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An optimized BRD4 inhibitor effectively eliminates NF-κB-driven triple-negative breast cancer cells

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ABSTRACT

Acetylation of NF-κB's RelA subunit at lysine-310 (AcLys310) helps to maintain constitutive NF-κB activity in cancers such as triple-negative breast cancer (TNBC). Bromodomain-containing factor BRD4 binds to acetylated RelA to promote the activity of NF-κB. Hence, interfering with the acetylated RelA-BRD4 interaction is a potential strategy for treating NF-κB-driven TNBC. Here, a new compound **13a** was obtained by structural optimization and modification of our previously reported compound. In comparison with the well-known BRD4 inhibitor (+)-JQ1, **13a** showed more potent anticancer activity in NF-κB-active MDA-MB-231 cells. Mechanistically, **13a** antagonized the protein–protein interaction (PPI) between BRD4 and acetylated RelA, decreased levels of IL-6, IL-8, Snail, Vimentin, and ZEB1, induced cell senescence and DNA damage, and weakened the adhesion, metastasis, and invasion ability of TNBC cells. Our results provide insights into avenues for the further development of potent BRD4-acetylated RelA PPI inhibitors. Moreover, our findings highlight the effectiveness and feasibility of blocking the interaction between BRD4 and acetylated RelA against NF-κB-active cancers, and of screening antagonists of this PPI.

1. Introduction

Triple-negative breast cancer (TNBC) is a subtype of heterogeneous and clinically aggressive breast cancer without targeted agents [1], while metastasis is a leading factor of morbidity and mortality in TNBC patients. Current chemotherapeutic strategies for TNBC treatment are limited by drug resistance and serious side effects such as organ damage, which increases the suffering of TNBC patients. Therefore, there is an urgent necessity to advance new remedies to treat this type of cancer.

Bromodomain and extra-terminal (BET) proteins are a family of epigenetic "readers" that regulate gene activity via their ability to recognize acetylated lysine motifs in histones as well as non-histone substrates, including transcription factors. Bromodomain-containing factor BRD4, a BET family member, has a key function in controlling the gene transcription and the cell cycle, and is also linked with tumorigenesis. BRD4 contains BD1, BD2 bromodomains, and an extra-terminal domain. BRD4 recognizes acetylated lysine motifs in histones or transcription factors using its BD1 and BD2 domains [2].

Nuclear factor-κB (NF-κB) plays an important role in the initiation, development, metastasis and resistance of human cancer. Because of the inflammatory microenvironment and various oncogenic mutations, many human cancers exhibit constitutive NF-κB activity, which promotes tumor cell proliferation, increases angiogenesis, inhibits apoptosis, and also induces the epithelial-mesenchymal transition (EMT) and distant metastasis [3]. Acetylation of NF-κB at lysine-310 (AcLys310) promotes NF-κB target gene expression [4–9] and helps to maintain constitutive NF-κB activity in cancers [3,10,11]. BRD4 binds to AcLys310 and functions as a coactivator regulating the transcriptional activity of NF-κB [3]. Thus, targeting BRD4 may embody a new strategy for treating cancers with constitutively active NF-κB [12–16].

(+)-JQ1, the first and most extensively used BET inhibitor, has been documented to inhibit the proliferation and transformation potential of A549 lung cancer cells and suppress the tumorigenicity of A549 cells in severe combined immunodeficiency mice [3], (+)-JQ1 also was found to inhibit constitutively active NF-κB in cancer cells and to relieve viral-induced airway inflammation via disturbing the BRD4-acetylated NF-

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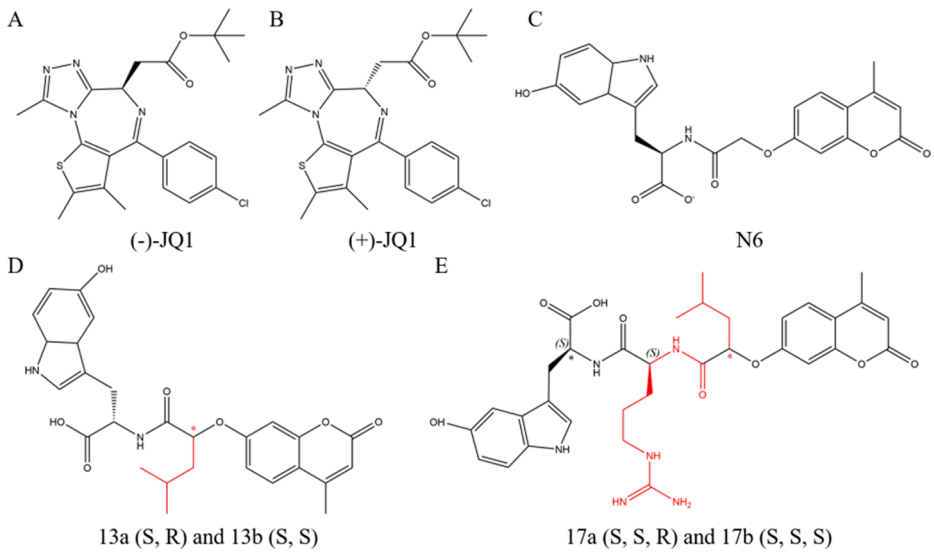
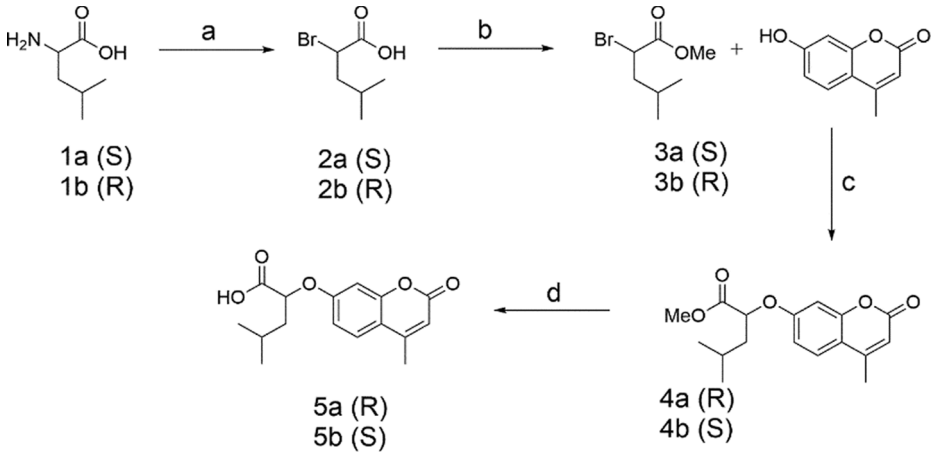


