Liver-targeted delivery of liposome-encapsulated curcumol using galactosylated-stearate

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Abstract. Liver-targeted drug delivery improves the efficacy of anti-liver cancer agents and reduces systemic toxicity by limiting the bioavailability of these drugs to within tumors. Liver targeting reagents with galactose residues, which selectively combine to asialoglyco protein receptors, have previously been used to improve liposome-encapsulated drug accumulation within liver cells. They lead to a reduction in liver cancer cell growth and have been used to cure certain hepatic diseases. In the present study, curcumol, which is the primary active component of Chinese traditional medicine *Rhizoma zedoariae,* was encapsulated in galactosylated-liposomes to enhance its anti-liver cancer efficacy. Galactosylated-liposomes and normal liposomes were labeled with propidium iodide. Galactosylated-liposomes with increasing concentrations of galactosylated-stearate (Gal-s) had a notably increased level of uptake in HepG2 cells (hepatoblastoma) compared with SGC-7901 (gastric cancer) and A549 (non-small cell lung cancer) cells. When the percentage of Gal-s reached 20%, liposome uptake plateaued. In the *in vitro* anti-liver cancer experiment, the anti-liver cancer efficacy of galactosylated-curcumol-liposomes increased significantly more compared with normal curcumol liposomes and free curcumol as indicated by cell survival rate and lactate dehydrogenase release rate. Collectively, these results demonstrate that galactosylated-liposomes are able to enhance the *in vitro* liver-targeting effect and anti-liver cancer efficacy of curcumol.

Introduction

Liver cancer is the sixth most prevalent type of cancer and has the second highest tumor associated mortality worldwide, with over half of all new cases and mortalities occuring in China (1). The current clinical treatments for liver cancer include surgery, radiotherapy and chemotherapy (1). For patients who miss the optimal timing required for surgery, chemotherapy is the recommended therapy (2). Although the majority of chemotherapy drugs inhibit the excessive proliferation of liver cancer cells, the side effects are may be severe (3-5). Novel drugs are required that have anti-liver cancer effects with minimal side-effects (6).

Curcumol (Cur), also known as turmeric alcohol, is a major component of essential oil (a type of oily liquid obtained by steam distillation) of the traditional Chinese medicine root of *Rhizoma zedoariae* that has been demonstrated to have anticancer, antibacterial, anti-inflammatory and antiviral effects (7,8). Previous studies have revealed that Cur has significant anti-liver cancer effects and serves an important role in liver protection (9,10). However, it is not as effective at treating liver cancer as traditional chemotherapeutic drugs, including fluorouracil (5-FU) and cisplatin (11,12). Cur is poorly soluble in water and a number of toxic solubilizer, such as Tween-80 have been previously used to enhance its solubility (13). These restrictions have limited the application of Cur within a clinical setting. In recent years, different dosing methods of Cur have been investigated (14). Cur liposomes have previously been investigated, however to the best of our knowledge Cur active targeting liposomes have not. To enhance the anti-liver cancer effect of Cur and minimize the efficacy gap between Cur and traditional chemotherapeutic drugs, active targeting liposomes may be used to encapsulate Cur.

Liposomes are novel carriers of targeting agents (15). The structure of liposomes is similar to that of biofilm and their membrane material is non-toxic in the human body (16).

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Liposomes may be used to precisely target and improve the stability of drugs within the human body without the need for complex structural modifications to the drugs themselves (17). Due to these characteristics, liposomes have previously been used as carriers of anticarcinogens (18-20). Galactosylated-liposomes, which specifically bind with the asialoglyco protein receptor (ASGPR) on the surface of liver cells are a type of liver targeting preparation (21). These liposomes may be transferred to liver cells via receptor-mediated endocytosis (RME) (22). A number of previous studies have demonstrated that drugs may be targeted to liver cells via galactosylated-liposomes (23,24).

In the present study, galactosylated-stearate (Gal-s) was used as a modifier to lead liposomes encapsulating Cur to HepG2 cells, with the aim of enhancing the anti-liver cancer efficacy of Cur. The *in vitro* uptake and anti-liver cancer efficacy of these liposomes were also investigated. The HepG2 cell line was originally thought to be a hepatocellular carcinoma cell line but was later revealed to derive from a hepatoblastoma (25). The HepG2 cell line has been previously used for the study of anti-liver cancer therapies (26) and was therefore selected for use in the present study to investigate liver-targeting liposomes.

