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Trisindoline 5 Compound Inhibits Human Breast Cancer Stem Cell Formation by Downregulating the BCL-2 Gene Expression

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ABSTRACT

Background: Breast cancer cells growth and survival are supported by breast cancer stem cells (bCSCs) mammosphere formation. bCSCs represent a subpopulation of undifferentiated cancer cells which associated with self-renewing abilities, tumor initiation, drug resistance and metastasis. Overexpression of B Cell Lymphoma 2 (BCL-2) family in many tumor cells contributes to apoptosis resistance. Trisindoline is an indole trimer alkaloid natural compound that provide a cytotoxic effect on cancer cells. A new modification of trisindoline has been synthesized into trisindoline 5 in 2021.

Objective: This study purposed to investigate the effect of trisindoline 5 compound against BCL-2 gene expression in bCSCs in vitro.

Methods: The bCSCs MDA-MB-231 were divided into control and treatment groups which futher analysed in gene expression using qPCR Livak method.

Results: Based on gene expression analysis, the results showed that trisindoline 5 may decrease the expression of BCL-2 in MDA-MB-231 cells.

Conclusion: This study concludes that trisindoline 5 could downregulate the antiapoptotic BCL-2 gene expression in bCSCs in vitro.

Keywords: BCL-2, Breast cancer stem cells, trisindoline 5

INTRODUCTION

Breast cancer prevalence is a leading cause of mortality among women worldwide.[1] Breast cancer cells growth and survival is supported by breast cancer stem cells (bCSCs) mamosphere formation.[2] bCSCs represent a subpopulation of undifferentiated cancer cells which associated with self-renewing abilities, tumor initiation, drug resistance and metastasis.[3] Individual CSCs are capable to grow into spheroids or tumor spheres under appropriate culture condition.[4] Recent studies, overexpression of B Cell Lymphoma 2 (BCL-2) family in many tumor cells contributes to apoptosis resistance.[5] BCL-2 and BCL-XL are two key inhibitors that has been identified in various apoptotic pathways.[6] The member of antiapoptotic BCL-2 family prevent mitochondria outer membrane permeabilization (MOMP) by binding and sequestration of the active forms of BAK and BAX and/ or the sequestration of the direct activators BH3-only proteins.[7,8]

Chemotherapy, radiotherapy and surgery as a breast cancer treatment are available, but it remains an obstacle such as drug resistance that leads to poor prognoses in patients.[9] Another study revealed that 5-fuorouracil (5-FU)-based chemotherapy as BCL-2 inhibitors is resistance in patients with colorectal cancer.[10] As a result, it is necessary to develop an alternative drug as anticancer that

focused on the role of BCL-2 and its family member.[5] Trisindoline is an indole trimer alkaloid natural compound that provide a cytotoxic effect on cancer cells which first isolated from *Vibrio* sp. symbiosis with sponge *Hyrtios altum* in Okinawa, Japan. [11, 12] The cytotoxic activity of trisindoline have shown in several types of cancer cells, including lung cancer A549 and liver cancer HepG2. The results showed that trisindoline compound is toxic against cancer cells, but not toxic against normal cells.[11]

The latest modification of trisindoline has been successfully synthesized into trisindoline 5-fluoro-3,3-di((methylindole-5-carboxylate)-3-yl)-2-indolon or known as trisindoline 5 in 2021. It combines the isatin with fluoro group and indole with methyl ester group. This compound was still tested on lung cancer cells A549, prostate cancer cells DU-145 and normal heart cells of mice H9C2 with IC50 25.69 µg/ml, 22.23 µg/ml and 201.65 µg/ml respectively.[13] However, the potential of trisindoline 5 as BCL-2 inhibitor in bCSCs is still not yet known. In this study, we aim to analyze the effect of trisindoline 5 compound against BCL-2 gene expression in bCSCs in vitro.

MATERIAL AND METHODS

Reagents and chemicals

Trisindoline 5 compound and doxorubicin-HCl (51000000-OBC-000540091, PT. Dankos Farma, Indonesia) as a positive control are obtained from Chemistry of Natural and Synthetic Materials Laboratory, Department of Chemistry, Institut Teknologi Sepuluh Nopember, Surabaya.

