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Design, synthesis and biological evaluation of β-boswellic acid based HDAC inhibitors as inducers of cancer cell death

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ABSTRACT

The synthesis and bio-evaluation of naturally occurring boswellic acids (BAs) as an alternate CAP for the design of new HDAC inhibitors is described. All the compounds were screened against a panel of human cancer cell lines to identify leads, which were subsequently examined for their potential to inhibit HDACs. The identified lead compound showed IC_{50} value of 6 μm for HDACs, found to induce G_1 cell cycle arrest at significantly low concentration (1 μM) and caused significant loss in mitochondrial membrane potential at 5 and 10 μM. Furthermore, specific interactions of the lead molecule inside the catalytic domain were also studied through in silico molecular modeling.

Histone deacetylase (HDAC) inhibitors are an emerging class of drugs that alter gene expression by inducing chromatin remodeling.[1] HDAC inhibitors have potential to reverse the epigenetic states related to cancer and other diseases[2] owing to their implication in a wide range of cellular phenomenon such as cell cycle, apoptosis, autophagy, angiogenesis, glucose metabolism and DNA damage.[3] The association of HDAC inhibition has got profound and emerging attention in the current anticancer therapeutic strategies owing to the observed aberrant acetylation status of tumor suppressor and cell death associated genes. Among the current targets for cancer chemotherapy, natural product based small molecule epigenetic disruptors are emerging as novel anti-cancer therapeutics.[4] Interestingly, dietary sources are also regarded as rich sources of HDAC's, for example, sulforaphane is an effective chemoprotective agent in carcinogen-induced animal models.[5] Due to the promising preclinical activity of these agents, several HDAC inhibitors are in the clinical trials (Fig. 1).[6,7] Two HDAC inhibitors, that is, SAHA and Romidepsin have also been approved for the treatment of cutaneous T-cell lymphoma.[8,9] The HDAC inhibitors generally have three characteristic structural features, that is, (1) a hydrophobic region that binds the surface of the active site referred as CAP, (2) a connecting unit, linker from the hydrophobic cap group to coordinating group and (3) a coordinating/enzyme inhibiting group (EIG) that chelates to Zn^{2+} at the bottom of the tubular active site.

Thus, based on our continuous efforts to develop new anticancer leads from natural products[10] we envisioned using boswellic acids (BAs) as an alternative CAP for the design and development of HDAC inhibitors. BAs comprise of four naturally occurring triterpenic acids, that is, β-boswellic acid (the main triterpenic acid), 11-keto-β-boswellic acid and their acetates (**1** and **2**).[11] Based on our previous work,[12] we exploited 3-α-acetoxy-4-β-amino-24-norurs-12-ene (**3**) and 3-α-acetoxy-4-β-amino-11-oxo-24-norurs-12-ene (**4**) as potential CAP in the design of HDAC inhibitors as they exhibited significant anticancer activity.

To access BAs analogs, a simple synthetic protocol was followed as outlined in Scheme 1. Synthesis began by subjecting **1** and **2** to Curtius reaction, involving treatment with thionyl chloride in toluene followed by addition of sodium azide to give the corresponding isocyanates in 90% yields. The isocyanates, thus formed were further rearranged to corresponding amines by treatment with TFA/H_2O (2:1) to produce 3-α-acetoxy-4-β-amino-24-norurs-12-ene (**3**) and 3-α-acetoxy-4-β-amino-11-oxo-24-norurs-12-ene (**4**) in 80% and 85% yields, respectively. The next step was to introduce linker of different carbon chain lengths to the **3** and **4** through amide bond. This was accomplished by coupling amine with

