Chinese Chemical Letters 34 (2023) 107923

Contents lists available at ScienceDirect

Chinese Chemical Letters

journal homepage: www.elsevier.com/locate/cclet

Identification of highly efficacious PROTACs targeting BRD4 against acute myeloid leukemia: Design, synthesis, and biological evaluations

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ARTICLE INFO

Article history:
Received 6 August 2022
Revised 12 October 2022
Accepted 18 October 2022
Available online 28 October 2022

Keywords:
Acute myeloid leukemia
BRD4
PROTACs
Protein degradation
Apoptosis

ABSTRACT

The abnormal activation of BRD4 accelerates the progression of acute myeloid leukemia (AML), developing more precise therapeutics to intervene BRD4 promise to be an excellent opportunity to avoid current limitations of chemotherapy in clinic. Herein, a range of small-molecule PROTACs with the privileged 8-methyl-pyrrolo[1,2-*a*]pyrazin-1(2*H*)-one scaffold were rationally designed, which harbored different carbon or ethylenedioxy chains to degrade BRD4 mediated by the E3 ubiquitin ligase CRBN. Among them, the most potential **B24** exhibited remarkable BRD4 degradation and excellent anti-proliferative activities in MV4-11 cells, with values of DC_{50} and IC_{50} for 0.75 nmol/L and 0.4 nmol/L, respectively, which were better than the BRD4 inhibitor **(+)-JQ-1**. Notably, this compound could time-dependently degrade the target protein in the BRD4-, CRBN-, and proteasome-dependent manner. Besides, **B24** dramatically decreased the level of proto-oncogene c-Myc, and induced cell apoptosis by arresting the cell cycle in G0/G1 phase, down-regulating Bcl-2 and up-regulating Bax to amplify apoptotic effectors. This proof-of-concept study also highlighted the feasibility of BRD4-based PROTACs as a more powerful strategy against AML.

Acute myeloid leukemia (AML) is an aggressive and clonally heterogeneous myeloid malignancy characterized by abnormal proliferation or accumulation of immature cells in the bone marrow, peripheral blood, and other tissues, with extremely poor prognosis and high recurrence [1,2]. In 2022, the American Cancer Society estimated that AML constituted the leading cause of annual deaths due to leukemias, and the number of new AML cases and deaths would up to 20,050 and 11,540, respectively [3]. Current first-line chemotherapeutic agents for AML rely on cytotoxic agents, such as cytarabine and daunorubicin, but the therapeutic efficacy is unsatisfied with low cure rate [4]. Hence, novel therapeutic strategies are desperately required to fulfill unmet medical needs of AML from drug discovery point of view.

Increasing evidence has demonstrated the epigenetic regulatory network mediates multiple cellular processes, including cell growth, differentiation, cycle, and apoptosis that closely related to several diseases, such as cancer [5,6]. Proteins of the bromodomain and extra-terminal (BET) domain family, comprised of BRD2, BRD3, BRD4, and BRDT, are epigenetic readers that specifically recognize acetylated histones through their bromodomains to regulate gene transcriptions [7]. Among these, BRD4 represents the most extensively characterized BET proteins implicated in a number of malignancies, including AML, and has been considered as an attractive drug target both in pharmaceutical and academic field [8]. Over the past decades, diverse privileged scaffolds that could mimic the acetylated lysine (KAc) interactions have been identified, of which **(+)-JQ-1** bearing the triazolodiazepine structure was the first potent inhibitor targeting BRD4-BD1/BD2 reported in 2010 [9]. Many other BRD4 inhibitors, such as **I-BET762** and **OTX-015**, have also subsequently entered the clinic against various diseases (Fig. 1) [10–15]. However, inevitable clinical drawbacks, including poor selectivity, unbearable side effects, and acquired drug resistance, have constituted additional worries that impede further development [16]. As a consequence, it is urgent to explore alternative methods that could recognize histone-acetylated lysine residues exactly.

The recent PROTACs technology has become a research hotspot by hijacking the endogenous ubiquitin-proteasome system to

