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Communication

Discovery of [1,2,4]triazolo[1,5-*a*]pyrimidine derivatives as new bromodomain-containing protein 4 (BRD4) inhibitors

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ABSTRACT

Targeting bromodomain-containing protein 4 (BRD4) has been proved to be an effective strategy for cancer therapy. To date, numerous BRD4 inhibitors and degraders have been identified, some of which have advanced into clinical trials. In this work, a focused library of new [1,2,4]triazolo[1,5-*a*]pyrimidine derivatives were discovered to be able to inhibit BRD4. **WS-722** inactivated BRD4 (BD1/BD2), BRD2 (BD1/BD2) and BRD3 (BD1/BD2) broadly with the IC_{50} values less than 5 μmol/L. Besides, **WS-722** inhibited growth of THP-1 cells with an IC_{50} value of 3.86 μmol/L. Like (+)-**JQ1**, **WS-722** inhibited BRD4 in a reversible manner and enhanced protein stability. Docking studies showed that **WS-722** occupied the central acetyl-lysine (Kac) binding cavity and formed a hydrogen bond with Asn140. In THP-1 cells, **WS-722** showed target engagement to BRD4. Cellular effects of **WS-722** on THP-1 cells were also examined, showing that **WS-722** could block c-MYC expression, induce G0/G1 phase arrest and p21 up-regulation, and promote differentiation of THP-1 cells. BRD4 inhibition by **WS-722** resulted in cell apoptosis and up-regulated expression of cleaved caspased-3/7 and PARP in THP-1 cell lines. The [1,2,4]triazolo[1,5-*a*]pyrimidine is a new template for the development of new BRD4 inhibitors.

As histone acetylation can be written by histone acetyltransferases (HATs) and erased by histone deacetylases (HDACs), the reading process of histone acetylation marks is much less studied until the discovery of potent and selective inhibitors of bromo and extra-terminal (BET) domain proteins [1,2]. As a unique protein domain, BET domain, a highly conserved domain composed of about 110 amino acids, can recognize the histone acetylation modification and mediate interaction of proteins. Proteins with BET domain can be divided into nine protein families due to the sequence similarity, of which BRD2, BRD3, BRD4 and BRDT are the largest and most studied. All members of BET family have two BRD active domains BD1 and BD2 and an extra-terminal (ET) region, which bind to acetylated lysine located in H3 and H4 as well as some other non-histone proteins [3,4]. Among the BET family members, BRD4, abnormally expressed in many tumor cancer cells, has been reported to be associated with various diseases [5–10], which makes it an important epigenetic target for disease treatment. To date, a large number of small-molecule inhibitors and degraders targeting BET proteins have been reported [11–19], some of which such as (+)-**JQ1**, **OTX-015**, **TEN-010**, **I-BET762**, **CPI-0610**, **I-BET151**, **PLX51107**, **ABBV-075**, **AZD5153**, **BMS-986158** and **INCB0543294** are currently undergoing clinical assessment at different phases for cancer therapy [20,21].

Our previous work has showed that the bicyclic triazole fused pyrimidines have possessed interesting biological activities [22–33]. Following our previous work, herein a focused library of [1,2,4]triazolo[1,5-*a*]pyrimidine-based BRD4 inhibitors were synthesized and evaluated for their inhibitory activity against BRD4. Among these compounds, **WS-722** effectively and reversibly inhibited BRD4 (BD1) and BRD4 (BD2) with the IC_{50} values of 2.15 and 4.36 μmol/L, respectively. Cellular target engagement in THP-1 cells was confirmed by the thermal shift assay. Cellular effects were also examined in THP-1 cells, indicating that **WS-722** induced differentiation of THP-1 cells, arrested cell cycle at G0/G1 phase and resulted in obvious cell apoptosis. The results suggest that the [1,2,4]triazolo[1,5-*a*]pyrimidine is a new starting point for the development of BRD4 inhibitors.

