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Epigenetic Blockade of Neoplastic Transformation by Bromodomain and Extra-Terminal (BET) Domain Protein Inhibitor JQ-1

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Abstract

The neoplastic transformation of cells and inflammation are processes that contribute to tumor initiation. Recently, emerging evidence has suggested that epigenetic alterations are also implicated in the early stages of carcinogenesis. Therefore, potent small molecules targeting epigenetic regulators have been developed as novel cancer therapeutic and preventive strategies. Bromodomain and extraterminal domain (BET) proteins are epigenetic readers that play key roles at the interface between chromatin modification and transcriptional regulation. In this study, we investigated the effect of the BET inhibitor JQ-1 on malignant transformation induced by 12-O-tetradecanoylphorbol-13-acetate (TPA) in mouse skin epidermal JB6 P+ cells. Treatment with JQ-1 effectively impaired TPA-induced colony formation *in vitro*. At the molecular level, the expression of several key TPA-induced pro-survival and pro-proliferative genes (Bcl2, Cyclin D1, and c-Myc) decreased rapidly after BET inhibition. In addition, JQ-1 treatment attenuated the activation of inflammatory NF-κB signaling triggered by TPA. Luciferase reporter assays using plasmids carrying different elements from the COX2 or IL6 promoters demonstrated that JQ-1 does not directly inhibit interactions between NF-κB and its binding sequence; rather, it affects CRE-element-associated transcriptional enhancement. Through siRNA gene silencing, we found that JQ-1 inhibits the p300-dependent transcriptional activation of COX2, which correlates with the results of the luciferase assay. Chromatin immunoprecipitation assays showed that TPA

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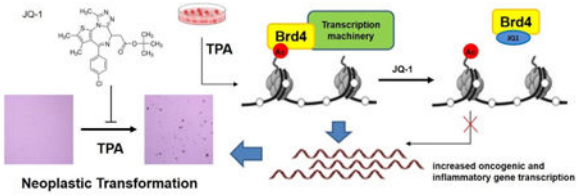
elevated H3K27Ac enrichment in the COX2 promoter region, which is mediated by p300, and Brd4. JQ-1 treatment did not change H3K27Ac levels but decreased the recruitment of Brd4 and RNA Polymerase II. Collectively, our study reveals that the BET inhibitor JQ-1 exerts potent anti-cancer and anti-inflammatory effects by interfering with the core transcriptional program of neoplastic transformation.

Graphical abstract



1. Introduction

Accumulating experimental evidence has suggested that epigenetic alterations are involved in cancer development. DNA methylation on 5-cytosine (5-mC) and covalent modifications to histone tails are the major mechanisms that maintain DNA stability and control gene transcription at the epigenetic level. Recent publications from our group and others have identified that aberrant DNA methylation is associated with altered gene expression in cancer models *in vitro* and *in vivo* [1, 2]. In addition, loss of 5-hydroxymethylcytosine (5-hmC) and ten-eleven translocation 2 (TET2) protein expression has been reported in malignant melanoma [3]. In addition, chromatin factors such as histone methyltransferases also play a role in tumorigenesis [4, 5]. Unlike genetic mutations, epigenetic alterations are heritable changes in gene expression not caused by alterations in the underlying DNA sequences; thus, they are potentially reversible by chemicals. Therefore, targeting epigenetics for cancer chemopreventive and therapeutic strategies is gaining growing interest. Among all of the epigenetic regulators, DNA methyltransferases (DNMTs) and histone deacetylases (HDACs) have been most extensively studied, and their inhibitors are on the market for the treatment of myelodysplastic syndromes and cutaneous T-cell lymphoma [6], respectively.

In addition to epigenetic writers such as DNMTs and HDACs, epigenetic readers refer to another group of proteins that recognize the covalent modifications on DNA or histones and perform key roles at the interface between epigenetic memory, chromatin remodeling and transcriptional regulation. The N-acetylation of lysine residues on histone tails is associated with an open chromatin structure and transcriptional activation [7]. More recent studies have demonstrated that the molecular recognition of acetyl-lysine is principally mediated by bromodomains (BrDs) [8]. In humans, there are estimated to be 46 different BrD-containing proteins that encode a total of 61 BrDs [9]. Despite large sequence diversities, all BrD-containing proteins share an evolutionarily conserved left-handed four-helix bundle, where the N-acetyl lysine is recognized and anchored by a hydrogen bond with an asparagine residue in a central hydrophobic cavity. This module is linked by diverse loop regions that discriminate between different acetylated lysine residues and determine substrate specificity.

The BrD and extraterminal domain (BET) family consists of BRD2, BRD3, BRD4 and BRDT, which share a common domain architecture characterized by two amino-terminal BrDs. BET proteins recognize acetylated lysine residues on histone tails, and recruit chromatin-modifying enzymes, transcription factors, transcriptional co-activators and transcriptional co-repressors, thereby coupling histone acetylation and gene expression [10].

Recent studies have built a compelling rationale for targeting BET proteins in cancer because of their critical roles in tissue development, inflammatory responses and tumor progression [10]. For example, high BRD2 levels have been found in human leukemic cell lines and primary leukemic blasts, whereas lymphoid-restricted constitutive expression of BRD2 leads to the development of aggressive B-cell lymphoma in mice [11]. Fusions of BRD4 or BRD3 with nuclear protein in testis (NUT) that generate an in-frame chimeric peptide with tandem N-terminal BrDs preceding the NUT protein result in a proliferation advantage and genetically define an aggressive form of human squamous cell carcinoma as NUT midline carcinoma (NMC) [12]. In addition, siRNA silencing of the BRD–NUT oncoproteins in NMC cell lines arrests the cell cycle and prompts squamous differentiation.

Subsequently, small-molecules with high potency and specificity toward BET proteins have been developed, including JQ-1, I-BET151, MS417 and PFI-1 [13-15]. These compounds competitively bind to acetyl-lysine recognition motifs, inhibit the recruitment of BET proteins to chromatin and repress downstream transcriptional programs [13]. In particular, inhibition of BET has been introduced as a novel strategy to target oncogenic c-Myc [16, 17], the transcription of which is associated with locally and globally enhanced acetylation of the histone lysine side chain [18, 19]. Furthermore, BET inhibitors have shown promising efficacy against solid tumor progression in recent reports, including lung adenocarcinoma, melanoma and hematological malignancies [20-22]. However, the role of BET proteins in the early stages of carcinogenesis has not yet been studied.

Tumor formation is a long-term process that typically comprises three different stages: initiation, promotion and progression [23, 24]. In this study, we investigated the anti-tumor activity of the Brd4 inhibitor JQ-1 with an emphasis on tumor initiation. Neoplastic transformation of cells is one of the major processes during early carcinogenic transformation. We assessed the efficacy of pharmacologically inhibiting BET proteins using the tumor promoter 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced JB6 P+ cell transformation model, and its underlying molecular events.

2. Materials and Methods

2.1 Materials

(±)JQ-1 was generously provided by the Structural Genomics Consortium (Toronto, ON). As manufactured, the compound comes as an enantiomer, and only (+)-JQ-1 is the active form. Bacteriological agar, Eagle's basal medium (BME), and TPA were purchased from Sigma-Aldrich (St. Louis, MO). Fetal bovine serum (FBS), minimum essential medium (MEM), Dulbecco's modified Eagle medium (DMEM), and trypsin-EDTA solution were obtained from Gibco Laboratories (Grand Island, NY).

