Original Article

Epigenetic heterogeneity promotes acquired resistance to BET bromodomain inhibition in ovarian cancer


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Abstract: BET bromodomain inhibitors (BETi) are promising therapeutic regimens for epithelial ovarian cancer (EOC). However, early-stage clinical trials indicate that drug tolerance may limit their anti-tumor efficacy. Here, we show that JQ1-refractory EOC cells acquire reversible resistance to BET inhibition and remain dependent on BRD4 function. The insensitivity is driven by a unique non-genetic mechanism that involves clonal selection for a pre-existing cell subpopulation with ample acetylated histones and sufficient nuclear phase-separated BRD4 droplets to counteract BETi antagonism. A vertical combination approach by co-blocking BET proteins and downstream Aurora kinases proves to achieve more complete responses than single inhibitors. Collectively, our study implicates epigenetic heterogeneity in therapeutic resistance to chromatin-targeted agents and proposes a rational strategy to address this anticipated clinical dilemma.

Keywords: Ovarian cancer, BET inhibitor, drug resistance, epigenetic heterogeneity, vertical combination therapy

Introduction

A growing emphasis of the current drug discovery program has focused on modulating the chromatin-modifying factors, including histone acetyltransferases (HAT), histone deacetylases (HDAC) and the bromodomain and extra-terminal (BET) family members [1-6]. Notably, as epithelial ovarian cancer lacks conventional drug-gable genetic alterations [7], specific inhibition of epigenetic regulators is increasingly recognized as an attractive therapeutic strategy [8-12]. For example, we and other groups recently reported that in preclinical studies, small molecule inhibitors of BET bromodomain proteins exhibited robust efficacy against ovarian cancer via inducing cell cycle arrest and apoptosis [8, 13, 14]. The selectivity of BET-targeting compounds arose from disproportionate localization of BET proteins to super-enhancer elements that transcriptionally regulated key oncogenes such as FOXM1, ALDH1A1 and MYC [6, 8, 15, 16]. However, early clinical trials of BET inhibitors (BETi) showed disappointing results in ovarian cancer cases, necessitating further investigations to overcome the limitations of these epigenomic agents [17].

Lessons from myriad molecular targeted therapies indicate that drug resistance represents a major obstacle to successful cancer treatment. Substantial efforts have revealed diverse mechanisms underlying BETi tolerance in a spectrum of advanced malignancies [18-23]. In
Acute myeloid leukemia, adaptive WNT signaling serves as an alternative pathway to circumvent BET inhibition [24, 25]. Likewise, receptor tyrosine kinase reprogramming leads to BETi resistance in multiple solid tumors [26-30]. On the other hand, BETi-tolerant triple-negative breast cancer cells preserve dependency on BRD4 function but develop bromodomain-independent chromatin recruitment machinery [31, 32]. Therefore, acquired resistance to BET inhibitors can occur through either bypass tracks or target reactivation.

In this study, using JQ1-responsive ovarian cancer as a prototype, we identified an underappreciated modality of BETi resistance involving epigenetic heterogeneity and clonal evolution. Our data demonstrated that JQ1-resistant ovarian cancer cells emerged from the therapeutic selection of pre-existing subpopulations with desirable basal levels of acetylated histone H3 lysine 27 (H3K27ac), and remained addicted to excessive chromatin-bound BRD4. Simultaneous suppression of BRD4 and its downstream Aurora kinases provided a rational combination approach yielding vertical pathway inhibition and synergistic anti-tumor effects. Together, these findings proposed a novel paradigm for epigenetic drug evasion and unveiled a unique opportunity to optimize the clinical efficacy of numerous BET inhibitors under development.

Materials and methods

Cell culture and reagents

Cell lines COV 413B (RRID: CVCL_2423), OVCA420 (RRID: CVCL_3935) and SKOV3 (RRID: CVCL_0532) were obtained from American Type Culture Collection (ATCC) and were cultured in RPMI1640 (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen) and 1% penicillin and streptomycin (Gibco). JQ1 was purchased from Millipore. C646, AZD1152, VX680, and MLN8237 were purchased from MedChemExpress. 1,6-hexanediol was purchased from Sigma. For visualization, cells were fixed with formalin and stained with crystal violet.

