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EFFECT OF IRAK1/4 INHIBITOR ON IL-1β, IL-6, INF-γ AND TNF-α EXPRESSION IN BREAST CANCER CELLS OF SEVERAL LINES

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Recent studies have shown that inflammation mediated via interleukin-1 receptor-associated kinases (IRAKs) is associated with cancer cells drug resistance. We aimed to evaluate the expression of inflammatory cytokines as the potential mechanism involved in the development of cancer cells resistance to conventional chemotherapy drugs. Breast cancer cells of BT549, BT20 and MB468 lines were treated with IRAK 1/4 inhibitor alone or in combination with chemotherapeutic agents methotrexate and topotecan. Expression of IL-1β, IL-6, TNF-α, and IFN-γ genes was quantified by real-time PCR. It was found that IRAK1/4 inhibitor suppressed IL-1β expression in BT549 cells at most and had minimal effect on IL-6 expression in MB468 cells. For the first time we showed that concomitant use of IRAK1/4 inhibitor with topotecan and methotrexate reduced IL-1β, IFN γ, TNF-α and IL-6 expression in BT-20, BT-549, MB-468 cell lines compared to the controls. It is suggested that specific IRAK inhibitors in combination with conventional chemotherapy can be used in cancer treatment to increase drug sensitivity and decrease the risk of tumor recurrence.

K e y w o r d s: breast cancer, methotrexate, topotecan, inhibitor of interleukin-1 receptor-associated kinase, real-time PCR.

According to World Health Organization (WHO) statistics in 2018, after lung cancer, breast cancer is the second leading cause of cancer death in the world. Among women, breast cancer has been diagnosed as the most common cancer, accounting for 11.6% of all cancer deaths [1]. Surgery is the first step in the treatment of breast cancer, which then continues with radiotherapy and chemotherapy [2], and therefore chemotherapy is one of the main ways to treat and prevent the cancer from returning. In chemotherapy, the drugs inhibit the growth of cancer cells, kill the cells or stop them from dividing [3, 4]. Drug resistance is considered as the biggest obstacle in the recovery of cancer patients and the appropriate response to treatment [5]. Studies show that the increase of some pro-inflammatory cytokines by cancer cells is involved in the development of primary and acquired drug resistance [2].

While acute inflammation is part of the immune response; chronic inflammation can lead to diseases such as cancer. Infiltration of leukocytes into the tumor is considered part of the innate immune mechanism against cancer, but inflammatory cells help tumors distribution, angiogenesis, growth, and invasion by secreting cytokines, growth factors, chemokines, and proteases [6]. Leukocytes secrete several cytokines such as interferon-gamma, interferon-alpha, interleukins 8, 2, 10, and TNFα, which have been shown to play an important role in breast

carcinogenesis [6]. NF-κB induces the expression of genes encoding pro-inflammatory cytokines, IL-6 and IL-8, and is involved in tumorigenesis, inhibition of apoptosis and drug resistance in cancer cells [7, 8]. Thus, inhibitors targeting NF-κB or upstream molecules of its signaling pathway can be useful in cancer treatment as a new class of anticancer drugs [9].

One of the key factors in toll like receptor (TLR) signaling and NF-κB activation is interleukin-1 receptor-associated kinases (IRAKs). IRAKs are recently identified factor that associated with drug resistance in cancer cells is. There are 4 IRAK genes in the human genome (IRAK-1, IRAK-2, IRAK-3 and IRAK-4) [10]. IRAK-1 interacts with MYD88 though its death domain. Phosphorylation and activation of IRAK-1 is a multi-step process. IRAK-1 is sequentially phosphorylated in roots in its activation loop. MYD88 binds only to non-phosphorylated IRAK-1. Phosphorylated IRAK-1 is isolated from the receptor complex, binds to TRAF6 and activates NF-κB, and NF-κB activation increases the expression of cytokines such as IL-6, IL-1β and TNF-α [11]. In normal cells, NF-κB is inactive and binds to the kappa B inhibitor protein (IκB). Cellular stimulation by TNF-α and various factors such as cytokines, mitogens, growth factor, bacterial and viral agents, and ultraviolet rays, lead to the activation of kappa B kinase inhibitors (IκKs), that result in phosphorylation and degradation of IκBs. This degradation leads to the release of NF-κB and its transfer from the cytoplasm to the nucleus [12].

