Biointerface Research in Applied Chemistry
Open-Access Journal (ISSN: 2069-5837)

Article
Volume 13, Issue 6, 2023, 593
https://doi.org/10.33263/BRIAC136.593

Inhibition of IRAK Signaling Can Improve Topotecan Sensitivity of Breast Cancer Cell Lines by Decrease of P-gp Gene Expression

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Received: 28.01.2023; Accepted: 24.02.2023; Published: 8.04.2023

Abstract: Breast cancer is the most prevalent malignancy among females. Drug resistance is one of the major problems in successfully treating cancer with chemotherapy. P-gp is the key factor in the development of drug resistance in breast and many other cancers. Also, drug resistance is influenced by inflammation, and for this reason, we examined the effect of suppression of inflammation by IRAK-inhibitor on Topotecan sensitivity. BT20, BT549, MB468, and MCF7 breast cancer cell lines were cultured, and then the various concentration of topotecan was added to the cell culture medium with or without IRAK inhibitor. The viability of the cells was measured by the WST1, and EC50 was computed. Cell apoptosis or necrosis was measured by flow cytometry, and expression of P-gp was evaluated by Real-Time PCR. IRAK1 inhibitor decreased IC50 in the four cell lines, especially in the MB-468 cell line. Flow-cytometry results indicate an apoptotic effect of IRAK inhibitor on all cell lines. Also, IRAK inhibitors reduced the gene expression of ABCB1 in four cell lines. Our results showed that inhibitors of IRAK can sensitize several breast cancer cell lines to topotecan by inhibiting inflammation and decreasing P-gp expression.

Keywords: topotecan; drug resistance; P-glycoprotein; breast cancer; IRAK inhibitor.

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1. Introduction

Breast cancer has become the most common type of malignancy and the second leading cause of cancer death in women worldwide [1, 2]. The Annual incidence of breast cancer is about 2.1 million new cases worldwide, and it has the highest rate of death from cancer [3].

Chemotherapy is one of the main procedures to treat breast tumors, which reduces the mortality of breast cancer patients and decreases its recurrence [2]. Two types of drug resistance, i.e., intrinsic and acquired drug resistance, have been identified in cancer. [4]. It is estimated that more than half of the patients have breast cancer do not respond to commonly used drugs (intrinsic resistance)[5], and in many cases, tumors that respond to primary drugs

recur and become insensitive to a wide range of drugs with different chemical structures and performances (acquired resistance)[6, 7]. Therefore, resistance to chemotherapy is one of the main obstacles to successful treatment, especially in some types of breast cancer, such as triple-negative breast cancer (TNBC)[8].

The ATP binding cassette (ABC) transporters, which are expressed on the cell membrane, play an important role in this phenomenon[9]. P-gp is the first member of the family B in the superfamily of ABC transporters, which is expressed by the MDR-1 gene in many tissues[10, 11]. Studies show that this protein causes drug resistance in several breast cancer subtypes against many antitumor agents, including Topotecan [12].

Topotecan, a topoisomerase 1 inhibitor, has strong anticancer effects on breast, cervical, ovarian, and small-cell lung cancers [13]. A strategy is required to increase its efficacy and limit its toxicity because of the toxicity related to Topotecan doses[14].

The interleukin-1 receptor-associated kinase (IRAK) is one of the newly known factors associated with chemoresistance in cancer cells. Among the four members of the IRAK family (IRAK1 / IRAK2 / IRAK3 / IRAK4), IRAK1 and IRAK4 have serine/threonine kinase activity[15, 16]. These proteins are essential mediators for Toll-like receptors (TLRs) signaling and IL-1 receptors[15, 16]. TLRs can also be stimulated by stress-induced endogenous signals or dying cells or by various exogenous stimuli such as bacteria, fungi, yeasts, and viruses[14]. Myeloid differentiation primary response 88(MYD88) plays an important role in the signaling process of TLRs/IL 1R, whose activity initiates IRAK1 and IRAK4 activity. The non-phosphorylated form of IRAK1 is isolated from MYD88 and activates the path of NFκB by binding to Tumor necrosis factor receptor (TNFR)-associated factor 6(TRAF6)[17].

Based on epidemiological findings, inflammation plays an important role in the development, progression, and drug resistance in cancer. Therefore, targeting molecules involved in inflammation can be a useful strategy to increase drug sensitivity in cancer[18]. IRAK is one of the multiple factors that result in inflammation and inflammatory responses by affecting many inflammatory genes in the immune cell[19, 20]. Studies on non-squamous cell lung cancer (NSCLC) patients have shown that tumor tissue significantly increases the expression of IRAK[21]. Also, increased expression of IRAK1 plays an important role in developing resistance to paclitaxel in breast cancer[22]. On the other way, Melanoma cell lines showed increased expression and activity of IRAK1 and IRAK4. Using specific siRNAs against IRAK1 and IRAK4 increases the sensitivity of the melanoma cell line to Vinblastine[23].

