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Growth Stimulating Effect on Queen Bee Larvae of Histone Deacetylase Inhibitors

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ABSTRACT: Royal jelly (RJ) is a widely used natural food. It is also a major source of nutrition for queen bees and plays a key role in their development. RJ is secreted from the hypopharyngeal and mandibular glands of young adult worker bees. The regulation of gene expression in these two glands may influence the development of queen bees by affecting the content of RJ. This study investigated the epigenetic effects in these two glands in young adult worker bees treated with histone deacetylase inhibitors (HDACis), a U.S. Food and Drug Administration-approved drug, suberoylanilide hydroxamic acid (SAHA), and NBM-HD-1, a novel compound synthesized in this laboratory. Western blot analyses indicated that the levels of acetyl-histone 3 and p21 protein expression in MCF-7 cells increased markedly after treatment with NBM-HD-1. The data proved that NBM-HD-1 was a novel and potent HDACi. Furthermore, a method of affecting epigenetic regulation of the *mrjp* family gene in the hypopharyngeal and mandibular glands of young adult worker bees was developed by feeding young adult worker bees HDACi. Epigenetic regulation produced several important biological effects. A marked change in the protein composition of the RJ secreted from these treated bees was found. Only the ratio of specific major royal jelly protein 3 (MRJP3) was significantly altered in the treated bees versus the untreated controls. Other MRJP family proteins did not change. This alteration in the ratio of royal jelly proteins resulted in a significant increase in the body size of queen bee larvae. The data seem to suggest that HDACis may play an important role in the epigenetic regulation of the hypopharyngeal and mandibular glands of young adult worker bees. They appear to change *mrjp3* gene expression and alter the ratio of MRJP3 protein in RJ. This study presents the first evidence that HDACis are capable of regulating the ratio of MRJP3 proteins in RJ, which has the potential to change the body size of queen bees during their development.

KEYWORDS: *NBM-HD-1, royal jelly, HDAC inhibitor, honeybee, queen bee larvae*

INTRODUCTION

Colony collapse disorder (CCD) is the term used to describe the phenomenon that results in an estimated loss of >20% of bee colonies every year.[1] CCD has been found at high rates in many European countries such as France, Greece, Italy, and Spain. Canada and at least 24 different states in the United States have reported at least one case of CCD. Honeybees are becoming more and more important in agriculture and ecology for their pollination. In 2000, the value of U.S. crops that were dependent on honeybee pollination exceeded U.S. $15 billion. The cause of CCD is not yet fully understood. Many factors may be involved, including varroa mites or insect diseases, environmental change-related stressors, pesticides, cell phone radiation, and malnutrition.[2,3] The exact mechanisms of CCD are still unclear. Two reports suggest that the CCD syndrome in honeybees may result from infection by the Israel acute paralysis virus (IAPV).[4,5]

Honeybees produce a wide range of nutritional products that are beneficial to human beings, such as royal jelly (RJ), honey, propolis, and pollen.[6] Honeybees live in matrilineal societies where the bee colony is controlled by a queen bee. Worker bees and queen bees develop from fertilized eggs, whereas drones develop from unfertilized eggs.[7] Queen bees are bigger in body size, and their lifespan is normally 10−15 times greater than that of worker bees. Typically, only one queen is present in a bee colony. The food fed to a larva by younger worker bees determines whether the larva develops into a worker bee or a queen bee. RJ is an essential substance in the maintenance of a bee colony.[8] Unlike all the other bees in the colony, the queen bee is fed RJ throughout its lifetime. RJ is secreted by the hypopharyngeal and mandibular glands of young adult worker bees. RJ is an opaque, thick liquid composed of 60−70% water, 12−15% proteins, 10−12% sugars, 3−5% lipids, and many trace elements and minerals.[10] Of the protein constituents, approximately 89−90% are water-soluble proteins. The composition of RJ may change at different developmental stages of the larvae. In general, such changes involve mostly the sugar rather than protein components of RJ.[11] The major royal jelly protein (MRJP) family accounts for the majority of the

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water-soluble proteins in RJ.[12] Although the amino acid sequences of these MRJP proteins have been determined, their functions remain unclear. MRJP1 (royalactin) was identified in the brain of bees and is assumed to be responsible for their behavior. In addition, it has been proven to be an important factor in RJ as it induces queen differentiation in honeybees.[9] MRJP1 accounts for 44−48% of the water-soluble proteins in RJ and is the most abundant isoform among the MRJP family proteins.[13,25] MRJP3, a highly polymorphic protein, has received the most attention lately as it is considered to play a major role in immune regulation.[14] MRJP3 accounts for 12% of the total water-soluble proteins in RJ and is the second-most abundant isoform of all the MRJP proteins (MRJP1−9). MRJP3 is distinct because of its antiallergic and anti-inflammatory activities.[14] Variants of MRJP3 proteins are 60−70 kDa and are products of the highly polymorphic MRJP gene.[12] MRJPs provide abundant essential amino acids and other nutrient components for queen bee development.[15] RJ is the only source of nutrition for queen bee larvae, and MRJPs are the most important proteins in RJ.

Epigenetic effects have outcomes that affect health, behavior, and appearance. Substances, such as drugs, nutrients, and natural products, have epigenetic effects.[16] Epigenetic modifications may include DNA methylation, histone methylation or acetylation, histone variants, and long-distance chromosomal interactions.[17] This may occur in two ways. First, methylation can directly interfere with the binding of transcription factors to recognition sites on DNA. Second, methylated sites attract methyl-CpG-binding domain proteins (MBPs) that can reinforce silencing by recruiting corepressor complexes harboring histone deacetylases or histone methyltransferases.[18] Epigenetic changes may alter the regulation of the enzymes involved not only in the methylation of DNA and histones but also in the acetylation of histones.[16] Histone acetyltransferases (HATs) and histone deacetylases (HDACs) are involved in the processes of histone acetylation and deacetylation, respectively, resulting in chromatin remodeling. Currently, HDAC inhibitors (HDACis) are believed to be promising targeted anticancer drugs.[19] The current study demonstrated that RJ is rich in DNA methyltransferase inhibitor, which results in triggering larvae development into queen bee.[22] It is a typical case of epigenetic effect in honeybees.

Here, we used our in-house synthesized HDACi NBM-HD-1 and an FDA-approved HDACi SAHA to test if HDACis could affect gene expression of the hypopharyngeal and mandibular glands of young adult worker bees to produce a more nutritious RJ. Indeed, after consuming our recipe with HDACi, the young adult worker bees secreted a royal jelly that allows queen bee larvae to grow more rapidly, with a body weight 2−3 times that of ordinary larvae.