We found in our previous study that IRAK1/4 inhibitor can increase the efficacy of chemotrapeutic drugs [13] and Based on the above evidence, and considering the expression of pro-inflammatory cytokines downstream of the NFκB pathway we designed this study to evaluate IRAK1/4 inhibition on expression of gene encoding of some pro-inflammatory cytokines. Also, because for the role of inflammation in develop of drug resistance, we evaluated the effects of IRAK1/4 inhibitor in combination with methotrexate and topotecan in several breast cancer cell lines to examine possible effects of these combinations on pro-inflammatory cytokines expression.

Materials and Methods

This study was approved by ethics committee of Kerman University of medical Sciences (IR.KMU.REC.1399.300).

Cell culture and treatment. Because triple negative breast cancer cell lines are more aggressive and become resistant to the drug sooner than the other breast cancer cell lines [14], we used triple negative cell lines of breast cancer including BT-549, BT-20 and MB-468 [15] in this study. All cell lines were obtained from the Biological Research Center. All cells were cultured in a humidified atmosphere of 5% CO_2 at 37°C and in their own dedicated culture media. The BT-549 cells were cultured in DMEM (Gibco, USA) medium and fetal bovine serum (FBS, Gibco,USA) 10% and 100 units/ml of penicillin, 100 μg/ml of streptomycin, and 2 mM L-glutamine. The BT-20 and MDA-MB-468 cells were cultured in Ham's F12 (Gibco, USA) medium plus 2 mM L-glutamine and FBS 10%, and 100 units/ml of penicillin, and 100 μg/ml of streptomycin. For assessing the effect of IRAK1/4 inhibitor on gene expression, each cell line was treated with IRAK1/4 inhibitor (1 μg/ml) for 6, 12, 24, 48, and 72 h. BT20, BT549 and MB468 cell lines were treated with IRAK1/4 inhibitor at a concentration (1 μg/ml) at different times of 6, 24, 12 and 72 h. Because 72 h was the most effective time in our previous study [13], therefore we used 72 h for simultaneous treatment of the cell lines with drugs (methotrexate or topotecan) plus IRAK1/4 inhibitor. All cell lines in their specific medium were treated with methotrexate (1 μg/ml) or topotecan (1 μg/ml) with or without IRAK1/4 inhibitor (1 μg/ml) for 72 h [13].

Table. Primer sequences used for real-time PCR

Gene	Forward primer (5′→3′)	Reverse primer (5′→3′)
IL-6	CCGGGAACGAAAGAGAAGCT	GCGCTTGTGGAGAAGGAGTT
TNF-α	TGGAGAAGGGTGACCGACTC	TGCCCAGACTCGGCAAAG
IL-1β	AATCTGTACCTGTCCTGCGTGTT	TGGGTAATTTTTGGGATCTACACTCT
IFN-γ	AATTGGAAAGAGGAGAGTGAC	GTTACCGAATAATTAGTCAG
β-actin	ATCAAGATCATTGCTCCTCCTGAG	CTGCTTGCTGATCCACATCTG

Quantitative real-time PCR. Total RNA from about three million cells of each cell line (BT-20, BT-549, MB-468) was extracted with the RNeasy mini kit (Qiagen, Germany) according to the manufacturer's guidelines. The RNA concentration was determined at 260 and 280 nm (ND-1000 Nanodrop). The quality of RNA was confirmed by Ethidium bromide staining of 18S and 28S ribosomal RNA bands after electrophoresis in a 2% agarose gel. The sequences of the primers which were used in this study are listed in Table. Changes of expression were calculated by the $2^{-\Delta\Delta CT}$ equation [13]. All experiments were performed as triplicate.

Statistical analysis. All data are presented as mean ± SEM. Statistical analyses were performed using the SPSS software version 20 for Windows. We used Students *t*-test analysis to evaluate the differences between groups. $P < 0.05$ was considered as statistically significant.

Results and Discussion

Effect of IRAK1/4 inhibitor alone or in combination with methotrexate or topotecan on TNF-α gene expression. TNF-α gene expression was increased in all cell lines treated with IRAK1/4 inhibitor without clear time-dependent manner, although *TNF-α* gene expression in the MB468 cell line for 72 h was not effective (Fig. 1).

The expression of TNF-α in the groups treated with methotrexate or topotecan significantly increased in comparison with control group. However, IRAK1/4 inhibitor was able to reduce the expression of TNF-α in all cell lines was treated with topotecan or methotrexate (Fig. 2).

Effect of IRAK1/4 inhibitor alone or in combination with methotrexate or topotecan on *IFN-γ* gene expression:

IFN-γ gene expression in all cell lines treated with IRAK1/4 inhibitor showed a significant reduction compared to the control group, independent of duration of treatments (Fig. 3).