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https://doi.org/10.1016/j.cclet.2022.107923
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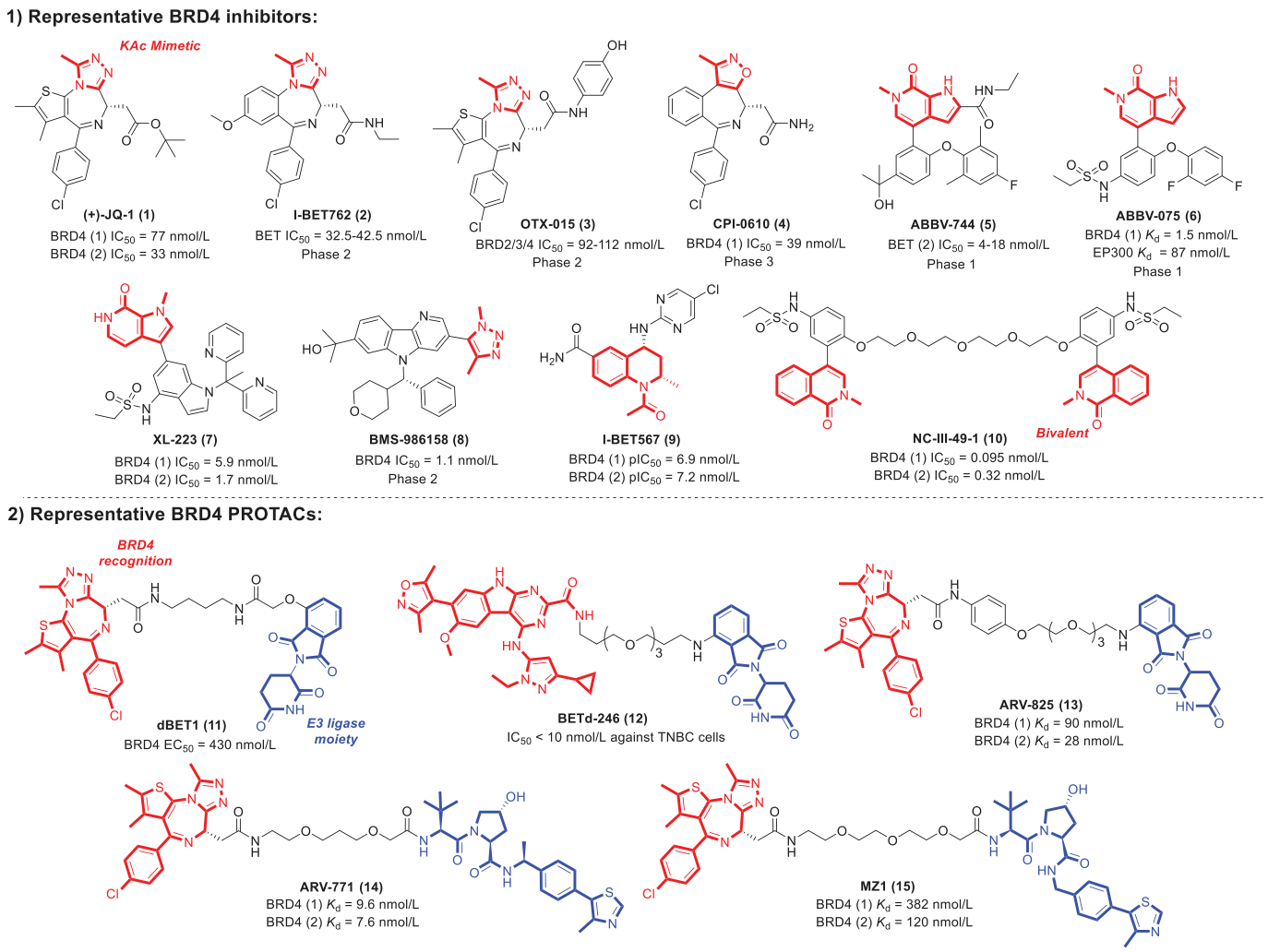


Fig. 1. Chemical structures for the representative small-molecule BRD4 inhibitors and PROTACs.

degrade disease-causing proteins [17]. PROTACs were heterobifunctional small molecules consisting of ligands for target proteins and E3 ubiquitin ligases connected by proper linkers, which simultaneously recognized the target protein and recruited E3 ubiquitin ligase to trigger the ubiquitination and subsequent degradation to offer a catalytic cycle [18]. Compared with classical inhibitors for the occupancy-driven pharmacology, PROTACs belonged to the event-driven that possessed distinctive advantages, such as high potency, improved selectivity, overcoming drug resistance, and targeting undruggable targets [19]. A growing number of researches have elucidated PROTACs targeting BRD4, exemplified by **dBET1**, **BETd-246**, **ARV-825**, **ARV-771** and **MZ1**, which could redirect the E3 ubiquitin ligase CRBN or VHL to degrade (Fig. 1) [20–24]. Nevertheless, extremely high consumption of synthetic resources, no significant improvement in activity, relatively long onset time, and poor druggability due to large molecular weight become inherent defects that cannot be ignored. Therefore, it is of practical significance to investigate diversified PROTACs both with excellent BRD4 degradation and relatively simplified structure that is convenient to prepare.

In our previous work, we were dedicated to optimizing BRD4 inhibitors for improved selectivity, increased activities, and favorable PK profiles [25–27]. Not long ago, Yu's team integrated the BRD4 inhibitor **ABBV-075** and CRBN ligand pomalidomide *via* flexible linkers, while structural diversity was insufficient and dispensable groups like fluorine atoms had little effects on affinity were not simplified [28]. In this study, we laid more emphasis on a class of efficient PROTACs with the 8-methyl-pyrrolo[1,2-*a*]pyrazin-1(2*H*)-one fragment as the KAc mimetic for recognizing BRD4 that distinct from **ABBV-075**. Meanwhile, non-essential motifs of the BRD4 binding moiety were excluded, which was expected to expand future therapeutic insights in AML.