Cell Culture

bCSCs were isolated from breast cancer cells MDA-MB-231 (ECACC #92020424) (Porton Down, Wiltshire, UK) using *magnetic-activated cell sorting* (MACS) (Miltenyi Biotec, Bergisch Gladbach, Germany). bCSCs validation is known based on the expression of $CD44^{+}/CD24^{-}$.[2,14] bCSCs MDA-MB-231 were plated in Dulbecco's Modified Eagle Medium F-12 (DMEM F-12) (Gibco, USA) supplemented with 3 ml fetal bovine serum (FBS) (Gibco, USA) 20%, 186 µl penicilin-streptomicin 1,24% (Gibco, USA), 153 µl glutamin 1,02% (Gibco, USA), 37,5 µl fungizone (Amphotericin B) (Gibco, USA) 0,25% and MammoCult 20%.

Analysis of gene expression by quantitative reverse transcription-PCR

About 5-10 x 10^6 cells/ well were plated into 6 well plates, treated with doxorubicin-HCl 10 µg/ml and trisindoline 5 compound with concentration of ½ IC_{50} and 1 IC_{50} for 24 hours incubation. Total RNA was extracted using RNA Isolation Kit (FavorPrep™ Tri-RNA). Then, the isolated RNA was quantify using Nanodrop instrument (Nanodrop ND-1000 Technologies). 2 µl of RNA was used to synthesize cDNA using the TOYOBO ReverTra Ace™ qPCR RT Master Mix with gDNA Remover No. FSQ-301. Quantitative reverse transcription-PCR (qRT-PCR) analyses were performed using Eco™ Real-Time PCR System. GAPDH was used as the housekeeping gene, and the fold change values were analyze based on ΔΔCT relative expression using Livak methods. The primer's sequences used were shown in Table 1.

Table 1. Primer's sequences used for real-time qPCR

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
BCL-2	GAGCGTAGACAAGGAGATGC	TCCGACTGAAGAGCGAAC
GAPDH	CCAGCCGAGCCACATCGCTC	ATGAGCCCCAGCCTTCTCCAT

Statistical Analysis

Data were represented as mean ± standard deviation (SD) (SPSS statistical analysis software, version 20.0) of three independent assays. All variables were analysed of descriptive test then non parametric test, Kruskal Wallis test was carried out and followed by the Mann Whitney test to determine the differences between each group. Statistical significance difference is illustrated as asterisks (*, $p < 0.05$; **, $p < 0.01$; *, $p < 0.001$).

RESULTS

The results of the analysis of BCL-2 gene expression after 24 hours incubation treated with trisindoline 5 shown in Figure 1. The results showed that trisindoline 5 decreases the expression of BCL-2 respectively in bCSCs (P<0,05). Using Post Hoc Mann Whitney analysis, it was found that the BCL-2 gene expression in the treatment of trisindoline 5 with IC_{50} concentration was significantly different from control and doxorubicin-HCl ($p < 0.05$), but not significant when compared to trisindoline 5 ½ IC_{50} concentration ($p > 0.05$). The data also showed that trisindoline 5 ½ IC_{50} concentration was not significantly different from the control ($p > 0.05$), but significantly different from the doxorubicin-HCl ($p < 0.05$).