Cell viability assays and combination matrices

Cell viability assay was performed using Cell Counting Kit-8 (Dojindo Laboratories). Cells were seeded in triplicates in 96-well plates and treated with indicated inhibitors for 96 h before measuring the absorbance at 450 nm according to the manufacturer’s instructions. For evaluating combination efficacy, cells were seeded in 96-well plates at 3000-5000 cells per well and treated with JQ1 and Aurora kinase inhibitors in 6×6 matrices. After 96 h, cell viability was determined using Cell Counting Kit-8. The Bliss synergy score was calculated by the equation (A+B) - A×B. A and B were the fractional growth inhibitions induced by agents A and B at a given dose.

Western blot

Cells were lysed in RIPA buffer (Tris pH 7.4 50 mM, NaCl 150 mM, NP-40 1%, SDS 0.1%, EDTA 2 μM) containing proteinase inhibitors (Roche) and phosphatase inhibitors (Roche), and subjected to SDS-PAGE and Western blot. Antibodies against the following proteins were used: BRD2, BRD3, BRD4 (Abcam); H3K27ac, FoxM1, AURKA, AURKB, H3, Actin, GAPDH (Cell Signaling Technology).

Quantitative PCR assays

Genomic DNA from tumor cells was extracted using QIAampDNA mini kit (Qiagen) according to the manufacturer’s protocol. The genomic DNA was used to perform the quantitative PCR on the Applied Biosystems VIIA7 machine. Three biological replicates were included for each condition. Data were normalized relative to MTHFR. The primer sequences used for PCR were as follows: MTHFR-F: CCATCTTCCTGCTGCTGTAACTG; MTHFR-R: GCCTTCTCTGCAACACTGTC; BRD4-exon1-F: GACCTCCAACCCTAACAA; BRD4-exon1-R: TTCCATAGTGCTTGAAGCA; BRD4-exon5-F: GAGATGTTTGCCAAGAAG; BRD4-exon5-R: TTGATGATGCACAGTAGT.

Immunofluorescent staining

Cells were plated in an 8-well culture dish for confocal laser scanning microscopy (Ibidi). For immunofluorescent staining, cells were washed with phosphate-buffered saline (PBS), fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100 and blocked with 4% BSA. The primary antibodies (1:200) were incubated overnight, followed by secondary antibodies (1:400) for 30 min and DAPI counterstaining for 5 min in the dark. Alexa Fluor 488 (Goat anti-
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Flow cytometry analysis

Cells were fixed and permeabilized using BD Cytofix/Cytoperm kit (BD Biosciences) according to the manufacturer's protocol. The primary antibodies (1:200) were incubated for 1 hour, followed by secondary antibodies (1:200) for 30 min in the dark. Flow cytometric analysis was performed on a FACS AriaII cytometer (BD Biosciences). Flow cytometry data were analyzed using FlowJo software.

Chromatin immunoprecipitation and sequencing

Chromatin precipitation was performed as previously described [33]. Cells were cross-linked with serum-free medium plus 1% formaldehyde for 10 min and quenched with 2.5 M glycine. Cell pellets were lysed and sonicated using Sonics Vibra-Cell 505 ultrasonicator (Sonics and Materials). 50 μl of sonicated DNA was taken as control, and the remaining DNA fragments were cleared and incubated overnight with magnetic beads coated with the H3K27ac antibody (Abcam). Precipitated complexes were rinsed and cross-links were reversed overnight. Samples were digested with RNase A and Proteinase K, and DNA was extracted with QIAquick PCR Purification Kit according to the manufacturer's instructions (Qiagen). DNA libraries were generated and sequenced on an Illumina Hiseq 2500 platform. All the downstream analyses were based on high-quality clean data. Index of the reference genome was built using Bowtie v2.2.3 and clean reads were aligned to the reference genome using TopHat v2.0.12. We used HTSeq v0.6.1 to count the reads mapped to each gene and further normalized read counts to the control ERCC reads. Differential expression analysis was performed using the DESeq R package (1.18.0). P-values were adjusted using the Benjamini-Hochberg procedure for controlling the false discovery rate. Genes with an adjusted P-value of < 0.05 were considered differentially expressed.

Plasmids and sgRNA

The BRD4 open reading frame was amplified from the genomic DNA of SKOV3 cells. Plasmids expressing GFP-tagged BRD4 or GFP were constructed using the Gibson Assembly Cloning Kit (New England Biolabs) according to the manufacturer’s protocol. Stable cell lines were established by infecting COV 413B, OVCA420, and SKOV3 with a lentiviral construct carrying pLVX-BRD4-GFP or pLVX-GFP. The CRISPR-Cas9 technology was employed to knock out indicated genes. Virally infected cells were selected with 2-5 μg/mL puromycin.