ABBV-075, a highly potent BRD4 inhibitor identified by Abbvie ($IC_{50} = 1.5$ nmol/L), and **38** bearing the favorable 8-methyl-pyrrolo[1,2-*a*]pyrazin-1(2*H*)-one fragment reported by Zhou's group ($IC_{50} = 2.6$ nmol/L) were fused to recognize the BRD4 moiety [29,30]. Selective BRD4 inhibitors generally comprised of acetylated lysine mimetics interacting with Asn 140 and Tyr 97 in BRD4-BD1, substituents occupying the ZA channel to improve their activity and selectivity, and an essential WPF shelf (Fig. 2A). Besides, lipophilic groups like phenyl or pyridinyl formed stable $\pi-\pi$ stacking interactions with Trp 81. Based on the above detailed analysis, it was suggested the external region adjacent to the ZA channel existed suitable sites for linkage an E3 ligase ligand for novel PROTACs design [10,28,31]. Unnecessary groups, such as fluorine atoms with little contribution to the binding affinity, were removed to obtain more simplified chemical structures. Compared with reported E3 ubiquitin ligase ligand VHL with molecular weight beyond 400, the CRBN series showed greater physiochemical and PK characteristics with relatively smaller molecular weight around 250 [32]. Hence, the most commonly used CRBN ligase ligand pomalidomide developed by Celgene company is more ideal

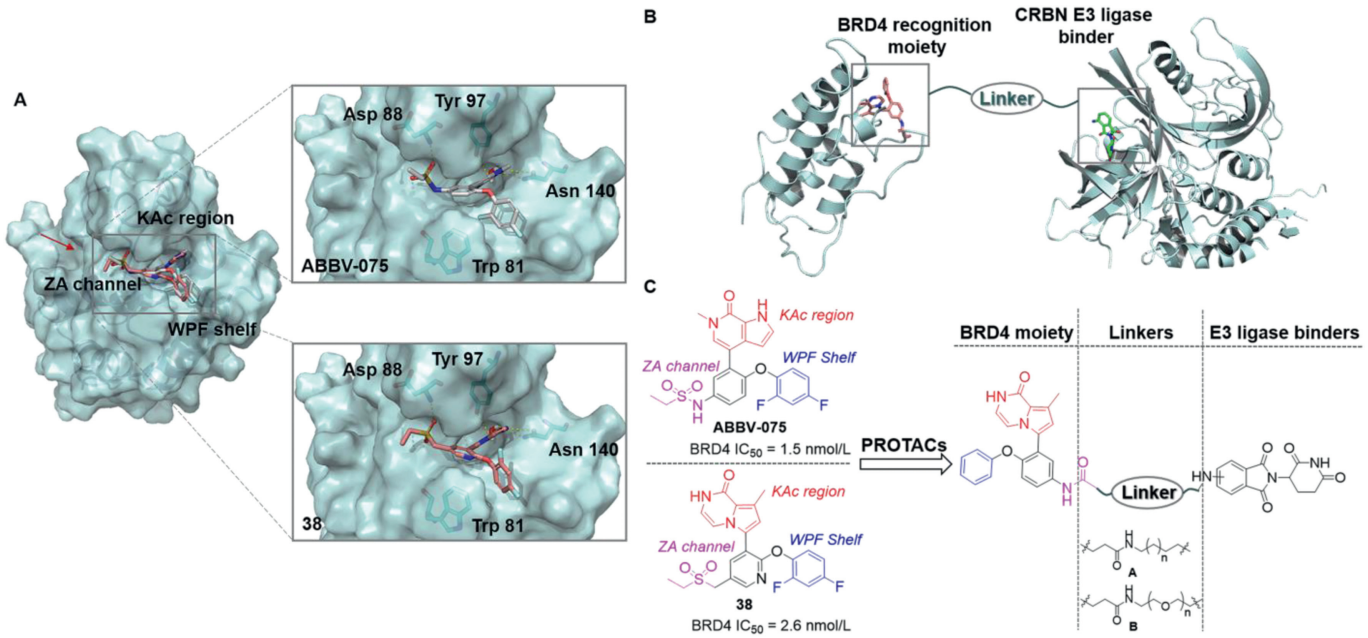
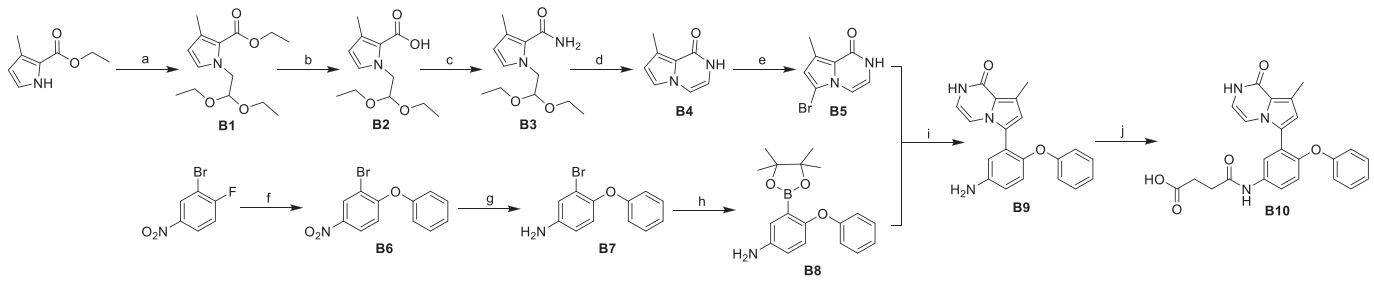


