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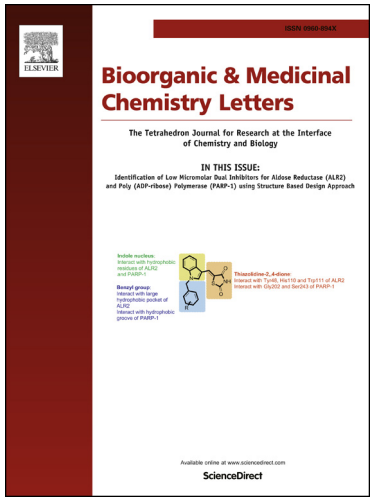

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Design, synthesis and biological evaluation of 4-piperazinyl-containing Chidamide derivatives as HDACs inhibitors

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Abstract:

The synthesis and biological evaluation of a variety of 4-piperazinyl-containing Chidamide derivatives is described. Some of these compounds were shown to inhibit HDAC1 with IC_{50} values below micromolar range, and inhibited proliferation of several human cancer cells, not possessing toxicity to human normal cells and hERG K^+ ion channels. Compound **9g,** proved to be the most potent and efficacious derivative in this series, was orally active in an HCT116 xenograft model *in vivo*.

Epigenetic mechanisms are essential for normal development and maintenance of tissue-specific gene expression patterns in mammals. Epigenetic defects can lead to alter gene function and malignant cellular transformation and have been linked to the progression of a number of diseases, such as cancer.[1, 2] The reversible acetylation of histones, which is controlled by histone acetyltransferases (HATs) and histone deacetylases (HDACs), has emerged as an important epigenetic modification implicated in cell proliferation and has been identified as a valuable target for anticancer drug design.[3-5] HDACs comprise a family of 18 genes, which are grouped into "classical HDACs" (Zn^{2+} for Classes I, II and IV) and sirtuins (NAD^+ for Class III) based on their catalytic mechanisms.[6, 7]While the biological functions of the many HDACs subtypes are still being defined, there is compelling evidence that class I HDACs (HDAC1, 2, 3, 8) in particular are overexpressed in human cancers and therefore are viable targets for cancer therapeutics.[8-11]

Up to date, four HDACs inhibitors (HDACIs）,Vorinostat (Zolinza; 2006), Romidepsin (Istodax; 2009), Belinostat (Beleodaq; 2014) and Panobinostat (Farydak; 2015) have been approved by the US FDA.[12] However, it turned out that most of the

currently used HDACIs inhibited either all or at least several members of the HDACs family, which may suffer from numerous side effects, low potenty, or low stability[13, 14]Meanwhile, a number of benzamide HDACIs, such as Entinostat (MS-275),[15]Mocetinostat (MGCD0103)[16] and Chidamide (CS055),[17] are currently undergoing clinical evaluation in solid tumors and hematological malignancies, which can selectively inhibit HDACs 1-3 but does not significantly inhibit the other HDACs isoforms. Recently, Chidamide was approved by the China FDA for the treatment of advanced peripheral T-cell lymphoma (PTCL) as the first orally available benzamide class of HDACIs .[18]

Despite the variety of structural characteristics, most HDACIs can be broadly described by a common pharmacophore according to the HDACs active site, as shown in **Figure 1**. This model is composed of a zinc binding group (ZBG), attached to a linker chain occupying the hydrophobic tunnel of the active site. This chain is terminated by a functional "cap" group, mainly aromatic,interacting with the HDACs external surface. The connecting unit between the cap and the linker chain can be modulated to improve interactions.

