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Cyclic Hydroxamic-acid-containing Peptide 31, a Potent Synthetic Histone Deacetylase Inhibitor with Antitumor Activity

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ABSTRACT

Cyclic hydroxamic-acid-containing peptide 1 (CHAP1), designed as a hybrid of trichostatin A and trapoxin, is a lead compound for the development of potent inhibitors of histone deacetylase (HDAC). In this study, we synthesized a series of CHAP derivatives and evaluated their biological activities by monitoring the potency of their inhibition of HDAC activity, their ability to augment the expression of MHC class-I molecules in B16/BL6 cells, and their effect on cell proliferation. A structure-activity relationship study using these three assay systems revealed several requirements of their structure for the strong inhibition of HDAC not only in the cell-free situation, but also in cells. When the structures of CHAP derivatives are represented as *cyclo*(-Asu(NHOH)-AA_2-AA_3-Pro or Pip-)$_n$, where Asu(NHOH) and Pip are ζ-hydroxamide-α-aminosuberic acid and pipecolic acid, respectively, (*a*) the tetrapeptide structure ($n = 1$) was better than the octapeptide one ($n = 2$); (*b*) AA_2 and AA_3 should be hydrophobic; and (*c*) the combination of amino acid chirality should be LDLD for the strongest inhibition of HDAC in cells (LDLD > LLLD, LDLL > LLDL). *cyclo*(-L-Asu(NHOH)-D-Tyr(Me)-L-Ile-D-Pro-) or CHAP31 was selected as one of the strongest CHAPs, and its biological activity was characterized further. CHAP31 was much more stable in the presence of cultured cells ($t_{1/2} > 3000$ h) than trichostatin A ($t_{1/2} = 14.7$ h) or trapoxin A ($t_{1/2} = 2.10$ h). CHAP31 exhibited antitumor activity in C57BL × DBA/2 F_1 ($BD2F_1$) mice bearing B16/BL6 tumor cells. Furthermore, CHAP31 inhibited the growth in four of five human tumor lines implanted into nude mice. These results suggest CHAP31 to be promising as a novel therapeutic agent for cancer treatment.

INTRODUCTION

HDAC[2] is a family of enzymes (1–6) playing an important role in gene expression. Because it has been reported that its inhibition brought about cell-cycle arrest and induced differentiation (7–13), HDAC is considered a target for new types of pharmaceuticals. A possible application of HDAC inhibitors would be treatment of cancers (14, 15). In fact, FK228, a naturally derived HDAC inhibitor (formerly known as FR901228, or depsipeptide; Ref. 16), is now under clinical trial at the National Cancer Institute (Frederick, MD) for cancer therapy. MS-275, a newly synthesized benzamide derivative, and suberoylanilide hydroxamic acid, a synthetic hybrid polar compound, were also reported to be HDAC inhibitors and to have marked *in vivo* antitumor activity in transplant models (17, 18). A differentiation therapy using HDAC inhibitor and RA has been proposed for the treatment of an RA-resistant type of acute promyelocytic leukemia, in which RA cannot release HDAC associated with the mutated retinoid-α receptor fused to the promyelocytic leukemia zinc finger protein from the promoter of RA-inducible genes (19–21). In fact, a recent trial study on the coadministration of RA with sodium phenylbutyrate, an HDAC inhibitor, for acute promyelocytic leukemia suggested its usefulness in such a combination therapy (22). Other applications of HDAC inhibitors than cancer treatment, such as antimalaria drugs (23) and additives for the efficient expression of adenovirus vectors for gene therapy (24, 25), are also promising.

Several structurally unrelated HDAC inhibitors have been isolated, *e.g.*, TSA (Fig. 1; Ref. 13), TPX (Fig. 1; Ref. 8), FK228 (16), HC-toxin I (26), chlamydocin (26), apicidin (23), and depudecin (27), from natural sources. However, we have only limited information about the structure suitable for strong inhibition of HDAC, both in cell-free and cellular situations, because no comparative studies have been reported on their biological activities. Recently, some synthetic compounds having HDAC inhibitory activity have been reported (17, 28–30), but their activity was about 10–1000-fold weaker than that of the naturally derived strong HDAC inhibitors such as TSA and TPX. The functional group of TSA responsible for the inhibition of HDAC is the hydroxamic acid moiety (12), which chelates a zinc ion in the active-site pocket, whereas that of TPX and other related compounds was suggested to be the epoxyketone structure at the end of the side chain of a constituent amino acid, Aoe (8). In a previous study (31), we synthesized cyclic peptide compounds having a hydroxamic acid side chain instead of the epoxyketone (CHAP), which corresponded to TPX, Cyl-1 and -2 (Fig. 1), WF-3161, chlamydocin, and HC-toxin, and showed that they all strongly and reversibly inhibited HDAC. These results suggest that the side chain of the Aoe-containing cyclic tetrapeptide is a substrate mimic, and that the replacement of the epoxyketone with the hydroxamic acid converted it to an inhibitor chelating the zinc like TSA.

Here we report a SAR study using both enzymatic and cellular assay systems. The biological activity of CHAPs varied by changing the number of the amino acids constituting the ring structure, the pattern of the combination of amino acid chirality, and the side chain structure of each amino acid. We also show that CHAP31, one of the most potent CHAPs, inhibited tumor growth in mice bearing murine or human cancer transplants, indicating that CHAP31 is a promising candidate for an anticancer drug having HDAC-inhibiting activity.

MATERIALS AND METHODS

Materials. Tritium-labeled acetic anhydride was purchased from Daiichi Pure Chemicals (Tokyo, Japan). BSA used in the present study was of RIA grade and was obtained from Sigma Chemical Co. (St. Louis, MO). CDDP was purchased from Nacalai Tesque (Osaka, Japan). Other chemicals used were of reagent grade.

Cell Lines. Murine melanoma B16/BL6, human melanoma LOX-IMVI, and human lung cancer DMS114 were obtained from the National Cancer Institute (Frederick, MD). Human stomach cancers MKN-7 and MKN-74, and

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[2] The abbreviations used are: HDAC, histone deacetylase; RA, retinoic acid; TSA, trichostatin A; TPX, trapoxin; Aoe, (2S, 9S)-2-amino-9, 10-epoxy-8-oxodecanoic acid; Asu(NHOH), ζ-hydroxamide-α-aminosuberic acid; CHAP, cyclic hydroxamic-acid-containing peptide; SAR, structure-activity relationship; FBS, fetal bovine serum; CDDP, *cis*-platinum (II) diamine dichloride; RP-HPLC; reverse-phase high-performance liquid chromatography; Pip, pipecolic acid; Asu, α-aminosuberic acid; Api, α-aminopirrelic acid; Aaz, α-aminoazelaic acid.

