Identification of 3,5-Dimethylisoxazole Derivatives as BRD4 Inhibitors for the Treatment of Colorectal Cancer

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ABSTRACT: Bromodomain-containing protein 4 (Brd4) plays a critical regulatory role in gene transcription that has been recently recognized as a promising strategy for cancer therapy. Based on the BRD4 protein containing two tandem bromodomain structures, BD1 and BD2, we designed and synthesized a series of 3,5-dimethylisoxazole derivative dimers targeting both bromodomains simultaneously to enhance protein binding potency. Among them, compound **22** significantly inhibited the proliferation of colorectal cancer cells HCT116 (IC_{50} = 162 nM), with a 20-fold increase in antiproliferative activity compared to inhibitor **14**. The results of WesternBlot showed that compound **22** could down-regulate c-MYC protein levels and up-regulate HEXIM1 expression and modulate apoptosis through intrinsic pathways. In addition, compound **22** exhibited outstanding antitumor efficacy in the CT-26 tumor mouse model with a tumor suppression rate of 56.1%. Taken together, 3,5-dimethylisoxazole derivative dimer **22** has remarkable protein inhibitory effect and antitumor activity in vitro and in vivo. A protein binding model of compound **22** is being further analyzed, which will facilitate the development of bivalent BRD4 inhibitors and probe the biological function of BRD4.

KEYWORDS: BRD4 inhibitors, colorectal cancer, bivalent ligands, epigenetics

The bromodomain (BRD)-containing protein plays a critical role in recognizing epigenetic acetylated lysine marks on histones.¹ In humans, 46 proteins contain bromodomains segregated into eight groups.² Among them, the bromodomain and extra-terminal domain (BET) family of proteins, consisting of BRD2, BRD3, BRD4, and testis-specific BRDT members share a common domain architecture, two N-terminal bromodomains and an extra-terminal domain.^{3–5} It has been proposed that BET proteins are crucial for cancer pathogenesis via modulating diverse transcriptional programs.^{6–8}

BRD4 has been identified highly enriched in large clusters of enhancers that drive the expression of genes involved in the pathogenesis of cancer.^{9,10} Recent studies have shown that BRD4 directly regulates the expression of MYC genes as a therapeutic target in cancer.^{11,12} Further, BRD4 protein plays an essential role in recruiting the positive transcription elongation factor p-TEFb complex (CDK9 and cyclin T1), which is required for RNA polymerase II-dependent transcription elongation.^{13–15} Recently, a number of BRD4 inhibitors which target BET family proteins for cancer therapy have been reported, including JQ1 (**1**), OTX015 (**2**), ABBV075 (**8**), etc. (Figure 1).^{16–19} To our knowledge, most of the reported BRD4 inhibitors do not show selectivity for individual family members and bind BRD4 protein in a monovalent fashion.²⁰ Almost at the same time, Tanaka and Waring disclosed the first bivalent BRD4 molecule inhibitor, MT1 (**6**) and AZD5153 (**7**), respectively, exhibiting promising biological activities in vitro and in vivo.^{17,20}

Figure 1. Selected examples of known BET inhibitors.

MT1 is an intramolecular bivalent BRD4 binder with more potent proliferation activity than the corresponding monovalent antagonist, JQ1.¹⁷ However, AZD5153, a clinical candidate bivalent BET inhibitor, is a single inhibitor molecule containing two acetyl lysine mimicking moieties simultaneously engaging both bromodomains.²¹ Given that no bivalent BRD4 inhibitors are currently available in clinics or in the market, developing

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bivalent BRD4 inhibitors with diverse core structures and excellent pharmacological profile is essential for therapeutic studies.

Here we detailed the identification and characterization of a new class of bivalent BET inhibitors containing the 3,5-dimethylisoxazole motif. We found that compound **22** was a highly potent and efficacious bivalent inhibitor against colorectal cancer, which was also stronger than its monovalent counterparts.

