Cell. Mol. Biol. (ISSN: 1165-158X)

Journal Homepage: www.cellmolbiol.org

Cellular and Molecular Biology

Original Article

Potentiation of growth suppression and modulation of multidrug resistance by gamma and beta interferons in MDA-MB-231 breast cancer cell line

Pouya Ghaderi[1,*], Hamid Reza Jalili[2], Mobin Mohammadi[2,3], Mohammad Reza Rahmani[2,4]

[1] *Department of Pathobiology, Faculty of Veterinary Medicine, Sanandaj Branch, Islamic Azad University, Sanandaj, Iran*
[2] *Department of Immunology, Faculty of Medicine, Kurdistan University of Medical Sciences, Sanandaj, Iran*
[3] *Cancer and Immunology Research Center, Research Institute for Health Development, Kurdistan University of Medical Sciences, Sanandaj, Iran*
[4] *Zoonosis Research Center, Research Institute for Health Development, Kurdistan University of Medical Sciences, Sanandaj, Iran*

Article Info

Article history:

Received: January 31, 2024
Accepted: April 03, 2024
Published: August 31, 2024

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Abstract

Multi-drug resistance (MDR) might be acquired by the cancer cells during chemotherapy, and ATP-binding cassette (ABC) transporters play a significant role in MDR. Interferon-γ (IFN-γ) and IFN-β can inhibit cancer cell proliferation; however, the effects and mechanism of these cytokines on the growth and MDR are still unclear. To investigate the effects of IFN-γ and IFN-β, alone or in combination, on viability, resistance, and the expression of ABC transporters of the MDA-MB-231 breast cancer cell line. Using the MDA-MB-231 cell line, we assessed the effects of 20, 100, and 500 IU/ml of IFN-γ and IFN-β, alone or in combination, on cell viability by methyl thiazolyl tetrazolium (MTT) assay; and then we performed the Uptake and Efflux experiment to evaluate the effect of these IFNs on the cell resistance. Then, using quantitative real-time PCR, we evaluated changes in the expression of ABCB1, ABCC1, and ABCG2 mRNA levels. We discovered that IFN-γ and IFN-β might both reduce viability, either alone or in combination. The combination of IFNs also displayed synergistic responses, particularly when utilizing equivalent dosages of 500 or 100 IU/ml. The combination of IFN-γ and IFN-β resulted in a significant increase in Doxorubicin accumulation and down-regulation of the ABCC1 gene at the mRNA level. Our study suggested that equal doses of IFN-γ and IFN-β in combination might result in potentiated responses against cancer, especially, along with chemotherapy agents.

Keywords: ATP-binding cassette transporter, Combinational therapy, Interferon beta, Interferon gamma, Multi-drug resistance.

1. Introduction

Resistance to chemotherapy agents was one of the major issues regarding cancer treatment [1]. It was reported that 90 percent of metastatic cancer chemotherapy failures are because some cancer cells acquire a multidrug resistance (MDR) phenotype during chemotherapy [2]. MDR occurs via diverse mechanisms and one of the most important mechanisms is to increase the efflux of chemotherapy agents from the intracellular compartment, which is mainly executed by ATP-binding cassette (ABC) transporters [1, 3, 4]. Pieces of evidence have shown that the poor clinical prognosis of patients with breast cancer is related to the up-regulation of ABC transporter proteins [5, 6]. ABCB, ABCC, and ABCG2 are the main members of the ABC superfamily, which are primarily related to chemotherapy resistance [3, 7, 8]. On top of that, ABCB1, ABCC1, and ABCG2 are the most detected ABC transporters among mentioned ABC transporters in breast cancer cells [5, 6, 9, 10]. Combination therapy, which involves utilizing several medications and treatment modalities, reduces the amount of chemotherapy needed while targeting different signaling pathways in cancer cells and reduces side effects associated with a particular drug regimen. Implementing this strategy can modulate MDR and tumor recurrence [11-15].

