The role of simvastatin in inhibiting migration and proliferation of breast cancer cells through Rho/ROCK signaling pathway

Erwin Danil Yulian1*, Muchlis Ramli1, Rianto Setiabudy2, Nurjati Chairani Siregar3, Arleni Bustami4 and Ricky Dosan4

Introduction
Breast cancer is the most common cancer in women and metastasis is responsible for 90% of breast cancer deaths. The poor prognosis associated with breast cancer is mainly related to bone and visceral metastasis, the major causes of tumor recurrence in breast cancer. The invasion and migration of cancer cells into the...
lymphatic or blood vessels facilitates secondary growth in distant organs. Cell migration is vital for the several steps involved in cancer metastasis such as invasion, intravasation, and extravasation. There is an urgent need for the development of therapeutic intervention specifically targeted to the metastatic process. The Rho/ROCK pathway is found to be involved in invasion and metastasis. Recent studies have revealed that the Rho/ROCK pathway plays a critical role in regulation of cancer cell migration and proliferation, making it a potential therapy target. Therefore, a therapy focused on preventing metastasis is needed [1].

Migration are hallmark characteristics of the metastasis of cancer cells [2,3]. Tumor cell migration and invasion play fundamental roles in cancer metastasis. The Rho family of small GTPases (Rho, Rac, and Cdc42) play well-characterized roles in the regulation of actin cytoskeleton organization and dynamics [4] furthermore, Rho GTPases act as important regulators of cellular homeostasis [5,7].

The Rho/ROCK pathway regulates the intracellular cascades related to cytoskeleton reorganization, cell motility, survival, and proliferation [8]. Rho, one of the RhoGTPase family, plays important role in formation of stress fiber, focal adhesion, and actomyosin contraction from the rear. Rho is differentiated into RhoA, RhoB, and RhoC. In breast cancer, RhoA strongly correlated with proliferation, and RhoC is strongly correlated with migration. High expression of RhoC strongly correlated with progression and metastasis of cancer. The Rho-associated (ROCK) serine/threonine kinases have emerged as central regulators of the actomyosin cytoskeleton, their main purpose being to promote contractile force generation. Amoeboid type cell migration which can be found in both neoplastic or non neoplastic cells is also induced by the Rho/ROCK pathway. CD44, major receptor for hyaluronic acid in solid tumors, activate RhoGTPase, Ras-MAPK and PI3K/AKT [8]. CXCR4 and its ligand, SDF-1, activate the Rho/ROCK pathway and control breast cancer homing [9,10] Recent studies have revealed that the Rho/Rho-associated protein kinases (ROCK) pathway plays a critical role in the regulation of cancer cell motility and invasion. ROCK signaling plays crucial roles in a range of human diseases and is now considered as a potential target for the treatment of several diseases, including diabetic nephropathy [11] as well as diseases of the central nervous and the cardiovascular system [12-14].

In addition, the Rho/ROCK pathway plays important roles in invasion and metastasis on the basis of its predominant function of cell cytoskeletal regulation in breast cancer. CXCR4 and its ligand, SDF-1, activate the Rho/ROCK pathway and control breast cancer homing [9,10].

According to the current understanding of tumor migration, there are two modes of tumor cell movement: mesenchymal and amoeboid. In addition, cancer cell movement can be interchangeable between the mesenchymal and amoeboid movements under certain conditions. Control of cell migration through the actin cytoskeleton creates the potential for regulating tumor cell metastasis. The isoprenoids demonstrate tumor-suppressive properties as regulators of important hallmarks of cancer, such as proliferation, migration, and angiogenesis [15].

Clinical studies showed that statin, in addition to its lipid lowering effect, also thought to inhibit the Rho/ROCK through its pleiotropic effect [16-18]. Statins are believed to work through suppressing HMGCR, thus reducing the level of mevalonate and isoprenoid intermediates (farnesyl pyrophosphate and geranylgeranyl pyrophosphate) [19]. The isoprenoids are needed by Rho for posttranslational prenilations. Simvastatin is the most commonly used statin in clinical practice. It is also known for its affordability and safety for long-term use in hypercholesterolemic patients.

Cholesterol is a structural component of the cell membrane, specially localized in lipid rafts-membrane microdomains that assemble the signal transduction machinery and associate to proteins implicated in key cellular signaling pathways. Many of these pathways closely associate with malignant transformation due to their influence in organization of the c toskeleton, cell polarity and angiogenesis [20,21]. Recently, hypercholesterolemia, an established comorbidity of obesity, has been idetified as an independent risk factor for breast cancer in postmenopausal women [4-6].

