A new anti-tumor cytotoxic triterpene from *Ganoderma lucidum*

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**ABSTRACT**

*Ganoderma lucidum* (*G. lucidum*) is a famous medicinal fungus used as a traditional medicine for generations in China. Among bioactivities that *G. lucidum* possesses, the anti-tumor effect has aroused extensive interests. In this study, one new triterpene (1) was isolated from 90% ethanol extract of the dried fruiting bodies of *G. lucidum*. Its structure was determined based on the analysis of its spectroscopic data, including 1D and 2D NMR and MS spectra. Furthermore, 1 elicited moderate anti-tumor activities (IC\(_{50}\) values of 15.38 ± 0.34 and 18.61 ± 0.55 μM for A549 and HepG2 cells, respectively) compared with cisplatin which was employed as the positive drug with IC\(_{50}\) values of 8.21 ± 0.17 and 5.36 ± 0.29 μM for A549 and HepG2 cells, respectively. The mechanism study by RT-qPCR and western blot analysis suggested that the p53/caspase-3 pathway is involved in the 1-induced apoptosis.

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1. Introduction

Lung and hepatic cancers are leading causes of death worldwide. Although the prevention of oncogenesis is the best strategy regarding cancers, the anti-cancer agents, especially targeted medicines that kills cancer cells, are still one of the main therapeutic methods for oncotherapy. Of note is that folk medicines and natural products...
have been reported to possess potent anti-tumor effects, especially for lung and hepatic cancers (Tong et al. 2018; Chanvorachote et al. 2016; Rawat et al. 2018). Therefore, exploring new natural anti-cancer agents from folk medicines, especially from those with anti-cancer effects, has been arousing extensive interests, for example, the famous commercial anti-cancer drug taxol, which was isolated from the bark of Taxus brevifolia in 1960s.

Ganoderma lucidum is a famous medicinal fungus and has been used as a traditional medicine in China for generations. The main effect of it is to treat physical frailty according to the theory of TCM (Zeng et al. 2019). Clinically, it is used in Asian countries for the treatment of various diseases, improving the quality of life for patients with chronic diseases. The polysaccharides from it have been reported to be able to treat cancers (Sohretoglu and Huang 2018; Jin et al. 2016; Unlu et al. 2016). Moreover, it is also reported to be capable of reversing multidrug resistance and eliciting chemopreventive effects on cancers (Cizmarikova 2017). Other pharmacological activities, such as immunomodulatory, anti-inflammatory, anti-atherosclerotic, analgesic, antiviral, hypolipidemic, antibacterial, anti-fibrotic, and hepatoprotective effects, are also reported for G. lucidum (Sanodiya et al. 2009; Wu et al. 2021). The basidiocarp, spores, and mycelia of G. lucidum produce over 400 active constituents, including triterpenoids, polysaccharides, sterols, nucleotides, and steroids, etc. (Sanodiya et al. 2009; Koo et al. 2019; Zhang et al. 2011; Ye et al. 2020). Recently, the extract which is rich in triterpenes from G. lucidum has been reported to display an anti-proliferative effect on lung cancer cells (Zolj et al. 2018; Li et al. 2013), suggesting that an investigation on triterpenes from G. lucidum might lead to the discovery of potent anti-cancer agents.

Therefore, in order to discover more anti-tumor agents from G. lucidum, a chemical study on fruiting bodies of G. lucidum was performed, leading to the isolation and identification of one anti-tumor triterpene. Herein, the isolation, structure

Figure 1. The structure of compound 1.
identification, \textit{in vitro} antitumor effects on A549 and HepG2 cells, and the underlying mechanism of the novel triterpene (1) (Figure 1) are reported.

