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FoxM1 inhibition by Thiostrepton downregulates DNA damage response genes, enhancing sensitivity of breast cancer cells to therapy

Thiostrepton ile FoxM1 inhibisyonu, DNA hasar yanıt genlerini baskılayarak meme kanseri hücrelerinin tedaviye duyarlılığını artırır

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ABSTRACT

Aim: Chemotherapy resistance, often linked to the development of resistance against genotoxic agents, is a major obstacle in cancer treatment. FoxM1, a transcription factor frequently overexpressed in malignancies such as breast cancer, is strongly associated with genotoxic therapy resistance. The aim of this study is to conduct a comparative analysis of the effects of thiostrepton (THIO), a FoxM1 inhibitor, on the DNA damage response in HUVEC cells (non-malignant) and MDA-MB-231 breast cancer cells (malignant)

Materials and Methods: THIO's impact on cell viability were evaluated in both cell lines using the MTT assay. Oxidative DNA damage levels were measured with the 8-OHdG kit, and apoptosis was assessed using the Caspase 3 ELISA kit. The expression levels of DNA damage response genes (BRCA-1, DNAPKC, FOXM1, RAD51, MRE11 and XRCC1) were analyzed by RT-PCR.

Results: MDA-MB-231 cells exhibited greater sensitivity to the cytotoxic effects of THIO than HUVEC cells. In HUVEC cells, THIO caused a significant increase in oxidative DNA damage, whereas no such effect was observed in MDA-MB-231 cell lines. Conversely, breast cancer cells showed a significant increase in Caspase 3 levels. RT-PCR results revealed a marked downregulation of DNA damage response genes, particularly BRCA-1, DNAPKC, MRE11, FOXM1, and XRCC1, in both cell types.

Conclusion: THIO has been shown to inhibit FoxM1 expression and downregulate DNA damage response genes in both malignant and non-malignant cells, demonstrating its potential to enhance the sensitivity of breast cancer cells to therapy by disrupting DNA repair pathways. However, its potential to induce oxidative damage in non-malignant cells underscores the need for further comprehensive studies to validate its therapeutic efficacy and assess its safety in normal tissues.

Keywords: FoxM1, resistance to chemotherapy, Thiostrepton, breast cancer, DNA damage response

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ÖZ

Amaç: *Kemoterapi direnci, genellikle genotoksik ajanlara karşı direnç gelişimiyle ilişkilendirilir ve kanser tedavisinde önemli bir engel teşkil eder. FoxM1, meme kanseri gibi malignitelerde sıkça aşırı eksprese edilen ve genotoksik tedavi direnci ile güçlü bir şekilde ilişkili olan bir transkripsiyon faktörüdür. Bu çalışmanın amacı, FoxM1 inhibitörü olan thiostrepton'un (THIO) malign olmayan HUVEC hücreleri ile malign MDA-MB-231 meme kanseri hücrelerinde, DNA hasar yanıtı üzerindeki etkilerini karşılaştırmalı olarak analiz etmektir.*

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Gereç ve Yöntem: *THIO'nun hücre canlılığı üzerine etkileri her iki hücre hattında MTT testi kullanılarak değerlendirildi. Oksidatif DNA hasar seviyeleri 8-OHdG kiti ile ölçülürken, apoptoz seviyeleri Caspase 3 ELISA kiti ile belirlendi. DNA hasar yanıt genlerinin (BRCA-1, DNAPKC, FOXM1, RAD5, MRE11, ve XRCC1) ekspresyon düzeyleri RT-PCR yöntemiyle analiz edildi.*

Bulgular: *THIO'nun, MDA-MB-231 hücrelerinde HUVEC hücrelerine kıyasla daha belirgin sitotoksik etki gösterdiği gözlendi. HUVEC hücrelerinde THIO, oksidatif DNA hasarında anlamlı bir artışa neden olurken, MDA-MB-231 hücrelerinde bu tür bir etki gözlenmedi. Buna karşın, meme kanseri hücrelerinde Caspase 3 seviyelerinde belirgin bir artış saptandı. RT-PCR analizleri, DNA hasar yanıt genlerinin, özellikle BRCA-1, DNAPKC, MRE11, FOXM1, ve XRCC1'in ekspresyonunda her iki hücre hattında anlamlı bir düşüş olduğunu gösterdi.*