Interferon (IFN) is a natural protein secreted by different cells, such as fibroblasts, natural killer cells, white blood cells, and epithelial cells in response to pathogens, cancer cells, as well as other foreign substances [16]. In humans, type 1 IFN comprises IFN-β and 17 other members, boosting immune responses at different levels [17, 18]. They have anti-cancer effects such as inducing acquired and innate immune responses simultaneously, reducing inhibitory immune responses, inhibiting proliferation and the induction of apoptosis in cancer cells [19], and enhancing the sensitivity of cancer cells to chemotherapy agents [20, 21]. Type 2 IFN only consists of IFN-γ, which can enhance immune responses against the microorganisms. Moreover, this cytokine can stimulate the immune system against cancer cells and help in the recognition and elimination of these cells [22]. IFN-γ is capable of the down-regulation of *ABCB1*, in both mRNA and protein levels, in colon carcinoma cells [21]. Studies showed that

* Corresponding author.
E-mail address: pouya.ghaderi752500@gmail.com (Pouya Ghaderi).
Doi: http://dx.doi.org/10.14715/cmb/2024.70.8.11

the combination of IFN-γ and IFN-β can synergistically inhibit the growth of resistant and sensitive cancer cells, and this inhibition is significantly higher than using IFN-γ or IFN-β alone [23, 24].

Even though the effect of IFN-γ and IFN-β on growth suppression of the cancer cells was extensively studied, the effects and mechanism of these cytokines, alone or in combination, on the growth and MDR of breast cancer cells are still unclear. Therefore, we assessed the effect of IFN-γ and IFN-β, alone or in combination, on cell viability in MDA-MB-231 cell line. Besides, to determine the effect of IFN-γ and IFN-β treatment, alone or in combination, on the MDR, we evaluated the chemotherapy agent accumulation in the MDA-MB-231 cells by performing Uptake and Efflux experiment. To determine whether these changes in Uptake and Efflux are related to the changes in the expression of *ABCB1*, *ABCC1*, and *ABCG2* mRNA levels, we performed the RT-qPCR experiment.

2. Materials and Methods

2.1. Cell lines and culture conditions

MDA-MB-231 cells (RRID: CVCL_0062) were purchased from Pasteur Institution (Iran). The cells were cultured in DMEM / F12 medium (Gibco, UK) supplemented with 10% (v/v) fetal calf serum (FBS) (Gibco, UK) at 37°C in a 5% CO2 humidified incubator. The medium was supplemented with 100 IU/mL penicillin (Shafa Farmed, Iran) and 100 µg/mL streptomycin (ShafaDarou Co, Iran). For maintaining, culture media were changed once in two days and cells were sub-cultured when they reached 80% confluence.

2.2. Interferons preparation and treatment

IFN-γ (Exir, Iran) and IFN-β (CinnaGen, Iran) were purchased, and their stock solutions were prepared at a concentration of 2×10^3 IU/ml in PBS, and then stored at 4 °C. The final concentrations were prepared from these stock solutions. For the initiation of IFN treatment, first, the cells were plated at the appropriate density, and following 24 hours culture media changed and then the cells were treated with IFN-γ or IFN-β, or IFN-γ plus IFN-β, or without any IFN (as a control group) in the culture media. After 48 and 96 hours, the culture medium change and cell treatment were then repeated. Cells were finally prepared for further studies seven days after being seeded. All experiments were carried out in quintuplicate.

2.3. Cell viability assay and combination index

To assess cell viability, the cells were treated with 20, 100, and 500 IU/ml IFN-γ or IFN-β, alone or in combination in 96-well plates. After the treatments, 100 µL of MTT solution (0.5 mg/mL) was added to each well; then the plates were incubated for 2 hours. Afterward, 50 µL DMSO was used to dissolve the blue-purple crystals, and the plates were incubated for 20 minutes in a shaking incubator. Finally, the optical density of formazan product was measured at 540 nm, and all measured optical densities were normalized to no drug control in percentage. To categorize synergy occurring among IFNs, CompuSyn V.1 software (ComboSyn, USA) was used, and then Combination Index (CI) was determined.