IFN-γ gene expression in the group treated with Topotecan alone in MB468 cell line compared with the control group showed a significant difference, while the effect of this drug in other cell lines was not significant. Also methotrexate did have no effect on *IFN-γ* gene expression in none of the cell lines. Use of IRAK1/4 inhibitor reduced *IFN-γ* gene expression in all three cell lines treated with methotrexate and topotecan, or in other words (Fig. 4).

Effect of IRAK1/4 inhibitor alone or in combination with methotrexate or topotecan on IL-1B gene expression:

IL-1B gene expression in all cell lines treated with IRAK1/4 inhibitor, with treatment periods of 12, 24 and 72 h, showed a significant difference com-

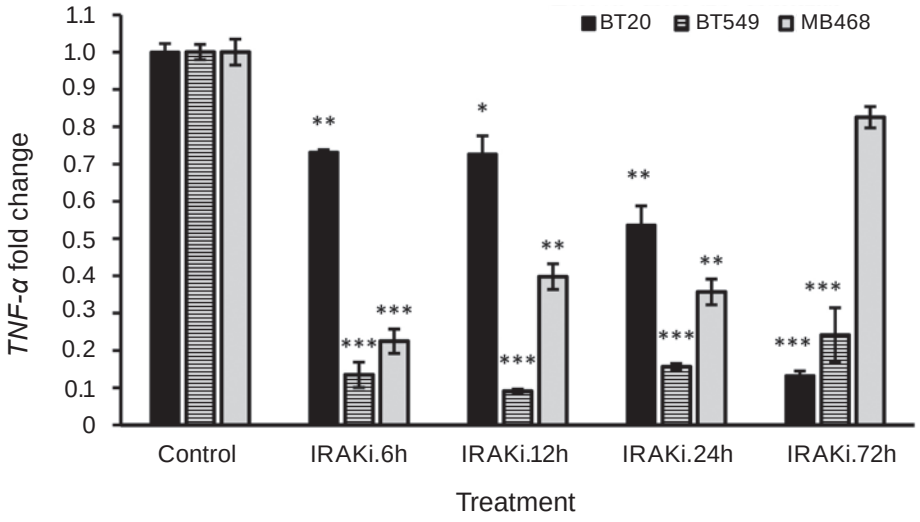

*Fig. 1. The effect of IRAK1/4 inhibitor (1 μg/ml) on TNF-α gene expression in three cell lines BT20, BT549, MB468. Cell lines BT549 and MB468 were cultured in DMEM medium and cell line BT20 in Hams F12 medium. Cells were treated with IRAK1/4 inhibitor concentration (1 μg/ml) at 6, 12, 24 and 72 h with IRAK1/4 inhibitor. The results were obtained by real-time PCR technique and were reported relative to the actin gene. Data are expressed as mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.0001 statistically significant compared to the control group*

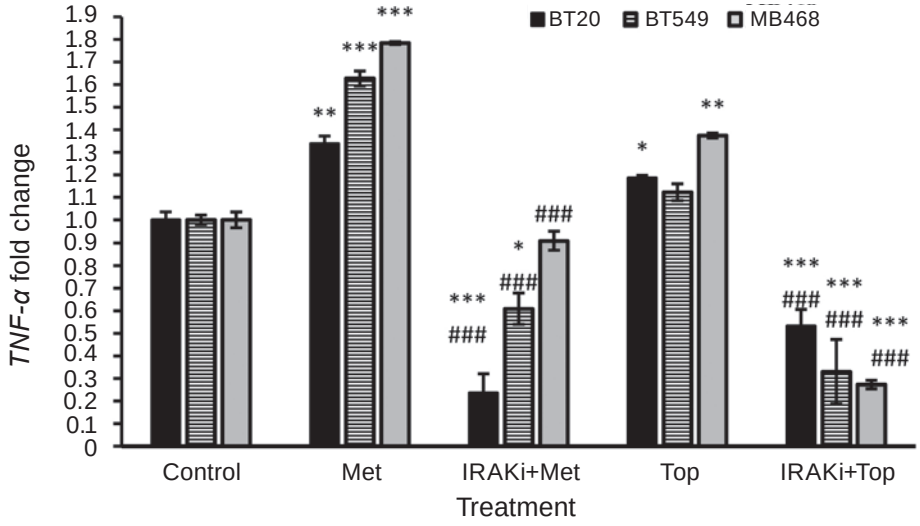

*Fig. 2. The effect of methotrexate and topotecan alone and in combination with IRAK1/4 inhibitor on TNF-α gene expression in three cell lines BT20, BT549, MB468. Cell lines BT549 and MB468 were cultured in DMEM medium and cell line BT20 in Hams F12 medium. The cells were then treated with a concentration (1 μg/ml) of methotrexate or topotecan alone or in combination with an IRAK1/4 inhibitor (1 μg/ml) for 72 h. The results were obtained by real-time PCR technique and were reported relative to the actin gene. Data are expressed as mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.0001 statistically significant compared to the control group, and #P < 0.05, ##P < 0.01, ###P < 0.0001 show statistical significant between the group treated with methotrexate or topotecan compared to the treatment group received methotrexate or topotecan and IRAK1/4 inhibitor*