Sonuç: *THIO, hem malign hem de malign olmayan hücrelerde FoxM1 ekspresyonunu inhibe ederek DNA hasar yanıtı genlerini baskılamış ve DNA onarım yollarını bozarak meme kanseri hücrelerinin tedaviye duyarlılığını artırma potansiyeli sergilemiştir. Bununla birlikte, malign olmayan hücrelerde oksidatif hasar oluşturabilme potansiyeli, bu ajanının terapötik etkinliğinin doğrulanması ve normal dokular üzerindeki güvenliğinin değerlendirilmesi için daha kapsamlı araştırmaların gerekliliğine işaret etmektedir.*

Anahtar sözcükler: *FoxM1, kemoterapi direnci, Thiostrepton, meme kanseri, DNA hasar yanıtı*

INTRODUCTION

Breast cancer ranks among the most prevalent cancers affecting women globally (1). The triple-negative breast cancer (TNBC) subtype is particularly challenging due to its poor prognosis, frequent recurrences, and limited treatment options (2). TNBC tumors are typically more sensitive to chemotherapy, but less responsive to targeted therapy than other breast cancer subtypes. Consequently, individuals diagnosed with TNBC face a greater likelihood of distant metastasis, shorter overall survival, and higher mortality rates (3). The diverse molecular characteristics and absence of specific therapeutic targets in TNBC present major obstacles for treatment development. Despite progress in understanding TNBC biology and discovering potential therapeutic targets, there remains a substantial need for effective therapies for TNBC patients.

Numerous studies have indicated that the FoxM1 transcription factor is critical in fostering resistance to chemotherapies that inflict DNA damage, and can facilitate the development of drug resistance if its activation or expression becomes aberrant (4,5). FoxM1 orchestrates DNA repair processes by regulating the transcription of various genes involved in detecting, mediating, signaling, and repairing DNA damage (6,7). FoxM1 has been shown to promote DNA repair by regulating the transcription of its direct target, EXO1, which helps protect ovarian cancer cells from apoptosis induced by cisplatin (8). Cells lacking FoxM1 show reduced levels of XRCC1 and BRCA2 gene expression. Additionally, phosphorylation of FoxM1 by checkpoint kinase 2 (Chk2) occurs in response to DNA damage (6). Notably, studies have shown that FoxM1 indirectly stabilizes the MRN subunits, which are essential for the homologous recombination repair process (5). Given its pivotal role in the DNA damage response, FoxM1 is a key regulator of genotoxic resistance and presents a promising target for therapies designed to overcome resistance to genotoxic agents

Thiazole antibiotics like Thiostrepton (THIO) and Siomycin A are known to reduce mRNA and protein levels of FoxM1, leading to cell death by inhibiting its transcriptional activity (9,10). THIO is a natural thiopeptide antibiotic isolated from Streptomyces species and is used in veterinary applications to prevent bacterial infections (11,12). In addition to its antibacterial properties, THIO exhibits anticancer activity by inducing apoptosis, autophagy, and proteotoxic stress (13,14). THIO is also a specific inhibitor of the FoxM1 transcription factor, which is overexpressed in cancer cells. It has been shown that THIO directly or indirectly suppresses cancer cell proliferation through FoxM1 inhibition, reduces the metastatic ability of cancer cells by preventing epithelial-mesenchymal transition, and inhibits tumor growth (15–17). Although THIO has been reported to have important effects in cancer studies, its cytotoxic and genotoxic effects on non-malignant healthy cells are unknown.

The current study aimed to explore and contrast the cytotoxic and genotoxic responses of HUVECs and MDA-MB-231 cells when exposed to THIO. By evaluating cell viability, DNA damage responses, and apoptotic changes, we intended to clarify whether THIO exerts different effects on healthy endothelial cells and aggressive breast cancer cells. Comprehending these differential impacts may be paramount in assessing the therapeutic possibilities of using THIO to inhibit FoxM1 and overcome resistance to genotoxic agents.