The primer sequences used for constructing plasmids were as follows:  **BRD4-F**: GCATGGA-GAGCTGTACAAGTCTAGAATGTCTGCGGAGAG-CGGCCC;  **BRD4-R**: GGAGGGAGAGGGGCGGGA-TCCTCAGAAAAAGATTTTCTTCAAATATTGACAAT;  **GFP-F**: TAGAGGATCTATTTCCGGTGATGGTGAGC-
AAGGGCGAG; GFP-R: CGTCAATGCTTGTGAATGTTTACTTTGACAGCCTCGTGCTCGAG.

The sgRNA sequences used for gene knockout were as follows: EGFP-sgRNA-F: CACCGGAGGCTGGACACCC; EGFP-sgRNA-R: AACTGCAAGCTGTGAGAC; BRD4-sgRNA-1F: CACCGGACACCATGTCGACTGACAG; BRD4-sgRNA-1R: AACAAGCGAAGCTGTGAGAC; BRD4-sgRNA-2F: CACCGGTCGTGCTGAGTCAG; BRD4-sgRNA-2R: AACTGCAAGCTGTGAGAC; BRD4-sgRNA-3F: GTGGCACTTCTGACAC; BRD4-sgRNA-3R: GAGTAGTGTCGACTGACAG.

Statistical analysis

Statistical analysis was performed with the R system version 3.6.0 or GraphPad Prism version 6.00. Cell counting and fluorescence intensity assessment were performed using Image-Pro Plus 6.0. In all experiments, comparisons between two groups were based on a two-sided Student's t-test. Pearson's correlation coefficient was used to measure the linear correlation between two variables. P-values of < 0.05 were considered statistically significant.

Results

JQ1-tolerant ovarian cancer cells exhibit reversible resistance to BET inhibition

To determine the molecular mechanisms of acquired resistance to BET inhibitors in ovarian cancer, we used multiple models to represent different genetic and epigenetic scenarios. Specifically, three ovarian cancer cell lines were selected, namely COV 413B, OVCA420 and SKOV3 which displayed diverse somatic profiles and super-enhancer landscapes as identified with chromatin immunoprecipitation followed by sequencing (Figure 1A; Supplementary Tables 1, 2, 3). We generated BETi-resistant cells by the long-term culture of these three models in escalating concentrations of JQ1 [3]. Consistent with our previous work [13], JQ1 treatment severely reduced parental cell viability (Figure 1B). In contrast, resistant clones maintained stable growth in the presence of 1 μM JQ1 and showed a greater than ten-fold increase in IC50 (Figure 1C). Cross-tolerance was observed between JQ1 and chemically distinct I-BET151 [6], indicating that both compounds might share common resistance mechanisms (Figure 1B and 1C). More importantly, the resistant phenotype was reversible upon culturing cells in JQ1-free media for over four weeks. Indeed, we found that all three JQ1-resistant cell lines gradually restored drug sensitivity (Figure 1D; Supplementary Figure 1A), and of interest, regained similar morphology of parental cells (Supplementary Figure 1B). Taken together, these findings suggested that JQ1-tolerant ovarian cancer cells exhibited reversible resistance to BET inhibition and the underlying mechanisms were most likely non-mutational.

