

Upregulation of p53 Gene Expression on Breast Cancer Stem Cells Treated with Trisindoline 5 Compound

Yofinta I Salsabila¹, Nadia Azhaar¹, Muhammad Fatoni^{1*}, Awik P D Nurhayati¹, Shabrina S Ghaissani¹, Vinda Aprilia Nurul Andifa¹, Mardi Santoso², Fahimah Martak²

*Correspondence:

muhammadfatoniofficial@gmail.com

¹Department of Biology, Faculty of Science and Data Analytics, Institut Teknologi Sepuluh Nopember, Surabaya 60111, Indonesia

² Department of Chemistry, Faculty of Science and Data Analytics, Institut Teknologi Sepuluh Nopember, Surabaya 60111, Indonesia

Received 22 September 2022

Accepted 28 September 2022

Available online on 30 September 2022

© 2022 The Authors. Published by Stem Cell and Cancer Research, Semarang, Indonesia. This is an open-access article under the terms of the Creative Commons Attribution-NonCommercial-ShareAlike License (CC BY-NC-SA 4.0), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

ABSTRACT

Background: Cancer stem cells (CSCs) are the subpopulation of cancer cells that have been demonstrated as the cause of tumor formation. The most common cancer for women and the second leading cause of death in breast cancer. Numerous genes have been involved in breast cancers, including p53. In cancer cells, p53 as well-known as the tumor suppressor gene plays an important role in directing cells with DNA damage into apoptosis. Trisindolines are heterocyclic nitrogen compounds consisting of an isatin core bearing two indole moieties that provide cytotoxic effects on cancer cells. Recently research had led to the development of the new modification of trisindoline compound into trisindoline 5

Objective: This study aims to investigate the effect of trisindoline 5 compounds against p53 gene expression in CSCs MDA-MB-231 in vitro.

Methods: CSCs MDA-MB-231 were divided into control and treatment groups which were further analyzed in gene expression using the qPCR Livak method.

Results: Based on gene expression analysis, trisindoline 5 increases the expression of p53 in CSCs MDA-MB-231.

Conclusion: This study informed that trisindoline 5 could upregulate the expression of tumor suppressor gene p53 in CSCs MDA-MB-231 in vitro.

Keywords: Breast cancer stem cells, p53, Trisindoline 5

INTRODUCTION

Cancer stem cells (CSCs) are the subpopulation of cancer cells that have been demonstrated as the cause of tumor formation.¹ CSCs within tumors holding stemness properties have been proposed to help drive tumorigenicity and inherent drug therapy resistance. CSCs could control the neighboring cells to provide the nutrients and collaborate in the elusion from the immune system, thus creating a favorable environment for tumor growth.² Many cancer patients with poor prognoses recurrence after treatment due to the permanence of CSCs.³ The most common cancer for women and the second leading cause of death in breast cancer. The existence of CSCs in breast cancer cells helps maintain the incurable metastasis and the current optimal management of this disease remains unclear.⁴ Thus, CSCs are emerging as a target for cancer therapy.⁵

Numerous genes have been involved in breast cancers, including P53.⁶ In normal cells, P53 has a wide range of functions such as in cell homeostasis, including cell cycle, cell proliferation, DNA maintenance, and apoptosis.² It has also been referred to as the guardian of the genome that protects the cell from cellular damage under stress conditions.⁷ Whether in cancer cells, P53 as well-known as the tumor suppressor gene plays an important role in directing cells with DNA damage into apoptosis.⁶

Apoptosis is a programmed cell death that results in the loss of cells without the release of harmful substances into the environment.⁸ Previous study showed that a higher level of p53 expression in CSCs MCF-7 compared to CSCs MDA-MB-231 after being treated with doxorubicin.⁹ Another research stated doxorubicin has been considered and approved as one of the most commercial drug administrations³. The treatment using doxorubicin leads to DNA alterations, cellular damage, and cell death⁴. However, this cellular damage not only occurs in cancer cells but also in healthy cells which are mainly a consequence of its toxicity.¹⁰

