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Synthesis and biological evaluation of Santacruzamate-A based analogues

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ABSTRACT

Several derivatives of Santacruzamate-A, a natural product that is structurally related to SAHA, were synthesized to explore the potential of carbamates and oxalylamides as novel biasing element for targeting the catalytic site of zinc-dependent histone deacetylases (HDACs). An additional class of Santacruzamate-A derivatives was synthesized to investigate the influence of the cap group and the linker element on HDAC inhibitory activity. All compounds were evaluated in dose response for their in vitro cytotoxic activity in MTT assay in HCT116 cells. HDAC inhibitory activity was evaluated in vitro by western blot analysis for histone hyperacetylation assay and biochemically for representative human HDACs isoforms. Two novel compounds were identified to exhibit potent time dependent anti proliferative activity. However, unlike hydroxamic acid analogues, the tested Santacruzamate-A derivatives showed no noticeable HDAC inhibitory activity. The ethylcarbamate moiety as unusual zinc-binding group displayed no ability to coordinate the zinc ion and thus, presumably, was not able to reproduce known inhibitor-substrate zinc-binding group interactions with the HDAC catalytic site. This study confirmed that the accommodation of the zinc-binding group is deeply critical of the positioning of the linker and the projection of the cap group toward the different surface pockets of the enzyme.

1. Introduction

There is growing evidence that gene expression, governed by epigenetic changes, is crucial for the onset and progression of various diseases, including cancer.[1,2] Epigenetic alterations can cause aberrant gene expression, thus leading to disease. These transcriptional changes are potentially reversible by pharmacologically targeting the enzymes responsible for the epigenetic modifications.[3] In the past decade dedicated drug development efforts in industry and academia have been devoted to providing a panel of next-generation anticancer agents that target the respective epigenetic master regulators responsible for these biological mechanisms.[4–7] Among these, epigenetic enzymes, such as histone deacetylases (HDACs) or histone acetyltransferases (HATs) are altered in tumor cells. The selective targeting of single HDAC isoform has several implications in the differentiation, apoptosis, cell cycle regulation, migration, susceptibility to chemotherapy and angiogenesis.[8,9] Natural products, such as the cyclopeptides FK228 [Romidepsin, Istodax®, **1**, FDA approved for the treatment of cutaneous T-cell lymphoma (CTCL)], Apicidin (**2**) or FR235222 (**3**),[10–12] are used as modulators of specific enzymes implicated in cancer cell proliferation (Fig. 1a) preventing tumorigenesis and cancer progression. These peptide analogues modulate the acetylation level on histones and non-histone proteins in cells, working as specific histone deacetylases inhibitors (HDACIs). The first in class FDA approved HDACI for CTLC is the synthetic pan-HDAC inhibitor SAHA (Vorinostat, Zolinza®, **4**, Fig. 1b). All these compounds are able to drive the silencing or the activation of gene transcription activity at low nanomolar concentrations; because of their pharmacokinetic liabilities (chemical and metabolic instability), new solutions in terms of structure modification are highly desired.[13]

Recently the isolation, structure elucidation and HDAC inhibitory activity of Santacruzamate-A (**5a**, SCA, Fig. 1b) was reported by Balunas and co-workers.[14] The chemical structure of SCA, a simple small molecule from a marine Panamanian cyanobacterium, resembles the SAHA scaffold containing the three canonical

Abbreviations: AcCN, acetonitrile; BnOH, benzyl alcohol; DMF, *N,N*-dimethylformamide; DMSO, dimethylsulphoxide; EtOAc, ethyl acetate; EtOH, ethanol; GABA, γ-aminobutyric acid; GADPH, glyceraldehyde 3-phosphate dehydrogenase; HBTU, *O*-(benzotriazol-1-yl)-*N,N,N′,N′*-tetramethyluronium hexafluorophosphate; MeOH, methanol; *n*-hex, *n*-hexane; NMM, 4-methylmorpholine; SD, standard deviation; TEA, triethylamine; THF, tetrahydrofuran.

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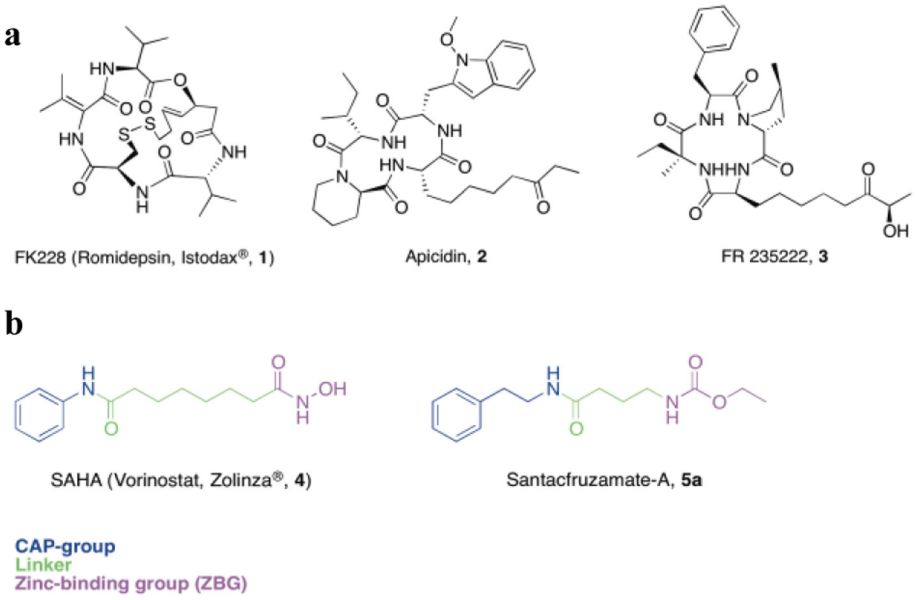


