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Discovery of thieno[2,3-d]pyrimidine based hydroxamic acid derivatives as bromodomain-containing protein 4/histone deacetylases dual inhibitors induce autophagic cell death in colorectal carcinoma cells

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Abstract: BRD4 and HDAC are both attractive epigenetics targets in cancer and other chronic diseases. Based on the integrated fragment-based drug design, synthesis, in vitro and in vivo evaluations, a series of novel thieno[2,3-d]pyrimidine-based hydroxamic acid derivatives are discovered as selective BRD4/HDAC dual inhibitors. Compound 17c is the most potent inhibitor for BRD4 and HDAC with IC_{50} values at nanomolar levels, as well as the expression level of c-Myc, and increases the acetylation of histone H3. Moreover, 17c presents inhibitory effects on the proliferation of colorectal carcinoma cells via inducing autophagic cell death. It also has a good pharmacokinetic profile in rats and oral bioavailability of 40.5%. In the HCT-116 xenograft in vivo models, 17c displays potent inhibitory efficiency on tumor growth by inducing autophagic cell death and suppressing IL6-JAK-STAT signaling pathways. Our results suggest the BRD4/HDAC dual inhibition might be an attractive therapeutic strategy for colorectal carcinoma.

Keywords: BRD4; HDAC; Dual inhibitor; Colorectal carcinoma; Autophagy

Introduction

Colorectal carcinoma (CRC) is one of the most common malignances worldwide and remains the second and third leading causes of cancer-related deaths in male and female adults, respectively.[1, 2] Although improvements in diagnostic and effective therapeutic methods have been developed for CRC, novel therapeutics for the treatment of advanced CRC are still urgently needed.[3-5] In addition, the clinically used molecular targeted agents of CRC, such as epidermal growth factor receptor (EGFR) inhibitors, cetuximab and bevacizumab, only appear to have limited activity in metastatic CRC (mCRC) patients, but they have dramatically increased therapeutic costs and have a heavy economic burden on the patient and medical system.[6-8] The epigenetic alterations of CRC are considered to be an important biomarker of mCRC, and among the epigenetic targets, histone acetylation in tumor tissues is one of the most frequent changes in mCRC.[9-12] The histone acetylation level detected by immunohistochemical (IHC) staining is strongly correlated with the stages, metastasis and prognosis of CRC.[13]

In the process of histone acetylation, two types of protein families are considered as potential drugable targets: bromodomain-containing protein 4 (BRD4) plays a role as a histone acetylated lysine “reader” and histone deacetylases (HDAC) act as an acetylated lysine “eraser”.[14] BRD4 is a member of the bromodomain and extra-terminal (BET) protein family, and it acts as a central element in the recognition of histone or non-histone substrates, which may regulate many molecular and cellular processes in epigenetic modification and gene transcription.[15-19] HDACs are actuators of deacetylation reactions of lysine residues, which have been recently deemed as potential therapeutic targets in human malignancies. [20-25] There are four main subgroups containing 11 isoforms of HDAC that have been recently categorized: HDACs 1, 2, 3,

and 8 are class I; HDACs 4, 5, 7, and 9 are class IIa; HDACs 6 and 10 are class IIb; and HDAC11 is class IV.[13, 26] In the past investigations on BRD4 and HDAC inhibition, a number of structurally diverse inhibitors have been reported, and some HDAC inhibitors were approved by the U.S. food and drug administration (FDA), or discovered in various clinical evaluation or preclinical stages for cancer therapy, e.g. vorinostat,[27] panobinostat,[28] mocetinostat,[29] trichostatin A,[30] romidepsin[31] and 9Z8,[32] etc. And some BRD4 inhibitors were evaluated in preclinical or clinical evaluation stages, such as JQ-1,[33] I-BET151,[34] I-BET726,[35] I-BET762,[36] XD14,[37] RVX-208,[38] PFI-1[39], ZL0420,[40] Mivebresib (ABBV-075),[41] Alobresib (GS-5829)[42], PLX51107[43] and BIC1,[44-47] etc. (Figure 1)

BRD4 and HDACs could synergistically down-regulate c-Myc expression and inhibit the progress and metastasis of CRC. Moreover, Hu et al. reported that Protein Phosphatase 1 alpha (PP1α) and class I histone deacetylase signaling pathways are essential for releasing chromatin-bound BRD4 for positive transcriptional elongation factor b (P-TEFb) recruitment.[48] Mishra et al. reported novel insights into the action mechanisms of HDAC inhibition by eliciting BRD4 and c-Myc and inducing a subset of gene expression in pancreatic ductal adenocarcinoma.[49] Recently, Zeng et al. reported that the BRD4 inhibitor JQ1 could sensitize breast cancer cells to HDAC inhibition by the LIFR-JAK-STAT (Leukemia Inhibitory Factor Receptor - Janus Kinase - Signal Transducer and Activator of Transcription) signaling pathways.[50] There have been several reports about the combination of HDAC and BRD4 inhibitors as potential synergistic therapeutics for various cancers, e. g. the suberoylanilide hydroxamic acid (SAHA) analogues of I-BET726 (DUAL946)[51], I-BET151 (Zhang et al.)[52] and (+)-JQ1 (He et al.).[53] The previous reports of our laboratory suggested that the combination of RVX-208 and SAHA also resulted enhanced anti-proliferative

effects in AML cells.[54]

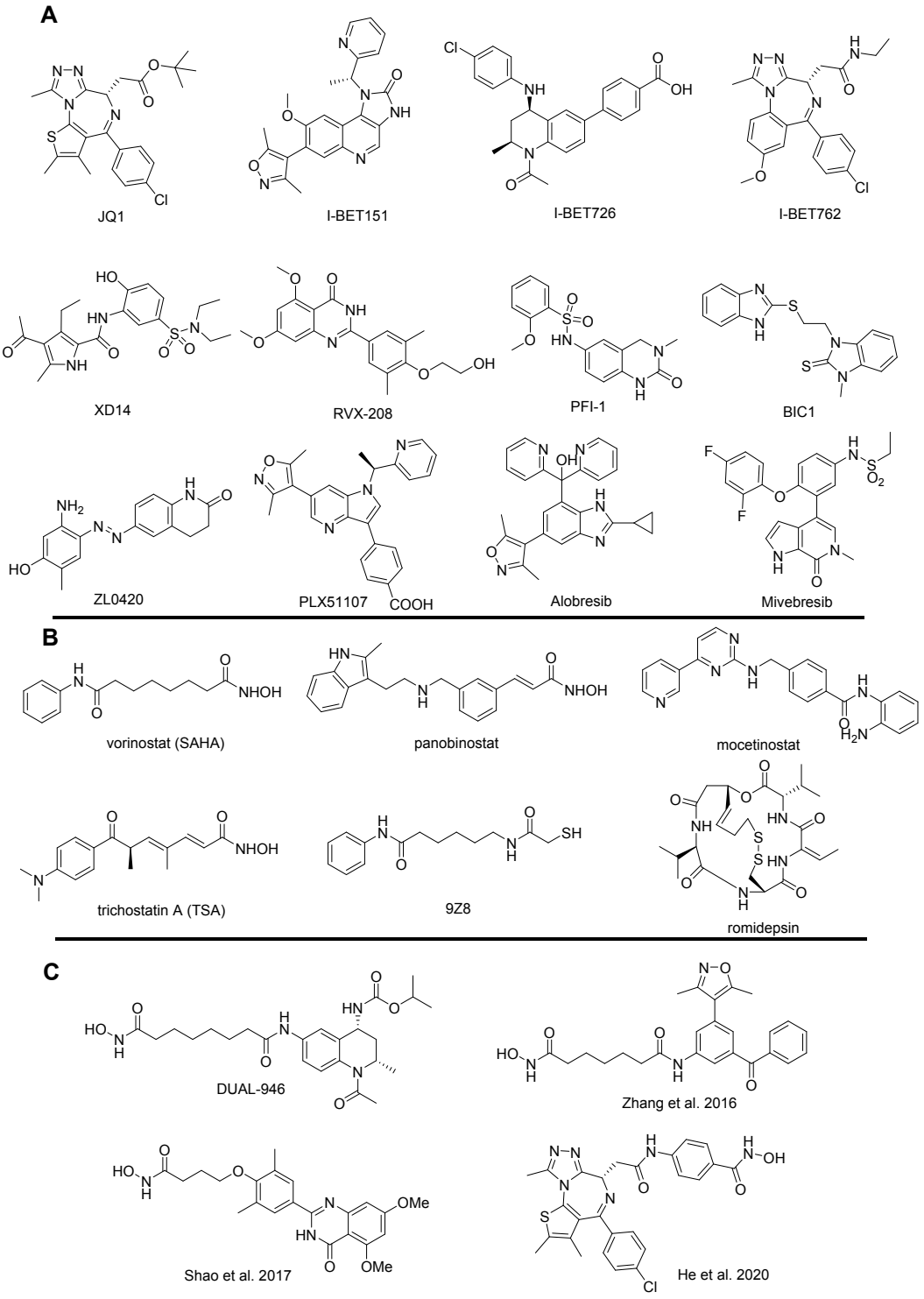


Figure 1. Structures of some known BRD4 and HDAC inhibitors: A. Selection of published BRD4 inhibitors with diverse scaffolds; B. Selection of published HDAC inhibitors with diverse ZBG; C. Structures of some reported BRD4-HDAC dual-inhibitors based on known BRD4 inhibitors.

As a part of our continuous work on discovering histone epigenetic modification inhibitors as autophagic regulators,[55] in the current study, we designed and synthesized a series of novel BRD4-HDAC dual inhibitors and performed a panel of subsequent in vitro and in vivo biological evaluations. Furthermore, compound **17c** induced apoptosis and autophagic cell death by downregulating c-Myc, deacetylation of histone H3, inhibiting apoptosis inhibitor protein Bcl-2 and interfering the formation of autophagolysosome in HCT-116 colorectal cancer cells. **17c** mainly inhibited HDAC 1, 2, 3 and 6, while both western blotting and IHC analyses revealed that **17c** potently inhibited the deacetylation of histone H3. In addition, **17c** displayed a good therapeutic efficiency on the HCT-116 colorectal cancer xenograft mouse and increased the cytotoxic T-cell infiltration via activation of IL6-JAK-STAT signaling pathways. In summary, we reported a series of novel small molecule BRD4-HDAC dual inhibitors, and **17c** exhibited a superior anti-proliferative capacity on colorectal cancer compared to those of RVX-208 and vorinostat, suggesting that **17c** is a potential anticancer lead compound via selective BRD4 and HDACs inhibition and subsequent autophagic cell death.

Results and discussion

Tissue microarray (TMA) and IHC analyses

The expressions of BRD4 and HDAC2 were evaluated by three independent pathologists to avoid diagnostic bias. The HDAC2 was used as a representative biomarker to evaluate the expression level of HDACs. The relative expression levels of BRD4 and HDAC2 in both tumor and matched normal tissues of each case are illustrated in Figure 2. There were significant differences in both BRD4 and HDAC2

expression levels between tumor and matched normal tissues. Moreover, there was strong correlation between BRD4 and HDAC2 in tumor tissues. In the HDAC2 staining, 35.56% (16/45) of stage I-II CRC tumor tissues showed moderate to strong staining, while in stage III-IV tissues, the percentage of moderate to strong staining was up to 58.97% (23/39) (Figure 2D). Additionally, there were 48.88% (22/45) and 61.54% (24/39) of tumor tissues that showed moderate to strong BRD4 staining in stage I-II and III-IV tissues, respectively. In brief, the BRD4 and HDAC2 expressions were up-regulated and correlated with the clinical stage and prognosis in CRC tissues, which suggested that BRD4 and HDAC may be potential targets for combined therapeutics.

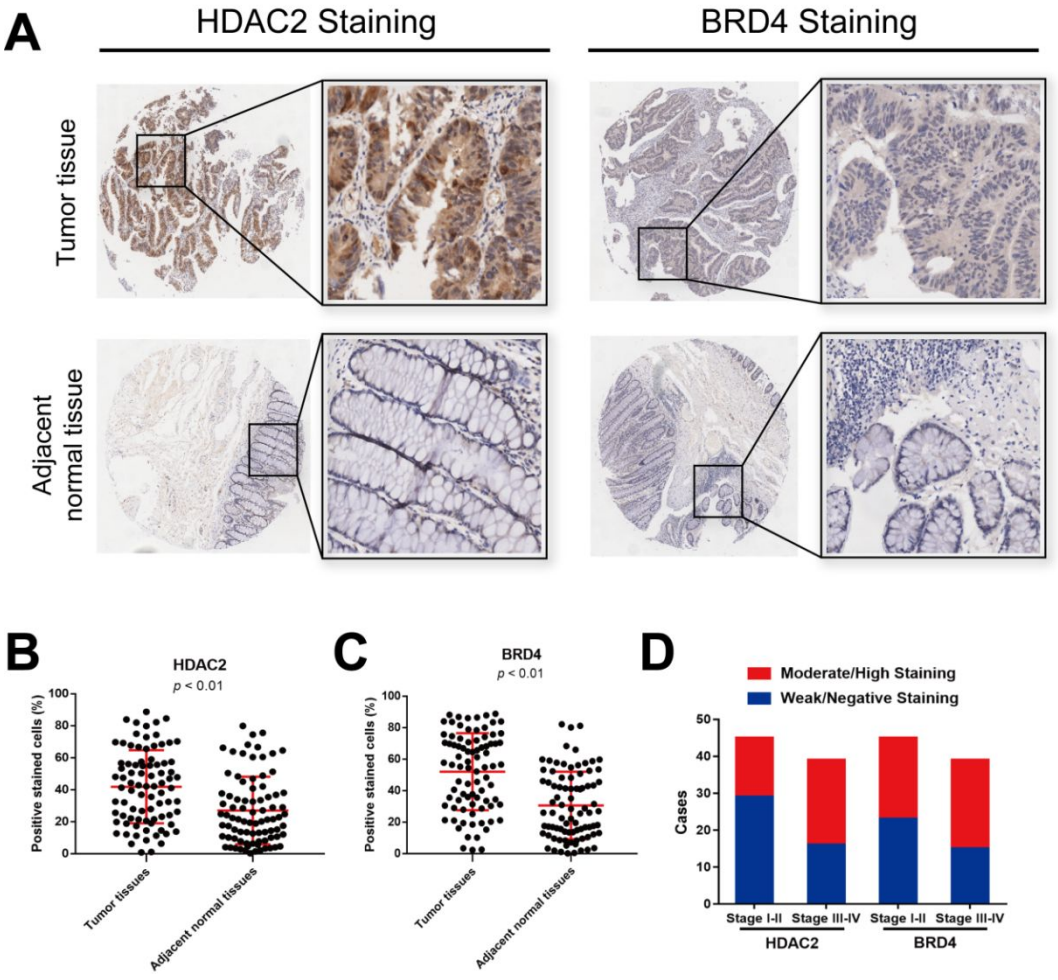


Figure 2. Identification of HDAC2 and BRD4 are significantly upregulated in CRC clinical samples**:** A. Representative immunoreactivity intensities of HDAC2 and BRD4

in CRC tissues and adjacent normal tissues; B. HDAC2 expression is upregulated in CRC tissues; C. BRD4 expression is upregulated in CRC tissues; D. The upregulated HDAC2 and BRD4 expression are related to the CRC stages.

Discovery of novel BRD4-HDAC dual inhibitors

A four-step strategy was performed for the discovery of small-molecule BRD4-HDAC dual-inhibitor candidates: the fragment-based design of the novel BRD4 inhibitory scaffold, the synthesis of BRD4 inhibitors and in vitro enzymatic evaluation, the design and synthesis of a novel BRD4-HDAC inhibitor, and the systemic evaluation of this novel BRD4-HDAC dual inhibitor as an anti-cancer lead compound both in vitro and in vivo. The BRD4 protein has two bromodomains, an ubiquitin binding domain and a carboxyl-terminal domain. A canonical BRD4 protein contains four left-handed alpha helices and two loops (ZA and BC loops) to form the binding site of the acetylated lysine (Kac) residue. Most of the BRD4 inhibitors were designed to mimic the interaction modes of Kac, thus giving them the ability to disrupt the interaction between an acetylated histone and the Kac binding site within a BRD protein. First, the fragments generated from known BRD4 inhibitors were embedded into the fragment-like library of the ZINC database and then individually docked into the two bromodomains of the BRD4 protein by the Libdock program, a rigid docking method. The top 200 hit fragments were subsequently screened by the Ligandfit program, a flexible docking method considering induced-fit effects. The top ten scoring fragments are shown in Figure 3, and there were three structural characteristics in the screened fragments: the stable hydrogen bond interactions with the ZA-loop residues; the van-der-walls and hydrogen bond interactions in the Kac binding pocket; and a hydrophobic fragment to fulfill the steric requirement on the outside of the binding site. Secondly,

based on these screened fragments, a focused library of twenty-four compounds was synthesized, and the BRD4 inhibitory activities of the compounds were characterized (Scheme 1 and 2, Table 1). The general synthetic route of the target molecules **4a-t, 9a-d** has been presented in Scheme 1 and Scheme 2, respectively. Commercially available cyclohexanone, cyclohexanone and N-substituted piperidine **1a-e** as the first starting material were reacted with ethyl cyanoacetate and sulfur to give **2a-e**, and then these five compounds were reacted with commercially available cyanobenzene and 3,4,5-substituted cyanobenzene (**3a-d**) to give **4a-t**. Similarly, tert-butyl 4-oxopiperidine-1-carboxylate was reacted with 2-cyanoacetamide and sulfur to give **5** by Gewald reaction, and then **6** was obtained through acylation. After the removal of protection, ring-closure reaction was completed under the catalysis of strong alkali to give **8**. Finally, compounds **9a-d** were prepared by nucleophilic substitution.

As shown in Table 1, the enzymatic assay results of compounds 4a-t demonstrated that the substitutes on the phenyl groups at the 2-position of thieno[2,3-b]pyrimidin-4-one played a key role for maintaining the BRD4 inhibitory activity. When the para-hydroxyl group was removed, the BRD4 inhibitory capacity was totally lost; moreover, methyl groups at the meta-position could obviously increase the potency of the inhibitor. The 3,5-dimethylisoxazole fragment displayed comparable BRD4 inhibitory capacity than that of 3,5-dimethyl-4-hydroxyl-phenyl fragment, the docked confirmations of 9a were highly similar to that of 4l (Figure S1). In addition, larger substitutes on the outside fragment could decrease the inhibitory activity.

Subsequently, compound **4l** was chosen as a novel BRD4 inhibitor scaffold for dual-inhibitor discovery. It was well known that a typical HDAC inhibitor contained three fragments: a cap fragment, a hydrophobic linker and a zinc binding group (ZBG) group. In the design of BRD4-HDAC dual-inhibitors, compound **4l** was used as the cap

fragment. There were two linkage sites in compound **4**: the hydroxyl group on the 3,5-dimethyl-4-hydroxyl-phenyl group and the N atom in the piperidine ring. According to the previous report of our laboratory, the BRD4/HDAC dual inhibitor based on RVX-208, the ZBG group could linked to the 3,5-dimethyl-4-hydroxyl-phenyl group. We chose the N-hydroxyhexanamide and phthalate fragments as model groups to explore the correct linking mode of the cap fragment, respectively. The model compounds were synthesized and characterized for their BRD4/HDAC inhibition as well as cell proliferation inhibitory activity in vitro (Scheme 3 and 4, Table 2). The N-hydroxyhexanamide and phthalate fragments were also attached to the piperidine ring of compound **9** to afford potential BRD4/HDAC dual inhibitor **21** and **22**. It was surprising that compound **20**, **21** and **22** displayed none significant BRD4 inhibitory capacity up to 10μM, and compound **17** was greatly superior to compound **20** in cell proliferation assays. In addition, the HDAC2 and HDAC6 inhibitory activities of compound **17c** were moderately superior to that of compound **20** (58 nM to 136 nM, 73 nM to 129 nM), respectively. Then, the different linkages and ZBG fragments were conjugated on the scaffold of compound **17** (Scheme 3, Table 2). The synthetic routes of these dual-inhibitors are outlined in Scheme 3-5. Compounds **11a-f** were reacted with 3,5-dimethyl-4-hydroxybenzonitrile to give **12a-f**, which were then hydrolyzed with NaOH to give **13a-f**. These compounds were treated with phthalates or NH_2OH to give **14a-c** or **15a-f**. Compounds **14a-c** were reacted with **2b** to give **16a-c**, and **15a-f** reacted with **2b** to afford **17a-f**. On the other hand, the co-crystallized structure of 4l to BRD4 suggested another binding mode, the N-methylpiperidine moiety exposed out of the binding pocket, providing another feasible linkage site for ZBG. As depicted in Scheme 4 and 5, commercially available piperidin-4-one as the first starting material was reacted with ethyl cyanoacetate and sulfur to give compound **18**, which was reacted

with methyl 6-bromohexanoate to give compound **19**, and then 4-hydroxy-3,5-dimethylbenzonitrile was reacted with the compound **19** in HCl/1,4-dioxane under room temperature for 48h, the resultant was treated with hydroxylamine aqueous solution in the mixed solvent CH_3OH and CH_2Cl_2 to give compound **20**. As described before, compounds 21 and 22 were prepared by amination and amidation of 9b-d, respectively.