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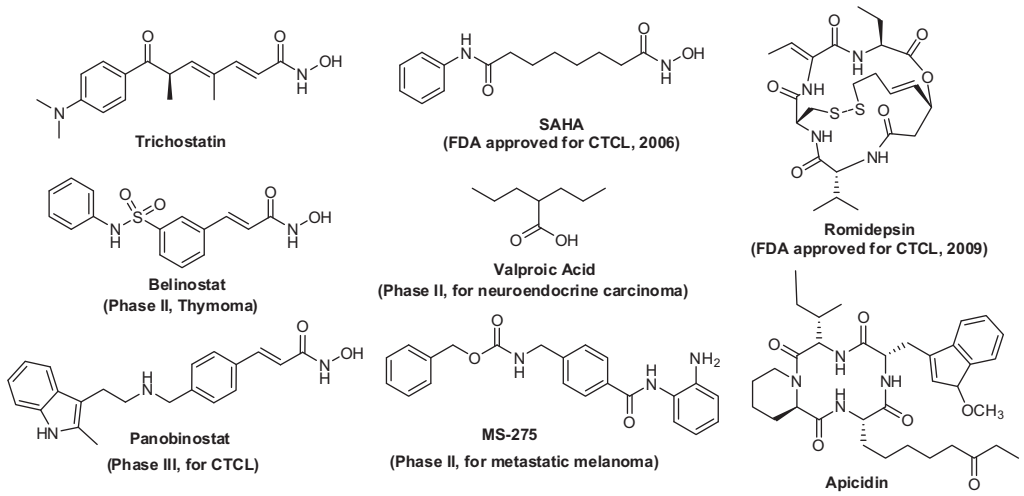
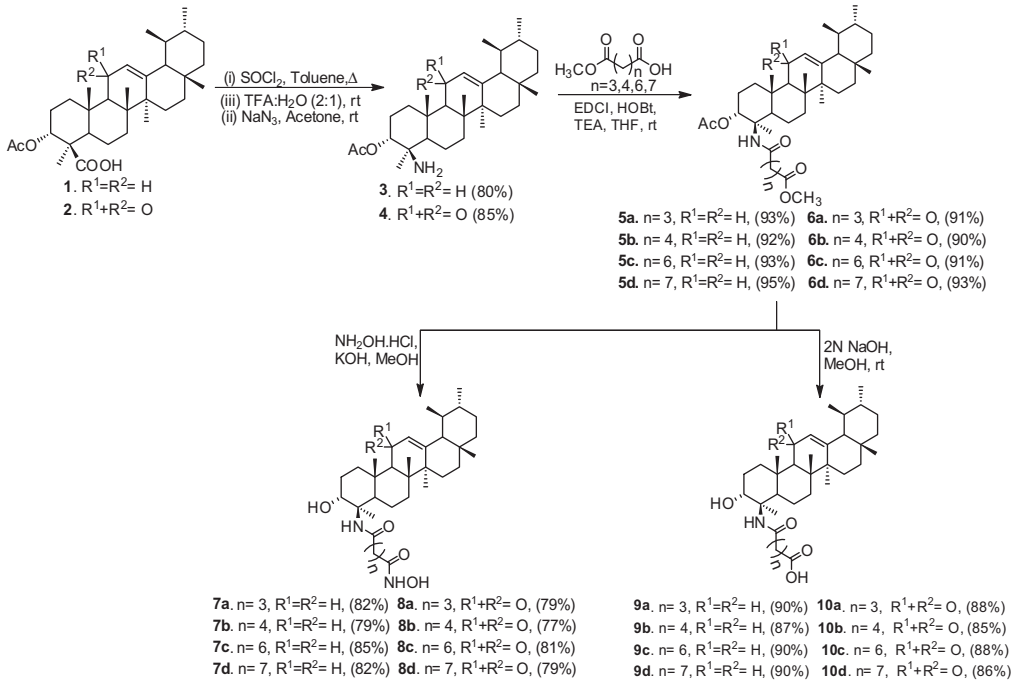


Figure 1. Structures of various HDAC inhibitors.