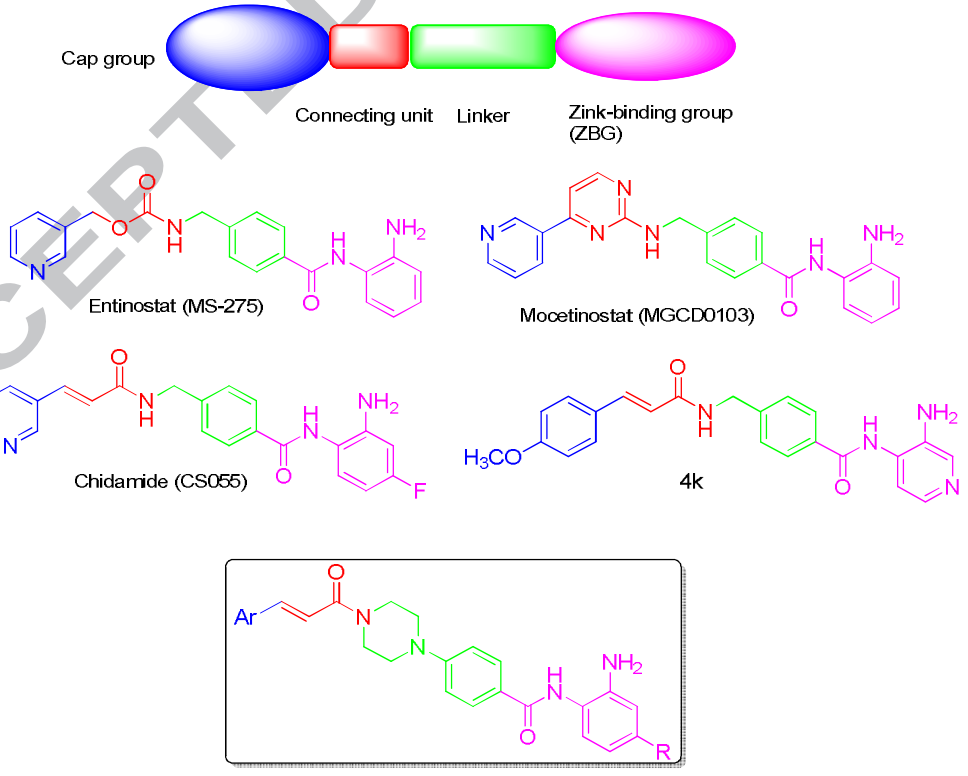
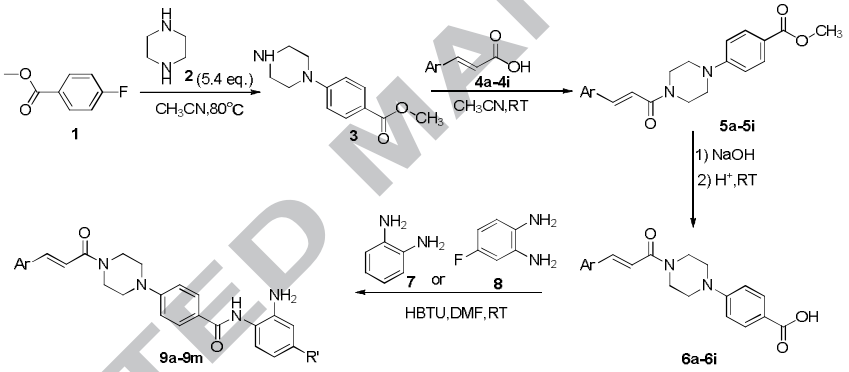


Figure 1. Chemical structures of HDACIs

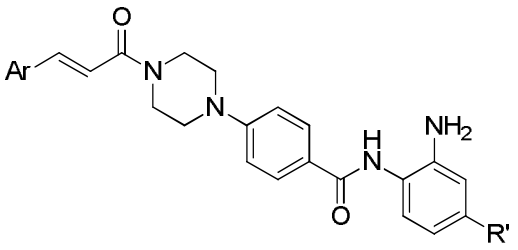
In our previous study, we prepared the Chidamide analog **4k** which had a *N*-(3-Aminopyridin-4-yl)-substituted residue as the ZBG. **4k** exhibited comparable HDAC1 inhibitory activity (IC_{50}=3.3 μM) as Chidamide (IC_{50}= 2.7 μM), though it had a problem with moderate antitumor activity against PC-3 cell xenografts in nude mice (TGI=39%, at 100 mg/kg).[19]Several efforts have been made to replace the linker chain with alternative chemical moieties, such as *N*-substituted piperazine ,which could lead to a significant enhancement in HDACs inhibitory activity over analogs.[20-22]Inspired by the structure-activity relationship (SAR) , herein we reported the design, synthesis, and preliminary bioactivity evaluation of 4-piperazinyl-containing Chidamide derivatives as HDACIs.