MATERIALS AND METHODS

Preparation of 3′,4′,5,7-*O*-Tetramethylpropolin G (2). To a solution of propolin G (**1**) (152 mg, 0.31 mmol) were added K_2CO_3 (431 mg, 3.1 mmol) and acetone (15 mL) with Me_2SO_4 (0.25 mL, 2.48 mmol), and the resulting solution was heated under N_2 for 24 h. After removal of the organic solvent, the residue was dissolved in CH_2Cl_2 (50 mL) and washed with distilled (dis) H_2O (50 mL × 3). The organic layer was dried over Na_2SO_4 and evaporated under reduced pressure. The residue obtained was purified by a silica gel column (EtOAc/*n*-hexane = 1:6) to give **2** (127 mg, yield = 75%): ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.26 (1H, d, J = 8.6 Hz), 6.86 (1H, d, J = 8.6 Hz), 6.27 (1H, s), 5.50 (1H, dd, J = 2.5, 13.5 Hz), 5.14−5.11 (1H, m), 5.11−5.10 (1H, m), 5.02−4.99 (1H, m), 3.87 (3H, s), 3.83 (3H, s), 3.79 (6H, s), 3.50 (1H, dd, J = 6.6, 15.2 Hz), 3.43 (1H, dd, J = 5.8, 15.2 Hz), 3.00 (1H, dd, J = 13.5, 16.7 Hz), 2.68 (1H, dd, J = 2.6, 16.7 Hz), 2.00−1.92 (2H, m), 1.75 (3H, s), 1.70 (3H, s), 1.65 (3H, s), 1.60 (3H, s), 1.52 (3H, s); ^{13}C NMR (100 MHz, $CDCl_3$) δ 189.4 (s), 164.0 (s), 163.3 (s), 159.6 (s), 153.0 (s), 147.2 (s), 135.6 (s), 134.2 (s), 131.5 (s), 131.3 (s), 129.8 (s), 124.1 (d), 122.8 (d), 122.7 (d), 122.1 (d), 118.2 (s), 110.3 (d), 108.7 (s), 95.6 (d), 75.9 (d), 61.8 (q), 60.7 (q), 55.7 (q), 55.7 (q), 45.1 (t), 39.6 (t), 26.6 (t), 25.7 (q), 25.6 (q), 24.9 (t), 22.0 (t), 17.7 (q), 17.6 (q), 16.3 (q); HREIMS m/z 548.3140 (calcd for $C_{34}H_{44}O_6$ 548.3142).

Preparation of NBM-HD-1. To a solution of **2** (200 mg, 0.37 mmol) in THF (6 mL) was added 49% H_2SO_4 (4 mL) in a dropwise manner in an ice bath. The reaction mixture was then stirred at room temperature for 8 h and diluted with H_2O. The aqueous layer was extracted with CH_2Cl_2 (50 mL × 3). The combined organic layers were dried over Na_2SO_4 and evaporated under reduced pressure to give a residue, which was purified by a silica gel column (EtOAc/*n*-hexane 1:6−1:1) to give the pure oil **3** (54 mg, yield = 25%): IR (KBr) ν_{max} cm^{-1} 3745, 3443, 2968, 1676, 1600, 1455; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.27 (1H, d, J = 8.6 Hz), 6.87 (1H, d, J = 8.6 Hz), 6.28 (1H, d, J = 2.6 Hz), 5.49 (1H, dd, J = 2.4, 13.6 Hz), 5.05 (1H, t, J = 6.0 Hz), 3.87 (3H, s), 3.84 (3H, s), 3.79 (6H, s), 3.52 (1H, dd, J = 5.5, 15.2 Hz), 3.43 (1H, dd, J = 7.6, 15.2 Hz), 2.97 (1H, dd, J = 8.0, 16.7 Hz), 2.70−2.59 (3H, m), 1.93 (92H, t, J = 6.4 Hz), 1.65 (3H, s), 1.64−1.60 (2H, m), 1.41−1.32 (4H, m), 1.25 (6H, s), 1.15 (3H, s), 1.14 (3H, s); ^{13}C NMR (100 MHz, $CDCl_3$) δ 189.4 (s), 164.0 (s), 163.3 (s), 159.6 (s), 153.0 (s), 147.2 (s), 135.6 (s), 134.2 (s), 131.5 (s), 131.3 (s), 129.8 (s), 124.1 (d), 122.8 (d), 122.7 (d), 122.1 (d), 118.2 (s), 110.3 (d), 108.7 (s), 95.6 (d), 75.9 (d), 61.8 (q), 60.7 (q), 55.7 (q), 55.7 (q), 45.1 (t), 39.6 (t), 26.6 (t), 25.7 (q), 25.6 (q), 24.9 (t), 22.0 (t), 17.7 (q), 17.6 (q), 16.3 (q); HREIMS m/z 584.3344 (calcd for $C_{34}H_{48}O_8$ 584.3338).

Cell Culture and Western Blot Assay. Human MCF-7 breast cancer and rat C6 glioma cells were purchased from the Food Industry Research and Development Institute (Hsinchu, Taiwan). Both cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco, Grand Island, NY, USA) containing 10% (v/v) fetal bovine serum (FBS), 1% (w/v) penicillin−streptomycin, and 2 mM L-glutamine. Cells were maintained at 37 °C in an atmosphere of 95% air and 5% CO_2. NBM-HD-1 was dissolved in dimethyl sulfoxide (DMSO). MCF-7 and C6 cells (1.0×10^6 per dish) were cultured in a 100 mm dish and incubated for 14 h before treatment with NBM-HD-1 at various concentrations (4.3−17.0 μM) for 2 or 3 days to evaluate the antiproliferative activity. It was also treated with a fixed concentration (17.0 μM) of NBM-HD-1 for 1−4 h to analyze the levels of Ac-histone 3 and p21 protein expression. The vehicle (DMSO) in the cell culture medium was fixed at a concentration of 2.0 μL/mL. After treatment, cells were collected and resuspended in a 100 μL lysis buffer. Equal amounts of protein (30.0 μg) were mixed with a 2× sample buffer and resolved using sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE) with a 12.5% gel for the detection of β-actin, p21, and Ac-histone 3 through Western blotting. Proteins were electrotransferred to an Immobilon polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA, USA). Equivalent protein loading was verified by staining the membrane with the reversible dye Amido Black (Sigma Chemical Co., St. Louis, MO, USA). This was followed by overnight blocking with a solution containing 20 mM Tris-HCl (pH 7.4), 125 mM NaCl, 0.2% Tween 20, and 3% bovine serum albumin (BSA) before probing with specific antibodies directed against human β-actin, p21, and Ac-histone 3. The specific antibodies used were anti-β-actin (1:1000 of mouse monoclonal antibodies; Sigma), anti-p21 antibody (1:1000 of mouse monoclonal antibodies; BD Pharmingen), and anti-Ac-histone 3 (1:1000 of rabbit polyclonal antibodies; Cell Signaling Technology). Antibodies were detected using chemiluminescence (ECL, Amersham, GE Healthcare, Buckinghamshire, U.K.).

Primary Astrocytes. Rat astrocytes were prepared from the cerebral cortexes of 17-day-old embryonic rats. The rat cerebral cortex was dissected and incubated with trypsin at room temperature for 5 min. Brain cortex cells were then mechanically dissociated in the

culture medium with a fire-narrowed Pasteur pipet and plated in a 100 mm dish at a density of 2.0×10^6 cells for 10 mL of culture medium. Brain cortex cells were cultured for 6 days in DMEM (Gibco) containing 10% FBS, a solution of 1% dilution of penicillin–streptomycin, and 2 mM glutamine. Brain cortex cells rapidly proliferated. Ninety-five percent of the brain cortex cells differentiated into astrocytes and expressed glial fibrillary acidic protein (GFAP). GFAP is an intermediate filament protein that is considered to be a specific biomarker for astrocytes in CNS. Astrocytes (3.0×10^5 per well) were seeded in 6-well plates and incubated for 14 h. They were then treated with NBM-HD-1 at various concentrations (4.3–17.0 μM) for 48 h.

Primary Cortical Neurons. Rat cortical neuron cultures were prepared from the cerebral cortexes of 17-day-old embryonic rats. The rat cerebral cortexes were dissected and incubated at room temperature for 5 min. Cells were then mechanically dissociated in culture medium with a fire-narrowed Pasteur pipet and plated at a density of 150 cells/mm^2 in 6-well dishes. Prior to use, all dishes were sequentially coated with 30 μg/mL poly-D-lysine and then with a neurobasal medium (Gibco) mixture containing B27 supplement (Gibco). The culture medium contained 100 U/mL penicillin, 100 μg/mL streptomycin, and 2 mM glutamine. Cells were cultured in the medium and treated with NBM-HD-1 at various concentrations (1.08–4.3 μM) for 3 days in vitro (DIV). Cultures were maintained at 37 °C in a 5% CO_2/95% air-humidified incubator. Neurite outgrowth and cortical neuron survival activity were determined using a microscope.

