Ganoderma lucidum extract reverses hepatocellular carcinoma multidrug resistance via inhibiting the function of P-glycoprotein in vitro and in vivo

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Abstract

Cancer is a leading cause of death globally. Chemotherapy still plays an indispensable role in the clinical treatment of cancer. However, the emergence of multidrug resistance (MDR) has greatly obstructed the further application of chemotherapy agents. Ganoderma lucidum (G. lucidum) is a traditional Chinese medicine as well as an edible mushroom. In this study, we first explored the effect of six extract samples derived from G. lucidum on the cell viability of adriamycin-resistant human hepatocellular carcinoma multidrug-resistant cell subline (HepG-2/ADM). All these samples showed no obvious toxicity to cells; however, only G. lucidum ethanol extract could reverse resistance to doxorubicin and paclitaxel. Then the P-glycoprotein (P-gp) function in hepatocellular carcinoma G2 (HepG-2) and HepG-2/ADM cells was determined after incubated with these samples, and we found that G. lucidum ethanol extract could inhibit P-gp function in vitro. Furthermore, G. lucidum ethanol extract could reverse resistance to paclitaxel in HepG-2/ADM tumor-bearing mice in vivo, while the protein expression level of P-gp was unchanged. Taken together, these results indicated the potential role of G. lucidum ethanol extract in reversing MDR in the clinical treatment of hepatocellular carcinoma.

Keywords: Ganoderma lucidum, hepatocellular carcinoma, multidrug resistance, paclitaxel, P-glycoprotein

Introduction

Cancer has become a leading cause of death globally, and has severely threatened the life expectancy of humans (Bray et al., 2021). It is estimated that about 19.3 million new cases and 10 million cancer deaths occurred worldwide in 2020 (Sung et al., 2021). Although different approach to treating cancer have developed successfully, the incidence and mortality are still increasing rapidly. The main cause of failure of anti-cancer therapy is the emergence of acquired resistance, among which multidrug resistance (MDR) has played a major role (Gottesman et al., 2002). MDR is a phenomenon in which cancer cells develop resistance to multiple chemotherapy agents with distinct structures or mechanisms (Kumar and Jaitak, 2019). P-glycoprotein (P-gp), encoded by adenosine triphosphate (ATP)-binding cassette (ABC) sub-family B member 1 (ABCB1) is the extensively studied factor in the development of MDR. ABCB1 could utilize the energy of ATP to actively transport drugs out of cytoplasm, resulting in the survival of cancer cells (Kumar and Jaitak, 2019). P-gp has been confirmed to be involved in the resistance to several anti-cancer agents, such as paclitaxel, doxorubicin, cisplatin, etoposide, etc. (Gottesman et al., 2002; Szakacs et al., 2006; Zeino et al., 2015). In spite of great efforts in exploring P-gp inhibitors, most of
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Materials and Methods

Materials and chemicals

Samples 1–6 were obtained from Guangzhou Hanfang Pharmaceutical Co. Ltd. (Guangzhou, China). Sample 1 was the major product of Ganoderma lucidum spore oil using supercritical fluid CO₂. Sample 2 was the product of Ganoderma lucidum spore oil using supercritical fluid CO₂ with modifier ethanol. Sample 3 was ethanol extract of the remains after using supercritical fluid CO₂. Sample 4 was water extract of the remains after using supercritical fluid CO₂. Sample 5 was the water extract of Ganoderma lucidum. Sample 6 was the ethanol extract of Ganoderma lucidum. Verapamil, paclitaxel, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), and rhodamine-123 (Rh-123) were purchased from Sigma-Aldrich (Deisenhofer, Germany). Doxorubicin (Dox) was obtained from Zhejiang HISUN Pharmaceuticals Co. Ltd. (Zhejiang, China).

Cell lines and culture

Human hepatocellular carcinoma (HCC) cell line HepG-2 and the multidrug-resistant P-gp overexpressing HCC cell line HepG-2/ADM were provided by Cancer Institute & Hospital, Chinese Academy of Medical Sciences (Beijing, China). All cell lines were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM; Corning, New York, US) containing 10% fetal bovine serum (FBS; Life Technologies, New York, US) and 1% penicillin-streptomycin solution (Life Technologies). Cell lines were maintained at 37°C in a humidified atmosphere with 95% air and 5% CO₂.