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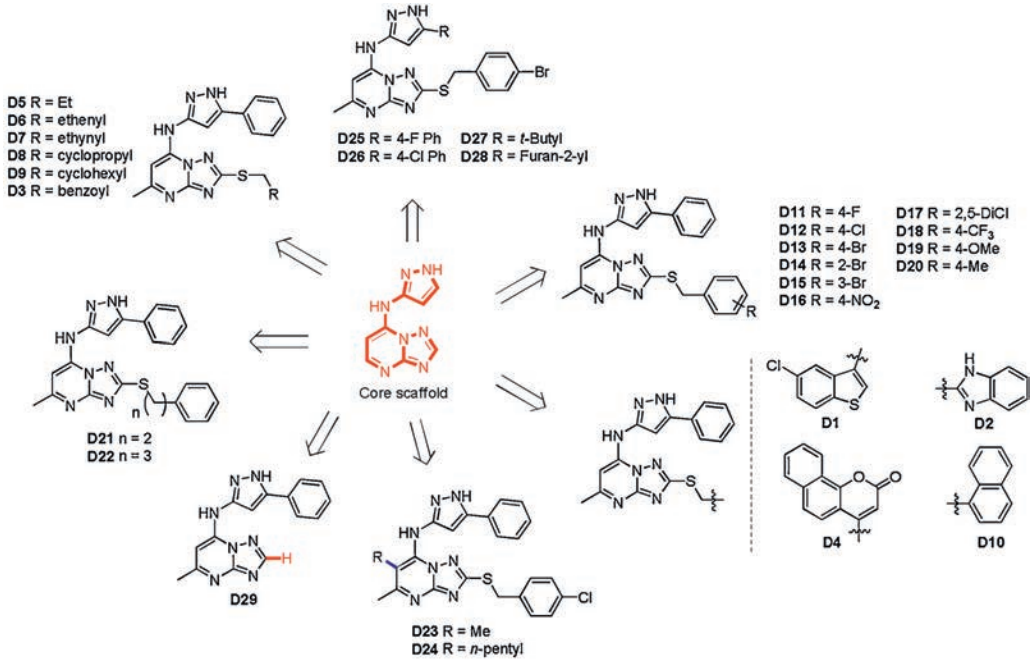

Fig. 1. Modifications based on the score scaffold for SARs studies.

The general synthetic route of compounds **D1**–**D29** is shown in Scheme S1 (Supporting information). The synthesized compounds are shown in Fig. 1 with the core scaffold highlighted in red. Clearly, modifications focused on the variations of substituents, generating a focused library of [1,2,4]triazolo[1,5-*a*]pyrimidine derivatives.

Based on the established TR-FRET protocol (Fig. S1 in Supporting information), we first screened the synthesized compounds for their inhibitory activity against BRD4 (BD1) using (+)-**JQ1** as a positive control and the results are shown in Fig. S2 (Supporting information). The compounds showed varied inhibitory activity against BRD4, indicating the importance of substituents for the activity. Particularly, nine of these compounds (Fig. S2 in Supporting information) exhibited acceptable inhibitory activity against BRD4 (BD1) at 10 μmol/L with the inhibitory rates over 39%. In view of their biochemical potency against BRD4 (BD1), these nine compounds were then further evaluated against BRD4 (BD2), the IC_{50} values of these compounds were shown in Table 1. Compound **D7** (also named as **WS-722**) displayed the best potency against BRD4 (BD1/BD2) with the IC_{50} values of 2.15 and 4.36 μmol/L, respectively. Compound **D13** bearing the *p*-bromobenzyl group had comparable inhibitory activity with **D7** against BRD4. In contrast, compounds **D1**, **D12**, **D14**, **D15**, **D18** and **D19** showed decreased inhibitory activity against BRD4. Compared to **D12**, introduction of the R group to the core scaffold did not significantly improve the inhibitory activity. **D24** inhibited BRD4 (BD1) and BRD4 (BD2) moderately with the IC_{50} values of 11.21 and 8.64 μmol/L, respectively.

Table 1
Inhibitory activity of selected compounds against BRD4.

