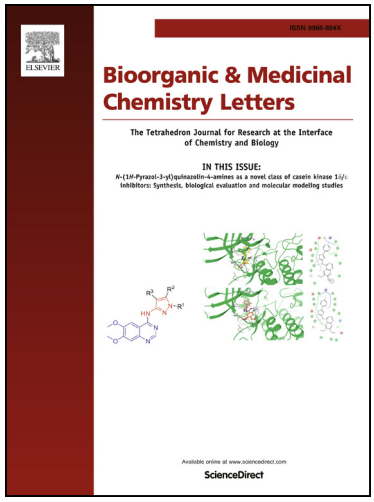

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Structure-based design, synthesis and in vitro antiproliferative effects studies of novel dual BRD4/HDAC inhibitors

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Bromodomain and extraterminal (BET) and histone deacetylase (HDAC), which are important epigenetic modulators, are influential targets in drug discovery and development. The BET family proteins, including Brd2, Brd3, Brd4, and BrdT, utilize tandem bromodomains (BD-1 and BD-2) to recognize specific acetylated lysine residues in the *N*-terminal tails of histone proteins[1], and play vital roles in the regulation of cellular proliferation and apoptosis by regulating the genes transcription[2,

[3]. HDACs are a family of enzymes that catalyze the deacetylation of lysine residues locating at the *N*-terminus of different protein substrates[4]. There are eighteen human HDACs enzymes divided into four different categories: class I (HDACs 1,2,3,8), class II (HDACs 4,5,6,7,9,10), class III (Sirtuins 1,2,3,4,5,6,7) and class IV (HDAC 11). The class III HDACs enzymes are NAD^+ dependent while the others belong to zinc dependent metal enzymes[5, 6]. Actually, only zinc dependent HDACs, especially class I and class II isozymes, are closely related to the proliferation, angiogenesis and differentiation of tumor cells.

BET bromodomain inhibitors (BET inhibitors) can block cancer cell proliferation and induce apoptosis in a wide range of tumor types[7]. To date, several classes of potent and specific BET inhibitors have been reported, such as JQ-1[8], I-BET-151[9], I-BET726[10] and RVX-208[11] (Fig. 1A). These prospective BET inhibitors have provided a set of powerful pharmacological tools for further investigation about the mechanism of BET proteins in essential cellular processes and the therapeutic potential of BET inhibition in human diseases[12].

As have been approved by FDA for the treatment of haematological and solid tumors (Fig. 1B), HDACs inhibitors have been becoming a promising therapy for aberrant epigenetic changes associated with cancer, inflammation and neurodegenerative diseases[13]. The hydroxamic acid vorinostat, (also known as SAHA or Zolinza) was the first-in-class FDA approved HDAC-inhibitor to treat cutaneous T-cell lymphoma (CTCL) in 2006[14]. Oral panobinostat (also known as LBH589 or

Farydak) combined with bortezomib and dexamethasone was approved by FDA for the treatment of recurrent multiple myeloma in 2015[15]. Accordingly, the pharmacophore of HDACs inhibitors (Fig. 1A, Vorinpstat) is usually composed of a capping group, a zinc-binding group (ZBG) and a proper linker. And hydroxamic acid is one of the most potent zinc^{2+} chelating groups among ZBGs which play important roles in the binding efficiency between HDACs inhibitors and enzyme[16].

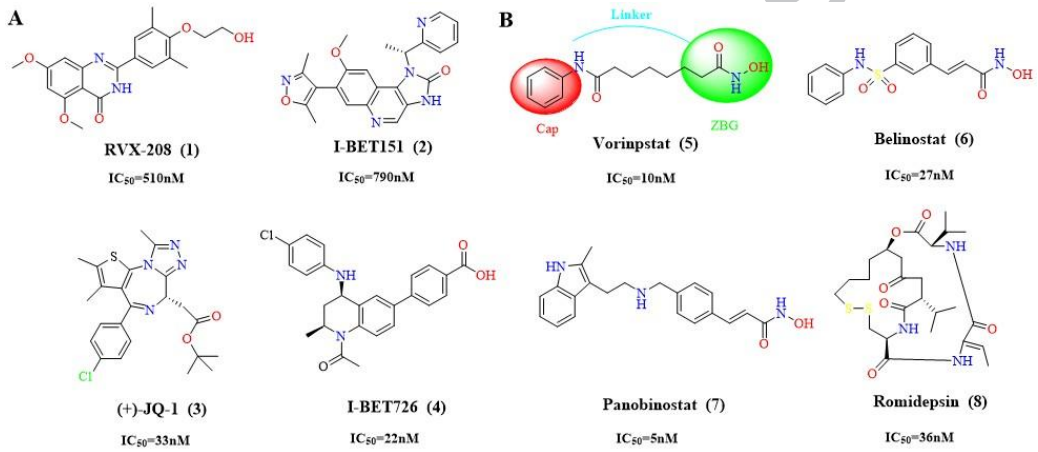


Figure 1. (A) Structures of representative inhibitors of BET bromodomains. (B) Structures of FDA approved HDAC inhibitors.

