Review

Explorative Study on Isoform-Selective Histone Deacetylase Inhibitors

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Histone deacetylases (HDACs) catalyze the deacetylation of the acetylated lysine residues of histones and non-histone proteins, and are involved in various fundamental life phenomena, such as gene expression and cell cycle progression. Thus far, eighteen HDAC family members (HDAC1—11 and SIRT1—7) have been identified, but the functions of the HDAC isoforms are not yet fully understood. In addition, some of the HDAC isoforms have been suggested to be associated with various disease states, including cancer and neurodegenerative disorders. Therefore, isoform-selective HDAC inhibitors are of great interest, not only as tools for probing the biological functions of the isoforms, but also as candidate therapeutic agents with few side effects. It was against this background that we initiated research programs to identify isoform-selective HDAC inhibitors. We designed HDAC inhibitors based on the three-dimensional structure of the enzyme and on the proposed catalytic mechanism of HDACs, and found several isoform-selective HDAC inhibitors. Furthermore, we elucidated the functions of HDAC6 by chemical genetic approaches using these inhibitors. The results of this research also suggested the feasibility of using isoform-selective HDAC inhibitors as therapeutic agents.

Key words histone deacetylase; isoform-selective inhibitor; chemical genetic approach

Introduction

Reversible protein acetylation by histone deacetylases (HDACs) and histone acetyl transferases is an important posttranslational modification that regulates the function of the proteins. For example, histone deacetylation causes transcriptional silencing,[1—3] whereas histone hyperacetylation arising from HDAC inhibition induces the transcriptional activation of genes such as *p21*$^{WAF1/CIP1}$,[4] *Gadd 45*,[5] *FAS* and *caspase-3*[6] which are associated with growth arrest and apoptosis in tumor cells. It has also been reported that reversible acetylation of non-histone proteins, such as p53 and α-tubulin, regulates cell functions.[7—10]

To date, eighteen HDAC family members have been identified. As shown in Table 1, they can be divided into two categories, *i.e.*, zinc-dependent enzymes (Class I, Class II and Class IV) and nicotinamide adenine dinucleotide (NAD^+)-dependent enzymes (Class III: sirtuins).[1,11—13] Class I HDACs include HDAC1,[14—17] HDAC2,[16,18,19] HDAC3[16,20—22] and HDAC8,[23,24] and show homology to the yeast protein reduced potassium deficiency 3. Class II HDACs include HDAC4,[25—30] HDAC5,[27,31,32] HDAC6,[33—37] HDAC7,[38—40] HDAC9[41,42] and HDAC10,[43] and are homologous to the yeast enzyme HDA1. Class II HDACs are further grouped into two subclasses, IIa (HDAC4, HDAC5, HDAC7, and HDAC9) and IIb (HDAC6 and HDAC10), according to their sequence homology and domain organization. HDAC11,[44—46] the most recently identified member, is classified into the new Class IV of HDACs. Sirtuins, including human sirtuin (SIRT)1—7,[47] show distinct homology with the yeast enzyme Sir2.[48] It was reported that SIRT1,[47,49—52] SIRT2,[47,53,54] SIRT3,[47,55—57] SIRT5[47] and SIRT6[47,58,59] induce deacetylation of histones or non-histone proteins, whereas SIRT4[47,60,61] and SIRT7[47,62] do not possess *in vitro* deacetylase activity.[53] Some of the HDAC isoforms have been reported to play important roles in cell functions (Table 1) and to be associated with a variety of disease states, including cancer, inflammation and neurosis.[63—66] Therefore, isoform-selective HDAC inhibitors are candidate therapeutic agents with few side effects, as well as being useful tools for probing the biological functions of the isoforms.[67,68] With these in mind, we have been working on research programs to find isoform-selective HDAC inhibitors, which are described in this review.

1. Isoform-Selective Inhibitors of Zinc-Dependent HDACs

1.1. Identification of Non-hydroxamate Zinc-Binding Groups for Isoform-Selective HDAC Inhibitors A number of zinc-dependent HDAC inhibitors have been identified thus far.[67] As depicted in Fig. 1, HDAC inhibitors typically possess a zinc-binding group (ZBG), which coordinates the

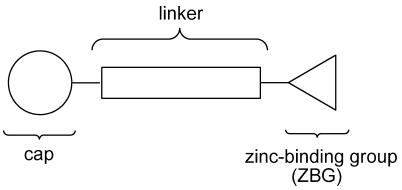

Fig. 1. Pharmacophoric Summary of the Structural Characteristics of HDAC Inhibitors

