Effects of BET Inhibitor JQ1 and Interleukin-6 on Breast Cancer Cells

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Research Article

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Abstract

Bromodomain and extra-terminal (BET) proteins are recognized acetylated lysine of histone 4 and act as scaffolds to recruit many other proteins to promoters and at enhancers of active genes, especially at the super-enhancers of key genes, driving the transcription process and have been identified as potential therapeutic targets in breast cancer. However, the efficacy of BET inhibitors such as JQ1 in breast cancer therapy is impeded by IL-6 through an as yet defined mechanism. We investigated the interplay between IL-6 and JQ1 in MCF-7 and MDA-MB-231 human breast cancer cells. Here we demonstrate that the efficacy of JQ1 on the inhibition of cell growth and apoptosis was stronger in MDA-MB-231 cells than in MCF-7 cells. Further, MCF-7 cells, but not MDA-MB-231 cells, exhibited increased expression of CXCR4 following IL-6 treatment. JQ1 significantly reduced CXCR4 surface expression in both cell lines and diminished the effects of IL-6 pre-treatment on MCF-7 cells. While IL-6 suppressed the extension of breast cancer stem cells (BCSCs) in MCF-7 cells, JQ1 impeded its inhibitory effect. In addition, in MCF-7 cells JQ1 increased the number of senescent cells in a time-dependent manner. Analysis of gene expression indicated that JQ1 and IL-6 synergistically increase SNAIL expression and decrease c-MYC expression in MCF-7 cells. So, the BET proteins are promising, novel therapeutic targets in late-stage breast cancers.

1. Introduction

Breast cancer is one of the most heterogeneous malignancies and the main cause of female cancer deaths worldwide [1]. In addition, the prevalence of breast cancer is increasing in developing countries [2]. Despite significant progress in the treatment of breast cancer, many patients experience disease recurrence and resistance to therapy [3]. Moreover, the tumor microenvironment plays a pivotal role in the response of the breast cancer cells to treatment [4]. Thus, identification of the factors involved in the behavior of tumors cells is intensively needed.

Previous studies reported that there is an increase in expression of interleukin-6 (IL-6) in the environment of cancerous breast tissue compared to normal breast tissue [5]. While the role of IL-6 in different biological activities such as inflammation and metabolism is well established [6], there are controversial reports regarding additional roles of IL-6. Some studies suggest IL-6 is a tumor suppressor since it induces cell cycle arrest [7]. On the other hand, several reports have indicated the role of IL-6 in the emergence of resistant breast cancer stem cells (BCSCs) or in tumor progression and metastasis through the induction of epithelial-to-mesenchymal transition (EMT) [8, 9]. Thus, understanding the molecular mechanisms underlying these activities of IL-6 would reveal a new method to increase therapeutic efficacy.

One hallmark of breast cancer, similar to other types of cancers, is the penetration of cancer cells into the peripheral blood and other bodily organs (metastasis), such as lymph nodes, liver, spleen, lung, skin, and breast, as well as the formation of a repertory of malignant cancerous cells. The CXCR4 receptor and its ligand, CXCL12, also known as Stromal-Derived Factor (SDF), play a critical role in metastasis in solid malignancies such as breast cancer[10, 11].
IL-6 has also been found to trigger the Jagged1 signaling pathway, which leads to excessive expression of the genes controlling the cell cycle and proliferation [12, 13]. Jagged1 expression occurs through association of the Jagged1 promoter with the BRD4 gene expression regulator [13], a member of the bromodomain and extra terminal (BET) protein family. BET proteins are an important epigenetic class of histone reader proteins that recognize acetylated lysine residues (bromodomains) on histone tails in chromatin in order to regulate gene expression [14]. BRD2, BRD3, BRD4, and a testis-specific form (BRDT) constitute the BET family of proteins [14]. In normal mammalian cells, BRD4 and BRD2 have crucial roles in cell cycle control and proliferation [15]. Accordingly, inhibition of these components to prevent excessive expression of the cell proliferation genes might be effective in cancer therapy. Owing to the role of epigenetic machinery in cancer biology, recent studies on the treatment of cancer have focused on blocking regulators of gene expression [16]. Bromodomain and Extra-Terminal motif (BET) inhibitors are a class of small molecule medicines that bind the BET proteins BRD2, BRD3, BRD4, and BRDT’s bromodomains in a reversible manner. These BET inhibitors work by preventing BET proteins from interacting with acetylated histones and transcription factors [17]. JQ1 is one of the first selective BET inhibitors shown to block BET proteins in malignancies, inflammation, and viral infections [18–21]. JQ1 is a thienotriazolodiazepine that is the tert-butyl ester of acetic acid and belongs to the thienotriazolodiazepine class. Anti-cancer and cardioprotective activities of a bromodomain-containing protein 4 inhibitors [22]. In addition, leukemia and lymphoma cell lines are sensitive to JQ1; however, leukemia stem cells are resistant to JQ1 [23, 24]. Breast cancer stem cells (BCSCs) are a subset of breast cancer cells that play a key role in the spread of the disease to other organs. BCSCs have the ability to self-renew and differentiate into the specialized cells present in cancer [25]. The effectiveness of BET and the role of JQ1 in BCSCs remains unclear. In this study, we evaluate the interplay between the effects of IL-6 and JQ1 on the differentiation of BCSCs (CD44+/CD24−), the expression of CXCR4 as a metastatic factor, and the induction of senescence or apoptosis in a triple negative breast cancer cell line (MDA-MB-231) and a luminal breast cancer cell line (MCF-7).