2.2 Cell culture and treatment

JB6 P+ (JB6 Cl 41-5a, ATCC CRL-2010) cells from American Type Culture Collection were maintained in MEM containing 5% FBS at 37°C in a humidified 5% CO_2 atmosphere. HaCaT (human keratinocyte), HCT116 cells were grown in DMEM supplemented with 10% FBS. The cells were grown to 60–80% confluency and seeded at a density of 1×10^5 cells/well in 6-well plates. After 24 h incubation, cells were treated with medium containing various concentrations of JQ-1 for 4 h. After 4 h of JQ-1 treatment, TPA was added to the indicated groups at a final concentration of 20 ng/ml. Next, cells were harvested 8 h after TPA treatment for mRNA extraction and 24 h after TPA treatment for western blotting. HCT116 cells were treated only with the indicated concentrations of JQ-1. DMSO (0.1%) was used as a vehicle control for all experiments.

2.3 Anchorage-independent cell transformation assay

Anchorage-independent cell transformation assay was carried out as previously described [25]. Briefly, Eagle's basal medium agar (3 ml of 0.5%) containing 10% FBS with or without JQ-1 was layered onto each well of the 6-well plates. JB6 P+ cells were treated with various concentrations of JQ-1 for 4 h; then, 8×10^3 pretreated cells were mixed with 1 ml of 0.33% Eagle's basal medium agar and seeded on the top of the 0.5% agar layer. The cells were maintained with 0.1% DMSO (vehicle control), TPA (20 ng/ml) alone or a combination of TPA (20 ng/ml) and designated concentrations of JQ-1 in a 5% CO_2 incubator at 37°C for 14 days, at which time colonies were photographed under a light microscope using the Nikon ACT-1 program (Version 2.20; LEAD Technologies) and counted using ImageJ software (Version 1.40g; NIH).

2.4 RNA isolation and quantitative real-time polymerase chain reaction

Total RNA was extracted using the RNeasy Mini Kit (QIAGEN, Valencia, CA). First-strand cDNA was synthesized from 1 μg of total RNA using TaqMan Reverse Transcription Reagents (Life Technologies, Grand Island, NY). The mRNA expression of specific genes was determined by quantitative real-time PCR using the first-strand cDNA as the template in the ABI79000HT system.

2.5 Western blotting

Whole-cell lysates were prepared from treated cells using RIPA buffer (Cell Signaling Technology, Danvers, MA) supplemented with protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO). The protein concentration was determined using the bicinchoninic acid (BCA) kit (Pierce, Rockford, IL). Proteins (20 μg from each sample) were resolved by 4 to 15% SDS-polyacrylamide gel electrophoresis (Bio-Rad, Hercules, CA) and electro-transferred to a PVDF membrane (Millipore, Bedford, MA). After blocking with 5% BSA in Tris-buffered saline-0.1% Tween 20 buffer, the membrane was sequentially incubated with specific primary antibodies and HRP-conjugated secondary antibodies. Finally, the blots were visualized using the SuperSignal enhanced chemiluminescence (ECL) detection system and documented using a Gel Documentation 2000 system (Bio-Rad, Hercules, CA). The intensity of the bands was analyzed by densitometry using the ImageJ program (Version 1.40g; NIH).

2.6 Cell cycle distribution analysis

Cell cycle distribution analysis was performed using propidium iodide staining method as previously reported [26]. HCT116 cells were seeded in 60-mm Petri dishes followed by serum-starvation for 24 h in serum free medium. Next, the cells were treated with complete DMEM containing either 0.1% DMSO (as a negative control) or the indicated concentration of JQ-1 (100 and 500 nM) for another 24 h. Following treatment, cells were harvested by trypsinization and washed twice in ice-cold phosphate buffered saline (PBS). Cells were collected by centrifugation at 500*g for 5 min, and cell pellets were then re-suspended in 300 μl of PBS and fixed by gradually adding 700 μl of ethanol. The fixed cells were then stained in 500 μl of PBS containing 10 μg/ml propidium iodide and 100 μg/ml RNase A at room temperature for 30 min in the dark. The cell cycle distribution was then analyzed by flow cytometry using the FACS/Cell Sorting Core Facility at EOHSI, Rutgers University.

2.7 Luciferase reporter activity assay

The genomic sequences of the human COX2 and IL6 promoter regions were retrieved from UCSC gene browser. The targeting sequences were amplified by PCR and cloned into the pCR2.1 TOPO vector (Life Technologies, Grand Island, NY). These sub cloning plasmids were amplified in *E. coli*, isolated and digested with KpnI and XhoI. The designated fragments were then inserted into the luciferase promoter region of pGL4.15 [luc2P/Hygro] vector (Promega, Madison, WI) as the plasmid for luciferase assay. Sequences of all recombinant plasmids were verified by sequencing (Genewiz, South Plainfield, NJ). For the luciferase activity assay, HaCaT or HEK293 cells were transfected with 400 ng of the indicated reporter plasmids using Lipofectamine 3000 (Life Technologies, Grand Island, NY) according to the manufacturer's manual. The cells were co-transfected with 100 ng of pGL4.75 [hRluc/CMV] plasmid (Promega, Madison, WI), which constitutively expresses Renilla luciferase as an internal control. The transfected cells were treated with 20 ng/ml TPA alone or in combination with 500 nM JQ-1 for 24 h and were then lysed in dual luciferase lysis buffer. The luciferase activity of each sample was measured using a Sirius luminometer (Berthold Detection System GmbH, Pforzheim, Germany). The firefly luciferase activities were normalized with corresponding Renilla luciferase activities, and results were presented as the induced fold change compared with the vehicle control.

2.8 RNA interference

RNAi duplex oligos (siRNA) targeting murine p300 were designed and synthesized from Integrated DNA Technologies (Coralville, IA). Transfection of siRNA was carried out using Lipofectamine 3000 (Life Technologies, Grand Island, NY) according to the manufacturer's instructions. Briefly, 5 μl of si-p300 stock solution (10 μM) in 125 μl of Opti-MEM was added to another 125 μl of Opti-MEM containing 5 μl of Lipofectamine 3000. Universal negative control siRNA was similarly prepared and used as siControl. After 15 min incubation at room temperature, the transfection mixtures were applied to JB6 P+ cells for 24 h in a 37°C incubator. Then, the cells were subjected to further treatments of 20 ng/ml TPA alone or in combination of 500 nM JQ-1.