Materials and methods

Preparation of liposomes. The preparation of Gal-s was performed as previously described (24). Briefly, D-galactose and vinyl stearate, which were dissolved in tetrahydrofuran, were used to synthesize galactose stearate under the catalysis of Novozym 435 immobilized lipase. The synthetic product was purified by silica gel column chromatography and analyzed using mass spectrometry and proton nuclear magnetic resonance. The content of the target product was detected using a high-performance liquid chromatography-evaporative light scattering detector, which was then used to confer the yield of the target product.

A total of 1.98 mg propidium iodide (PI; purity ≥95%; cat. no. P-4170; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) was dissolved in 100 ml PBS to obtain the PI solution. Using the thin film dispersion method as previously described (27), yolk lecithin (cat. no. PC-98T; injection grade) and cholesterol (cat. no. CHO-HP; injection grade) (both Advanced Vehicle Technology Pharmaceutical Co., Ltd., Shanghai, China) were used at a ratio of yolk lecithin: cholesterol, 2:1 to prepare Gal-s liposomes containing PI. The lipid film was rehydrated using PI solution at 45°C for 50 min. Galactosylated PI liposomes (Gal-PI-L) with different percentages of Gal-s (0, 5, 10, 15, 20 and 25%) were prepared, which were denoted as 0% Gal-PI-L, 5% Gal-PI-L, 10% Gal-PI-L, 15% Gal-PI-L, 20% Gal-PI-L and 25% Gal-PI-L, respectively. Normal liposomes were free of Gal-s.

Based on previous studies (7,28), the concentration of Cur in the liposomes used in the present study was set as 4 g/l. Using the two-step emulsification method (29), Gal-s, Cur (purity 95%; Nanjing Jingzhu Bio-technology Co., Ltd., Nanjing, China), yolk lecithin, cholesterol and glycerol trioleate (purity 60%; Aladdin Shanghai Biochemical Technology Co., Ltd., Shanghai, China) were used at a mass ratio of yolk lecithin: cholesterol, 2:1 to prepare galactosylated-Cur-liposomes (Gal-Cur-L). These lipids and Cur were completely dissolved in

4 ml diethyl ether. The total concentration of glycerol trioleate was 1 g/l. Poloxamer 188 (Shanghai Civi Chemical Technology Co. Ltd., Shanghai, China) was used as an emulgator in the second emulsification step. Normal Cur liposomes (Cur-L) contained 0% Gal-s and Blank liposomes (Gal-L) were devoid of Cur. The percentage of Gal-s in Gal-Cur-L was determined by a liposome *in vitro* uptake assay. The content of Cur in Gal-Cur-L was detected by the vanillin chromogenic method as previously described (30). In addition, the entrapment efficiency of these liposomes was calculated using the petroleum ether extraction method (30). The particle diameter of these liposomes was determined using laser scattering equipment (Delsa™ Nano Beckman Coulter, Inc., Brea, CA, USA).

Cell culture. HepG2 (hepatoblastoma), SGC-7901 (gastric cancer) and A549 (non-small cell lung cancer) were purchased from the cell bank of the Chinese Academy of Medical Science (Beijing, China) and maintained in RPMI 1640 medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.) in the thermostat incubator at 37°C with 5% CO_2. When cells were in the logarithmic growth phase, trypsin-EDTA solution (0.25:0.02%; Nanjing SenBeiJia Biological Technology Co., Ltd., Nanjing, China) was used to digest them. The cells were seeded in 12- or 96-well plates at a density of $5x10^5$ or $2x10^4$ cells/well, respectively with RPMI 1640 medium and 10% FBS in a thermostat incubator at 37°C with 5% CO_2 for 24 h prior to further experimentation.

Liposomes in vitro uptake assay. The nutrient solution in the 12-well plates was removed and a mixture of Gal-PI-L, PBS and RPMI 1640 medium (excluding FBS) was added. Following 20 min incubation in thermostat incubator at 37°C with 5% CO_2, the mixture was removed and cells were washed twice with PBS. A laser confocal inverted fluorescence microscope (LSM710; Zeiss AG, Oberkochen, Germany) was used to capture images of the cells (magnification, x200). The cells in the 96-well plate (fluorescence detection plate) were handled according to the above method and a fluorescence microplate reader (Gemini XPS; Molecular Devices, LLC, Sunnyvale, CA, USA) was used to analyze the fluorescence intensity of the cells. The volume of PBS, liposome, and RPMI 1640 medium used in the 96-well plate was 10, 40 and 50 µl, respectively for each group. The volumes used in the 12-well plate were 100, 400 and 500 µl, respectively.

