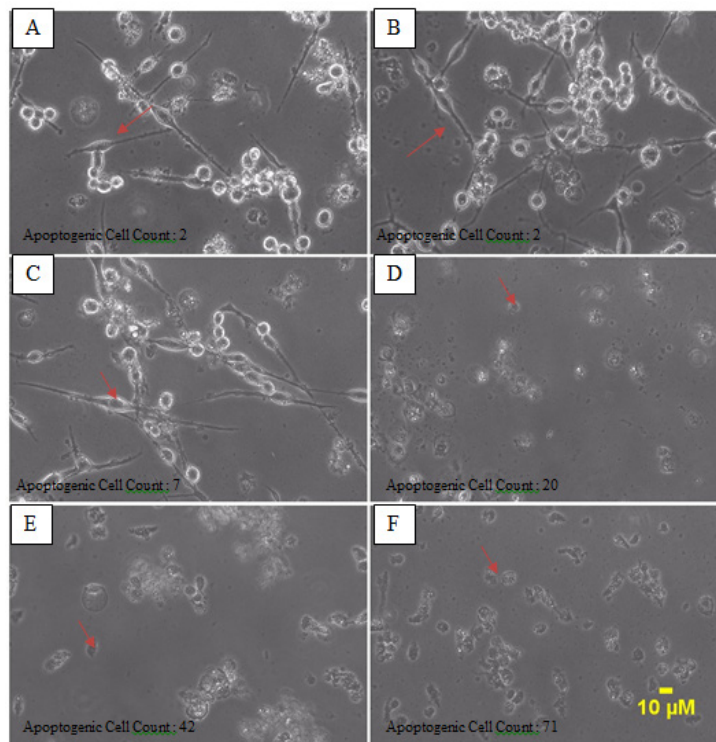


Figure 6. Morphological Studies by Inverted Microscope at Actual Magnification 100× on MDA-MB-231. At concentration of Simvastatin (A) 1μM, (B) 2μM, and (C) 5μM, intact MDA-MB-231 breast cancer cell morphology was seen. At concentration of (D) 9μM, (E) 15μM, and (F) 30μM, lysed MDA-MB-231 breast cancer cell morphology was seen.

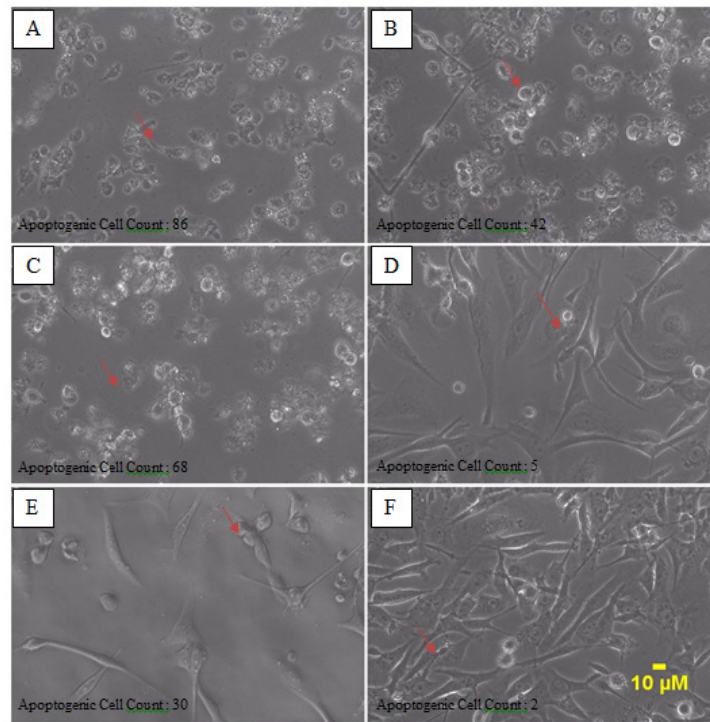


Figure 7. Morphological Studies by Inverted Microscope at Actual Magnification 100 $\times$  on MDA-MB-231. At concentration of (A) 50 $\mu$ M Simvastatin, (B) 5 $\mu$ M Simvastatin + 5 $\mu$ M Doxorubicin, (C) 5 $\mu$ M Simvastatin + 10 $\mu$ M Doxorubicin, (D) 5 $\mu$ M Doxorubicin (positive control), and (E) 10 $\mu$ M Doxorubicin (positive control) lysed MDA-MB-231 breast cancer cell morphology were seen. (F) Negative control of MDA-MB-231 breast cancer cell lines were 80-90% confluent after incubation for 48 hours.

to negative controls. Increasing the dose of Simvastatin caused an increase in cell death in both cancer cell lines (dose-dependent manner and time-dependent manner) (Table 1).