Analysis of the Cell Cycle. Rat C6 cells (1.0×10^6/dish) in 100 mm dishes were treated with various concentrations of NBM-HD-1 (4.3–17.0 μM) for 72 h. Cells were trypsinized and collected in ice-cold PBS. They were then resuspended in 200 μL of PBS and fixed by adding 800 μL of 100% ethanol before being incubated overnight at −20 °C. Cell pellets were collected using centrifugation, resuspended in 1.0 mL of hypotonic buffer (0.5% Triton X-100 in PBS and 1.0 μg/mL RNase A), and incubated at 37 °C for 30 min. Then, 1.0 mL of propidium iodide (PI) solution (50.0 μg/mL) was added, and the mixture was allowed to stand at 4 °C for 30 min. The cellular DNA content was then analyzed using FACScan cytometry (Becton Dickinson).

HDACs 1–11 Activity Assay. Determination of HDACs 1–11 enzyme activity was performed by Reaction Biology Corp. (Malvern, PA, USA). NBM-HD-1 and trichostatin A (TSA, a pan-HDACi as a positive control) were tested in the platform. Both compounds were assayed in serial dilution concentrations ranging from a starting concentration at 20 to 0.08 μM. The general substrate was fluorogenic peptide from p53 residue 379–382 (RHKKAc). The fluorescence intensity was measured using a fluorometric reader with excitation at 360 nm and emission at 460 nm.

Collection of RJ and Queen Bee Larvae. The experiment was carried out at the bee farm of NatureWise Biotech & Medicals Corp. located in Hualian, Taiwan. Bees in beehives with similar expression levels of the MRJP3 protein (3–7, 12, 18, 25, 26, and 30) were identified and selected for use as shown in Figure 4a. Two beehives were tested in each control and treatment group. On the first day, the honey originally contained in the beehives was removed by shaking, and the worker bees (*Apis mellifera*) were fed on formulated sugar water (640 g of sugar plus 360 g of H_2O) for the first time. The formulated sugar water for the treated group contains added component of either NBM-HD-1 or SAHA dissolved in a vehicle (Figure 1a) at various amounts. The young adult worker bees of the control group were fed sugar water and blank vehicle. On the second day, for the treated group, the young adult worker bees were fed the same formulated sugar water. On the third day, 1.5-day-old larvae (which were offspring from the same queen) were inserted into the beehives. The control group worker bees were fed for the last time with sugar water and the treatment group bees were fed for the last time with treated sugar water. After 24, 48, and 72 h, 96 larvae per beehive and the RJ in their cells were collected for analysis. Figure 1b shows the full treatment scheme.

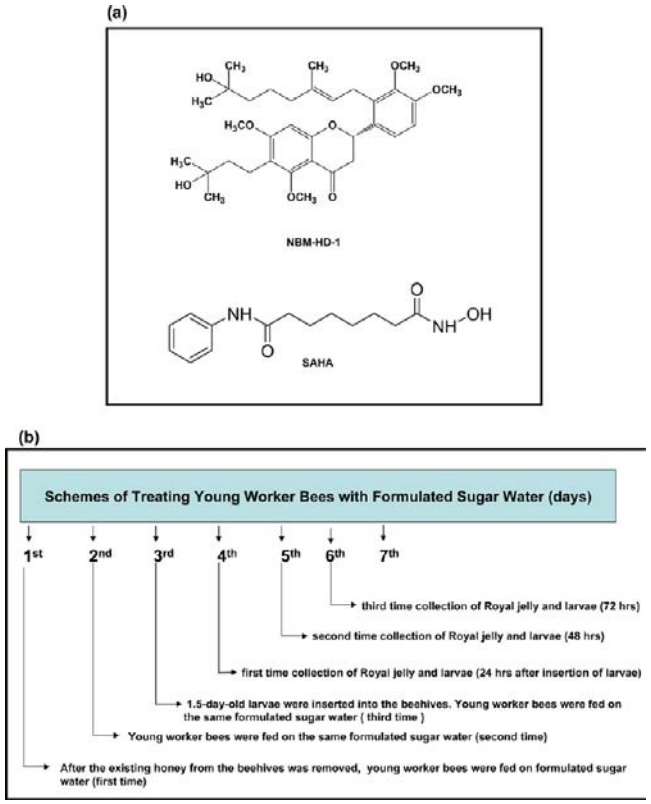

Figure 1. Structures and schemes: (a) structures of NBM-HD-1 and SAHA; (b) schemes of HDAC inhibitors on the treatment of beehive. Details are given under Materials and Methods.

Analysis of Royal Jelly. RJ and sterilized water were mixed at a ratio of 1:10 (w/w) for extraction. A sample of the proteins in the aqueous layer was removed for determination of protein concentration using the Bradford dye-binding method (Bio-Rad protein assay, Bio-Rad Laboratories, Inc., Hercules, CA, USA). The absorption at 595 nm of the protein samples was determined with an ELISA Reader (Bio-TEK). Ten micrograms of the protein samples was electrophoresed on a 12.5% SDS-PAGE gel at 60 V for 30 min followed by 120 V for 2 h to separate proteins of different molecular weights. The gel was stained with Coomassie Blue (Coomassie Brilliant Blue R-250, Bio Basic Inc., Markhan, ON, Canada) for 15 min and destained in a destain buffer (methanol/acetic acid/ddH_2O, 20:7:73) to make the background of the gel transparent.

Analysis of the Queen Bee Larvae. The larvae were washed with 1× PBS to remove residual RJ before being ground. After grinding, a suitable amount of sterilized water was added for protein extraction. A sample of the aqueous layer was removed to determine protein concentration. Ten micrograms of protein samples was analyzed on a 12.5% SDS-PAGE gel as described above. The gel was stained with Coomassie Blue for 15 min and destained in the above-mentioned destain buffer until the background of the gel became transparent.

Measurement of the Weight of Queen Bee Larvae. RJ and queen bee larvae were collected from different hives in the control or experimental group at various points in time (24, 48, or 72 h). The larvae were weighed and photographed for analysis and comparison.

Proteomic Analysis. Protein extracts of the queen bee larvae were vacuum-dried using a rotary evaporator (Speed Vac) and redissolved in a dehydration buffer. The protein concentration was determined using the Bradford dye-binding method. Afterward, 100.0 μg of queen bee larvae samples were analyzed with two-dimensional gel electrophoresis (2-DE) as follows. The samples were first separated by isoelectric focusing (IEF) on a strip of pH 3–10. This was followed by equilibration and then separation by SDS-PAGE (stacking gel, 30% acrylamide (Bio-Rad), Tris, pH 6.8, 10% APS, ddH_2O, 1% SDS, TEMED; separating gel, 30% acrylamide (Bio-Rad), Tris, pH 8.8, 10%