Fig. 1. Chemical structures for screening BRD4-acetylated RelA PPI inhibitors in this paper. The red parts are modified groups compared to compound **N6**.



Scheme 1. Synthesis of 4-methyl-2-(4-methyl-2-oxo-2*H*-chromen-7-yloxy) pentanoic acid **5(a & b)**. Reagents and conditions: (a) $NaNO_2$/HBr (48%), 0 °C to RT, Yield = 98% (**2a)** & 95% (**2b)**; (b) Con. H_2SO_4/MeOH, Yield = 90% (**3a)** & 92% (**3b)**; (c) Cs_2CO_3/CH_3CN, Yield = 78% (**4a)** & 75% (**4b)**; d) LiOH, THF/H_2O (1:1), 2 h, 0 °C to RT, Yield = 93% (**5a)** & 90% (**5b)**.

κB PPI mediated signaling [3]. CPI-061, a BET antagonist with preference for the first bromodomain, has advanced into Phase II human clinical trials for myelofibrosis [17]. RVX-208, a BET antagonist selective for the second bromodomain, has entered into Phase III human clinical trials for cardiovascular diseases [18,19].

Our group have previously identified two single-domain BRD4 inhibitors with good anti-melanoma activity [20,21]. However, while a number of BRD4 inhibitors are under preclinical or clinical trials [22,23], no BRD4 inhibitor has yet been approved in clinic. Most reported BRD4 inhibitors are pan-BET inhibitors that lack selectivity [22,24]. Thus, there is an urgent need to develop a more novelty, effective and selective BRD4 inhibitor.

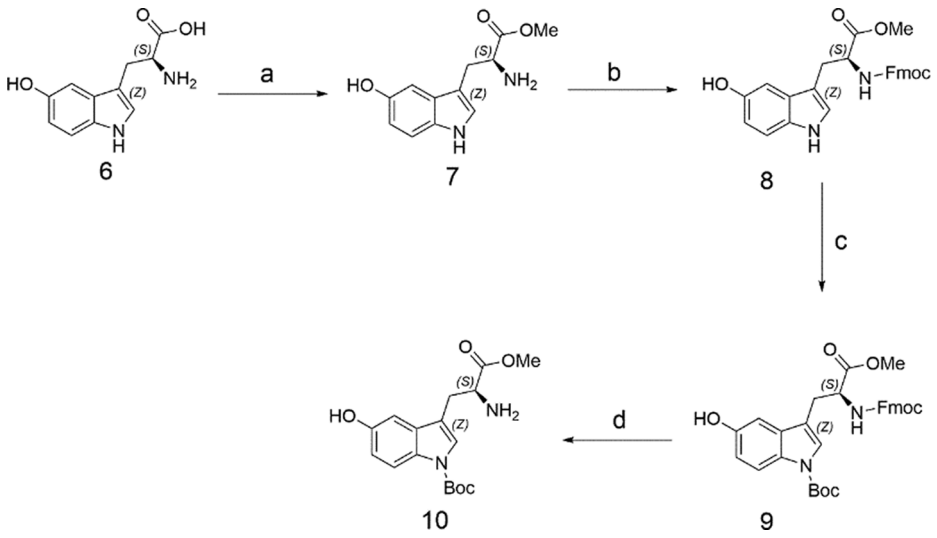
2. Results and discussion

(+)-JQ1 (Fig. 1A–B) exhibits excellent *in vitro* and *in cellulo* activity against BET proteins [3]. Based on the X-ray crystal structure of BRD4 BD1 with (+)-JQ1 (PDB: 3MXF), our previous study identified (R)-3-(5-hydroxy-1H-indol-3-yl)-2-(2-((4-methyl-2-oxo-2H-chromen-7-yl)oxy) acetamidopropanoic acid (**N6**) as a BRD4 inhibitor (Fig. 1C) by virtual screening and hit validation [21]. This compound exhibited potent inhibitory activity against BD1 and BD2 domain of BRD4 with an IC_{50} value of 0.062 μM and 0.081 μM, respectively. Mechanistically, compound **13a** (Fig. 1D) blocked the interaction between BRD4 and RelA, leading to the deactivation of NF-κB and reducing the expression of NF-κB target genes. Compound **13a** is an attractive scaffold for the further development of anticancer agents that can suppress metastasis and induce senescence in NF-κB-driven cancer cells.