Using bioactive natural compounds from marine sponges as a new cancer therapeutic strategy has special attention because it has a rich source of secondary metabolites and is widespread in a tropical reefs.¹¹ Trisindolines are heterocyclic nitrogen compounds consisting of an isatin core bearing two indole moieties which were first isolated from the cultured marine bacterium *Vibrio* sp. symbiosis with marine sponge *Hyrtios altum* from Okinawa Japan.¹² Trisindoline was reported to have anticancer activity against many cancer cell lines, including colorectal adenocarcinoma cells (HCT-15), lung cancer cells (A-549), uterine sarcoma cells (MES-SA), and breast cancer cells (MCF-7).¹³ Recently, a research had led to the development of the latest new modification of trisindoline compound into trisindoline 5-fluoro-3,3-di((methylindole-5-carboxylate)-3-yl)-2-indolon or trisindoline 5 which combines the isatin with a fluoro group and indole with a methyl ester group⁵. This compound was still tested in cytotoxicity in vitro on lung cancer cells (A-549), 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.¹⁴ Therefore, in this study, we explore the potential of trisindoline 5 to increase the expression of p53 in CSCs. This study aims to investigate the effect of trisindoline 5 compounds against p53 gene expression in CSCs MDA-MB-231 in vitro.

MATERIAL AND METHODS

Reagents and Chemicals

The Trisindoline 5 compound and doxorubicin as a positive control are obtained from the Chemistry of Natural and Synthetic Materials Laboratory, Department of Chemistry ITS Surabaya.

MDA-MB-231 Cells culture

CSCs 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). CSCs validation is known based on the expression of CD44⁺/CD24⁻. CSCs 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 penicillin-streptomycin 1,24% (Gibco, USA), 153 µl glutamine 1,02% (Gibco, USA), 37,5 µl fungizone (Amphotericin B) (Gibco, USA) 0,25% and MammoCult 20%.

p53 gene expression by qRT-PCR

About 5-10 x 10⁶ cells/ well were plated into 6 well plates, treated with doxorubicin 10 µg/ml and trisindoline 5 compounds with a concentration of ½ IC₅₀ and 1 IC₅₀ for 24 hours incubation. Total RNA was extracted using RNA Isolation Kit (*FavorPrep™ Tri-RNA*). Then, the isolated RNA was quantified using a 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*. The effect of trisindoline 5 on the expression level of marker genes p53 and GAPDH was analyzed by RT-PCR. A comparative threshold cycle was used for the estimation of gene expression. The primer sequences used were shown in Table 1. In summary, the CT (threshold cycle) was obtained from triplicate amplification throughout the exponential stage of the amplification. Next,

the CT value obtained for the reference gene (GAPDH) was deducted from the CT values obtained for the p53 and was calculated for every single gene. Following the calculation of $\Delta\Delta CT$ for each sample, the relative expression of each gene was evaluated using the ratio formula (ratio = $2^{-\Delta\Delta CT}$).

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

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
p53	GCTCAGATAGCGATGGTCTGGC	AGTGGATGGTGGTACAGTCAGAG
GAPDH	CCAGCCGAGCCACATCGCTC	ATGAGCCCCAGCCTTCTCCAT

Statistical Analysis

Data were represented as mean $\pm$ standard deviation (SD) (SPSS statistical analysis software, version 20.0) of three independent assays. All variables were analyzed for normality, homogeneity, and parametric testing one-way analysis of variance (ANOVA), followed by post hoc analysis using Mann Whitney. The statistical significance difference is illustrated as asterisks (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

RESULTS

Effect of Trisindoline 5 on p53 gene expression

The result showed that there was an increase in p53 gene expression significantly in all treatment groups compared to the control. The optimum increasing p53 gene expression occurs in trisindoline 5 with IC₅₀ dose and doxorubicin. This study showed a significant effect of trisindoline 5 on p53 gene expression in CSCs MDA-MB-231.