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Table 1. HDAC Family

Zinc-dependent enzymes		Localization	Function	References
Class I (RPD3 homologue)				
	HDAC1	Nucleus	Transcriptional repression	14—17
	HDAC2	Nucleus	Transcriptional repression	16, 18, 19
	HDAC3	Nucleus, cytoplasm	Transcriptional repression	16, 20—22
	HDAC8	Nucleus, cytoplasm	Smooth muscle cell contractility	23, 24
Class II (HDA1 homologue)				
IIa	HDAC4	Nucleus, cytoplasm	Transcriptional repression Muscle differentiation block Retinal protection	25—30
	HDAC5	Nucleus, cytoplasm	Transcriptional repression Muscle differentiation block	27, 31, 32
	HDAC7	Nucleus, cytoplasm	Transcriptional repression Muscle differentiation block	38—40
	HDAC9	Nucleus, cytoplasm	Transcriptional repression Muscle differentiation block	41, 42
IIb	HDAC6	Cytoplasm	Regulation of microtubule stability and function Regulation of molecular chaperone function Regulation of aggresome function	33—37
	HDAC10	Cytoplasm	Unknown	43
Class IV				
	HDAC11	Nucleus	Transcriptional repression	44—46

NAD^+-dependent enzymes		Localization	Function	References
Class III (Sir 2 homologue)				
	SIRT1	Nucleus	Functional regulation of p53 Transcriptional repression	47, 49—52
	SIRT2	Nucleus, cytoplasm	Cell cycle regulation	47, 53, 54
	SIRT3	Mitochondria	Activation of mitochondrial functions Thermogenesis in brown adipose Activation of acetyl-CoA synthethase 2	47, 55—57
	SIRT4	Mitochondria	Insulin secretion	47, 60, 61
	SIRT5	Mitochondria	Unknown	47
	SIRT6	Nucleus	DNA repair and genomic stability Regulation of telomere metabolism and function	47, 58, 59
	SIRT7	Nucleus	Activation of RNA polymerase I transcription	47, 62

zinc ion in the active site, a cap substructure, which interacts with amino acids at the entrance of the *N*-acetylated lysine binding channel, and a linker connecting the cap and the ZBG at a proper distance. Most of the previously reported HDAC inhibitors are hydroxamic acid derivatives, typified by suberoylanilide hydroxamic acid (SAHA) **1**[69,70] and Trichostatin A (TSA) **2**[71,72] (Fig. 2), which are thought to chelate the zinc ion in the active site in a bidentate fashion through its CO and OH groups.[73—75] Although hydroxamic acids are frequently employed as ZBGs, most hydroxamate HDAC inhibitors inhibit all of the HDAC isoforms. We hypothesized that the non-isoform selectivity of hydroxamate HDAC inhibitors is due to their high ability to coordinate the zinc ion in the active site of HDACs, and the discovery of non-hy-

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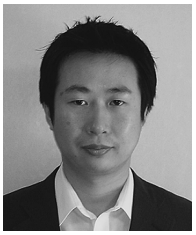

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droxamate HDAC inhibitors could lead to the identification of isoform-selective HDAC inhibitors.

We designed non-hydroxamate HDAC inhibitors based on the three dimensional structure of the enzyme and on the proposed catalytic mechanism of HDACs.

On the basis of the three-dimensional structure of the active site of the enzyme, SAHA-based non-hydroxamates were designed and synthesized as HDAC inhibitors (Fig. 3). As mentioned above, the co-crystal structure of an archaebacterial HDAC-like protein (HDLP)/hydroxamate or HDAC8/hydroxamate made it clear that the hydroxamic acid group chelates the zinc ion in a bidentate fashion and forms hydrogen bonds with tyrosine and two histidines (Fig. 4a).[73—75] As bidentate ZBGs, we designed SAHA-based hydroxyurea (**3**), semicarbazide (**4**) and hydroxysulfonamide (**5**),[76,77] which could coordinate the zinc ion bidentately and could also form hydrogen bonds with tyrosine and two histidines like hydroxamic acid (Fig. 4b). We also designed monodentate ZBGs. Thiol **6**, thioacetate **7** and methylsulfide **8** were designed based on the high thiophilicity of zinc ion (Fig. 3).[77,78] In particular, thiol could interact not only with zinc ion but with amino acid residues in the active site. Another newly designed mondentate ZBG is sulfoxide **9** (Fig. 3).[79] Since sulfoxide has a partial negative charge on its oxygen, it is estimated to chelate zinc ion and inhibit HDACs.

We designed SAHA-based non-hydroxamates based on the proposed catalytic mechanism for the deacetylation of acetylated lysine residues (Fig. 5). We initially designed substrate analogues based on the proposed deacetylation mechanism whereby a zinc-chelating water molecule activated by histidine makes a nucleophilic attack on the carbonyl carbon of an acetylated lysine substrate (Fig. 6a).[73] With this mechanism, the HDACs would supposedly be inhibited if the water molecule is forcibly removed from the zinc ion, and then heteroatom-containing substrate analogues **10**—**13** were designed (Fig. 5).[77,80] These analogues would be recognized as substrates by HDACs and be easily taken into the active site where they would force the water molecule off the zinc ion and the reactive site for deacetylation through chelation of the heteroatom to the zinc ion, and behave as HDAC inhibitors (Fig. 6b).