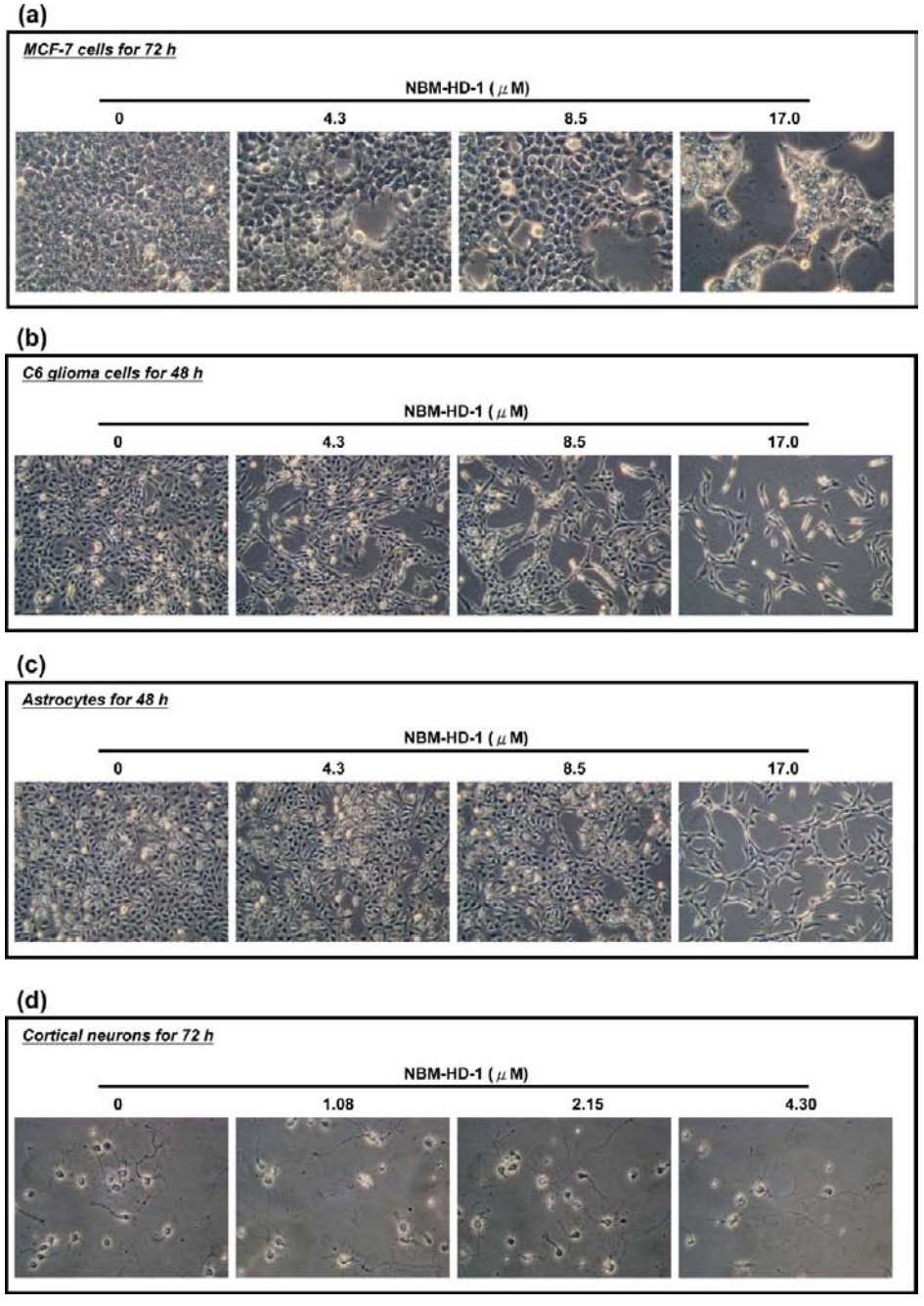

Figure 2. Effects of NBM-HD-1 on several cell types. (a) MCF-7 cells were treated with NBM-HD-1 at various concentrations of 4.3−17.0 μM for 72 h. (b) C6 glioma cells were treated with NBM-HD-1 at various concentrations of 4.3−17.0 μM for 48 h. (c) Astrocytes were cultured from embryonic rat cortex, and the details are given under Materials and Methods (cells were treated with NBM-HD-1 at various concentrations of 4.3−17.0 μM for 48 h). (d) Cortical neurons were cultured from the same source of embryonic rat cortex (neurons were treated with NBM-HD-1 at various concentrations of 1.08−4.3 μM for 72 h).

APS, ddH_2O, 1% SDS, TEMED). Images of the gel were taken and analyzed using Image Master 2D Platinum 6.0 software. The spots of interest on the gel were subjected to in-gel trypsin digestion and were analyzed with MS/MS on a LC-ESI-Q-TOF mass spectrometer. The data obtained were then compared against the database for protein identification using Mascot software.

Densitometric Analyses for Protein Expression. SDS-PAGE was scanned with BioImaging system (UVP, Inc., Upland, CA, USA). In each case, bands were subjected to multiple exposures on the data to ensure that the band density was in the linear range. The data are presented in terms of fold change over internal control (MRJP1 and MRJP2) for each treatment. All data presented were from two to three independent experiments with similar results.

Statistical Analysis. The RJ and queen bee larvae analysis results are presented as the mean ± SD. Student's *t* test was used to calculate the statistical significance of differences between each group and the control group.

RESULTS

Semisynthesis of NBM-HD-1. Propolin G was isolated from Taiwanese green propolis (TGP). Our previous study suggested that propolin G induced growth inhibition and apoptosis of a brain cancer cell line, possibly due to modulation of the expressions of cell cycle-regulator genes and further activation of caspases and mitochondrial pathways.[20] Further-

