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Histone deacetylase as a new target for cancer chemotherapy

Abstract Trichostatin A (TSA) and trapoxin (TPX), inhibitors of the eukaryotic cell cycle and inducers of morphological reversion of transformed cells, inhibit histone deacetylase (HDAC) at nanomolar concentrations. Recently, FK228 (also known as FR901228 and depsipeptide) and MS-275, antitumor agents structurally unrelated to TSA, have been shown to be potent HDAC inhibitors. These inhibitors activate the expression of p21Waf1 in a p53-independent manner. Changes in the expression of regulators of the cell cycle, differentiation, and apoptosis with increased histone acetylation may be responsible for the cell cycle arrest and antitumor activity of HDAC inhibitors. TSA has been suggested to block the catalytic reaction by chelating a zinc ion in the active site pocket through its hydroxamic acid group. On the other hand, an epoxyketone has been suggested to be the functional group of TPX capable of alkylating the enzyme. We synthesized a novel TPX analogue containing a hydroxamic acid instead of the epoxyketone. The hybrid compound, called cyclic hydroxamic-acid-containing peptide 1 (CHAP1) inhibited HDAC at low nanomolar concentrations. The HDAC1 inhibition by CHAP1 was reversible, as is that by TSA, in contrast to irreversible inhibition by TPX. Interestingly, HDAC6, but not HDAC1 or HDAC4, was resistant to TPX and CHAP1, while TSA inhibited these HDACs to a similar degree. CHAP31, the strongest HDAC inhibitor obtained from a variety of CHAP derivatives, exhibited antitumor activity in BDF1 mice bearing B16/BL6 tumor cells. These results suggest that CHAP31 is promising as a novel therapeutic agent for cancer treatment, and that CHAP may serve as a basis for new HDAC inhibitors and be useful for combinatorial synthesis and high-throughput screening.

Keywords Transcription · Histone · Acetylation · HDAC · Trichostatin · Trapoxin

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Introduction

The organization of chromatin is crucial for the regulation of gene expression. In particular, both the positioning and properties of nucleosomes influence promoter-specific transcription in response to extracellular or intracellular signals [8]. The nucleosome core contains DNA of 146 bp tightly wrapped around a central histone octamer comprising two molecules of each of the core histones. The four core histones (H2A, H2B, H3, and H4) are subject to a variety of enzyme-catalyzed posttranslational modifications, thereby modulating the function of the chromatin. Of the modifications, acetylation has been the most extensively studied. The primary sites of histone acetylation are specific lysine residues in the positively charged N-ter-

minal tails that protrude from the octamer, which are important for both histone-DNA and histone-nonhistone protein interactions. The neutralization of the positive charge by acetylation has long been thought to lead to loosening of histone-DNA contacts, which facilitates the accessibility of a variety of factors to DNA (Fig. 1). The recent description of the enzymes controlling histone acetylation and deacetylation confirms that acetylation of histones is an important step in transcription [16, 31].

Acetylation and deacetylation are catalyzed by specific enzyme families, histone acetyltransferases (HATs) and deacetylases (HDACs), respectively. HATs have been identified as transcriptional coactivators and include GCN5 [3], CBP/p300 [25], and PCAF [44], as well as the p160 family proteins. On the other hand, HDACs have been found to be yeast transcriptional regulators related to Rpd3 [32], Hda1 [28], and Sir2 [11]. A number of transcriptional repressors and corepressors, such as Sin3, SMRT, and N-CoR, have been shown to recruit the HDAC complex to the promoter regions [17, 33].

We have identified HDACs as the target of trichostatin A (TSA) and trapoxin (TPX), both of which are microbial metabolites that induce cell differentiation, cell cycle arrest, and reversal of transformed cell morphology [48]. Several phytopathogenic and antifungal compounds related to TPX, such as chlamydocin and HC-toxin, have also been shown to inhibit HDAC [2]. Since aberrant histone acetylation has been linked to malignant diseases in some cases, HDAC inhibitors have potential as new drugs due to their ability to modulate transcription and to induce differentiation and apoptosis [22]. FK228 [24] and MS-275 [29], potent antitumor agents under clinical investigation, have been shown to inhibit HDACs.

