Liposomes co-loaded with ursolic acid and ginsenoside Rg3 in the treatment of hepatocellular carcinoma

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**Objective:** Liposomes co-loaded with ursolic acid and ginsenoside Rg3 (UA+Rg3-LIP) were prepared to study their effects on the proliferation, apoptosis and cell cycle of hepatocellular carcinoma (HCC) cells. Methods: Liposomes were prepared by reverse evaporation, and then UA+Rg3-LIP were prepared by the pH gradient method, and followed by liposome characterization. Next, the effects of UA+Rg3-LIP on the proliferation, apoptosis and cell cycle of HepG2 cells were investigated by MTT method and flow cytometry at the cell level. Results: The entrapment efficiency of UA in UA+Rg3-LIP was 78.52% and that of Rg3 was 71.68%, as assayed by low-temperature ultracentrifugation. The \textit{in vitro} release rates of UA+Rg3-LIP and UA+Rg3 detected by the dialysis membrane method were 1–10 h. The release rate of UA+Rg3 was close to 100%, that of UA+Rg3-LIP was decreased after 10 h and approached 100% after 24 h. It was further confirmed by cell experiments that UA+Rg3-LIP could significantly reduce cells’ viability while at the same time increase their apoptosis rate and raise the proportion of cells in the G0/G1 phase. Conclusion: Liposomes co-loaded with ursolic acid and ginsenoside Rg3 could affect cell proliferation, apoptosis and cell cycle, thus slowing down the \textit{in vitro} drug release ability of HCC.

**Keywords:** ursolic acid; ginsenoside Rg3; liposome; hepatocellular carcinoma; HepG2; \textit{in vitro} drug release

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**Abbreviations:** HCC, hepatocellular carcinoma; UA, ursolic acid

**INTRODUCTION**

Hepatocellular carcinoma (HCC) is a common tumor with high malignancy, low cure rate, and a mortality rate as high as 90% (Jiao \textit{et al.}, 2017). At present, the preferred methods for the treatment of HCC are still surgical resection or chemotherapy, but patients have a high recurrence rate and a low survival rate after surgery (Elmore, 2007).

In recent years, it has been found that natural Chinese herbal plants can improve the body’s immunity and have certain anti-tumor activity. Ursolic acid (UA) (Fig. 1A) is a ursolane-type pentacyclic triterpenoid acid that is ubiquitous in the leaves and fruits of natural medicinal plants, such as the leaves of \textit{Ligustrum lucidum} Ait., \textit{Eriobotrya japonica} (Thunb.) Lindl., \textit{Paulownia tomentosa} (Thunb), Steud., and \textit{Eclipta prostrata} L. (L.) Spreng, the whole plant of \textit{Prunella vulgaris} L., and fruits with waxy protection, like apples, pears, and plums (Zhan & Yi, 2008; Ngo \textit{et al.}, 2011). It possesses extensive pharmacological activities and especially has favorable effects on human diseases (Laszczyn, 2009). Research has shown that UA has a variety of biological effects, such as sedation, anti-inflammation, antibiosis, anti-diabetes, hypoglycemia, anti-proliferation, pro-apoptosis and anti-angiogenesis in \textit{in vitro} and \textit{in vivo} cancer models (Shanmugam \textit{et al.}, 2012). In addition, UA can inhibit HepG2 cell growth (Yie \textit{et al.}, 2015), and increase apoptotic death in HCC cells (Kim \textit{et al.}, 2019).

Ginsenoside Rg3 (Rg3) (Fig. 1B), a tetracyclic triterpenoid saponin present in the natural medicine ginseng, is one of the main active components of ginseng (Hua & Hua, 2012). Studies have shown that ginsenoside Rg3 has a certain inhibitory effect on many malignant tumors, such as the lung cancer, gastric cancer, intestinal cancer, liver cancer, and breast cancer, while it can also enhance the immune function in patients (Zhang \textit{et al.}, 2006; Lu \textit{et al.}, 2008; He \textit{et al.}, 2011). Although UA and Rg3 have been more experimentally studied in inhibiting the development of various tumors, the poor water solubility and low tumor targeting of these two drugs limit their efficacy (Lee \textit{et al.}, 2014; Khan \textit{et al.}, 2018). It has been reported that liposomal carriers can significantly improve the delivery efficiency of non-oral administration of hydrophobic drugs (Musacchio & Torchilin, 2011). The combination of liposomal carriers has an excellent anti-tumor effect, good targeting, low toxicity and small side effects (Jiang \textit{et al.}, 2018). A study by Sun and others (Sun \textit{et al.}, 2020) confirmed that hyaluronic acid (HA)-coated nanostructured lipid carriers (NLC) simultaneously loaded with oleanolic acid (OA), UA and Rg3 have more potent antitumor activity than 5-Fu in liver cancer. However, specific functional studies have not been reported.