The cytotoxic effect of Simvastatin on MCF-7 and MDA-MB-231 cancer cells can be seen using the MTT assay. The results showed that Simvastatin treatment with an incubation period of 24, 48, and 72 hours has cytotoxic properties and triggered MCF-7 cell death. Half-maximal inhibitory concentration ( $IC_{50}$ ) values calculated from the results were 30.6 $\mu$ M ( $P < 0.001$ ), 8.9 $\mu$ M ( $P < 0.001$ ),

and 1.1 $\mu$ M ( $P < 0.001$ ) respectively for each incubation period. Simvastatin is also shown to have cytotoxic effects on MDA-MB-231 cells which caused cell death with an incubation period of 24, 48, and 72 hours. Half-maximal inhibitory concentration ( $IC_{50}$ ) values calculated from the results were 26.7 $\mu$ M ( $P < 0.001$ ), 4.5 $\mu$ M ( $P < 0.001$ ), and 1.7 $\mu$ M ( $P < 0.001$ ) respectively for each incubation period. Declining  $IC_{50}$  values with longer incubation period showed that the dose needed to cause the death of both breast cancer cells will be lower in prolonged

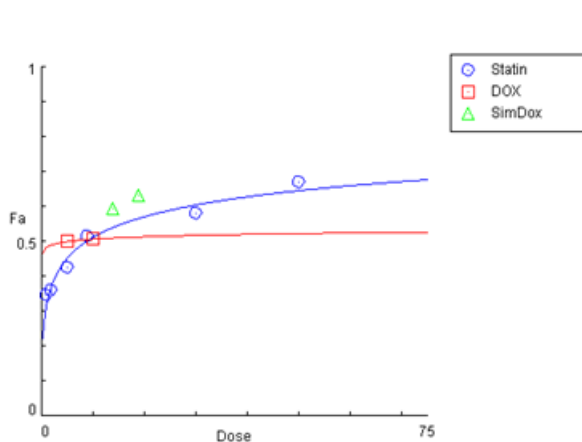


Figure 8. Combination of the Simvastatin with Doxorubicin Induces Synergism in MCF-7 cells. MCF-7 cells were treated as indicated concentration with Simvastatin only, Doxorubicin only, and Simvastatin-Doxorubicin Combination for 48 hours. Combination index was analyzed using Compusyn Software.

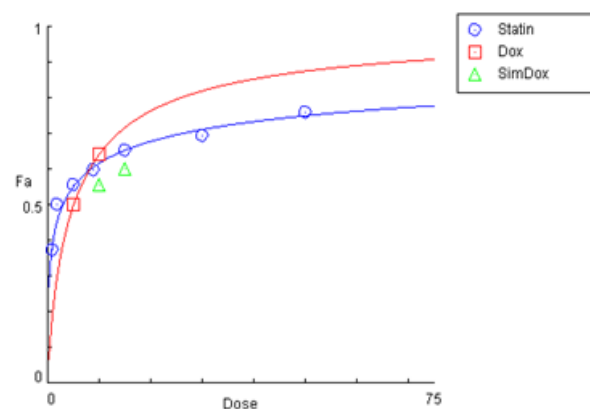


Figure 9. Combination of the Simvastatin with Doxorubicin Induces Antagonism in MDA-MB-231 Cells. MDA-MB-231 cells were treated as indicated concentration with Simvastatin only, Doxorubicin only, and Simvastatin-Doxorubicin Combination for 48 hours. Combination index was analyzed using Compusyn Software.

Table 3. Cell Viability and Cell Death in MCF-7 Breast Cancer Cell Lines Treated with Simvastatin for 48 hours. Results were obtained from Trypan Blue test.

Concentration ( $\mu\text{M}$ )	Cell Viability (%)	Cell Death (%)
Sim1	65.4	34.6
Sim2	63.6	36.2
Sim5	57.3	42.7
Sim9	48.5	51.5
Sim30	41.9	58.1
Sim50	32.8	67.2
Dox5	50.0	50.0
Dox10	49.2	50.7
Sim9+Dox5	40.6	59.4
Sim9+Dox10	36.6	63.4
Control Cell	96.4	3.6

incubation period, such as 48 and 72 hours, compared to only 24 hours. These results indicate that the longer the cell is exposed to Simvastatin, cell death will be more prominent (Table 2) (Figure 1).

