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Design, synthesis, and biological activity evaluation of a series of novel sulfonamide derivatives as BRD4 inhibitors against acute myeloid leukemia

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ABSTRACT

Accumulating researches have contributed much effect to discover novel chemotherapeutic drug for leukemia with expeditious curative effect, of which bromodomain-containing protein 4 (BRD4) inhibitor is considered as a eutherapeutic drug which has presented efficient cell proliferation suppression effect. In this study, we disclosed a series of phenylisoxazole sulfonamide derivatives as potent BRD4 inhibitors. Especially, compound **58** exhibited robust inhibitory potency toward BRD4-BD1 and BRD4-BD2 with IC_{50} values of 70 and 140 nM, respectively. In addition, compound **58** significantly suppressed cell proliferation of leukemia cell lines HL-60 and MV4-11 with IC_{50} values of 1.21 and 0.15 μM. In-depth study of the biological mechanism of compound **58** exerted its tumor suppression effect via down-regulating the level of oncogene c-myc. Moreover, in vivo pharmacokinetics (PK) study was conducted and the results demonstrated better pharmacokinetics features versus (+)-JQ1. In summary, our study discovers that compound **58** represents as a novel BRD4 inhibitor for further investigation in development of leukemia inhibitor with potentiality.

1. Introduction

Histone covalent modification is a class of sophisticated epigenetics modification that, in the context of chromatin biology, partakes in regulating the structure of chromatin and the transcriptional stimulation of oncogene[1,2]. Bromodomain-containing protein 4 (BRD4) is a protein that considered as a member of bromodomain and extra-terminal domain (BET) family, which serves as a "reader" to recognize the specific ε-*N* acetylated lysine residues on histone tails[3]. The recognition and binding of the acetylated lysine sequences on histones of BRD4 could modulate multifarious downstream cell processes, including cell cycle, proliferation and apoptosis[4,5]. After combining to acetylate histones, BRD4 interacts with the positive transcription elongation factor complex (P-TEFb) and thereby affects the activity of RNA polymerase II [6]. The constitution of BRD4 includes two bromodomains (BD1 and BD2), *N*-terminal extra-terminal (NET) domain, and a C-terminal domain (CTD)[7]. Each bromodomain comprises the active acetyl-lysine-binding pocket which is formed by ZA loop and BC loop[8]. Interfering the interaction between BRD4 and histone via BRD4 inhibition is recently recognized as a promising therapeutic strategy for various types of carcinomas[5,9–11], inflammation[12,13], and HIV [14,15]. For its notably "druggable" site of acetyl-lysine-binding pockets, BRD4 has been seen as a promising target for the further development of small molecular inhibitor[8].

Many BRD4 inhibitors with different scaffolds have been reported in the literature, such as the compounds **1**–**12** (Fig. 1). (+)-JQ-1 (**1**) with triazolothienodiazepine was the first potent BET inhibitor with an IC_{50} value of 77 nM against BRD4 in the AlphaScreen assay[16]. Its analog I-BET762 (**2**) has entered clinical trials for cancer treatment[17]. I-BET151 (3) bearing with isoxazoloquinaoline was discovered by GSK through fragment-based approach and it exhibited in vivo efficacy against MLL-fusion leukemia [18,19]. By the application of the same

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approach, ABBV-075 (**7**) and PFI-1 (**8**) were identified as the selective sulfonamide inhibitors with IC_{50} values of 20 nM and 220 nM against BRD4, respectively [20,21]. As an alternative way, structrue-based screening methods have also been widely used to find BRD4 inhibitors for different chemotypes. Compound **10** with 3,5-dimethylisoxazole scaffold[22] was described as a specific BRD4 inhibitor with significant activity, high selectivity and good pharmacokinetics profile derived from the optimization of a structrue-based screening hit. Studies recently reported two series of potent inhibitors **11** and **12**, which were identified as BRD4 inhibitors through the application of high-throughput screening and crystallography[23,24].

So far, majority of researchers have done much effort to discover that the inhibition of BRD4 is a curative strategy for human acute myeloid leukemia (AML)[25]. However, drug-resistance against BRD4 inhibitor gradually occurred with the long-term usage in clinical therapy. Lucubration of the concrete mechanism of BRD4 inhibitor demonstrated that NF-κB signaling activation[26], overexpression of DUB3[27], and the aberrant suppression of PRC2 complex[28] all contribute to the primary and acquired BRD4 inhibitor resistance. Therefore, seeking for effective BRD4 inhibitor with novel chemical structure is still of great importance for the AML therapeutic field. Although there are many reported inhibitors, the discovery of novel potent inhibitors still attracts much attention due to their therapeutic potentiality for various human diseases. The diverse inhibitors will also provide pharmacological tools to investigate the fine structure and the different functions of BRD4 protein. Here in this study, we disclosed a novel serious of compounds bearing 1,2,3-triazole and sulfonamide groups as potential BRD4 inhibitors for acute myelogenous leukemia (AML).

2. Results and discussion

2.1. Design strategy for the development of novel BRD4 inhibitors

Based on our previous literature investigational study, we noted that the representatively typical drug for BRD4 inhibition and the compounds published recently, all collectively presented that the nitrogen heterocycles (including methylisoxazole, methylpyridine, thiazolidinone and triazole) are a kind of crucial and functional groups that are necessary for activity, the core structure of the compound is highly conserved, and monocyclic, bicyclic and tricyclic rings could retain the activity of inhibiting BET proteins. The cores of these compounds also contain hydrophobic side chains. The main purpose of our research is to design and synthesize a series of BRD4 inhibitor with high selectivity and novel chemical structures with various kinds of new functional groups appended to a known scaffold with effective combination ability to BET proteins. Compound **11** bearing 3,5-dimethylisoxazole was discovered using computational research and it showed combination ability toward BET family proteins including BRD2, BRD3 and BRD4 [29]. Here in this study, compound **11** was selected as our lead compound for the development of potential BRD4 inhibitor via structural modification mainly for two reasons. The first reason is that compound **11** has been validated to own BET affinity toward BRD2/3/4 and the binding mode of **11** has been well investigated via crystallographic data. [29] The second reason is that compound **11** with simple structure provides a platform that is easy to investigate the effect of alteration of functional group distinctly. According to the reported research by Christopher et al[30] we noted that 1,2,3-triazole could be used as potential acetyl-lysine mimetic heterocycle to improve the molecular interaction with BRD4 protein. Herein, based on compound **11**, the 1,2,3-triazole five-ring system with multifarious hydrogen-bond

