Histone Deacetylase Inhibitory Activity and Antiproliferative Activity of the Cultured Medium of *Aspergillus niger* strain TS1

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ABSTRACT

Inhibition of histone deacetylase (HDAC) activity in cancer cells is a promising mechanism for cancer treatment. A screening program for fungi capable of producing HDAC-inhibiting molecules would lead to discovery of potential anticancer agents. The objective of this study was to isolate and characterize fungi capable of producing HDAC inhibitors from soil samples. Only one out of 48 soil fungal isolates exhibited HDAC inhibitory activity. Based on its morphological characteristics and the internal transcribed spacer (ITS) sequence of ribosomal RNA gene, the TS1 fungal isolate was identified as *Aspergillus niger* and named as *A. niger* TS1. The cultured medium of strain TS1 significantly inhibited HDAC activity both *in vitro* and in mammalian cells. The cultured medium of strain TS1 exhibited antiproliferative activity against human acute T cell leukemia cell line (Jurkat cells), human cervical cancer cell line (HeLa cells), and human colon cancer cell line (HCT116 cells). The growth inhibitory effects appeared to be mediated by induction of apoptosis. The current study demonstrates that the filamentous soil fungus *A. niger* TS1 can serve as a natural source of HDAC inhibitors, which could lead to the discovery of a novel anticancer agent.

Keywords: HDAC inhibitory activity, apoptosis, *Aspergillus niger*, anticancer activity

1. INTRODUCTION

Histone deacetylase (HDAC) inhibition by HDAC inhibitors is a promising strategy for cancer therapy especially in combination with chemotherapeutic agents and in combination with radiotherapy [1, 2]. HDAC inhibitors constitute a new class of chemotherapeutic drug currently in several clinical trials with promising results as anticancer agents [1, 3-5]. The major consequences usually observed upon treatment with HDAC inhibitors include
growth arrest, apoptosis, and inhibition of angiogenesis [1]. Induction of cancer cell apoptosis is one downstream mechanism upon HDAC inhibitor treatments, which is usually caused by a shift in the balance of pro- and anti-apoptotic genes toward apoptosis [6]. Because of their low toxicity, HDAC inhibitors constitute a promising treatment for cancer therapy, especially in combination with other chemotherapeutic agents [6, 7]. To date, two HDAC inhibitors, Vorinostat and Romidepsin, have been granted approval by the US Food and Drug Administration (FDA) for the treatment of cutaneous T-cell lymphoma (CTCL) [1, 8].

Recently, the development and search for novel HDAC inhibitors have become a popular research focus on discovering safe and effective anticancer agents [9, 10]. Both natural and synthetic HDAC inhibitors have been identified that are able to inhibit the activity of class I, II, and IV HDACs [1]. HDAC inhibitors can be classified into structurally different classes based on their chemical structure and mechanism of action, including hydroxamic acid-based derivatives, carboxylates (short chain fatty acids), benzamides, electrophilic ketones, and cyclic peptides [11]. Several HDAC inhibitors are made by fungi, including HC-toxin from Cochliobolus carbonum, Chlamydocin from Diheterospora chlamydosporia, Trapoxin from Helicoma ambiens, Apicidin from Fusarium pallidoroseum, and Depudecin from Alternaria brassicicola [12, 13]. The establishment of screening programs for fungi capable of producing HDAC inhibitor molecules from soil samples could lead to discovery of natural, safe, and potential anticancer agents. Here, the filamentous soil fungus Aspergillus niger strain TS1 capable of producing HDAC inhibitor(s) was isolated, and the HDAC inhibitory activity and antiproliferative activity of its secreted product was investigated.

2. MATERIALS AND METHODS
2.1 Isolation of Soil Fungi
Soil samples were collected from Khon Kaen province, Thailand. Soil suspensions (10 g of dried soil in 90 mL sterile water) were made by serial dilution method, and 100 μL of 10⁻² and 10⁻³ dilutions was spread over nutrient agar plate (Martin’s medium). After incubation at 28 ± 2°C for 3-5 days, the fungal colonies were isolated and streaked separately on potato dextrose agar (PDA) plate.