However, it has not been examined the relationship between ATP-binding cassette transporters and drug resistance yet, and it seems that the increase of ATP-binding cassette transporters is one of the mechanisms of IRAK to increase drug resistance, which this research aims to examine. In this case, IRAK inhibitors may be combined with common chemotherapy drugs to increase drug sensitivity. The current study examined the effects of IRAK inhibition on the sensitivity to topotecan, which is the P-gp substrate. We selected different cell lines to measure IRAK inhibition signaling on drug sensitivity due to the different characteristics of tumors in malignancy and drug sensitivity.

2. Materials and Methods

2.1. Cell culture.

All breast cancer cell lines, including MCF-7, BT-549, MDA-MB-468, BT-20 were provided by the Iranian Biological Research Center. MCF-7 cells were cultured in the DMEM medium (Gibco, 12800-017), BT-549 in the DMEM medium with 2 mM L-glutamine, and BT-20 and MDA-MB-468 cells in Ham's F12 medium with L-glutamine (2 mM) in an incubator with 5% CO2 at 37°C, all of them were supplemented with FBS 10% and 1% penicillin/streptomycin. The cells were passaged at least 10 times before performing the experiments.

2.2. Reagents.

Topotecan (Sigma, T2705) was procured in dimethyl sulfoxide (DMSO). The concentrations required for treatment (100,10,1,0.1,0.01,0.001 μg/ml) were made using a complete medium from the original stock. IRAK1/4 (Sigma, I5409) specific inhibitor stock solution was also provided in DMSO. Each treatment used an inhibitor stock volume that made the concentration of 1 μg/ml [16] for this inhibitor in the treatment medium.

2.3. Cell survival assessment.

A total of 10,000 cells with a viability of 100% and a volume of 200 μl from the cell plate obtained from the cell culture centrifuge were added to the wells of a 96-well plate, providing the time required for cells to adhere to the well bottom 2 hours before treatment. The supernatant was replaced with fresh medium after 24 hours, and each cell line treated differently with the expressed concentration pattern: the control group has not been treated, one topotecan-treated group, the topotecan+ IRAK1/4 inhibitor-treated group, and the IRAK1/4 inhibitor-treated group. 20 μL of the WST-1 reagent solution (Roche, 05015944001) was added to each well after 72 hours. The amount of surviving cells was measured by WST1 and compared to non-medicated controls after 2 hours of incubation. In this test, the tetrazolium salt was converted to formazan by the dehydrogenase enzymes' activity in the cells' mitochondria, whose absorption was measured at 460 nm wavelengths.

2.4. Apoptosis assessment.

The IRAK1/4 inhibitor effect on apoptosis was measured using apoptosis analysis due to the increased drug sensitivity to Topotecan[21]. Briefly, MCF-7, BT-20, BT-549, and MB-468 cells were cultured in a 12-well plate at a density of 2X 105 cells/well. And 24 hours later, incubated with the desired concentration of 1 μg/ml of topotecan and with 1 μg/ml of IRAK-specific inhibitor (Sigma: I5409) for 72 hours. The cells were collected by trypsin-EDTA solution and centrifuge after incubation. Then the cells were washed with PBS, and the Annexin V apoptosis detection kit (BD Biosciences, USA) was used to stain the BT-20, BT-549, and MB-468 cells in accordance with the manufacturer's instructions. The early stage of apoptosis was defined with positive Annexin V and negative 7AAD, and the late stage as cells with positive Annexin V and 7AAD. The MCF-7 cells were subjected to flow cytometry using a Propidium iodide (PI) staining solution.

2.5. Quantitative Real-time PCR.

Quantitative Real-time PCR: Using the RNeasy mini kit from Qiagen in accordance with the manufacturer's instructions, total RNA from each cell line (MCF-7, BT-20, BT 549, and MB-468) was extracted. The absorbance of ultraviolet (UV) light at 260 nm and 280 nm wavelengths was used to calculate the RNA concentration. (ND-1000 Nanodrop). After electrophoresis in a 2% agarose gel, the 18S and 28S ribosomal RNA bands were stained with ethidium bromide to validate the RNA quality.