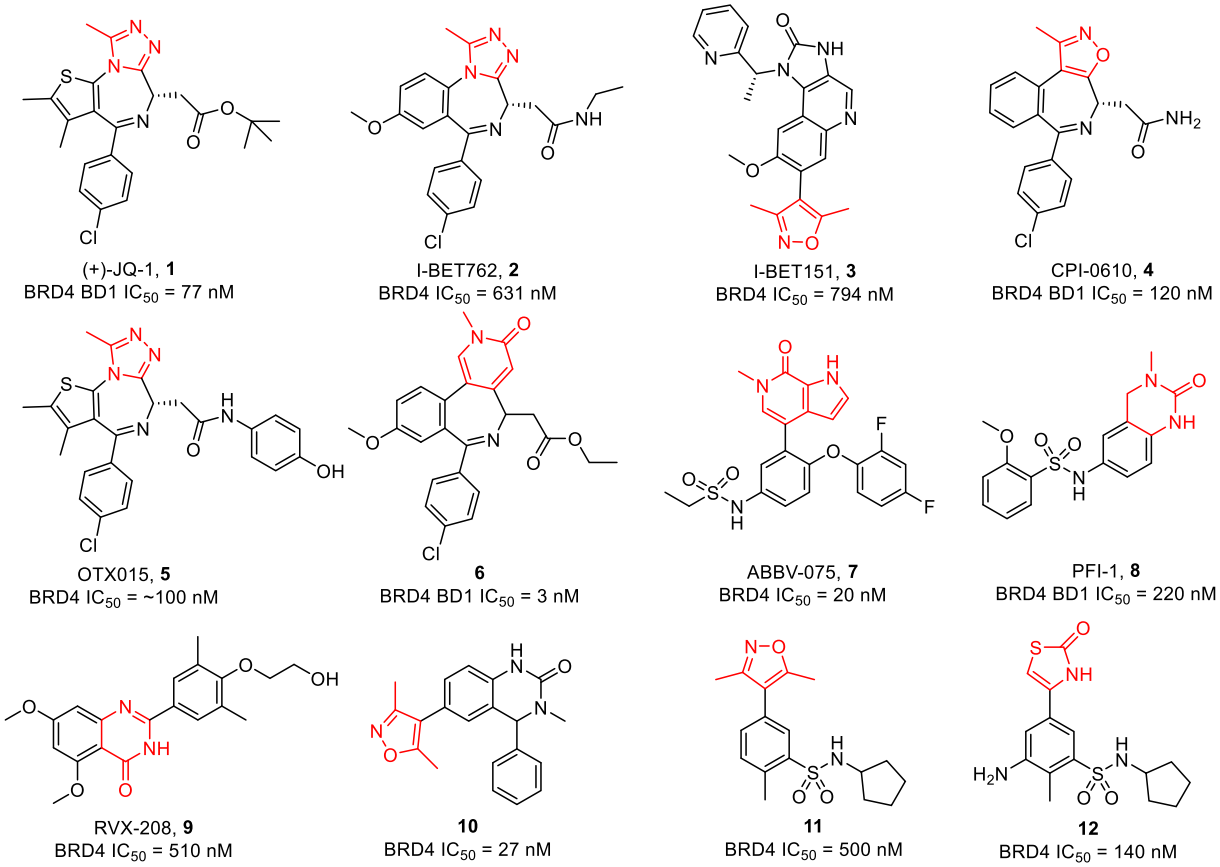


Fig. 1. Representative BRD4 inhibitors. KAc (Acetylated lysine) mimicking motif highlighted in red. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

receptors was introduced on the side chains to aim at improving its binding potency with BRD4 protein via promoting the formation of a hydrogen bond (Fig. 2). Simultaneously, the 1-position of 1,2,3-triazole moiety was found probable to be extended into the ZA channel, which might further increase the affinity to BRD4 protein, and the introduction of a 1,2,3-triazole five-ring system might improve the stability in liver microsomes[31] and thereby improve the pharmacokinetics feature. Different substituents were also introduced into the 1-position of the 1,2,3-triazole to investigate structure–activity relationship. Subsequently, using the principle of bioelectricity isotactic, the structure of acetylated lysine active center was modified, of which 3,5-dimethyl isoxazole structure was replaced by *N*-methylpyrimidine parent nucleus. Based on these two structural modification strategies, we designed and synthesized a series of compounds (compounds **19**–**40**, **51**–**65**) for further evaluation of the biological function.

2.2. Chemistry

The synthetic routes of target compounds **19**–**40** and **51**–**65** were outlined in Schemes 1-2. Flash chromatography was conducted for the purification of compounds and the purity was verified using HPLC (purity was > 95%). ^{1}H NMR, ^{13}C NMR spectrum, infrared spectrum, and mass spectrometry were applied to further confirm the chemical structure.