2. Methods

2.1. Cell culture

The human breast cancer cell lines, MCF-7 and MDA-MB-231, were obtained from the Pasteur Institute of Iran and were cultured in RPMI1640 (Gibco, Invitrogen, UK) containing 10% fetal bovine serum (FBS) and 1% Penicillin-Streptomycin. All cultures were maintained in a humidified incubator at 37°C with 5% CO₂. In regard to the IL-6 treatment, the MCF-7 and MDA-MB-231 cells were cultured with/without 50 ng/ml recombinant human IL-6 (Peprotech, Rocky Hill, NJ, USA) for 14 days [9]. After two weeks, the cultures were incubated with or without JQ1 for 48 or 72 hours under the same conditions.

2.2. MTT assay for dose response curve

Untreated or IL-6 pretreated MCF-7 (9000 cells per well) and MDA-MB-231 (6000 cells per well) cells were seeded in 96-well plates and after 24 hours the cells were treated with/without JQ1 for 48 or 72 hours.
Cell viability was determined by the MTT assay. The cells were incubated with the MTT buffer reagent (5 mg/ml of PBS) for four hours at 37 °C. Then, 100 µl of dimethyl sulfoxide (DMSO, Duksan, Gyeonggi-do, South Korea) was added to each well for 15 minutes. The absorbance was measured at 595 nm and background absorbance was determined at 630 nm using an ELISA microplate reader (Labsystem Multiskan, Labsystem, Helsinki, Finland). Each experiment was conducted in triplicate.

2.3. Flow cytometry

MCF-7 and MDA-MB-231 cells were cultured in 75 cm$^2$ flasks in media with or without IL-6 for two weeks. Then, IL-6 treated and untreated cells were exposed to 20 or 16.5 µM JQ1 for 48 and 72 hours, respectively, based on our initial studies to establish the IC50. The cells were then trypsinized and washed with PBS and cell suspensions were divided into three groups. The first group of cells was dissolved in binding buffer and then co-stained with FITC-conjugated Annexin V and propidium iodide (PI) using the apoptosis detection kit (BD Biosciences, USA) according to the manufacturer’s protocol. The second group was used for identifying the breast cancer stem cell markers (CD44+ / CD24-). Thus, the cells were stained with FITC-conjugated anti-CD24 (BD Biosciences, USA) and PE-conjugated anti-CD44 antibodies (BD Biosciences, USA) for 30 minutes at room temperature. The third group was used to detect the surface expression of CXCR4 via staining with PE-conjugated anti-CXCR4 monoclonal antibody. All samples were prepared in triplicate and analyzed using the Partec ML flow cytometer.