Liposome in vitro anti-liver cancer study. Cur-suspension and Cur-blank-suspension were prepared as previously described (31). Fluorouracil (5-Fu; Shanghai Xudong Haipu Pharmaceutical Co., Ltd., Shanghai, China), which is a current drug used for cancer therapy was used for comparison in the present study, was diluted to the desired concentration using saline. An MTT reagent kit (Nanjing Jiancheng Bioengineering Institute Co., Ltd., Nanjing, China) was used to determine the viability of HepG2 cells. In the MTT assay dimethyl sulfoxide was used to dissolve the purple formazan. The absorbance value of each well was measured at 490 nm wavelength by a microplate reader. A lactate dehydrogenase (LDH) assay kit (Nanjing Jiancheng Bioengineering Institute Co., Ltd.) was used to analyze the LDH release rate of HepG2 cells.

These assays were performed according to the manufacturer's protocol. The drug concentrations used in each experimental group are listed in Table I.

Statistical analysis. Data are expressed as the mean + standard deviation. Multiple group comparisons were performed using one-way analysis of variance followed by Dunnett's post hoc test to detect inter-group differences. SPSS 17.0 (SPSS, Inc., Chicago, IL, USA) was used for statistical analysis of the data. Each experiment was repeated a minimum of six times. P<0.05 was considered to indicate a statistically significant difference.

Results

Characterization of liposome particle size. Fig. 1 indicates the average particle diameter of the liposomes in each group. As the percentage of Gal-s increased, the average diameter of these liposomes also gradually increased. All liposomes were <200 nm, which fulfilled the requirements for the present study.

Liver-targeted drug delivery enhances PI delivery to HepG2 cells. Quantitative analysis of liposome uptake was performed *in vitro* using a fluorescence microplate reader (Fig. 2). As the percentage of Gal-s increased the fluorescence intensity of HepG2 cells signifiacntly increased (P<0.05). No statistically significant differences were observed between the 20% Gal-PI-L group and the 25% Gal-PI-L group, which may indicate that the fluorescence intensity of HepG2 cells plateaued at its maximum. No significant changes in the fluorescence intensity of the SGC-7901 and A549 cells were observed. The fluorescence intensity of the PI solution groups was significantly decreased compared with all other groups (P<0.05).

Images captured of liposome uptake in HepG2, SGC-7901 and A549 cells following fluorescent staining are presented in Figs. 3-5, respectively. In the 0% Gal-PI-L group, a few dyed cells were present for all cell types. In the 20% Gal-PI-L group, a notable increase in the number of dyed cells was observed in the HepG2 group (Fig. 3B-b); however, there was no notable increase in the number of dyed cells in the SGC-7901 and A549 groups (Figs. 4B-b and 5B-b). In the PI solution group, very few dyed cells were observed for all cell types.

Liver-targeted drug delivery enhances the anti-liver cancer effect of Cur in HepG2 cells. MTT and LDH assays were performed to determine the viability and LDH release rate of each cell type (Fig. 6). The viability of the Cur-blank-suspension group was significantly increased compared with all other groups with the exception of Gal-L (P<0.05; Fig. 6A). Cell viability was significantly decreased in the Cur-L and all the Gal-Cur-L groups compared with the Cur-suspension group (P<0.05). Cell viability was significantly decreased in the Gal-Cur-L Med and High groups compared with the Cur-L group (P<0.05), which indicates that Gal was able to target drug delivery and result in cell death.

The LDH release rates were the reverse of the MTT assay results (Fig. 6B). The LDH release rate in all groups except the Gal-L group were significantly increased compared with the Cur-blank-suspension group (P<0.05). Furthermore, the LDH release rate in the Gal-Cur-L high and Gal-Cur-L medium groups were significantly increased compared with the Cur-L

Table I. Group of *in vitro* anti-liver cancer study.