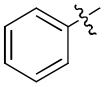
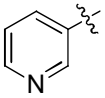
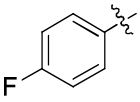
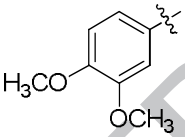
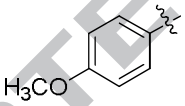
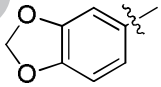
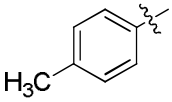
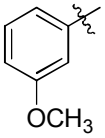
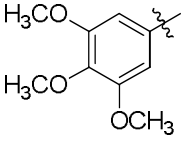


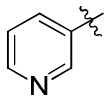
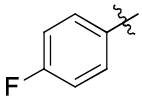
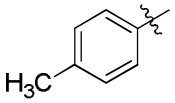
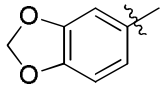
Scheme 1. General synthesis of 4-piperazinyl-containing Chidamide derivatives 9a-9m

The preparation of target compounds was shown in **Scheme 1**. The intermediate methyl 4-(piperazin-1-yl)benzoate **3** could be obtained via the phenyl fluoride displacement of commercially available methyl 4-fluorobenzoate with excess piperazine (5.4 equiv) at 80□ in CH_3CN, which was subsequently reacted with (*E*)-3-(pyridin-3-yl)acrylic acid or cinnamic acid analogs in the presence of HBTU to afford the key intermediates **5a–5i**. **5a–5i** were then converted by hydrolysis of the methyl ester with NaOH to intermediates **6a–6i** and further by HBTU coupling in the presence of excess phenylenediamine or 4-fluorophenylenediamine to obtain the desired compounds **9a-9m**.

Table 1. The chemical structures and HDAC1 inhibitory activities of 4-piperazin-containing derivatives 9a-9m



Compound	Ar	R'	% Inhibition [a]	
			1 μM	10 μM
9a		H	34.30	66.82
9b		H	18.11	53.82
9c		H	52.02	79.05
9d		H	46.52	79.24
9e		H	50.18	87.82
9f		H	49.63	75.50
9g		H	56.30	94.22
9h		H	45.33	66.89
9i		H	21.82	60.54

9j		F	4.71	19.22
9k		F	36.03	58.83
9l		F	17.43	64.31
9m		F	18.01	53.37
Chidamide			55.12	81.27

[a] Assays done in replicates (n⩾3) as described in reference.[23] Mean values are shown, and the standard deviations are <30% of the mean.

All of the target compounds were initially screened for their ability to inhibit recombinant human HDAC1 at two concentrations (1 μM and 10 μM). The preliminary results showed that most of 4-piperazinyl-containing derivatives showed potent HDAC1 inhibitory activity but not signficiantly superior to Chidamide. As presented in **Table 1**, analogs containing 2-amino-4-fluorophenyl group in the ZBG positions, such as **9j, 9k, 9l** and **9m**, were typically less potent than derivatives (**9b,9c, 9g** and **9f**) with 2-aminophenyl substitution at the same positions. Interestingly, introduction of more polar groups such as 3-pyridyl in the "cap" domain (**9b** and **9j**) led to reduce binding affinity to HDAC1 versus phenyl substituted analogues, indicating that the electrostatic properties of the substitutions on the "cap" group may show critical effect on inhibitory activities. Three compounds (**9c, 9e, 9g**) were further evaluated for the IC_{50} of HDAC1 inhibition and showed promising activities with IC_{50} values in the nanomolar range (**Table 2**). The IC_{50} values of **9c, 9e** and **9g** are 0.983, 0.903 and 0.798 μM, respectively, whereas Chidamide exhibited IC_{50} values of 0.864 μM.

Table 2. Profiles of selected compounds 9c, 9e and 9g *in vitro*

Compound	IC_{50}[a] (μM)	IC_{50}[a] (μM)					IC_{50}[b] (μM)
	HDAC1	cell lines					hERG
		HCT116	A549	K562	PC-3	MRC-5	

9c	0.983	1.977	0.811	1.102	2.142	>30.0	32.6
9e	0.903	0.765	0.530	2.963	8.706	>30.0	25.1
9g	0.798	0.069	0.135	4.856	3.964	>30.0	27.1
Chidamide	0.864	0.202	0.665	0.975	2.093	>30.0	32.0

[a] Assays done in replicates (n⩾3). Mean values are shown, and the standard deviations are <30% of the mean.