Compound	BRD4 (BD1) IC_{50} (μmol/L)	BRD4 (BD2) IC_{50} (μmol/L)
D1	5.59 ± 0.16	7.71 ± 0.21
D7 (**WS-722**)	2.15 ± 0.23	4.36 ± 0.13
D12	15.65 ± 0.58	10.76 ± 0.63
D13	3.75 ± 0.68	1.56 ± 0.51
D14	25.35 ± 0.81	15.73 ± 0.89
D15	4.61 ± 0.61	9.82 ± 0.71
D18	13.43 ± 0.72	8.37 ± 0.18
D19	4.53 ± 0.37	5.24 ± 0.67
D24	11.21 ± 0.76	8.64 ± 0.79
(+)-**JQ1**	0.131 ± 0.002	0.037 ± 0.002

In view of the acceptable potency of **WS-722** toward BRD4 (Fig. 2A), we further evaluated its selectivity against BRD2 and BRD3. As shown in Figs. 2B and C, **WS-722** also moderately inhibited BRD2 and BRD3, indicating that **WS-722** is a pan-BRD inhibitor *in vitro*. A dilution assay was then used to test the reversibility using (+)-**JQ1** as the control compound. We found that 100-fold dilution of the BRD4/**WS-722** mixture resulted in the recovery of BRD4 activity (Figs. 2D and E). Similarly, the BRD4 activity of (+)-**JQ1** can also be recovered after dilution (Figs. 2D and E). These results indicate that like (+)-**JQ1**, **WS-722** reversibly bound to BRD4 (BD1) and BRD4 (BD2) (Figs. 2D and E). The melting temperature (T_m) is defined as the temperature at which half of the protein is native and the other half denatured, and could be used to describe the stability of a protein. The positive changes in T_m upon ligand binding indicate thermal stabilization. To further evaluate the binding property of **WS-722** to BRD4, the protein thermal shift assay [34] was used to detect the ΔT_m between the T_m (BRD4 + 25 μmol/L **WS-722**) value and the reference T_m (BRD4 + DMSO) using the (+)-**JQ1** as the control compound. As indicated in Fig. 2F, for BRD4 (BD1) and BRD4 (BD2), the ΔT_m increased upon **WS-722** and (+)-**JQ1** treatment. The results suggest that (+)-**JQ1** and **WS-722** could enhance BRD4 protein stability.

Recent studies have shown that BRD4 has played key roles in the maintenance of aberrant chromatin states in AML, acute lymphoblastic leukemia (ALL), myeloma and lymphoma, and treatment with BRD4 inhibitors could recapitulate anti-leukemic effects in several AML cell lines [35–37]. Initially, the antiproliferative activity of **WS-722** was evaluated against THP-1 cells. As shown in Fig. 3A, after treatment for 7 days, **WS-722** moderately inhibited growth of THP-1 cells with an IC_{50} value of 3.86 μmol/L. To confirm whether **WS-722** could abrogate BRD4 activity in acute leukemia cell lines, we used the cellular thermal shift assay to study thermal stability of BRD4 upon **WS-722** treatment in THP-1