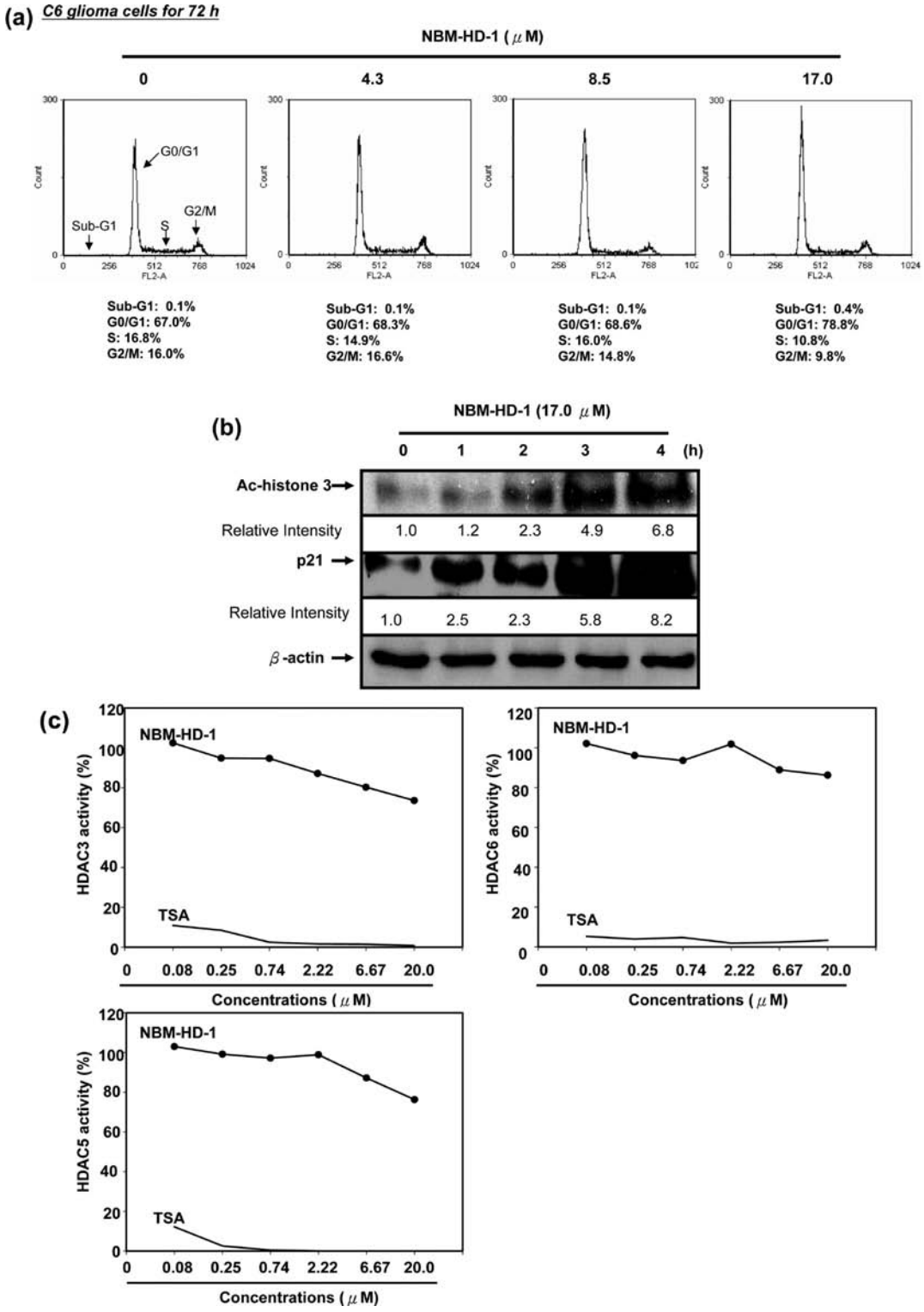

Figure 3. NBM-HD-1 was an HDAC inhibitor. (a) Flow cytometric analysis was performed on NBM-HD-1-treated C6 glioma cells stained with propidium iodide. The detailed process is described under Materials and Methods. Following a flow cytometric analysis, the cellular DNA profile was analyzed by Cell Quest software; all tests were performed in three independent experiments. A representative example of the three replicates is shown. (b) MCF-7 cells were treated with a fixed concentration of 17 μM for 1, 2, 3, and 4 h. Cell lysates were prepared and subjected to SDS-PAGE and immunoblotting using respective specific antibodies. Ac-histones 3 and p21 HDAC inhibitor biomarkers were determined by a Western blot assay as described under Materials and Methods. (c) HDACs 1−11 enzyme activity was determined by NBM-HD-1. NBM-HD-1 and TSA (HDACi, as a positive control) were tested in this platform. Both compounds were assayed in serial dilution concentrations (from 20.0 to 0.08 μM). The general substrate was fluorogenic peptide from p53 residue 379−382 (RHKKAc) as described under Materials and Methods.

more, we also found that propolin G possesses a weak HDACi property. For this reason, we were interested in the development of a more potent HDACi from propolin G. The detailed process was presented under Materials and Methods.

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Table 1. Weight of Larvae Fed RJ Secreted by Young Adult Worker Bees Fed NBM-HD-1 (n = 12)

group	av wt of each larva (mg)		
	at 24 h	at 48 h	at 72 h
control (U-1)	2.28 ± 0.5	7.78 ± 1.1	19.34 ± 2.5
TGP[a] extract (T-1) (2.5 g/kg)	2.79 ± 0.4	12.73 ± 1.4	51.87 ± 5.8
NBM-HD-1 (H1-1) (50 mg/kg)	4.58 ± 0.6	18.02 ± 2.5	37.43 ± 4.0
NBM-HD-1 (H2-1) (100 mg/kg)	3.47 ± 0.4	14.29 ± 1.5	79.93 ± 9.6
NBM-HD-1 (H3-1) (200 mg/kg)	2.10 ± 0.3	17.82 ± 1.9	82.55 ± 11.2

[a]TGP, Taiwanese green propolis.

We found that NBM-HD-1[26] and NBM-HD-3[21] were active compounds that could inhibit the growth of several types of cancer cell lines.

Effects of NBM-HD-1 on the Different Types of Cell Lines. Through a two cancer cell line (MCF-7 and C6) growth inhibition assay (Figure 2a,b), we found that NBM-HD-1 significantly induced a cytotoxic effect in human MCF-7 breast cancer cells for 72 h (Figure 2a). NBM-HD-1 markedly induced a cytotoxic effect at a concentration of 17.0 μM. Rat C6 glioma cells treated with NBM-HD-1 at the same concentration showed markedly suppressed cell growth. Cell growth may have been inhibited by cancer cell differentiation (Figure 2b). C6 glioma cells treated with NBM-HD-1 at a concentration 34.0 μM markedly induced cytotoxic effects (data not shown). To evaluate the effect of NBM-HD-1 in normal brain cells, rat astrocytes were cultured in our laboratory. These astrocytes were treated with NBM-HD-1 at various concentrations (4.3−17.0 μM) for 48 h. Figure 2c shows that, at a concentration of 17.0 μM, NBM-HD-1 markedly suppressed cell growth and induced differentiation. Dendrite or neurite outgrowth occurred in a high concentration (17.0 μM) of NBM-HD-1-treated cells. Neurite outgrowth was an important biomarker for neural cells. Neurons and astrocytes were generated from neural stem cells (neurosphere). To investigate the effect of NBM-HD-1 on rat primary cortical neurons, cortical neurons were treated with NBM-HD-1 at low concentrations (1.08−4.3 μM) for 72 h. Figure 2d shows that neurite outgrowth was induced in cortical neurons after treatment with NBM-HD-1 at concentrations of 1.08−4.3 μM. Cortical neurons were sensitive to the serum medium. Thus, a serum-free medium was used in the experiment as described under Materials and Methods.

NBM-HD-1 as an HDACi. Inhibition of cell proliferation was clearly present in NBM-HD-1-treated cells (Figure 2a−c). To evaluate the cell cycle, rat C6 glioma cells were treated with NBM-HD-1 at various concentrations (4.3−17.0 μM) for 72 h. Flow cytometry was used to determine whether NBM-HD-1-induced cell growth suppression (10000 event/case) was caused by regulation of the cell cycle. Figure 3a shows that no significant increase in the apoptotic cell population (sub-G1 phase) occurred following NBM-HD-1 (0−17.0 μM) treatment. This suggests that NBM-HD-1-inhibited cell growth may be via the differentiation mechanism. Cytotoxicity was not found in rat C6 glioma cells (Figure 2b). In contrast, the cell population at the G0/G1 phase significantly increased at a NBM-HD-1 concentration of 17.0 μM (Figure 3a). To determine whether NBM-HD-1 was a potent HDACi, MCF-7 cells were treated with NBM-HD-1 at a fixed concentration (17.0 μM) for 1, 2, 3, and 4 h. A Western blot assay was used to determine the levels of HDACi-triggered biomarker protein expression. Figure 3b shows that the protein levels of Ac-histone 3 and p21 markedly increased after a 2−4 h treatment with NBM-HD-1. The Ac-histone 3 level was up-regulated approximately 6.8 times more than in untreated cells (0 h). Ac-histone 3 was an important biomarker for the HDACi. The level of P21 protein, which was another important biomarker for the HDACi, was also significantly up-regulated after cells were treated with NBM-HD-1. Increasing the p21 gene or protein expression may trigger cell cycle arrest in the G0/G1 phase (Figure 3a). The enzymatic activity of 11 HDACs was evaluated using NBM-HD-1. Figure 3c shows that the enzymatic activities of HDAC3, HDAC5, and HDAC6 were mildly inhibited by NBM-HD-1. However, other HDACs were not markedly inhibited. NBM-HD-1 seems to possess a selective property. Trichostatin A (TSA) is a more potent pan-HDACi than NBM-HD-1 as shown in Figure 3c.