Therefore, based on the synergistic effect of multi-components of traditional Chinese medicine, UA and Rg3 were encapsulated in liposomes to prepare a carrier

![Figure 1. The chemical structures of Ursolic acid (A) and Ginsenoside Rg3 (B)](https://example.com/figure1.png)
for the two components (UA+Rg3-LIP) in this study. We also chose an artificial intestinal fluid to simulate the intestinal environment to investigate the in vitro drug release of UA+Rg3-LIP on HCC and its effects on HepG2 cell proliferation, apoptosis and cell cycle, so as to provide a reference basis for the research and development of insoluble multi-component drug delivery system of traditional Chinese medicine for the treatment of HCC.

MATERIALS AND METHODS

Cell line and treatment

The human hepatocellular carcinoma cell line HepG2 was purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China), which was cultured in the DMEM medium containing 10% fetal bovine serum (FBS) and two antibiotics (100 U/ml penicillin and streptomycin), in a 5% CO2 incubator at 37°C. The experimental groups were divided based on the concentration of UA+Rg3-LIP as follows: Control group (without UA+Rg3-LIP intervention in HepG2 cells), 0.25 mg/mL group (with 0.25 mg/mL UA+Rg3-LIP intervention in HepG2 cells), 0.5 mg/mL group (with 0.5 mg/mL UA+Rg3-LIP intervention in HepG2 cells), and 1 mg/mL group (with 1 mg/mL UA+Rg3-LIP intervention in HepG2 cells).

Reagents

UA and MTT kit were purchased from Sigma, USA; ginsenoside Rg3 was purchased from Ruijin Biotechnology Co., Ltd.; phospholipids, cholesterol and DMEM medium were all purchased from Beijing Solarbio Technology Co., Ltd.; fetal bovine serum was purchased from Amresco; AnnexinV-FITC/PI apoptosis kit was purchased from Nanjing KeyGEN BioTECH Corp., Ltd; and artificial intestinal fluid was obtained according to the methods in the literature (Commission, 2015).

Preparation of UA+Rg3-LIP

Liposomes were prepared according to the methods described in (Ou et al., 2011). That is, lecithin, cholesterol and DSPE-PEG-GA were dissolved in ether. UA and ginsenoside Rg3 were dissolved in ethyl alcohol. After being mixed well, the mixture was injected at a constant speed into 20 mL of PBS at 65°C in a water bath, then placed on a constant-temperature magnetic stirrer to stir slowly and uniformly, and passed through a 0.8 μm filter membrane. After that, the liposomes were kept away from light for 24 hours, dialyzed against distilled water for another 48 hours and finally lyophilized and stored. At room temperature, the particle size and surface potential of an appropriate amount of UA+Rg3-LIP and liposomes (LIP) were measured by confocal microscopy and potentiometric analyzer after 50-fold dilution with distilled water.

Entrapment efficiency of UA+Rg3-LIP

The entrapment efficiency of liposomes co-loaded with UA and Rg3 was determined according to the method described in (Ma et al., 2019). Briefly, six milliliters of co-loaded liposomes were taken for ultra-centrifugation and placed in six tubes for ultra-high speed centrifuges. After being sealed, they were centrifuged in an ultra-high speed centrifuge at 3000 r/min for 30 min. Afterwards, the drug-loaded liposomes precipitated to the bottom of the centrifuge tubes, and the free drug without being encapsulated by phospholipids was present in the supernatant. Finally, concentration of the drug in the supernatant was determined and the entrapment efficiency was then calculated.

In vitro release rate

The in vitro release rate was determined according to the methods described in (Wang, 2010). 1.000 g of the sample was accurately weighed and placed in a dialysis bag with both ends tightly tied to remove bubbles. The bag was tied to the lower end of the stirring paddle and immersed in 500 mL of PBS (pH 6.5) dialysis medium containing 50% ethanol by volume fraction. It was rotated at a speed of 50 rpm/min in a circulating water bath (37°C). 1 mL of the sample was taken at time points of 0.25, 0.5, 1, 4, 8, 10, 15, 24 and 48 h, while in the meantime the same amount of isothermal dialysis medium was added, to determine the liposome drug content and calculate the cumulative release amount Q. The calculation formula was: Q (%) = (cumulative drug concentration in the release solution at each time point × volume of release medium/liposome drug content) × 100%. In artificial intestinal fluid medium, the release data of berberine sulfate were fitted with zero-order and first-order kinetic equation, as well as the Higuchi equation for UA+Rg3-LIP (Commission, 2015).

