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Pioglitazone enhances cisplatin's impact on triple-negative breast cancer: Role of PPARγ in cell apoptosis

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ABSTRACT

Peroxisome proliferator-activated receptor-gamma (PPARγ) has been recently shown to play a role in many cancers. The breast tissue of triple-negative breast cancer (TNBC) patients were found to have a significantly lower expression of PPARγ than the other subtypes. Furthermore, PPARγ activation was found to exert anti-tumor effects by inhibiting cell proliferation, differentiation, cell growth, cell cycle, and inducing apoptosis. To start with, we performed a bioinformatic analysis of data from OncoDB, which showed a lower expression pattern of $PPAR_{\gamma}$ in different cancer types. In addition, high expression of $PPAR_{\gamma}$ was associated with better breast cancer patient survival. Therefore, we tested the impact of pioglitazone, a PPARγ ligand, on the cytotoxic activity of cisplatin in the TNBC cell line. MDA-MB-231 cells were treated with either cisplatin (40 μM) with or without pioglitazone (30 or 60 μM) for 72 h. The MTT results showed a significant dose-dependent decrease in cell viability as a result of using cisplatin and pioglitazone combination compared with cisplatin alone. In addition, the protein expression of Bcl-2, a known antiapoptotic marker, decreased in the cells treated with cisplatin and pioglitazone combination at doses of 40 and 30 μM, respectively. On the other hand, cleaved- poly-ADP ribose polymerase (PARP) and -caspase-9, which are known as pro-apoptotic markers, were upregulated in the combination group compared with the solo treatments. Taken together, the addition of pioglitazone to cisplatin further reduced the viability of MDA-MB-231 cells and enhanced apoptosis compared with chemotherapy alone.

1. Introduction

Breast cancer (BC) is the most widely diagnosed type of cancer worldwide, accounting for approximately 12 % of 7.8 million cancer-diagnosed women in 2020 (Evelina Arzanova et al., 2022). One of the most challenging subgroups of breast tumors is triple-negative breast cancer (TNBC) (Yin et al., 2020). In fact, TNBC is challenging to treat because of limited targeted therapy options, and a significant proportion of patients show resistance to conventional therapy. About 10–20 % of breast cancer patients fall into this subgroup (Martini et al., 2022; Tzikas et al., 2020). Moreover, TNBC is characterized by aggressiveness, high proliferation, remodeling and stiffening of the extracellular matrix (ECM), and a greater distant metastatic potential (Bou Zerdan and Maroun, 2022; Drain et al., 2021; Karim et al., 2023). Furthermore, the mortality rate during the first 5 years following the diagnosis of TNBC is 40 %, which is higher than that of other subtypes of breast cancer (Yin et al., 2020). Therapy resistance leads to poor clinical outcomes, as evidenced by increased recurrence rates and lower survival rates (Yin et al., 2020,Nedeljković and Damjanović, 2019).

The ability of cancer cells to resist chemotherapy treatments can be attributed to a range of mechanisms. These mechanisms include increasing drug efflux or decreasing its influx, altering drug targets, improving DNA repair abilities, and increasing the detoxification system (Chen and Chang, 2019; Wang et al., 2021). In addition, avoiding apoptosis is another important mechanism by which cancer cells can exhibit treatment resistance and promote tumor growth. For instance, resistance to cisplatin can be induced by escaping apoptosis (Tchounwou et al., 2021) and may result from the dysfunction of apoptotic proteins and signaling pathways (Lugones et al., 2022; Zhu et al., 2016). However, other pathways have been proven to mediate apoptosis in

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cancer cells, activation of which may relieve chemoresistance. For instance, when activated by their ligands, peroxisome proliferator-activated receptors (PPARs) can either repress cell growth or induce apoptosis (Wagner and Wagner, 2022; Zhao et al., 2022). In this group, the transcription factor PPARγ stands out for its established role in regulating various genes associated with glucose metabolism and lipid homeostasis (Augimeri et al., 2020a). In general, PPARγ ligands can be divided into endogenous substances, including long-chain poly-unsaturated fatty acids and synthetic agents like thiazolidinedione (Augimeri et al., 2020b).