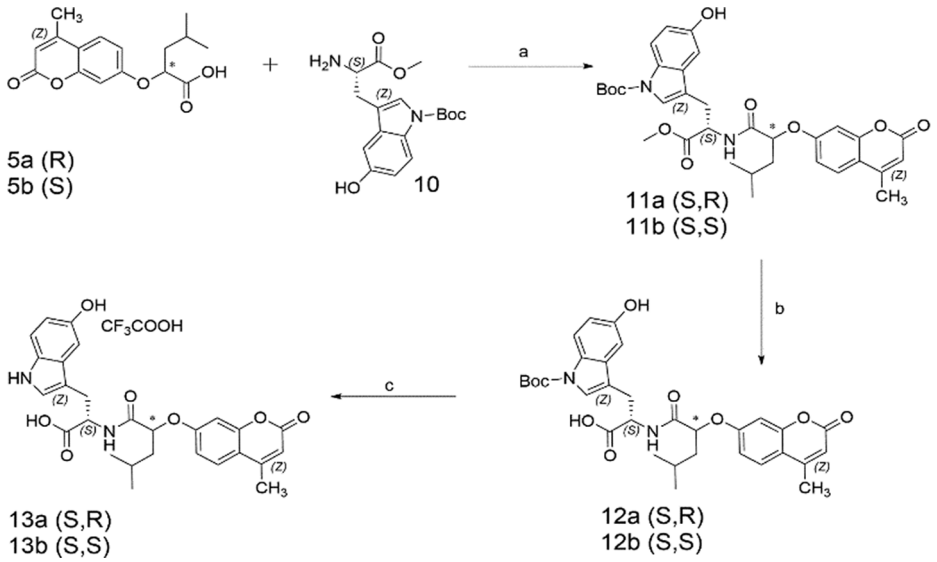
The key reaction intermediates 4-methyl-2-(4-methyl-2-oxo-2H-chromen-7- yloxy) pentanoic acid (**5**) and (*S*)-*tert*-butyl 3-(2-amino-3-methoxy-3-oxopropyl)-5-hydroxy-1H-indole-1-carboxylate (**10**) were synthesized in four steps from enantiopure isoleucine (**1a** and **1b**) (Scheme 1) and (*S*)-tryptophan 6, respectively (Scheme 2).

Compounds **5** were then reacted separately with compound **10** to generate the diastereomers **13** (Scheme 3). Alternatively, compound **10** could be reacted first with enantiopure **14** to introduce a guanidine group, before subsequent reaction with **5** to generate diastereomers **17**, respectively (Scheme 4). All the synthetic reaction intermediates and targeted compounds were characterized using ^{1}H NMR, ^{13}C NMR, high-resolution mass spectrometry (HRMS), and elemental analysis (Fig. S1–S8).

Enantiomers of chiral drugs can exhibit marked differences in biological activities such as pharmacokinetics, pharmacology, metabolism, and toxicology [25]. The enantiomers of JQ1 also exhibited different



Scheme 2. Synthesis of (S)-*tert*-butyl 3-(2-amino-3-methoxy-3-oxopropyl)-5-hydroxy-1*H*-indole-1-carboxylate **10**. Reagents and conditions: (a) $SOCl_2$ /MeOH, 0 °C to RT, Yield = 98%; (b) Fmoc-Cl/10% solution of Na_2CO_3, 1,4-dioxane, Yield = 95%; (c) Boc_2O, DMAP (cat)/CH_3CN, Yield = 75%; (d) DBU (1 eq), DCM, RT, Yield = quantitative.



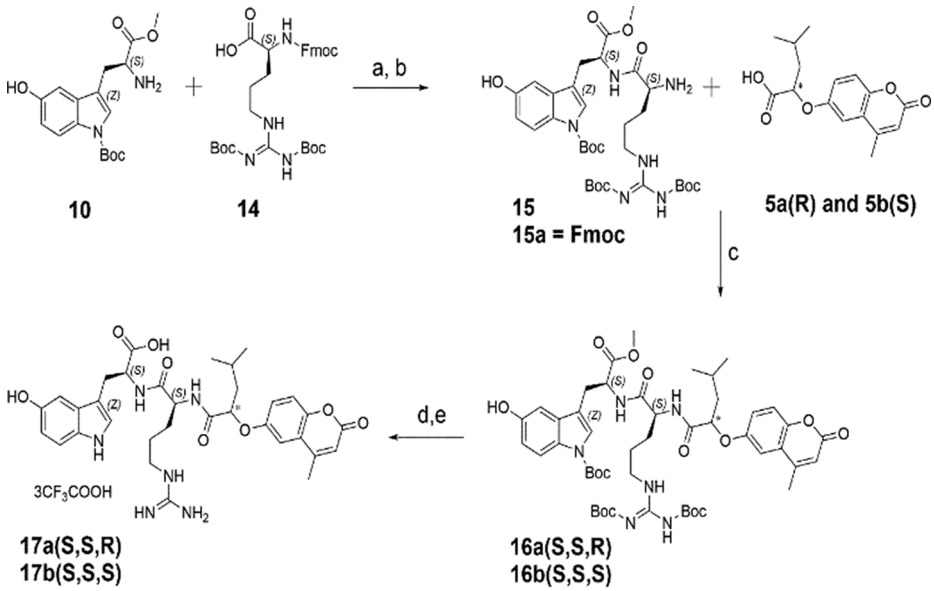
Scheme 3. Synthesis of compound **13 (a & b)**. Reagents and conditions: (a) EDCI/HOBt /DCM, 0 °C to RT, Yield = 78% (**11a**) & 80% (**11b**); (b) LiOH, THF/H_2O (1:1), 2 h, 0 °C to RT, Yield = 94% (**12a**) & 97% (**12b**); (c) TFA, DCM Yield = 93% (**13a**) & 95% (**13b**).