Cell viability assay

Cells were seeded in 96-well plates at a density of 4×10⁴ cells per well and cultured overnight. The cells were treated with various concentrations of samples 1–6 or compounds in the presence or absence of verapamil for 48 h. MTT, 20 μL, was added into each well and incubated for an additional 4 h. Finally, purple formazan crystals were dissolved in 150-μL dimethyl sulfoxide (DMSO) and the absorbance was observed at 490 nm by multimode microplate reader (Bio-Rad Laboratories, CA, US).

Rhodamine-123 intracellular accumulation assay

Cells were seeded in six-well plates at a density of 1×10⁵ cells per well and cultured overnight. The cells were treated with 500 μg/mL of samples 1–6 for 48 h and incubated with 10-μM Rh-123 for an additional 4 h in dark at 37°C. Then the cells were collected, washed with phosphate-buffered saline (PBS) thrice, and analyzed by flow cytometer (BD Bioscience, San Jose, CA, US). The data were analyzed using the FlowJo 7.6.1 software.

Xenograft mouse model experiments

BALB/c nude mice were purchased from Guangdong Province Medical Animal Center and monitored under specific pathogen-free conditions. All animal experiments complied with the National Institute of Health Guide for the Care and Use of Laboratory Animals, 8th edition (National Institutes of Health, 2011). HepG-2/ADM cells (1×10⁸ in 200 μL) were collected and injected subcutaneously into the right flank of mice. Treatments were initiated when tumors reached a mean volume of 100 mm³. Mice were randomly divided into the following four groups (n = 5): (1) control group (mice were given normal saline [NS]); (2) paclitaxel group (mice were given 18-mg/kg paclitaxel); (3) sample 6 group (mice were given 400-mg/kg sample 6); and (4) sample 6 + paclitaxel
group (mice were given 400-mg/kg sample 6 + 18-mg/kg paclitaxel). Mice were daily administrated sample 6 orally. Mice were injected with paclitaxel intraperitoneally every 2 days. Body weight and tumor volume were determined every 2 days, and tumor volume was calculated using the following formula:

\[ V = \pi \times (\text{length} \times \text{width}^2)/6. \]

Animals were euthanized with CO2 when the average tumor volume in the control group reached 1,300 mm³, and tumors were collected and weighed for the following experiments.

**Results**

**G. lucidum samples showed no obvious toxicity to HepG-2/ADM cells**

We measured the viability of HepG-2/ADM cells after incubation with various concentrations of samples 1–6 using MTT assay to evaluate whether the six samples derived from *G. lucidum* possessed toxicity to multidrug-resistant HCC cells. As shown in Figure 1, all samples showed little toxicity for HepG-2/ADM cells. Over 90% cells were alive even when the concentration reached 1,000 μg/mL. The results indicated that these samples derived from *G. lucidum* had no obvious toxicity to multidrug-resistant HCC cells, which could be candidates for investigating the effect of *G. lucidum* extract on MDR.

**G. lucidum ethanol extract reversed multidrug resistance in HepG-2/ADM cells**

In order to investigate the effect of *G. lucidum* extract on the MDR of HepG-2/ADM cells, we measured cell viability after incubation with doxorubicin or paclitaxel in combination with samples 1–6 or a classic inhibitor of P-gp (verapamil). As shown in Table 1, verapamil could potently suppress the function of P-gp and diminish cell viability at the 50% inhibitory concentration (IC\(_{50}\)) of doxorubicin and paclitaxel, which proved the MDR characteristic of HepG-2/ADM cells. To our surprise, we also observed that only sample 6 could effectively reverse resistance to doxorubicin and paclitaxel at IC\(_{50}\) and 20% inhibitory concentration (IC\(_{20}\)) and further reduced the viability of HepG-2/ADM cells. The fold reversal of resistance to doxorubicin and paclitaxel was almost three times at IC\(_{50}\) while that was nearly five to seven times at IC\(_{20}\). These data indicated that *G. lucidum* ethanol extract could reverse MDR in HCC cells.