2.4. Uptake and Efflux

This experiment was performed to evaluate the effect of IFN-γ and IFN-β, alone or in combination, on the residual uptake amounts of chemotherapy agents in the intracellular compartment of the cells following chemotherapy treatments. For this aim, the cell culture media were changed to fresh DMEM/F12 containing 800 nM Doxorubicin (DOX) (Sobhan, Iran), a fluorescent chemotherapeutic drug, after the treatment of the cells with 100 IU/ml of each IFN, alone or in combination, according to the given parameters. After 1 hour of incubation, the cell media were replaced with the fresh medium of equal volume without DOX for a further 1 hour to allow DOX efflux. Afterward, the cells were detached from tissue culture dishes with 0.25% Trypsin and 0.02% EDTA solution (Sigma, USA), washed three times with ice-cold FACS buffer, and fixed with 4% paraformaldehyde. Finally, the fluorescent emission of the residual uptake amounts of DOX, which was not effluxed from the intracellular compartment of the cells, was measured by BD FACSCalibur flow cytometer, and the Geometric Mean of the samples was used for the statistical analysis.

2.5. Quantitative real-time PCR (qRT-PCR)

To determine the effect of IFN-γ and IFN-β, alone or in combination, on the expression level of *ABCB1*, *ABCC1*, and *ABCG2* mRNA by qRT-PCR, total RNA was isolated from the cells according to the kit protocol (SINACLON, Iran) by the phenol-chloroform method after the treatment of cells with 100 IU/ml of each IFN, alone or in combination, according to the mentioned conditions. RNA integrity was assessed using agarose gel electrophoresis, and RNA concentration and purity were quantified using a 2000 Nanodrop spectrophotometer (Thermo Scientific, USA). Reverse transcription was used to create the cDNA using 1000 ng of total RNA in accordance with the kit's instructions (Sinaclon, Iran). PCR experiment was performed using EPPENDORF MASTERCYCLER 5331 thermal cycler (Eppendorf, Germany) in a 96-well format. Then, using agarose gel electrophoresis, the gene expression was examined, and primers were validated. Afterward, quantitative RT-PCR reactions with SinaGreen HS-qPCR Mix (Sinaclon, Iran) were performed using Rotor-Gene Q real-time PCR cycler (Qiagen, USA). Standard curves were generated for each primer pair by serial dilution of the starting template. Then, the mean relative expression was calculated based on the related standard curves. The endogenous *β-actin* was used for normalization. The primer sequences are given in Table 1.

2.6. Statistical analysis

To analyze the data statistically, the results of various

Table 1. The designed primers for qRT-PCR experiment.

Target gene	Forward primer sequence	Reverse primer sequence
ABCB1	CGAAGAGTGGGCACAAACC	CCATCAACACTGACCATCCC
ABCC1	CAGAGATTGGCGAGAAGGG	CTGTGCGTGACCAAGATCC
ABCG2	GCCGTGGAACTCTTTGTGG	CACTCTGACCTGCTGCTATGG

tests were analyzed by SPSS V.23 software (IBM Analytics, USA). Kolmogorov-Smirnov test examined data normality, and statistical analysis was performed by One-way ANOVA and Post Hoc-Tukey tests. Statistical significance was regarded as $p<0.05$.

3. Results

3.1. IFN-γ and IFN-β, alone or in combination, significantly decreased MDA-MB-231 cell viability

To assess the effect of IFN-γ and IFN-β, alone or in combination, we performed an MTT assay, measured the optical density of formazan product, and then calculated the percentage of the viability compared to the control cells considering them as 100%. Afterward, the CI was determined using CompuSyn software (Table 2).

We found that IFN-γ and IFN-β, alone or in combination, could decrease the viability of MDA-MB-231 cells significantly compared to the control cells (p=0.00) (Fig. 1). Furthermore, there were no high differences among the antiproliferative effects of IFN-γ and IFN-β when compared at equal concentrations. The antiproliferative effects of the combinations of both IFNs were significantly higher than that of either IFN alone (p=0.00 and CI<1). Among all the groups treated with the combination of IFNs, most groups treated with non-equal doses of IFNs had a significantly higher CI compared to the groups treated with equal doses of IFNs (p=0.00). Moreover, the group receiving the combination of 500 IU/ml IFN-γ plus 500 IU/ml IFN-β had the lowest cell viability and CI, as shown in Table 2 (p=0.00). Furthermore, after this group, the lowest CI belongs to the group treated with 100 IU/ml IFN-γ plus 100 IU/ml IFN-β (p=0.00) which the cell viability did not have a high difference compared to the groups treated with 500 IU/ml IFN-γ plus 20 IU/ml IFN-β or 20 IU/ml IFN-γ plus 500 IU/ml IFN-β, that their total doses were more. We found that there was no significant difference between the cell viability of groups treated with non-equal doses of IFNs in combination but with the same total aggregate IFN concentrations.

