The suppression of rotenone-treated human breast cancer stem cell survival using survivin inhibitor YM155 related to oxidative stress modulation

Septelia Inawati Wanandi1,*, Resda Akhra Syahrani2, Elvira Yunita3, Go Aulia4
1Department of Biochemistry and Molecular Biology, Faculty of Medicine, Universitas Indonesia
1,2Molecular Biology and Proteomics Core Facilities, IMERI Faculty of Medicine, Universitas Indonesia
3,4Master Program in Biomedical Sciences, Faculty of Medicine, Universitas Indonesia

*Corresponding Author
septelia@gmail.com, septelia@ui.ac.id

Despite the recent progress in molecular-targeted therapies, breast cancer remains the first-leading cause of cancer-death among women over the world. Nowadays, breast cancer stem cells (BCSCs) is believed to be responsible for therapy resistance and recurrence of cancer. Very recently, we have demonstrated that human BCSCs (CD24-/CD44+) could survive better than their counterpart non-BCSCs (CD24+/CD44-) when treated with rotenone, which might be due to lower ROS levels production, high expression of antioxidant manganese superoxide dismutase (MnSOD) and anti-apoptosis survivin. The aim of this study was to verify the role of survivin on the survival of human BCSCs under oxidative stress modulation by suppressing its expression using a survivin inhibitor YM155. Human BCSCs (ALDH+ cells) were firstly treated with YM155 for 24 hours prior to rotenone treatment for another 6 hours. We determined the intracellular superoxide level using dihydroethidium assay, MnSOD expression using qRT-PCR, as well as cell viability using trypan blue exclusion and acridine orange/ ethidium bromide apoptosis assay. This study found that the suppression of survivin expression using YM155 could reduce the survival of rotenone-treated BCSCs, which may be associated with the oxidative stress modulation, as shown by the increased ROS levels and decreased MnSOD expression. In conclusion, we confirm that survivin is responsible for maintaining the BCSC survival under oxidative stress modulation. Furthermore, the present study reported for the first time that YM155 could modulate the oxidative stress in BCSCs by reducing the MnSOD expression and increasing the ROS levels. Thus, we propose that YM155 treatment may overcome the BCSC resistance to oxidative stress-based anti-cancer.

Key Words: Breast cancer, Stem cell, YM155, Survivin

Introduction

The cancer stem cell (CSC) hypothesis proposes that tumors are initiated and maintained by a small fraction of cells, but the origin of these tumorigenic cells are actually not known. The stem cell theory of cancer proposes two major concepts. One theory claims that CSCs arise from mutated stem cells which then expand in such a manner that the mutation is shared by many of the descendants.1 An alternative theory proposes that transformed and differentiated cells acquire stem cell-like characteristics.2 It is also believed that CSCs may be responsible for resistance against therapeutic approaches and tumor recurrence.3

Breast cancer is the most common cancer and the second leading cause of cancer-related mortalities among women after lung cancer. Breast cancer treatments include chemotherapy, hormone therapy, as well as surgery and radiotherapy. Despite the recent progress in molecular-targeted therapies, breast cancer remains the principal cause of cancer death in women due to high incidence of recurrence.4 Breast CSCs (BCSCs) are heterogeneous and could be identified based on the presence of several surface antigen markers, such as CD44+, CD24−, ESA+, and CD133+.5,6 As well as other CSCs, BCSCs are considered responsible for resistance to chemo-radiation therapy, disease recurrence, and metastasis.7,8,9 Consequently, the development of effective breast cancer therapy should pay more attention on targeting the eradication of BCSCs.8

One of the general strategies of breast cancer therapy is to treat cancer cells excessively with free radicals.9 As a consequence, the balance between free radicals and endogenous antioxidant defense mechanisms in cancer cells will be disturbed, known as oxidative stress, leading to various types of cell death such as apoptosis and autophagy.10,11 Chemo-radiation therapy modulates oxidative stress in cancer cells which induces cellular adaption responses such as cell survival and antioxidant defense mechanisms.12,13,14 Our recent study has demonstrated that human BCSCs (CD24-/CD44+) could survive better than their counterpart non-BCSCs (CD24+/CD44-) when treated with rotenone, which might be due to lower ROS levels production, high expression of antioxidant MnSOD and anti-apoptosis survivin.15

