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# Targeting Brd4 for cancer therapy: inhibitors and degraders

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Bromodomain-containing protein 4 (Brd4) plays an important role in mediating the expression of genes involved in cancers and non-cancer diseases such as inflammatory diseases and acute heart failure. Inactivating Brd4 or downregulating its expression inhibits cancer development, leading to the current interest in Brd4 as a promising anticancer drug target. Numerous Brd4 inhibitors have been studied in recent years and some of them are currently in various phases of clinical trials. Recently, selective degradation of target proteins by small bifunctional molecules (PROTACs) has emerged as an attractive drug discovery approach owing to the advantages it could offer over traditional small-molecule inhibitors. A number of Brd4 degraders have been reported and showed more efficient anticancer activities than just protein inhibition. In this review, we will discuss recent findings in the discovery and development of small-molecule inhibitors and degraders that target Brd4 as a potential anticancer agent.

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## Introduction

Reversible lysine acetylation plays an essential role in the epigenetic regulation of chromatin structure and transcription of genes *via* modification of histone proteins and transcription factors. These changes in gene expression are modulated by three categories of epigenetic regulatory proteins, which are commonly known as "writers", "erasers", and "readers". Histone acetyltransferases (HATs) function as "writers" to acetylate lysine residues on histone tails,[1] while histone deacetylases (HDACs) act as "erasers" to remove the acetyl group from acetylated lysine (Kac).[2,3] The third kind of epigenetic regulatory protein is the bromodomain family of proteins that selectively bind to acetylated lysines, thus functioning as "readers" of the lysine acetylation state.[4]

The bromodomain and extra-terminal (BET) family is a subset of 46 bromodomain-containing proteins found only in the human genome.[5] BET proteins are composed of four proteins, namely bromodomain-containing protein 2 (Brd2), Brd3, Brd4 and bromodomain testis-specific protein (BrdT).[6] Each BET family member contains tandem N-terminal bromodomains (BD1 and BD2) and an extra C-terminal domain (ET) exhibiting high levels of sequence conservation.[7] Each bromodomain of Brd4 shares a conserved fold comprising a left-hand bundle of four antiparallel α helices (αA, αB, αC and αZ) linked by two hydrophobic loop structures, namely the ZA loop formed between the αZ and αA helices and the BC loop formed between the αB and αC helices. The loop regions of Brd4 BD1 (Brd4(1)) and BD2 (Brd4(2)) diverge slightly in sequence and length which contributes to acetyllysine binding specificity.[8] Cocrystal structures between Brd4(1) and peptidic substrates show that helices αB and αC and the ZA loop form a hydrophobic Kac-recognition pocket at the helical bundle terminus. The top of the pocket usually contains a well-conserved asparagine residue 140 (Asn140), which engages in a direct hydrogen bonding to the Kac. Simultaneously, a second hydrogen bond is formed between an acetyl carbonyl oxygen atom and the phenol of a conserved Tyr97 *via* a structured water molecule. A conserved hydrophobic region, the WPF shelf, formed among the ZA loop, BC loop and αZ, is also important for Brd4 binding affinities.[6,9] Brd4 regulates gene expression through its ability to bind to Kac residues of histone tails, followed by recruiting the positive transcription elongation factor b (P-TEFb) to phosphorylate RNA polymerase II (RNA Pol II).[10]

Deregulation of BET proteins, in particular Brd4, has been implicated in the development of diverse diseases, especially cancers.[11] Zuber *et al.* demonstrated that Brd4 played an important role in maintaining c-Myc expression to promote aberrant self-renewal of AML cells. Knockdown of Brd4 using shRNAs or pharmacologic inhibition of Brd4 with a small-molecule inhibitor resulted in induction of terminal differentiation and elimination of leukemia stem cells and showed

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potent anti-leukemic effects in a variety of human AML cell lines and primary patient-derived cells.[12,13] Hunter and colleagues identified *Brd4* as an inherited susceptibility gene that robustly predicted progression, metastasis and survival for breast cancer. Brd4 dynamically regulated breast cancer metastasis through modulation of the extracellular matrix gene expression.[14,15] Malley *et al.* demonstrated that BET protein inhibition potently suppressed the growth of tamoxifen-resistant breast cancer cells. Co-treatment with BET inhibitor and the ER degrader fulvestrant in a tamoxifen-resistant breast cancer xenograft mouse model displayed a strong long-lasting anticancer effect.[16] In NSCLC tissues and NSCLC cell lines with higher invasion and metastasis potentials, Brd4 expression was significantly up-regulated. Suppression of Brd4 expression in NSCLC cell lines impaired cell invasion, inhibited cell proliferation, and accelerated cell apoptosis. More importantly, a high level of Brd4 was closely correlated with the poor prognosis of NSCLC patients.[17] Brd4 was highly over-expressed in primary and metastatic melanoma tissues and essential for melanoma tumor growth. Treatment with Brd4 inhibitors rapidly down-regulated key cell-cycle genes, including *SKP2*, *ERK1* and c-*Myc*, and strongly attenuated melanoma cell proliferation *in vitro* and tumor growth and metastatic behavior *in vivo*. Individual silencing of Brd4 mostly recapitulated the potent anti-leukemic effects of Brd4 inhibitor-mediated suppression. Notably, Brd4 inhibitor treatment remained robustly effective against *BRAF* or *NRAS* mutant melanoma cells.[18] Ayad *et al.* demonstrated that Brd2 and Brd4 were significantly elevated in glioblastoma (GBM). Depletion of Brd4 using siRNA in GBM cells significantly reduced GBM cell proliferation by arresting cell cycle progression at the $G_1$/S phase. Similarly, treatment with the BET protein inhibitor **I-BET151** inhibited GBM cell proliferation *in vitro* and *in vivo*.[19] In castration-resistant prostate cancer (CRPC), knockdown of Brd2, 3 and 4 led to significant inhibition of cell proliferation and invasion, phenocopying the BET bromodomain inhibition treatment. In CRPC xenograft mouse models, BET inhibitors showed powerful anticancer activity and were more efficacious than direct AR antagonists.[20]

In view of the intimate link between Brd4 expression and cancers, Brd4 has been considered as a promising therapeutic target in many malignancies.[21,22] Significant efforts have been made to develop pharmacological inhibitors of Brd4, and a number of Brd4 inhibitors have progressed to clinical and preclinical evaluation.[23,24] Recently, Brd4 degraders based on protein proteolysis-targeting chimera (PROTAC) technology have emerged as a novel approach for the epigenetic therapy of cancers and displayed exciting antitumor efficiency. Herein, we mainly focus on discussing recent advances in small-molecule inhibitors and degraders of Brd4 developed for cancer therapy.

# 1 Brd4 inhibitors

Since the first Brd4 inhibitor (+)-**JQ1** was developed in 2010, numerous Brd4 inhibitors have been discovered in the past few years. According to the interaction mode between BDs and inhibitors, there are two classes of Brd4 inhibitors: monovalent and bivalent. Monovalent Brd4 inhibitors bind to each bromodomain of Brd4 protein separately. In contrast, bivalent Brd4 inhibitors are capable of engaging both bromodomains simultaneously within Brd4. The most widely studied Brd4 inhibitors are monovalent Brd4 inhibitors, which are mainly divided into the following eight categories according to the similarity of their chemical structures: triazoloazepine derivatives, isoxazole derivatives, pyridine derivatives, tetrahydroquinoline derivatives, triazolopyrazine derivatives, 4-acyl pyrrole derivatives, 2-thiazolidinone derivatives and others (Table 1). Moreover, several Brd4 inhibitors with disclosed structure have been enrolled into different phases of human clinical trials.[25]

## 1.1 Monovalent Brd4 inhibitors

**1.1.1 Triazoloazepine-based Brd4 inhibitors.** Inspired by the observation from Mitsubishi Pharmaceuticals that simple thienodiazepines possessed binding activity for Brd4, Bradner *et al.* reported the first potent and selective BET protein inhibitor (+)-**JQ1** (**1**) containing a thieno-triazolo-1,4-diazepine scaffold (Fig. 1). The co-crystal structure showed that (+)-**JQ1** entirely occupied the acetyl-lysine binding pocket by forming a hydrogen bond between the methyltriazole moiety and the conserved asparagine140. (+)-**JQ1** strongly inhibited Brd4(1) with an $IC_{50}$ value of 77 nM and a $K_d$ value of 50 nM, determined by alpha-screen and isothermal titration calorimetry (ITC) assays, respectively. In contrast, its enantiomer (−)-**JQ1** showed no significant interaction with any BET proteins. In several NUT midline carcinoma xenograft models, remarkable tumor regression and prolonged overall survival were observed after treatment with (+)-**JQ1** for 18 days at a well-tolerated dose. (+)-**JQ1** also showed robust antitumor efficacy in several other xenograft models including multiple myeloma and AML.[11,26] The development of (+)-**JQ1** revealed novel insights into the therapeutic potential of inhibiting Brd4 and aroused an upsurge of research on Brd4 inhibitors.

Exploring the effect of the substituent on the chain moiety of (+)-**JQ1** led to a number of thienodiazepine-based Brd4 inhibitors. The representative compounds **2–6** are shown in Fig. 1. Among them, the two best known are **OTX015** (**2**)[27–29] and **TEN-010** (**3**),[30] which have entered a phase I clinical trial for the treatment of hematological malignancies and other cancers. Compound **4**, patented by Tensha Therapeutics,[31] and compound **5**, patented by Bayer,[32] exhibited potent inhibition against Brd4(1) with an $IC_{50}$ value of 0.43 nM and 27 nM, respectively. Compound **6** was generated from a phenotypic drug discovery study on thienodiazepine derivatives and showed impressive Brd4(1) inhibitory activity with an $IC_{50}$ value of 34 nM. In addition to robust *in vivo* antitumor efficacy with a tumor growth inhibition (TGI) of 80% at a dose of 10 mg $kg^{-1}$ twice a day, compound **6** also displayed potent immunosuppressive activity in a mouse collagen-induced arthritis model.[33]

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**Table 1** Overview of small-molecule Brd4 inhibitors

| | Structure features | Compounds | Compounds in clinical trials | Clinical phase | NCT identifier | Ref. |
|---|---|---|---|---|---|---|
| Monovalent Brd4 inhibitors | Triazoloazepines | **1–15** | **2 (OTX015)** | I | NCT02698189<br>NCT02698176<br>NCT01713582<br>NCT02259114 | 27–29, 128 |
| | | | **3 (TEN-010)** | I | NCT02308761 | 30 |
| | | | **10 (GSK525762, I-BET762)** | I/II | NCT01943851<br>NCT01587703<br>NCT02964507 | 20, 35, 36 |
| | Isoxazoles | **16–42** | **19 (CPI-0610)** | I | NCT01949883<br>NCT02157636<br>NCT02158858 | 41 |
| | | | **28 (I-BET151)** | I | NCT02630251 | 47, 48 |
| | | | **36 (PLX51107)** | I | NCT02683395 | 53 |
| | | | **38 (INCB0543294)** | I/II | NCT02431260 | 55 |
| | Pyridines | **43–55** | **46 (ABBV-075)** | I | NCT02391480 | 60, 61 |
| | Tetrahydroquinolines | **56–62** | | | | |
| | Triazolopyrazines | **63–69** | **BI 894999**[a] | I | NCT02516553 | |
| | 4-Acyl pyrroles | **70–74** | | | | |
| | Thiazolidinones | **75–77** | | | | |
| | Others | **78–85** | **82 (BMS-986158)** | I/II | NCT02419417 | 86 |
| Bivalent Brd4 inhibitors | Triazolopyridazines | **86–89** | **89 (AZD5153)** | I | NCT03205176 | 94, 95 |
| | Triazoloazepines | **90** | | | | |

[a] Specific chemical structure is not disclosed.

A parallel but independent study from GlaxoSmithKline described a series of compounds with different substitutions on benzodiazepine as potent Brd4 inhibitors (Fig. 2).[34] Compound 7 was initially developed as an ApoA1 upregulator with an $EC_{170}$ of 0.22 μM ($EC_{170}$ is the concentration of compound resulting in a 70% increase in luciferase activity). However, subsequent research studies confirmed that the ApoA1 upregulation was caused by directly targeting BET proteins with a $pIC_{50}$ of 6.3 for Brd4. Various chemical modifications on compound 7 were carried out to explore more potent BET inhibitors. Compound 8 with potent Brd4 inhibition ($pIC_{50}$ of 6.4) was generated; however, the remarkable Brd4 inhibition potency was counteracted by the fact that compound 8 was prone to undergo a ring-opening and hydrolysis reaction in an acidic aqueous solution ($T_{1/2}$ = 0.23 h at pH 2). It was found that compound 8 bound to Brds in a highly stereospecific manner, as exemplified by the (*R*)-enantiomer (+)-8, which is 251 times more potent than the (*S*)-enantiomer

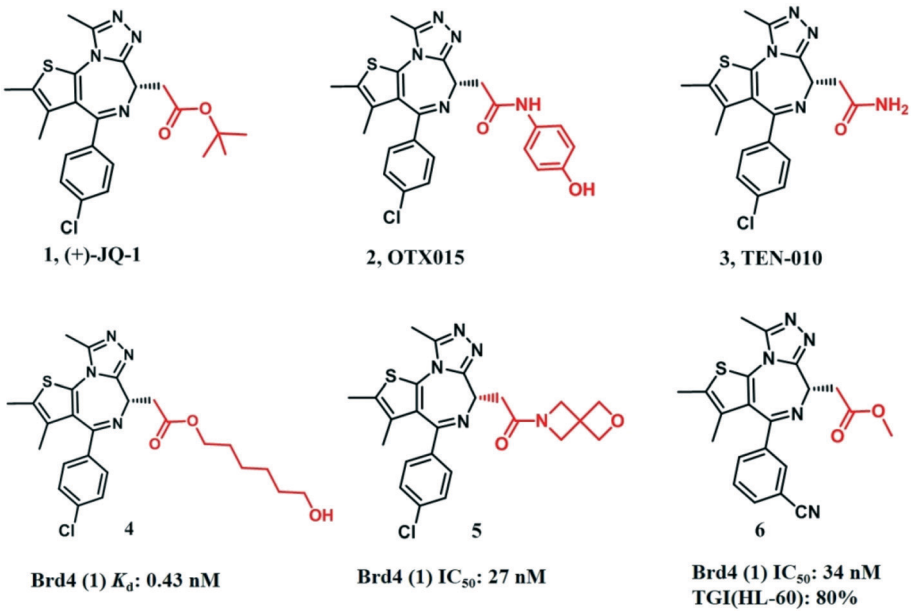


**Fig. 1** Reported thienotriazolodiazepine-based Brd4 inhibitors.

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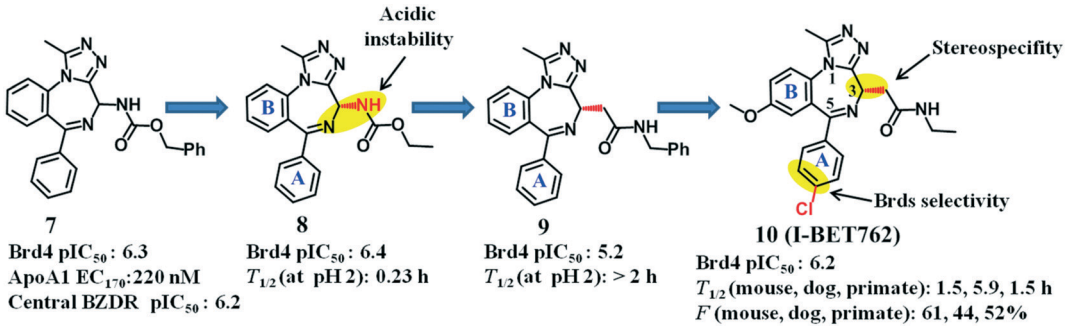


Fig. 2 Discovery and development of Brd4 inhibitor **I-BET762**.

against Brd4. Compound **9** with improved acidic stability ($T_{1/2} > 2$ h at pH 2) was achieved by changing the carbamate group of compound **8** to an amide, but a 10-fold decrease in inhibition for Brd4 was observed.