Group	Final concentration of drug (mg/l)
Cur-blank-suspension	0.0
Gal-L	0.0
5-Fu	1.7
Cur-suspension	27.0
Cur-L	27.0
Gal-Cur-L low dosage	13.5
Gal-Cur-L medium dosage	27.0
Gal-Cur-L high dosage	54.0

Cur, curcumin; Gal, galactosylated; L, liposome; 5-Fu, fluorouracil.
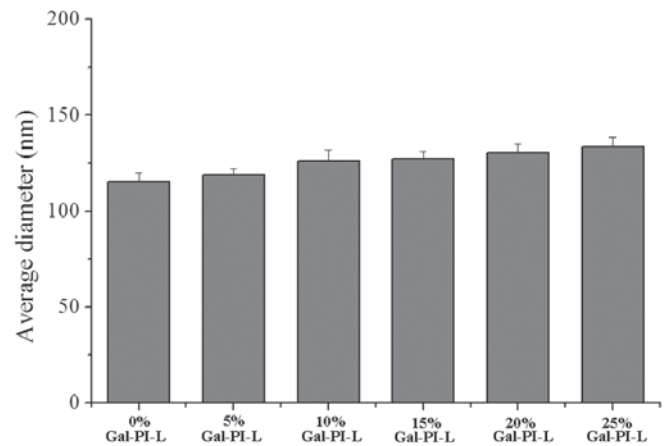

Figure 1. Results of particle diameter determination. Data are presented as the mean ± standard deviation (n=6). Gal, galactosylated; L, liposome; PI, propidium iodide.

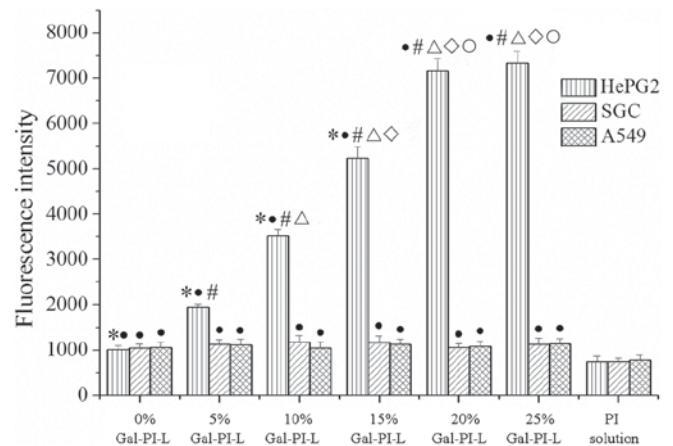Figure 2. Liposome *in vitro* uptake assay. Data are presented as the mean ± standard deviation (n=6). *P<0.05 vs. 25% Gal-PI-L group; •P<0.05 vs. the PI group; #P<0.05 vs. 0% Gal-PI-L group; △P<0.05 vs. 5% Gal-PI-L group; ◇P<0.05 vs. 10% Gal-PI-L; ○P<0.05 vs. 15% Gal-PI-L group. Gal, galactosylated; L, liposome; PI, propidium iodide.

group (P<0.05). The Cur-L group and all Gal-Cur-L groups had significantly increased LDH release rates compared with the Cur-suspension group (P<0.05; Fig. 6B). These results
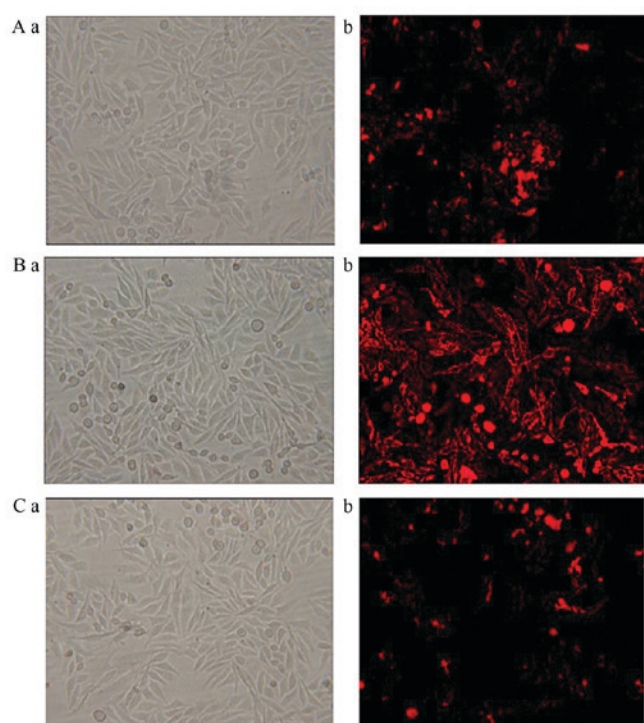
Figure 3. Fluorescent images of HepG2 cells. (A-a) Normal and (A-b) fluorescent images of 0% Gal-PI-L. (B-a) Normal and (B-b) fluorescent images of 20% Gal-PI-L. (C-a) Normal and (C-b) fluorescent images of PI solution. Magnification, x200. PI, propidium iodide; Gal, galactosylated; L, liposome.