The other design was based on the transition state (TS) structure of HDAC deacetylation, which was estimated to include a tetrahedral carbon[73] (Fig. 7a) as with other zinc proteases.[81] Sulfone-based SAHA analogues[77,80] could be TS analogue inhibitors because they have sufficient similarity with the TS of amide bond hydrolysis (Fig. 7b), both from a steric and an electronic point of view.[82] Then, compounds **14** and **15** (Fig. 5), in which a hydroxamic acid of SAHA is replaced by sulphonamide and sulfone, respectively, were designed as TS analogues. We also designed boronic acid **16**.[83] The hydrated form of the boronic acid **16** could act as a TS analogue and coordinate the zinc ion in the active site of HDACs (Fig. 7c).

Compounds **1**—**16** were tested with an *in vitro* assay using

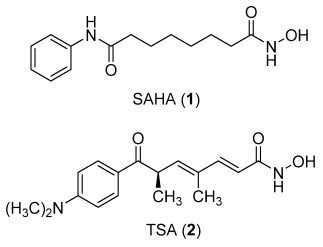


Fig. 2. Hydroxamate HDAC Inhibitors

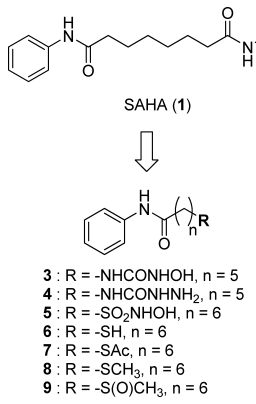


Fig. 3. SAHA-Based Non-hydroxamate Compounds Designed Based on the Three-Dimensional Structure of HDAC

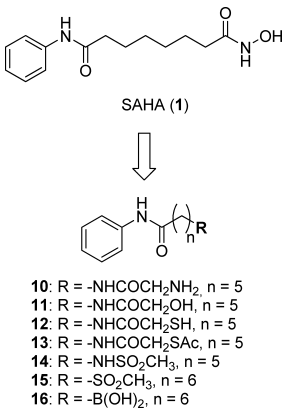


Fig. 5. SAHA-Based Non-hydroxamates Designed Based on the Catalytic Mechanism for the Deacetylation of Acetylated Lysine Substrates

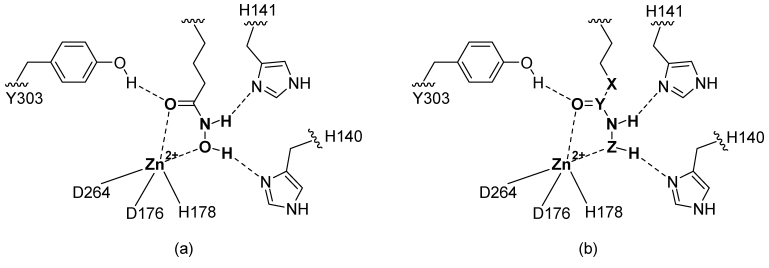


Fig. 4. Model for the Binding of SAHA (a) and Hydroxamic Acid Mimics **3**—**5** in the Catalytic Core of HDAC1

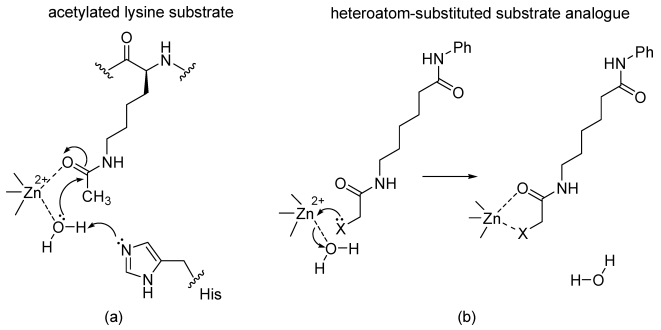


Fig. 6. Mechanism Proposed for the Deacetylation of Acetylated Lysine Substrate (a), and Model for the Binding of Hetero Atom-Containing Substrate Analogues to Zinc Ion (b)

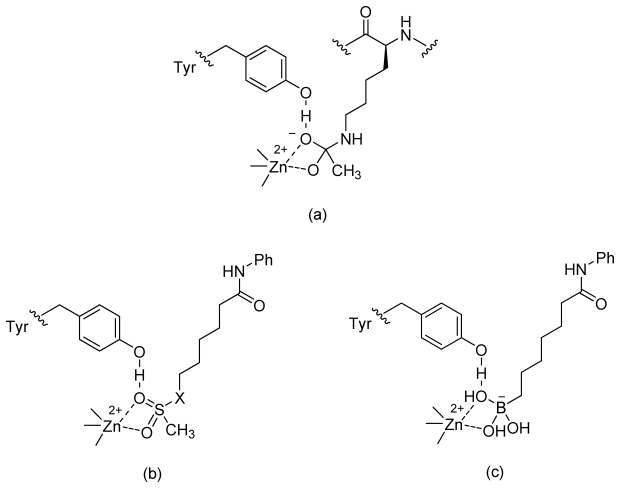
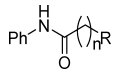