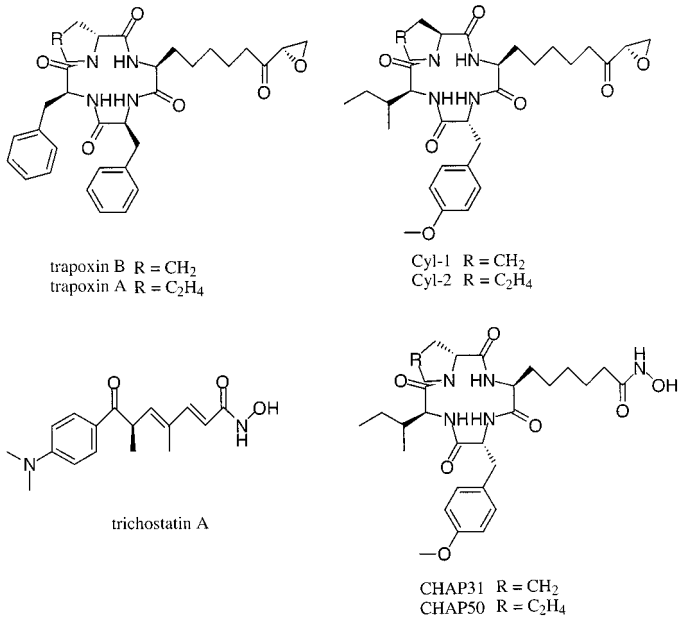

Fig. 1. Structures of TPX A and B, Cyl-1 and -2, TSA, CHAP31, and CHAP50.

human breast cancer BSY-1 were described elsewhere (32, 33). B16/BL6 cells were cultured in Eagle's MEM supplemented with 10% FBS, and the human lines were cultured in RPMI 1640 supplemented with 5% FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin under a 5% CO_2 atmosphere at 37°C in a humidified chamber. Mouse myeloma SP2/*O*-Ag14 was obtained from the RIKEN cell bank (Tsukuba, Ibaraki, Japan) and cultured in RPMI 1640 supplemented with 10% FBS.

Synthesis of CHAPs. CHAPs used in this study were synthesized according to one of the following 3 strategies: strategy 1, liquid-phase synthesis of linear peptide and subsequent liquid-phase cyclization; strategy 2, solid-phase synthesis of linear peptide and subsequent cyclization upon cleavage from resin; and strategy 3, solid-phase synthesis of linear peptide and subsequent cleavage from resin and then liquid-phase cyclization. The cyclic peptide precursors were converted to corresponding hydroxamates (CHAPs) by side chain modification (31, 34, 35). The resulting CHAPs were purified by RP-HPLC (column, YMC-Pack ODS A323; 10 × 250 mm; elution, isocratic with 25% CH_3CN/0.1% TFA) and characterized by fast atom bombardment mass spectrometry. The purity of all CHAPs synthesized in the present study was determined by RP-HPLC (column, Wako Pak C_{18}; 4.6 × 150 mm; elution, 10- 100% linear gradient of CH_3CN/0.1% TFA over 30 min) to be >95%. The details of the synthesis of CHAPs will be reported elsewhere.

HDAC Assay. The activity of CHAPs and related compounds toward HDAC was measured using HDAC prepared from mouse melanoma B16/BL6 cells as an enzyme source and a radio-acetylated histone H4 peptide as its substrate. HDAC of B16/BL6 was prepared according to the method reported previously (13), with minor modifications. Approximately 1×10^8 cells were suspended in 5 ml of buffer-A [15 mM potassium phosphate, 5% glycerol, 0.2 mM EDTA, and 1 mM 2-mercaptoethanol (pH 7.5)], homogenized by a Teflon homogenizer, and centrifuged (at 2,500 × *g* for 10 min). The pellet was resuspended in 2 ml of buffer-A containing 1 M $(NH_4)_2SO_4$, sonicated by a tip-type sonicator (TAITEC ULTRS Homogenizer VP-5S), and centrifuged (at 100,000 × *g* for 1 h). The concentration of $(NH_4)_2SO_4$ in the supernatant was elevated to 3.5 M, and the insoluble materials were collected by centrifugation (at 100,000 × *g* for 1 h). After the pellet had been resuspended in buffer-A (0.5 ml), the solvent was changed to buffer-A (12 ml) by gel filtration (PD-10; Amersham Pharmacia Biotech, Buckinghamshire, England) to yield a partially purified HDAC.

The NH_2-terminal peptide of histone H4 with an added COOH-terminal cysteine residue (SGRGKGGKGLGKGGAKRHRKVC) was purchased from Sawady Technology (Tokyo, Japan). This peptide (1 mg) dissolved in 4 ml of 20 mM $Na_2B_4O_7$ (pH 9.3) was mixed with 23 MBq of [^{3}H]acetic anhydride (NET018A; NEN) in dry dioxane (100 μl) under ice cooling and stirred for 30 min. After 6 ml of 50 mM acetic acid had been added, the reaction mixture was applied onto a SepPack C_{18} Cartridge (Waters Co., Milford, MA) that had been conditioned with 50 mM acetic acid. The cartridge was washed with 30 ml of 50 mM acetic acid, and then the [^{3}H]-labeled substrate was eluted by 2 ml of ethanol:water (1:1).

HDAC inhibitory activity of CHAPs was determined as follows. A solution of partially purified HDAC (80 μl) and inhibitors (dissolved in 10 μl of buffer-A containing 0.1% BSA) were mixed and preincubated for 30 min on ice. The enzymatic reaction was started by adding 10 μl of the [^{3}H]-labeled substrate diluted with buffer-A containing 0.1% BSA at the final concentration of ~5 μg/ml. After the reaction mixture had been incubated for 3 h at 37°C, the reaction was stopped by adding 25 μl of an acid solution (1 M HCl and 0.2 M acetic acid). The reaction was linearly dependent on time and the amount of enzyme under these conditions. The released [^{3}H]-acetic acid was extracted with 1 ml of ethyl acetate, and 0.8 ml of the solvent layer was mixed with 4 ml of liquid scintillator solution (ClearSol I; Nacalai Tesque, Osaka, Japan) for determination of its radioactivity. The degree of HDAC inhibition of compounds was calculated based on the control inhibition by 10 μM TSA (100% inhibition) and compared in terms of their IC_{50}s.