Interestingly, studies showed that PPARγ mRNA and protein levels in human breast tumors are lower than those in normal breast tissue (Badawi and Badr, 2003; Jiang et al., 2003). The expression of PPARγ in the MDA-MB-231 cell line was significantly lower than that in other breast cancer cell lines. For instance, the MCF-7 cell line (luminal A) exhibited the highest PPARγ expression when compared to the other tumor cell lines (zhao et al., 2022, Jiang et al., 2011).

Notably, earlier research has shown that the activation of PPARγ can exert anti-tumor effects in breast cancer by inhibiting cell proliferation, differentiation, cell growth, and cell cycle and inducing apoptosis in different in vitro and in vivo models(Bonofiglio et al., 2017; Elstner et al., 1998; Li et al., 2015; Moon et al., 2010; Catalano et al., 2011 Rovito et al., 2013). However, human clinical trials have shown no beneficial effect of using PPARγ ligands as monotherapy in several advanced human malignancies. (Burstein et al., 2003; Kulke et al., 2006; Smith et al., 2004). According to a preclinical study, troglitazone, a $PPAR_{\gamma}$ ligand, has a synergistic anticancer effect when combined with either cisplatin or paclitaxel in non-small-cell lung cancer (NSCLC). Interestingly, the synergistic impact of the combination was only observed when troglitazone was administered after chemotherapy, and not vice versa (Reddy et al., 2008).

As a possible mechanism, PPARγ exerts these favorable effects by increasing the expression of genes that encode proteins responsible for promoting apoptosis. In a preclinical study, PPARγ agonist and paclitaxel doubled the apoptotic index compared with paclitaxel alone in vitro and minimized anaplastic thyroid carcinoma (ATC) tumor growth in vivo (Copland et al., 2005). Moreover, PPARγ downregulates the expression of anti-apoptotic Bcl-2 genes (Tan et al., 2021). Furthermore, it was reported that PPARγ activation in thyroid carcinoma cells significantly increased the expression of the pro-apoptotic gene C-myc (Ohta et al., 2001). Furthermore, stimulation of PPARγ increases the expression of genes associated with apoptosis, such as growth arrest and DNA damage-inducible 153 (GADD153), in colon cancer cells (Shimada et al., 2002).

To the best of our knowledge, no reports have explored the combined effects of a PPARγ ligand and cisplatin on TNBC. This study aimed to investigate the potential role of PPARγ stimulation by pioglitazone in enhancing the efficacy of cisplatin therapy in TNBC. We assessed the impact of the pioglitazone and cisplatin combination on MDA-MB-231 cell viability and examined apoptotic markers to elucidate its mechanism of action.

2. Methods

2.1. Cells and cell culture

The MDA-MB-231 human breast cancer cell line, obtained from the American Type Culture Collection (ATCC, Rockville, USA), was cultured in RPMI 1640 medium (Sigma-Aldrich, Massachusetts, USA) in a T-75 tissue culture flask. The culture medium was supplemented with 10 % fetal bovine serum (FBS) and 1 % penicillin–streptomycin (Thermo Fisher Scientific, Massachusetts, USA). The cells were maintained in an environment with 5 % CO2 at 37 °C and 95 % relative humidity, and the culture medium was refreshed every 48–72 h. When the cells reached 80 % confluency, passage was performed as a part of the experimental procedure.

2.2. Cell treatment

To study the possible sensitizing effect of pioglitazone on the cisplatin effect in TNBC, MDA-MB-231 cells were seeded in six-well plates and allowed to attach overnight. The following day, the media was removed and replaced with fresh media containing either cisplatin 40 μM or a combination of cisplatin and different concentrations of pioglitazone (Cis 40 μM + Pio 30 μM) or (Cis 40 μM + Pio 60 μM) (Nadarajan et al., 2016). The dose of pioglitazone was selected on the basis of a previous scientific report that studied its effects on the mRNA expression of PPARα and other associated genes in MDA-MB-231 cells. The control group was treated with the control vehicle Dimethyl Sulfoxide (DMSO) only. The dose of cisplatin was selected on the basis of the MTT results where 40 μM was identified as the submaximal dose and implemented in the subsequent experiments. After 72 h, the cell viability assay and western blotting were performed.

