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Identification of a potent and stable antiproliferative agent by the prodrug formation of a thiolate histone deacetylase inhibitor

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Abstract—To identify prodrugs of a thiolate histone deacetylase inhibitor NCH-31 that show potent antiproliferative activity and are stable in human plasma, we synthesized several candidate prodrugs of NCH-31. Among these compounds, *S*-2-methyl-3-phe-nylpropanoyl compound **2** showed more potent antiproliferative activity and higher stability in human plasma than *S*-isobutyryl compound NCH-51.

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The dynamic homeostasis of the nuclear acetylation of histones is regulated by the opposing activity of the enzymes histone acetyl transferases and histone deacetylases (HDACs).^{1,2} Deacetylation of histone lysine residues is associated with a condensed chromatin structure resulting in transcriptional repression, whereas acetylation of histones is associated with a more open chromatin configuration and activation of transcription.³ Aberrant activation of HDACs results in the transcriptional repression of oncoprotein and is linked to the malignant phenotypes of tumors.^{4,5} Inhibition of HDACs causes histone hyperacetylation which leads to the disruption of the chromatin structure and the transcriptional activation of genes associated with cancer.^{3,6} In addition, it has recently been reported that inhibition of an HDAC exerts antitumor effects through the non-transcriptional pathway in addition to the intervention of transcriptional regulation.^{7,8} Indeed, HDAC inhibitors such as trichostatin A (TSA) and suberoylanilide hydroxamic acid (SAHA) (Fig. 1) have potent anticancer effects in vitro and in vivo.9,10

In the course of our study of HDAC inhibitors, we found that a series of thiol-based analogues, including

NCH-31 (Fig. 2), are potent HDAC inhibitors.¹¹ Thiols are thought to inhibit HDACs by coordinating the zinc ion in the active site which is required for deacetylation of the acetylated lysine substrate. Further, the *S*-isobutyryl prodrug NCH-51 (Fig. 2), which is thought to be hydrolyzed to free thiol within cells, showed antiproliferative activity against cancer cells,¹² however, NCH-51 has stability problems. NCH-51 was vulnerable to human plasma metabolism at the remaining rate of approximately 50% after 24 h of incubation. We therefore decided to search for prodrugs that are more stable in human plasma and exert more potent antiproliferative activity than NCH-51.

Since prodrugs with a less hindered acyl group tended to exhibit less potent antiproliferative activity,¹² we designed S-acyl prodrugs 1–4 which bear an acyl group more bulky than NCH-51. Four candidate prodrugs of NCH-31 were synthesized as shown in Scheme 1. The sulfhydryl group of NCH-31 was acylated with the corresponding carboxylic acid to give the desired compounds 1–4.

The compounds synthesized in this study were initially tested in antiproliferative activity assays using human lung cancer NCI-H460 cells and human breast cancer MDA-MB-231 cells (Table 1).¹³ Although *S*-2-meth-oxy-2-phenylacetyl compound **3** and 3-phenyl-2-(phenylmethyl)propanoyl compound **4** were less active than thiol NCH-31 and its *S*-isobutyryl prodrug NCH-51,

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Figure 1. Structures of TSA and SAHA.





NCH-51

Figure 2. Structures of NCH-31 and NCH-51.



Scheme 1. Reagents and conditions: (a) RCOCl (for 1 and 4), DMAP, pyridine, 0 °C to rt, 41% for 1, 54% for 4; (b) RCOOH (for 2 and 3), EDCI, DMAP, DMF, rt, 63% for 2, 23% for 3.

S-2-phenylpropanoyl compound **1** showed similar activity against NCI-H460 cells and S-2-methyl-3-phenylpropanoyl compound **2** also displayed similar activity against both NCI-H460 cells and MDA-MB-231 cells when compared with NCH-51. Furthermore, we examined proliferation inhibition by NCH-51, compounds **1** and **2**, against eight solid cancer cell lines. As can be seen from Table 2, compound **2** showed potent proliferation inhibition against various cancer cells representing a 2-fold improvement over NCH-51 (average EC_{50} of **2** 2.4 μ M, NCH-51 4.9 μ M).