MHC Class-I Molecule Up-Regulating Assay. The activity of CHAPs and related compounds to induce the expression of MHC class-I molecules was determined by the method reported previously (11). Briefly, 24 h after 5000 B16/BL6 cells had been introduced into each well of a 96-well microplate (200 μl), test samples were added. After an additional 72 h of incubation, the cell-surface expression of the MHC class-I molecules was measured by a cell ELISA method. The degree of MHC class-I up-regulating activity of the compounds was compared with respect to their concentrations for 2-fold up-regulation ($C_{\times 2}$) of the MHC class-I molecules.

Cell Proliferation Assay. The cell proliferation inhibitory activity of CHAPs and related compounds was determined by measuring the bioreducing activity of viable cells by using a Promega CellTiter 96 Aqueous Non-radioactive Cell Proliferation Assay kit. The degree of cell proliferation inhibitory activity of compounds was expressed as their IC_{50}s.

Detection of Histone Acetylation. Effects of CHAPs on the degree of core histone acetylation were determined by Western blotting using an anti-acetyllysine monoclonal antibody. A hybridoma AL3D5 producing an anti-acetyllysine monoclonal antibody was obtained by immunizing female BALB/c mice with an acetyllysine-containing peptide (Gly-acetyllysine-ϵ-aminocaproic acid-Cys) conjugated with keyhole limpet hemocyanin and fusing their spleen cells with myeloma SP2/*O*-AG14 (36, 37). The antibody was purified by affinity gels immobilized with acetyllysine-containing peptides. After treatment with CHAPs, cells were harvested with 62.5 mM Tris-HCl and 2% SDS (pH 6.8), sonicated, boiled with 2-mercaptoethanol, and the lysates were then applied to SDS-12% PAGE. After being transferred to a polyvinylidene difluoride membrane, acetylated histones were probed with AL3D5 monoclonal antibody and detected using an ECL system (Amersham) according to the manufacturer's instructions. No large changes in histone contents in cells were observed even after the treatment with CHAPs (data not shown).

Degradation of HDAC Inhibitors in the Presence of Cultured Cells. The stability of HDAC inhibitors in the presence of cultured cells was determined by measuring the residual MHC class-I up-regulating activity in the cell-cultured media. One hundred pmol of TSA, TPX A, or CHAP31 were mixed with 1.5×10^5 B16/BL6 cells in 1 ml of culture medium and incubated at 37°C in a humidified chamber in the presence of 5% CO_2. The culture media were sampled at various time points and frozen. The up-regulation activity of MHC class-I molecules in each sample was evaluated as described above.

***In Vivo* Antitumor Activity.** To evaluate the antitumor activity of compounds against murine melanoma B16/BL6, we inoculated C57BL × DBA/2 F_1 (hereafter called $BD2F_1$) mice (males, 7 weeks of age; Japan Charles River, Inc.) s.c. with 10^6 cells. Six days after the implantation, the mice were divided into groups consisting of animals bearing almost the same size of tumor (day −1). To evaluate the antitumor activity of CHAP31 against human tumor lines, we inoculated nude mice (BALB/c genetic background; females, 7 weeks of age; Japan Charles River, Inc.) s.c. with 3 mm^3 tumor fragments, which had been grown as s.c. tumors in the nude mice. When the tumors had reached 100–300 mm^3 in volume, the animals were divided randomly into groups consisting of six mice per group (day 0) as reported previously (38). Test compounds were dissolved in PBS containing 10% polyoxyethylated hydrogenated castor oil (Uniox HC-60; NOF Corporation, Tokyo, Japan), and injected i.v. via a tail vein on days 0, 3, 6, and 9. We evaluated the tumor size

by measuring its length and width (in mm) to calculate the tumor weight by using the following formula: tumor volume (mm^3) = 0.5 × (length) × (width)^2. The statistical significance of the effects of drugs *versus* control was analyzed by Dunnett's multiple comparison test.

RESULTS

Comparison of Activity between Cyclic Tetrapeptides and Octapeptides. Because the framework of all naturally occurring cyclic peptide antibiotics containing Aoe is a tetrapeptide, we examined the merit of the structure. We synthesized a set of cyclic tetrapeptides and octapeptides having the hydroxamic acid instead of the epoxyketone at the end of the side chain of Aoe, and evaluated their ability to inhibit partially purified mouse HDAC *in vitro* by comparing their IC_{50}s. To evaluate their cellular activity, we determined the concentration required for 2-fold increased expression of MHC class-I molecules in B16/BL6 melanoma cells (C_{x2}), because HDAC inhibitors were shown to markedly induce MHC gene expression in B16/BL6 cells (11), and cellular histone acetylation levels of B16/BL6 cells correlated well with their MHC class-I expression levels (see below). Because the octapeptides (CHAP19 and CHAP14) are the dimers of the corresponding tetrapeptides (CHAP1 and CHAP30, respectively), the amino acids surrounding Asu(NHOH) are the same as those of the tetrapeptides. As shown in Table 1, the IC_{50}s of the octapeptides for HDAC enzyme inhibition were 10–25-fold larger than those of the corresponding tetrapeptides. On the other hand, when we examined their effect on the expression of MHC class-I molecules in B16/BL6 cells, no activity of cyclic octapeptides was detected. These results suggest that the cyclic tetrapeptide framework is superior to the octapeptide one in inhibiting HDAC, both in cell-free and cellular situations. On the basis of these results, we used tetrapeptides in the following SAR study.

Comparison of Isomers with Different DL Combinations. We next examined the appropriate combination of optically different amino acids that constitute CHAPs. As shown in Table 1, of the four combinations, the LDLD (CHAP27, -57, -31, and -50), LLLD (CHAP1 and -56), and LDLL (CHAP38, -58, -30, and -49) isomers inhibited the enzyme activity of HDAC to almost the same extent. For these three isomers, the largest difference was only 1.75-fold (CHAP27 *versus* CHAP1). However, the LLDL isomer (CHAP38) was much weaker than the others by about two orders of magnitude. In contrast to the enzyme inhibition, the activity to induce MHC class-I molecule expression and growth inhibition was largely different among the isomers having different DL combinations: the order of efficacy was LDLD > LLLD > LDLL ≫ LLDL for the TPX type; and LDLD > LDLL for the Cyl-1/2 type (>, ~10-fold; and ≫, ~100-fold). This is probably attributable to the higher membrane permeability of the configurations with more hydrophobicity, because the retention times of the LDLD isomers in RP-HPLC analysis were longer than those of the other isomers (Table 1).