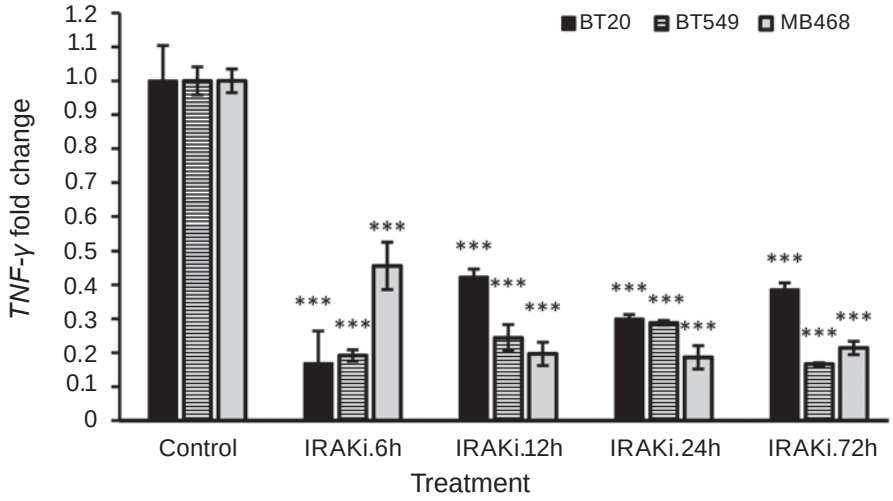

*Fig. 3. The effect of IRAK1/4 inhibitor (1 μg/ml) on IFN-γ gene expression in three cell lines BT20, BT549, MB468. Cell lines BT549 and MB468 were cultured in DMEM medium and cell line BT20 in Hams F12 medium. Cells were treated with IRAK1/4 inhibitor concentration (1 μg/ml) at 6, 12, 24 and 72 h with IRAK1/4 inhibitor. The results were obtained by real-time PCR technique and were reported relative to the actin gene. Data are expressed as mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.0001 statistically significant compared to the control group*

pared to the control group. But it was not effective in MB468 and BT20 cell lines in the time of 6 h (Fig 5).

After treatment with Topotecan, *IL-1B* gene expression decreased in BT20 and BT549 cell lines. Methotrexate didn't have any effect on transcription

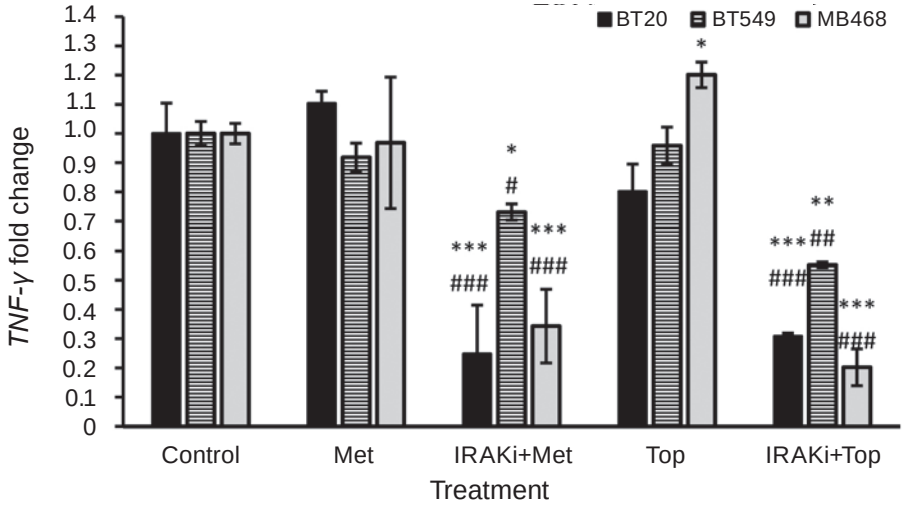

*Fig. 4. The effect of methotrexate and topotecan alone and in combination with IRAK1/4 inhibitor on IFN-γ gene expression in three cell lines BT20, BT549, MB468. Cell lines BT549 and MB468 were cultured in DMEM medium and cell line BT20 in Hams F12 medium. The cells were then treated with a concentration (1 μg/ml) of methotrexate or topotecan alone or in combination with an IRAK1/4 inhibitor (1 μg/ml) for 72 h. The results were obtained by real-time PCR technique and were reported relative to the actin gene. Data are expressed as mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.0001 statistically significant compared to the control group, and #P < 0.05, ##P < 0.01, ###P < 0.0001 show statistical significant between the group treated with methotrexate or topotecan compared to the treatment group received methotrexate or topotecan and IRAK1/4 inhibitor*

