Cancer Management and Research

Dovepress

open access to scientific and medical research

Open Access Full Text Article

ORIGINAL RESEARCH

Combination BET Family Protein and HDAC Inhibition Synergistically Elicits Chondrosarcoma Cell Apoptosis Through RAD51-Related DNA Damage Repair

This article was published in the following Dove Press journal:
Cancer Management and Research

Songwei Huan[1,*]
Tao Gui[1,*]
Qiutong Xu[1,*]
Songkuan Zhuang[2]
Zhenyan Li[1]
Yuling Shi[3]
Jiebin Lin[3]
Bin Gong[1]
Guiqiang Miao[1]
Manseng Tam[4]
Huan-Tian Zhang[1]
Zhengang Zha[1]
Chunfei Wu[3]

[1]Institute of Orthopedic Diseases and Department of Bone and Joint Surgery, The First Affiliated Hospital, Jinan University, Guangzhou 510630, Guangdong, People's Republic of China; [2]School of Life Science, Xiamen University, Xiamen, Fujian 361005, People's Republic of China; [3]Department of Orthopedics, The Third Affiliated Hospital, Guangzhou University of Chinese Medicine, Guangzhou, Guangdong 510405, People's Republic of China; [4]IAN WO Medical Center, Macao Special Administrative Region, People's Republic of China

*These authors contributed equally to this work

Correspondence: Zhengang Zha; Chunfei Wu
Tel +86-20-38688617;
Tel +86-13610075186
Fax +86-20-38688000;
Email tbri@jnu.edu.cn;
wuchunfei666@163.com

Background: Chondrosarcoma is the second-most common type of bone tumor and has inherent resistance to conventional chemotherapy. Present study aimed to explore the therapeutic effect and specific mechanism(s) of combination BET family protein and HDAC inhibition in chondrosarcoma.

Methods: Two chondrosarcoma cells were treated with BET family protein inhibitor (JQ1) and histone deacetylase inhibitors (HDACIs) (vorinostat/SAHA or panobinostat/PANO) separately or in combination; then, the cell viability was determined by Cell Counting Kit-8 (CCK-8) assay, and the combination index (CI) was calculated by the Chou method; cell proliferation was evaluated by 5-ethynyl-2′-deoxyuridine (EdU) incorporation and colony formation assay; cell apoptosis and reactive oxygen species (ROS) level were determined by flow cytometry; protein expressions of caspase-3, Bcl-XL, Bcl-2, γ-H2AX, and RAD51 were examined by Immunoblotting; DNA damage was determined by comet assay; RAD51 and γ-H2AX foci were observed by immunofluorescence.

Results: Combined treatment with JQ1 and SAHA or PANO synergistically suppressed the growth and colony formation ability of the chondrosarcoma cells. Combined BET and HDAC inhibition also significantly elevated the ROS level, followed by the activation of cleaved-caspase-3, and the downregulation of Bcl-2 and Bcl-XL. Mechanistically, combination treatment with JQ1 and SAHA caused numerous DNA double-strand breaks (DSBs), as evidenced by the comet assay. The increase in γ-H2AX expression and foci formation also consistently indicated the accumulation of DNA damage upon cotreatment with JQ1 and SAHA. Furthermore, RAD51, a key protein of homologous recombination (HR) DNA repair, was found to be profoundly suppressed. In contrast, ectopic expression of RAD51 partially rescued SW 1353 cell apoptosis by inhibiting the expression of cleaved-caspase-3.

Conclusion: Taken together, our results disclose that BET and HDAC inhibition synergistically inhibit cell growth and induce cell apoptosis through a mechanism that involves the suppression of RAD51-related HR DNA repair in chondrosarcoma cells.

Keywords: chondrosarcoma, JQ1, HDAC, apoptosis, RAD51, DNA repair

Introduction

Chondrosarcoma is the second-most frequent primary malignant tumor of bone and is characterized by the production of a cartilage-like extracellular matrix.[1] Poor vascularity and abundant hyaline-dense cartilage matrix have been elucidated as the fundamental factors leading to the inherent resistance of chondrosarcoma to

submit your manuscript | www.dovepress.com
DovePress
http://doi.org/10.2147/CMAR.S254412

conventional chemotherapy and radiotherapy.[2,3] Accumulated evidence has suggested that increased expression of antiapoptotic proteins such as the Bcl-2 family members and survivin play a critical role in the chemotherapy resistance of chondrosarcoma cells.[4,5] Therefore, therapeutics aimed at modulating the apoptotic signaling pathway have increasingly been recognized as a promising treatment strategy for the chemo-resistant chondrosarcoma cells.

During the past decade, most therapeutic strategies have been developed based on genetic mutations, while recent advances have dramatically shifted towards targeting epigenetic regulators, including the bromodomain and extraterminal domain (BET) protein[6], [Zhang et al, 2017]. Some small-molecule inhibitors targeting BET proteins, including JQ1, have been undergoing clinical trials and have exhibited excellent efficacy in suppressing cell growth in a wide range of cancers.[7,8] Mechanistically, the role of JQ1 in treating cancers is achieved via displacement of the bromodomain containing 4 (BRD4) protein from chromatin, which inhibits the expression of several oncogenes, such as c-Myc and YAP, as well as impairs the homologous repair (HR) DNA repair signaling pathway.[9–13] We have recently demonstrated that JQ1 remarkably inhibits chondrosarcoma cell growth via the YAP/p21 signaling axis, yet little cell apoptosis is induced (Zhang et al, 2017). Understanding how JQ1 affects DNA damage repair might suggest a way to increase chondrosarcoma cell apoptosis.

Histone acetylation is another form of the epigenetic landmark related to gene regulation, and its dysfunction is frequently observed in chondrosarcoma.[14,15] In general, the abundance of histone acetylation marks is fine-tuned by histone acetyltransferases (HATs), histone deacetylases (HDACs), and BET proteins. HDACs are frequently overexpressed in cancers, including sarcoma, and inhibitors targeting HDACs have been reported to induce growth arrest, apoptosis, and differentiation in chondrosarcoma cells.[16,17] Despite their efficacy, HDAC inhibitors (HDACIs) have shown only a modest benefit in early clinical trials as a single agent.[18–20] Combination HDAC inhibition and BET inhibition is known to be an effective strategy for several cancers, but the synergistic effect has not been investigated in chondrosarcoma. In the current study, we demonstrated that combination BET family protein and HDAC inhibition synergistically inhibits chondrosarcoma cell growth, induces DNA damage, and subsequent cell apoptosis. Mechanistically, JQ1 synergizes with the HDACIs to impair the RAD51-related HR repair signaling.

Materials and Methods

Cell Culture and Reagents

Two chondrosarcoma cell lines (SW 1353 cells and Hs 819.T cells) were purchased from American Type Culture Collection and cultured as we described previously (Zhang et al, 2017). HDACIs (SAHA and PANO) were purchased from Selleck (Shanghai, China). BET bromodomain inhibitor (JQ1) and caspase-3 inhibitor (Z-DEVD-FMK) were obtained from MedChem Express (Beijing, China). JQ1 and HDACIs stock solutions were prepared by dissolving the compounds in dimethyl sulfoxide (DMSO, MP Biomedicals, USA) according to the manufacturer's instructions and were stored at −20 °C.

Cell Counting and Viability Assays

After seeding chondrosarcoma cells for 24 h, the cells were treated with DMSO, JQ1 (20 μM), SAHA (1 μM for SW 1353 cells and 2 μM for Hs 819.T cells) or their combination for 24, 48, and 72 h, respectively. Then, cells were counted at each time point using an Automatic Cell Counter (AMQAF1000, Countess II FL, USA).