Fig. 2. Design strategy for novel BRD4 PROTACs. (A) **ABBV-075** and **38** were aligned in the three-dimensional surface diagram of co-crystal BRD4 (PDB: 6KEE), and the red arrow indicated this site was appropriate for constructing novel PROTACs. (B) Docking pose of the BRD4 recognition moiety to targeted protein (PDB: 6KEE) and the co-crystal structure of **pomalidomide** to CRBN (PDB: 4CI3). (C) Design of target PROTACs based on **ABBV-075** and **38**.



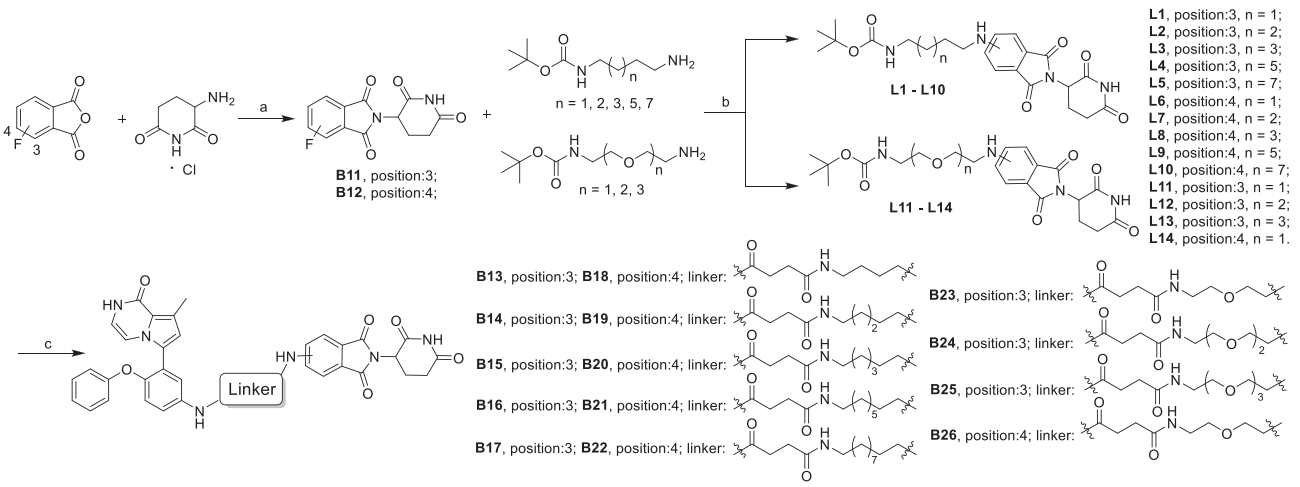
Scheme 1. General synthetic routes for the intermediate **B10**. Reagents and conditions: (a) Bromoacetaldehyde diethyl acetal, Cs_2CO_3, DMF, 110 °C, 24 h, 87.0%; (b) LiOH·H_2O, EtOH, H_2O, 75 °C, 12 h, 83.2%; (c) NH_4Cl, HATU, DIPEA, DMF, 25 °C, 12 h, 85.5%; (d) AcOH, 105 °C, 83.7%; (e) NBS, TFA, DCM, 0 °C, 1 h, 92.4%; (f) Phenol, K_2CO_3, DMF, 90 °C, 2 h, 95.6%; (g) Fe, AcOH, EtOH, 85 °C, 2 h, 44.6%; (h) Bis(pinacolato)diboron, Pd(dppf)Cl_2, KOAc, anhydrous 1,4-dioxane, 100 °C, N_2, 12 h, 48.1%; (i) Pd(PPh_3)$_4$, K_2CO_3, 1,4-dioxane, H_2O, 100 °C, N_2, 10 h, 75.4%; (j) Succinic anhydride, toluene, 115 °C, 5 h, 88.4%.

for BRD4 PROTACs that tether with aforementioned BRD4 recognition portion through two kinds of linkers, *i.e.*, ethylenedioxy and carbon chains, with different length and substituted positions to explore the potential degradation ability (Figs. 2B and C).

