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Histone deacetylase inhibitors from the rhizomes of *Zingiber zerumbet*

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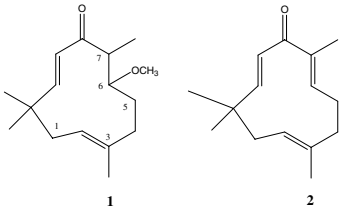
Histone acetylation and deacetylation play fundamental roles in the modulation of chromatin topology and the regulation of gene transcription. Histone deacetylase (HDAC) inhibitors that inhibit proliferation and induce differentiation and/or apoptosis of tumor cells in culture and in animal models have been identified. A number of structurally diverse histone deacetylase inhibitors have shown potent antitumor efficacy with little toxicity *in vivo* in animal models. In the context of our natural product chemistry program dealing with the development of new potent anticancer agents, we have examined the isolation from *Zingiber zerumbet* as leads for novel HDAC inhibitors. *Zingiber zerumbet* (L.) J. E. Smith (Zingiberaceae) is a wild ginger that typically grows widely in Southeast Asia. Isolation of the *n*-hexane soluble fraction from *Zingiber zerumbet* yielded two major sesequiterpenoids, 6-methoxy-2*E*,9*E*-humuladien-8-one (**1**) and zerumbone (**2**). The structures were elucidated on the basis of spectroscopic data. The histone deacetylase (HDAC) activities of compounds **1** and **2** were determined *in vitro* against HDAC enzyme assay. Compound **1** exhibited growth inhibitory activity on six human tumor cell lines, and showed potential inhibitory activity in histone deacetylase (HDAC) enzyme assay ($GI_{50} = 1.25\ \mu M$). It also exhibited growth inhibitory activity on five human tumor cell lines and more sensitive inhibitory activity on the MDA-MB-231 breast tumor cell line ($IC_{50} = 1.45\ \mu M$). Further structure-activity relationships of position C-6 and C-7 from aromatic ring will be reported in due course.

1. Introduction

The turnover of histone acetylation is regulated by the opposing activities of histone acetyltransferases (HATs) and histone deacetylases (HDACs) (Vigushin and Coombes 2002). Histone deacetylase (HDAC) inhibitors have been identified that inhibit proliferation and induce differentiation and/or apoptosis of tumor cells in culture and in animal models. A number of structurally diverse histone deacetylase inhibitors have shown potent antitumor efficacy with little toxicity *in vivo* in animal models (Mai et al. 2005). Recently, HDAC inhibitors are emerging as an exciting new class of potential anticancer agents for the treatment of solid and hematological malignancies (Imre et al. 2006). A number of natural and synthetic HDAC inhibitors have shown an anti-proliferative activity on tumor cells. Among them, trichostatin A (TSA) (Vigushin and Coombes 2004), apicidin (Han et al. 2000), trapoxin B (TPX) (Kijima et al. 1993) and FK-228 (Ueda et al. 1994) were classified as natural substances, while suberoylanilide hydroxamic acid (SAHA) (Butler et al. 2000) and other TSA or SAHA-like analogues were reported as synthetic HDAC inhibitors (Mai et al. 2003).

Zingiber zerumbet (L.) J. E. Smith (Zingiberaceae) is a wild ginger that typically grows widely in Southeast Asia. The rhizomes have been used as an anti-inflammatory and/or anticancer in traditional medicine (Farnsworth and Bunyapraphatsara 1992). In particular, a monocyclic sesquiterpene, zerumbone (2*E*,6*E*,10*E*-humulatrien-1-one), is a monocyclic sesquiterpene found as the major component of the essential oil of wild ginger, has been studied intensively for potential use in anti-inflammatory, bacterial chemopreventive, and chemotherapeutic strategies (Kitayama et al. 2001; Murakami et al. 2002; Tanaka et al. 2001). Previous phytochemical studies on this plant resulted in the isolation of several sesquiterpenoids and flavonoids (Masuda et al. 1991; Matthes et al. 1980). Recently, Jang et al. (2005) reported two isomers of sesquiterpenoids from the hexane-soluble fraction of the MeOH extract of the rhizomes of *Z. zerumbet*.

