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Antiproliferative and Apoptotic Effects of Tempol, Methotrexate, and Their Combinations on the MCF7 Breast Cancer Cell Line

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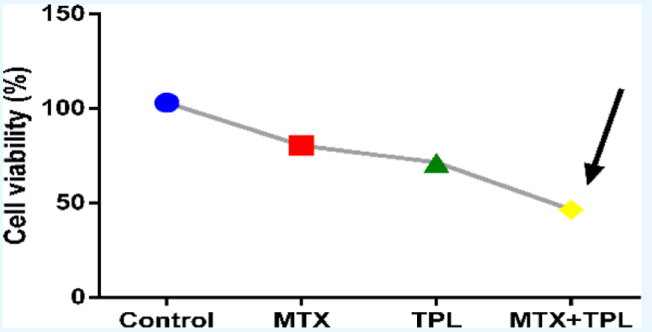
ABSTRACT: Breast cancer holds the top position among the cancers occurring in women. Despite the utilization of surgical removal, chemotherapy, and radiation therapy, there is currently no conclusive treatment available to prevent breast cancer. New treatment approaches are being studied since traditional chemotherapeutics also damage healthy cells. Tempol (TPL) is a potent antioxidant agent that has been shown to exhibit anticancer activity. The objective of this research was to examine the impacts on cell proliferation and apoptosis by using methotrexate (MTX) and TPL individually and in combination on MCF7 breast cancer cells. MCF7 cells were exposed to TPL, MTX, and MTX + TPL for 48 h. The effects of the administered drugs on cell viability were determined using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Enzyme-linked immunosorbent assay analysis was conducted to assess the levels of the antiapoptotic protein Bcl-2, the pro-apoptotic protein Bax, and the activity of caspase-3 in MCF7 cells. Increasing concentrations of TPL and MTX significantly decreased the proliferation in MCF7 cells in both solo and combined use. Solo and combined use of TPL and MTX significantly increased caspase-3 activity and Bax levels and significantly decreased Bcl-2 levels in the cells. This study revealed that the solo use of TPL and MTX inhibited proliferation and increased apoptotic activity in the cells. In addition, TPL increased the antiproliferative and apoptosis efficiency of MTX on cancer cells as a result of the combined use of these drugs.



1. INTRODUCTION

Cancer is an important disease with a high mortality rate which causes changes in the normal cell cycle by uncontrolled division of cells.[1] Specifically, breast cancer stands out as one of the most prevalent cancer types and holds the primary position among all cancers diagnosed in women globally (24.5%).[2] High incidence and mortality rates cause the studies on the treatment of this disease to increase everyday.[3]

Presently, while surgical resection, chemotherapy, and radiotherapy are employed in the management of breast cancer, there is still no conclusive treatment available to proactively prevent the onset of breast cancer.[4] Chemotherapeutic agents used in the treatment cause significant damage to cell deoxyribonucleic acid (DNA) by affecting healthy cells as well as cancer cells.[5] Since the mutations in cell DNA can cause cancer formation, destroying these cells through apoptosis is important.[6]

One of the significant challenges encountered in chemotherapy is the development of resistance in cancer cells against apoptosis.[5] Apoptosis is the controlled death of senescent, harmful, or damaged cells.[7] The development of resistance to apoptosis causes tumor formation by reducing the loss of damaged cells.[6] This situation leads to inadequacy of chemotherapeutic drugs used in the treatment of cancer. For this reason, treatments that target the destruction of tumor cells while having few side effects on healthy cells are being investigated.[4] The use of noncross-resistant drug combinations is more effective than single-drug treatment to overcome apoptosis resistance in breast cancer.[8]