2.9 Chromatin immunoprecipitation assay

Chromatin immunoprecipitation (ChIP) assays were conducted using ChIP Kit - One Step (Abcam, Cambridge, MA) following the manufacturer's instructions. Briefly, JB6 P+ cells were treated with 20 ng/ml TPA for 4 h in the presence or absence of 500 nM JQ-1 pretreatment. After washing twice with PBS, chromatin was cross-linked with 1% formaldehyde for 10 min at room temperature. Next, 1.25 M glycine was added to quench the excess formaldehyde. The cells were pelleted by centrifugation, re-suspended in lysis buffer and sonicated to generate 200- to 500-bp DNA fragments. The cross-linked chromatin fragments were subjected to immunoprecipitation with specific antibodies against Ac-H3K27, Histone H3, BRD4 and RNA Polymerase II (Pol II) (Abcam, Cambridge, MA) to capture protein-DNA complexes. After precipitation, 2 μl of each purified DNA was used as a template for qPCR quantification. Before immunoprecipitation, a small proportion was kept from each chromatin sample as input. These input samples were treated in parallel as the precipitated samples expect for that no antibody was added in the immunoprecipitation procedure. Purified input DNA were geometrically diluted into a series of concentration then used for standard curve in qPCR. The relative enrichment was calculated by locating the precipitated DNA sample on its self-input standard curve. Then the relative enrichment was normalized to the vehicle control group as fold change. For Ac-H3K27 enrichment quantification, additional normalization to the Histone H3 C-terminal enrichment of each sample was done prior to the comparison with the control group. Non-specific IgG was used as a control to verify the specificity of the ChIP assays.

2.10 Statistical analysis

The data are presented as the mean ± SD. The statistical analyses were performed using Student's t-test or one-way analysis of variance (ANOVA) followed by Dunnett's test. Statistically significant differences among the means were set at *$P < 0.05$, **$P < 0.01$, and #$P < 0.001$.

3. Results

3.1 JQ-1 inhibits TPA-induced JB6 P+ cell transformation

JQ-1 treatment was tested on the TPA-induced anchorage-independent growth of JB P+ cells in the soft agar assay. As shown in Fig. 1, JB6 P+ cell by itself cannot form colonies in soft agar (anchorage-independent grow condition) unless stimulated with TPA. Both colony number and size were markedly decreased by JQ-1 treatment. Compared with the TPA-treated group, 100 or 500 nM JQ-1 treatment decreased the mean number of colonies by approximately 61% ($P < 0.05$) and 74% ($P < 0.01$), respectively. These results show that JQ-1 significantly suppressed the TPA-induced colony formation of JB6 P+ cells.

3.2 BET inhibition decreases TPA-induced oncogenic signaling

To investigate the cellular processes affected by BET inhibition, we analyzed a series of cell cycle and proliferation markers in cells treated with JQ-1 in the context of TPA stimulation. Consistent with previous reports, TPA triggered the phosphorylation of the mitogen-activated protein kinases (MAPKs) ERK1/2 (Fig. 2A). Accordingly, elevated levels of anti-

apoptotic and pro-growth oncogenes such as Bcl2, Cyclin D1, and c-Myc were observed in response to 20 ng/ml TPA treatment (Fig. 2B&C). Since JB6 P+ cell line is derived from mouse epidermis and skin cancer is the most commonly diagnosed cancer in the United States, we also evaluated the translational potential of these results on human keratinocyte HaCaT cell line (Fig. 2D&E). In accordance with the anchorage-independent cell growth assay, JQ-1 treatment reduced TPA-induced Bcl2 expression in both JB6 P+ and HaCaT cells (Fig. 2C&E). Furthermore, TPA-stimulated Cyclin D1 and c-Myc expression was decreased at both the mRNA and protein levels in a dose-dependent manner in response to JQ-1. Surprisingly, JQ-1 also attenuated the levels of phosphorylated ERK1/2.

3.3 JQ-1 induces G1 cell cycle arrest in HCT-116 cells

It has been reported that BRD4 influences mitotic progression by binding to the transcriptional start sites of genes expressed during cell cycle transition [8], which is in agreement with our results above. In order to evaluate whether there exists a common set of transcriptional events mediated by BET in different stage of cancers, such as the cell cycle regulatory genes, we next examined the effect of JQ-1 on cell cycle regulatory genes in a cancer cell line HCT-116. The cells were treated with the indicated concentrations of JQ-1 for 24 h. As shown in Fig. 3A&B, the cells treated with 100 or 500 nM JQ-1 showed a remarkable decrease in c-Myc, Cyclin A, and Cyclin D1 expression at both the mRNA and protein levels. Cell cycle distribution was then analyzed by flow cytometry after JQ-1 treatment. Accordingly, the results showed a significant increase in the percentage of the cells in G1 phase from 47.7% (0μM) to 70.7% (500 nM) (Fig. 3C).

3.4 JQ-1 alters the expression of HDACs in HCT116 cells

Other epigenetic-modifying compounds such HDAC inhibitors (HDACis) also cause cycle arrest in cancer cells. Interestingly, a recently study revealed the similarity of gene expression alterations induced by BET inhibitors (BETis) and HDACis in murine lymphoma cells [27]. Therefore, we investigated a potential interaction between these two levels of epigenetic regulation by performing western blotting to evaluate the effect of JQ-1 treatment on the expression of HDACs. We found that the protein levels of HDAC2, HDAC3, and HDAC4 were significantly reduced in HCT116 cells after JQ-1 treatment, whereas there were no changes in the expression of HDAC1 (Fig. 4).

3.5 JQ-1 down-regulates the mRNA and protein levels of NF-κB target genes

Activation of NF-κB signaling is an important mechanism implicated in tumor progression that is promoted by TPA [28]. In our study, 20 ng/ml TPA treatment increased the mRNA expression of NF-κB target genes in both JB6 P+ and HaCaT cells. Compared with the TPA-alone group, cells treated with JQ-1 at concentrations of 100 and 500 nM showed significantly decreased transcription activities of these TPA-induced genes, including COX2 and IL6 (Fig. 5A&C). The protein level of COX2 was further determined in cells treated with TPA alone or in combination with JQ-1 by western blotting. Corresponding to the qPCR results, the protein expression of COX2 was up-regulated by TPA, whereas JQ-1 reduced the TPA-boosted levels of COX2 (Fig. 5B&D). These results indicate that the inhibition of Brd4 may block the activation of NF-κB signaling by TPA.

3.6 JQ-1 inhibits CRE-associated transcription in luciferase reporter activity

To evaluate how JQ-1 affects the transcription activities of NF-κB target genes, luciferase reporters driven by different regions of the COX2 and IL6 promoters were constructed as shown in Fig. 6A. In addition to NF-κB binding sites, both the COX2 and IL6 promoters have cAMP response elements (CREs), which are recognized by cAMP response element-binding proteins (CREBs). CREB is a transcription factor that binds to CREB-binding protein (CBP) and its paralog p300, which enhances gene expression by relaxing the chromatin structure through its intrinsic histone acetyltransferase (HAT) activity and recruiting Pol II to the promoter. It has been reported that p300 is widely involved in NF-κB-driven transcription [29, 30]; therefore, we established plasmids carrying NF-κB binding sequences alone or both NF-κB and CREB binding elements (Fig. 6A). After transient transfection into the cells, 20 ng/ml TPA substantially increased the luciferase activities among all four reporter constructs at 20 h. Interestingly, 500 nM JQ-1 could not inhibit the luciferase activities induced by TPA in the cells transfected with plasmids carrying NF-κB binding sites alone (COX2-321 and IL6-105). By contrast, 500 nM JQ-1 abolished the luciferase activities (82% decreases) stimulated by TPA in the cells transfected with the reporter constructs containing both NF-κB and CREB binding sites (Fig. 6B). The results indicate that inhibition of Brd4 might influence the transcriptional activities of NF-κB target genes by indirectly associating with co-activators rather than by blocking the direct translocation and DNA-binding of NF-κB.