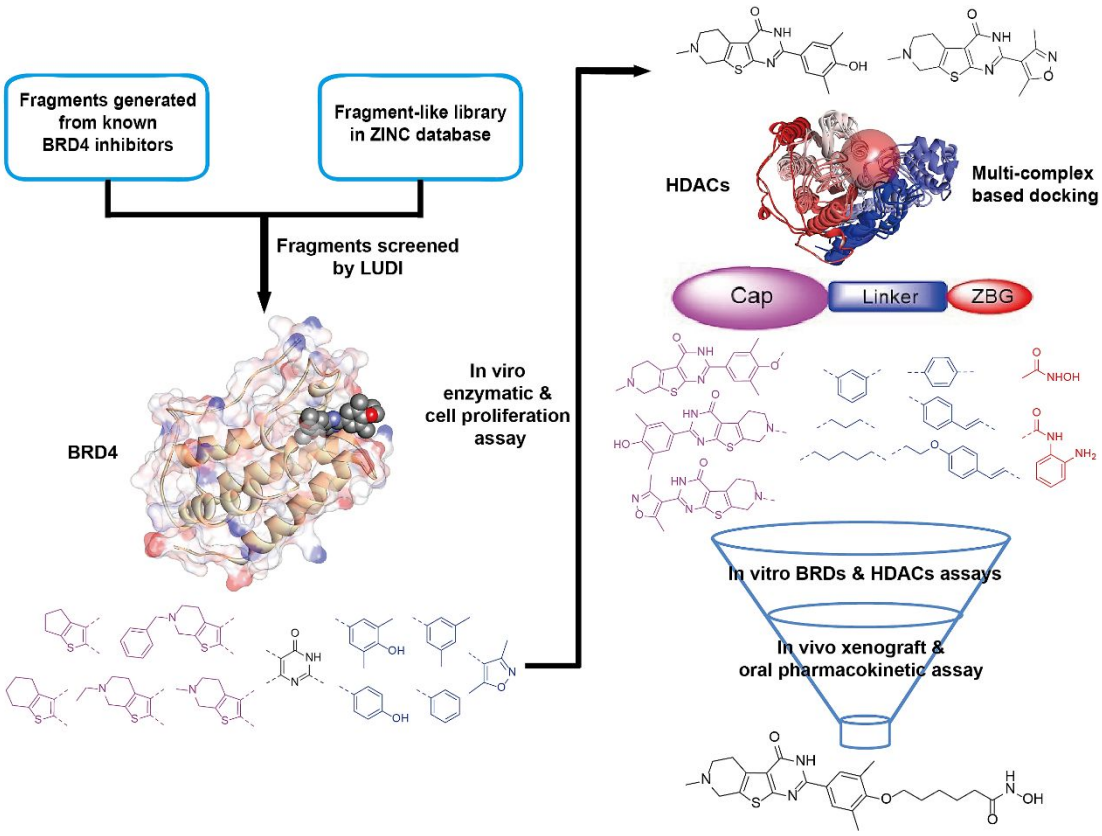
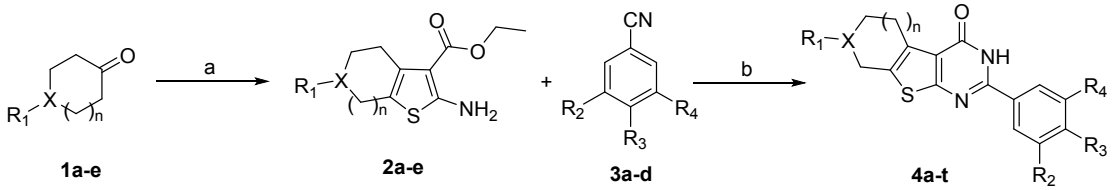
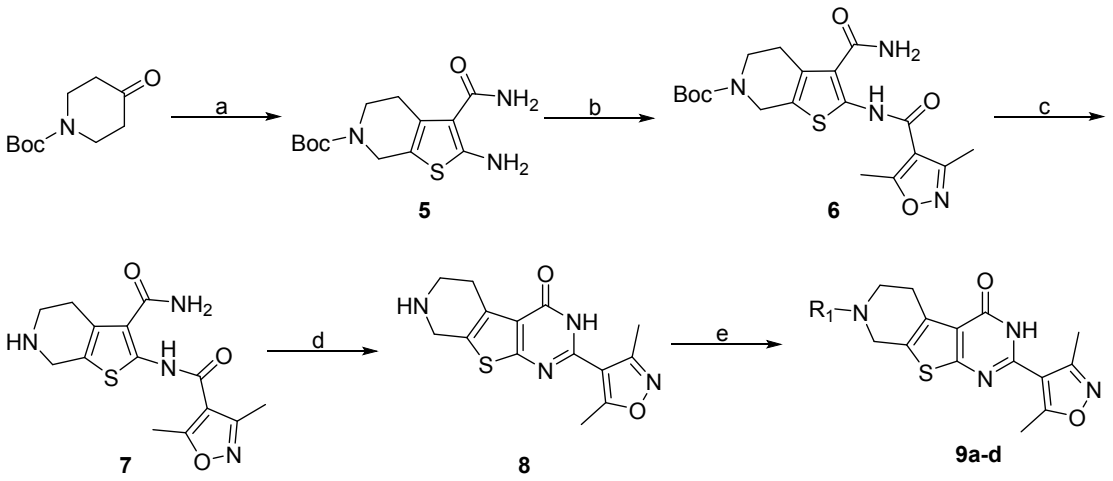


Figure 3. Fragment based drug design protocols for the identification of the novel BRD4-HDAC dual inhibitors (PDB ID: 4ZW1 for BRD4 BD1, PDB ID: 2YEM for BRD4 BD2, PDB ID: 4LXZ for HDAC2, PDB ID: 5WGI for HDAC6 and PDB ID: 3C0Z for HDAC7).



Scheme 1. Reagents and conditions: (a) $NCCH_2CO_2Et$, S_8, Et_3N, EtOH, reflux, 12h; (b) HCl-1,4-dioxane, 25℃, overnight.

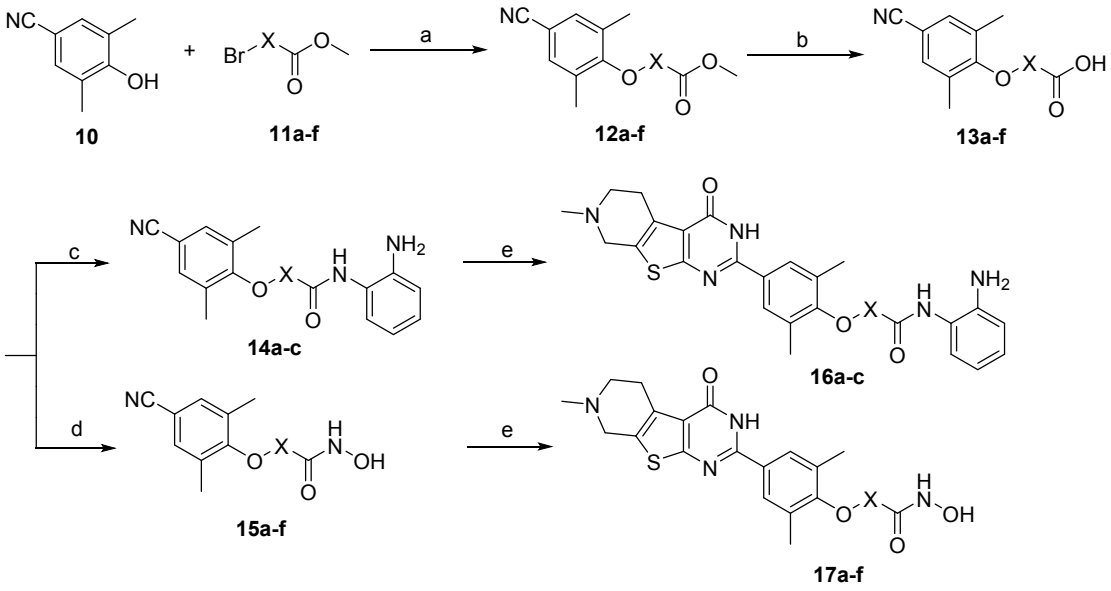


Scheme 2. Reagents and conditions: (a) $NCCH_2CONH_2$, S_8, Et3N, EtOH, reflux, 12h; (b) 3,5-dimethylisoxazole-4-carbonyl chloride, pyridine, 3h, 25℃; (c) 4M HCl/dioxane, 4h, 25℃; (d) NaOH, EtOH, 24h, 100℃; (e) K_2CO_3, 1,4-dioxane, 25℃ or reflux, 6h.

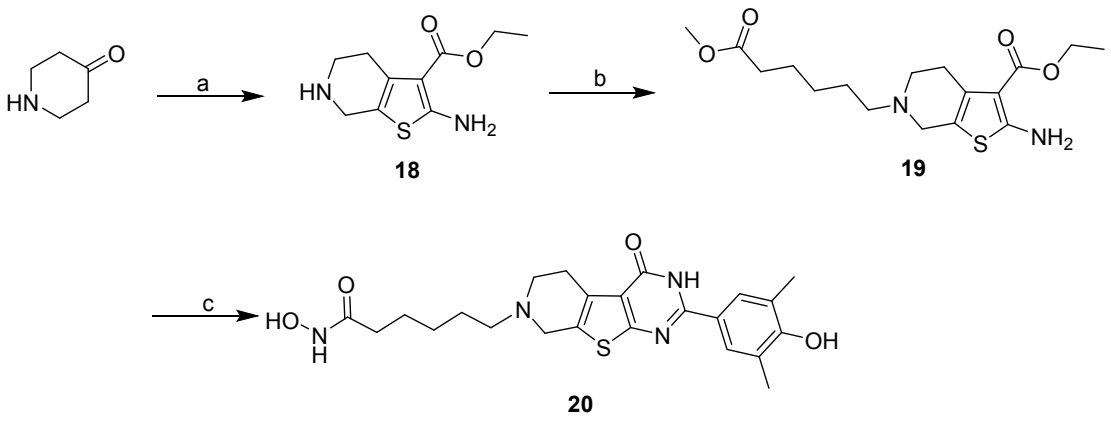
Table 1. In vitro enzymatic and HCT-116 cell proliferation evaluations of compounds **4a-t, 9a-d.**

No.	n	X	R_1	R_2	R_3	R_4	BRD4 IC_{50} (μM)	HCT-116 IC_{50} (μM)
4a	0	CH	H	H	H	H	>10	>10
4b	0	CH	H	H	OH	H	9.28±1.37	>10
4c	0	CH	H	CH_3	H	CH_3	>10	>10
4d	0	CH	H	CH_3	OH	CH_3	2.64±0.54	4.85±0.74
4e	1	CH	H	H	H	H	>10	>10

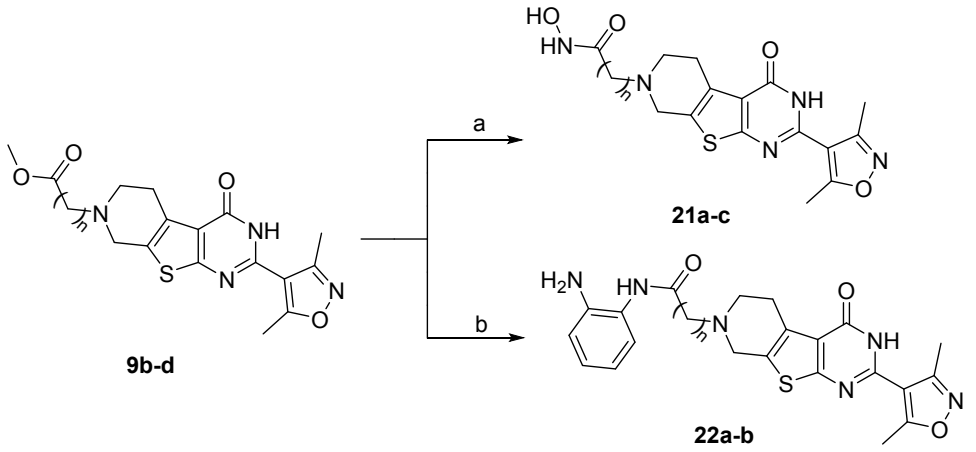
4f	1	CH	H	H	OH	H	5.77±0.63	9.73±3.16
4g	1	CH	H	CH_3	H	CH_3	>10	>10
4h	1	CH	H	CH_3	OH	CH_3	1.81±0.23	5.41±0.63
4i	1	N	CH_3	H	H	H	>10	>10
4j	1	N	CH_3	H	OH	H	6.31±1.08	8.68±0.91
4k	1	N	CH_3	CH_3	H	CH_3	>10	>10
4l	1	N	CH_3	CH_3	OH	CH_3	0.85±0.16	1.33±0.52
4m	1	N	CH_3CH_2	H	H	H	>10	>10
4n	1	N	CH_3CH_2	H	OH	H	>10	>10
4o	1	N	CH_3CH_2	CH_3	H	CH_3	>10	>10
4p	1	N	CH_3CH_2	CH_3	OH	CH_3	5.60±1.92	8.91±1.97
4q	1	N	Bn	H	H	H	>10	>10
4r	1	N	Bn	H	OH	H	>10	>10
4s	1	N	Bn	CH_3	H	CH_3	>10	>10
4t	1	N	Bn	CH_3	OH	CH_3	>10	>10
9a	-	-	CH_3	-	-	-	2.41±0.31	9.77±1.05
9b	-	-	CH_3OOCCH_2	-	-	-	6.68±1.08	16.21±2.04
9c	-	-	$CH_3OOC(CH_2)_3$	-	-	-	5.93±0.56	12.70±1.09
9d	-	-	$CH_3OOC(CH_2)_5$	-	-	-	3.62±0.53	17.03±2.58
RVX-208	-	-	-	-	-	-	2.45±0.36	12.83±2.40



Scheme 3. Reagents and conditions: (a) HCl-1,4-dioxane, 25℃, 48 h; (b) 1,4-dioxane, NaOH, 30 min; (c)DCC/HOBt, DCM, 24 h; (d) Ethyl chloroformate, N-Methylmorpholine, 1,4-dioxane, 15 min, then NH_2OH, CH_3OH, 20 min; (e) compound 4l, HCl-1,4-dioxane, 25℃, overnight.

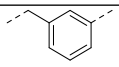
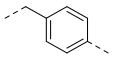
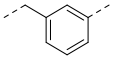
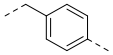
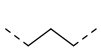
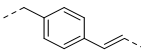
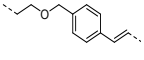


Scheme 4. Reagents and conditions: (a) $NCCH_2CO_2Et$, S_8, Et_3N, EtOH, reflux, 12h; (b) methyl 6-bromohexanoate, DBU, THF, reflux, overnight; (c) 4-hydroxy-3,5-dimethylbenzonitrile, HCl-1,4-dioxane, rt, 48h, then CH_3OH, CH_2Cl_2, $HONH_2$, 2h.



Scheme 5. Reagents and conditions: (a) NaOH/CH_3OH, hydroxylamine aqueous solution (50:50, w/w), 4M HCl/dioxane; (b) 2N NaOH, 4M HCl/dioxane and thenbenzene-1,2-diamine, EDCI, HOBt, CH_2Cl_2, 12h, 25℃.

Table 2. In vitro enzymatic assays and colorectal carcinoma cell proliferation evaluations of compounds **16a-c**, **17a-f**, **20**, **21a-c** and **22a-b**.

		IC_{50} (μM)						
No.	**X or n**	**BRD4**	**HDAC2**	**HDAC6**	**HDAC7**	**HCT-116**	**SW620**	**DLD1**
16a		3.48±0.48	0.86±0.09	>10	>10	4.53±0.32	>10	>10
16b		2.55±0.35	0.14±0.05	>10	>10	4.32±0.39	5.49±0.40	>10
16c		2.19±0.29	0.17±0.04	>10	>10	6.93±0.78	9.87±0.71	>10
17a		1.93±0.33	0.251±0.031	0.78±0.09	>10	5.49±0.75	7.83±0.96	9.16±1.23
17b		1.77±0.20	0.105±0.012	0.188±0.023	>10	1.07±0.05	3.87±0.66	6.24±0.59
17c		0.71±0.09	0.058±0.004	0.073±0.005	>10	0.45±0.06	1.78±0.31	2.11±0.38
17d		1.17±0.21	0.16±0.04	0.25±0.09	>10	2.08±0.33	3.83±0.59	9.72±0.89
17e		1.19±0.18	0.097±0.011	0.202±0.016	>10	0.92±0.18	2.34±0.25	3.67±0.42
17f		0.98±0.07	0.085±0.006	0.159±0.008	>10	0.69±0.11	3.85±0.43	3.04±0.38
20	-	>10	0.136±0.009	0.129±0.010	>10	>10	15.82±1.43	>10
21a	1	>10	0.489±0.036	0.455±0.049	>10	22.80±2.75	19.60±1.58	22.55±2.26
21b	3	>10	0.282±0.039	0.464±0.055	>10	17.99±1.87	9.86±0.73	19.64±2.11

21c	5	>10	0.135±0.011	0.156±0.016	>10	15.29±2.02	8.47±0.59	16.79±1.62
22a	1	>10	0.352±0.045	0.391±0.058	>10	28.29±3.75	15.25±2.23	15.22±1.62
22b	5	>10	0.112±0.014	0.196±0.014	>10	15.23±2.09	11.08±1.25	14.83±2.10
RVX-208	-	2.45±0.36	ND[a]	ND[a]	ND[a]	12.83±2.46	>10	>10
SAHA	-	ND[a]	0.035±0.004	0.047±0.007	>10	>10	6.94±0.78	>10
RVX-208+SAHA [b]	-	2.54±0.28	0.031±0.006	0.049±0.005	>10	5.85±1.02	4.80±0.65	>10

[a] ND: Not determined.

[b] The molar ratio of RVX-208 and SAHA was set to 1:1.

The synthesized dual-inhibitors were assayed for their inhibitory activities on BRD4 as well as HDAC2, HDAC6 and HDAC7 by using RVX-208 and SAHA as positive controls, respectively. Compounds **17a-f,** which used hydroxamic acid as the ZBG fragment and various types of linker group positions, had better HDAC inhibitory activities than those of compounds **16a-c** with o-phenylenediamine as the ZBG fragment. Moreover, the inhibitory potencies of all of the compounds on HDAC2 and HDAC6 were greatly superior to those on HDAC7; these results suggested that the novel dual-inhibitors might selectively inhibit class I and IIb type HDACs rather than class IIa. In addition, compounds **16a-c** and **17a-f** also displayed good BRD4 inhibitory capacities at a sub-micromolar scale, which suggested that the ZBG and linker moiety only slightly affected the BRD4 inhibitory effects. Intriguingly, the linkage of ZBG moiety on the piperidine ring resulted in the totally loss of BRD4 inhibitory activities in compounds **20-22**. The most potent compound **17c**, with IC_{50} values of 58 and 73 nM on HDAC2 and HDAC6, respectively, displayed good selectivity for HDAC 2/6 versus HDAC7. Compound **17c** also displayed a moderate inhibitory capacity on BRD4, with an IC_{50} value of 710 nM, which was better than that of the positive control RVX-208.

Table 3. HDACs and BRDs inhibitory profiling of compound **17c**.

Target	IC_{50} (μM)		
	17c	**JQ-1**	**SAHA**
	Class I HDAC		
HDAC1	0.046±0.003	ND[a]	0.017±0.003
HDAC2	0.058±0.004	ND[a]	0.035±0.004
HDAC3	0.075±0.009	ND[a]	0.049±0.003
HDAC8	0.167±0.029	ND[a]	0.195±0.031
	Class IIa HDAC		
HDAC4	>10	ND[a]	>10
HDAC5	>10	ND[a]	>10
HDAC7	>10	ND[a]	>10
HDAC9	>10	ND[a]	>10
	Class IIb HDAC		
HDAC6	0.073±0.005	ND[a]	0.047±0.007
HDAC10	0.923±0.005	ND[a]	0.278±0.056
	Class IV HDAC		
HDAC11	>10	ND[a]	>10
	BRDs[b]		
BRD2(BD1)	16.4±1.13	0.155±0.005	ND[a]
BRD2(BD2)	>20	0.017±0.003	ND[a]
BRD3(BD1)	>20	0.064±0.004	ND[a]
BRD3(BD2)	>20	0.039±0.002	ND[a]
BRD4(BD1)	1.10±0.14	0.022±0.005	ND[a]
BRD4(BD2)	2.05±0.71	0.069±0.007	ND[a]
BRDT(BD1)	>20	0.134±0.011	ND[a]

[a] ND: Not determined; [b] the BRDs inhibitory profiling were performed by an Alphascreen based method reported in our previous study and using JQ-1 as a positive control.[56]

Since compound **17c** demonstrated both potent enzymatic and anti-proliferative activity in vitro, it was further tested for its isoform selectivity on the other isoforms of BRDs and HDACs. As shown in Table 3, compound **17c** potently inhibited HDAC1-3 and HDAC6 with IC_{50} values from 45 to 482 nM and at the sub-micromolar level against HDAC8 and HDAC10, respectively. And there were almost no inhibitory effects of compound **17c** on class IIa and class IV HDAC isoforms up to a concentration of 10 μM. Moreover, it was notable that compound **17c** displayed better activities on the BRD4 than on the other isoforms of BRD proteins by using the alphascreen assays, compared to the positive control compound JQ-1, compound **17c** showed higher selectivity to BRD4 with moderate IC_{50} values. We then evaluated the anti-proliferative capacity of 17**c** on human colorectal carcinoma HCT-116, SW620 and DLD1 cells, and 17**c** displayed good activities with IC_{50} values of 0.45, 1.78 and 2.11 μM on HCT-116, SW620 and DLD1 cells, respectively. In summary, the above results suggested that **17c** deserved further development with potent BRD4-HDAC dual inhibition.

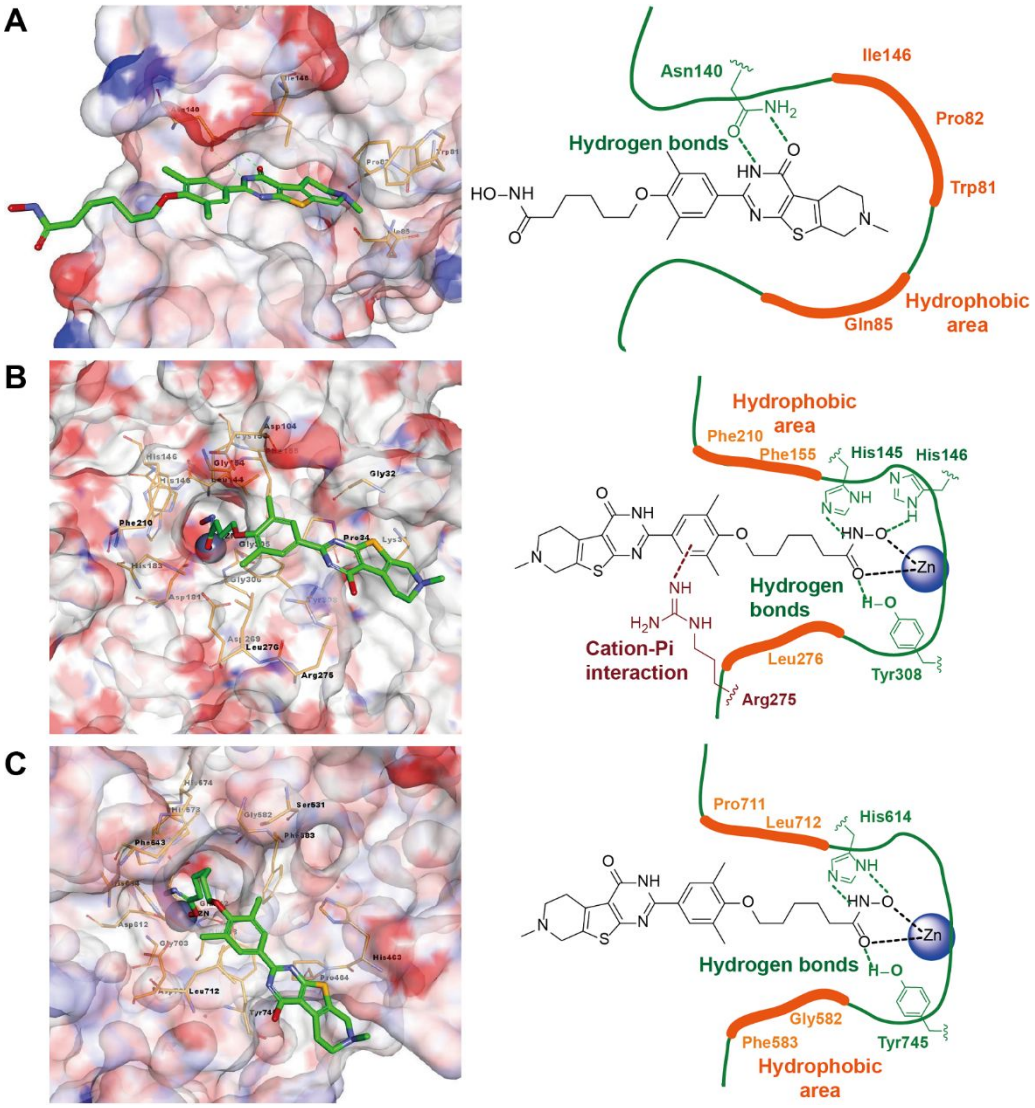


Figure 4. The docked conformations and binding modes of **17c** to BRD4 (A, PDB ID: 4ZW1), HDAC2 (B, PDB ID: 4LXZ) and HDAC6 (C, PDB ID: 5WGI), respectively. The proteins were illustrated by solvent-accessible surface with charges (blue for positive, red for negative), the binding site residues were highlighted.