Fig. 1. (a) Structure of natural HDAC inhibitors. (b) Structure of SAHA, **4** and Santacruzamate-A, **5a** and their structure similarities based on HDAC inhibitor pharmacophore model.

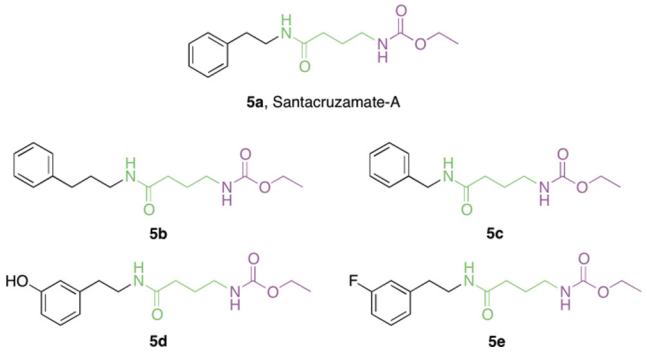


Fig. 2. Chemical structure of class I SCA-like small molecules **5a**–**e**.

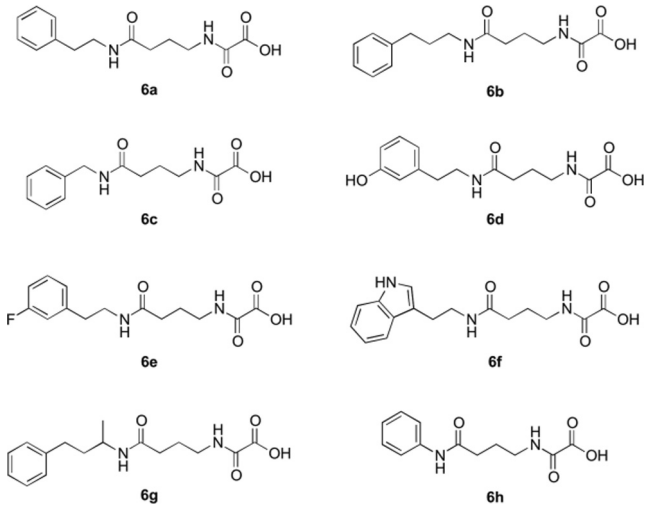


Fig. 3. Chemical structure of class II SCA-like small molecules **6a**–**h**.

structural motifs of HDAC inhibitor pharmacophore model including a zinc-binding group (ZBG), an aliphatic linker and a cap group (CAP) for additional enzyme interactions.[15–17] Initially, we were intrigued by the ethylcarbamate moiety, as unusual ZBG, reported to inhibit histone deacetylases in the nanomolar range. Therefore, we synthesized a small collection of SCA-analogues herein reported as class I SCA-like small molecules (**5b**–**e**, Fig. 2) following synthetic procedure previously described.[14]

Then, in an effort to re-evaluate the previously reported antiproliferative and HDAC inhibitory activities, we pursued the synthesis of a class II SCA-like small molecules (**6a**–**h**) introducing a terminal oxamic acid as new zinc chelating moiety on γ-aminobutyric acid-linker (GABA-linker), with different CAP groups (Fig. 3).[18]

2. Results and discussion

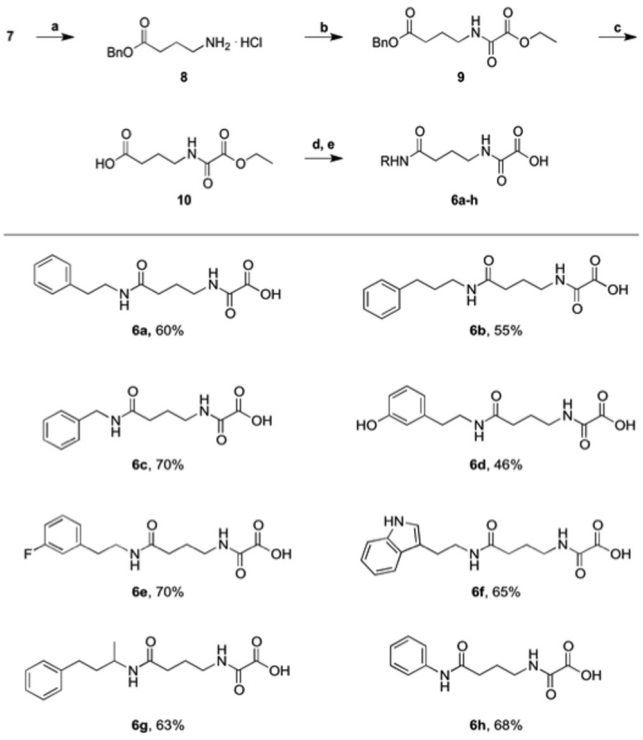
2.1. Chemistry

Class I SCA-like small molecules (**5b**–**e**) were obtained according to the synthetic procedure reported by Balunas and co-workers (see also Supporting information, Scheme 1SI).[14]