The synthesis of intermediate **B10** was briefly outlined in Scheme 1. Substitution of the starting material ethyl 3-methyl-1*H*-pyrrole-2-carboxylate provided **B1**, which was then hydrolyzed under basic conditions and went through amide condensation to yield compound **B3**. Cyclization of **B3** under acidic conditions and following bromination of the resulting **B4** with NBS afforded **B5**. Treatment of the commercially available 3-bromo-4-fluoronitrobenzene with phenol in DMSO furnished the title product **B6** that was further reduced by Fe/HOAc to yield corresponding aniline **B7**. The subsequent palladium-catalyzed Suzuki-Miyaura reaction delivered the coupling product **B9**, which continued to react with succinic anhydride to obtain **B10**.

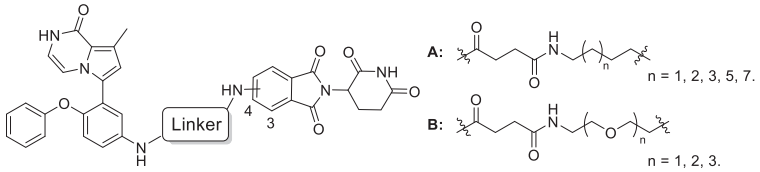
The CRBN ligand pomalidomide conjugated linkers (**L1**–**L14**) were prepared as illustrated in Scheme 2. Fluoro-substituted phthalic anhydrides in different positions were treated with 3-aminopiperidine-2,6-dione hydrochloride in the solvent of acetic acid to give **B11** or **B12**, which was converted into diverse *N*-Boc-protected ethylenedioxy and ethylidene linkers through nucleophilic substitution reactions. Further removal of the Boc protecting group and consecutive amide condensation with **B10** gained the final compounds **B13**–**B26** in acceptable yields.

To seek for the suitable category, length and connection site for linkers, Western blot assay was initially adopted to evaluate the degradation efficacy by synthesized PROTACs in MV4-11 cells with **(+)-JQ-1** as a reference to compare the different effects between degraders and inhibitors. It was found that all compounds exhibited different potential against BRD4 and c-Myc, ranging from moderate to high efficacy in a dose-dependent manner. When single carbon chains were employed to construct the target PROTACs, better potency could be observed for **B13**-**B17** than that of **B18**-**B22** occupying at the position C4 of pomalidomide instead of C3, such as the 61.9% BRD4 degradation of **B14** *versus* 27.7% of **B19** @ 1 μmol/L (Table 1 and Fig. S1 in Supporting information). Besides, the length of carbon linkers made little difference to the BRD4 and c-Myc degradation. Since ethylenedioxy linker has ever been reported to be effective for PROTACs, the BRD4 recognition moiety also linked to pomalidomide through distinct ethylenedioxy linkers. Encouragingly, **B23**, **B24** and **B25** maintained excellent degradation against BRD4 even at the concentration of 10 nmol/L (40.5%, 61.1%, and 43.9%, respectively). Howbeit, **B26** presented negative feedback on the degradation of BRD4 at 1 μmol/L, which also con-



Scheme 2. General synthetic routes for target compounds **B13**–**B26**. Reagents and conditions: (a) NaOAc, AcOH, 115 °C, 12 h, 74.4% and 65.7%, respectively; (b) DIPEA, NMP, 110 °C, 10 h, 25.9%–71.8%; (c) 1) TFA, DCM, 25 °C, 1 h; 2) **B10**, HATU, DIPEA, DMF, 25 °C, 12 h, two steps 36.5%–55.7%.

Table 1
The degradation efficiency of BRD4 and c-Myc in MV4-11 cells and *in vitro* antiproliferative activities against various AML cell lines for designed PROTACs.