2.3. Cell viability assay

Cell viability was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay. MDA-MB-231 cells were cultivated in 96-well plates and incubated for 24 h. Following this, the cells were treated as indicated. Subsequently, 40 μl of a fresh MTT solution (5 mg/ml) was introduced into each well, and the cells were incubated at 37 °C for 4 h. DMSO (Sigma-Aldrich, Massachusetts, USA). was then added to each well, and the absorbance was quantified using a microplate reader at a wavelength of 549 nm (Thermo Fisher Scientific, Massachusetts USA). Finally, the median inhibitory concentration (IC50) was determined through a nonlinear regression analysis of the plotted data using GraphPad Prism.

2.4. Immunoblotting

The cells were treated with either a control vehicle, 40 μM cisplatin, or combinations of cisplatin and pioglitazone (Cis 40 μM + Pio 30 μM) or (Cis 40 μM + Pio 60 μM). Following this, the cellular lysates were analyzed by immunoblotting to explore variations in the levels of various apoptotic markers. In brief, the cells were lysed using radioimmunoprecipitation assay (RIPA) Lysis Buffer (Thermo Fisher Scientific, Massachusetts USA) supplemented with protease inhibitors. Subsequently, the proteins were separated via sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride (PVDF) membranes (Sigma-Aldrich, Massachusetts, USA). The membranes were then obstructed with 5 % non-fat milk for 2 h. Following this, the membranes were exposed to primary antibodies targeting cleaved PARP (sc-56196), caspase-9 (sc-56076), and Bcl-2 (sc-7382), which were sourced from Santa Cruz (Santa Cruz, Texas, USA), and incubated overnight at 4 °C. After rinsing with buffer, the membranes were incubated for 1 h with a secondary antibody linked to horseradish peroxidase at room temperature. Protein bands were visualized using ECL reagent (Sigma-Aldrich, Massachusetts, USA) with the BioRad Molecular Imager (BioRad, California USA). Expression of GAPDH, a housekeeping gene, served as a loading control.

2.5. Bioinformatics analysis

Box plots were used for comparing the gene expression distribution of different groups i.e. in cancer vs normal samples, by mining The Cancer Genome Atlas (TCGA) RNA-seq using the OncoDB online database (https://www.oncodb.org/). RNA-Seq data is normalized, using Trusted Platform Module (TPM) according to the website. Kaplan–Meier plot was used to compare survival probabilities over time among different groups. ImageJ software (NIH) was used to analyze the blot images converted to grayscale.

2.6. Statistical analysis

Regression analysis was used to calculate the MTT results in the different treatment groups. The data obtained from TCGA are presented as the mean ± SEM and the comparisons were conducted using Student's *t*-test. Linear regression analysis was used to determine the statistical significance of dose–response curves.

3. Results

3.1. PPARγ expression level in various types of cancer

Further investigation of the status of PPARγ expression among different types of cancer was performed through the OncoDB online database (https://www.oncodb.org/). Mining TCGA RNA-seq data by generating box plots (Fig. 1) showed that the expression of PPARγ is significantly lower in all patients' samples compared with that in normal individuals across different types of cancer. Notably, among these types is Breast Cancer gene (BRCA)-associated breast cancer.

3.2. Pioglitazone enhances the cytotoxic effect of cisplatin

The MTT assay was conducted to study the effect of combining cisplatin with pioglitazone on TNBC cell viability. After treatment of MDA-MB-231 cells with cisplatin (40 μM) with or without pioglitazone (30 or 60 μM) for 72 h, the MTT results showed a dose-dependent decrease in cell viability (Fig. 2A). Accordingly, this finding indicates a possible enhancement effect of pioglitazone on cisplatin-induced cytotoxicity. As shown in Fig. 2A, the Lethal Dose 50 % (LC_{50}) of cisplatin was reduced from 60 to 20 μM after the addition of pioglitazone at different doses.