Figure 5. Fluorescent images of A549 cells. (A-a) Normal and (A-b) fluorescent images of 0% Gal-PI-L. (B-a) Normal and (B-b) fluorescent images of 20% Gal-PI-L. (C-a) Normal and (C-b) fluorescent images of PI solution. Magnification, x200. PI, propidium iodide; Gal, galactosylated; L, liposome.

Discussion

Fluorescent imaging of HepG2, SGC-7901 and A549 cells revealed that free PI, a type of water-soluble fluorescent dye, did not readily enter the cells under normal growth conditions. Only a few cells, which were in terminal apoptosis or dead, were visible with fluorescence under normal conditions. Gal-PI-Ls were delivered to the liver cancer cells, whereas they were not delivered to the gastric or lung carcinoma cells. The liver-specific delivery was regulated by galactose residues, which specifically bind with ASGPR on the surface of liver cells (32). Galactosylated-liposomes, which have galactose residues, may be transferred to liver cells through RME (33). ASGPRs are primarily expressed on the surface of mammal liver parenchyma cells (34). The surfaces of stomach and lung cancer cells do not express ASGPR (34), which explains why there was no clear targeting of galactosylated-liposomes to SGC-7901 and A549 cells.

As the phospholipid membrane shares certain similarities with the cell membrane, a few PI-L (0% Gal-PI-L) may penetrate the cell membrane and enter the cells. The results of quantitative fluorescent analysis supported this suggestion. When the percentage of Gal-s in the liposomes reached 20%, liposome uptake appeared to plateau. Therefore, 20% was selected as the percentage of Gal-s in galactosylated-liposomes to be used in the present study.

A cell viability assay combined with an LDH release rate assay comprehensively evaluated the anti-tumor activity of the liposomes. These results revealed that Cur-suspension, Cur-L and Gal-Cur-L all had certain *in vitro* anti-tumor activity, whereas blank liposomes did minimal damage to the cells.
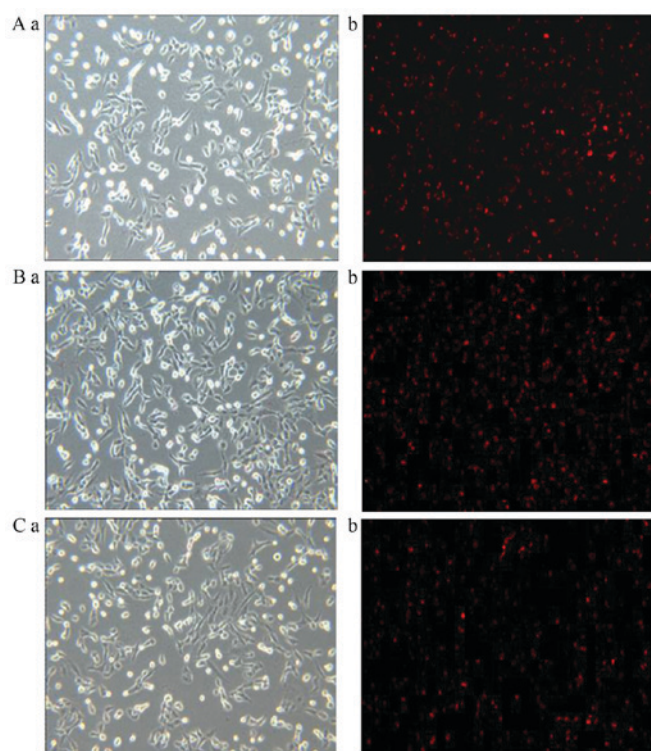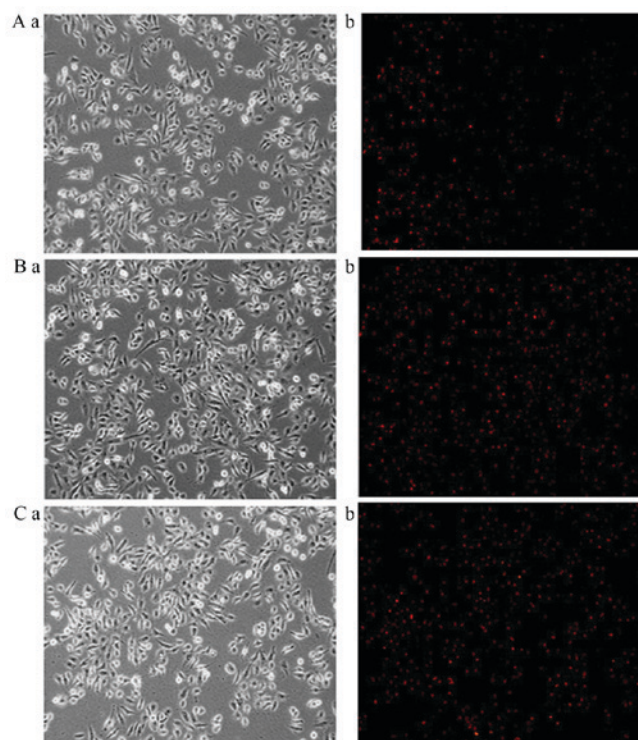