3.2. IFN-γ and IFN-β, alone or in combination, increase the accumulation of DOX in MDA-MB-231 cells

To determine whether IFN-γ and IFN-β treatment, alone or in combination, can affect the accumulation of chemotherapy agents in the cancer cells, we measured the mean fluorescence emission of DOX by BD FacsCalibur flow cytometer after treating the cells with IFN followed by DOX according to mentioned condition. The accumulation of DOX in the IFN-γ plus IFN-β group was significantly higher compared to the control, IFN-γ, or IFN-β group (p=0.00) (Fig. 2). Besides, we observed that the accumulation of DOX in the IFN-γ or IFN-β group was significantly increased compared with the control group (p=0.00).

3.3. IFN-γ and IFN-β, only in combination, reduced the ABCC1 gene transcription of MDA-MB-231 cells

To determine the effect of IFN-γ and IFN-β, alone or in combination, on the *ABCB1*, *ABCC1*, and *ABCG2* gene transcript levels, we treated cells with IFN based on the mentioned conditions. Afterward, we performed RT-qPCR, and then the mean relative expression was calculated. The group receiving 100 IU/ml IFN-γ plus 100 IU/ml IFN-β expressed the *ABCC1* gene mRNA at almost a

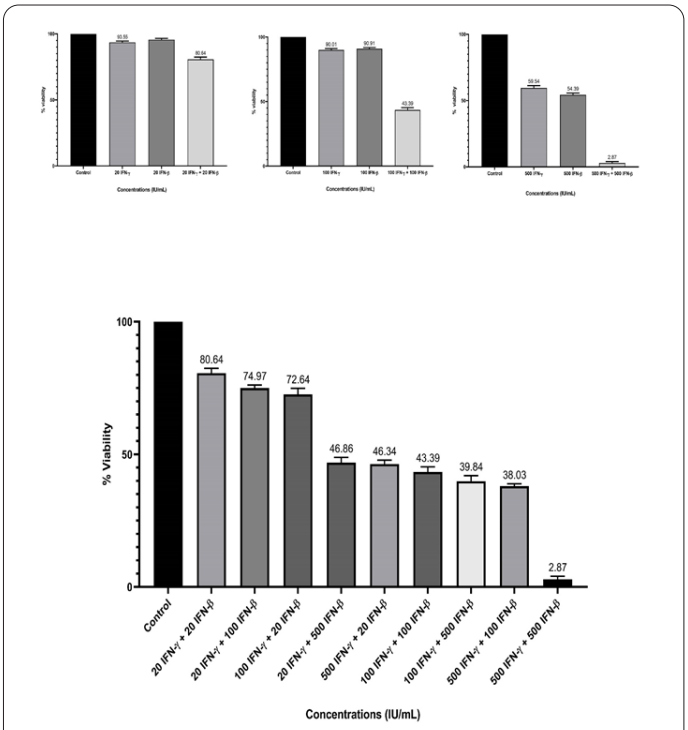

Fig. 1. The effect of IFN-γ and IFN-β treatment, alone or in combination, on the viability of MDA-MB-231 cells. Cells were seeded at the 96-well plates; 24 hours later, the medium changed, and cells were treated with or without IFNs. 48 and 96 hours later, the medium was replaced, and the cells were treated with or without IFNs again. 7 days after seeding, cell viability was measured by the MTT assay. Data represent mean ± SD of 5 independent experiments. Control: cells treated with the culture media without IFNs. IFN, Interferon.

Table 2. Combinational Index data of the combination of IFN-γ plus IFN-β.