Then cDNA synthesis was performed using QuantiTect Reverse Transcription Kit (Qiagen). Relative qPCR was performed on a Qiagen Thermal Cycler (Mic PCR, Bimolecular system) using the comparable QuantiFast SYBR Green PCR kit (Qiagen). The cycle threshold (CT) was calculated for each sample. Results were calculated by the equation: $2^{-\Delta\Delta CT}$.The sequence of primers used in this study are Forward (5'CGGGAGCAGTCATCTGTGGT3') and Reverse (5'CAAAGAGAGCGAAGCGGCTG3') for P-gp AND Forward (5'GACTACGAGACCGAGCTCCAGGAGT3') and Reverse (5'TGGACACCTCCGAAGTCCTTGCCCAA3') for Actin.

2.6. Statistical analysis.

All data are presented as Mean±SEM. Probit Regression Analysis was performed for the calculation of IC50. Students' t-test is used to examine the differences in IC50 and gene expression between topotecan and topotecan+IRAKi treated groups. The SPSS software version 20 for Windows was used to perform the statistical analyses. P<0.05 were considered statistically significant.

3. Results and Discussion

3.1. IRAK inhibitor decreases Topotecan IC_{50}.

Our results indicated that IRAK inhibitor (1 µg/ml) alone does not affect cell viability compared to the control group in all breast cancer cell lines.

As Figure 1 shows, the results of the WST-1 test indicated that in the MCF-7 cell line, the IC50 values were 188 µg/ml for topotecan in the 72-hour treatment. By adding IRAK inhibitor (1 µg/ml) to the culture medium, the IC50 of topotecan diminished to 78 µg/ml.

IC50 values in the BT-20 cell line for topotecan were 70 µg/ml, and the addition of IRAK inhibitor (1 µg/ml) to the medium decreased the IC50 to 31 µg/ml for topotecan (Figure 1).

As it is observed in Figure 1, IRAK inhibitor (1 µg/ml) can decrease IC50 of Topotecan from 132 to 60 µg/ml of the culture medium in the BT-549 cell line.

Finally, as it is clear in Figure 1, the IC50 of topotecan alone was 111 µg/ml for the MB-468 breast cancer cell line, and simultaneous treatment of cells with topotecan+IRAKi showed 14 µg/ml IC50 for topotecan.

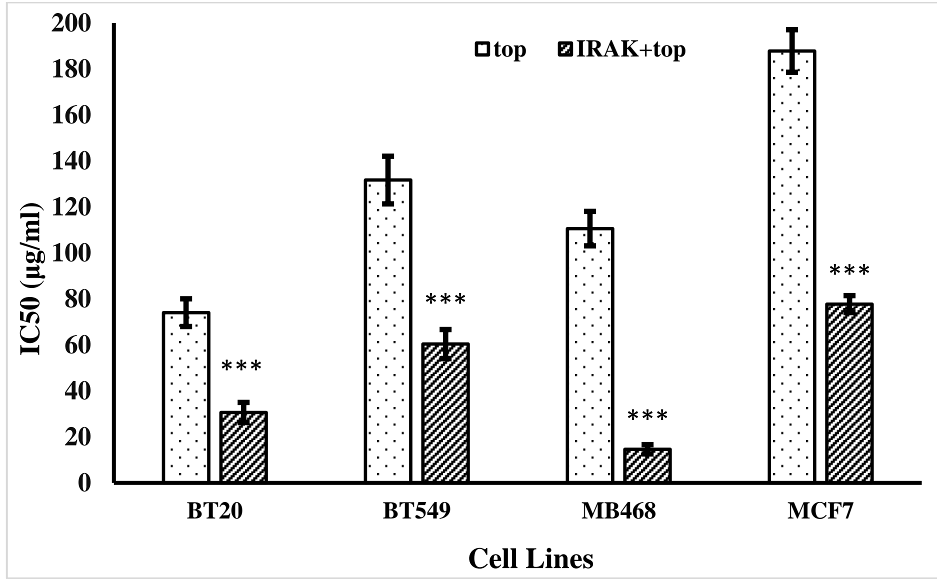


Figure 1. The effect of IRAK inhibitor on the reduction of IC_{50} of Topotecan in different breast cancer cell lines. The cells were treated with different topotecan concentrations (0.001, 0.01, 0.1, 1, 10, and 100 µg/ml) with a constant amount of IRAK inhibitor (1 µg/ml) for 72 hours. Then, the rate of cell death was measured using the WST1 kit, and IC50 was calculated by Probit regression analysis.

3.2. IRAK inhibitor effect against breast cancer cell lines

The type of cell death was analyzed by PE Annexin V Apoptosis Detection Kit to ensure that IRAK inhibitor, in combination with topotecan, induces apoptosis, not necrosis in BT-20, BT-549, MB-468, and MCF-7 breast cancer cell lines.