Compd.	Linker	Position	BRD4 (%) [a]		c-Myc (%) [a]		IC_{50} ± SD (nmol/L) [b]		
			1 μmol/L	0.01 μmol/L	1 μmol/L	0.01 μmol/L	MV4-11	THP-1	HL-60
B13	A, n = 1	3	67.4	45.9	72.8	30.6	22.4 ± 4.0	262.9 ± 37.3	215.1 ± 14.4
B14	A, n = 2	3	61.9	44.4	77.9	36.1	10.9 ± 0.8	134.3 ± 28.1	44.9 ± 10.6
B15	A, n = 3	3	55.7	27.0	76.7	34.2	25.3 ± 2.0	104.6 ± 14.7	38.2 ± 8.6
B16	A, n = 5	3	64.3	48.3	80.6	40.7	92.6 ± 30.1	477.1 ± 80.6	411.9 ± 37.2
B17	A, n = 7	3	66.5	39.2	77.4	18.3	94.1 ± 21.0	763.3 ± 146.2	138.1 ± 27.3
B18	A, n = 1	4	19.4	14.8	42.0	-0.7	732.1 ± 46.7	534.5 ± 129.9	376.28 ± 49.7
B19	A, n = 2	4	27.7	6.2	63.0	25.8	3316.7 ± 939.8	133.0 ± 31.2	577.53 ± 61.5
B20	A, n = 3	4	28.4	28.3	54.7	4.9	1066.7 ± 55.2	856.9 ± 115.8	687.2 ± 95.5
B21	A, n = 5	4	23.0	-10.5	60.5	7.5	1195.3 ± 170.1	375.4 ± 43.8	122.5 ± 16.4
B22	A, n = 7	4	29.2	-13.6	66.7	35.6	447.3 ± 72.7	54.6 ± 4.6	240.2 ± 51.8
B23	B, n = 1	3	89.8	40.5	76.7	18.7	19.5 ± 2.3	86.7 ± 17.3	31.8 ± 6.5
B24	B, n = 2	3	92.3	61.1	82.8	26.3	0.4 ± 0.1	22.9 ± 3.8	45.6 ± 8.3
B25	B, n = 3	3	84.5	43.9	81.6	18.0	66.7 ± 11.6	16.8 ± 2.5	54.7 ± 9.5
B26	B, n = 1	4	31.0	62.3	44.0	11.0	2598.7 ± 430.4	1841.7 ± 492.3	466.2 ± 58.7
(+)-JQ-1	/	/	17.6	5.5	57.2	11.4	150.1 ± 18.1	210.2 ± 30.5	282.8 ± 70.6

[a] Degradation efficiency of BRD4 and c-Myc against MV4-11 cell lines are expressed as mean values from two independent experiments.
[b] IC_{50}s, compound concentrations required to inhibit the tumor cell proliferation by 50%, and data are shown as the mean ± SD of three independent experiments with GraphPad Prism 8.0 software.

firmed the C4 position of pomalidomide was not beneficial for further structural optimization.

Subsequently, antiproliferative activities of **B13-B26** in diverse AML cell lines were evaluated. As shown in Table 1, most compounds displayed significant inhibition against MV4-11, HL-60 and THP-1 cells with IC_{50} values at the nanomolar range. The suppressed proliferation against AML cell lines could be explained by the degradation of BRD4 and c-Myc. When the substitution position for the linker was C4 instead of C3 at pomalidomide, a significant decrease appeared, such as the inhibition of **B15** against MV4-11 cells was up to forty times *versus* **B20** (IC_{50} = 25.3 nmol/L and 1066.7 nmol/L, respectively). Similar SARs also existed among other derivatives, whose linkers occupying different sites of the E3 ubiquitin ligase ligand generally caused distinct biological effects. Besides, **B13**-**B17** and **B23**-**B25** possessed favorable antiproliferative activities against MV4-11 cells, which indicated the length of linker moiety had little influence. To our delight, PROTAC **B24** with the linker containing 2 PEG chains exhibited the most potent antiproliferative activities towards the MV4-11 cell line (IC_{50} = 0.4 nmol/L), as well as excellent BRD4 degradation efficacy (92.3% BRD4 degradation at 1 μmol/L). Nevertheless, **B24** exhibited weaker proliferative inhibition in other solid cancer cell lines with IC_{50} values at the micromolar range, which verified compound **B24** functioning as a highly efficacious BRD4 PROTAC that could selectively and effectively inhibit the proliferation of AML cell lines (Table S1 in Supporting information).

BRD4 is well-known for its role in super-enhancer organization and transcription activation of several prominent oncogenes including c-Myc, which is an important member of the Myc oncogene family [33]. BRD4 inhibitors generally exert anti-tumor efficacy by down-regulating the expression of downstream c-Myc, and PROTACs serve as heterobifunctional molecules that extending the accessibility to undruggable proteins from the aspect of degradation [34]. To further validate the potency of **B24**, the rela-