The search for alternative treatments against cancer is still ongoing, and the data obtained are extremely promising. There are many substances that are effective against cancer.[9] Methotrexate (MTX) is an antiproliferative folic acid antagonist, which is considered a basic drug in oncology. It works by inhibiting dihydrofolate reductase, which plays a role in the synthesis of DNA and ribonucleic acid.[10−12] MTX is used in large doses in cancer treatment. However, high doses of MTX cause serious damage to healthy cells.[12] Therefore, although its clinical use continues, it is not the first choice for the treatment of breast cancer. Reducing the cytotoxic effect of MTX on healthy cells by the combined use of it with different substances can be effective in preventing the disease.[10] Various

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antioxidants are used to minimize the cytotoxicity of MTX against healthy cells such as omega-3 fatty acids and vitamin C.[13] In this study, Tempol (4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl) (TPL), which has strong antioxidant properties, was used.

TPL, which has superoxide dismutase (SOD) mimetic activity, is a nitroxyl antioxidant that can cross biological membranes. It protects tissues against oxidative stress by preventing the formation of hydroxyl radicals.[14] Alongside the antioxidant activity of TPL, its high concentrations act as a pro-oxidant that inhibits the growth of cancer cells by increasing the production of cellular reactive oxygen species (ROS).[15]

The objective of this study was to explore the impact of using TPL, known for its robust antioxidant properties, and MTX, a cancer treatment agent, both individually and in combination, on cell viability and apoptosis.

2. RESULTS

Figure 1 depicts the outcomes of cell viability (%) when MCF7 breast cancer cells were subjected to different levels of MTX

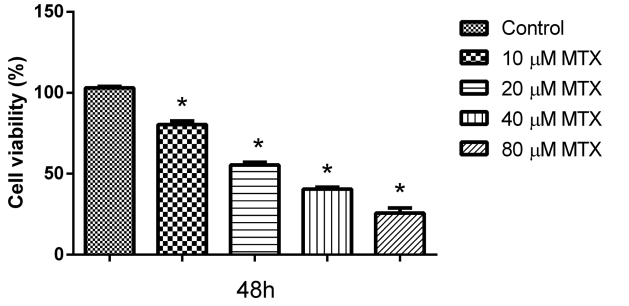

Figure 1. Viability levels in MCF7 cells following the administration of increasing doses of MTX. [n = 7, mean ± standard error of mean (SEM)] (*P < 0.05, when the cell viability is compared with that of the control).

treatment (10, 20, 40, and 80 μM) for a 48 h period. It is evident that higher doses of MTX resulted in a notable decrease in cell proliferation compared to the control group after the 48 h time frame (*p < 0.05).

Figure 2 depicts the outcomes of cell viability (%) when MCF7 breast cancer cells were subjected to different levels of TPL (2, 4, and 8 mM) for a 48 h period. It is evident that higher doses of TPL resulted in a notable decrease in cell proliferation compared to the control group after the 48 h time frame (*P < 0.05).

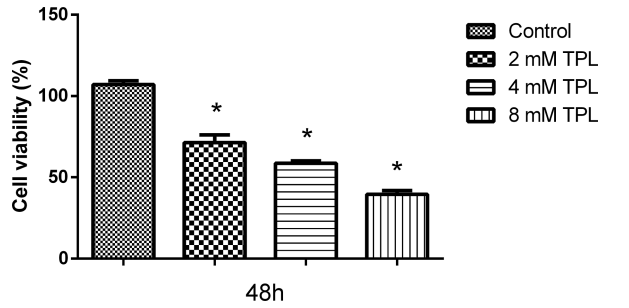

Figure 2. Viability levels in MCF7 cells following the administration of increasing doses of TPL. (n = 7, mean ± SEM) (*P < 0.05, when the cell viability is compared with that of the control).