Binding modes of 17c to BRD4, HDAC2 and HDAC6

The compound **17c** was docked into the binding pocket of BRD4, HDAC2 and HDAC6 proteins, respectively. The docking results were illustrated in Figure 4. The hydroxamic group of compound **17c** stable chelated with the zinc atom in the binding sites of

HDACs. Moreover, the thieno[2,3-d]pyrimidine fragment of compound **17c** fitted into the surface groove and occupied the cap region well. The carbonyl fragment of hydroxamic group could form hydrogen bonds interaction with Tyr308 residues of HDAC2 and Tyr745 residues of HDAC6, respectively. The hydroxylamine fragment of **17c** interacted with His145/His146 residues of HDAC2 and His614 residue of HDAC6 via stable hydrogen bonds, respectively. And the phenyl substituted at 2-position of thieno[2,3-d]pyrimidine formed a cation-π interaction with Arg275 residue of HDAC2. In addition, the 3-nitrogen atom and 4-carbonyl group of thieno[2,3-d]pyrimidine could form H-bond with Asn140 residues of BRD4. The N-methylpiperidine group was placed between the WPF stack (Trp81, Pro82 and Phe83). Furthermore, **17c** derived supplementary activities by deploying the long alkanes chain and hydroxamic group into the ZA channel. In general, the molecular docking results displayed good agreement with the results of enzymatic assays.

17c induces cell cycle arrest, apoptosis and autophagic cell death and in colorectal carcinoma cells

The cytotoxicity of **17c** was screened against a panel of colorectal carcinoma cells. As shown in Figure 5, the IC_{50} of **17c** was remarkably higher in CRC cells than in normal colorectal epithelial cells. Moreover, the cell cycles of HCT-116 cells after being treated with **17c** were determined by a flow cytometry method. As shown in Figure 5B, the cell cycles results displayed obviously sub-G1 peaks in the 0.2 μM and 0.5 μM of compound **17c** treated HCT-116 cells, which indicated that the apoptotic cell death occurred. The death subroutines of **17c**-treated HCT-116 cells were detected by flow cytometry analysis using the Annexin V/PI staining kit (Figure 5C and D). The percentages of Annexin V-positive apoptotic cells in the **17c** treatment groups were

33.2 ± 5.30% (0.2 μM) and 45.3 ± 6.8% (0.5 μM), which were significantly higher than in the control (6.3 ± 0.4%, $p < 0.05$) group. However, there were significant differences in apoptotic cells between the 0.2 μM and 0.5 μM of **17c** treated groups. These results suggest that **17c** induced apoptotic death in a dose-dependent manner. The nuclei morphology after Hoechst 33258 staining (Figure 5E) in **17c**-treated HCT-116 cells revealed that apoptotic cell death was apparent after 0.2 μM and 0.5 μM **17c** treatments. The colony formation of HCT-116 cells was also potently suppressed after **17c** incubation for 24 hours (Figure 5F).

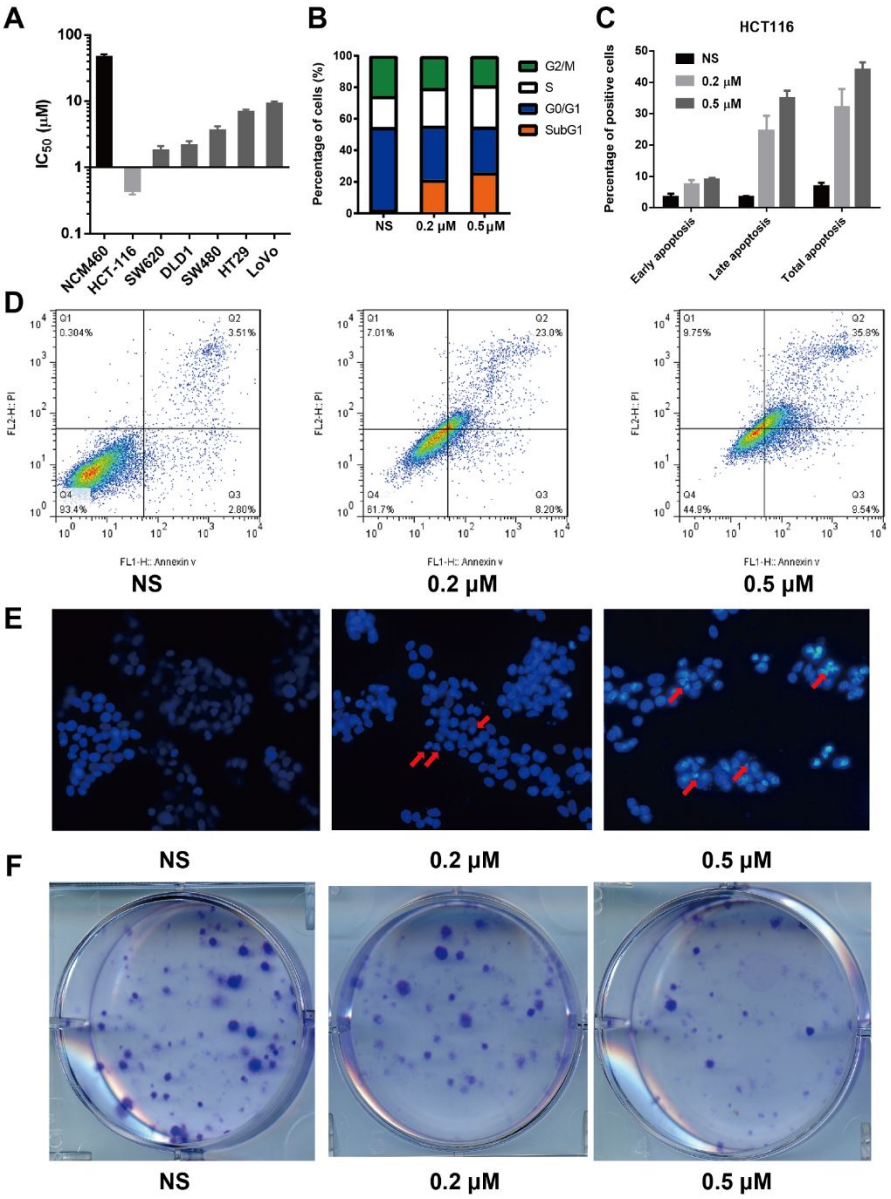


Figure 5. Compound **17c** induced apoptotic cell death in HCT-116 cells: A. The cell proliferation IC_{50} values were measured for compound **17c** on a panel of colorectal carcinoma cells by MTT assay; B. The cell cycle analysis of HCT-116 cells after 0.2μM and 0.5μM of **17c** treated; C and D. The apoptosis assay of HCT-116 cells after 0.2μM and 0.5μM of **17c** treated by Annexin V/PI dual-staining; E. The nuclei morphology of HCT-116 cells after 0.2μM and 0.5μM of **17c** treated; F. The colony formation assay of HCT-116 cells after 0.2μM and 0.5μM of **17c** treated.

To determine the effects of **17c**, RVX-208 and SAHA on the expression level of histone H3 as well as acetylated histone H3, the biomarkers of HDAC inhibition in HCT-116 cells were determined via the western blotting method. As shown in Figure 6, in agreement with their relative potencies in enzymatic assays, **17c** and SAHA upregulated Ac-H3 levels in a dosage-dependent manner, and the expression levels of histone H3 were not affected, while both H3 and Ac-H3 were not disturbed by RVX-208 (Data not shown). Moreover, the expression levels of BRD4 were declined by the addition of **17c** or RVX-208, and **17c** treatment suppressed the downstream c-Myc proteins more efficiently than that of RVX-208. As expected, **17c** induced more cleavage of PARP, caspase-3 and caspase-9, which suggested more mitochondrial apoptosis was induced by BRD4/HDAC dual inhibitor.

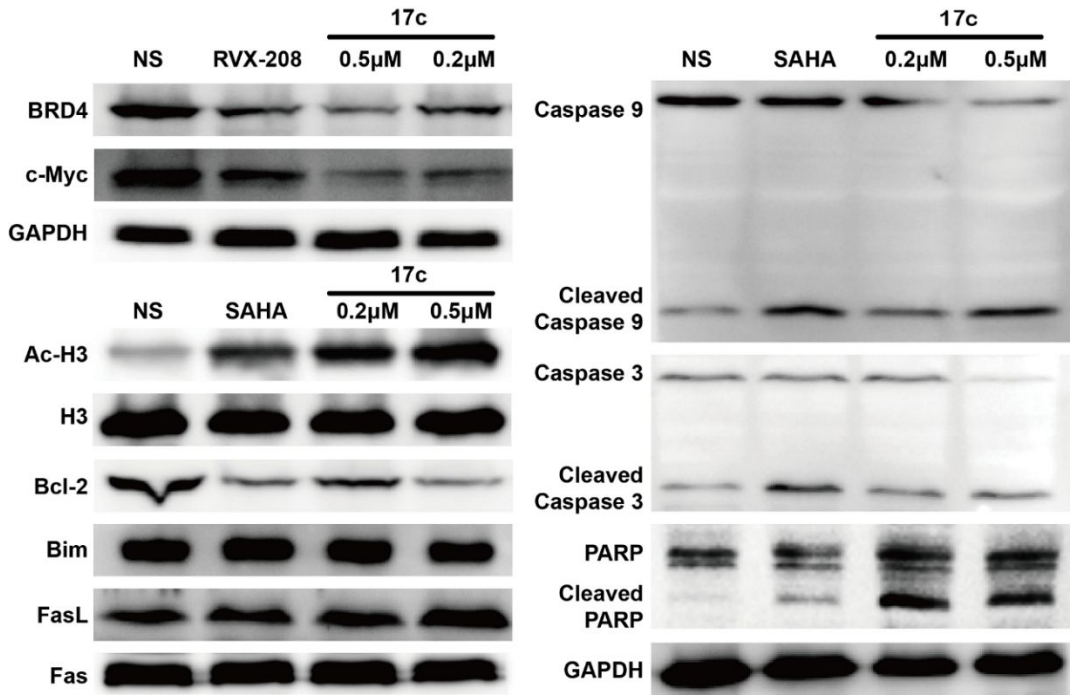


Figure 6. Compound **17c** induced mitochondrial apoptosis via BRD4-HDAC dual inhibition. HCT-116 cells were treated with 0.2 or 0.5μM of compound **17c**, positive control RVX-208 or SAHA for 24 hours, respectively. Then the expression levels of BRD4, c-Myc, Histone H3, Ac-H3, Bim, Bcl-2, Fas, FasL, PARP, Caspase-3 and Caspase-9 were detected by western blot analysis.

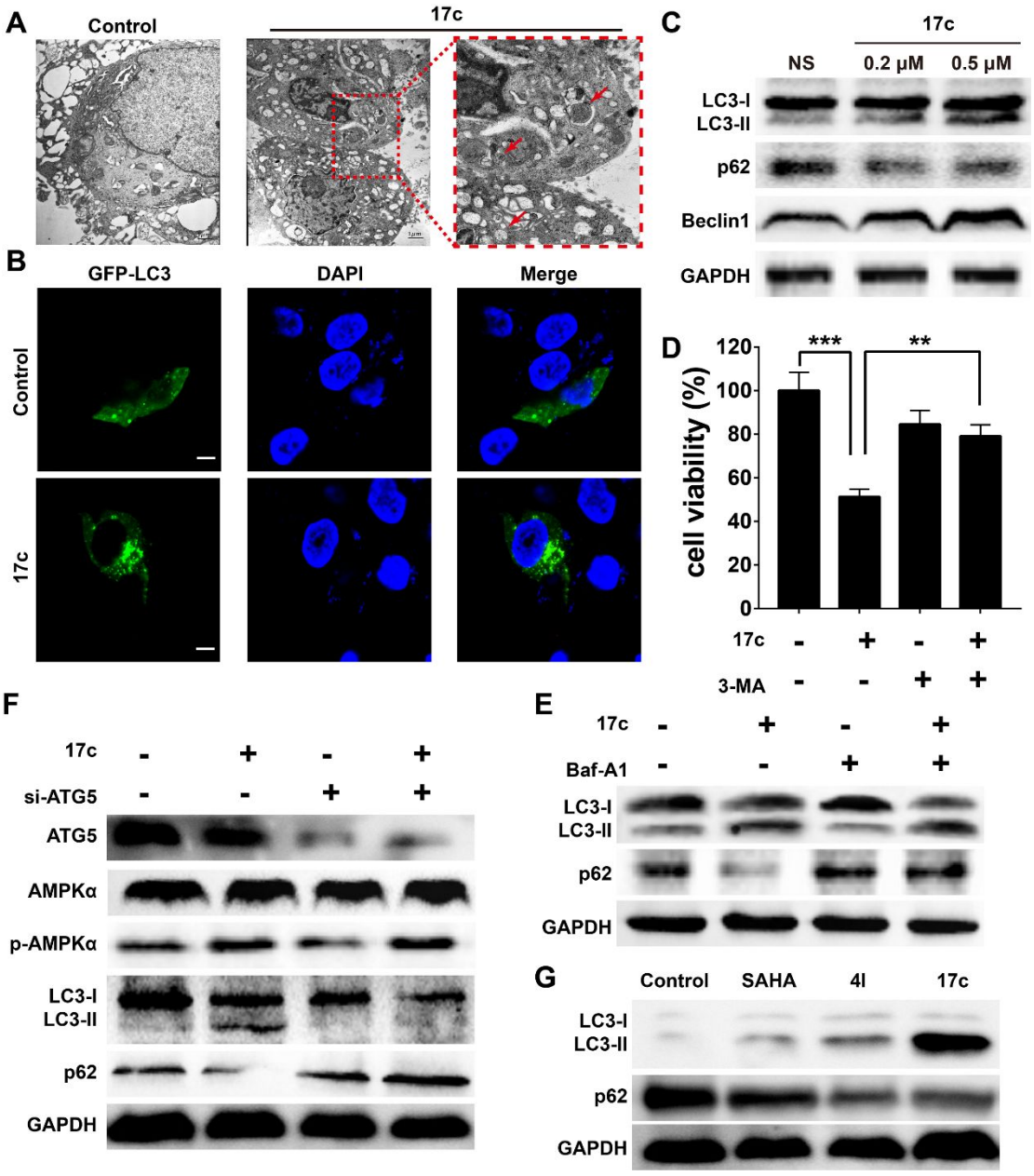


Figure 7. Compound **17c** induces autophagic cell death in colorectal carcinoma cells. (A) HCT-116 cells were incubated with 0.5 μM of 17c for 24 hours, then the cells were fixed and imaged under transmitted electron microscopy; scale bar = 1 μm. (B) HCT-116 cells were transfected with a GFP-LC3 plasmid, followed by treatment with 0.5 μM 17c, the formation of autophagosomes were observed under a confocal fluorescence microscope, scale bar = 6 μm. (C) After 17c incubation for 24 hours, the protein expression of LC3, p62, and beclin-1 were detected by WB analysis. (D) HCT-116 cells were treated with 0.5 μM of 17c and/or 500 μM of 3-MA, then the cell

viability was determined by MTT assay. *** indicated $p < 0.001$ and ** indicated $p < 0.01$. (E) HCT-116 cells were treated with 0.5 μM of 17c with or without Bafilomycin-A1 for 24 hours, the protein expression of LC3 and p62 were detected by WB analysis. (F) HCT-116 cells were transfected with ATG5 siRNA or scramble siRNA, respectively. After RNA silence, with 0.5 μM of 17c or normal saline incubation, the protein expression of ATG5, AMPKα, p-AMPKα, p62, and LC3 were determined by WB analysis. (G) HCT-116 cells were treated with 0.5 μM 17c, 2.0 μM 4l or 10 μM SAHA for the 24 hours, respectively. Then the protein expression of LC3 and p62 were detected by WB analysis.

There were several reports suggested that BRD4 protein was an autophagy suppressor in cancer cells, therefore inhibition of BRD4 might resulted in autophagy initiation.[55, 57-59] In current study, we observed **17c** induced the formation of cytoplasm autophagic vacuole in HCT-116 cells under transmission electron microscope (Figure 7A), and the increased LC3 puncta in GFP-LC3 transfected HCT-116 cells were also observed after 0.5 μM of **17c** incubation for 24 hours, these results suggested the initiation of autophagy in HCT-116 cells (Figure 7B). The **17c**-induced autophagy was further confirmed by the WB analysis of autophagy protein markers, we found that **17c** incubation resulted in the declined expression of p62, upregulation of beclin-1 and dosage-dependent cleavage of LC3-I to LC3-II in HCT-116 cells (Figure 7C). To further validate the cytotoxic or cyto-protective effects of **17c**-induced autophagy, 3-MA (3-methyladenine), an inhibitor of type III phosphatidylinositol 3-kinases (PI3KIII) was employed to block the initiation of autophagic flux. As depicted in Figure 7D, the cytotoxicity of **17c** were potently reversed by the addition of 500 μM 3-MA ($p < 0.01$). In addition, an autophagolysosome inhibitor bafilomycin A1 (BafA1) was utilized to

validate the effects of **17c** on autophagic flux (Figure 7E). Both LC3-II and p62 were accumulated after the combined treatment of BafA1 and **17c**. These results suggest that compound **17c** induced autophagic cell death in HCT-116 colorectal carcinoma cells. In recent reports, the BRD4-AMPK-mTOR-ULK signaling axis was suggested as an upstream autophagy signaling pathway in cancer cells.[55, 59, 60] To further determine whether **17c**-induced autophagy was depended on ATG5, the ATG5 mRNA was interfered by a specific siRNA with or without the combination of **17c** incubation (Figure 7F). In agreement with our previous report, the mRNA knockdown of ATG5 did not interfere the **17c**-induced AMPK activation, however, the cleavage of LC3 and degradation of p62 were obviously suppressed. Moreover, the autophagy induction capacities of **17c** and the corresponding BRD4i and HDACi fragments were detected by WB analysis (Figure 7G). The high concentration of SAHA (10 μM) only led to marginal autophagy, and 0.5 μM **17c** incubation resulted stronger autophagy than that of 2.0 μM 4l. Taken together, above results indicated that **17c** induces autophagic cell death via BRD4-AMPK signaling pathway.

17c suppresses IL6-JAK-STAT pathway in colorectal carcinoma cells

Recent studies suggested that the activation of IL6-JAK-STAT signaling pathway might be a vital factor of drug resistant to HDAC inhibitors in cancer cells.[50] And the addition of BRD4 siRNA or inhibitor could reverse this drug resistance via suppressing the activation of IL6 family cytokines. In the current study, we also observed the transcriptional activation of four IL-6 family cytokines by RT-PCR, including interleukin 6, oncostatin M(OSM), leukemia inhibitory factor (LIF) and cardiotrophin 1 (CTF1) were stimulated by 10 μM SAHA incubation (Figure 8A). And the 4l treatment did not change the expression of these cytokines, 17c treatment only slightly

elevated these cytokines. The WB analysis of representative protein markers in IL6-JAK-STAT signaling pathways also indicated that SAHA could activate IL6 family cytokines and downstream JAK-STAT signaling axis, and these effects of HDAC inhibitors were not observed in 17c treated group (Figure 8B). These results suggested that these BRD4/HDAC dual inhibitors possibly overcome the drug resistance mechanism of single HDAC inhibition.

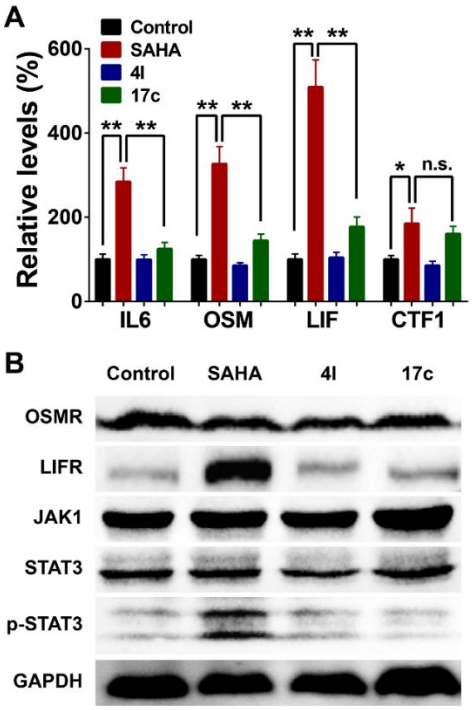


Figure 8. (A) The change of IL6, OSM, LIF and CTF1 mRNA levels in HCT-116 cells after incubated with 0.5 μM 17c, 2.0 μM 4l or 10 μM SAHA for the 24 hours, respectively. (B) HCT-116 cells were treated with 0.5 μM 17c, 5.0 μM 4l or 10 μM SAHA for the 24 hours, respectively. Then the protein expression of OSMR, LIFR, JAK1, STAT3 and phosphorylated STAT3 were detected by WB analysis.

17c inhibits tumor growth in xenograft models

We subsequently determined that **17c** bears potent inhibitory effects on the BRD4-HDAC pathways in vivo, and then, HCT-116 human colorectal carcinoma xenograft models were established to evaluate the efficacy of **17c**. The mean tumor data after 19

days of oral administration of SAHA or **17c** are presented in Figures 9. Both **17c** and SAHA exhibited potent antitumor activity at the dosage tested, and the average percentage of tumor growth inhibition in the **17c** groups was 68.8%. Moreover, the **17c**-treated groups displayed a dose-dependent decrease in tumor volume compared with the saline control (Figure 9B), with total treatment-to-control ratios (T/C) in the **17c** 15 mg/kg and 30 mg/kg groups of 42.7% and 68.8%, respectively. As expected, there was a significant difference between the **17c** group and the SAHA group. Therefore, **17c** was effective in reducing the growth of HCT-116 tumors in a xenograft model in vivo. Furthermore, to confirm whether the molecular mechanism of **17c** was based on the BRD4 and HDAC pathways in vivo, the changes in the expression levels of Ac-H3 and c-Myc were determined by immunohistochemical methods as well as apoptosis marker TUNEL (Terminal deoxynucleotidyl transferase dUTP nick end labeling) and Ki-67 staining. As shown in Figure 9D, the expression levels of Ac-H3 and c-Myc in HCT-116 xenograft tumor tissues in the vehicle- and **17c**-treated groups were measured by IHC analysis. The expression level of c-Myc was reduced in the **17c**-treated group, and Ac-H3 was elevated. Ki-67 is a proliferation marker with prognostic and predictive potential in colorectal carcinoma. The results of the IHC analysis and statistical analysis were also presented in Figure 9D. A significant reduction in the expression of Ki-67 was observed upon treatment with **17c**. Histological assessment of apoptosis by TUNEL staining revealed that TUNEL-positive apoptotic nuclei were significantly increased by **17c**. In agreement to the in vitro experiments, the autophagy marker LC3-II was elevated in 4l and **17c** treated groups. The protein expression of LIFR and phosphorylated STAT3 were also activated in SAHA treated group, and these phenomena were not observed in 17c treated group. In addition, there were no significant changes observed in the H&E (hematoxylin and eosin) staining sections of

the main organs as well as body weights of mice in the **17c**-treated groups (Figure S2).