3.3. The impact of PPARγ gene expression on breast cancer patients' survival

Moreover, according to Kaplan–Meier analysis of breast cancer

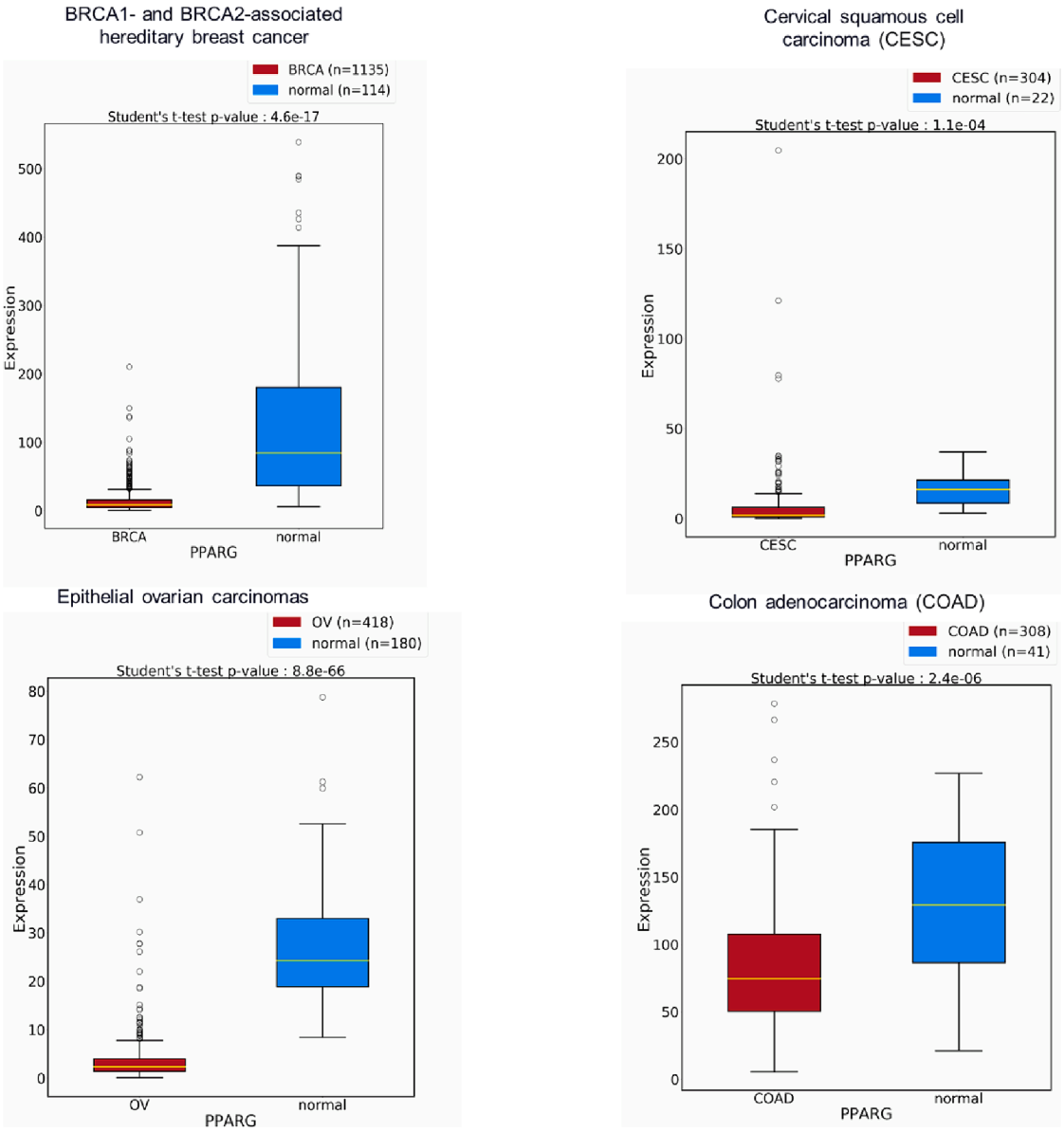

Fig. 1. PPARγ expression level in various types of cancer. A comparison of the gene expression levels of PPARγ in cancer vs normal samples in various types of cancer by mining TCGA RNA-seq data through generating box plots using OncoDB online database. RNA-Seq data is normalized, using Trusted Platform Module (TPM) according to the website. The patients' data are presented as the mean ± SEM and the comparisons were conducted using Student's *t* test.

