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Targeting STAT3 With C188-9 Enhances Doxorubicin Cytotoxicity and Alters Monocyte Surface Marker Expression in Triple-negative Breast Cancer

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ABSTRACT

Triple-negative breast cancer (TNBC) is an aggressive breast cancer subtype associated with early relapse, poor survival, and limited therapeutic options. Doxorubicin (DOX) is a standard treatment for TNBC; however, its clinical use is restricted by dose-limiting toxicities. Signal transducer and activator of transcription 3 (STAT3) is frequently activated in TNBC and contributes to tumor progression and immune modulation. We investigated whether inhibition of STAT3 with the small-molecule inhibitor C188-9 could enhance the antitumor efficacy of DOX and modulate monocyte phenotype in vitro.

MDA-MB-231 cells, a single TNBC cell line, were treated with DOX (7.4 μg/mL), C188-9 (14.2 μg/mL), or their combination (2 μg/mL DOX plus 7.1 μg/mL C188-9). Cell viability was evaluated using the MTT assay. Apoptosis and migration were assessed using Annexin V/propidium iodide staining and a wound-healing assay, respectively. To examine tumor-immune interactions, primary human monocytes from three healthy donors were exposed to conditioned medium (CM) from treated cancer cells, and the CD86/CD206 ratio was analyzed by flow cytometry.

The DOX/C188-9 combination significantly reduced viability and increased apoptosis compared with either drug alone. STAT3 inhibition also attenuated cell migration. CM from cancer cells treated with a STAT3 inhibitor increased the CD86/CD206 ratio in monocytes, and this effect was greatest with combination therapy.

Targeting STAT3 sensitized TNBC cells to DOX and modulated monocyte surface marker expression toward an M1-like phenotype. These findings suggest a potential immunomodulatory effect of STAT3 inhibition, but further in vivo studies are required to validate its therapeutic relevance.

Keywords: C188-9 compound; Doxorubicin; STAT3 transcription factor; Triple-negative breast cancer; Tumor microenvironment

INTRODUCTION

Breast cancer is the most common malignant tumor and the second leading cause of cancer-related death among women worldwide.[1] It is a heterogeneous disease composed of distinct molecular subtypes. Triple-

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negative breast cancer (TNBC) is an aggressive clinical subtype defined by the absence of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2) expression.[2] TNBC constitutes approximately 20% of all breast cancer cases and is characterized by aggressive behavior, poor survival, and a high recurrence rate. Despite available treatments, including chemotherapy and immunotherapy, metastasis and multidrug resistance (MDR) remain critical challenges in managing TNBC.[3,4] Emerging evidence highlights the critical role of the tumor microenvironment (TME) in cancer development and progression. The TME is a complex network comprising extracellular matrix, stromal and immune cells, cytokines, and signaling factors. Ultimately, tumor suppression or progression depends on the dynamic feedback loops and cellular interactions between cancer and heterogeneous cells that occur in this microenvironment. Therefore, disrupting key regulatory loops within the TME is a promising therapeutic strategy.[5-8] Several studies have shown that constitutive activation of signal transducer and activator of transcription 3 (STAT3) serves as a key regulatory hub within the TME. It is activated in various cancers, such as breast cancer and plays a pivotal role in regulating multiple features of tumor biology, including cell proliferation, migration, immunosuppression, and resistance to chemotherapy.[9] In addition, the secretion of interleukin-6 (IL-6), interleukin-8 (IL-8), and other growth factors by cancer and stromal cells in the TME leads to STAT3 signaling pathway activation in tumor-associated immune cells. This activation drives the expansion of myeloid-derived suppressor cells (MDSCs) and tumor-associated macrophages (TAMs), inhibits dendritic cell maturation, and suppresses cytotoxic T-cell function, thereby impairing antitumor immunity.[10-14] Activation of STAT3 in monocytes promotes M2 macrophage polarization by upregulating the expression of interleukin-10 (IL-10), arginase 1 (ARG1), and the interleukin-4 receptor α, while concurrently suppressing pro-inflammatory cytokines such as tumor necrosis factor α (TNF-α) and interleukin-12 (IL-12). In contrast, inhibition of STAT3 skews macrophages toward an M1-like phenotype, characterized by increased surface expression of CD86 and major histocompatibility complex (MHC) class II, thereby enhancing antigen presentation and T-cell activation. This phenotypic switch is mediated by the downregulation of STAT3-dependent target genes, including *c-Myc* and *HIF-1α*, which are known to drive the metabolic reprogramming of macrophages toward an immunosuppressive state.[15,16] A recent study in multiple myeloma directly demonstrated that inhibition of STAT3 with the small-molecule inhibitor C188-9 reversed tripartite motif-containing protein 21 (TRIM21)-induced M2 polarization by restoring suppressor of cytokine signaling 3 (SOCS3) expression and inhibiting the Janus kinase (JAK)-STAT pathway. Together, these findings further support the immunomodulatory role of STAT3 inhibition.[17] Furthermore, STAT3 activity has been shown to play a key role in chemoresistance to agents such as doxorubicin (DOX) in patients with breast cancer.[18-20] Notably, DOX induces DNA damage and inhibits topoisomerase II in cancer cells, triggering a signaling cascade that upregulates proinflammatory mediators such as granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-6, and IL-8. This leads to the activation of transcription factors, including STAT3 and nuclear factor κB (NF-κB), creating a feedforward loop that exacerbates immunosuppression.[21-23] Previous studies have demonstrated that inhibition of STAT3 enhances chemosensitivity in TNBC models.[24] For instance, STAT3 knockdown using small interfering RNA (siRNA)[24] or treatment with the small-molecule inhibitors Stattic[25] and S3I-201[26] sensitized MDA-MB-231 cells to doxorubicin, paclitaxel, and cisplatin. Importantly, beyond its role in tumor progression and drug resistance, STAT3 phosphorylation in macrophages has been identified as a mechanism contributing to DOX-induced cardiomyopathy (DCM), suggesting that inhibition of this pathway may also mitigate associated cardiac damage.[27] Considering the pivotal role of STAT3 in shaping an immunosuppressive TME, its inhibition represents a promising strategy to restore antitumor immunity and break the cycle of immunosuppression.[28] Over the past decades, targeting STAT3 directly or indirectly through the development of novel therapeutic agents has attracted considerable attention in cancer treatment strategies. Although no drugs have yet been approved by the US Food and Drug Administration, these approaches show promise for future clinical application. The major limitations of current STAT3 inhibitors include poor oral bioavailability, off-target effects, and dose-limiting toxicities. Many first-generation inhibitors (eg, Stattic, S3I-201) also suffer from low specificity and need to be used at relatively high concentrations, which limits their