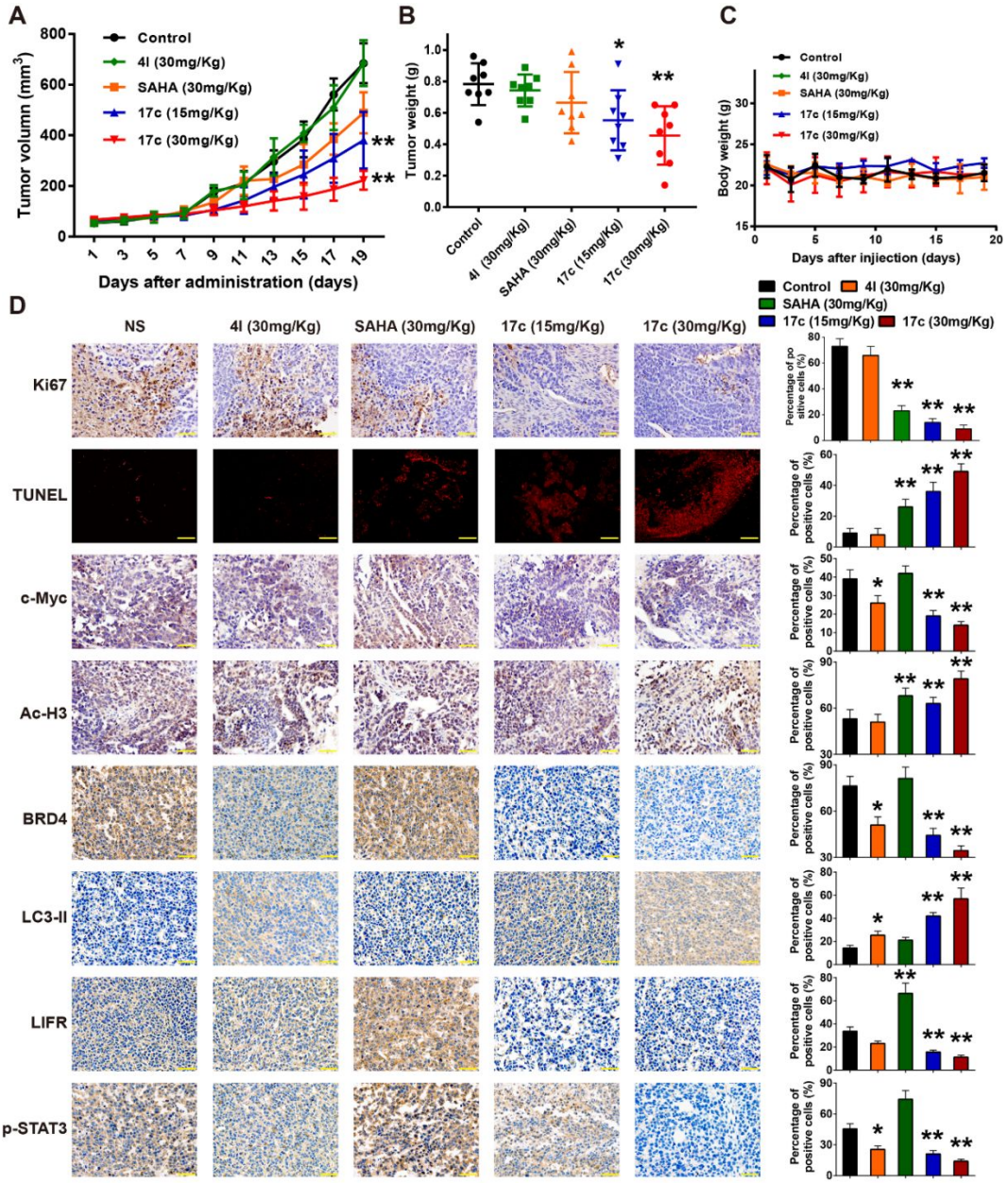


Figure 9. In vivo anticancer effect of compound **17c** on the HCT-116 xenograft models: A. The tumor volume changes with oral administration 15 or 30 mg/Kg of compound **17c**, 30mg/Kg of **4l** or SAHA, **: $p < 0.01$ compared to control group; B. The tumor weights analysis after the end of therapy; C. The body weight changes of mice; D. Representative images of immunohistochemical analysis of proliferative marker Ki-67, c-Myc, Ac-H3, BRD4, LC3-II, LIFR, p-STAT3 and immunofluorescent analysis of apoptosis marker TUNEL in different groups; scale bar = 50μm; *: $p < 0.05$ compared

to control group, **: $p < 0.01$ compared to control group.

Oral pharmacokinetics and preliminary safety evaluation

Compound **17c** (encapsulated in HP-β-CD for intravenous administration and 0.5% CMC solution for oral administration) was administered to SD rats intravenously (i.v.) or orally at a 30 mg/kg dosage. The drug concentrations of **17c** in the plasma were analyzed on a Waters UPLC-MS system. As shown in Table 4, the oral AUC value, in vivo half-life and oral bioavailability of **17c** were 63279.5 μg•L^{-1}•h, 1.53h and 40.5%, respectively. **17c** could enriched in the tumor tissues and prolong its antitumor effects (Figure S3). The predicted ADMET descriptors also suggested that the physicochemical properties of **17c** located in a reasonable range (Figure S4). These results indicated that compound **17c** possesses good PK profile, which provides the foundation for further development.

Table 4. Pharmacokinetic Parameters Tested in Vivo

	17c	
route	*i.v.*	*p.o.*
N [a]	5	5
dose (mg/Kg)	30	30
$AUC_{0\text{-}inf}$ (μg•L^{-1}•h) [b]	156245.5	63279.5
C_{max} (μg•L^{-1}) [c]	-	5361.3
T_{max} (h) [d]	0.12	1.53
$T_{1/2}$ (h) [e]	2.48	4.80
F (%) [f]	-	40.5

[a] Numbers of rats. [b] Area under the curve following intravenous dosing, integrated drug concentration with respect to time and integrated drug concentration with respect to time following oral dosing. [c] Maximum plasma concentration after oral administration. [d] Time to archive maximum

plasma concentration after oral administration. [e] plasma half-life. [f] Percentage of oral bioavailability.

Conclusion

To develop novel BRD4-HDAC dual inhibitors against colorectal carcinoma, a series of hydroxamic acid derivatives of thieno[2,3-d]pyrimidine were designed, synthesized, and evaluated for their activities in vitro and in vivo. The structure-activity relationship (SAR) analysis based on the enzymatic inhibition capacities on the class I and IIa isoform HDACs and BRDs of these synthesized compounds indicated that the main goal of finding novel small molecule BRD4-HDAC dual-inhibitors was successfully achieved. The most potent compound **17c** demonstrated good inhibitory activity and selectivity on HDACs and BRDs. The cell proliferation results suggested that **17c** suppressed the growth of colorectal carcinoma cells with IC_{50} values at sub-micromolar levels. Subsequently, the western blotting as well as flow cytometry analyses further confirmed the molecular mechanisms of **17c**. It also displays potent inhibitory efficiency on tumor growth by inducing autophagic cell death and suppressing IL6-JAK-STAT signaling pathways in the in vitro and in vivo colorectal carcinoma models. Moreover, compound **17c** could be orally administrated with an oral bioavailability of 40.5% in rats, and it displayed dose-dependent antitumor activity in xenograft colorectal carcinoma models. Taken together, these integrated design, synthesis, in vitro and in vivo studies indicate that the small molecule BRD4-HDAC dual inhibitor **17c** is a potential lead compound for the novel chemotherapeutics of colorectal carcinoma.

Experimental Section

Tissue microarray (TMA) and IHC staining

A human colorectal cancer TMA was purchased from Shanghai Outdo Biotech (OD-HColA180Su11). This TMA contained 180 tumors and matched adjacent normal tissues from 84 patients, including 70 adenocarcinomas, seven mucinous adenocarcinoma and seven canalicular adenocarcinoma, respectively. The protocol of IHC staining was perform by using rabbit monoclonal antibodies against BRD4 (Abcam, USA, 1:50 dilution) and HDAC2 (Abcam, USA, 1:200 dilution) as in our previously described studies unless otherwise stated. The results were interpreted in a semi-quantitative manner, and the expressions of BRD4 and HDAC2 were identified as the percentage of positive tumor cells by three independent pathologists to avoid different thresholds of positively stained tumor cells.

Fragment based molecular design

The coordinate files of BRD4 and HDACs were retrieved from the PDB database. Three crystallographic structures of HDACs, HDAC2 in class I (PDB No. 4LXZ), HDAC7 in class IIa (PDB No. 3C0Z) and HDAC6 in class IIb (PDB No. 5WGI), were utilized for the molecular docking studies. The three HDAC proteins complexed with SAHA were aligned and superposed by sequence similarity. The BRD4 protein contained two bromodomains, and the first bromodomain (PDB No. 4ZW1) and the second bromodomain (PDB No. 2YEM) were also aligned together by sequence similarity. All hydrogen atoms were minimized in the Accelrys Discovery Studio package by using the CHARMM27 force field. The fragments were generated from the known BRD4 inhibitors and then embedded into the ZINC fragment-like library. By using the MCSS (Multiple Copy Simultaneous Search) program in the Discovery Studio Package, the fragments were docked and scored into the binding pockets of BRD4 proteins. The top-scoring fragments were selected as novel scaffolds, merged or

optimized by the LUDI program in the Discovery Studio Package, and in situ minimized by the Libdock program in the Discovery Studio Package. The 1000 top-ranked molecules according to docking scores were evaluated by Verber drug-likeness filters, the Lipinski's "Rule-of-Five" and the ADMET (absorption, distribution, metabolism, excretion and toxicity) filter developed by our library to remove improper molecules. The optimized molecules were synthesized, and their BRD4 binding potencies were initially evaluated on HTRF® assays. The screened molecules were individually linked to the zinc binding group (ZBG) of the HDAC inhibitor and then docked into the binding pockets of three HDAC proteins.

Chemistry

All of the chemicals were purchased as analytically pure and used without further purification. The ^{1}H NMR and ^{13}C NMR spectra were performed on a Bruker Avance III 400 MHz spectrometer (Bruker Co. Ltd., Germany). Chemical shifts were given in ppm (parts per million) with TMS as an internal standard. Mass spectra were recorded on a Waters Q-TOF mass spectrometer (Micromass, Manchester, U.K.). The purity of each compound (>95%) was determined on an Shimadzu LC-20 instrument with a GL-C18 reverse-phase column (4.6 mm × 150 mm, 5 μm) with methanol, acetonitrile and water as the mobile phase.

General Procedures of Method for the Synthesis of 4a-4t.

Ethyl 2-amino-5,6-dihydro-4H-cyclopenta[b]thiophene-3-carboxylate (2a). To a mixture of cyclopentanone **(1a) (**4.20g, 50mmol), ethyl cyanoacetate (5.66 g, 100.0 mmol), and sulfur (1.60 g, 50.0 mmol) in absolute ethanol (100 mL) was added triethylamine (10mL) and refluxed for 12 h; the reaction mixture was concentrated and

the residue was partitioned between water and ethyl acetate. The organic layer was separated, and concentrated, and the crude product was purified by silica gel column chromatography using a mixture solvent of petroleum ether: ethylacetate (8:1), to give **2a.** Yield 76%, white powder. ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.18 (s, 2H, **NH_2**), 4.13 (q, J = 7.2 Hz, 2H, O**CH2**CH3), 2.70 (t, J = 7.2 Hz, 2H, S-C-**CH2**), 2.62 (t, J = 7.2 Hz, 2H, **CH_2-**Thiophene), 2.22 (p, J = 7.2 Hz, 2H, CH_2**CH_2**CH_2), 1.23 (t, J = 7.2 Hz, 3H, **CH_3**).

Ethyl 2-amino-4,5,6,7-tetrahydrobenzo[b]thiophene-3-carboxylate (2b). To a mixture of cyclohexanone**(1b) (**4.90g, 50mmol), ethyl cyanoacetate (5.66 g, 100.0 mmol), and sulfur (1.60 g, 50.0 mmol) in absolute ethanol (100 mL) was added triethylamine (10mL) and refluxed for 12 h; the reaction mixture was concentrated and the residue was partitioned between water and ethyl acetate. The organic layer was separated, and concentrated, and the crude product was recrystallized with ethanol (100ml), to give **2b.** Yield 72%, light yellow crystal. ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.20 (s, 2H, **NH_2**), 4.14 (q, J = 7.2 Hz, 2H, O**CH2**CH3), 2.59 (t, J = 5.6 Hz, 2H, S-C-**CH2**), 2.41 (t, J = 5.6 Hz, 2H, **CH_2-**Thiophene), 1.74-1.58 (m, 4H, CH_2**CH_2**CH_2**CH_2**), 1.24 (t, J = 7.2 Hz, 3H, **CH_3**).

Ethyl 2-amino-6-methyl-4,5,6,7-tetrahydrothieno[2,3-c]pyridine-3-carboxylate (2c). Compound 2c was synthesized from N-methyl-4-piperidone **(1c)**, in a manner similar to 2b. Yield 79%, yellow powder. ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.23 (s, 2H, **NH_2**), 4.15 (q, J = 7.2 Hz, 2H, O**CH_2**CH_3), 3.22 (t, J = 2.0 Hz, 2H,N-**CH_2-**Thiophene), 2.66 (t, J = 5.2 Hz, 2H,N-**CH_2-**CH_2), 2.53 (t, J = 6.0Hz, 2H,N-CH_2-**CH_2**), 2.29 (s, 3H, N-**CH_3**), 1.24 (t, J = 7.2 Hz, 3H, CH_2**CH_3**).

Ethyl 2-amino-6-ethyl-4,5,6,7-tetrahydrothieno[2,3-c]pyridine-3-carboxylate (2d). Compound 2d was synthesized from N-ethyl-4-piperidone **(1d)**, in a manner

similar to 2b. Yield 72%, yellow powder. ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.23 (s, 2H, **NH_2**), 4.14 (q, J = 7.2 Hz, 2H, O**CH_2**), 3.27 (s, 2H, N-**CH_2**-Thiophene), 2.65 (t, J = 5.9 Hz, 2H, N-**CH_2**-CH_2), 2.59 (t, J = 5.2 Hz, 2H, N-CH_2-**CH_2**), 2.45 (q, J = 7.2 Hz, 2H, N**CH_2**), 1.24 (t, J = 7.2 Hz, 3H, OCH_2**CH_3**), 1.04 (t, J = 7.2 Hz, 3H, NCH_2**CH_3**).

Ethyl 2-amino-6-benzyl-4,5,6,7-tetrahydrothieno[2,3-c]pyridine-3-carboxylate (2e). Compound 2e was synthesized from N-benzyl-4-piperidone **(1e)**, in a manner similar to 2b. Yield 69%, yellow powder. ^{1}H NMR (400 MHz, Chloroform-d) δ 7.39-7.26 (m, 5H), 5.93 (s, 2H, Ar-**CH_2**), 4.25 (q, J = 7.2 Hz, 2H, O**CH2**CH3), 3.67 (s, 2H, N-**CH_2**-Thiophene), 2.82 (t, J = 5.6 Hz, 2H, N-**CH_2**-CH_2), 2.74 (t,J = 5.6 Hz, 2H, N-CH_2-**CH_2**), 1.31 (t, J = 7.2 Hz, 3H, **CH_3**).

2-Phenyl-3,5,6,7-tetrahydro-4H-cyclopenta[4,5]thieno[2,3-d]pyrimidin-4-one (4a). Compound 2a (105.50mg, 0.5mmol) and cyanobenzene(3a, 51.50mg, 0.5mmol) were placed in 25 mL flask, and then 5ml 1,4-dioxane saturated by HCl was added, The reaction mixture was stirred for 48 h at room temperature, the reaction solvent was concentrated, the residues was added 10ml H_2O, this solvent was neutralized by $NaHCO_3$to pH=7 to give a suspension, centrifugated(4500 rounds/minute), the resultant solids was dried, fanally washed with ethylacetate(5ml) and ethanol(5ml) respectively to give **4a**. Yield 58%, off-white powder. mp: 284.4 –286.9℃. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.26-8.15 (m, 2H,Ar-**H**), 7.51-7.36 (m, 3H, Ar-**H**), 2.96-2.82 (m, 4H,2**CH_2**.Thiophene), 2.35 (p, J = 7.2 Hz, 2H, CH_2-**CH_2**-CH_2).^{13}C NMR (100 MHz, CF_3COOD) δ 157.34, 155.13, 152.64, 145.20, 141.72, 136.42, 130.33 , 127.49 , 123.09, 119.22, 28.39, 27.59, 27.40.HRMS(ESI): calcd. for$C_{15}H_{12}N_2NaOS^+$ $[M+Na]^+$, 291.0563; found 291.0566.

2-(4-Hydroxyphenyl)-3,5,6,7-tetrahydro-4H-cyclopenta[4,5]thieno[2,3-d] pyrimidin-4-one (4b). Compound (4b) was synthesized from (2a) and 4-

hydroxybenzonitrile (3b), in a manner similar to (4a). Yield 39%, gray powder. mp>320℃, dec.^{1}H NMR (400 MHz, DMSO-d_6) δ 8.10-8.01 (m, 2H, Ar-**H**), 6.84-6.73(m, 2H, Ar-**H**), 2.94-2.81 (m, 4H, 2**CH_2**-Thiophene), 2.35 (p, J = 7.2 Hz, 2H, CH_2-**CH_2**-CH_2).^{13}C NMR (100 MHz, CF_3COOD) δ 162.94, 157.67, 154.27, 152.97, 144.47, 141.69, 130.32 , 124.90, 117.66 , 115.14, 28.41, 27.66, 27.50.HRMS(ESI): calcd. for$C_{15}H_{12}N_2NaO_2S^+$[M+Na]$^+$, 307.0512; found 307.0516.

2-(3,5-Dimethylphenyl)-3,5,6,7-tetrahydro-4H-cyclopenta[4,5]thieno[2,3-d] pyrimidin-4-one (4c). Compound (4c) was synthesized from (2a) and 3,5-dimethylbenzonitrile (3c), in a manner similar to (4a). Yield 42%, off-white powder. mp>320℃, dec.^{1}H NMR (400 MHz, DMSO-d_6) δ 7.86 (s, 2H, Ar-**H**), 7.02 (s, 1H, Ar-**H**), 2.92-2.81 (m, 4H, 2**CH_2**-Thiophene), 2.35 (p, J = 7.2 Hz, 2H, CH_2-**CH_2**-CH_2), 2.32 (s, 6H, 2Ar-**CH_3**).^{13}C NMR (100 MHz, CF_3COOD) δ 157.53, 155.29, 152.76, 144.91, 141.73 , 141.64, 138.32, 124.92 , 122.86, 119.05, 28.36, 27.57, 27.40, 19.13 .HRMS(ESI): calcd. for$C_{17}H_{16}N_2NaOS^+$[M+Na]$^+$, 319.0876; found 319.0880.

2-(4-Hydroxy-3,5-dimethylphenyl)-3,5,6,7-tetrahydro-4H-cyclopenta[4,5] thieno[2,3-d]pyrimidin-4-one (4d). Compound (2d) was synthesized from (2a) and 4-hydroxy-3,5-dimethylbenzonitrile (3d), in a manner similar to (4a). Yield 45%, off-white powder. mp>320℃, dec.^{1}H NMR (400 MHz, DMSO-d_6) δ 7.82 (s, 2H, Ar-**H**), 2.94-2.84 (m, 4H, 2**CH_2**-Thiophene), 2.36 (p, J = 7.2 Hz, 2H, CH_2-**CH_2**-CH_2), 2.21 (s, 6H, 2Ar-**CH_3**).^{13}C NMR (100 MHz, CF_3COOD) δ 160.44, 154.40, 153.13, 144.09, 141.68, 128.53 , 127.31 , 124.94, 118.43, 114.15, 28.43, 27.69, 27.56, 14.10 .HRMS(ESI): calcd. for$C_{17}H_{16}N_2NaO_2S^+$[M+Na]$^+$,335.0825; found335.0828.

2-Phenyl-5,6,7,8-tetrahydrobenzo[4,5]thieno[2,3-d]pyrimidin-4(3H)-one (4e). Compound (4e) was synthesized from (2b) and cyanobenzene (3a), in a manner similar to (4a). Yield 55%, off-white powder.mp: 279.6-282.2℃^{1}H NMR (400 MHz, DMSO-

d_6) δ 8.31-8.22 (m, 2H,Ar-**H**), 7.43-7.29 (m, 3H,Ar-**H**), 2.91 (t, J = 5.6 Hz, 2H, Thiophene-**CH_2**), 2.68 (t, J = 5.6 Hz, 2H, Thiophene-**CH_2**), 1.85-1.68 (m, 4H, CH2-**CH2-CH2-**CH2).^{13}C NMR (100 MHz, CF_3COOD) δ 157.46, 155.20, 148.63, 139.78, 136.51, 133.05, 130.38 , 127.52 , 123.05, 122.25, 24.36, 24.23, 21.59, 20.64.HRMS(ESI): calcd. for$C_{16}H_{14}N_2NaOS^+$[M+Na]$^+$, 305.0719; found 305.0725.

2-(4-Hydroxyphenyl)-5,6,7,8-tetrahydrobenzo[4,5]thieno[2,3-d]pyrimidin-4(3H)-one (4f). Compound (4f) was synthesized from (2b) and 4-hydroxybenzonitrile (3b), in a manner similar to (4a). Yield 45%, dark gray powder.mp: 270.2-272.1℃. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.06-7.99 (m, 2H, Ar-**H**), 6.85-6.78 (m, 2H, Ar-**H**), 2.90 (t, J = 5.6 Hz, 2H, Thiophene-**CH_2**), 2.76-2.67 (t, J = 5.6 Hz, 2H, Thiophene-**CH_2**), 1.90-1.65 (m, 4H, 4H, CH2-**CH2-CH2-**CH2). ^{13}C NMR (100 MHz, CF_3COOD) δ 162.95, 157.67, 154.24, 148.88, 138.97, 132.92, 130.30 , 121.53, 117.62 , 115.03, 24.40, 24.19, 21.65, 20.71.HRMS(ESI): calcd. for $C_{16}H_{14}N_2NaO_2S^+$ [M+Na]$^+$, 321.0668; found 321.0675.

2-(3,5-Dimethylphenyl)-5,6,7,8-tetrahydrobenzo[4,5]thieno[2,3-d]pyrimidin-4(3H)-one (4g). Compound (4g) was synthesized from (2b) and 3,5-dimethylbenzonitrile (3c), in a manner similar to (4a). Yield 49%, off-white powder.mp: 289.5-290.6℃^{1}H NMR (400 MHz, DMSO-d_6) δ 12.36 (s, 1H, Pyrimidine-**H**), 7.76 (s, 2H,Ar-**H**), 7.20 (s, 1H, Ar-**H**), 2.91 (t, J = 5.6 Hz, 2H, Thiophene-**CH_2**), 2.76 (t, J = 5.6 Hz, 2H,Thiophene-**CH_2**), 2.35 (s, 6H, 2Ar-**CH_3**), 1.87-1.73 (m, 4H, CH2-**CH2-CH2-**CH2).^{13}C NMR (100 MH, CF_3COOD) δ 157.58, 155.25, 148.68, 141.69 , 139.39, 138.30, 132.88, 124.89 , 122.77, 121.99, 24.29, 24.13, 21.53, 20.59, 19.12 .HRMS(ESI): calcd. for $C_{18}H_{18}N_2NaOS^+$ [M+Na]$^+$, 333.1032; found 333.1036.