2.2 Screening of HDAC Inhibitor Producing Fungi by in vitro HDAC Inhibition Activity Assay
The 48 fungal isolates were grown in potato dextrose broth (PDB) at 30°C with agitation at 120 rpm for 10 days. The 0.2 g (wet weight) of mycelium from each isolates was ground in a microcentrifuge tube containing sterile sand and 100 μL of dimethyl sulfoxide (DMSO), and then centrifuged at 5,000 × g for 15 min. The supernatant was collected as a crude fungal extract and kept at 4°C until use. HDAC inhibitory activity of the fungal extracts was determined by using the Fluor-de-Lys HDAC activity assay kit (Biomol, Enzo Life Sciences International, Inc., Plymouth Meeting, PA, U.S.A.). The assay was performed according to the manufacturer’s instructions. HeLa nuclear extract was used as a source of the HDAC enzymes, which is a rich source of HDACs 1 & 2 for use as a positive control or as a source of HDAC activity for screening. Fluorescence was measured using a spectra Max Gemini XPS microplate spectrofluorometer (Molecular Devices, Sunnyvale, CA, U.S.A.) with excitation at 360 nm and emission at 460 nm. Inhibition of HDAC activity was monitored by a decrease in fluorescence signal.
2.3 Morphological Characterization

The morphological characteristics of the HDAC inhibitor-producing strain were examined under a light microscope (Olympus, Model CH30RF200, Olympus Optical Co., LTD, Tokyo, Japan). The isolated fungus was identified by comparing its morphological characteristics with previously published data [14, 15].

2.4 Sequencing of ITS Sequence of Ribosomal RNA Gene and Phylogenetic Analysis

Genomic DNA of strain TS1 was extracted and the ITS (internal transcribed spacer) sequence of ribosomal RNA gene was determined by KU Vector Custom DNA Synthesis Service Unit (Department of Microbiology, Faculty of Science, Kasetsart University, Bangkhen campus, Bangkok 10900, Thailand). The ITS sequence of ribosomal RNA gene of strain TS1 was submitted to the DDBJ database (accession no. JF412766). Similarity searches were done by NCBI-BLAST. Phylogenetic tree was generated using Phylogeny.fr program for non-specialist [16].

2.5 Preparation of a Crude Ethanolic Extract and Fractionated Solvent Extracts

The fungal isolate possessing HDAC inhibitory activity was grown in potato dextrose broth (PDB) at 30°C with agitation at 120 rpm for 10 days. Five grams (wet weight) of mycelium was ground in a mortar containing sterile sand and extracted in 50 mL of ethanol for 48 h at room temperature. The suspension was filtered through the filter paper and centrifuged at 5,000 × g for 15 min. The supernatant was lyophilized to obtain the water soluble fraction. The remaining precipitate was redissolved in 50 mL of methanol, ethyl acetate, and hexane, sequentially. Each suspension was repeatedly extracted as described above to get methanol, ethyl acetate and hexane soluble fractions, respectively. These soluble fractions were air-dried and the residue of each solvent fraction was kept at 4°C until use and reconstituted in DMSO before testing.

2.6 Cell Culture

HeLa cells were obtained from the National Cancer Institute, Bangkok, Thailand. Jurkat and HCT116 cells were kindly provided by Dr. M. Leid (Oregon State University, Oregon, U.S.A.) and Dr. O. Tetsu (University of California, San Francisco, U.S.A.), respectively. Cells were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 mg/ml) (Gibco-BRL). The cells were incubated at 37°C in a humidified atmosphere with 5% CO₂.

2.7 Extraction of Histone Proteins

Cells grown in a 4.5-cm dish were treated with either solvent control or the sample for 6 hours, and the histone proteins were then isolated according to the Abcam’s protocol (Abcam Inc., Cambridge, MA, U.S.A.) with some modifications. In brief, cells were harvested by trypsinization, washed with PBS, and then resuspended in Triton Extraction Buffer (TEB; PBS containing 0.5% (v/v) Triton X-100, 2 mM phenylmethylsulfonyl fluoride, 0.02% (w/v) NaN₃) at a cell density of 10⁵ cells/mL. The cells were incubated on ice and agitated periodically for 10 min.
The suspension was centrifuged at 7,500 rpm for 10 min at 4°C to spin down the nuclei and the supernatant was discarded. The nuclei pellet was resuspended in 0.2 M HCl at a density of 10^6 nuclei/mL and incubated overnight at 4°C. The suspension was centrifuged at 7,500 rpm for 10 min at 4°C and the supernatant containing histone proteins was collected. Protein concentration was measured by using a Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA, U.S.A.) based on the Bradford method.