$\text{IC}_{50}$  results from this study also indicate that MDA-MB-231 cell death required a lower dose of Simvastatin compared to MCF-7 cells. This happens because p53 in MCF-7 and MDA-MB-231 cells have a synergistic contribution to the effect of cell death. MDA-MB-231 cells have a p53 mutation, so MDA-MB-231 cells and thus are more susceptible to cell death than MCF-7 cells. This happened, presumably because the damage that occurred in the DNA of MDA-MB-231 cells at various stages of the cell cycle cannot be repaired by the mutated p53 (Rezano et al., 2013).

The effect of Simvastatin on the increase in both cell death can be proven by the similarity of effects between the treatment of Simvastatin and Doxorubicin, which were a conventional chemotherapy. The effect on the increase in cell death that was not significantly different ( $P > 0.05$ ) between cells treated with Simvastatin and cells treated with Doxorubicin showed that both drugs had the same effect. The results showed that treatment of Simvastatin at an indicated dose of 5, 10, 20, and 50  $\mu\text{M}$  for 24 and 48 hours on MDA-MB-231 cells showed no effect on the number of cell death that was not significantly different from Doxorubicin-treated cells at a dose of 5  $\mu\text{M}$  and 10  $\mu\text{M}$  ( $P > 0.05$ ). The administration of Simvastatin at a dose of 50  $\mu\text{M}$  for 24 hours and a dose of 5  $\mu\text{M}$ , 10  $\mu\text{M}$ , and 20  $\mu\text{M}$  for 48 hours on MCF-7 cells showed no significant difference in efficacy when compared to Doxorubicin at a dose of 5  $\mu\text{M}$  and 10  $\mu\text{M}$  ( $P > 0.05$ ). MDA-MB-231 cells that were treated with Simvastatin at a dose of 3, 5 and 10  $\mu\text{M}$  for 72 hours also showed no significant effect against cells treated with Doxorubicin at a dose of 5  $\mu\text{M}$  and 10  $\mu\text{M}$  ( $P > 0.05$ ). The administration of Simvastatin at a dose of 10  $\mu\text{M}$  on MCF-7 cells for 72 hours showed an effect that was also not significantly different from cells given Doxorubicin at a dose of 5  $\mu\text{M}$  ( $P > 0.05$ ).

The effect of Simvastatin on MCF-7 and MDA-MB-231 cell death can be strengthened by trypan blue exclusion

Table 4. Cell Viability (%) and Cell Death (%) in MDA-MB-231 Breast Cancer Cell Lines Treated with Simvastatin for 48 hours. Results were obtained from Trypan Blue test.

Concentration ( $\mu\text{M}$ )	Cell Viability (%)	Cell Death (%)
Sim1	62.4	37.5
Sim2	50.0	50.0
Sim5	44.4	55.6
Sim9	40.1	59.8
Sim15	34.5	65.4
Sim30	30.3	69.6
Sim50	23.8	76.2
Dox5	50.0	50.0
Dox10	35.7	64.3
Sim5+Dox5	44.4	55.6
Sim5+Dox10	40.0	60.0
Control Cell	95.0	5.0

test. The  $\text{IC}_{50}$  of Simvastatin which was 8.9  $\mu\text{M}$  and 4.5  $\mu\text{M}$  for MCF-7 and MDA-MB-231 had a very significant effect on cell death when incubated for 48 hours ( $P < 0.001$ ). The effect can also be proven by comparing with the positive control. The results showed that the administration of Simvastatin with  $\text{IC}_{50}$  dose until 50  $\mu\text{M}$  and combination drug caused cell death on both cancer cell lines ( $P < 0.05$ ).

The death of MCF-7 and MDA-MB-231 cell lines also proven by microscopic examination. Morphological changes that were unique to cancer cell lines such as cell shrinking, cell rounding, cell detaching, and cell lysis were displayed when cells were treated with Simvastatin at a dose of  $\text{IC}_{50}$  until dose of 50  $\mu\text{M}$  and combination drug on MCF-7 (Table 3, Figure 4, Figure 5) and MDA-MB-231 (Table 4, Figure 6, Figure 7). Microscopic images also showed an evidence of cell death, with apoptogenic cell counting values indicate significant cell death ( $P < 0.05$ ).