To investigate the difference in activity between (R)-2 and (S)-2, optically active (R)-2 and (S)-2 were prepared from (S)-4,5,5-trimethyloxazolidin-2-one 5¹⁴ as outlined in Scheme 2. The chiral auxiliary 5 was converted to compounds 6 and 7 by *N*-propionylation and *N*-3-phenylpropionylation, respectively. Stereoselective enolate benzylation of 6 and methylation of 7, and the subsequent hydrolysis of 8 and 9 gave optically active 2methyl-3-phenylpropanoic acids (R)-10 and (S)-10, respectively. The condensation of carboxylic acids

 Table 1. Proliferation inhibition data on NCI-H460 cells and MDA-MB-231 cells for NCH-31, NCH-51, and compounds 1–4^a

		H /\	0
\bigcirc	$\begin{pmatrix} N \\ S \end{pmatrix}$	N H()	`S [/] R 6

Compound	R	EC ₅₀ (μM) NCI-H460 MDA-MB-231 7.6 >20 2.1 4.4		
		NCI-H460	MDA-MB-231	
NCH-31	-H	7.6	>20	
NCH-51	∖ Me Me	2.1	4.4	
1	∖ Ph Me	3.2	>20	
2	Me Ph	1.5	1.9	
3	→ Ph OMe	11	>20	
4	Ph	>20	>20	

^a Values are means of at least two experiments.

(*R*)-10 and (*S*)-10 with NCH-31 in the presence of EDCI and DMAP afforded optically active (*R*)-2 and (*S*)-2, respectively. The enantiomeric excess of both (*R*)-2 and (*S*)-2 was determined to be 90% by chiral column chromatography.¹⁵

We examined the antiproliferative activity of (R)-2 and (S)-2 against four cancer cell lines, and there was not much difference between the activities of the two stereo-isomers (Table 3).

Next, we investigated the in vitro HDAC inhibitory activity of compound **2** (Table 4).¹⁶ Although NCH-31 exhibited potent inhibitory activity against HDAC1 ($IC_{50} = 0.048 \ \mu$ M), the *S*-2-methyl-3-phenylpropanoyl prodrug **2** did not inhibit HDAC1 at a concentration of 100 μ M.

Compound 2 was evaluated for the accumulation of acetylated histone H4 using Western blot analysis (Fig. 3).¹⁷ Treatment of HCT116 cells with compound 2 produced an increase in the accumulation of acetylated histone H4, which indicated that the antiproliferative activity of compound 2 significantly correlates with the inhibition of HDACs. Since S-2-methyl-3-phenylpropanoyl prodrug 2 was totally inactive in an enzyme assay

Table 2. Proliferation inhibition data on eight human solid cancer cell lines for NCH-51, compoun	unds 1 and 2
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Cell		NCH-51, EC ₅₀ (µM)	1, EC ₅₀ (µM)	2 , EC ₅₀ (µM)
T-47D	Breast cancer	8.3	>100	4.3
MDA-MB-231	Breast cancer	4.4	27	1.9
NCI-H460	Lung cancer	2.1	3.2	1.5
A498	Renal cancer	6.8	26	2.6
PC-3	Prostate cancer	9.5	37	3.2
DLD-1	Colon cancer	2.3	86	1.8
HCT116	Colon cancer	1.3	23	1.2
MALME-3M	Melanoma	4.3	33	3.0
Mean		4.9	42	2.4

^a Values are means of at least two experiments.



Scheme 2. Reagents and conditions: (a) i—*n*-BuLi, THF, -78 °C; ii—propionyl chloride (for 6) or 3-phenylpropionyl chloride (for 7), THF, -78 °C, 71% for 6, 83% for 7; (b) i—LDA, THF, -78 °C; ii—BnBr (for 8) or MeI (for 9), THF, -78 °C to rt, 71% for 8, 63% for 9; (c) LiOH·H₂O, THF, H₂O, 0 °C to rt, 77% for (*R*)-10, 78% for (*S*)-10; (d) NCH-31, EDCI, DMAP, CH₂Cl₂, rt, 57% for (*R*)-2, 52% for (*S*)-2.

Table 3. Proliferation inhibition data on four human solid cancer cell lines for (*R*)-**2** and (*S*)- 2^{a}

Cell		(<i>R</i>)- 2 , EC ₅₀ (μM)	(S)-2 EC ₅₀ (μM)	
MDA-MB-231	Breast cancer	3.1	4.0	
NCI-H460	Lung cancer	3.2	3.0	
PC-3	Prostate cancer	9.9	4.4	
HCT116	Colon cancer	0.75	0.62	

^a Values are means of at least two experiments.