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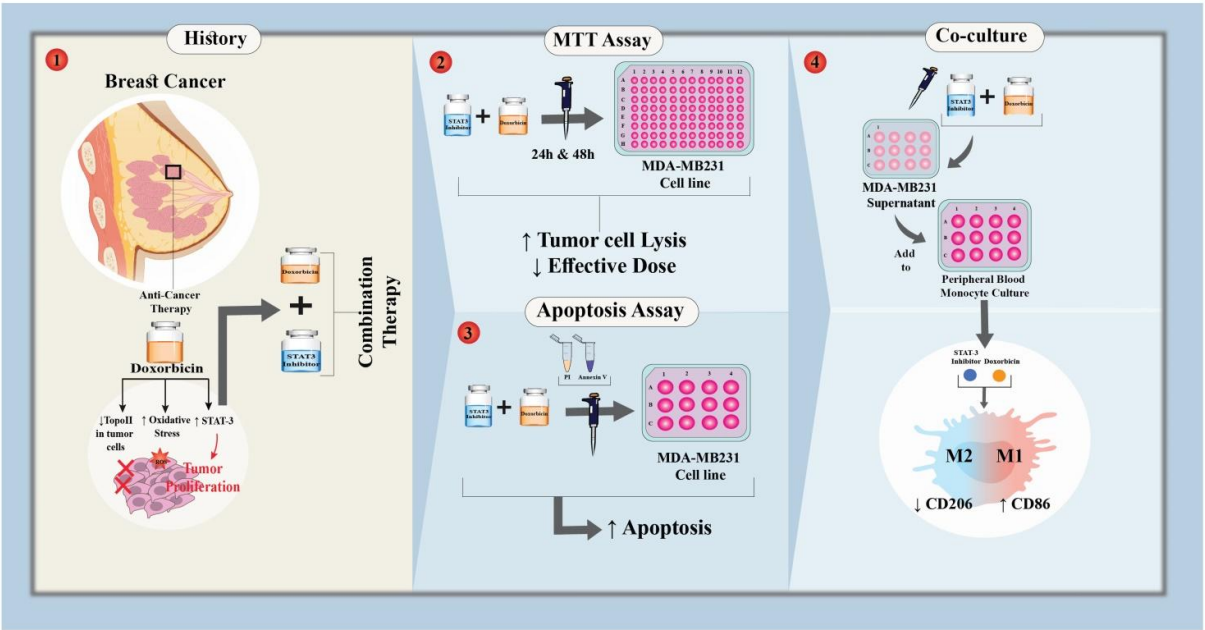
clinical application. Furthermore, some small-molecule STAT3 inhibitors have caused serious adverse events such as lactic acidosis and peripheral neuropathy due to mitochondrial dysfunction. In contrast, C188-9 (TTI-101) does not affect mitochondrial respiration and even alleviates chemotherapy-induced neuropathic pain,[29] making it a more suitable candidate for combination therapy.[30,31] C188-9 directly binds to the STAT3 Src homology 2 (SH2) domain, blocking its dimerization, phosphorylation, and nuclear translocation.[32] This compound has shown promising antitumor efficacy in several cancers, including hepatocellular carcinoma,[33] non-small cell lung cancer,[34] head and neck squamous cell carcinoma (HNSCC),[35] and pancreatic cancer.[36] Moreover, *in vitro* treatment with C188-9 enhanced the responsiveness of HNSCC cells to chemotherapeutic agents.[35] However, studies on the use of C188-9 for treating TNBC, and its combination with DOX in any cancer, remain limited, and no such studies have yet been reported. The aim of this study was to explore a novel strategy to enhance the antitumor efficacy of DOX in the aggressive TNBC cell line MDA-MB-231 by combining it with C188-9. Using *in vitro* experiments, we found that the DOX/C188-9 combination had stronger antitumor effects than either agent alone, allowing for a substantial reduction in the effective dose of DOX. The enhanced efficacy correlated with increased apoptosis in cancer cells and changes in monocyte surface marker expression, suggesting a trend toward an M1-like phenotype. These findings suggest that STAT3 inhibition may modulate tumor–immune cell interactions in this *in vitro* setting; however, further *in vivo* studies are required to determine whether this translates into an immunostimulatory TME.