2. Results and discussion

2.1. Structural identification

Compound 1 was obtained as a white powder, with a molecular formula C$_{33}$H$_{42}$O$_{9}$ as determined by the HR-ESI-MS that revealed a pseudo-molecular ion peak at $m/z$ 605.2721 [M + Na]$^+$ (calcd. 605.2727 for C$_{33}$H$_{42}$O$_{9}$Na) (Figure S1). $^1$H NMR spectrum of 1 gave six tertiary methyl signals at $\delta_H$ 0.85, 0.89, 1.06, 1.34, 1.75, and 2.13, one olefinic proton signal at $\delta_H$ 6.12, and one methoxyl group at $\delta_H$ 3.68 (Figure S2). The $^{13}$C NMR spectrum of 1 revealed the presence of six carbonyls at $\delta_C$ 176.4, 193.7, 198.0, 198.5, 204.7, 218.1 and one acetyl group at $\delta_C$ 21.4 and 171.5 (Figure S3). All the NMR data of 1 resembled those of the methyl ganoderate H (Kikuchi et al. 1985), except for the occurrence of one additional double bond ($\delta_C$ 126.2 and 154.2) and one carbonyl group ($\delta_C$ 218.1) (Figure S4). In the HMBC spectrum, correlations from H-28/29 to C-3 ($\delta_C$ 218.1) and from H-21 to C-20/22 were observed, leading to the determination of the location of the additional carbonyl group ($\delta_C$ 218.1) and the C-20/C-22 double bond (Figure S5). The correlations of H-29/H-19, H-12/H-30/H-17, and H-28/H-5 were detected in the NOESY spectrum of 1, which established the stereo-configurations of 1 (Figure S6). Moreover, $E$ geometry for the $\Delta^{20(22)}$ was also determined by NOESY, whereby interactions of H-22/H-16 and H-21/H-17 were observed. Therefore, the structure of 1 was determined as 12$\beta$-acetoxy-3,7,11,15,23-pentaoxolanosta-8,20E(22)-dien-26-oic acid methyl ester.

2.2. Cell viability assay

Cytotoxic effects of 1 on A549 and HepG2 cell lines were assessed. After 48-h treatment, 1 significantly suppressed A549 and HepG2 cell growth with IC$_{50}$ values of 15.38 $\pm$ 0.34 and 18.61 $\pm$ 0.55 $\mu$M, respectively (cisplatin was employed as the positive drug with IC$_{50}$ values of 8.21 $\pm$ 0.17 and 5.36 $\pm$ 0.29 $\mu$M for A549 and HepG2 cells, respectively) (Figure S7). Moreover, a Live/Dead cell assay was conducted to examine the cytotoxic effect of 1. 1 potently decreased the live-cell density and increased the density of dead cells at two indicated concentrations (5 and 15 $\mu$M) dose-dependently. Both A549 and HepG2 living cells significantly decreased at a concentration of 15 $\mu$M compared with the control group (Figure S8).

2.3. Apoptosis-induced by 1 in A549 and HepG2 cell lines

To explore the possible mechanism of the cytotoxicity of 1, the Hoechst dye 33258 was used to assess the apoptosis-inducing ability of 1. The results showed that compound 1 could cause A549 and HepG2 cell apoptosis at 5 $\mu$M and a potent apoptosis was observed both in A549 and HepG2 cell lines after the treatment of 1 for 24 h (Figure S9), suggesting that 1 could elicit significant cytotoxicity through the induction of apoptosis.
2.4. \textit{P53/caspase-3 pathway is involved in the apoptosis induced by 1}

To further understand the mechanism of apoptosis induced by 1, mRNA levels of p53 and expression levels of apoptosis-related proteins (Bcl-2, Bax, and caspase-3) were assessed in A549 cells. The results showed that 1 (15 \mu M) could increase the p53 gene expression (Figure S10A) and increase protein levels of proapoptotic bax and cleaved caspase-3 while decreasing anti-apoptotic Bcl-2 and procaspase-3 protein levels (Figure S10B). All these results suggested that p53/caspase-3 pathway was involved in the apoptosis caused by 1.

Triterpenoids are the main components in \textit{G. lucidum} indicated the various bioactivities such as anti-tumor, anti-inflammatory et al (Koo et al. 2019). Although many natural products derived from the herb have demonstrated potent anti-tumor activities on the cell lines of HL-60 and CA46 (Li et al. 2013; Wu et al. 2021), their mechanisms of action, to a certain extent, remain unclear. For example, recent studies have shown that non-coding RNAs and RNA-binding proteins are crucial in the pathogenesis of cancers. Therefore, studies of 1 on the modulatory effect on novel therapeutic targets for cancers, such as miRNAs, should be performed in the future.

Meanwhile, the traditional effects of \textit{G. lucidum} is to strengthen the human body according to the theory of TCM, which suggests that the cancer-preventive effects of components from \textit{G. lucidum} is another important direction of bioactive research. Therefore, the lung and hepatic cancer-preventive effects of 1 are also needed to be further assessed.