2.3. Structure-activity relationship study

To develop compounds with improved affinity for BRD4-BD1, we focused on the ZA channel region and KAc (Acetylated lysine) pocket region, and performed an extensive structure activity relationship (SAR) study in an effort to enhance the protein–ligand interactions. The BRD4 protein binding affinity of compounds (**19**–**40**, and **51**–**65**) were evaluated utilizing TR-FRET assay *in vitro*. **(+)-JQ-1** and **I-BET151** were applied as positive control. The binding affinity against BRD4-BD1 at a concentration of 1 μM was estimated and the results were presented in Table 1. Obviously, the synthetic compounds **19**–**40**, and **51**–**65** all indicated conspicuously higher affinity to BRD4-BD1 compared to the previously reported compound **11**. Comparing our synthetic novel derivatives to **11** (lead compound), the introduction of 1,2,3-triazole significantly potentiated the inhibitory effect toward BD1, which indirectly validated our previous assumption that the introduction of triazole side chain could improve the selectivity toward BRD4. Especially compound **58** with an IC_{50} value of 0.07 μM toward BRD4-BD1 is considered as the best among all preferred compound and it exerted similar activity to **(+)-JQ-1** and better activity than **I-BET151**. Docking study further demonstrated that compound **58** exposed to the ZA channel via triazole side chain, which further explains that compounds with 1,2,3-triazole group own augmented binding affinity with BRD4 (Fig. 3). Additionally, the experimental consequences exhibited that compounds **51**–**65** bearing methylpyridine manifested roughly 10 folds of potentiated binding potency with BRD4-BD1 versus compounds **19**–**40** bearing methylisoxazole. These results collectively illustrated that methylpyridine group acts as a KAc mimic group with higher binding affinity with BRD4-BD1 versus methylisoxazole. Next, the introduction of 1,2,3-triazole group enabled the compound to extend to the ZA channel and thereby enhancing its combination ability to BRD4.

To understand the binding mode of the series of methylpyridine compounds, Schrödinger was adopted to analyze the possible interaction mechanism with BRD4-BD1. As shown in Fig. 3**A**, compound **58** was docked into BRD4-BD1 crystal complex (PDB ID: 4BJX), and the analysis of the docking conformation of **58** with BRD4-BD1 revealed that **58** binds in well-defined pocket and forms interactions with ASN140 and a second interaction with TYR97, via a structured water molecule. Meanwhile, it has a second interaction with ASP81, which is similar to the binding mode of the lead compound **11** (Fig. 3**B**). Altogether, compound **58** exhibits efficient binding affinity to BD1 domain of BRD4, which may support its efficiently inhibitory effect against BRD4.

2.4. Compound *58* presented promising BRD4 inhibitory effect in AML cells

BRD4-BD2 binding affinity of the preferred compounds was also detected. Compound **58** showed relatively lower inhibitory rate against BRD4-BD1 than other compounds, which suggested that the phenyl group was important for compound activity. R_1 was replaced with *N*-methyl pyridone-2(1*H*)-one, of which the inhibition data identified it as the optimal substituent. Furthermore, compounds with high BRD4-BD1 affinity were selected to further investigate their IC_{50} values against BRD4-BD2, of which compound **58** exhibited better affinity toward BRD4-BD2 compared with **I-BET151**, and similar affinities toward BRD4-BD2 versus (+)-JQ1 (Table 2).

Further investigation of the effect of compound **58** on AML was conducted via estimating the anti-proliferative capacity on AML cell lines HL-60 and MV4-11 (Table 3). The reported sensitive cell lines human promyelocytic leukemia HL-60 and acute myeloid leukemia MV4-11 were utilized to test the cellular proliferation inhibition effects. Our compounds manifested reasonable potency against HL-60 and MV4-11 cells. Overall, considering the data from the above assays, compound **58** has good profiles for further evaluation.

To study the specific mechanism of **58**, we subsequently applied flow cytometry (FCM) assay to explore its capacity to induce cell cycle arrest and apoptosis in AML cells (MV4-11). As shown in Fig. 4**A and 4B**, compound **58** had the effect in inducing cell cycle arrest in G1 phase in a dose dependent, and generating cell apoptosis with the increasing concentration. Furthermore, multiple studies demonstrated that BET bromodomain proteins act as upstream modulatory factors for c-myc[32],

