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Cytotoxic activity of simvastatin in T47D breast cancer cell lines and its effect on cyclin D1 expression and apoptosis

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ABSTRACT

Statins is HMG-CoA inhibitors which used for decreasing plasma cholesterol levels and preventing coronary artery disease. Preclinical and clinical studies showed that statin could decrease the risk of cancer. This study was performed to evaluate the cytotoxic activity of simvastatin on T47D breast cancer cell lines and its effect in cyclin D_1 expression and apoptosis. This was quasi experiment using post test with non-equivalent control group design. Simvastatin cytotoxic activity was evaluated using MTT assay. Furthermore, the effect of simvastatin on cyclin D_1 expression and apoptosis were evaluated using flow cytometry using antibody monoclonal anti-cyclin D_1 and annexin V-Pi, respectively. The results showed that simvastatin had cytotoxic activity on T47D breast cancer cell lines with an IC_{50} value of 25.25 ± 1.61 μg/mL. Moreover, simvastatin in concentration range from 6.31 to 50.5 μg/mL decreased the cyclin D1 expression with an EC_{50} value of 18.96 ± 4.42 μg/mL and induced apoptosis with an EC_{50} value of 26.96 ± 6.05 μg/mL. In conclusion, simvastatin inhibits T47D breast cancer cell growth through reduction of cyclin D_1 expression and induction of apoptosis.

ABSTRAK

Statin (HMG-CoA *inhibitor*) merupakan golongan obat yang digunakan untuk menurunkan kadar kolesterol plasma dan untuk mencegah jantung koroner. Penelitian praklinik dan klinik menunjukkan bahwa terapi statin dapat menurunkan risiko terjadinya kanker. Penelitian ini bertujuan mengkaji aktivitas sitotoksik simvastatin terhadap kultur sel kanker payudara T47D dan efeknya terhadap ekspresi *cyclin* D_1 dan apoptosis. Jenis penelitian ini adalah eksperimenal semu dengan rancangan *post test with non equivalent control group.* Aktivitas sitotoksi simvastatin diuji dengan metode MTT *assay*. Selanjutnya efek simvastatin terhadap ekspresi *cyclin* D_1 dan apoptosis dikaji menggunakan *flowcytometry* menggunakan *antibody monoclonal anti-cyclin* D_1 dan annexin V-Pi. Hasil penelitian menunjukkan simvastatin mempunyai aktivitas sitotoksik terhadap sel kanker payudara T47D dengan nilai IC_{50} sebesar 25,25 ± 1,61 μg/mL. Simvastatin dengan kisaran konsentrasi 6,31 sampai 50,5 μg/mL mampu menurunkan ekspresi *cyclin* D_1 dengan nilai EC_{50} sebesar 18,96 ± 4,42 μg/mL dan menginduksi apoptosis dengan nilai EC_{50} sebesar 26,96 ± 6,05 μg/mL. Sebagai kesimpulan, simvastatin menghambat pertumbuhan sel kanker payudara T47D dengan menurunkan ekspresi *cyclin* D_1 dan menginduksi apoptosis.

Keywords: simvastatin - cytotoxic activity - cyclin D_1 - apoptosis activity - T47D breast cancer cell lines

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INTRODUCTION

Cancer can be defined as the rapid growth of body cells cause disorders thus growth beyond the limits of necessity and then invade and spread to other parts of the body. Breast cancer is one of the most common cancers that cause death in women in the world. In 2013 Ministry of Health, Republic of Indonesia reported that the prevalence of breast cancer was the second highest in Indonesia, approximately 0.5% of total population, with the highest prevalence in Yogyakarta Special Region (2.4%).[1]

Statin (HMG-CoA Inhibitors) is drugs used for decreasing cholesterol level and prevent heart coroner disease.[2] In Indonesia, 82.3% patients with hypercholesterolemia still use statin as first choice drug with the most frequently used is simvastatin (42.8%) followed by rosuvastatin (27.9%) and atorvastatin (19.2%).[3] Statin use is associated with 20% risk reduction of cancer. Statin have protective effect on cancer survivors therapy for more than 4 years.[4] Cohort study found that using simvastatin in patients diagnosed with breast cancer, could reduce the death rate of breast cancer.[5]

Furthermore, the statins such as fluvastatin, atorvastatin and simvastatin can inhibit cell proliferation associated with decreasing synthesis of DNA and cell cycle arrest in G_1 and G_2/M by increasing the expression of p53 and p21 proteins and induces cell death with oxidative stress.[6,7] Simvastatin could also induce antiproliferative effects and increase expression of caspase 3 in order to stimulate apoptosis.[8,9] This study was conducted to prove the cytotoxicity of simvastatin against T47D breast cancer cell lines and its effect on cyclin D_1 expression and apoptosis.