2.8 Acid-Urea Triton X-100 Polyacrylamide Gel Electrophoresis (AUTPAGE)

Hyperacetylation of cellular histones was analyzed by gel electrophoresis using acid urea/Triton-X-100 (AUT) gels [17]. The upper gel consisted of 5% acrylamide/bis-acrylamide containing 0.9 M acetic acid, 8 M urea. The resolving gel was 15% acrylamide/bis-acrylamide containing 0.9 M acetic acid, 8 M urea, and 0.37% Triton X-100. The running buffer was 0.9 M acetic acid. In this buffer system, positively charged proteins migrate toward the cathode. Electrophoresis was performed in a Mini PAGE System (Select BioProducts, Edison, NJ, U.S.A.). Gels were pre-run at 150 volts for 4 h at the ambient temperature. Wells were then loaded with the second pre-run solution (1 M cysteamine (Sigma-Aldrich), 8 M urea, 0.9 M acetic acid) to scavenge the residual free radicals and the gel was pre-run at 150 volts for a further 40 min. Histone samples solubilized in loading buffer (8 M urea, 10% glycerol, 0.9 M acetic acid, 5% β-mercaptoethanol and 0.25% methylene blue) were boiled for 5 min before being loaded and gels were run at 90 volts for 6 h. Gels were silver-stained by using PageSilver™ Silver Staining Kit (Fermentas, Burlington, Ontario, Canada), dried, and photographed.

2.9 Antiproliferative Activity Assay

Cell growth inhibition was determined by MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) assay [18] with some modifications. Briefly, cells were seeded in a 96-well plate at cell density of 10^4 cells/well and incubated for 24 h. Cells were treated with different concentrations (1-5%, v/v) of 3 kDa-filtered cultured medium for 24, 48, and 72 h. Control (0%, v/v) was added with uncultured medium. After exposure of the cells to the strain TS1 cultured medium, the medium was removed, and the cells were incubated with MTT (Sigma Chemical Co., St Louis, MO) (0.5 mg/mL in PBS) for 2 h. Formazan was dissolved in DMSO, and the absorbance at 550 nm was measured with a microtiter plate reader (Bio-Rad Laboratories, Hercules, CA, U.S.A.). The absorbance at 655 nm was used as a reference wavelength. The number of viable cells was proportional to the production of formazan.

2.10 Apoptosis Analysis

Apoptosis analysis was performed by using a Vybrant Apoptosis Assay Kit #2 (Molecular Probes, Invitrogen Corporation, Carlsbad, CA, U.S.A.) according to the manufacturer’s instructions. Briefly, cells were seeded at 1.2 × 10^6 cells/4 mL in a 4.5-cm dish, incubated for 24 h, and treated with different concentrations of the samples for 6 h. Cells were harvested by trypsinization, washed with cold PBS, and resuspended in the annexin-binding buffer. Cell density was determined and diluted in the annexin-binding buffer to 10^5 cells per assay. Cells were incubated with Alexa Fluor 488 annexin V and propidium iodide (PI) at room temperature for 15 min. Following the incubation, cells were analyzed by flow cytometry using a Beckman Coulter Cytomics FC500 MPL flow cytometry (Beckman Coulter, Miami, FL, U.S.A.). The flow
cytometry results were compared with conventional cell count and morphology under a fluorescence microscope.

2.11 Statistical Analysis

Data are expressed as means ± standard deviation (SD) from three independent experiments. Tests for significant differences between vehicle controls and sample treated cells were carried out using one-way ANOVA with Duncan’s post hoc test. The criterion for statistical significance was set at $p < 0.05$.