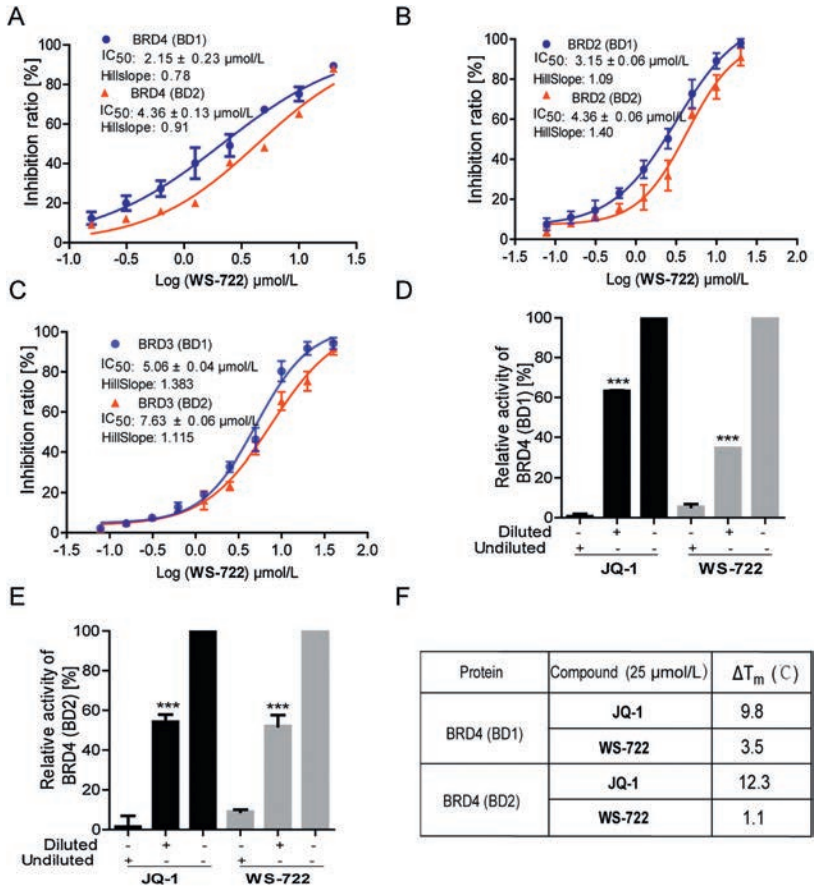

Fig. 2. Biochemical characterization of **WS-722**. (A, B, C) Inhibitory activity of **WS-722** against BRD2/3/4 (BD1) and BRD2/3/4 (BD2); (D) Dilution assay of **WS-722** to BRD4 (BD1) with TR-FRET assay, (+)-**JQ1** was used as the positive control; (E) Dilution assay of **WS-722** to BRD4 (BD2) with TR-FRET assay, (+)-**JQ1** was used as the positive control; (F) BRD4 (BD1) and BRD4 (BD2) recombinant protein thermal shift assays were performed when treated with **WS-722** and (+)-**JQ1**. d(Fluorescence)/dT and ΔT_m was analyzed. Data are the mean ± SD. Each experiment was repeated three times. *** $P < 0.001$.