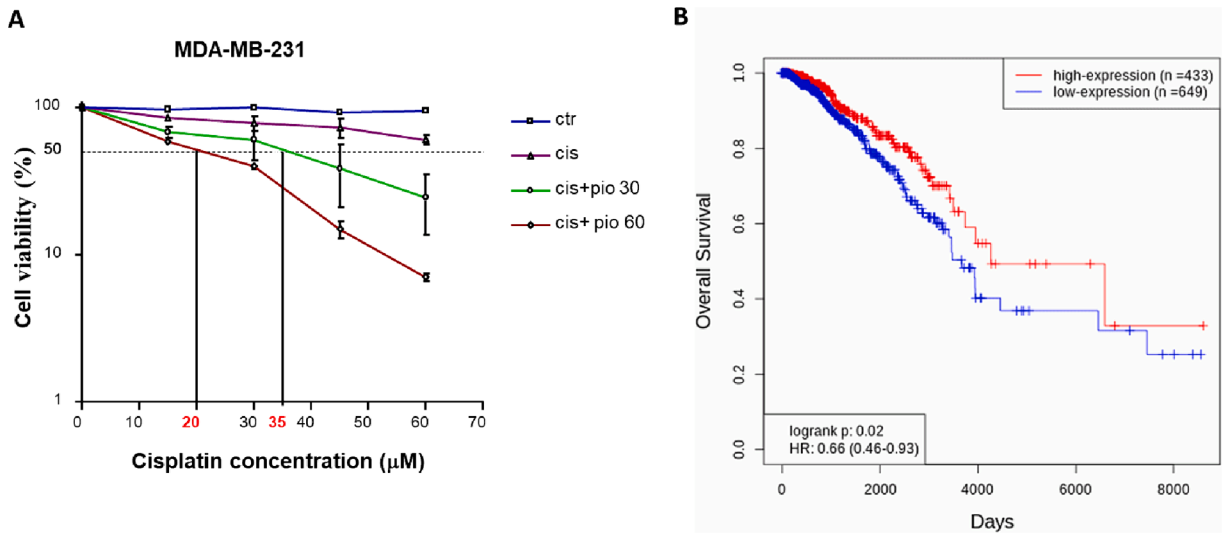

Fig. 2. Pioglitazone augments the cytotoxic effect of cisplatin(A) The cytotoxicity was determined based on MDA-MB-231 cells viability after treatment with either cisplatin (cis) alone at a dose of (40 μM) or its combination with pioglitazone (pio) at two doses (30 or 60 μM) for 72 h using the MTT test. The arrows indicate the LC50 for each treatment group. The results are presented as mean ± SEM (n = 3). Linear regression analysis was used to determine the statistical significance of dose–response curves (B) A Kaplan–Meier plot of 1082 breast cancer patients, the recurrence-free survival is significantly longer in patients with high PPARγ expression. The significance level was expressed as the log-rank p-value of 0.02 with a cutoff threshold of 60 %.

patients, the expression of PPARγ gene significantly influenced recurrence-free survival. Overall survival was greater in patients with high PPARγ expression (Fig. 2B).

3.4. Combination of cisplatin and pioglitazone enhances MDA-MB-231 cell apoptosis

Next, we sought to investigate the impact of the combination therapy at the molecular level, more precisely on apoptosis. Interestingly, the expression of Bcl-2, a known antiapoptotic marker, was decreased in cells treated with cisplatin and pioglitazone combination at doses of 40 and 30 μM, respectively (Fig. 3A). In the combination group, there was an observed trend of upregulation in apoptotic mediators, including cleaved-PARP and caspase-9, compared with the solo treatment groups (Fig. 3B).

4. Discussion

The activation of PPARγ was considered as a potential therapeutic strategy for various types of cancer. Numerous studies have reported that PPARγ can inhibit tumorigenesis in different types of cancer, including breast, pancreatic, lung, and colon cancers (Tan et al., 2021). Notably, the bioinformatic results in this study supported this premise where the expression of PPARγ is downregulated in almost all types of cancer studied. Our data showed that the expression of PPARγ is significantly lower in all patients' samples compared with normal individuals across different types of cancer. Notably, among these types are BRCA1- and BRCA2-associated breast cancer. Furthermore, according to Kaplan–Meier analysis of breast cancer patients, the expression of PPARγ gene significantly influenced recurrence-free survival. Overall survival is greater in patients with high PPARγ expression.

As mentioned earlier, the use of PPARγ agonists in breast cancer has been found to effectively hinder tumor growth by inhibiting cell proliferation, differentiation, cell growth, cell cycle progression, and inducing apoptosis in different in vitro and in vivo models (Bonofiglio et al., 2017; Elstner et al., 1998; Li et al., 2015; Moon et al., 2010; Catalano et al., 2011, Rovito et al., 2013). In fact, PPARγ could be regulated by many epigenetic effectors, including non-coding RNAs, epigenetic enzymes, histone modifiers, and DNA methyltransferases, as reviewed by Porcuna et al. (Porcuna et al., 2021). In addition, BRCA1/2 dysregulation in breast cancer, including TNBC, may influence PPARγ expression indirectly via modifications in the cellular environment.