In the context of our natural product chemistry program dealing with the development of new potent anticancer agents, we examined sesquiterpenoid compounds as leads for novel HDAC inhibitors. Herein, we describe



1 **2**

the isolation, enzyme inhibition, and cancer cell growth inhibition of one such sesquiterpenoids-based HDAC inhibitor.

2. Investigations, results and discussion

We evaluated the HDAC inhibitory activities of the newly isolated compounds on partially purified HDAC enzyme obtained from HeLa cell lysate and their anti-proliferative effects using PC-3 cells (Nishino 2004). Compound **1** was active in the HDAC enzyme assay and showed potential growth inhibitory activity on the PC-3 cell line (Table 1). Growth inhibitory activities of **1**, **2** and SAHA were evaluated in six human tumor cell lines. Cell proliferation assays were also performed on six human tumor cell lines and one types of human primary cells (hepatocytes) (Table 2). Compounds **1** and **2** were tested against all cancer cell lines, but with a two- to ten-fold lower activity than SAHA. **1** and **2** did exhibit cytotoxicity effects in the cancer cells tested. This result is consistent with the enzymatic and histone acetylation assay results. The toxicity of **2** against hepatocytes is comparable to that of SAHA, while **1** (6-methoxy-2*E*,9*E*-humuladien-8-one) has increased cytotoxicity against hepatocytes ($GI_{50} = 383\ \mu M$). Growth inhibition (GI_{50}) was measured by the MTT assay of these HDAC inhibitors. The tumor cell lines are listed in Table 2. Compound **1** exhibited growth inhibitory activity on six human tumor cell lines and showed the most potent inhibitory activity against the MDA-MB-231 breast tumor cell line ($IC_{50} = 1.45\ \mu M$). We have found that **1** has improving growth inhibiting potency *in vitro*. Further studies to investigate the mode of action of C-6 and C-7 from the aromatic ring are in progress.

Taken together, our findings provide important information of the structural features that influence the functional activities of this class of compounds, and offer new possibilities to further improve its potency.

Table 1: HDAC enzyme and growth inhibition by 6-methoxy-2*E*,9*E*-humuladien-8-one (1), zerumbone (2) and SAHA

Compd.	IC_{50} (μM) Enzyme	GI_{50} (μM) PC-3
1	1.25 ± 0.05	4.63 ± 0.13
2	8.35 ± 0.91	14.24 ± 0.23
SAHA	0.15 ± 0.06	0.72 ± 0.08

* Values are means of a minimum of five experiments

3. Experimental

3.1. General

NMR: Bruker AMX 400 spectrometer (Bruker, USA), the chemical shifts being represented as ppm with tetramethylsilane as a internal standard; column chromatography: silica gel 60 (70–230 and 230–400 mesh, Merck), HPLC (HP, USA) and Supelcosil ABZ+Plus (12 nm, S-75 mm, Supelco); TLC: pre-coated silica gel 60 F_{254}. Suberoylanilide hydroxamic acid was purchased from Sigma Chemical Company (St. Louis, MO, USA).