Scheme 1. Synthesis of BAs derivatives.

carboxylic acid group of commercially available monomethyl esters of glutaric, adipic, suberic and azelaic acid. The reaction was carried out in the presence of 1-ethyl-3-(3-dimethyllaminopropyl)carbodiimide hydrochloride (EDCI), hydroxybenzotriazole (HOBt) and triethylamine in THF at 0 °C to afforded esters **5a**–**d** and **6a**–**d** in high yields (90–95%). To meet structural requirements of HDAC inhibitors our next objective was to introduce EIGs viz., hydroxamic acids and carboxylic acids to the **5a**–**d** and **6a**–**d**. Thus, esters **5a**–**d** and **6a**–**d** converted to desired hydroxamic acids **7a**–**d** and **8a**–**d** in 77–85% yields by treatment with excessive hydroxylamine hydrochloride and KOH in methanol at 0 °C. And, to introduce carboxylic acid as EIG, esters **5a**–**d** and **6a**–**d** were hydrolyzed with 2 N NaOH solution and kept on stirring overnight at room temperature to give **9a**–**d** and **10a**–**d** in 85–90% yields, respectively.

All the synthesized molecules were initially evaluated for in vitro cytotoxicity at 20 and 0.25 μM concentrations against a panel of human cancer cell lines of different tissue origins. Molecules which were found to be significantly cytotoxic at 20 μM were evaluated for 4-dose assay to generate their IC_{50} values. Hydroxamic acid based molecules **7a**–**d** which have β-boswellic acid as cap were significantly cytotoxic at 20 μM concentration against HL-60 and mostly inactive against other cancer cell lines. Apart from HL-60, **7b** was active against A549, HCT-116 and MIA-PaCa-2 also. Whereas another class of hydroxamic acid based molecules **8a**–**d** which employ 11-keto-β-boswellic acid as cap were found to be active against all cell lines in the panel except **8d** which was found to be active only against HCT-116 and MCF-7. Apart from hydroxamate based molecules, some intermediates were also found cytotoxic against various cell lines as shown in Table 1. Compounds **3** and **4** were found to be cytotoxic against most of the cell lines at 20 μM concentration. Esters **5a**–**d** which contain β-boswellic acid based cap were found to be inactive against most of the cell lines in the panel whereas esters **6a**–**d** having 11-keto-β-boswellic acid derived cap were found to be significantly cytotoxic against

Table 1
IC_{50} values of different compounds against human cancer cell lines

Compd	Cell type					
	HL-60	A549	HCT-116	MIAPaCa-2	MCF-7	fR-2
	IC_{50} values (μM)					
3	10	13.2	8.7	7.2	6.9	13.9
4	6.1	6	15.3	15.1	14	7.1
5a	17.1	>20	19	11.4	8.7	>20
5b	>20	>20	>20	>20	13.8	>20
5c	>20	>20	>20	>20	>20	>20
5d	>20	>20	>20	>20	17	>20
6a	17.6	7.4	>20	>20	>20	>20
6b	>20	15.1	15.1	15.1	15.1	>20
6c	>20	2	15.7	8	<0.25	>20
6d	10	10	18	6.1	2	17.2
7a	11.7	>20	>20	8.8	3.6	>20
7b	>20	>20	>20	11.8	4.4	>20
7c	>20	>20	>20	>20	>20	>20
7d	12.4	15	11.8	11.9	6.4	>20
8a	1.6	13	15.1	15.1	7.1	1.9
8b	6.6	3.1	>20	13.1	>20	7.2
8c	3	6.9	5.2	8.7	4	4.3
8d	>20	>20	12	>20	11	>20
9a	13.4	>20	>20	11	14.3	>20
9b	>20	>20	>20	>20	>20	>20
9c	>20	>20	>20	>20	>20	>20
9d	>20	>20	>20	15.9	16.5	>20
10a	>20	6.6	7.1	6	11.1	>20
10b	>20	19.1	>20	14.1	18	>20
10c	>20	2.9	>20	8.6	>20	>20
10d	7.5	>20	>20	>20	15.1	16.6

Note: HL-60 = leukemia; A549 = lung; HCT-116 = pancreas; MIAPaCa-2 = pancreas; MCF-7 = breast; fR-2 = normal epithelial cells.

most of the cell lines in the panel. Among the carboxylic acid based molecules, **9a–d** having β-boswellic acid based cap were again found to be inactive against most of the cell lines in the panel whereas, **10a–d** with 11-keto-β-boswellic acid based cap were found to be significantly cytotoxic against some of the cell lines in the panel (please see Table S1 Supporting info.) but all molecules of **10a–d** series did not exhibit significant cytotoxicity against normal cell line fR-2.