Class II SCA-like analogues were synthesized starting from GABA benzyl ester **8**,[19] which was readily converted into corresponding amide-ester **9**. The debenzylation of **9** with H_2 and Pd/C provided access to the corresponding carboxylic acid **10** in very good yield. Coupling with various aromatic amines[20] and subsequent one-pot saponification furnished the target compounds **6a**–**h** in good yields and purity (Scheme 1).

2.2. Biology

To evaluate the antiproliferative activity of class I and II SCA analogous (**5a**–**e** and **6a**–**h**), we treated HCT116 colorectal cancer cells in dose response MTT assay (Table 1). As expected, we found that SAHA significantly inhibited the viability of HCT116 cells in a dose and time dependent manner after 24 h (IC_{50} 5 μM), 48 h (1 μM < IC_{50} < 5 μM) and 72 h (IC_{50} < 1 μM) of treatment (Table 1). HDAC inhibitory activity was tested by western blot analysis using histone hyperacetylation as well established biomarker (Fig. 4) and inhibitory activity for various HDAC isoforms was determined in biochemical assays[21] (data not shown/see Supporting information). Consistent with recently published results and contrary to



Scheme 1. Synthesis of class II SCA-like compounds **6a**–**h**. Reagents and conditions: (a) BnOH, $SOCl_2$, 3 h, 0 °C to r.t., 80%; (b) ethylchloroxacetate, TEA, dry DMF, 1.5 h, 0 °C to r.t., 79%; (c) H_2, Pd/C, MeOH, r.t., 45 min, 97%; (d) RNH_2, HBTU, NMM, dry AcCN, 4 h, r.t.; (e) LiOH, THF/H_2O (1:1, v/v), 4 h, r.t., 46–70%.

Table 1
MTT Assay results of SCA-like molecules class I and II in HCT116 cell line.

Entry	Compounds	IC_{50}[a,c] (μM)		
		24 h	48 h	72 h
SCA-like Class I compounds				
1	[d]**5a**, **SCA**	>50	>50	>50
2	**5b**	>50	>50	>50
3	**5c**	>50	n.d.[b]	0.615 ± 0.049
4	**5d**	>50	0.476 ± 0.042	1 < IC_{50} < 5
5	**5e**	>50	0.825 ± 0.054	5 < IC_{50} < 10
SCA-like Class II compounds				
6	**6a**	>50	>50	n.d.
7	**6b**	>50	n.d.	n.d.
8	**6c**	n.d.	n.d.	n.d.
9	**6d**	>50	>50	n.d.
10	**6e**	>50	n.d.	n.d.
11	**6f**	<50	n.d.	n.d.
12	**6g**	n.d.[b]	n.d.	n.d.
13	**6h**	0,814 ± 0,069	>50	n.d.
14	[d]**SAHA**	5	1 < IC_{50} < 5	0.877 ± 0.019

[a] Anti-proliferative activity of compounds **5a**–**e** and **6a**–**h** in HCT116 cell line is expressed as cell viability. Results are expressed as mean of three independent experiments ± s.e.m.

[b] n.d.: not determined; indicates no relevant cytotoxic activity or registered values are not fit in a IC_{50} curve.

[c] Compounds are tested by using serial dilution starting from 50 μM, 10 μM, 5 μM, 1 μM.

[d] Control compounds (SAHA e SCA) were tested by using serial dilution starting from 50 μM.

the original report,[22–24] we found that Santacruzamate-A (**5a**) had no significant antiproliferative activity in HCT116 cells. Furthermore, we did not observe inhibition of HDAC activity in HCT116 (using histone acetylation as biomarker) and in biochemical assays probing individual HDAC isoforms (see Supplementary Material for

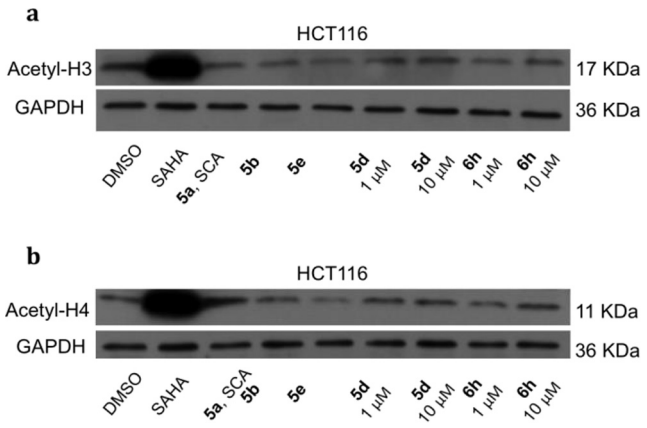


Fig. 4. Representative western blot analysis of total acetyl-histone-H3 (a) and total acetyl-histon-H4 (b) expression in total protein lysates from HCT116 cells untreated (DMSO) or treated for 24 h with the compounds (10μM if not otherwise specified) (mean ± SD). GAPDH served as the loading control. Data are representative of three different experiments with similar results.