IFN-γ dose (IU/ml)	IFN-β dose (IU/ml)	CI[a]
20	20	0.25 ± 0.04
20	100	0.51 ± 0.04
20	500	0.58 ± 0.05
100	20	0.41 ± 0.06
100	100	0.16 ± 0.01
100	500	0.46 ± 0.05
500	20	0.37 ± 0.03
500	100	0.29 ± 0.01
500	500	0.02 ± 0.01

[a] Combinational Index.

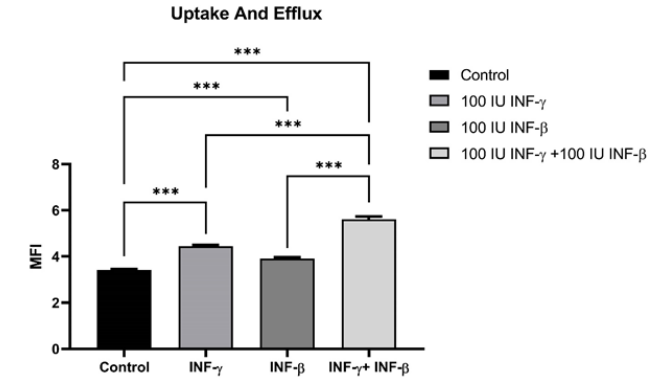

Fig. 2. The effect of IFN-γ and IFN-β treatment, alone or in combination, on MDR phenotypes in MDA-MB-231 cells. Cells were seeded at the appropriate density; 24 hours later, the medium changed, and cells were treated with or without IFNs. 48 and 96 hours later, the medium was replaced, and the cells were treated with or without IFNs again. 7 days after seeding, the cell culture media were replaced with new media containing 800 nM Doxorubicin. After 1 hour of incubation, the cell media were replaced with the fresh medium of equal volume without Doxorubicin for a further 1 hour to allow Doxorubicin efflux. Finally, the fluorescent emission of the residual uptake amounts of Doxorubicin, which was not effluxed from the intracellular compartment of cells after Doxorubicin treatment, was measured. Data are represented as mean fluorescence intensity ± SD of 5 independent experiments. Control: cells treated with the culture media without IFNs. *** shows a significance level of below 0.001. IFN, Interferon; MFI, mean fluorescence intensity.

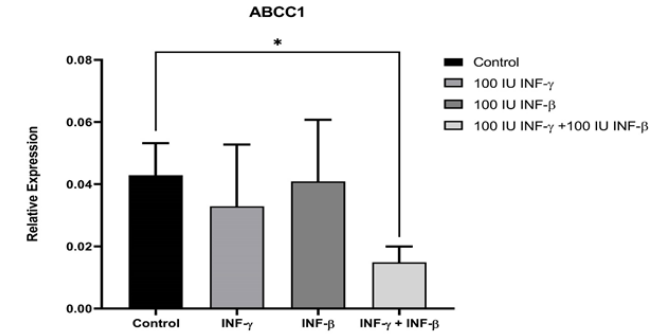

Fig. 3. The effect of IFN-γ and IFN-β treatment, alone or in combination, on the *ABCC1* gene transcription of MDA-MB-231 cells. Cells were seeded at the appropriate density; 24 hours later, the medium changed, and the cells were treated with or without IFNs. 48 and 96 hours later, the medium was replaced, and the cells were treated with or without IFNs again. 7 days after seeding, to quantify the level of transcripts for *ABCC1* gene, and the reference gene (β-actin), quantitative RT-PCR reactions were carried out. Absolute copy numbers for the transcripts were quantified based on related standard curves, and the mean relative expression was calculated. Data represent mean ± SD of 5 independent experiments. Control: cells treated with the culture media without IFNs. * shows a significance level of below 0.05. IFN, Interferon.

2.9-fold lower level than control cells ($p<0.05$), and there was no significant change in the mRNA level of *ABCC1* gene among those groups receiving IFN-γ or IFN-β alone (Fig. 3). No *ABCB1* transcript was detected in all experiment groups. Moreover, there was no significant change in the mRNA level of *ABCG2* gene among the experiment groups.