Based on preliminary screening lead molecules were chosen for determination of IC_{50} values against the cell lines. Molecule **8a** was found to be most cytotoxic against HL-60 (IC_{50} = 1.6 μM). Also, **8a** was found to be significantly toxic against normal cancer cell line, fR-2 (IC_{50} = 1.9 μM) and had less cytotoxicity against A549, HCT-116, MIAPaCa-2 and MCF-7 cell lines having IC_{50} values of 13, 15.1, 15.1 and 7.1 μM, respectively.

The most specific and potent molecules, that is, **7a**, **7c–d** and **8a–d** having hydroxamic acids attached to their linker group were selected for enzyme based HDAC inhibition evaluation. The **7a**, **7c–d** exhibited IC_{50} values at concentrations 34, 30 and >50 μM. Molecules **8a–d** showed IC_{50} values at concentrations 6, 42, >50 and 48 μM, respectively (Table 2). From the structure and activity data (Table S2, Supporting info.), it becomes clear that glutaric acid based 3C long chain (present in **7a** and **8a**) is the linker that can enhance the anticancer potency of these molecules; however the molecule with 11-keto-β-boswellic acid derived cap (present in **8a**) exhibit better in vitro activity. Compound **3** which does not contain enzyme inhibitor group as well as characteristic linker did not show significant HDAC inhibition even at 50 μM, also the compound **6a** and **10c** having carboxylic ester and acid respectively as a substitute of hydroxamic acid results in significant loss of activity (IC_{50} > 50 μM).

Cancer cells are characterized by high growth rate owing to deregulations in the cell cycle. DNA content of cells varies in different phases of cell cycle. DNA content during G_0/G_1 phase is half

Table 2
HDAC inhibition by different molecules

Molecule	Concn (μM)	% HDAC inhibition	IC_{50} (μM)
7a	50	54 ± 1	34
	30	46 ± 0	
7c	50	56 ± 1	30
	30	50 ± 2	
7d	50	45 ± 1	>50
	30	34 ± 0	
8a	50	88 ± 0	6*
	30	86 ± 1	
	20	70 ± 1	
	10	65 ± 0	
	1	23 ± 2	
8b	50	57 ± 1	42
	30	37 ± 2	
8c	50	39 ± 0	>50
	30	12 ± 0	
8d	50	52 ± 0	48
	30	31 ± 0	

* For determination of IC_{50} values, lower concentrations were used.

that of during G_2/M and during S-phase DNA content is intermediate between G_0/G_1 and G_2/M phases. Using fluorescent DNA intercalating dye propidium iodide as a probe, the percentage of cells in different phases of cell cycle can be detected on flow cytometer. Molecule **8a** which showed least IC_{50} of 6 μM against HDACs and causes 50% HL-60 cell death at 1.6 μM and prominent G_1 arrest at 1 μM concentration in HL-60 cell line. Interestingly, at concentrations of above 1 μM, that is, 5 and 10 μM more prominent cell death was observed (Figs. 2a and b).

It is well known that the mitochondria plays a dual role in cellular physiology; it helps in keeping cell alive by playing pivotal role in aerobic metabolism of the cell and is also involved in apoptotic cell death under such demanding conditions.[13] During apoptotic cell-death, loss of membrane potential across the inner mitochondrial membrane may occur and Rh-123 dye is usually used to measure this.[14] The fluorescence level of Rh-123 decreases with increase in loss of membrane potential. We observed that **8a** induced significant loss in mitochondrial membrane potential at 5 and 10 μM concentrations (Fig. 3). In order to find, whether HDAC specific inhibition by **8a** has any direct role in G_1 arrest, cell death and loss in mitochondrial membrane potential as observed in the present study, another molecule, that is, **7c** was used. Molecule **7c** which was found to be inactive against HL-60 cells and showed IC_{50} of 34 μM against HDACs did not cause any change in percentage of population in G_1 phase and caused only 4.5% and 36% apoptosis at 5 and 10 μM concentrations as against 92.2% and 97.5% at similar concentrations (Figs. 2a and b). **7c** also causes significantly lesser loss of mitochondrial membrane potential as compared to **8a** at the corresponding concentrations (Fig. 3). Altogether, this suggests that the potent HDAC inhibition as induced by **8a** is responsible for cancer cell death.