Cholesterol is implicated in cancer metabolism, both as energy source and as building blocks for lipid rafts formation in cell membrane that harbors oncogenic receptors. Aside from lowering cholesterol, statins may inhibit metastasis which accounts for more than 90% of breast cancer mortality. Statins inhibit cancer migration by disrupting Rho/ROCK signalling that lead to inhibition of intracellular cascades controlling cytoskeleton reorganization and actin-myosin contraction [16,18].

Besides reducing cholesterol biosynthesis by inhibiting 3-hydroxy-3-methylglutaryl coenzyme A reductase, statins also decrease the formation of isoprenoids intermediates essential for mediating the Rho/ROCK signalling. Statin is thought to inhibit the Rho/ROCK pathway and is safe for long-term use. Also statins are relatively cheap and can contribute to decrease the high cost of cancer treatment.

To date, there are no studies focusing on the effects of simvastatin in preventing metastasis in human breast cancer. This study aimed to determine the effect of 40 mg/day simvastatin taken orally for four to six weeks on preventing breast cancer metastasis especially in migration and proliferation focussed on the Rho/ROCK pathway.

Ethics, consent and permission
The protocol of this study has been approved by Medical Ethics Committee of Universitas Indonesia (No. 717/UN2.F1/ETIK/2014). Informed consents were obtained from the patients before initiation of this trial.

Methods
This research was an interventional perioperative “window” trial, parallel unmatching, randomized, double-blinded, and placebo-controlled study, conducted from November 2014 to July 2015. This study took place in Jakarta, involving Cipto...
Mangunkusumo Hospital, Koja Regional Hospital, Gatot Subroto Army Hospital and Persahabatan Hospital.

Thirty breast cancer patients were randomized evenly to simvastatin 40 mg/day and placebo group for 4-6 weeks followed by surgery. From cell cultures, fresh tissue, and immunohistochemistry specimens, we collected data on migration index, ROCK activity, RhoC mRNA expression, CXCR4 mRNA expression, CD44 mRNA expression, and Ki67 expression. Samples were collected twice for each subject; the first was from biopsy before the start of simvastatin treatment, while the second was taken when the subjects underwent mastectomy. Cell migration index was measured with Boyden chamber assay on primary culture of the breast cancer tissues using three different mediums, which were Dulbecco's modified Eagle's Medium (DMEM) medium, DMEM and Fetal bovine serum (FBS) medium, and DMEM and Stromal-derived factor-1 (SDF-1) medium. The expression of RhoC, CD44, and CXCR4 mRNA were assessed by Real time quantitative polymerase chain reaction (RT-qPCR) on fresh frozen breast cancer tissue specimens. The ROCK activity was evaluated by Enzyme Linked Immuno Assay (ELISA) on fresh frozen breast cancer tissue specimens. The expression of Ki67 was determined using immunohistochemistry during pathological examination of the breast cancer tissues.

Changes from data obtained from biopsy and mastectomy specimens were compared statistically. Relationships of significant factors with tumor grade, ER/PR status, and HER-2 status were analyzed. Saphiro Wilk test is used for normality test, if p>0.05 the data distribution is normal and the data will be present in “mean±deviation standard”, if not then the data distribution is not normal and the data will be present in “median (minimum–maximum)”. Unpaired T-Test or Mann-Whitney test is used for analitical test for comparing simvastatin and placebo group depends on the normality test.

Results
A total of thirty female breast cancer patients participated in this study. Each of the simvastatin and placebo group had 15 participants. 53.33% of the subjects had hypercholesterolemia, 60% had stage II breast cancer, 50% had high tumor grade, 60% had HER-2 amplification, and 63.33% were ER/PR positive (Table 1).

### Effects of simvastatin on cell migration index

We assessed the pre and post therapy migration index including the delta between pre and post therapy on simvastatin and placebo group. By comparing the simvastatin and placebo group, we found significant decline in delta of cell migration index on DMEM medium (p=0.031) (Table 2) and DMEM with FBS medium (p=0.006) (Table 3), but not on DMEM with SDF-1 medium (p=0.095) (Table 4).

### Effects of simvastatin on RhoC mRNA expression

We assessed the pre and post therapy RhoC mRNA expression including the delta between pre and post therapy on simvastatin and placebo group. Significant difference in declines between the simvastatin and placebo group of RhoC mRNA expression was not found (p=0.163) (Table 5). In the simvastatin group, a visual trend of declined RhoC mRNA expression after simvastatin therapy was observed.