Fig. 7. Transition State Proposed for HDACs (a), and Models for the Binding of Sulfone Derivatives (b) and Hydrated Boronic Acid **16** (c)

a HeLa nuclear extract rich in HDAC activity (Table 2). Among compounds **3**—**5**, which were designed as compounds with bidentate ZBGs, hydroxyurea **3** and semicarbazide **4** showed inhibitory activity although they were much less effective than SAHA.[76,77] As for the compounds with monodentate ZBGs (**6**—**9**), the activity of thiol **6** was far greater than expected. Although the inhibitory ability of monodentate ZBGs was thought to be less than that of bidentate ZBGs such as hydroxamic acid, a pronounced inhibitory effect ($IC_{50}=0.21\ \mu M$) was observed with thiol **6**,[77,78] which was as potent as SAHA. The transformation of thiol into thioacetate (**7**) and methylsulfide (**8**) led to an inhibitor that was about 30-fold less potent and a compound devoid of anti-HDAC activity, respectively. These results suggest that the thiolate anion generated under physiological conditions is intimately involved in the interaction with the zinc ion in the active site. In addition, sulfoxide **9**, the other compound with a monodentate ZBG, inhibited HDACs with an IC_{50} of 48 μM.[79] Among compounds **10**—**13** which were designed based on the catalytic mechanism of HDACs, potent inhibition was observed with mercaptoacetamide **12**, while aminoacetamide **10** and hydroxyacetamide **11** did not possess inhibitory activity.[77,80] Mercaptoacetamide **12** exhibited an IC_{50} of 0.39 μM, and its activity was comparable to that of SAHA. As expected, the transformation of thiol into thioacetate (**13**) led to a much less potent inhibitor. These results

Table 2. HDAC Inhibition Data for SAHA **1** and SAHA-Based Non-hydroxamtes **3**—**16**



Compd.	R	*n*	IC_{50} (μM)[a]
SAHA (**1**)	–CONHOH	6	0.28
3	–NHCONHOH	5	80
4	$-NHCONHNH_2$	5	150
5	$-SO_2NHOH$	6	>100
6	–SH	6	0.21
7	–SAc	6	7.1
8	–SMe	6	>100
9	–S(O)Me	6	48
10	$-NHCOCH_2NH_2$	5	>100
11	$-NHCOCH_2OH$	5	>100
12	$-NHCOCH_2SH$	5	0.39
13	$-NHCOCH_2SAc$	6	22
14	$-NHSO_2Me$	5	7500
15	$-SO_2Me$	6	230
16	$-B(OH)_2$	6	>100

a)

suggest that the ease of ionization of thiol is an important factor in the inhibition of HDACs like the case of thiol **6**. Sulfone derivatives **14**, **15** and boronic acid **16**, which were designed as TS analogues, were found to be less potent inhibitors.[77,80,83]

As a result, we identified thiol as a ZBG suitable for HDAC inhibitors. As for the zinc-binding ability of thiol, the results of density functional theory calculations suggested that the zinc-binding affinity of thiol is lower than that of hydroxamic acid.[84,85] On the hypothesis that discovery of a ZBG having zinc-binding affinity lower than hydroxamic acid could lead to the identification of isoform-selective HDAC inhibitors, we chose thiol as ZBG for further explorative study on isoform-selective HDAC inhibitors.

1.2. Identification of NCH-51, a Prodrug of the HDAC6-Insensitive Inhibitor NCH-31, and Its Application Optimization of the linker part and aromatic cap part of compound **6** led to the identification of NCH-31 **17** and its *S*-isobutyryl prodrug NCH-51 **18** (Fig. 8).[77] Interestingly, while the histone-acetylating activity of NCH-51 **18** was as strong as that of SAHA **1**, the α-tubulin or HSP90 acetylating activity of NCH-51 **18** was much weaker than that of SAHA **1**.[86] Because HSP90, as well as α-tubulin, was reported to be deacetylated by HDAC6,[33—36] these results indicated that NCH-31 **17** does not inhibit HDAC6. Furthermore, NCH-51 **18** inhibited the cell growth of a variety of lymphoid malignant cells more effectively than SAHA **1**.[86] To understand the differences in action mechanisms between HDAC6-insensitive inhibitor NCH-51 **18** and pan-inhibitor SAHA **1**, DNA microarray analysis and proteome analysis were performed. Gene expression profiles showed that NCH-51 **18** and SAHA **1** similarly upregulated the cell cycle regulators *p19* and *p21* and downregulated anti-apoptotic molecules such as *survivin*, *bcl-w* and *c-FLIP*. Proteome analysis and Western blot analysis revealed that SAHA **1** upregulated anti-oxidant molecules, including peroxiredoxin 1 (PRDX1), PRDX2 and glutathione *S*-transferase, at the protein level more potently than NCH-51 **18**. In accordance with these re-