Figure 3 depicts the outcomes of cell viability (%) when MCF7 breast cancer cells were subjected to MTX (10 μM),

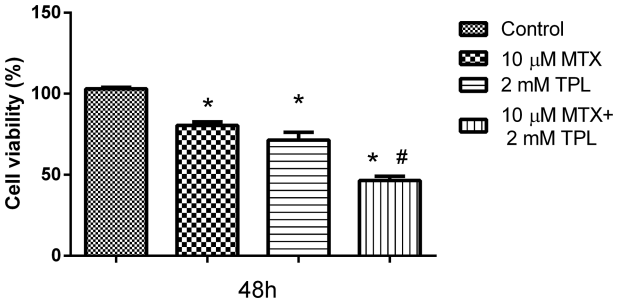

Figure 3. Viability levels in MCF7 cells following the administration of MTX, TPL, and MTX + TPL. (n = 7, mean ± SEM) (*P < 0.05, when the cell viability is compared with that of the control).

TPL (2 mM), and MTX + TPL (10 μM + 2 mM) for a 48 h period. Both individual and combined drug administrations led to a significant reduction in cell proliferation by the end of the 48 h period (*P < 0.05, #P < 0.05).

In this study, the combination of MTX and TPL (MTX + TPL) notably augmented the expression of caspase-3 and the pro-apoptotic protein Bax within the MCF7 breast cancer cells when compared to the control group (#P < 0.05) (Figure 4). Conversely, the MTX + TPL combination considerably diminished the expression of the antiapoptotic protein Bcl-2 in the cells as compared to the control group (#P < 0.05) (Figure 4).

The administrations of MTX and TPL resulted in a notable increase in the levels of caspase-3 and Bax proteins, accompanied by a significant decrease in Bcl-2 levels within the cells, in comparison to the control group (*P < 0.05) (Figure 4).

3. DISCUSSION

In this study, the antiproliferative and apoptotic efficiency of MTX and TPL on MCF7 cells were investigated. TPL is a powerful antioxidant that prevents the formation of hydroxyl radicals by inhibiting the Fenton reaction and has therapeutic potential for disorders resulting from oxidative stress.[16] Apart from its application in treating hypertension and inflammation, it is also employed in addressing neurodegenerative disorders like Alzheimer's disease and Parkinson's disease.[14] In many studies, it has been stated that the use of TPL alone or in combination with drugs used in cancer treatment suppresses proliferation in cancer cells and may be effective in cancer treatment by increasing apoptosis.[14,15,17]

TPL's antioxidant properties stem from its ability to mimic SOD activity. SOD is an enzyme that converts superoxide radicals to hydrogen peroxide and oxygen. Hydrogen peroxide is then reduced to water by other antioxidant enzymes, such as catalase and glutathione peroxidase. In this way, TPL reduces oxidative stress by lowering the levels of ROS in the cells.[18] However, high concentrations of TPL can have a pro-oxidant effect by increasing ROS production in the cells. This can lead to oxidative damage that inhibits the growth of cancer cells and induces apoptosis.[18] Therefore, TPL's anticancer potential depends on the regulation of ROS levels. TPL can be protective for normal cells under oxidative stress but toxic for cancer cells.

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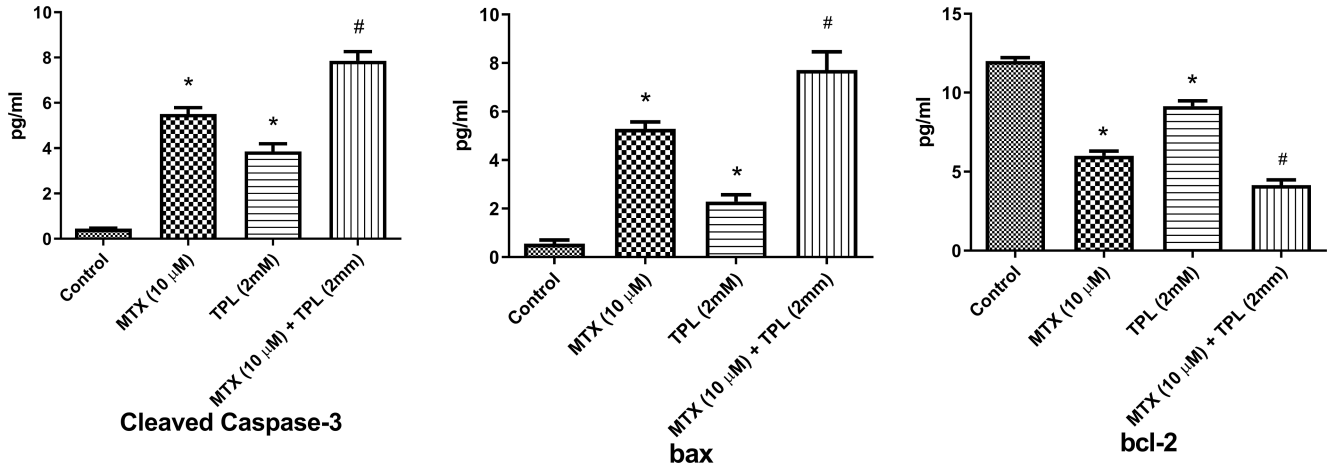