In order to predict the binding conformation and mode of inhibition of most potent analogs **7c** and **8a**, molecular modeling studies of β-boswellic acid derivatives carried out with HDAC8–SAHA complex retrieved from Protein data bank (PDB ID: 1T69)[15] where in this complex, protein is homotrimeric, consisting three identical chains (377 residues) with zinc as cofactor and SAHA molecule as ligand. Therefore, grid file for docking was constructed considering SAHA ligand as centroid of grid box which has been replaced by compound **7c** and **8a** during the docking studies. Considering both ligand and protein flexible in nature, induce fit docking method has been applied under the default conditions[16] (for details please see Supporting info.). Results demonstrates that zinc binding group,

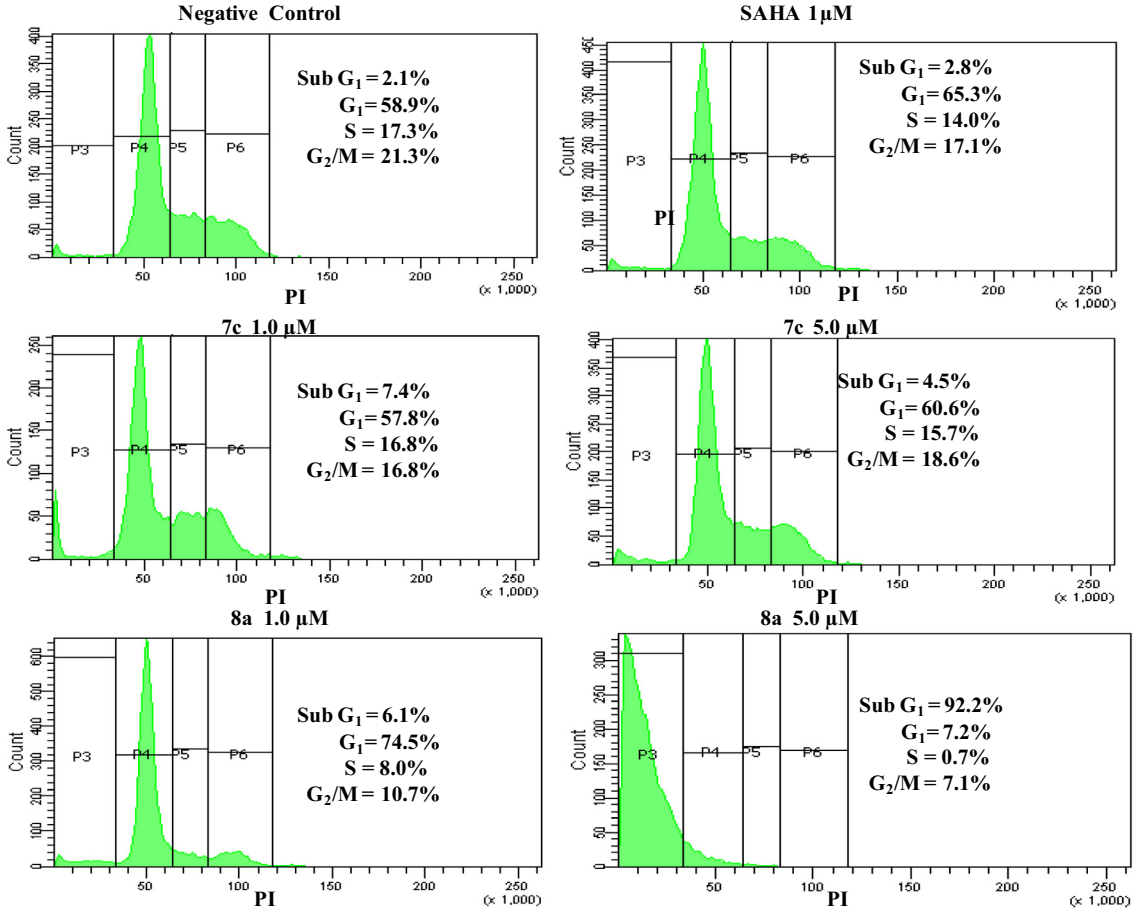


Figure 2a. Effect on distribution of HL-60 cells in different phases of cell cycle on treatment with **7c** and **8a**.

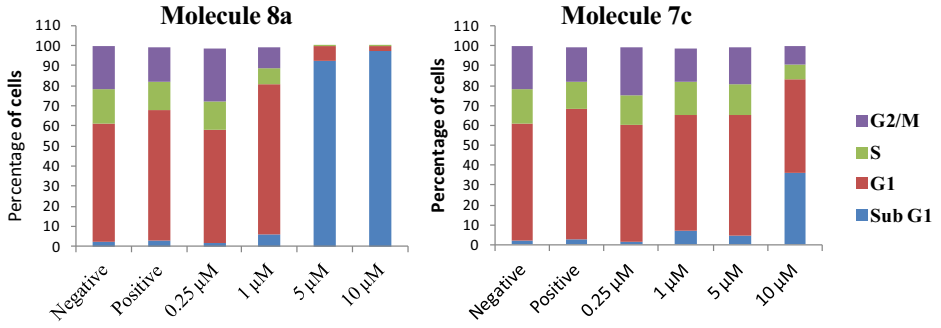


Figure 2b. Graphical representation of distribution of cells in different phases of cell cycle on treatment with **8a** and **7c**.

that is, terminal hydroxamic acid of **7c** and **8a** molecule have identical orientation and interactions in comparison to SAHA molecule except, it does not interact and stabilize the Zn^{2+} ions buried inside catalytic domain probably it might be the reason for moderate loss of inhibitory potency in comparison to SAHA. **7c** interacts with Gly151, Asp101 and Tyr306 residues by H-bonding while **8a** interacts with the Phe208 and Tyr306 by H-bonding. Interestingly, furthermore difference in potency of **7c** and **8a** is attributed to the hydrophobic interactions of linker region and solvent exposed surface interacting cap group conformations (Fig. 4).

In conclusion, biological potential of BAs as a surface receptor has been studied in HDAC inhibition. A simple synthetic methodology has been followed which provide rapid access to the library of molecules. BAs in combination with hydrophobic linker of three carbon chain length and hydroxamic acid as enzyme inhibiting group was found to be the potential molecule showing IC_{50} values of 6 µM against total HDACs. This has tremendous significance considering the role of HDAC inhibition in cancer epigenetic therapy as well recognized but yet less understood. Furthermore, molecule **8a** induces G_1 cell cycle arrest at 1 µM and significant loss of mitochondrial membrane potential at 5 and 10 µM. Further studies at **8a** are aimed to elucidate its mode of inhibition. Notably, several other molecules were found to be significantly cytotoxic against different cancer cell lines, which may be exploited to develop target based anticancer leads of therapeutic significance.

