DOI 10.1002/mnfr.200700155 Mol. Nutr. Food Res. 2007, *51*, 1472 – 1477

Research Article

The anti-invasive effect of lucidenic acids isolated from a new *Ganoderma lucidum* strain

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Ganoderma lucidum is a well-known mushroom with various pharmacological effects that has been used for health and longevity purposes. The objective of this study was to investigate the anti-invasive effect of lucidenic acids isolated from a new *G. lucidum* strain (YK-02) against human hepatoma carcinoma ($HepG_2$) cells. Triterpenoid components in the ethanol extract of *G. lucidum* (YK-02) were separated by means of a semi-preparative RP HPLC. Four major peaks were separated and crystallized from triterpenoids fraction, and were identified as lucidenic acids A, B, C, and N according to their spectroscopic values of ^1H NMR and MS. Treatment of the lucidenic acids (50 μM) in the presence of 200 nM phorbol 12-myristate 13-acetate (PMA) after 24 h of incubation all resulted in significant inhibitory effects on PMA-induced MMP-9 activity and invasion of $HepG_2$ cells. The results indicate that the lucidenic acids isolated from *G. lucidum* (YK-02) are anti-invasive bioactive components on hepatoma cells.

Keywords: Anti-invasion / *Ganoderma lucidum* / $HepG_2$ cells / Lucidenic acid / MMP-9 activity

Received: April 23, 2007; revised: May 27, 2007; accepted: June 3, 2007

1 Introduction

Cancer metastasis refers to the spread of cancer cells from the primary neoplasm to distant sites and the growth of secondary tumors at sites distant from a primary tumor. Metastasis occurs through a complex multistep process consisting of invasion into the circulation from a primary tumor, immigration to distant organs, adhesion to endothelial cells, and infiltration into the tissue. Metastasis is responsible for the majority of failures in cancer treatment, and is the major cause of death in various cancer patients [1]. When cancer is diagnosed, additional chemotherapy may be recommended to prevent a local recurrence of primary tumor and spread of tumor cells. However, severe side effects may be induced at effective therapeutic doses. Therefore, in addition to minimize the growth of existing tumors, treatments that limit their spread to new sites and blockade their invasion have been considered to enhance survival of cancer patient [2, 3].

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Abbreviations: **GLE**, *Ganoderma lucidum* extract; **LA**, lucidenic acid; **MMP**, matrix metalloproteinase; **MTT**, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; **PMA**, phorbol 12-myristate 13-acetate

Ganoderma lucidum, a well-known mushroom with pharmaceutical effects, has been used in China for thousands of years for health and longevity purposes. A number of pharmacological effects of the dried powder and aqueous/ethanol extracts of *G. lucidum* are used for preventing or treating various types of human diseases such as allergy, bronchitis, hyperglycemia, inflammation, nephritis, hepatopathy, arthritis, hypertension, neurasthenia, and chronic hepatitis [4–6]. *G. lucidum* is also used to reduce the likelihood of invasion and metastasis, and prevent occurrence or reoccurrence of various types of cancer [4, 7]. Different compounds with various pharmacological activities were extracted from mycelia, fruiting bodies, or spores of *G. lucidum*. Nowadays, studies on fungus have demonstrated the presence of many interesting biologically active compounds such as polysaccharides and triterpenoids, especially triterpenoids in *Ganoderma* species [7–9]; some of them are linked to possible therapeutic effects. Although many biological activities of triterpenoids fraction in *G. lucidum* have been studied, the types of bioactive triterpenoids still have not been fully identified. Due to the difficulties of isolation and purification as well as low extraction yields, literatures regarding the identification of bioactive triterpenoids and pharmaceutical effect of individual triterpenoid component of *G. lucidum* are limited. Therefore, the purposes of this study were to isolate and identify the triter-

penoid components from the fruiting body of a new *G. lucidum* (YK-02) strain, and to test their anti-invasive abilities against human hepatoma carcinoma ($HepG_2$) cell line.