JQ1-resistant tumor cells retain oncogene addiction to BRD4

To gain insights into potential molecular underpinnings of the reversible resistance to BET inhibition, we initially probed JQ1-induced differential gene expression by performing RNA sequencing (RNA-seq) of ovarian cancer models. To our surprise, the drastic response of gene transcripts to JQ1 exposure in parental cells was also observed in resistant lines (Figure 2A; Supplementary Tables 4, 5, 6, 7, 8, 9), indicating that BRD4 and its bromodomain remained functionally indispensable for global transcriptional programs. The majority of differentially expressed genes exhibited downregulation upon JQ1 treatment, with considerable overlap between sensitive and resistant clones. Nevertheless, the diversity of significantly altered genes was noticeably reduced in all three resistant cell lines relative to their respective parental cell lines (Figure 2B). These results suggested that albeit to a less extent, BRD4 might be still required in JQ1-resistant cells. Indeed, we observed a significant decrease of cell viability upon BRD4 knockout using the clustered regularly interspaced short palindromic repeats (CRISPR)-Cas9 system (Figure 2C, 2D). Consistently, BRD4 depletion...
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Figure 1. JQ1-tolerant ovarian cancer cells exhibit reversible resistance to BET inhibition. A. Enhancers in COV 413B, OVCA420, and SKOV3 cell lines ranked by increasing normalized H3K27ac signal (length \times density). Dashed gray lines marked cutoffs distinguishing typical from super-enhancers. These three cell lines harbored different somatic mutations as labeled on the top left of each graph. B. JQ1-sensitive and JQ1-resistant cells (COV 413B, OVCA420, and SKOV3) were treated with DMSO, JQ1 (500 nM), or I-BET151 (500 nM) for 10 days. The remaining cells were stained with crystal violet. C. Cell viability of indicated cells treated with various concentrations of JQ1 or I-BET151 for 5 days. Data were presented as mean ± standard deviation of four biological replicates. D. Cell viability of indicated cells treated with various concentrations of JQ1 for 5 days. Data were presented as mean ± standard deviation of four biological replicates.
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Figure 2. JQ1-resistant cells retain oncogene addiction to BRD4. A. Heatmaps of global gene expression values in COV 431B, OVCA420, and SKOV3 cells that were treated with JQ1 (500 nM for 6 hours) versus DMSO control. B. Venn diagram showing the overlap of JQ1-induced differentially expressed transcripts between sensitive and resistant cells. C. Immunoblotting of BRD4 in BRD4-depleted cells using the CRISPR-Cas9 system. D. Relative cell viability of BRD4 knockout cells as compared to control cells (*P < 0.05, unpaired Student’s t test). E. Immunoblotting of BRD4 in cells treated with various concentrations of ARV825. F. Cell viability of indicated cells treated with ARV825 for 5 days. Data were presented as mean ± standard deviation of four biological replicates. G. Indicated cells were treated with ARV825 for 5 days and the remaining cells were stained with crystal violet.

with ARV825 (Figure 2E), a BET protein proteolysis-targeting chimera (PROTAC) [34], effectively inhibited resistant cell growth (Figure 2F) in a dose-dependent manner (Figure 2G). Taken together, JQ1-resistant tumor cells retained oncogene addiction to BRD4.

JQ1-resistant ovarian cancer cells display excessive chromatin-bound BRD4

The coexisting JQ1 resistance and BRD4 addiction in ovarian cancer seemed counterintuitive and prompted us to hypothesize that BRD4 might be upregulated in resistant cells to counteract the effects of BET inhibitors. As expected, we found that BRD4 levels (Figure 3A), most obviously the chromatin-associated protein (Figure 3B), were consistently elevated in all three resistant cell lines regardless of JQ1 treatment. Immunofluorescence assay also demonstrated a relative enrichment of BRD4 signals within the nuclei of resistant cells (Figure 3C), whereas in sensitive cells, a substantial proportion of BRD4 molecules resided in the cytoplasm (Figure 3D). In line with these results, restoration of drug sensitivity upon taking off JQ1 was accompanied by decreased chromatin-bound BRD4 protein (Figure 3E). More importantly, reminiscent of recent data showing that BET proteins and other coactivators formed condensates at super-enhancers via liquid-liquid phase separation [35], BRD4 was visualized as discrete puncta within the nuclei of ovarian cancer cells (Figure 3F), and brighter and larger spots accumulated in resistant cells relative to sensitive controls (Figure 3G), indicating that JQ1-tolerant cells were likely dependent on more condensed phase-separated BRD4 to overcome drug inhibition. To support this notion, we treated JQ1-resistant cells with 1,6-hexanediol, a compound known to diminish liquid-like clusters [35]. Following the disruption of BRD4 puncta by pre-exposure to 1,6-hexanediol, JQ1 sensitivity was restored in drug-tolerant cells (Supplementary Figure 2A, 2B). To validate the role of BRD4, we tested whether exogenous BRD4 expression was sufficient to induce JQ1 resistance using a GFP-tagging assay. Specifically, ovarian cancer cells overexpressing BRD4-GFP chimera or GFP alone (Supplementary Figure 2C, 2D) were mixed with parental cells at a starting fraction of approximately 10%. We observed that the proportion of GFP-positive cells remained roughly stable in DMSO (Figure 3H). However, upon JQ1 treatment, the percentage of BRD4-GFP, but not GFP, cell population increased significantly (Figure 3I). Hence, we concluded that high amount of chromatin-bound BRD4 formed phase-separated clusters and conferred BETi resistance in ovarian cancer.