Here we discuss the mechanisms by which these known inhibitors block the catalytic reaction of HDAC. Furthermore, we report that a novel hybrid compound of TPX and TSA called cyclic hydroxamic-acid-containing peptide 1 (CHAP1), in which the 2-amino-8-oxo-9,10-epoxy-decanoic acid (Aoe) in TPX B is replaced by Asu(NHOH), strongly inhibits HDAC1. CHAP1 will be a unique lead for the development of specific HDAC inhibitors.

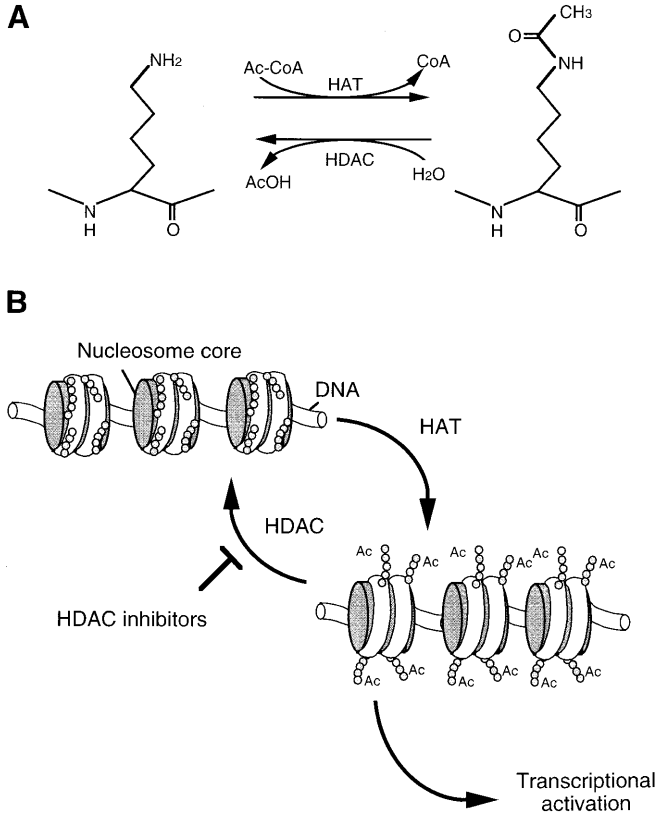

Fig. 1A, B Histone acetylation. **A** Acetylation and deacetylation of the ϵ-amino group at the specific lysine residues in the N-terminal tails of histones. The enzymatic reaction is catalyzed by histone acetyltransferase (HAT) and deacetylase (HDAC). **B** A model for the regulation of nucleosome structure and transcriptional activity mediated by reversible histone acetylation. Acetylation of the lysine residues in the histone tails looses the contact with DNA. Inhibition of HDAC leads to histone hyperacetylation

HDAC1 and related enzymes

In 1996, Schreiber and colleagues succeeded in the molecular cloning of HDAC1 by using its affinity to the specific inhibitor TPX [32] (see below). HDAC1 is remarkably similar to RPD3, a yeast transcriptional regulator, which had been identified in genetic screenings as a positive and negative factor for a subset of yeast genes [41]. In a separate study, biochemical fractionation of yeast extracts led to the discovery of two distinct yeast deacetylation activities, HDA (a 350-kDa complex) and HDB (a 600-kDa complex). Protein microsequencing and subsequent cloning demonstrated that the catalytic subunits for these deacetylase complexes were encoded by *HDA1* and *RPD3* genes, respectively. These two proteins share 49% similarity. Moreover, three other yeast open-reading frames (ORFs) have been found to be homologous to HDA1 (HOS1–3). To date, at least eight different mammalian enzymes related to HDAC1 have been described, and they are divided into class I (similar to Rpd3) [4, 10, 18, 32] and class II (similar to Hda1) [7, 13, 39, 42].