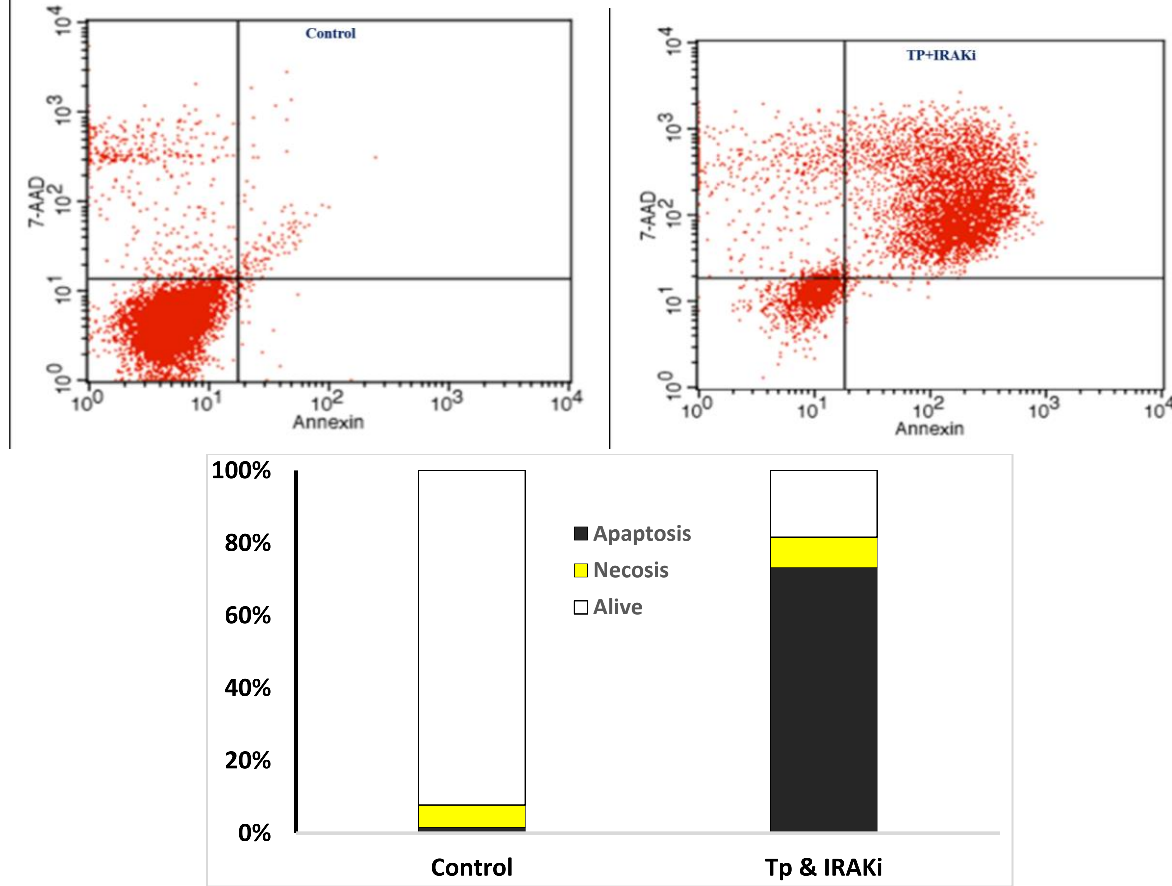


Figure 2. Comparison of the percentage of living cells, necrosis, and apoptosis in BT-20 cell line. The cells were exposed to topotecan (1 µg/ml) and IRAK1/4 inhibitor (1 µg/ml) for 72 hours and were compared to the control (untreated cells).

Annexin negative/7-AAD negative cells are considered living cells; Annexin positive/7-AAD negative cells are at early stages of apoptosis; Annexin positive/7-AAD positive cells are at late stages of apoptosis; and Annexin negative/7-AAD positive cells are considered to be necrotic.

As shown in Figures 2–5, incubation of BT-20, BT-549, MB-468, and MCF-7 cell lines with IRAK inhibitors and topotecan for 72 hours significantly increased annexin V and annexin V/7AAD-positive breast cancer cells.