activity as BET inhibitors [3,26,27]. Hence, we designed two pairs of enantiomers based on the structure of **N6**, a BRD4 inhibitor identified in our previous work [21]. Compared with **N6**, compounds **13a/13b** contains an additional isobutyl group to increase hydrophobicity, which could potentially improve engagement of hydrophobic pockets in BRD4. Meanwhile, an arginine residue was added in diastereomers **17a/17b** (Fig. 1D and E) to increase the number of hydrogen bonds that could potentially be formed with the target.

The inhibitory activity of the compounds against BRD4 *in vitro* was assessed by a TR-FRET assay that measures the binding of BRD4 to its acetylated substrates. The results showed that all the derivatives generally exhibited better inhibitory activity against BRD4 than **N6** (Table 1). Compound **13a** was the top dual-domain BRD4, with IC_{50} values of 0.023 μM and 0.016 μM for BRD4 BD1 and BD2, respectively. Interestingly, just as with JQ1, the diastereomers of each pair also exhibited different activity to each other.

Then, the *in cellulo* activity of five hit compounds (including **N6**) was further investigated in a human TNBC cell line (MDA-MB-231). After 24 h treatment, the levels of downstream genes of the BRD4-acetylated RelA PPI were detected using real-time quantitative PCR (Fig. 2A–F), enzyme-linked immunosorbent assay (Fig. 2G, H), and Western blotting (Fig. 2I). Compound **13a** exhibited the highest BRD4 inhibitory activity at a concentration of 3 μM, as indicated by decreased transcriptional and translational levels of downstream genes (IL-6, IL-8, Snail, Vimentin, and ZEB1) of BRD4-NF-κB signaling, with little effect on c-Myc, a gene regulated by the interaction BRD4 and its histone substrates.

The cytotoxicity of five candidate BRD4 inhibitors against four breast cancer cell lines (MDA-MB-231, MCF-10A, T47D, and MCF-7) and a human normal liver cell line (LO2) was also tested (Table 1). **13a** exhibited the greatest cytotoxicity against MDA-MB-231 cells (IC_{50} = 0.36 μM), which constitutively express NF-κB. For the other cell lines which do not have constitutively active NF-κB, IC_{50} values were increased by at least 22.5-fold (Table 1). Moreover, as shown using a luciferase reporter assay, **13a** significantly reduced NF-κB-driven activity in a dose-dependent fashion in MDA-MB-231 cells and was significantly more potent than (+)-JQ1 at the same concentration



Scheme 4. Synthesis of compound **17** (**a** and **b**). Reagents and conditions: (a) EDCI/HOBt /DCM, 0 °C to RT, Yield = 68% (**15** Fmoc protected); (b) DBU (1 eq), DCM, RT, Yield = quantitative for (**15** amine); (c) EDCI/HOBt /DCM, 0 °C to RT, Yield = 75% (**16a**) & 80% (**16b**); (d) LiOH, THF/H_2O (1:1), 2 h, 0 °C to RT, Yield = 95% (**16a**) & 93% (**16b**); (e) TFA, DCM Yield = 70% (**17a**) & 73% (**13a**).

Table 1
The 50% inhibiting concentration (IC_{50}, μM) of **N6** and its derivatives in *in vitro* and *in cellulo*. The inhibitory activity of our compounds on the binding ability of the two BDs of BRD4 with their acetylated substrates were detected by TR-FRET assay *in vitro*, while the cytotoxicity of these five compounds were analyzed by MTT assay.

Compounds (μM)	BRD4 BD1	BRD4 BD2	MDA-MB-231	MCF-7	MCF-10A	T47D	LO2
N6	0.062	0.081	4.68	1.21	>10	>10	>10
13a	0.023	0.016	0.36	8.16	>10	>10	>10
13b	0.054	0.013	8.73	>10	>10	>10	>10
17a	0.073	0.049	6.15	>10	>10	>10	>10
17b	0.032	0.014	9.26	>10	>10	>10	>10

(Fig. S1). The cytokines IL-8 and IL-6 directly regulated by NF-κB signaling induce a self- and cross-reinforcing inflammatory environment which promotes tumorigenesis in breast cancer cells [28]. Interestingly, **13a** not only lead to cytotoxicity but also impaired the release of IL-8 and IL-6 (Fig. 2G and 2H), which suggested that **13a** may, at least in part, mediate the cytotoxicity via BRD4-NF-κB signaling.

To further investigate if BRD4 is directly targeted by **13a** in MDA-MB-231 cells, the cellular thermal shift assay was performed (Fig. 3A). **13a** stabilized BRD4 in cell lysates (ΔTm: 5.7 °C) relative to DMSO-treated controls (Fig. 3B), while it had little effect on the thermal stability of acetylated RelA (Fig. 3C). This suggested that **13a** directly and selectively engaged BRD4 (rather than its partner acetylated RelA) *in vitro* and *in cellulo* to inhibit NF-κB signaling. BRD4 binds to acetylated lysine-310 of RelA to function as a coactivator of NF-κB signaling, and this helps to maintain constitutive NF-κB activity in cancers [3–11]. The mode of action of **13a** was studied using co-immunoprecipitation experiments. The results revealed that **13a** blocked the interaction between BRD4 and acetylated RelA without affecting BRD4 and acetylated RelA levels in MDA-MB-231 cells (Fig. 4A). Meanwhile, the levels of acetylated RelA were sharply decreased due to degradation of nuclear RelA by the ubiquitin system (Fig. S4), which is in accordance with a previous report [3].