For cell viability assay, cells were treated with JQ1, SAHA, PANO, or their combinations for 48 h, followed by the incubation with 10 μL enhanced Cell Counting kit-8 (CCK-8, C0042, Beyotime, China) solution for 2 h at 37 °C. Subsequently, the cell viability was examined at 450 nm using a spectrophotometer (Varioskan™ LUX, Thermo Scientific, USA). Relative cell viability was analyzed from at least three independent experiments.

Calculation of Drug Synergy

For the determination of drug synergy, JQ1 and HDACIs were used in fixed-dose ratios. For each cell line, at least five different combinations of concentrations were applied. The IC50 values of JQ or HDACIs were shown in Table S1, while the combination index (CI) was calculated by the Chou-Talalay algorithm with CompuSyn software 1.0 (ComboSyn Inc.). A CI value of less than 1 was considered synergism.[21]

Immunoblotting (IB) Analysis

IB was performed as we previously described.[13] The primary antibodies used in this study were listed as follows: anti-phospho-histone H2AX (20E3, 1:1000, CST), anti-Bcl-2 (D17C4, 1:1000, CST), anti-cleaved-caspase-3 (5A1E, 1:1000, CST), and anti-Bcl-XL (54H6, 1:1000, CST), the secondary antibodies were HRP-conjugated

(1:1000–1:2000, CST). Antibodies against RAD51 (PC130, 1:2500, Merck) was obtained from Merck, and anti-acetyl-histone H3 (Lys9, H3K9, #3079121, 1:500) was purchased from Millipore. Anti-β-actin (8H10D10, 1:2000, CST) was used as an internal control. The IB images captured by the Automatic chemiluminescence image analysis system (5200, Tanon, China) were further quantified by Image J software.

5-Ethynyl-2′-Deoxyuridine (EdU) Incorporation Assay

Briefly, cells were treated with individual agents or their combinations for 48 h, after that, the cells were incorporated with 20 μM EdU solution (EdU, C10310-1, Ribobio, Guangzhou, China) for another 2 h. After fixation and permeabilization, the cells were stained with EdU solution at room temperature (RT) for 30 min followed by the treatment with Hoechst 33342 for another 30 min. The images were captured with a microscope (Olympus IX71, Tokyo, Japan). The EdU-positive cells were counted from ten random areas, with a minimal cell number of more than 500.

Colony Formation Assay

SW 1353 or Hs 819.T cells were seeded in a 6-well plate at a density of 1000 cells/well and cultured in complete medium for 48 h; next, the cells were treated with JQ1, SAHA, PANO, or their combinations for another 48 h. Then, the crystal violet solution was added to each well after cells were further cultured with fresh medium for 5 d. The colonies with more than 50 cells were counted under a SZ760 series microscope (Chongqing Optec Instrument Co., China).

Analysis of Apoptosis by Flow Cytometry

After treatment with JQ1, SAHA, or their combination for 48 h, $1\sim5\times10^5$ cells were collected and resuspended in 500 μL binding buffer. Then, the cells were stained with Annexin V-FITC/propidium iodide (PI) Apoptosis Detection Kit (KGA107, KeyGEN BioTECH, China) at RT for 15 min. Finally, the cells were subjected to flow cytometry (BD Accuri^{TM} C6 PLUS, USA) to determine the proportions of apoptotic cells.

Reactive Oxygen Species (ROS) Assay

In brief, chondrosarcoma cells were cultured until they reach 85% confluence. Then the cells were treated with 10 μM DCFH-DA (KGT010, KeyGEN BioTECH, China). After that, cells were collected and resuspended in ice-cold PBS, and the DCF fluorescence intensity (FI) was detected by flow cytometry.

DNA Damage Comet Assay

Chondrosarcoma cells were treated with DMSO, JQ1, SAHA or their combinations for 48 h. Then, the cells were harvested and analyzed using the comet assay as we described previously.[22] The Olive tail moment (OTM) was analyzed by Open comet software.[23]

Determination of DNA Damage and Repair Foci

SW 1353 cells were cultured on slides and fixed with 4% paraformaldehyde after the above treatments. After permeabilization and blocking, cells were incubated with the RAD51 antibody at RT for 2 h, followed by incubation with the secondary antibody (Alexa Fluor 555-labeled anti-rabbit IgG, 1:100, CST) and 4,6-diamidino-2-phenylindole (DAPI, 1:1000, Life Technologies, CA, USA) at 4 °C overnight. The RAD51 foci were captured with a confocal microscope (ZEISS LSM 700, Germany).

Reverse Transcription and Real-Time Polymerase Chain Reaction (PCR)

Reverse transcription was performed according to our previous protocols.[13] Briefly, total RNA from SW 1353 cells was purified, and then reverse-transcribed into complementary DNA (cDNA). Real-time PCR was conducted in a QuantStudio™ 3 Real-Time PCR Instrument (No.A28132, Thermo Fisher Scientific, Singapore). The primers used for RAD51 amplification were listed as follows: 5′-CTCTGGCAGTGATGTCCTGG-3′ (sense) and 5′-TGTTCTGTAAAGGGCGGTGG-3′ (antisense). The primers used for RUVBL1 amplification were 5′-TGCTGGACATTGAGTGCTTCACC-3′ (sense) and 5′-TGATGACACAGTTGCCTCGGTTG-3′ (antisense). GAPDH was used as the endogenous control to calculate the relative mRNA levels.

Plasmids Construction and Transfection

Genomic DNA from SW 1353 cells was used to amplify the RAD51 coding region by regular PCR using a high-fidelity polymerase. The primers used for amplification were 5′-TCTGTCGACAATGGCAATGCAGATGCAGCT-3′ and 5′-TAAAGCGGCCGCCCAATGATTCAGTC

TTTGGCAT-3′. Then the PCR product was subcloned into the HA-CMV vector at XhoI and EcoRI sites. The HA-RAD51 construct was verified by sequencing and the expression check.

Statistical Analysis

Results from each experiment were presented as mean±SD from three independent experiments. Differences were tested for significance using ANOVA among groups or unpaired *t*-test for two groups in the GraphPad Prism 7 software (Graphpad Software, IL, USA). P values less than 0.05 were considered statistically significant.

Results

JQ1 and HDACIs Inhibit Chondrosarcoma Cell Growth in a Synergistic Manner

Our previous study has demonstrated that JQ1 substantially inhibits cell proliferation, accompanied by limited apoptosis in chondrosarcoma cells (Zhang et al, 2017). Given that HDACIs play a key role in regulating cell apoptosis in several cancers,[17,24,25] we sought to examine whether HDACIs synergize with JQ1 in suppressing cell growth and/or inducing apoptosis in chondrosarcoma cells. SW 1353 and Hs 819.T cells were treated with different concentrations of JQ1 and SAHA at a fixed-ratio for 48 h. As shown in Figure 1A and B, in each cell line tested, the combination treatment of JQ1 and SAHA resulted in a sharp dose-dependent decline in relative cell viability when compared to treatment with the single agent. The combined treatment with JQ1 and SAHA showed synergistic anticancer effects in chondrosarcoma cells (CI value < 1),[21] as determined by the Chou and Talalay method (Figure 1C). A similar synergistic effect of JQ1 with another HDACI, PANO, was observed in both chondrosarcoma cell lines (Figure 1D-F). Next, we compared the effect of JQ1 or SAHA alone or in combination on chondrosarcoma cell growth. As expected, compared to treatment with DMSO, treatment with JQ1 or SAHA alone remarkably reduced the total cell numbers of chondrosarcoma, and that cotreatment with JQ1 and SAHA further reduced the total cell numbers compared to treatment with the single inhibitor (Figure 1G and H). The expression of H3K9, which indicates enhanced histone acetylation upon treatment with SAHA, was confirmed as the internal control (Figure 1I and S1A).