Effects of Methylene Chain Length between Hydroxamic Acid and Cyclic Tetrapeptide Core. We previously reported that CHAP with a five-carbon-long spacer between the carbonyl group of their

Table 1 *SAR of CHAPs*

Compound	Cyclo[a]	Configuration[b]	Chain length[c]	Sequence[d]	HDAC[e] IC_{50} (nM)	MHC[f] $C_{\times 2}$ (nM)	GI[g] IC_{50} (nM)	Retention time[h] (Min)
(TPX B type)								
CHAP1	4	LLLD	5	*cyclo*(-L-Asu(NHOH)-L-Phe-L-Phe-D-Pro-)	6.03 ± 1.51 (3)[i]	98.2 ± 23.3 (11)[i,j]	257 ± 35 (3)[i]	16.43
CHAP19	8	LLLD	5	*cyclo*(-L-Asu(NHOH)-L-Phe-L-Phe-D-Pro-)$_2$	83.7 ± 33.4 (3)	>40,000 (3)	N.T.[k]	
CHAP27	4	LDLD	5	*cyclo*(-L-Asu(NHOH)-D-Phe-L-Phe-D-Pro-)	3.44 ± 0.63 (3)	3.01 ± 1.26 (7)	18.0 ± 2.5 (3)	19.35
CHAP38	4	LDLL	5	*cyclo*(-L-Asu(NHOH)-D-Phe-L-Phe-L-Pro-)	5.32 ± 1.64 (3)	558 ± 97 (4)	3,757 ± 1,165 (3)	16.94
CHAP39	4	LLDL	5	*cyclo*(-L-Asu(NHOH)-L-Phe-D-Phe-L-Pro-)	226 ± 63 (3)	65,800 ± 6,050 (3)	>100,000 (1)	16.16
CHAP18	4	LLLD	4	*cyclo*(-L-Api(NHOH)-L-Phe-L-Phe-D-Pro-)	150 ± 56 (3)	10,900 ± 900 (3)	59,300 ± 17,300	
CHAP17	4	LLLD	6	*cyclo*(-L-Aaz(NHOH)-L-Phe-L-Phe-D-Pro-)	24.7 ± 9.3 (3)	990 ± 168 (3)	1,260 ± 210 (3)	
CHAP131	4	LDLD	5	*cyclo*(-L-Asu(NHOH)-D-Phe-L-Pro-D-Phe-)	91.6 ± 57.7 (3)	6,740 ± 1,780 (5)	N.T.[k]	
CHAP132	4	LDLD	5	*cyclo*(-L-Asu(NHOH)-D-Pro-L-Phe-D-Phe-)	25.5 ± 18.2 (6)	323 ± 55 (7)	N.T.[k]	
CHAP80	4	LLLD	5	*cyclo*(-L-Asu(NHOH)-L-Ala-L-Phe-D-Pro-)	1.95 ± 0.90 (3)	325 ± 10 (3)	N.T.[k]	
CHAP88	4	LDLD	5	*cyclo*(-L-Asu(NHOH)-D-Ala-L-Phe-D-Pro-)	3.23 ± 0.87 (3)	75.5 ± 9.6 (3)	N.T.[k]	
CHAP45	4	LDLD	5	*cyclo*(-L-Asu(NHOH)-D-Phe-L-Ala-D-Pro-)	1.69 ± 0.54 (3)	23.1 ± 3.5 (3)	N.T.[k]	
(TPX A type)								
CHAP57	4	LDLD	5	*cyclo*(-L-Asu(NHOH)-D-Phe-L-Phe-D-Pip-)	2.91 ± 0.81 (3)	2.75 ± 0.54 (4)	33.7 ± 17.6 (3)	19.66
CHAP56	4	LLLD	5	*cyclo*(-L-Asu(NHOH)-L-Phe-L-Phe-D-Pip-)	4.78 ± 0.49 (3)	29.9 ± 5.1 (4)[j]	89.0 ± 13.0 (3)	18.59
CHAP58	4	LDLL	5	*cyclo*(-L-Asu(NHOH)-D-Phe-L-Phe-L-Pip-)	4.18 ± 1.37 (3)	250 ± 48 (4)	1,048 ± 265 (3)	18.37
(Cyl-1 type)								
CHAP30	4	LDLL	5	*cyclo*(-L-Asu(NHOH)-D-Tyr(Me)-L-Ile-L-Pro-)	3.31 ± 0.32 (3)	16.9 ± 8.2 (4)[j]	112 ± 3 (3)	17.18
CHAP14	8	LDLL	5	*cyclo*(-L-Asu(NHOH)-D-Tyr(Me)-L-Ile-L-Pro-)$_2$	85.0 ± 14.3 (3)	>10,000 (3)	N.T.[k]	
CHAP31	4	LDLD	5	*cyclo*(-L-Asu(NHOH)-D-Tyr(Me)-L-Ile-D-Pro-)	3.32 ± 1.47 (3)	1.41 ± 0.51 (6)	5.43 ± 0.39 (3)	18.63
CHAP42	4	LDLD	4	*cyclo*(-L-Api(NHOH)-D-Tyr(Me)-L-Ile-D-Pro-)	53.8 ± 16.4 (3)	512 ± 247 (5)	4,550 ± 860 (3)	
CHAP43	4	LDLD	6	*cyclo*(-L-Aaz(NHOH)-D-Tyr(Me)-L-Ile-D-Pro-)	33.9 ± 6.4 (3)	155 ± 66 (5)	610 ± 60 (3)	
CHAP44	4	LDLD	5	*cyclo*(-L-Asu(NHOH)-D-Tyr(Me)-L-Ala-D-Pro-)	3.38 ± 0.34 (3)	59.6 ± 10.9 (3)	N.T.[k]	
CHAP77	4	LDLD	5	*cyclo*(-L-Asu(NHOH)-D-Tyr-L-Ile-D-Pro-)	3.38 ± 0.34 (3)	59.6 ± 10.9 (3)	N.T.[k]	
(Cyl-2 type)								
CHAP50	4	LDLD	5	*cyclo*(-L-Asu(NHOH)-D-Tyr(Me)-L-Ile-D-Pip-)	3.96 ± 1.17 (3)	1.41 ± 0.44 (7)	5.44 ± 0.37 (3)	20.15
CHAP49	4	LDLL	5	*cyclo*(-L-Asu(NHOH)-D-Tyr(Me)-L-Ile-L-Pip-)	4.81 ± 1.71 (3)	5.30 ± 2.16 (9)[j]	65.9 ± 14.1 (3)	17.94