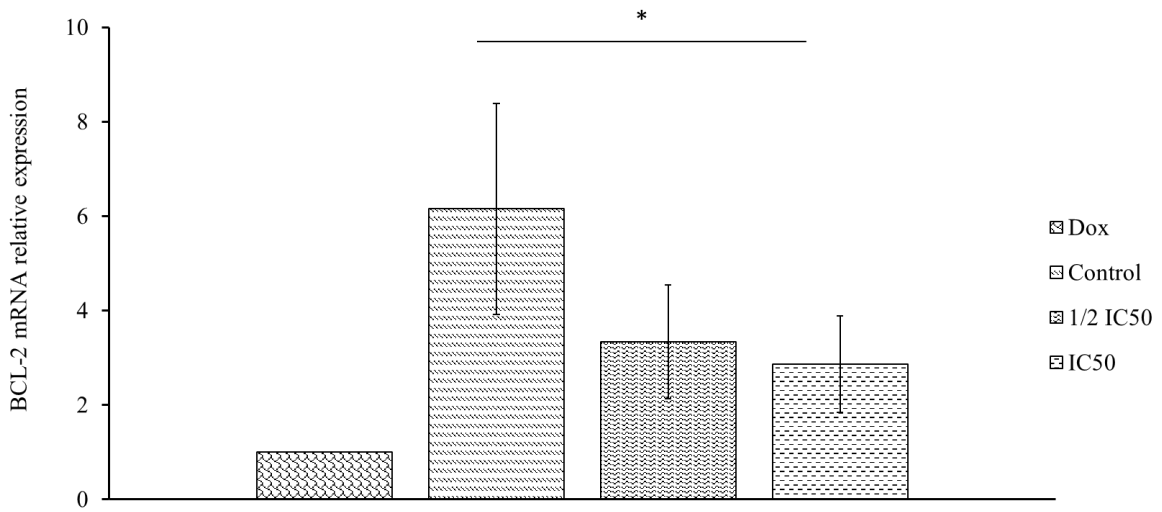

Figure 1. mRNA relative expression of BCL-2 in each group. **Control :** Untreated MDA-MB-231 cells , **½ IC50 :** CSCs treated with trisindoline 5 in 1/2 IC_{50} dose, **IC50 :** CSCs treated with trisindoline 5 in IC_{50} dose, and **Dox :** CSCs treated with doxorubicin. * Indicates a significant difference from the control group ($P < 0.05$); indicates a significant difference from the control ($P < 0.05$; $n = 3$ per group). The data represent mean ± standard deviation (SD).

DISCUSSION

Previous study stated that trisindoline compound had the potential to become anti-cancer drugs because able to trigger the apoptosis process via intrinsic (BH-3 and BAX) and also extrinsic ((Tumor Necrosis Factor receptor 1 (TNFR1), Fas, Death Receptor-5 (DR5)) by multiple signaling pathways. The enhanced expression of B-cell lymphoma/leukemia-2 (BCL-2) family can prevent the cancer cells from damage induce by a cytotoxic agent.[5] It was first discovered in B-cell malignancies. The main function of antiapoptotic BCL-2 proteins is to restrain proapoptotic BAX/ BAK, thus preserving

mitochondrial outer membrane integrity. The BCL-2 could direct binding and sequestration of proapoptotic BH3-only proteins that possess the ability to directly or indirectly activate BAX/ BAK.[16] Moreover, BCL-2 is able to prevent the early mitochondrial features of apoptosis.[17] The BCL-2, which is one of the antiapoptotic member in the initiation of cell death process, is localized in the nucleus, mitochondria and estrogen receptor. Many of the BCL-2 family members are currently being explored as markers for target treatments several types of cancer.[5] Another study also showed that overexpression BCL-2 is resistance to apoptosis induced by paclitaxel treatment. Another apoptosis-inducing agent used such as etoposide, doxorubicin, arabinofuranosylcytosine could disrupt the mitochondrial transmembrane, which lead to the nuclear DNA fragmentation.[17]

Our results showed that trisindoline 5 treatment might be act as BCL-2 inhibitors by blocked the expression of the antiapoptotic members BCL-2 and BCL-XL and also induce apoptosis by increasing the expression levels of proapoptotic members such as BAX, BAK, BAD, BIK and BIM in bCSCs. BCL-2 family proteins are the regulator of apoptosis that able to interact with inhibitors or inducers of cell death[18,19]. Together they regulate and mediate the process by which mitochondria contribute to cell death known as the intrinsic apoptosis pathway.The activation of apoptotic pathways could lead to the activation of caspase which classified into initiators (caspase-8 and -9) and effectors (caspase-3, -6 and -7) caspase.[20] The exact mechanisms of BCL-2 family proteins resulting from the trisindoline 5 treatment have not been fully explained.[18,20] Thus, inhibiting BCL-2 resulting in the reduced of survival the bCSCs.

CONCLUSION

Finally, this study showed that trisindoline 5 compound could downregulate the antiapoptotic BCL-2 gene expression in human MDA-MB-231 in vitro.

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AUTHORS' CONTRIBUTIONS

Wrote the first draft of the manuscript: MF and YIS. Contributed to the writing of the manuscript: APDN and SSG. Agree with manuscript results and conclusions: APDN and MS. Jointly developed the structure and arguments for the paper: MF, YIS and SSG. Made critical revisions and approved final version: MS and FM. All authors reviewed and approved of the final manuscript.

COMPETING INTERESTS

The authors declare that there was no conflict of interest.

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