These HDACs, together with the prokaryotic enzymes acetylspermine deacetylase (ASD) and acetoin utilization protein (acuC), comprise a deacetylase superfamily (Fig. 2). Accumulating data suggest that each member of the HDAC family is a component of a physical complex playing a distinct role in gene expression. For example, a large protein complex containing HDAC1 binds the E2F transcription factor via association with the retinoblastoma tumor suppressor protein [1, 21], while HDAC4 and HDAC5 associate specifically with the myocyte enhancer factor MEF2A and repress MEF2A-dependent transcription [19, 23]. Of these HDACs, HDAC6 is a unique isoform, in which the catalytic domain is internally duplicated [7, 39].

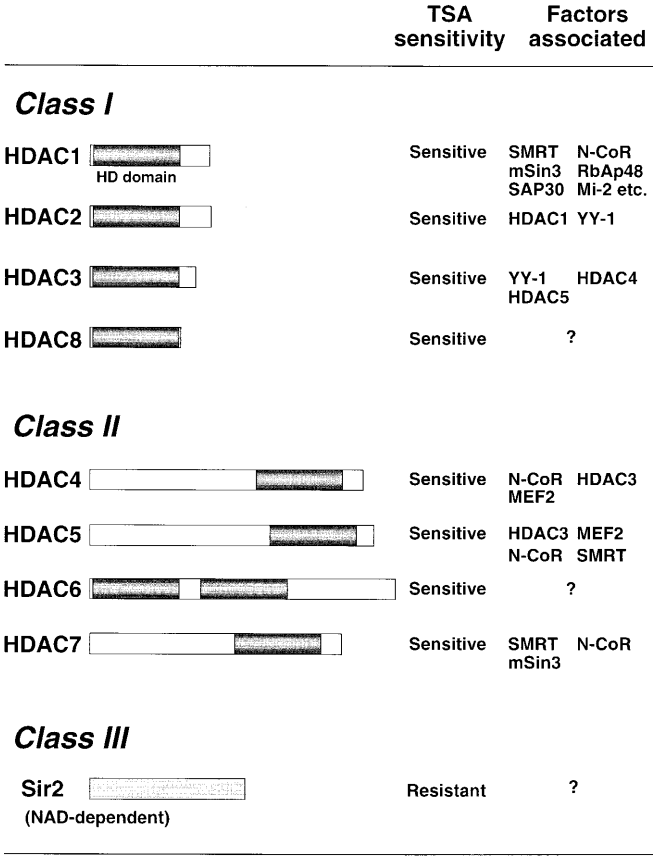

Fig. 2 Family of mammalian HDAC enzymes. The class I enzymes are similar to yeast RPD3, while the class II enzymes are related to yeast HDA1. The class I and II enzymes possess one or two histone deacetylase domains necessary for enzyme activity. Sir2 is a novel class of HDAC without homology to the class I and II enzymes whose the activity is dependent on NAD

Furthermore, it is normally localized in the cytoplasm and only a fraction of the protein relocalizes into the nucleus in response to stimuli for differentiation, suggesting that its natural substrates include nonhistone acetylated proteins [40]. Most recently, Sir2, a yeast silencer protein, and its human homologue have been shown to be a new type of HDAC without any homology with known HDACs. The HDAC activity of Sir2 is dependent on nicotinamide adenine dinucleotide (NAD) [11]. The specific roles of these enzymes and their target genes remain to be elucidated.

Molecular mechanism of HDAC inhibition by TSA

TSA and its glucopyranosyl derivative (trichostatin C) were originally isolated from *Streptomyces hygroscopicus* as antifungal antibiotics active against *Trichophyton* spp (Fig. 3) [34, 35]. About 10 years thereafter, the potent activity of trichostatins in inducing differentiation of Friend murine erythroleukemia (MEL) cells and in inhibiting proliferation of mammalian cells was reported [45, 46]. Exposure of MEL cells to nanomolar concentrations of TSA for 4–5 days produces hemoglobin-positive cells detected with high efficiency by benzidine staining. A clue to understanding the target of TSA was incidentally obtained from the analysis of the modification of histones. Acid urea triton (AUT) gel electrophoresis, which can separate core histone molecules with different degrees of acetylation, revealed that histones in the cells treated with TSA are acetylated to unusually high degrees.

