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Synthesis and biological evaluation of santacruzamate A and analogs as potential anticancer agents†

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Santacruzamate A, a recently discovered natural product from a Panamanian marine cyanobacterium *Symploca* sp., features a similar structure to the clinically used histone deacetylase (HDAC) inhibitor vorinostat (**SAHA**). We have synthesized the natural product and a small set of analogues for SAR studies. To our surprise, the synthetic natural product santacruzamate A (**1a**) and the analogues did not show an obvious inhibition even at 2 μM in HDAC enzyme assays while the IC_{50} value was 0.12 nM in the original report. However, a novel compound, **5**, containing a terminal thiourea motif was found to inhibit the growth of malignant cells at submicromolar concentrations. Moreover, **5** was not cytotoxic to normal human colonic epithelial cells CCD841, suggesting that its cytotoxicity was specific to cancer cells. Further investigation indicated that the compound induced apoptosis, affected cell cycle progression and increased ROS production. We believe its mechanism of action is unrelated to HDAC inhibition and the original activity reported for santacruzamate needs to be reevaluated.

Tumors, the result of abnormal cells with uncontrolled, rapid and pathological proliferation, cause one of the most formidable afflictions globally.[1,2] Apart from the use of surgical treatment and irradiation, chemotherapy still remains the main therapeutic strategy to treat cancer.[3,4] However, one of the major hurdles in cancer chemotherapy is attributed to the prevalence of drug and multidrug resistance and the need for selectivity against normal cells.[5–7] Therefore, considerable efforts have been made on the design and discovery of new anticancer agents, focusing on the search of novel chemical entities for the successful treatment of cancer.[8,9]

Nature has been continuously providing humans with important leads and natural product medicines for the treatment of a wide spectrum of diseases.[10–12] Natural products have been a particularly important source of anticancer chemotherapeutic medicines including taxanes, *Vinca* alkaloids and camptothecin that act upon the mitotic spindle.[13] This trend is likely to continue as natural products are identified that modulate specific signaling pathways in cells. One example is the relatively new field of epigenetics relating to chromatin modelling *via* structural modifications of the DNA and histone proteins. A variety of natural products have already been reported that inhibit the enzymes involved in epigenetics.[14] Among these, the histone deacetylase (HDAC) family of enzymes has received much attention. HDACs are the enzymes that hydrolyse acetyl-lysine amino acid residues in proteins back to lysine and play crucial roles in diverse cellular functions, while their overexpression or mutation is widely observed in cancer cells.[15–17] A number of potent natural product HDAC inhibitors such as trichostatin A and apicidin are used as biological tools while the depsipeptide FK228 (romidepsin) has received FDA approval for the treatment of cutaneous T-cell lymphoma (CTCL).

Recently, Balunas and co-workers reported the isolation of santacruzamate A (**1a**) from a marine Panamanian cyanobacterium resembling the genus *Symploca*.[18] Santacruzamate A shares some structural similarity with the synthetic HDAC inhibitor vorinostat (**SAHA**), the first clinically approved drug in this class (Fig. 1). It was reported that santacruzamate A specifically inhibited the isoform HDAC2 with an IC_{50} of 0.12 nM. This was a surprising result given the established SAR of HDAC inhibitors in which a zinc-binding group, such as the hydroxamic acid in vorinostat, is important for reversible binding to the enzyme active site.[19] Instead, santacruzamate A contains a carbamate and amide-functional groups that have not been previously associated with potent HDAC inhibition. The natural product azumamide A, for example, contains a

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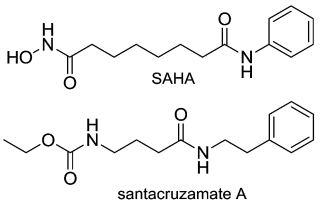


Fig. 1 The structures of santacruzamate A and SAHA.

carboxamide zinc-binding group and is only micromolar in HDAC inhibition.[20,21] Furthermore, the high selectivity of santacruzamate A for HDAC2 was intriguing as **SAHA** itself is a non-selective inhibitor active against both Class I and Class II HDAC isoforms. With this background, it was of interest to synthesize santacruzamate A as well as a series of analogues to investigate the structure–activity relationships (SAR) of this new lead or the development of anticancer agents. This has led to the identification of a potent cytotoxic compound **5** that we nevertheless believe acts by a HDAC-independent mechanism of action.

To investigate the importance of the linker in santacruzamate A between the carbamate and amide structural features, our SAR strategy was to move around the position of the amide and also to replace the terminal ethoxycarbonyl group with other bioisosteric functional groups. Thus, a series of compounds were designed and prepared alongside with the natural product santacruzamate A itself (**1a**) (Fig. 2).