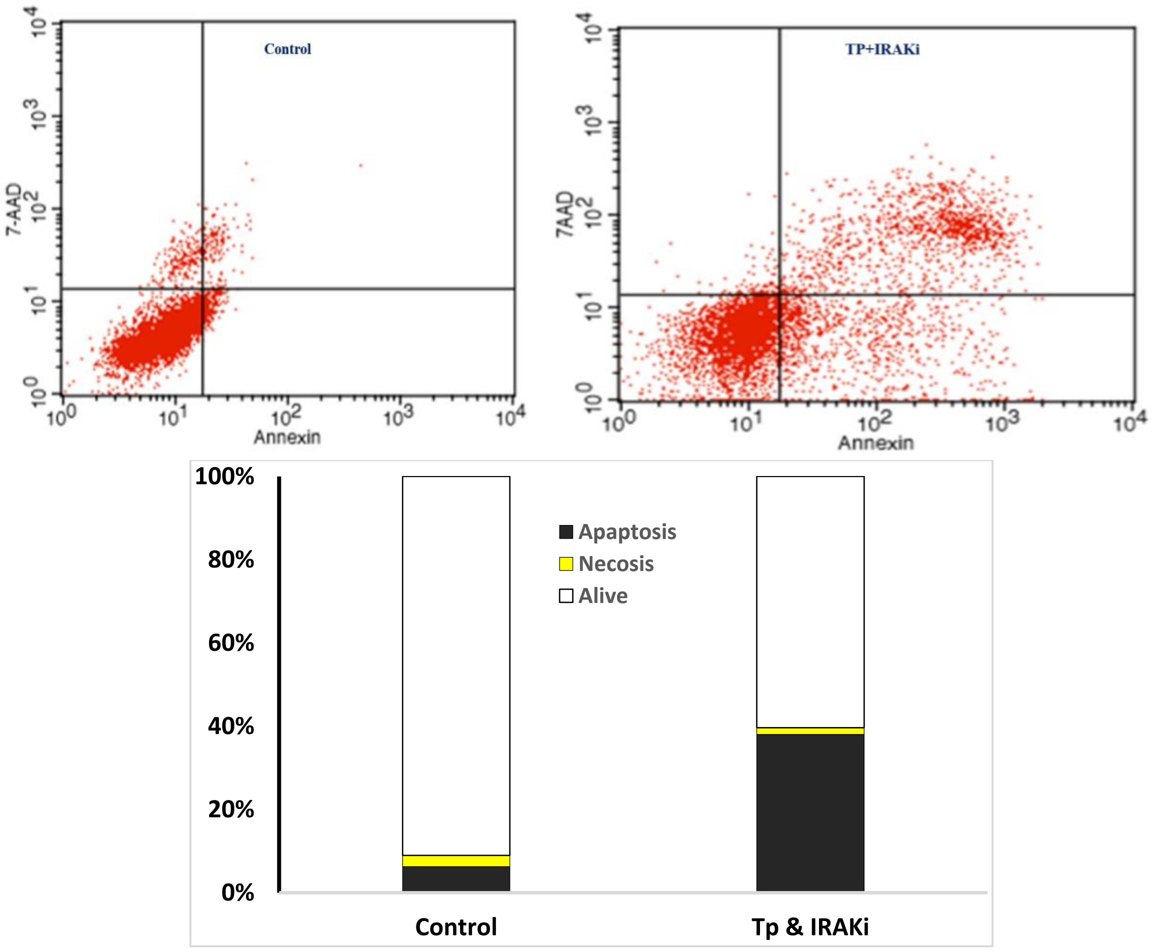
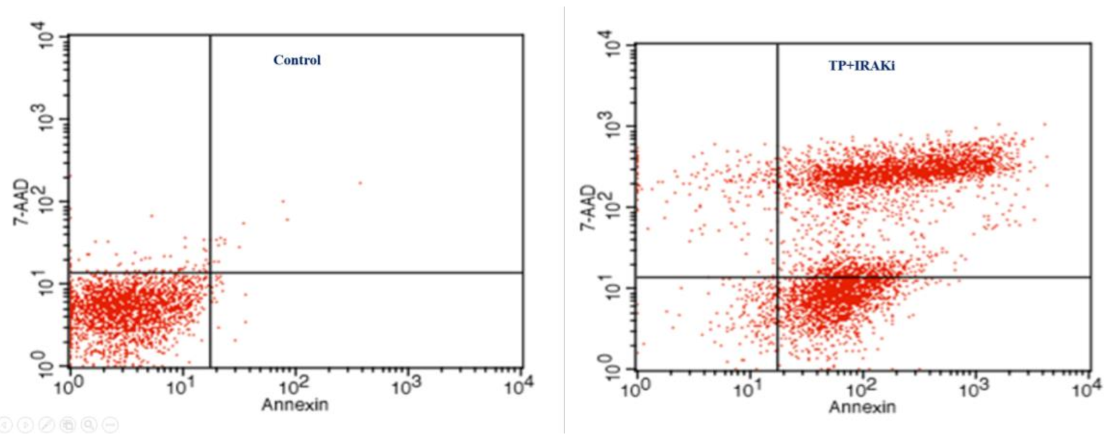


Figure 3. Comparison of the percentage of living cells, necrosis, and apoptosis in the BT-549 cell line. The cells were exposed to topotecan (1 μg/ml) and IRAK1/4 inhibitor (1 μg/ml) for 72-hour and were compared to the control (untreated cells).