(Table 4), it could possibly permeate the cell membrane and be converted to active free thiol NCH-31 by enzymatic hydrolysis within the cell.

Finally, we assessed the stability of compound 2 in human plasma (Table 5).¹⁸ Interestingly, compound 2

Table 4. HDAC1 inhibition data for TSA, NCH-31, and compound 2^a

Compound	IC ₅₀ (µM)
TSA	0.026
NCH-31	0.048
2	>100 ^b

^a Values are means of at least three experiments.

^b 19% inhibition at 100 μM.



Figure 3. Western blot analysis of histone hyperacetylation in HCAT116 cells produced by compound **2** and by reference compound SAHA.

was found to be tolerant to human plasma metabolism. Nearly 100% of the intact compound was observed even after 24-h incubation in human plasma.

In summary, to identify potent prodrugs of a thiolate HDAC inhibitor, NCH-31, that are more stable in human plasma than NCH-51, we designed and prepared several candidate prodrugs of NCH-31. Among these compounds, S-2-methyl-3-phenylpropanoyl compound **2** showed more potent antiproliferative activity than S-isobutyryl compound NCH-51. Compound **2** also displayed high stability in human plasma. Further study pertaining to compound **2** is underway to investigate the therapeutic efficacy against human cancers.

Table 5. Stability of NCH-51 and compound 2 in human plasma^a

Compound			% R ^b		
	0h	2h	4h	8h	24h
NCH-51	100	86	71	63	49
2	100	100	99	99	100

^a Values are means of at least three experiments.

^b The percentage of the parent compound remaining (%*R*) was measured after 2, 4, 8, and 24 h incubation at 37 °C.

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- 13. The antiproliferative activity assay was performed as follows. Cancer cells were plated in 96-well plates at initial density of 1500 cells/well and incubated at 37 °C. After 24 h, cells were exposed to test compounds at various concentrations in 10% FBS-supplemented RPMI-1640 medium at 37 °C in 5% CO₂ for 48 h. The medium was removed and replaced with 200 μ L of 0.5 mg/mL of methylene blue in RPMI-1640 medium, and cells were incubated at room temperature for 30 min. Supernatants were removed from the wells, and methylene blue dye was dissolved in 100 μ L/ well of 3% aqueous HCl. Absorbance was determined on a microplate reader (Bio-Rad) at 660 nm.
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- Analytical conditions of chiral column chromatography; column: CHIRALCEL OA (Daicel Chemical Industries), eluent: *n*-hexane/isopropanol = 19:1, flow rate: 1 mL/min; retention time: 27.4 min ((*R*)-2), 29.3 min ((*S*)-2).
- 16. The inhibitory activities of test compounds against partially purified HDAC1 were assayed according to a method reported in the literature: Furumai, R.; Komatsu, Y.; Nishino, N.; Khochbin, S.; Yoshida, M.; Horinouchi, S. Proc. Natl. Acad. Sci. U.S.A. 2001, 98, 87.
- 17. Western blot analysis was performed as follows. HCT-116 cells (5×10^5) were treated for 8 h with 5 µM of SAHA or compound 2 in 10% FBS-supplemented McCoy's 5A medium and then collected and extracted with SDS buffer. Protein concentrations of the lysates were determined using a Bradford protein assay kit (Bio-Rad Laboratories); equivalent amounts of proteins from each lysate were resolved in 15% SDS-polyacrylamide gel and then transferred onto nitrocellulose membranes (Bio-Rad Laboratories). After blocking for 30 min with Tris-buffered saline (TBS) containing 3% skim milk, the transblotted membrane was incubated overnight at 4 °C with hyperacetylated histone H4 antibody (Upstate Biotechnology) (1:4000 dilution) in TBS containing 3% skim milk. After probing with the primary antibody, the membrane was washed twice with water, then incubated with goat antirabbit IgG-horseradish peroxidase conjugates (diluted 1:5000) for 2 h at room temperature, and further washed twice with water. The immunoblots were visualized by enhanced chemiluminescence.
- 18. NCH-51 and compound **2** (100 μ M) were incubated at 37 °C in human plasma. The percentage of the parent compound remaining (%*R*) was determined by HPLC.