Recent studies have indicated that BET and HDAC inhibitors have similar target genes and biological effects, thus synergizing to kill in Myc-induced murine lymphoma[17]. Combination of BRD4 antagonist and histone deacetylase inhibitor have been proven effective to against human acute myelogenous leukemia cells[18]. Therefore, incorporation of the active group of BET and HDAC inhibitors into one molecule will be a new way for cancer drug development. Atkinson et al.[19] have reported a dual active BRD/HDAC small molecule probe by fusing a BRD active tetrahydroquinoline (THQ) core with a hydroxamic acid HDACi motif, while Zhang et al.[16] have also been synthesizing a series of novel 3, 5-dimethylisoxazole

derivatives as BRD4/HDAC dual inhibitors. These dual BET/HDACs inhibitors were demonstrated to be efficacious and promising, suggesting that the drugs can be rationally designed for disease applications. In addition, further investigation is necessary for better effectiveness and specificity of BET/HDACs dual inhibitors.

For the treatment of atherosclerosis-associated cardiovascular diseases, Resverlogix Corporation has designed the quinazolone RVX-208[20, 21] which has been entering clinical trials on Alzheimer's disease recently[22]. RVX-208 is a derivative of resveratrol (3, 4', 5-trihydroxy-transstilbene), which has good biological effects on the BET proteins. Besides, RVX-208 prefer to binds with the second bromodomain of BET proteins, presenting better selectivity on BD1 up to 23-fold[23]. It indicates that quinazolone derivatives are worth of further investigations.

RVX-OH is another compound closely related to RVX208, but it lacks of selectivity against BD1 and BD2. In order to maintain the efficacy of RVX-208 on the BET proteins during the development of BRD4/HDAC dual inhibitors, we have observed the previously reported X-ray crystal structure of RVX-208 and RVX-OH within the first and second bromdomains of human BET proteins (**Fig. 2**). According to the analysis of the crystal structure of the second bromodomain of BRD2, we find that RVX-208 bound to the acetyl-lysine binding pocket in a peptide-competitive manner and the hydroxy-ethylether moiety of RVX-208 is outside of the acetyl-lysine binding pocket and rarely contacts with the bromodomain surface[23]. The binding mode of RVX-OH is similar to RVX-208 in the crystal structure of the second

bromodomain of BRD2 (**Fig. 2A**). Interestingly, RVX-OH reverses its binding mode in the crystal structure of the first bromodomain of BRD4, the free hydroxyl group that is from the phenyl ring system acts as an acetyl-lysine mimetic moiety, thus forming a hydrogen bond with N140, while RVX-208 has a similar interaction where the quinaxolinone functions as the acetyl-lysine mimetic moiety (**Fig. 2B**). Hence, in order to form a series of dual BRD/HDAC inhibitors, we used various types of linkers to link the ZBG group with the free hydroxyl group of the phenol ring (**Fig. 3**). The fused molecules were expected to retain the essential interactions with both proteins to exert desired biological functions.