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**Table 1. Subject characteristics.**

<table>
<thead>
<tr>
<th></th>
<th>Simvastatin (n=15)</th>
<th>Placebo (n=15)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age (years)</strong></td>
<td>51 (30–64)**</td>
<td>49 (37–65)**</td>
</tr>
<tr>
<td><strong>Menopause</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>Post</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td><strong>Body Mass Index (kg/m²)</strong></td>
<td>24.7 (15.5–30.5)**</td>
<td>26.2 (21.09–34.9)**</td>
</tr>
<tr>
<td><strong>Total Blood Cholesterol (mg/dL)</strong></td>
<td>204.4 (138–282)**</td>
<td>214 (144–327)**</td>
</tr>
<tr>
<td><strong>Stage</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IIA</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td>IIB</td>
<td>9</td>
<td>8</td>
</tr>
<tr>
<td>IIA</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td><strong>Grade</strong></td>
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<td></td>
</tr>
<tr>
<td>Low</td>
<td>8</td>
<td>7</td>
</tr>
<tr>
<td>High</td>
<td>7</td>
<td>8</td>
</tr>
<tr>
<td><strong>Tumor Type</strong></td>
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<td></td>
</tr>
<tr>
<td>Ductal</td>
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<td>9</td>
</tr>
<tr>
<td>Lobular</td>
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<td>2</td>
</tr>
<tr>
<td>Mixed</td>
<td>9</td>
<td>4</td>
</tr>
<tr>
<td><strong>Length of Therapy (day)</strong></td>
<td>32 (28–39)**</td>
<td>32 (28–47)**</td>
</tr>
<tr>
<td><strong>ER/PR</strong></td>
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<td></td>
</tr>
<tr>
<td>Positive</td>
<td>10</td>
<td>8</td>
</tr>
<tr>
<td>Negative</td>
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<td>7</td>
</tr>
<tr>
<td><strong>HER-2</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Amplification</td>
<td>9</td>
<td>6</td>
</tr>
<tr>
<td>No amplification</td>
<td>6</td>
<td>9</td>
</tr>
<tr>
<td><strong>Ki67(%)</strong></td>
<td>75(30–90)**</td>
<td>30(15–40)**</td>
</tr>
<tr>
<td>Migration Index</td>
<td>1.44±0.31*</td>
<td>0.953±0.23*</td>
</tr>
<tr>
<td>(absorbance level)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RhoC (ng/µL)</td>
<td>0.15(0.05–2.33)**</td>
<td>0.14(0.08–1.48)**</td>
</tr>
<tr>
<td>CD44 (ng/µL)</td>
<td>6.54(0.97–99.55)**</td>
<td>14.31(0.5–293.32)**</td>
</tr>
<tr>
<td>CXCR4 (ng/µL)</td>
<td>12.89(0.46–81.83)**</td>
<td>14.94(1.35–30.06)**</td>
</tr>
<tr>
<td>ROCK (ng/µL)</td>
<td>0.963±0.608*</td>
<td>0.6235±0.275*</td>
</tr>
</tbody>
</table>

*Mean±SD
**Median (minimum–maximum)

**Table 2. Pre and post therapy differences in migration index in DMEM medium.**

<table>
<thead>
<tr>
<th></th>
<th>Simvastatin (n=3)</th>
<th>Placebo (n=3)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre</td>
<td>0.82 (0.74–0.87)</td>
<td>0.98 (0.53–0.99)</td>
<td>--</td>
</tr>
<tr>
<td>Post</td>
<td>0.44 (0.34–0.54)</td>
<td>1.24 (0.83–1.31)</td>
<td>--</td>
</tr>
<tr>
<td>Delta</td>
<td>0.31 (0.28–0.33)</td>
<td>-0.32 ((-0.71)–0.15)</td>
<td>0.031*</td>
</tr>
</tbody>
</table>

*Mann Whitney; *p<0.05
Effects of simvastatin on ROCK activity

We assessed the pre and post therapy ROCK activity including the delta between pre and post therapy on simvastatin and placebo group. Significant difference in declines of ROCK activity between the simvastatin and placebo group was found (p=0.002) (Table 6). The decrease in ROCK activity was related to high cholesterol (p=0.008), low tumor grade (p=0.019) and HER-2 amplification (p=0.009).