3.7 JQ-1 decreases the recruitment of Brd4 and Pol II to the COX2 transcription starting site

As shown above, it appears to be that JQ-1 inhibits the transcription of NF-κB targeted gene indirectly. Therefore, ChIP assay was employed to further examine the COX2 transcription regulatory mechanism on the scope of histone modification in JB6 P+ cells during TPA-induced neoplastic transformation. As shown in Fig. 7, 20 ng/ml TPA increased the enrichment of Ac-H3K27 on the COX2 promoter, subsequently recruiting Brd4 and Pol II to the COX2 transcription starting site (TSS). JQ-1 treatment did not change the level of Ac-H3K27 associated with the COX2 promoter but did decrease the TPA-induced recruitment of both Brd4 and Pol II to this region, which is proposed to be essential for transcription initiation and elongation. The specificity of ChIP assays was verified by using non-specific IgG in the precipitation procedure as a negative control, which has no specific amplification in qPCR (data not shown).

3.8 JQ-1 inhibits the p300-dependent transcription activation of COX2

We conducted gene silencing using p300 siRNA to confirm that JQ-1 influences the NF-κB-mediated COX2 gene transcription through histone modifications, rather than interfering with the direct NF-κB-DNA binding. The efficiency of siRNA delivery into cells was confirmed by qPCR and western blotting. The transfection of negative control (NC) siRNA, which has no homology to any known mammalian gene, was performed in parallel with that of p300 siRNA. We found that the knockdown of p300 attenuated the TPA-stimulated enrichment of Ac-H3K27 on the promoter region of COX2, accompanied with reduced Brd4 on TSS (Fig. 8A). Correspondingly, lower mRNA and protein levels of COX2 were

observed in samples from the si-p300 group, compared with the si-NC group. However, after RNA interference of p300, 500 nM JQ-1 did not show any further inhibitory effects on COX2 induction (Fig. 8 B&C).

4. Discussion

In this study, we found that the inhibition of Brd4 by JQ-1 blocked TPA-induced JB6 P+ cell transformation, indicating a potential role for BET proteins in promoting the early stages of carcinogenesis. JB6 P+ is a promotion-sensitive murine epidermal cell line that undergoes neoplastic transformation after environmental challenges, including TPA, EGF, and UVB [31]. It has been demonstrated to be a useful model to study tumor promoter-induced carcinogenic processes at the molecular level [32]. As shown in Fig. 2, TPA promoted the transformation of JB6 P+ cells, which was accompanied by elevated p-ERK1/2, Bcl2, Cyclin D1, and c-Myc. Although HaCaT cells are not sensitive to TPA stimulation for anchorage-independent growth (data not shown), similar molecular alterations were observed in this cell line after TPA and JQ-1 treatment.

Notably, JQ-1 down-regulated the mRNA and protein expression of c-Myc, which is one of the most commonly deregulated oncogenes in human cancers [33, 34]. The direct inhibition of c-Myc has not proven successful since the lack of a ligand-binding domain [35]. Given that chromatin is a platform for c-Myc signal transduction [36], selective small-molecule inhibitors were developed to inhibit Myc transcription and function through the competitive displacement of chromatin-bound BET proteins. These compounds showed evidence of anti-tumor efficacy in several preclinical models [16, 17, 37-40]. Their growth inhibitory effects against cancer cells were mainly attributed to the suppression of c-Myc protein levels as well as the Myc-dependent transcriptional network. In our study, JQ-1 attenuated the TPA-induced expression of anti-apoptotic and pro-proliferative oncogenes during neoplastic transformation of JB6 P+ cells by disrupting Brd4 activity. An unexpected observation was that JQ-1 treatment reduced the levels of phosphorylated ERK1/2, a finding that was also found in several previous reports. Segura et al. noted that this effect may result from the reduced transcription of ERK1 following BRD4 inhibition in melanoma cells [21]. However, a plausible mechanistic explanation remains to be fully elucidated.

In addition to this neoplastic transformation model, we assessed the influence of JQ-1 in cancer cell line HCT116 with a special interest on cell cycle. Similarly, JQ-1 treatment resulted in significant decreases in c-Myc, Cyclin A, and Cyclin D1 expression in HCT116 cells, which are associated with G1 cell cycle arrest (Fig. 3). These results of our study are consistent with those in human acute myelogenous leukemia (AML) cells [41] and melanoma cells [21] in response to JQ-1 or other BET inhibitors, indicating that a common set of transcriptional events mediated by BET may exist among different stage of cancers.

The combination of small-molecule inhibitors targeting chromatin remodeling enzymes has recently become an attractive strategy that may have lower toxicity and stronger inhibitory effects in several cancer types than using these inhibitors alone [42, 43]. Specifically, it has been reported that the HDAC inhibitor SAHA synergizes with JQ-1 to achieve a more potent suppressive effect in advanced pancreatic ductal adenocarcinoma (PDAC) [44]. Surprisingly,

as mentioned previously, there is an overlap of genes induced by BETis and HDACis. It was hypothesized that the induction of those genes is due to the relocation of the transcription elongation factor p-TEFb to other transcriptional complexes upon BETi and HDACi treatment [27]. However, the exact molecular mechanism has not been elucidated. In our study, we examined the expression of HDACs and found that JQ-1 decreased the protein level of several HDACs, partially explaining the similarity between genes induced by BETis and HDACis.

In addition to alterations of cell cycle- and apoptosis-related genes, activation of NF-κB has been observed in response to a wide variety of extracellular stimuli such as TPA, growth factors, cytokines, and UV radiation [45]. Activation of NF-κB has been implicated in the development of skin, colon, and prostate cancer in human clinical cases and mouse models [46-48]. Therefore, the inflammation-associated NF-κB signaling pathway is essential in tumor promoter-induced transformation and the development of tumors. Our results also demonstrated that TPA up-regulated the expression of NF-κB target genes such as COX2, IL6 and uPAR in JB6 P+ cells (Fig. 5), whereas inhibition of Brd4 by JQ-1 reduced TPA-induced inflammatory gene expression. Nicodeme et al. reported that the synthetic compound I-BET (a BET inhibitor) suppressed the inflammatory response in activated macrophages [49]. By interfering with the binding of BET proteins to acetylated histones, I-BET disrupts the assembly of the chromatin complexes that are essential for initiating mRNA transcription of inflammatory genes. The common anti-inflammatory effects of JQ-1 and I-BET between our experiments and previous reports indicate that targeting Brd4 may serve as a novel strategy in the regulation of NF-κB downstream gene expression.

To determine how JQ-1 influences the transcription of NF-κB target genes, we performed luciferase reporter assays driven by various fragments from the COX2 and IL6 promoters. The transcriptional activation of NF-κB involves a series of cofactors, particularly CBP/p300 that possesses HAT activity [50]. For instance, the induction of COX2 expression has been shown to require both the NF-κB and CREB binding sites in bronchial airway epithelial cells stimulated with lipopolysaccharide and TPA [51]. The activated CREB protein binds to a CRE region and then the KIX domain of CBP/p300, relaxes the chromatin structure and recruits the transcriptional machinery, including Pol II, resulting in the enhanced transcription of target genes [52, 53]. In our study, JQ-1 inhibited luciferase expression from reporter constructs carrying both NF-κB and CREB binding sites but had minimal effect on those only containing NF-κB elements. These results suggest that BET proteins may be implicated in the CREB-CBP/p300-mediated transcriptional enhancement of NF-κB target genes. Interestingly, in vascular smooth muscle cells, NF-κB is required for both basal and Angiotensin II (Ang II)-mediated IL6 expression, whereas CREB is required only for Ang II-induced effects [54]. This observation suggests the inhibition of BET proteins might have the potential to selectively block pathological alterations of NF-κB target gene expression under stress conditions.