MATERIALS AND METHODS

Materials

Simvastatin, 4-(2-hydrocyethyl)-piperazine-ethane) sulphonic acid (HEPES), RPMI 1640, fetal bovine serum (FBS) 10%, amphotericin B, penicillin-streptomycin, and trypsin were obtained from Sigma-Aldrich®. The T47D breast cancer cell lines was obtained from the Laboratory of Parasitology, Faculty of Medicine, Universitas Gadjah Mada, Yogyakarta. Other materials were doxorubicin (Ebewe), sodium dodecyl sulfate (SDS) (Gibco®), sodium bicarbonate (Nacalai Tesque), phosphate buffer saline (PBS) (Invitrogen®), MTT [3-(4,5-dimethylthiazol-2-yl)-2.5-diphenyltetrazolium bromide] (Bio Basic Inc.®), HCl (Merck®), cyclin D1-PE (A-12) monoclonal antibody (Santa cruz biotechnology, INC), and annexin V fluos staining kit (Roche®).

Cytotoxic activity assay

The T47D breast cancer cell lines at 1 x 10^5/mL were plated in 96 well microplate with 100 µL each well and incubated for 24 hours in starvated media. One hundred µL of simvastatin solution in concentration 50; 25; 12.5; 6.25; 3.125; and 1.5625 µg/mL were added in triplicate and then incubated for 24 hours. The media in each well was changed with 100 µL new media and 10 µL MTT reagent was added in each well and then incubated for 4-6 hours in a CO_2 incubator at 37°C. Followed after incubation, 100 µL SDS in HCl 10% was added in each well and incubated at room temperature for 12 hours. The absorbance of each well was recorded using ELISA reader at λ of 595 nm. The absorbance of each well was used to calculate the percentages of proliferation inhibition by comparing with the control cells without any compounds. The concentration inhibiting

50% (IC_{50}) of the cell lines was determined by probit analysis.

Expression of cyclin D_1 assay

The T47D breast cancer cell lines were cultured in 6 well plates at $5x10^5$ cells per well. Each well was added with 2000 µL of each concentration series of simvastatin 0; 6.31; 12.62; 25.25 and 50.48 µg/mL, and 400 µL of flowcytometry reagent. Cells suspension was transferred into the flowcytometry-tube through filter (nylon/glass cloth fabrics) using 1 mL micropipette. The profile of cyclin D_1 expression was measured with flowcytometer and analyzed using FASC-Calibur program. Data were displayed in percentage of cyclin D_1 expression in each treatment groups. The effect on cyclin D_1 expression was assessed on the concentration inhibiting 50% expression (EC_{50}) which determined by probit analysis.

Apoptosis induction assay

The T47D breast cancer cell lines were cultured at $5x10^5$ in 6 well plates. Each well was added with 2000 µL from each concentration series of simvastatin 0; 6.31; 12.62; 25.25 and 50.48 µg/mL and doxorubicin 0.15 µg/mL. Solutions were prepared using incubation buffer 10 mM Hepes/NaOH pH 7.4; 140 mM NaCl and 5 mM CaCl2. Labeling solution for sample consisting of 15 to 30 µL of annexin V plus 1.5 mL of incubation buffer and 30 µL of PI. Incubation cells were placed at the dark room for 10-15 minutes at a temperature of 15-25°C. Apoptosis was analyzed using FACS-Calibur program to obtain distribution of the survived cell, apoptotic and necrotized cell on all treatment group. The apoptosis induction activity was assessed on the concentration inducing 50% apoptosis (EC_{50}) which determined by probit analysis.

Statistical analysis

Data of IC_{50} an EC_{50} were presented as mean ± SD. Data of cyclin D1 expression and apoptosis induction were presented as percentage and compared using analysis of variance (ANOVA) followed by post hoc Bonferroni or Tamhane. A p value <0.05 was considered as statistically significant.

RESULTS

Cytotoxic activity assay

The inhibitory concentration 50% (IC_{50}) of simvastatin and doxorubicin on T47D breast cancer cell lines were 25.25±1.61 and 0.15±0.016 µg/mL, respectively (TABLE 1). These results showed that simvastatin had cytotoxic activity on T47D breast cancer cell lines, although its activity was lower than that doxorubicin as positive control ($p<0.05$).