Our previously reported quinoxalinone BRD4 inhibitor, compound **5i**, effectively inhibited BRD4 protein (IC_{50} = 73 nM).²² The docking model showed that the ethyl in compound **5i** extended into the solvent region outside the pocket. Compound **5i** had no room for optimization and was not suitable for further development as a lead compound (Figure 2).

Figure 2. Design and modification strategy of our lead compound **14**.

We used the skeleton hopping technique to replace the quinoxalinone with a quinazolinone motif to obtain compound **14** with BRD4 protein activity IC_{50} = 32 nM, which was remarkably higher than that of compound **5i**. Molecular docking models implied that the 3-nitrogen atom of compound **14** faced the extrapocket solvent region, which provided an advantage for further design of bivalent ligand (Figure 3). Therefore, the above results suggested that compound **14** deserved further development for the design of dimerized BRD4 inhibitors.

Figure 3. Docking pose for compound **14** without specific chirality (yellow, A) in BRD4(1) protein. (B) Compounds **14** without specific chirality (yellow) and **5i** (green) were superimposed in the docking models (PDB code 2YEL).

First, we selected carbon chains, polyethylene glycol chains, and triazole polyethylene glycol chains as the linkers and obtained dimeric BRD4 inhibitors **15–22** by connecting two molecules of compounds **14**. Interestingly, compounds containing triazole as linker (**21**, **22**) exhibited outstanding activities against BRD4 protein, which was greatly superior to that of other types of dimer inhibitors. Subsequently, compounds **28–32** were designed and synthesized by further optimization, including the removal of methyl group, the introduction of carbon chains, etc. It was noteworthy that, compared to compounds **21** and **22**, compounds **28–32** showed considerably more potent BRD4 inhibition activities (IC_{50} < 2.1 nM).

Compounds **15–22** were synthesized as shown in Scheme 1. Bromination of the starting material 2-aminobenzophenone,

followed by amine reduction with *tert*-butyl subsulfinate, led to the formation of intermediate **10**. Then, compound **10** was reduced using sodium borohydride to give **11**, and subsequent treatment with triphosgene afforded the quinazolinone **12**, which was then subjected to methylation to give the key intermediate compound **13**. Finally, the target compound **14** was prepared by Suzuki cross-coupling reaction to introduce the 3,5-dimethylisoxazole group. Compound **14** was treated with 1,4-dibromobutane, 1,5-dibromopentane, 1,6-dibromohexane, and 1,10-dibromodecane, respectively, to gain compounds **15–18**. In addition, compound **14** reacted with triethylene glycol ditoluenesulfonate or tetraethylene glycol ditoluenesulfonate to obtain compounds **19–20**. Compounds **21–22** were yielded by the reaction of compound **14** with 3-bromopropyne, followed by a click reaction with N3-PEG1-N3 or N3-PEG3-N3.

The synthesis of compounds **28–32** was described in Scheme 2. Compound **9** was used as the starting material to obtain compound **23** via Suzuki cross-coupling reaction, which was treated with *tert*-butylenesulfinate to produce intermediates **24**. Then, compound **24** was reduced by sodium borohydride to produce compound **25**, followed by substitution via 3-bromopropyne to generate intermediate **26**. The key intermediate **27** was prepared by treatment of compound **26** with triphosgene. Lastly, intermediate **27** was reacted with different linkers to obtain the target compounds **28–32** via click reaction.

In the initial phase of our study, compounds **15–22** were designed and synthesized using compound **14** as the lead compound. The results of Alphascreen BRD4 assay demonstrated that most of the compounds showed more potent activities than the lead compound **14**. Among them, the compounds (**21**, **22**) containing triazoles displayed prominent inhibitory activities with BRD4(1) IC_{50} = 4.2 nM and 7.7 nM, respectively. The BRD4 inhibitory activities of compounds **19** and **20** using polyethylene glycol as the linker were more valid than that of analogs with other linkers. Furthermore, compounds **15–18** with carbon chains as the linker exhibited relatively weak inhibitory activities, except for compound **15** (Table 1).