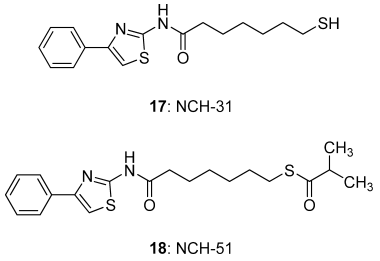


Fig. 8. Structures of NCH-31 **17** and NCH-51 **18**

sults, NCH-51 **18** increased the accumulation of reactive oxygen species (ROS), whereas SAHA **1** continuously decreased ROS. In addition, it was reported that HDAC6 is responsible for the transportation and degradation of cellular proteins such as anti-oxidant proteins.[37] Taking these results together, the action mechanism of NCH-51 **18** and SAHA **1** and the reason why NCH-51 **18** showed more potent antiproliferative activity than SAHA **1** were considered to be as follows. 1) NCH-51 **18** and SAHA **1** similarly downregulate anti-apoptotic molecules and upregulate cell cycle regulators at both the mRNA and protein levels by inhibiting nuclear HDACs, leading to cell cycle arrest and apoptosis. 2) NCH-51 **18** and SAHA **1** also upregulate anti-oxidant molecules at both the mRNA and protein level by inhibiting nuclear HDACs. 3) Since SAHA **1** blocks the transportation and degradation of anti-oxidant molecules by inhibiting HDAC6, the expression of anti-oxidant molecules at the protein level continuously increases, leading to a decrease in ROS accumulation. 4) On the other hand, since NCH-31 **17** does not block the transportation and degradation of anti-oxidant molecules because of its weaker HDAC6-inhibitory activity, the expression of anti-oxidant molecules at the protein level decreases, which leads to an increase in ROS accumulation. 5) Therefore, NCH-51 **18** exhibits cytotoxicity *via* mitochondrial injury by sustaining ROS at a higher level than that in the case of SAHA **1**. These findings revealed the relationship between HDAC6 and ROS accumulation, and suggested that HDAC6-insensitive inhibitors should be more potent anticancer agents than pan-HDAC inhibitors.

1.3. Identification of HDAC6-Selective Inhibitors and Their Applications In order to uncover HDAC6-selective inhibitors, we designed and prepared a series of thiolate analogues based on the structure of an HDAC6-selective substrate **19**[87] (Fig. 9) and evaluated their properties by Western blotting and enzyme assays. The conversion of the acetamide of **19** to thiol and further structural optimization led to the identification of NCT-10a **20** and NCT-14a **21** (Fig. 9), which showed selectivity for HDAC6 over HDAC1 and HDAC4 in enzyme assays.[88,89] *S*-Isobutyryl prodrugs NCT-10b **22** and NCT-14b **23** (Fig. 9) induced a dose-dependent increase in acetylation of α-tubulin, one of the substrates of HDAC6,[33,34] without causing a major increase in acetylated histone H4. These results indicated that compounds NCT-10b **22** and NCT-14b **23** selectively inhibit HDAC6 in preference to nuclear HDACs in cells. Investigation of the structure–selectivity relationship revealed that the presence of a bulky alkyl group, such as adamantyl or cycloalkyl and a *tert*-butylcarbamate group in these compounds is important for HDAC6-selective inhibition.

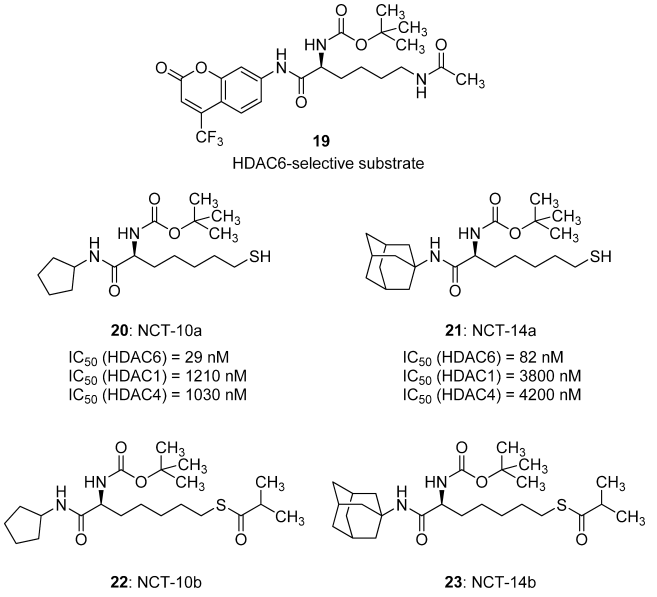


Fig. 9. Structures of Compound **19**, NCT-10b **22**, and NCT-14b **23** and Structures and Selectivity of NCT-10a **20** and NCT-14a **21**

In biological experiments, although NCT-10b **22** and NCT-14b **23** did not show cancer cell growth-inhibitory activity at the concentrations at which they displayed distinct α-tubulin acetylation effects, the combination of NCT-10b **22** or NCT-14b **23** and paclitaxel caused a synergistic inhibition of cancer cell growth.[89] The synergistic effect of these HDAC6-selective inhibitors may allow for a reduction of the required paclitaxel dosage with consequently fewer side effects. In addition, NCT-10b **22** and NCT-14b **23** also showed inhibition of the estrogen-stimulated growth of human breast cancer MCF-7 cells,[89] which can be explained by the finding that the acetylation of HSP90 owing to HDAC6 inhibition and the subsequent inhibition of chaperone function of HSP90 cause the attenuation of ERa.[90] These findings suggested that HDAC6-selective inhibitors have potential as anticancer agents.