**G. lucidum ethanol extract inhibited the function of P-gp in HepG-2/ADM cells**

Rh-123 is a specific fluorescent substrate of P-gp used to monitor the efflux function of P-gp. Therefore, we first treated HepG-2 cells and HepG-2/ADM cells with Rh-123 to validate their difference in P-gp function. As shown in Figure 2A, the fluorescent intensity of Rh-123 was significantly higher in HepG-2 cells than that in

**Statistical analysis**

Data were expressed as mean ± SD. Comparison of differences between multiple groups was performed using one-way ANOVA. Two-tailed Student’s *t*-test was used to compare difference between two groups. Statistical analysis was performed using the SPSS 22.0 software; *p* < 0.05 were considered statistically significant.
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We established HepG-2/ADM tumor-bearing mice models and administrated with paclitaxel and sample 6. As shown in Figures 3A–3C, paclitaxel treatment alone failed to inhibit the growth of tumor, indicating that resistance to paclitaxel was maintained in the xenografted tumor model. Furthermore, although treatment with sample 6 alone did not inhibit the growth of HepG-2/ADM tumors in vivo, it was apparent that sample 6 could...
Table 1. The reversal effect of *G. lucidum* ethanol extract on the MDR of HepG-2/ADM cells (n = 5).

<table>
<thead>
<tr>
<th></th>
<th>Cell viability ± SD (%) (fold-reversal)</th>
</tr>
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<tbody>
<tr>
<td><strong>Doxorubicin (IC&lt;sub&gt;50&lt;/sub&gt;)</strong></td>
<td></td>
</tr>
<tr>
<td>+Sample 6 (500 µg/mL)</td>
<td>57.393 ± 4.889</td>
</tr>
<tr>
<td>+Sample 6 (250 µg/mL)</td>
<td>16.118 ± 4.093*** (3.561)</td>
</tr>
<tr>
<td>+Verapamil (5 µmol)</td>
<td>15.402 ± 3.538*** (3.085)</td>
</tr>
<tr>
<td><strong>Doxorubicin (IC&lt;sub&gt;20&lt;/sub&gt;)</strong></td>
<td></td>
</tr>
<tr>
<td>+Sample 6 (500 µg/mL)</td>
<td>84.807 ± 3.378</td>
</tr>
<tr>
<td>+Sample 6 (250 µg/mL)</td>
<td>11.054 ± 3.366*** (7.672)</td>
</tr>
<tr>
<td>+Verapamil (5 µmol)</td>
<td>13.922 ± 11.810*** (6.092)</td>
</tr>
<tr>
<td><strong>Paclitaxel (IC&lt;sub&gt;50&lt;/sub&gt;)</strong></td>
<td></td>
</tr>
<tr>
<td>+Sample 6 (500 µg/mL)</td>
<td>47.507 ± 10.849</td>
</tr>
<tr>
<td>+Sample 6 (250 µg/mL)</td>
<td>15.402 ± 3.538*** (3.085)</td>
</tr>
<tr>
<td>+Verapamil (5 µmol)</td>
<td>16.279 ± 4.660*** (2.918)</td>
</tr>
<tr>
<td><strong>Paclitaxel (IC&lt;sub&gt;20&lt;/sub&gt;)</strong></td>
<td></td>
</tr>
<tr>
<td>+Sample 6 (500 µg/mL)</td>
<td>83.588 ± 11.879</td>
</tr>
<tr>
<td>+Sample 6 (250 µg/mL)</td>
<td>16.311 ± 2.058*** (5.841)</td>
</tr>
<tr>
<td>+Sample 6 (250 µg/mL)</td>
<td>16.989 ± 4.604*** (4.920)</td>
</tr>
</tbody>
</table>

Data are presented as mean ± SD, and significant differences are indicated as ***p < 0.001 vs the doxorubicin group, ###p < 0.001 vs the paclitaxel group.