Based on the above activity results, we further optimized the bivalent inhibitors (**21**, **22**) using linkers containing a triazole group. The methyl on the nitrogen atom was also removed, and the polyethylene glycol chain was replaced by a carbon chain to produce compounds **28–32**. The BRD4(1) protein activities of compounds **28–32** (BRD4(1) IC_{50} < 2.1 nM) were much superior to those of other bivalent compounds (Table 1).

The binding model of compound **14** and BRD4BD1 protein was predicted, and the molecular docking model showed that the 3,5-dimethylisoxazole group in compound **14** could bind to the Kac pocket of the BRD4 bromodomain as a Kac mimic. The nitrogen atom in 3,5-dimethylisoxazole formed a hydrogen bond to the key amino acid ASN140 located in the Kac pocket, anchoring compound **14** in the Kac pocket. The benzene ring at position 4 extended into the WPF hydrophobic pocket formed by Trp81, Pro82, and Phe83. In addition, the nitrogen atom at the 3-position of compound **14** faced the solvent region outside the pocket, providing a modification site for postmodification optimization. Compound **14** overlapped perfectly with our previously announced compound **5i**. The monomer moiety (compound **14**) of bivalent ligand **22** could manage to bind to the BRD4Kac pocket, which was similar to the binding pattern of single-molecule compound **14**. 3,5-Dimethylisoxazole filled the Kac pocket forming a hydrogen bond with Asn140, and the

Scheme 1^a

^aReagents and conditions: (a) NBS, DCM, 0 °C; (b) *tert*-butanesulfinamide, tetraethyl titanate, THF, reflux; (c) NaBH₄, THF, H₂O, r.t.; (d) bis(trichloromethyl) carbonate, THF, r.t.; (e) dimethylsulfate: Cs₂CO₃, acetonitrile, r.t.; (f) phenylboronic acids, Pd(P(Ph)₃)₄, Na₂CO₃, N₂, H₂O/EtOH/toluene, 80 °C; (g) diverse linkers, NaH, DMF, 0 °C to r.t.; (h) 3-bromopropyne, Cs₂CO₃, DMF, 0 °C to r.t.; (i) diverse linkers, CuSO₄, sodium erythorbate, MeOH, H₂O, r.t.

Scheme 2^a

^aReagents and conditions: (a) phenylboronic acids, Pd(P(Ph)₃)₄, Na₂CO₃, N₂, H₂O/EtOH/toluene, 80 °C; (b) *tert*-butanesulfinamide, tetraethyl titanate, THF, reflux; (c) NaBH₄, THF, H₂O, r.t.; (d) 3-bromopropyne, NaH, DMF, 0 °C to r.t.; (e) bis(trichloromethyl) carbonate, THF, r.t.; (f) diverse linkers, CuSO₄, sodium erythorbate, MeOH, H₂O, r.t.

4-position benzene ring extended into the WPF hydrophobic pocket formed by Trp81, Pro82, and Phe83. In addition, the triazole moiety formed weak π - π stacking interactions with the 4-position benzene ring located in the WPF shelf and Trp81. Another monomer moiety connected with nitrogen atoms at the 3-position via a linker extended into the outer solvent region of the BRD4Kac pocket (Figure 4). Moreover, we speculated that

ligand **22** could bind to another bromodomain in tandem according to a previous report, enhancing protein binding activity.¹⁷

Considering that the above compounds have noticeable BRD4 protein inhibitory activities, we evaluated the anti-proliferative activities of all compounds **14–22** and **28–32** at the cellular level. Primary screening tests were performed on

Table 1. BRD4(1) Inhibitory Activities of Compounds 14–22 and 28–32

Compound No.	Linker	R ₁	BRD4(1) 0.5 μM (%)	BRD4(1) IC ₅₀ ^{a,b} μM
15		Me	100	0.013±0.011
16		Me	91	0.056±0.003
17		Me	78	0.042±0.012
18		Me	9	NT ^c
19		Me	98	0.031±0.008
20		Me	98	0.019±0.010
21		Me	100	0.0042±0.0012
22		Me	100	0.0077±0.0006
28		H	100	<0.0021
29		H	100	<0.0021
30		H	100	<0.0021
31		H	100	<0.0021
32		H	100	<0.0021
14			100	0.032±0.006
JQ1			100	0.042±0.004
ABBV-075			100	0.0025±0.0011

^aData are expressed as the mean ± SD from the dose–response curves of at least three independent experiments. ^bIC₅₀ values are shown as mean values of at least three determinations unless specified otherwise. ^cNot tested.