Summary: C188-9 and doxorubicin synergistically induce apoptosis in triple-negative breast cancer cells, shift monocyte surface marker expression toward an M1-like phenotype, and may enhance antitumor immunity.

MATERIALS AND METHODS

Cell Culture and Drug Treatment

The human breast cancer cell line MDA-MB-231 was obtained from the National Cell Bank of Iran, Tehran, Iran. Cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS; Gibco, Germany) and antibiotics (streptomycin, 100 ng/mL; penicillin, 100 U/mL) at 37°C in a humidified 5% CO_2 atmosphere. Stock solutions of the STAT3 inhibitor C188-9 (TTI-101; Cayman Chemical Company, USA) and doxorubicin hydrochloride (Pfizer, Australia) were prepared in dimethyl sulfoxide (DMSO; Merck, Germany) and sterile phosphate-buffered saline (PBS), respectively, according to their manufacturer's

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instructions. Working concentrations were prepared in complete culture medium immediately prior to use. For conditioned medium (CM) preparation, MDA-MB-231 cells (2×10^5 cells/well) were seeded in 6-well plates and allowed to adhere overnight at 37 °C with 5% CO_2. Cells were then treated with DOX, C188-9, or their combination for 24 hours. Following treatment, the culture medium was collected, centrifuged at 300*g* for 5 minutes to remove cell debris, and the supernatant was collected as CM. Primary human monocytes were incubated with the CM for 24 hours to assess the effects of tumor-derived factors on monocyte polarization by flow cytometry.

Isolation of Peripheral Blood Monocytes

Peripheral blood mononuclear cells (PBMCs) were isolated from blood samples from three independent healthy donors by density gradient centrifugation using Lymphodex (Inno-Train, Germany), as described elsewhere.[37] Briefly, blood was diluted 1:1 with sterile PBS and carefully layered over Lymphodex solution. After centrifugation at 350*g* for 30 minutes at 20°C, the PBMC layer (buffy coat) was collected and washed three times with PBS (10 minutes at 300*g*). To isolate monocytes, the PBMC suspension was transferred to culture flasks and incubated for three hours at 37°C in a 5% CO_2 humidified incubator to allow monocyte adhesion. Nonadherent cells were then removed and the adherent monocytes were used for subsequent experiments. Monocytes were identified by forward-scatter and side-scatter (FSC/SSC) gating, and then the expression of CD86 and CD206 was analyzed on the gated monocyte population. Data are presented as mean±standard deviation (SD), and donor-to-donor variability is included in the error bars. A representative gating strategy is shown in Supplementary Figure S1.

Cell Proliferation and Viability Assay

Cell viability was assessed by measuring the formazan crystals produced via the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) by mitochondrial dehydrogenases in viable cells. Briefly, MDA-MB-231 cells (4×10^3 cells/well) were seeded in 96-well plates and allowed to adhere overnight. The following day, cells were treated with various concentrations of C188-9 and DOX. For combination treatments, DOX was tested at fractions of its IC_{50} (25%, 50%, 75%, and 100%) together with equivalent fractions of the C188-9 IC_{50}. The resulting 4 × 4 dose-response matrix is shown in Supplementary Table S1. Vehicle control wells received an equivalent volume of C188-9 solvent (DMSO). After 24 or 48 hours, the supernatant was carefully removed, and 200 µL of MTT solution (5 mg/mL) was added to each well. Plates were then incubated for 4 hours at 37°C in a 5% CO_2 atmosphere. Subsequently, the resulting formazan crystals were dissolved in 100 µL of DMSO. Absorbance was measured at 492 nm with a reference wavelength of 690 nm using an ELISA microplate reader (Stat Fax 2100, Awareness Technology, Palm City, FL, USA). All experiments were performed in triplicate. Dose–effect data from single-agent and combination experiments were assessed by calculating the combination index (CI) through the Chou–Talalay method using CompuSyn software (v1.0). A fraction affected (Fa)-CI plot was generated to characterize the interaction. CI<1 indicates synergy, CI=1 indicates an additive effect, and CI>1 indicates antagonism.