Effects of simvastatin on CXCR4 mRNA expression

We assessed the pre and post therapy CXCR4 mRNA expression including the delta between pre and post therapy on simvastatin and placebo group. Significant difference in declines of CXCR4 mRNA expression between the simvastatin and placebo group was found (p=0.045) (Table 7). The decrease of CXCR4 mRNA expression was related to high cholesterol (p=0.024), positive ER/PR (p=0.013), and HER-2 amplification (p=0.018).

Discussion

Effects of simvastatin on cell migration index

We only use 6 samples because we found that it's not easy to culture cells from primary tumor. Primary breast cancer cell tends to be heterogen and the cell cycle will stop after the G0 phase. We also use early stage cancer which will need a long time to assess the migration index using boyden chamber assay. At least 2 month's were needed for cancer cells to grow until can be assessed using the boyden chamber assay from mastectomy's breast cancer tissue. Longer time will be needed for biopsy's breast cancer tissue [22].
The result of this study showed significant difference in declines on cell migration index on DMEM medium and DMEM with FBS medium. This result supported the inhibitory effect of simvastatin towards breast cancer cell migration through the Rho/ROCK pathway. This result was supported by Mandall’s [18] and Denoyel’s [23] findings that found inhibition on cell migration on MDA-MB-231 cell line. Kidera [19] and Zanfardino [24] found that simvastatin inhibited melanoma cell migration on xenograft through Rho/ROCK axis. Schramm [25] found that in vivo simvastatin 5mg/kg therapy in mice halted leukocyte migration. Wu [26], Gliemroth [27], and Zohrabain [28] found that simvastatin inhibited glioma migration, especially on high concentrations.

This study found no significant difference in declines on cell migration index on DMEM with SDF-1 medium, though there was a trend of decreased cell migration index in the simvastatin group. This result indicates that 40 mg of simvastatin daily is not adequate to inhibit migration through the CXCR4/SDF-1 pathway. This inadequacy could be caused by cancer behavior, insufficient simvastatin dose and length of therapy. In contrast, Masori [29] found that simvastatin inhibited CXCR4 expression in monocyte cell line and endothelial cell SDF-1 production. Sameermahmood [30] found that the increase and decrease of cell migration through SDF-1 is dependent on simvastatin dose. Aside from CXCR4, SDF-1 also binds with CXCR7 receptor in modulating metastasis. Liu [31] found that CXCR7 was inhibited significantly by atorvastatin, but other statins donot exhibit similar effects. Drugs that suppress CXCR7 don’t necessarily affect CXCR4, and vice versa.

**Effects of simvastatin on RhoC mRNA expression**

This study did not find significant difference in declines of RhoC mRNA expression between the simvastatin and placebo group, although there was a trend of declined RhoC mRNA expression in the simvastatin group. Matuszewicz [32] stated that different effects of drugs in different studies could be caused by differences in cancer types, statin types, drug dosages, and duration of drug administration. There is a chance that longer duration of therapy could have resulted in a significant result. Prolonging simvastatin treatment in this study’s subject would delay their surgery plans, and therefore avoided for both ethical and safety concerns.

This result differs from that of Collison’s [33], which found that atorvastatin, another lipidophilic statin, decreased RhoC expression of melanoma cells in a dose-dependent manner. Another form of Rho GTPase, RhoA, was found to promote tumor growth more than RhoC, while RhoC promotes metastasis more than RhoA. This study’s subjects were breast cancer patients without metastasis, therefore their expression of RhoC might be less prominent than their RhoA expression.

**Effects of simvastatin on ROCK activity**

We only use 26 samples because 4 of our samples for the ROCK assay were damaged. We have tried to run the ELISA and found that the result unacceptable.

There was a significant difference in ROCK activity declines between the simvastatin and placebo group. Liu [34] observed similar inhibitory effects of simvastatin in leukocyte ROCK activity after four weeks of simvastatin administration. Liu’s result stayed significant even after controlling cholesterol levels, suggesting that this effect was independent from statin’s effects on cholesterol. Nohria [35] also found that 80mg/day of atorvastatin inhibited Rho/ROCK pathway on leukocyte.

Mean reduction of ROCK activity was greater on subjects with hypercholesterolemia, low grade tumor, and HER-2 positive expression. Hsu [36] found that ROCK activity was higher on breast cancer with negative hormonal receptor and HER-2 amplification, the latter of which supported this study’s findings. On the other hand, Lane [37] found that higher grade breast cancers exhibited higher ROCK activity, which did not correlate with this study’s finding.