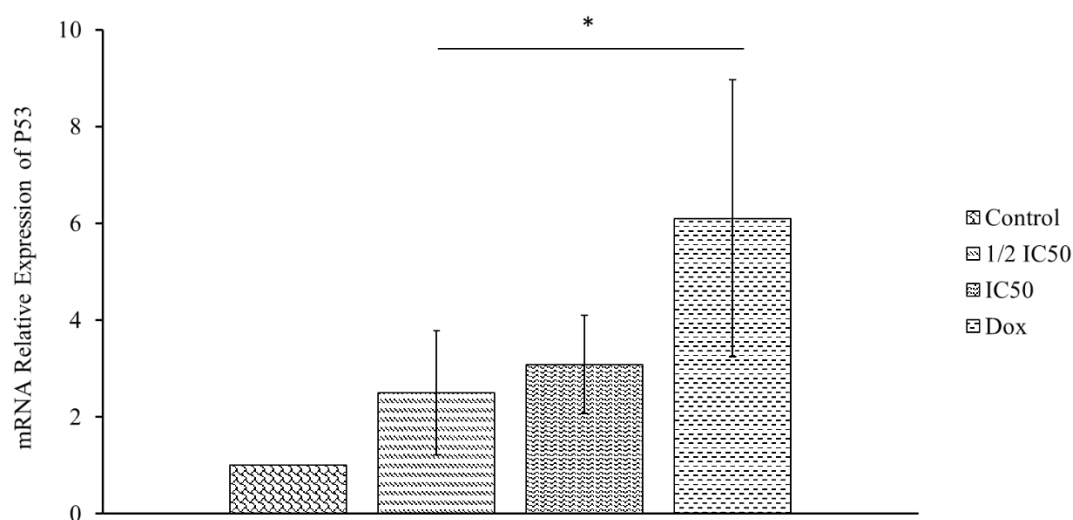


Figure 1. Real-time polymerase chain reaction (RT-PCR) relative expression of p53 on CSCs MDA-MB-231. **Control :** Untreated MDA-MB-231 cells, **1/2 IC₅₀ :** CSCs treated with trisindoline 5 in 1/2 IC₅₀ dose, **IC₅₀ :** CSCs treated with trisindoline 5 in IC₅₀ dose, and **Dox :** CSCs treated with doxorubicin. * 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 $\pm$ standard deviation (SD).

DISCUSSION

The tumor suppressor p53 plays a crucial role in ensuring genomic integrity of embryonic stem cells and controls their proliferation, differentiation, and apoptosis.¹⁵ p53 expression in normal cells is maintained at a low level due to the disruption of the activity of E3-ubiquitin ligases, such as MDM2, HDM2, and TRIM24 by ubiquitylation and protein degradation.¹⁶ DNA damage, UV radiation, hypoxia, and hyper-proliferation in embryonic stem cells could lead to p53 activation.^{6,17} In response to mild or severe DNA damage, p53 differentially regulates cell fate decisions. Under mild damage conditions, p53 is phosphorylated at Ser15 and Ser20 leading to cell cycle arrest and DNA repair. While under severe damage conditions, p53 is phosphorylated not only at Ser15 and Ser20, but also at Ser46 resulting in cell death.¹⁸

The activity of cyclin D and CDK4/6 which regulate the G1 to S phase is decreased when DNA is damaged by decreasing the amount of Rb protein. This results in the inhibition of the E2F transcription factor and triggers cell cycle arrest. The cyclin D will enter the damaged DNA and stimulate the activation of proteins involved in DNA repair mechanisms, such as RAD51 and BRCA2.^{19,20}

The damaged DNA also will initiate a signaling pathway by activating the ATM/ ATR kinase.²¹ These proteins then phosphorylate the Chk1/ Chk2 kinase to activate p53 as the main target.²² The activated p53 will stimulate the transcription process of the p21 that acts as a CDK inhibitor.²³ The p21 protein then binds to G1/S-CDK to stop the cell cycle progression from G1 to the S phase.^{20,24} On the other hand, p53 protein also could directly activate several pro-apoptotic target genes, such as BAX and BAK²⁵. Then, BAX and BAK will release cytochrome c from mitochondria. The cytochrome c will bind to ATP, APAF-1, and procaspase-9 to form a complex called apoptosome^{24,25}. The apoptosome then activates caspase 9 and will initiate the apoptosis.²⁶ Therefore, in this study, trisindoline 5 compounds might be potential as an alternative anticancer candidate on CSCs MDA-MB-231 to minimize the side effects from the chemotherapy agents through increasing the expression of tumor suppressor gene p53.