TABLE 1. The percentage of T47D cell lines proliferation inhibition (%) and the IC_{50} (mean ± SD) of simvastatin and doxorubicin

Drug	Concentration (µg/mL)	Replication						Mean IC_{50}
		1		2		3		
		Inhibition	IC_{50}	Inhibition	IC_{50}	Inhibition	IC_{50}	
Simvastatin	50	89.45		90.44		91.39		
	25	51.28		45.73		58.52		
	12.5	21.25	27.03	20.66	24.78	20.92	23.92	25.25±1.61
	6.3	12.68		26.45		18.60		
	3.1	1.98		26.22		11.76		
	1.6	2.46		5.56		9.06		
Doxorubicin	0.25	75.71		80.53		80.15		
	0.13	38.51		69.17		56.64		
	0.06	16.19	0.17	14.67	0.14	5.14	0.15	0.15±0.016
	0.03	2.46		2.74		-6.25		
	0.02	-10.84		-9.49		-9.02		

Expression of cyclin D_1 assay

The cyclin D_1 expression of T47D breast cancer cell line after incubation with simvastatin is presented in FIGURE 1. At the left side showed population of cells that did not express cyclin D_1, and the right side showed population of cells that expressed cyclin D_1.

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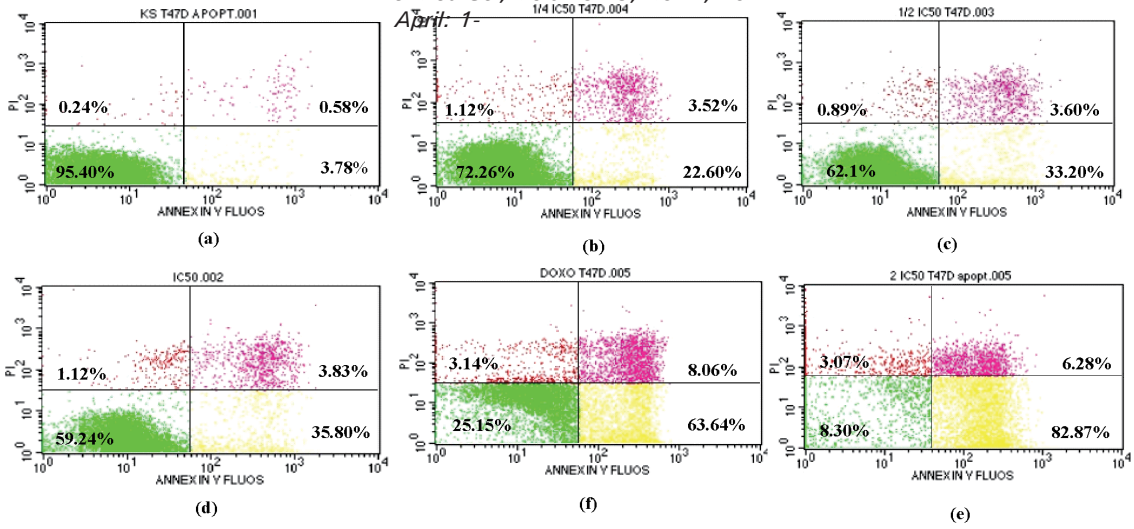

FIGURE 1. Cyclin D1 expression measured with flowcytometry in (a) control group without simvastatin, (b) simvastatin 6.31 µg/mL, (c) simvastatin 12.62 µg/mL, (d) simvastatin 25.25 µg/mL (e) simvastatin 50.48 µg/mL.

TABLE 2. Cyclin D_1 expression of T47D cells (%) after incubation with simvastatin for 24 hours.

Treatment	Concentration (µg/mL)	Cyclin D_1 expression (Mean ± SD %)	p	EC_{50} (µg/mL)
Control	0	65.89±0.73		
Simvastatin	6.31	59.98±1.81[b]		18.96±4.42
	12.62	54.97±3.42[b]	0.000*	
	25.25	45.01±3.96[ab]		
	50.48	25.23±13.72[a]		

*:ANOVA; a: p<0.05, ANOVA followed by post hoc Bonferroni, compared with control;
b: p<0.05, ANOVA followed by post hoc Bonferroni, compared with simvastatin 50.48 µg/mL.

Apoptosis induction assay

The apoptosis of T47D breast cancer cell line after incubation with simvastatin is presented in FIGURE 2. Lower left showed live cells population, lower right showed apoptotic cells, and upper right showed necrotic cells.