Apoptosis Assay

Induction of apoptosis was assessed by flow cytometry using Annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI; BD Pharmingen) according to the manufacturer's instructions. Briefly, MDA-MB-231 cells (2×10^5 cells/well) were seeded in 6-well plates and treated with DOX at its IC_{50} (7.4 µg/mL), C188-9 at its IC_{50} (14.2 µg/mL), or the combination (2 µg/mL DOX plus 7.1 µg/mL C188-9) for 24 hours. After treatment, cells were washed twice with PBS and resuspended in 1× Annexin V binding buffer. Cells were then stained with 5 µL of Annexin V-FITC for 15 minutes at room temperature in the dark, followed by the addition of PI immediately before analysis. Samples were analyzed using a BD flow cytometer, and data were processed with FlowJo software (v10).

Cell Migration and Invasion

Cell migration was assessed using a wound-healing assay. Briefly, MDA-MB-231 cells (2×10^5 cells/well) were seeded in 6-well plates and cultured for 24 hours until they were 95% confluent. A uniform, cell-free scratch was created in the center of each well using a sterile 200 µL micropipette tip. After washing twice with PBS, the cells were treated with DOX (7.4 µg/mL), C188-9 (14.2 µg/mL), or the combination (2 µg/mL DOX plus 7.1 µg/mL C188-9) for 24 hours. Vehicle control wells received an equivalent volume of C188-9 solvent (DMSO). Images were captured immediately

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after wounding (0 hours) and 48 hours later using an inverted microscope at 10× magnification. The width of the scratch was measured using Fiji (ImageJ; National Institutes of Health, USA) and the percentage of wound closure was calculated.

Statistical Analysis

All experiments were repeated at least three times. Data are presented as mean±SD. Statistical comparisons were made using one-way analysis of variance (ANOVA) for multiple groups or Student t test for two groups, with significance defined as $p<0.05$. GraphPad Prism (version 6.07) was used for all analyses.

RESULTS

C188-9 Synergistically Enhances the Cytotoxic and Apoptotic Effects of Doxorubicin in MDA-MB-231 Cells

To determine whether combining C188-9 with DOX enhances cytotoxicity compared to either agent alone, MDA-MB-231 cells were treated using a combination matrix based on the previously determined IC_{50} values. Specifically, DOX was tested at fractions of its IC_{50} (25%, 50%, 75%, and 100%) in combination with equivalent fractions of the C188-9 IC_{50}. This nonconstant-ratio design generated a comprehensive dose-response surface, enabling a robust quantitative assessment of drug interactions, whether synergistic, additive, or antagonistic. The single-agent activities of C188-9 and DOX in MDA-MB-231 cells showed dose-dependent growth inhibition, with 24-hour IC_{50} values of 14.2 and 7.4 µg/mL, respectively (Figure 1). However, co-treatment with C188-9 significantly enhanced DOX cytotoxicity. Combination index (CI) values were calculated at multiple effect levels (Fa=0.50, 0.75, and 0.90). At a low C188-9 concentration (3 µg/mL), the combination yielded predominantly antagonistic effects (CI>1); however, combinations using higher C188-9 concentrations (7, 10, and 14 µg/mL) produced CI values <1, indicating strong synergistic effects (Figure 1C). Complete CI data for all tested concentration pairs are provided in Supplementary Table S2. The therapeutic benefit of this synergy was quantified by the dose reduction index (DRI), which showed that the effective dose of DOX could be reduced by up to 4-, 8.4-, and 11.1-fold when combined with 7, 10, and 14 µg/mL C188-9, respectively (detailed values are provided in Supplementary Table S3). The combination of 0.25× the DOX IC_{50} with 0.5× the C188-9 IC_{50} was selected as the optimal ratio for further studies. These findings demonstrate that C188-9 significantly enhances DOX cytotoxicity, providing a strategy to lower its effective dose and potentially alleviate the adverse effects of high-dose chemotherapy. To explore the mechanism underlying the observed synergy, apoptosis was assessed by Annexin V/PI staining and flow cytometry. Representative quadrant plots are shown in Figure 2A, and the quantitative data are summarized in Supplementary Table S4. Compared to the control, the DOX/C188-9 combination significantly increased early apoptosis (from 3% to 17%) and late apoptosis (from approximately 2% to approximately 24%) after 24 hours, while necrosis remained low (from 3% to 7%). The proportion of total apoptotic cells induced by the DOX/C188-9 combination was approximately 42%, which was significantly higher ($p<0.001$) than that induced by either agent alone (approximately20%) (Figure 2B). These findings indicate that the synergistic cytotoxicity is mediated, at least in part, through enhanced activation of the apoptotic pathway.

The Doxorubicin/C188-9 Combination Synergistically Inhibits Migration and Invasion of MDA-MB-231 Cells

Because cell motility is a critical factor in tumor metastasis, the effect of the DOX/C188-9 combination on MDA-MB-231 cell migration was evaluated using a wound-healing assay. As shown in Figure 3A, untreated control cells exhibited rapid migration, achieving 85.3±4.1% wound closure after 48 hours. Monotherapy with DOX or C188-9 moderately reduced closure to 62.7±5.8% ($p<0.01$ vs control) and 41.2±6.3% ($p<0.001$ vs control), respectively. In contrast, the DOX/C188-9 combination nearly abolished cell movement, limiting wound closure to only 18.9±3.5% ($p<0.001$ vs control and all other groups) (Figure 3). The calculated CI for inhibiting migration was 0.72, confirming a synergistic interaction between the two agents. These results suggest that the combination therapy may suppress migration associated with local invasion and metastasis.