Pulse-chase experiments have revealed that histone hyperacetylation induced by TSA is due not to increased acetylation but to decreased deacetylation of histones. In vitro experiments using partially purified histone deacetylase from mouse mammary tumor cells (cell line FM3A) have shown that TSA is a potent inhibitor with a Ki value of 3.4 n*M*. We have also derived a mutant cell line called TR303 from FM3A which is resistant to TSA alone and have found that the histone deacetylase from the mutant cells possesses a markedly increased Ki value of 31 n*M*, indicating that the enzyme itself has become resistant to TSA. This genetic evidence confirms that histone deacetylase is the primary intracellular target of TSA [47].

Recent crystallographic studies [5] have shown a zinc-dependent acetamide cleavage reaction by a bacterial enzyme related to HDAC (histone deacetylase-like protein, HDLP). Cocrystallization of this enzyme with TSA or suberoylanilide hydroxamic acid (SAHA) [27] has demonstrated that these inhibitors mimic the substrate, and that chelation of the zinc in the catalytic pocket by the hydroxamic acid group is the main mechanism of inhibition [5]. TSA is thought to consist of a dimethylamino-phenyl group as a cap group and a hydroxamic acid as a functional group for enzyme inhibition (Fig. 3). The aromatic ring group serves as a cap necessary for packing the inhibitor at the rim of the tube-like active-site pocket (Fig. 4). Conservation of the amino acid sequences of the loops that form the active-site pocket among HDLP and class I and class II HDACs strongly suggests the same catalytic reaction and TSA inhibition of HDAC as HDLP. This mechanism of inhibition is consistent with the reversible enzyme inhibition by TSA.

TPX: an irreversible inhibitor

TPX A and B (Fig. 3) were isolated from fungal metabolites as cyclic tetrapeptides inducing morphological reversion of v-*sis*-transformed NIH3T3 cells by Itazaki et al. [12] in 1990. Since TSA has also been reported to have similar activity in inducing morphological reversion of v-*sis*-transformed cells, we examined the similarity between the molecular action of these agents. TPX was found to cause accumulation of highly acetylated core histones in a variety of mammalian cells. In vitro experiments using partially purified histone deacetylase showed that a low concentration of TPX irreversibly inhibits deacetylation of acetylated histone molecules, in