Further modification of acid-stable compound **9** was focused on improving its potency and physiochemical properties. Substitution at the *p*- or *m*-position of ring A abolished the off-target GABA receptor activity, which was derived from the 1,4-benzodiazepine motif found in many marketed GABA receptor-positive allosteric modulators. Higher potency and better drug-like properties were obtained by introducing a methoxyl substituent at the 8 position of the B ring and replacing the benzyl group with alkyl groups. All the favored modifications were incorporated into compound **9** and created a more druggable potent Brd4 inhibitor, compound **10** (namely **I-BET762** or **GSK525762**), with excellent physicochemical and pharmacokinetic properties. **I-BET762** displayed enhanced metabolic stability, good on-target Brd4 activity, excellent solubility, good tissue distribution, and good oral bioavailability (44–61% in mice, dogs, and primates). Potent anticancer activity was observed for **I-BET762** as an oral agent in several xenograft models.[20,35] **I-BET762** is currently under evaluation in a phase I/II clinical trial for the treatment of different cancers.[36]

By replacing the amide at the 3-position of **I-BET762** with 1,3,4-oxadiazole, Bayer disclosed compound **11** (Fig. 3).[37] Compound **11** showed potent anti-Brd4(1) activity ($IC_{50}$ = 20 nM) and was very active against MOLM-13 cancer cells ($EC_{50}$ = 140 nM). Bayer also filed a patent for compounds **12** and **13**, which were featured with triazolopyrazolodiazapine and triazolopyrrolodiazapine scaffolds, respectively.[38] Compounds **12** and **13** were potent against Brd4(1) ($IC_{50}$ = 140 and 20 nM, respectively) and showed marked growth inhibition in multiple cell lines. Interestingly, researchers from Bayer found that

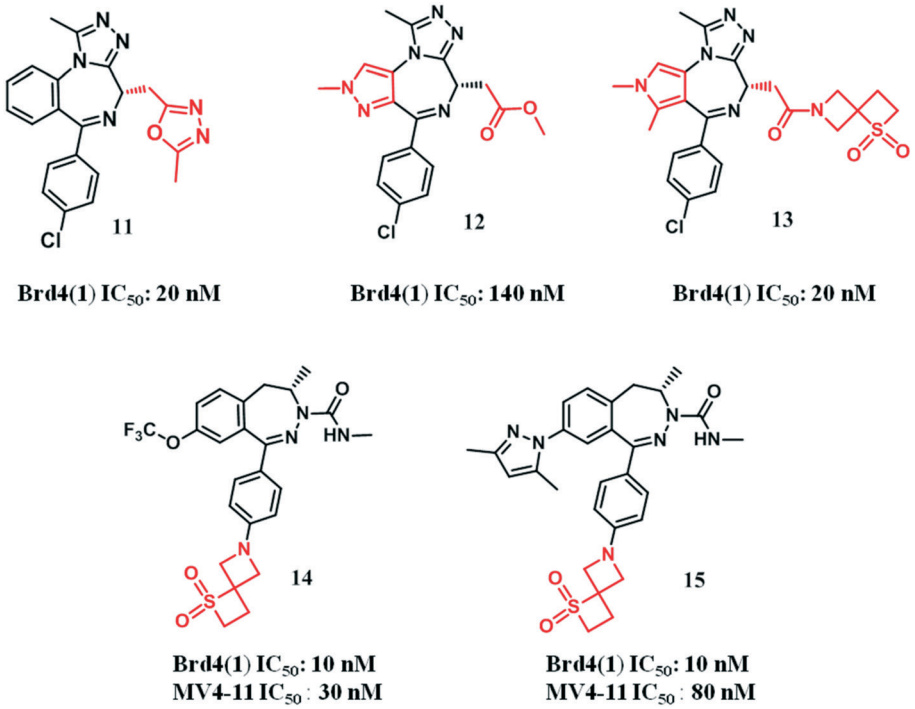


Fig. 3 Chemical structures of compounds **11–15**.

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isomeric benzodiazepine analogs without triazoles retained potency against Brd4.[39] Two representative compounds, **14** and **15**, demonstrated the same potent Brd4(1) inhibition with $IC_{50}$ values of 10 nM and inhibited the growth of MV4-11 cancer cells with $IC_{50}$ values less than 100 nM.

**1.1.2 Isoxazole-based Brd4 inhibitors.** A fragment screen study from Albrecht *et al.* identified amino-isoxazole **16** as a weak Brd4(1) inhibitor with an $IC_{50}$ of 33 μM. The cocrystal structure of Brd4(1) with compound **16** revealed that compound **16** could bind to Brd4(1) in a mode similar to that of (+)-**JQ1**. Inspired by this finding, novel isoxazole azepine compound **17** was developed by hybridizing the isoxazole motif with an azepine scaffold, which displayed potent Brd(1) inhibition ($IC_{50}$ = 290 nM) and moderate c-Myc suppression activity in Raji cells ($IC_{50}$ = 2.1 μM) (Fig. 4).[40] Further modification of this compound led to **18**, which contained an amino group in place of the *tert*-butoxy group in compound **17**.[41] Compound **18** demonstrated around 10-fold better potency than compound **17** against Brd4(1) ($IC_{50}$ = 26 nM) and c-Myc ($IC_{50}$ = 140 nM). Compound **18** exhibited favorable pharmacokinetic profiles in rats with a $T_{1/2}$ of 1.4 h and a bioavailability of 31%. P.O. dosing with compound **18** (10, 30, and 100 mg $kg^{-1}$) dose-dependently inhibited c-Myc mRNA expression *in vivo*, with an up to 75% reduction in c-Myc levels in the tumor at 4 h after a 100 mg $kg^{-1}$ dose. With the goal of avoiding the potential issue of metabolic instability, the thiophene ring in **18** was replaced with a more metabolically stable phenyl ring, and clinical candidate compound **19** (**CPI-0610**) was gained.[41] Compound **19** was proved to have similar potency to **18** against Brd4 and more desirable pharmacokinetic parameters. *In vivo*, oral treatment of an MV4-11 xenograft model with compound **19** caused substantial suppression of tumor growth with a maximum of 80% TGI at a dose of 30 mg $kg^{-1}$ twice daily and showed no obvious body weight loss. In the same model, co-treatment with compound **19** (10 mg $kg^{-1}$ subcutaneously twice daily) and doxorubicin (2 mg $kg^{-1}$ intravenously twice weekly) for 28 days resulted in complete inhibition of tumor growth. In patients with heavily pretreated diffuse large B-cell lymphoma and follicular lymphoma, treatment with compound **19** led to meaningful antitumor effects. Introducing an acetamide-substituted pyrazole to the 8-position of compound **19** produced compound **20**.[42] Compound **20** was potent against Brd4(1) ($IC_{50}$ = 17 nM) and c-Myc in MV4-11 cells ($IC_{50}$ = 32 nM). In comparison to compound **19**, compound **20** showed a longer half-life and greater bioavailability. In MV4-11 tumor xenografts in Balb/c nude mouse models, compound **20** effectively attenuated c-Myc mRNA levels at doses of 5 (50% reduction) and 15 mg $kg^{-1}$ (75% reduction) twice a day.

A parallel but separate work from Heightman's group disclosed the 3,5-dimethylisoxazole motif as a Brd4 inhibitor.[43] It was observed that compound **20** bound in the acetylated-lysine recognition pocket of the Brd4(1) by forming a key hydrogen bond between the oxygen of the dimethylisoxazole and $NH_2$ of the conserved Asn 140 residue. Moreover, the dimethylisoxazole nitrogen atom interacts with the phenol group of Tyr-97 *via* the structured water molecule. The ethyl ether binds within the ZA channel and the methyl group attached to the secondary alcohol binds within the WPF shelf.

Replacing the methyl group with larger substituents such as aromatic rings to enhance the WPF shelf binding gave compounds **21** and **22**. As expected, compared to compound

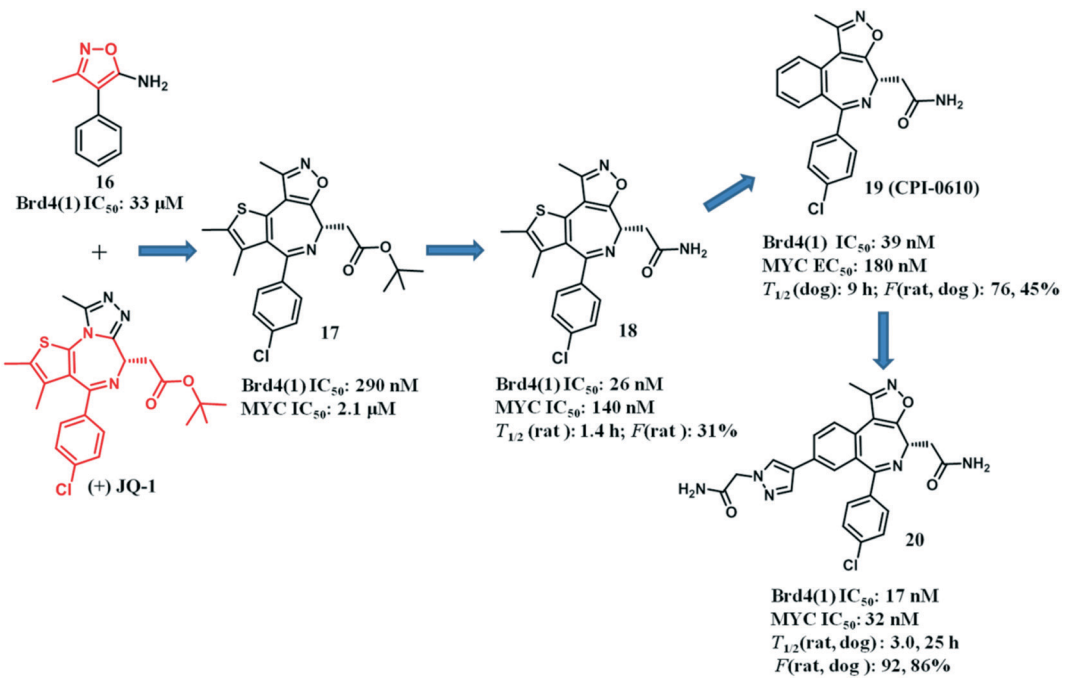


**Fig. 4** Discovery and development of Brd4 inhibitor **CPI-0610**.

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**20**, both **21** and **22** had significant improvement in Brd4 binding affinity with $IC_{50}$ values of 382 and 371 nM, respectively.[44]

Researchers from GSK developed compound **23** through structure-guided rational optimization of the privileged dimethylisoxazole chemotype.[45] Compound **23** had an $IC_{50}$ value of 0.5 μM against Brd4 and showed good anti-inflammatory activity in cellular assays, but poor solubility was observed. With the goal to improve the solubility, introduction of a polar group to the phenyl ring *para* to the isoxazole of **23** led to compound **24**, which had a slightly decreased potency ($IC_{50}$ = 2.6 μM) but remarkable improved solubility as compared to compound **23**.

Compound **25** was achieved using fluorous-tagged multicomponent reactions and showed robust Brd4 binding ability with a $K_d$ of 550 nM and cellular potency ($IC_{50}$ = 724 nM) in Brd4-dependent lines.[46] Notably, compound **25** potently bound to two other bromodomain-containing proteins including TAF1 ($IC_{50}$ = 560 nM) and TAF1L ($IC_{50}$ = 1.3 μM) (Fig. 5).

Most studies on 3,5-dimethylisoxazole-based Brd4 inhibitors have focused on introducing various heteroaromatic scaffolds to the 4-position of isoxazole.