4. Discussion

Combination treatment has the benefit of employing lower effective dosages of chemotherapeutic drugs and targeting numerous signaling pathways in cancer cells. This approach has also shown its efficacy in lowering MDR [11-15]. Among all those various substances that can modulate MDR phenotype, cytokines have been of interest the most and may increase tumor response rates in combination with conventional chemotherapy agents [25]. IFN is one of those cytokines that exert significant effects on growth and MDR phenotypes in various cancer cell lines.

The main anti-tumor effect of IFN was shown to be owing to its direct antiproliferative effect [26]. Several studies found that IFN-β is more potent than IFN-γ [26, 27]; however, our findings were inconsistent and showed that there were no high differences between the antiproliferative effects of IFN-γ and IFN-β when compared at equal concentrations. The present study showed that the combinations of IFN-γ and IFN-β could synergistically enhance growth suppression and were significantly more effective than using each IFN alone, and this finding was consistent with other studies [23, 24, 26, 27]. We found that the majority of the groups in our research that got equal doses of IFNs in combination, despite receiving lower overall aggregate concentrations of IFNs, were able to potentiate antiproliferation more effectively than the groups that received non-equal doses of IFNs in combination. Besides, among those that received equal doses of IFNs in combination; even though the group treated with 500 IU/ml IFN-γ and IFN-β showed the highest synergy, few cells remained due to the high growth suppression (2.87±1.14%). Therefore, we were not able to use this combination to perform further experiments. The combination of 100 IU/ml IFN-γ plus 100 IU/ml IFN-β had the closest synergy index to the latter combination and could reduce cell viability by approx. 64 to 69%, which made using this combination possible to perform further experiments.

All members of type 1 IFN bind to a common cell-surface receptor which is known as the type 1 IFN receptor [18], and type 2 IFN binds to a different cell-surface receptor, known as the type 2 IFN receptor [28]. Both IFN receptors interact with Janus-activated kinase (JAK) family members [29]. After IFNs bind to their receptors, JAKs must phosphorylate and activate in order for the effects of IFNs to begin. When JAKs are activated by IFN, multiple downstream cascades and signaling pathways are either directly or indirectly regulated [17]. IFNs induce the expression of hundreds of genes mediating multiple biological responses [30], and some of these genes are regulated by type 1 IFN, some by type 2 IFN, and some by both types of IFN [17, 30]. The first shown IFN-activated signaling pathway was the JAK-signal transducer and activator of the transcription (STAT) pathway [31-33]. JAK-STAT model plays a significant role in the induction of many IFNs effects. Nevertheless, the JAK-STAT pathway is insufficient to mediate all of the IFN biological effects, and other IFN-regulated pathways are involved.

Among the IFN-regulated pathways, some are independent of JAK-STAT, whereas others cooperate with STATs optimizing the regulation of target gene transcription, and no signaling cascade alone is sufficient to generate any given biological end-point. Furthermore, numerous IFN-induced signaling pathways are necessary for IFNs to have

an antiproliferative impact [17]. Either IFN-γ or IFN-β can augment cancer cell sensitivity to the other IFN [27], and our results provide evidence that it is possible that IFN-γ and IFN-β mutually cooperate to exert their antiproliferative effects; because there was no significant difference among the cell viability of the groups treated with non-equal doses of IFNs in combinations, but with the same total aggregate IFN concentrations. We found that most of the groups that received equal doses of IFNs in combination could potentiate antiproliferative effect more efficiently compared to the groups that received non-equal doses of IFNs in combination, even with higher total aggregate concentration. The main reason behind this was probably a mediating element (or elements), which is responsible for the induction of synergistic antiproliferative response and effectively induced by an equal proportion of IFN-γ signal and IFN-β signal.