2 Materials and methods

2.1 Materials

The *G. lucidum* strain (YK-02), which was preserved in Mycelium Center of Biotechnology R & D Institute (Shnang Hor Group, Tainan Hsien, Taiwan), was grown to fruiting body according to the report of Chen *et al*. [10]. It has been identified by Dr. Shen-Hwa Wu (National Museum of Natural Science, Taiwan), using morphology characterization and DNA profiles. Phorbol 12-myristate 13-acetate (PMA) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma Chemical (St. Louis, MO). Methanol, ethanol, acetone, ethyl acetate, ACN (HPLC grade), and DMSO were purchased from Tedia Co. (Fairfield, OH). DMEM was purchased from HyClone (Logan, UT). Fetal bovine serum (FBS) was purchased from Gibco BRL (Grand Island, NY).

2.2 Preparation of *G. lucidum* (YK-02) extracts

Dried fruit body of *G. lucidum* (YK-02) were ground into powders that were extracted with ethanol (1:10, w/v) in a shaking incubator at room temperature for 8 h. After cooling, the extracts were filtered and the filtrate was evaporated to dryness under vacuum using a rotary evaporator, and then stored at $-20^{\circ}C$ until use.

2.3 Preparation of crude triterpenoids from *G. lucidum* (YK-02)

Crude triterpenoids of *G. lucidum* (YK-02) were separated according to the method of Chen *et al*. [10]. Briefly, *G. lucidum* (YK-02) powder was extracted with 50% ethanol for 1 h. The mixture was filtered, and the filtrate was evaporated to dryness with a rotary evaporator. The residue was extracted with $CHCl_3/H_2O$ (1:1, v/v) three times. The $CHCl_3$ layer was further extracted with saturated $NaHCO_3$ three times, and the alkali solution was collected and acidified with 6N HCl to a pH of 3–4. The solution was then extracted with ethyl acetate three times. After the solvent was evaporated off, the residue was dried in an oven to yield an acidic-ethyl acetate-soluble material containing fraction (crude triterpenoids).

2.4 Isolation of lucidenic acids from crude triterpenoids of *G. lucidum* (YK-02)

Isolation of lucidenic acids from the ethanol solution of crude triterpenoids was performed according to the method of Chen *et al*. [10]. Briefly, 2 g of crude triterpenoids were dissolved in 5 mL of 95% ethanol. Then, the sample was subjected to semi-preparative reverse-phase HPLC (Hitachi, Tokyo, Japan), consisting of a model L-6050 pump, a model D-2500 integrator, and a diode array detector (Hitachi, L-4200 model) set at 252 nm in one injection. A 250×25 mm id, 7 μM, Lichrosorb RP-18 column (Merck, Darmstadt, Germany) was used for separation at a flow rate of 7.8 mL/min. The elution solvents were (A) ACN (CH_3CN) and (B) 2% acetic acid (CH_3COOH). The solvent gradient program used was 0–5 min, 100% A; 5–10 min, 80% A; 10–20 min, 70% A; 20–30 min, 50% A; 30–40 min, 40% A; 40–50 min, 20% A; 50–70 min, 100% B. A monitoring UV detector (model L-4200) was set at 252 nm. Seven major peaks were obtained by this separation condition. The seven peaks were collected and concentrated in a rotary evaporator, and were stood for 4 to 8 days for crystallization. Only the compounds of peaks 1, 2, 5, and 6 were isolated as transparent needle crystals, which were collected and weighted to determine yields and further identified by means of spectrometry.

2.5 Spectrometry

The UV-vis absorption spectra of active compounds in methanol were recorded by a Hitachi UV-3210 spectrometer. The mass spectra of different components were obtained using the EI-MS mode at 30 eV with a VG 70-250S mass spectrometer (VG. Analytical, Manchester, UK). The temperature was raised by steps of $30^{\circ}C$/min from 60 to $240^{\circ}C$. The nuclear magnetic resonance (NMR) spectra were measured in $CDCl_3$ and methanol-*d*4 with a Bruker AMX-500 FT-NMR spectrometer (Billerica, MA, USA) operating at 500.13 MHz for ^1H NMR. The sweep width, pulse angle, repetition delay, and acquisition time for ^1H NMR were 6510.417 Hz, 9.20 μs, 6.50 μs and 1.26 s, respectively. The chemical shifts are reported as δ values in parts per million (ppm) from tetramethylsilane.