The BRD4-acetylated RelA PPI increases cellular adhesion, metastasis, and invasion by occupying the promoters of IL-6, IL-8, Snail, Vimentin, and ZEB1, and increasing their expression [3–8]. The chromatin immunoprecipitation assay and RT-qPCR were conducted to test the ability of **13a** interfere with the binding activity of BRD4 to promoters of its downstream genes (primers in Supplementary Table S1). The results showed that **13a** weakened the binding ability between BRD4 and the promoters of the IL-6, IL-8, Snail, Vimentin, and ZEB1 (Fig. 4B–F), thus downregulating those genes at both the transcriptional and translational levels (Fig. 4G–I and K–O). To further explore the effect of acetylated site of RelA on the interaction between BRD4 and acetylated RelA, p300 protein, an acetyltransferase that acetylates RelA at lysine-310 [29–31], was knocked down. The results indicated that both knocking down p300 and **13a** treatment the BRD4-acetylated RelA PPI, and moreover, p300 knockdown impaired the inhibitory effect of **13a** his PPI (Fig. 4J). Taken together, these data suggested that **13a** exhibited its anticancer activity via blocking the interaction between BRD4 and its substrate acetylated RelA K310Ac, resulting in the transcriptional repression of downstream genes (IL-6, IL-8, Snail, Vimentin, and ZEB1).

BRD4 is overexpressed in MDA-MB-231 cells, contributing to constitutive NF-κB activity and high metastatic potential [32–34]. Upregulation of Snail, Vimentin, and ZEB1 genes are hallmark features of the epithelial-mesenchymal transition, which maintains the metastasis phenotype of epithelial cancer cells [18]. To further investigate the anticancer mechanism of **13a**, its effects on the adhesion, metastasis, and invasion abilities of MDA-MB-231 cells were investigated. The results showed that **13a** weaken the adhesion (Fig. S2A), metastasis