In specific, elevated levels of COX2 have been observed frequently in neoplasms of epithelial origin. In skin cancer, experimental evidence has suggested that high levels of COX2 expression and PGE_2 production play a role in tumorigenesis [55, 56]. Therefore, the suppression of prostaglandin synthesis by inhibiting COX2 expression was suggested as a

promising chemopreventive strategy [57]. Our results showed that JQ-1 suppressed TPA-induced COX2 expression in JB6 P+ cells (Fig. 5). We further evaluated how JQ-1 treatment regulated the transcription of COX2 by siRNA silencing of p300 and ChIP assays. Interestingly, despite the attenuated level of COX2 induced by TPA, JQ-1 had a negligible effect on COX2 expression in the context of p300 silencing (Fig 8B&C). This result is in agreement with the luciferase activity assays described above. A previous study demonstrated that the intrinsic HAT activity of p300 plays an important role in opening up the chromatin structure and activating COX2 transcription [58]. In our study, we found that TPA treatment increased the enrichment of acetyl-H3K27 on the promoter region of the COX2 gene, which is proposed to be specifically mediated by CBP/p300 [59]. Correspondingly, our results have shown that p300 siRNA treatment attenuates the TPA-induced Ac-H3K27 enrichment at COX2 promoter, accompanied with the decrease of Brd4 enrichment at gene TSS (Fig. 8A). On the other hand, JQ-1 did not change the level of acetyl-H3K27 but decreased the recruitment of Brd4 and Pol II (Fig. 7). Given that Brd4 functions as an adaptor that binds to acetylated chromatin and promotes transcriptional elongation, it is possible that JQ-1 blocks COX2 overexpression in JB6 P+ cells during neoplastic transformation underlying a histone modification-related epigenetic mechanism.

Collectively, we have demonstrated that the inhibition of BET proteins by JQ-1 blocks JB6 P + cell neoplastic transformation focusing on its potent anti-proliferative and anti-inflammatory effects. Indeed, cancer initiation involves more complicated cellular and molecular oncogenic alterations, even in the same model as the present work [60, 61]. It would be helpful to broaden the understanding of pharmacological BET inhibition by exploring the effect on those pathways in the future work. On the other hand, it has been reported that JQ-1 could inhibit other oncogenic signals by regulating the transcriptional events, such as prostate cancer with constitutively AR activation [62] and non-small cell lung cancer driven by Kras mutant [39]. In addition, these in vivo studies also have shown JQ-1 is well tolerated in animals at effective doses, which implies a potential for further clinical investigation. In summary, epigenetic modifications clearly affect cancer initiation and progression. A variety of emerging small molecules that modify the tumor epigenetic landscape have shown promising clinical and preclinical results [63]. Our study suggests that BET proteins could be potential targets for chemopreventive or therapeutic strategies for early-stage carcinogenesis.

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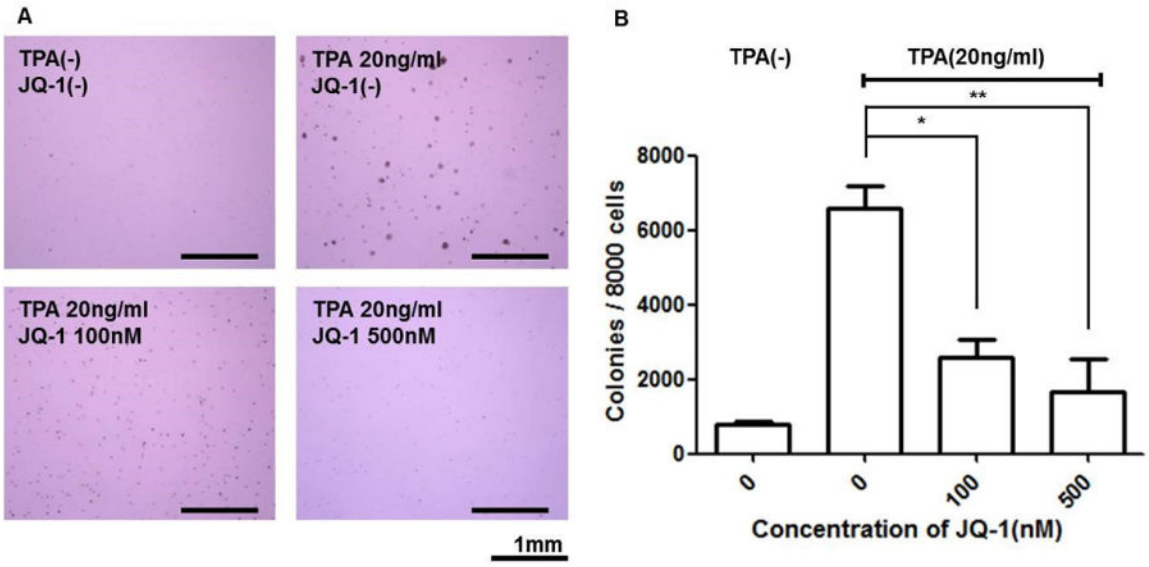

Figure 1. Inhibitory effects of JQ-1 on TPA-induced transformation of JB6 P+ cells

Cells were seeded in soft agar containing DMSO (vehicle control), 20ng/mL TPA or a combination of TPA and indicated concentration of JQ-1 in 6-well plates and were allowed to grow for 14 days. The colonies exhibiting anchorage-independent growth were imaged under a microscope, and the colony numbers were counted using ImageJ software. A) Representative images of each treatment group. B) Graphical data of colony numbers in each group are presented as the mean ± SD from three independent experiments. JB6 P+ cells in the vehicle control group rarely formed colonies in agar plate unless treated with TPA. JQ-1 significantly inhibited the TPA-induced anchorage independent growth of JB6 P+ cells. *P<0.05 and **P<0.01 indicate significant differences between the JQ-1-treated group and cells treated with TPA alone.