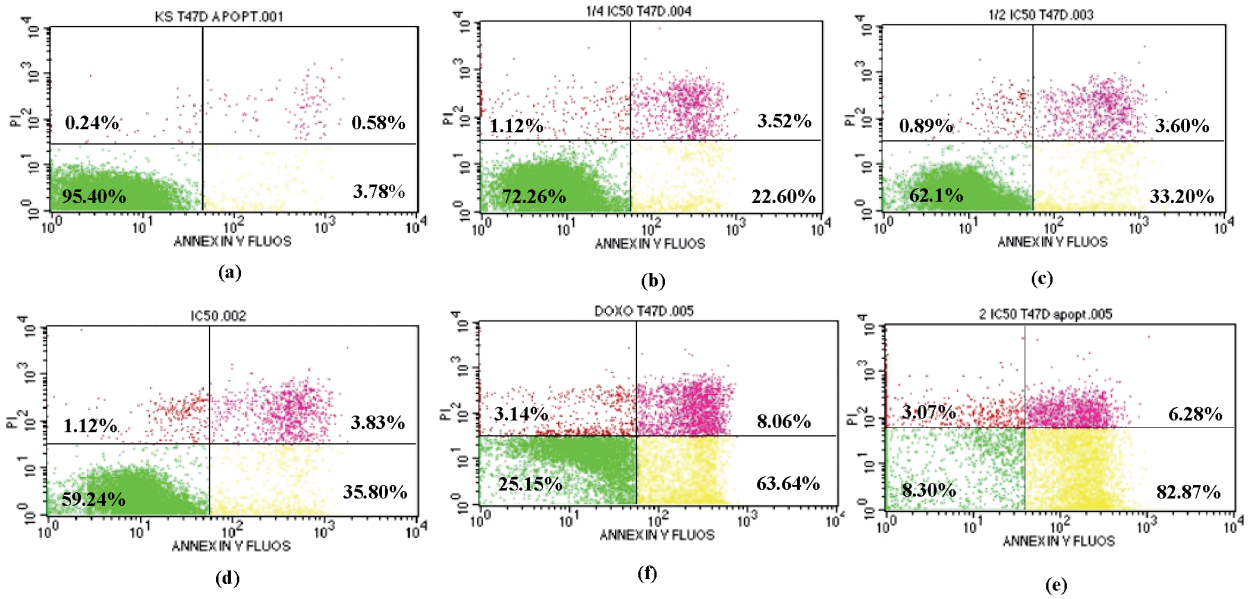

FIGURE 2. Apoptosis induction measured with flowcytometry after 24 hours incubation in (a) control group without simvastatin, (b) simvastatin 6.31 µg/mL, (c) simvastatin 12.62 µg/mL, (d) simvastatin 25.25 µg/mL, (e) simvastatin 50.48 µg/mL, and (f) doxorubicin 0.15 µg/mL as positive control. Lower left: live cells population, lower right: apoptotic cells, upper right: necrotic cells

TABLE 3 shows the T47D breast cancer cells apoptosis after incubation with simvastatin for 24 hours. The simvastatin IC_{50} value (25.25 µg/mL) obtained from the cytotoxic activity assay was also used as one of the tested concentration for apoptosis

assay. Simvastatin increased cells apoptosis in a concentration-dependent manner with the EC_{50} value of 26.96 ± 6.05 µg/mL. This finding indicated that incubation with simvastatin induces apoptosis in T47D breast cancer cells.

TABLE 3. Mean of apoptotic cell of T47D cells (%) after giving simvastatin or doxorubicin for 24 hours

Treatment	Concentration (µg/mL)	Mean of apoptotic T47D cells (%) ± SD	p	EC_{50} (µg/mL)
Control	0	4.82±0.92		
Simvastatin	6.31	20.87±4.55		26.96± 6.05
	12.62	19.60±11.79	0.000*	
	25.25	31.60±8.62		
	50.48	82.39±0.54[ab]		
Doxorubicin	0.15	66.94±2.97[ab]		

*: ANOVA Test; a: p<0.05, ANOVA continued post hoc Tamhane, compared with control; b: p<0.05, ANOVA continued post hoc Tamhane, compared with simvastatin 6.31 µg/mL.