STAT3 Inhibition in Tumor Cells Promotes Proinflammatory Monocyte Polarization

To better understand the influence of the TME on immune cells, we employed an indirect coculture system. Primary human monocytes were exposed for 24 hours to CM collected from MDA-MB-231 cell cultures

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treated with DOX, C188-9, or the DOX/C188-9 combination. Surface expression of M1 and M2 markers, CD86 and CD206, respectively, was measured by flow cytometry. Because both antibodies were phycoerythrin (PE)-conjugated, CD86 and CD206 were stained and analyzed in separate tubes. Monocytes were identified by their characteristic forward and side scatter profile (Figure 4A). Stimulation with CM from C188-9-treated tumor cells increased the frequency of CD86-positive monocytes and decreased CD206-positive cells. The CD86/CD206 ratio increased from approximately 0.31 in control monocytes to 0.95 in the DOX group, 1.61 in the C188-9 group, and 1.79 in the DOX/C188-9 group (Figure 4B). These results indicate that STAT3 inhibition in cancer cells can shift monocyte surface marker expression toward a pro-inflammatory, M1-like phenotype, suggesting a potential mechanism by which STAT3-targeted interventions may modulate the TME from immunosuppressive to immunostimulatory. However, the CD86/CD206 ratio should be interpreted as an approximate measure because the markers were analyzed in separate tubes.

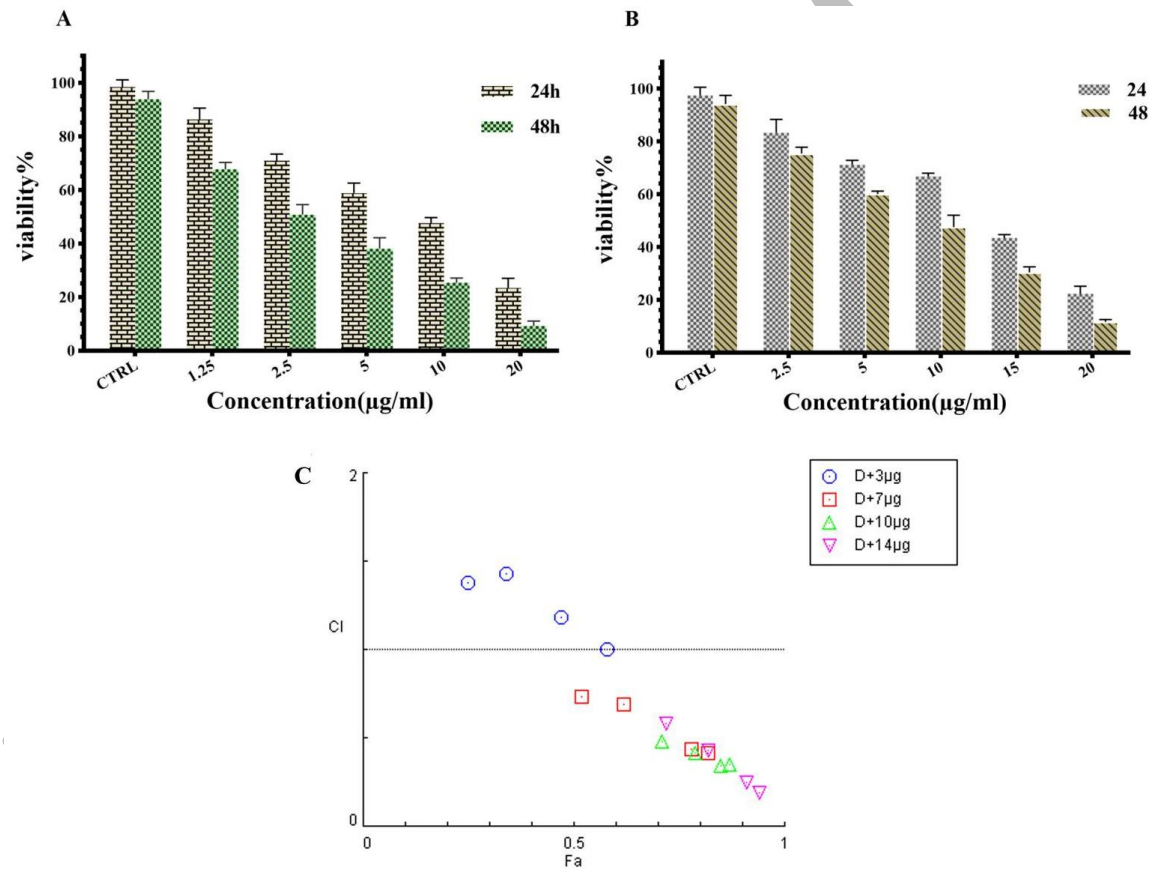

Figure 1. Effect of C188-9 and doxorubicin on the viability of MDA-MB-231 triple-negative breast cancer cells. A. and B. Cell viability was assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay at 24 and 48 hours after treatment with doxorubicin or C188-9 alone. C. Fraction affected-combination index plot after 24 hours of treatment with fixed C188-9 concentrations (3, 7, 10, and 14 µg/mL) and increasing doxorubicin concentrations (1.8, 3.7, 5.5, and 7.4 µg/mL; 25% to 100% of the half-maximal inhibitory concentration). A combination index <1 indicates synergism; =1, an additive effect; and >1, antagonism. CTRL indicates vehicle control (dimethyl sulfoxide). Data are mean±standard deviation (n=3).