2.4. Real-time PCR

Total RNA was isolated using the TRIZOL reagent (Invitrogen) and the RNA purity was verified by measuring the 260/280 absorbance ratio. Then, cDNA was synthesized using the RevertAid Reverse Transcriptase kit (Fermentas, USA) according to the manufacturer’s protocol. Real-time PCR was conducted using the SYBR Green master mix (Takara, Japan) in Rotor-Gene 6000 (Corbett Life Science, Qiagen). The amplification reactions were subjected to the following condition: 95 °C for 6 minutes, followed by 40 cycles of denaturation at 95 °C for 10 seconds, annealing at 60 °C for 20 seconds, extension at 72 °C for 30 seconds, and finalized with the melting curve. The primer sequences used in this study include: GAPDH, forward 5′- ACA CCC ACT CCT CCA CCT TTG -3′, reverse 5′-GTC CAC CAC CCT GTT GCT GTA -3′; c-MYC, forward 5′-AAT GAA AAG GCC CCC AAG GTA GTT ATC C-3′, reverse 5′-GTC GTT TCC GCA ACA AGT CCT CCT-3′; SNAIL, forward 5′-ACC ACT ACT CCT CCT TTG -3′, reverse 5′-GTC GTT TCC GCA ACA AGT CCT CCT-3′; SLUG, forward 5′-ACC ACT ATG CGC TCT T-3′, reverse 5′-GTC GTT TCC GCA ACA AGT CCT CCT-3′. All primer pairs were checked for primer-dimer formation using the three-step protocol described above without the addition of the template. The relative mRNA expression of the selected genes was normalized to the GAPDH expression and quantified using the $2^{-\Delta\Delta CT}$ method.

2.5. Cellular senescence assay

Detection of cellular senescence was based on the β-galactosidase activity in senescent cells. IL-6 treated or untreated cells were seeded in 6-well plates. After the indicated treatments had been performed, the cells were stained for detection of β-galactosidase activity according to the senescence detection kit (Abcam, USA) instructions. Briefly, the cells were washed and fixed with a fixative solution for 15 minutes.
Finally, all cells were washed again and stained with X-gal staining solution for 24 hours at 37 ºC inside a zip-lock bag in order to avoid any effects from ambient CO₂.

2.6. Statistical analysis

The statistical analysis was performed using GraphPad Prism 6.0 statistical software. Comparisons among the various treatment groups and the control group were performed using the student’s t test and one-way ANOVA. The results are presented as mean ± SD and the statistical significance was defined at $P < 0.05$.

3. Results

3.1. Effect of IL-6, JQ1, and their combination on cell viability and apoptosis in MDA-MB-231 and MCF-7 cells

We first wanted to investigate the cytotoxic effect of JQ1 on breast cancer cells. To this end, both TNBC (MDA-MB 231) and ER+ (MCF-7) breast cancer cell lines were exposed to increasing concentrations of JQ1 for 48 and 72 hours. Cell viability was then assessed by MTT assay. JQ1 inhibited the growth of MDA-MB231 cells after 48 and 72 h of treatment, with IC50 values of 20 µM and 16.5 µM, respectively (Fig. 1A). Conversely, MCF-7 cells were found to be resistant to the cytotoxic effects of JQ1 (Fig. 1A).

Therefore, the JQ1 IC50 values effective at inducing growth inhibition of MDA-MB-231 cells were used for both cell lines in subsequent experiments. Then, we investigated the effect of IL-6 or JQ1 alone and in combination on the viability of MDA-MB-231 and MCF-7 cells after 48 and 72 h. JQ1 significantly reduced the cell viability of both untreated and IL-6-treated MDA-MB-231 cells (Fig. 1B, $P < 0.05$). In addition, JQ1 significantly decreased the cell viability of IL-6 pretreated MCF-7 cells in a time-dependent manner (Fig. 1C). Next, we investigated whether the reduction in viable cells caused by JQ1 was induced by proliferation arrest or apoptosis induction. As indicated in Figure 1D, JQ1 induced marked apoptosis (early and late apoptosis) in MDA-MB-231 cells compared to that in MCF-7 cells. Interestingly, pre-treatment with the IL-6 decreased the effect of JQ1 on apoptosis induction in MDA-MB-231 cells after 72 h and in the MCF-7 cells after 48 h (Fig. 1D).