Our results showed that adding PPARγ agonist to cisplatin significantly enhanced the latter cytotoxicity. The addition of pioglitazone to cisplatin further dose-dependently reduced the viability of MDA-MB-231 cells compared with monotherapy. In addition, we showed that

Fig. 3. The combination of cisplatin and pioglitazone enhances MDA-MB-231 apoptosis. MDA-MB-231 cells were treated with DMSO (CTR) or either with cisplatin alone (40 mM) or a combination (COM) of cisplatin (Cis, 40 μM) and pioglitazone (Pio, 30 μM) for 72 h. Subsequently, cell were harvested, and proteins were used for western blot analysis using the (A) Bcl-2 or (B) cleaved-caspase 9 and cleaved-PARP antibodies. The band intensities were semi-quantified using ImageJ software (NIH).

the overall survival of breast cancer patients is significantly higher in PPARγ high-expression group than in the low-expression group. The addition of pioglitazone reduced the LC50 of cisplatin, which has substantial clinical implications. These include enhanced efficacy of cisplatin at lower doses, reduced toxicity, and potential for improved patient outcomes. Similar findings were reported in 2008 by Geoffrey et al, who observed that the combination of rosiglitazone and carboplatin had a synergistic effect on inhibiting tumor development in various genetically engineered mouse models of lung carcinogenesis. Importantly, the combination did not increase systemic toxicity (Girnun et al., 2008). In addition, pretreatment with rosiglitazone reduces cisplatin-induced renal damage in dimethylbenz[a]anthracene (DMBA)-induced breast cancer and synergistically increases the anticancer effectiveness of cisplatin (Tikoo et al., 2009).

As a possible molecular mechanism, we investigated the probable involvement of the apoptotic pathway in this effect. According to our findings, PPAR-γ activation through pioglitazone enhanced apoptosis as evidenced by lowering the expression of the antiapoptotic protein Bcl-2 and increasing caspase-9 levels. This finding goes in parallel with many previous studies that have documented that PPAR-γ agonists exert apoptotic effects. For instance, another PPARγ agonist, troglitazone, was documented to mediate apoptosis in MDA-MB-231 cells in a dose-dependent pattern (Park et al., 2006). Indeed, different mechanisms have been implicated in this action either by decreasing the expression of anti-apoptotic genes expression, like Bcl-2, or by promoting pro-apoptotic factors (e.g., Bax) (Wu et al., 2021). Moreover, Chern et al.. suggested that thymoquinone, through acting as a PPARγ ligand, downregulates the expression of the Bcl-2 gene and exerts strong anti-proliferative effects on MDA-MB-231 cells (Woo et al., 2011). Indeed, the decrease in Bcl-2 expression is possibly a consequence of both the direct effect of the combination treatment and the modulation of different cellular processes. Our data show a significant reduction in Bcl-2 expression following the combination treatment with pioglitazone and cisplatin compared with the control group, which provides evidence of the direct effect of the combination treatment on Bcl-2. However, this is not an exclusive fact since besides PPARγ activation, the combination treatment may precipitate cross-talk with other signaling pathways and dynamic cellular responses. This would require further investigations into the precise molecular mechanisms relating PPARγ activation to Bcl-2.

In addition, we found that the protein expression of cleaved-PARP was increased after adding pioglitazone to cisplatin. Poly(ADP-ribose) polymerase (PARP) is a caspase cleavage substrate and is a known hallmark of apoptosis (Chaitanya et al., 2010). In addition, cleavage of PARP by caspases results in its inactivation, which in turn terminates its action in promoting DNA repair, resulting in cancer cell apoptosis. Similar to our finding, rosiglitazone treatment of A549 (lung carcinoma epithelial) cells with and without carboplatin resulted in a significant elevation of cleaved PARP (Khandekar et al., 2018). In addition, in one study using 6-iodolactone (6IL) supplement, a derivative of iodinated arachidonic acid (AA) that possesses antitumoral effects, resulted in significant stimulation of PPARγ and triggers apoptosis in MCF-7 cells through both Bax caspases and apoptosis inducing factor (AIF)/PARP-1 (Arroyo-Helguera et al., 2008).