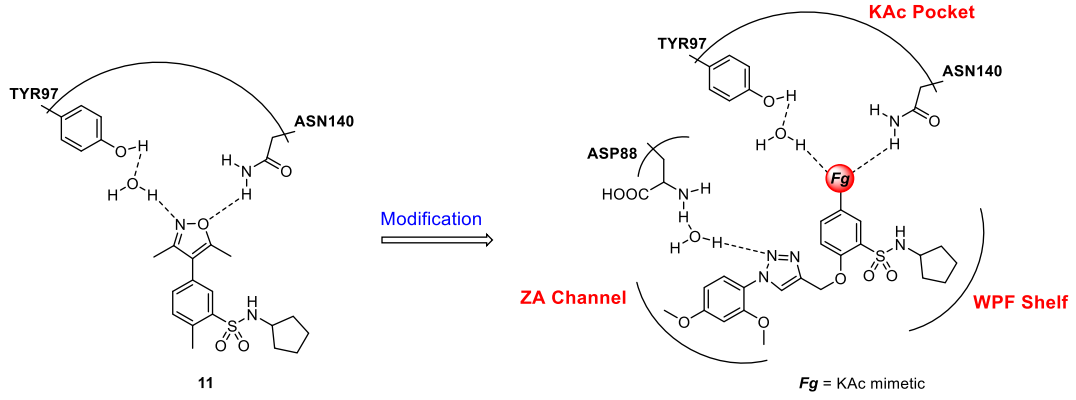
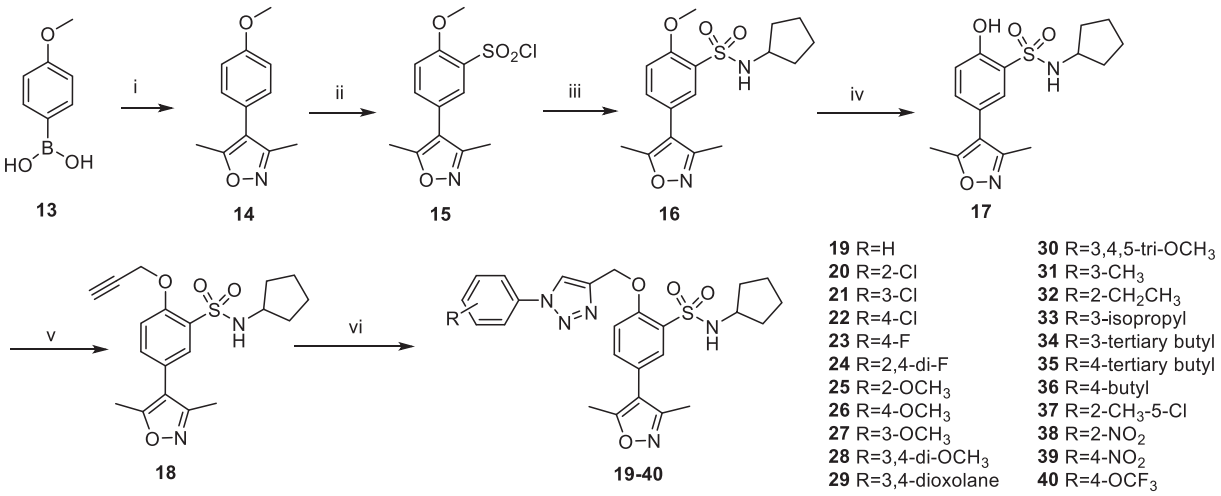
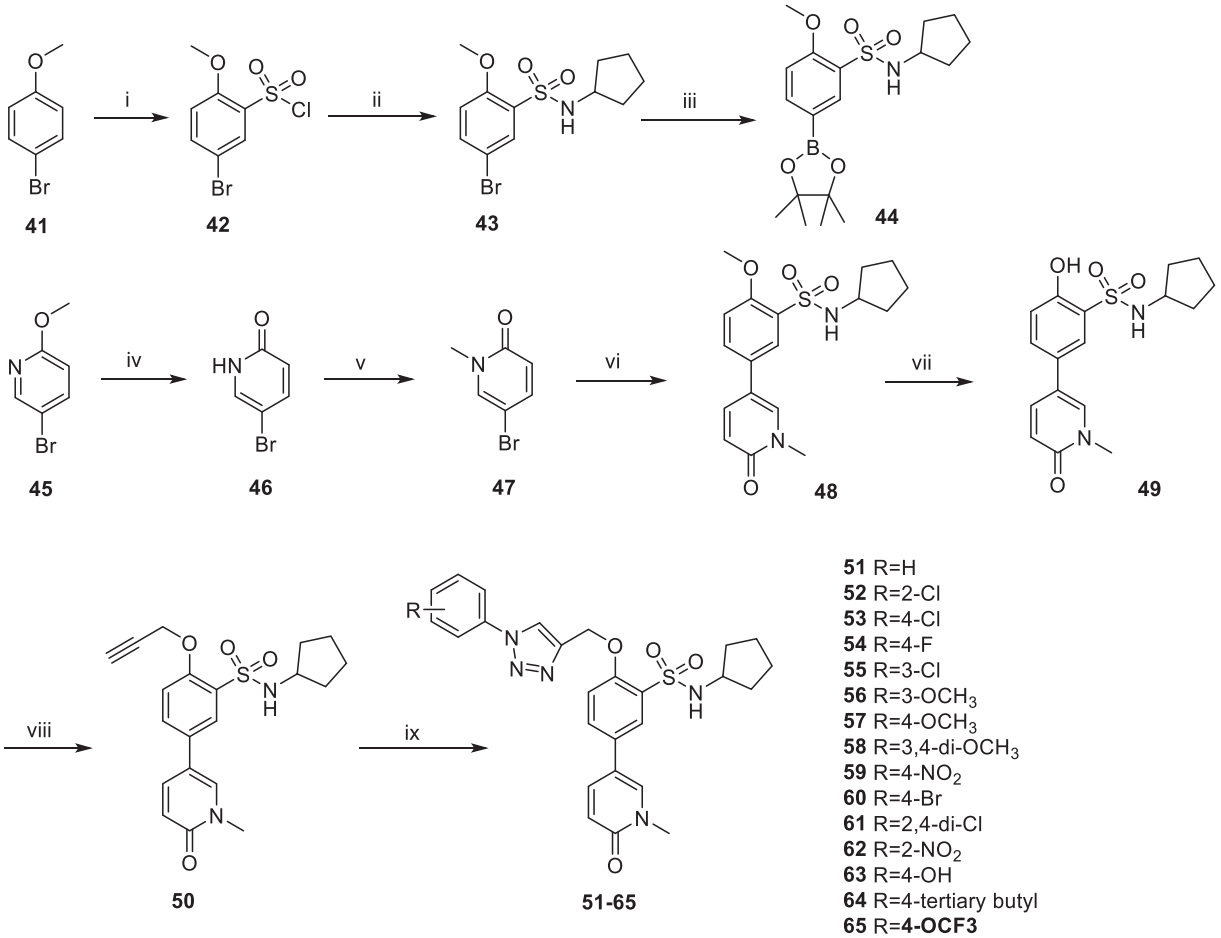


Fig. 2. Schematic representation of key KAc (Acetylated lysine) mimetic binding interaction of **11** with BRD4-BD1 and SAR investigations on the KAc mimicking group and ZA Channel region. ***Fg*** represents functional groups.



Scheme 1. General synthesis of 3,5-dimethylisoxazole derivatives **19**–**40**. Reagents and conditions: (i) 4-iodo-3,5-dimethylisoxazole, $Pd(PPh_3)_4$, K_2CO_3, 1,4-dioxane/H_2O (4/1), 110℃; (ii) $ClSO_3H$, PCl_5, CH_2Cl_2, 0℃; (iii) cyclopentylamine, pyridine, CH_2Cl_2, rt; (iv) BBr_3, CH_2Cl_2, 0℃; (v) 3-bromo-1-propyne, K_2CO_3, acetonitrile, reflux; (vi) azides, $CuSO_4{\cdot}5H_2O$, sodium L-ascorbate, H_2O/CH_3OH (3:1), r.t., 6–15 h.