3. RESULTS AND DISCUSSION

3.1 Isolation and Characterization of Aspergillus niger TS1

Strain TS1 was the only one fungal isolate possessing HDAC inhibitory activity identified from 48 soil fungal isolates. The macroscopic morphology identification of strain TS1 was primarily based on colony pigmentation. Colonies on potato dextrose agar at 30°C were wooly, initially white and rapidly turning black with formation of black conidiospores (Figure 1a). The microscopic identification was based on morphology of the conidial head (Figure 1b). Conidial structure was examined under light microscope (Figure 1c, 1d) and its morphological characteristics were compared with previously published data (Table 1). Conidial characteristics were similar to that observed for A. niger strains in previous reports [14, 15]. However, the metula, a structure of biseriate sterigmata, of strain TS1 was strikingly larger than that observed in previous reports (Table 1). This difference may be due to a high level of genetic variation in an asexual fungus A. niger [19]. In this study, strain TS1 was the only one A. niger isolate from the total of three morphologically identified A. niger isolates that possessed HDAC inhibitory activity, confirming the presence of different genotypes within A. niger strains.

The ITS sequence of ribosomal RNA gene (GenBank accession no. JF412766) of strain TS1 was compared with sequences in the GenBank. A phylogenetic tree based on known representatives of A. niger strains and other HDAC inhibitor producing fungi including Metacordyceps chlamydospora, Neurospora crassa, Alternaria brassicicola strain EEB 2232, and Cochliobolus carbonum, is shown in Figure 2. Strain TS1 showed high similarity with the strains from the genera of Aspergillus and Eurotiomycetes, and the highest similarity of 99% was with A. niger strain FP1 (GenBank accession no. GU216310). Based on the above characteristics, strain TS1 was identified as A. niger and named as A. niger TS1.

Figure 1. Morphological characteristics of Aspergillus niger TS1. (a) Colonies grown for 3 days on PDA plate at 30°C. Scale bar, 2 cm. (b) Conidial head and conidiophore. Scale bar, 100 μm. (c) Conidial structure displaying vesicle and sterigmata. Scale bar, 50 μm. (d) Sterigmata (metula, phialides) and conidia. Scale bar, 40 μm.
3.2 Inhibition of Human HDACs by *Aspergillus niger* TS1

The crude extract, partial purified fractions, and culture medium of strain TS1 were investigated for their HDAC inhibitory activity. As presented in Figure 3a, crude extract, partial purified water fraction, and cultured medium of strain TS1 exhibited *in vitro* HDAC inhibitory activity. These results suggested that HDAC inhibitor molecule(s) produced by strain TS1 was secreted into a culture medium. However, purification and characterization of the HDAC inhibitor molecule(s) in a cultured medium remains to be explored. Preliminary study regarding some properties of the secreted HDAC inhibiting molecules revealed that the 3 kDa-filtered cultured medium of strain TS1 inhibited *in vitro* HDAC activity comparable to that of the non-filtered cultured medium (Figure 3a), suggesting that the molecular weight of the secreted HDAC-inhibiting molecule(s) would be smaller than 3,000 dalton. The secreted HDAC inhibiting molecules appeared to be nonprotein molecules and possess a high degree of polarity according to the solubility in the cultured medium. Purification by preparative Thin-Layer chromatography failed to separate the compound(s) with high degree of polarity (data not shown). It is therefore challenging issue in exploration of a suitable technique for future purification of the secreted HDAC inhibitor(s). Although HDAC inhibitor molecule(s) remains to be identified, AUT polyacrylamide gel electrophoresis of acetylated histone H4 revealed that the strain TS1 3 kDa-filtered cultured medium inhibited HDAC activity in mammalian cells (Figure 3b). Hyperacetylation of histone H4 upon

### Table 1. Conidial characteristics of *Aspergillus niger* TS1 compared with published data.

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<td>Conidia (diameter; μm)</td>
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<td>Metula (length × width; μm)</td>
<td>(30.0 - 40.0) × (7.5 - 10.0)</td>
<td>(20.0 - 30.0) × (5.0 - 6.0)</td>
<td>(12.0 - 20.0) × (3.0 - 6.0)</td>
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<td>Phialide (length × width; μm)</td>
<td>(7.5 - 12.5) × (3.0 - 4.0)</td>
<td>(7.0 - 10.0) × (3.0 - 3.5)</td>
<td>(7.0 - 10.0) × (3.0 - 4.0)</td>
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<td>Vesicle (diameter; μm)</td>
<td>40.0 - 50.0</td>
<td>45.0 - 75.0</td>
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**Figure 2.** Phylogenetic relationship between *Aspergillus niger* TS1 and related species based on the ITS (internal transcribed spacer) sequence of ribosomal RNA gene. The filamentous fungus *Aspergillus nidulans* was used as outgroup. The accession numbers are in parentheses. Scale bar represents substitutions per nucleotide position.
inhibition of HDAC enzymes in HeLa cells was evidently observed when treating the cells with 2.5 % (v/v) of strain TS1 3 kDa-filtered cultured medium.