Recent studies have provided valuable insights into the apoptotic pathways involving PPARγ and caspase activation. Activation of PPARγ leads to an increase in the activity of caspase-3, −8, and −9 through receptor-meditated signaling pathways such as the receptor for advanced glycation end products (RAGE), Toll-like receptor-4 (TLR4)-dependent MAPK, Phosphatase and tensin homolog (PTEN)-Akt, mammalian target of rapamycin (mTOR), and Nuclear factor kappa B (NF-kB). This caspase activation leads to PARP cleavage and ultimately results in apoptosis (Chi et al., 2021). The activation of RAGE by advanced glycation end products (AGEs) induces oxidative stress and promotes apoptosis in various cells (Ishibashi et al., 2013). This occurs in a cascade of downstream signaling, including MAPK, JNK/p38, and NF-κB (Chi et al., 2021). Subsequently, activated caspase-9 triggers the intrinsic apoptotic pathway, which is correlated with Bcl-2 family proteins. Thus, the downregulation of Bcl-2 may be an outcome of the amplification of caspase-mediated apoptotic signaling. Moreover, the combination we used may interrupt the balance of pro- and anti-apoptotic Bcl-2 family members, favoring a proapoptotic environment. In addition, activation of PPARγ and RXR was shown to induce anti-tumor effects in MCF7 cells by stimulating the intrinsic apoptotic pathway through the production of cytochrome *c* and subsequent cleavage and activation of caspase-9 (Bonofiglio et al., 2009).

In our study, we focused on key apoptotic markers; however, further exploration of potential apoptotic pathways and markers for future investigation is warranted to better understand the exact molecular mechanisms of our findings. These include; extrinsic apoptotic pathway (Fas/FasL or TRAIL signaling), other Bcl-2 family members (Bax, Bak), death receptors such as Fas (CD95) or TNF receptor 1 (TNFR1), mitochondrial membrane potential (ΔΨm), DNA fragmentation, and endoplasmic reticulum (ER) stress. In addition, the caspase-independent pathway of apoptosis involving AIF that causes DNA fragmentation and chromatin condensation is of great importance for further investigation.

In conclusion, the activation of PPARγ by using its ligand pioglitazone further enhances the effect of cisplatin and inhibits MDA-MB-231 cell viability compared with chemotherapy alone. Such an effect can be attributed to the enhancement of apoptosis by lowering the expression of the antiapoptotic protein Bcl-2 and increasing caspase-9 and cleaved-PARP levels. The synergy between PPARγ activation by pioglitazone and cisplatin's cytotoxic effects probably involves broader cellular processes and pathways. Possible examples include DNA damage and repair, cellular metabolism and energetics, and epigenetic regulation. In addition, PPARγ is known for its anti-inflammatory properties, and the role of inflammation and immune modulation in cancer progression and treatment response has been well documented (Moon et al., 2010; Rovito et al., 2013; Shimada et al., 2002). Although our study focused on the MDA-MB-231 cell line, we acknowledge the importance of evaluating the reproducibility of the effect of combined treatment across multiple TNBC cell lines to establish the broader applicability of our findings. Another limitation of our study is the lack of cell cycle analysis. Indeed, cell cycle analysis and Annexin V apoptosis assay would provide a more comprehensive understanding of the mechanisms and enrich our methods section. Further exploration in preclinical and clinical trials will be vital to confirm these findings and verify the translational relevance of pioglitazone and cisplatin combination therapy in the clinical management of TNBC. Given the well-established side effects of cisplatin, such as nephrotoxicity and neurotoxicity, its combination with pioglitazone may introduce additional considerations because both agents could contribute to a cumulative effect of certain toxicities. Therefore, an in vivo study would be needed to assess the safety of such a combination and would be considered for our future work.

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CRediT authorship contribution statement

Qamraa Hamad Alqahtani: Writing – review & editing, Writing – original draft, Validation, Supervision, Resources, Project administration, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Layla Abdullah Alkharashi:** Validation, Supervision, Software, Resources, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Hanaa Alajami:** Validation, Supervision, Methodology, Investigation. **Ishraq Alkharashi:** Validation, Software, Methodology, Investigation, Data curation. **Layan Alkharashi:** Validation, Supervision, Software, Resources, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Shoug**

Nasser Alhinti: Writing – review & editing, Writing – original draft, Validation, Software, Data curation.

Declaration of competing 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.

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