Compound **26**, which was initially developed as an ApoA1 up-regulator by utilizing a high-throughput screen approach, was proved to be a Brd4 inhibitor by researchers at GSK (Fig. 6).[47,48] Optimizations of **26** to compound **27** created a more potent Brd4 inhibitor with an $IC_{50}$ of 794 nM but as well frustrating potent CYP2C9 and CYP3A4 inhibition ($IC_{50}$ = 2.8 and 3.9 μM, respectively). Improvement of selectivity over CYP450 was achieved by eliminating $CONH_2$ and freezing the intramolecular hydrogen bond between C3 and C4 through a cyclization strategy to create a series of imidazolone analogues. Among them, **I-BET151** (**28**) was the most promising Brd4 inhibitor ($IC_{50}$ = 794 nM) with low CYP2C9 and CYP3A4 inhibition ($IC_{50}$ = 9.9 and 9.7 μM, respectively) and favorable PK properties suitable for oral exposure. **I-BET151** demonstrated remarkable potency in two distinct mouse models of murine MLL-AF9 and human MLL-AF4 leukemia.

Compounds **29–31** with tricyclic scaffolds similar to that of **I-BET151** were reported as potent Brd4 inhibitors in a patent from Trillium Therapeutics, with $IC_{50}$ values in the range of 10–21 nM.[49] It was found that the most potent compound **31** ($IC_{50}$ = 10 nM) was quite unstable in both mouse and human liver microsomes due to the active $CH_2$ position which was prone to metabolism.

With the goal of expanding the chemical diversity of Brd4 inhibitors and enhancing the solubility of previously developed isoxazoloquinoline Brd4 inhibitors (**27**, **28**), Mirguet *et al.* synthesized a series of 1,5-naphthyridine derivatives by replacing the benzene ring of compound **20** with a naphthyridine system.[50] Among them, compounds **32** and **33** showed the best $pIC_{50}$ values for Brd4 (6.5 and 6.8, respectively) as well as good oral availability and solubility. Compounds **32** and **33** also showed efficacy in an acute inflammatory BALB/c mouse model.

Wang *et al.* reported a series of γ-carboline-containing compounds as novel small-molecule BET inhibitors (Fig. 6).[51] The most potent inhibitor compound **34** bound to Brd2–4 proteins with $K_i$ values of 3.2–24.7 nM as well as CREBBP protein with a $K_d$ of 670 nM and showed good specificity over eight other non-BET bromodomain-containing proteins ($K_d$ values ≥10 000 nM). Notably, compound **34** demonstrated remarkable cell growth inhibition activity and excellent cellular specificity in AML cell lines. Compound **34** potently inhibited the viability of MV4-11 and MOLM-13 cells containing the MLL1 fusion gene, with $IC_{50}$ values of 20 and 66 nM, respectively, while it showed no obvious inhibitory potency ($IC_{50}$ > 2 μM) against the K562 cell line harboring a Bcr-Abl fusion protein.

Recently, Wang's group developed novel BET inhibitors containing a 9*H*-pyrimido[4,5-*b*]indole tricyclic core structure by inserting one extra nitrogen atom into the 5*H*-pyrido[4,3-

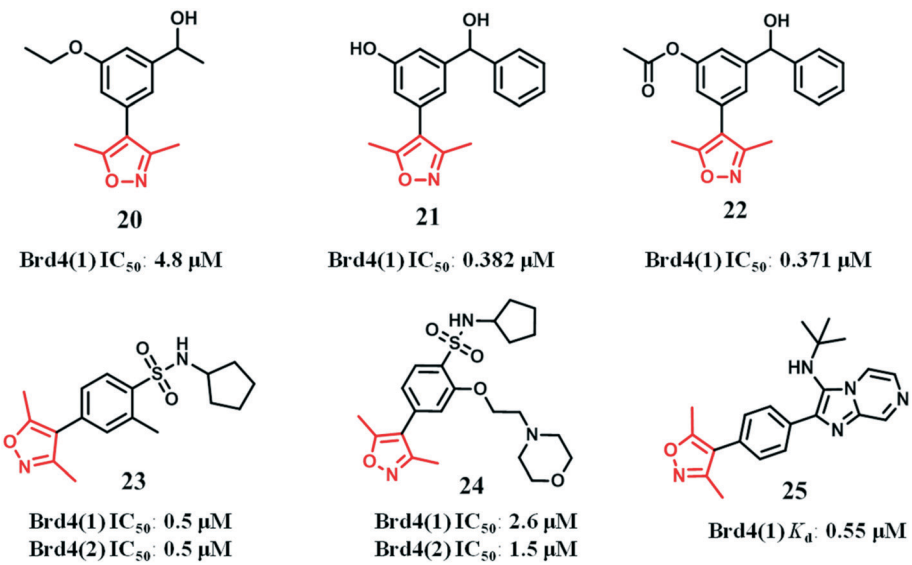


Fig. 5 Chemical structures of compounds **20–25**.

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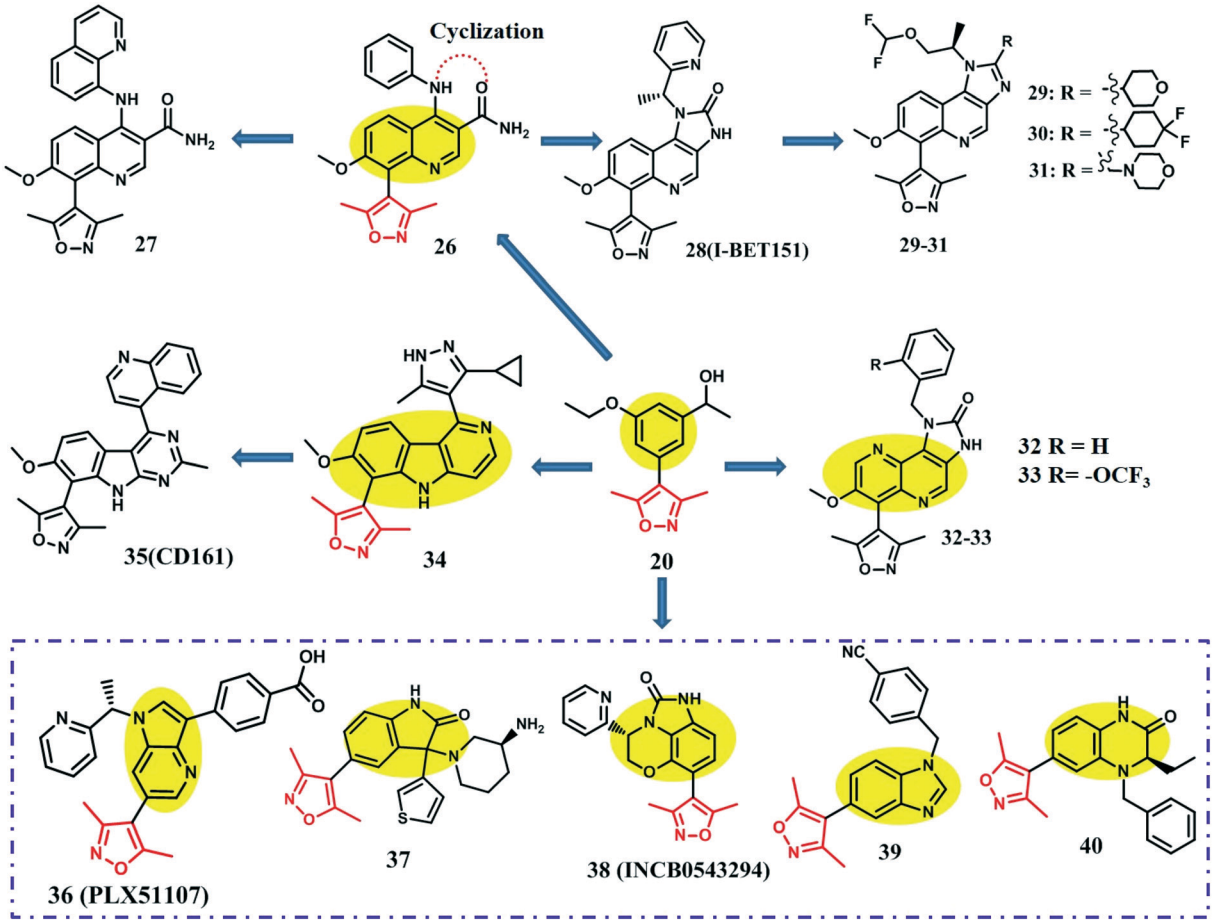


Fig. 6 Reported isoxazole-based Brd4 inhibitors.

*b*]indole of compound **34**.[52] A systematic SAR study led to the discovery of compound **35**, which bound to Brd4(1) and Brd4(2) with $K_i$ values of 8.2 nM and 1.4 nM, respectively. Compound **35** showed significant activities in inducing the down-regulation of Brd4-downstream c-*Myc* gene and up-regulation of p21 related to apoptosis in MV4-11 leukemia cells. This excellent Brd4 inhibition of compound **35** translated into dramatic antiproliferative effects against MV4-11 and MOLM-13 cell lines with $IC_{50}$ values of 26 and 53 nM, respectively. In addition to acute leukemia cell lines, compound **35** also potently inhibited cell growth in nine breast cancer cell lines with $IC_{50}$ values of $<1$ μM. Compound **35** was found to have good solubility, microsomal stability in human microsomes and excellent oral bioavailability (93% in rat). In *in vivo* experiments, daily oral administration with compound **35** led to >80% and complete tumor regression in the MV4-11 and MDA-MB-231 mouse xenograft model, respectively, without inducing any toxicity or decline in weight gain.

Hatice *et al.* described compound **36** (**PLX51107**) to be a potent Brd4 inhibitor. **PLX51107** potently bound to Brd4(1) and Brd4(2) with $K_d$ values of 1.7 nM and 6.1 nM, respectively.[53] **PLX51107** could significantly suppress the CpG-induced proliferation of primary chronic lymphocytic leukemia (CLL) cells with $IC_{50}$ values in the range of 0.156 μM to 10 μM. In a *de novo* mouse model of CLL, **PLX51107** significantly reduced leukemic disease burden in peripheral blood and spleen at 20 mg $kg^{-1}$ (oral daily) and was more effective than 50 mg $kg^{-1}$ **OTX015**. **PLX51107** is currently in phase I clinical studies for solid tumor, AML and myelodysplastic syndrome.

BeiGene Ltd. has patented a series of substituted 5-(3,5-dimethylisoxazol-4-yl)indoline-2-one compounds including compound **37** as novel Brd4 inhibitors. Compound **37** inhibited Brd4 with an $IC_{50}$ value of 8 nM in a TR-FRET methodology and showed promising antiproliferation cellular activity against the MV4-11 cell line ($IC_{50}$ = 34 nM).[54]

Phillip *et al.* described the preclinical activity of a novel Brd4 inhibitor compound **38** (**INCB054329**) with low nanomolar potency against Brd4 for the potential treatment of malignant diseases.[55] In both AML and lymphoma cell lines, **INCB054329** remarkably inhibited expression of c-Myc, induced apoptosis consistent with increased expression of pro-apoptotic regulators, and suppressed cell growth with potencies of less than 200 nM. Oral administration of **INCB054329** showed potent *in vivo* antitumor efficiency in several models of hematologic cancers. At present, **INCB054329** is being tested in patients with any advanced solid malignancy or lymphoma by Incyte.

In pursuit of a potent and rapidly accessible Brd4 inhibitor, Brennan's group developed benzimidazole-substituted

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3,5-dimethylisoxazoles as novel Brd4 inhibitors, which could be easily gained by three-step regioselective synthesis.[56] The most potent compound, **39**, had a Brd4(1) $IC_{50}$ of 180 nM and showed marked selectivity for CREBBP as well as seven other tested bromodomain-containing proteins.

Yang *et al.* reported compound **40** as a potent Brd4 inhibitor by replacing the benzo[*d*]imidazole core of compound **39** with a dihydroquinoxalinone skeleton.[57] This modification conferred an extra interplay with the ZA channel region and a stronger interaction with the WPF shelf, which were reflected in superior Brd4 inhibition activity ($IC_{50}$ = 70 nM) and excellent antiproliferative activity in MV4-11 cancer cells ($IC_{50}$ = 258 nM) of compound **40**.

More recently, Xu's group described a series of benzo[*d*]isoxazole-containing compounds as potent Brd4 inhibitors.[58] Among them, compounds **41** and **42** showed the highest binding affinities to Brd4(1) with $K_d$ values of 82 and 81 nM, respectively (Fig. 7). Thermal stability shift assay and BROMOscan revealed that compounds **41** and **42** exhibited excellent selectivity over other non-BET subfamily members. Compounds **41** and **42** demonstrated a good inhibitory effect against acute leukemia cell lines ($IC_{50}$: 0.4–0.68 μM) and AR-positive prostate cancer cell lines ($IC_{50}$: 0.29–2.62 μM), while they showed weak cytotoxicity in the AR-negative prostate cancer cells and normal lung fibroblast cell line HFL-1. Consistent with the cell viability assays, compounds **41** and **42** significantly inhibited the colony formation of C4-2B and 22Rv1 cells at 0.5 μM. In a C4-2B CRPC xenograft tumor model treated intraperitoneally five times a week with compounds **41** and **42** for 25 days at a dose of 50 mg $kg^{-1}$, TGI values of 70% and 51% were achieved, respectively.

**1.1.3 Pyridone-based Brd4 inhibitors.** Researchers at AbbVie identified a novel phenylpyridazinone fragment, compound **43**, as a weak Brd4 binder ($K_i$ = 160 μM) by a two-dimensional NMR fragment screen.[59] Compound **43** occupied the acetylated lysine binding pocket of Brd4(2) through two key interactions: hydrogen bonding of pyridone carbonyl oxygen to the amino group of conserved Asn433 and pyridone *N*-methyl substituent in the amphoteric pocket. Sustained efforts to achieve structural optimization of the privileged phenylpyridazinone moiety led to the discovery of a number of potent Brd4 inhibitors.