The ^1H NMR spectrum of compound 1 (3,7,12-Trihydroxy-4,4,14-trimethyl-11,15-dioxo-5-chol-8-en-24-oic acid) showed the following chemical shifts: 4.78 (1H, dd, J = 8.7, 8.8 Hz, H-7), 4.37 (1H, s, H-12), 3.22 (1H, dd, J = 5.0, 10.7 Hz, H-3), 0.80 (3H, s, H-18), 0.87 (3H, s, H-27), 1.04(3H, s, H-26), 1.14 (3H, d, J = 6.7 Hz, H-21), 1.31 (3H, s, H-19), 1.44 (3H, s, H-25). The EI-MS of compound 1 gave an M^+ ion peak at 476, suggesting a molecular weight of 476. EI-MS, *m/z* 476 (calculated for $C_{27}H_{40}O_7 = 476$).

The ^1H NMR spectrum of compound 2 (3,7-Dihydroxy-4,4,14-trimethyl-11,15-dioxo-5-chol-8-en-24-oic acid) showed the following chemical shifts: 4.80 (1H, dd, J = 8.6, 9.0 Hz, H-7), 3.21 (1H, dd, J = 5.4, 10.8 Hz H-3), 0.86 (3H, s, H-18), 0.98 (3H, d, J = 6.3 Hz, H-21), 0.98 (3H, s, H-27), 1.04 (3H, s, H-26), 1.22 (3H, s, H-19), 1.35 (3H, s, H-25). The EI-MS of compound 2 gave an M^+ ion peak at 460, suggesting a molecular weight of 460. EI-MS, *m/z* 460 (calculated for $C_{27}H_{40}O_6 = 460$).

The ^{1}H NMR spectrum of compound 5 (7,12-Dihydroxy-4,4,14-trimethyl-3,11,15-trioxo-5-chol-8-en-24-oic acid) showed the following chemical shifts: 4.83 (1H, dd, J = 8.7, 8.6 Hz, H-7), 4.37 (1H, s, H-12), 0.83 (3H, s, H-18), 1.13 (3H, s, H-27), 1.14 (3H, d, J = 6.6 Hz, H-21), 1.16 (3H, s, H-26), 1.44 (3H, s, H-19), 1.44 (3H, s, H-25). The EI-MS of compound 5 gave an M^+ ion peak at 474, suggesting a molecular weight of 474. EI-MS, *m/z* 474 (calculated for $C_{27}H_{38}O_7 = 474$).

The ^{1}H NMR spectrum of compound 6 (7-Hydroxy-4,4,14-trimethyl-3,11,15-trioxo-5-chol-8-en-24-oic acid) showed the following chemical shifts: 4.85 (1H, dd, J = 8.0, 9.0 Hz, H-7), 0.98 (3H, d, J = 6.4 Hz, H-21), 1.00 (3H, s, H-18), 1.11 (3H, s, H-27), 1.13 (3H, s, H-26), 1.26 (3H, s, H-19), 1.34 (3H, s, H-25). The EI-MS of compound 6 gave an M^+ ion peak at 458, suggesting a molecular weight of 458. EI-MS, *m/z* 458 (calculated for $C_{27}H_{38}O_6$: 458).

2.6 Cell culture

Human hepatoma cells ($HepG_2$ cells) were obtained from the Bioresource Collection and Research Center (BCRC, Food Industry Research and Development Institute, Hsin Chu, Taiwan). Cells were grown in DMEM, supplemented with 10% v/v FBS, 100 units/mL penicillin, 100 μg/mL streptomycin, 0.37% w/v $NaHCO_3$, 0.1 mM NEAA (nonessential amino acid), and 1 mM sodium pyruvate at 37°C, in a humidified atmosphere of 95% air and 5% CO_2. In the invasive and metastatic experiments, the cells were cultured in serum-free medium.