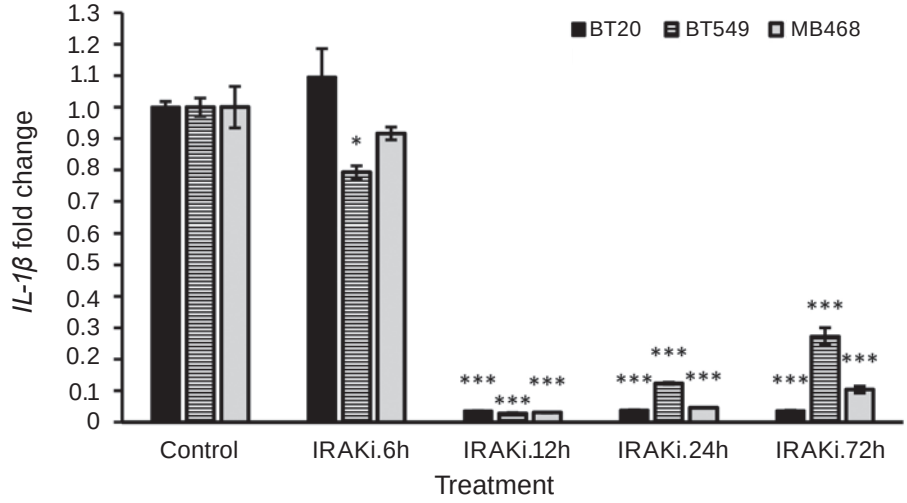

*Fig. 5. The effect of IRAK1/4 inhibitor (1 μg/ml) on IL-1B gene expression in three cell lines BT20, BT549, MB468. Cell lines BT549 and MB468 were cultured in DMEM medium and cell line BT20 in Hams F12 medium. Cells were treated with IRAK1/4 inhibitor concentration (1 μg/ml) at 6, 12, 24 and 72 h with IRAK1/4 inhibitor. The results were obtained by Real time PCR technique and were reported relative to the actin gene. Data are expressed as Mean ± SEM. *P <0.05, **P < 0.01, ***P < 0.0001 statistically significant compared to the control group*

of *IL-1* gene in all three cell lines. Use of IRAK1/4 inhibitor reduced IL-1 gene expression in all three cell lines treated with Methotrexate and Topotecan (Fig. 6).

Effect of IRAK1/4 inhibitor alone or in combination with Methotrexate or Topotecan on IL-6 gene expression. *IL-6* gene expression in all cell lines treated with IRAK1/4 inhibitor, with treatment pe-

riods of 6, 12, 24 and 72 significantly reduced compared to control group with exception of MB468 cell line in the time of 72 h which there was no significant differences (Fig. 7).

IL-6 gene expression in the group treated with methotrexate or topotecan alone in the two cell lines BT20, BT549 significantly increased compared to the control group. But MB468 cell line in the same groups showed significant decrease in *IL-6* gene expression compared to the control group. Also, in combination use of these drugs with IRAK1/4 inhibitor, all three cell lines BT20, BT549 and MB468 showed a significant decrease in *IL-6* gene expression compared to the control group (Fig. 8).

The results of the present study showed that *IL-6* gene expression was significantly reduced in all cell lines treated with IRAK inhibitor, with treatment periods of 6, 12, 24 and 72 compared to the control group. It has been shown that activation of IRAK causes resistance to chemotherapy drugs. *IL-6* gene expression increased significantly in methotrexate and topotecan alone in BT20 and BT549 cell lines compared with the control group, but MB468 cell line decreased significantly compared to the control group. The results of the present study are consistent with the study of Olsen et al. which showed that methotrexate increased the expression of interleukin-6 in U937 medium, which is consistent with the results obtained in the BT20 and BT549 cell lines in the present study. The mechanism of this increase seems to be due to the activation of the NFκB pathway [16].