Compound **44** was found to potently bind to Brd4 with a $K_i$ value of 13 nM and exhibited significant antiproliferative activity against MX-1 cancer cells with an $EC_{50}$ of 47 nM. A mouse study showed that an OPM-2 mouse xenograft model, dosed orally Q.D. with compound **44** for 21 days consecutively at doses of 1 mg $kg^{-1}$ and 3 mg $kg^{-1}$, demonstrated a TGI of 66% and 73%, respectively. Further chemical modification led to the macrocycle compound **45**, which showed superior potency than compound **44** both in the cellular assay and *in vivo* assay.[59]

By fusing a pyrrole ring with the pyridine core of compound **44**, McDaniel *et al.* described a series of novel Brd4 inhibitors featured with a pyrrolopyridone scaffold, which could bidentately interact with the conserved asparagine residue of the Brd4 protein, as exemplified by the clinical candidate compound **46** (**ABBV-075**).[60] Compound **46** exhibited superior potency than compound **44** both in Brd4 binding assays ($K_i$ = 1.5 nM) and antiproliferation effect on MX-1 cancer cells ($EC_{50}$ = 13 nM). Compound **46** displayed favorable PK profiles with moderate oral bioavailability (50%) and a fairly good oral half-life ($T_{1/2}$ = 25 h) in humans. Compound **46** achieved a TGI of 99% administered orally Q.D. at a dose of 1 mg $kg^{-1}$ for 25 days with acceptable tolerability (weight loss ≤10%) in a Kasumi-1 AML mouse xenograft model, more

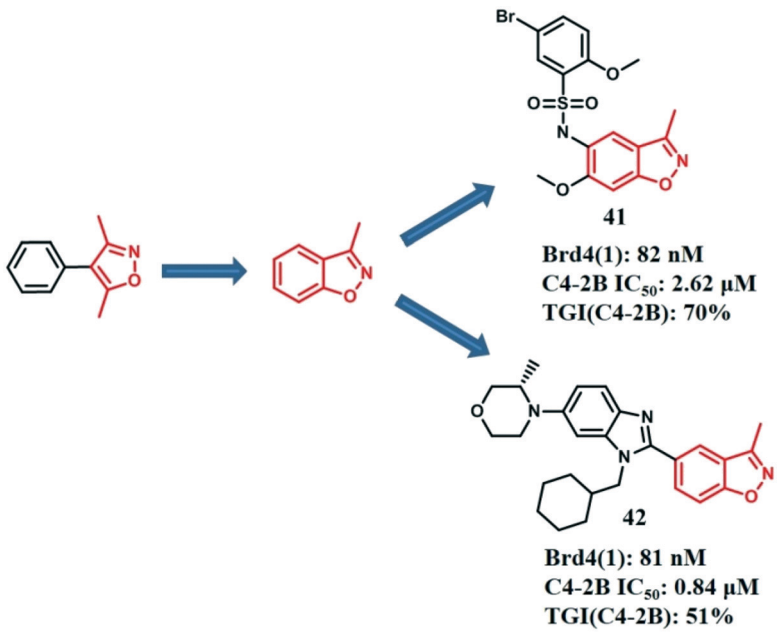


Fig. 7 Reported benzo[*d*]isoxazole-based Brd4 inhibitors.

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potent than 5-azacitidine with a TGI of 78% at its maximum tolerated dose. Compound **46** at doses ranging from 1 to 2 mg $kg^{-1}$ also demonstrated robust *in vivo* antitumor efficacy against both hematologic and solid tumors.[61]

A number of other pyridone-based potent Brd4 inhibitors including compounds **47–51** with $IC_{50}$ values in the low nanomolar level have also been reported in patents (Fig. 8).[62,63]

AbbVie and Jubilant Biosys filed patents for a number of fused polycyclic pyridones as potent Brd4 inhibitors.[64,65] Compounds **52–55**, representatives of this design, demonstrated Brd4(1) $IC_{50}$ values in single-digit nanomolar levels, and their good protein potency translated into the cellular assay. Both compounds **52** and **53** demonstrated an $EC_{50}$ of 16 nM in an MX-1 cellular assay and showed impressive efficacy in mouse tumor xenograft models. Compounds **54** and **55** displayed remarkable MV4-11 antiproliferative activity with $EC_{50}$ values of 2.0 nM and 8.0 nM, respectively (Fig. 9).

**1.1.4 Tetrahydroquinoline-based Brd4 inhibitors.** Compounds **56** and **57** were identified as novel *N*-acetyllysine mimetics by Chung and co-workers *via* fragment-based screenings and were considered as appealing starting points to exploit novel Brd4 inhibitors due to their chemical tractability.[66]

GlaxoSmithKline developed a tetrahydroquinoline-based Brd4 inhibitor, compound **58** (**I-BET726**), on the basis of *N*-acetyllysine mimetics compound **56**.[67] Compound **58** showed high binding affinities to Brd4 with $K_d$ values of 23 and 4.4 nM, confirmed by surface plasmon resonance (SPR) and isothermal titration calorimetry (ITC), respectively. Compound **58** demonstrated a high degree of selectivity against non-BET bromodomain-containing proteins as well as nonbromodomain targets. Moreover, it achieved a moderate elimination half-life and excellent oral bioavailability. Compound **58** strongly inhibited the proliferation of a panel of neuroblastoma cell lines with a median $IC_{50}$ value of 75 nM. Oral administration of compound **58** to mouse xenograft models of human CHP-212 neuroblastoma resulted in down-regulation of BCL2 and MYCN and a TGI of 82%.[68]

Novel tetrahydroquinoline-based Brd4 inhibitor compound **59** has been patented by GlaxoSmithKline and showed marked inhibition against Brd4(2) with an $IC_{50}$ value of less than 100 nM; however, it showed weak inhibition against Brd4(1).[69] Compounds **60** and **61**, patented by Forma Therapeutics[70] and Bayer,[71] respectively, also displayed potent Brd4 inhibitory activity with $IC_{50}$ values in the nanomolar range.

Fish *et al.* described the discovery of compound **62** through optimization of the hit compound **57**.[72] Compound **62** displayed an $IC_{50}$ value of 220 nM against Brd4(1) and inhibited IL-6 production in human blood mononuclear cells

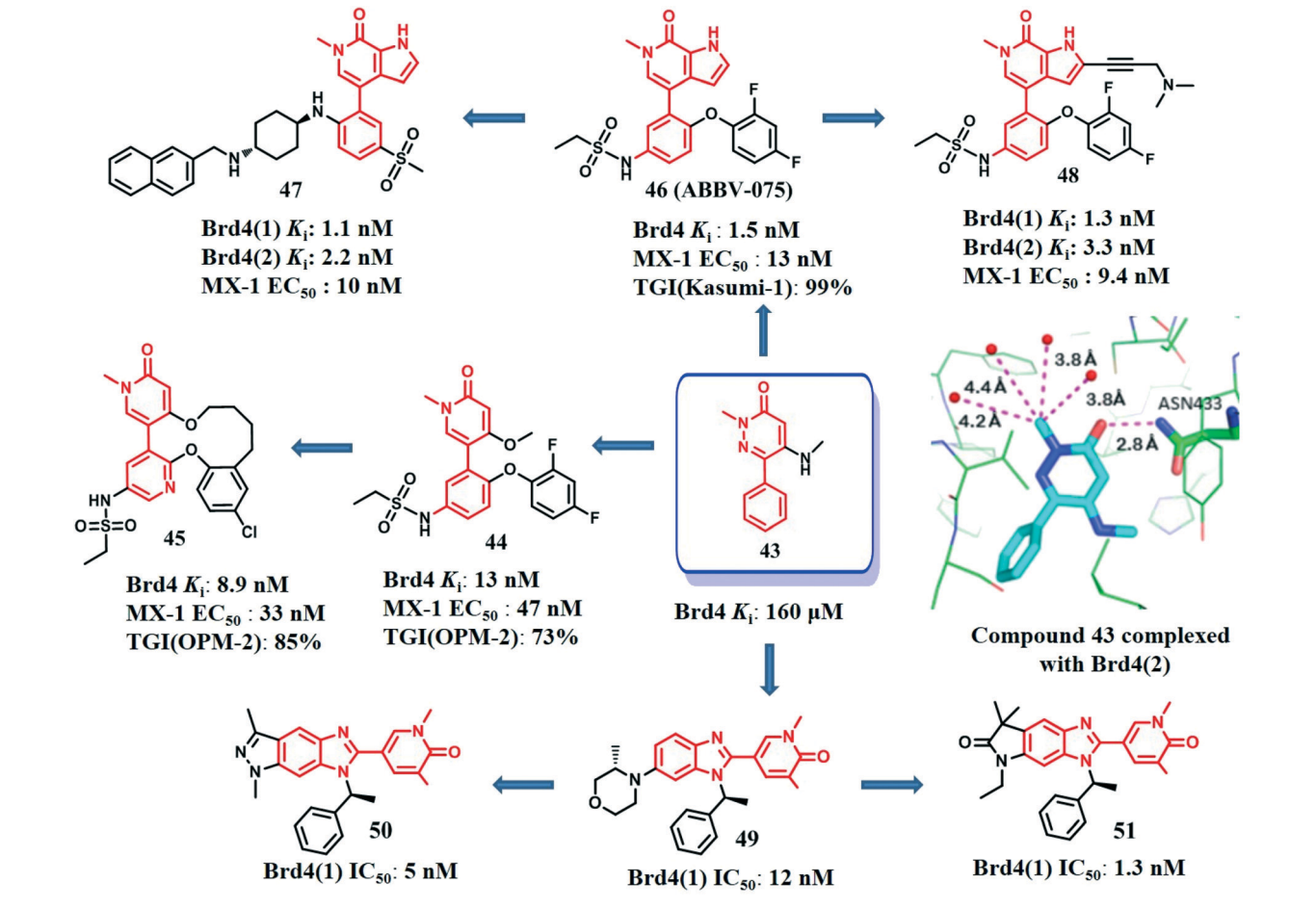


Fig. 8 Reported pyridone-based Brd4 inhibitors.

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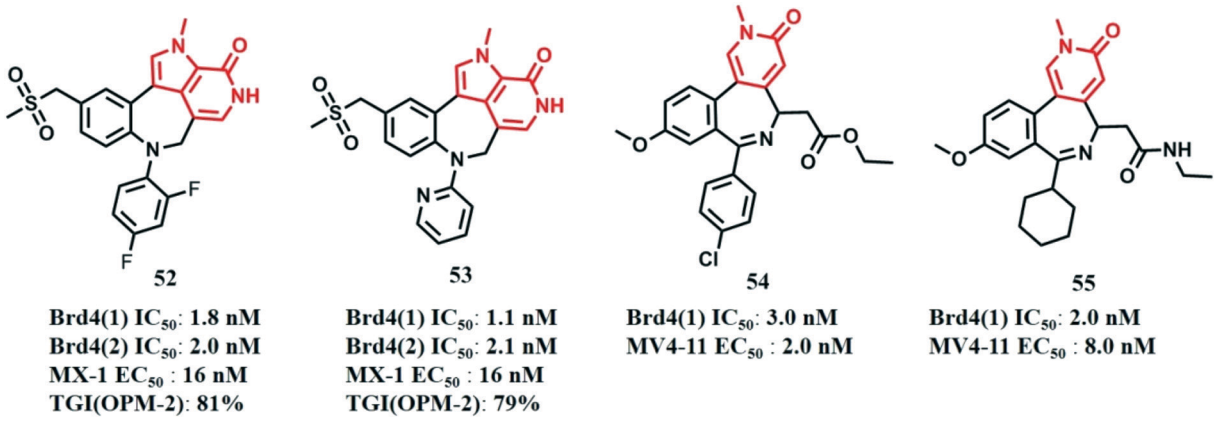


Fig. 9 Reported fused polycyclic pyridone-based Brd4 inhibitors.

stimulated by LPS with an $EC_{50}$ value of 1.89 μM. Compound **62** showed thermal shifts of 2.1–6.5 °C against BET proteins, while it showed thermal shifts of less than 1 °C against three other tested bromodomain-containing proteins, indicating good selectivity for the BET bromodomains (Fig. 10).

**1.1.5 Triazolopyrazine-based Brd4 inhibitors.** A Boehringer Ingelheim Gmbh patent described Brd4 inhibitors predominantly extended on the triazolopyrazine scaffold; the representative compounds included compounds **63–66**, which were recently highlighted in *ACS Med. Chem. Lett.*[73] Compounds **63–66** displayed potent Brd4(1) inhibition with remarkable $IC_{50}$ values in the single-digit nanomolar range (Fig. 11).[74]

Compound **67** and compounds **68–69** were obtained and patented by using a triazolopyridazine core[75] or a triazolopyridine core[76] as a bioisostere of triazolopyrazine, respectively. Compounds **67–69** showed low nanomolar potencies in inhibition of Brd4 and MV4-11 cancer cell growth.