Scheme 2. General synthesis of *N*-methyl pyridone-2(1*H*)-one derivatives **51**–**65**. Reagents and conditions: (i) $ClSO_3H$, PCl_5, CH_2Cl_2, 0℃; (ii) cyclopentylamine, pyridine, CH_2Cl_2, rt; (iii) bis(pinacolato)diboron, KOAc, $Pd(dppf)_2Cl_2$, 1,4-dioxane, 95 °C; (iv) 6 M HCl, reflux for 8 h; (v)CH_3I, NaH, DMF, rt; (vi) the intermediate **44**, $Pd(PPh_3)_4$, K_2CO_3, 1,4-dioxane/H_2O (4/1), 110℃; (vii) BBr_3, CH_2Cl_2, 0℃; (viii) 3-bromo-1-propyne, K_2CO_3, DMF, reflux; (ix) azides, $CuSO_4{\cdot}5H_2O$, sodium L-ascorbate, H_2O/CH_3OH (3:1), r.t., 6–15 h.

and BRD4 inhibitor (+)-JQ1 induces reduction on c-myc expression to make an anti-proliferative effect[33]. Hence, to further assess whether compound **58** exerted its effect via impeding the expression of c-myc, we conducted the western blot experiment to study the cellular effect related to c-myc, and (+)-JQ-1 was used as positive control. As shown in Fig. 4**C**, the abundance of c-myc was presented in dose-dependent reduction both in (+)-JQ1 group and compound **58** group when compared with negative control, which indicated that compound **58** is

Table 1
Structures and BRD4-BD1 binding affinity of compounds **19**–**40** and **51**–**65**.

Compd	R^1	R^2	BRD4-BD1 IC_{50} (μM) [a]	Compd	R^1	R^2	BRD4-BD1 IC_{50} (μM) [a]
19		H	1.61 ± 0.06	**39**		4-NO_2	1.22 ± 0.06
20		2-Cl	1.22 ± 0.07	**40**		4-OCF_3	2.61 ± 0.13
21		3-Cl	3.15 ± 0.01	**51**		H	0.11 ± 0.01
22		4-Cl	2.01 ± 0.03	**52**		2-Cl	0.21 ± 0.01
23		4-F	1.18 ± 0.06	**53**		4-Cl	0.39 ± 0.02
24		2,4-di-F	2.12 ± 0.03	**54**		4-F	0.14 ± 0.01
25		2-OCH_3	0.89 ± 0.06	**55**		3-Cl	0.42 ± 0.02
26		4-OCH_3	1.87 ± 0.04	**56**		3-OCH_3	0.18 ± 0.01
27		3-OCH_3	1.85 ± 0.17	**57**		4-OCH_3	0.16 ± 0.01
28		3,4-di- OCH_3	0.56 ± 0.01	**58**		3,4-di-OCH_3	0.070 ± 0.01
29		3,4-dioxolane	4.72 ± 0.05	**59**		4-NO_2	0.24 ± 0.01
30		3,4,5-tri-OCH_3	1.12 ± 0.02	**60**		4-Br	0.27 ± 0.03
31		3-CH_3	1.93 ± 0.11	**61**		2,4-di-Cl	0.29 ± 0.01
32		2-CH_2CH_3	1.54 ± 0.01	**62**		2-NO_2	0.20 ± 0.01
33		3-isopropyl	1.93 ± 0.02	**63**		4-OH	0.26 ± 0.01
34		3-tertiary butyl	6.42 ± 0.12	**64**		4-tertiary butyl	0.45 ± 0.04
35		4-tertiary butyl	5.58 ± 0.17	**65**		4-OCF_3	0.48 ± 0.03
36		4-butyl	2.10 ± 0.04	**11**	–	–	>10
37		2-CH_3-5-Cl	2.87 ± 0.08	**(+)-JQ-1**	–	–	0.062 ± 0.01
38		2-NO_2	1.04 ± 0.05	**I-BET151**	–	–	0.086 ± 0.02

[a] The IC_{50} in the table was calculated from the TR-FRET assay. The data were expressed as the means ± SD, representing the data from at least three independent experiments.

an effective BRD4 selective inhibitor via down-regulating c-myc.

2.5. Assessment of pharmacokinetics (PK) properties

According to the conspicuous binding affinity toward BD1 and BD2 as well as tumor inhibitory function of compound 58, further investigation of the in vivo pharmacokinetics study was conducted to estimate its metabolic feature in rats. 10 mg/kg dose and 20 mg/kg dose of compound 58 for intravenous injection (i.v.) and oral administration (p.o.), respectively. Subsequently, LC-MS/MS was applied for the analysis

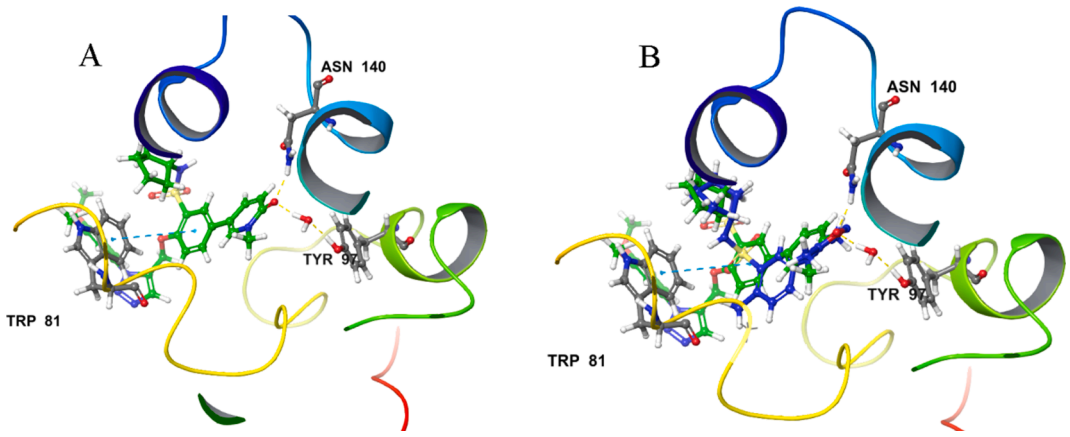


Fig. 3. (A) Docking conformation of **58** in BRD4-BD1 (PDB ID: 4BJX). (B) Overlay of compound lead compound **11** (Blue) and **58** (Green) bound to BRD4-BD1 (PDB ID: 4BJX). Key residues are labeled in red, and hydrogen bonding interactions are represented by green dashed lines. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 2
The affinity to BRD4-BD2 of the selected preferred compounds.