2-(4-Hydroxy-3,5-dimethylphenyl)-5,6,7,8-tetrahydrobenzo[4,5]thieno[2,3-d]pyrimidin-4(3H)-one (4h). Compound (4h) was synthesized from (2b) and 4-

hydroxy-3,5-dimethylbenzonitrile (3d), in a manner similar to (4a). Yield 41%, off-white powder. mp>320℃, dec.^{1}H NMR (400 MHz, DMSO-d_6) δ 12.12 (s, 1H, Pyrimidine-**H**), 8.97 (s, 1H, **OH**), 7.80 (s, 2H, Ar-**H**), 2.93-2.85 (t, J = 5.6 Hz, 2H,Thiophene- **CH_2**), 2.79-2.69 (t, J = 5.6 Hz, 2H, Thiophene-**CH_2**), 2.22 (s, 6H,2Ar-**CH_3**), 1.88-1.69 (m, 4H, CH2-**CH2-CH2-**CH2).^{13}C NMR (100 MHz, CF_3COOD) δ 160.23, 157.80, 154.18, 148.99, 138.52, 132.77, 128.42 , 127.13 , 121.20, 114.00, 24.33, 24.09, 21.61, 20.65, 13.98 .HRMS(ESI): calcd. for $C_{18}H_{18}N_2NaO_2S^+$ $[M+Na]^+$, 349.0981; found 349.0986.

2-Phenyl-7-methyl-5,6,7,8-tetrahydropyrido[4',3':4,5]thieno[2,3-d]pyrimidin-4(3H)-one(4i). Compound (4i) was synthesized from (2c) and cyanobenzene (3a), in a manner similar to (4a). Yield 65%, light yellow powder. mp: 266.4-268.2℃1H NMR (400 MHz, DMSO-d6) δ 8.26-8.19 (m, 2H, Ar-**H**), 7.44-7.39 (m, 3H, Ar-**H**), 3.51 (s, 2H, N-**CH_2**-Thiophene), 2.97 (t, J = 5.6 Hz, 2H, N-**CH_2**-CH_2), 2.64 (t, J = 5.6 Hz, 2H, N-CH_2-**CH_2**), 2.37 (s, 3H, N-**CH_3**).^{13}C NMR (100 MHz, CF_3COOD) δ 157.58, 156.99, 151.47, 137.01, 130.35 , 128.95, 127.82 , 126.77, 122.80, 120.63, 51.83, 51.59, 42.84, 22.00.HRMS(ESI): calcd. for $C_{16}H_{15}N_3NaOS^+$ $[M+Na]^+$, 320.0828; found 320.0836.

2-(4-Hydroxyphenyl)-7-methyl-5,6,7,8-tetrahydropyrido[4',3':4,5]thieno[2,3-d]pyrimidin-4(3H)-one (4j). Compound (4j) was synthesized from (2c) and 4-hydroxybenzonitrile (3b), in a manner similar to (4a). Yield 43%, yellow powder. mp>320℃, dec.^{1}H NMR (400 MHz, DMSO-d_6) δ 8.09-8.00 (m, 2H, Ar-**H**), 6.84-6.76 (m, 2H, Ar-**H**), 3.51 (s, 2H, N-**CH_2**-Thiophene), 2.95 (t, J = 5.6 Hz, 2H, N-**CH_2**-CH_2), 2.64 (t, J = 5.6 Hz, 2H, N-CH_2-**CH_2**), 2.36 (s, 3H, N-**CH_3**).^{13}C NMR (100 MHz, CF_3COOD) δ 163.95, 157.19, 156.35, 151.36, 130.73 , 128.90, 125.92, 119.79, 117.70 , 114.22, 51.73, 51.58, 42.78, 21.93.HRMS(ESI): calcd. for $C_{16}H_{16}N_3O_2S^+$ $[M+H]^+$, 314.0958; found 314.0961.

2-(3,5-Dimethylphenyl)-7-methyl-5,6,7,8-tetrahydropyrido[4',3':4,5]thieno[2,3-d]pyrimidin-4(3H)-one (4k). Compound (4k) was synthesized from (2c) and 3,5-dimethylbenzonitrile (3c), in a manner similar to (4a). Yield 41%, yellow powder. mp: 292.3-293.5℃^{1}H NMR (400 MHz, DMSO-d_6) δ 7.92 (s, 2H, Ar-**H**), 6.96 (s, 1H, Ar-**H**), 3.47 (s, 2H, N-**CH_2**-Thiophene), 2.96 (t, J = 5.6 Hz, 2H, N-**CH_2**-CH_2), 2.62 (t, J = 5.6 Hz, 2H, N-CH_2-**CH_2**), 2.36 (s, 3H, N-**CH_3**), 2.32 (s, 6H, 2Ar-**CH_3**). ^{13}C NMR (100 MHz, CF_3COOD) δ 157.78, 157.20, 151.22, 142.00 , 139.22, 129.08, 126.81, 125.44 , 122.49, 120.66, 51.82, 51.62, 42.83, 22.10, 19.19. HRMS(ESI): calcd. for $C_{18}H_{19}N_3NaOS^+$ $[M+Na]^+$, 348.1141; found 348.1149.

2-(4-Hydroxy-3,5-dimethylphenyl)-7-methyl-5,6,7,8-tetrahydropyrido[4',3':4,5]thieno [2,3-d]pyrimidin-4(3H)-one (4l). Compound (4l) was synthesized from (2c) and 4-hydroxy-3,5-dimethylbenzonitrile (3d), in a manner similar to (4a). Yield 47%, yellow powder. mp>320℃, dec.^{1}H NMR (400 MHz, DMSO-d_6) δ 7.81 (s, 2H, Ar-**H**), 3.53 (s, 2H, N-**CH_2**-Thiophene), 2.95 (t, J = 5.6 Hz, 2H, N-**CH_2**-CH_2), 2.66 (t, J = 5.6 Hz, 2H, N-CH_2-**CH_2**), 2.37 (s, 3H, N-**CH_3**), 2.22 (s, 6H, 2Ar-**CH_3**).^{13}C NMR (100 MHz, CF_3COOD) δ 157.52, 156.36, 151.50, 129.18 , 129.05, 127.45 , 125.95, 124.90, 119.76, 113.34, 51.93, 51.74, 42.92, 22.20, 14.08 .HRMS(ESI): calcd. for $C_{18}H_{19}N_3NaO_2S^+$ $[M+Na]^+$, 364.1090; found 364.1095.

7-Ethyl-2-phenyl-5,6,7,8-tetrahydropyrido[4',3':4,5]thieno[2,3-d]pyrimidin-4(3H)-one (4m). Compound (4m) was synthesized from (2d) and cyanobenzene (3a), in a manner similar to (4a). Yield 61%, light yellow powder.mp: 257.3-259.1℃. ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.58 (br, 1H, Pyrimidine-**H**), 8.20-8.13 (m, 2H, Ar-**H**), 7.54-7.45 (m, 3H, Ar-**H**), 3.61 (s, 2H, N-**CH_2**-Thiophene), 2.96 (t, J = 5.6 Hz, 2H, N-**CH_2**-CH_2), 2.72 (t, J = 5.6 Hz, 2H, N-CH_2-**CH_2**), 2.55 (q, J = 7.2 Hz, 2H, CH_3-**CH_2**-N), 1.09 (t, J = 7.2 Hz, 3H, **CH_3**-CH_2-N).^{13}C NMR (100 MHz, CF_3COOD) δ 158.50,

157.77, 152.32, 137.90, 131.25 , 130.17, 128.71 , 127.79, 123.71, 121.49, 53.87, 50.42, 50.27, 22.86, 8.84.HRMS(ESI): calcd. for $C_{17}H_{18}N_3OS^+$ $[M+H]^+$, 312.1165; found 312.1173.

7-Ethyl-2-(4-hydroxyphenyl)-5,6,7,8-tetrahydropyrido[4',3':4,5]thieno[2,3-d]pyrimidin-4(3H)-one (4n). Compound (4n) was synthesized from (2d) and 4-hydroxybenzonitrile (3b), in a manner similar to (4a). Yield 35%, yellow powder.mp: 307.3-308.1℃^{1}H NMR (400 MHz, DMSO-d_6) δ 8.10-8.03 (m, 2H, Ar-**H**), 6.84-6.78 (m, 2H, Ar-**H**), 3.56 (s, 2H, N-**CH_2**-Thiophene), 2.94 (t, J = 5.6 Hz, 2H, N-**CH_2**-CH_2), 2.70 (t, J = 5.6 Hz, 2H. N-CH_2-**CH_2**), 2.58-2.52 (q, J = 7.2 Hz, 2H, CH_3-**CH_2**-N), 1.09 (t, J = 7.2 Hz, 3H, **CH_3**-CH_2-N). ^{13}C NMR (100 MHz, CF_3COOD) δ 163.96, 157.22, 156.30, 151.35, 130.87 , 129.32, 126.34, 119.89, 117.75 , 114.27, 53.03, 49.61, 49.42, 22.07, 8.02.HRMS(ESI): calcd. for $C_{17}H_{17}N_3NaO_2S^+$ $[M+Na]^+$, 350.0934; found 350.0938.

7-Ethyl-2-(3,5-dimethylphenyl)-5,6,7,8-tetrahydropyrido[4',3':4,5]thieno[2,3-d]pyrimidin-4(3H)-one (4o). Compound (4o) was synthesized from (2d) and 3,5-dimethylbenzonitrile (3c), in a manner similar to (4a). Yield 48%, yellow powder.mp: 261.7-263.2℃^{1}H NMR (400 MHz, DMSO-d_6) δ 7.89 (s, 2H,Ar-**H**), 6.98 (s, 1H,Ar-**H**), 3.53 (s, 2H, N-**CH_2**-Thiophene), 2.94 (t, J = 5.6 Hz, 2H, N-**CH_2**-CH_2), 2.68 (t, J = 5.6 Hz, 2H, N-CH_2-**CH_2**), 2.53 (q, J = 7.2 Hz 2H, CH_3-**CH_2**-N), 2.32 (s, 6H, 2Ar-**CH_3**), 1.09 (t, J = 7.2 Hz, 3H, **CH_3**-CH_2-N).^{13}C NMR (100 MHz, CF_3COOD) δ 157.71, 157.12, 151.20, 141.91 , 139.12, 129.33, 126.87, 125.33 , 122.44, 120.52, 53.07, 49.64, 49.48, 22.06, 19.11 , 8.01.HRMS(ESI): calcd. for $C_{19}H_{21}N_3NaOS^+$ $[M+Na]^+$, 362.1298; found 362.1304.

7-Ethyl-2-(4-hydroxy-3,5-dimethylphenyl)-5,6,7,8-tetrahydropyrido[4',3':4,5] thieno[2,3-d]pyrimidin-4(3H)-one (4p). Compound (4p) was synthesized from (2d)

and 4-hydroxy-3,5-dimethylbenzonitrile (3d), in a manner similar to (4a). Yield 43%, yellow powder. mp: 260.5-263.7℃ ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.83 (s, 2H, Ar-**H**), 3.57 (s, 2H, N-**CH_2**-Thiophene), 2.93 (t, J = 5.6 Hz, 2H, N-**CH_2**-CH_2), 2.70 (t, J = 5.6 Hz, 2H, N-CH_2-**CH_2**), 2.58-2.52 (q, J = 7.2 Hz , 2H, CH_3-**CH_2**-N), 2.21 (s, 6H, 2Ar-**CH_3**), 1.09 (t, J = 7.2 Hz, 3H, **CH_3**-CH_2-N).^{13}C NMR (100 MHz, CF_3COOD) δ 157.50, 156.30, 151.46, 129.35, 129.13 , 127.41 , 126.11, 127.87, 119.69, 113.31, 53.08, 49.70, 49.50, 22.16, 14.04 , 8.09.HRMS(ESI): calcd. for $C_{19}H_{21}N_3NaO_2S^+$ $[M+Na]^+$, 378.1247; found 378.1251.

7-Benzyl-2-phenyl-5,6,7,8-tetrahydropyrido[4',3':4,5]thieno[2,3-d]pyrimidin-4(3H)-one (4q). Compound (4q) was synthesized from (2e) and cyanobenzene (3a), in a manner similar to (4a). Yield 55%, light yellow powder.mp: 253.7-254.3℃^{1}H NMR (400 MHz, DMSO-d_6) δ 8.31-8.20 (m, 2H, Ar-**H**), 7.43-7.23 (m, 8H, Ar-**H**), 3.69 (s, 2H, Ar -**CH_2**), 3.55 (s, 2H, N-**CH_2**-Thiophene), 2.96 (t, J = 5.6 Hz, 2H, N-**CH_2**-CH_2), 2.74 (t, J = 5.6 Hz, 2H, N-CH_2-**CH_2**).^{13}C NMR (100 MHz, CF_3COOD) δ 157.53, 157.01, 151.43, 137.02, 131.16, 130.37 , 130.13 , 129.56 , 129.23, 127.78 , 127.05, 126.01, 122.82, 120.57, 61.53, 49.59, 49.21, 22.02.HRMS(ESI): calcd. for $C_{22}H_{19}N_3NaOS^+$ $[M+Na]^+$, 396.1141; found 396.1145.

7-Benzyl-2-(4-hydroxyphenyl)-5,6,7,8-tetrahydropyrido[4',3':4,5]thieno[2,3-d]pyrimidin-4(3H)-one (4r). Compound (4r) was synthesized from (2e) and 4-hydroxybenzonitrile (3b), in a manner similar to (4a). Yield 49%, yellow powder.mp: 259.1 –262.3℃^{1}H NMR (400 MHz, DMSO-d_6) δ 8.15-8.05 (m, 2H, Ar-**H**), 7.40-7.31 (m, 4H, Ar-**H**), 7.30-7.24 (m, 1H, Ar-**H**), 6.80-6.71 (m, 2H, Ar-**H**), 3.68 (s, 2H, Ar -**CH_2**), 3.53 (s, 2H,N-**CH_2**-Thiophene), 2.96 (t, J = 5.6 Hz, 2H, N-**CH_2**-CH_2), 2.73 (t, J = 5.6 Hz, 2H, N-CH_2-**CH_2**).^{13}C NMR (100 MHz, CF_3COOD) δ 164.02, 157.30, 156.31, 151.37, 131.27, 130.93 , 130.38 , 129.66 , 129.37, 126.60, 126.18, 119.96, 117.82 ,

114.35, 61.62, 49.68, 49.32, 22.23.HRMS(ESI): calcd. for $C_{22}H_{19}N_3NaO_2S^+$ $[M+Na]^+$, 412.1090; found 412.1098.

7-Benzyl-2-(3,5-dimethylphenyl)-5,6,7,8-tetrahydropyrido[4',3':4,5]thieno[2,3-d]pyrimidin-4(3H)-one (4s). Compound (4s) was synthesized from (2e) and 3,5-dimethylbenzonitrile (3c), in a manner similar to (4a). Yield 42%, yellow powder.mp: 265.8-267.2℃^{1}H NMR (400 MHz, DMSO-d_6) δ 7.87 (s, 2H, Ar-**H**), 7.39-7.32 (m, 4H, Ar-**H**), 7.30-7.25 (m, 1H, Ar-**H**), 6.99 (s, 1H, Ar-**H**), 3.68 (s, 2H, Ar -$\mathbf{CH_2}$), 3.53 (s, 2H, N-$\mathbf{CH_2}$-Thiophene), 2.94 (t, J = 5.6 Hz, 2H, N-$\mathbf{CH_2}$-CH_2), 2.73 (t, J = 5.6 Hz, 2H, N-CH_2-$\mathbf{CH_2}$), 2.31 (s, 6H,2Ar-$\mathbf{CH_3}$).^{13}C NMR (100 MHz, CF_3COOD) δ 157.63, 157.11, 151.14, 141.85 , 139.05, 131.12, 130.11 , 129.53 , 129.22, 126.86, 126.02, 125.22 , 122.40, 120.41, 61.50, 49.57, 49.19, 22.00, 19.07 .HRMS(ESI): calcd. for $C_{24}H_{23}N_3NaOS^+$ $[M+Na]^+$, 424.1454; found 424.1458.

7-Benzyl-2-(4-hydroxy-3,5-dimethylphenyl)-5,6,7,8-tetrahydropyrido[4',3':4,5]thieno[2,3-d]pyrimidin-4(3H)-one (4t). Compound (4t) was synthesized from (2e) and 4-hydroxy-3,5-dimethylbenzonitrile (3d), in a manner similar to (4a). Yield 46%, yellow powder. mp>320℃, dec.^{1}H NMR (400 MHz, DMSO-d_6) δ 12.19 (s, 1H, Pyrimidine-**H**), 8.99 (s, 1H, **OH**), 7.81 (s, 2H, Ar-**H**), 7.40-7.32 (m, 4H, Ar-**H**), 7.31–7.25 (m, 1H, Ar-**H**), 3.72 (s, 2H, Ar -$\mathbf{CH_2}$), 3.61 (s, 2H, N-$\mathbf{CH_2}$-Thiophene), 2.95 (t, J = 5.6 Hz, 2H, N-$\mathbf{CH_2}$-CH_2), 2.79 (t, J = 5.6 Hz, 2H, N-CH_2-$\mathbf{CH_2}$), 2.22 (s, 6H, 2Ar-$\mathbf{CH_3}$).^{13}C NMR (100 MHz, CF_3COOD) δ 157.47, 156.31, 151.45, 131.26, 130.26 , 129.67 , 129.28, 129.02 , 127.43 , 126.18, 126.12, 126.07, 119.57, 113.32, 61.65, 49.74, 49.34, 22.16, 14.00 .HRMS(ESI): calcd. for $C_{24}H_{23}N_3NaO_2S^+$ $[M+Na]^+$, 440.1403; found 440.1408.

Tert-butyl 2-amino-3-carbamoyl-4,7-dihydrothieno[2,3-c]pyridine-6(5H)-carboxylate (5). To a solution of tert-butyl 4-oxopiperidine-1-carboxylate(3.98g, 20.0

mmol), 2-cyanoacetamide (1.68 g, 20.0 mmol), and sulfur (641mg, 20.0 mmol) in absolute ethanol (25 mL), triethylamine (4 mL) was added and then the miture was refluxed for 12 h; the reaction mixture was concentrated and the residue was washed with cool methanol(20 ml) to give **5**, Yield 82%, white powder.^{1}H NMR (400 MHz, Chloroform-d) δ 6.17 (s, 2H, CO**NH$_2$**), 5.36 (s, 2H, Thiophene-**NH$_2$**), 4.38 (s, 2H, N-**CH$_2$**-Thiophene), 3.68 (t, J = 5.2 Hz, 2H, N-**CH$_2$**-CH$_2$), 2.74 (t, J = 5.2 Hz, 2H, N-CH$_2$-**CH$_2$**), 1.48 (s, 9H, Boc-**H**).

Tert-butyl 3-carbamoyl-2-(3,5-dimethylisoxazole-4-carboxamido)-4,7-dihydrothieno[2,3-c]pyridine-6(5H)-carboxylate (6). Compound 5 (594 mg, 2 mmol) and pyridine (193 µl, 2.4 mmol) was dissolved in 1,4-dioxane (10 mL) and then 3,5-dimethylisoxazole-4-carbonyl chloride (381 mg, 2.4 mmol) was added into above solution. The mixture was stirred for 3 h at room temperature. After the reaction was complete, the miture were removed in vacuo and the residue was washed with methanol (8 ml) to give 6, Yield 86.9%, off-white powder.^{1}H NMR (400 MHz, DMSO-d$_6$) δ 12.22 (s, 1H, CO**NH**), 7.40 (br, 2H, CO**NH$_2$**), 4.49 (s, 2H, N-**CH$_2$**-Thiophene), 3.57 (t, J = 5.6 Hz, 2H, N-**CH$_2$**-CH$_2$), 2.83 (t, J = 5.6 Hz, 2H, N-CH$_2$-**CH$_2$**), 2.70 (s, 3H, Isoxazole-**CH$_3$**), 2.47 (s, 3H, Isoxazole-**CH$_3$**), 1.43 (s, 9H, Boc-**H**).

N-(3-carbamoyl-4,5,6,7-tetrahydrothieno[2,3-c]pyridin-2-yl)-3,5-dimethylisoxazole-4-carboxamide (7). Compound 6(2.10 g, 5ml mmol) was added into 4M HC1/dioxane (10 ml, 40 mmol) at 0℃, the reaction miture was sterred for 4 hours at room temperature. The reaction solution was added with appropriate water and neutralized with NaHCO$_3$. The precipitated product was collected by vacuum filtration and washed with additional water (30 ml) and dichloromethane (20 ml) to give 7, Yield 83.3%, yellow solid. ^{1}H NMR (400 MHz, DMSO-d$_6$) δ 7.13 (br, 2H, CO**NH$_2$**), 4.00 (s, 2H, N-**CH$_2$**-Thiophene), 3.16 (s, 2H, N-**CH$_2$**-CH$_2$), 2.97 (s, 2H, N-CH$_2$-**CH$_2$**), 2.63 (s,

3H, Isoxazole-**CH_3**), 2.41 (s, 3H, Isoxazole-**CH_3**).

2-(3,5-dimethylisoxazol-4-yl)-5,6,7,8-tetrahydropyrido[4',3':4,5]thieno[2,3-d]pyrimidin-4(3H)-one (8). Compound 7 (300mg, 0.94 mmol) and NaOH (600 mg, 15 mmol) was dissolved in absolute ethanol (5ml). The mixture was sterred at 100℃ for 24 h. The reaction mixture was concentrated and the residue was purified by silica gel column chromatography using a mixture of dichloromethane: methanol (15:1, v/v), to give 8, Yield 57.2%, brown powder. mp: 255.9 – 257.2℃ ^{1}H NMR (400 MHz, DMSO-d_6) δ 3.90 (s, 2H, N-**CH_2**-Thiophene), 2.97 (t, J = 5.6 Hz, 2H, N-**CH_2**-CH_2), 2.87 (t, J = 5.6 Hz, 2H, N-CH_2-**CH_2**), 2.54 (s, 3H, Isoxazole-**CH_3**), 2.34 (s, 3H, Isoxazole-**CH_3**).^{13}C NMR (101 MHz, DMSO-d_6) δ 170.27, 163.43 , 159.71 , 158.92 , 147.21 , 132.12 , 130.21 , 121.30 , 111.77 , 44.50 , 42.65 , 26.92 , 12.55 , 10.95 .HRMS(ESI): calcd. for $C_{14}H_{15}N_4O_2S^+$ $[M+H]^+$, 303.0910; found 303.0912.

2-(3,5-dimethylisoxazol-4-yl)-7-methyl-5,6,7,8-tetrahydropyrido[4',3':4,5]thieno[2,3-d]pyrimidin-4(3H)-one (9a). Compound 8 (90 mg, 0.30 mmol), K_2CO_3(83 mg, 0.60 mmol) were suspended in 1,4-dioxane (5 ml), and then iodomethane (42 mg, 0.30 mmol) was added into the suspension. The mixture was stirred for 4 h at room temperature. The suspension was concentrated and the residue was purified by thin layer chromatography using a mixture of dichloromethane: methanol (20:1), to give 9a, Yield 26.5 %, yellow solid. mp: 227.5 – 230.2℃.^{1}H NMR (400 MHz, Chloroform-d) δ 11.93 (s, 1H, Pyrimidine-**H**), 3.66 (s, 2H, N-**CH_2**-Thiophene), 3.06 (t, J = 5.8 Hz, 2H, N-**CH_2**-CH_2-Thiophene), 2.78 (t, J = 5.8 Hz, 2H, N-CH_2-**CH_2**-Thiophene), 2.67 (s, 3H, Isoxazole-**CH_3**), 2.52 (s, 3H, N**CH_3**), 2.50 (s, 3H, Isoxazole-**CH_3**).^{13}C NMR (101 MHz, Chloroform-d) δ 170.39 , 164.89, 160.40 , 158.85 , 158.44 , 145.66 , 139.66 , 131.54 , 129.29 , 120.49 , 110.83, 53.73 , 51.88 , 45.55 , 25.89 , 12.69 , 11.08 .HRMS(ESI): calcd. for $C_{15}H_{17}N_4O_2S^+$ $[M+H]^+$,317.1067; found

317.1062.