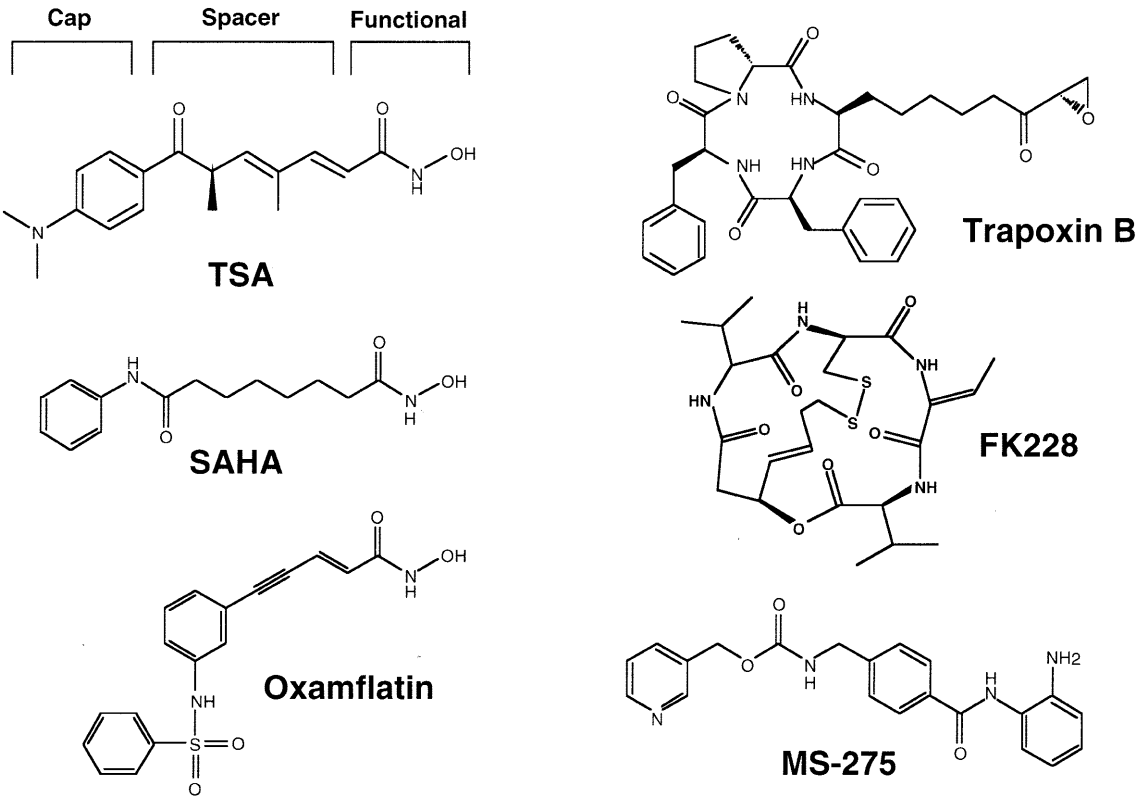

Fig. 3 Chemical structures of HDAC inhibitors. Most inhibitors comprise cap, spacer, and functional groups

contrast to the reversible inhibition by TSA. Kinetic analysis indicates that TPX can be classified as a "slow-binding inhibitor".

TPX contains an unusual amino acid, 2-amino-8-oxo-9,10-epoxy-decanoic acid (Aoe), which may act as an acetylated lysine substrate mimic, since TSA can compete with TPX for binding to HDAC1 [32]. Since only the epoxyketone group in the Aoe residue is a chemically reactive moiety, this moiety appears likely to play a role in forming a covalent bond between TPX and the enzyme molecule. This idea is supported by chemical reduction of the epoxide group. The reduced TPX loses its inhibitory activity, suggesting that TPX inactivates the histone deacetylase by binding the enzyme via its epoxide group. TR303 shows cross-resistance to TPX. Thus TPX is an additional specific inhibitor of HDAC [14]. As described above, human HDAC1 was first isolated using its affinity to a TPX analogue.

CHAP: a potent and reversible inhibitor of HDAC1

If the aliphatic chain of Aoe in TPX (*cyclo*[Aoe-Phe-Phe-D-Pro]), acts as a substrate analogue, then replacement of the epoxyketone group of TPX with the hydroxamic acid group of TSA should convert it to a reversible-type inhibitor that chelates the catalytic center zinc. To test this possibility, we synthesized a novel cyclic tetrapeptide compound containing L-Asu(NHOH) instead of Aoe and evaluated its ability to inhibit HDAC1. The compound containing L-Asu(NHOH) was a strong inhibitor, blocking the enzyme activity with a 50% inhibitory concentration (IC_{50}) at the nanomolar level. This is the first example of a synthetic HDAC inhibitor stronger than TSA (Table 1). We called the compound CHAP1.

To test whether the effect of CHAP1 is reversible, we incubated HDAC1 immobilized on agarose beads with CHAP1, TSA, or TPX for 20 min, washed the beads thoroughly, and then analyzed the enzyme activity associated with the bead-conjugated HDAC1 in the absence of the inhibitors. The activity of the enzyme that had been treated with CHAP1 recovered to almost the initial level, while that of the enzyme treated with TPX B did not (data not shown). These results clearly demonstrate that the replacement of the epoxyketone group with the hydroxamic acid converted the inhibitor to a reversible one.