Compounds	BRD4-BD2 IC_{50} (μM) [b]	Compounds	BRD4-BD2 IC_{50} (μM) [b]
26	1.65 ± 0.07	**59**	0.51 ± 0.03
29	1.12 ± 0.03	**60**	0.46 ± 0.01
51	0.26 ± 0.01	**61**	0.57 ± 0.04
52	0.55 ± 0.06	**62**	0.44 ± 0.03
53	0.94 ± 0.02	**63**	0.57 ± 0.07
54	0.22 ± 0.03	**64**	0.86 ± 0.06
55	0.76 ± 0.04	**65**	0.84 ± 0.03
56	0.42 ± 0.03	**(+)-JQ-1**	0.12 ± 0.04
57	0.30 ± 0.02	**I-BET151**	NT
58	0.14 ± 0.05		

NT = not tested.
[b] The IC_{50} in the table was calculated from the TR-FRET assay. The data were expressed as the means ± SD, representing the data from at least three independent experiments.

Table 3
The IC_{50} values of 17 optimization compounds against HL-60 and MV4-11 cell lines.

Compounds	HL-60 IC_{50} (μM)[a]	MV4-11 IC_{50} (μM) [a]	Compounds	HL-60 IC_{50} (μM) [a]	MV4-11 IC_{50} (μM) [a]
26	>10	1.47 ± 0.11	**59**	4.45 ± 0.22	0.44 ± 0.05
29	2.85 ± 0.21	0.86 ± 0.07	**60**	>10	0.89 ± 0.11
51	1.56 ± 0.15	0.42 ± 0.08	**61**	6.42 ± 0.16	0.48 ± 0.06
52	1.55 ± 0.09	0.37 ± 0.14	**62**	4.57 ± 0.08	0.42 ± 0.07
53	5.54 ± 0.14	0.65 ± 0.22	**63**	6.57 ± 0.10	0.54 ± 0.12
54	2.10 ± 0.22	0.28 ± 0.02	**64**	>10	0.93 ± 0.15
55	3.12 ± 0.13	0.58 ± 0.13	**65**	5.86 ± 0.23	1.18 ± 0.22
56	1.76 ± 0.05	0.37 ± 0.06	**(+)-JQ-1**	1.12	0.13
57	1.87 ± 0.11	0.32 ± 0.10	**I-BET151**	NT	NT
58	1.21 ± 0.02	0.15 ± 0.02			

NT = not tested.
[a] The IC_{50} value in the table were expressed as the means ± SD, representing the data from at least three independent experiments.

of compound 58 plasma concentrations. Depicted in Table 4 were the analytical results of compound 58. Data showed that the C_{max} value was 88.73 ng/ml, the AUC value was 553.38 ng*h /ml, the $T_{1/2}$ value was 10.85 h, and the oral bioavailability was 36.88%. Conclusively, our experimental data exhibited that compound 58 is a potential BRD4 inhibitor with better pharmacokinetics features compared to those of (+)-JQ1[34].

3. Conclusion

In summary, we designed and synthesized a series of novel 3,5-dimethylisoxazole derivatives as BRD4 inhibitors via structural modification of a previously reported molecule. In vitro experiments demonstrated conspicuous anti-proliferative capacity of compound **58**, and **58** also exhibited robust binding affinity toward both BRD4-BD1 and BRD4-BD2 with IC_{50} values of 70 and 140 nM, respectively. Docking studies were performed to determine structure–activity relationships. Moreover, compound **58** presented proliferative effect toward HL-60 and MV4-11 leukemia cells with IC_{50} values of 1.21 and 0.15 μM, respectively. In addition, it can further down-regulated c-myc levels in MV4-11 cells. These findings demonstrate that compound **58** might be a potent BRD4 inhibitor in the cellular environment and is worthy of further investigation.

4. Experimental section

4.1. Chemistry

4.1.1. General

The hydrogen and carbon nuclear magnetic resonance (NMR) spectra were measured by Bruker ACF300/400 MHz nuclear magnetic resonance instrument, the internal standard was tetramethylsilane (TMS); the mass spectrometry (MS) was measured by Waters UPLC/MS liquid-mass spectrometry system; experiment The middle column chromatography uses 200–300 mesh silica gel produced by Qingdao Ocean Chemical Co., Ltd. as the stationary phase, and thin layer chromatography (GF254) plates are purchased from Yantai Jiangyou Silica Gel Development Co., Ltd. The chemical reagents are all commercially available analytical or chemically pure products. Unless otherwise specified, reagents are used directly without treatment.

4.1.2. Synthetic procedures

Compounds **13**, **41** and **45** were purchased. Other compounds were prepared by one of two schemes.