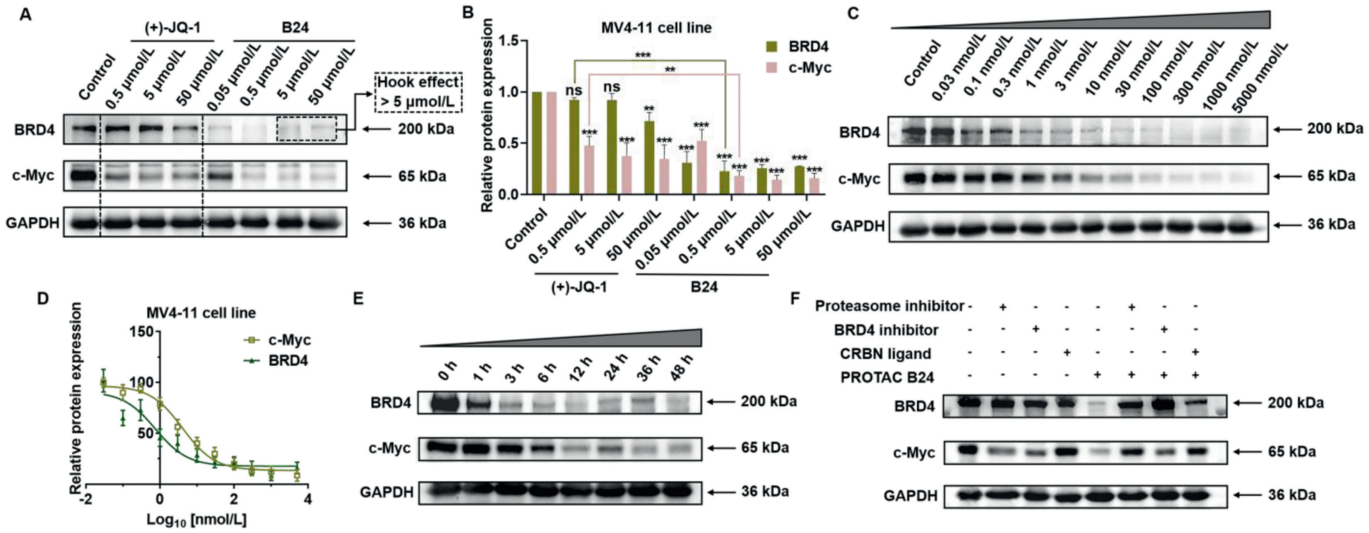


Fig. 3. Western blot analysis of BRD4 and c-Myc protein expressions in MV4-11 cells after different treatments. (A, B) Relative protein levels after treatment with **(+)-JQ-1** and **B24** for 24 h, respectively. (C, D) Relative protein levels after treatment with **B24** at different concentrations for 24 h. (E) Relative protein levels after treatment with **B24** (0.3 μmol/L) at different timelines. (F) The determination of degradation mechanism for BRD4 with **B24**. Cells were pre-treated with proteasome inhibitor **MG-132** (10 μmol/L), BRD4 inhibitor **(+)-JQ-1** (10 μmol/L), and CRBN ligand pomalidomide (10 μmol/L) for 3 h, and then treated with **B24** (0.3 μmol/L) for another 12 h. Relative protein expressions were quantified by the ImageJ software and normalized to GAPDH. Data are expressed as the mean ± SD of three independent experiments. ns: $P > 0.05$, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, *vs.* control.