2.7 Cell viability assay

The cell viability was determined with the MTT assay. $HepG_2$ cells were seeded onto 96-well plates at a concentration of 1×10^6 cells/well in DMEM without FBS. After 24 h of incubation, the cells were treated with various concentrations of lucidenic acids in 0.1% DMSO for further incubation. The controls were treated with 0.1% DMSO alone. The dye solution (10 μL; 5 mg/mL PBS), specific for the MTT assay, was added to each well for an additional 60 min of incubation at 37°C. After the addition of DMSO (100 μL/well), the reaction solution was stood for 30 min in dark. The absorbance at 570 nm (formation of formazan) and 630 nm (reference) were recorded with a Fluostar Galaxy plate reader. The percent viability of the treated cells was calculated as follows:

$$(A_{570\,nm} - A_{630\,nm})_{sample} / (A_{570\,nm} - A_{630\,nm})_{control} \times 100.$$

2.8 Gelatin zymography

$HepG_2$ cells were incubated in serum-free DMEM with or without lucidenic acids in the presence of indicated PMA for a given time and the conditioned medium were collected as samples. The samples were separated by electrophoresis on 8% SDS/polyacrylamide gels containing 0.1% gelatin. After electrophoresis, the gels were washed twice in washing buffer (2.5% v/v Triton X-100 in dH_2O) for 30 min at room temperature to remove SDS, then incubated in reaction buffer (10 mM $CaCl_2$, 0.01% NaN_3, and 40 mM Tris-HCl, pH 8.0) at 37°C for 12 h to allow proteolysis of the gelatin substrate. Bands corresponding to activity were visualized by negative staining using CBB R-250 (Bio-Rad, Richmond, CA) and molecular weights were estimated by reference to pre-stained SDS-PAGE markers.

2.9 Cell invasion assay

Cell invasion assay was performed according to the method of Repesh [11]. $HepG_2$ cells to be tested for invasion were detached from the tissue culture plates, washed with PBS buffer, and resuspended in serum-free DMEM medium (5×10^4 cells/200 μL) in the presence or absence of drugs (PMA and lucidenic acids), and then seeded onto the upper chamber of matrigel-coated filter inserts (8-μm pore size) purchased from Becton Dickinson (Franklin Lakes, NJ, USA). Serum-free DMEM medium (500 μL) was added to the lower chamber. After 24 h of incubation, filter inserts were removed from the wells and the cells on the upper surface of the filter were wiped with a cotton swab. Filters were fixed for 10 min with methanol and stained with Giemsa dye, and then the cells that invaded the lower surface of the filter were counted under a microscope.

2.10 Protein content determination

The protein content was determined by the method of Bradford [12] with BSA as a standard.

2.11 Statistical analysis

Data are indicated as mean ± SD of three different determinations. Differences between variants were analyzed by the Student's *t*-test for unpaired data. Values of $p < 0.05$ were regarded as statistically significant.

3 Results and discussion

3.1 Isolation and identification of triterpenoids from *G. lucidum* (YK-02)

To isolate and identify the active components of *G. lucidum* (YK-02), triterpenoid components in the ethanol extract was analyzed and separated by means of an RP HPLC. After the extraction and purification processes, 31.4 g of crude triterpenoids was obtained from 1 kg of *G. lucidum* (YK-02) (extraction yield = 3.1 g/100 g). Of crude triterpenoids dissolved in 5 mL of 95% ethanol 2 g was purified and separated by a semi-preparative HPLC. The results