MATERIALS AND METHODS

Cell culture and test chemicals

This study utilized MDA-MB-231 cells, which are a malignant breast cancer cell line, and HUVEC cells, representing a non-malignant cell line. Both cell types were grown in RPMI 1640 medium, which was enriched with 10% fetal bovine serum (FBS, sourced from the US), 100 units/mL of penicillin, and 100 µg/mL of streptomycin. The cultures were kept at 37°C in an atmosphere with 5% CO_2, ensuring their optimal growth and maintenance. THIO was procured from Enzo Life Sciences and dissolved in DMSO to prepare a 10 mg/ml stock solution, with the required dilutions made for all experiments. DMSO (Dimethyl sulfoxide) was obtained from Sigma-Aldrich (St. Louis, MO, USA).

Cell viability assay

The assessment of cell viability was carried out using the MTT assay. Cells were seeded at a concentration of $5x10^3$ cells in each well of 96-well plates, followed by exposure to different THIO concentrations (1.25µM-40 µM) at 37°C for durations of 24 and 48 hours. At the highest concentration of THIO applied to the cells, the DMSO percentage was 0.67%, which remains below 1% and is widely considered safe for most cell lines in terms of cytotoxicity. Notably, the highest concentration of DMSO was used in our control group. After completing the treatment, MTT solution (5 mg/ml) was added to each well and incubated for 3–4 hours. Following incubation, the medium was carefully removed, and 100 µL of DMSO was added to each well to dissolve the formazan crystals. The plates were subsequently placed on an orbital shaker for 5 minutes, and the absorbance was measured utilizing a spectrophotometer. The viability of control cells (those not treated with THIO) was designated 100%, and the viability of the cells exposed to THIO was expressed as a percentage relative to this control.

Real-time PCR analysis

Following a 24-hour treatment with 5 µM THIO, total RNA was extracted by employing TRIzol reagent (Invitrogen), with slight modifications to the originally published protocol (Chomczynski and Sacchi, 1987). The purity and concentration of the RNA were assessed using a Nanodrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Complementary DNA (cDNA) was subsequently synthesized utilizing a cDNA Reverse Transcription Kit (Thermo Fisher Scientific, Waltham, MA, USA). Real-time PCR amplification was executed using a light cycler instrument (Bio-Rad) with HOT FIREPol EvaGreen qPCR Mix Plus (no ROX) (SolisBioDyne, Inc.). Primers specific to BRCA1, DNAPKC, FoxM1, H2AX, MRE11, RAD51, and XRCC1 genes were designed with Oligo7 software, and their sequences were as follows: BRCA1 forward primer - GACAGGCTGTGGGGTTTCTC; BRCA1 reverse primer - TCACACTTTGTGGAGACAGGT; DNAPKC forward primer - TAGGGGAAAGCCATTGCCAG; DNAPKC reverse primer - GGGATCACTCAGGTAAGCCG; FoxM1 forward primer - AGGGTCTCCACAATTGCCCG; FoxM1 reverse primer - CCCAGGGGGAGTTCGGTTTT; H2AX forward primer - TTCACCGGTCTACCTCGCTA; H2AX reverse primer - CGGGCCCTCTTAGTACTCCT; MRE11 forward primer - GGACGCCGTTCTCTCCC; MRE11 reverse primer - TGGGACCAGGTTCTTCCTCA; RAD51 forward primer - AGACCGAGCCCTAAGGAGAG; RAD51 reverse primer - AGTTGCAGTGGTGAAACCCA; XRCC1 forward primer - CCACCAGTGCTCCAGGAAG; XRCC1 reverse primer - TGTCCGTGTTCTCATCCGTG. GAPDH served as the internal reference gene. All experiments were performed in triplicate. Gene expression levels were normalized relative to GAPDH (ΔCt values), and fold changes in mRNA expression were quantified employing the 2^-ΔΔCt method.