Survivin is a member of the inhibitor of apoptosis protein (IAP) family. Retrospective studies revealed that up-regulation of survivin correlates with decreased survival rates, increased relapse, and higher frequency of metastases in breast cancer patients.16,17 Moreover, survivin is highly expressed in other cancer cells in which its expression confers resistance to apoptosis induced by various chemotherapeutic agents.18,19 Previous study has demonstrated that a novel small molecule, YM155, could suppress survivin expression with minimal effect on expression levels of other IAP family members or Bcl-2 related proteins.20

In this study, we aimed to verify the role of survivin on the survival of human BCSCs under oxidative stress modulation by suppressing its expression using a survivin inhibitor YM155. Additionally, the in vitro antitumor efficacy of
YM155 was evaluated through the survival and oxidative stress modulation of rotenone-induced BCSCs.

Methods

A. Cell Culture

Breast cancer specimens (~1 gram) were obtained within approximately 1 hour after surgical removal of the tumor. The tumor was then soaked in a sterile DMEM (Gibco®) medium, mechanically dissociated and homogenated with a sterile scalpel blade. To obtain a single cell suspension, homogenate was mixed with 0.14% collagenase IV in PBS buffer and incubated at 37°C for 3-4 hours. Every 15-20 minutes, cell suspension was mixed. After incubation, collagenase was inactivated by adding 10% heat-inactivated Fetal Bovine Serum (FBS) in DMEM (Gibco®) into cell suspension. Subsequently, cell suspension was washed in PBS and centrifuged at 300g for 10 min. After re-suspension of cell pellets, single cell suspension was filtered using 40-µm cell strainer, washed with PBS and finally cultured with DMEM (Gibco®) supplemented 1% Penicillin-Streptomycin, 1% Fungizone, and 10% FBS.

The BCSCs (ALDH+) were obtained using FACS sorting. All of the sorts cells were cultured in different media. BCSCs were grown in non-serum high glucose DMEM/F12 medium (Gibco®) and supplemented with 1% Penicillin-Streptomycin and 1% Fungizone. Meanwhile, non-BCSCs (ALDH-) were grown in high glucose DMEM (Gibco®) supplemented 1% Penicillin-Streptomycin, 1% Fungizone, and 10% FBS. The medium was replaced every 2-3 days. The cells were subcultured with 0.25% Trypsin-EDTA whenever the cultures reached 80-90% of confluence. The standard conditions for all cell cultures were 5% CO2 and 20% O2 at 37°C.

B. YM155 and Rotenone Treatment

YM155 (Cayman Chemicals®) was dissolved in dimethyl sulfoxide (DMSO) and diluted at 100mM with serum-free culture medium [final concentration of DMSO in culture medium: 0.0009% (v/v)]. On the other hand, rotenone (Sigma Aldrich®) was dissolved in DMSO and diluted at various concentrations (0.5µM; 5µM; 50µM) using serum-free culture medium [final concentration of DMSO in culture medium: 0.2% (v/v)].

A total of 10^5 BCSCs per well in 12-well plate (TPP®) were treated with 100nM YM155, while the control with 0.0009% DMSO for 24 hours. The cells were then treated with rotenone at various concentrations while treating the control with 0.2% DMSO. After 6 hours of incubation, the cells were harvested for further analysis.

C. In vitro assay for cell viability and ROS detection

Cell viability was determined using trypan blue exclusion method, while measurement of intracellular ROS production was conducted using superoxide sensitive probe dihydroethidium (DHE) assay. Briefly, 2x10^4 cells were collected and washed with sterile PBS 1x twice. Cells were suspended in 500µL of PBS 1x and loaded with 20µM DHE dye for 30 minutes at 37°C in the dark. Fluorescence was measured immediately with fluorometer (Varioskan™ Flash multi-mode Reader by Thermo Scientific) with the excitation at 480nm and emission at 585nm.