HDAC inhibitor production of strain TS1 reached its maximum after 5 days incubation at 30°C. As shown in Figure 3c, HDAC inhibitory activity increased with the increase of biomass concentration. HDAC inhibitor production was steadily maintained until at least 10 days of culture as HDAC inhibitory activity was observed at high level during 5-10 days of culture (Figure 3c). The strain TS1 secreted product was found to be toxic to itself at early stage of growth before conidial formation (data not shown). The mechanisms by which strain TS1 protects itself against its own toxic HDAC inhibitor molecule(s) have not been elucidated. The filamentous fungus C. carbonum has at least two mechanisms for protecting itself from its HDAC inhibitor, HC-toxin; first by a rapid efflux of HC-toxin out of the cytoplasm by the MFS drug transporter [20], and second by producing protective factor that binds to and protects HDAC enzymes from HC-toxin [12]. Accordingly, one could envision that one possible protection mechanism that strain TS1 protects itself against its own HDAC inhibitor molecule(s) could be an efflux of HDAC inhibitor(s) out of the cytoplasm, as the HDAC inhibitory activity could be observed in the medium of strain TS1 culture (Figure 3a, 3c).

Figure 3. HDAC inhibitory activity of crude extract, partial purified fractions, and cultured medium of Aspergillus niger strain TS1. (a) In vitro HDAC inhibitory activity of ethanolic crude extract (200 μg), partial purified fractions (200 μg), and 10 day-cultured medium (5%; v/v) of strain TS1. H₂O and DMSO (5%; v/v) represent solvent control treatments, whereas TSA (0.25 μM) represents a positive control treatment. DMSO was used as a solvent for dissolving both crude extract and partial purified fractions (methanol, ethyl acetate and hexane). HeLa nuclear extract was used as a source of the HDAC enzymes. (b) HDAC inhibitory activity of 10 day cultured medium (3 kDa-filtrate; 0.63, 1.25, 2.50%; v/v) of strain TS1 in mammalian cells (HeLa cells). Control represents the level of histone acetylation in cells treated with PD broth (uncultured medium). TSA (10 nM) treatment was used as a positive control. The degree of histone acetylation of histone H4 is indicated as follows: 0, nonacetylated; 1, monoacetylated; 2, diacetylated; 3, triacetylated; and 4, tetraacetylated. (c) Time-course of HDAC inhibitor production and dry weight biomass of strain TS1. Five microliters of cultured medium was used for HDAC activity assay.
3.3 Effect of 3 kDa-filtered Cultured Medium of Strain TS1 on Proliferation of Cancer Cells

In order to test if human cancer cells are sensitive to the 3-kDa filtrate of strain TS1 cultured medium possessing HDAC inhibitory activity, Jurkat, HCT116, and HeLa cell lines representing T-cell leukemia, colorectal carcinoma, and cervical carcinoma, respectively, were chosen. Surviving cells after treatment with a series of filtrate concentrations for 24, 48, and 72 h were analyzed using MTT assay. As presented in Figure 4, the 3-kDa filtrate of 10-day cultured medium inhibited proliferation of all human cancer cell lines in a time- and dose-dependent manner with the half maximal inhibitory concentration (IC50) values in the small amount of strain TS1 cultured medium. In contrast, the medium control showed no effect on proliferation of all cancer cell lines (data not shown). All cell lines appeared to be very sensitive to the strain TS1 cultured medium. In particular, human colon cancer cell line (HCT116 cells) appeared to be the most sensitive cell line to the strain TS1 cultured medium for 72 h-treatment (IC50 < 1.0 %; v/v). These findings suggest a promising application in the treatment of cancer using strain TS1 culture medium.