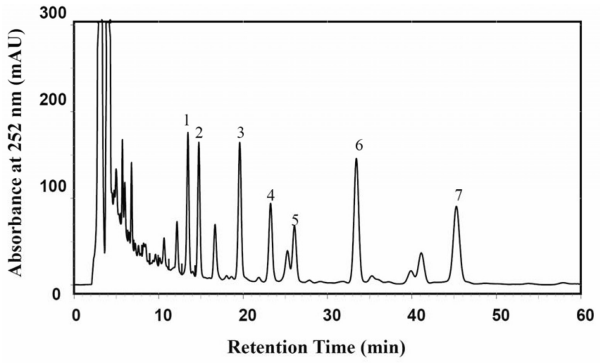
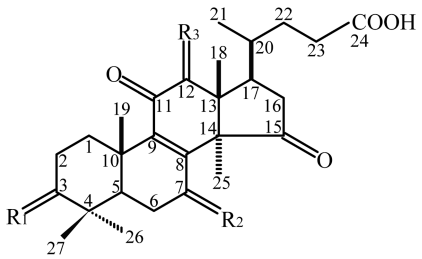


Figure 1. HPLC profile of crude triterpenoids separated from *G. lucidum* (YK-02). The retention times of seven major peaks are 13.48, 14.73, 19.62, 23.27, 26.10, 33.43, and 45.26 min.



Lucidenic acid A	R_1=O, R_2=β-OH, R_3=H
Lucidenic acid B	R_1=O, R_2=R_3=β-OH
Lucidenic acid C	R_1=R_2=R_3=β-OH
Lucidenic acid N	R_1=R_2=β-OH, R_3=H

Figure 2. Structures of lucidenic acids A, B, C, and N.

showed that there are seven major peaks in the HPLC profile with retention times of 13.48, 14.73, 19.62, 23.27, 26.10, 33.43, and 45.26 min, respectively (Fig. 1). The seven peak fractions were collected, concentrated in a rotary evaporator and stood for 4 to 8 days for crystallization. Only the compounds of peaks 1, 2, 5, and 6 were isolated as transparent needle crystals and the yields were 2.1, 1.2, 3.3, and 7.8 g/100 g, respectively. The crystals of compounds 1, 2, 5, and 6 were collected and further identified by comparing their ^{1}H NMR and MS data with those of the published data [13, 14]. The spectroscopic properties obtained from compounds 1, 2, 5, and 6 were consistent with lucidenic acids C, N, B, and A reported in the literatures, respectively. Hence, the four triterpenoids isolated from *G. lucidum* (YK-02) were identified as lucidenic acids A, B, C, and N. The chemical structures of lucidenic acids A, B, C, and N are shown in Fig. 2.

The extraction yields of lucidenic acids separated from 1 kg *G. lucidum* (YK-02) (or 31.4 g crude triterpenoids) were in an order of lucidenic acid A (2449 mg) > lucidenic acid B (1021 mg) > lucidenic acid C (659 mg) > lucidenic acid N (376 mg). Based on the reports of Nishitoba *et al.* [13] and Wu *et al.* [14], 68 g crude triterpenoids were separated from 6 kg dried fruiting body of common *G. lucidum* (yield =1.13 g/100 g) and three triterpenoids, including lucidenic acids A (241 mg), C (5 mg), and N (23 mg) were obtained from 603 g air-dried fruiting body of common *G. lucidum*. In the present study, both crude triterpenoids and lucidenic acids extracted from *G. lucidum* (YK-02) were three and ten times, respectively, higher than those of common *G. lucidum.* The results revealed that *G. lucidum* (YK-02) is a new strain of *Ganoderma* with high content of triterpenoids and lucidenic acids. The lucidenic acids isolated from *G. lucidum* (YK-02) were used for the following investigation.