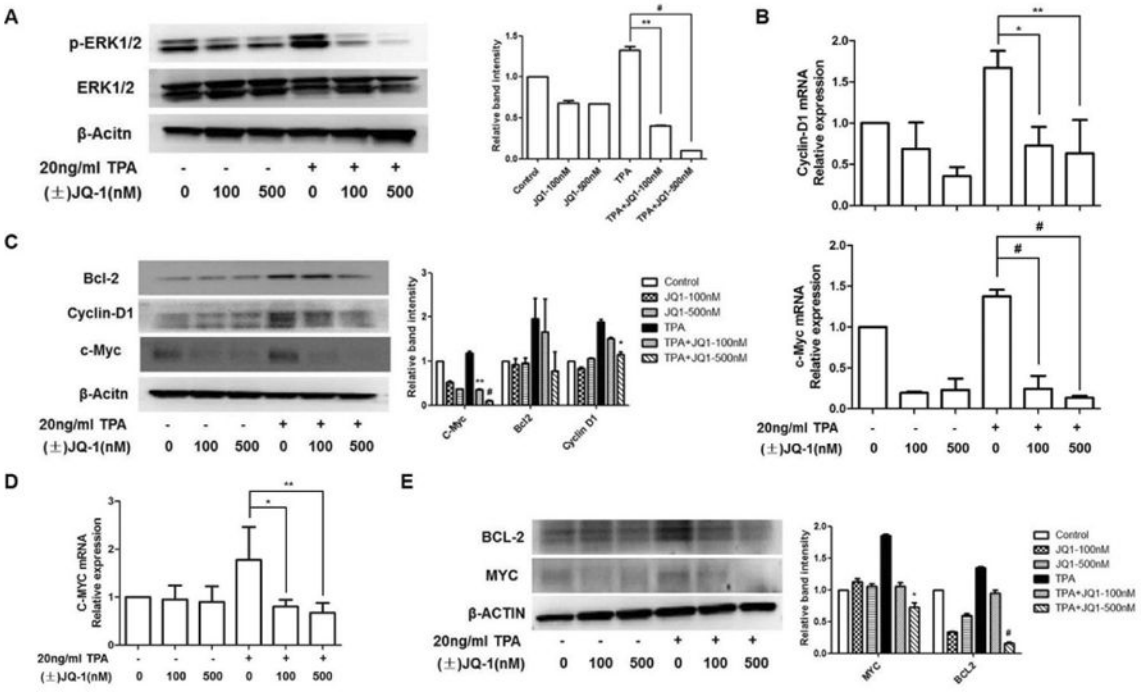

Figure 2. Effect of JQ-1 on the mRNA and protein expression of oncogenic genes induced by TPA

JB6 P+ or HaCaT cells were treated with the indicated concentrations of JQ-1 for 4 h, followed by 20 ng/ml TPA treatment. Cells were harvested 8 h post TPA treatment for RNA extraction and 24 h for protein sample preparation. A) JQ-1 attenuated the TPA-induced phosphorylation of ERK1/2 in JB6 P+ cells; band intensity of the blots were digitized and normalized to the vehicle control as fold change, presented as the mean ± SD in the right panel. B) JQ-1 treatment decreased the TPA-induced transcription of Cyclin D1 and c-Myc in JB6 P+ cells. C) Western blotting images of Bcl2, Cyclin D1, and c-Myc in JB6 P+ cells after JQ-1 treatment; right panel shows the bar graph of relative band intensity as the mean ± SD. D) JQ-1 treatment attenuated c-MYC mRNA levels in HaCaT cells. E) JQ-1 treatment decreased c-MYC and Bcl2 protein levels in HaCaT cells; right panel shows the bar graph of relative band intensity. **P*<0.05, ***P*<0.01, and #*P*<0.001 indicate significant differences between the JQ-1-treated group and the cells treated with TPA alone.

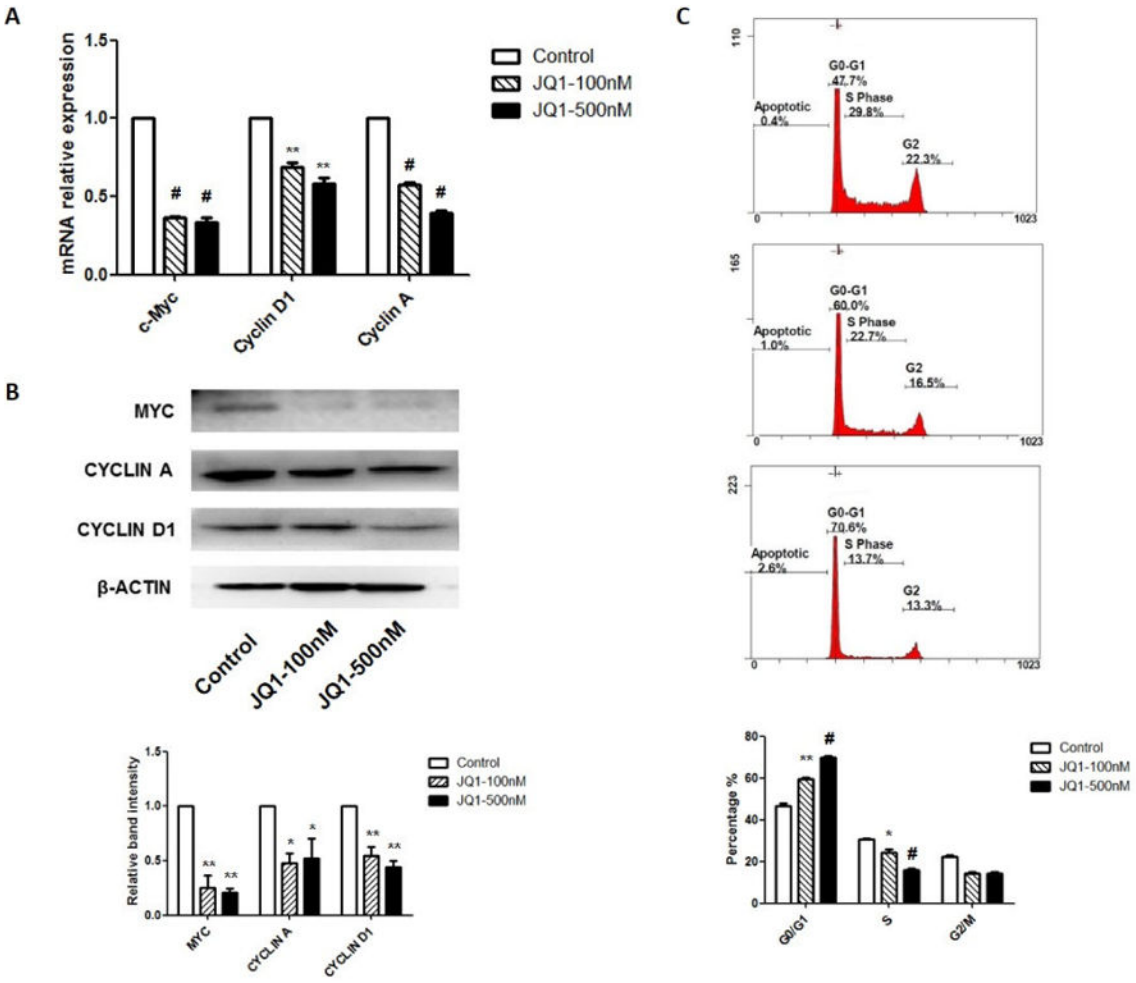

Figure 3. The effect of JQ-1 on G1 arrest of HCT-116 cells

HCT116 cells were treated with the indicated concentrations of JQ-1 for 24 h. A, B) JQ-1 suppressed cell cycle-related markers such as c-Myc and Cyclins A and D1 at both the mRNA and protein levels; relative mRNA expression or blot band intensity are presented as the mean ± SD; C) JQ-1 significantly induced G1 cell cycle arrest in HCT116 cells. Representative results from three independent experiments are provided in the upper panel whereas the lower panel shows the cell percentage in each phase as the mean ± SD. The statistical analyses were performed using one-way ANOVA with post-hoc Dunnett's test. *P<0.05, **P<0.01, and #P<0.001 indicate significant differences between the JQ-1-treated group and the vehicle control.