MTT assay

HepG2 cells in the logarithmic growth phase were inoculated on a 96-well culture plate (100 μL/well) with 5×104 cells/mL, and were cultured under 5% CO2 at 37°C. After the cells in each well were completely adherent, they were treated with 0.25 mg/mL, 0.5 mg/mL and 1 mg/mL of UA+Rg3-LIP for 48 h and then cultured for another 12, 24, 48, and 72 h, when 10 μL of 5 mg/mL MTT solution was added to each well, as previously described (Jiang et al., 2011). Finally, after 4 h culture in the CO2 incubator, DMSO (100 μL/well) was added to dissolve the formed purple crystals. The 570 nm wavelength absorbance value (OD) of each well was detected by a microplate reader.

Cell apoptosis and cell cycle

For the cell apoptosis assay, HepG2 cells were treated for 48 h and then collected by centrifugation at 1500 rpm for 5 min. Cells were stained with AnnexinV-FITC/PI at 4°C for 30 min. The apoptosis rate was measured with the FACSCalibur Flow Cytometer (Becton Dickinson, USA), and analyzed by using the FlowJo software.

Cell cycle was detected using PI staining. After 48 h of incubation, cells were collected and fixed in pre-cooled 70% ethanol, and kept at 4°C overnight. Subsequently, the cells were washed and stained with PI for 30 min in the dark at room temperature. The cells were then analyzed with the FACSCalibur Flow Cytometer.

Statistical analysis

All experimental data were statistically analyzed with the SPSS 26.0 software, and expressed as mean ± standard deviation (S.D.). Comparisons between two groups (when there were only two groups) were analyzed by t-test, while pairwise comparisons among multiple groups
Table 1. Characteristics of liposomes: particle size, zeta-potential and entrapment efficiency (n=3)

| Group          | Size (nm)    | zeta-potential (mV) | EE% (UA) | EE% (Rg3) |
|----------------|--------------|---------------------|----------|-----------|
| UA+Rg3-LIP     | 181.64±4.08  | −33.35±1.49         | 78.52 %  | 71.68 %   |
| LIP            | 163.31±4.05  | −18.43±2.11         | −        | −         |

EE, entrapment efficiency.

Table 2. Results of fitting models of in vitro release

| Item          | Mathematical model | Fitting equation | r       |
|---------------|--------------------|------------------|---------|
| UA+Rg3-LIP    | 0 order            | Q=3.322t+33.317   | 0.8381  |
|               | First order        | Q=98.16 (1-e^{-0.166t}) | 0.9622  |
|               | Higuchi            | Q=18.929t^{1/2}+12.893 | 0.9833  |

RESULTS

Characteristics of UA+Rg3-LIP

As shown in Table 1, the particle size of UA+Rg3-LIP was 181.7±4.36 nm and that of LIP was 163.5±3.92 nm, which were measured by confocal microscopy. The surface potential of UA+Rg3-LIP detected by the potential analyzer was −33.7±1.48 mV, and that of LIP was −18.5±2.17 mV. Moreover, the results of low-temperature ultracentrifugation revealed that the entrapment efficiency of UA and Rg3 in UA+Rg3-LIP were 78.52% and 71.68%, respectively.

In vitro release properties of UA+Rg3-LIP

The experimental results with the dialysis membrane method showed that at 1–10 h, the in vitro release rates of UA+Rg3-LIP and UA+Rg3 were relatively fast. The release rate of UA+Rg3 was close to 100% at 12 h; and, the in vitro release rate of UA+Rg3-LIP was decreased after 10 h but approached 100% after 24 h (Fig. 2). Furthermore, the fitting test showed that the release behavior of UA+Rg3-LIP and UA+Rg3 conformed to the first-order kinetic equation, and the best model for UA+Rg3-LIP was the Higuchi equation (Table 2).

Effect of UA+Rg3-LIP on proliferation of HepG2 cells

MTT results (Fig. 3) demonstrated that when compared to the Control group, the proliferation rates of cells in the 0.25 mg/mL, 0.5 mg/mL and 1 mg/mL groups were obviously decreased in a concentration-dependent manner, which confirmed that the inhibitory effect of UA+Rg3-LIP on proliferation of HepG2 cells was enhanced over time.