Scheme 1 Firstly, we used 4-methoxyphenylboronic acid (**13**) as raw

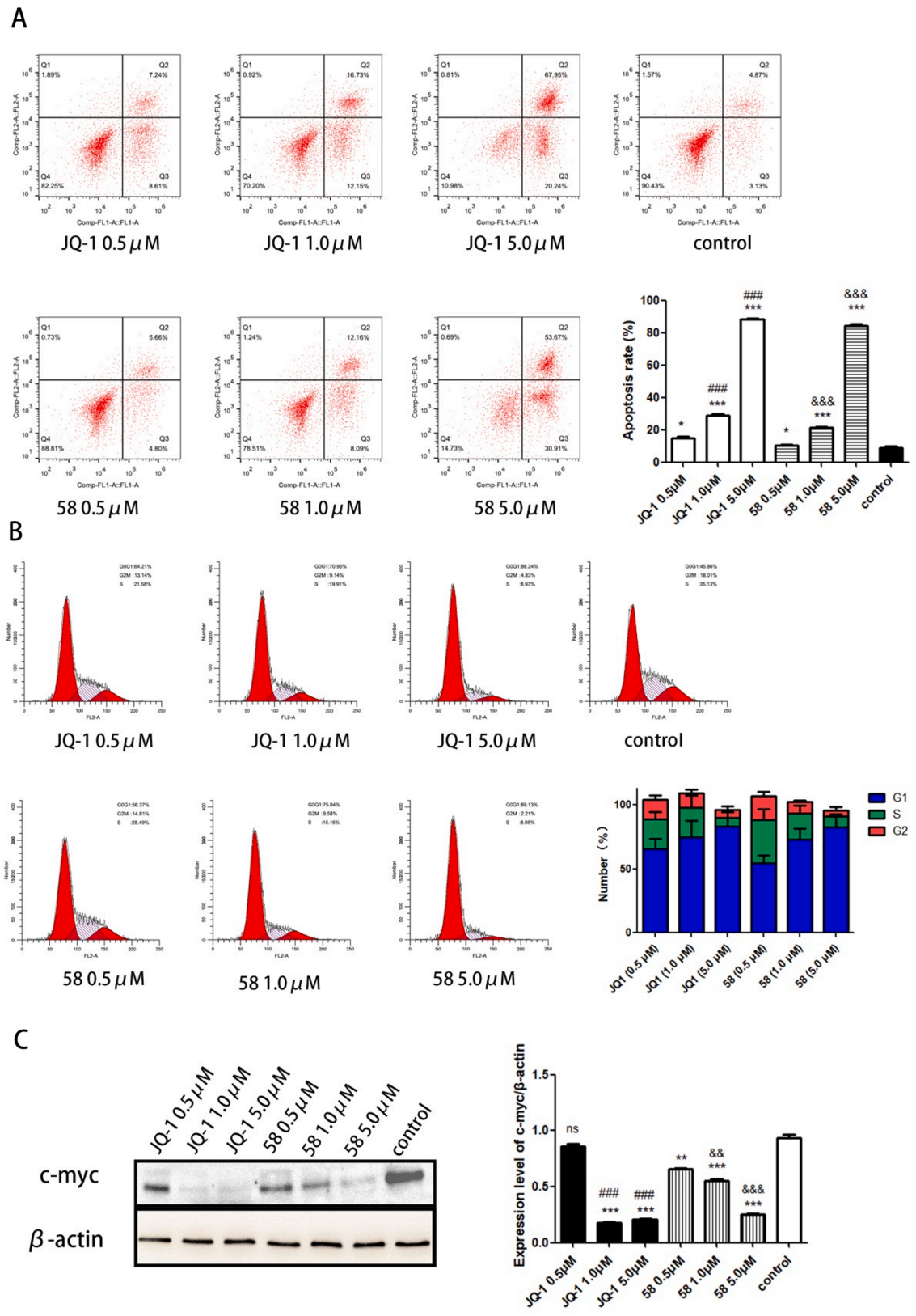


Fig. 4. Compound **58** was validated the effects of cell apoptosis promotion, G1 phase arrest, and c-myc silencing. (A) Compound **58** caused apoptosis in MV4-11 cells after 48 h of incubation. (B) Compound **58** caused cell cycle arrest in MV4-11 cells after 24 h of incubation. (C) Western blot assay indicated that the down-regulation of c-myc is presented concentration dependent with compound 58. *$p < 0.05$, ***$p < 0.001$ compared with control group. #$p < 0.05$, ###$p < 0.001$ compared with JQ-1 0.5 μM group. &$p < 005$, &&&$p < 0.001$ compared with 58 0.5 μM group. ns means no significant difference.

Table 4
In vivo pharmacokinetic (PK) data for compound **58**.

Route of administration	Dose (mg/kg)	T_{max} (h)	C_{max} (ng/mL)	AUC_{0-t} (ng*h/mL)	$T_{1/2}$ (h)	CL (ng*h/mL)	F (%)
p.o.	20	0.083	88.73	553.38	10.85	0.030	36.88
i.v.	10	2	302.35	768.98	10.65	0.011	–

material, which reacted with 4-iodo-3,5-dimethylisoxazole via Suzuki coupling reaction to obtain compound **14**. Then, in the presence of chlorosulfonic acid and PCl_5, compound **14** underwent ortho chlorosulfonation of benzene ring to give intermediate **15** of benzenesulfonyl chloride, of which compound 15 was reacted with cyclopentamine to form compound **16** of benzenesulfonamide. Then we used boron tribromide to demethylate to give compound **17**. The intermediate **18** was obtained by nucleophilic substitution of **17** with 3-bromopropyne. Finally, the target compound **19**–**40** was obtained by click reaction. In the process of the synthesis of compound 19–40, we first selected 25% methanol as the reaction solvent for the click chemistry reaction. In this reaction solvent, the click chemistry reaction was catalyzed by anhydrous copper sulfate and sodium ascorbate.

Scheme 2 Compound **51**–**65** was synthesized from 4-methoxybromobenzene (**41**) by chlorosulfonation to give compound **42**, which was then reacted with cyclopentamine to give compound **43**. Finally, the key intermediate 44 of Suzuki coupling reaction was obtained by **43** and pinacol diboride. On the other hand, compound **46** was synthesized by refluxing 5-bromo-2-methoxypyridine (**45**) in 6 M hydrochloric acid. Then, compound **46** was methylated to give compound **47**. Intermediate **44** and **47** was mixed to perfume Suzuki reaction to give **48**. Finally, compound **49** was obtained by compound **48** demethylation. After nucleophilic substitution of compound **49**, compound **50** was obtained. The target compounds **51**–**65** were obtained by click reaction.