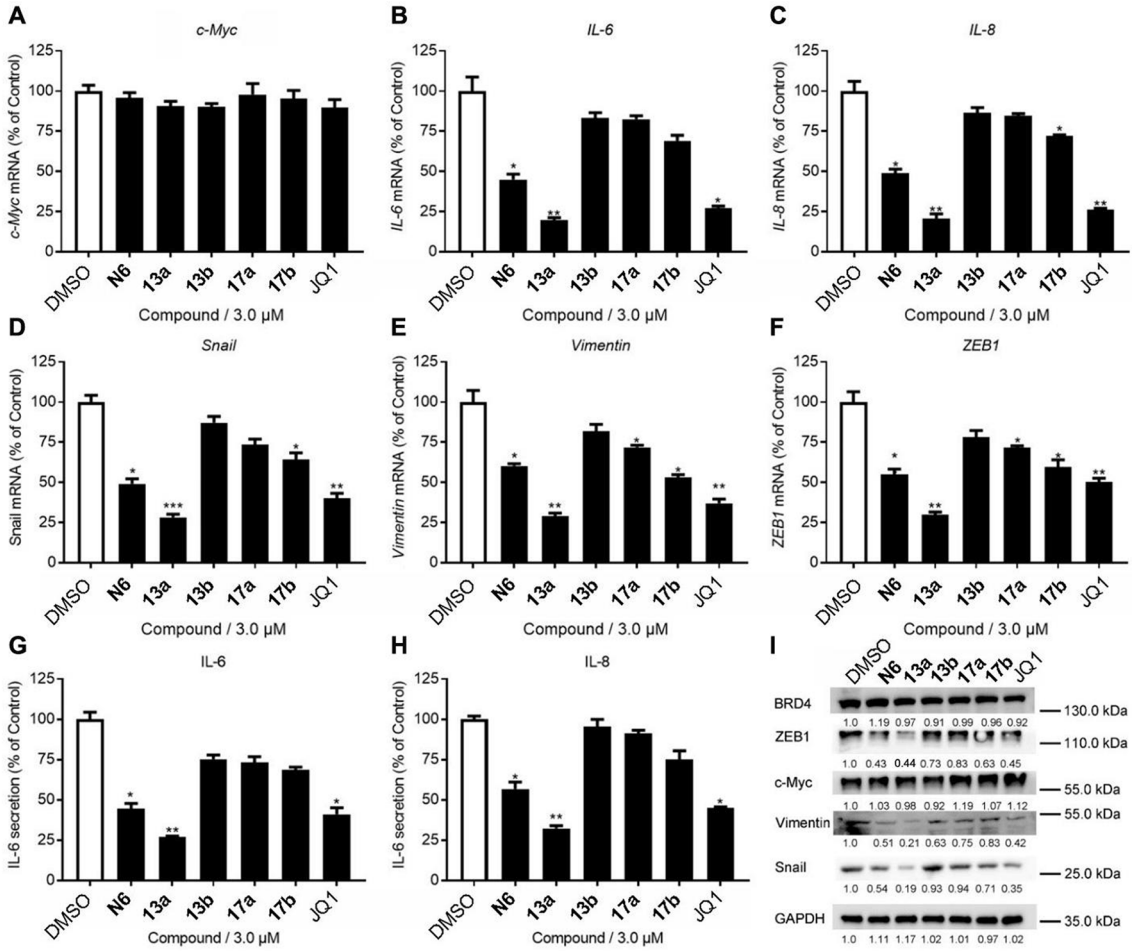


Fig. 2. Compound **13a** selectively inhibits BRD4-NF-κB signaling. (A) The effect of the five hit compounds and JQ1 on gene levels of BRD4-NF-κB signaling and c-Myc level was determined by RT-qPCR assay. (B-F) The effect of the five hit compounds and JQ1 on downstream gene levels of BRD4-NF-κB signaling was determined by RT-qPCR assay. (G-I) The effect of the five hit compounds and JQ1 on downstream protein levels of BRD4-NF-κB signaling by ELISA and Western blotting.

(Fig. S2B and S2C), and invasion (Fig. S2D and S2E) abilities of MDA-MB-231 cells. BRD4 also insulates chromatin from DNA damage signaling via promoting the secretion of cytokines IL-6 and IL-8 [30,31]. Hence, the effect of **13a** on DNA damage was evaluated using immunofluorescence and western blotting. The results showed that both **13a** and JQ1 induced DNA damage in MDA-MB-231 cells as indicated by the accumulation of γ-H2AX based on immunofluorescence assay (Fig. 5A and B) and western blotting (Fig. 5C), a biomarker positively correlated with DNA damage [35]. Importantly, **13a** showed more potent effects at inducing DNA damage than JQ1 at the same concentration. At the same time, DNA damage often triggers senescence in cancer cells [36], while BRD4 relieves cell senescence via downregulating p21 levels in head and neck cancer [37].

In this study, **13a** induced cell senescence in MDA-MB-231 cells in a dose-dependent fashion, as revealed by an increase of β-galactosidase (SA-β-gal), a senescence-associated biomarker (Fig. S3A and B), and the upregulation of p21 (Fig. S3C). Taken together, the anticancer activity of **13a** could be associated, at least in part, with its ability to inhibit the BRD4-acetylated RelA PPI thus leading to cell senescence.

3. Conclusion

In summary, we have identified compound **13a** as a new BRD4 inhibitor. Compound **13a** inhibited NF-κB signaling via blocking the interaction between BRD4 and acetylated RelA, thus impeding the transcriptions of downstream genes. Moreover, compound **13a** induced senescence, and impaired adhesion, metastasis, and invasion of MDA-MB-231 cells. We anticipate that the discovery of **13a** may provide a novel molecular scaffold for the future development of more efficacious and selective BRD4 inhibitors or theranostic probes against NF-κB-driven cancers.

4. Experimental section

4.1. Reagents and cell lines

Normal cell lines (HEK293T and LO2) and breast cancer cell lines (MDA-MB-231, MCF-10A, T47D, and MCF-7 cells) used in this paper were cultured at 37 °C with 5% CO2 in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum and 1% penicillin/streptomycin. Compounds **N6**, **1a**, **1b**, **6**, **14**, and (+)-JQ1 were purchased from J&K Scientific Ltd. (Hong Kong, China) and dissolved in DMSO. Two TR-FRET assay kits for single bromodomain of BRD4 were bought from Cayman Chemical (Carlsbad, CA, USA). The resources of all the antibodies and related reagents used here were shown in this paper.

4.2. Molecular modeling

The initial model of BRD4 BD1 in complex with (+)-JQ1 and BRD4 BD2 in complex with A-1457066 were derived from the X-ray crystal structures (PDB: 3MXF and 5UVV, respectively) using the molecular conversion procedure implemented in the ICM-pro 3.6-1d program (Molsoft, San Diego, CA, USA). The molecular conversion procedure and high throughput molecular docking were administrated as previous

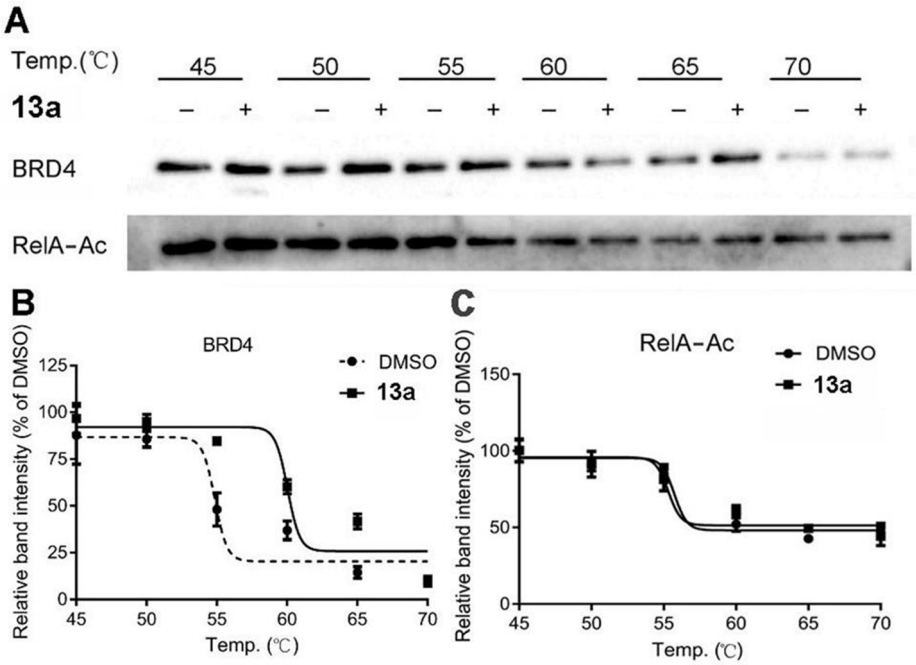


Fig. 3. Compound **13a** increases the thermal stability of BRD4 in cell lysates. (A) Lysates of MDA-MB-231 cells were treated with **13a** at 3.0 μM. Proteins in the soluble fraction were visualized by Western blotting (B–C). Densitometry analysis of BRD4 and Rel-Ac content. Data are represented as mean ± SD.

reports [38,39].