Epigenetic heterogeneity underlies drug resistance to BET inhibition

Since BRD4 gene copy number (Supplementary Figure 3A) and mRNA level (Supplementary Figure 3B) were not necessarily elevated, we sought to explore the alternative mechanisms underlying increased BRD4 accumulation and recruitment to chromatin in the nuclei of JQ1-resistant cells. BET family proteins are epigenetic readers that can bind to distal enhancers via acetylated histone H3 lysine 27 (H3K27ac) [16, 36]. Intriguingly, resistant cells exhibited a consistent increase in nuclear H3K27ac abundance along with BRD4 protein enrichment (Figure 4A). Flow cytometry (FACS) analysis confirmed this finding and showed that higher expression of H3K27ac and BRD4 were detected in all three resistant models (Figure 4B; Supplementary Figure 4). We performed immunofluorescence staining of ovarian cancer cells (Figure 4C), and found that H3K27ac and BRD4 fluorescent signals (normalized to DAPI) significantly correlated with each other at the single-cell level (Figure 4D). Notably, ovarian tumor cells displayed considerable diversity of these two proteins even at baseline, and their relative distribution in dot plots shifted upwards in resistant cells (Figure 4E). Hence, pre-existing epigenetic heterogeneity might
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Figure 3. JQ1-resistant ovarian cancer cells display excessive chromatin-bound BRD4. A. Western blot analysis of total BRD4, BRD2 and BRD3 in sensitive and resistant cells treated with DMSO or JQ1 (1 μM). B. Western blot analysis of chromatin-bound BRD4, BRD2 and BRD3 in sensitive and resistant cells treated with DMSO or JQ1 (1 μM). C. Immunofluorescence imaging (40×) of BRD4 in sensitive and resistant cells. The fluorescence signal of BRD4 was shown alone or merged with fibrous actin and DAPI staining. Scale bar = 20 μm. D. Bar charts with dot plots represented the ratio of nuclear BRD4 to total BRD4 calculated by fluorescence intensity in 50 sensitive and resistant cells. E. Western blot analysis showed the reduced levels of chromatin-bound BRD4 upon JQ1 withdrawal. F. Representative images (63×) of BRD4 puncta in JQ1-sensitive, JQ1-resistant, and JQ1-withdrawn ovarian cancer cells. Scale bar = 10 μm. G. Violin plots represented the quantification of BRD4 puncta in JQ1-sensitive, JQ1-resistant, and JQ1-withdrawn cells (~20 cells per group). H. Representative images (20×) were shown at 7 days after initial cell seeding of approximately 10% BRD4-GFP- or GFP-expressing tumor cells with DMSO or JQ1 treatment. Nuclear DNA was labeled with TO-PRO-3. Scale bar = 25 μm. I. Bar graphs showing the percentage of GFP-positive cells (3 images per group, *P < 0.05, unpaired Student’s t test).

lead to differential JQ1 responses and positive selection for functionally robust subclones under the therapeutic pressure. To corroborate this point, we used histone acetyltransferase inhibitor C646 to eliminate H3K27ac heterogeneity. Following C646 treatment, we observed an obvious reduction of H3K27ac and the disappearance of BRD4 puncta (Figure 4F; Supplementary Figure 5A). When we washed out the compounds, BRD4 phase separation was largely recovered, reinforcing the specific effects of this epigenetic drug (Figure 4F). More importantly, H3K27ac disequilibrium upon inhibitor administration substantially narrowed down BRD4 distribution (Supplementary Figure 5B) and consequently sensitized ovarian cancer cells to JQ1 treatment (Figure 4G).

Consistently, we observed that knocking out EP300 and CREBBP using the CRISPR-Cas9 system (Figure 4H) could disperse phase-separated puncta and render ovarian cancer cells more sensitive to JQ1 (Figure 4I; Supplementary Figure 5G). These data highlighted the intrinsic heterogeneity of epigenetic machinery in mediating resistance to chromatin-targeted therapies.