[b] hERG Patch clamp screen as described in reference.[24] IC_{50} values represent the concentration to inhibit 50% of hERG current (IKr). Numbers represent IC_{50} values generated from 3-point concentration response relationships in duplicate.

Encouraged by their potent anti-HDAC1 profiles, anti-proliferative effects of more potent compounds (**9c, 9e** and **9g**) against HCT116 cells, A549 cells, K562 cells and PC-3 cells were tested using MTT assay as described in reference.[25] As shown in **Table 2**, three selected compounds exhibited good antiproliferative activities against all the tested cancer cells with IC_{50} values in the micromolar or sub-micromolar range. Compound **9g** showed significant growth inhibition with IC_{50} values of 0.069 and 0.135μM against HCT116 and A549 cells respectively, which was obviously superior to Chidamide. To investigate the selective cytotoxicity of this new set of compounds, **9c**, **9e** and **9g** were tested over the human fetal lung fibroblast (MRC-5) normal cell lines (**Table 2**). Interestingly, all the tested compounds including Chidamide exhibited high IC_{50} values (>30.0 μM), which indicated their differential growth inhibitory activities towards human cancer cells rather normal cell lines.

In vitro cardiovascular safety was assessed in a patch-clamp hERG K^+ channels screen (**Table 2**). Compounds **9c, 9e** and **9g** were inactive in hERG binding assay with a IC_{50} > 20.0 μM (reducing incidence of QT interval prolongation).

In order to gain insights into the possible interactions of **9g** with the active site of HDAC1(PDB code: 4BKX), the molecular docking was performed using MOE software (Molecular Operating Environment, Version 2015.1001) (**Figure 2**).[26]

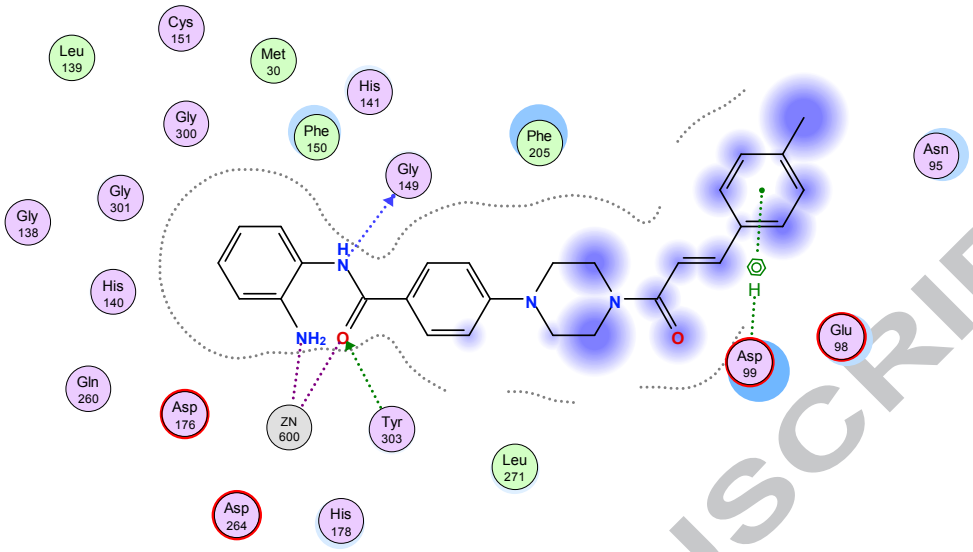


Figure 2. Binding modes of 9g at the active site of HDAC1

From the docking result, we found that compound **9g** could insert into HDAC1 pocket, and the 2-aminophenyl moiety anchored onto the bottom of the pocket, which was coordinated to the zinc ion, and formed hydrogen bonds with Tyr303 and Gly149. Meanwhile, the phenyl ring formed a H-π interaction with residues of Asp99 in the cap region, which could help to establish electrostatic interaction with the malleable protein surface. Interestingly, according to our docking modes, the introduction of piperazine group in the linker chain may not be favorable due to the lack of interaction with residues of the tunnel of the HDAC1 active site. This was proved to be consistent with experimental datas as shown that most 4-piperazinyl-containing derivatives exhibited moderate HDAC1 inhibitory activity compared to Chidamide. We could presume that the size and position of the substituents on the piperazine ring may play an important role in enhancing the binding affinity, which could provide invaluable information for further design of novel piperazin-containing derivatives.