HDAC inhibition enzymatic assay) (entry 1, Table 1 IC_{50} > 50 μM). Interestingly, the novel class I SCA-like molecules **5d** and **5e** showed potent time dependent antiproliferative activity at both 48h and 72h treatment (entry 4 and 5, Table 1). Similar results were obtained for the class II SCA-like compound **6h**, which was most pronounced after a 24 h (IC_{50} = 1 μM) treatment (entry 13, Table 1).

To evaluate the role of HDAC-inhibition in the anti-proliferative effects, the total amount of acetyl-histone-H3 and acetyl-Histone-H4 were analysed in HCT116 cell line treated with selected molecules (10 μM for 24 h, Fig. 4a, b). Compounds **5d** (10 μM) and **6h** (10 μM) did not significantly increase of histone H3 and H4 acetylation compared to vehicle treated cells, while SAHA treatment strongly increased acetylation of H3 and H4 (Fig. 4).

These data are in agreement with the absence of significant biochemical HDAC inhibitory activity for representative class I and class II HDAC isoforms (HDAC1,3,6,8) (see Supplementary figure "HDAC 16 h preincubation"). Together, these results suggested that class I SCA-like molecules exert their cytotoxic activity by an HDAC independent mechanism. Additional experiments to elucidate the underlying mode of action of the antiproliferative activity of compounds **5d**, **5e**, **6h** are currently underway.

3. Conclusion

In conclusion, aiming to clarify and better elucidate the SAR of Santacruzamate-A the putative HDAC inhibitory activity, we pursued the synthesis of natural product SCA **5a**, class I (**5b**–**e**) and class II (**6a**–**h**) SCA-like analogues. We modified alternatively the CAP, by using several aromatic amines, and the unusual ethyl-carbamate moiety (**5b**–**e**) or oxamic acid group (**6a**–**h**) as ZBG and identified class I SCA-like molecules (**5d** and **5e**), and compound **6h**, featuring a novel oxamic acid as ZBG, to have potent antiproliferative activity in HCT116 cancer cells. However, biochemical profiling, in vitro MTT viability studies and western blot analysis for histone hyperacetylation in HCT116 cells confirmed that Santacruzamate-A and analogues lack HDAC inhibitory activity. These results may have a structural interpretation based on the absence in Santacruzamate-A and its derivatives of a moiety involved in zinc coordination in the HDAC catalytic site. The hydroxamic acid of SAHA is replaced in Santacruzamate-A by an ethyl-carbamate moiety devoid of any zinc coordination ability. With this study, we confirmed that the accommodation of the ZBG into the HDAC catalytic site is a crucial step of the inhibition process and is finely

controlled by its zinc coordination ability and by key interactions with the surrounding protein residues.

4. Experimental section

4.1. Chemistry

All reagents were purchased from Sigma-Aldrich (Milan, Italy) in the highest available purity and were used as received. All reactions involving air or moisture sensitive reagents were carried out under a dry nitrogen atmosphere using dry solvents. Dry DMF, AcCN and MeOH were purchased and used without further distillation. When necessary, compounds were dried *in vacuo* over P_2O_5 or by azeotropic removal of water with toluene under reduced pressure. Reaction temperatures were measured externally; reactions were monitored by TLC on Merck silica gel plates (0.25 mm) and visualized by UV light, $KMnO_4$, *p*-anisaldehyde, or ninhydrin solutions and drying. Flash chromatography was performed on Merck silica gel 60 (particle size: 0.040–0.063 mm) and the solvents employed were of analytical grade. Yields refer to chromatographically and spectroscopically (1H and 13C NMR) pure compounds. NMR spectra were generally recorded at room temperature, on Bruker Avance series 400 and 300 spectrometer. Chemical shifts (δ) are reported in ppm relatively to the residual solvent peak ($CHCl_3$, δ: 7.26, $13CDCl_3$, δ: 77.0; CD_2HOD, δ: 3.35, $13CD_3OD$, δ: 49.0) and the multiplicity of each signal is designated by the following abbreviations: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; br, broad; app, apparent. Coupling constants (*J*) are quoted in Hz. High resolution mass spectra (HRMS) were recorded on a high resolution mass spectrometer equipped by electrospray (ESI) and nanospray sources, and a quadrupole-time of flight hybrid analyser, coupled with capillary UPLC system (Q-TOF Premier/nanoAquity, Waters) in positive mode, and either protonated molecular ions $[M+H]^+$ were used for empirical formula confirmation, unless otherwise stated.