[a] The number of amino acids constituting cyclic peptides.
[b] Configuration of each compound is indicated by the combination of the chirality of the constituent amino acids (D or L).
[c] Methylene chain length between hydroxamic acid and cyclic peptide core.
[d] Amino acid sequences are expressed from amino terminal side by using the ordinary three letter codes with the indication of their chirality (D or L), except for Asu^2 (α-aminosuberic acid), Pip^2 (pipecolic acid), Api^2 (α-aminopimelic acid), and Aaz^2 (α-aminoazelaic acid). Tyr(Me) is the amino acid whose phenolic OH is replaced by OMe. The expression "*cyclo*" denotes that their amino- and COOH-terminals are combined by an amido bond. NHOH in parenthesis denotes that a hydroxamic acid residue was added to the end of the side chain of Asu, Api, or Aaz. The subscript number "2" indicates that these sequences were repeated to form the cyclic octapeptide structures.
[e] HDAC inhibitory activity was evaluated by using HDACs prepared from B16/BL6 cells; the IC_{50} with the SD.
[f] Concentration for 2-fold increased expression ($C_{\times 2}$) of MHC class-I molecules in B16/BL6 cells (MHC), along with the SD.
[g] IC_{50} for inhibition of cell proliferation of B16/BL6 with the SD.
[h] Retention times of CHAPs in an RP-HPLC analysis, whose elution condition was indicated in "Materials and Methods."
[i] Numbers in parentheses, the number of separate experiments.
[j] Values reported previously (31).
[k] N.T., not tested.

hydroxamic acid and the cyclic tetrapeptide core was much more effective than that with six or four carbons in inhibiting recombinant HDAC1 (31). Also, to confirm that the CHAP with the five-carbon-long spacer is better than CHAPs with other chain lengths for inhibition in cells, we compared the MHC-inducing and cell proliferation inhibitory activities of CHAPs with different methylene chain lengths (four, five, and six). As shown in Table 1, CHAPs with the chain length of five (CHAP1 and CHAP31) were the most effective, followed by those with the length of six (CHAP17 and CHAP43) and then by those with four (CHAP18 and CHAP42). These results indicate that one of the four amino acids constituting CHAP should be Asu(NHOH).

Comparison of the Proline Position. All natural Aoe-containing cyclic peptide antibiotics contain one Pro or pipecolic acid (Pip^2) at the second (HC-toxin) or the fourth position, when Aoe is fixed at the first position. We therefore investigated the appropriate position of Pro in CHAP (Table1; CHAP27, 131, and 132). When Asu(NHOH) instead of Aoe was fixed at the first position, Pro at the fourth position (CHAP27) was the best for both cell-free HDAC inhibition and cellular MHC class-I molecule expression.

Effects of Amino Acid Replacement at the Second and Third Positions. Because we concluded that Asu(NHOH) and Pro should be located at the first and fourth positions, respectively, next we analyzed the effect of amino acid replacement at the second and third positions (Table 1). When the hydrophobic residues of D-form at the second position (D-Phe in CHAP27 and D-Tyr(Me) in CHAP31) were replaced with less hydrophobic ones (D-Ala in CHAP88 and D-Tyr in CHAP77), their MHC-inducing activities significantly decreased (25–42-fold), whereas no change in HDAC inhibitory activity was observed. On the other hand, when L-Phe at the second position in CHAP1 was replaced with L-Ala (CHAP80), its HDAC inhibitory activity was augmented (~3-fold), but MHC-inducing activity was weakened (~3-fold). The hydrophobic amino acids at the third position (L-Phe in CHAP27 and L-Ile in CHAP31) were also important for MHC-inducing activity, because the replacement of them with L-Ala reduced their MHC-inducing activity (7.7–25-fold) without significant influence on the HDAC inhibitory activities. These results indicate that hydrophobic D- and L-amino acids are appropriate for the second and the third positions, respectively, to constitute strong HDAC inhibitors effective in cells.

Histone Acetylation Induced by CHAPs. To confirm the ability of CHAPs to inhibit HDAC in cells, we used Western blotting to analyze histone acetylation in B16/BL6 cells treated with CHAPs. We here compared the effect of isomers having different DL combinations (CHAP27, -1, -38, and -39) after 6 h of treatment. As shown in Fig. 2*A*, CHAP27 markedly augmented the acetylation of cellular histones at 10 nM but not at 1 nM. CHAP1 and CHAP38 were effective at 100 and 1000 nM, respectively. CHAP39 could not induce acetylation of histones even at 1000 nM. These effective concentrations correlate well with the up-regulation of their MHC molecules (Table 1), confirming that MHC-inducing activity serves as one of the most sensitive markers for the detection of HDAC inhibition in cells. Fig. 2*B* illustrates the effects of CHAP31, which is the most effective compound, both in enzyme and cellular assays (Table 1), on histone acetylation in B16/BL6 cells. As shown in this figure, CHAP31 induced cellular histone acetylation at 10 nM, indicating its potent activity on HDAC in cells.

Stability of CHAP31 in the Presence of Cultured Cells. We chose CHAP31 {*cyclo*[-L-Asu(NHOH)-D-Tyr(Me)-L-Ile-D-Pro-]; Fig. 1} for additional study because of its strong activity *in vitro*. To compare the biological stability of CHAP31 with that of TSA and TPX A, we incubated the inhibitors (100 pmol) with B16/BL6 cells (1.5×10^5 cells) in 1 ml of culture medium, and the residual activity in the medium to up-regulate MHC class-I molecules was determined

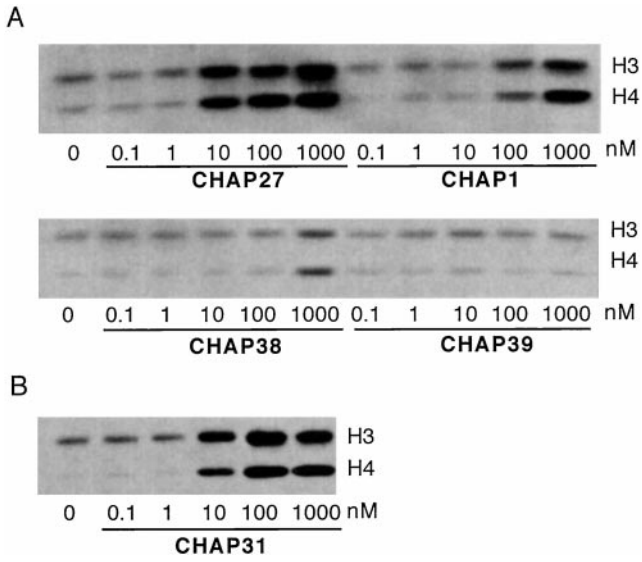

Fig. 2. Histone acetylation of B16/BL6 cells induced by CHAPs. After B16/BL6 cells had been treated with CHAP27, -1, -38, -39 (*A*), or -31 (*B*) at the indicated concentrations for 6 h, 4 μg each of the total cellular protein was subjected to SDS-12% PAGE. After being transferred to a polyvinylidene difluoride membrane, acetylated histones were detected by Western blotting as described in "Materials and Methods." *H3* and *H4*, positions of histones H3 and H4, respectively.

at various time points. As shown in Fig. 3, the MHC-inducing activity of TPX A decreased very quickly, and that of TSA also decreased, but less rapidly. In contrast, almost no decrease in the activity of CHAP31 was detected up to 72 h of incubation. The half-lives of TSA, TPX A, and CHAP31 under the present conditions were 14.7, 2.10, and >30000 h, respectively. These results suggest that TSA and TPX A are subject to rapid degradation or inactivation, and that CHAP31 is stable in the presence of cultured cells.