Considering the drug efficiency and toxicity, the final drug concentrations used for subsequent experiments were given in Table S2, and the treatment time was 48 h. In support of the above findings, combined treatment with JQ1 and SAHA also significantly attenuated the percentage of EdU-incorporated cells, indicating their inhibitory role in chondrosarcoma cell proliferation (Figure 2A and B). Further, we did show that combined BET bromodomain and HDAC inhibition substantially suppressed colony formation of chondrosarcoma cells, when compared to the DMSO or single-agent groups (Figure 2C-F). These results together suggest that JQ1 and HDACIs synergistically inhibit chondrosarcoma cell growth.

BET Bromodomain and HDAC Inhibition Synergistically Cause Cell Apoptosis

Next, we investigated whether combination treatment with JQ1 and HDACIs has a synergistic effect on chondrosarcoma cell apoptosis. As shown in Figure 3A and B, treatment with JQ1 or SAHA alone increased the percentage of apoptotic cells modestly (12.37% and 11.26%, respectively), while combined treatment with JQ1 and SAHA dramatically elevated the percentage of apoptotic cells to 44.1%. ROS is one of the most important contributing factors of cell apoptosis.[21] In agreement with this, we also found that cotreatment with JQ1 and SAHA remarkably enhanced the relative DCF-fluorescence intensity (FI), which reflects the ROS level (Figure 3C and D). Furthermore, we examined the changes of apoptotic signaling proteins including cleaved-caspase-3, Bcl-2, and Bcl-XL, by IB analysis. Compared with JQ1 or HDACIs treatment alone, combination treatment with JQ1 and HDACIs significantly increased the expression of cleaved-caspase-3 (Caspase-3) and decreased the expressions of Bcl-2 and Bcl-XL in chondrosarcoma cells (Figure 3 E-G). The caspase-3 inhibitor, Z-DEVD-FMK partially rescued the cell apoptosis induced by the combination treatment with JQ1 and SAHA (Figure S1B), indicating caspase-3-dependent apoptosis. Similarly, cotreatment with JQ1 and PANO also enhanced chondrosarcoma cell apoptosis (Figure 3H and I). Thus, we conclude that JQ1 and HDACIs act synergistically in inducing apoptosis of chondrosarcoma cells.

Cotreatment with JQ1 and HDACIs Induces DNA Damage and Impairs HR Signaling

Given that combined treatment with JQ1 and HDACIs significantly induced ROS level and promoted cell

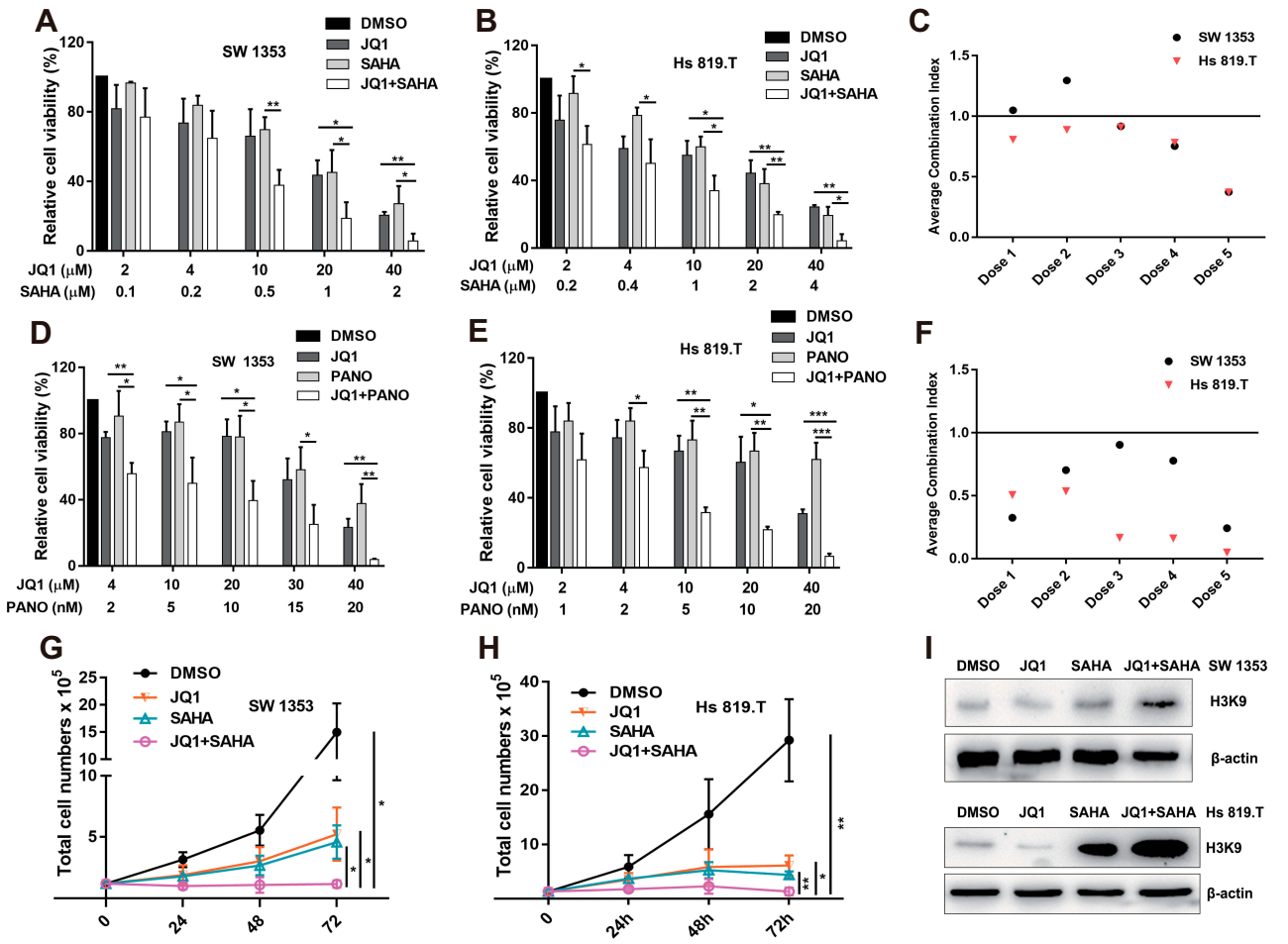

Figure 1 The synergistic effect of JQ1 and HDACIs on chondrosarcoma cells. (**A**–**B**) Synergy analysis of JQ1 and SAHA in chondrosarcoma cells. SW 1353 and Hs 819.T cells were treated with the indicated concentrations of JQ1 and SAHA at a fixed-ratio for 48 h, and then the relative cell viability was measured by the CCK-8 assay. (**C**) The combination indexes (CIs) in (**A**) and (**B**) were calculated by the Chou method. CI < 1 was considered to represent a synergistic anti-cancer effect. (**D**–**E**) SW 1353 and Hs 819.T cells were treated with the indicated concentrations of JQ1 and PANO at a fixed-ratio for 48 h, and then the relative cell viability was measured. (**F**) The combination indexes (CIs) in (**D** and **E**) were calculated. (**G**–**H**) JQ1 synergizes with SAHA in suppressing chondrosarcoma cell growth in a time-dependent manner. SW 1353 and Hs 819.T cells were treated with 20 μM JQ1, 1 μM SAHA (2 μM for Hs 819.T), or a combination for 24, 48 or 72 h, and then the total cell number was counted and analyzed. (**I**) SW 1353 and Hs 819.T cells were treated as in (**G**) and (**H**) for 48 h, and the expression of H3K9 was evaluated by immunoblotting. **P* <.05; ***P* <.01; ****P* <.001.

apoptosis, we next investigated the underlying regulatory mechanisms, eg, triggering DNA damage or impairing DNA repair. As shown in Figure 4A-C, cotreatment with JQ1 and SAHA significantly induced DSBs in chondrosarcoma cells, as evidenced by the comet assay. DNA damage markers, such as the formation of γ-H2AX foci, were further investigated. The results showed that combined treatment with JQ1 and SAHA robustly promoted the formation of γ-H2AX foci (Figure 4D). Consistently, the expression of γ-H2AX was significantly increased (Figure 4E and S1C), indicating that JQ1 in combination with HDACIs indeed triggers more severe DNA damage than either single inhibitor.