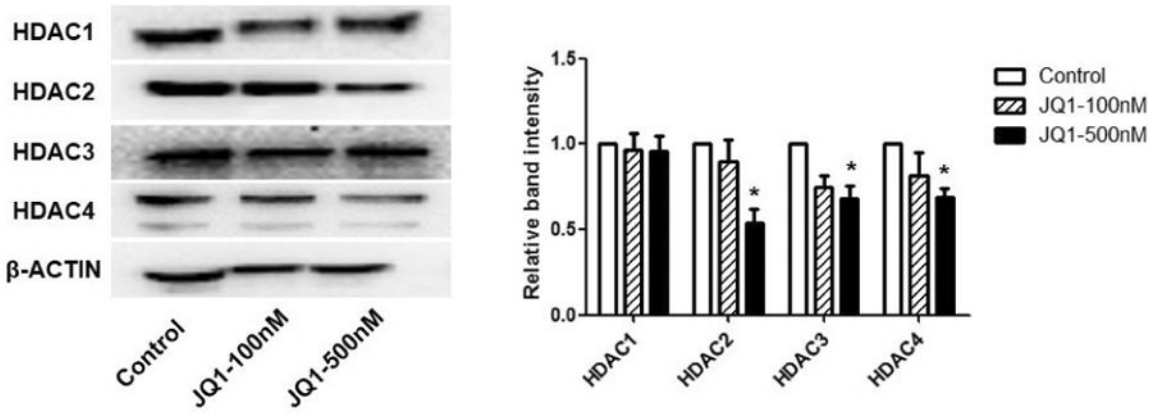

Figure 4. JQ-1 altered the protein expression of HDACs in HCT116 cells

The protein expression of HDAC1–4 was determined by western blotting. The relative band intensity (fold change) was calculated by normalizing the intensity of each sample to the vehicle control. Representative bands are shown in the left panel, whereas the bar chart in the right panel presents the mean ± SD of three independent experiments. *P<0.05 indicates significant differences between the JQ-1-treated group and the vehicle control.

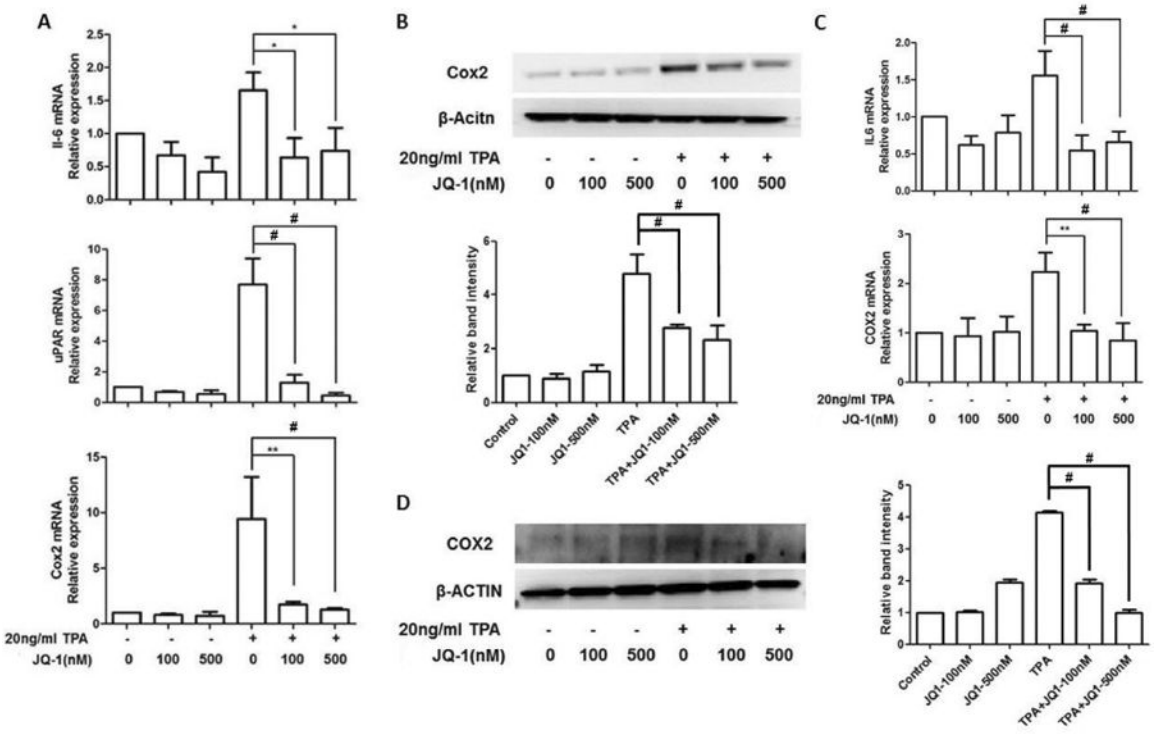

Figure 5. Effect of JQ-1 on the activation of NF-κB target genes triggered by TPA

A) JQ-1 treatment decreased the TPA-induced transcription of IL6, uPAR, and COX2 in JB6 P+ cells. B) Western blot images of COX2 expression in JB6 P+ cells after JQ-1 treatment. C) JQ-1 treatment attenuated IL6 and COX2 mRNA levels in HaCaT cells. D) JQ-1 treatment decreased COX2 protein levels in HaCaT cells. *P<0.05, **P<0.01, and #P<0.001 indicate significant differences between the JQ-1-treated group and cells treated with TPA alone.