Methyl 2-(2-(3,5-dimethylisoxazol-4-yl)-4-oxo-3,5,6,8-tetrahydropyrido[4',3':4,5]thieno[2,3-d]pyrimidin-7(4H)-yl)acetate (9b). Compound 8 (90 mg, 0.30 mmol) and methyl 2-bromoacetate (55 mg, 0.36 mmol) were dissolved in 1,4-dioxane (5 ml), and then K_2CO_3(83 mg, 0.60 mmol) was added into the solution; The mixture was refluxed for 6 h. The reaction mixture was concentrated and the residue was purified by silica gel column chromatography using a mixture of dichloromethane: methanol (80:1), to give 9b, Yield 47.6%, yellow solid.mp: 174.5 – 180.2℃. ^{1}H NMR (400 MHz, Chloroform-d) δ 11.17 (s, 1H, Pyrimidine-**H**), 3.91 (s, 2H, N-**CH_2**-Thiophene), 3.77 (s, 3H, O**Me**), 3.50 (s, 2H, MeCOO**CH_2**N), 3.09 (t, J = 5.6 Hz, 2H, N-**CH_2**-CH_2-Thiophene), 3.00 (t, J = 5.6 Hz, 2H, N-CH_2-**CH_2**-Thiophene), 2.68 (s, 3H, Isoxazole-**CH_3**), 2.50 (s, 3H, Isoxazole-**CH_3**).^{13}C NMR (101 MHz, Chloroform-d) δ 170.69, 170.47, 164.55, 159.85, 158.29, 145.61, 131.01, 129.38, 119.98, 110.67, 58.04, 51.90, 51.13, 49.68, 25.55, 12.79, 11.20.HRMS(ESI): calcd. for $C_{17}H_{19}N_4O_4S^+$ $[M+H]^+$,375.1122; found 375.1118.

Methyl 4-(2-(3,5-dimethylisoxazol-4-yl)-4-oxo-3,5,6,8-tetrahydropyrido[4',3':4,5]thieno[2,3-d]pyrimidin-7(4H)-yl)butanoate(9c). Compound 9c was synthesized from Compound 8 and methyl 4-bromobutanoate, in a manner similar to 9b, Yield 28.5%, yellow solid.mp: 57.8 – 60.2℃.^{1}H NMR (400 MHz, Chloroform-d) δ 11.78 (s, 1H, Pyrimidine-**H**), 3.70 (s, 2H, N-**CH_2**-Thiophene), 3.67 (s, 3H, O**Me**), 3.02 (t, J = 5.6 Hz, 2H, N-**CH_2**-CH_2-Thiophene), 2.83 (t, J = 5.6 Hz, 2H, N-CH_2-**CH_2**-Thiophene), 2.67 (s, 3H, Isoxazole-**CH_3**), 2.61 (t, J = 7.2 Hz, 2H, MeCOO**CH_2**), 2.50 (s, 3H, Isoxazole-**CH_3**), 2.45 – 2.39 (m, 2H, MeCOOCH_2CH_2**CH_2**), 1.98 – 1.89 (m, 2H, MeCOOCH_2**CH_2**CH_2).^{13}C NMR (101 MHz, Chloroform-d) δ 173.94, 170.39, 164.87, 160.29, 158.42, 145.58, 131.78, 129.70, 120.50, 110.81, 56.69,

51.76, 51.58, 49.92, 31.86, 25.77, 22.56, 12.71, 11.09.HRMS(ESI): calcd. for $C_{19}H_{23}N_4O_4S^+$ $[M+H]^+$,403.1435; found 403.1435.

Methyl 6-(2-(3,5-dimethylisoxazol-4-yl)-4-oxo-3,5,6,8-tetrahydropyrido[4',3':4,5]thieno[2,3-d]pyrimidin-7(4H)-yl)hexanoate(9d).

Compound 9d was synthesized from Compound 8 and 6-bromohexanoate, in a manner similar to 9b, Yield 43.2 %, yellow solid. mp: 121.5 – 123.8℃.^{1}H NMR (400 MHz, Chloroform-d) δ 11.99 (s, 1H, Pyrimidine-**H**), 3.73 (s, 2H, N-**CH_2**-Thiophene), 3.68 (s, 3H, O**Me**), 3.05 (t, J = 5.8 Hz, 2H, N-**CH_2**-CH_2-Thiophene), 2.86 (t, J = 5.8 Hz, 2H, N-CH_2-**CH_2**-Thiophene), 2.67 (s, 3H, Isoxazole-**CH_3**), 2.61 (t, J = 7.6 Hz, 2H, MeCOO**CH_2**), 2.49 (s, 3H, Isoxazole-**CH_3**), 2.35 (t, J = 7.6 Hz, 2H, N-**CH_2**-CH_2-CH_2), 1.73 – 1.61 (m, 4H, N-CH_2-**CH_2**-CH_2-**CH_2**), 1.44 – 1.37 (m, 2H, N-CH_2-CH_2-**CH_2**).^{13}C NMR (101 MHz, Chloroform-d) δ 174.10, 170.42, 165.00, 160.39, 158.46, 158.45, 145.73,129.61, 120.44, 110.83, 57.40, 51.81, 51.52, 49.88, 33.97, 29.70, 26.98, 26.85, 24.81, 12.70, 11.07. HRMS(ESI): calcd. for $C_{21}H_{27}N_4O_4S^+$ $[M+H]^+$,431.1748; found 431.1748.

General Procedures of Method for the Synthesis of 16a-c and 17a-f.

Methyl 3-((4-cyano-2,6-dimethylphenoxy)methyl)benzoate (12a). Compound (12a) was synthesized from 4-hydroxy-3,5-dimethylbenzonitrile (10) and methyl 3-(bromomethyl) benzoate (11a), in a manner similar to (12b). Yield 40%, white powder.mp: 68.6-69.7℃。^{1}H NMR (400 MHz, DMSO-d_6) δ 8.09 (t, J = 1.6 Hz, 1H, Ar-**H**), 7.97 (dt, J = 7.8, 1.6 Hz, 1H, Ar-**H**), 7.78 (dt, J = 7.8, 1.6 Hz, 1H, Ar-**H**), 7.59 (t, J = 7.6 Hz, 1H, Ar-**H**), 7.58 (s, 2H, Ar-**H**), 4.98 (s, 2H, **CH_2**), 3.88 (s, 3H, O**CH_3**), 2.27 (s, 6H, 2Ar-**CH_3**).^{13}C NMR (100 MHz, DMSO-d_6) δ 166.50 , 159.68 , 138.20 , 133.22(5C) , 130.29 , 129.50 , 129.32 , 128.91 , 119.31 , 107.04 , 73.29 , 52.71 , 16.35 . HRMS(ESI): calcd. for $C_{18}H_{17}NNaO_3^+$ $[M+Na]^+$, 318.1101; found 318.1104.

Methyl 4-((4-cyano-2,6-dimethylphenoxy) methyl)benzoate (12b). Compound (12b) was synthesized from4-hydroxy-3,5-dimethylbenzonitrile (10) and methyl 4-(bromomethyl) benzoate (11b), in a manner similar to (12d), but the crude product was recrystallized by a mixture solvent of ethyl acetate and ethanol (50ml, v/v=1:1). Yield 53%, white crystal. mp: 118.4-120.3℃. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.02 (d, J = 8.4 Hz, 2H, Ar-**H**), 7.64 (d, J = 8.0 Hz, 2H, Ar-**H**), 7.58 (s, 2H, Ar-**H**), 4.98 (s, 2H, **CH_2**), 3.88 (s, 3H, O**CH_3**), 2.27 (s, 6H, 2Ar-**CH_3**).^{13}C NMR (100 MHz, DMSO-d_6) δ 166.44 , 159.65 , 142.76 , 133.21(4C) , 129.80 , 129.69 , 128.35 , 119.28, 107.09 , 73.23 , 52.62 , 16.32 .HRMS(ESI): calcd. for $C_{18}H_{17}NNaO_3^+$ $[M+Na]^+$, 318.1101; found 318.1107.

Methyl 6-(4-cyano-2,6-dimethylphenoxy) hexanoate (17c). Compound (**17c**) was synthesized from4-hydroxy-3,5-dimethylbenzonitrile (10) and methyl 6-bromohexanoate (11c), in a manner similar to (12d). Yield 68%, light yellow oil. ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.53 (s, 2H, Ar-**H**), 3.79 (t, J = 6.4 Hz, 2H, O**CH_2**CH_2), 3.59 (s, 3H, O**CH_3**), 2.34 (t, J = 7.3 Hz, 2H, **CH_2**$COOCH_3$), 2.24 (s, 6H, 2Ar-**CH_3**), 1.74 (p, J = 6.6 Hz, 2H, OCH_2**CH_2**), 1.61 (p, J= 7.2 Hz, 2H, C=OCH_2**CH_2**), 1.52-1.42 (m, 2H, OCH_2CH_2**CH_2**).

Methyl 4-(4-cyano-2,6-dimethylphenoxy) butanoate(12d). 4-hydroxy-3,5-dimethyl benzonitrile (10) (1.47g, 10mmol), methyl 4-bromobutanoate(11d) (2.17g, 12mmol) were dissolved in THF(100ml), and then DBU (3.04g, 20mmol) was added into the mixed solution; The mixture was refluxed for 24 h. The reaction mixture was concentrated and the residue was partitioned between water and ethyl acetate. The organic layer was separated, and concentrated, and the crude product was purified by silica gel column chromatography using a mixture solvent of petroleum ether: ethylacetate (12:1), to give 12d. Yield 70%, light yellow oil. ^{1}H NMR (400 MHz,

DMSO-d_6) δ 7.53 (s, 2H, Ar-**H**), 3.82 (t, J = 6.4 Hz, 2H, O**C**$\mathbf{H_2}$), 3.62 (s, 3H, O**C**$\mathbf{H_3}$), 2.55 (t, J = 7.4 Hz, 2H, **C**$\mathbf{H_2}$COOCH$_3$), 2.23 (s, 6H, 2Ar-**C**$\mathbf{H_3}$), 2.01 (p, J = 6.8 Hz, 2H, OCH$_2$**C**$\mathbf{H_2}$).

Methyl (E)-3-(4-((4-cyano-2,6-dimethylphenoxy) methyl)phenyl)acrylate (12e). Compound (12e) was synthesized from4-hydroxy-3,5-dimethylbenzonitrile (10) and methyl (E)-3-(4-(bromomethyl) phenyl)acrylate (11e), in a manner similar to (12b). Yield 47%, white powder.mp: 152.3-153.8℃. ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.77 (d, J = 8.2 Hz, 2H, Ar-**H**), 7.69 (d, J = 16.0 Hz, 1H, **C**H=CH-C=O), 7.58 (s, 2H, Ar-**H**), 7.53 (d, J = 8.0 Hz, 2H, Ar-**H**), 6.68 (d, J = 16.0 Hz, 1H, CH=**C**H-C=O), 4.92 (s, 2H, **C**$\mathbf{H_2}$), 3.74 (s, 3H, O**C**$\mathbf{H_3}$), 2.27 (s, 6H, 2Ar-**C**$\mathbf{H_3}$).^{13}C NMR (100 MHz, DMSO-d_6) δ 167.10, 159.77 , 144.54 , 139.75 , 134.28 , 133.23 , 133.20 , 128.95 , 128.91 , 119.32, 118.53 , 106.97 , 73.55 , 51.97 , 16.38. HRMS (ESI): calcd. for $C_{20}H_{19}NNaO_3^+$ $[M+Na]^+$, 344.1257; found 344.1261.

Methyl (E)-3-(4-((2-(4-cyano-2,6-dimethylphenoxy)ethoxy)methyl)phenyl) acrylate (12f). Compound (12f) was synthesized from4-hydroxy-3,5-dimethylbenzonitrile (10) andmethyl (E)-3-(4-((2-bromoethoxy) methyl) phenyl)acrylate (11f), in a manner similar to (12b). Yield 42%, white powder. mp: 75.1 –77.8℃. ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.71 (d, J = 8.0 Hz, 2H, Ar-**H**), 7.66 (d, J = 16.0 Hz, 1H, Ar-**C**H=CH), 7.53 (s, 2H, Ar-**H**), 7.38 (d, J = 7.9 Hz, 2H, Ar-**H**), 6.64 (d, J = 16.0 Hz, 1H, Ar-CH=**C**H), 4.60 (s, 2H, O**C**$\mathbf{H_2}$Ar$_)$, 4.07-3.99 (m, 2H, ArO**C**$\mathbf{H_2}$CH$_2$), 3.81-3.74 (m, 2H, ArOCH$_2$**C**$\mathbf{H_2}$), 3.73 (s, 3H, O**C**$\mathbf{H_3}$), 2.26 (s, 6H, 2Ar-**C**$\mathbf{H_3}$).^{13}C NMR (100 MHz, DMSO-d_6) δ 167.13 , 160.03 , 144.70 , 141.42 , 133.60 , 133.08 , 132.99 , 128.79 , 128.13 , 119.36, 118.06 , 106.62 , 72.09 , 72.07 , 69.71 , 51.91 , 16.22. HRMS(ESI): calcd. for $C_{22}H_{23}NNaO_4^+$ $[M+Na]^+$, 388.1519; found 388.1522.

3-((4-Cyano-2,6-dimethylphenoxy) methyl)benzoicacid (13a). Compound (13a)

was synthesized from(12a), in a manner similar to (13b). Yield 72%, white powder. mp: 162.6-164.2℃. ^{1}H NMR (400 MHz, DMSO-d_6) δ 13.07 (s, 1H, **COOH**), 8.07 (t, J = 1.6 Hz, 1H, Ar-**H**), 7.95 (dt, J = 7.8, 1.4 Hz, 1H, Ar-**H**), 7.75 (dt, J = 7.8, 1.4 Hz, 1H, Ar-**H**), 7.58(s, 2H, Ar-**H**), 7.56(t, J = 7.8 Hz, 1H, Ar-**H**), 4.96 (s, 2H, **CH_2**), 2.28 (s, 6H, 2Ar-**CH_3**).^{13}C NMR (100 MHz, DMSO-d_6) δ 167.57, 159.70, 137.93, 133.24, 133.21 , 132.85 , 131.45 , 129.48 , 129.29 , 129.20 , 119.32 , 107.01 , 73.43 , 16.35. HRMS(ESI): calcd. for $C_{17}H_{15}NNaO_3^+$ $[M+Na]^+$, 304.0944; found 304.0949.

4-((4-Cyano-2,6-dimethylphenoxy) methyl)benzoic acid (13b). To a solution of 12b (1.48g, 5mmol) in CH_3OH (30ml) and 1.4-dioxane (30ml) was added 1M NaOH(30ml), the reaction mixture was stirred for 30 min at room temperature. At the end of the reaction, the resulting mixture was acidized by hydrochloric acid to pH=3, the white precipitate was filtered, wash with CH_3OH(10ml) to give **(13b)**. Yield 77%, white powder. mp: 208.4-209.3℃. ^{1}H NMR (400 MHz, DMSO-d_6) δ 13.00 (s, 1H, COOH), 8.00 (d, J = 8.2 Hz, 2H, Ar-**H**), 7.62 (d, J = 8.2 Hz, 2H, Ar-**H**), 7.58 (s, 2H, Ar-**H**), 4.97 (s, 2H, **CH_2**), 2.27 (s, 6H, 2Ar-**CH_3**).^{13}C NMR (100 MHz, DMSO-d_6) δ 167.53, 159.71, 142.27, 133.23(4C), 130.88, 129.95, 128.27, 119.31, 107.05, 73.34, 16.34. HRMS(ESI): calcd. for $C_{17}H_{16}NO_3^+$ $[M+H]^+$, 282.1125; found 282.1132.

6-(4-Cyano-2,6-dimethylphenoxy) hexanoic acid (13c). Compound (13c) was synthesized from(**17c**), in a manner similar to (13d). Yield 80%, white powder. ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.00 (s, 1H, **COOH**), 7.53 (s, 2H, Ar-**H**), 3.79 (t, J = 6.4 Hz, 2H, O**CH_2**CH_2), 2.24 (s, 6H, 2Ar-**CH_3**), 2.24 (t, J = 7.2 Hz, 2H, HOOC**CH_2**), 1.74 (p, J = 6.6 Hz, 2H, OCH_2**CH_2**), 1.58 (p, J = 7.2 Hz, 2H, C=OCH_2**CH_2**), 1.52-1.43 (m, 2H, OCH_2CH_2**CH_2**).

4-(4-Cyano-2,6-dimethylphenoxy)butanoic acid (13d). To a solution of 12d (1.24g, 5mmol) in CH_3OH(30ml) was added 1M NaOH (30ml), the reaction mixture was

stirred for 30 min at room temperature. At the end of the reaction, the resulting mixture was acidized by hydrochloric acid to pH=3, the white precipitate was filtered, wash with CH_3OH to give **(13d)**. Yield 76%, white powder. ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.19 (brs, 1H, **COOH**), 7.54 (s, 2H, Ar-**H**), 3.81 (t, J = 6.4 Hz, 2H, O**CH$_2$**), 2.45 (t, J = 7.2 Hz, 2H, **CH$_2$**COOH), 2.24 (s, 6H, 2Ar-**CH$_3$**), 1.97 (p, J = 6.8 Hz, 2H, OCH$_2$**CH$_2$**).

(E)-3-(4-((4-cyano-2,6-dimethylphenoxy) methyl)phenyl)acrylic acid (13e). Compound 13e was synthesized from 12e, in a manner similar to 13c. Yield 70%, white powder.mp: 201.3-203.4℃^{1}H NMR (400 MHz, DMSO-d_6) δ 12.43 (s, 1H, **COOH**), 7.74 (d, J = 8.2 Hz, 2H, Ar-**H**), 7.62 (d, J = 16.0 Hz, 1H, **CH**=CH-C=O), 7.58 (s, 2H, Ar-**H**), 7.53 (d, J = 8.0 Hz, 2H, Ar-**H**), 6.56 (d, J = 16.0 Hz, 1H, CH=**CH**-C=O), 4.91 (s, 2H, **CH$_2$**), 2.27 (s, 6H, 2Ar-**CH$_3$**). ^{13}C NMR (100 MHz, DMSO-d_6) δ 167.99 , 159.78 , 143.91 , 139.45 , 134.52 , 133.24 , 133.20 , 128.93 , 128.78 , 119.95, 119.33 , 106.96 , 73.59 , 16.39 .HRMS(ESI): calcd. for $C_{19}H_{17}NNaO_3^+$ $[M+Na]^+$, 330.1101; found 330.1108.

(E)-3-(4-((2-(4-cyano-2,6-dimethylphenoxy) ethoxy)methyl)phenyl)acrylic acid (13f). Compound 13f was synthesized from 12f, in a manner similar to 13c. Yield 68%, white powder. mp:183.2-185.4℃. ^{1}H NMR (400 MHz, DMSO-d_6) δ7.62-7.53 (m, 4H, Ar-**H**), 7.43 (d, J = 15.8 Hz, 1H, Ar-**CH**=CH), 7.36 (d, J = 7.8 Hz, 2H, Ar-**H**), 6.48 (d, J = 15.8 Hz, 1H, Ar-CH=**CH**), 4.60 (s, 2H, O**CH$_2$**Ar), 4.07-4.01 (m, 2H, ArO**CH$_2$**CH$_2$), 3.83-3.76 (m, 2H, ArOCH$_2$**CH$_2$**), 2.25 (s, 6H, 2Ar-**CH$_3$**).^{13}C NMR (100 MHz, DMSO-d_6) δ 163.25, 161.01 , 140.45 , 138.25 , 135.01 , 133.05 , 132.81 , 128.50 , 127.76 , 119.57 , 119.28 , 106.79 , 72.10 , 72.20 , 69.45 , 16.25 .HRMS(ESI): calcd. for $C_{21}H_{21}NNaO_4^+$ $[M+Na]^+$, 374.1363; found 374.1369.

N-(2-aminophenyl)-3-((4-cyano-2,6-dimethylphenoxy) methyl)benzamide (14a). Compound 14a was synthesized from 8a and benzene-1,2-diamine, in a manner similar

to 14b. Yield 65%, white powder.mp: 150.9-152.3℃^{1}H NMR (400 MHz, DMSO-d_6) δ 9.74 (s, 1H, **NH**), 8.11(s, 1H, Ar-**H**), 8.01 (d, J = 7.8 Hz, 1H, Ar-**H**), 7.70 (d, J = 7.6 Hz, 1H, Ar-**H**), 7.59(s, 2H, Ar-**H**), 7.58 (t, J = 7.6Hz, 1H, Ar-**H**), 7.18 (d, J = 7.8 Hz, 1H, Ar-**H**), 6.99 (td, J = 7.6, 1.5 Hz, 1H, Ar-**H**), 6.80 (dd, J = 8.0, 1.3 Hz, 1H, Ar-**H**), 6.61 (td, J = 7.6, 1.4 Hz, 1H, Ar-**H**), 4.97 (s, 2H, **CH_2**), 4.91 (s, 2H, **NH_2**), 2.31 (s, 6H, 2Ar-**CH_3**).^{13}C NMR (100 MHz, DMSO-d_6) δ 165.56 , 159.79, 143.68 , 137.63 , 135.34 , 133.28 , 133.23(4C) , 131.39 , 128.94 , 127.95 , 127.23 , 127.03 , 123.70 , 119.34, 116.73 , 116.60 , 107.02 , 73.70 , 16.42 .HRMS(ESI): calcd. for $C_{23}H_{21}N_3NaO_2^+$ $[M+Na]^+$, 394.1526; found 394.1532.

N-(2-aminophenyl)-4-((4-cyano-2,6-dimethylphenoxy)methyl)benzamide (14b). To a solution of 13b (281mg, 1mmol) and benzene-1,2-diamine (432mg, 4mmol) in anhydrous CH_2Cl_2(15ml) was added EDCI(192mg, 1mmol), HOBt(135mg, 1mmol), the reaction mixture was stirred for 24h at room temperature. The reaction mixture was partitioned between water and dichloromethane, the organic layer was separated, and concentrated, and the crude product was purified by silica gel column chromatography using a mixture solvent of petroleum ether: ethylacetate (5:1), to give 14b. Yield 67%, white powder.mp: 194.3-195.5℃^{1}H NMR (400 MHz, DMSO-d_6) δ 9.69 (brs, 1H, **NH**), 8.03 (d, J = 7.8 Hz, 2H, Ar-**H**), 7.62 (d, J = 8.2 Hz, 2H, Ar-**H**), 7.59 (s, 2H, Ar-**H**), 7.18 (dd, J = 7.8, 1.6 Hz, 1H, Ar-**H**), 6.79 (dd, J = 7.8, 1.6 Hz, 1H, Ar-**H**), 6.61 (td, J = 7.2, 1.4 Hz, 1H, Ar-**H**), 4.98 (s, 2H, **CH_2**), 4.91 (brs, 2H, **NH2**), 2.29 (s, 6H,2Ar-**CH_3**).^{13}C NMR (100 MHz, DMSO-d_6) δ 165.47 , 159.82 , 143.64 , 140.69 , 134.80 , 133.23(4C) , 128.44 , 128.18 , 127.19 , 126.99 , 123.71 ,119.34, 116.71 , 116.59 , 106.99 , 73.53 , 16.43 .HRMS(ESI): calcd. for $C_{23}H_{22}N_3O_2^+$ $[M+H]^+$, 372.1707; found 372.1711.