3. Experimental

3.1. General experimental procedures

The NMR spectra were measured on a Bruker AV-600 NMR spectrometer (BRUCK USA Inc., Houston, TX USA). A Thermo Scientific\textsuperscript{TM} Talos\textsuperscript{TM} F200C transmission electron microscopy (TEM, Thermo Fisher Scientific Inc., Waltham, MA USA) was used for the observation of cells. HR-MS analysis was performed on an Agilent 1260 UPLC system with 6550 Q-TOF MS. Apoptosis rate assay was evaluated by an Attune NxT flow cytometer (Thermo Fisher Scientific Inc., Waltham, MA USA). The 96-well plates were studied by Tecan Sunrise microplate reader (Tecan Trading AG, Switzerland). The chromatographic column for HPLC is YMC Pack ODS-A (10 \mu m, 250 \times 10 mm, YMC Co., Ltd, Kyoto, Japan).

3.2. Materials

\textit{G. Lucidum} were purchased from Benherb Biotechnology Co., Ltd., (Xi’an, China), and were identified by Dr. Zhongwen Yuan from The Third Affiliated Hospital of Guangzhou Medical University. A voucher specimen (GL20180013) has been deposited at the Department of Pharmacology, The Third Affiliated Hospital of Guangzhou Medical University. Silica gel (200-300 mesh) for column chromatography were from Marine Chemical Factory (Qingdao, China). All the organic reagents for the HPLC and analytic grade were from Yuwang Chemical Co., Ltd. (Shandong, China). All antibodies were purchased from Cell Signaling Technology, United States. A549 and HepG2 cells...
were purchased from Shanghai Institute of Cell Research, Chinese Academy of Sciences (Beijing, China) and stored at −80 °C.

3.3. Extraction and isolation

The dried fruiting bodies of *G. lucidum* (5 kg) were smashed by sieve of 50 mesh and extracted with 90% ethanol (40 L) under reflux thrice for 2.5 h each time. The extract (0.9 kg) obtained by concentrating under reduced pressure was dispersed in H2O and then extracted with petroleum ether and ethyl acetate to obtain petroleum ether (85 g) and ethyl acetate (190 g) fractions. For the ethyl acetate fraction, eight sub-fractions were obtained by silica gel column chromatography with petroleum ether-ethyl as mobile phase (100:0-0:100). Fraction 4 was subjected to further separation using ODS column chromatography eluted with a gradient of MeOH-H2O to give five fraction. And the second fraction (40%, v/v) was purified by HPLC (acetonitrile-water 18:82 as mobile phase) to yield Compound 1 (26.4 mg).

12β-acetoxy-3,7,11,15,23-pentaoxolanosta-8,20E(22)-dien-26-oic acid methyl ester (1): white powder. HR-ESI-MS m/z 605.2721 [M + Na]+ (calcld. 605.2727 for C33H42O9Na). 1H-NMR (600 MHz, CDCl3): 6.12 (1H, s, H-22), 5.72 (1H, s, H-12), 3.68 (3H, s, -OCH3), 3.34 (1H, t, J = 9.0 Hz, H-17), 2.94 (1H, m, H-25), 2.93 (1H, m, H-1a), 2.92 (1H, m, H-24a), 2.75 (1H, overlapped, H-16a), 2.70 (1H, m, H-6a), 2.61 (1H, dd, J = 14.4, 1.8 Hz, H-6b), 2.55 (1H, m, H-2a), 2.51 (1H, br d, J = 12.6 Hz, H-24b), 2.39 (1H, dd, J = 18.6, 9.0 Hz, H-16b), 2.15 (3H, s, 12-OOCCH3), 2.13 (3H, s, H-21), 1.75 (3H, s, H-30), 1.66 (1H, m, H-2b), 1.61 (1H, overlapped, H-5), 1.53 (1H, m, H-1b), 1.34 (3H, s, H-19), 1.19 (3H, d, J = 7.2 Hz, H-27), 1.06 (3H, s, H-28), 0.89 (3H, s, H-29), 0.85 (3H, s, H-18). 13C-NMR (150 MHz, CDCl3): 218.1 (C-3), 204.7 (C-15), 198.5 (C-7), 198.0 (C-23), 193.7 (C-11), 176.4 (C-26), 170.5 (12-OOCCH3), 154.2 (C-20), 152.0 (C-9), 146.0 (C-8), 126.2 (C-22), 78.7 (C-12), 58.0 (C-14), 52.1 (-OCH3), 51.8 (C-5), 49.0 (C-17), 48.8 (C-13), 48.0 (C-24), 47.1 (C-4), 37.8 (C-16), 37.3 (C-10), 36.5 (C-6), 35.1 (C-1), 34.8 (C-25), 34.2 (C-2), 28.1 (C-28), 21.5 (C-21), 21.4 (12-OOCCH3), 20.7 (C-30), 17.9 (C-19), 17.3 (C-27), 15.6 (C-29), 13.4 (C-18).