Figure 4. Docking pose for compound 22 without specific chirality (purple, A) in BRD4(1) protein. Compounds 14 without specific chirality (yellow) and 22 without specific chirality (purple) were superimposed in the docking models (PDB code 2YEL).

pancreatic cancer cells BxPc3, Mia-capa2, and colorectal cancer cells HCT116, respectively.^{23,24} As a result, it was found that both pancreatic and colon cancer cells were sensitive to most compounds.

In particular, compounds 17 and 22 exhibited exceptional activities against all three tumor cell lines, with an ideal inhibition rate of 59.7% and 83.6% at 2 μM against colon cancer cell HCT116, respectively. However, compound 14 only exhibited a 14% inhibition rate against HCT116 at 2 μM. Therefore, antiproliferative activities of compounds 17 and 22 against HCT116 cells were further tested at gradient

concentrations. The results indicated that compounds **17** and **22** had antiproliferative potency with an IC_{50} value of 1.1 μ M and 0.16 μ M against HCT116 cells, respectively (Table 2). Both

Table 2. Anti-proliferation Activities of Compounds 14–22 and 28–32

Compound No.	BxPc3 2.5 μ M (%)	Mia-capa2 2.5 μ M (%)	HCT116 2.0 μ M (%)	HCT116 IC_{50} (μ M) ^{a,b}
15	46.4	68.2	59.3	NT ^c
16	27.9	71.3	52.0	NT
17	44.0	70.9	59.7	1.101 $\pm$ 0.023
18	45.2	65.2	48.8	NT
19	7.1	67.3	43.4	NT
20	42.1	73.4	35.3	NT
21	23.2	64.0	49.6	NT
22	61.3	68.4	83.6	0.162 $\pm$ 0.012
28	53.0	62.2	60.3	NT
29	42.2	66.4	60.4	NT
30	21.8	63.2	60.5	NT
31	52.1	54.4	55.6	NT
32	37.1	57.2	60.1	NT
14	11.2	59.1	14.0	3.341 $\pm$ 0.011
JQ1	9.1	56.2	23.2	4.212 $\pm$ 0.015
ABBV-075	36.8	64.3	69.5	0.550 $\pm$ 0.009

^aData are expressed as the mean $\pm$ SD from the dose–response curves of at least three independent experiments. ^b IC_{50} values are shown as mean values of at least three determinations unless specified otherwise. ^cNot tested.

compounds **17** and **22** exhibited significantly more potent antiproliferative activities than the lead compound **14**, and particularly the inhibitory activity of compound **22** was 20-fold more than that of compound **14**. In addition, the antiproliferative activity of compound **22** was better than that of the positive compounds JQ1 and ABBV-075. Thus, our next task was to further investigate the biological mechanisms of compound **22**.

The cell cycle arrest potency of compound **22** was evaluated in HCT116 cells. Cell cycle arrest experiments revealed that compound **22** dramatically increased the percentage of HCT116 cells in the G0/G1 phase at 0.16 μ M for 48 h. The G0/G1 phase arrest effect of compound **22** was significantly more than that of the lead compound **14** (Figure 5).

We further explored the potency of compound **22** on clone-forming against HCT116 cells. The results showed that **22** significantly inhibited the proliferation of HCT116 cells in a concentration-dependent manner for 12 days. In addition, the effect of compound **22** on suppressing the proliferation of

Figure 5. Effects of compounds **22** and **14** on the cell cycle arrest of HCT116 cells.

HCT116 cells notably exceeded the lead compound **14** (Figure 6).

Figure 6. Plate clone-forming effects of compounds **22** and **14** in HCT116 cell lines.