4.1.1. Synthetic procedures

4.1.1.1. Benzyl 4-aminobutanoate hydrochloride, ***8***. To a ice-cold solution of benzyl alcohol (5 mL) and GABA **7** (516 mg, 5 mmol), $SOCl_2$ (547 μL, 7.5 mmol) was added dropwise and the reaction stirred at room temperature for 3 h under N_2 dry atmosphere. After that period the uncoloured solution passed through a pale yellow solution. The solvent was evaporated up to the formation of a white solid. Crystallization of the crude in EtOH/*n*-hex (1:1, v/v) at 4 °C furnished the pure hydrochloride **9** (920 mg, 80%). ^{1}H NMR (400 MHz, Deuterium Oxide) δ: 7.48 (d, *J* = 3.7 Hz, 3H), 5.20 (s, 2H), 3.05 (t, *J* = 7.8 Hz, 2H), 2.58 (t, *J* = 7.3 Hz, 2H), 1.99 (p, *J* = 7.4 Hz, 2H) ppm. ^{13}C NMR (101 MHz, Deuterium Oxide) δ: 174.72, 135.57, 128.88, 128.79, 128.71, 128.38, 67.19, 38.70, 30.76, 22.03 ppm. HRMS (ESI-Q-TOF) *m*/*z* $[M+H]^+$ Calcd. for $C_{11}H_{16}NO_2$ 194.1176; Found 194.1170.

4.1.1.2. Benzyl 4-(2-ethoxy-2-oxoacetamido) butanoate, ***9***. In an oven dried round bottom flask, to an ice-cold DMF solution (5 mL) of **9** (300 mg, 1.31 mmol) ethylchloroxacetate (146 μL, 1.31 mmol) and TEA (548 μL, 3.93 mmol) were added and the reaction stirred under N_2 dry atmosphere for 30 min at 0 °C and then the mixture was allowed to reach room temperature for an additional 1 h. The reaction was monitored by TLC up to starting material disappeared. The solvent was removed *in vacuo* and the mixture washed with $NaCl_{ss}$ and extracted with EtOAc. The organic fractions were collected, dried under Na_2SO_4 and filtered. The crude was purified by column chromatography (*n*-hex/EtOAc 7:3) to furnish **10** as yellowish oil (304 mg, 79%). ^{1}H NMR (400 MHz, Methanol d_4) δ: 7.36 (dd, *J* = 13.4, 3.8 Hz, 4H), 5.14 (s, 2H), 4.33 (q, *J* = 7.1 Hz, 2H), 3.36 (s, 2H), 2.45 (t, *J* = 7.3 Hz, 3H), 1.90 (p, *J* = 7.1 Hz, 3H), 1.37 (t, *J* = 7.1 Hz, 4H) ppm. ^{13}C NMR (101 MHz, Methanol d_4) δ: 174.50, 161.47, 159.46, 137.61, 132.39, 129.86, 129.53, 129.18, 67.33, 63.80, 40.04, 32.29, 25.32, 14.23 ppm. HRMS (ESI-Q-TOF) *m*/*z* $[M+H]^+$ Calcd. for $C_{15}H_{20}NO_5$ 294.1336; Found 294.1345.

4.1.1.3. 4-(2-Ethoxy-2-oxoacetamido) butanoic acid, ***10***. In an oven dried round bottom flask, to a solution of **10** (300 mg, 1.03 mmol) in 20 mL of MeOH, catalytic amount of Pd/C was added. After three vacuum/H_2 (1 atm) atmosphere cycling operations, the reaction was stirred at room temperature for 45 min till starting material disappearing. The mixture was then filtered (*attention: the residue Pd may be pyrophoric*) by Celite and the solvent evaporated *in vacuo* to furnish compound **11** as white solid (197 mg, 97%). ^{1}H NMR (400 MHz, Methanol d_4) δ: 4.33 (q, *J* = 7.1 Hz, 2H), 3.34 (t, *J* = 7.0 Hz, 2H), 2.36 (t, *J* = 7.4 Hz, 2H), 1.87 (p, *J* = 7.2 Hz, 2H), 1.36 (t, *J* = 7.1 Hz, 3H) ppm. ^{13}C NMR (101 MHz, Methanol d_4) δ: 63.80, 40.12, 32.20, 25.34, 14.22 ppm. HRMS (ESI-Q-TOF) *m*/*z* $[M+H]^+$ Calcd. for $C_8H_{14}NO_5$ 204.0866; Found 204.0861.