Combined BRD4 and Aurora kinases inhibition eradicates JQ1-resistant ovarian cancer cells

Previous work by our laboratory identified Forkhead box protein M1 (FoxM1) as the functional target of BET inhibitors in ovarian cancer [13]. Based on the aforementioned nongenetic mechanism of acquired resistance, we reasoned that FoxM2 might display distinct kinetics during JQ1 exposure. Indeed, the immunoblots revealed that all three resistant lines exhibited higher expression levels of FoxM1 and its key transcriptional targets Aurora kinases at baseline and upon treatment (Figure 5A). Remarkably, the combination of JQ1 and MLN-8237 [37, 38], the most clinically advanced Aurora kinase inhibitor, demonstrated a synergistic reduction in resistant cell growth (Figure 5B). The synergy was confirmed by crystal violet staining and Bliss independence assay using two other Aurora kinase agents, AZD1152 and VX680 (Figure 5C; Supplementary Figure 6). Therefore, combined BRD4 and Aurora kinases inhibitors acted synergistically in eradicating JQ1-resistant ovarian cancer cells.

Discussion

Recent investigations have unequivocally revealed transcriptional addiction of ovarian cancer [33, 39, 40], and nominated BET inhibitors as new transcription-targeted therapeutic candidates. To realize their clinical promise, the molecular determinants of drug sensitivity need to be rigorously elucidated. Here, we documented that BETi resistant clones might emerge from pre-existing cell subsets that transiently acquired increased H3K27ac and chromatin-bound BRD4. As a result, vertical inhibition of BRD4 and its downstream Aurora kinases could deliver synergistic benefit, enabling effective treatment of ovarian cancer (Figure 5D). These findings have important implications for interpreting epigenetic heterogeneity and clonal selection as an unprecedented etiology of BETi resistance and providing rational combination approaches to improve therapeutic efficacy in future studies.

Triggered by early promising clinical trials of multiple BET inhibitors, recent work has started to evaluate the possible mechanisms of anticipated drug resistance using preclinical models. Different trajectories of acquired BETi resistance have been proposed in various cancer types [22, 32, 41-45]. For example, it has been demonstrated that WNT pathway activa-
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Figure 4. Epigenetic heterogeneity underlies drug resistance to BET inhibition. A. Western blot analysis showed the increased levels of H3K27ac and chromatin-bound BRD4 in JQ1-resistant cells, and the reduced levels of H3K27ac and chromatin-bound BRD4 upon JQ1 withdrawal. B. Sensitive cells and resistant cells were labeled with BRD4 and H3K27ac antibodies and analyzed by flow cytometry (FACS) analysis. FACS showed a correlation between H3K27ac and BRD4 signals and an enrichment of resistant cells with higher expression of H3K27ac and BRD4. C. Immunofluorescence imaging (40×) of H3K27ac and BRD4 in sensitive and resistant cells. Scale bar = 20 μm. D. Fluorescent signals of H3K27ac and BRD4 (50 cells per group) were quantified and normalized to DAPI. The data were presented in scatter plots and the corresponding regression lines reflected the positive associations between H3K27ac and BRD4. R indicated the Pearson’s correlation coefficient. E. Fluorescent signals of H3K27ac and BRD4 were quantified and presented in dot charts ranked by increasing levels of H3K27ac or BRD4. F. Immunofluorescence imaging (63×) of H3K27ac and BRD4 in sensitive and resistant cells upon treatment of histone acetyltransferase inhibitor C646 (15 μM). Scale bar = 20 μm. G. Cells were treated with JQ1 (0.5 μM) and C646 (15 μM) as indicated. The remaining cells were stained with crystal violet. H. EP300 and CREBBP were depleted using the CRISPR-Cas9 system. Western blot analysis showed knockout efficiency and reduced H3K27ac. I. Cell viability of indicated cells treated with various concentrations of JQ1 for 5 days. Data were presented as mean ± standard deviation of four biological replicates.

...condensates. We further uncovered that nuclear BRD4 enrichment correlated with elevated H3K27ac marks which were also reversible and attributable to stochastic cellular levels. Hence, we proposed a new pattern for BETi resistance that dynamic regulation of epigenetic heterogeneity provided a reservoir of tumor cells surviving potentially lethal treatment. This model was supported by a serial of evidence. First, H3K27ac along with nuclear BRD4 varied across individual cells at baseline. Second, the diversity distribution of these two proteins allowed for a therapeutic selection of fitness-enhanced cells, leading to the establishment of a new homeostatic state. Third, reducing the variation in basal H3K27ac expression by histone acetyltransferase inhibitors or gene editing could dissipate phase-separated BRD4 droplets and preclude the emergence of the resistant phenotype. Taken together, our findings went beyond the well-defined role of resistance-conferring genetic heterogeneity and implicated an analogous mechanism of non-mutational epigenetic heterogeneity in acquired drug tolerance to chromatin-targeted therapeutics [49-51].