Effects of NBM-HD-1 on the Expression of MRJP3. In 2008, Maleszka et al. stated that honeybee reproductive status could be controlled by regulating DNA methylation.[22] DNA methylation, as well as histone acetylation, can regulate epigenetics. Histone acetyltransferases (HATs) and histone deacetylases (HDACs) are involved in the processes of histone acetylation and deacetylation, respectively, which result in chromatin remodeling. Thus, HDACis may influence epigenetic regulation of the hypopharyngeal and mandibular glands of young adult worker bees. The influence of RJ on MRJP protein expression was investigated. NBM-HD-1 at various concentrations was added to sugar water to form formulated sugar waters. Young adult worker bees were fed formulated sugar water containing 50, 100, or 200 mg/kg of NBM-HD-1. The queen bee larvae and RJ produced by the young adult worker bees were collected for analysis at 24, 48, and 72 h after insertion of the 1.5-day-old larvae as shown in Figure 1b. The weight of queen bee larvae and RJ were analyzed; details are presented under Materials and Methods. As shown in Table 1 and Figure 4b, NBM-HD-1 at 50−200 mg/kg significantly induced the growth of larvae. Because propolins have been proven to possess weak HDACi properties, TGP extract, which contains high levels of propolins, was used as a positive control (Table 1). From 24 to 48 h, the larvae of the control group grew from 2.28 to 7.78 mg, which was a weight increase of approximately 2.40-fold. Meanwhile, the larvae fed by the young adult worker bees treated with a medium dose of NBM-HD-1 (100 mg/kg) grew from 3.47 to 14.29 mg, which was a weight increase of approximately 3.11-fold. From 48 to 72 h, the larvae of the control group grew from 7.78 to 19.34 mg, a weight increase of approximately 1.49-fold. Meanwhile, the larvae fed by the worker bees with a medium dose of NBM-HD-1 (100 mg/kg) grew from 14.29 to 79.93 mg, a weight increase of approximately 4.59-fold. Thus, the HDACi appeared to induce the growth of queen bee larvae.

Effects of NBM-HD-1 on the Expression of MRJP3 Protein and Larvae Growth. Young adult worker bees were fed formulated sugar water containing 50, 100, or 200 mg/kg of NBM-HD-1/kg. Queen bee larvae and RJ produced by young adult worker bees were collected for analysis at 24, 48, and 72 h after insertion of the 1.5-day-old larvae. The levels of MRJP protein expression in RJ were investigated. MRJPs are a major water-soluble protein in RJ. Figure 4c shows that the expression of the water-soluble protein MRJP3 in the altered RJ (from the treated groups, H1-1 to H3-1) notably differed from that of

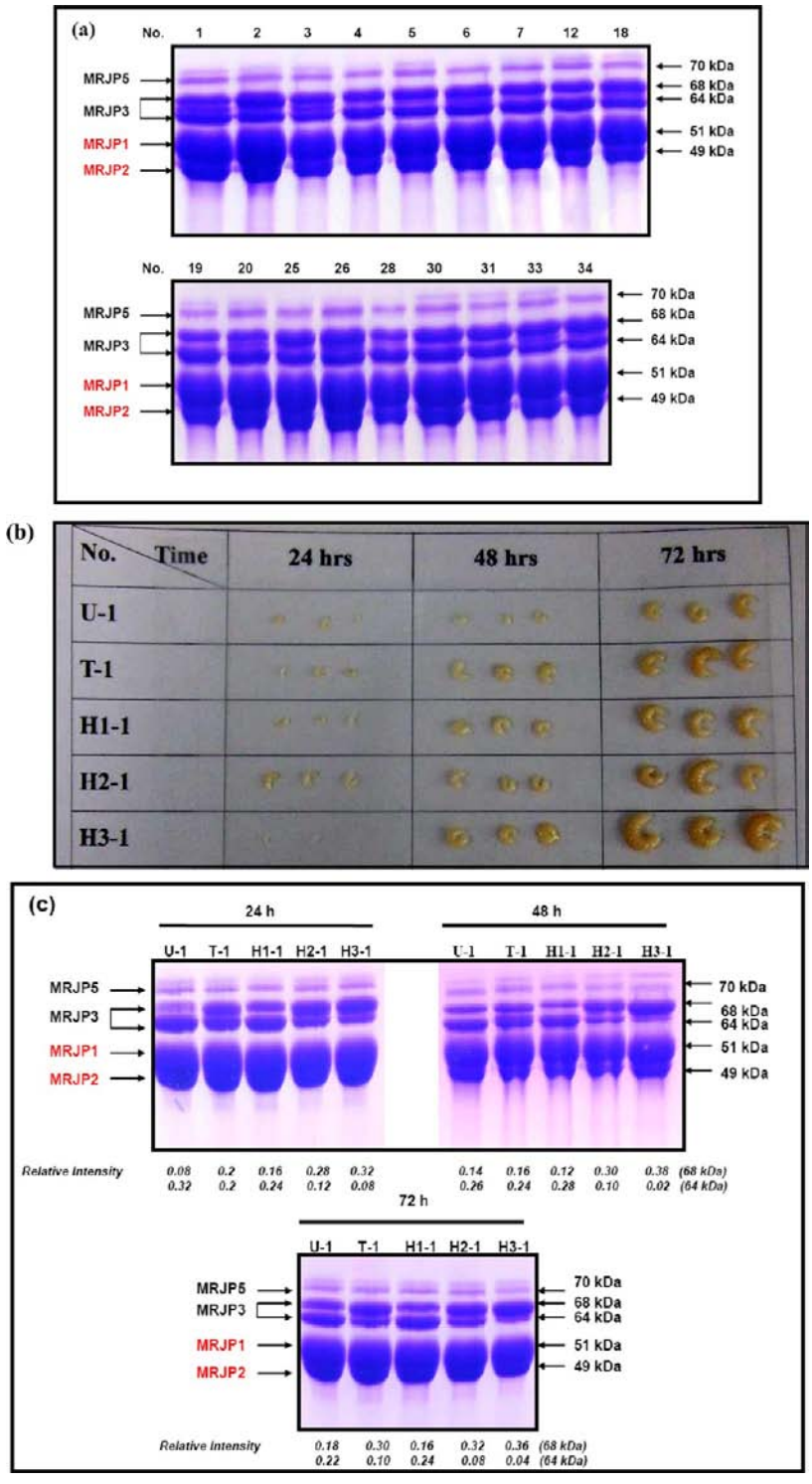

Figure 4. Effects of NBM-HD-1 on queen bee larvae and RJ. (a) Ten colonies of bees in beehives with similar expression levels of the MRJP3 protein (3, 4, 5, 6, 7, 12, 18, 25, 26, and 30) were identified and selected for study in this experiment. Two beehives each were tested in each control and treatment group. (b) Young adult worker bees fed NBM-HD-1 secreted altered RJ, which induced rapid growth of the larvae. The larvae were collected at 24, 48, and 72 h. U-1, control group; T-1, treated with 2.5 g/kg TGP extract; H1-1, treated with 50 mg/kg NBM-HD-1; H2-1, treated with 100 mg/kg NBM-HD-1; H3-1, treated with 200 mg/kg NBM-HD-1. (c) Samples of RJ were collected at different time points and different groups. U-1, control group; T-1, 2.5 g/kg TGP extract; H1-1, 50 mg/kg NBM-HD-1; H2-1, 100 mg/kg NBM-HD-1; H3-1, 200 mg/kg NBM-HD-1. Details are given under Materials and Methods.

normal RJ (from the control group, U-1). From 24 to 72 h, NBM-HD-1 significantly increased the level of protein expression of 68 kDa MRJP3 and decreased that of 64 kDa MRJP3 (Figure 4c). During the same period, levels of MRJP1, MRJP2, and MRJP5 did not change significantly. *Mrjp3* possesses highly polymorphic properties. The data suggest that NBM-HD-1 markedly induced a ratio change in 68 and 64 kDa MRJP3 proteins (Figure 4c). Neither changes in the ratio