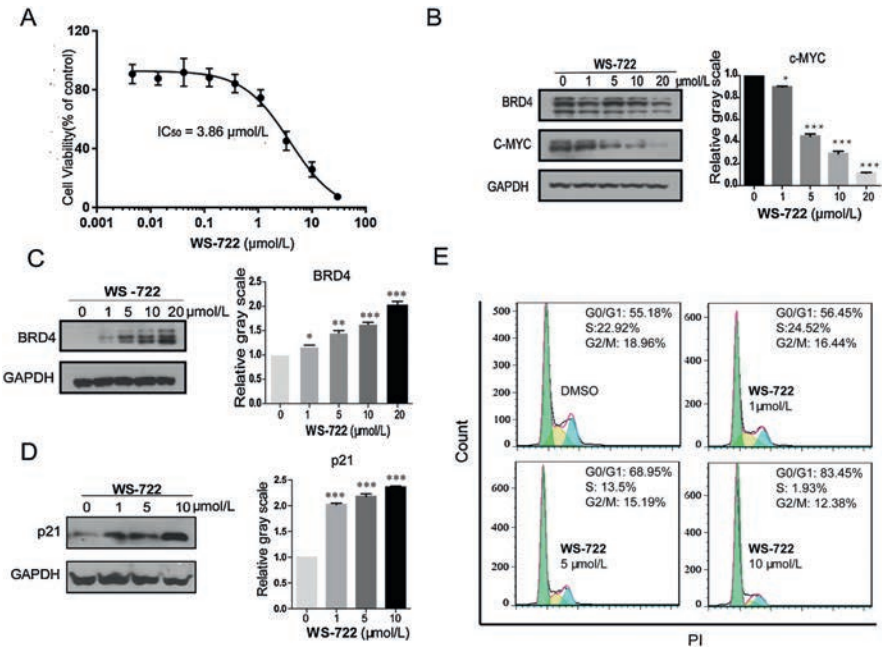

Fig. 3. WS-722 treatment decreases c-MYC and induces cell cycle in THP-1 cell line. (A) The effect of compound **WS-722** on the viability of THP-1 cells for 7 days; (B) Enhancement of thermal stability of BRD4 in THP-1 cells; (C) BRD4 and c-MYC expression in THP-1 cells when treated with **WS- 722** for 24 h. GAPDH was used as the loading control; (D) Expression of p21 when THP-1 cells were treated with **WS-722** for 48 h. GAPDH was used as the loading control; (E) Effect of **WS-722** on the cell cycle of THP-1 cells. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