Because compound **22** exhibited excellent biological activity at both protein and cellular levels, we moved on to understand its intrinsic mechanism of inducing apoptosis in HCT116 cells. After treatment of HCT116 cells with compound **22** for 48 h, the contents of relevant proteins such as Bcl-2, HEXIM1, BRD4, and c-Myc were determined by WesternBlot. The results showed that compound **22** was able to significantly down-regulate c-Myc protein expression and decreased Bcl-2 protein expression in HCT116 cells compared to lead compound **14**. In addition, compound **22** remarkably up-regulated the expression level of HEXIM1 protein and enriched BRD4 protein, consistent with the findings reported in previous literature (Figure 7).^{25,26} Moreover, compound **22** could activate

Figure 7. Effects of compounds **22** (1 μ M), **14** (1 μ M), and ABBV-075 (1 μ M) on protein expression in HCT116 cancer cells.

downstream apoptotic effectors such as caspase 3 (Figure S1). Mechanism studies indicated that compound **22** promoted apoptosis in HCT116 cells via an intrinsic pathway, which was conducive to further study of the mechanism of BET protein in cells.

Based on the inhibitory activity of **22** against human colorectal cancer cell line HCT116, the effect of compound **22** on tumor growth inhibition in vivo was further investigated in the CT-26 (mouse colorectal cancer cell line) model mouse. The results showed that the intraperitoneal administration of compound **22** at 20 mg/kg for 14 days prominently inhibited tumor growth in CT-26 model mice, and the tumor suppression rate was 56.1%, significantly superior to 11.5% of the lead compound **14** (Figures 8 and S2). There were also no serious toxic side effects in the tumor mice and no obvious weight loss in the rats, confirming the feasibility of bivalent BET inhibitors. In addition, we conducted immunohistochemical analysis of tumor tissue in sections. The results demonstrated that compound **22** notably promoted the expression of HEXIM1 and decreased the expression of c-Myc and Ki67.^{27–29} Meanwhile, HE staining

Figure 8. Anticancer effects of compound 22 in CT-26 xenograft model of colorectal cancer.

Figure 9. Immunohistochemistry of CT-26 tumor tissues treated with compound 22.

implied the promotion of morphological changes in cell necrosis (Figure 9). In addition, the pharmacokinetic parameters of compound 22 were predicted using SwissADME and Discovery Studio 3.0, which indicated that compound 22 showed poor absorption and bioavailability (Figure S3 and Table S1).^{30,31}

Taken together, based on the BRD4 inhibitor compound 5i previously reported by our group, we designed and synthesized the lead compound 14 utilizing the skeleton-hopping principle, and the BRD4(1) protein activity of compound 14 significantly increased with an IC_{50} value of 32 nM. Since the structural characteristics of BRD4 contained two tandem bromodomains, we designed and synthesized a series of bivalent BRD4 inhibitors by using different types and lengths of linkers for connecting two molecules 14. Among them, compound 22 exhibited excellent BRD4 protein inhibitory activity with an IC_{50} value of 7.7 nM and showed significant antiproliferative activity with an IC_{50} value of 162 nM against HCT116 cells. Meanwhile, compound 22 resulted in cell cycle arrest at the G0/G1 phase. WesternBlot results indicated that compound 22 interfered with apoptosis via intrinsic pathways. CT26 mouse model in vivo experiments implied that compound 22 effectively inhibited tumor growth in vivo with a tumor suppression rate of 56.1%. In addition, immunohistochemistry results showed that compound 22 achieved antitumor effects in vivo by inhibiting the BRD4 signaling pathway and thus might be a powerful chemical probe for further investigation of BRD4 bromodomains.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acsmchemlett.0c00294>.

Compound synthesis, spectroscopic characterization, and biology experimental procedures (PDF)

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Author Contributions

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Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS

Brd4, bromodomain-containing protein 4; HEXIM1, hexamethylene bis-acetamide inducible protein; MYC, myelocytomatosis oncogene; pTEFb, positive transcription elongation factor b; BET, bromodomain and extraterminal domain

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