D. Total RNA preparation and real time-PCR

Total RNA was isolated from the cells using TriPure Isolation Kit (Roche®) according to the manufacturer's instruction. Total RNA concentration was quantified using spectrophotometry at λ 260nm (Varioskan™ Flash multi-mode Reader by Thermo Scientific). Quantitative Real-time PCR was performed using KAPA SYBR® qPCR (Kapa Biosystem®) in the Exicycler™ 96 (Bioneer®), according to the manufacturer's instruction. Result of amplifications was represented by Ct value and normalized to 18S rRNA. All sets of reactions were conducted in triplicate. The relative expression levels were expressed as percentage of the indicated control.

Table 1. Sequences of the primers

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>18S rRNA</td>
<td>Forward</td>
<td>5'-GACTGCTCCACATCCAGAG-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5'-GGCACACGCTGTTATGACTA-3'</td>
</tr>
<tr>
<td>Survivin</td>
<td>Forward</td>
<td>5'-GCCAGATGAGCCACCATAGAGA-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5'-TCGATGGCCAGCGGCACCTT-3'</td>
</tr>
<tr>
<td>MnSOD</td>
<td>Forward</td>
<td>5'-GGACTTAGACAGCATGTAGAC-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5'-ACTCTTCCTCGGTTGACGTT-3'</td>
</tr>
</tbody>
</table>

E. Determination of Survivin Total Protein

Total protein isolation from human BCSCs was performed using RIPA lysis buffer (Invitrogen®) according to manufacturer’s instruction. The total protein concentration was quantified using spectrophotometry at λ 280nm (Varioskan™ Flash multi-mode Reader by Thermo Scientific). A total of 20µL protein lysate was used to determine the survivin protein using PathScan® Total Survivin (Cell Signaling Technology®), as described in the manufacturer’s protocol. The absorbance was measured using spectrophotometry at λ 450nm (Varioskan™ Flash multi-mode Reader by Thermo Scientific). The absorbance was divided by total protein concentration.

F. Analysis of MnSOD Specific Activity

Total protein was isolated from harvested cell culture of breast CSCs and non-CSCs using TriPure Isolation kit (Roche®) according to manufacturer’s protocol. MnSOD enzyme activity was measured using xanthine oxidase inhibition assay (RanSOD® kit), as previously described. To inhibit the Cu/ZnSOD, natrium cyanide (5 mM) was firstly added into each sample and the mixture was incubated for 5 minutes at room temperature. Xanthine oxidase was then added to the mixture and the color change was measured by spectrophotometer at λ 505nm after 30 seconds and 3 minutes. The enzyme activity was calculated as a percentage inhibition of the samples plotted to the standard curve. The specific activity of MnSOD enzyme was calculated as enzyme activity (in Unit) per mg protein. Protein concentration was measured using spectrophotometer at λ 280nm and plotted to the Bovine Serum Albumin (BSA) standard curve.
G. Determination of Apoptosis using Acridine Orange/Ethidium Bromide

Two microliters of AO/EB solution were added to 25µL of cell suspension and mixed gently. The mixture was incubated in the dark for 3 minutes. Ten microliters of the mixture was placed onto a microscope slide, covered with a glass coverslip, and examined at least 100 cells in a fluorescence microscope (Olympus®).

H. Statistical Analysis

All values obtained were compared with those of control cells and presented as mean ± standard deviation (SD). Statistical evaluation of significant differences was performed using the Student's t-test.