**G. lucidum** ethanol extract showed no effect on the protein expression level of P-gp

In order to investigate the underlying mechanism of the reversal effect of sample 6 on MDR of HCC tumor, we extracted the total protein of tumor tissues and carried out Western blot assay. As shown in Figure 4, administration of paclitaxel could slightly increase the expression level of P-gp; however, sample 6 showed no additional effect on P-gp expression level. The results indicated that the reversal effect of *G. lucidum* ethanol extract on MDR reverse resistance to paclitaxel and decrease tumor volume and weight combined with paclitaxel. H&E staining of tumor sections showed that after treatment with paclitaxel + sample 6, tumor tissues demonstrated large areas of nuclear consolidation, cell vacuolization, and necrosis, with lymphocyte-infiltrated hyperemia in Figure 3E. We also measured the body weight and serum levels of ALT and AST in mice, and the results showed that neither sample 6 nor paclitaxel was toxic to mice as shown in Figures 3D and 3F. The results showed that *G. lucidum* ethanol extract could reverse resistance to paclitaxel in HepG-2/ADM tumor-bearing mice without additional toxicity.

Figure 2. *G. lucidum* ethanol extract inhibited the function of P-gp in HepG-2/ADM cells. (A) HepG-2 and HepG-2/ADM cells were incubated with Rh-123, and the fluorescent intensity was measured by flow cytometry. (B) HepG-2/ADM cells were treated with samples 1–6 and incubated with Rh-123, and the fluorescent intensity was measured by flow cytometry.
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**Discussion**

In the current study, the effect of six extract samples derived from *G. lucidum* on the viability of HepG-2/ADM human hepatocellular carcinoma multidrug-resistant cells was determined, and they exhibited no obvious toxicity. However, *G. lucidum* ethanol extract could reverse resistance to doxorubicin and paclitaxel. Then the P-gp function in HepG-2 and HepG-2/ADM cells was determined after incubated with these samples, and it was found that *G. lucidum* ethanol extract could inhibit P-gp function in vitro. Furthermore, *G. lucidum* ethanol extract could reverse resistance to paclitaxel in HepG-2/ADM tumor-bearing mice in vivo, without
P-gp, a member of the ABC transporter family, has been identified as a major factor that leads to MDR via utilizing the energy of two ATP molecules. P-gp could fulfill one catalytic cycle and the following conformational change, and pump the substrate out of the cytoplasm, resulting in resistance to several cytotoxic chemotherapy agents (Zeino et al., 2015). The overexpression of P-gp has been confirmed in different malignancies, such as hepatocellular carcinoma (Komori et al., 2014), breast carcinoma (Ding et al., 2021), colorectal carcinoma (Hu et al., 2014), and chronic myeloid leukemia (Ammar et al., 2020). Although great efforts have been invested in the research of P-gp inhibitors, most candidates failed because of inevitable toxicity and frustrating efficacy. Nowadays, natural products have demonstrated promising capabilities in regulating the function of P-gp while providing safer outcomes (Kumar and Jaitak, 2019).

In this study, we first applied a classic P-gp inhibitor, verapamil, and found that it could dramatically reverse the resistance of HepG-2/ADM cells to doxorubicin or paclitaxel, implying the role of P-gp in the MDR of HepG-2/ADM cells. Interestingly, we further measured the reversal effect of *G. lucidum* extract samples and discovered that only the ethanol extract sample 6 could significantly reverse resistance to doxorubicin and paclitaxel. Therefore, in order to investigate the effect of *G. lucidum* components on P-gp function, we applied Rh-123, a specific fluorescent substrate of P-gp, and performed flow cytometry experiments. Our results showed that, compared to HepG-2 cells, the multidrug-resistant HepG-2/ADM cells affecting the protein expression level of P-gp, indicating that *G. lucidum* ethanol extract might directly inhibit the function of P-gp.

In the past decades, cancer has become one of the major threats to human health worldwide, with increased incidence and mortality (Sung et al., 2021). Currently, in the clinical treatment of cancer, chemotherapy still plays an indispensable role because of good response and wide utility. Unfortunately, the development of MDR has severely limited the use of chemotherapeutic drugs, such as paclitaxel, doxorubicin, etoposide, and cisplatin (Robey et al., 2018).

*G. lucidum*, a basidiomycete, has been recognized as a traditional Chinese medicine with a history of hundreds of years in ancient China. It was believed that *G. lucidum* could promote immunity and extend life expectancy (Chan et al., 2021). Recently, researchers have discovered that *G. lucidum* exhibits multiple biological activities, including antioxidant, anticancer, anti-inflammatory, and immunomodulatory effects (Cor et al., 2018).