3.2. Effect of IL-6, JQ1, and their combination on the BCSC population in MDA-MB-231 and MCF-7 cells

Previous studies have clearly demonstrated the role of BCSCs in inducing resistance to therapy or recurrence of breast cancers. As such, we investigated the percentage of BCSCs (CD44⁺/24⁻) in all cultures. As illustrated in Figure 2, our treatments had no significant effect on the population of the BCSCs in the MDA-MB-231 cells; however, in the MCF-7 line the percentage of the BCSCs increased significantly in the JQ1 treatment groups ($P < 0.05$, Fig. 2). Notably, the exposure of MCF-7 cells to IL-6 for
two weeks significantly suppressed the progression of the breast cancer cells (P < 0.05, Fig. 2) in comparison with untreated MCF-7 cells (control group).

### 3.3. Inhibition of BET results in suppression of CXCR4 expression on breast cancer cells

One of the major players in the metastasis of breast cancer is the CXCR4 chemokine receptor. Thus, we extended our analyses to assess the effects of IL-6 and the BET inhibitor JQ1 on CXCR4 surface expression. As illustrated in Figure 3, the percentage of CXCR4-positive MDA-MB-231 and MCF-7 cells decreased significantly (P < 0.01) after treatment with JQ1 for 48 and 72 hours compared to their respective control groups (Fig. 3). Notably, the IL-6 treated MCF-7 cells exhibited a significant enhancement in the number of CXCR4 positive cells (P < 0.01, Fig. 3). In addition, IL-6 pretreatment diminished the inhibitory effect of JQ1 in MCF-7 cells. In result, the proportion of CXCR4-positive cells significantly decreased in both cell line treated with a combination of IL-6 and the BET inhibitor (P < 0.01, Fig. 3).

### 3.4. Inhibition of BET increases MCF-7 cell senescence in a time-dependent manner

In order to investigate the effects of the treatments on the induction of senescence during cycle arrest, we evaluated expression of a well-known senescence marker (Fig. 4). The effect of the BET inhibitor on the induction of cellular senescence in MDA-MB-231 cells did not follow a specific pattern. After 48 hours, β-galactosidase was increased in JQ1-treated MDA-MB-231 cells; however, at 72 hours it decreased below baseline (Fig. 4). Interestingly, IL-6 pretreatment reduced the effect of JQ1 on this time-dependent increase in senescence of MCF-7 cells (Fig. 4).

### 3.5. Effect of IL-6, JQ1, and their combination on SLUG, SNAIL, and c-MYC gene expression

Since it is known that the expression of SLUG, SNAIL, and c-MYC increases tumor promotion, we evaluated the effect of our treatments on the expression of these genes using real-time PCR. Administration of JQ1 alone significantly reduced the expression of SLUG mRNA in MDA-MB-231 cells, while it did not exert an effect on SLUG mRNA levels in MCF-7 cells (Fig. 5A). In addition, treatment with IL-6 for two weeks resulted in a significant reduction in the level of SLUG expression in MDA-MB-231 cells (Fig. 5A), whereas, it did not affect expression of this gene in MCF-7 cells (Fig. 5A). Notably, JQ1 significantly decreased the expression of SLUG in all cultures pretreated with IL-6 (Fig. 5A and 5B). The expression of SNAIL increased (about four-fold) significantly in the MDA-MB-231 cells after treatment with only JQ1 for 72 h (Fig. 5B). Although the SNAIL expression was not affected in MDA-MB-231 cells under any of the other treatment conditions. In addition, expression of SNAIL was significantly enhanced in all treated MCF-7 cells (Fig. 5B). The expression of c-MYC did not change significantly in the MDA-MB-231 cells except for the cells pretreated with IL-6 and then exposed to JQ1 for 72 h (P < 0.05, Fig. 5C). On
the other hand, in the MCF-7 cells all treatments resulted in a reduction of c-MYC expression, and JQ1 and IL-6 had a synergistic effect (Fig. 5C). Indeed, JQ1 reduced surface expression of CXCR4 even in IL-6 driven cells, indicating that it is a potent anti-metastatic factor.

4. Discussion

Here we validate the role of BET proteins in the cell cycle and on protein expression in human cancer cells. Given the importance of BET proteins they could be good target candidates for treatment of breast cancer. Currently specific BET inhibitors, such as JQ1, are available; however, their effectiveness in the presence of the environmental tumor cytokines is unknown. In agreement with previous findings [26], we show that JQ1 reduces cell viability in both MDA-MB231 and MCF-7 breast cancer cell lines. However, further studies will be necessary to determine if JQ1 inhibits proliferation of breast cancer cells or enhances their apoptosis.