**1.1.6 4-Acyl pyrrole-based Brd4 inhibitors.** Lucas *et al.* performed a high-throughput virtual screening campaign on Brd4 to identify novel inhibitors. This screening led to 22 hits whose binding affinity towards Brd4(1) was determined by ITC assay, identifying compound **70** bearing a 4-acyl pyrrole motif as the most potent hit against Brd4(1) with a $K_d$ value of 237 nM.[77] Among 56 cell lines tested, compound **70** showed strong

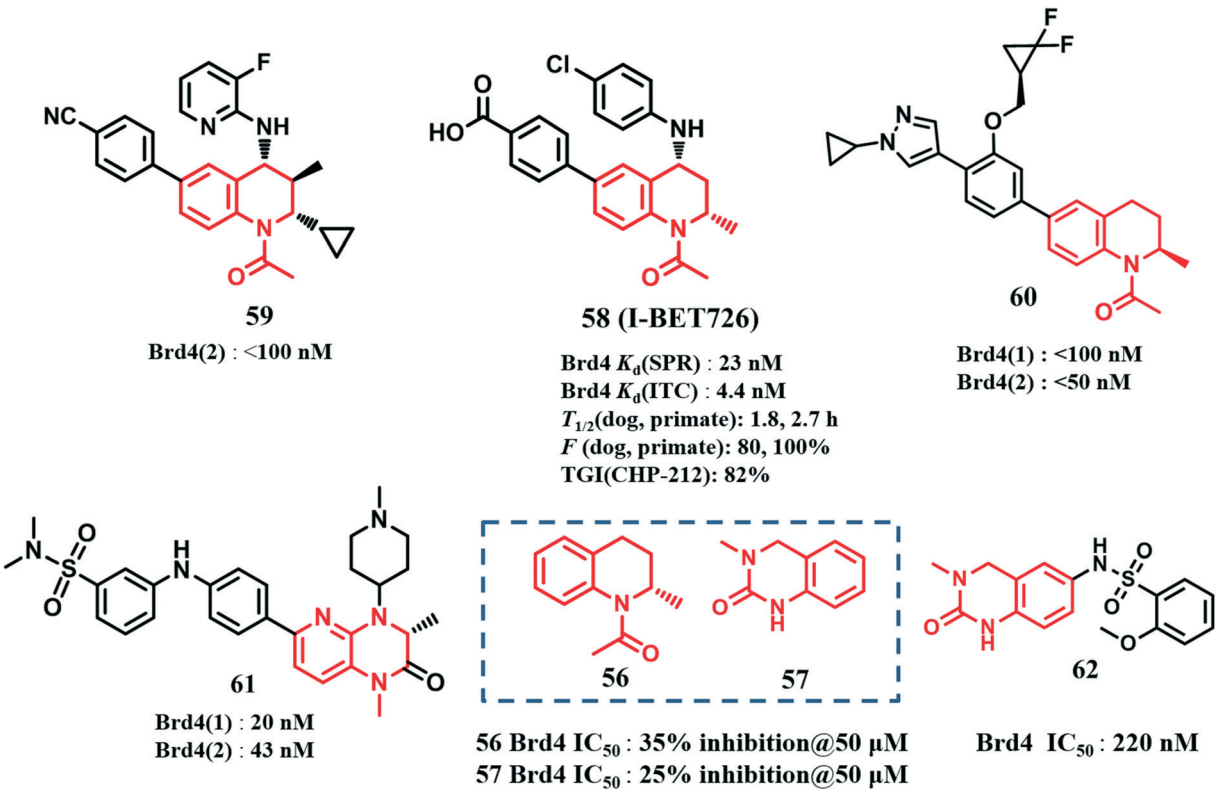


Fig. 10 Reported tetrahydroquinoline-based Brd4 inhibitors.

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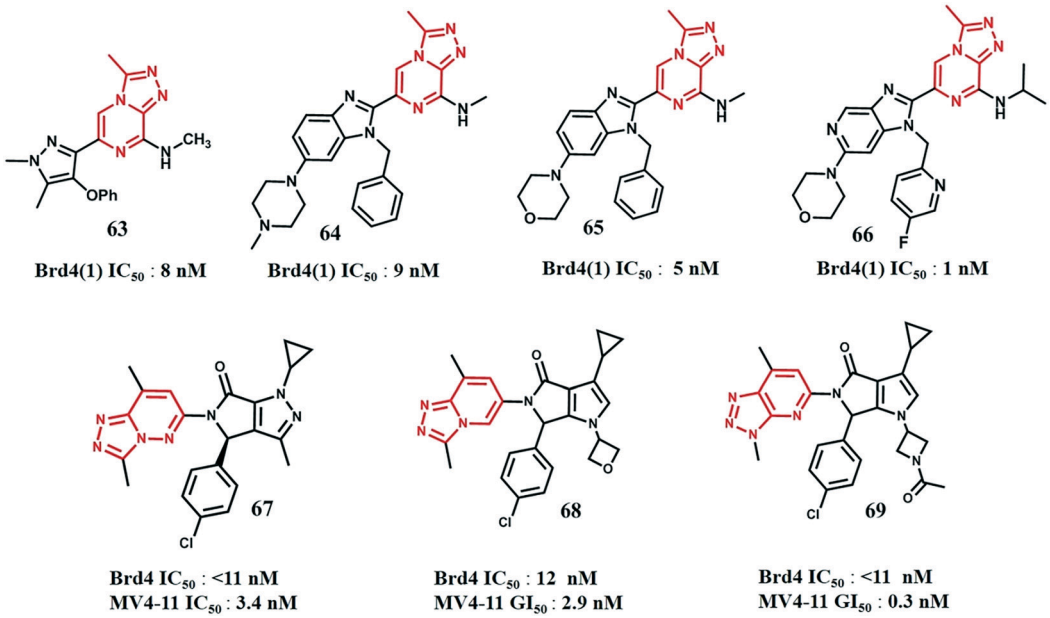


Fig. 11 Reported triazolopyrazine-based Brd4 inhibitors.

inhibitory potency and selectivity towards leukemia cells, with a maximum 65% growth inhibition at 10 μM against HL-60 cancer cells. A structure-guided modification of compound **70** performed based on the cocrystal structure of Brd4(1)/**70** led to Brd4 inhibitors **71** and **72** with $K_d$ values of 0.46 and 0.81 μM, respectively; however, both were less potent than compound **70** (Fig. 12).[78]

AbbVie published a patent application describing 4-acyl pyrrole-based Brd4 inhibitors, as represented by compound **73**, which was potent both biochemically [Brd4(1) $K_i$ = 38 nM, Brd4(2) $K_i$ = 225 nM] and in the MX-1 cell line ($EC_{50}$ = 433 nM).[79] Compound **74**, patented also by AbbVie, exhibited $K_i$ values of 15 nM and 43 nM against Brd4(1) and Brd4(2), respectively. Compound **74** was potently active against the MX-1 cell line with an $EC_{50}$ value of 160 nM, which translated into *in vivo* efficacy with a TGI of 80% at a dose of 100 mg $kg^{-1}$ in an MX-1 mouse xenograft model.[80]

**1.1.7 2-Thiazolidinone-based Brd4 inhibitors.** Shen's group described compound **76** as a novel Brd4 inhibitor, which was synthesized based on the novel *N*-acetyllysine mimic compound **75** featured with a 2-thiazolidinone core.[81] Compound **76** displayed Brd4(1) inhibition with an $IC_{50}$ of 4.1 μM and had good metabolic stability, but showed weak proliferation inhibition activity in HT-29 cancer cells with a $GI_{50}$ of 47.8 μM. Subsequent optimization by replacing the sulfonylaminophenyl substitution of **76** with an amino sulfonyl

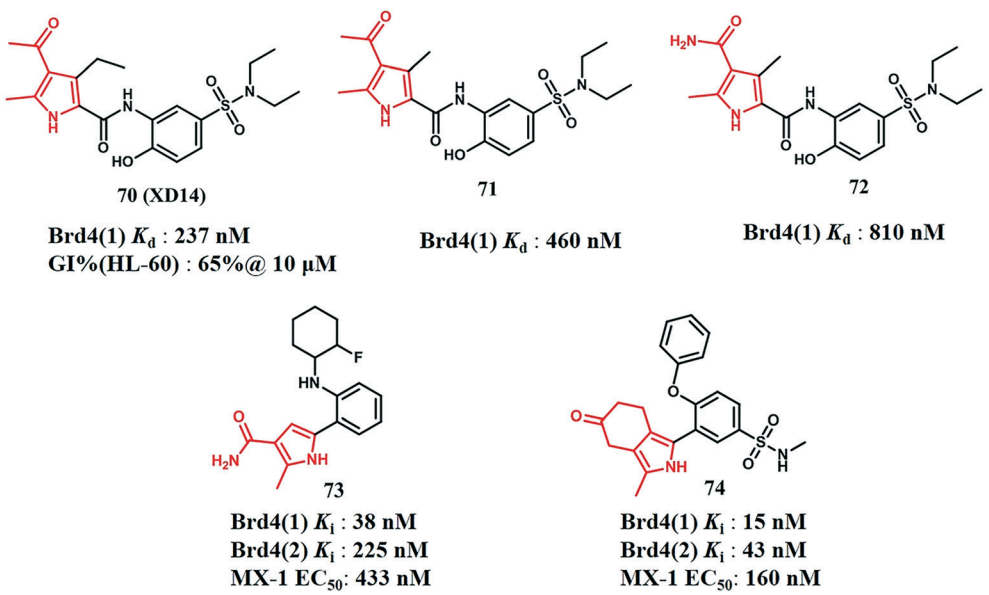


Fig. 12 Reported 4-acyl pyrrole-based Brd4 inhibitors.

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phenyl group gave compound 77 with an $IC_{50}$ of 140 nM, which was about 30 times more potent than compound 76.[81] Compound 77 was found to exhibit potent activity against the proliferation of two leukemia cell lines and one colon cancer cell line with $IC_{50}$ values ranging from 184 to 860 nM (Fig. 13).

**1.1.8 Other reported Brd4 inhibitors.** Other selected examples of Brd4 inhibitors are listed in Fig. 14. Xu and coworker reported the discovery of the benzo[*cd*]indol-2(1*H*)-one scaffold as a new class of BET bromodomain inhibitors *via* a structure-based virtual screening approach and subsequent extensive chemical optimization.[82] Among them, representative compound 78 showed robust binding affinity to Brd4(1) with a $K_d$ value of 137 nM and excellent selectivity over other non-BET bromodomain-containing proteins in the ITC assay. In MV4-11 leukemia cells and HL-60 cancer cells, compound 78 exhibited reasonable antiproliferation effects with $IC_{50}$ values of 1.30 μM and 2.99 μM, respectively. Oral administration with compound 78 in rats demonstrated good pharmacokinetic profiles with high oral bioavailability (76.8%) and a moderate half-life ($T_{1/2}$ = 3.95 h).

William's group disclosed dihydropyridopyrimidine derivative 79 as a novel Brd4 inhibitor, which was facilely generated in a single step from commercially available starting materials.[83] Compound 79 had a $K_i$ of 110 nM for Brd4(1) and strongly inhibited the growth of MM1.S cells with an $IC_{50}$ value of 0.46 μM. Compound 79 also demonstrated potent binding affinity to BrdT(1) with a $K_i$ of 200 nM. Compound 79 was identified as the best Brd4 inhibitor ($IC_{50}$ = 0.43 μM) with good selectivity over other BET family proteins.

Compound 80 markedly inhibited Brd4 and its downstream target c-Myc, induced ATG5-dependent autophagy *via* blocking Brd4–AMPK (AMP-activated protein kinase) interaction and displayed potent antiproliferative activity in breast cancer cell lines (MCF-7, $IC_{50}$ = 1.62 μM; MDA-MB-231, $IC_{50}$ = 3.27 μM). Moreover, in *in vivo* efficacy evaluations of MCF-7 and MDA-MB-231 xenograft models, compound 80 showed substantial antitumor activity with a TGI of 80% and 76% after intragastric administration of 100 mg $kg^{-1}$, respectively, without causing significant loss of body weight and toxicity.[84]

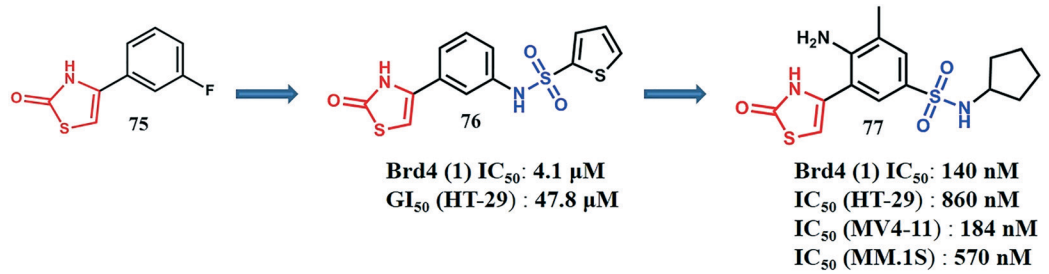


Fig. 13 Reported 2-thiazolidinone-based Brd4 inhibitors.

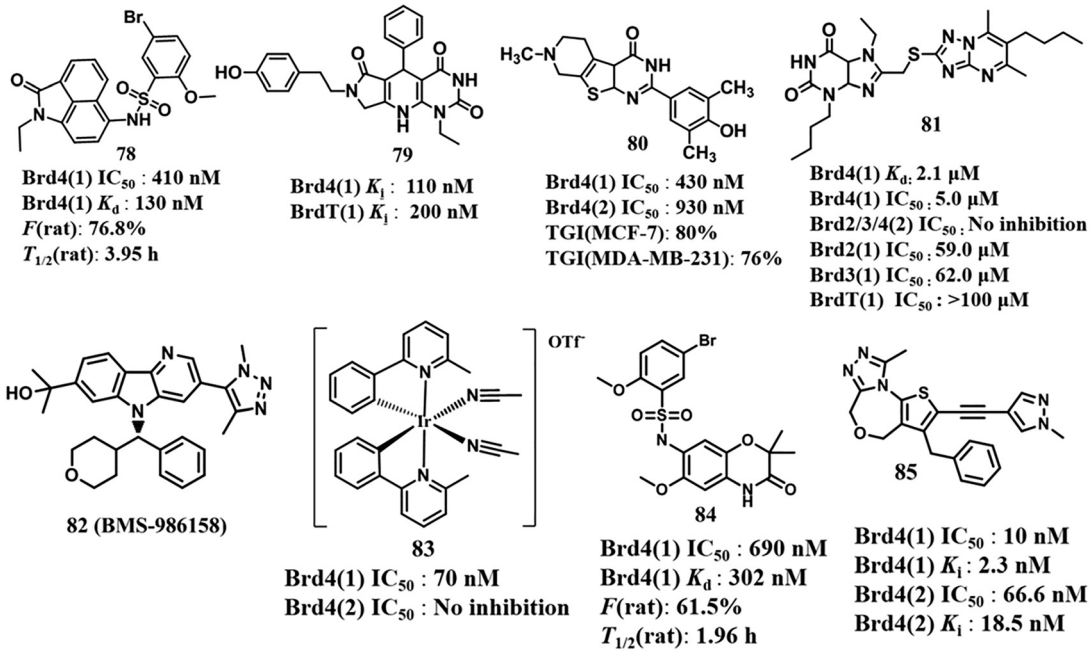


Fig. 14 Other reported Brd4 inhibitors.