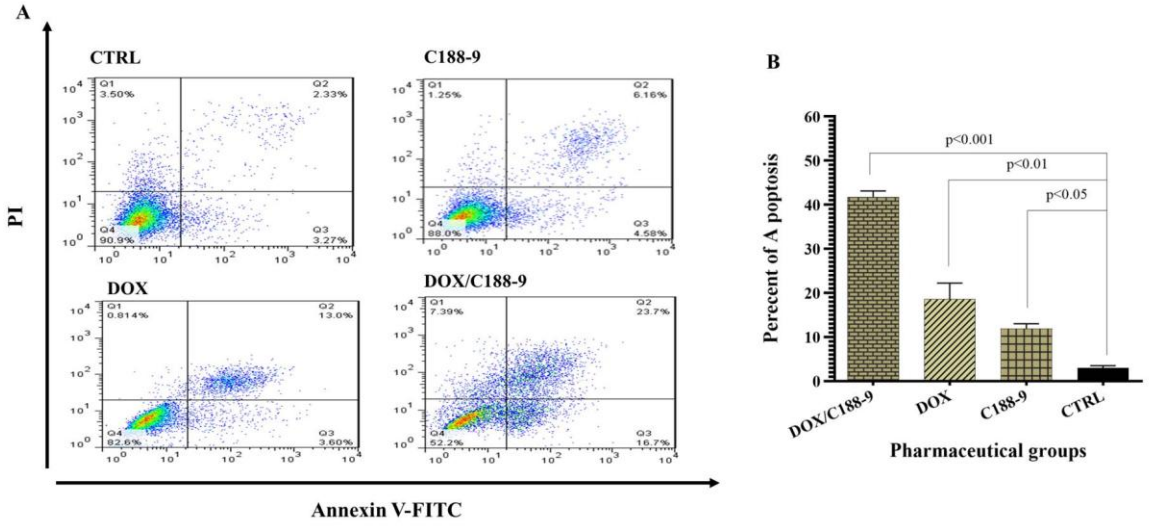

Figure 2. Apoptotic effects of C188-9 and doxorubicin on MDA-MB-231 triple-negative breast cancer cells. A. Representative flow cytometry plots showing Annexin V-fluorescein isothiocyanate and propidium iodide staining after 24 hours of treatment with dimethyl sulfoxide (control), doxorubicin (7.4 µg/mL), C188-9 (14.2 µg/mL), or the combination (2 µg/mL doxorubicin plus 7.1 µg/mL C188-9). B. Quantification of the total percentage of apoptotic cells. Data are mean±standard deviation (n=3). Statistical significance is indicated as *p<0.05, **p<0.01, and ***p<0.001.

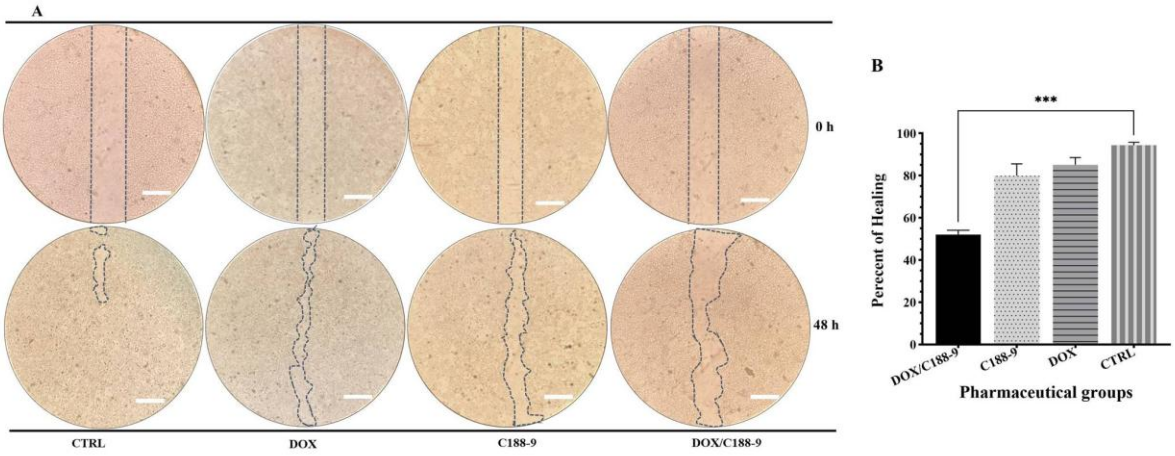

Figure 3. Effect of C188-9 and doxorubicin on MDA-MB-231 triple-negative breast cancer cell migration. A. Representative images of the wound-healing assay at 0 and 48 hours after treatment with dimethyl sulfoxide (control), doxorubicin, C188-9, or their combination. B. Quantification of wound closure. Scale bar, 200 µm. Data are mean±standard deviation (n=3). Statistical significance is indicated as *p<0.05, **p<0.01, and ***p<0.001.