IFN can enhance the sensitivity of cancer cells to the chemotherapy agents [20, 21, 34]. In most cases, an increased accumulation of fluorescent ABCB1 substrates, like DOX, was measured in cancer cells after cytokine treatment, reflecting the sensitization of the tumor cells [21]. To determine whether IFN treatment can change the sensitivity of MDA-MB-231 cells to chemotherapy agents and modulate MDR, we performed Uptake & Efflux experiment. Our results showed that IFN-γ and IFN-β, alone or in combination, can increase the accumulation of DOX. It shows that besides the potentiated direct antiproliferative activity, 100 IU/ml IFN-γ plus 100 IU/ml IFN-β can also efficiently help eliminate cancer cells by increasing chemotherapy agent accumulation in cancer cells and sensitizing them. We found that when we treated MDA-MB-231 cells with 100 IU/ml IFN-γ plus 100 IU/ml IFN-β, it seems there was a relative connection between the amount of the antiproliferative activity change and DOX accumulation compared to the control group, and this probably indicates that the antiproliferative effect of the combination of IFNs on MDA-MB-231 cells results from mechanisms related to the cancer cells sensitizing and their MDR phenotype.

In breast cancer cells, ABCB1, ABCC1, and ABCG2 are the most detected transporters among ABC transporters which are primarily related to chemotherapy resistance [3, 5-10]. Therefore, we speculated that the expression changes in these transporters might be one of the main reasons why the IFN treatment, alone or in combination, induced DOX accumulation in MDA-MB-231 cells. Our results indicated that the significant reduction in the mRNA level of the *ABCC1* gene, by 100 IU/ml IFN-γ and 100 IU/ml IFN-β in combination, is one of the possible mechanism by which this IFN combination help DOX accumulation in MDA-MB-231 cells. As our results indicate, the *ABCB1* gene does not express in MDA-MB-231 cells at all, and IFN-γ and IFN-β, alone or in combination, cannot increase DOX accumulation in MDA-MB-231 cells via changing of mRNA level of *ABCG2* gene. These inconsistencies between the IFN-induced DOX accumulation and the changes in the mRNA levels of the mentioned genes suggest that there are other causing elements to the IFN-induced DOX accumulation in MDA-MB-231 cells, for instance, reduction in the other ABC transporters. Changes in the ABCC1 and ABCG2 expression in the protein levels might have caused DOX accumulation in MDA-MB-231 cells, as there are shreds of evidence regarding post-transcriptional regulation by IFNs [17, 35].

5. Conclusions

In conclusion, we showed that IFN-γ and IFN-β treatment, alone or in combination, suppress the expansion of MDA-MB-231 cells. This suppression is applied more effectively by the combination of equal doses of IFN-γ and IFN-β than the combination of unequal doses. Furthermore, we showed that alone or combined treatment of IFN-γ and IFN-β can sensitize MDA-MB-231 cells to chemotherapy agents. Besides, equal doses of IFN-γ and IFN-β in combination significantly increased chemotherapy agent accumulation in cells and reduced *ABCC1* gene expression. Hence, combining equal doses of IFN-γ and IFN-β might help overcome a major obstacle to cancer treatment, MDR, by enhancing the accumulation of chemotherapy agents in cancer cells. Our study indicated that equal doses of IFN-γ and IFN-β in combination can enhance the effectiveness of cancer treatment considerably and lessen the adverse effects by reducing the chemotherapy agent's effective dose. Additionally, when combined with chemotherapeutic drugs, this combinational cytokine therapy may provide superior outcomes than many pricey current cancer therapies in clinical settings.

Conflicts of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Consent for publications

Pouya Ghaderi: I read and approved the final manuscript for publication.
Hamid Reza Jalili: I read and approved the final manuscript for publication.
Mobin Mohammadi: I read and approved the final manuscript for publication.
Mohammad Reza Rahmani: I read and approved the final manuscript for publication.

Availability of data and material

Pouya Ghaderi: I embedded all data in the manuscript.
Hamid Reza Jalili: I embedded all data in the manuscript.
Mobin Mohammadi: I embedded all data in the manuscript.
Mohammad Reza Rahmani: I embedded all data in the manuscript.

Authors' contributions

P.Gh: Writing – original draft, Investigation, Formal analysis. **H.R.J**: Investigation, Conceptualization. **M.M**: Writing – review & editing, Methodology. **M.R.R**: Project administration, Resources, Supervision.

Funding

There is nothing to declare.

Acknowledgments

We acknowledge Shohreh Fakhari (the Medical University of Kurdistan, Cellular and Molecular Research Center) for his continuous scientific support during this project.

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