N-(2-Aminophenyl)-6-(4-cyano-2,6-dimethylphenoxy)hexanamide (14c). Compound 14c was synthesized from 13c and benzene-1,2-diamine, in a manner

similar to 14b. Yield 75%, white powder.mp:111.2-112.5℃. ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.10 (s, 1H, **NH**), 7.53 (s, 2H, Ar-**H**), 7.15 (dd, J = 7.9, 1.6 Hz, 1H, Ar-**H**), 6.89 (td, J = 7.6, 1.6 Hz, 1H, Ar-**H**), 6.71 (dd, J = 8.0, 1.4 Hz, 1H, Ar-**H**), 6.53 (td, J = 7.6, 1.4 Hz, 1H, Ar-**H**), 4.81 (s, 2H, **NH$_2$**), 3.81 (t, J = 6.4 Hz, 2H, ArO**CH$_2$**), 2.36 (t, J = 7.2 Hz, 2H, C=O-**CH$_2$**), 2.25 (s, 6H, 2Ar-**CH$_3$**), 1.78 (p, J = 6.5 Hz, 2H, ArOCH$_2$**CH$_2$**), 1.68 (p, J = 7.4 Hz, 2H, C=O-CH$_2$**CH2**), 1.57-1.48 (m, 2H, ArOCH$_2$CH$_2$**CH$_2$**).^{13}C NMR (100 MHz, DMSO-d_6) δ 171.50 , 160.23 , 142.35 , 133.12 , 132.98, 126.15 , 125.73 , 124.01 , 119.39, 116.61 , 116.33 , 106.52 , 72.45 , 36.15 , 30.11 , 25.67 , 25.59 , 16.22 .HRMS(ESI): calcd. for $C_{21}H_{26}N_3O_2^+$ $[M+H]^+$, 352.2020; found 352.2023.

3-((4-Cyano-2,6-dimethylphenoxy)methyl)-N-hydroxybenzamide (15a)

Compound 15a was synthesized from 13a and hydroxylamine hydrochloride, in a manner similar to 15d. Yield 56%, off-white powder. mp: 171.0-173.2℃. ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.29 (s, 1H, OH), 9.07 (s, 1H, **NH**), 7.90 (t, J = 1.7 Hz, 1H, Ar-**H**), 7.75 (dt, J = 7.6, 1.4 Hz, 1H, Ar-**H**), 7.64 (dt, J = 7.6, 1.4 Hz, 1H, Ar-**H**), 7.59 (s, 2H, Ar-**H**), 7.50 (t, J = 7.6 Hz, 1H, Ar-**H**), 4.92 (s, 2H, **CH$_2$**), 2.28 (s, 6H, 2Ar-**CH$_3$**).^{13}C NMR (100 MHz, DMSO-d_6) δ 164.40 , 159.72 , 137.69 , 133.47 , 133.27 , 133.22 , 131.22 , 129.02 , 127.17 , 126.97 , 119.33 , 106.99 , 73.64 , 16.37 .HRMS(ESI): calcd. for $C_{17}H_{16}N_2NaO_3^+$ $[M+Na]^+$, 319.1053; found 319.1061.

4-((4-Cyano-2,6-dimethylphenoxy)methyl)-N-hydroxybenzamide (15b)

Compound 15b was synthesized from 13b and hydroxylamine hydrochloride, in a manner similar to 15d. Yield 58%, off-white powder.mp: 191.2-193.1℃. ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.26 (s, 1H, **OH**), 9.06 (s, 1H, **NH**), 7.80 (d, J = 8.2 Hz, 2H, Ar-**H**), 7.60-7.54 (m, 4H, Ar-**H**)), 4.93 (s, 2H, CH_2), 2.27 (s, 6H, 2Ar-**CH$_3$**).^{13}C NMR (100 MHz, DMSO-d_6) δ 164.38 , 159.75 , 140.43 , 133.23 , 133.21 , 132.96 , 128.28 ,

127.53 , 119.32 , 107.00 , 73.45 , 16.37 .HRMS(ESI): calcd. for $C_{17}H_{16}N_2NaO_3^+$ $[M+Na]^+$, 319.1053; found 319.1058.

6-(4-Cyano-2,6-dimethylphenoxy)-N-hydroxyhexanamide (15c). Compound 15c was synthesized from 13c and hydroxylamine hydrochloride, in a manner similar to 15d. Yield 63%, off-white powder. mp: 93.3-95.2℃. ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.36 (brs, 1H, **OH**), 8.67 (brs, 1H, **NH**), 7.53 (Ar-**H**), 3.78 (t, J = 6.4 Hz, 2H, O**CH$_2$**CH$_2$), 2.24 (s, 6H, 2Ar-**CH$_3$**), 1.99 (t, J = 7.2 Hz, 2H, C=O-**CH$_2$**), 1.73 (p, J = 6.6 Hz, 2H, OCH$_2$**CH$_2$**), 1.57 (p, J = 7.2 Hz, 2H, C=OCH$_2$**CH$_2$**), 1.39-1.49 (m, 2H, OCH$_2$CH$_2$**CH$_2$**).^{13}C NMR (100 MHz, DMSO-d_6) δ 169.45 , 160.20 , 133.11 , 132.96 , 119.38 , 106.52 , 72.38 , 32.65 , 30.01 , 25.55 , 25.40 , 16.20 .HRMS(ESI): calcd. for $C_{15}H_{20}N_2NaO_3^+$ $[M+Na]^+$, 299.1366; found 299.1371.

4-(4-Cyano-2,6-dimethylphenoxy)-N-hydroxybutanamide (15d). Hydroxylamine hydrochloride (345mg, 5mmol) was added to a stirred solution of sodium methoxide (270mg,5mmol) in methanol (10 mL). The mixture was stirred for 30 min at the same temperature and the precipitated sodium chloride was filtered and the filtrate was used as such. Meanwhile, To a solution of 13d (233mg, 1mmol) in 1.4-dioxane (10mL) ethyl chloroformate (130mg,1.2mmol) and N-methylmorpholine(130mg, 1.3mmol) were added and the mixture was stirred for 15 min. The solid was filtered and the filtrate was added to above prepared hydroxylamine (165mg, 5 mmol) in methanol. The reaction mixture was stirred at room temperature for 20 min. The solvent was evaporated and the residue was washed by ether, and purified by silica gel column chromatography using a mixture solvent of dichloromethane: acetone (5:1) to give **15d** Yield 60%, off-white powder. mp: 137.9-138.6℃. ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.44 (brs, 1H, **OH**), 8.75 (brs, 1H, **NH**), 7.53 (s, 2H, Ar-**H**), 3.80 (t, J = 6.4 Hz, 2H,O-**CH$_2$**), 2.24 (s, 6H, 2Ar-**CH$_3$**), 2.20 (t, J = 7.6 Hz, 2H,C=O-**CH$_2$**), 1.97 (p, J = 6.8 Hz,

2H, CH_2-**CH_2**-CH_2).^{13}C NMR (100MHz, DMSO-d_6) δ 169.02, 160.04, 133.13 , 132.98 , 119.35, 106.63, 71.81, 29.12, 26.38, 16.21 .HRMS(ESI): calcd. for $C_{13}H_{16}N_2NaO_3^+$ $[M+Na]^+$, 271.1053; found 271.1060.

(E)-3-(4-((4-cyano-2,6-dimethylphenoxy)methyl)phenyl)-N-hydroxyacrylamide (15e). Compound 15e was synthesized from 13e and hydroxylamine hydrochloride, in a manner similar to 15d Yield 52%, off-white powder. mp: 186.2-187.1℃. ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.80 (s, 1H, **OH**), 9.08 (s, 1H, **NH**), 7.67-7.43 (m, 7H, Ar-**H,** Ar-**CH**=CH), 6.51 (d, J = 15.8 Hz, 1H, Ar-CH=**CH**), 4.90 (s, 2H, **CH_2**), 2.27 (s, 6H, 2Ar-**CH_3**).^{13}C NMR (100 MHz, DMSO-d_6) δ 163.14 , 159.78 , 138.62 , 138.36 , 135.14 , 133.24 , 133.19 , 129.06 , 128.05 , 119.85 , 119.33 , 106.94 , 73.66 , 16.39 .HRMS(ESI): calcd. for $C_{19}H_{18}N_2NaO_3^+$ $[M+Na]^+$, 345.1210; found 345.1213.

(E)-3-(4-((2-(4-cyano-2,6-dimethylphenoxy)ethoxy)methyl)phenyl)-N-hydroxyacrylamide (15f) Compound 15f was synthesized from 13f and hydroxylamine hydrochloride, in a manner similar to 15d. Yield 49%, brown oil. ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.75 (s, 1H, **OH**), 9.04 (s, 1H, **NH**), 7.60-7.51 (m, 4H, Ar-**H**), 7.45 (d, J = 15.8 Hz, 1H, Ar-**CH**=CH), 7.37 (d, J = 7.8 Hz, 2H, Ar-**H**), 6.46 (d, J = 15.8 Hz, 1H, Ar-CH=**CH**), 4.59 (s, 2H, O**CH_2**Ar), 4.05-4.00 (m, 2H, ArO**CH_2**CH_2), 3.80-3.74 (m, 2H, ArOCH_2**CH_2**), 2.26 (s, 6H, 2Ar-**CH_3**).^{13}C NMR (100 MHz, DMSO-d_6) δ 163.20, 160.07 , 140.25 , 138.50 , 134.45 , 133.10 , 133.01 , 128.30 , 127.90 , 119.39 , 119.38 , 106.59 , 72.16 , 72.09 , 69.67 , 16.24 .HRMS(ESI): calcd. for $C_{21}H_{22}N_2NaO_4^+$ $[M+Na]^+$, 389.1472; found 389.1478.

N-(2-aminophenyl)-3-((2,6-dimethyl-4-(7-methyl-4-oxo-3,4,5,6,7,8-hexahydro pyrido[4',3':4,5]thieno[2,3-d]pyrimidin-2-yl)phenoxy)methyl)benzamide (16a). Compound 16a was synthesized from 14a and 2c, in a manner similar to 4a. Yield 35%, yellow powder. mp:243.7-245.8℃. ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.39 (brs, 1H,

Pyrimidine-**H**), 9.80 (s, 1H, **CONH**), 8.15 (s, 1H, Ar-**H**), 8.02 (d, J = 7.6 Hz, 1H, Ar-**H**), 7.92 (s, 2H, Ar-**H**), 7.72 (d, J = 7.6 Hz, 1H, Ar-**H**), 7.57 (t, J = 7.6 Hz, 1H, Ar-**H**), 7.19 (d, J = 7.8 Hz, 1H, Ar-**H**), 6.98 (t, J = 7.6 Hz, 1H, Ar-**H**), 6.80 (d, J = 8.0 Hz, 1H, Ar-**H**), 6.61 (t, J = 7.6 Hz, 1H, Ar-**H**), 4.95 (s, 4H, O-**CH_2**, **NH_2**), 3.56 (s, 2H, N-**CH_2**-Thiophene), 2.97 (t, J = 5.6 Hz, 2H, N-**CH_2**-CH_2), 2.67 (t, J = 5.6 Hz, 2H, N-CH_2-**CH_2**), 2.38 (s, 3H, N-**CH_3**), 2.35 (s, 6H, 2Ar-**CH_3**).^{13}C NMR (100 MHz, DMSO-d_6) δ 165.61, 164.26, 160.45, 158.33, 153.33, 143.66, 137.97, 135.27, 131.45, 131.28, 129.40, 129.30, 128.88, 128.81 , 128.58, 127.93, 127.89, 127.24, 126.93, 123.82, 120.46, 116.68 , 116.65, 73.60, 53.56, 51.79, 45.56 , 26.33 , 16.77 .HRMS(ESI): calcd. for $C_{32}H_{32}N_5O_3S^+$ $[M+H]^+$, 566.2220; found 566.2224.

N-(2-aminophenyl)-4-((2,6-dimethyl-4-(7-methyl-4-oxo-3,4,5,6,7,8-hexahydro pyrido[4',3':4,5]thieno[2,3-d]pyrimidin-2-yl)phenoxy)methyl)benzamide (16b). Compound (16b) was synthesized from 14b and 2c, in a manner similar to 4a. Yield 38%, yellow powder. mp:239.8-241.8℃. ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.38 (brs, 1H, Pyrimidine-**H**), 9.71 (s, 1H, **CONH**), 8.04 (s, 2H, Ar-**H**), 7.91 (s, 2H, Ar-**H**),7.64 (s, 2H, Ar-**H**), 7.19 (s, 1H, Ar-**H**), 6.98 (s, 1H, Ar-**H**), 6.80 (s, 1H, Ar-**H**), 6.61 (s, 1H, Ar-**H**), 4.97 (s, 2H, O-**CH_2**), 4.92 (s, 2H, **NH_2**), 3.56 (s, 2H, N-**CH_2**-Thiophene), 2.97 (s, 2H, N-**CH_2**-CH_2), 2.67 (s, 2H, N-CH_2-**CH_2**), 2.38 (s, 3H, N-**CH_3**), 2.33(s, 6H, 2Ar-**CH_3**).^{13}C NMR (100 MHz, DMSO-d_6) δ 165.50, 164.01, 159.23 , 158.57 , 152.52 , 143.64 , 141.05, 134.69, 131.60 , 130.16 , 129.37 , 128.81 , 128.42 , 128.09 , 127.72, 127.19 , 126.97 , 123.75 , 120.62 , 116.71 , 116.59 , 73.44, 53.50, 51.73 , 45.53 , 26.24 , 16.76. HRMS(ESI): calcd. for $C_{32}H_{32}N_5O_3S^+$ $[M+H]^+$, 566.2220; found 566.2224.

N-(2-aminophenyl)-6-(2,6-dimethyl-4-(7-methyl-4-oxo-3,4,5,6,7,8-hexahydro pyrido[4',3':4,5]thieno[2,3-d]pyrimidin-2-yl)phenoxy)hexanamide (16c). Compound 16c was synthesized from 14c and 2c, in a manner similar to 4a. Yield 37%,

yellow powder. mp:222.2-223.8℃. ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.34 (brs, 1H, Pyrimidine-**H**), 9.13 (s, 1H, **CONH**), 7.87 (s, 2H, Ar-**H**), 7.16 (dd, J = 8.0, 1.6 Hz, 1H, Ar-**H**), 6.89 (td, J = 7.6, 1.6 Hz, 1H, Ar-**H**), 6.71 (dd, J = 8.0, 1.6 Hz, 1H, Ar-**H**), 6.54 (td, J = 7.6, 1.6 Hz, 1H, Ar-**H**), 4.82 (s, 2H, **NH_2**), 3.80 (t, J = 6.4 Hz, 2H, O-**CH_2**), 3.55 (s, 2H, N-**CH_2**-Thiophene), 2.95 (d, J = 5.6 Hz, 2H, N-**CH_2**-CH_2), 2.66 (t, J = 5.6 Hz, 2H, N-CH_2-**CH_2**), 2.39-2.34 (m, 5H, N-**CH_3**,**CH_2**CONH), 2.28 (s, 6H, 2Ar-**CH_3**), 1.80 (p, J = 6.8 Hz, 2H,OCH_2**CH_2**$CH_2CH_2CH_2$), 1.69 (p, J = 7.2 Hz, 2H, $OCH_2CH_2CH_2$**CH_2**CH_2), 1.54 (m, 1.60 –1.50, 2H, OCH_2CH_2**CH_2**CH_2CH_2).^{13}C NMR (100 MHz, CF_3COOD) δ 178.18, 163.31 , 156.55 , 150.17 , 149.69 , 135.10 , 129.59 , 129.27 , 128.99 ,128.86, 128.31, 126.98 , 126.52 , 126.35 , 118.03, 114.38 , 111.56 , 73.12 , 51.71 , 51.56, 42.75 , 35.50 , 29.00 , 25.40 , 24.95 , 21.93 , 14.82 .HRMS(ESI): calcd. for $C_{30}H_{36}N_5O_3S^+$ $[M+Na]^+$, 546.2533; found 546.2540.

3-((2,6-Dimethyl-4-(7-methyl-4-oxo-3,4,5,6,7,8-hexahydropyrido[4',3':4,5] thieno[2,3-d]pyrimidin-2-yl)phenoxy)methyl)-N-hydroxybenzamide (17a). Compound 17a was synthesized from 15a and 2c, in a manner similar to 4a. Yield 41%, yellow powder. mp: 184.1-186.1℃. ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.39 (brs, 1H, Pyrimidine-**H**), 11.29 (brs, 1H, **OH**), 9.07 (brs, 1H, C=O**NH**), 7.93 (s, 1H, Ar-**H**), 7.90 (s, 2H, Ar-**H**), 7.75 (d, J = 7.5 Hz, 1H, Ar-**H**), 7.65 (d, J = 7.4 Hz, 1H, Ar-**H**), 7.51 (t, J = 7.6 Hz, 1H, Ar-**H**), 4.91 (s, 2H, O**CH_2**), 3.58 (s, 2H, N-**CH_2**-Thiophene), 2.96 (t, J = 5.7 Hz, 2H, N-**CH_2**-CH_2), 2.68 (t, J = 5.7 Hz, 2H, N-CH_2-**CH_2**), 2.39 (s, 3H, N-**CH_3**), 2.32 (s, 6H, 2Ar-**CH_3**).^{13}C NMR (100 MHz, DMSO-d_6) δ 164.02 , 159.19 , 158.50 , 152.52 , 138.03 , 133.46 , 132.99, 131.63 , 131.12 , 130.08 , 129.35 , 128.99 , 128.81 , 127.69, 127.07 , 126.86 , 120.60 , 73.57 , 53.46 , 51.70 , 45.48 , 26.20 , 16.72 .HRMS(ESI): calcd. for $C_{26}H_{27}N_4O_4S^+$ $[M+H]^+$, 491.1748; found 491.1752.

4-((2,6-Dimethyl-4-(7-methyl-4-oxo-3,4,5,6,7,8-hexahydropyrido

[4',3':4,5]thieno[2,3-d]pyrimidin-2-yl)phenoxy)methyl)-N-hydroxybenzamide (17b). Compound 17b was synthesized from 15b and 2c, in a manner similar to 4a. Yield 43%, yellow powder. mp: 213.0-215.3℃. ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.93 (s, 2H, Ar-**H**), 7.81 (s, 2H, Ar-**H**), 7.58 (s, 2H, Ar-**H**), 4.90 (s, 2H, O**C**$\mathbf{H_2}$), 3.53 (s, 2H, N-**C**$\mathbf{H_2}$-Thiophene), 2.97 (s, 2H, N-**C**$\mathbf{H_2}$-CH_2), 2.66 (s, 2H, N-CH_2-**C**$\mathbf{H_2}$), 2.37 (s, 3H, N-**C**$\mathbf{H_3}$), 2.30 (s, 6H, 2Ar-**C**$\mathbf{H_3}$).^{13}C NMR (100 MHz, DMSO-d_6) δ 164.58, 131.22 , 131.09 , 129.27 , 128.80 , 128.76 , 128.16 , 120.31 , 73.37 , 53.68 , 51.95 , 45.63 , 16.75. HRMS(ESI): calcd. for $C_{26}H_{27}N_4O_4S^+$ $[M+H]^+$, 491.1748; found 491.1750.

6-(2,6-Dimethyl-4-(7-methyl-4-oxo-3,4,5,6,7,8-hexahydropyrido [4',3':4,5]thieno[2,3-d]pyrimidin-2-yl)phenoxy)-N-hydroxyhexanamide (17c). Compound **17c** was synthesized from 15c and 2c, in a manner similar to 4a. Yield 38%, yellow powder. mp: 173.6-176.8℃. ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.42 (brs, 1H, **OH**), 8.75 (s, 1H, **NH**), 7.90 (s, 2H, Ar-**H**), 3.75 (t, J = 6.4 Hz, 2H, ArO**C**$\mathbf{H_2}$), 3.51 (s, 2H, N-**C**$\mathbf{H_2}$-Thiophene), 2.96 (t, J = 5.8 Hz, 2H, N-**C**$\mathbf{H_2}$-CH_2), 2.64 (t, J = 5.8 Hz, 2H, N-CH_2-**C**$\mathbf{H_2}$), 2.36 (s, 3H, N-**C**$\mathbf{H_3}$), 2.27 (s, 6H, 2Ar-**C**$\mathbf{H_3}$), 2.03-1.96(m, 2H, **C**$\mathbf{H_2}$C=O), 1.74 (p, J = 7.2 Hz, 2H, ArOCH$_2$**C**$\mathbf{H_2}$), 1.58 (p, J = 7.2 Hz, 2H, **C**$\mathbf{H_2}$CH$_2$C=O), 1.51-1.40 (m, 2H, ArOCH$_2$**C**$\mathbf{H_2}$CH$_2$).^{13}C NMR (100 MHz, DMSO-d_6) δ 169.45, 165.12 , 157.84, 130.51, 129.11 , 128.81 , 128.70 , 128.66, 126.49, 119.87 , 72.10 , 53.79 , 52.10 , 45.68 , 32.69 , 30.12 , 26.67 , 25.69 , 25.48 , 16.59 .HRMS(ESI): calcd. for $C_{24}H_{31}N_4O_4S^+$ $[M+H]^+$, 471.2061; found 471.2057.

4-(2,6-Dimethyl-4-(7-methyl-4-oxo-3,4,5,6,7,8-hexahydropyrido [4',3':4,5] thieno[2,3-d]pyrimidin-2-yl)phenoxy)-N-hydroxybutanamide (17d). Compound 17d was synthesized from 15d and 2c, in a manner similar to 4a. Yield 35%, yellow powder.mp: 190.9-192.1℃^{1}H NMR (400 MHz, DMSO-d_6) δ 12.17(brs, Pyrimidine-**H**), 10.45 (s, 1H, **OH**), 7.85 (s, 2H, Ar-**H**), 3.79 (t, J = 6.3 Hz, 2H, O**C**$\mathbf{H_2}$), 3.55 (s, 2H,

N-**CH$_2$**-Thiophene), 2.95 (t, J = 5.7 Hz, 2H, N-**CH$_2$**-CH$_2$), 2.67 (t, J = 5.7 Hz, 2H, N-CH$_2$-**CH$_2$**), 2.37 (s, 3H, N-**CH$_3$**), 2.28 (s, 6H, 2Ar-**CH$_3$**), 2.21 (t, J = 7.4 Hz, 2H, **CH$_2$**C=O), 1.99 (q, J = 6.8 Hz, 2H, OCH$_2$**CH$_2$**).^{13}C NMR (100 MHz, DMSO-d$_6$) δ 169.06 , 164.03 , 159.22 , 158.77, 152.56 , 131.35, 130.05 , 129.33 , 128.72 , 127.37 , 120.54 , 71.65 , 53.48 , 51.71 , 45.51 , 29.22 , 26.47 , 26.23 , 16.57 .HRMS(ESI): calcd. for $C_{22}H_{27}N_4O_4S^+$ $[M+H]^+$, 443.1748; found 443.1750.