Figure 4. Effect of MTX and TPL on caspase-3, Bax, and Bcl-2 expressions in MCF7 cells. (n = 7, mean ± SEM) (*P < 0.05, when the expression levels are compared with that of the control; #P < 0.05, when the expression levels are compared with that of the control and other groups).

In a study focused on examining the antiproliferative impact of TPL on OVCAR3 and SKOV3 human ovarian cancer cell lines, the findings revealed that the proliferation of cells was inhibited in a manner that depended on the dosage used.[15] TPL has also been documented to enhance the inhibition of proliferation caused by cisplatin, a clinically employed treatment for ovarian cancer, specifically in the OVCAR3 cells. The combined administration of TPL and cisplatin induced apoptosis in OVCAR3 cells, achieved by reducing the expression ratio of Bcl-2 to Bax.[15] In another study, high-dose TPL administration to the lung cancer cell line significantly inhibited cell growth.[14]

Apoptosis is known as programmed cell death, which acts in physiological or pathological processes.[6] Apoptosis, a process regulated by specific genes, serves dual roles: as a homeostatic mechanism safeguarding cells during developmental and aging processes and as a defensive response when cells encounter damage, including conditions like autoimmune disorders, neurodegenerative ailments, and notably cancer.[6,19] This shows that genes acting in apoptosis have both an inducing and suppressive character in cell death.[19] Control and regulation of apoptosis is organized by members of the Bcl-2 protein family, whose main mechanism of action is altering mitochondrial membrane permeability.[20] Bcl-2 from the Bcl-2 protein family is an antiapoptotic protein that suppresses cell death. Bax, the antagonist of Bcl-2, is a pro-apoptotic protein. Bax also counteracts the apoptosis-suppressing activity of Bcl-2.[19] Apart from these proteins, apoptosis is regulated by the balance between pro-apoptotic proteins such as Bad, Bak, Bcl-xs, and pro-caspases and antiapoptotic proteins such as Bcl-xl and Bcl-w.[6] Caspase-3, also recognized as the "executioner caspase", is triggered by initiator caspases such as caspase-8, caspase-9, or caspase-10. It holds a crucial role in the apoptotic pathway by aiding in the breakdown of various proteins within the cell, among which is poly(ADP-ribose) polymerase.[5,6] It was reported that increasing doses of TPL administration induced apoptosis by significantly increasing caspase-3 activation in As4.1 juxtaglomerular cells.[21]

The apoptotic effects of TPL on cancer cells may also occur by inducing oxidative stress in cells.[20] Studies demonstrated that TPL administered to the HL60 human promyelocytic leukemic cell line induces apoptosis in a manner that correlates with the dosage by disrupting mitochondrial functions in cells.[17] It has been reported that apoptosis resulted from induced free radicals, causing oxidative stress. Mitochondria need high glutathione (GSH) levels to prevent oxidative damage.[17] TPL induces oxidative stress by depleting both cellular and mitochondrial GSH levels, which leads to apoptosis. As a result, TPL sensitizes cancer cells to the pro-apoptotic effects of agents used in chemotherapy. While it was observed that 1 mM TPL administered had an antiproliferative effect on HL60 cells at 48 h, higher doses had a cytotoxic effect on cells. In the TUNEL analysis, it was reported that there was an increase in the rate of apoptotic cells at the end of the 24 h due to the increase in TPL concentration.[17]

In this study, we observed that the administration of TPL and MTX at high doses suppressed cell proliferation and triggered apoptosis, which was similar to the results of the studies stated above.