Contrary to the results of the present study, Nishina et al. showed that methotrexate significantly reduced the level of interleukin-6, CRP in the serum of patients with rheumatoid arthritis but had no effect on the level of TNF-α in the serum of patients [17]. The reason for this difference is probably due to the difference in the type of sample and concomitant use of these drugs with IRAK inhibitors in the three cell lines (BT20, BT549 and MB468). The results of the Zhang study in 2018 showed that more than 50% of breast cancer patients had high levels of TNF-α and that TNF-α expression was positively associated with anthracyclines resistance. It has been shown that altered expression of TNF-α caused sensitivity of breast cancer cells and breast cell lines to doxorubicin. Our results also confirm the findings of this study that the simultaneous administration of IRAK1 inhibitor with anticancer drugs significantly decreased inflammatory chemokines in the NF-KB pathway. The expression of IFN-γ and TNFα in all

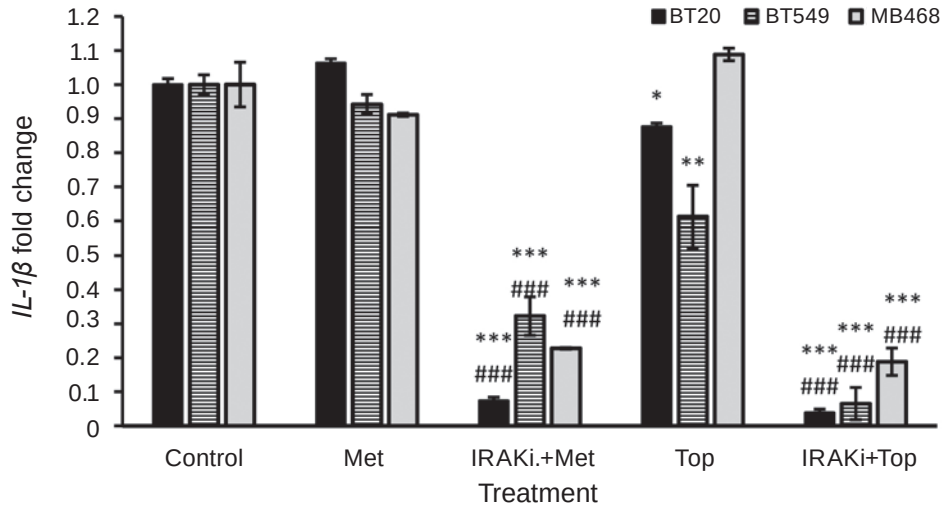

*Fig. 6. The effect of methotrexate and topotecan alone and in combination with IRAK1/4 inhibitor on IL-1 gene expression in three cell lines BT20, BT549, MB468. Cell lines BT549 and MB468 were cultured in DMEM medium and cell line BT20 in Hams F12 medium. The cells were then treated with a concentration (1 μg/ml) of methotrexate or topotecan alone or in combination with an IRAK1/4 inhibitor (1 μg/ml) for 72 h. The results were obtained by real-time PCR technique and were reported relative to the actin gene. Data are expressed as mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.0001 statistically significant compared to the control group, and #P < 0.05, ##P < 0.01, ###P < 0.0001 show statistical significant between the group treated with methotrexate or topotecan compared to the treatment group received methotrexate or topotecan and IRAK1/4 inhibitor*

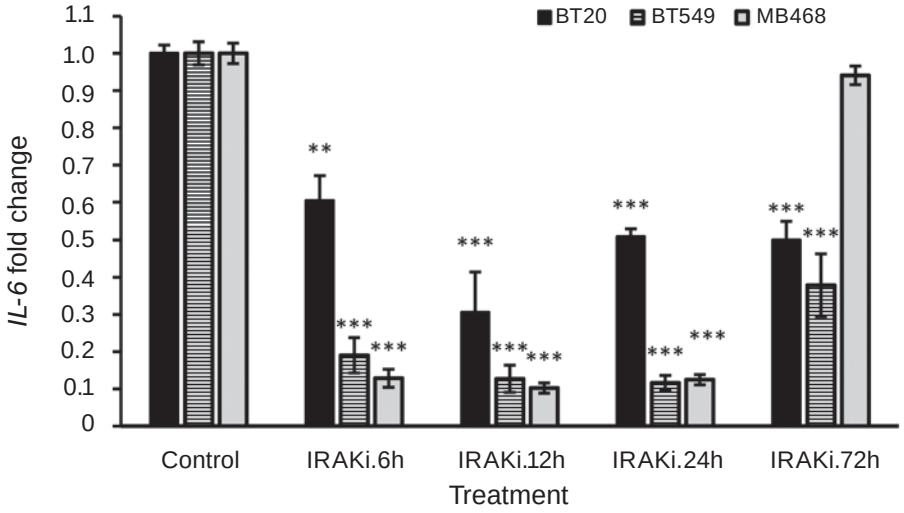

*Fig. 7. The effect of IRAK1/4 inhibitor (1 μg/ml) on IL-6 gene expression in three cell lines BT20, BT549, MB468. Cell lines BT549 and MB468 were cultured in DMEM medium and cell line BT20 in Hams F12 medium. Cells were treated with IRAK1/4 inhibitor concentration (1 μg/ml) at 6, 12, 24 and 72 h with IRAK1/4 inhibitor. The results were obtained by real-time PCR technique and were reported relative to the actin gene. Data are expressed as mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.0001 statistically significant compared to the control group*