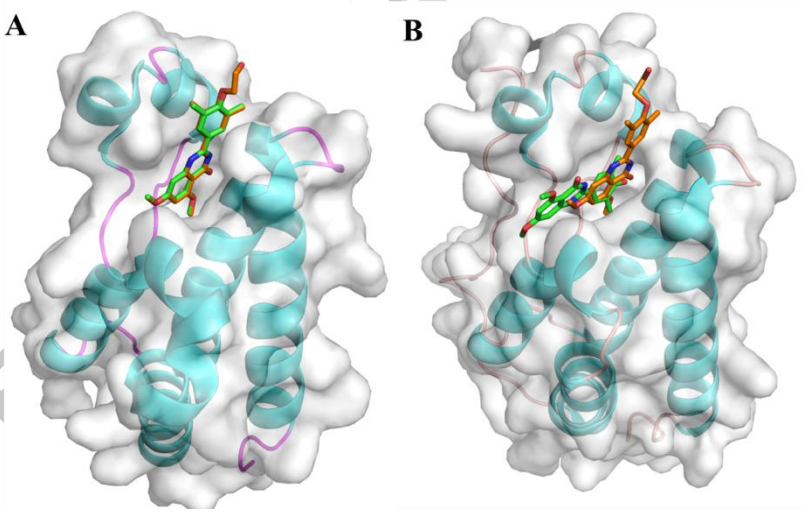


Figure 2. Design of dual inhibitors of BRD4/HDAC (A) Overview of RVX-208 and RVX-OH binding onto BD2 of BRD2 (PDB entry: 4mr6 and 4mr5). (B) Overview of RVX-208 and RVX-OH binding onto BD1 of BRD4 (PDB entry: 4mr4 and 4mr3). The proteins are shown as white surface and blue cartoon. Orange sticks represent RVX-208 and green sticks represent RVX-OH.

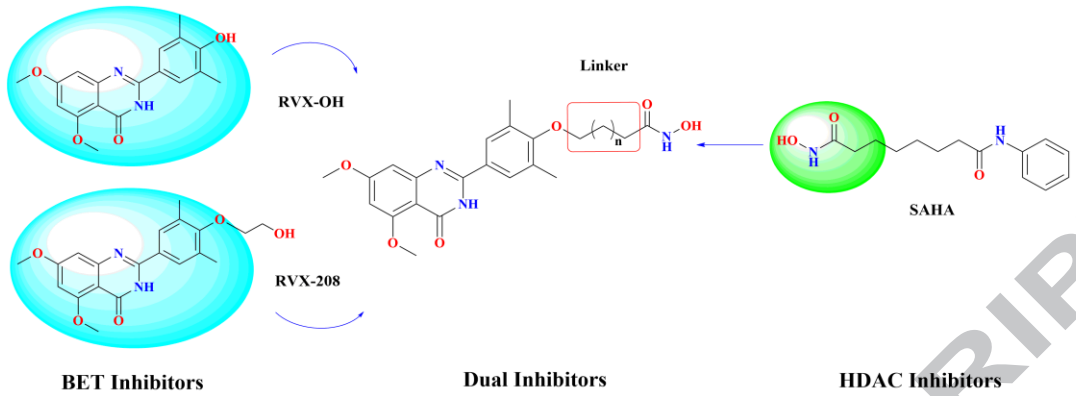
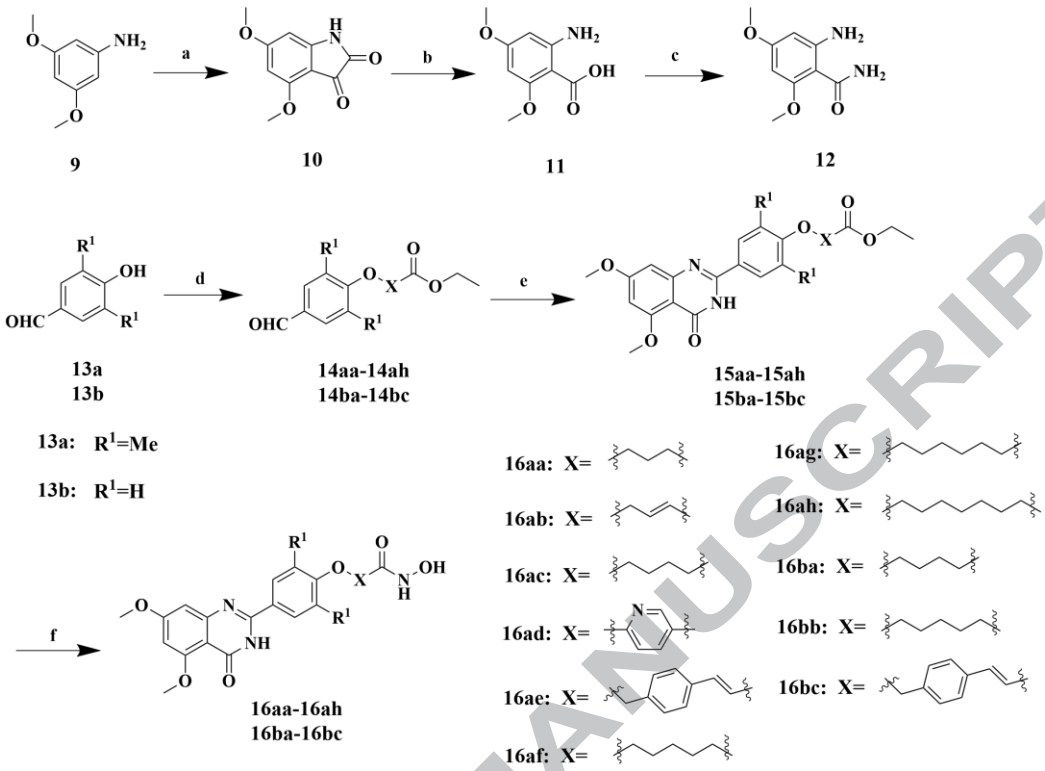