Effects of UA+Rg3-LIP on apoptosis rate and cell cycle of HepG2 cells

DISCUSSION

HCC is one of the common malignant tumors, and at present mainly occurs in the Asia-Pacific region (Lui, 2011). Although chemotherapy is one of the most widely studied methods in anticancer therapy, its efficacy and safety remain a major concern because of its serious side effects. In recent years, research on the use of natural herbal plants as anti-cancer drugs has gradually increased, and traditional Chinese medicine has played an essential role in the comprehensive treatment of cancer.

Multiple studies show that UA can control the growth, apoptosis and differentiation of HepG2 cells, with inhibition of their proliferation in a concentration-
and time-dependent manner, which is currently considered to be an effective anticancer drug in vitro (Ramos et al., 2008; Liu et al., 2014; Yie, Zhao et al., 2015). On the other hand, ginseng is a natural Chinese herbal plant, from which an effective chemical component called ginsenoside Rg3, one of the most common natural medicines, is extracted (Tan et al., 2011). Previous studies have shown that Rg3 has significant anticancer effects on various malignant tumors, such as the colon cancer (Kim et al., 2009), prostate cancer (Kim et al., 2004), breast cancer (Zhang et al., 2008), and lung cancer (Liu et al., 2009). In addition, ginsenoside Rg3 can inhibit the proliferation, adhesion, invasion, as well as metastasis of hepatoma cells, and induce cell death (Wen et al., 2008; Dogu & Díaz, 2009; Yin & Wang, 2015). Liposomes are biodegradable phospholipids without toxic and side effects on the human body. They have good targeting and permeability, which can minimize the toxic side effects of drugs and improve the efficacy of anti-cancer drugs. They are currently used as a more ideal drug carrier. In recent years, the development of Chinese medicine liposomes has become a hot topic in the study on the dosage forms of traditional Chinese medicine (Wu Y et al., 2006).

In the study presented here, UA+Rg3-LIP were successfully prepared. The particle size and surface potential of co-loaded liposomes and single liposomes were measured by confocal microscopy and potential analyzer, respectively. The assay results showed that the particle size of UA+Rg3-LIP was 181.7±3.96 nm and that of LIP was 163.5±3.92 nm; the surface potential of UA+Rg3-LIP was −33.7±1.48 mV and that of LIP was 163.5±3.92 nm; the surface potential of UA+Rg3-LIP was −18.5±2.17 mV, which met the requirements of a passive targeting drug delivery system. Upon determination of the entrapment efficiency, it was found that the entrapment efficiency of UA in the co-loaded liposomes was 78.52%, and that of Rg3 was 71.68%. The release process of UA+Rg3-LIP prepared in this study revealed that the in vitro release rate of co-loaded liposomes in the artificial intestinal fluid was faster at 1–10 h, but it slowed down after 10 h and approached 100% after 24 h. This release behavior confirmed to the first-order kinetic equation and the optimal model for UA+Rg3-LIP was the Higuchi equation. Then, the effect of co-loaded liposomes on liver cancer was examined by in vitro experiments, in which MTT and flow cytometry revealed that co-loaded liposomes could effectively and efficiently inhibit the growth and proliferation of liver cancer cells, and significantly increase the apoptosis rate of HepG2 cells, while at the same time markedly raise the proportion of cells in the G0/G1 phase and decrease their proportion in the S phase, showing a certain concentration dependence. The above results illustrated that UA+Rg3-LIP could inhibit proliferation and induce apoptosis of the hepatoma cells to achieve the anti-hepatoma killing effect. Studies have shown that UA or ginsenoside Rg3 inhibits HepG2 cell proliferation and promotes apoptosis by regulating the expression balance of Bel-2, Bel-xl and Bax of Bel-2 family members (Tan et al., 2011; Zhang et al., 2012; Shanmugam et al., 2013). However, the specific mechanism of action of UA+Rg3-LIP on HCC needs further study.

CONCLUSION

In conclusion, in this study we have successfully prepared liposomes co-loaded with UA and Rg3, which can effectively inhibit proliferation of HepG2 and promote cell apoptosis. These liposomes also have a potential to serve as an efficient tumor-targeted drug delivery system.

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Figure 4. Effects of different concentrations of UA+Rg3-LIP on apoptosis rate and cell cycle of the HepG2 cells. After being treated with different concentrations of UA+Rg3-LIP (0.25 mg/mL, 0.5 mg/mL, and 1 mg/mL) for 48 h, apoptosis (A) and cell cycle (B) were detected by flow cytometry. *P<0.05 vs Control group; **P<0.01 vs Control group; ***P<0.001 vs Control group.
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