The functional HR DNA repair pathway is an important hallmark against cell apoptosis and DNA damage. Since BET proteins, such as BRD4, were recently found to be involved in HR-mediated DNA damage repair,[26] we hypothesized that combination treatment with JQ1 and SAHA also plays a role in regulating HR repair signaling. Although JQ1 alone significantly downregulated the mRNA expression of DNA repair genes such as TIP60, EZH2, BRCA1, and BRCA2; combined treatment with SAHA did not further suppress the expression of these genes (Figure S1D). Notably, we found that cotreatment with JQ1 and SAHA significantly inhibited RAD51 mRNA expression when compared with treatment with JQ1 or SAHA alone (RUVBL1 as control, Figure 5A). It is well-recognized that RAD51 is a highly conserved protein that catalyzes HR DNA repair thus directly modulating cellular sensitivity to DNA-damaging treatments.[27,28] We next sought to quantify RAD51 foci formation and its expression

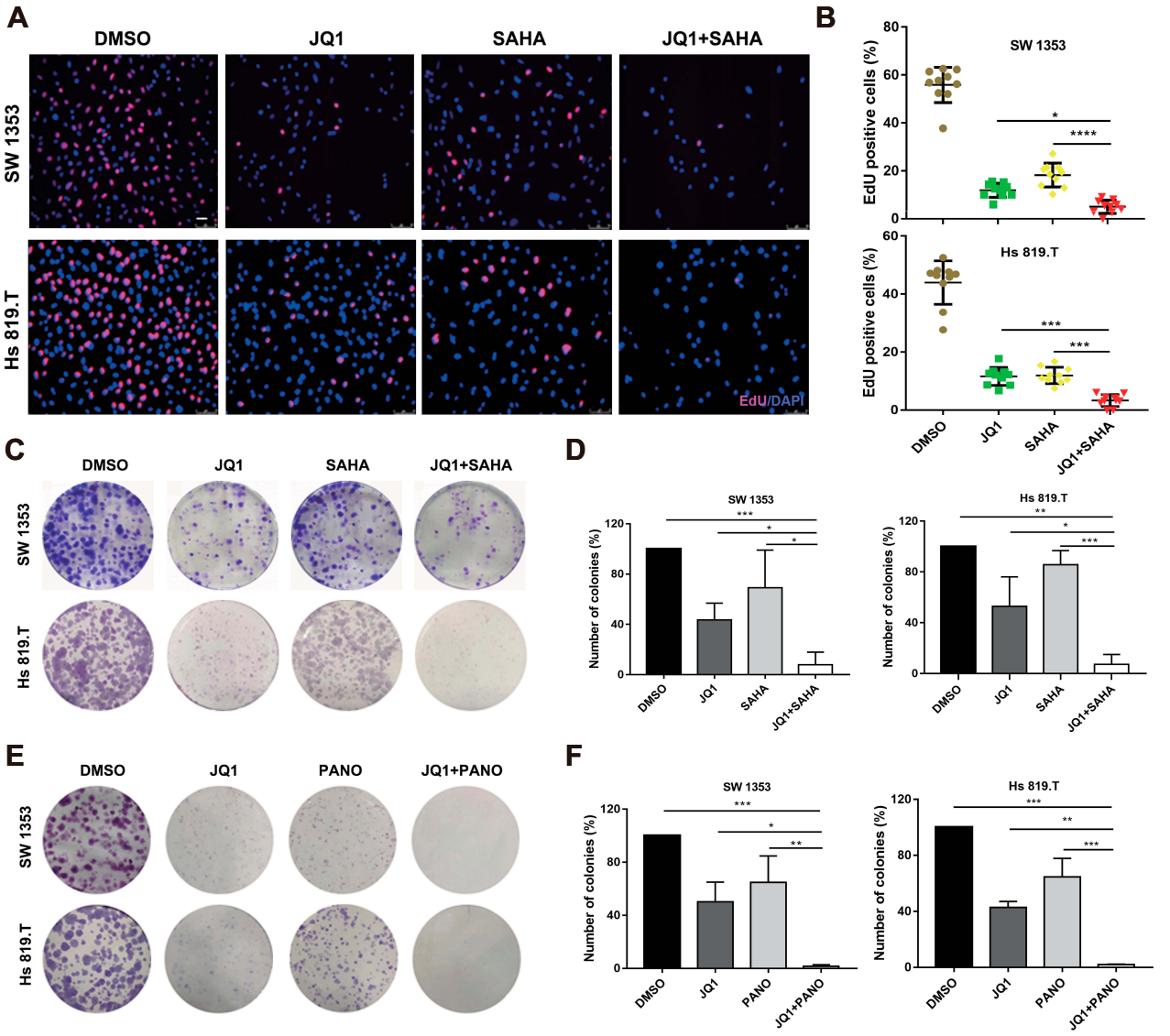

Figure 2 Combination treatment with JQ1 and HDACIs inhibits cell proliferation and colony formation. (**A**–**B**) SW 1353 and Hs 819.T cells were treated with DMSO, JQ1 (20 μM), SAHA (1 μM or 2 μM) or their combination for 48 h, and cell proliferation was determined by the EdU incorporation assay. The percentages of EdU-positive cells were calculated from ten random fields and the results are presented. Scale bar = 50 μm. (**C** and **E**) SW 1353 or Hs 819.T cells were seeded into 6-well plates and treated with JQ1 (20 μM), SAHA (1 μM for SW 1353 and 2 μM for Hs 819.T)/PANO (10 nM for both cell lines), or a combination of both for 48 h. The colonies were stained with crystal violet solution after incubation with fresh medium for 5 d. (**D** and **F**) The number of colonies (more than 50 cells) was manually counted from three independent experiments. *P <.05; **P <.01; ***P <.001. ****P <.0001.

in SW 1353 cells. As shown in Figure 5B-E, RAD51 foci formation along with its protein expression was dramatically compromised upon cotreatment with JQ1 and SAHA, indicating severe impairment of the DNA repair capacity. Nevertheless, ectopic expression of RAD51 in SW 1353 cells by transfection with a plasmid encoding HA-RAD51 partially compromised the apoptosis elicited by the cotreatment with JQ1 and SAHA (Figure 5F and G). Overall, we conclude that combined treatment with JQ1 and HDACIs induces DNA damage and impairs HR signaling by suppressing the RAD51 protein, which is pivotal for the induction of chondrosarcoma apoptosis in a caspase-3-dependent manner (Figure 5H).

Discussion

Chondrosarcoma represents the second-most frequent primary bone malignancy and, is poorly responsive to conventional chemotherapy and radiotherapy.[29] Understanding the underlying mechanisms will lead to new treatment options for chondrosarcoma resistance. Recently, targeting epigenetic readers, such as BET proteins, by specific inhibitors, was shown to be essential for