Antitumor Activity of CHAP31. We next examined the antitumor activity of CHAP31 in $BD2F_1$ mice inoculated with B16/BL6 melanoma. As shown in Fig. 4*A*, the growth of B16/BL6 tumor was inhibited by CHAP31 administered i.v. every 3 days at doses of 1, 3, and 10 mg/kg, although no clear dose-dependency was observed within this dose range. The normal increase in the body weight was suppressed by the treatment with CHAP31, but no body-weight loss was observed at these doses (Fig. 4C). Although CDDP inhibited the tumor growth dose-dependently, and about 90% inhibition was observed at a dose of 10 mg/kg (Fig. 4*B*), a significant (>25%) body-weight loss occurred (Fig. 4*D*), and no mice survived until day 14 at this highest dose. The efficacy of CHAP31 at the doses examined was almost the same as for CDDP at 3 mg/kg. Although we also examined other administration schedules, *i.e.*, four times daily or every 2 days, no merit over the present schedule was observed, and a single injection of a high dose (100 mg/kg) of CHAP31 was not effective (data not shown).

Fig. 5 shows the antitumor activity of CHAP31 in nude mice bearing various human cancer lines, including BSY-1 breast cancer, DMS114 lung cancer, LOX-IMVI melanoma, and MKN-7 and -74 stomach cancers. In four of five tumor lines examined, statistically significant antitumor effects were observed. Especially, BSY-1 breast and DMS114 lung cancers responded well to CHAP31, even at the lower dose (1 mg/kg). For the LOX-IMVI melanoma, only a slight antitumor effect was observed during the period of drug administration (Fig. 5*C*). As for the MKN-7 stomach cancer line, the tumor size in two and four out six mice in the 1 and 10 mg/kg groups, respectively, regressed during the period of drug administration (not shown). It is therefore conceivable that this cancer is a good responder to CHAP31, although the large animal-to-animal deviation did not con-

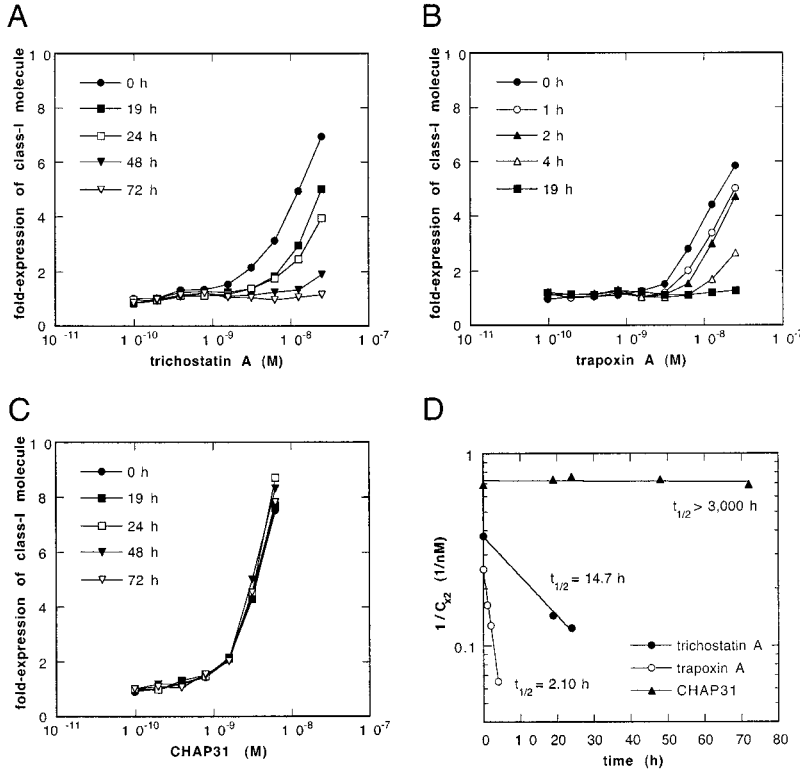

Fig. 3. Degradation of HDAC inhibitors in cell culture medium. One hundred pmol of TSA (*A*), TPX A (*B*), or CHAP31 (*C*) was mixed with 1.5×10^5 B16/BL6 cells in 1 ml of culture medium, and incubated at 37°C in a humidified chamber in the presence of 5% CO_2. At the times indicated in the figures (no incubation (●), 1 h (○), 2 h (▲), 4 h (△), 19 h (■), 24 h (□), 48 h (▼), and 72 h (▽), culture media were sampled and frozen. Activity for up-regulation of MHC class-I molecules in each sample was evaluated as described in "Materials and Methods," and the expression levels of MHC molecules were plotted against the original concentrations, assuming the basal expression level without additives to be unity. *D*, Concentrations for 2-fold expression of MHC class-I molecules (C_{x2}) were determined for TSA (●), TPX A (○), and CHAP31 (▲) from *A*, *B*, and *C*, respectively; and their reciprocal values (indexing their relative activities) were plotted against time of incubation by using the semilogarithmic coordinate. Data points were fitted with single exponential curves, and half-lives ($t_{1/2}$) of activities were calculated: TSA, *14.7 h*; TPX A, *2.10 h*; and CHAP31, *>3000 h*. Each point represents a single determination.

fer its statistical significance (Fig. 5*D*). With MKN-74 stomach cancer cells, the 10 mg/kg group tended to respond to CHAP31 treatment, although it was not statistically significantly (Fig. 5*E*). The maximum decrease in body weight was within 0.1–5% and 10–20% in the 1 and 10 mg/kg groups, respectively. No deaths caused by the drug administration were observed.