4.1.2. General procedure for the synthesis of class II SCA-like molecules ***6a–h***

4.1.2.1. 2-Oxo-2-((4-oxo-4-(phenethylamino)butyl)amino)acetic acid, ***6a***. In an oven dried round bottom flask, to a ice cold solution of **11** (20 mg, 0.098 mmol) in 1 mL of dry AcCN, HBTU (48 mg, 0.13 mmol) and NMM (38.0 μL, 0.34 mmol) were added. After 5 min magnetic stirring, phenylethylamine (11.9 mg, 0.098 mmol, 12 μL) was added at 0 °C and then the mixture was stirred at room temperature over night under N_2 dry atmosphere. After that period, the mixture was monitored via TLC and at the end of the reaction the mixture was washed with twice with NH_4Cl_{ss}, Na_2CO_{3ss}, $NaCl_{ss}$ and extracted with DCM. The organic phase were collected and dried under Na_2SO_4, and filtered. The crude was directly dissolved in H_2O/THF mixture (1:1 v/v, 1.6 mL) then LiOH added (47 mg, 1.96 mmol) and the reaction kept stirred for additional 14 h. After this period, the mixture was acidified up to pH 7 with HCl 2 N and then extracted twice with EtOAc. The organic fractions were collected, dried under Na_2SO_4 and filtered. Purification by column chromatography (DCM/MeOH 95:5) furnished compound **6a** as a white solid (16 mg, 60%). ^{1}H NMR (400 MHz, Methanol d_4) δ: 7.29 (t, *J* = 7.3 Hz, 1H), 7.26–7.17 (m, 2H), 3.42 (t, *J* = 7.4 Hz, 2H), 3.27 (t, *J* = 6.9 Hz, 2H), 2.81 (t, *J* = 7.3 Hz, 2H), 2.21 (t, *J* = 7.4 Hz, 2H), 1.83 (p, *J* = 7.1 Hz, 2H) ppm. ^{13}C NMR (101 MHz, Methanol d_4) δ: 175.22, 159.11, 140.51, 129.81, 129.47, 127.34, 41.96, 40.22, 36.50, 34.30, 26.30. HRMS (ESI-Q-TOF) *m*/*z* $[M+H]^+$ Calcd. for $C_{14}H_{19}N_2O_4$ 279.1339; Found 279.1349.

4.1.2.2. 2-Oxo-2-((4-oxo-4-((3-phenylpropyl)amino)butyl)amino) acetic acid, ***6b***. The synthesis of compound **6b** was carried out following the general procedure for the synthesis of class II SCA-like molecules using **11** (20 mg, 0.098 mmol) in 1 mL dry AcCN and HBTU (48.3 mg, 0.13 mmol), NMM (38.0 μL, 0.34 mmol), propylphenylamine (14 μL, 0.098 mmol), LiOH (47 mg, 1.96 mmol) in H_2O/THF mixture (1:1 v/v, 1.6 mL) furnishing final product **6b** as a white solid (16 mg, 55%). ^{1}H (400 MHz, Methanol d_4) δ: 7.27 (t, *J* = 7.4 Hz, 1H), 7.24–7.13 (m, 2H), 3.35–3.29 (m, 3H), 3.21 (t, *J* = 7.1 Hz, 2H), 2.65 (t, *J* = 7.7 Hz, 2H), 2.24 (t, *J* = 7.4 Hz, 2H), 1.85 (dp, *J* = 15.9, 7.3 Hz, 4H) ppm. ^{13}C NMR (101 MHz, Methanol d_4) δ: 175.26, 166.68, 159.12, 142.99, 129.40, 126.88, 40.27, 40.10, 34.31, 34.21, 32.21, 26.32 ppm. HRMS (ESI-Q-TOF) *m*/*z* $[M+H]^+$ Calcd. for $C_{15}H_{21}N_2O_4$ 293.1496; Found 293.1488.

4.1.2.3. 2-((4-(Benzylamino)-4-oxobutyl)amino)-2-oxoacetic acid, ***6c***. The synthesis of compound **6c** was carried out following the general procedure for the synthesis of class II SCA-like molecules using **11** (20 mg, 0.098 mmol) in 1 mL dry AcCN and HBTU (48.3 mg,

0.13 mmol) e NMM (38.0 μL, 0.34 mmol), benzylamine (11 μL, 0.098 mmol), LiOH (47 mg, 1.96 mmol) in H_2O/THF mixture (1:1 v/v, 1.6 mL) furnishing final product **6c** as a white solid (18 mg, 70%). ^{1}H NMR (400 MHz, Methanol d_4) δ: 7.47–7.11 (m, 4H), 3.35–3.30 (m, 3H), 2.30 (t, J = 7.5 Hz, 2H), 1.89 (p, J = 7.1 Hz, 2H) ppm. ^{13}C NMR (101 MHz, Methanol d_4) δ: 175.14, 161.96, 159.13, 139.96, 129.54, 128.56, 128.20, 128.15, 44.14, 40.27, 34.27, 26.31 ppm. HRMS (ESI-Q-TOF) m/z $[M+H]^+$ Calcd. for $C_{13}H_{17}N_2O_4$ 265.1183; Found 265.1176.