Target enzyme specificity of HDAC inhibitors

Although a number of natural and synthetic compounds have been described as HDAC inhibitors, little is known about their target enzyme specificity. Class I deacetylases related to yeast Rpd3 include HDAC1 [32], HDAC2 [18], HDAC3 [4], and HDAC8 [10], while class II related to Hda1 contains HDAC4 [7, 42], HDAC5 [7, 39], HDAC6 [7, 39], and HDAC7 [13]. It has been reported that HDAC1 and HDAC2 are found in the same complex [18], while HDAC4 and HDAC5 are associated

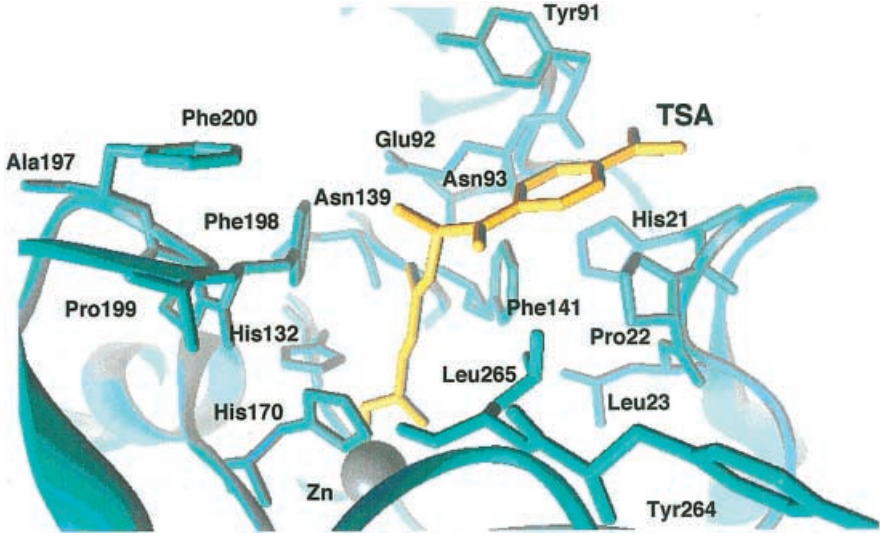

Fig. 4 Structure of the HDLP-TSA complex [5]. This figure was prepared with the program WebLab ViewerLite 3.2 (Molecular Simulations, San Diego, Calif.) using the coordinate 1C3R in the Brookhaven Protein Data Bank

Table 1 Effects of TPX, TSA, and CHAPs on isoforms of HDAC (*NT* not tested)

Compound	IC_{50} (n*M*)			HDAC6/HDAC1
	HDAC1	HDAC4	HDAC6	
TSA	6.0	38	8.6	1.4
TPX B	0.11	0.30	360	3300
CHAP1	1.90	2.70	19	10
CHAP31	0.38	NT	13	34

with HDAC3 [7]. HDAC6, a particular isoform that contains two deacetylase domains, has not been reported to be associated with other known HDACs. We chose HDAC1 as a class I enzyme and HDAC4 and HDAC6 as class II deacetylases, and compared the effects of these inhibitors.

TSA inhibited all the deacetylases tested to a similar degree, although HDAC4 was slightly resistant when compared with HDAC1 and HDAC6 (Table 1). TPX also strongly inhibited the enzyme activity of HDAC1 and HDAC4 at subnanomolar concentrations. Surprisingly, however, HDAC6 was highly resistant to TPX (Table 1). A similar but smaller degree of resistance was also observed with CHAP1 for HDAC6. HDAC1 was the most sensitive to CHAP1 among the HDACs tested, and the IC_{50} value for HDAC6 was tenfold greater than that for HDAC1. These results suggest that the cyclic tetrapeptide structure is responsible, at least in part, for the weak inhibition of HDAC6 by TPX.

TSA and SAHA, simple analogues of acetylated lysine with small cap groups, may cause nonselective inhibition of class I and II HDACs. On the other hand, the cyclic tetrapeptide structure, which probably makes extensive contacts at the rim of the pocket and in the shallow grooves surrounding the pocket entrance, may mimic the substrate structure surrounding acetylated lysine. If so, it would be likely that the target enzyme specificity of CHAP can be modulated by changing amino acids in the cyclic tetrapeptide. The fact that CHAP1 and TPX were less active against HDAC6 than HDAC1 supports the idea that the cyclic tetrapeptide structure is responsible for the target enzyme specificity. The cyclic tetrapeptide structures of CHAPs synthesized in this study may be distant from the natural substrates for HDAC6.

