KAPLAT Achievement Report
Design, Synthesis, and Biological Evaluation of HDAC6-Selective Inhibitors

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1. Introduction

Histones deacetylase 6 (HDAC6) is a unique HDAC family member that regulates cell proliferation, metastasis, invasion, and mitosis in tumors (1, 2). In addition, HDAC6 was also reported to regulate several critical factors in the immune system (3). For example, HDAC6 regulates myeloid differentiation primary response 88 (MyD88) as a key regulator-dependent canonical toll like receptor (TLR) enabling to control cytokine levels (3). Therefore, HDAC6 has emerged as a promising drug target against cancer and immune diseases. To date, a number of HDAC6 inhibitors have been reported, however, most of them have a hydroxamate group which leads to mutagenicity. In addition, the very tight binding potency of the hydroxamate moiety to the zinc ion at the active site of HDACs also leads to the lack of HDAC isozyme selectivity, which could cause toxicity (4). The aim of this present study is to identify a non-hydroxamate HDAC6-selective inhibitor by structure-based drug design, which could reduce the toxicity of hydroxamate HDAC inhibitors (5).

2. Results and Discussion

Design and Synthesis. We cannot disclose the structure of the compound we designed because it has not been published yet. After we drew the structure of compound A by ChemDraw, we applied it to a molecular modeling study using Maestro 11.9 to see the predicted interaction between HDAC6 and compound A. This technique has been widely used in the field of drug discovery. The molecular docking study showed that compound A could interact with some amino acid residues of HDAC6. Interestingly, compound A shared the same binding site with Nextrastat A, a previously reported HDAC6 inhibitor. Therefore, compound A was subjected to further investigations. Next, the chemical synthesis of compound A was carried out and the structure and purity of compound A were determined by NMR, MS, and HPLC analysis. The yield of compound A was 34.76%, and the HPLC analysis revealed that it had a high purity (97.19%) (Figure 1). Moreover, the NMR and MS data supported the structure of compound A. Based on all the results, we were confident that the compound we synthesized was the target compound A.

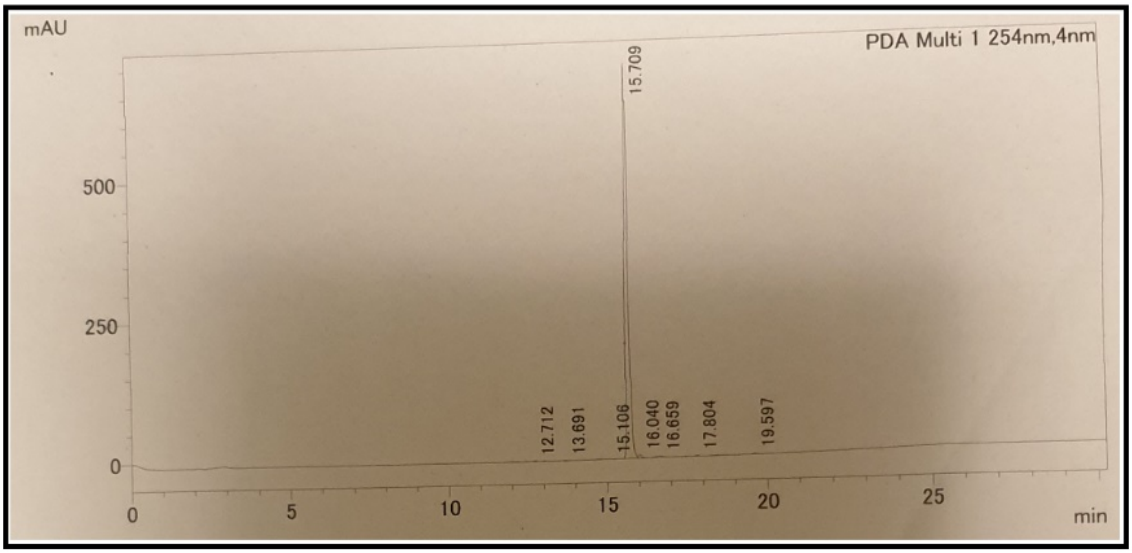

Figure 1. The HPLC data of compound A.