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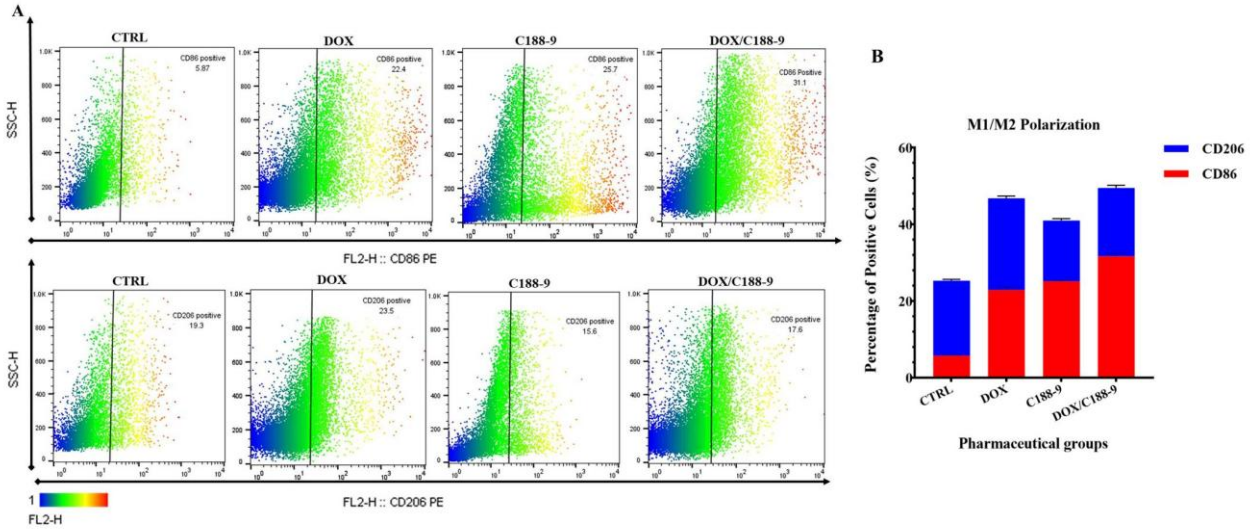

Figure 4. Effect of C188-9 and doxorubicin on monocyte polarization. A. Representative flow cytometry plots showing CD86 and CD206 expression in primary human monocytes after 24 hours of incubation with conditioned medium from MDA-MB-231 cells treated with dimethyl sulfoxide (control), doxorubicin (7.4 µg/mL), C188-9 (14.2 µg/mL), or the combination (2 µg/mL doxorubicin plus 7.1 µg/mL C188-9). B. The CD86/CD206 ratio indicated an M1-like shift, with the greatest change induced by the combination. Data are mean±standard deviation (n=3).

DISCUSSION

Currently, there are no targeted therapies available for patients with TNBC.[38] Furthermore, immunotherapy with atezolizumab is applicable only to the subset of TNBC patients whose tumors express PD-L1.[39,40] Consequently, systemic chemotherapy with DOX remains the primary treatment option, despite its associated side effects.[38] Constitutively active STAT3 is detected in over 50% of primary breast tumors and established cell lines.[41] Moreover, elevated STAT3 expression or activity correlates strongly with reduced survival rates in breast cancer patients.[42] Several STAT3 inhibitors, including AZD9150, C188-9, OPB-31121, and OPB-51602, are being evaluated in clinical trials.[43] Notably, Kong et al previously demonstrated that C188-9 enhanced the therapeutic efficacy of low-dose 5-aza-2′-deoxycytidine (DAC) in pancreatic cancer.[36] In this study, we investigated whether STAT3 inhibition could enhance the antitumor efficacy of DOX in the MDA-MB-231 TNBC cell line *in vitro*. Our results indicated that the DOX/C188-9 combination significantly inhibited MDA-MB-231 cell growth compared to either agent alone, with strong synergistic cytotoxicity (CI<1). This synergy allowed a substantial reduction in the effective DOX dose: 4-, 8.4-, and 11.1-fold when combined with 7, 10, and 14 µg/mL C188-9, respectively, while maintaining equivalent growth inhibition. In addition, MDA-MB-231 cells treated with DOX/C188-9 exhibited significantly higher apoptosis compared to those treated with DOX alone. This finding suggests that STAT3 inhibition sensitizes TNBC cells to DOX-induced apoptosis. To our knowledge, this study is among the first to demonstrate synergistic efficacy between C188-9 and DOX in a TNBC model. These *in vitro* findings suggest that combining STAT3 inhibition with conventional chemotherapy may represent a promising strategy that warrants further investigation in appropriate *in vivo* models. Migration is a key step in cancer metastasis, and evidence suggests that chemotherapeutic agents, including DOX, can enhance cell migration in certain cancer models.[44] STAT3 has been established as a key regulator of MMP-9 expression, promoting cell migration and invasion in various cancer models.[45] In our study, scratch assay results indicated that inhibition of STAT3 by C188-9, either alone or in combination with DOX, reduced the migratory potential of the cells, with the combination treatment showing the most pronounced effect. These findings highlight that STAT3 inhibition could reduce chemotherapy-induced cell migration. The interaction of monocytes with MDA-MB-231 cells increases secretion of invasion-related factors and shifts monocyte function toward a pro-inflammatory but potentially tumor-