Figure 3. Schematic diagram of construct novel dual BRD4/HDAC inhibitors**.**

As shown in **Schemes 1**, all compounds were synthesized via the general route. Intermediate **10** was synthesized from the commercial available material 3,5-dimethoxyaniline (**9**) and 5-dimethoxyaniline, and together with the freshly made HCl (g) to produce the corresponding hydrochloride salt, which was followed condensed with oxalyl chloride to obtain the cyclization adduct. The 1*H*-pyrrole-2, 3-dione of compound **10** was hydrolyzed under the basic condition to give the *o*-aminobenzoic acid derivative **11**. The key intermediate **12** was synthesized by the amidation of **11** with NH_3 (g) in the presence of common peptide condensing agents EDC and HOBt. The other important intermediates **14aa-14ah** were simply made by refluxing of 4-hydroxy-3, 5-dimethylbenzaldehyde (**13a**) with various alkyl bromides in acetonitrile. Then **12** and **14aa-14ah** were treated with sodium hydrogen sulfite and PTSA under the high boiling point solvent DMAc to give compounds **15aa-15ah**, whose ester groups reacted with freshly prepared hydroxylamine in methanol to produce target compounds **16aa-16ah**. The rest compounds **16ba-16bc** were prepared in the same way from the distinct reagent 4-hydroxybenzaldehyde.



Scheme 1. Synthesis of Compounds **16aa-16ah** and **16ba-16bc**. Reaction conditions: (a) HCl (g), ether, 0 °C, 2 h, oxalyl chloride, 70 °C, 1.5 h, MeOH, reflux, 1 h, 81%; (b) NaOH (33% in water), H_2O_2 (30% in water), 1 h, 70 °C, 33% (c) EDC, HOBT, NMM, THF, 4 h, NH_3 (g), 1 h, rt, 75%; (d) K_2CO_3, alkyl bromide, acetonitrile, reflux, 24 h, 80–85%; (e) PTSA, $NaHSO_3$, DMAc, 120 °C, 16 h, 75-89%; (f) 50% NH_2OH aq, NaOH, CH_2Cl_2/MeOH (1:2), rt, 80–85%.

To explore the biological activity, compounds **16aa-16ah** were evaluated the inhibitory effects for human BRD4/BD2 at 5 μM and 0.5 μM (**Table 1**). All of them could inactive BRD4/BD2 over 50% at 5 μM, and the majority still inhibited BRD4/BD2 over 50% ration at 0.5 μM. This proposes that our compounds have maintained the biological activity to BRD4/BD2. We also evaluated compounds **16aa-16ah** for their inhibition of HDAC1 at 1 μM and 0.1 μM (**Table 1**). In accord with the results, we found that RVX-OH had no biological activity on HDAC1 as SAHA had no biological activity on BRD4/BD2. Almost all synthesized compounds,

except for **16ad**, inhibited 50% HDAC1 at 1 μM, whereas only **16ac, 16af** and **16ah** inhibited HDAC1 over 50% rate at 0.1 μM. This indicates that the compounds which use various alkanes as linkers have a better biological activity for HDAC1. Above all, **16ac** and **16af** showed powerful inhibitory function against HDAC1 and BRD4.

Table 1. Inhibition ratio of compounds **16aa-16ah** on BRD4/BD2 and HDAC1.