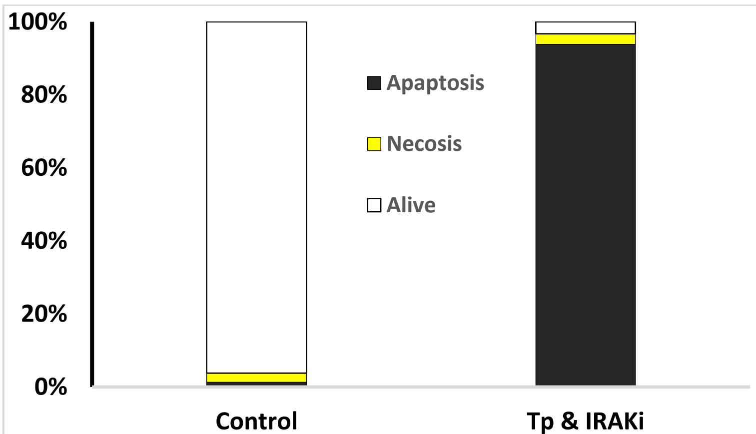


Figure 4. Comparing the percentage of living cells, necrosis, and apoptosis in +the MB-468 cell line. The cells were exposed to topotecan (1 μg/ml) and IRAK1/4 inhibitor (1 μg/ml) for 72 hours and were compared to the control (untreated cells).