(E)-3-(4-((2,6-dimethyl-4-(7-methyl-4-oxo-3,4,5,6,7,8-hexahydro pyrido[4',3':4,5]thieno[2,3-d]pyrimidin-2-yl)phenoxy)methyl)phenyl)-N-hydroxy acrylamide 17e. Compound 17e was synthesized from 15e and 2c, in a manner similar to 4a. Yield 37%, yellow powder. mp >320℃, dec. ^{1}H NMR (400 MHz, DMSO-d$_6$) δ 8.03 (d, J = 8.7 Hz, 2H, Ar-**H**), 7.91 (s, 2H, Ar-**H**), 7.73 (d, J = 20.6 Hz, 2H, Ar-**H**), 5.18 (s, 2H, O**CH$_2$**Ar), 4.20-3.54 (m, 4H, Piperidline-**H**), 3.36 (d, J = 9.6 Hz, 2H, Piperidline-**H**), 2.56-2.36 (m, 9H, 2Ar-**CH$_3$,** N-**CH$_3$**). HRMS(ESI): calcd. for $C_{28}H_{29}N_4O_4S^+$ $[M+H]^+$, 517.1904; found 517.1912.

(E)-3-(4-((2-(2,6-dimethyl-4-(7-methyl-4-oxo-3,4,5,6,7,8-hexahydropyrido [4',3':4,5]thieno[2,3-d]pyrimidin-2-yl)phenoxy)ethoxy)methyl)phenyl)-N-hydroxy acrylamide (17f). Compound 17f was synthesized from 15f and 2c, in a manner similar to 4a. Yield 33%, yellow powder. mp>320℃, dec. ^{1}H NMR (400 MHz, DMSO-d$_6$) δ 7.90-7.74(m, 3H, Ar-**H,** Ar-**CH**=CH), 7.56 (s, 2H, Ar-**H**), 7.41 (s, 2H, Ar-**H**), 6.48 (s, 1H, Ar-CH=**CH**), 4.61 (s, 2H, O**CH$_2$**Ar), 4.01 (s, 2H, ArO**CH$_2$**CH$_2$), 3.78 (s, 2H, ArOCH$_2$**CH$_2$**), 3.56 (s, 2H, Piperidline-**H**), 2.96 (s, 2H, Piperidline-**H**), 2.67 (s, 2H, Piperidline-**H**), 2.42-2.22 (m, 9H, 2Ar-**CH$_3$,** N-**CH$_3$**). HRMS(ESI): calcd. for $C_{30}H_{33}N_4O_5S^+$ $[M+Na]^+$, 583.1986; found 583.1995.

Ethyl 2-amino-4,5,6,7-tetrahydrothieno[2,3-c]pyridine-3-carboxylate (18). ^{1}H NMR (400 MHz, DMSO-d$_6$) δ 7.22 (s, 2H, **NH$_2$**), 4.14 (q, J = 7.1 Hz, 2H, O**CH$_2$**CH$_3$),

3.56 (t, J = 2.0 Hz, 2H, N-**CH_2**-Thiophene), 2.85 (t, J = 5.8 Hz, 2H, N-**CH_2**-CH_2), 2.56 (d, J = 5.4 Hz, 2H, N-CH_2-**CH_2**), 1.24 (t, J = 7.1 Hz, 3H, OCH_2**CH_3**).

Ethyl 2-amino-6-(6-methoxy-6-oxohexyl)-4,5,6,7-tetrahydrothieno[2,3-c] pyridine-3-carboxylate (19). yellow powder. mp 281℃, dec. ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.82 (s, 2H, Ar-**H**), 3.58 (s, 3H, O**Me**), 3.53 (s, 2H, N-**CH_2**-Thiophene), 2.91 (t, J = 5.5 Hz, 2H, N-**CH_2**-CH_2-Thiophene), 2.68 (t, J = 5.6 Hz, 2H, N-CH_2-**CH_2**-Thiophene), 2.45 (t, J = 6.9 Hz, 2H, N-**CH_2**), 2.31 (t, J = 7.4 Hz, 2H, MeCOO**CH_2**), 2.20 (s, 6H, 2Ar-**CH_3**), 1.60-1.47 (m, 4H, N-CH_2-**CH_2**-CH_2**CH_2**), 1.35-1.29(m , 2H, N-CH_2-CH_2-**CH_2**).

N-hydroxy-6-(2-(4-hydroxy-3,5-dimethylphenyl)-4-oxo-3,5,6,8-tetrahydropyrido [4',3':4,5]thieno[2,3-d]pyrimidin-7(4H)-yl)hexanamide (20). yellow powder. mp. 318℃ dec. ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.16 (s, 1H, Pyrimidine-**H**), 10.34 (s, 1H, NH**OH**), 9.00 (s, 1H, Ar-**OH**), 8.65 (s, 1H, **NH**OH), 7.81 (s, 2H, Ar-**H**), 3.77 (s, 2H, N-**CH_2**-Thiophene), 2.99 (s, 2H, N-**CH_2**-CH_2-Thiophene), 2.87 (s, 2H, N-CH_2-**CH_2**-Thiophene), 2.61 (s, 2H, N-**CH_2**), 2.23 (s, 8H, 2Ar-**CH_3**, CO**CH_2**), 1.62-1.50 (m, 4H, N-CH_2-**CH_2**-CH_2**CH_2**), 1.36-1.28 (m, 2H, N-CH_2-CH_2-**CH_2**). ^{13}C NMR (100 MHz, DMSO-d_6) δ 174.92, 169.50, 164.62, 159.22, 157.15, 153.13, 129.45, 128.47, 124.80, 122.58, 119.88, 56.93, 51.27, 49.82, 34.08, 32.68, 26.76, 25.46, 24.84, 17.08. HRMS (ESI): calcd. for $C_{23}H_{28}N_4NaO_4S^+$ $[M+Na]^+$, 479.1723; found 479.1720.

2-(2-(3,5-dimethylisoxazol-4-yl)-4-oxo-3,5,6,8-tetrahydropyrido[4',3':4,5]thieno[2,3-d]pyrimidin-7(4H)-yl)-N-hydroxyacetamide (21a). Compound 9b (86 mg, 0.20 mmol) and NaOH (40 mg, 1.0 mmol) were dissolved in methanol (5 ml), and then hydroxylamine (330 mg, 10 mmol, 50% in water) was added into the solution. The mixture was stirred for 15 min at room

temperature and hen acidified with 4M HC1/dioxane to pH 2-3, followed by filtration, concentration and the crude product was purified by thin layer chromatography using a mixture of dichloromethane: methanol (5:1), to give21a, Yield 76.6%, yellow solid. mp: 205.9 – 207.2℃.^{1}H NMR (400 MHz, DMSO-d_6) δ 10.50 (s, 1H, NH**OH**), 8.79 (s, 1H, **NH**OH), 3.74 (s, 2H, N-**CH_2-**Thiophene), 3.11 (s, 2H, CO**CH_2**N), 2.94 (t, J = 5.2 Hz, 2H, N-**CH_2-**CH_2-Thiophene), 2.80 (t, J = 5.2 Hz, 2H, N-CH_2-**CH_2-**Thiophene), 2.51 (s, 3H, Isoxazole-**CH_3**), 2.31 (s, 3H, Isoxazole-**CH_3**).^{13}C NMR (101 MHz, DMSO-d_6) δ 170.46, 166.25, 163.95, 163.65, 159.07, 158.89, 130.30, 129.54, 121.01, 111.45, 58.14, 51.38, 49.69, 25.88, 12.50, 10.83. HRMS(ESI): calcd. for $C_{16}H_{18}N_5O_4S^+$ $[M+H]^+$,376.1074; found 376.1073.

4-(2-(3,5-dimethylisoxazol-4-yl)-4-oxo-3,5,6,8-tetrahydropyrido[4',3':4,5]thieno[2,3-d]pyrimidin-7(4H)-yl)-N-hydroxybutanamide (21b). Compound 21b was synthesized from Compound 9c and hydroxylamine, in a manner similar to 21a, Yield 73.1%, yellow solid. mp: 215.2 – 217.7℃.^{1}H NMR (400 MHz, DMSO-d_6) δ 10.36 (s, 1H, NH**OH**), 8.68 (s, 1H, **NH**OH), 3.63 (s, 2H, N-**CH_2-**Thiophene), 2.95 (s, 2H, N-**CH_2-**CH_2-Thiophene), 2.73 (t, J = 5.2 Hz, 2H, N-CH_2-**CH_2-**Thiophene), 2.54 (s, 3H, Isoxazole-**CH_3**), 2.47 (t, J = 7.2 Hz, 2H, N-**CH_2**), 2.34 (s, 3H, Isoxazole-**CH_3**), 2.02 (t, J = 7.2 Hz, 2H, CO**CH_2**), 1.81 – 1.67 (m, 2H, N-CH_2-**CH2**-CH2). ^{13}C NMR (101 MHz, DMSO-d_6) δ 170.40, 169.53, 163.66, 159.23, 158.89, 146.95, 130.63, 129.75, 120.96, 111.54, 56.53, 51.56, 49.75, 30.60, 26.06, 23.22, 12.51, 10.86.HRMS(ESI): calcd. for $C_{18}H_{22}N_5O_4S^+$ $[M+H]^+$,404.1387; found 404.1391.

6-(2-(3,5-dimethylisoxazol-4-yl)-4-oxo-3,5,6,8-tetrahydropyrido[4',3':4,5] thieno[2,3-d]pyrimidin-7(4H)-yl)-N-hydroxyhexanamide (21c). Compound 21c was synthesized from Compound 29d and hydroxylamine, in a manner similar to 21a,

Yield 72.5 %, yellow solid. mp:183.6 – 185.4℃.^{1}H NMR (400 MHz, DMSO-d_6) δ 12.37 (s, 1H, Pyrimidine-**H**), 10.33 (s, 1H, NH**OH**), 8.65 (s, 1H, **NH**OH), 3.62 (s, 2H, N-**CH_2-**Thiophene), 2.94 (t, J = 4.8 Hz, 2H, N-**CH_2-**CH_2-Thiophene), 2.73 (t, J = 4.8 Hz, 2H, N-CH_2-**CH_2-**Thiophene), 2.54 (s, 3H, Isoxazole-**CH_3**), 2.47 (t, J = 7.2 Hz, 2H, N-**CH_2**), 2.34 (s, 3H, Isoxazole-**CH_3**), 1.96 (t, J = 7.2 Hz, 2H, CO**CH_2**), 1.58 – 1.44 (m, 4H, N-CH_2-**CH_2-**CH_2-**CH_2**), 1.32 – 1.23 (m, 2H, N-CH_2-CH_2-**CH_2**).^{13}C NMR (101 MHz, DMSO-d_6) δ 175.15, 174.29, 168.38, 163.96, 163.64, 151.68, 135.45, 134.49, 125.73, 116.29, 61.98, 56.38, 54.67, 37.48, 31.70, 31.53, 30.91, 30.29, 17.26, 15.61.HRMS(ESI): calcd. for $C_{20}H_{26}N_5O_4S^+$ $[M+H]^+$,432.1700; found 432.1702.

N-(2-aminophenyl)-2-(2-(3,5-dimethylisoxazol-4-yl)-4-oxo-3,5,6,8-tetrahydropyrido[4',3':4,5]thieno[2,3-d]pyrimidin-7(4H)-yl)acetamide (22a). Compound 9b (86 mg, 0.20 mmol) was added into the mixed solution of THF (3 ml) and 2N NaOH (1 ml). The solution was stirred for 4 h at room temperature. The mixture was acidified with 4M HC1/dioxane to pH 2-3, followed by concentration to give a crude product. The above product, benzene-1,2-diamine(108 mg, 1.0 mmol) EDCI(76.8 mg, 0.40 mmol), and HOBt(54 mg, 0.4 mmol) was dissolved in anhydrous CH_2Cl_2(10ml), the reaction mixture was stirred for 12 h at room temperature, followed by filtration and concentration, the crude product was purified by thin layer chromatography using a mixture of dichloromethane: methanol (20:1), to give 22a, Yield 28.3%, yellow solid.mp: 118.6 – 120.2℃.^{1}H NMR (400 MHz, DMSO-d_6) δ 12.39 (s, 1H, Pyrimidine-**H**), 9.19 (s, 1H, **NH**CO), 7.30 (dd, J = 7.9, 1.2 Hz, 1H, Ar-**H**), 6.94 – 6.88 (m, 1H, Ar-**H**), 6.76 (dd, J = 7.9, 1.2 Hz, 1H, Ar-**H**), 6.62 – 6.56 (m, 1H, Ar-**H**), 4.78 (s, 2H, Ar-**NH_2**), 3.88 (s, 2H, N-**CH_2-**Thiophene), 3.38 (s, 2H, CO-**CH_2-**N), 3.03 (t, J = 5.6 Hz, 2H, N-**CH_2-**CH_2-Thiophene), 2.94 (t, J = 5.6 Hz, 2H, N-CH_2-**CH_2-**Thiophene), 2.54 (s, 3H, Isoxazole-**CH_3**), 2.34 (s, 3H, Isoxazole-**CH_3**).^{13}C NMR (101

MHz, DMSO-d_6) δ 170.46, 168.66, 163.72, 159.11, 158.89, 146.91, 142.07, 130.32, 129.60, 126.19, 125.27, 124.20, 121.03, 117.14, 116.80, 111.47, 60.56, 51.49, 49.84, 26.01, 12.50, 10.84. HRMS(ESI): calcd. for $C_{22}H_{23}N_6O_3S^+$ $[M+H]^+$, 451.1547; found 451.1540.

N-(2-aminophenyl)-6-(2-(3,5-dimethylisoxazol-4-yl)-4-oxo-3,5,6,8-tetrahydropyrido[4',3':4,5]thieno[2,3-d]pyrimidin-7(4H)-yl)hexanamide(22b).

Compound 22b was synthesized from Compound 9d and benzene-1,2-diamine, in a manner similar to 22a, Yield 24.5%, yellow solid. mp: 88.6 – 92.2℃.^{1}H NMR (400 MHz, Chloroform-d) δ 7.46 (s, 1H, Ar-**H**), 7.13 (d, J = 7.7 Hz, 1H, Ar-**H**), 7.02 (t, J = 7.1 Hz, 1H, Ar-**H**), 6.76 – 6.72 (m, 2H, Ar-**H, NH**CO), 3.69 (s, 2H, N-**CH_2**-Thiophene), 3.02 (t, J = 5.4 Hz, 2H, N-**CH_2**-CH_2-Thiophene), 2.82 (t, J = 5.4 Hz, 2H, N-CH_2-**CH_2**-Thiophene), 2.65 (s, 3H, Isoxazole-**CH_3**), 2.59 (t, J = 7.2 Hz, 2H, N-**CH_2**), 2.48 (s, 3H, Isoxazole-**CH_3**), 2.43 (t, J = 7.2 Hz, 2H, CO-**CH_2**), 1.80 (p, J = 7.2 Hz, 2H, CO-CH_2-**CH_2**), 1.71 – 1.63 (m, 2H, N-CH_2-**CH_2**), 1.52 –1.45 (m, 2H, N-CH_2-CH_2-**CH_2**).^{13}C NMR (101 MHz, Chloroform-d) δ 171.78, 170.40, 164.92, 160.51, 158.53, 145.67, 140.90, 131.65, 129.65, 127.10, 125.33, 124.35, 120.49, 119.46, 118.14, 110.90, 57.30, 51.99, 49.86, 36.82, 29.70, 26.94, 25.52, 14.12, 12.65, 11.03. HRMS(ESI): calcd. for $C_{26}H_{31}N_6O_3S^+$ $[M+H]^+$,507.2173; found 507.2176.

BRD4 and HDACs enzymatic assays

The in vitro BRD4 and HDACs assays were performed according to the previous reports and manufacturer's protocols. For the BRD4 assay, the HTRF® assay used a GST-tagged bromodomain of the BRD4 protein, and then, the biotinylated Kac peptide of histone H4 (1-21) and two HTRF® detection reagents were added. The HTRF® signal was proportional to the amount of interaction between GST-tagged BRD4 protein and biotinylated Kac peptide. For the HDACs assays, the HTRF® assay used the

biotinylated Kac peptide of histone H3 (1-21), an Eu^{3+} cryptate-labeled anti-H3K9 monoclonal antibody and fluorescence-conjugated streptavidin. The HTRF® signal was proportional to the concentration of deacetylated H3 (1-21) peptide. The BRDs enzymatic profiling assays were performed by the Alphascreen method, the detailed experimental procedures were reported previously.[56]

Cell proliferation and apoptosis assays

The colorectal carcinoma cell lines SW620 and HCT-116 were obtained from the ATCC (American Type Culture Collection) and cultured in the state key laboratory of biotherapy, west china hospital, Sichuan University. The SW620 and HCT-116 cells were cultured in DMEM supplemented with 10% fetal bovine serum and maintained at 37 °C with 5% CO_2 in atmosphere. The cell proliferation assay was measured by using the MTT method. Cells treated with DMSO were set as negative control. In brief, about $5*10^3$ cells were incubated with test compounds in 96-well plate for 24-48 hours, then 10 μL MTT (5 mg/ml) was added and incubated for an additional 4 hours, added the extraction buffer and incubated overnight, the absorbance values were measured at 570nm on a microplate reader (Thermo Scientific Multiskan, Finland). The Annexin V-propidium iodide (PI) dual-staining method was used as apoptosis assay. HCT-116 cells with or without compounds incubated were harvested and washed twice with cold PBS. The early and late apoptosis cells were identified on a flowcytometry instrument (BD Biosciences, San Jose, CA, USA) by staining with FITC-conjugated annexin V-PI kit according to the manufacturer's instructions (Keygen, Nanjing, China). In addition, HCT-116 cells were plated in six-well plates, the cells were growing and adhered for 24 hours, then incubated with 1.5, 2, or 3 μM of **17c** for an additional 12 hours followed by Hoechst 33258 addition. The morphology of nuclei was visualized under an

Olympus fluorescence microscope.

Western blot analysis

The western blot (WB) analysis were performed according to our previously reports. In brief, the equivalent concentrations of total proteins in cell lysate were separated by SDS-PAGE and then transferred to PVDF (poly-vinylidene difluoride membrane, Millipore, MA, USA). After blocked by 0.5-1% of BSA under 4^{o}C overnight, the PVDF membranes were incubated by corresponding primary antibodies at 4^{o}C overnight or room temperature for two hours. The PVDF membranes were washed twice by TBST solution and then incubated with HRP-conjugated second antibodies. The immunoblotting slides were collected by using ECL (enhanced chemiluminescence) method according to the manufacturer's instruction.

In vivo pharmacokinetics study

The in vivo antitumor activity, oral pharmacokinetics and preliminary safety of **17c** were carried out according to the Guidelines for the Care and Use of Laboratory Animals that were approved by the Institutional Animal Care and Use Committee and Committee of Ethics of Animal Experimentation of Sichuan University. Compound **17c** was encapsulated in hydroxypropyl-β-cyclodextrin aqueous solution for intravenously administration and 0.5% sodium carboxyl methyl cellulose (CMC-Na) aqueous solution for oral administration. The Sprague-Dawley rats with 200−250 g body weights were obtained from Beijing HFK Bioscience Co. Ltd. The compound **17c** was intravenously administered to a group of eight rats (four male and four female rats), by a bolus injection (30 mg/kg dose) to the tail vein or oral administration. At time points 0 (prior to dosing), 5, 15, 30, 45, 60, 120, 240, 360, 480, 600, 720 and 1440 minutes

after dosing, the blood sample was collected from each animal respectively and separated plasma by refrigerated centrifugation and then stored in a freezer under liquid nitrogen. All samples were separated and determined the plasma concentration compound **17c** by LC−MS/MS (Waters Acquity UPLC-Q-TOF instrument).

Xenograft tumor models and primary safety evaluation

The six to eight weeks old SPF (specific pathogen-free) male nude mice (Balb/cAnNCrl) were purchased from Beijing Vital River Biotechnology Co., Ltd, and were randomly grouped by weighed and coded (n=8 per group). After adapted for five to eight days, the mice were subcutaneously grafted into the dorsal flank with 0.1 mL of phosphate buffer containing 2×10^6 HCT-116 cells. When the tumors grew to a size of approximate leg diameter of 6 mm, the mice were initially treated and orally administered with **17c** or saline on days 1-19, and were monitored on a daily basis during treatment (tumor volume and body weights). On the last day, the animals were sacrificed; tumors and main organs were isolated and weighed. The tumor and various organs tissues were sectioned, then fixed in 4% paraformaldehyde in PBS for immunohistochemistry analysis and stained with TUNEL, Ki67, Ac-H3, c-Myc, BRD4, LC3-II, LIFR and p-STAT3 antibodies as the previous studies stated.

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Abbreviations used: BRD4, Bromodomain-containing protein 4; HDAC, Histone deacetylases; CRC, Colorectal carcinoma; EGFR, epidermal growth factor receptor; IHC, immunohistochemical; BET, bromodomain and extra-terminal; FDA, U.S. food and drug administration; PP1α, Protein Phosphatase 1 alpha; P-TEFb, positive transcriptional elongation factor b; LIFR, Leukemia Inhibitory Factor Receptor; JAK, Janus Kinase; STAT, Signal Transducer and Activator of Transcription; SAHA, suberoylanilide hydroxamic acid; TMA, Tissue microarray; Kac, acetylated lysine; ZINC, ZINC is not commercial; ZBG, zinc binding group; TR-FRET, Time-resolved fluorescence energy transfer; MRT, mean residence time; T/C, treatment to control ratios; TUNEL, Terminal deoxynucleotidyl transferase dUTP nick end labeling; H&E, Hematoxylin and eosin stain; SAR, structure-activity relationship; MCSS, Multiple Copy Simultaneous Search; ADMET, absorption; distribution; metabolism; excretion and toxicity; ECL, enhanced chemiluminescence.

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#Zhaoping Pan and Xiang Li contributed equally to this work.

Supporting Information

Additional figures containing additional figures related biological studies; NMR

spectra of final compounds and HPLC chromatogram of final compounds (PDF); Molecular formula strings (CSV). This material is available free of charge via the Internet at http://pubs.acs.org.

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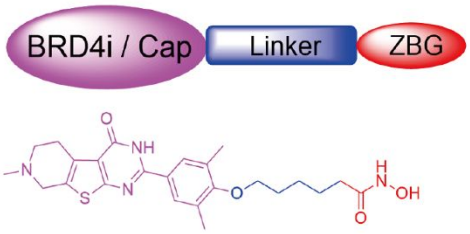
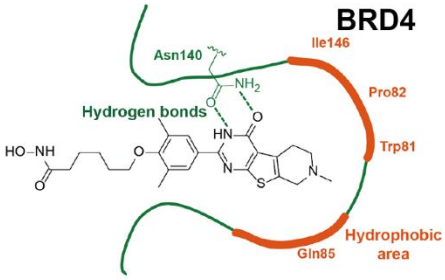
Table of Contents graphic





BRD4 IC_{50} = 0.71 μM

Class I/IIb HDACs IC_{50} Ranges 0.046 - 0.923 μM

Oral Bioavailibility = 40.5%

Enhanced Autophagic Cell Death in CRC cells