4.3. Time resolved fluorescence resonance energy transfer (TR-FRET)

The effect of tested compounds on the interaction between single domain of BRD4 and its acetylated ligand peptides was detected using kits according to the manufacturer's protocols (Cayman Chemical, Carlsbad, CA, USA) [20]. The results were read using a multimode plate reader (PerkinElmer, Waltham, MA, USA) via detecting the dual emissions at 620 nm and 670 nm using an excitation at 340 nm. Data analysis was conducted using the TR-FRET ratio (670 nm emission/620 nm emission).

4.4. Cellular thermal shift assay

The cellular thermal shift assay was conducted to monitor BRD4 engagement in MDA-MB-231 cells using BRD4 antibody as described previously [40].

4.5. Western blotting analysis

MDA-MB-231 cells were lysed using RIPA lysis buffer containing 1% PMSF (100 mM). 20 mg of extracted total cellular proteins were loaded into each well and separated using SDS-polyacrylamide gel electrophoresis. After electroblotting, the membranes were successively incubated with corresponding primary antibodies and horseradish peroxidase-conjugated second antibody (1:1000), and developed with the enhanced chemiluminescent method. GAPDH protein was used as an international reference protein.

4.6. Real-time quantitative PCR assay

RT-qPCR assay was conducted in a ViiA 7™ Real-Time PCR system (Applied Biosystems, Foster City, CA, USA). After extraction xfrom MDA-MB-231 cells using RNAiso Plus reagents (TaKaRa, Dalian, China), the total RNA transcribed into the cDNA using a First-stand cDNA Synthesis kit (Beyotime, Shanghai, China). The transcriptional changes of target genes were detected and the housekeeping GAPDH was used as a control for normalization. Each sample was conducted in triplicate in a BeyoFast™ SYBR Green qPCR Mix (Beyotime, Shanghai, China). The reaction procedures and results were analyzed following previous methods [40–42], and the related primers for these genes were documented in Table S1.

4.7. Co-immunoprecipitation (co-IP) assay

The co-IP assay was performed as previous procedures [40,43]. Briefly, 1×10^6 MDA-MB-231 cells were plated into a 6-well plate and incubated for 24 h with the indicated concentrations of **13a**. Cells were lysed and the protein samples were extracted. The protein concentration of each sample was measured using the Bradford method. 30 μg total protein for each sample was incubated overnight with 10 μL anti-BRD4 (Abcam, ab128874, Cambridge, MA, USA) magnetic beads according to the manufacturer's procedures. The complex was washed five times to remove non-specific and non-cross-linked antibodies. Then, the precipitated proteins were separated by SDS-PAGE and detected by western blotting with anti-H4K5Ac (Abcam, ab51997, USA) anti-Twist1 (Absin abs136367, Shanghai, China), and anti-Acetylated-lysine antibody (CST 9441, Danvers, MA, USA).

4.8. Enzyme-linked immunosorbent assay (ELISA)

The release of cytokines IL-8 and IL-6 in MDA-MB-231 cells after treatment with compound was detected by ELISA using human IL-6 ELISA kit and human IL-8 ELISA kit (NeoBioscience, Shenzhen, China) [33,34].

4.9. Gene knockdown assay

MDA-MB-231 cells were seeded in 10 cm dishes at 80% confluence in DMEM medium with 10% fetal calf serum and a mixture of penicillin and streptomycin (1%) for 24 h. After 5 min preincubation with 250 μL serum-free medium, Lipo3000 reagent, BRD4, p300 or control siRNA were gently mixed and incubated at room temperature for 20 min. Then, the growth medium was removed from the wells and replaced with 0.5 mL of fresh medium. Then, the knockdown mixture was added to each