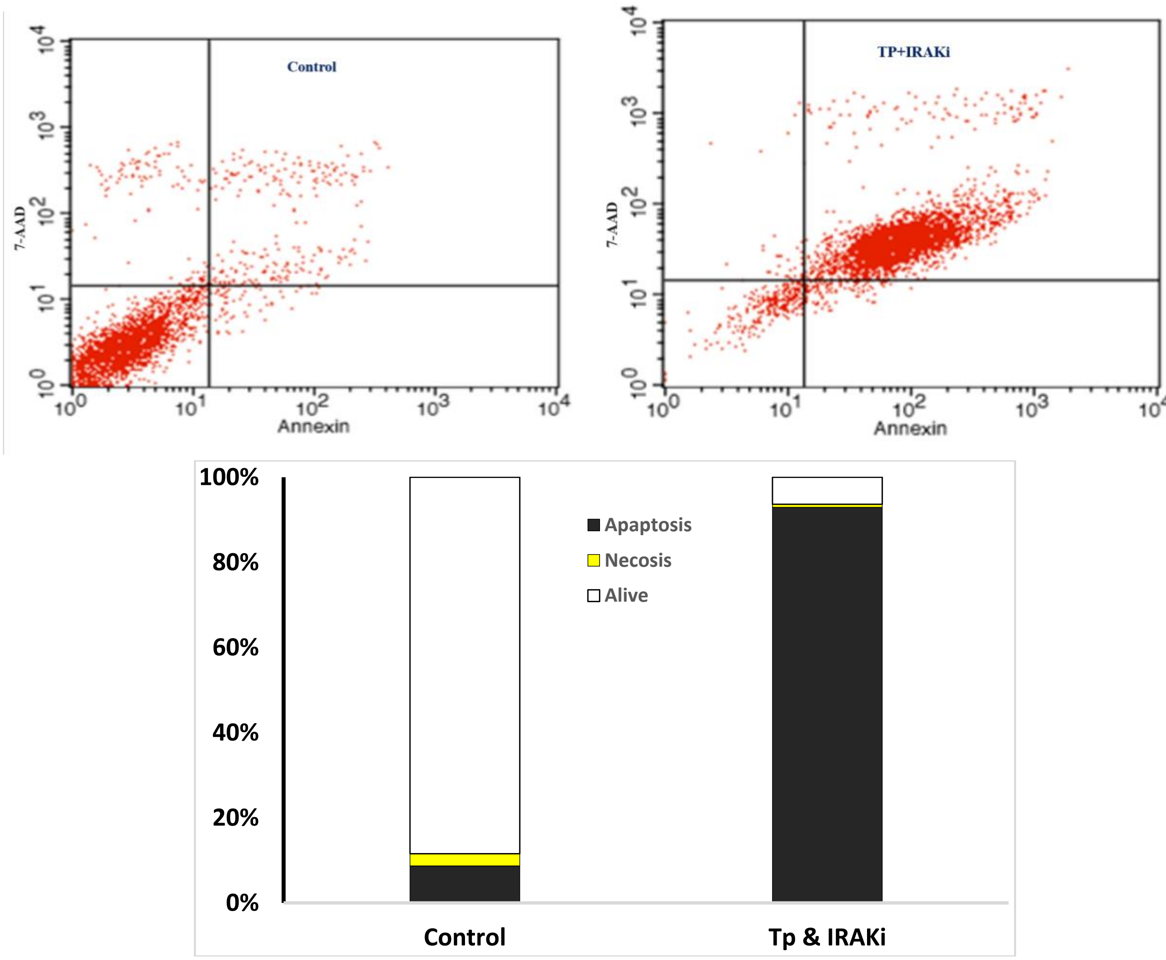


Figure 5. Comparison of the percentage of living cells, necrosis, and apoptosis in MCF-7 cell line. The cells were exposed to topotecan (1 μg/ml) and IRAK1/4 inhibitor (1 μg/ml) for 72-hour and were compared to the control (untreated cells).

3.3. The effect of IRAK inhibitor on the transcription of ABCB1 gene.

As shown in Figure 6 IRAK inhibitors significantly decrease transcription of the ABCB1 gene, which codes P-gp, in four breast cancer cell lines including BT-20 and BT-549, MB-468, and MCF-7.

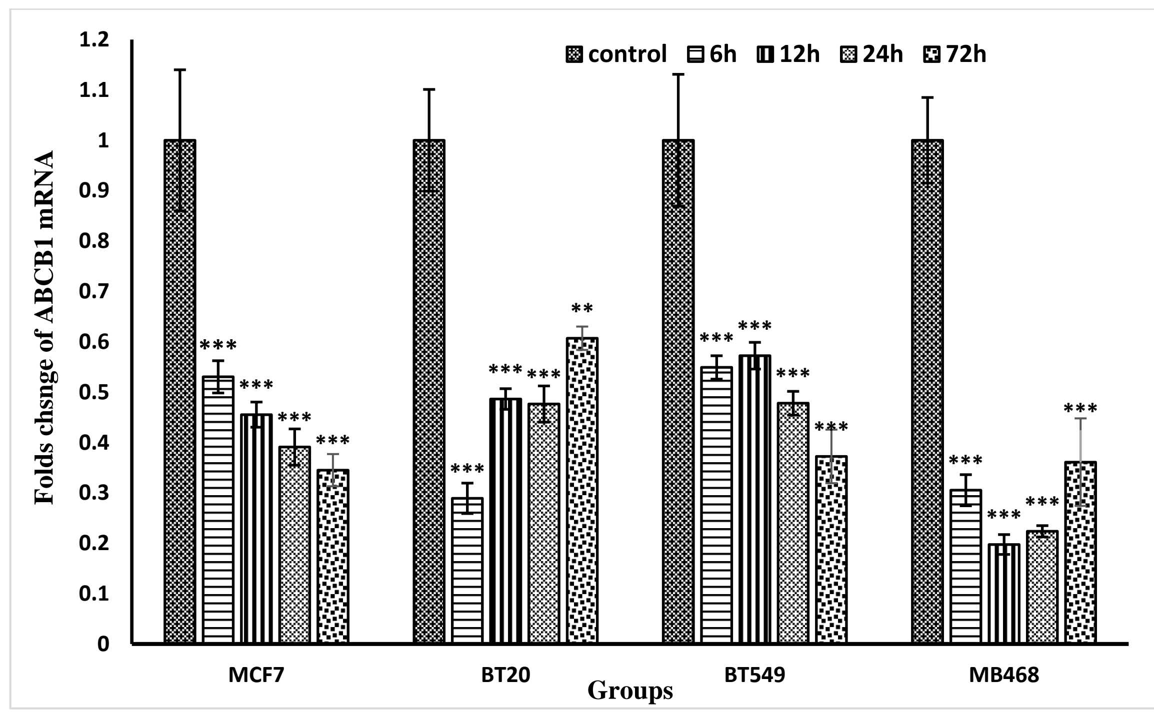


Figure 6. The effect of IRAK1/4 inhibitor on ABCB1 gene expression in Breast Cancer cell lines. All cells were exposed to IRAK1/4 inhibitor (1µg/ml) for 6, 12, 24, or 72 hours, then total RNA was extracted, and qPCR with specific primers was performed. Relative mRNA expression of ABCB1 compared to the control group (no IRAK inhibitor). Results are presented in three replicates. (P<0.0001***).