CONCLUSION

Our results demonstrated that Trisindoline 5 could upregulate the expression of tumor suppressor gene p53 in CSCs MDA-MB-231 in vitro.

FUNDING

None

ACKNOWLEDGEMENTS

The authors acknowledge to Department of Biology ITS, Department of Chemistry ITS, and SCCR Indonesia for continuously supporting this research.

AUTHORS' CONTRIBUTIONS

All authors made substantial contributions to the conception and interpretation of the work. All authors were involved in drafting the work, revising it critically for content, and approving the final version for publication.

COMPETING INTERESTS

The authors declare that there was no conflict of interest.

REFERENCES

1. Sun HR, Wang S, Yan SC, Zhang Y, Nelson PJ, Jia HL, Qin LX, Dong QZ. Therapeutic Strategies Targeting Cancer Stem Cells and Their Microenvironment. *Front Oncol.* 2019;9(1104):1-14. doi:10.3389/fonc.2019.01104
2. Aponte PM, Caicedo A. Stemness in Cancer: Stem Cells, Cancer Stem Cells, and Their Microenvironment. *Stem Cells Int.* 2017;5619472:1-17. doi:10.1155/2017/5619472
3. Rasti A, Mehrazma M, Majd Z, Abolhasani M, Zanjani LS, Asgari M. Co-expression of Cancer Stem Cell Markers OCT4 and NANOG Predicts Poor Prognosis in Renal Cell Carcinomas. *Sci Rep.* 2018;8(11739):1-11. doi:10.1038/s41598-018-30168-4
4. Kim SY, Rhee JG, Song X, Prochownik EV, Spitz DR, Lee YJ. Breast cancer stem cell-like cells are more sensitive to ionizing radiation than non-stem cells: role of ATM. *PLoS One.* 2012;7(11):e50423. doi:10.1371/journal.pone.0050423
5. Liu TJ, Sun BC, Zhao XL, Zhao XM, Sun T, Gu Q, Yao Z, Dong XY, Zhao N, Liu N. CD133+ cells with cancer stem cell characteristics associated with vasculogenic mimicry in triple-negative breast cancer. *Oncogene.* 2013;32(5):544-53. doi:10.1038/onc.2012.85
6. Kaabinejadian S, Fouladdel Sh, Ramezani M, Azizi E. p53 Expression in MCF7, T47D and MDA-MB 468 Breast Cancer Cell Lines Treated with Adriamycin Using RT-PCR and Immunocytochemistry. *Journal of Biological Science.* 2008;8(2):380-385. doi:10.3923/jbs.2008.380.385
7. Tanaka T, Watanabe M, Yamashita K. Potential therapeutic targets of TP53 gene in the context of its classically canonical functions and its latest non-canonical functions in human cancer. *Oncotarget.* 2018;9(22):16234-16247. doi:10.18632/oncotarget.24611
8. Igney FH, Krammer PH. Death and anti-death: tumor resistance to apoptosis. *Nat Rev Cancer.* 2002;2(4):277-288. doi:10.1038/nrc776
9. Ashour F, Awwad MH, Sharawy HEL, Kamal M. Estrogen receptor-positive breast tumors resist chemotherapy by the overexpression of P53 in cancer stem cells. *Journal of the Egyptian National Cancer Institute.* 2018;30(2):45-48. doi:10.1016/j.jnci.2018.04.002
10. Varela-López A, Battino M, Navarro-Hortal MD, Giampieri F, Forbes-Hernández TY, Romero-Márquez JM, Collado R, Quiles JL. An update on the mechanisms related to cell death and toxicity of doxorubicin and the protective role of nutrients. *Food Chem Toxicol.* 2019;134:110834. doi:10.1016/j.fct.2019.110834