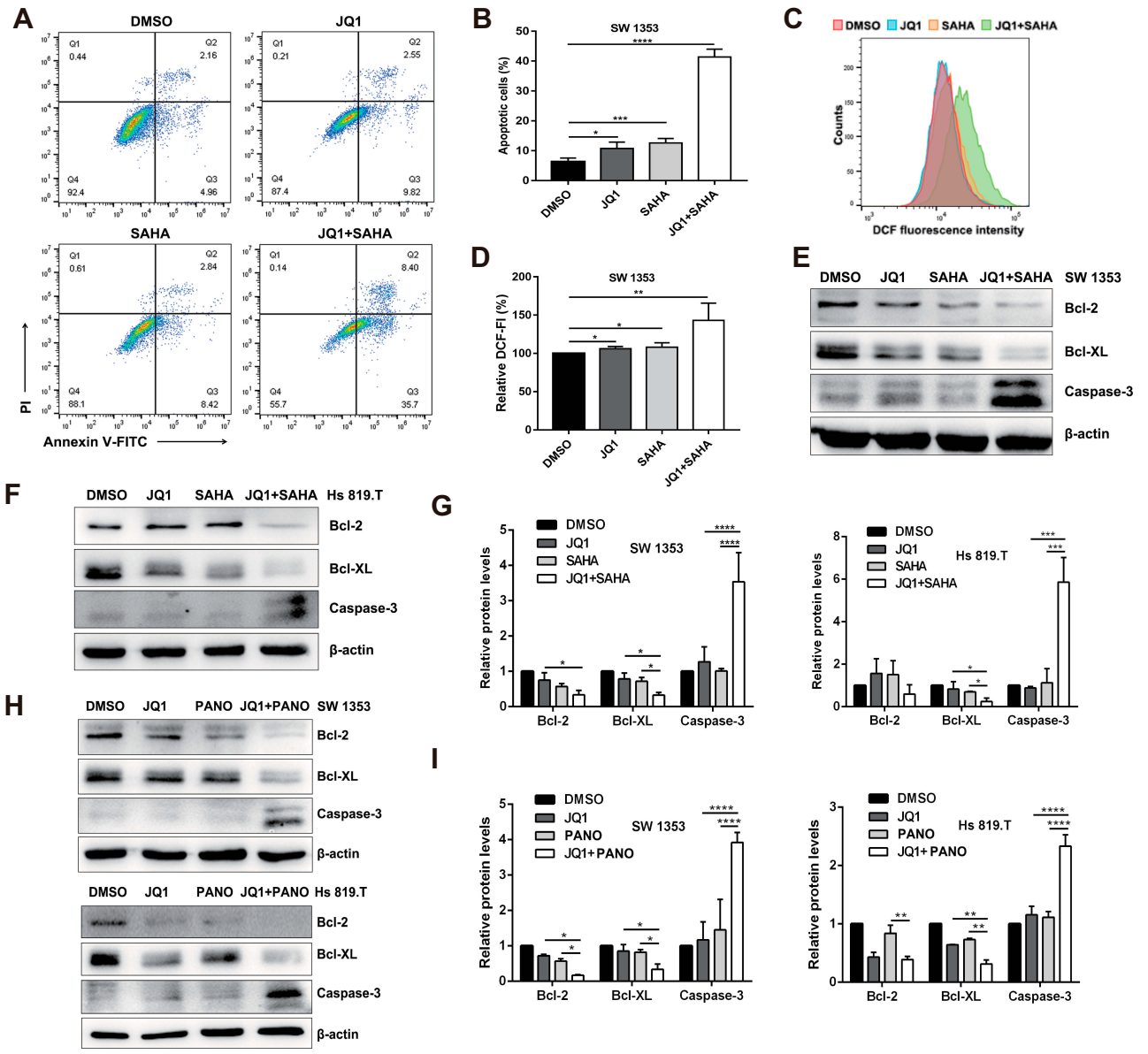

Figure 3 Combination treatment with JQ1 and HDACIs leads to apoptosis of chondrosarcoma cells. (**A**) SW 1353 cells were treated with the indicated inhibitors (JQ1: 20μM, SAHA: 1 μM) for 48 h, and then apoptotic cells were assessed by flow cytometry using the Annexin V-FITC/PI kit. (**B**) The percentage of apoptotic cells was calculated from three independent experiments. (**C**–**D**) After the indicated treatment, SW 1353 cells were incubated with 10 μM DCFH-DA in serum-free medium at 37 °C for 20 min. DCF fluorescence intensity (FI) was detected by flow cytometry, the relative DCF-FI was calculated and presented. (**E**–**I**) SW 1353 and Hs 819.T cells were treated with JQ1 (20 μM), SAHA (1 μM for SW 1353 and 2 μM for Hs 819.T)/PANO (10 nM for both cell lines) or their combinations for 48 h. The total cell lysate was prepared and the expression levels of Bcl-2, Bcl-XL, and caspase-3 were determined by immunoblotting analysis, and the protein expression was quantified using the Image J software (n = 3). **P* <.05; ***P* <.01; ****P* <.001; *****P* <.0001.

suppressing cancer cell growth both in vitro and in vivo, and the strategies mainly worked by modulating the cell cycle, facilitating differentiation and inducing cell apoptosis.[7–9,30,31] In our previous work, we showed that JQ1 efficiently inhibited chondrosarcoma cell proliferation, yet targeting YAP/p21 signaling did not elicit pronounced cell apoptosis in chondrosarcoma (Zhang et al, 2017). In the current study, we present evidence that a BET inhibitor and HDACIs inhibit the growth and induce the apoptosis of chondrosarcoma cells synergistically. Mechanistically, the apoptosis induced by combination treatment with JQ1 and HDACIs was attributed to an accumulation of DNA damage and impairment of HR repair via suppression of RAD51 expression.

HDACs are critical epigenetic gene expression and chromatin structure modulators during cell proliferation, differentiation, and apoptosis.[17,32,33] Substantial evidence has shown that HDACIs can induce apoptosis in a variety