4. Conclusions

Breast cancer is one of the most prevalent cancers; about 2.3 million new cases are diagnosed yearly [24]. Although chemotherapy has a significant effect at the start of tumor treatment, chemotherapy resistance is an important challenge in this direction. Multidrug resistance contributes to the death of more than 90 percent of patients who receive chemotherapy[25].

In our study, WST-1 results for the first time showed that using the concomitant of IRAK and Topotecan can reduce the IC50 of this anticancer agent in breast cancer cell lines, including BT-20, BT-549, MB-468, and MCF-7. Among breast cancer cells, the most expression level of IRAK is reported in Triple Negative Breast Cancer (TNBC) cells [22, 26]; thus, reducing IC50 in BT-20, BT-549, and MB-468 cell lines that are TNBC is higher than MCF-7 cell line, which is not in this group of breast cancer cells.

The greatest ability of IRAKi to reduce IC50 of topotecan was observed in MB-468 cell line. When a tumor metastasizes, the expression and activity of IRAK increase in it and thus becomes more sensitive to IRAK inhibitors [22]. Therefore, it is expected that a further decrease in the IC50 of topotecan will be observed as a result of IRAK inhibitor treatment in the MB-468 cell line.

IRAK1 is likely the key factor causing acquired chemotherapy resistance. IRAK1/4 activation can induce various downstream kinases and transcription factors, including NF-κB, P-38, and MAPK [27]. NF-κB- related cytokines such as IL-6, IL-1β, and CXCL play a significant role in the growth, metastasis, and drug resistance of TNBC [22]. On the other hand, because the direct targeting of NF-κB in patients has been proven to cause severe toxicity [28] and safe and effective NF-κB inhibitors are still clinically unavailable[29], upstream mediators in the activation of NF-κB will be a good target for inhibition of NF-κB pathway.

Also, the P-38 signaling pathway is related to apoptosis in cancer cells, and inhibition of P-38 MAPK is related to increased sensitivity to doxorubicin [30]. Wee *et al*. showed that both groups of cytokine-induced by NF-κB and P-38 signaling have an important role in developing paclitaxel resistance, and drug-targeting to IRAK-1 may be an effective option to treat advanced metastatic TNBC because these factors are downstream of IRAK-1 [22]. Therefore, it can be said that IRAK inhibitors can increase drug sensitivity and induce apoptosis through the suppression of MAPK and NF-κB signaling pathways. These results can be compared to the study conducted by Srivastava R *et al*. that indicated the inhibition of IRAK1 and IRAK4 by specific siRNA increases the drug sensitivity to vinblastine in melanoma cell lines[23].

It also has been specified that NF-κB activation results in changes in the activity of P-gp, which is an important factor in multidrug resistance[31]. Since topotecan is a substrate of P-gp [32], we evaluated the effect of IRAK inhibitor on ABCB1 gene expression, for one of the IRAK inhibiting mechanisms in increasing drug resistance may be mediated by ABC transporters.

RT-qPCR test results showed that IRAK inhibitor reduces the ABCB1 gene expression in 4 cell lines of MB-468, BT-20, BT-459, and MCF-7. NF-κB translocation to the nucleus to bind to the promoter region of the MDR-1 induced MDR-1 expression and affected P-gp expression [33]. A study conducted on patients with hepatocellular carcinoma has shown that increased expression of NF-κB is related to multidrug resistance. Interference with the activation of NF-κB can inhibit P-gp expression and increase the sensitivity of hepatocellular carcinoma to anticancer drugs[34]. Also, Zhou Xu *et al*. showed that inhibition of P-38 MAPK signaling reduced the expression of P-gp and increased sensitivity to doxorubicin in the MCF-7 cell line. Cells with drug resistance have high levels of phosphorylated P-38, which is related to increased expression of P-gp in the MCF-7 cell line[30]. IRAK inhibitor can result in suppression of ABCB1 transcription, and decreased P-gp can induce Topotecan sensitivity via indirect inhibition of P-38 MAPK and NF-κB results.

In conclusion, Breast cancer is one of the most prevalent cancers, and this study showed that IRAK inhibitor, as an inhibitor of the inflammation pathway, can increase the sensitivity of some breast cancer cell lines to topotecan by decreasing the expression of P-gp. Also, our results suggested that the combination of IRAK inhibitor and topotecan causes cell death via apoptosis and not necrosis. This study showed that IRAK inhibitor is a good option to increase chemotherapy efficiency, but further studies are required to prove it.

Funding

This research was funded by the Vice Chancellor for Research and Technology, Kerman University of Medical Sciences, Kerman, Iran, grant number 950088.

Acknowledgments

We would like to appreciate the Kerman Medical Unversity and Kerman Research Council's help and support in conducting this study.

Conflicts of Interest

The authors have no acknowledgments to declare.

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