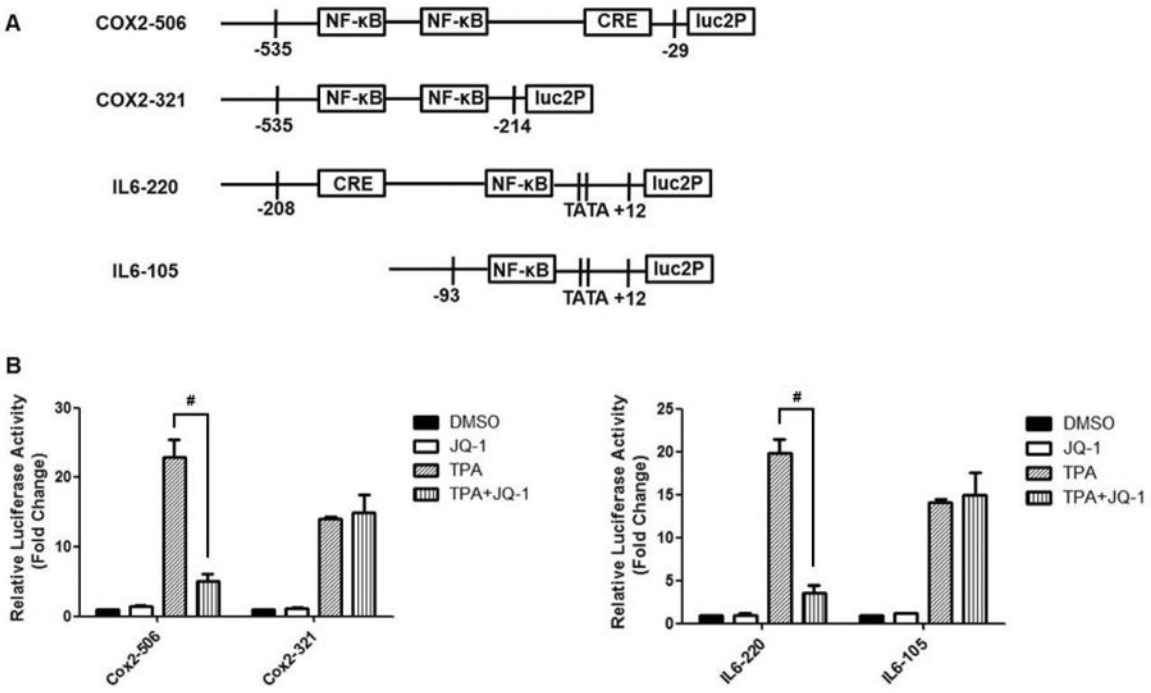

Figure 6. JQ-1 is not a direct inhibitor of NF-κB

A) Different constructs of luciferase reporter genes. B) HaCaT cells were co-transfected with 400 ng of the designated pGL4.15 firefly luciferase reporters and 100 ng of pGL4.75 [hRluc/CMV] Renilla luciferase plasmids as an internal control. The transfected cells were treated with DMSO, 20 ng/ml TPA, 500 nM JQ-1, or the combination for 20 h. The firefly luciferase readings were normalized to the Renilla luciferase reading first; then, the fold changes were calculated by comparing each treatment group to the vehicle control. JQ-1 inhibits the luciferase activity in the cells transfected with the plasmids carrying CRE elements (#P<0.001). No significant changes were observed after JQ-1 treatment in the cells transfected with the plasmids carrying the NF-κB binding sequence alone.

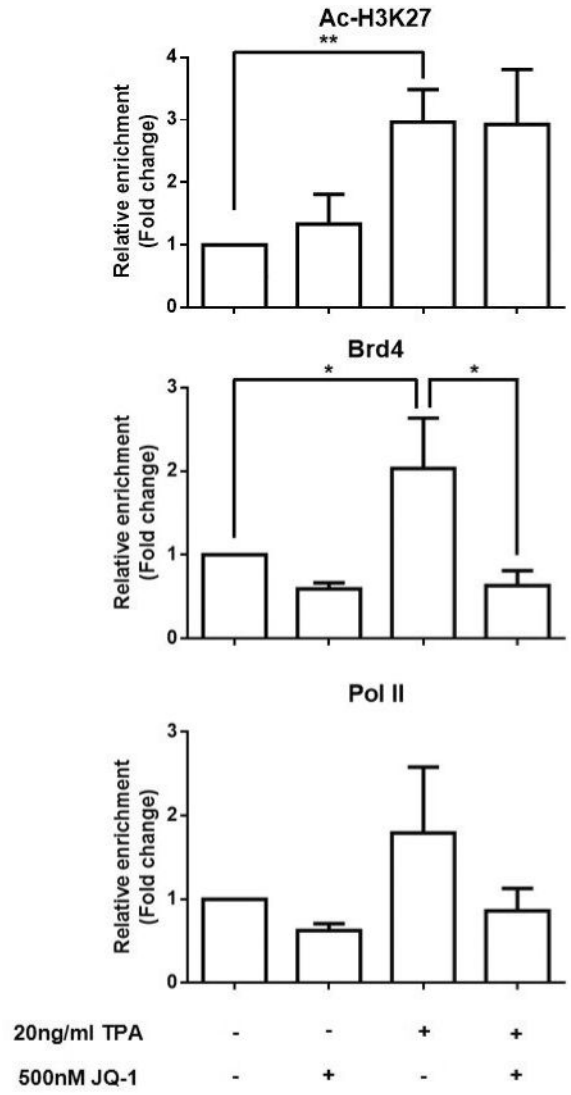

Figure 7. Effect of JQ-1 on the recruitment of the transcriptional machinery to COX2 under TPA stimulation

ChIP assays were performed to analyze the enrichment of H3K27Ac, Brd4, and Pol II on the promoter or transcription start site of COX2 in JB6 P+ cells. The immunoprecipitated DNA was used as a template for qPCR and the enrichment was quantified as the proportion ratio of its self- input. Relative fold change was then calculated by normalizing the ratio to that of the vehicle control. *P<0.05 and **P<0.01 indicate significant differences between the TPA-treated group and the indicated groups.

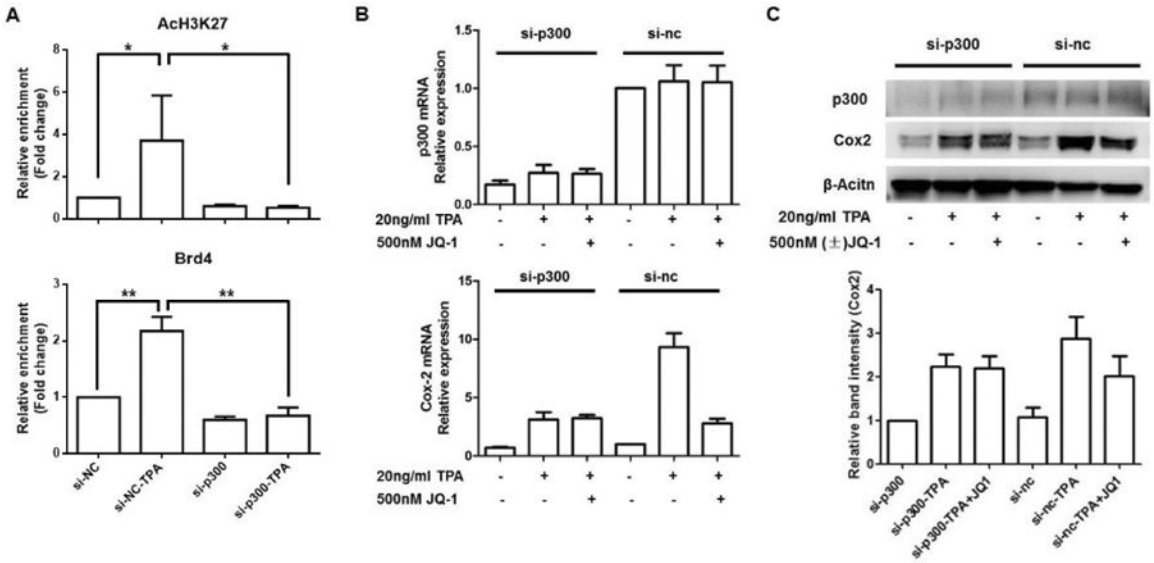

Figure 8. JQ-1 inhibits the p300-dependent activation of COX2

JB6 P+ cells were transfected with siRNA against p300 or non-specific negative control, and the cells were then further treated with DMSO, 20 ng/ml TPA alone or 20 ng/ml TPA in combination with 500 nM JQ-1 for 24 h. ChIP assays were performed to examine the enrichment of H3K27Ac and Brd4 after TPA stimulation in the p300 or negative control siRNA treated cells. The mRNA and protein levels of COX2 were assessed respectively. A) TPA increased the Ac-H3K27 enrichment on COX2 gene promoter and Brd4 enrichment on TSS. Knock down of p300 attenuated these TPA induced alterations. B) The mRNA levels of p300 and COX2 were determined by quantitative PCR. C) Representative western blot images of p300 and COX2 protein levels. Transfection of p300 siRNA attenuated TPA-induced COX2 expression at both the mRNA and protein levels. JQ-1 treatment did not show further inhibitory effects after p300 knockdown.