There are studies that used TPL to induce the antiproliferative and apoptotic effects of drugs used clinically in breast cancer and to support its use in treatment. A significant study assessed the antiproliferative and apoptotic impacts of doxorubicin (DOX), TPL, and their combined applications in both human breast adenocarcinoma MCF7 wild type (WT) cells and MCF7 Adr^R cells, which represent the multidrug resistance (MDR) variant of MCF7 WT cells.[22] MCF7 Adr^R cells exhibit a MDR phenotype resulting from the exposure of MCF7 WT cells to DOX. Notably, MCF7 Adr^R cells from both DOX-treated cell lines have demonstrated higher resistance levels compared with MCF7 WT cells. Furthermore, there are reports suggesting that the combined administration of drugs renders MCF7 Adr^R cells more susceptible to the antiproliferative impacts of DOX than MCF7 WT cells. The reason for this is that TPL induces oxidative stress by decreasing intracellular GSH levels and increases the accumulation of DOX in resistant cells. Decrease in GSH levels increases oxidative stress in the cells and causes lipid peroxidation.[23] This triggers a cell death mechanism called ferroptosis.[24] In DOX-resistant cells, the entry and accumulation of DOX into the cell decrease.[25] This reduces the anticancer efficacy of DOX.[26] The causes of DOX resistance include changes in the expression of drug transporter proteins in the cell membrane, impairments in intracellular drug metabolism, and dysregulations in apoptosis pathways.[27] Decrease in GSH levels and increase in oxidative stress can

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enhance the efficacy of DOX by inducing ferroptosis in DOX-resistant cells. Also, TPL's reaction with GSH can facilitate the entry of DOX into the cell by preventing GSH from inhibiting drug transporter proteins.[28] In this way, a decrease in GSH levels can lead to more DOX accumulation in resistant cells. It has been noted that TPL heightens the susceptibility of MCF7 Adr^R cells to apoptosis by elevating the expression levels of pro-apoptotic proteins p21 and Bax, while concurrently reducing the expression level of the antiapoptotic protein Bcl-2.[22] In a different research, various amounts of TPL were introduced to Calu-6 and A549 lung cancer cell lines, alongside normal lung WI-38 VA-13 cells and primary normal HPF cells, over a 48 h period which reduced the growth of the cells.[14] In addition, it was stated that the application of TPL (2 mM) to Calu-6, A549, and WI-38 VA-13 cell lines prevented cell growth by causing an increase in the level of the O_2^- levels. It has been reported that TPL triggers apoptosis in cells by augmenting the levels of caspase-3, a key player in the apoptotic process.[14]

Studies of damaged cell lines determined that the administration of TPL at low doses preserved cell viability and decreased apoptosis. However, the administration of TPL at high doses decreases cell viability, similar to the results in our study. TPL administration to H9c2 cells, whose viability decreased as a result of exposure to hypoxia, increased cell viability significantly and had a protective effect against apoptosis by decreasing caspase-3 expression and Bax/Bcl-2 protein expression ratio.[16] In another notable study, the administration of TPL at low concentrations was found to mitigate the damage caused by $Aß_{1-42}$ in human SH-SY5Y neuroblastoma cells. This resulted in an increase in cell viability and a decrease in the rate of apoptosis. However, it has been documented that when TPL was administered at high concentrations, it led to a decrease in cell viability.[29]

4. CONCLUSIONS

In conclusion, this study revealed that TPL can be used as a potential anticancer agent in breast cancer treatment as it induces the antiproliferative and apoptotic effects of MTX on the human breast cancer cell line MCF7. Moreover, since it is thought that this combination may generate similar effects in other cancer cell lines, it was concluded that our data form the basis for future studies.