Figure 4. Fluorescent images of SGC-7901 cells. (A-a) Normal and (A-b) fluorescent images of 0% Gal-PI-L. (B-a) Normal and (B-b) fluorescent images of 20% Gal-PI-L. (C-a) Normal and (C-b) fluorescent images of PI solution. Magnification, x200. PI, propidium iodide; Gal, galactosylated; L, liposome.

further clarify that galactosylated-liposomes enhance the anti-liver cancer effect of Cur.
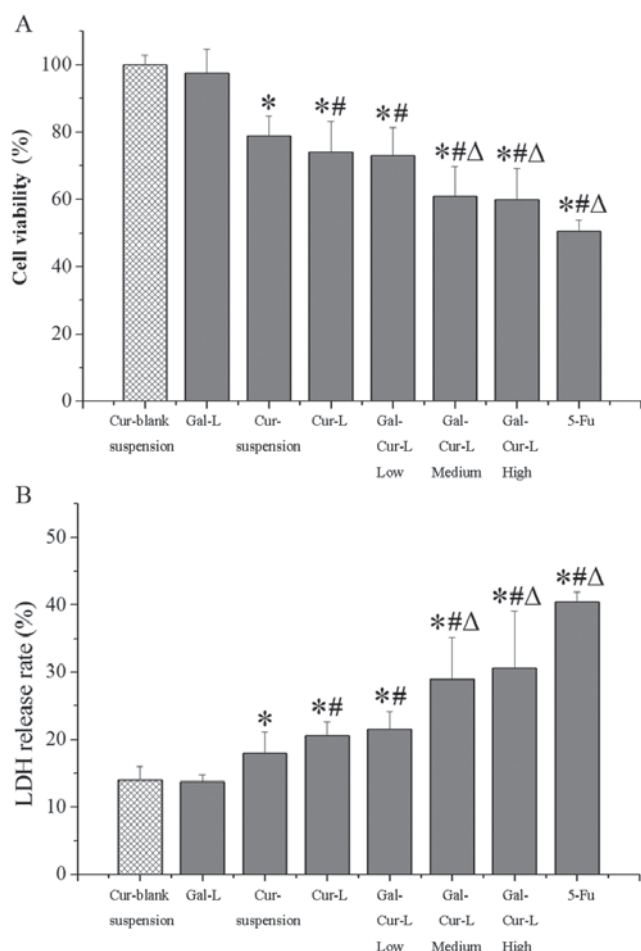
Figure 6. Anti-liver cancer effect of liposomes *in vitro*. (A) Viability of HepG2 cells. (B) LDH release rate of HepG2 cells. Data are represented as the mean ± standard deviation (n=6). *P<0.05 vs. Cur-blank suspension group; #P<0.05 vs. Cur-suspension group; ΔP<0.05 vs. Cur-L group. LDH, lactate dehydrogenase; Gal, galactosylated; L, liposome; Cur, curcumin.

When Cur was encapsulated by liposomes, its anti-liver cancer efficacy was significantly enhanced. The galactosylated-liposomes significantly increased the anti-tumor activity compared with the normal liposomes.

In conclusion, the results of the present study demonstrate that liposomes modified with Gal-s may be easily and effectively delivered to liver cancer cells. The anti-liver cancer efficacy of Cur was significantly enhanced by the use of galactosylated-liposomes. Future research based on the present study should use small animals to investigate the *in vivo* distribution of galactosylated-liposomes. Poly (2-ethyl-2-oxazoline) may also be used to enhance permeability and avoid the rapid clearance of the liposomes by macrophages (35).

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

WJL, YC and HL participated in research design. YWL, QSG, NL and WJL conducted the experiments. WXL and YBH performed data analysis. WJL wrote the manuscript.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors confirm that they have no competing interests.

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