It should be noted that cytokines present in the tumor microenvironment not only affect cell fate, but can also alter drug efficacy. Dethlefsen et al. reported that intratumorally IL-6 has a major role in tumor cell growth and metastasis [6]. As chronic inflammation is known to increase progression of breast cancer [12], it is key to survey the effects of IL-6 after prolonged (14 days) treatment with any therapeutic.

In the present study, we report that a triple-negative breast cancer cell line, MDA-MB-231, is more sensitive to JQ1, a well-known BET inhibitor, than estrogen receptor-positive MCF-7 cells (Fig. 1A, 1B, and 1C). Following 48 hours of JQ1 treatment at 20 µM or 72 hours at 16.5 µM, MDA-MB-231, cells exhibited a significant reduction in proliferation (∼50%). In addition, we demonstrated that stimulation of MDA-MB-231 cells with IL-6 for two weeks reduces the cell viability as compared to untreated cells. In contrast, JQ1 had no effect on the proliferation of untreated MCF-7 cells; however, pretreatment with IL-6 increased the sensitivity of these cells to JQ1 after 48 h. Therefore, in this study we demonstrate that IL-6 may influence the efficacy of JQ1 on the induction of apoptosis in breast cancer cell lines.

Recently, it has been demonstrated that IL-6 stimulation can increase tumor metastasis and the invasiveness of cancers [27, 28]. CXCR4 is the main agent involved in breast cancer metastasis [10, 29], and here we demonstrate that IL-6 increases the proportion of CXCR4-positive MCF-7 cells, but not of MDA-MB-231 cells. Conversely, analysis of CXCR4 surface expression revealed that the inhibition of the BET proteins by JQ1 also inhibited surface expression of the CXCR4, especially in triple-negative breast cancer cells. Furthermore, combinatorial treatment revealed that JQ1 antagonizes the effect of IL-6 on the expression of CXCR4, and in MCF-7 cells JQ1 inhibits the effects of IL-6 effect in a time dependent manner. These findings indicate that the role of BET proteins is more crucial in MDA-MB-231 cells than in MCF-7 cells. In addition, it seems that to IL-6 exerts a strong effect on MCF-7 cells than on MDA-MB-231 cells. Because, the expression of the IL-6R in MCF-7 is higher than MDA-MB-231 [30].While a previous study demonstrated that JQ1 inhibits the secretion of IL-6 [31], in the present study we demonstrate for the first time that JQ1 antagonizes the effects of IL-6 in a manner dependent on the cell line and length of exposure.
Many studies have demonstrated the role of cancer stem cells (CSCs) in drug resistance, metastasis, and cancer recurrence [32, 33]. Also, the BET inhibitors were introduced as an effective therapy for different types of cancers [16, 19, 23]. However, our investigation in regard to the effect of the BET inhibitor (JQ1) on the CSC population of two human breast cancer cell lines, MDA-MB-231 and MCF-7, yielded contrasting results. The MDA-MB-231 cell line was found to have the highest percentage of BCSCs (about 90 percent) and treatment with JQ1 did not change this population. However, treatment with JQ1 enriched the BCSC population in MCF-7 cells. In addition, IL-6 pre-treatment decreased the percentage of BCSCs, while combined treatment with IL-6 and JQ1 increased the percentage of BCSCs in both cell lines. In contrast, neither JQ1 nor IL-6 affected the expression of BCSC markers in triple-negative breast cancer cells. In addition, BCSCs are resistant to JQ1 therapy and JQ1 diminished the inhibitory effects of IL-6 on BCSC differentiation [30]. Recently, the induction of phosphorylation of BRD4 by casein kinase II (CK2) was reported to enhance the stability of BRD4 in JQ1-resistant cells [34]. Subsequently, another study revealed that the activator of enzymatic activity against PP2A phosphatase phenothiazine (PTZ) has an opposite function to that of CK2 and can dephosphorylate BRD4, which increases the sensitivity of the resistant cells to JQ1 [35]. Therefore, a combination therapy using JQ1 and a suitable JQ1 sensitizer, such as PTZ, may synergistically increase the therapeutic effects. In addition, treatment of MCF-7 cells with JQ1 causes the expression of the SNAIL gene to increase significantly in a manner that correlates with the increase of the BCSC population in MCF-7 cells ($R = 77\%, p < 0.05$). Therefore, this suggests that increased expression of SNAIL may be responsible for the conversion of non-BCSCs to BCSCs in MCF-7 cells. Another explanation for this occurrence could be survival of BCSCs after treatment with JQ1, since they are resistant to JQ1.