5. METHODS

5.1. Cell Culture. Human breast cancer cell line MCF7 was purchased commercially from the American Type Culture Collection (ATCC). MCF7 cells were grown in T25 flasks in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco) supplemented with 10% fetal bovine serum, 1% L-glutamine, and 1% penicillin-streptomycin (HyClone). Cells were incubated at 37 °C in humidified conditions with 5% CO_2 and routinely passaged every 4−5 days. TPL (Sigma-Aldrich) and MTX (Sigma-Aldrich) were dissolved in dimethyl sulfoxide (DMSO). Cells reaching 80% density were seeded into six-well plates (1×10^5). When the cells became monolayer, the control group was treated with fresh medium and the other groups were treated with TPL, MTX, and TPL + MTX for 48 h.[14]

The MCF7 cell line is the most widely used human breast cancer cell line in breast cancer research. The popularity of this cell line stems from its retention of many characteristics specific to the mammary epithelium.[30] MCF7 cells reflect many aspects of breast cancer in the clinical setting.[31] Especially in the management of postmenopausal women with hormone receptor-positive breast cancer, MCF7 cells have served as a valuable model system to elucidate other pathways of hormone response and resistance.[30] Therefore, choosing the MCF7 cell line is a reasonable decision to understand and treat the molecular heterogeneity of breast cancer.

5.2. MTT Assay. The impact of TPL and MTX on the proliferation of cultured cancer cells was assessed by using the MTT assay. Cells were seeded into 96-well plates at a density of 1×10^4 cells per well. Following a 48 h exposure to varying concentrations of TPL (2, 4, and 8 mM), MTX (10, 20, 40, and 80 μM), and the combination MTX + TPL (10 μM + 2 mM), the cell culture medium was removed. Subsequently, the cells were treated with 100 μL of MTT solution (dissolved in 0.5 mg/mL DMEM) and incubated at 37 °C with 5% CO_2 for 2 h. Afterward, the MTT solution was discarded, and 100 μL of DMSO was introduced into each well. The optical density (OD) was measured at 550 nm by using a microplate reader (EL800, Bio-Tek Instruments, Inc.). The viability was calculated by determining the ratio of the average OD from each measurement outcome to that of the control group.

5.3. Cell Homogenization. Cells treated with TPL, MTX, and TPL + MTX for 48 h in the six-well plates were transferred to 15 mL tubes. Following centrifugation at 2000 rpm at 4 °C for 10 min, the liquid fraction was aspirated. Subsequently, 5 mL of phosphate-buffered saline (PBS) was added to the tubes, and centrifuged again at 4 °C at 2000 rpm for 10 min. The PBS was then removed. To the cells were added 250 μL of radio-immunoprecipitation assay (RIPA) buffer, along with 2.5 μL of phenylmethylsulfonyl fluoride (200 mM), 2.5 μL of sodium vanadate (100 mM), and 2.5 μL of protease inhibitor. Homogenates were prepared from the cells using an ultrasonic homogenizer while maintaining a cold environment. These homogenates were subsequently subjected to centrifugation at 10,000 rpm for 10 min, leading to the separation of supernatants, while the remaining sediment (pellets) was discarded.

5.4. Enzyme-Linked Immunosorbent Assay. The activity of caspase-3 and expressions of Bcl-2 and Bax protein (Abcam) were determined with the purchased enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer's protocols.

5.5. Statistical Analysis. The significance of differences in protein expressions and MTT levels between the groups was evaluated using Student's *t*-test. Statistical significance was acknowledged when the p-value was less than 0.05 ($p < 0.05$).

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.3c07624.

Data obtained from experiments used for analysis and to form graphs (XLSX)

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Notes

The authors declare no competing financial interest.

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