The synthetic route to obtain these compounds is shown in Scheme 1. Conventional acylation of the terminal amino groups of commercial available amino acids **6** with ethyl chloroformate gave compounds **7a–d**, while protection of **6c** with di(*tert*-butyl) carbonate (Boc_2O) afforded **8**. Amidation of the carboxylic acid groups of **7a–d** and **8** with amines or aniline afforded the desired target products **1a–d**, **2** and **3**. After removal of the Boc group from **3** under acidic conditions, the obtained free amine **9** underwent subsequent treatment with either isocyanatoethane or ethyl isothiocyanate to give two additional analogues **4** and **5**.

After the synthesis of the designed compounds **1a–d**, and **3–5**, they were subsequently screened for their cellular biological activity. To our surprise, synthetic santacruzamate A, *i.e.* **1a** did not show cytotoxicity against human colon cancer cells HCT-116 even at 100 μM while most of the other synthetic analogues were inactive (Table 1). However, compound **5** inhibited the growth of HCT-116 cells and human myeloblastic leukemia cells ML-1 with IC_{50} values of 6.0 and 9.4 μM. To our delight, **5** was not cytotoxic to normal cells (CCD841) even at 100 μM. Indeed, it is high desirable to obtain a compound with a high selectivity to kill malignant cancer cells because most of clinical anticancer drug also kill normal cells during therapeutical treatment. For example, **SAHA** was very cytotoxic to CCD841 cells at 20 μM although with its lower IC_{50} value to cancer cells.

Next, we performed mechanistic enzyme assays against both total HDACs isolated from cell lysates and the individual recombinant isoform HDAC2, using **SAHA** as a positive control. When **SAHA** showed the expected activity with IC_{50} 79.7 nM against HDAC2 (Fig. S1†), none of our synthesized compounds

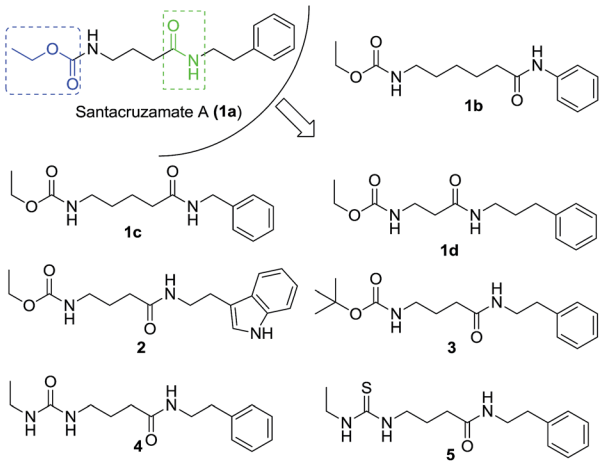
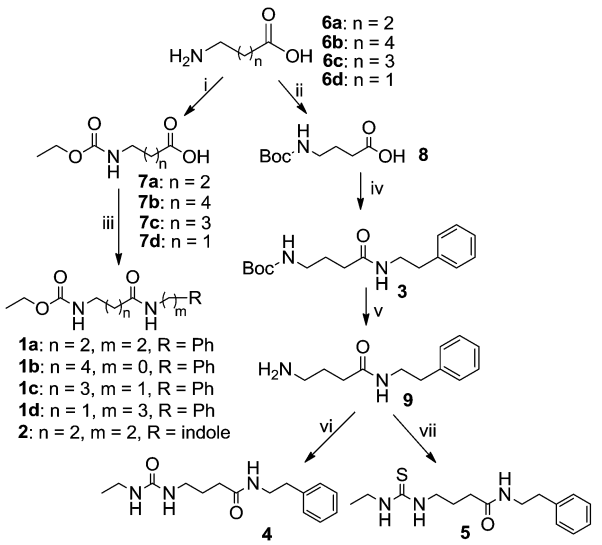


Fig. 2 The strategy to design analogues of santacruzamate A.

showed significant HDAC2 inhibition at the concentration of 2 μM while the IC_{50} value of santacruzamate A was reported to be 0.12 nM.[18] The assay was repeated three times on different dates, and the results were consistent. We have also double checked the spectrum of ^1H NMR of our synthetic santacruzamate A (**1a**), and verified that our synthetic sample matches the reported data for the natural product (Fig. 3). To exclude a possibility that **1a** might be decomposed under the enzymatic assay conditions, a solution of **1a** in DMSO was diluted in the buffer employed for the enzymatic assay and incubated for several hours. The sample was finally taken for mass spectroscopy detection, and the clean major peak (*m*/*z* 301.0)