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Through a midthroughput screen, a xanthine derivative compound **81** was identified as a Brd4 inhibitor with a $K_d$ of 2.1 μM determined by ITC. Despite compound **81** showing weak cellular activity against Jurkat T cells with an $EC_{50}$ of 27 μM, impressive selectivity for Brd4(1) ($IC_{50}$ = 5.0 μM) over Brd2/3(1) ($IC_{50}$ > 50 μM) and BrdT(1) ($IC_{50}$ > 100 μM) was observed; more unexpectedly, compound **81** showed no detectable inhibition against the BD2 counterparts of all BET proteins tested.[85]

Compound **82** (**BMS-986158**) is a novel Brd4 small-molecule inhibitor developed by Bristol-Myers Squibb and is currently being tested as monotherapy or in combination with nivolumab in subjects with selected advanced solid tumors or hematologic malignancies. However, to date, no clinical data on **BMS-986158** have been published in peer-reviewed journals.[86]

Zhong *et al.* identified an iridium(III) complex as the first metal-based, irreversible Brd4 inhibitor, compound **83**.[87] Compound **83** strongly inhibited Brd4(1) with an $IC_{50}$ value of 70 nM in a TR-FRET assay, while it showed no significant inhibition against Brd4(2). ChIP analysis and immunoblotting analysis revealed that compound **83** could disrupt the binding of Brd4 to the c-Myc and Bcl-2 promoters and reduced the expression of c-Myc and Bcl-2 in A375 and A2058 cancer cells. In a xenograft mouse model of human A375 melanoma cells, compound **83** significantly repressed tumor growth (a 40% reduction of tumor volume) without causing visible toxicity at a dose of 100 mg $kg^{-1}$ once daily for 16 days.

Xu's group designed and synthesized a series of 2,2-dimethyl-2*H*-benzo[*b*][1,4]oxazin-3(4*H*)-one derivatives and evaluated their Brd4 inhibitory activities, obtaining several compounds as potent Brd4 inhibitors with $IC_{50}$ values in the nanomolar range.[88] The representative compound **84** potently inhibited Brd4(1) with an $IC_{50}$ value of 690 nM and a $K_d$ value of 302 nM determined by an ITC experiment. Compound **84** showed good inhibition against a panel of prostate cancer cell lines, including C4-2B, LNCaP, and 22Rv1, with $IC_{50}$ values in the range of 3.23–4.51 μM, and potently inhibited 22Rv1 cancer cell colony formation in a dose-dependent manner. Oral dosing with compound **84** in rats demonstrated reasonable pharmacokinetic properties with a $T_{1/2}$ value of 1.96 h and an oral bioavailability of 61.5%.

Wang's group described a series of [1,4]oxazepine derivatives as a new class of Brd4 inhibitors. The most potent, compound **85**, bound to Brd4(1) with a $K_i$ value of 2.3 nM and an $IC_{50}$ value of 10 nM and was more potent than **OTX015**. Consistent with its high binding affinities to Brd4, compound **85** was an effective inhibitor of cell growth, with $IC_{50}$ values of 55.8, 207, and 173 nM in the MV4-11, MOLM-13, and RS4-11 cell lines, respectively.[89]

### 1.2 Bivalent Brd4 inhibitors

Waring and colleagues demonstrated a series of triazolopyridazine-containing compounds, which were capable of binding both Brds in a single Brd4 protein simultaneously.[90] The first bivalent Brd4 inhibitor, compound **87**, was accidentally generated based on the structure optimization of **AZD3514** (**86**), an androgen receptor (AR) modulator (Fig. 15).[91,92] Despite its increased AR down-regulation potency, however, compound **87** induced estrogen receptor-α (ERα) down-regulation, indicating that the direct target of compound **87** might not be AR. Considering the structural similarity between compound **87** and Brd4 inhibitor **I-BET762**, a panel of Brd inhibition assays were performed. Compound **87** displayed potent inhibition against Brd4(1) and Brd4(2) with p$K_d$ values of 7.2 and 6.1, respectively. Compound **87** suppressed c-Myc levels, inhibited cell growth and induced apoptosis more effectively in several Brd4-sensitive

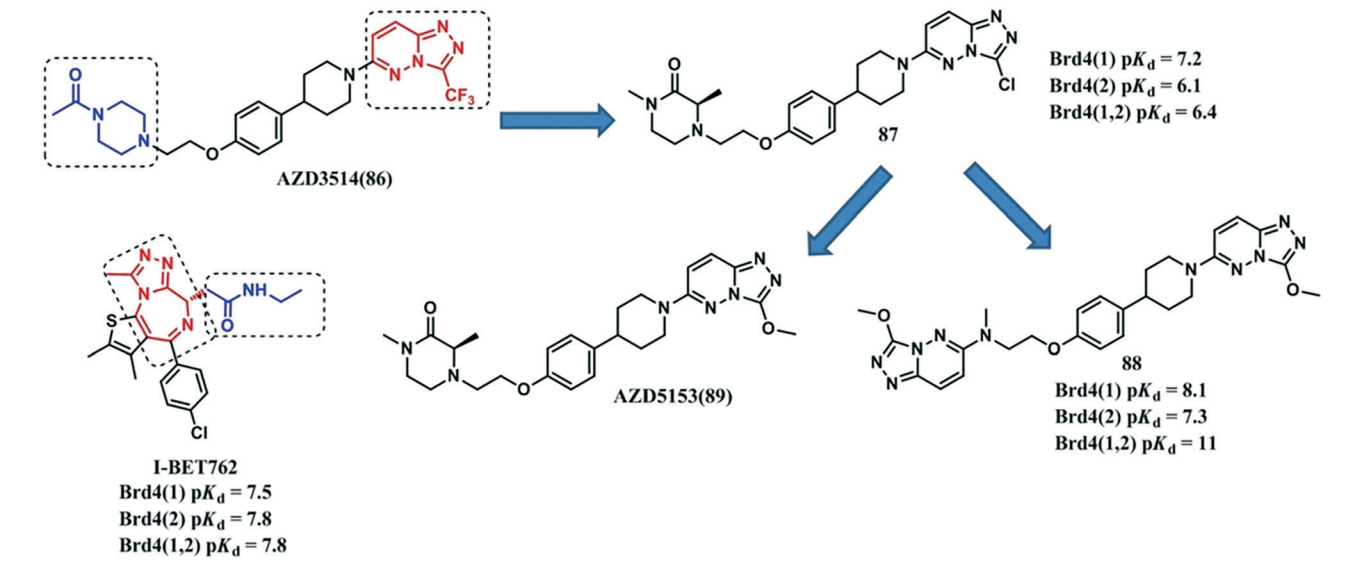


Fig. 15 Discovery and development of bivalent Brd4 inhibitor **89**.

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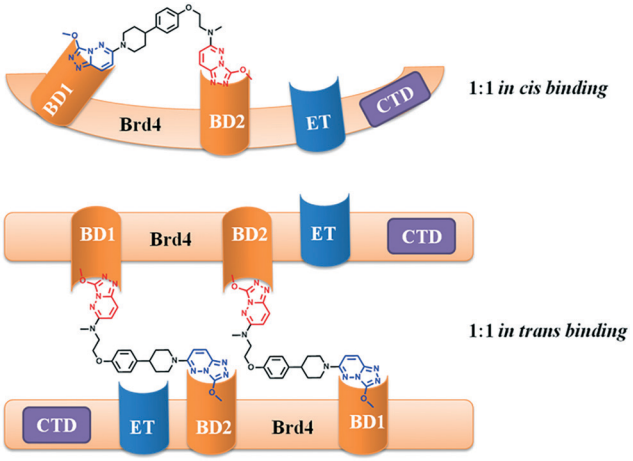


Fig. 16 Binding modes for bivalent Brd4 inhibitor **88**.

cancer cells than less sensitive cancer cells, consistent with its marked Brd4 inhibition. The cocrystal structure of Brd4(1) with compound **87** revealed that compound **87** could remarkably induce dimerization of two Brd4(1) by spanning both the acetyl-lysine binding pocket *via* the triazolopyridazine motif and the piperazinone group.

Further modification of compound **87** by changing the piperazinone group or chloro-substituted triazolopyridazine with a methoxy-substituted triazolopyridazine, a more potent acetyl-lysine pocket binding scaffold, gave compounds **88** and **89** (**AZD5153**), respectively.[93] Compound **88** was identified as the most potent Brd4 inhibitor reported to date with p$K_d$ values of 8.1 and 7.3 against Brd4(1) and Brd4(2), respectively. It was also found that compound **88** showed few off-target effects on cellular receptors and ion channels, more than 100 of which were tested. The increased Brd4 inhibition potency was reflected in its cellular activity; compound **88** displayed highly potent antiproliferation activity against both MV4-11 and MM.1S cells with a p$IC_{50}$ of 9.5, which was 1000-fold more potent than that of **I-BET762**. The therapeutic advantages of bivalent Brd4 inhibition by compound **88** was also evidenced by the near-complete cell killing in RS4-11 cancer cells, compared to **I-BET762** with no full inhibition at even higher concentrations. Compound **88** was proved to be more significant and sustained in suppressing the c-Myc expression, which might be responsible for its enhanced antiproliferation activity.

Chen's group reported the anticancer effects of **AZD5153** against hematologic malignancies. **AZD5153** potently inhibited Brd4 with $IC_{50}$ values of 5 nM against full-length Brd4 and 1.6 μM against Brd4(1). **AZD5153** was more potent than the monovalent inhibitor **I-BET762** in down-regulating c-Myc, with a c-Myc protein modulation $IC_{50}$ of 5.4 nM for **AZD5153** compared with an $IC_{50}$ of 329 nM for **I-BET762**. **AZD5153** displayed an excellent pharmacokinetic profile and tumor growth inhibition in xenograft studies. In a xenograft model of MV4-11 cancer cells, daily treatment with 1 mg $kg^{-1}$ **AZD5153** gave a TGI of 72%, while 5 mg $kg^{-1}$ daily oral doses of **AZD5153** led to tumor repression.[94] **AZD5153** is now in a phase I clinical trial by AstraZeneca for treatment of malignant solid tumors and lymphoma.[95]

Compound **88** engaged two Brd4(1)s concurrently in a fashion similar to that observed with compound **87** and reduced a Brd4(1) dimer confirmed by X-ray. A variety of biophysical experiments including NMR spectroscopy, analytical ultracentrifugation, and small-angle X-ray scattering were performed to elucidate the binding mode of **88** with full-length Brd4 in cells. The results showed that compound **88** simultaneously bound both BD1 and BD2 of a single Brd4 in *cis* binding fashion and brought the two Brds into close proximity *via* folding the Brd4 protein (Fig. 16).

Bradner *et al.* described a series of bivalent Brd4 inhibitors by tethering two (+)-**JQ1** molecules *via* a variable-length polyethylene glycol linker. Among them, compound **MT1** (**90**) was identified as a more potent Brd4 bivalent inhibitor with improvement of exposure time and PK properties *in vivo* compared to (+)-**JQ1** (Fig. 17).[96] Size-exclusion chromatography (SEC) with bromodomain mutant experiments supported that one **MT1** molecule simultaneously inhibited both BD1 and BD2 of Brd4 in *cis* binding mode, as observed with compound **88**. In cellular assays, 100 nM **MT1** remarkably and rapidly depleted c-Myc protein, up-regulated HEXIM, and showed a 10-fold higher potency in reducing apoptosis in MV4-11 cells than the corresponding monovalent inhibitor, (+)-**JQ1**. Mouse studies exhibited that a half-equivalence of **MT1** significantly delayed leukemia progression in an aggressive disseminated leukemia mouse model (mCherry+, luciferase+ MV4-11) compared to (+)-**JQ1**. The findings of SEC, ITC and nanomaterial-based proximity assays supported that the improved antitumor activity of **MT1** was associated with its ability to dimerize Brds.

## 2 Brd4 degraders

While Brd4 inhibitors have demonstrated their promising therapeutic potential in a variety of c-*Myc*-driven malignancies,

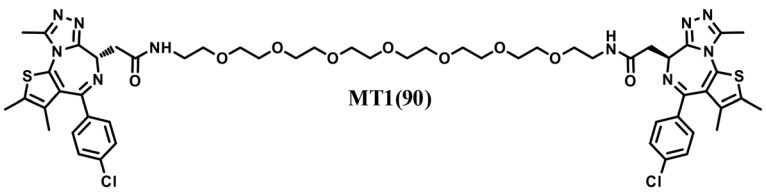


Fig. 17 The structure of bivalent Brd4 inhibitor **MT1**.

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several recent studies showed that Brd4 inhibitors led to significant compensatory accumulation of Brd4 protein in several cancers including Burkitt's lymphoma, lung cancer and prostate cancer, which may account for their inefficient *c-Myc* suppression, modest apoptotic induction and antiproliferative activity.[97,98] Moreover, drug resistance against triazoloazepine-based Brd4 inhibitors **I-BET762** and (+)-**JQ1** has been described.[99]

In addition to target protein inhibition, selective induced target protein degradation was emerging as a novel drug discovery strategy.[100,101] One promising approach to degrade protein is to design proteolysis-targeting chimeras (PROTACs), which has draw extensive attention from medicinal chemists and pharmaceutical firms.[100,102–105] PROTAC, initially proposed 15 years ago by Deshaies *et al.*,[106] was a heterofunctional bispecific small molecule. It comprises three components: a target protein-specific ligand, an E3 ubiquitin ligase binder and a linker that couples these two functionalities. The PROTACs forms a ternary complex by binding to both the target protein and a component of E3 ubiquitin ligase in a spatially favorable presentation to promote the target protein ubiquitination by the E3 ligases, thereby eliciting its ubiquitination and subsequent proteasomal degradation (Fig. 18).[107,108]

Theoretically, degradation of oncoproteins by PROTACs exhibited potential advantages over protein inhibition in cancer treatment. Firstly, removal of the entire protein is expected to be more effective compared to inhibiting an individual activity site, which leaves other parts and domains of the proteins still functional; secondly, PROTACs could act catalytically to degrade superstoichiometric amounts of the target protein; thirdly, "undruggable" proteins including transcription factors could be targeted by PROTACs.[109,110]

PROTACs have recently been used to degrade a variety of proteins including tyrosine kinases,[111] estrogen receptor α,[112] CDK9,[113] Bcr-Abl[114,115] and so on. Considering the therapeutic potential of epigenetic regulator Brd4 in cancers and compensatory protein up-regulation often observed upon Brd4 inhibition, several groups sought to utilize PROTACs to degrade Brd4 to treat cancers. Recently reported Brd4-targeted PROTACs were mainly designed based on thalidomide derivatives (**91–93**) identified as small ligands of cereblon (CRBN), a component of the $CRL4^{CRBN}$ E3 ligases,[116] and **VHL-2** (**94**) and **VH-032** (**95**), two high-affinity ligands of the $CRL2^{VHL}$ E3 complex (Table 2) (Fig. 19).[117,118]

### 2.1 $CRL4^{CRBN}$ E3-based Brd4 degraders

$CRL4^{CRBN}$ E3-based PROTACs targeting Brd4 were synthesized by connecting thalidomide derivatives to various Brd4 small-molecule inhibitors *via* different linkers (Fig. 20). The aryl ring of thalidomide derivatives and the carboxyl scaffold on Brd4 inhibitors can tolerate chemical substitution and so were chosen as suitable connecting points for a linker.