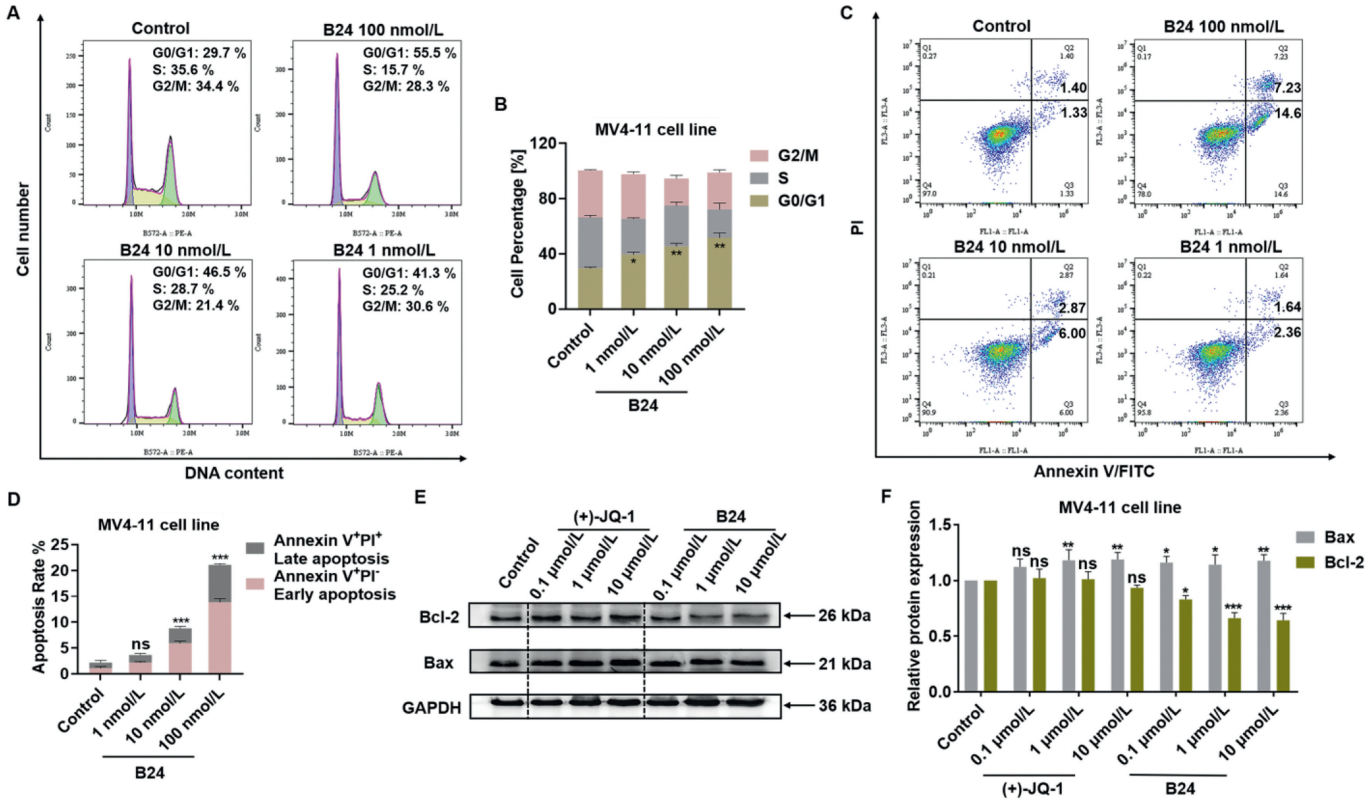


Fig. 4. Cell cycle arrest and apoptosis assays after treatment with **B24** in MV4-11 cells. (A, B) **B24** induced the G0/G1 cell cycle arrest by flow cytometry analysis. (C, D) Pro-apoptotic activity of **B24** determined by flow cytometry analysis. (E, F) Effects of **(+)-JQ-1** and **B24** on the Bcl-2 and Bax by Western blot analysis. Relative protein expressions were quantified by the ImageJ software and normalized to GAPDH. Data are expressed as the mean ± SD of three independent experiments. ns: $P > 0.05$, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, *vs.* control.

tive protein expression of BRD4 and c-Myc were analyzed by Western blot in the MV4-11 cell line after 24 h treatment with **(+)-JQ-1** and **B24**, respectively (Figs. 3A and B). It was quite obvious that **B24** served as a superior BRD4 PROTAC that possessed more potential on BRD4 and c-Myc degradation than the BRD4 inhibitor **(+)-JQ-1**, even at the concentration of 0.5 μmol/L. However, due to unstable interactions for the PROTAC-mediated ternary complex between the target protein BRD4 and E3 ubiquitin ligase CRBN, the hook effect of **B24** appeared at the high concentrations more than 5 μmol/L. Besides, compound **B24** effectively induced the degradation of BRD4 and c-Myc in a concentration-dependent manner, with DC_{50} values of 0.75 ± 0.16 nmol/L, 14.06 ± 1.09

nmol/L, and D_{max} more than 95%, respectively (Figs. 3C and D). In terms of mechanism, **B24** could time-dependently degrade the specific disease-causing protein in the BRD4-, CRBN-, and proteasome-dependent manner (Figs. 3E and F).

We continued to investigate the intracellular anti-proliferative activity of **B24** through cell cycle arrest and apoptosis analysis in MV4-11 cell line by flow cytometry. As depicted in Figs. 4A–D, **B24** could obviously induce the G0/G1 phase cell cycle arrest and cell apoptosis in diverse degrees at different concentrations. To clarify whether compound **B24** could amplify apoptotic signallings through apoptosis-related signal factors, the relative expression of pro-apoptotic Bax and anti-apoptotic Bcl-2 were further determined (Figs. 4E and F). As a result, **B24** could dose-dependently down-regulate the level of Bcl-2 and up-regulate the expression of Bax to induce the apoptosis of AML cells. The up-regulation of the pro-apoptotic protein Bax was also dose-dependent in MV4-11 cells mediated by **(+)-JQ-1**, while the inhibitory effects on the anti-apoptotic protein Bcl-2 made no obvious differences.

In summary, a series of novel and highly potent BRD4-based PROTACs were designed and synthesized based on the pharmacophore hybridation of BRD4 inhibitor **ABBV-075** and **38**. Particularly, the preferred compound **B24** displayed excellent degradation against BRD4 in the MV4-11 cell, with the DC_{50} value of 0.75 ± 0.16 nmol/L and D_{max} > 95%, respectively. Mechanistic studies revealed that the degradation of target protein was presented in the BRD4-, CRBN-, proteasome-, and time dependent manner. In addition, compound **B24** displayed better inhibitory activities than the classical BRD4 inhibitor **(+)-JQ-1** in the MV4-11 cell (IC_{50} = 0.4 ± 0.1 nmol/L and 150.1 ± 18.1 nmol/L, respectively). It was worth mentioning that **B24** inhibited the growth of AML cancer cells through degradation of BRD4, reduction of c-Myc, arresting the cell cycle in G0/G1 phase, and induction of apoptosis. This study not only highlighted the advantages of BRD4 PROTACs as novel and potential anti-AML agents, but also expanded the applications of PROTACs in the field of AML therapy.

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.

Acknowledgments

We thank the National Science Foundation of China (Nos. 81872733, 82173674, and 81872734), and the Research & Development Project in Key Areas of Guangdong Province (No. 2019B020203003) for supporting this study.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.cclet.2022.107923.

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