promoting state.[46] Breast cancer cells can also educate macrophages toward an immunosuppressive M2 phenotype, which correlates with aggressive tumor features and poor prognosis.[47] Given the central role of STAT3 in mediating intercellular interactions within the TME, we hypothesized that its inhibition could reverse this tumor-promoting immune polarization. Our study shows that conditioned medium from cancer cells treated with a STAT3 inhibitor increased the CD86/CD206 ratio in primary human monocytes, suggesting a shift toward a proinflammatory M1-like phenotype. Notably, the DOX/C188-9 combination further enhanced this effect, indicating an additive impact on monocyte polarization. CD86 and CD206 were measured in separate tubes because both antibodies were PE-conjugated. Therefore, the CD86/CD206 ratio is an approximate measure of the M1/M2 balance. Despite this limitation, a consistent trend toward an M1-like phenotype was observed across replicates, particularly with the combination treatment. Taken together, these findings suggest that STAT3 inhibition in breast cancer cells may modulate monocyte phenotype toward a more pro-inflammatory profile in this *in vitro* setting. However, we acknowledge that additional markers (eg, CD80, HLA-DR, and CD163) would be required to fully characterize the polarization state in future studies. We acknowledge that all experiments in this study were performed *in vitro* using a single TNBC cell line (MDA-MB-231). Therefore, the findings should be considered preliminary and cannot be directly extrapolated to *in vivo* conditions or clinical settings. Future studies in appropriate animal models are essential to evaluate the therapeutic efficacy and safety of the DOX/C188-9 combination before any clinical application can be considered.

This study has several limitations. First, only one TNBC cell line (MDA-MB-231) was used; therefore, the generalizability of our findings to other TNBC models remains unknown. Second, all experiments were performed *in vitro*, and no animal model was included; thus, the *in vivo* efficacy and safety of the DOX/C188-9 combination cannot be inferred from the present data. Third, while C188-9 is a well-established STAT3 inhibitor based on previous reports, we did not directly measure STAT3 phosphorylation or downstream target gene expression; therefore, the observed functional effects are indirectly attributed to STAT3 inhibition rather than experimentally confirmed. Fourth, monocyte polarization was evaluated using only two surface markers (CD86 and CD206); a more comprehensive panel including CD80, HLA-DR, CD163, ARG1, and IL-10, as well as functional assays, would provide stronger evidence for M1/M2 polarization. Fifth, the wound-healing assay may be confounded by reduced proliferation or increased apoptosis; proliferation-independent migration assays were not performed. Although C188-9 is considered a selective STAT3 inhibitor, potential off-target effects cannot be ruled out. Furthermore, it has demonstrated activity against STAT1 in head and neck squamous cell carcinoma (HNSCC).[48] Notably, a phase 1 clinical trial of C188-9 reported no significant toxicity and showed no evidence of mitochondrial dysfunction, a side effect associated with some other STAT3 inhibitors.[29] While our study did not directly assess these off-target interactions, we acknowledge this limitation and suggest that comprehensive kinome-wide selectivity profiling should be performed in future studies to fully characterize the specificity of C188-9 in the context of TNBC.

In summary, our *in vitro* findings indicate that STAT3 inhibition in triple-negative breast cancer cells is associated with enhanced cytotoxic and proapoptotic effects of doxorubicin, as well as reduced chemotherapy-induced cell migration and a trend toward an M1-like monocyte phenotype. The DOX/C188-9 combination showed the most pronounced effects, highlighting a synergistic impact on both tumor cells and the tumor microenvironment. Although these findings suggest that targeting STAT3 in combination with conventional chemotherapy may offer a promising approach for aggressive breast cancers by concurrently affecting tumor cells and modulating the immune microenvironment, further *in vivo* studies are essential to confirm its therapeutic potential and safety.

STATEMENT OF ETHICS

This study was approved by the Ethics Committee of Tarbiat Modares University (IR.MODARES.AEC.1403.033) on October 5, 2024.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

ACKNOWLEDGMENTS

The authors have no acknowledgments to declare.

DATA AVAILABILITY

The data that support the findings of this study are included within the article and its supplementary information files. Raw data are available from the corresponding author upon reasonable request

AI ASSISTANCE DISCLOSURE

ChatGPT (OpenAI) was used only for translation an d language editing. The authors reviewed and approved all content.

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