3.4. Cell culture

A549 and HepG2 cells were cultured using Gibco high glucose DMEM (10% fetal bovine serum) (5% CO2 at 37 °C). A549 and HepG2 at the exponential growth phase were digested and resuspended. Later the suspensions were dispensed into 6-well or 96-well Corning plates.

3.5. Cell viability assay

A549 and HepG2 cells at the density of 1 × 10^5 cells/mL were cultured in 96-well plates for 24 h at 37 °C, and then different concentrations (1, 10, 30, 60, 100, 200 μM) of 1 were added into cells and incubated for 48 h at 37 °C. After the 48-h incubation, 10 μL of MTT (5 mg/mL) was added per well and after further 4-h incubation, the medium was removed and 150 μL of DMSO was added to solubilize the MTT formazan salt. The absorbance of the solution was measured by a microplate reader at 570 nm.
3.6. Live/dead cell assay

Staining assay was achieved according to the manufacturer’s instruction of a Live/Dead Viability/Cytotoxicity Kit. Briefly, cells were seeded in a 96-well plate with a density of $1 \times 10^4$ cells per well and incubated for 24 h. Then, cells were treated with 1 at concentrations of 5 and 15 μM for 24 h at 37°C. Subsequently, cells were stained with calcein-AM (5 μM) and ethidium homodimer (5 μM) for 30 min at 37°C. Finally, the analysis was achieved by fluorescence microscope.

3.7. Western blot analysis

Cells were washed with ice-cold PBS and lysed with lysis buffer. Then cells were centrifuged at 12 000 $\text{rpm}^{-1}$ for 15 min and the protein concentration was measured with BCA protein concentration kit. Subsequently, the protein sample was denatured at 100°C for 5 min. Protein samples of different groups were electrophoresed and transferred to a PVDF membrane, blocked the PVDF membrane in a 5% skim milk powder for 1.5 h at RT (25°C). Proteins and the standard were separated by 10% SDS-PAGE gels. The blots were incubated with primary antibodies for 2 h at room temperature and subsequently washed with TBST three times, followed by horseradish peroxidase (HRP)-conjugated secondary antibodies for 1 h at room temperature. $\beta$-actin was used as a loading control. Immunoreacticity was detected by enhanced chemiluminescence.

3.8. Rt-qPCR

Quantitative real-time PCR was performed using SYBR Premix EXtaqII (TaKaRa, Dalian, China) in the PRISM 7900HT system (Applied Biosystems, Carlsbad, CA, United States). RNA was reverse transcribed by using the high-capacity cDNA archive kit (Applied Biosystems). Primers were designed by RiboBio (Guangzhou, China). The qRT-PCR results were expressed relative to GAPDH expression levels at the threshold cycle (Ct), which were then converted to fold changes ($2^{-\Delta\Delta Ct}$).

3.9. Statistics

Data are represented as mean ± S.D. in three independent experiments. The one-way ANOVA analysis of Prism 6 was used to demonstrate significant values, and $^*P < 0.05$ indicates the statistical significance.

4. Conclusions

In this paper, one new triterpene (1) with anti-tumor activities ($IC_{50}$ values of 15.38 ± 0.34 and 18.61 ± 0.55 μM for A549 and HepG2 cells, respectively) are reported. Through the mechanism study, the p53/capase-3 pathway is considered to be involved in the 1-induced apoptosis, suggesting that 1 is potentially a new natural anti-tumor agent for lung and hepatic cancers.
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Declaration of interest statement

The Authors Report No Declarations Of Interest.

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