Furthermore, we also found a synergistic interaction between IL-6 and JQ1 in regard to the reduction of c-MYC and SLUG in the MCF-7 and MDA-MB-231 cells, respectively. These findings are consistent with previous results, which demonstrated that BET inhibitors decrease the expression of c-MYC in acute leukemia cells [36]. Further research will be needed to decipher the precise mechanisms through which JQ1 functions for the treatment of breast cancer.

5. Conclusion

The results of this study confirm that JQ1 is a viable candidate for breast cancer therapy, as previously described [16], with potential specifically for treatment of resistant triple-negative breast cancer. In addition, we observed that JQ1 is a powerful anti-metastatic factor as it inhibited surface expression of CXCR4, even in IL-6 stimulated cells. Investigation of the BCSC population led to the conclusion that chronic exposure to IL-6 reduces the proportion of BCSCs, whereas JQ1 diminishes the inhibitory effects of IL-6. Further studies are required to clarify the molecular pathways through which this effect on the effects of IL-6 is mediated.

Overall, BET inhibitors similar to JQ1 show promise as therapeutic candidates for breast cancers, especially when triple-negative breast cancer cells are increased and/or tumor promoting factors like IL-6 exist in the tumor microenvironment.
Declarations

Declaration of Competing Interest

The authors declare that they have no conflict of interest.

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Author contributions statement

MGS, MSJ and HS conceived the study; ASH designed research; ASH, MH, AS performed research; ASH, MGS, MS analyzed data; and ASH and BAJ wrote the manuscript with a minor contribution from other authors. All authors read and approved the final manuscript.

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Figures
Figure 1

Effect of IL-6, JQ1, and their combination on cell viability.

(A) MDA-MB-231 and MCF-7 breast cancer cell lines were cultured for 48 and 72 h with different doses of JQ1. Cell viability was determined by MTT assay. IC50 values were calculated for each culture. (B and C) JQ1 significantly inhibited the growth of the IL-6 pretreated/untreated human MDA-MB-231 cells after 48
or 72 h while MCF-7 cells were more resistant to treatment with JQ1. (D) Annexin V-FITC/PI staining assay to measure the effects of the treatments on apoptosis. Human breast cancer cells lines (MDA-MB-231 and MCF-7) were treated with DMSO as a control. * = p < 0.05, ** = p < 0.01, and *** = p < 0.001 compared with single or combination treatment of IL-6 and JQ1 with the DMSO-treated controls.

**Figure 2**

Reduction of the BCSC population in IL-6 treated MCF-7 cells is inhibited by JQ1. The proportion of the CD44+/CD24- stem cells was evaluated by the flow cytometry. Dot plots show the percentage of the BCSCs in the (A) MCF-7 and (B) MDA-MB-231 cell lines. (C) The graph represents the effect of JQ1 and/or IL-6 on the BCSC population in the MDA-MB-231 and MCF-7 cell lines. Data shown are means ± SD of three assays. * = p<0.05 and *** = p<0.001 compared with DMSO-treated controls.
Figure 3

JQ1 inhibits CXCR4 expression.

Cell lines were treated with IL-6 (10 ng/ml) and/or JQ1 (20 µM for 48 h / 16.5 µM for 72 h) and analyzed using flow cytometry. The expression of cell surface CXCR4 was determined by staining of (A) MDA-MB-231 and (B) MCF-7 cells with PE-conjugated anti-CXCR4. (C) Data presented are means ± SD of three assays. ***p < 0.001 compared with DMSO-treated controls.
Figure 4

IL-6 antagonizes JQ1 in the induction of cell senescence.

Senescent cells are blue in color
Figure 5

The effect of JQ1, IL-6, and IL-6 plus JQ1 on SLUG, SNAIL, and c-MYC gene expression.

Data is expressed as fold change relative to the untreated cells (control). The columns represent the means ± SD of three independent experiments. *p < 0.05, **p < 0.01 ***p < 0.001.