Measurement of 8-OH-dG and Caspase-3 levels in cell lysates

The quantification of oxidative DNA damage was carried out utilizing the 8-OHdG ELISA kit (BT Lab

no.: EA0048Hu), whereas caspase 3 activity was assessed with the caspase ELISA kit (Sunred Bio Cat No:201-12-2249). For both measurements, cells were seeded into 6-well plates at a density of $5x10^5$ cells per well. Subsequent to a 24-hour incubation with 5 µM THIO, the cells were transferred into sterile tubes and centrifuged. The cell pellets were then rinsed with phosphate buffered saline, and cell lysis was achieved through three successive freeze-thaw cycles. After centrifugation (2000 rpm for 20 min), the lysate supernatants were collected and stored at -20°C. Finally, the analyses were performed based on the kit instructions, with spectrophotometric measurements at a wavelength of 450 nm.

Statistical Analysis The statistical differences observed between the experimental groups and the control group were evaluated using Student's t-test (GraphPad Software, Boston, Massachusetts USA). Statistical significance was defined as a p-value under 0.05.

RESULTS

Assessment of cell viability

The anti-proliferative effects of THIO on HUVEC and MDA-MB-231 cell lines were evaluated using the MTT assay (Figure 1a, 1b). After incubation with the specified concentrations of THIO, the viability of MDA-MB-231 cells dropped below 50% at distinct concentrations depending on the time of exposure. At 24 hours, cell viability decreased below 50% at a concentration of 40 µM, reaching 50.97%. In contrast, after 48 hours of incubation, cell viability dropped below 50% at a much lower concentration of 2.5 µM, further emphasizing the time-dependent inhibitory effects of THIO on cell viability. In contrast, HUVEC cells exhibited a dose- and time-dependent decrease in viability, but the concentrations required to reduce viability below 50% were higher compared to MDA-MB-231 cells. At 24 hours, cell viability remained above 50% at all tested concentrations, with the lowest recorded viability being 71.24% at 40 µM. However, after 48 hours, cell viability dropped below 50% at a concentration of 5 µM, reaching 30.62%. This highlights the relatively greater resistance of HUVEC cells to THIO compared to MDA-MB-231 cells, demonstrating that malignant cells are more sensitive to THIO.

Figures and Legends

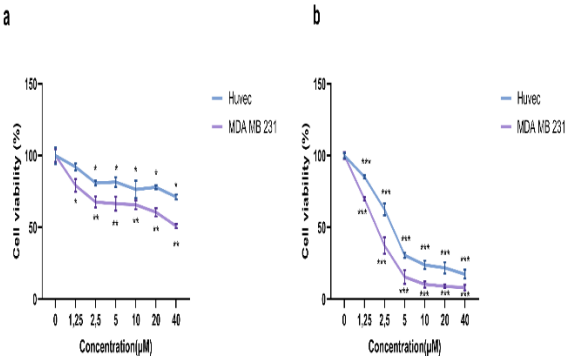

Figure-1. Effects of THIO on the cell viability of HUVEC and MDA-MB-231 cells after 24 hours (a) and 48 hours (b) of treatment. Data are shown as the mean ± SEM obtained from four separate experiments (*p<0.05, **p<0.01, and ***p<0.0001).

Evaluation of 8-OhDG and Caspase-3 level in cell lysates

Figure-2a illustrates the levels of 8-OHdG and Caspase-3 in lysates derived from HUVEC and MDA-MB-231 cells that were treated with 5 µM THIO. The findings revealed that exposure to 5 µM THIO resulted in a notable increase in oxidative DNA damage within HUVEC cells (p-value = 0.0496). Conversely, the breast cancer cells demonstrated a non-significant reduction in oxidative DNA damage compared to the control (p = 0.5972). As depicted in Figure 2b, treatment with 5 µM THIO did not induce a significant change in Caspase-3 levels in HUVEC cells. However, a noticeable increase in Caspase-3 was observed within the breast cancer cells (p = 0.0072).