In light of the persistent BRD4 dependence of JQ1-tolerant cells, this research offered mechanism-based combination strategies to overcome BETi resistance in ovarian cancer. Specifically, we identified that pharmacological perturbation of key downstream targets via Aurora kinase inhibitors, which have reached phase 3 trials [52-54], sensitized resistant cells to BRD4-targeted treatment. Although early clinical experience suggests that single BETi agents can be given safely [17], toxicity liabilities must be carefully considered when they are concurrently administered with other therapies. The proposed approach here substantiates the recently described concept of vertical pathway
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MLN8237
AZD1152
VX680
MLN8237
AZD1152
VX680
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Figure 5. Combined BRD4 and Aurora kinases inhibition eradicates JQ1-resistant ovarian cancer cells. A. Western blot analysis of FoxM1, AURKA and AURKB in sensitive and resistant cells treated with JQ1 (1 μM). B. Cells were treated with JQ1 (1 μM) and Aurora kinase inhibitor MLN8237 (30 nM) as indicated. The remaining cells were stained with crystal violet. C. Heatmaps of bliss synergy scores demonstrated synergistic activities of JQ1 and Aurora kinase inhibitors in JQ1-sensitive and JQ1-resistant cells. A higher score represented a more potent synergistic efficacy. D. A schematic summary of the study, showing that BETi resistance might emerge from epigenetic heterogeneity and vertical inhibition of BRD4 and its downstream Aurora kinases could overcome drug tolerance in ovarian cancer.
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inhibition, which might elicit powerful anti-tumor synergy even at suboptimal doses with minimal side effects [55, 56]. Furthermore, in addition to reactively treating resistant disease, we envision that preventive upfront polytherapy might be more effective to forestall the onset of drug tolerance in patients. Our results call for delicately designed clinical studies testing the combination of BET protein and Aurora kinase inhibitors in advanced ovarian cancer and potentially other human malignancies.

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Disclosure of conflict of interest

None.

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**Supplementary Figure 1.** A. JQ1-resistant, and JQ1-withdrawn cells (COV 413B, OVCA420 and SKOV3) were treated with DMSO or JQ1 for 10 days. The remaining cells were stained with crystal violet. B. Phase-contrast imaging of JQ1-resistant, JQ1-resistant, and JQ1-withdrawn ovarian cancer cells.
Supplementary Figure 2. A. Representative images (63×) of JQ1-resistant ovarian cell lines before and after treatment with 1% hexanediol for 15 min. Scale bar = 10 μm. B. Resistant cells were pre-treated with 1,6-hex (1%) for 15 min followed by JQ1 (1 μm) treatment for 5 days. The remaining cells were stained with crystal violet. C. Western blot analysis of GFP- and BRD4-GFP-expressing COV 413B, OVCA420 and SKOV3 cells. D. GFP-expressing cells showed fluorescent signals in both cytoplasm and nuclei. BRD4-GFP-expressing cells showed fluorescent signals only in the nuclei, which formed phase-separated puncta. Scale bar = 10 μm.
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Supplementary Figure 3. A. Quantitative real-time PCR on genomic DNA extracted from sensitive and resistant cells. B. The comparisons of BRD4 mRNA expression between sensitive and resistant cells.

Supplementary Figure 4. H3K27ac and BRD4 expression histograms obtained by flow cytometry. The resistant models showed a cell population with high levels of H3K27ac and BRD4.
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Supplementary Figure 5. A. Western blot analysis of EP300, CREBBP and H3K27ac in sensitive and resistant cells upon C646 treatment. B. Fluorescent signals of H3K27ac and BRD4 were quantified and presented in dot charts ranked by increasing levels of H3K27ac or BRD4. C. Immunofluorescence images (63×) of BRD4 and H3K27ac in EP300/CREBBP-knockout cells and control cells. Scale bar = 10 μm.
Supplementary Figure 6. Cells were treated with JQ1 (1 μM) and Aurora kinase inhibitors AZD1152 or VX680 (100 nM) as indicated. The remaining cells were stained with crystal violet.