NCT-10b **22** was used to increase the efficiency of gene transfer.[91] The success of viral and nonviral gene delivery relies on the ability of DNA-based vectors to traverse the cytoplasm and reach the nucleus. It has been reported that plasmids utilize the microtubule network and its associated motor proteins to traffic toward the nucleus.[92—94] While disruption of microtubules with nocodazole was shown to greatly inhibit cytoplasmic plasmid trafficking, it did not abolish it. It has been demonstrated that a pool of stabilized posttranslationally acetylated microtubules exists in cells, and that this acetylation may play a role in protein trafficking. In order to determine whether this modification could account for the residual DNA trafficking in nocodazole-treated cells, we inhibited HDAC6 (α-tubulin deacetylase) using NCT-10b **22**, thereby generating higher levels of acetylated microtubules. Electroporation of plasmids into cells with HDAC6 inhibited by NCT-10b **22** resulted in increased gene transfer. This increased transfection efficiency was not because of increased transcriptional activity, but rather, because of increased cytoplasmic trafficking. This result suggested that modulation of HDAC6 and the microtubule network can increase the efficiency of gene transfer.

2. Isoform-Selective Inhibitors of NAD^+-Dependent HDACs

2.1. 2-Anilinobenzamide, a SIRT1-Selective Inhibitor In contrast to Class I and Class II HDACs, which are zinc-dependent deacetylases, deacetylation *via* Class III HDACs is dependent on NAD^+.[95,96] In the deacetylation reaction of SIRTs, it is believed that NAD^+ is hydrolyzed to release nicotinamide and the acetyl group of the acetylated lysine substrate is transferred to cleaved NAD^+, generating *O*-acetyl-ADP ribose.[97,98] To date, several classes of Sir2 or SIRT inhibitors have been reported (Fig. 1).[99] Among these, nicotinamide is a potent SIRT inhibitor and it has been proposed that it inhibits SIRTs by binding to a conserved pocket adjacent to the NAD^+ binding pocket, thereby blocking NAD^+ hydrolysis.[96,100]

We evaluated the SIRT1-inhibitory activity of compounds in an in-house library comprised of nicotinamide and benzamide derivatives, which were expected to bind to the NAD^+- or nicotinamide-binding pocket of SIRTs, and found that 2-anilinobenzamide (NCS-7, **24**) (Fig. 10) is a SIRT1-selective inhibitor (SIRT1 $IC_{50}=17\,\mu M$; SIRT2 $IC_{50}=74\,\mu M$; SIRT2 $IC_{50}=235\,\mu M$).[101] NCS-7 **24** does not inhibit class I and II HDACs at a concentration of $1000\,\mu M$. Kinetic enzyme assays made it clear that NCS-7 **24** competes with the acetylated lysine substrate. Molecular modelling suggested the significance of the conformation of NCS-7 **24** and the formation of hydrogen bonds between NCS-7 **24** and SIRTs. NCS-7 **24** caused p53 acetylation in cells, presumably owing to SIRT1 inhibition.

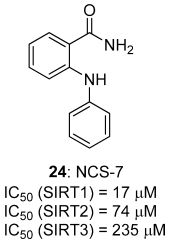


Fig. 10. Structure and Selectivity of NCS-7 **24**

2.2. An Acetylated Lysine Analogue, a SIRT1-Selective Inhibitor We designed SIRT inhibitors based on the proposed catalytic mechanism for the deacetylation of acetylated lysine residues.[102] Marmorstein and co-workers reported a high-resolution structural analysis of the ternary complex of yeast Hst2 (homologue of SIRTs) with an acetylated histone H4 peptide and a stable NAD^+ analogue, carba-NAD^+, as well as a ternary complex with ADP-ribose.[98] These crystal structures and detailed analysis of the catalytic mechanism utilizing various acetylated lysine analogues[103,104] have led to a solid understanding of the catalytic mechanism of the deacetylation of acetylated lysine substrates by sirtuins (Fig. 11). First, the oxygen of the acetylated lysine substrate attacks the 1′-carbon of the ribose ring of NAD^+ *via* an S_N2-like mechanism, releasing nicotinamide.[103,105] Next, the resulting imido ester intermediate is attacked by the 2′-OH group of the ADP ribose activated by His 135 (Hst2 numbering) to form a cyclic intermediate.[104] Finally, cleavage of the acetal analogue by water coordinated to Asn 116 and protonated His 135 affords deacetylated lysine and 2′-*O*-acetyl-ADP-ribose. This proposed catalytic mechanism for the deacetylation of acetylated lysine substrates provides a basis for the design of selective sirtuin in-

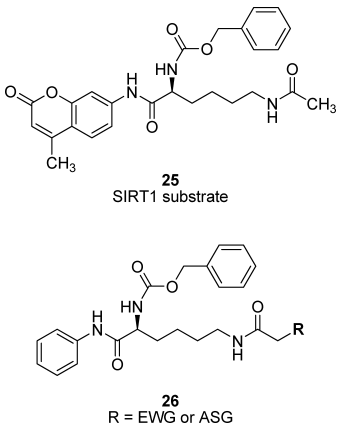


Fig. 12. Structures of Small-Molecular SIRT1 Substrate **25** and Acetylated Lysine Analogues **26**