HDAC Isozyme Inhibition Assay. The inhibitory activity of compound A against HDAC1, HDAC4, and HDAC6 was evaluated by using the previously reported fluorescent HDAC assays (6). In this experiment, the RHKK(Ac)-based substrate (BML-KI177-0005) was used for HDAC1 and HDAC6 assays, while the Boc-Lys-TFA-AMC was used for HDAC4 assays. The IC_{50} values for each HDAC isozyme are shown in Figure 3. As a result, compound A inhibited HDAC6 with an IC_{50} value of 456 nM, and the HDAC6 selectivity over HDAC1 of compound A was 50-fold (IC_{50} for HDAC1 = 23042 nM) and that over HDAC4 was 16-fold (IC_{50} for HDAC4 = 7204 nM). Although this selectivity is lower than that of TubastatinA, a positive control (HDAC1 IC_{50}/ HDAC6 IC_{50} = 1919, HDAC4 IC_{50}/ HDAC6 IC_{50} = 2235), compound A could be a non-hydroxamate lead compound from which more potent and selective HDAC6 inhibitors can be developed.

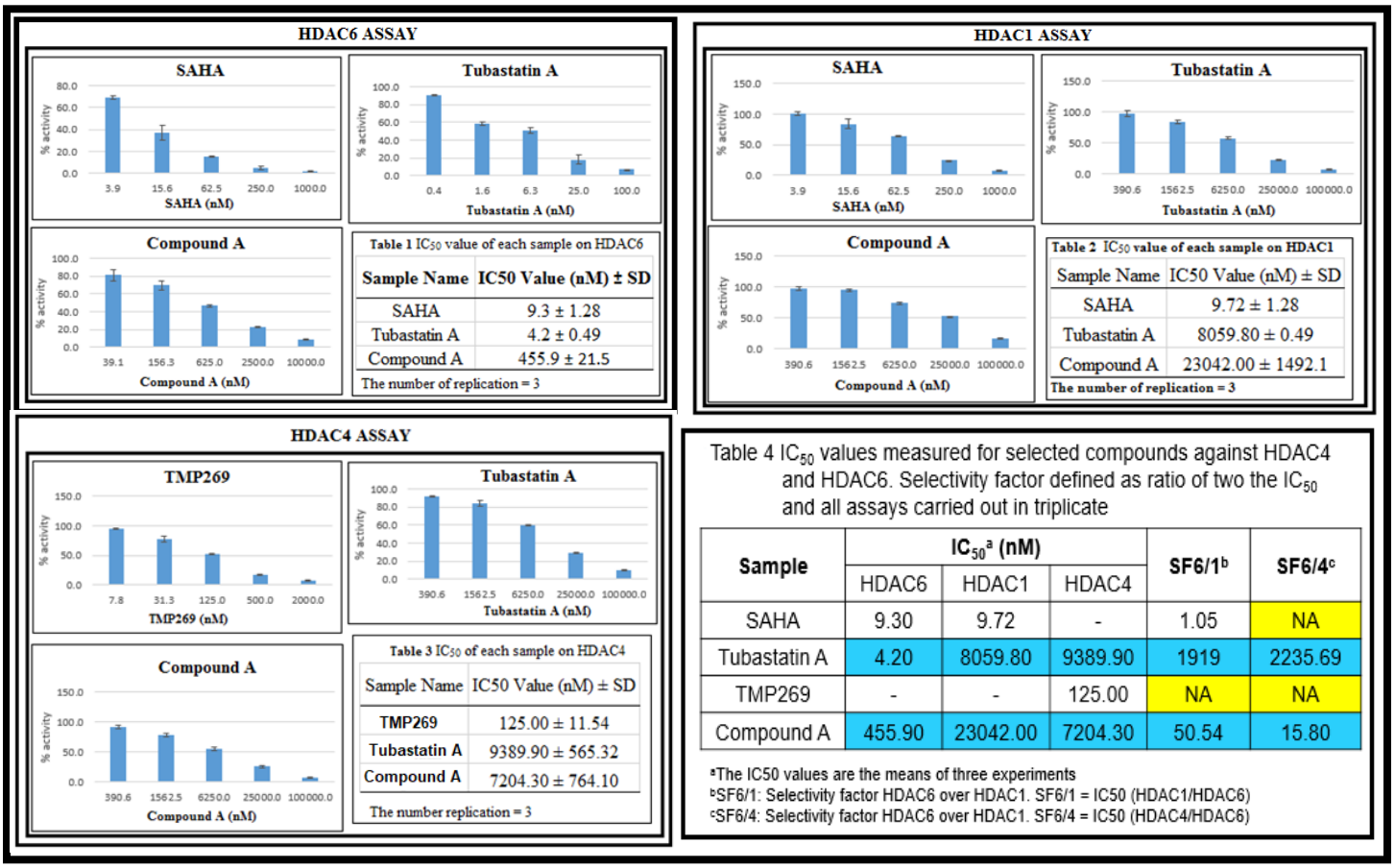

Table 4 IC_{50} values measured for selected compounds against HDAC4 and HDAC6. Selectivity factor defined as ratio of two the IC_{50} and all assays carried out in triplicate