Results

In this present study, we have succeeded to isolate BCSCs (ALDH+) and non-BCSCs (ALDH-) cells from human breast cancer tissue. Both cells were grown as monolayer cultures. In order to maintain stemness property and prevent differentiation, we particularly treated ALDH+ cells, a putative breast CSCs, with DMEM/F12 without FBS, in comparison with the medium used to culture ALDH- (DMEM supplemented with FBS). As demonstrated in Figure 1, human breast cancer ALDH+ cells tended to stick together forming mammospheres (Figure 1A), whereas the morphology of ALDH- cells was dominated by fibroblast-like cells (Figure 1B). In addition, we observed that the growth of ALDH+ cells was slower than their counterpart, ALDH- cells. In a parallel study, we could confirm that the isolated human breast cancer ALDH+ have considerably higher expression of Oct-4, a major pluripotent gene, as well as higher mammosphere forming unit, as a proof of tumorigenic capability compared with ALDH- cells (data not shown). Hence, we imply the ALDH+ cells as BCSCs while the ALDH- cells as non-BCSCs.

Optimization of YM155 treatment

To verify the role of YM155 in inhibiting survivin, we first optimized YM155 by treating BCSCs with various concentrations and various incubation times. The optimization of concentration was conducted using 10nM, 50nM, and 100nM YM155 for 24 hours. The result showed that 100nM YM155 significantly decreased the viable cells by 64%.
compared to control cells treated with DMSO (Figure 2A). To inhibit survivin expression, we also optimized the incubation time of 100nM YM155 by treating BCSCs for 6, 24, 30, 48 hours. Figure 2B demonstrates that 24-hour incubation of 100nM YM155 could significantly decrease survivin expression in BCSCs (Figure 2B).

The effect of YM155 treatment on the survivin expression and protein level of rotenone-induced BCSCs

Modulation of oxidative stress in BCSCs and non-BCSCs was performed using rotenone - a complex I electron transport chain inhibitor – in order to simulate chemoradiation therapy based on ROS generation particularly superoxide radicals. Rotenone concentrations used in this study were 0.5 μM, 5 μM, and 50 μM based on our previous study that performed rotenone treatment for T98G glioblastoma cell line. Another recent study using human breast adenocarcinoma (MCF-7) cell line observed a significant decrease of cell viability following treatment of 50 μM rotenone. In addition to those results, incubation period of rotenone within 4 to 8 hours significantly increased the generation of ROS in human neuronal stem cells.

We also treated the cells with DMSO in order to control the effects arising from the solvent DMSO on the modulation of oxidative superoxide. DMSO is generally used as a cryoprotectant for the preservation of cells and also used as an organic solvent. Although DMSO has been established to have a low level of toxicity at low concentration, recent study has revealed that DMSO may modulate oxidative stress in the cell cultures when given at high concentration. DMSO concentration used in this study (0.2%) did not affect the viability of BCSCs, as well as of their counterpart non-BCSCs.

We further analysed the involvement of survivin as anti-apoptotic factor on the survival of BCSCs after rotenone treatment. Survivin plays a definite role in inhibiting the activation of many pro-apoptotic proteins such as caspase-9 and caspase activators. Prior to comparing the survivin mRNA expression level between BCSCs and non-BCSCs, the expression levels in rotenone-treated cells were normalized to DMSO-treated cells. In this study, we showed that the expression level of survivin mRNA in the BCSCs following rotenone treatment was drastically increased (~5-fold at rotenone 0.5μM, ~5-fold at 5μM, and ~10.7-fold at 50μM) compared to that in non-BCSCs, indicating the role of survivin on the viability of BCSCs. The survivin expression after YM155 treatment on rotenone-induced BCSCs exhibited a significant suppression to the level of those in non-BCSCs.

![Figure 3. The effect of YM155 treatment on the survivin expression and protein level of rotenone-induced BCSCs.](image-url)
The effect of YM155 treatment on the viability of rotenone-induced BCSCs

To determine the ability of cells in maintaining or recovering their viability after rotenone treatment, we used trypan blue exclusion assay. Comparative analysis of viability between BCSCs and non-BCSCs was performed based on the ratio level of rotenone-treated cells to control (DMSO-treated cells). The results revealed that rotenone treatment could decrease cell viability in both BCSCs and non-BCSCs. However, the viability of BCSCs was significantly higher than those of the non-BCSCs, particularly at rotenone concentration of 50 µM (~1.36-fold, p<0.05). We then evaluated the effect of YM155 on cell viability. YM155 treatment, in a concentration-dependent manner, could reduce the viability of rotenone-induced BCSCs more prominently compared to those without YM155 treatment (~1.42-fold, p<0.05), even lower than those in non-BCSCs (Figure 4).