In this study, to further investigate the anticancer characteristics of *G. lucidum*, we prepared six samples with different isolation methods, and measured their effect on the viability of HepG-2/ADM cancer cells. The results revealed that, within a range of experimental dose, none of the six samples showed any inhibitory effects on cell growth, indicating that they posed no direct toxicity to HepG-2/ADM cancer cells.

![Figure 4](image-url)

**Figure 4.** *G. lucidum* ethanol extract showed no effect on the protein expression level of P-gp *in vivo*. The protein expression level of P-gp was assessed in HepG-2/ADM xenografts (*n* = 3). Data are presented as mean ± SD.

P-gp, a member of the ABC transporter family, has been identified as a major factor that leads to MDR via utilizing the energy of two ATP molecules. P-gp could fulfill one catalytic cycle and the following conformational change, and pump the substrate out of the cytoplasm, resulting in resistance to several cytotoxic chemotherapy agents (Zeino et al., 2015). The overexpression of P-gp has been confirmed in different malignancies, such as hepatocellular carcinoma (Komori et al., 2014), breast carcinoma (Ding et al., 2021), colorectal carcinoma (Hu et al., 2014), and chronic myeloid leukemia (Ammar et al., 2020). Although great efforts have been invested in the research of P-gp inhibitors, most candidates failed because of inevitable toxicity and frustrating efficacy. Nowadays, natural products have demonstrated promising capabilities in regulating the function of P-gp while providing safer outcomes (Kumar and Jaitak, 2019).

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ADM cells accumulated less Rh-123 in cytoplasm, indicating that the elevated function of P-gp contributed to MDR in HepG-2/ADM cells. Moreover, *G. lucidum* ethanol extract sample 6 could inhibit P-gp and elevate the level of Rh-123 in HepG-2/ADM cells, which further validated our previous results in the assay of reversal effect.

Based on the above results, xenograft HepG-2/ADM tumor-bearing mouse models were created and administered with paclitaxel and sample 6 to investigate whether *G. lucidum* ethanol extract could reverse MDR in HepG-2/ADM cells *in vivo*. The results demonstrated that paclitaxel had little effect on the growth of HepG-2/ADM tumors, confirming the MDR characteristic of xenografted tumor model. Further, *G. lucidum* ethanol extract sample 6 could reverse the MDR of xenografted tumors in combination with paclitaxel, resulting in the suppression of tumor volume and weight, accompanied with deteriorated tumor pathologic conditions. The results indicated the reversal effect of *G. lucidum* ethanol extract on MDR *in vivo*. Meanwhile, we also measured the body weight and serum levels of ALT and AST, and the data showed that the administration of sample 6 did not alter body weight and liver functions of mice, implying that *G. lucidum* ethanol extract had no obvious toxicity *in vivo*. Further, we extracted the total protein of tumor tissues and determined the expression level of P-gp in each treatment group. However, the results showed that sample 6 did not change the protein expression level of P-gp, while paclitaxel could slightly increase the expression of P-gp *in vivo*. Based on these results, we deduced that sample 6 could exert the reversal effect of MDR by directly inhibiting the function of P-gp, rather than affecting protein expression level. Therefore, our results indicated that *G. lucidum* ethanol extract could exert the reversal effect on MDR in HCC cells by directly inhibiting the function of P-gp both *in vitro* and *in vivo*.

**Conclusion**

Our study explored the effects of *G. lucidum* extract on multidrug-resistant HCC cells, and established that *G. lucidum* ethanol extract could reverse MDR in HCC cells by inhibiting the function of P-gp both *in vitro* and *in vivo* without severe adverse reactions. These findings implied the promising role of *G. lucidum* ethanol extract in reversing MDR because of chemotherapy agents in the clinical treatment of HCC.

**Acknowledgments**

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**Conflict of interest**

The authors stated that they had no conflict of interest to declare.

**Author Contributions**

Renwang Jiang, Juyan Liu, and Jing Li designed the study. Lin Cao wrote the manuscript. Jing Li, Cheng Yuan, Wendong Xu, Yaming Han, and Juyan Liu revised the manuscript. Jing Li, Hongfai Cai, Liang Chen, and Qin Zhang carried out the experiments. Lin Cao, Zhaoqin Jiang, and Hongfai Cai analyzed the data. All authors approved the final version of the paper.

**References**