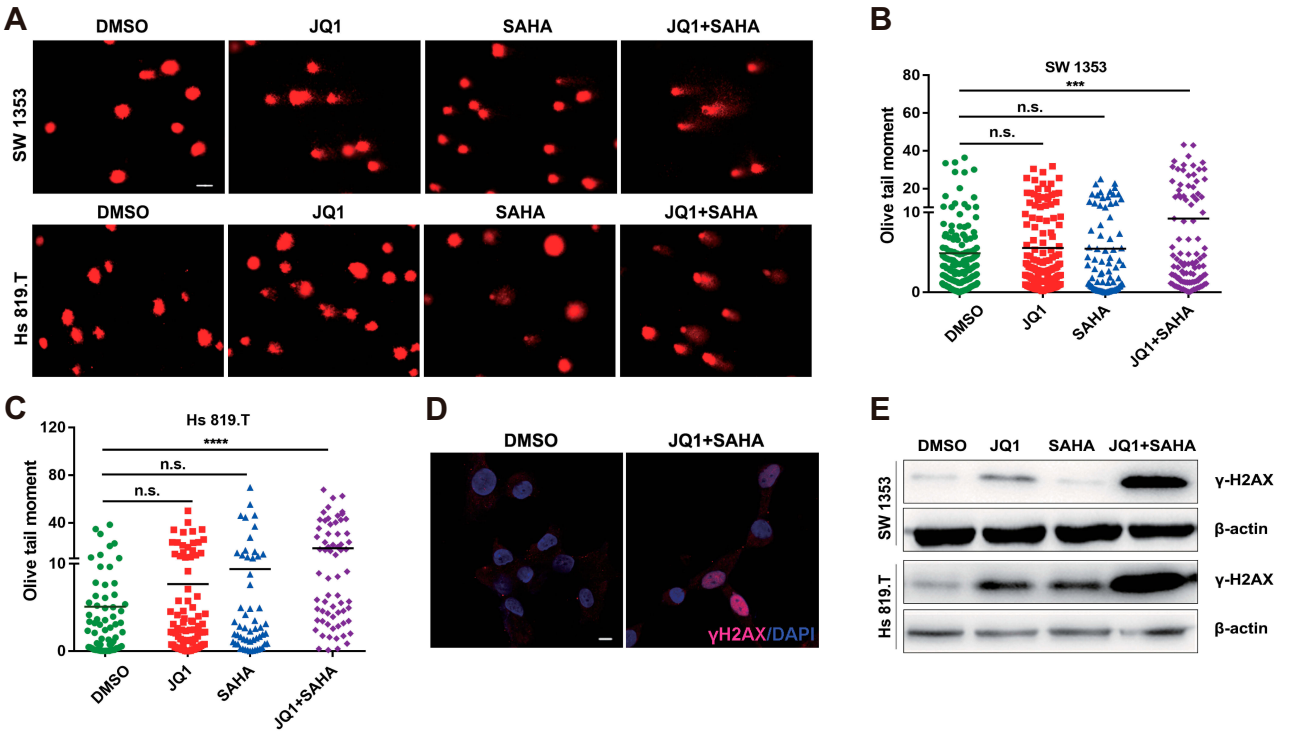

Figure 4 Combination treatment with JQ1 and SAHA elicits DNA damage in chondrosarcoma cells. (**A**) SW 1353 and Hs 819.T cells were treated with JQ1 (20 μM) or SAHA (1 μM for SW 1353 and 2 μM for Hs 819.T) alone or their combinations for 48 h, and then DNA damage was visualized by the comet assay. Scale bar = 50 μm. (**B**–**C**) The Olive tail moment (OTM) in A was analyzed by Open comet software. (**D**) Representative images of γ-H2AX foci formation (red) in SW 1353 cells under treatment with DMSO or the combination. The nucleus was stained with DAPI (blue). Scale bar = 10 μm. (**E**) After treatment as indicated, γ-H2AX expression in SW 1353 and Hs 819.T cells was analyzed by immunoblotting. n.s., no significance; ***P <.001; ****P <.0001.

of cell types including chondrosarcoma cells,[1,16] emphasizing their potential for applications in cancer therapy. However, as a single agent, HDAC inhibitors show a limited clinical benefit for patients with solid tumors, prompting the investigation of rational drug combination strategies to improve efficacy.[34] Several attempts have been made using combination treatments with a BET inhibitor and an HDAC inhibitor in several cancer types. Meng et al found that cotreatment with PANO and JQ1 or OTX015 synergistically suppressed cell proliferation and caused apoptosis in glioblastoma cells.[35] Shahbazi et al also reported that JQ1 and PANO synergistically reduced LIN28B gene and N-Myc protein expression, and synergistically induce growth inhibition and apoptosis in neuroblastoma cells.[36] Fiskus et al found that cotreatment with JQ1 and the HDAC inhibitor PANO synergistically greatly attenuated oncogenes, such as c-MYC and BCL2.[37] These studies have suggested that the true therapeutic potential of HDACIs is most likely lies in combination with other anticancer drugs, eg, the BET inhibitor, in chondrosarcoma. Notably, our studies suggest that HDACIs synergize with JQ1 in suppressing cell proliferation and inducing apoptosis in chondrosarcoma (Figures 1–3), indicating the promise of combination treatments targeting an epigenetic reader and eraser as novel strategies for chemotherapy-refractory chondrosarcoma.

The generation of γ-H2AX upon DNA damage, together with other histone modifications, is essential for the recruitment of HR DNA repair proteins, including RAD51, which in turn promotes the repair of the original lesion.[38] Accumulated evidence has suggested that enhanced DNA repair signaling enables cancer cells to survive the DNA damage induced by chemotherapeutic drugs and that inhibition of a specific DNA repair pathway can favor cancer cells undergoing apoptosis upon chemotherapy.[39,40] King and colleagues recently illustrated that RAD51 overexpression contributed to the resistance of glioblastoma cells to radiation,[41] and HR defects caused by impaired RAD51 expression may sensitize the affected tumors to DNA-damaging agents.[42] Therefore, it is reasonable to assume that RAD51 is closely related to sensitivity to chemotherapeutic agents. We next sought to explore the mechanisms underlying the apoptosis-induced by cotreatment with JQ1 and HDACIs. As shown in Figures 4 and 5,