The effect of YM155 treatment on the superoxide anion levels of rotenone-induced BCSCs

To specifically monitor the level of superoxide radicals (O$_2^-$) in the cells produced upon rotenone treatment, we performed dihydroethidium (DHE) assay. DHE is able to freely permeate through cell membranes and reacts with superoxide anions to form fluorescent product 2-hydroxyethidium which can be detected by spectrofluorometer. In determining the effect of rotenone on superoxide level in BCSCs, DHE intensity of rotenone-treated cells was normalized to DMSO-treated cells as a control. As shown in Figure 5, superoxide anion levels in BCSCs after YM155 treatment were significantly higher than those without YM155 treatment. Due to the fact that YM155 can induce superoxide production, MnSOD antioxidant was also needed to be analysed to evaluate the adaptive response of BCSCs.

The effect of YM155 treatment on MnSOD expression level and specific activity of rotenone-induced BCSCs

MnSOD is a major endogenous antioxidant enzyme that eliminates superoxide radicals (O$_2^-$) generated by the mitochondrial electron transport chain. MnSOD mRNA expression level and specific activity of MnSOD may be altered under several physiological and pathophysiological conditions including environmental factors such as availability of manganese ions. Analysis of MnSOD mRNA expression level in BCSCs were performed based on the relative expression normalized to the control. In this study, we found that mRNA MnSOD expression level in BCSCs following YM155 treatment-only was decreased significantly about 0.6-fold compared with that in control (Figure 6A). We also found that the suppression not only in expression, but also in specific activity about 0.68-fold compared to control in BCSCs YM155 treatment only (Figure 6B).

The effect of YM155 treatment on apoptosis of rotenone-induced BCSCs

Apoptosis, a type of programmed cell death, is an active process. It is a normal component of the development and health of multicellular organisms. Rotenone provokes overproduction of superoxide radicals in mitochondria, leading to stimulation of cell apoptosis through intrinsic pathway. We further analyzed the effect of YM155 on the apoptosis and necrosis of rotenone-treated BCSCs using AO/EB staining. The result was then visualized using fluorescence microscope. Viable cells have a normal green nucleus, early apoptotic cells have bright green nucleus with condensed or fragmented chromatin; late apoptotic cells and condensate nucleus, early apoptotic cells have bright green nucleus with condensed or fragmented chromatin; late apoptotic cells have bright green nucleus with condensed or fragmented chromatin; late apoptotic cells have condensed or fragmented chromatin; late apoptotic cells have bright green nucleus with condensed or fragmented chromatin; late apoptotic cells have condensed condensed and fragmented orange chromatin; cells that have died from direct necrosis have a structurally normal orange nucleus.
Figure 7 shows various apoptosis and necrosis levels between each treatments. Interestingly, BCSCs treated with 100nM YM155, and 100nM 0.5µM demonstrated higher necrosis compared to apoptosis, in contrast to those treated with 100nM 5µM and 100nM 50µM.

Discussion

The success of cancer therapy is basically assessed by its ability to affect the survival of cancer cells. When cancer cells are sensitized to any conventional cancer therapy, such as chemotherapy, radiation, as well as hormonal therapy, it is expected that the cancer cell survival is decreased leading to eradication of the cancer cells. Conventional regimen of anti-cancer therapy is generally based on the assumption that all cancer cells have the same potential of malignancy, without considering the presence of breast CSCs. A number of recent scientific reviews and studies have enthusiastically described CSCs as a minor population of breast cancer cells that play a crucial role in tumor progression and metastasis.
roles on high survival rate and resistance of cancer cells to anti-cancer therapies. Therefore, the development of an anti-cancer therapy targeted to breast CSCs is urgently required so that cancer can be eradicated from the root.