DISCUSSION

Although a variety of structurally unrelated natural inhibitors of HDAC have been isolated (8, 13, 16, 23, 26, 27), no comparative SAR study has been described. In this study, we synthesized a series of CHAP compounds, and elucidated their structural requirements for

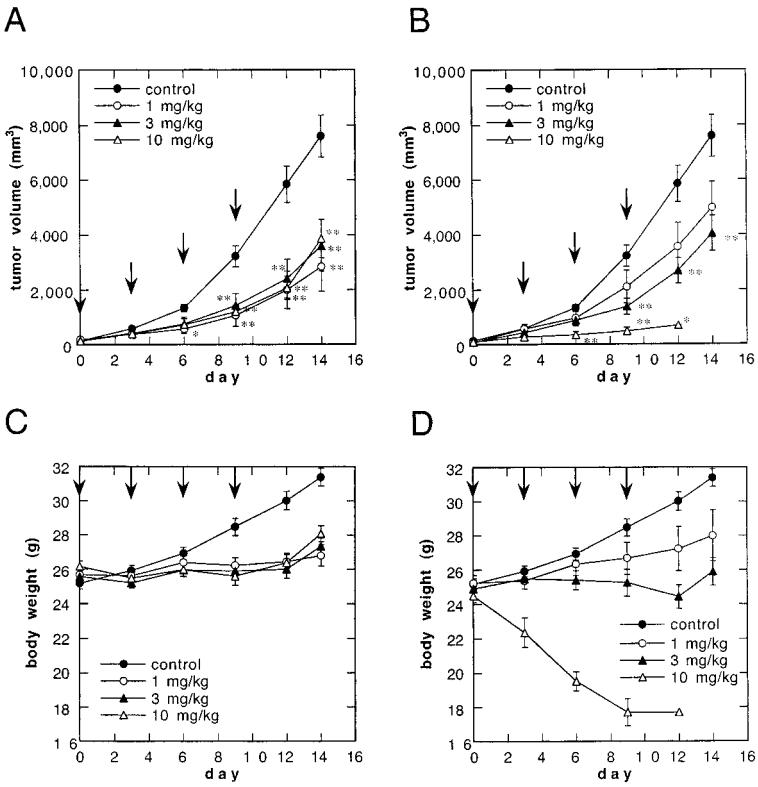

Fig. 4. *In vivo* antitumor effects of CHAP31 and CDDP in $BD2F_1$ mice bearing B16/BL6 melanoma. Tumor growth (*A* and *B*) and body weight changes (*C* and *D*) in tumor-bearing mice treated with CHAP31 (*A* and *C*) or CDDP (*B* and *D*) are depicted. Seven days after the s.c. inoculation with B16/BL6 cells (10^6 cells/mouse), i.v. administration of CHAP31 or CDDP at a dose of 1 (○), 3 (▲), or 10 mg/kg (△) was started (day 0), and it was repeated another three times every 3 days (days 3, 6, and 9). Vehicle solution of the compound was administered to the control group (●). *Arrows* indicate the days of drug administration. Each point represents the mean ± SE of 10 (*control*), 4 (CDDP, *1 mg/kg*), or 6 animals. *, $P < 0.05$; and **, $P < 0.01$ *versus* control group by Dunnett's test. All animals in the 10-mg/kg group of CDDP died before day 14, whereas no other deaths were observed.

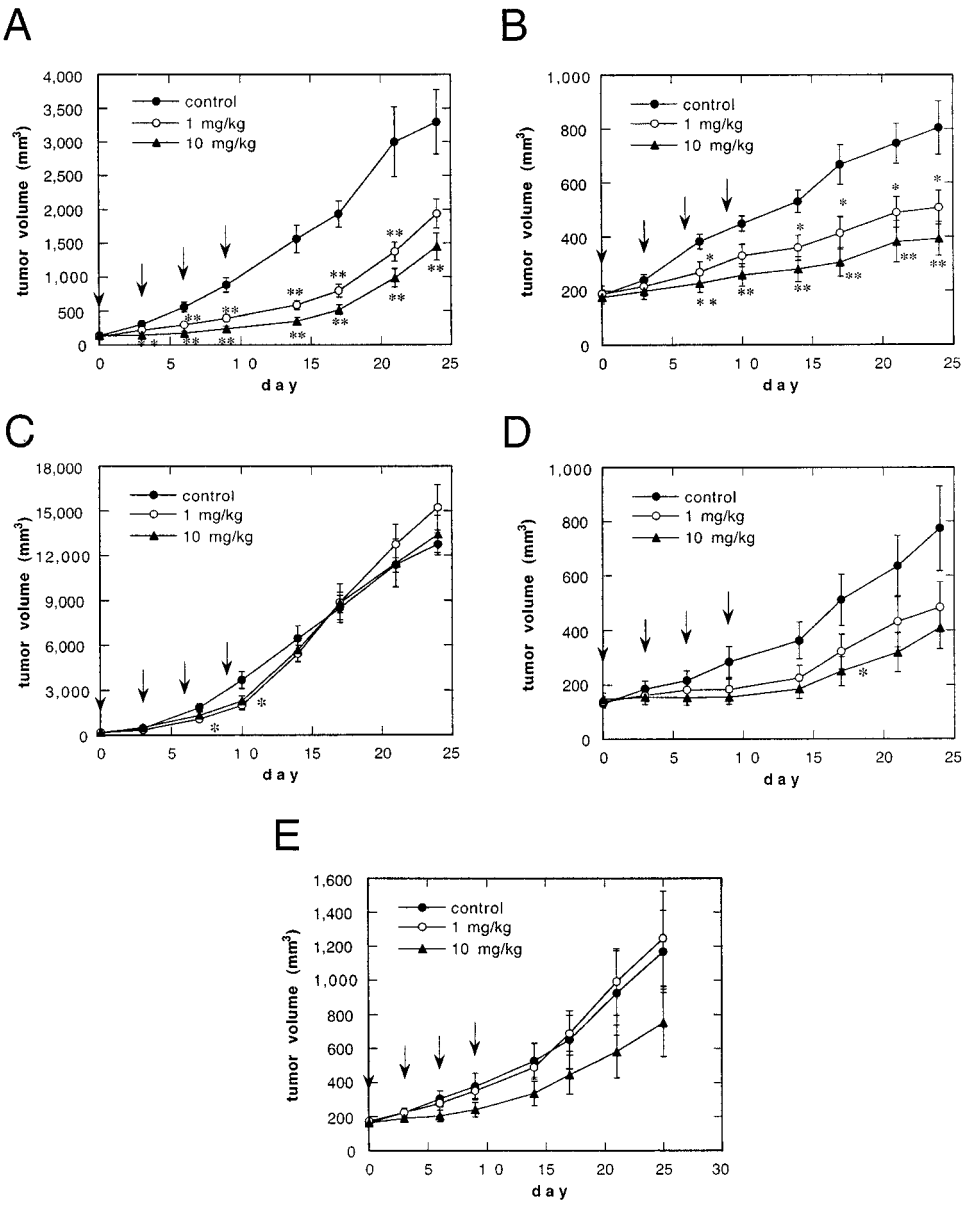

Fig. 5. *In vivo* antitumor effects of CHAP31 in nude mice bearing various human cancer xenografts as follows: *A*, BSY-1 breast cancer; *B*, DMS114 lung cancer; *C*, LOX-IMVI melanoma; *D*, MKN-7 stomach cancer; and *E*, MKN-74 stomach cancer. Inoculation with tumor was carried out as described in "Materials and Methods." CHAP31 was administered on days 0, 3, 6, and 9 (*arrows*) at a dose of 0 (●, control), 1 (○), or 10 mg/kg (▲). Each point represents the mean ± SE of six animals. *, $P < 0.05$; **, $P < 0.01$, *versus* control group by Dunnett's test.