Sample	IC_{50}[a] (nM)			SF6/1[b]	SF6/4[c]
	HDAC6	HDAC1	HDAC4		
SAHA	9.30	9.72	-	1.05	NA
Tubastatin A	4.20	8059.80	9389.90	1919	2235.69
TMP269	-	-	125.00	NA	NA
Compound A	455.90	23042.00	7204.30	50.54	15.80

[a]The IC50 values are the means of three experiments
[b]SF6/1: Selectivity factor HDAC6 over HDAC1. SF6/1 = IC50 (HDAC1/HDAC6)
[c]SF6/4: Selectivity factor HDAC6 over HDAC1. SF6/4 = IC50 (HDAC4/HDAC6)

Figure 2. The results of HDAC Assay

Western Blot Assay. Since HDAC6 has been reported to be an α-tubulin deacetylase, inhibition of HDAC6 leads to the accumulation of acetylated α-tubulin. In this study, not only α-tubulin acetylation but also histone acetylation (a marker of HDAC1/2/3 inhibition) levels were evaluated to examine the in-cell HDAC6 selectivity of compound A. After the treatment of breast cancer MCF-7 cells with compound A, western blotting assay was performed. After SDS-page, a membrane was placed on the gel, to which separated proteins in the gel were electrophoretically transferred. The membrane with transferred proteins was then probed with a primary antibody (an antibody specific for the target protein), washed, and reacted with a secondary antibody labeled with a horse radish peroxidase. The protein level was detected by chemiluminescent method. The results of the western blot assay are shown in Figure 3.

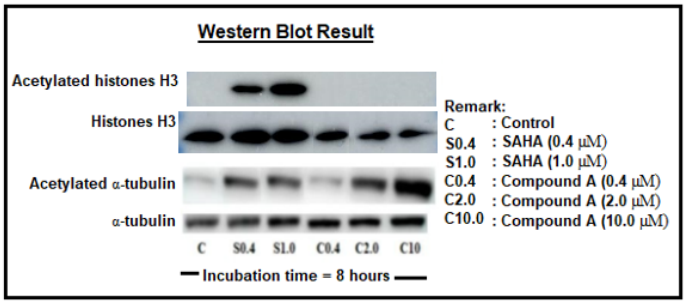

Figure 3. The results of western blot assay

As shown in Figure 3, compound A selectively increased α-tubulin levels with a negligible effect on the levels of acetylated histone H3 in MCF-7 cells. On the other hand, SAHA, a hydroxamate pan-HDAC inhibitor, showed both α-tubulin and histone H3 acetylation. These results suggested that the compound A selectively inhibited HDAC6 in MCF-7 cells.

References

1. *Histone deacetylase 6 in cancer*. **Li, Ting, et al.** 111, 2018, Journal of Hematology & Oncology, Vol. 11, pp. 1-10.
2. *Role of Histone Deacetylases in Carcinogenesis: Potential Role in Cholangiocarcinoma.* **Pant, Kishor, et al.** 780, 2020, Cells, Vol. 9, pp. 1-14.
3. *Histone deacetylase 6 regulates endothelial MyD88-dependent canonical TLR signaling, lung inflammation, and alveolar remodeling in the developing lung.* **Menden, Heather, et al.** 3, 2019, American Journal of Physiology Lung Cellular and Molecular Physiology, Vol. 317, pp. L332–L346.
4. *Why Hydroxamates May Not Be the Best Histone Deacetylase Inhibitors—What Some May Have Forgotten or Would Rather Forget?* **Shen, Sida and Kozikowski, Alan P.** 2016, ChemMedChem, Vol. 11, pp. 15-21.
5. *Recent advances in small molecular modulators targeting histone deacetylase 6.* **Xiao, Yufeng and Zhang, Xuan.** 4 (FDD53), 2020, Future Drug. Discov., Vol. 2, pp. 2631-3316.
6. *A Fluorogenic Histone Deacetylase Assay Well Suited for High-Throughput Activity Screening.* **Wegener, Dennis, et al.** 2003, Chemistry & Biology, Vol. 10, pp. 61-68.