Winter *et al.* demonstrated a small-molecule $CRL4^{CRBN}$ E3-based degrader compound **96** (**dBET1**), which consisted of a Brd4 inhibitor (+)-**JQ1** linked to a thalidomide derivative, which binds CRBN.[119] On treatment with the MV4-11 cell line for 2 hours at a concentration as low as 100 nM, **dBET1** could entirely degrade Brd4 and significantly reduced its transcriptional target c-Myc, with minimal off-target degradation. Degradation of Brd4 by **dBET1** induced a more potent and superior inhibitory effect than (+)-**JQ1** against MV4-11 cells, DHL4 lymphoma cells and primary blasts from patients with leukemia. The therapeutic opportunity of **dBET1** *in vivo* was evaluated in a murine hind-limb xenograft model of human MV4-11 leukemia cells and an aggressive disseminated leukemia mouse model (mCherry+ MV4-11). In the AML model, daily treatment with **dBET1** significantly attenuated tumor progression and decreased tumor weight in two weeks, together with Brd4 degradation and c-Myc down-regulation. Notably, in the disseminated model, **dBET1** treatment caused a greater decrease in leukemic burden in bone marrow than did (+)-**JQ1**.

Concurrent with Winter's study, Crews *et al.* reported another small-molecule $CRL4^{CRBN}$ E3-based PROTAC **ARV-825** (**97**), which was capable of degrading Brd4 rapidly at picomolar potencies in Burkitt's lymphoma cells.[97] **ARV-825** was synthesized by connecting a small-molecule Brd4 inhibitor (**OTX015**)

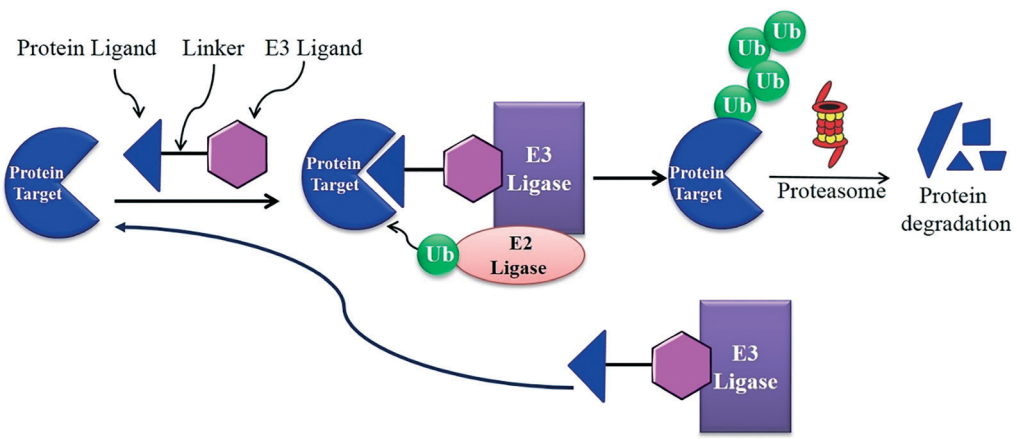


Fig. 18 Mechanism of protein degradation by PROTACs.

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Table 2 Overview of small-molecule Brd4 degraders

| Comp. | Brd4 inhibitor | E3 ligand | E3 ligase | Cellular activity | *In vivo* activity | Ref. |
|---|---|---|---|---|---|---|
| **96 (dBET1)** | **(+)-JQ1** | Thalidomide | $CRL4^{CRBN}$ | Entirely depleted Brd4 in MV4-11 cells at 100 nM with a 2 h treatment | Significantly decreased leukemic burden in bone marrow<br>mCherry+ MV4-11 xenografts model: 63.8 μM $kg^{-1}$, once daily for 19 days | 119 |
| **97 (ARV-825)** | **OTX015** | Pomalidomide | $CRL4^{CRBN}$ | Remarkably reduced the levels of Brd4 and c-Myc with a 2 h treatment<br>Namalwa cells: $DC_{50} < 1$ nM<br>Ramos cells: $DC_{50} < 1$ nM<br>Efficient and sustained depletion of Brd4 with an 18 h treatment<br>Mino cells: 500 nM<br>Z138 cells: 500 nM<br>Induction of apoptosis<br>$IC_{50}$ (mino) = 16 nM<br>$IC_{50}$ (ibrutinib-resistant Z138) = 327 nM<br>$IC_{50}$ (sAML SET2) = 14.5 nM<br>$IC_{50}$ (sAML UKE1) = 256 nM | Not reported | 97, 120, 121 |
| **98 (BETd-246)** | **HJB97** | Lenalidomide | $CRL4^{CRBN}$ | Completely reduced the levels of Brd4 with a 3 h treatment<br>MDA-MB-453: 30 nM<br>Cell growth inhibition<br>$IC_{50}$ (MDA-MB-453) < 10 nM<br>$IC_{90}$ (MDA-MB-453) < 100 nM | TGI: 85%<br>MDA-MB-453 xenograft model: i.v., 5 mg $kg^{-1}$, 3 times per week for 3 weeks | 122 |
| **99 (BETd-260)** | **HJB97** | Lenalidomide | $CRL4^{CRBN}$ | Remarkably reduced the levels of Brd4 and c-Myc with a 24 h treatment<br>RS4-11: < 30 pM, 0.1 nM<br>Cell growth inhibition<br>$IC_{50}$ (MOLM-13) = 2.3 nM<br>$IC_{50}$ (RS4-11) = 51 pM | Rapid tumor regression with a maximum of >90% tumor regression<br>RS4-11 xenograft model: i.v., 5 mg $kg^{-1}$, three times a week for 3 weeks | 122, 123 |
| **100 (QCA570)** | **QCA276** | Lenalidomide | $CRL4^{CRBN}$ | Remarkably reduced the levels of Brd4 and c-Myc with a 3 h treatment<br>RS4-11: < 10 pM<br>MV4-11: < 10 pM, 30 pM<br>Cell growth inhibition<br>$IC_{50}$ (MV4-11) = 8.3 pM<br>$IC_{50}$ (MOLM-13) = 63 pM<br>$IC_{50}$ (RS4-11) = 32 pM | Complete and long-lasting tumor regression<br>RS4-11 xenograft model: i.v., 1, 2.5, and 5 mg $kg^{-1}$, three times a week for 3 weeks<br>MV4-11 xenograft model: i.v., 5 mg $kg^{-1}$, three times per week for 2 weeks | 89 |
| **101 (ARV-771)** | **OTX015** | **VHL-2** | $CRL2^{VHL}$ | Potently reduced the levels of Brd4 with a 16 h treatment<br>22Rv1: $DC_{50} < 5$ nM<br>VCaP: $DC_{50} < 5$ nM<br>LnCaP95: $DC_{50} < 5$ nM<br>Efficient and sustained depletion of Brd4 with an 18 h treatment<br>Mino cells: 500 nM<br>Z138 cells: 500 nM<br>Induction of apoptosis<br>$IC_{50}$ (mino) = 17 nM<br>$IC_{50}$ (ibrutinib-resistant Z138) = 142 nM | Tumor regression<br>22Rv1 xenograft model: s.c., 30 mg $kg^{-1}$, once daily for 15 days<br>TGI: 60%<br>VCaP xenograft model: s.c., 30 mg $kg^{-1}$, one time per 3 days for 15 days<br>Significantly improve the median and overall survival of the NSG mice<br>MCL Z138/Luc xenografts in NSG mice: s.c., 30 mg $kg^{-1}$, daily × 5 days per week for 3 weeks | 124, 125 |
| **102 (MZ1)** | **(+)-JQ1** | **VH-032** | $CRL2^{VHL}$ | Completely depleted Brd4 with a 4 h treatment<br>HeLa cells: 1 μM<br>U2OS: 5 μM | Not reported | 126 |
| **104 (AT1)** | **(+)-JQ1** | **90 (VH-032)** | $CRL2^{VHL}$ | Remarkable Brd4-selective depletion in HeLa cells with a 24 h treatment at 1 μM<br>Potently reduced the levels of Brd4 in HeLa cells with a 24 h treatment | Not reported | 107 |

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Table 2 *(continued)*

| Comp. | Brd4 inhibitor | E3 ligand | E3 ligase | Cellular activity | *In vivo* activity | Ref. |
|---|---|---|---|---|---|---|
| **106 (MZP-54)** | **I-BET726** | **90 (VH-032)** | $CRL2^{VHL}$ | $pDC_{50}$ (Brd4 short) = 8.0<br>$pDC_{50}$ (Brd4 long) = 7.6<br>Cell growth inhibition<br>$pEC_{50}$ (MV4-11) = 7.31<br>$pEC_{50}$ (HeLa) = 6.57 | Not reported | 127 |

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to pomalidomide *via* a flexible polyethylene glycol linker. Treatment of Burkitt's lymphoma cells with **ARV-825** led to 50% Brd4 protein degradation within 2 hours, with a $DC_{50}$ (50% of maximum degradation) below 1 nM. Given that the $K_d$ values of **OTX015** and pomalidomide to their respective targets, Brd4 and cereblon, were 10 nM and 3 μM, respectively, **ARV-825** showed sub-stoichiometric characteristics in degrading Brd4.[120] In comparison to the Brd4 inhibitors (+)-**JQ1** and **OTX015**, **ARV-825** provided a more prolonged and pronounced suppression of c-Myc levels even at lower concentrations and showed no Brd4 accumulation which was associated with Brd4 inhibition. This sustained suppression of c-Myc translated into superior antiproliferative and apoptotic effects of the **ARV-825** against Burkitt's lymphoma cells compared with the Brd4 inhibitors. They also found that **ARV-825** was more profound and longer-lasting than **OTX015** in depleting Brd4, c-Myc, CDK4/6, JAK2, pSTAT3/5, PIM1 and Bcl-xL in cultured and patient-derived $CD34^+$ post-MPN sAML cells, including those expressing JAK2-V617F and mutant TP5, consistent with higher **ARV-825**-induced apoptosis and lethality in sAML cells.[121]

**BETd-246** (**98**) was developed by Wang's group using an azacarbazole-containing Brd4 inhibitor **HJB97** coupled to lenalidomide *via* a polyethylene glycol linker.[122] The majority of Brd4 was depleted after treatment with **BETd-246** for three hours at 10 to 30 nM in several triple-negative breast cell lines. **BETd-246** strongly suppressed the growth of 9 TNBC cell lines with $IC_{50} < 10$ nM, and was 50-fold more potent than **HJB97**. In both Washington Human in Mouse (WHIM) and MDA-MB-453 xenograft models, **BETd-246** achieved significant antitumor activity and induced partial tumor regression at well-tolerated dose schedules. However, **BETd-246** showed very limited or no antitumor activity in MDA-M-231 and MDA-MB-468 xenograft models, respectively, due to limited drug exposure in both models.

**BETd-260** (**99**) was generated by further structure optimization of **BETd-246**.[123] **BETd-260** showed a more potent antiproliferative activity than **BETd-246** in TNBC cells, and more importantly, **BETd-260** exhibited a much higher drug exposure in both MDA-MB-231 and MDA-MB-468 xenograft models and exerted a much stronger antitumor activity than **BETd-246** without inducing observable toxic effects.[122] **BETd-260** was capable of effectively reducing the level of Brd4 protein at concentrations as low as 30 pM with a 24 hour treatment in the RS4-11 leukemia cell line, accompanied by strong down-regulation of c-Myc protein, while **HJB97** showed no effect on the level of Brd4 proteins even at 1 μM. **BETd-260** significantly inhibited RS4-11 cell line and MOLM-13 cell line proliferation with $IC_{50}$ values of 51 pM and 2.3 nM, respectively. *In vivo*, treatment of the RS4-11 xenograft model with **BETd-260** induced rapid tumor regression with a maximum of >90% regression observed.

More recently, Wang's group reported an exceptionally potent small-molecule BET degrader **QCA570** (**100**), which was synthesized using a novel [1,4]oxazepine-based BET inhibitor developed by Wang's group and known as cereblon ligand.[89] After 3 h of treatment, **QCA570** was capable of effectively reducing the levels of Brd4 and c-Myc at concentrations as low as 10 pM in the RS4-11 cell line and 30 pM in the MV4-11 cell line, while the corresponding Brd4 inhibitor failed to abate the levels of Brd4 and c-Myc at a concentration of 10 μM. Consistent with its extraordinary ability to suppress Brd4, **QCA570** showed significant potency in inhibition of cell growth in MV4-11, MOLM-13, and RS4-11 cell lines with $IC_{50}$ values of 8.3, 62, and 32 pM, respectively. A direct comparison of cell growth inhibitory potency in these three leukemia cell lines between **QCA570** and previously published Brd4 degraders including **dBET1**, **ARV-825** and **ARV-771** was performed. The results demonstrated that **QCA570** was the most potent and efficacious Brd4 degrader to date. Significantly, in both the MV4-11 and RS4-11 acute leukemia xenograft models, **QCA570** achieved complete and long-lasting tumor regression at 5 mg $kg^{-1}$ without apparent toxicity (Fig. 21).