DISCUSSION

Cytotoxic activity of simvastatin

The T47D breast cancer cell lines was used in this study due to its high homogeneity and its simplicity replaceable with frozen stock contamination observed. Therefore these cells are often used for *in vitro* cancer studies.[10] The results showed that incubation with simvastatin at concentrations from 50 to 1.5625 µg/mL (119.45-3.73 µM) for 24 hours inhibited T47D cell lines proliferation. This finding was consistent with other *in vitro* studies conducted by some authors. Lee *et al.*[11] showed that incubation with simvastatin at concentrations from 0 to 500 µM (0-209.3 µg/mL) for 24 and 48 hours inhibited the bile duct cancer cells proliferation. The incubation with simvastatin at concentration from 15 to 120 µM (6.27 to 50.22 µg/mL) for 24 and 28 also inhibited lung cancer A549 cells proliferation.[12,13] Furthermore, Huang *et al.*[14] reported that incubation with simvastatin at concentration from 2 to 16 µM for 24-72 hours inhibited the HepG2 and Huh7 cell line growth. The proliferation inhibition of ECC-1 and Ishikawa cell lines were also observed after incubation with simvastatin at concentration from 0.01 to 50 µM (0.0041 to 20.92 µg/mL).[15]

According to American National Cancer Institute (NCI), a compound is considered to have cytotoxic effect on cancer cell lines if its IC_{50} lower than 50 µg/mL.[16] Furthermore, a compound could be classified as a very active, active and moderate potential anticancer compound if they have IC_{50} value < 5, 5-10 and 11-30 µg/mL, respectively.[17] Based on this criteria, simvastatin with an IC_{50} value of 25.25±1.61 µg/mL could be classified as a moderate potential anticancer compound. The IC_{50} of statin group on several cancer cell lines in different incubation period has been reported in the previous studies. The IC_{50} of simvastatin on MDM-231, SKBr3 and MCF7 cancerous cell varied from 1.26 to 91 µM,[18] whereas on A549 cancerous cells was 45 µM and on HeLa cell line was 9.14 µM.[12,19] Other statin drug, fluvastatin also showed cytotoxic effect against C6 glioma

cancer cells.[18] In addition, atorvastatin was active against NCI-H292 cancer cells with an IC_{50} value of 5.54 µg/mL[20] and lovastatin was active against MDAMB468 and MDAMB231 cancer cells with IC_{50} values of 8 µg/mL and 5 µg/mL, respectively.[21]

Effect of simvastatin on cyclin D_1 expression

Simvastatin decreased cyclin D_1 expression of T47D breast cancer cell lines in a concentration-dependent manner (TABLE 2). This finding was similar with study conducted by Liang *et al.* [22] which showed that simvastatin at concentration range from 12.5 to 50 µM (5.23 to 20.92 µg/mL) induced cell cycle arrest of NCI-H460 cancer cells through reduction of cyclin D_1 and CDK4 expression and enhancement of P21 inhibitors CKD expression. Simvastatin also decreased cyclin D_1 expression and CDK on hepatocellular cancer cell lines (Hep3B and Huh-7).[23] Another study reported that simvastatin induced apoptosis in the HepG2 and Huh7 cancer cell lines and its activity was accompanied by inhibition of CDK and cyclin D_1, whereas CDK inhibitors p19 and p27 were enhanced.[24] Other statins such as lovastatin also could affect the cell cycle by decreased cyclin D1-CDK 4 expression and increased $p21^{WAF1/CIP1}$ expression on MCF-7 cells.[2]

Apoptosis induction activity of simvastatin

Simvastatin increased cells apoptosis in a concentration-dependent manner. This finding indicated that simvastatin induced apoptosis in T47D breast cancer cell lines. This finding similar with previous studies conducted by some authors. Gopalan *et al*.[25] demonstrated that incubation with simvastatin at concentration range from 0.625-5.0 µM for three days induced apoptosis of MCF7 and MDA-MB-231 cancer cell line via activation of JNK/CHOP/DR5 signaling pathway. Ghosh-Choudury *et al*.[26] also demonstrated that simvastatin attenuated the antiapoptotic Bcl_{XL} expression and induced depression of phosphatase and tensin homologous (PTEN) expression through NFκB to inhibit breast cancer growth. In addition, Koyuturk *et al.*[27] reported that simvastatin induced apoptosis through involvement of JNK in breast cancer cells independent of their ER or p53 expression status. Simvastatin also suppressed PI3K/Akt/mTOR pathway by enhancing PTEN expression and by further sequentially dephosphorylating downstream cascades including Akt, mTOR, p70S6K, S6RP and 4E-BP1. Furthermore, simvastatin inhibited MAPK/ERK pathway by dephosphorylating sequential cascades such as c-Raf, MEK1/2 and ERK1/2.[28]

CONCLUSION

Simvastatin shows cytotoxic activity on T47D breast cancer cell lines with an IC_{50} value of 25.25 µg/mL. Furthermore, simvastatin decrease cyclin D1 expression with an EC_{50} value of 18.96 µg/mL, and induce apoptosis with an EC_{50} value of 26.96 µg/mL.

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