Compd	Avg. Inh% (BRD4/BD2)		Avg. Inh% (HADC1)	
	5μM	**0.5μM**	**1μM**	**0.1μM**
16aa	90±1	42±3	61±3	5±1
16ab	93±2	41±5	66±4	20±3
16ac	91±2	73±3	96±1	79±3
16ad	93±1	51±2	14±4	-2±2
16ae	83±3	62±2	74±3	24±4
16af	97±1	90±1	92±2	56±2
16ag	88±3	29±5	81±3	36±5
16ah	99±1	59±2	89±2	56±3
RVX-OH	98±1	87±1	5±1	0±0
SAHA	10±3	2±1	99±1	96±1

Based on the results of inhibition rate, compounds **16ac** and **16ae** were evaluated for their BRD4/BD1, BRD4/BD2 and HDAC1 inhibitory activity in vitro (**Table 2**). The results have shown that the activity of compound **16ac** and **16ae** were decreased comparing to the positive control, but still remained at a good level against BRD4/BD2 and HDAC1. Similar to RVX-208, our compounds showed good selectivity when they effect on BRD4/BD1 and BRD4/BD2, and the selectivity of

16ac and **16ae** are more pronounced than RVX-208 due to the larger groups introduced on the free hydroxyl group of the phenol ring. This indicates that our designed compounds not only retains the original activity, but also enhances their selectivity for BRD4/BD2.

Table 2. IC_{50} values of compounds **16ac** and**16ae** on BRD4/BD1, BRD4/BD2 and HDAC1.

Compd	IC_{50} (nM)		
	BRD4/BD1	**BRD4/BD2**	**HDAC1**
16ac	>5000	225±32	32±10
16ae	>5000	401±21	204±21
RVX-208	1985±233	67±9	-
SAHA	-	-	10±3

BET and HDAC inhibitors have been reported synergize to kill in Myc-induced murine lymphoma, hence the antiproliferative activities against 3 kinds of human acute myelogenous leukemia (AML) cell lines MV4-11, OCI-AML2 and OCI-AML3 of the representative compounds were evaluated by MTT. In order to investigate whether the two meta-methyl groups on benzene have influence in the biological activity of those compounds, the optimal compounds based on the previous result of **Table 1** and **16ba-16bc** were selected to further tests. As shown in **Table 3**, RVX-208 and SAHA were used as positive controls. However, compounds **16ba-16bc** did not significantly inhibit the proliferation of human AML cell lines. It indicated that the

two meta-methyl on benzene really affected biological effects of the compounds. As expected, compounds **16ac** and **16af** still exhibited remarkable anti-proliferative activities. But the compound **16ae** which contains the structure of styrene showed better biological activity than compounds **16ac** and **16af**. The positive control RVX-208 showed an inhibitory effect against AML cell lines, but the activity is not very good. Compound **16ae** showed better biological activity than RVX-208 and SAHA, and it suggested that the dual inhibitors are more effective than the single target inhibitors against AML cell lines.

Table 3. In vitro cell growth inhibitory effects of compounds on human AML cell lines

Compd	IC_{50} (μM)		
	MV4-11	**OCI-AML2**	**OCI-AML3**
16aa	5.00±0.32	4.53±0.48	5.53±0.34
16ac	1.67±0.21	1.52±0.21	1.09±0.18
16ad	1.39±0.21	6.36±0.37	3.04±0.23
16ae	0.56±0.09	0.38±0.08	0.43±0.18
16af	0.92±0.12	0.98±0.17	0.67±0.10
16ah	2.22±0.36	2.21±0.39	1.83±0.21
16ba	9.88±0.45	12.25±1.26	9.31±0.38
16bb	4.28±0.31	13.60±1.33	11.13±0.23
16bc	1,97±0.35	1.78±0.36	1.80±0.17
RVX-208	4.48±0.21	8.31±0.32	7.17±0.34
SAHA	0.98±0.27	0.78±0.32	0.85±0.29

In order to verify whether our compounds inhibit Myc in human ALM cell lines, the most promising compound **16ae** was selected to measure its bioactivity in OCI-AML2 and OCI-AML3 cells by Western blot. As shown in Figure 4, corresponding with the relative potency in MTT assays, **16ae** induced Myc in a concentration-dependent manner. JQ1 and SAHA, the single-target inhibitors, have weaker effect to reduce Myc level than **16ae** at high concentration. The results demonstrated that the designed dual inhibitors have achieved the desired goals.

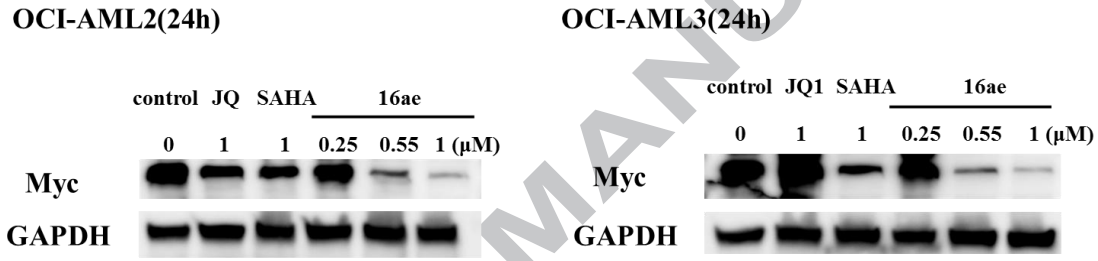


Figure 4. Western blot analysis of Myc in OCI-AML2 and OCI-AML3 cell lines after 24 h of treatment with compound 16ae at 250, 500, 1000 nM, JQ1 and SAHA at 1000 nM. GAPDH was used as a loading control.