It has been reported (Liao, 2002) that cancer cells usually exhibit elevated levels of HMG-CoA and low-density lipid receptor to satisfy their demand for isoprenoid and lipids, suggesting them to be more sensitive than normal cells to statins. Moreover, master mevalonate pathway genes, HMGCR and HMGCS2 were also overexpressed in breast cancer tissue at the level of gene transcript and protein. Therefore, these clues strongly indicated high dependency of breast cancer on mevalonate pathway (Ponten et al, 2011; Bai et al, 2019).

The effect of Simvastatin on MCF-7 and MDA-MB-231 cells is likely due to the inhibition of the mevalonate pathway which subsequently inhibited the production of various end products, such as cholesterol, dolichol, ubiquinone, isopentenyl adenine, geranylgeranyl pyrophosphate (GGPP) and farnesyl pyrophosphate (FPP). These were important components of various cell functions such as cell proliferation, differentiation, and survival, both for normal cells and cancer cells. Thus, the administration of Simvastatin will result in cell cycle arrest, cell proliferation inhibition, and even cell death via apoptosis and necrosis (Chan et al., 2003; Moon and



Bogle, 2010; Ostad and Parsa, 2011; Wang et al., 2016; Putra et al., 2017). The process can be explained in previous studies on lung and prostate cancer cell treatment with Simvastatin (Goc et al., 2012; Wang et al., 2018).

In previous studies, it was proven that the administration of Simvastatin to cancer cells will reduce the production of GGPP and FPP and thus caused apoptosis (Goc et al., 2012; Wang et al., 2016; Putra et al., 2017), anti-proliferative effects (Feldt et al., 2015; Wang et al., 2015; Beckwitt et al., 2018), and deactivation of the signaling pathway in breast cancer cells (Wang et al., 2016; Beckwitt et al., 2018). The reduced production of GGPP and FPP will also decrease RAS translocation from cytoplasm to cell membranes, causing RAS signal transduction to decrease or even stop. This resulted in the inactivation of two important pathways in cell proliferation, the MAPK pathway, and the PI3K/Akt pathway. Statin drugs also inhibited cell proliferation by decreasing cyclin D1 expression and increasing p27 expression which can cause cell cycle arrest in G1 phase. Statins can also cause cell apoptosis through intrinsic and extrinsic signaling pathways by increasing caspase 9, 10, 8, and 3 division (Goc et al., 2012).

Simvastatin mediated effects on breast cancer cell viability and cell death was similar to the effects of doxorubicin. Our data confirmed that both simvastatin and doxorubicin significantly induced both cell death and lysed cell morphology. Doxorubicin has shown great efficacy in cancer cell killing but the emergence of drug resistance and adverse effects are major limitation for successful breast cancer treatment. The combination treatment in this study was then interpreted based on the classification of drug interactions, in which  $CI < 1$  showed synergism,  $CI = 1$  showed additive effect and  $CI > 1$  showed an antagonistic effect (Chou, 2010). The results from combination treatment in MCF-7 cancer cell lines showed a synergistic effect, unlike the effect on MDA-MB-231 cancer cell lines which showed an antagonistic effect. This could be explained by the different drug interactions that occur in these cell lines due to the different amounts of drug receptors of these cell lines, resulting in receptor competition. In MDA-MB-231, the effect of combination treatment is more antagonistic with increases of doxorubicin concentration, unlike in MCF-7. Various biological mechanism involves in cancer treatment including drug resistance. The key effect of drug resistance is the cellular drug transporter system that may involve in triple negative breast cancer MDA-MB-231 cells.

Moreover, recent study shows that simvastatin increase oxidative stress and DNA damage by upregulating NRF1 and miR-140 expression in breast cancer cells (Bai et al., 2019). The response to DNA damage has a profound effect on tumor chemosensitivity and chemoresistance. Signaling pathways have evolved to stop cell cycle following DNA damage to allow time for DNA repair. If the DNA damage is too extensive, the cells will undergo apoptosis. The relationship between cell cycle arrest and apoptosis following chemotherapy is complex and dependent on both the chemotherapy used and the molecular phenotype of the tumor cells.

Having a promising data of Simvastatin in breast cancer cells, further study is conducting to advance analyze molecular mechanism of this drug inhibiting cell proliferation and inducing cell death via apoptosis.

In conclusion, our data showed that Simvastatin may have potential anti-cancer activity in breast cancer cells, with potent cytotoxic effect resulting in cell death and may serve as chemotherapeutic agent for treatment of breast cancer, possibly in combination with chemotherapy.

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## Conflict of Interests

The authors declare that they have no conflict of interests regarding researches, authorship, and/or publication of this article. None of the authors was affiliated with the companies mentioned in this article.

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