**Effects of simvastatin on CXCR4 mRNA expression**

This study found a significant difference of CXCR4 mRNA expression reduction between the simvastatin and placebo group. This finding is supported by Masori’s [29] study, which found that 10 µm of simvastatin in vitro decreased CXCR4 expression significantly. Kucia [9] also reported that statin inhibited the CXCR4/SDF-1 axis through lipid raft disruption. CXCR4 mRNA expression was reduced significantly in the subgroups with hypercholesterolemia, positive hormone receptors, and positive ER/PR. Boudot [38] reported that CXCR4 expression was higher in tumor with higher grade, which was not reflected in this study’s findings.

**Effects of simvastatin on CD44 mRNA expression**

This study did not find a significant CD44 mRNA expression difference between the simvastatin and placebo group. Mandall [18], in opposite, found an in vivo decrease in mRNA CD44 expression in mice and in vitro in the MDA-MB231 cell line after simvastatin treatment. This study was conducted using samples from human breast cancer cells. The heterogeneity of human breast cancer cells are hard to compare with specimens derived from homogenous cell lines or xenografts. Sokalska [39] found that simvastatin reduced mRNA CD44 expression in endometriosis patients. Aside from cell line and xenograft use, the different results could be caused by different usage of cancer subtypes, the ethicity of patients, statin subtypes, drug dosages, and study duration.

**Effects of simvastatin on Ki67 expression**

This study found significant difference of Ki67 expression declines between the simvastatin and placebo group. Gliemroth [27] found similar decrease of proliferation resulting from simvastatin treatment in human glioma GaMG and U87Mg cell lines. Wu [26] also observed similar inhibition of proliferation in U251 and U87 cell lines. Bjarnadottir [40], working with breast cancer, found that high doses of atorvastatin (80mg/day) reduced...
Ki67 expression, although it was not statistically significant. The result was significant in the subgroup of subjects with positive HMGCR expression.

This study found that simvastatin was decreased in subjects with hypercholesterolemia, both low and high tumor grade, both with or without HER-2 amplification, and both negative and positive hormone receptors expression. Mueck [41] also found that lipophilic statins, including simvastatin, inhibited in vitro breast cancer proliferation both in ER positive breast cancers and ER negative breast cancers. Garwood [42] reported better inhibitory effects of fluvastatin in higher grade tumors and ER negative tumors. Vinayak [43] observed that lipophilic statins had better efficacy in ER/PR negative tumors. Kumar [44] also supported this finding that statin worked better in ER negative phenotypes of breast cancers, which was their base for suggesting statin use as adjuvant of anti-estrogen therapy (tamoxifen and aromatase inhibitors).

Safety considerations
Simvastatin's known side effects are generally mild, including reversible muscle aches, diarrhea, myalgia, asthenia, constipation, myopathy, and flatulence. Rarer side effects range from joint pain, memory loss, muscle cramps, and rhabdomyolysis [19,45,46]. In this study, 4 patients reported side effects from the simvastatin group and 3 from the placebo group, all with a grade 1 severity. There was one report of memory loss, though it was transient and was not present any more at the last follow up.

There was concern of the effects of statin on subjects with normcholesterolemia. This study found that there was a significant decrease in cholesterol level in subjects with prior normal blood cholesterol level. This finding warrants further considerations for prescribing statins for this group of breast cancer patients.

A comparison of side effect reports from the simvastatin and placebo group revealed no significant differences aside from a reduction of blood cholesterol levels of the simvastatin group. Inferring from this study's data, it was concluded that administration of 40 mg simvastatin daily for 4-6 weeks has a tolerable margin of safety.

Conclusion
In conclusion, administration of 40 mg oral simvastatin daily for four to six weeks inhibits migration and proliferation in operable stage breast cancer. This study suggests the use of simvastatin as an adjuvant agent for breast cancer patients. Subgroups of breast cancer patients who should reap the most benefits from simvastatin treatment are the ones with hypercholesterolemia, HER-2 amplification, low grade tumor, and positive hormone receptors expression.

Competing interests
The authors declare that they have no competing interests.

Authors' contributions
The author's responsibility were as follows. Erwin Danil Yulian conducted the research and had primary responsibility for the content of the manuscript. Erwin Danil Yulian, Muchlis Ramli, Rianto Setiabudy and Nurjati Chairani Siregar designed the manuscript. Nurjati Chairani Siregar provided the laboratory testing in Anatomical Pathology Laboratory. Arleni Bustami provided the laboratory testing in Integrated Laboratory especially the Migration Assay. Erwin Danil Yulian and Ricky Dosan wrote the manuscript. Ricky Dosan analyzed the data and edited the manuscript. All authors read and approved the final manuscript.

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