*4.1.2.4. 2-((4-((3-Hydroxyphenethyl)amino)-4-oxobutyl)amino)-2-oxoacetic acid, **6d**.* The synthesis of compound **6d** was carried out following the general procedure for the synthesis of class II SCA-like molecules using **11** (40 mg, 0.196 mmol) in 2 mL dry AcCN and HBTU (97.08 mg, 0,256 mmol) e NMM (75 μL, 0.686 mmol), 3-(2-aminoethyl)phenol hydrochloride (33 mg, 0.196 mmol), LiOH (94 mg, 3.92 mmol) in H_2O/THF mixture (1:1 v/v, 3.2 mL) furnishing final product **6d** as a white solid (27 mg, 46%). ^{1}H NMR (400 MHz, Methanol d_4) δ: 7.08 (t, J = 7.8 Hz, 1H), 6.65 (dt, J = 15.1, 7.9 Hz, 2H), 3.38 (t, J = 7.4 Hz, 2H), 3.26 (t, J = 6.9 Hz, 2H), 2.71 (t, J = 7.3 Hz, 2H), 2.19 (t, J = 7.4 Hz, 2H), 1.81 (p, J = 7.2 Hz, 2H) ppm. ^{13}C NMR (101 MHz, Methanol d_4) δ: 175.23, 161.95, 159.11, 158.55, 141.98, 130.45, 121.00, 116.65, 114.27, 41.92, 40.22, 36.45, 34.33, 34.30, 26.31 ppm. HRMS (ESI-Q-TOF) m/z $[M+H]^+$ Calcd. for $C_{14}H_{19}N_2O_5$ 295.1288; Found 295.1296.

*4.1.2.5. 2-((4-((3-Fluorophenethyl)amino)-4-oxobutyl)amino)-2-oxoacetic acid, **6e**.* The synthesis of compound **6e** was carried out following the general procedure for the synthesis of class II SCA-like molecules using **11** (20 mg, 0.098 mmol) in 1 mL dry AcCN and HBTU (48.3 mg, 0.13 mmol) e NMM (38.0 μL, 0.34 mmol), 3-fluorophenylethylamine (33 mg, 0.196 mmol), LiOH (47 mg, 1.96 mmol) in H_2O/THF mixture (1:1 v/v, 1.6 mL) furnishing final product **6e** as a white solid (20 mg, 70%). ^{1}H NMR (400 MHz, Methanol d_4) δ: 7.28 (q, J = 7.4 Hz, 1H), 7.03 (d, J = 7.5 Hz, 1H), 7.00–6.88 (m, 2H), 3.41 (t, J = 7.1 Hz, 2H), 3.25 (t, J = 6.7 Hz, 2H), 2.80 (t, J = 7.1 Hz, 2H), 2.19 (t, J = 7.3 Hz, 2H), 1.81 (p, J = 6.8 Hz, 2H) ppm. ^{13}C NMR (101 MHz, Methanol d_4) δ: 175.26, 165.55, 163.13, 161.96, 159.10, 143.42, 143.35, 131.16, 131.07, 125.74, 125.71, 116.57, 116.36, 114.12, 114.10, 113.91, 113.88, 41.59, 40.20, 36.16, 34.31, 34.26, 26.29 ppm. HRMS (ESI-Q-TOF) m/z $[M+H]^+$ Calcd. for $C_{14}H_{18}FN_2O_4$ 297.1245; Found 297.1253.

*4.1.2.6. 2-((4-((2-(1H-Indol-3-yl)ethyl)amino)-4-oxobutyl)amino)-2-oxoacetic acid, **6f**.* The synthesis of compound **6f** was carried out following the general procedure for the synthesis of class II SCA-like molecules using **11** (20 mg, 0.098 mmol) in 1 mL dry AcCN and HBTU (48.3 mg, 0.13 mmol) e NMM (38.0 μL, 0.34 mmol), tryptamine (15.7 mg, 0.098 mmol), LiOH (47 mg, 1.96 mmol) in H_2O/THF mixture (1:1 v/v, 1.6 mL) furnishing final product **6f** as a white solid (20 mg, 65%). ^{1}H NMR (400 MHz, Methanol d_4) δ: 7.55 (d, J = 7.8 Hz, 1H), 7.32 (d, J = 8.0 Hz, 1H), 7.11–7.03 (m, 2H), 6.99 (t, J = 7.4 Hz, 1H), 3.47 (t, J = 7.2 Hz, 2H), 3.24 (t, J = 6.9 Hz, 2H), 2.94 (t, J = 7.2 Hz, 2H), 2.18 (t, J = 7.4 Hz, 2H), 1.80 (p, J = 7.1 Hz, 2H) ppm. ^{13}C NMR (101 MHz, Methanol d_4) δ: 175.22, 161.94, 159.07, 138.15, 128.81, 123.59, 123.52, 123.43, 122.29, 119.56, 119.27, 113.25, 112.27, 112.21, 41.42, 41.32, 40.23, 34.36, 26.27, 26.20 ppm. HRMS (ESI-Q-TOF) m/z $[M+H]^+$ Calcd. for $C_{16}H_{20}N_3O_4$ 318.1448; Found 318.1454.