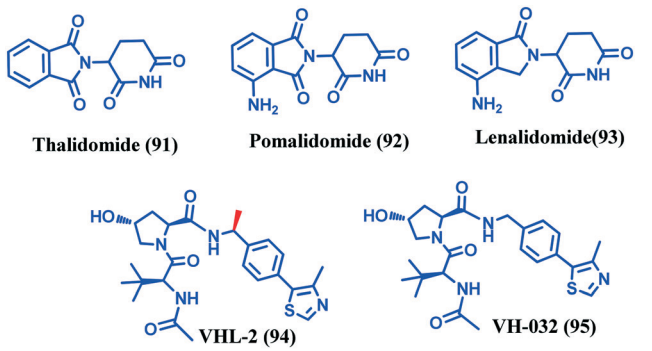


Fig. 19 Reported $CRL4^{CRBN}$ E3 and $CRL2^{VHL}$ E3 small-molecule ligands.

### 2.2 $CRL2^{VHL}$ E3-based Brd4 degraders

Inspection of VHL and its ligand crystal structures showed that the methyl group of the terminal acetyl groups in compounds

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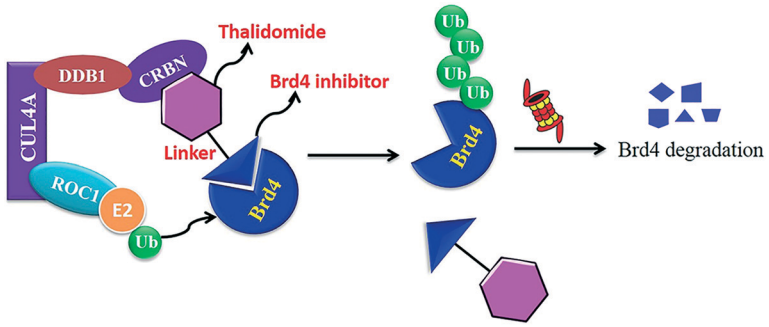


Fig. 20 Mechanism of Brd4 degradation by CRL4$^{CRBN}$ E3-based Brd4 degraders.

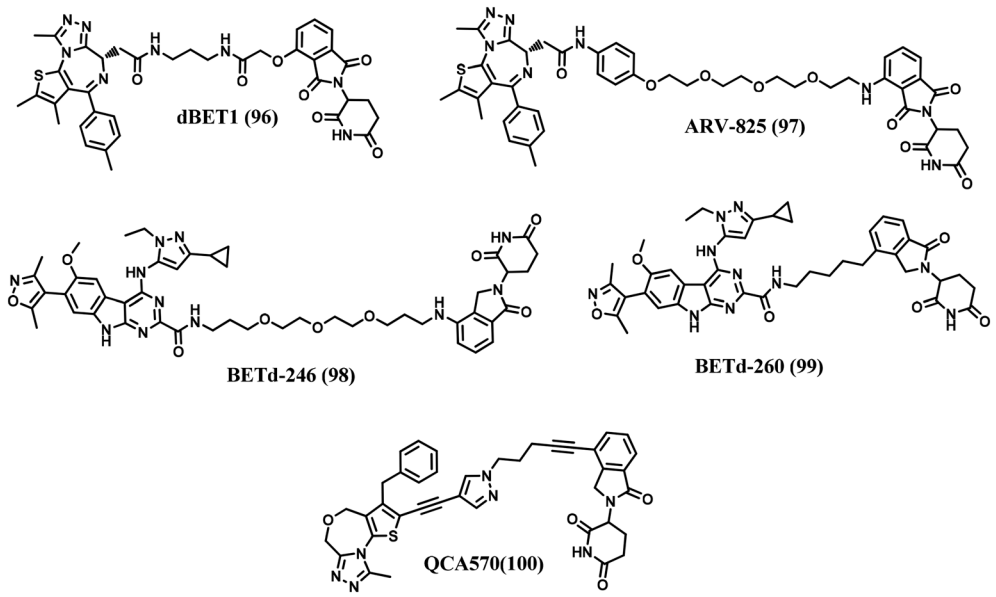


Fig. 21 Reported Brd4 degraders based on CRL4$^{CRBN}$ E3 ligase.

**VHL-2** and **VH-032** was solvent exposed, indicating that it could provide a suitable connecting point for a linker. By conjugating the terminal acetyl groups of **VHL-2** or **VH-032** with various Brd4 inhibitors through different linkers, CRL2$^{VHL}$ E3-based Brd4 degraders were obtained (Fig. 22).

**ARV-771** (**101**) is a VHL-based PROTAC using **OTX015** for the BET inhibitor portion and **VHL-2** as a ligand for the VHL E3 ligase.[124] **ARV-771** specifically degraded Brd4 as well as Brd2 and Brd3 with a $DC_{50}$ of <5 nM and led to the depletion of c-Myc protein with an $IC_{50}$ < 1 nM in several cellular models of castration-resistant prostate cancer. In addition, despite possessing $K_d$ values comparable with those of the (+)-**JQ1**, **ARV-771** demonstrated a more than 10 times higher efficacy in decreasing c-Myc levels. This strongly supports

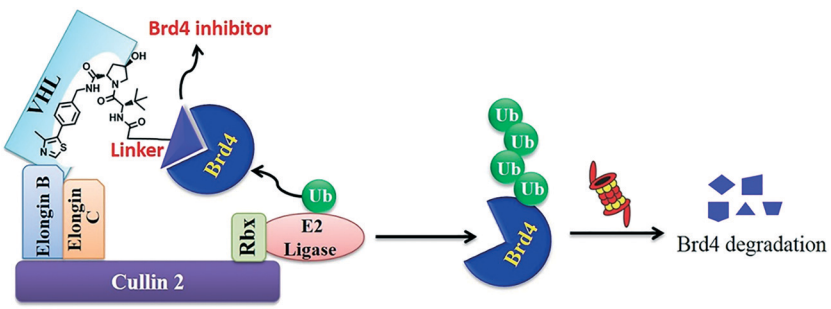


Fig. 22 Mechanism of Brd4 degradation by CRL2$^{VHL}$ E3-based Brd4 degraders.

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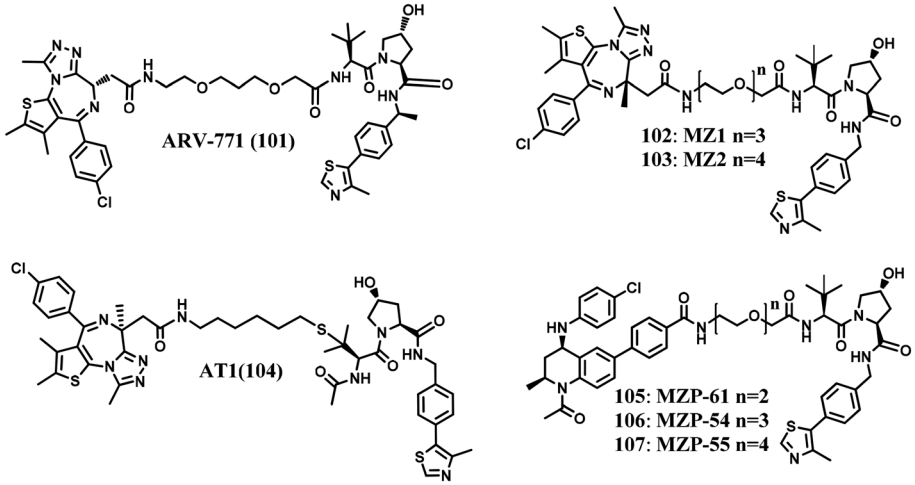


Fig. 23 Reported Brd4 degraders based on $CRL2^{VHL}$ E3 ligase.

that **ARV-771** behaved catalytically in degrading Brd4, consistent with previous findings by Winter *et al.* and Crews *et al.*[119,120] **ARV-771** showed significant antiproliferative activity towards prostate cancer cells with 10- to 500-fold higher potency than the inhibitors (+)-**JQ1** or **OTX015**, in line with its robust effects on apoptotic induction. *In vivo* experiments indicated that **ARV-771**, rather than inhibitor **OTX015**, effectively attenuated Brd4 and c-Myc levels and suppressed tumor growth and, more notably, induced partial tumor regression in a 22Rv1 xenograft model. **ARV-771** also showed superior tumor suppression and induced greater survival improvement than **OTX015** in immune-depleted mice engrafted with ibrutinib-resistant MCL cells.[125]

Ciulli and colleagues developed several PROTACs using (+)-**JQ1** and **VH-032**, a high-affinity ligand that had been designed against the VHL E3 ligase.[126] Treatment with **MZ1** (**102**), the most potent compound, for 24 h at a concentration of 1 μM completely depleted Brd4 proteins in HeLa cells, and no protein could be detected even after 48 h, indicating that **MZ1** was a powerful and long-lasting Brd4 degrader. Interestingly, despite (+)-**JQ1** showing no selectivity for individual BET family members and **MZ1** having comparable affinities to BET bromodomains, **MZ1** could induce potent and preferential depletion of Brd4 over its homologues Brd2 and Brd3 at suitable concentrations in both HeLa cells and U2OS osteosarcoma cells, which partly accounted for the different protein depletion profile and pharmacological response between **MZ1** and (+)-**JQ1**.

Recently, the crystal structure of the VHL E3–**MZ1**–Brd4 ternary complex and its impact on target degradation selectivity was elucidated by Ciulli *et al.* According to their work, **AT1** (**103**) was synthesized by attaching (+)-**JQ1** to the *tert*-Leu group of VHL ligand *via* a short linker, which was different from other VHL-based PROTACs. Treatment with 1 μM **AT1** for 24 h in all tested cancer cells depleted most of the Brd4, while the decrease of Brd2 and Brd3 as well as other 5671 detected proteins was negligible. This result suggested that **AT1** exhibited a more remarkable selectivity in depleting Brd4 than **MZ1**.[107]

Most recently, Ciulli's group has reported three novel VHL-recruiting PROTACs, **MZP-61** (**105**), **MZP-54** (**106**) and **MZP-55** (**107**), which were derived from a potent tetrahydroquinoline-based Brd4 inhibitor, **I-BET726**.[127] **MZP-54** proved to be the most powerful degrader, which was capable of inducing marked depletion of both BET and c-Myc proteins concentration-dependently in HeLa cells with a certain degree of selectivity for Brd4 and Brd3 over Brd2. However, despite **I-BET726** ($K_d$ = 4 nM) being a more potent Brd4 inhibitor than (+)-**JQ1** ($K_d$ = 100 nM), a direct comparison showed that **MZP-54** was a less powerful Brd4 degrader than **MZ1** due to negative cooperativities of ternary complex formation confirmed by ITC assay. Given being negatively cooperative, **MZP-54** still effectively degraded Brd4 at nanomolar concentrations and showed remarkable antiproliferative activity in two tested cell lines, suggesting that substoichiometric catalytic degradation was powerful (Fig. 23).

## Conclusion

Over the past decade, researchers have identified the cellular functions of BET proteins and their important roles in the development of many malignancies and other diseases including inflammation, HIV infection, and cardiovascular diseases. Among them, Brd4 is the most extensively studied member. Brd4 inhibitors with various scaffolds are being explored as therapeutic agents in numerous hematopoietic and solid tumor types, and some of them have entered into human clinical trials. Despite the exciting results of preliminary clinical trials using Brd4 inhibitors in malignancies, there are issues to resolve. Most of the reported Brd4 inhibitors bind non-selectively to BD1 and BD2 and also show low selectivity for individual BET family members. BET family members have different downstream gene regulatory profiles, and pan-BET inhibition makes an impact on numerous transcriptional pathways and leads to side effects, which has been observed in clinical trials of compound **OTX015**.[128] Thus, significant efforts are urgently needed to develop Brd4 inhibitors

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with high specificity and affinity. Moreover, drug resistance against triazoloazepine Brd4 inhibitors has been reported.[99] Considering the dominant position of triazoloazepine-based Brd4 inhibitors in clinical trials, novel Brd4 inhibitors with different chemical chemotypes deserve to be explored to address this issue as well as to elucidate the resistance mechanisms. Bivalent Brd4 inhibitors show profoundly enhanced potency than the parent monovalent inhibitor and may provide a new strategy for overcoming drug resistance. However, the bivalent Brd4 inhibitors reported now are mainly achieved by linking two monovalent inhibitors with a flexible linker, causing a large molecular weight and compromised drug-likeness; thus, there exists a large chemical space to develop structurally diverse bivalent Brd4 inhibitors.

In addition to functional inhibition by small molecules, Brd4 has been successfully targeted for degradation using PROTACs. Several PROTACs selectively and sub-stoichiometrically degraded Brd4 and demonstrated a more potent antitumor activity than the corresponding Brd4 inhibitors. Albeit PROTAC-induced Brd4 degradation has achieved impressive efficacy in several mouse models of cancer, the clinical potential of this technology remains to be explored. Challenges, including unfavorable physicochemical properties and low bioavailability owing to their relatively large molecular mass and scarce small E3 ligands, must be overcome in order to advance PROTACs into the clinic.

Overall, both traditional small-molecule Brd4 inhibitors and newly-developed Brd4 degraders have shown promising results in various human cancers. There is great hope that with sustained efforts on the drug discovery of Brd4, novel anticancer drugs targeting Brd4 will be achieved in the near future.

## List of abbreviations

| | |
|---|---|
| Brd4(1) | Bromodomain 1 of Brd4 |
| Brd4(2) | Bromodomain 2 of Brd4 |
| $IC_{50}$ | Half-maximal inhibitory concentration |
| $EC_{50}$ | Half-maximal effective concentration |
| $GI_{50}$ | Half-maximal growth inhibitory concentration |
| TGI | Tumor growth inhibition |
| $K_d$ | Dissociation constant |
| $K_i$ | Inhibition constant |
| $T_{1/2}$ | Elimination half-life |
| $F$ | Bioavailability |
| Q.D. | Once a day |

## Conflicts of interest

There are no conflicts of interest to declare.

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