Histone acetylation and cancer

Molecular analyses of human diseases have suggested that changes in acetylation may play a role in the uncontrolled cell growth of cancer. The Rubinstein-Taybi syndrome (RTS) is a well-defined syndrome with facial abnormalities, broad thumbs, broad first toes, and mental retardation as the main clinical features. In addition, an unusual incidence of neoplasms as well as the formation of keloid, a hyperproliferative response of fibroblasts to dermal injury, in RTS indicate the relevance of the susceptible gene of this disease to malignancy. Many patients with RTS have been shown to have breakpoints in, or deletions of, chromosome 16p13.3. Petrij et al. [26] have identified the region of the breakpoints where the CBP gene is mutated. Since the patients are heterozygous for the mutations, the loss of one functional copy of the CBP gene underlies the abnormalities in RTS. One can assume that the decrease in the gene dosage of CBP may cause insufficient histone acetylation in some specific chromatin regions and disturb correct gene expression, thereby promoting tumor incidence.

Several leukemogenic transcription factors repress expression of specific genes because of aberrant recruitment of HDACs. This repression of gene expression appears to be an important step in the leukemogenic action of these transcription factors. For example, aberrant recruitment of HDAC activity has been reported in cell lines derived from patients with acute promyelo-

cytic leukemia (APL) [6, 9, 20]. The oncoprotein encoded by the translocation-generated fusion gene in APL (promyelocytic leukemia–retinoic acid receptor-α) represses transcription by recruitment of HDAC1. Furthermore, resistance to the differentiating actions of all-*trans*-retinoic acid in a patient with APL has been overcome by cotreatment with an inhibitor of HDAC [43].

Antitumor activity of HDAC inhibitors

Although TSA and TPX are potent inhibitors of HDAC, they do not show sufficient antitumor activity, probably due to their in vivo instabilities. However, oxamflatin, an aromatic sulfonamide derivative with a hydroxamic acid-inhibiting HDAC (Fig. 3), has been shown to have in vitro antiproliferative activity against various mouse and human tumor cell lines, and in vivo antitumor activity against B16 melanoma [15]. Recently, the molecular mechanism of action of FR901228 (FK228), a potent antitumor substance (Fig. 3), has also been shown to be HDAC inhibition [24].

FK228 was isolated from *Chromobacterium violaceum* as an agent inducing morphological reversion of H-*ras*-transformed NIH3T3 cells [30, 36, 37]. It strongly inhibits proliferation of tumor cells in vitro by arresting cell cycle transition at the G_1 and G_2/M phases [38]. In addition, FK228 suppresses the growth of transplanted tumors in mice. Thus FK228 is now under clinical trials for cancer therapy.

MS-275, a newly synthesized benzamide derivative with HDAC inhibitory activity, has also been reported to have marked in vivo antitumor activity in transplant models [29]. Most recently, we have isolated an additional antitumor HDAC inhibitor from CHAP derivatives. Based on the potency of HDAC1 inhibition (Table 1), we selected CHAP31 and further characterized it. CHAP31 brings about hyperacetylation of core histones, and its stability in the presence of cultured cells is better than that of TSA or TPX. CHAP31 inhibits the growth of tumors in mice inoculated with the B16/BL6 murine melanoma line (data not shown). Thus CHAP31 is one of the promising candidates for antitumor drugs with strong HDAC-inhibiting activity.

Conclusions

HDAC inhibitors show potential as new antitumor drugs due to their ability to modulate transcription and to induce differentiation and apoptosis. We have shown potent and specific inhibition of HDAC1 by TSA, TPX, and its hybrid compounds CHAP. Our data suggest that the cyclic tetrapeptide portion affects both enzyme inhibitory potency and specificity, whereas the hydroxamic acid group acts as a potent enzyme-inhibiting group with in vivo stability better than the epoxyketone. One of the CHAP derivatives has shown strong antitumor activity in animal models. We have also shown the possibility of developing isoform-specific inhibitors based on CHAP. Since a marked structural diversity can be obtained by combinatorial synthesis of the cyclic tetrapeptide, CHAP is a promising basis on which to develop isoform-specific HDAC inhibitors. These specific HDAC inhibitors may be important for improving the therapeutic potential of transcription therapy and chemotherapy.

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