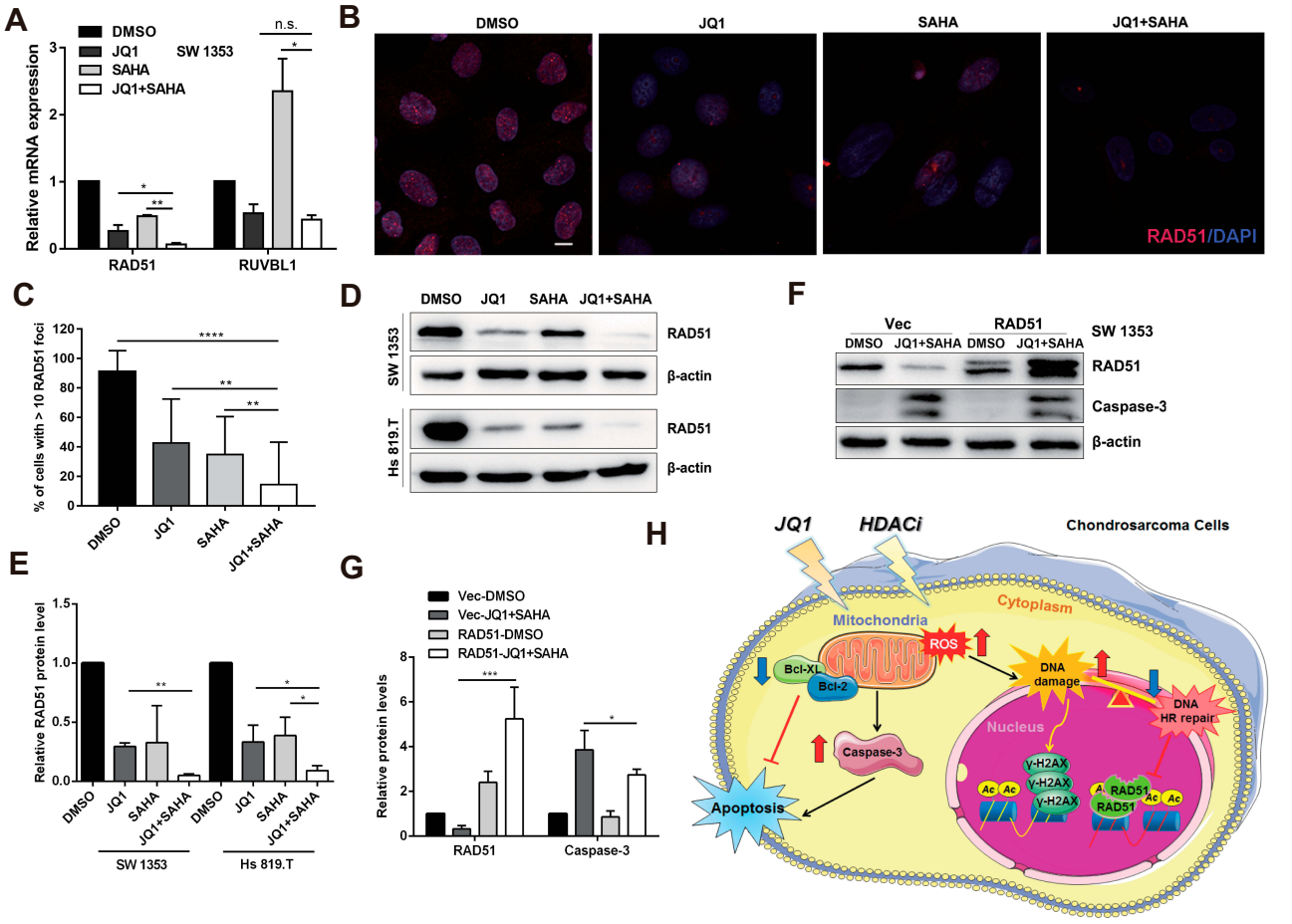

Figure 5 Combined treatment of JQ1 with HDACIs impairs the homologous repair (HR) pathway by targeting RAD51. (**A**) SW 1353 cells were treated with the indicated concentrations of DMSO, JQ1 (20 μM), SAHA (1 μM) or their combinations for 48 h, and the mRNA expressions of RAD51 and RUVBL1 were examined by real-time PCR. n.s., no significance; *P <.05; **P <.01. (**B**) Representative images of RAD51 (red) staining in SW 1353 cells are shown. The nucleus was stained with DAPI (blue). Scale bar = 20 μm. (**C**) The percentage of cells with more than 10 RAD51 foci per nucleus was calculated from three different experiments. **P <.01; ****P <.0001. (**D**) After treatment as indicated, RAD51 expression in SW 1353 and Hs 819.T cells was analyzed by immunoblotting. (**E**) A plasmid encoding HA-RAD51 was transfected into SW 1353 cells for 48 h. Then, the cells were treated with DMSO, JQ1 (20 μM) plus SAHA (1 μM) for 24 h, and the expression levels of apoptotic-related markers caspase-3 and RAD51 were evaluated by immunoblotting. (**F**–**G**) Relative protein expression levels in (**D**) and (**E**) from three independent experiments were quantified by Image J software (n = 3). *P <.05; **P <.01; ***P <.001. (**H**) Upon treatment with JQ1 and HDACIs (SAHA and PANO), the ROS level was elevated, accompanied by a downregulation of Bcl-XL and Bcl-2, while the expression of caspase-3 increased, together triggering apoptosis. The combined treatment also elicited pronounced nuclear γ-H2AX expression and foci formation, along with the impairment of HR DNA repair signaling via suppression of RAD51. The imbalance of accumulated DNA damage and HR repair, in turn, contributed to cell apoptosis.

combination BET inhibition and HDAC inhibition favors DNA damage while impairing HR DNA repair signaling by targeting RAD51, and ectopic RAD51 expression partially abolished the sensitivity of chondrosarcoma cells to JQ1 and SAHA. Our findings are in line with previous studies showing HDACIs and JQ1 as single-agent plays an important role in the proper assembly and expression of RAD51 and subsequent HR DNA repair in several cancer cells.[10,43,44] Interestingly, we noticed that although combined treatment with JQ1 and HDACIs robustly abolished the expression of endogenous RAD51, the expression of exogenous HA-RAD51 (under a CMV promoter) was essentially unaffected. RAD51 expression can be regulated via transcription, miRNAs including miR-96, −99, −107, −222 and −155, and protein stability,[45] further studies are warranted to disclose the detailed regulatory mechanism of JQ1 combined with HDACIs in regulating RAD51 expression in chondrosarcoma.

In summary, we have demonstrated that the combination of the BET inhibitor JQ1 and HDACIs leads to profound synergistic anti-cancer activity against chondrosarcoma cells by suppressing RAD51-related HR DNA repair (Figure 5F). These findings indicate that the combinatorial targeting of an epigenetic reader and an eraser may represent a promising novel strategy for treating chondrosarcoma.

Highlights

- BET inhibitors and HDACIs inhibit cell growth and induce apoptosis of chondrosarcoma cells in a synergistic manner
- Combination BET and HDAC inhibition elicits DNA damage in chondrosarcoma cells
- BET inhibition and HDAC inhibition synergize to impair RAD51-related HR repair signaling

Acknowledgments

This work was financially supported by the National Natural Science Foundation of China (81602360, 81672224, and 81871809), the National Natural Science Foundation of Guangdong Province (2017A030313665 and 2017A030313556), the China Postdoctoral Science Foundation (2016M602606 and 2017T100661), Major Science and Technology Planning Projects of Tianhe District (2018YZ001), the Science and Technology Planning Project of Guangzhou (201707010493), and the Medical Scientific Research Foundation of Guangdong Province (A2016502 and A2017485).

Author Contributions

All authors contributed to data analysis, drafting or revising the article, gave final approval of the version to be published, and agreed to be accountable for all aspects of the work.

Disclosure

All authors have declared no conflicts of interest.

References

1. Polychronidou G, Karavasilis V, Pollack SM, Huang PH, Lee A, Jones RL. Novel therapeutic approaches in chondrosarcoma. *Future Oncol*. 2017;13:637–648. doi:10.2217/fon-2016-0226
2. Italiano A, Mir O, Cioffi A, et al. Advanced chondrosarcomas: role of chemotherapy and survival. *Ann Oncol*. 2013;24(11):2916–2922. doi:10.1093/annonc/mdt374
3. Van Maldegem AM, Gelderblom H, Palmerini E, et al. Outcome of advanced, unresectable conventional central chondrosarcoma. *Cancer*. 2014;120(20):3159–3164. doi:10.1002/cncr.28845
4. de Jong Y, van Oosterwijk JG, Kruisselbrink AB, et al. Targeting survivin as a potential new treatment for chondrosarcoma of bone. *Oncogenesis*. 2016;5:e222. doi:10.1038/oncsis.2016.33
5. van Oosterwijk JG, Herpers B, Meijer D, et al. Restoration of chemosensitivity for doxorubicin and cisplatin in chondrosarcoma in vitro: BCL-2 family members cause chemoresistance. *Ann Oncol*. 2012;23(6):1617–1626. doi:10.1093/annonc/mdr512
6. Mery B, Espenel S, Guy JB, et al. Biological aspects of chondrosarcoma: leaps and hurdles. *Crit Rev Oncol Hematol*. 2018;126:32–36. doi:10.1016/j.critrevonc.2018.03.009
7. Andrieu G, Belkina AC, Denis GV. Clinical trials for BET inhibitors run ahead of the science. *Drug Discov Today Technol*. 2016;19:45–50. doi:10.1016/j.ddtec.2016.06.004
8. White ME, Fenger JM, Carson WE 3rd. Emerging roles of and therapeutic strategies targeting BRD4 in cancer. *Cell Immunol*. 2019;337:48–53.
9. Loven J, Hoke HA, Lin CY, et al. Selective inhibition of tumor oncogenes by disruption of super-enhancers. *Cell*. 2013;153(2):320–334. doi:10.1016/j.cell.2013.03.036
10. Miller AL, Fehling SC, Garcia PL, et al. The BET inhibitor JQ1 attenuates double-strand break repair and sensitizes models of pancreatic ductal adenocarcinoma to PARP inhibitors. *EBioMedicine*. 2019;44:419–430. doi:10.1016/j.ebiom.2019.05.035
11. Sabo A, Amati B. BRD4 and MYC-clarifying regulatory specificity. *Science*. 2018;360(6390):713–714. doi:10.1126/science.aat6664
12. Sun C, Yin J, Fang Y, et al. BRD4 inhibition is synthetic lethal with PARP inhibitors through the induction of homologous recombination deficiency. *Cancer Cell*. 2018;33(401–416):e8. doi:10.1016/j.ccell.2018.01.019
13. Zhang H-T, Zhang D, Zha Z-G, Hu C-D. Transcriptional activation of PRMT5 by NF-Y is required for cell growth and negatively regulated by the PKC/c-Fos signaling in prostate cancer cells. *Biochim Biophys Acta*. 2014;1839:1330–1340. doi:10.1016/j.bbagrm.2014.09.015
14. Dawson MA, Kouzarides T. Cancer epigenetics: from mechanism to therapy. *Cell*. 2012;150:12–27. doi:10.1016/j.cell.2012.06.013
15. Speetjens FM, de Jong Y, Gelderblom H, Bovee JV. Molecular oncogenesis of chondrosarcoma: impact for targeted treatment. *Curr Opin Oncol*. 2016;28:314–322. doi:10.1097/CCO.0000000000000300
16. Sakimura R, Tanaka K, Yamamoto S, et al. The effects of histone deacetylase inhibitors on the induction of differentiation in chondrosarcoma cells. *Clin Cancer Res*. 2007;13(1):275–282. doi:10.1158/1078-0432.CCR-06-1696
17. Tang F, Choy E, Tu C, Hornicek F, Duan Z. Therapeutic applications of histone deacetylase inhibitors in sarcoma. *Cancer Treat Rev*. 2017;59:33–45. doi:10.1016/j.ctrv.2017.06.006
18. Lee EQ, Reardon DA, Schiff D, et al. Phase II study of panobinostat in combination with bevacizumab for recurrent glioblastoma and anaplastic glioma. *Neuro Oncol*. 2015;17(6):862–867. doi:10.1093/neuonc/nou350
19. Luu TH, Morgan RJ, Leong L, et al. A phase II trial of vorinostat (suberoylanilide hydroxamic acid) in metastatic breast cancer: a California Cancer Consortium study. *Clin Cancer Res*. 2008;14(21):7138–7142. doi:10.1158/1078-0432.CCR-08-0122
20. Woyach JA, Kloos RT, Ringel MD, et al. Lack of therapeutic effect of the histone deacetylase inhibitor vorinostat in patients with metastatic radioiodine-refractory thyroid carcinoma. *J Clin Endocrinol Metab*. 2009;94:164–170. doi:10.1210/jc.2008-1631
21. Chou T-C. Drug combination studies and their synergy quantification using the Chou-Talalay method. *Cancer Res*. 2010;70(2):440–446. doi:10.1158/0008-5472.CAN-09-1947
22. Yang J, Li YH, He MT, et al. HSP90 regulates osteosarcoma cell apoptosis by targeting the p53/TCF-1-mediated transcriptional network. *J Cell Physiol*. 2020;235(4):3894–3904. doi:10.1002/jcp.29283
23. Gyori BM, Venkatachalam G, Thiagarajan P, Hsu D, Clement M-V. OpenComet: an automated tool for comet assay image analysis. *Redox Biol*. 2014;2:457–465. doi:10.1016/j.redox.2013.12.020
24. Enßle JC, Boedicker C, Wanior M, Vogler M, Knapp S, Fulda S. Co-targeting of BET proteins and HDACs as a novel approach to trigger apoptosis in rhabdomyosarcoma cells. *Cancer Lett*. 2018;428:160–172. doi:10.1016/j.canlet.2018.04.032
25. Holscher AS, Schulz WA, Pinkerneil M, Niegisch G, Hoffmann MJ. Combined inhibition of BET proteins and class I HDACs synergistically induces apoptosis in urothelial carcinoma cell lines. *Clin Epigenetics*. 2018;10:1. doi:10.1186/s13148-017-0434-3