Scheme 1 Synthesis of santacruzamate A (**1a**) and the analogues. *Reagents and conditions*: (i) ethyl chloroformate, K_2CO_3, THF/H_2O, 0 °C to rt., (ii) Boc_2O, NaOH, THF/H_2O, 0 °C to rt., (iii) amine, Et_3N, EDCI, cat. DMAP, CH_2Cl_2, 0 °C to rt., (iv) phenethylamine, Et_3N, EDCI, cat. DMAP, CH_2Cl_2, 0 °C to rt., (v) TFA, CH_2Cl_2, rt., (vi) isocyanatoethane, THF, rt., (vii) isothiocyanate, THF, rt. Note: EDCI, 1-ethyl-3-(3-dimethyllaminopropyl)carbodiimide hydrochloride; DMAP, 4-dimethylaminopyridine.

Table 1 MTS assay to evaluate the effect of compounds on the proliferation of two cancer cell lines: HCT-116 and ML-1 and normal colonic epithelial cell line CCD841

	IC_{50}[a] (μM) ± SEM		
Compound	HCT-116	ML-1	CCD841
1a	>100	>100	NT
1b	90.8 ± 6.9	>100	NT
1c	>100	>100	NT
1d	94.3 ± 13.8	>100	NT
2	>100	>100	NT
3	86.0 ± 9.0	>100	NT
4	>100	79.5 ± 3.8	NT
5	6.0 ± 1.2	9.4 ± 3.8	>100
SAHA	1.4 ± 0.0	2.9 ± 1.1	20.8 ± 0.17

[a] IC_{50} is the drug concentration effective in inhibiting 50% of the cell growth measured by the MTS assay. NT, not tested.

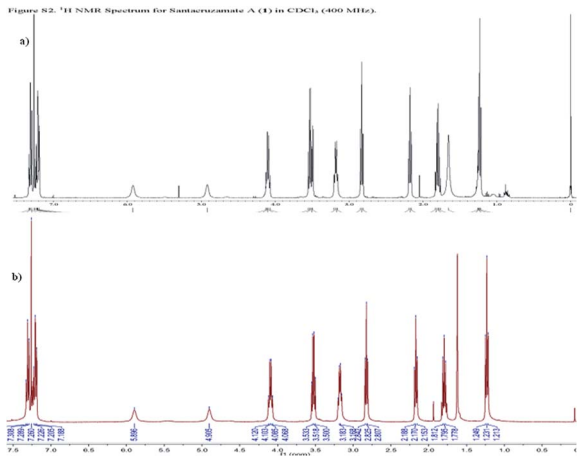


Fig. 3 The ^{1}HNMR spectra of (a) the reported santacruzamate A and (b) our synthetic **1a**.

corresponded to **1a**, indicating that **1a** tolerated the enzymatic assay conditions (Fig. S2†). These solid results lead us to believe that the original report need to be further reexamined.

While the mechanism of action of our compound **5** does not involve HDAC inhibition, further studies were carried out to profile its biological activity. At a concentration of 5 μM, **5** was able to effectively suppress colony formation of HCT-116 cells in a concentration dependent manner (Fig. 4) and to induce cell cycle arrest at the G2/M phase of HCT-116 cells but not ML-1 cells (Fig. 5).

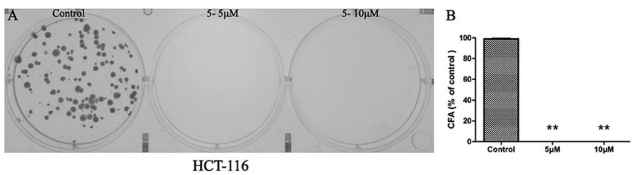


Fig. 4 Inhibition of colony formation of HCT-116 cells by **5**.

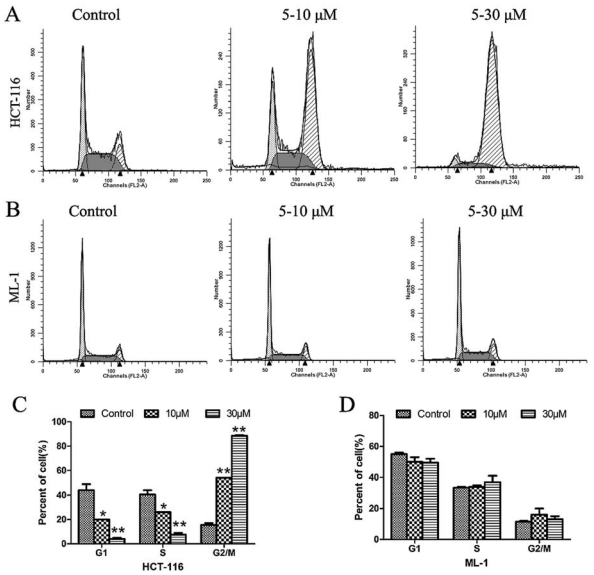


Fig. 5 The effect of **5** on cell cycle progression of (A) HCT-116 cells and (B) ML-1 cells; (C) and (D) the normalization of the effects above.