3.2. Plant material, purification and identification

The rhizomes of *Z. zerumbet* were purchased in April 2004 at local market in Okinawa prefecture, Japan, and voucher specimens (KKU-00532) have been deposited in the Herbarium of the College of Medicine, Kon Kuk University (Seoul, South Korea). The botanical identification was made by Dr. Kim Tae-Jin, KRIBB (Dae-Jeon, South Korea). The rhizomes of *Z. zerumbet* (2 kg) were freeze-dried for 7 days and then extracted with MeOH (3 times, 5 L for 2 weeks) at room temperature. The extracts were combined and concentrated *in vacuo* at 30 °C. The residue (315.6 g) was diluted with water and partitioned against *n*-hexane (82.2 g). The *n*-hexane-soluble extract (80 g) was chromatographed on silica gel (10 × 40 cm, 230–400 mesh) eluting as stationary phase using a solvent system [cyclohexane (5 L), cyclohexane-CH_2Cl_2 (1 : 1 v/v, 5 L), CH_2Cl_2 (5 L), CH_2Cl_2-MeOH (19 : 1 v/v, 5 L), MeOH (2 L)] to afford 8 pooled fractions (Fr: 01-Fr: 08). Fraction F07 [eluted with CH_2Cl_2-MeOH (19 : 1 v/v); 17 g] was chromatographed over silica gel (5 × 40 cm, 230–400 mesh; *n*-hexane-EtOAc gradient from 20 : 1 to 1 : 1 v/v, final 100% MeOH) resulting in seventeen subfractions (Fr: 09-01 ∼ Fr: 09-17) that were collected on the basis of TLC profiles. Fractions Fr: 09-02 and Fr: 09-04 [eluted with *n*-hexane-EtOAc (19 : 1 v/v); 3.5 g] were combined, and then was further chromatographed over silica gel (4 × 30 cm, 230–400 mesh; *n*-hexane-EtOAc = 19 : 1 v/v) to give compounds **1** (32 mg) and **2** (15 mg). Compounds were identified as **1** and **2** by comparing its spectral data with those previously reported (Kitayama 2001; Jang 2005).

3.3. Cell culture

Cell lines purchased from ATCC (Manassas, VA, USA) were maintained in Dulbecco's modified Eagle's media (DMEM) supplemented with 10% horse serum and 5% fetal bovine serum and incubated in a CO_2 incubator (5%) at 37 °C.

3.4. MTT Assay

Cells were serum-deprived by three washes of PBS and resuspended in DMEM. The suspended cells were plated on 96-well plates (1×10^4 cells/well) and treated with the indicated reagent(s). After treatment for 21 h, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was added to the medium (0.5 mg/mL), and the mixture was incubated at 37 °C for another 3 h. After discarding the medium, DMSO (100 mL) was then applied to the well to dissolve the formazan crystals, and the absorbances at 570 and 630 nm.

3.5. Histone deacetylase assay

HDAC fluorescent activity assays using a Fluror de Lys™ Substrate (Biomol, Plymouth Meeting, PA, USA), which contains an acetylated lysine side chain, were performed according to manufacturer's instructions. In brief, HeLa nuclear extracts, which were used as an HDAC enzyme source, were incubated at 25 °C with 250 mM of Fluror de Lys™ Substrate and various concentrations of each sample. Reactions were stopped after 20 min with Fluror de Lys™ Developer and fluorescence was meas-

Table 2: GI_{50} and origin type for cells treated with 6-methoxy-2*E*,9*E*-humuladien-8-one (1), zerumbone (2) and SAHA

Cell line	Origin	Growth inhibition (μM)		
		1	**2**	SAHA
ACHN	Kidney	4.24 ± 0.24	16.34 ±0.13	0.61 ± 0.04
NCI-H23	Lung	6.21 ± 0.24	15.24 ± 0.32	1.31 ± 0.06
PC-3	Prostate	4.34 ± 0.26	14.35 ± 0.35	0.68 ± 0.04
MDA-MB-231	Breast	1.45 ± 0.43	11.48 ± 0.13	0.81 ± 0.03
LOX-IMVI	Melanoma	4.21 ± 0.12	12.95 ± 0.23	1.65 ± 0.09
HCT-15	Colon	3.74 ± 0.23	15.21 ± 0.42	0.94 ± 0.05
Hepatocyte	Hepatocytes[1]	383	> 500	> 500

* Values are means of a minimum of five experiments
[1] Human primary cells

ured using a microplate spectrofluorometer (BioRad) with excitation at 360 nm and emission at 460 nm.

3.6. Statistical analysis

The 50% inhibitory concentration (IC_{50}) values for compound **1** and SAHA were obtained from the dose–response curves, using non-linear dose response curve fitting analysis with Sigma Pro software. Statistical significance was determined using the Student's t-tests. Results are presented by means ± standard error of mean (SEM). All p values quoted are two-tailed and were accepted as significantly different when p was ≤ 0.05.

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