26. Mio C, Gerratana L, Bolis M, et al. BET proteins regulate homologous recombination-mediated DNA repair: bRCAness and implications for cancer therapy. *Int J Cancer*. 2019;144:755–766. doi:10.1002/ijc.31898
27. Gachechiladze M, Škarda J, Soltermann A, Joerger M. RAD51 as a potential surrogate marker for DNA repair capacity in solid malignancies. *Int J Cancer*. 2017;141(7):1286–1294. doi:10.1002/ijc.30764
28. Tarsounas M, Davies AA, West SC. RAD51 localization and activation following DNA damage. *Philos Trans R Soc of Lond B Biol Sci*. 2004;359:87–93. doi:10.1098/rstb.2003.1368
29. Onishi AC, Hincker AM, Lee FY. Surmounting chemotherapy and radioresistance in chondrosarcoma: molecular mechanisms and therapeutic targets. *Sarcoma*. 2011;2011. doi:10.1155/2011/381564
30. Fowler T, Ghatak P, Price DH, et al. Regulation of MYC expression and differential JQ1 sensitivity in cancer cells. *PLoS One*. 2014;9(1):e87003. doi:10.1371/journal.pone.0087003
31. Zou Z, Huang B, Wu X, et al. Brd4 maintains constitutively active NF-kappaB in cancer cells by binding to acetylated RelA. *Oncogene*. 2014;33(18):2395–2404. doi:10.1038/onc.2013.179
32. Bolden JE, Shi W, Jankowski K, et al. HDAC inhibitors induce tumor-cell-selective pro-apoptotic transcriptional responses. *Cell Death Dis*. 2013;4:e519. doi:10.1038/cddis.2013.9
33. Wagner JM, Hackanson B, Lübbert M, Jung M. Histone deacetylase (HDAC) inhibitors in recent clinical trials for cancer therapy. *Clin Epigenetics*. 2010;1(3–4):117–136. doi:10.1007/s13148-010-0012-4
34. Thurn KT, Thomas S, Moore A, Munster PN. Rational therapeutic combinations with histone deacetylase inhibitors for the treatment of cancer. *Future Oncol*. 2011;7:263–283. doi:10.2217/fon.11.2
35. Meng W, Wang B, Mao W, et al. Enhanced efficacy of histone deacetylase inhibitor combined with bromodomain inhibitor in glioblastoma. *J Exp Clin Cancer Res*. 2018;37:241. doi:10.1186/s13046-018-0916-y
36. Shahbazi J, Liu PY, Atmadibrata B, et al. The bromodomain inhibitor JQ1 and the histone deacetylase inhibitor panobinostat synergistically reduce N-Myc expression and induce anticancer effects. *Clin Cancer Res*. 2016;22:2534–2544. doi:10.1158/1078-0432.CCR-15-1666
37. Fiskus W, Sharma S, Qi J, et al. Highly active combination of BRD4 antagonist and histone deacetylase inhibitor against human acute myelogenous leukemia cells. *Mol Cancer Ther*. 2014;13:1142–1154. doi:10.1158/1535-7163.MCT-13-0770
38 Ceccaldi R, Rondinelli B, D'Andrea AD. Repair pathway choices and consequences at the double-strand break. *Trends Cell Biol*. 2016;26:52–64. doi:10.1016/j.tcb.2015.07.009.
39. Puigvert JC, Sanjiv K, Helleday T. Targeting DNA repair, DNA metabolism and replication stress as anti-cancer strategies. *FEBS J*. 2016;283(2):232–245. doi:10.1111/febs.13574
40. Torgovnick A, Schumacher B. DNA repair mechanisms in cancer development and therapy. *Front Genet*. 2015;6:157. doi:10.3389/fgene.2015.00157
41. King HO, Brend T, Payne HL, et al. RAD51 is a selective DNA repair target to radiosensitize glioma stem cells. *Stem Cell Rep*. 2017;8(1):125–139. doi:10.1016/j.stemcr.2016.12.005
42. Birkelbach M, Ferraiolo N, Gheorghiu L, et al. Detection of impaired homologous recombination repair in NSCLC cells and tissues. *J Thorac Oncol*. 2013;8:279–286. doi:10.1097/JTO.0b013e31827ecf83
43. Adimoolam S, Sirisawad M, Chen J, Thiemann P, Ford JM, Buggy JJ. HDAC inhibitor PCI-24781 decreases RAD51 expression and inhibits homologous recombination. *Proc Natl Acad Sci U S A*. 2007;104(49):19482–19487. doi:10.1073/pnas.0707828104
44. Yin L, Liu Y, Peng Y, et al. PARP inhibitor veliparib and HDAC inhibitor SAHA synergistically co-target the UHRF1/BRCA1 DNA damage repair complex in prostate cancer cells. *J Exp Clin Cancer Res*. 2018;37:153. doi:10.1186/s13046-018-0810-7
45. Song L, McNeil EM, Ritchie AM, Astell KR, Gourley C, Melton DW. Melanoma cells replicate through chemotherapy by reducing levels of key homologous recombination protein RAD51 and increasing expression of translesion synthesis DNA polymerase zeta. *BMC Cancer*. 2017;17:864. doi:10.1186/s12885-017-3864-6
46. Simon HU, Haj-Yehia A, Levi-Schaffer F. Role of reactive oxygen species (ROS) in apoptosis induction. *Apoptosis*. 2000;5:415–418. doi:10.1023/A:1009616228304
47. Zhang HT, Gui T, Sang Y, et al. The BET bromodomain inhibitor JQ1 suppresses chondrosarcoma cell growth via regulation of YAP/p21/c-Myc signaling. *J Cell Biochem*. 2017;118:2182–2192. doi:10.1002/jcb.25863