Despite the sub-optimal potency *in vitro,* **9g** was demonstrated to be the most potent compound in this study and was selected for further biological evaluation.

The pharmacokinetic parameters of compound **9g** were evaluated in female Sprague Dawley rats after single iv (2 mg/kg) and po (10 mg/kg) administration. The terminal phase half-life after iv dosing was 1.07 h in rats. The clearance was 0.45

L/h/kg and the steady state volume of distribution was 0.11 L/kg. **9g** was quickly absorbed after oral dosing, with a T_{max} of 0.43 h. The oral bioavailability was found to be 36.3% in rats.

Table 3. Pharmacokinetic evaluation of compound 9g[a]

Compound	$T_{1/2}$ iv (h)	CL iv (L/h/kg)	V_{ss} iv (L/kg)	T_{max} po (h)	C_{max} po(μg/L)	AUC po(h*μg/L)	F (%)
9g	1.07	0.45	0.11	0.43	2509	8907	36.3

[a]For pharmacokinetic study, blood was collected from rats at various time points up to 6 h, and plasma samples were analyzed using an Agilent 1200 HPLC system coupled with Agilent 6410 B triple quadruple mass spectrometer. A solution of 0.05 N HCl in saline was used as the vehicle for both iv and po dosing.

9g was further tested in a mouse xenograft model (HCT116 colon) at daily oral doses of 25 mg/kg, 50 mg/kg and 100mg/kg for 21 days (**Fig. 3**), and dose-dependent inhibition of tumor growth was observed. At a dose of 25 mg/kg administered po the inhibitor was tolerated and a 42.4% tumor growth inhibition(TGI) was observed without significant weight loss (less than 5% body weight loss). The 50 and 100 mg/kg groups gave a 55.1% and 76.3% TGI, respectively. Unfortunately, mice treated with **9g** at 50mg/kg and 100 mg/kg oral dosage experienced significant and dose proportionate weight loss (11.3% loss in the 50 mg/kg ,19.2% loss in the 100 mg/kg).Adverse effects caused termination of the 100 mg/kg study by 12 days. Despite having a unsatisfactory therapeutic margin, compound **9g** represents a promising discovery lead. Inhibitors possessing an improved therapeutic index will be the focus of future disclosures.

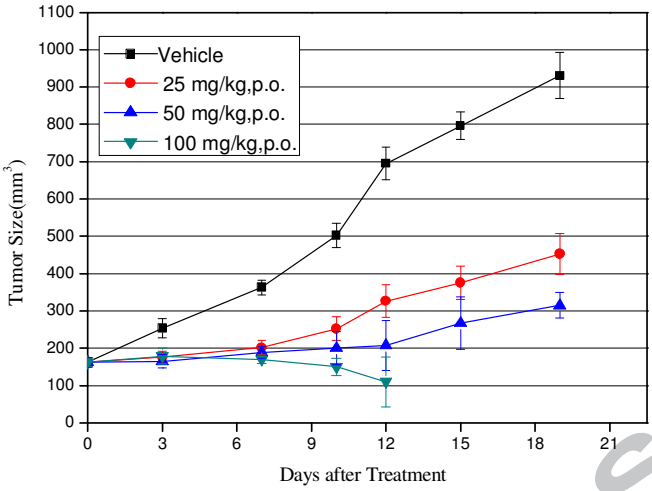


Figure 3. Effects of 9g on HCT116 cell-inoculated xenografts *in vivo*

In summary, we designed and synthesized a series of novel HDACIs with 4-piperazine group in the linker chain of Chidamide. Among them, **9c**, **9e** and **9g** exhibited similar or higher inhibitory activities in both enzymatic inhibitory activity and cellular anti-proliferative activity assay compared with Chidamide, not possessing significant toxicity to primary human cells and the patch clamp hERG K^+ ion channel. Molecular docking showed that optimisation of the linker remained an important challenge. Compound **9g** displayed good profiles *in vitro* , possessed favorable pharmacokinetic characteristics and exhibited potent orally antitumor activity in an HCT116 human colon carcinoma mouse xenograft model study. However, intolerable body weight loss of experimental mice at high doses has limited the further development of **9g** . Efforts to identify a safe, orally efficacious alternative of **9g** will be the subject of future communications.