Figure 4. Antiproliferative effect of a 3-kDa filtrate of 10-day cultured medium on three human cancer cell lines. Antiproliferative activity of the 3-kDa filtrate on (a) human T cell lymphoblast-like cell line (Jurkat cells), (b) human colon cancer cell line (HCT116 cells), and (c) human cervical cancer cell line (HeLa cells), treated for 24, 48, and 72 h. Data are calculated as percentages of cell growth with respect to the untreated control, which was defined as 100% growth, and the results are shown as mean ± S.D. (n = 4). The half maximal inhibitory concentration (IC50 value) from each experiment was estimated by plotting x-y and fitting the data with a straight line (linear regression). The average of IC50 values from 3 independent experiments was calculated and presented along with a line graph.
3.4 Induction of Cancer Cell Apoptosis by 3 kDa-filtered Cultured Medium of Strain TS1

HDAC inhibitors induce apoptosis in a number of cancer cell types and through various mechanisms [21]. To test if induction of apoptosis underlies antiproliferative effect of strain TS1 cultured medium, its capacity to induce apoptosis in cancer cell lines were examined. As shown in Figure 5, strain TS1 cultured medium exhibited a significant effect on induction of apoptosis in all cancer cell lines tested even only 6 h of exposure time. The treatment of Jurkat, HCT116, and HeLa cells with 5% (v/v) of strain TS1 cultured medium resulted in the increase of early apoptotic cells (panels B4) up to 8.6%, 71.8% and 9.4%, respectively. In contrast, the control Jurkat, HCT116, and HeLa cells had only 1.0%, 0.3% and 3.0% of apoptotic cells, respectively. Apparently, HCT116 cells appeared to be the most sensitive cells to the 6 h-induction of apoptosis by the strain TS1 cultured medium. These results suggest that strain TS1 cultured medium possessing HDAC inhibitory activity suppresses the growth of Jurkat, HCT116, and HeLa cells at least in part through induction of apoptosis. The mechanism by which strain TS1 cultured medium brings about apoptosis is of an interesting issue for elucidation. The treatment of HeLa cells with strain TS1 cultured medium resulted in hyperacetylation of histone proteins, indicating aberrant changes in the homeostasis of histone acetyltransferases (HATs) and HDACs. Histone hyperacetylation appeared to be an early signaling event in the execution of apoptosis of neuronal cells [22]. In the current study, the strain TS1 cultured medium could induce apoptosis of all cancer cell lines tested, especially human colon cancer cell line (HCT116 cells) (Figure 5b). Consistent with this finding, hyperacetylation of histone proteins induced by HDAC inhibitors appeared to promote apoptosis of various cancer cells [23-25], thus beneficial in cancer therapy.

Figure 5. Apoptotic death of three human cancer cell lines induced by a 3-kDa filtrate of strain TS1 10-day cultured medium. Dot plots display the apoptotic death of Jurkat, HCT116, and HeLa cells treated with the indicated concentrations. After 6 h treatment, cells labeled with Alexa Fluor 488 Annexin V and Propidium iodide (PI) were analyzed by flow cytometry to determine the percentage of cells displaying an increase in early apoptosis (panels B4). Cells treated with PD broth (1%, v/v) and Camptothecin (50-100 μg/mL) were used as negative and positive controls, respectively.
4. CONCLUSIONS

The present study reports the isolation and characterization of a novel strain of A. niger capable of producing HDAC inhibitor(s) isolated from soil in Khon Kaen province, Thailand. Comparison of its conidial characteristics revealed a strikingly larger size of the metula, a structure of biseriate sterigmata, than that of A. niger strains observed in previous published data. The strain TS1 cultured medium exhibited HDAC inhibitory activity both in vitro and in mammalian cells, indicating the presence of HDAC inhibitor molecule(s) in the cultured medium. To the best of our knowledge, this is the first report showing that Aspergillus species could serve as a natural source of HDAC inhibitor(s). The HDAC inhibitor molecule(s) produced by this fungus appeared to be a nonprotein molecule(s) with molecular weight of less than 3,000 dalton and exhibited a very high degree of polarity. Further isolation and structure determination of the HDAC inhibitor molecule(s) as well as determination of HDAC isoform selectivity are needed, which could lead to the discovery of a novel anticancer compound(s). The growth inhibitory effects on three cancer cell lines of the strain TS1 cultured medium were in accordance with its capability to induce cancerous cell apoptosis. These findings may open the door to new anticancer drug development and also raise the possibility that the strain TS1 cultured medium may be used effectively in killing cancer cells.

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