11. Shady NH, El-Hossary EM, Fouad MA, Gulder TAM, Kamel MS, Abdelmohsen UR. Bioactive Natural Products of Marine Sponges from the Genus *Hyrtios*. *Molecules* 2017;22(5):781. doi:10.3390/molecules22050781
12. Kobayashi M, Aoki S, Gato K, Matsunami K, Kurosu M, Kitagawa I. Marine Natural Products. XXXIV.1) trisindoline, a new antibiotic indole trimer, produced by a bacterium of *Vibrio* sp. Separated from the marine sponge *Hyrtios altum*. *Chem. Pharm. Bull.* 1994;42(12):2449-2451.
13. Wati FA, Santoso M, Moussa Z, Fatmawati S, Fadlan A, Judeh ZMA. Chemistry of trisindolines: natural occurrence, synthesis, and bioactivity. *RSC Advances.* 2021;11:25381-25421. doi:10.1039/d1ra03091d
14. Wati FA. Development of nitrogen heterocyclic compounds as antituberculosis and anticancer. *Doctoral thesis.* 2021. Institut Teknologi Sepuluh Nopember, Chemistry Department.
15. Ghatak D, Das Ghosh D, Roychoudhury S. Cancer Stemness: p53 at the Wheel. *Front. Oncol.* 2021;10:604124. doi:10.3389/fonc.2020.604124
16. Jain AK, Barton MC. p53: emerging roles in stem cells, development and beyond. *Development.* 2018;145(8):1-13. doi:10.1242/dev.158360
17. Putra A, Ignatius R, Suharto, Taat P, Indra W. Typhonium flagelliforme extract induce apoptosis in breast cancer stem cells by suppressing survivin. *J Cancer Res Ther.* 2018;14(7):1525-1534. doi:10.4103/jcrt.JCRT
18. Masyithah Darlan D, Munir D, Karmila Jusuf N, Putra A, Ikhsan R, Alif I. In vitro regulation of IL-6 and TGF- β by mesenchymal stem cells in systemic lupus erythematosus patients. *Med Glas (Zenica).* 2020;17(2):408-413. doi:10.17392/1186-20
19. Sahoo D, Mitra T, Chakraborty K, Sarkar P. Remotely controlled electro-responsive on-demand nanotherapy based on amine-modified graphene oxide for synergistic dual drug delivery. *Mater Today Chem.* 2022;25:100987. doi:10.1016/J.MTCHEM.2022.100987
20. Nugraha A, Putra A. Tumor necrosis factor- α -activated mesenchymal stem cells accelerate wound healing through vascular endothelial growth factor regulation in rats. *Universa Med.* 2018;37(2):135. doi:10.18051/univmed.2018.v37.135-142
21. Yang R, He Y, Chen S, Lu X, Huang C, Zhang G. Elevated expression of WWP2 in human lung adenocarcinoma and its effect on migration and invasion. *Biochem Biophys Res Commun.* 2016;479(2):146-151. doi:10.1016/j.bbrc.2016.07.084
22. Lin T, Lin Y. p53 switches off pluripotency on differentiation. *Stem Cell Res Ther.* 2017;8(44):1-7. doi:10.1186/s13287-017-0498-1

23. Liebl MC, Hoffmann TG. Cell Fate Regulation upon DNA Damage: p53 Serine 46 Kinases Pave the Cell Death Road. *BioEssays*. 2019;1900127:1-11. doi:10.1002/bies.201900127
24. Jirawatnotai S, Hu Y, Livingston DM, Sicinski P. Proteomic identification of a direct role for cyclin d1 in DNA damage repair. *Cancer Res*. 2012;72(17):4289-93. doi: 10.1158/0008-5472
25. A, B, Bray D, Hopkin K, Johnson A, Lewis J, Raff M, Roberts K, Walter P. *Essential Cell Biology Third Edition*. 2010. Garland Science.
26. Aubrey BJ, Kelly GL, Janic A, Herold MJ, Strasser A. How does p53 Induce Apoptosis and How Does This Relate to p53-mediated tumour suppression?. *Cell Death and Differentiation*. 2018;25:104-113. doi:10.1038/cdd.2017.169