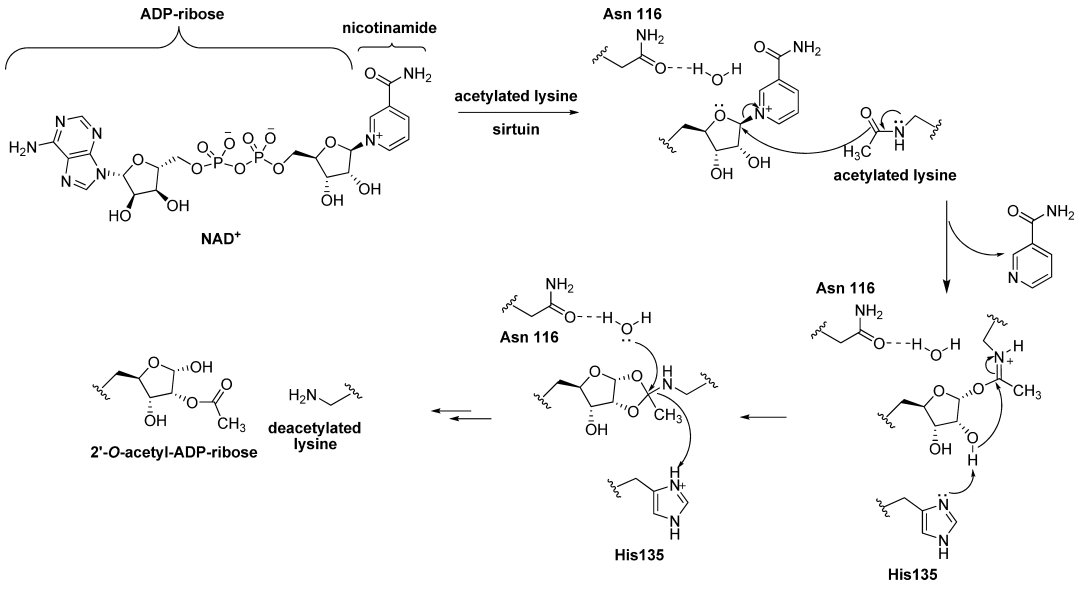


Fig. 11. Proposed Catalytic Mechanism for the Deacetylation of Acetylated Lysine by Sirtuins

hibitors. In designing selective SIRT inhibitors, we focused initially on a small-molecular SIRT1 substrate **25**.[87] Based on the structure of **25**, we designed acetylated lysine analogues **26** (Fig. 12) in which the coumarin structure is replaced with a simple benzene ring and various electron-withdrawing groups (EWGs) or anion-stabilizing groups (ASGs) are introduced at the α-position of the acetamide. The introduction of EWG or ASG at this position would enhance enol or anion formation of the acetylated lysine analogues, which in turn would increase the nucleophilicity of the α-carbon. If the nucleophilicity of the α-carbon is higher than that of the oxygen of acetamide, nucleophilic attack on NAD^+ should occur at the α-carbon (Fig. 13). The formation of the carbon–carbon bond, which is thermodynamically highly stable, would generate a stable acetylated lysine-ADP-ribose conjugate. We anticipated that this *in situ* generation of the conjugate might lead to potent SIRT inhibition, because the *in situ*-generated analogue would occupy both the acetylated lysine-binding pocket and the NAD^+-binding pocket of SIRTs. Among the compounds thus designed and synthesized, we found that NCS-12k **27** (Fig. 14), which contains an ethoxycarbonyl group at the α-position to the acetamide of acetylated lysine substrate analogue **25**, showed potent inhibitory activity in *in vitro* assay using recombinant SIRT1, with high selectivity over SIRT2 and SIRT3 (IC_{50} for SIRT1$=$3.9 μM; IC_{50} for SIRT2$=$65 μM; IC_{50} for SIRT3$>$300 μM). Mechanistic study by means of kinetic analysis, mass spectroscopy, and computation indicated that the enol form of NCS-12k **27** nucleophilically attacks NAD^+ in the active site of SIRTs to afford the stable NCS-12k **27**-ADP-ribose conjugate **28**, leading to inhibition of the enzyme activity (Fig. 15). NCS-12k **27** also caused a dose-dependent increase of p53 acetylation in human colon cancer HCT116 cells, indicating inhibition of SIRT1 in the cells. These results have implications for the development of selective sirtuin inhibitors by means of mechanism-based drug design.