For extended syntheses methods please see **Supplementary materials**.

4.2. Docking studies

All ligand molecules were drawn in ChemDraw 2014, and saved as sdf style. Then ligands were processed at a simulated pH of 7.4 ± 1.0 to generate all possible tautomers, stereoisomers, and protonation states and were finally minimized at the OPLS 2005 force field with Ligand preparation protocol of Maestro 10.2. The crystal complex (PDB id: 4BJX) was selected as the BRD4-BD1 docking protein. Docking study was applied to investigate the molecular interaction mechanism via Schrödinger software. Using Protein Data Bank, the published crystal structure of BRD4 (PDB ID: 4O74) was obtained and prepared via Protein Preparation Wizard based on the force field OPLS3 (optimized potentials for liquid simulations). The related 3 dimension structures of compound **58** were computed through the LigPrep module. Ultimately, the schrödinger XP precision was adopted for the calculation and the chosen structure with the lowest-energy was used for the result.

4.3. Biological evaluation

4.3.1. Binding affinities toward BRD4-BD1 and BRD4-BD2 from TR-FRET assay

TR-FRET Assay Kit for BRD4 bromodomain 1 (Item No. 600520) and BRD4 bromodomain 2 (Item No. 600520) (Cayman, Ann Arbor, MI, USA) were applied for the BRD4 binding affinity evaluation.

The BRD4 inhibition assay was performed by using BRD4 bromodomain 1 TR-FRET Assay Kit (Item No. 600520) and BRD4 bromodomain 2 TR-FRET Assay Kit (Item No. 600520) obtained from Cayman (Ann Arbor, MI, USA). Dilute the sample solution with the buffer solution, and prepare the sample concentrations at 100 μM, 50 μM, 20 μM, 10 μM, 5 μM, 1 μM, 0.5 μM, 0.2 μM, 0.1 μM, respectively. Add 5 μL to each well, and set up 3 replicate wells for each concentration gradient. Add 10 μL of BRD4-BD1 europium chloride solution to each well of a 384-well plate. After incubating for 15 min at room temperature in the dark, 5 μL of BRD4 ligand/APC receptor complex was added to each well. The plate was read by a microplate reader after incubating at ambient temperature for 1 h avoids light. The difference between the absorbance of the negative control and the compound group is compared with the absorbance of the negative control, which is the BRD4 inhibition rate. The concentration of small molecule compounds corresponding to 50% inhibition rate is the half inhibitory concentration (IC_{50}).

4.3.2. Cell culture and proliferation inhibition assays.

HL-60 or MV4-11 cells were seeded in 96-well plates at a concentration of 1×10^4 cells per well. Cells were grown in 100 μL of IMDM containing 20% fetal bovine serum. After 12 h, 50 μL of which containing various concentrations of compounds (triple diluted) was added. The measurement was conducted 72 h after seeding, and 10 μL of Cell-counting kit-8 (CCK-8) reagent was added to each well and incubated in 37 °C for 4 h. The spectrophotometric absorbance of each well was measured by a multi-detection microplate reader at a wavelength of 450 nm. The inhibition rate was calculated as ((A_{450} treated - A_{450} blank)/(A_{450} control - $A4_{50}$ blank)) × 100. The IC_{50} was calculated by GraphPad Prism 5 statistical software.

4.3.3. Cell cycle and apoptosis analysis.

Flow cytometry (FCM) was used to analyze the effects on cell cycle and apoptosis treated by compounds. Annexin V-FITC Apoptosis Detection Kit (Cat.NO: KGA107) and Cell Cycle Detection Kit (Cat.NO: KGA512) purchased from KeyGEN BioTECH were used to complete the cell cycle experiment and apoptosis evaluation based on the provided protocol. Cells treated with the preferred compound for 24 h were subsequently applied for the FCM examination.

4.3.4. Western blotting

MV4-11 cells in the logarithmic phase were harvested and BCA Protein Assay Kit (Beyotime) was adopted for the calculation of extracted proteins' concentration. After that, SDS-polyacrylamide gel was prepared for protein separation. After transferring the protein strap onto the PVDF membrane, TBST solution of 5% fat-free milk was used to block the non-specific binding sites. Subsequently, primary antibodies against c-myc and β-actin was diluted and used to deal with the PVDF membrane, following the co-incubation of secondary-antibody. Finally, proteins were developed and the gray density was subsequently analyzed using ImagJ and Graphpad software.

4.3.5. In vivo PK study

Compound **58** dissolved in 1% Tween80/water to a concentration of 1 mg/mL, and was given to ICR mice (Male, 180–220 g, n = 3) by gavage administration. Blood samples were collected at 0.25, 0.5, 1, 2, 4, 8, and 24 h after administration (anticoagulant: EDTA-Na2)0.100 mL of solvent of methanol: acetonitrile (1:1, v/v) with internal standard was added to 10 mL of plasma and vortexed thoroughly. It was centrifuged for 5 min, and then 20 mL of the supernatant was mixed with 20 mL of water for analysis. Samples were analyzed by Xevo TQ-S triple quadrupole mass spectrometer (Waters, USA). The ACQUITY UPLC BEH C18 (1.7 mm, 2.0 mm × 50 mm, Waters, USA) was used for the analysis. Gradient elution was applied consisting of 5 mM ammonium acetate aqueous solution containing 0.1% formic acid and acetonitrile

containing 0.1% formic acid. After analyzing the concentrations of these compounds, the value of AUC last, AUCINF_obs and MRTINF_obs were calculated from time - concentration curves in each animal using Phoenix WinNonlin (CERTARA, USA). C_{max} was determined as the maximum plasma concentration, and T_{max} was the time to reach the maximum concentration.

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 data

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