Acknowledgement

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Supplementary data

Supplementary data associated with this article can be found, in the online version.

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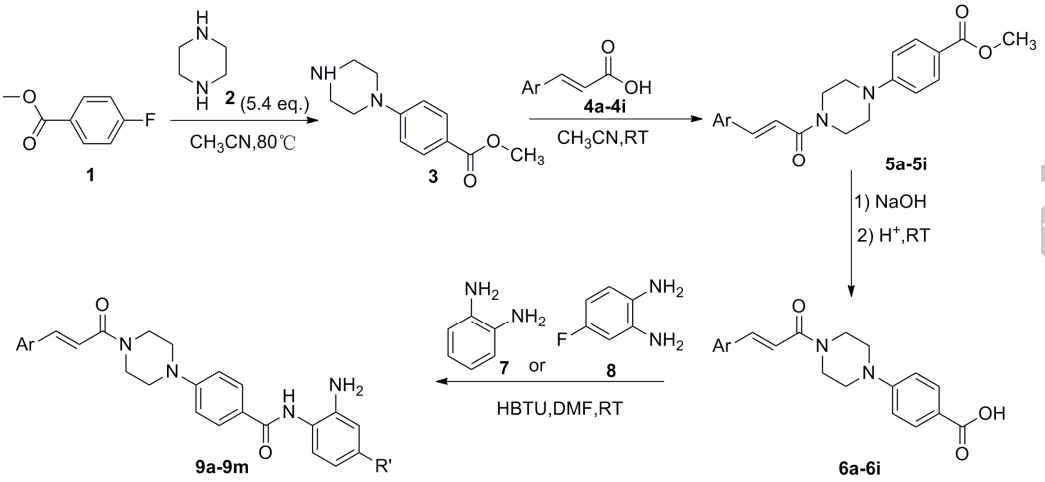
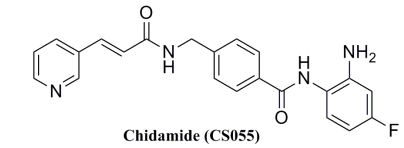
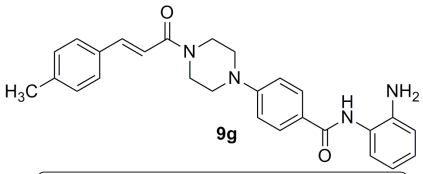


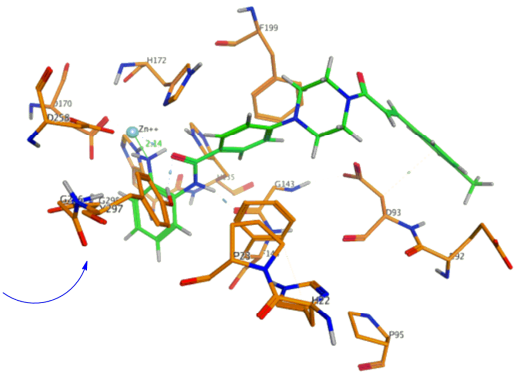


Chidamide (CS055)



9g

HDAC1 IC_{50}: 0.798μ M
Cell lines (HCT116,A549) IC_{50} :0.069, 0.135μ M
hERG IC_{50}:>30 μ M
TGI(HCT116 xenografts):42.4% ,55.1%, 76.3% at 25 , 50 and 100mg/kg q.d.



Highlights

- A series of 4-piperazinyl-containing Chidamide derivatives were synthesized.
- Target compounds were screened for their HDAC1 inhibitory activity.
- The promising compound **9g** displayed good profiles *in vitro* and had low affinities for hERG channels.
- **9g** possessed favorable pharmacokinetic characteristics.
- **9g** exhibited potent orally antitumor activity *in vivo*.