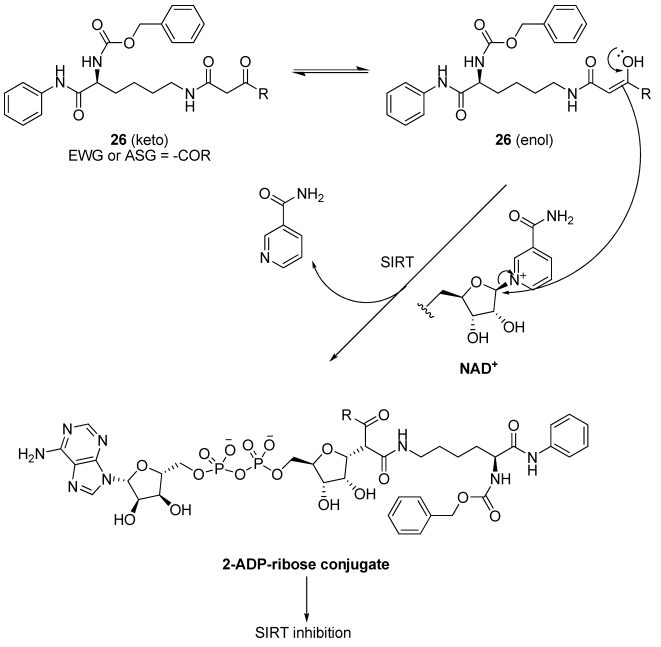


Fig. 13. Possible Mechanism of Sirtuin Inhibition by *in Situ* Formation of a Substrate Analogue-ADP-Ribose Conjugate

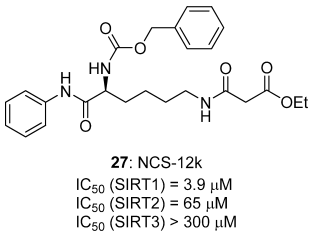


Fig. 14. Structure and Selectivity of NCS-12k **27**

Conclusion

As shown in this review, we have identified several isoform-selective inhibitors. As for isoform-selective zinc-dependent HDAC inhibitors, we designed non-hydroxamate ZBGs and found that hydroxamic acid can be replaced by

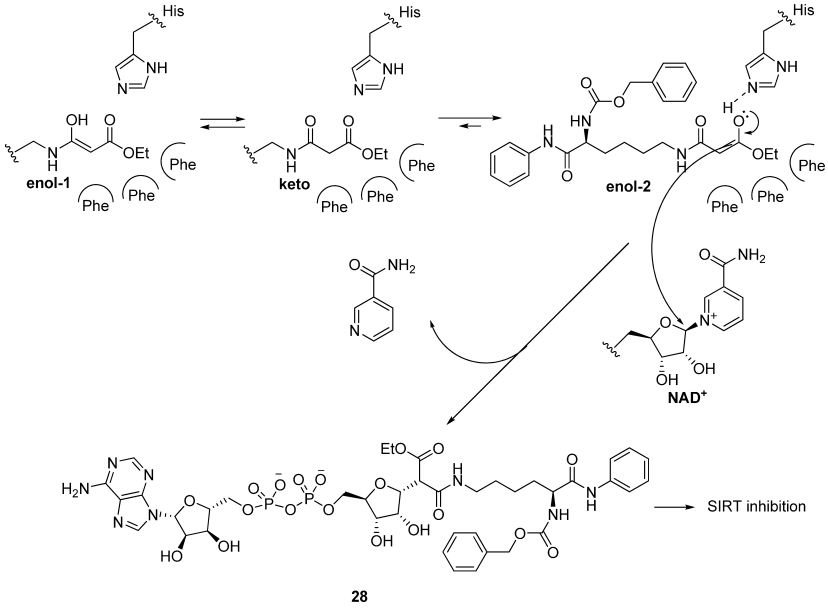


Fig. 15. Proposed Mechanism of Inhibition of SIRTs by NCS-12k **27**

thiol or mercaptoacetamide. Based on the thiol structure, NCH-31, an HDAC6-insensitive inhibitor, and NCT-10 and NCT-14, HDAC6-selective inhibitors, were identified. In addition, on the basis of our research, several isoform-selective zinc-dependent HDAC inhibitors such as HDAC3- and HDAC6-selective inhibitors have been identified by other groups.[106,107] With respect to isoform-selective NAD^+-dependent HDAC inhibitors, we identified SIRT1-selective inhibitors, which were designed based on the crystal structure of the enzyme and the catalytic deacetylation mechanism. Furthermore, we elucidated the functions of HDAC6 by chemical genetic approaches using NCH-51 **18** and NCT-10b **22**. The function of SIRT1 can be analyzed by our SIRT1-selective inhibitors and chemical genetic research using the SIRT1-selective inhibitors is currently underway.

Although many HDAC inhibitors have been found to date, there are only a limited number of isoform-selective inhibitors.[68,108,109] These selective inhibitors are intriguing both chemically and biologically, and are of interest as candidate therapeutic agents having few side effects. To date, X-ray crystal structures of human HDAC4,[110] HDAC7,[111] HDAC8,[73—75] SIRT2[112] and SIRT5[113] have been published. These crystal structures should pave the way for the discovery of novel isoform-selective inhibitors, which will be useful not only as tools for the detailed elucidation of the biological functions of the isoforms, but also in the development of therapeutic agents with few side effects.[67,68,114]

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