the strong inhibition of HDAC enzyme activity. The basic CHAP structure appropriate for HDAC inhibition was the cyclic tetrapeptide containing Asu(NHOH) and Pro or Pip, which is represented as *cyclo*(-Asu(NHOH)-AA_2-AA_3-Pro or Pip-). In addition, DL combinations of the constituent amino acids should be LDLD, LLLD, or LDLL. By crystallographic studies using a bacterial enzyme related to HDAC (HDAC-like protein; Ref. 39), TSA was shown to mimic the substrate and chelate the zinc in the catalytic pocket through its hydroxamic acid group. The dimethylamino-phenyl group of TSA serves as a cap necessary for packing the inhibitor at the rim of the tube-like active-site pocket. TPX and other related natural compounds may also consist of a cap group, *i.e.,* the cyclic tetrapeptide, and a functional group for enzyme inhibition, *i.e.,* an epoxyketone in Aoe. It is therefore reasonable that the cyclic tetrapeptide portion of CHAP acts as a cap, whereas the aliphatic chain with the hydroxamic acid group is inserted into the tube-like active-site pocket of HDAC, thereby chelating the zinc ion. If so, the weak activity of octapeptide derivatives may reflect the insufficient capping, probably attributable to its too-large ring structure. Although it is currently unclear why CHAP with the LLDL combination is weak, it is possible that one of the neighbors of Asu(NHOH) has to be a D-form amino acid for tight association with the entrance to the active-site pocket. In the previous study (31), we examined isozyme specificity of some CHAPs corresponding to natural Aoe-containing compounds and found that the concentrations of these CHAPs required for inhibiting HDAC1 were lower than those required for HDAC6. Our preliminary data showed that the IC_{50}s of CHAP31 for inhibiting HDAC1 and -6 were 0.38 and 13 nM, respectively, suggesting that the HDAC1 preference of CHAPs is kept even in this structurally optimized compound.

The present study also provides some fundamental requirements for the inhibition of cellular HDAC by CHAP. Previously we showed that CHAP with an aliphatic chain length of five, which corresponds to that of acetylated lysine, is stronger than those with other lengths in inhibiting the recombinant HDAC1 enzyme (31). Here we showed that this rule was also fulfilled for the MHC-inducing and proliferation-inhibiting activities in cells (Table 1). On the other hand, the activity of the LDLD isomer was significantly stronger than that of LDLL or LLLD, although they showed almost the same enzyme-inhibiting activity (Table 1). Because the LDLD configuration has not been found in the natural Aoe-containing counterparts (*e.g.,* LLLD for TPX and

LDLL for Cyl-1/2), these results suggest that CHAPs corresponding to the natural products are not always appropriate for the inhibition of HDAC in cells (see Table 1; CHAP1 *versus* CHAP27, CHAP56 *versus* CHAP57, CHAP30 *versus* CHAP31, and CHAP49 *versus* CAHP50). Because the retention times of LDLD isomers in RP-HPLC were longer than those of the corresponding LDLL and LLLD isomers (Table 1), the more hydrophobic nature of LDLD isomers might be more beneficial in allowing them to penetrate the cell membrane more efficiently than other isomers. In accordance with this speculation, replacement of the second or the third amino acid of CHAPs by a less hydrophobic one reduced their cellular activity without a decrease in the HDAC-inhibiting activity (Table 1). However, because CHAPs that had almost the same HDAC-inhibiting activity and retention times (*e.g.*, CHAP31 *versus* CHAP56) elicited cellular activities with different potency (Table 1), other factors distinct from simple hydrophobicity may also contribute to the differences in cellular activity induced by CHAPs. Examining the precise explanation for the differences between enzyme inhibitory activity and cellular activity will be the next task.

Recently, FK228 (16) and MS-275 (17), potent antitumor agents under clinical investigation, were shown to inhibit HDACs. Thus, HDAC is considered as one of the important targets for new anticancer drugs. However, TSA and TPX, known potent inhibitors of HDAC, do not show sufficient antitumor activity, probably because of their instability *in vivo*. On the basis of the potency of HDAC inhibition and MHC-inducing activity, we selected CHAP31, which fulfills all of the above requirements for strong HDAC inhibitors. CHAP31 brought about acetylation of core histones in B16/BL6 cells (Fig. 2*B*), and its stability in the presence of cultured cells was much better than that of TSA or TPX A (Fig. 3). Because the effective concentration of CHAP31 in plasma had been estimated to be maintained for several hours when administered i.v. at a dose of several mg/kg, based on our preliminary pharmacokinetic study in rats (a half-life of β-phase was ~50 min, and the plasma concentration 3 h after 10 mg/kg i.v. administration was ~330 nM, as determined by liquid chromatography-mass spectrometry), we expected this compound to be effective in tumor-bearing murine models. In fact, CHAP31 inhibited the growth of tumor in mice inoculated with the B16/BL6 murine melanoma line (Fig. 4) or with several human cancer lines (Fig. 5). These data confirm that HDAC inhibitors bear great potential as antitumor agents. Thus, CHAP31 is a promising candidate for antitumor drugs having strong HDAC-inhibiting activity. Although the most probable mechanism of antitumor effects of CHAP31 in transplant models is the inhibition of HDAC in tumor cells, it is possible that some other factors such as its antivessel toxicity and other host toxicity may contribute to the antitumor activity to some extent. The cause of the difference in the sensitivity among tumor lines in transplant models is currently unclear. Comparison of histone acetylation among tumors will be helpful to answer these questions. In the present animal study, toxicity on body weight gain was observed at every effective dose of CHAP31. Although we have not determined the inhibitory potency against the HDAC isoforms other than HDAC1 and -6, a relatively wide spectrum of CHAP31 might be responsible for such a low response/toxicity ratio. Additional *in vivo* studies will be needed to elucidate a detailed correlation between *in vitro* activities and *in vivo* antitumor effects of CHAPs.

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