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Table 2. Weight of Larvae Fed RJ Secreted by Young Adult Worker Bees Fed SAHA (n = 12)

group	av wt of each larva (mg)		
	at 24 h	at 48 h	at 72 h
control (U-1)	1.90 ± 0.3	13.3 ± 2.0	38.4 ± 4.6
TGP extract (T-1) (5.0 g/kg)	2.90 ± 0.4	24.1 ± 2.7	194.7 ± 29.0
NBM-HD-G (G-1)[a] (150 mg/kg)	3.6 ± 0.5	41.2 ± 4.8	178.6 ± 22.4
SAHA (S1-1) (5 mg/kg)	4.0 ± 0.5	28.2 ± 3.4	149.3 ± 17.8
SAHA (S2-1) (15 mg/kg)	7.3 ± 0.6	41.0 ± 5.5	149.2 ± 19.3

[a]A proprietary compound under investigation.

of other MRJPs nor new proteins were found. These data suggest that the change in the ratio of the different isoforms (68 and 64 kDa) of the MRJP3 proteins may account for the rapid growth of queen bee larvae, as demonstrated by the increase in the larval body weight. In short, feeding young adult worker bees with sugar water containing NBM-HD-1 resulted in the production of altered RJ, and the larvae fed on the altered RJ exhibited more rapid growth than controls.

Effects of SAHA on the Expression of the MRJP3 Protein and Larvae Growth. Next, SAHA was investigated to determine whether it would produce similar results. In 2006, the U.S. Food and Drug Administration (FDA), for the first time, approved SAHA, an HDACi, for the treatment of cancer.[19] For this reason, SAHA was tested on the growth of larvae. Table 2 and Figure 5a show the growth results after treatment with formulated sugar water containing 5 or 15 mg/kg SAHA. A pure active ingredient, termed NBM-HD-G, was used as another positive control. NBM-HD-G was purified from Taiwanese green propolis (Table 2). From 48 to 72 h, the larvae of the control group grew from 13.3 to 38.4 mg, a weight increase of approximately 1.89-fold. The larvae of the group treated with formulated sugar water containing 15 mg/kg SAHA grew from 41.0 to 149.2 mg, a weight increase of approximately 2.64-fold. SAHA, at a concentration of 5 mg/kg, significantly promoted the growth of queen bee larvae. Both NBM-HD-1 and SAHA showed similar results. However, SAHA displayed more potent activity than NBM-HD-1. SAHA is one of the most promising HDACis, inhibiting several HDACs such as HDAC1, HDAC2, HDAC3, HDAC6, HDAC8, and HDAC10 in nanomolar ranges.[23] These data suggest that HDACis can regulate epigenetic processes that cause RJ protein composition to change and indirectly promote the growth of queen bee larvae. MRJP3 protein expression in RJ was also analyzed. Figure 5b shows that SAHA and the positive control markedly up-regulated 68 kDa MRJP3 protein expression in comparison with the control group. In contrast, the MRJP3 (64 kDa) protein significantly decreased in

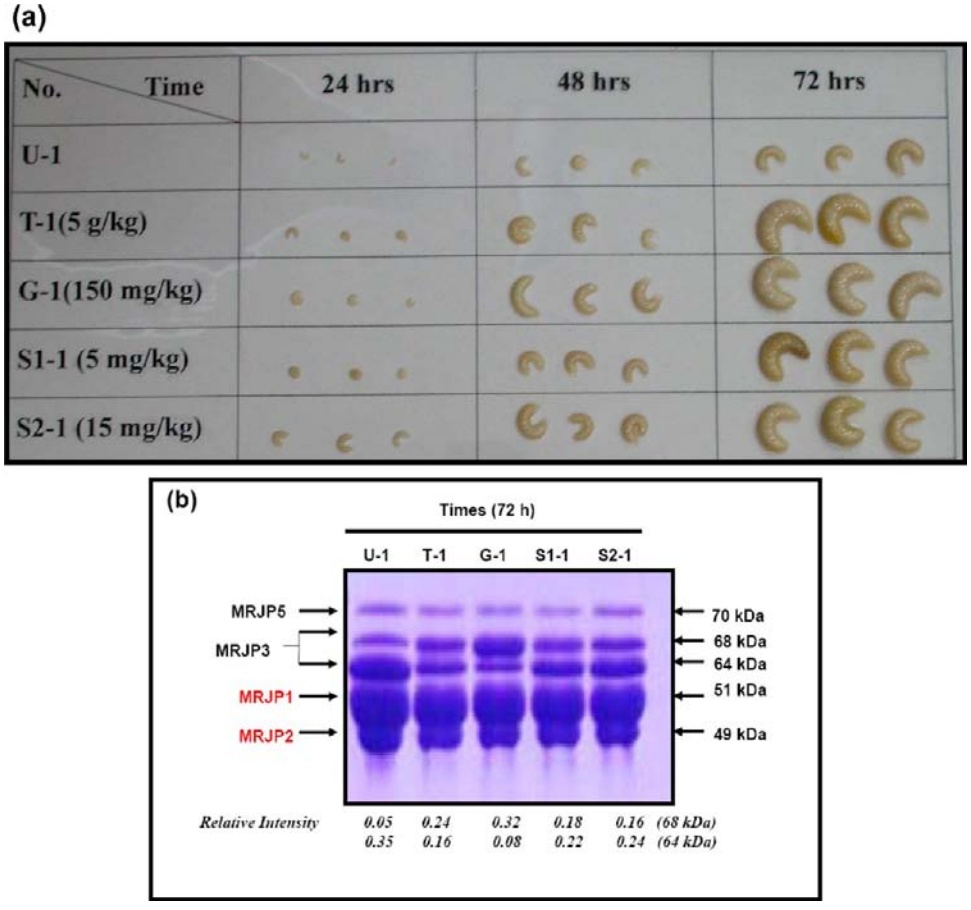

Figure 5. Effects of SAHA on queen bee larvae and RJ. (a) Young adult worker bees fed SAHA secreted altered RJ, which induced rapid growth of the larvae. The larvae were collected at different time points at 24, 48, and 72 h. U-1, control group; T-1 (TGP extract), treated with 5.0 g/kg TGP extract; G-1, treated with 150 mg/kg NBM-HD-G; S1-1, treated with 5 mg/kg SAHA; S2-1, treated with 15 mg/kg SAHA. (b) Samples were collected at 72 h. U-1, control group; T-1, 5.0 g/kg TGP extract; G-1, 150 mg/kg NBM-HD-G; S1-1, 5 mg/kg SAHA; S2-1, 15 mg/kg SAHA. Details are given under Materials and Methods.