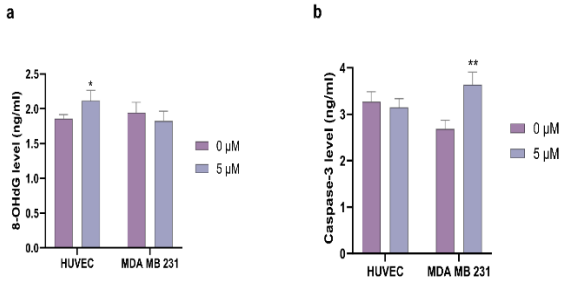

Figure-2. Levels of 8-OHdG (a) and Caspase-3 (b) in HUVEC and MDA-MB-231 cells treated with THIO for 24 hours. The values are reported as the mean ± SD (*p<0.05, **p<0.01).

Assessment of DNA Damage Response Gene Expression

Figure-3 demonstrates the alterations in the expression patterns of DNA damage response genes and FoxM1 in both HUVEC and MDA-MB-231 cells after a 24-hour exposure to 5 µM THIO. In line with expectations, THIO significantly reduced FoxM1 expression in both cell lines; the reduction was more marked in the malignant cell line, with a 10.10-fold decrease, compared to a 1.82-fold decrease in HUVEC. Notably, THIO's impact on DNA damage response genes varied between cell lines. In HUVEC cells, THIO induced suppression of BRCA1, DNAPKC, MRE11, and XRCC1 expression, with reductions of 3.37-, 2.38-, 2.01-, and 3.29-fold, respectively. Conversely, in MDA-MB-231 cells, all assessed DNA damage response genes (BRCA1, DNAPKC, H2AX, MRE11, Rad51, and XRCC1) experienced significant downregulation, with fold changes spanning from 1.52 to 3.04.

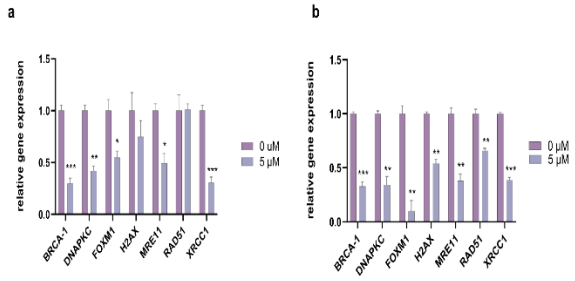

Figure-3. Expression levels of DNA damage response genes in (a) HUVEC and (b) MDA-MB-231 cells following treatment with THIO. The mean ± SD is used to present the data (*p<0.05, **p<0.01, and ***p<0.001).

DISCUSSION

This investigation focused on contrasting the potential of FoxM1 inhibitor THIO in influencing DNA damage responses across both non-malignant and malignant cells. Cell viability was markedly decreased by THIO treatment in both cell lines, with a clear dependence on both dose and duration. In various studies evaluating the cytotoxic effects of THIO, the IC50 value has been observed to range between 3-10 µM in cancer cells (18–20). Consistent with the literature, we found that 2.5-5 µM THIO significantly affected cell viability, especially after 48-hour treatment. However, THIO showed a higher cytotoxic effect on MDA-MB-231 cells than on HUVEC. These findings suggest that THIO's cytotoxic impact may vary in malignant (MDA-MB-231) and non-malignant (HUVEC) cell lines, with MDA-MB-231 cells displaying higher sensitivity or susceptibility to THIO. The stronger effect observed in malignant cell lines such as MDA-MB-231 may support the idea that THIO could be an effective agent against cancer cells. However, observing cytotoxic effects at 48 h of treatment on HUVEC cells also indicates the need to consider potential side effects during treatment or the risk of affecting normal tissues.