3.2 Lucidenic acids A, B, C, and N reduce PMA-induced MMP-9 activity as well as the invasion of $HepG_2$ cells

Lucidenic acids isolated from the fruiting body of *G. lucidum* are known to possess cytotoxicity on tumor cell lines [14]. In the present study, we first determined the cytotoxicities of the lucidenic acids A, B, C, and N on $HepG_2$ cells by a MTT assay. These four lucidenic acids were treated on $HepG_2$ cells at various concentrations (0, 25, 50, or 100 μM) for 24 h, and did not exhibit any significant cytotoxicities (Table 1). Therefore, the dose of lucidenic acids and time of treatment were used for the following MMP activities and invasion assays. In gelatin zymograhpy, we found that untreated $HepG_2$ cells were originally expressed MMP-2 only, and the invasive activity was not affected by lucidenic acids treatment (data not shown). As PMA is a well-known selective activator of a tumor promoter, MMP induction by PMA is proved in various cell types. $HepG_2$ cells were then incubated in a serum-free medium containing 200 nM PMA with or without lucidenic acids A, B, C, or N for 24 h, and the MMP-9 and MMP-2 activities of $HepG_2$ cells were determined by gelatin zymography. The results showed that $HepG_2$ cells were stimulated to express MMP-9 by PMA, and lucidenic acids A, B, C, or N sup-

Table 1. Effects of lucidenic acids A (LAA), B (LAB), C (LAC), and N (LAN) on viability of $HepG_2$ cells

Concentration (μM)[a)]	Cell viability (%)			
	LAA	LAB	LAC	LAN
0	100 ± 4	100 ± 5	100 ± 2	100 ± 5
25	102 ± 4	99 ± 4	99 ± 5	104 ± 6
50	99 ± 5	100 ± 6	96 ± 5	98 ± 4
75	98 ± 1	97 ± 6	93 ± 4	99 ± 5
100	87 ± 6	88 ± 4	90 ± 6	92 ± 6

a) Cells were incubated in a medium containing various concentrations (0, 25, 50, 75, or 100 μM) of lucidenic acids for 24 h. Viability of cells was analyzed against control (100%), and is presented as % of control. Data are expressed as mean ± SD from three independent experiments.

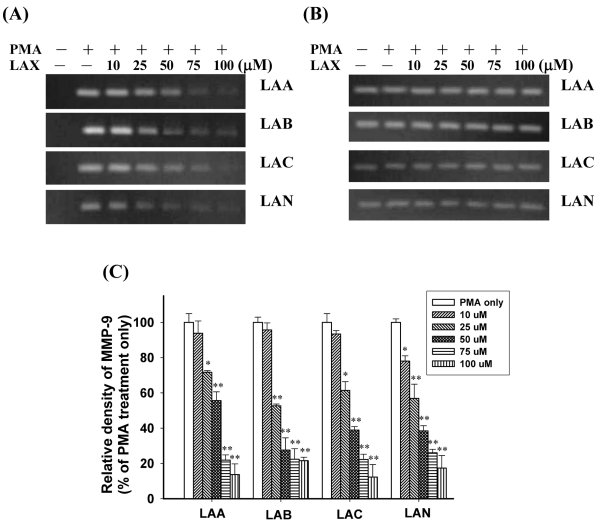


Figure 3. Effects of lucidenic acids A, B, C, and N on the activities of PMA-induced MMP-9 and MMP-2 in $HepG_2$ cells. $HepG_2$ cells were treated with 200 nM PMA and lucidenic acids A (LAA), B (LAB), C (LAC) or N (LAN) in various concentrations (0, 10, 25, 50, 75, or 100 μM) for 24 h. MMP-9 (A) and MMP-2 (B) activities of $HepG_2$ cells were determined by gelatin zymography. The bar graph (C) represents the relative density of the band obtained from gelatin zymography by densitometry. Data are the mean ± SD of three independent experiments. Results were statistically analyzed with Student's t-test (*p <0.05; **p < 0.01).