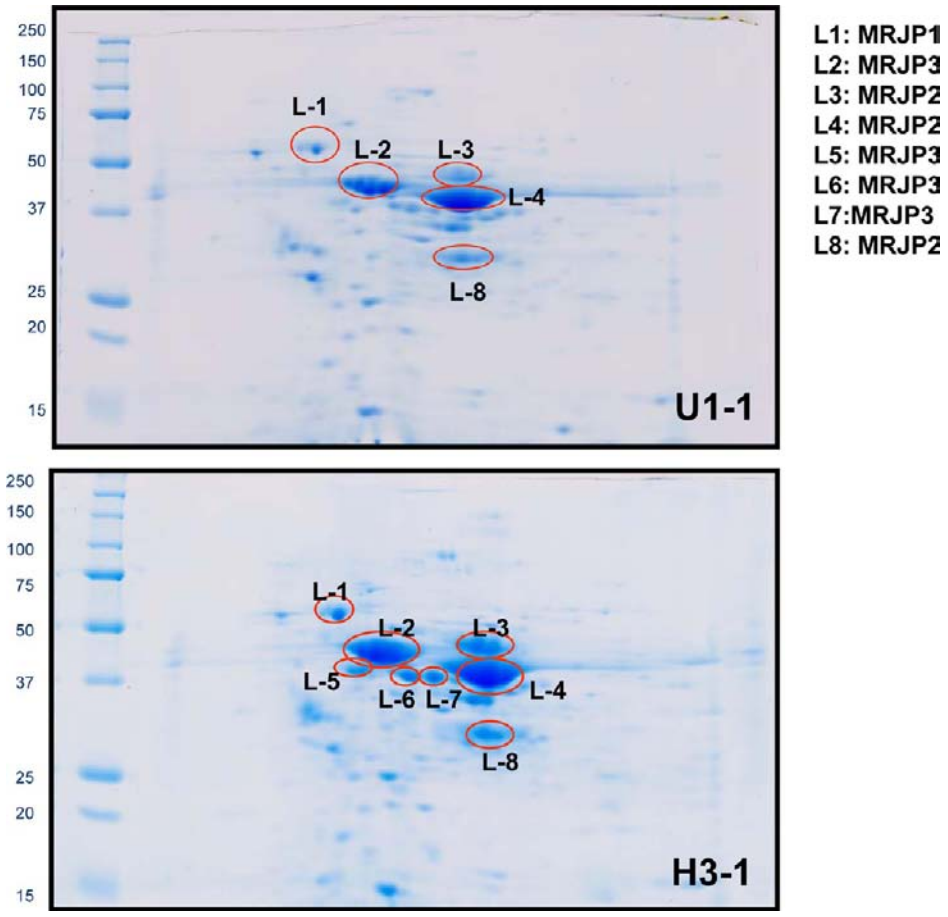

Figure 6. Effects of NBM-HD-1 on MRJP content in the extra large larvae of the queen bee. 2-D gel electrophoresis and mass spectrometric analysis of the water-soluble proteins after treatment with or without NBM-HD-1 (H3-1, 200 mg/kg) revealed an increase in the protein level of MRJP1,MRJP2, and MRJP3 (especially MRJP3) in the larvae from the treated group. L-1, MRJP1; L-2, MRJP3; L-3, MRJP2; L-4, MRJP2; L-5, MRJP3; L-6, MRJP3; L-7, MRJP3; L-8, MRJP2; U1-1, control group; H3-1, treated with 200 mg/kg NBM-HD-1.

comparison with the control group. MRJP1, MRJP2, and MRJP5 showed no significant change.

Effects of NBM-HD-1 on MRJP Content in the Extra Large Larvae of the Queen Bee. 2-D gel electrophoresis and mass spectrometric analysis of the water-soluble proteins expressed after treatment with NBM-HD-1 (H3-1, 200 mg/kg) revealed an increase in the protein levels of MRJP1, MRJP2, and MRJP3 (especially MRJP3) in the larvae from the treated group (Figure 6). These results suggest that NBM-HD-1 induces an increase in the body weight of the queen bee larvae as well as increases the expression of MRJP1, MRJP2, and MRJP3 in these larvae.

DISCUSSION

We first studied NBM-HD-1 for its anticancer activities and found it to have many features of HDACis.[26] Our laboratory also studied the effect of TGP on the health of bee colonies. We noticed that young adult worker bees fed both NBM-HD-1 and TGP extract could raise queen bee larvae to an exceptionally large size (Figures 4 and 5). We suspected that the effect might come from HDAC inhibition because we also found TGP to have many features of HDACis (unpublished results). We then tested our hypothesis by using the known HDACi, SAHA, and discovered it had the same effect. The experiments were carried out in the field. Due to the fragility and rapid growth of the larvae, starting all of the experiments with larvae close to the same size and weight was difficult. However, the magnitude of the size increase in the treated group compared to the control was noticeable. The same phenomena were seen in their developing stages (prepupa to mature queen bee, data not shown), which minimized the impact of the variation of early-phase larvae size. In this study, we observed that, although the ratio of 68 and 64 kDa MRJP3 proteins was highest in the group treated with the highest doses, the size of larvae did not proportionally increase. Thus, an optimal dose range had probably been achieved (Figure 5).

Currently, HDACis are divided into several classes on the basis of their core chemical structures:[24] (a) short-chain fatty acids (sodium butyrate, sodium phenyl butyrate, and valproic acid); (b) cyclic tetrapeptides (trapoxin, FK-228, and apicidin); (c) hydroxamic acids (TSA, SAHA, and LAQ-824); and (d) benzamides (MS-275). Because of its structure, NBM-HD-1 cannot be classified into any of these four classes. Therefore, it is considered to be a novel HDACi.

MRJP1 has been proven to have a specific role in triggering queen bee larvae development.[9] However, the MRJP3 protein has not been studied with regard to its specific capacity to trigger queen bee larvae growth and development. In our present study, the MRJP1 protein level did not change much. We therefore believe that MRJP1 in RJ may be required simply to maintain basic growth for queen bee larvae, whereas MRJP3 may play a more important role in enhancing the growth rate

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and size of queen bee larvae. MRJP3 protein possesses highly polymorphic properties. Currently, several isoforms have been identified. In our studies, we used different classes of DNA methyltransferase inhibitors (DMTis) or HDAC inhibitors (HDACis) to test their effects on gene expression. Our study results demonstrated that some inhibitors to these enzymes enhance the growth of queen bee larvae. We further found that there is a significant change in the ratios of MRJP3 proteins in the RJ that the larvae feed on. We propose that this change may indeed be caused by the effect of these inhibitors on gene expression. There are other factors such as MRJP1 affecting the growth of queen bee larvae; however, we were surprised to find the magnitude of the enhanced growth of queen bee larvae and the resulting size of queen bees by the inhibitors to DMT or HDAC. At this time we are unsure if this change of ratio was the only factor in influencing the queen bee larvae's rapid growth. It would be interesting to study the individual effect of each isoforms of MRJP3 on queen bee larva development and growth or whether they have other functions. To identity whether different isoforms of MRJP3 protein have different biological functions, specific MRJP3 antibodies must be developed and produced. This may be our next study interest.

CCD has become a pervasive global problem. In this study, we have shown that HDACis can regulate the protein composition of RJ, which in turn stimulates the growth of queen bee larvae. An exceptionally large queen bee can be generated using this method. This extra large queen bee may have enhanced reproductive capacity, which could quickly replenish depleted bee colonies. Our future studies will focus on characterizing the filial generation of worker bees and evaluating the reproductive capacity of the extra large queen bee. Such findings have the potential for significantly alleviating the severe effects of CCD.

In conclusion, this is the first report suggesting that HDACis are capable of affecting chromatin remodeling in the hypopharyngeal and mandibular glands of young adult worker bees, resulting in the selective altered expression of the 68 and 64 kDa MRJP3 proteins. Changes in the ratio of 68 and 64 kDa MRJP proteins were identified in each enlarged larva. This indicates that HDACis can induce alternative splicing of polymorphic *mrjp3*, which in turn changes the expression of the 68 and 64 kDa isoforms. This change in expression, which results in the altered protein content of the RJ, is most likely what promotes the increase in growth during queen bee development.

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Notes

The authors declare the following competing financial interest(s):NatureWise Biotech & Medicals Corporation is the developer and holds patents on the NBM-HD-1 and its derivative compounds in several countries. None of the authors has financial interest in this study.

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