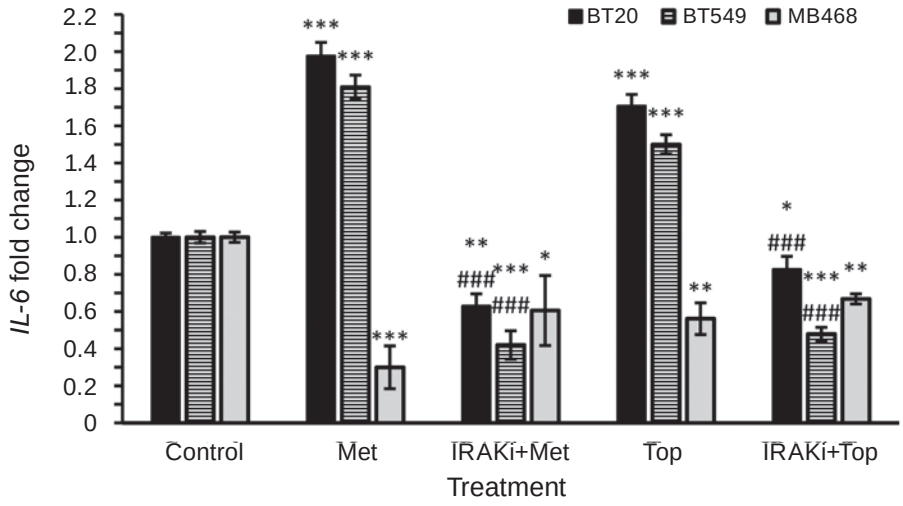

*Fig. 8. The effect of methotrexate and topotecan alone and in combination with IRAK1/4 inhibitor on IL-1 gene expression in three cell lines BT20, BT549, MB468. Cell lines BT549 and MB468 were cultured in DMEM medium and cell line BT20 in Hams F12 medium. The cells were then treated with a concentration (1 μg/ml) of methotrexate or topotecan alone or in combination with an IRAK1/4 inhibitor (1 μg/ml) for 72 h. The results were obtained by real-time PCR technique and were reported relative to the actin gene. Data are expressed as mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.0001 statistically significant compared to the control group, and #P < 0.05, ##P < 0.01, ###P < 0.0001 show statistical significant between the group treated with methotrexate or topotecan compared to the treatment group received methotrexate or topotecan and IRAK1/4 inhibitor*

three cell lines to which IRAK inhibitor and topotecan or methotrexate were added had a significant decrease compared to the drug-free control group [18].

IL-1β expression is increased in a wide range of cancers, including breast, prostate, colon, lung and neck cancers, and melanoma. Also it was docu-

mented that the patients with tumors that produce IL-1β have a worse prognosis [19]. IL-1β directly or indirectly mediates inflammatory cells and angiogenic pathways around the tumor cell, causing tumor growth and metastasis [20]. Therefore, inhibition of IL-1β can be an effective method for tumor treatment [21].

Activation of IRAK1 appears to play a key role in drug resistance. The IRAK1 signaling pathway has been shown to be important in the development and growth of cancer and to promote cancer growth and resistance to anticancer drugs in various cancer cell lines such as breast cells, melanoma, ovaries, skin and lung. The study by Srivastava et al, confirmed that inhibition of IRAK can inhibit drug resistance. The results of this study showed that inhibition of IRAK1 and IRAK4 by specific siRNAs increases drug sensitivity to Vinblastine in melanoma cell lines [22]. In addition, in another study it was showed that IRAK1 inhibition sensitizes hepatocellular carcinoma cells to Doxorubicin and Sorafenib by suppressing the apoptotic cascade *in vitro* [23]. In many cancers with elevated levels of IRAK1 and IRAK4, resistance to chemotherapy is observed, which highlights the role of IRAK in drug resistance [24]. Similar to the present study, a study by Ni et al. (2018) showed that the chemical substance, R191, inhibited IRAK1/4, reduced cell cycle and ultimately reduced NF-kB activity in Waldenström's Macroglobulinemia tumors, a type of lymphoid disease. IRAK1/4 reduces inflammatory pathway signaling. This study also showed that concomitant use with new anticancer drugs such as Afuresertib, Bortezomib and Ibrutinib could potentiate its effects [25].