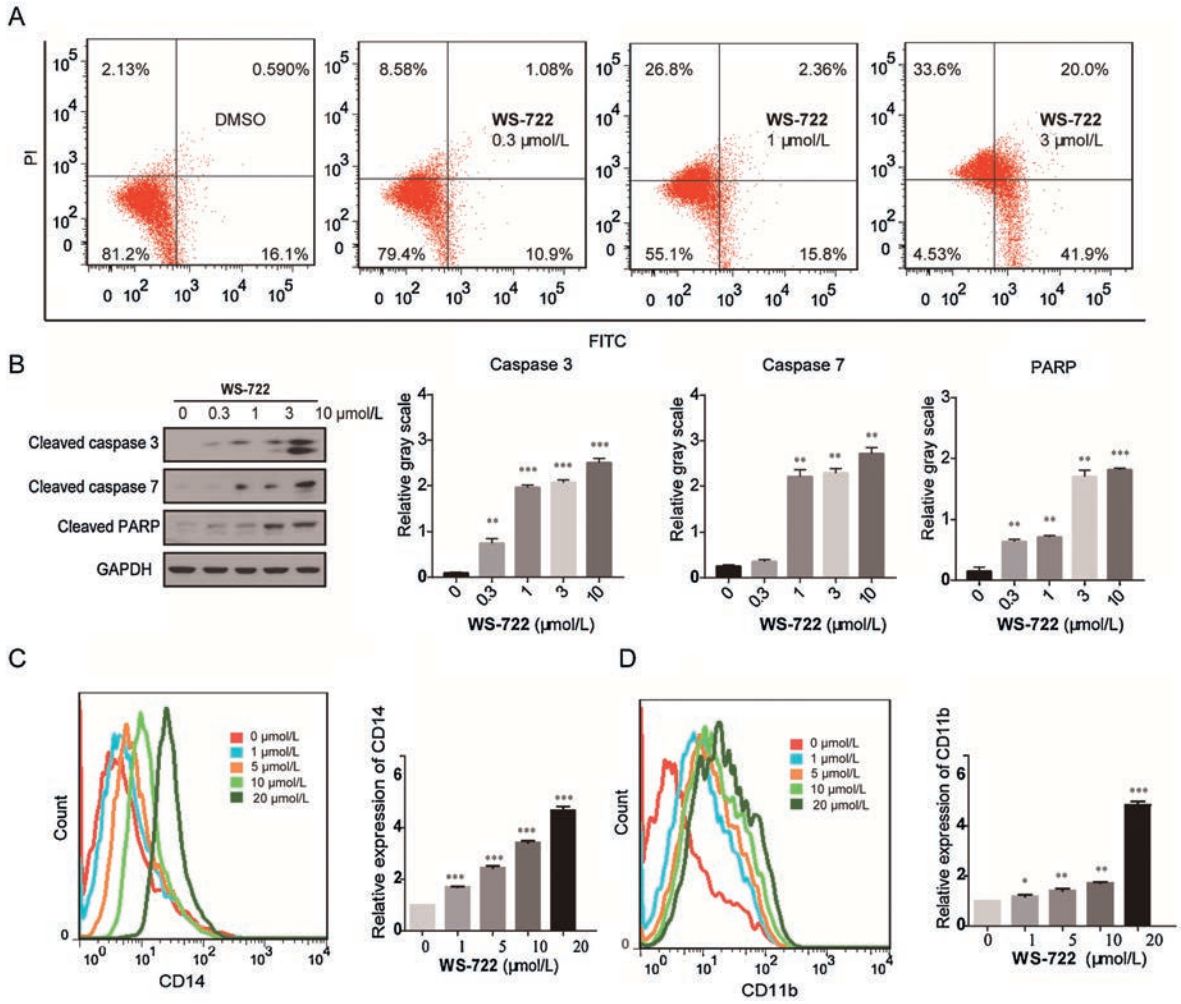

Fig. 4. Effect of **WS-722** on cell apoptosis and differentiation in THP-1 cell line. (A) THP-1 cell apoptosis when treated with **WS-722** at doses from 0.3 μmol/L to 3.0 μmol/L for 48 h. (B) Cleaved caspase-3, caspase-7 and PARP expression when treated with **WS-722** for 48 h. (C and D) Flow cytometry analysis of CD11b (C) and CD14 (D) in THP-1 cells treated with **WS-722**. Data are the mean ± SD. Each experiment was repeated three times. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

cell line. THP-1 cells were treated with **WS-722** and then heated for 3 min at 50 °C. After freezing in liquid nitrogen and thawing on ice, equal amounts of supernatant were removed and blotted with the BRD4 antibody. Our results suggested that **WS-722** stabilized BRD4 in a concentration-dependent manner, suggesting cellular target engagement of **WS-722** to BRD4 in THP-1 cells (Fig. 3B). It has been reported that BET-bromodomain inhibition potently suppresses MYC gene expression in leukemia by blocking its transcription [38]. Hence, to further explore the biological role of our BRD4 inhibitor **WS-722** in THP-1 cells, THP-1 cells were treated with **WS-722** and then subjected to western blotting analysis. As shown in Fig. 3C, treatment with **WS-722** dose-dependently decreased expression of c-MYC, without impact on BRD4 expression (Fig. 3C). To examine higher-order influences over biological networks regulated by c-MYC, which can influence cell cycle and p21 (also known as cyclin-dependent kinase inhibitor 1 or CDK-interacting protein 1) expression [39]. Expression of cell cycle regulatory protein p21 was also examined after treatment with **WS-722** for 48 h. As shown in Fig. 3D, **WS-722** treatment concentration-dependently induced accumulation of p21 in THP-1 cells. p21 is an universal cell-cycle inhibitor directly controlled by p53 and p53-independent pathways [40,41]. Then, the cell cycle of THP-1 cells was analyzed with flow cytometry after treatment with **WS-722**. As shown in Fig. 3E, **WS-722** arrested cell cycle of THP-1 cells at G0/G1 phase in a concentration-dependent manner, which was consistent with previous reports [42–44]. All these results confirmed that pharmacological inhibition of BRD4 could block c-MYC expression and then induce G0/G1 phase arrest and p21 up-regulation.

To study the effect of **WS-722** on cell apoptosis, THP-1 cells were treated with **WS-722** at doses from 0.3 μmol/L to 10 μmol/L for 48 h. We found **WS-722** induced apoptosis at high concentration (3.0 μmol/L) as detected by annexin V staining and PI uptake (Fig. 4A). Then samples were taken for the induction of apoptosis by measuring caspase-3, caspase-7 and PARP activity. As shown in Fig. 4B, after treatment of THP-1 cells with **WS-722** for 48 h, cleaved caspase 3, caspase 7 and PARP increased in a concentration-dependent manner. It has been reported that MYC (a family of regulator genes and proto-oncogenes that code for transcription factors) arrests the differentiation of embryonic stem cells and various neoplasms [45]. So, we assessed whether **WS-722** could promote differentiation of THP-1 cells by quantifying the expression of two differentiation related markers: CD14 and CD11b. As shown in Figs. 4C and D, **WS-722** induced expression of CD11b and CD14 in THP-1 cells, which promoted differentiation of leukemia cells dose-dependently. To conclude, these results suggest that **WS-722** down-regulated expression of c-MYC, induced cell cycle arrest, and promoted differentiation of THP-1 cells.