It should be noted that most of chemo-radiation therapy acts by modulating oxidative stress of cancer cells. In this study, rotenone is used to stimulate the effect of free radical-based anti-cancer therapies. Rotenone blocks electron flow from NADH to co-enzyme Q at complex I of respiratory chain in mitochondria. It is able to increase the production of superoxide radicals, and further induced cytotoxicity. The production of rotenone on cell survival was examined by cell viability. Even though viability of both BCSCs and non-BCSCs investigated in the present study were reduced after rotenone treatment compared with untreated cells, we should remark that BCSCs were more viable than non-BCSCs when treated with increasing concentrations of rotenone. Combined with the result of survivin mRNA expression, substantially higher expression of survivin in BCSCs is thought to precede the higher viability of BCSCs compared with those in non-BCSCs. Thus, we presumed that the increase of survivin mRNA expression level might be another mechanism owned by BCSCs to survive from the impact of oxidative stress modulation.

Furthermore, we evaluated the potency of survivin as an ideal target for breast cancer treatment. Survivin is an intriguing protein that facilitates tumor growth, inhibits cancer apoptosis, and function as an effector protein downstream of Her2 signaling in breast cancer. Considering the fact that survivin is overexpressed, not only in breast cancer but also metastatic human tumors and low expression (or absence) in most normal tissues, it is suggested that dysregulation of survivin expression may confer an ability to induce apoptosis. Significant efforts have been focused on developing strategies to use survivin as a target for therapies in cancer. Developing drugs that target survivin might initially seem difficult because survivin is not an enzyme nor it is a cell surface protein. However, considerable progress has been made to achieve optimal efficiency in suppressing survivin. In order to inhibit survivin expression, we used YM155, survivin suppressant. Previous study has indicated that YM155 suppresses survivin through direct binding to its promoter. This study confirmed that YM155 decreased the expression of survivin mRNA and protein levels. We also found that the YM155 treatment resulted in inhibition of BCSC proliferation.

Modulation of oxidative stress in BCSCs and non-BCSCs was performed using rotenone, a complex I electron transport chain inhibitor, in order to simulate chemo-radiation therapy based on ROS generation particularly superoxide radicals. Interestingly, we found that YM155 can also increase ROS production, which has never been reported before. We suggested that this mechanism is due to ubiquinone in YM155 structure which can produce superoxide radicals.

MnSOD is a major endogenous antioxidant enzyme that eliminates superoxide radicals (O2•−) generated by the mitochondrial electron transport chain. MnSOD mRNA expression level and specific activity of MnSOD may be altered under several physiological and pathophysiological conditions including environmental factors such as availability of manganese ions. The suppression of MnSOD expression in ovarian cancer cells enhanced superoxide level which further induced cell proliferation in vitro and tumor growth in vivo. It is also crucial to analyze its specific activity since MnSOD is synthesized in the cytoplasm as a precursor and post-translationally transported into the mitochondrial matrix via an amino-terminal targeting sequence. We demonstrated that BCSCs treated with only YM155 can also suppress MnSOD expression, both at mRNA levels and protein activity. The mechanism of MnSOD inhibition remains to be elucidated. However, the present study reported for the first time that YM155 could modulate the oxidative stress in BCSCs by suppressing the MnSOD expression and increasing the ROS levels.

It should be noted that most of chemo-radiation therapy acts by modulating oxidative stress of cancer cells. In this study, rotenone is used to simulate the effect of free radical-based anti-cancer therapies. The impact of rotenone on cell survival can be examined by analysing cell proliferation, cell viability and apoptosis. This finding is in accordance to the fact that rotenone provokes overproduction of superoxide radicals in mitochondria, leading to stimulation of cell apoptosis through intrinsic pathway. This suggests that the treatments done in this study may have alteration between apoptosis and necrosis pathway of BCSCs depends on the concentration treatment.

In conclusion, we confirm that survivin plays a remarkable role on BCSC survival through oxidative stress modulation. Furthermore, we propose that YM155 treatment may offer a novel therapeutic option for the eradication of BCSC and overcome BCSC resistance to oxidative stress-based anti-cancer.

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References