Bhaumik and colleagues investigated the effect of microRNA-146 inhibitor on the reduction of NF-kB activity in MDA-MB-231 breast cancer cell line, which reduces IRAK1 and TNF receptor-associated factor 6 in the NF-KB pathway and ultimately caused decreased expression of 1L-6, IL-1β and IL-8. This study suggests that microRNA-146 could be a therapeutic potential in suppressing and reducing the invasion of breast cancer metastasis [26]. Our study, as in previous studies, shows that inhibiting IRAK with chemicals or a variety of RNAs reduces the production of inflammatory cytokines by inhibiting the signaling pathway of inflammation, thereby reducing anticancer drug resistance and reducing inflammation. The Yang and coworkers (2019) investigated the role of IRAK1 in breast cancer patients treated with Neoadjuvant Chemotherapy (NAC) method. In this study, no correlation was found between IRAK expressions before and after of NAC, but a significant correlation was found between tumor size and IRAK reduction. IRAK1 expression was directly related to NAC reduction and therefore decreased IRAK1 expression following NAC efficacy is associated with decreased tumor size. This study suggests that IRAK could be a diagnostic indicator for assessing NAC for breast cancer patients [27]. This study was performed to enhance the treatment of breast cancer patients by inhibiting IRAK1 and reducing inflammation and reducing the size of the tumor. This is similar to our study of IRAK 1/4 inhibitors at different hours of cell culture, which ultimately reduces the expression of pro-inflammatory cytokines in cancer cells which was treated anticancer drugs Methotrexate and Topotecan. It is possible this effect of IRAK1/4 inhibitor is a mechanism for increasing cytotoxic effects of these drugs. Although cytokine levels not only depend on gene transcription and it was better to measure protein levels and that was the main limitation of our study.

Conclusion. Overall, the results of real-time PCR in this study showed for the first time that concomitant use of IRAK inhibitor with Topotecan or Methotrexate reduced the expression of IL-1β, IFN-γ, TNF-α and IL-6 in BT-20 , BT-549, and MB-468 cell lines relative to the control group. The highest and lowest effects of IRAK inhibitors on cytokine expression are on BT549 cell line for IL-1β and MB468 for IL-6, respectively. This study could guide further studies of other inflammatory mechanisms and improve anticancer drugs for patients.

Conflict of interest. Authors have completed the Unified Conflicts of Interest form at http://ukr-biochemjournal.org/wp-content/uploads/2018/12/coi_disclosure.pdf and declare no conflict of interest.

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ІНГІБІТОР ЗАЛЕЖНОЇ ВІД РЕЦЕПТОРА ІНТЕРЛЕЙКІНУ-1 КІНАЗИ ВПЛИВАЄ НА ЕКСПРЕСІЮ *IL-1β, IL-6, INF-γ* ТА *TNF-α* В КЛІТИНАХ РАКУ ГРУДНОЇ ЗАЛОЗИ

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Недавні дослідження показали, що запалення, опосередковане через дію інтерлейкін-1-рецепторзалежної кінази (IRAK), пов'язане з резистентністю ракових клітин до лікарських засобів. Метою роботи було оцінити експресію прозапальних цитокінів як потенційного механізму, що бере участь у розвитку стійкості ракових клітин до хіміотерапевтичних ліків. Клітини раку грудної залози BT549, BT20 та MB468 культивували в середовищі DMEM та інкубували з інгібітором IRAK1/4 окремо, або у поєднанні з хіміотерапевтичними препаратами: метотрексатом або топотеканом. Виявлено, що інгібітор IRAK1/4 найбільшою мірою пригнічує експресію IL-1β у клітинах BT549 та має найменший вплив на експресію IL-6 у клітинах MB468. Вперше показано, що одночасне застосування інгібітора IRAK1/4 із топотеканом або метотрексатом знижувало експресію IL-1β, IFN γ, TNF-α та IL-6 у клітинних лініях BT-20, BT-549, MB-468 порівняно з контролем. Зроблено припущення щодо можливого поєднання специфічних інгібіторів IRAK та традиційних хіміотерапевтичних препаратів у лікуванні раку для підвищення чутливості до ліків та зменшення ризику рецидиву пухлини.

Ключові слова: рак молочної залози, , інгобітор IRAK1/4, метотрексат, топотекан, ПЛР у режимі реального часу.

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