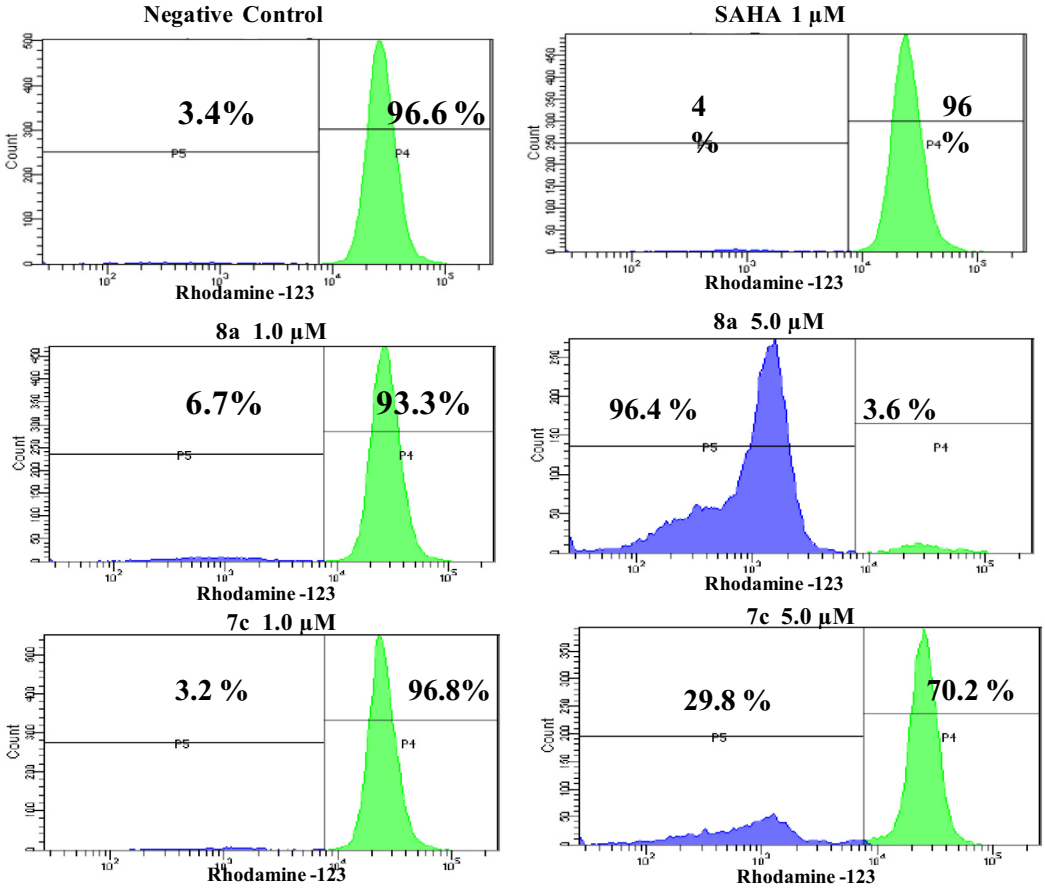


Figure 3. Loss in mitochondrial membrane potential induced by **8a** and **7c**.

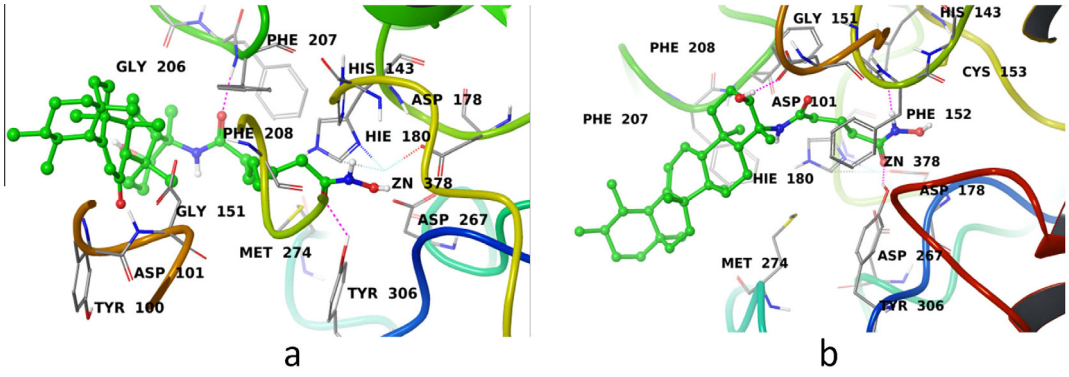


Figure 4. Molecular modeling of potent inhibitors with HDAC-8 (a) interactions for **7c** and (b) interactions for **8a**.

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Supplementary data

Supplementary data (experimental procedures and compound characterization data (^{1}H and ^{13}C NMR)) associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2014.08.007.

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