The molecular docking with compound **16ae** in BRD4/BD2 and HDAC1 were performed and the results are shown in Figure 4. From the bonding mode of **16ae** with BRD4/BD2(Fig. 5A), we found that the compound bound to the acetyl-lysine binding pocket of BRD4/BD2 in a peptide-competitive manner, and one of the nitrogen atoms of the quinazolinone ring formed a hydrogen bond with the residue Asn-433, which was similar to the crystal structure of RVX208. The docking mode of **16ae** in complex with HDAC1 is shown in Figure 5B. The hydroxamic acid group of **16ae** entered into the active site by chelating the essential catalytic zinc ion. The xylene group of compound **16ae** occupies the cap region and comfortably locks into the

surface groove, while the quinaxolinone moiety points out of the binding pocket and makes only a few contacts with the HDAC1 surface. This outcome was consistent with the results of biological activity experiments.

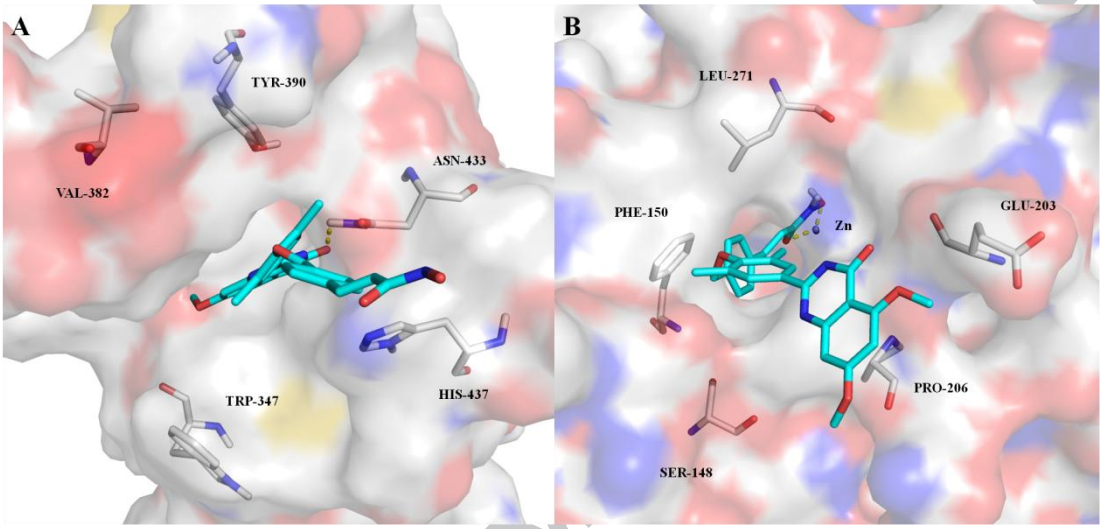


Figure 5. (A) Compound **16ae** docked into BRD4/BD2 (PDB entry: 5u2c), (B) Compound **16ae** docked into HDAC1 (PDB entry: 4kbx). Protein is shown as surface. Blue stick represents compound **16ae**. Zinc ion is shown as black sphere, and the hydrogen bonds were denoted by yellow dash lines.

In conclusion, utilizing structure-based design approach, we have successfully generated a series of novel BRD4/HDAC dual inhibitors composed of 3, 4', 5-trihydroxy-transstilbene with a hydroxamate group which is essential for chelation with the zinc ion in the active site of HDAC. As expected, most of compounds exhibited powerful inhibitory function against HDAC1 and BRD4. Subsequently, we found **16ae** exhibit remarkable effects on anti-proliferative activities *in vitro*. The Western blot analysis further confirmed the inhibitory effect of compound **16ae** on Myc, and it induced Myc in a concentration-dependent manner in human AML cell

lines. All these observations manifest that **16ae** is a potential and promising dual BET and HDAC inhibitor to kill the Myc-induced murine lymphoma.

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