We then further explored whether this inhibition of cell growth and cell cycle arrest by **5** was attributed to the induction of apoptosis. Annexin V/PI double-staining assay was used to study whether **5** could directly induce apoptotic cell death in HCT-116 and ML-1 cells (Fig. 6). The results indicated that **5** significantly increased the percentage of apoptotic cells (Annexin-V-positive) in a dose-dependent manner. No obvious change was observed in necrotic cells (only PI stained) as compared to control at 48 h (data not shown).

It has been widely recognized that increased endogenous reactive oxygen species (ROS) generation can selectively eliminate cancer cells, mainly by raising oxidative stress over the threshold of toxicity to abnormal cancer cells.[22] Recently, Schreiber and co-

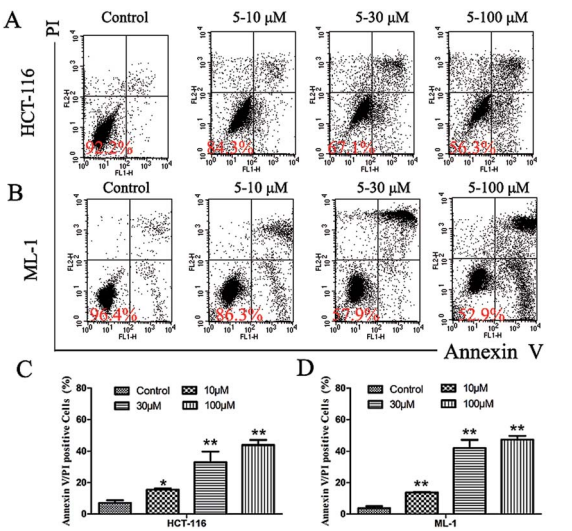


Fig. 6 Appotosis of (A) HCT-116 and (B) ML-1 cells induced by **5**; (C) and (D) the normalization of the induced appoptosis above.

workers have discovered that a natural product piperlongumine selectively killed cancer cells by targeting the stress response to ROS.[23] Thus, it was of our interest to examine ROS level in HCT-116 and ML-1 cells treated with **5** (Fig. S3†). The data indicated that a treatment with **5** at 10 μM induced a significant increase in ROS levels both in HCT-116 and ML-1 cells ($P < 0.05$). Our results implied that selective killing of cancer cells but not normal cells by **5** might result from the ROS generation. Further study to investigate its exact mechanism is under way.

Conclusions

In summary, a novel series of compounds were designed and synthesized based on the natural product santacruzamate A. The SAR study demonstrated that most of these analogues as well as synthetic santacruzamate A showed weak cytotoxicity against the two tested cancer cell lines, HCT-116 and ML-1. It is noteworthy that synthetic santacruzamate A did not inhibit either total HDACs or HDAC2 in enzyme assays. While this is in stark contrast to the original publication, it is consistent with the known SAR of HDAC inhibitors and it is likely that the earlier report was in error.[18] However, one analogue, **5** was found to exhibit anti-proliferative activity against HCT-116 ($IC_{50} = 6.0$ μM) and ML-1 ($IC_{50} = 9.4$ μM) cell lines. In addition, **5** did not cause damage to normal human colorectal cells, suggesting that **5** selectively killed the abnormal cancer cells. It is phenomenal that such a simple compound with a terminal thiourea has gained submicromolar anticancer activity with low toxicity to normal cells although the thiourea motif are reported in some biologically active compounds.[24] Further studies showed that **5** inhibited colony formation of HCT-116, induced apoptosis of both cancer cells HCT-116 and ML-1, and arrested cell cycle of HCT-116 at G2/M phase. Finally, ROS generation was observed in both cancer cell lines HCT-116 and ML-1, implying that this might be the reason why **5** selectively eliminated cancer cells. Further study to investigate its exact mechanism of action is underway. Due to its simple structure and selective killing of cancer cells, **5** might provide a useful scaffold for anticancer drug development.

Acknowledgements

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