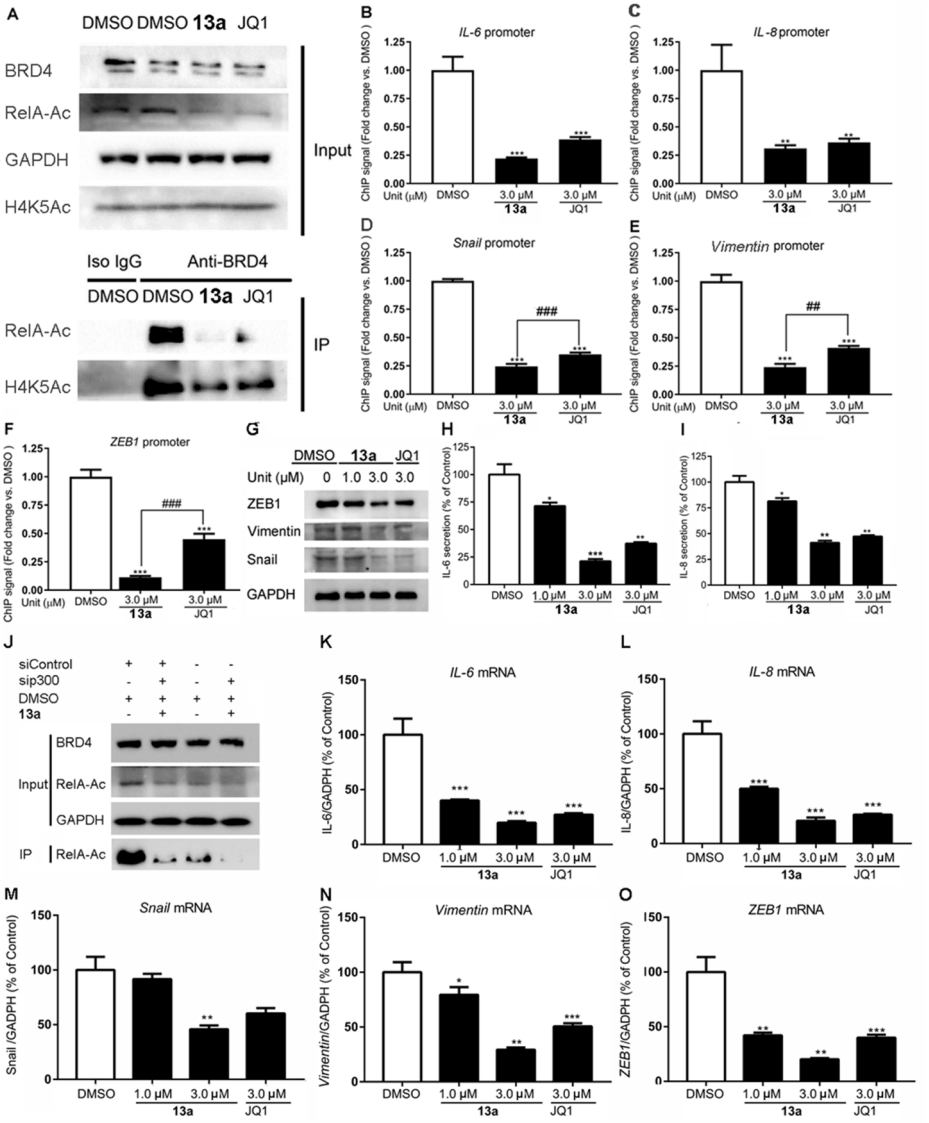


Fig. 4. **13a** impairs expression of downstream gene levels of NF-κB signaling by blocking the interaction between BRD4 and acetylated RelA in MDA-MB-231 cells.

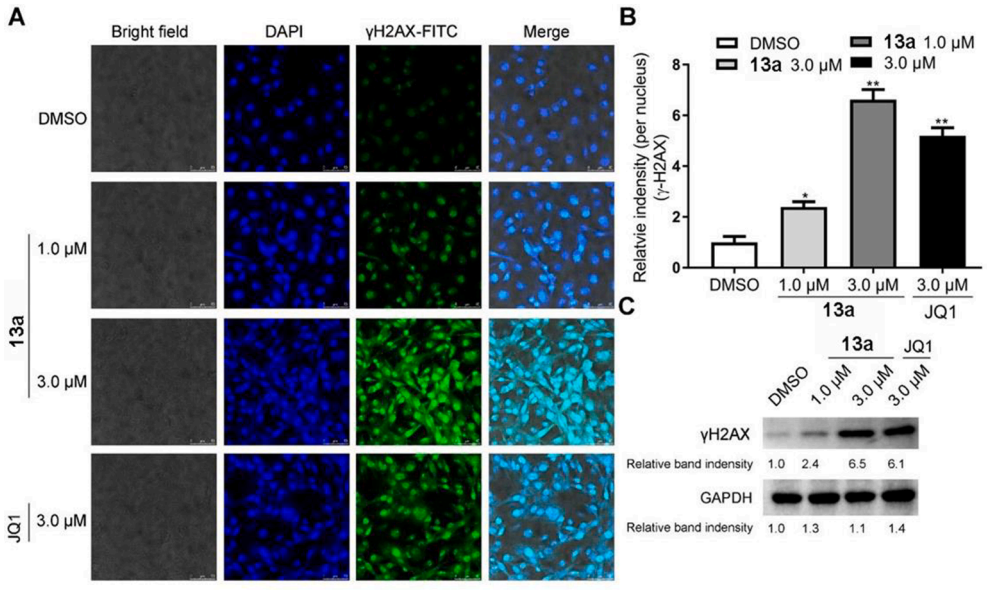


Fig. 5. The effect of **13a** on DNA damage of MDA-MB-231 cells. (A, B) The effect of **13a** on DNA damage in the MDA-MB-231 cells. (C) The effect of **13a** on γ-H2AX levels. Data are represented as mean ± SD. * $p < 0.05$, * $p < 0.01$ versus DMSO-treated groups.

well and the cells were continued incubated at 37 °C for 48 h in a cell incubator before further study.

CRediT authorship contribution statement

Guan-Jun Yang: Investigation, Validation, Project administration, Data curation, Writing - review & editing. **Ying-Qi Song:** Writing - original draft, Project administration. **Wanhe Wang:** Project administration. **Quan-Bin Han:** Writing - review & editing. **Dik-Lung Ma:** Compound synthesis, Funding acquisition, Resources, conceptuation, methods, Writing - review & editing. **Chung-Hang Leung:** Supervision, Funding acquisition, Resources, conceptuation.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bioorg.2021.105158.

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