(+)-**JQ1** features a novel thieno-triazolo-1,4-diazepine scaffold and is a well characterized cell permeable BET family inhibitor. (+)-**JQ1** strongly blocks binding of a tetra-acetylated histone H4 peptide to BRD4 and potently inhibits the first and second bromodomains (BD1 and BD2) of BRD4 with IC_{50} values of 77 and 33 nmol/L, respectively [46]. To explain the observed potency of compound **WS-722** against BRD4, an *in silico* docking simulation was carried out using the MOE 2015.10 software package. The crystal structure of the first bromodomain (BD1) of human BRD4 in complex with (+)-**JQ1** (PDB code: 3MXF) was therefore used as a docking receptor for computational studies. Similar to interactions observed in acetyl-lysine (Kac) complexes [47], (+)-**JQ1** (Fig. S3A in Supporting information) was fitted well into the hydrophobic Kac binding site with an extraordinary shape complementarity [46]. As revealed by the co-crystal structure of BRD4 (BD1)/(+)-**JQ1** complex (Fig. S3C in Supporting information), the triazole ring of (+)-**JQ1** formed a hydrogen bond with surrounding residue Asn140 in BRD4 (BD1), and the conserved BET residues stabilized the binding of (+)-**JQ1** through hydrophobic interactions. As depicted in Figs. S3A and S3B (Supporting information), like (+)-**JQ1**, **WS-722** occupied the central acetyl-lysine (Kac) cavity and formed a hydrogen bond with the key residue Asn140 as well (Fig. S3D in Supporting information). The binding models may be responsible for the observed potency of **WS-722** against BRD4 (BD1) (IC_{50} = 2.15 μmol/L). We also found that **WS-722** did not fit into the regions occupied by the bulky *t*-butyl ester group and *p*-chlorophenyl group (Fig. S3A), the bulky *t*-butyl ester group is predicted to mitigate binding to the central benzodiazepine receptor [48]. Additionally, the hydrophobic propargyl group was directed to a hydrophilic region (Fig. 3SB), which is not occupied by (+)-**JQ1**. These observations may explain the weaker potency of **WS-722** against BRD4 (BD1) than (+)-**JQ1**. As demonstrated in Fig. S3B, the phenyl ring in **WS-722** was oriented to the solvent region, suggesting that further structural modifications at this site may be allowed for improved potency and/or PD/PK properties. We believe that the structural basis may facilitate further structure-based drug design (SBDD) for more potent [1,2,4]triazolo[1,5-*a*]pyrimidine-based BRD4 inhibitors.

In summary, a focused library of new [1,2,4]triazolo[1,5-*a*]-pyrimidine derivatives were synthesized and evaluated for their inhibitory activity against BRD4. The shortlisted compound **WS-722** broadly inactivated BRD4 (BD1/BD2), BRD2 (BD1/BD2) and BRD3 (BD1/BD2) with the IC_{50} values less than 5 μmol/L. Besides, **WS-722** inhibited growth of THP-1 cells with an IC_{50} value of 3.86 μmol/L. Like (+)-**JQ1**, **WS-722** inhibited BRD4 in a reversible manner and enhanced protein stability as indicated in the protein thermal shift assay. Docking studies showed that **WS-722** had a similar binding model with (+)-**JQ1**, occupying the central acetyl-lysine (Kac) binding cavity and forming a hydrogen bond with the key residue Asn140. In THP-1 cells, **WS-722** showed cellular target engagement to BRD4. Cellular effects of **WS-722** in THP-1 cells were also examined, showing that **WS-722** could block c-MYC expression, induce G0/G1 phase arrest and p21 up-regulation, and promote differentiation of leukemia cells. What is more, inhibition of BRD4 by **WS-722** in THP-1 cell lines resulted in obvious cell apoptosis and up-regulated the expression levels of cleaved caspased-3/7 and PARP. The [1,2,4]triazole[1,5-*a*]pyrimidine may be served as a new scaffold for the development of novel BRD4 inhibitors.

Acknowledgments

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.cclet.2019.08.029.

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