*4.1.2.7. 2-Oxo-2-((4-oxo-4-((4-phenylbutan-2-yl)amino)butyl)amino) acetic acid, **6g**.* The synthesis of compound **6g** was carried out following the general procedure for the synthesis of class II SCA-like molecules using **11** (20 mg, 0.098 mmol) in 1 mL dry AcCN and HBTU (48.3 mg, 0.13 mmol) e NMM (38.0 μL, 0.34 mmol), 4-phenylbutan-2-amine (16 μL, 0.098 mmol), LiOH (47 mg, 1.96 mmol) in H_2O/THF mixture (1:1 v/v, 1.6 mL) furnishing final product **6g** as a white solid (19 mg, 63%). ^{1}H NMR (400 MHz, Methanol d_4) δ: 4.33 (q, J = 7.1 Hz, 2H), 3.34 (t, J = 7.0 Hz, 2H), 2.36 (t, J = 7.4 Hz, 2H), 1.87 (p, J = 7.2 Hz, 2H), 1.36 (t, J = 7.1 Hz, 3H) ppm. ^{13}C NMR (101 MHz, Methanol d_4) δ: 63.80, 40.12, 32.20, 25.34, 14.22 ppm. HRMS (ESI-Q-TOF) m/z $[M+H]^+$ Calcd. for $C_{16}H_{23}N_2O_4$ 307.1652; Found 307,1661.

*4.1.2.8. 2-Oxo-2-((4-oxo-4-(phenylamino)butyl)amino)acetic acid, **6h**.* The synthesis of compound **6h** was carried out following the general procedure for the synthesis of class II SCA-like molecules using **11** (20 mg, 0.098 mmol) in 1 mL dry AcCN and HBTU (48.3 mg, 0.13 mmol) e NMM (38.0 μL, 0.34 mmol), aniline (7.5 μL, 0.098 mmol), LiOH (47 mg, 1.96 mmol) in H_2O/THF mixture (1:1 v/v, 1.6 mL) furnishing final product **6h** as a white solid (17 mg, 68%). ^{1}H NMR (400 MHz, Methanol d_4) δ: 7.53 (d, J = 8.0 Hz, 2H), 7.29 (t, J = 7.8 Hz, 2H), 7.08 (t, J = 7.4 Hz, 1H), 3.37 (d, J = 6.9 Hz, 2H), 2.41 (t, J = 7.4 Hz, 2H), 1.94 (p, J = 7.1 Hz, 2H) ppm. ^{13}C NMR (101 MHz, Methanol d_4) δ: 173.66, 161.93, 159.17, 139.81, 129.75, 125.15, 125.08, 121.32, 121.27, 40.27, 35.13, 26.13 ppm. HRMS (ESI-Q-TOF) m/z $[M+H]^+$ Calcd. for $C_{12}H_{15}N_2O_4$ 251.1026; Found 251, 1019.

4.2. Biology

4.2.1. Cell cultures, treatments and cell viability assay

Human colorectal cancer cell (CRC) lines HCT116 and DLD1 were obtained from the Interlab Cell Line Collection (IST, Genoa, Italy) and grown in McCoy's 5A and RPMI medium respectively, at 37 °C in a 5% CO_2 atmosphere. Cells were seeded at a density of 1×10^5 cells/well and exposed to increasing concentrations of compounds. To evaluate cell viability the colorimetric MTT (3-(4,5 di-methylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) metabolic activity assay was performed as described previously.[25] Briefly, MTT stock solution (5 mg/mL in PBS, Sigma) was added to each well and incubated for 4 h at 37 °C in humidified CO_2. The formazan crystals were solubilized with acidic isopropanol (0.1 N HCl in absolute isopropanol). MTT conversion to formazan by metabolically viable cells was monitored by spectrophotometer at an optical density of 595 nm. Each data point represents the average of three separate experiments in triplicate.

4.2.2. Western blot analysis

Cells were seeded in 60-mm dishes, scraped and washed with ice-cold phosphate buffer saline (PBS). After treatments at indicated time points, western blot analysis was performed as previously described.[26] Briefly, total protein extracts were obtained through lysis in buffer A (50 mM Tris–HCl pH 8.0 buffer containing 150 mM NaCl, 1% Nonidet P-40, 2 mg/mL aprotinin, 1 mg/mL pepstatin, 2 mg/mL leupeptin, 1 mM Na_3VO_4). Protein concentration was determined by the Bradford assay using bovine serum albumin as standard. About 30 μg of proteins were loaded on 15% SDS–polyacrylamide gels under reducing conditions. After SDS–PAGE, proteins were transferred to nitrocellulose membranes that were blocked with 5% milk (Bio-Rad Laboratories, Inc.) and incubated with specific antibodies, anti-acetyl-Histone H4 (Santa Cruz Biotechnology), anti-acetyl-Histone H3 (Millipore) and GAPDH (Cell Signaling). Filters were washed and incubated with horseradish peroxidase-conjugated secondary antibodies. Membranes were stained using a chemoluminescence system (ECL-Amersham Biosciences, Glattbrugg, CH) and then exposed to X-ray film (Kodak, Rochester, NY).

A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.bmc.2017.10.026.

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