Oxidative DNA damage may occur due to various factors. Free radicals, which are reactive molecules generated during metabolic processes or from external sources, such as radiation, certain chemicals, mitochondrial dysfunction, and pollutants, can attack DNA molecules and cause oxidative damage. (21). There are several cellular pathways responsible for repairing oxidative DNA damage, such as base excision repair (BER), single-strand break repair (SSBR), and nucleotide excision repair (NER)(22). Our findings revealed that oxidative DNA damage levels were higher in HUVEC cells compared to malignant MDA-MB-231 cells. Notably, the malignant cells exhibited no significant alterations in oxidative DNA damage, suggesting differential responses to THIO treatment between the two cell types. Previous studies have demonstrated that silencing FoxM1 can induce oxidative stress in cells(23,24). In our study, although THIO treatment significantly suppressed FoxM1 mRNA expression in both cell types, it is possible that THIO may have an indirect effect on oxidative damage and repair in these cells, independent of FoxM1. Additionally, cancer cells have been reported to exhibit high ROS-scavenging activity (23,25), which may contribute to their ability to mitigate oxidative stress. This may explain the lack of significant changes in oxidative DNA damage observed in our study in malignant MDA MB 231 cells. Furthermore, the differential metabolic activity and antioxidant defense mechanisms between malignant and non-malignant cells could also play a role, as cancer cells often adapt their redox homeostasis to support survival under stress. (26). Further investigations are essential to elucidate the comprehensive effects of THIO on oxidative DNA damage and repair pathways, as well as its implications for therapeutic applications in cancer and normal cells.

The difference in caspase 3 levels is also noteworthy. The increased level of caspase 3 in

breast cancer cells suggests that THIO may induce apoptosis in these cells. Studies have shown that THIO induces apoptosis in certain cancer cell lines (13, 19, 27). This suggests that THIO may promote apoptosis in these cells, leading to inhibition of uncontrolled cell growth. The change in caspase 3 levels in HUVEC cells was not statistically significant, indicating that THIO may not effectively activate apoptotic pathways in these non-malignant cells. This finding aligns with our cytotoxicity analyses, which demonstrate that breast cancer cells exhibit a markedly more sensitive response to THIO treatment. The exact mechanism by which THIO affects caspase 3 levels is not yet fully understood. However, THIO may interfere with specific pathways or signaling molecules involved in caspase activation and apoptosis (28).

Our real-time PCR results indicated that THIO significantly suppressed the expression of BRCA1, DNAPKC, MRE11, and XRCC1, which play a crucial role in the DNA damage response, in both cell lines. However, we found that H2AX and RAD51 damage-response genes were also suppressed in breast cancer cells. THIO, a FoxM1 inhibitor, decreased its expression in both cell types. Recent studies have revealed that the oncogenic transcription factor, FoxM1, plays a pivotal role in chemotherapy resistance associated with DNA damage. Aberrant activation or expression of FoxM1 has been shown to potentially promotes drug resistance (4,5). The ability of FoxM1 to induce DNA repair encompasses transcriptional control of a network of genes involved in detecting, mediating, signaling, and repairing DNA damage (6,7). Kuthetuher et al. revealed that targeting FoxM1 using miR-4521 mimics could potentially serve as a novel therapeutic approach in breast cancer by modulating the DNA damage response (29). Wang et al. demonstrated that inhibiting FoxM1, either through shRNA or FDI-6 inhibitor, sensitizes BRCA- expressing TNBC cells to the PARP inhibitor olaparib by reducing DNA damage repair (30). In another study evaluating the anticancer effects of niclosamide on prostate cancer, it was found that inhibition of FoxM1 significantly reduced the expression of genes involved in DNA repair, particularly those associated with homologous recombination (31). These findings indicate that FoxM1 regulates the expression of genes critical for the cellular DNA damage response and that inhibition of FoxM1 may enhance the therapeutic sensitivity of cancer cells through suppression of DNA repair.

CONCLUSION

In conclusion, our study highlights the differential effects of THIO on DNA damage response and cellular mechanisms in malignant and non-malignant cell lines. While the findings provide valuable insights into the therapeutic potential of targeting FoxM1 via THIO in cancer treatment, the study is based on gene expression analyses. Protein-level validation of these findings is crucial to achieving a more in-depth understanding of the mechanisms underlying THIO's effects and its therapeutic potential in cancer treatment. Further research is needed to fully elucidate the complex interplay between THIO, FoxM1, and DNA damage response pathways.

Author contributions

FDK was involved with the conceptualization of the study, developing the methodology, and was responsible for the initial draft of the manuscript. ZD contributed to the conceptualization and methodology of the study, as well as the preparation of the manuscript. FMI was the supervisor of the project.

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Declarations

Conflict of interests

The authors declare that they have no conflicts of interest.

Conflicts of interest: Authors declared no conflict of interest.

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