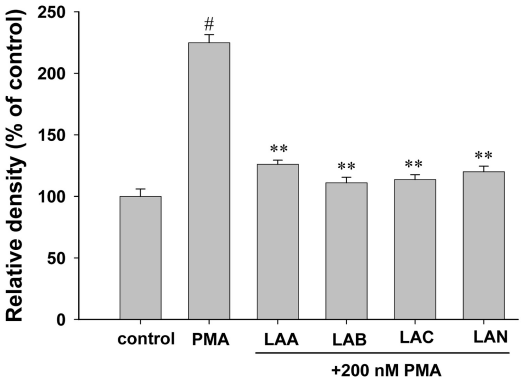


Figure 4. Effects of lucidenic acids A, B, C, and N on PMA-induced invasion of $HepG_2$ cells. A Matrigel invasion assay was performed with 50 μM of lucidenic acids A, B, C, or N in the presence of 200 nM PMA, as described in Section 2. $HepG_2$ cells that treated without PMA and lucidenic acids were used as the control. After 24 h of incubation, the total number of cells that invaded the lower surface of the filter was counted. Data represent mean ± SD of three independent experiments. Results were statistically analyzed with Student's *t*-test (# p < 0.01 compared with the control; **p < 0.01 compared with the PMA treatment only).

pressed PMA-induced MMP-9 activity in a dose-dependent manner (Figs. 3A and C). Interestingly, MMP-2 activity in $HepG_2$ cells was not inhibited by the lucidenic acids A, B, C, or N treatment (Fig. 3B). In a cell-invasion assay with matrigel-coated filter inserts, it was shown that the invasion of $HepG_2$ cells was significantly ($p < 0.05$) enhanced after a treatment with 200 nM PMA. The subsequent invasion experiment showed that the four lucidenic acids at a concentration of 50 μM after 24 h of incubation all resulted in significant ($p < 0.05$) inhibitory effects on PMA-induced invasion of $HepG_2$ cells (Fig. 4).

PMA is a well-known selective activator of protein kinase C (PKC) and a tumor promoter [15], and it can be used to induce matrix metalloproteinase (MMP)-9 expression [16, 17]. *G. lucidum* could exert anti-metastatic effects in various *in vivo* and *in vitro* models [18, 19]. The present results showed that PMA increased an invasion of $HepG_2$ cells, which meant that the invasion of $HepG_2$ cells could be promoted by environmental stimulation. Treatment of lucidenic acids A, B, C, and N for 24 h demonstrated that they did not induce cytotoxicity while they exerted an inhibitory effect on PMA-induced MMP-9 activity as well as an invasion of highly metastatic $HepG_2$ cells. MMP-2 and MMP-9 are the enzymes that can degrade type IV collagen, which is a major constituent of the basement membrane. Secretion of MMP-9 has been reported in lung, colon, and breast cancer [20] and MMP-2 expression is found on the highly metastatic human lung cancer cells, A549 cells [21]. Both of the MMP are presumed associating with the progression and invasion of different types of cancer cells. According to the present results, it was found that the lucidenic acids A, B, C, and N were the bioactive components in *G. lucidum* possessing anti-invasive activity on $HepG_2$ cells, and their anti-invasive activities might be through suppressing MMP-9 activity of $HepG_2$ cells. Further investigation of these lucidenic acids on the MMP expression in hepatoma cells is currently underway in our laboratory.

4 Concluding remarks

Many reports have indicated the pharmaceutical effects including anti-cancer, anti-metastasis, and anti-invasion of the aqueous/ethanol extracts of *Ganoderma* species [4, 22]. Fungal triterpenoids were found to be one of the biologically active compounds responsible for these pharmaceutical effects. Nevertheless, the individual bioactive ingredient of triterpenoids corresponding to the biological effects is still unclear. In this study, we have isolated lucidenic acids A, B, C, and N from a new strain of *G. lucidum* (YK-02), which possesses high level of lucidenic acids. Our results demonstrated that lucidenic acids A, B, C, and N are the bioactive components of *G. lucidum* possessing anti-invasive effects on hepatoma cells. Since the anti-invasive compounds of *G. lucidum* have been identified, further study is currently underway to investigate the molecular mechanism and signal pathway of anti-invasive effects of these lucidenic acids on hepatoma cell.

This research work was partially supported by the Council of Agriculture, Republic of China, under grant 96AS-3.1.3-FD-Z1(1).

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