TPGS-modified liposomes for the delivery of ginsenoside compound K against non-small cell lung cancer: formulation design and its evaluation in vitro and in vivo

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Abstract
Objective This work aimed at preparing ginsenoside compound K (GCK)-loaded liposomes modified with TPGS (GCKT-liposomes) to enhance solubility and targeting capability of GCK, as well as inhibit the efflux of GCK from tumour cells.

Methods GCKT-liposomes were prepared by the thin-film hydration method and characterized by particle size, polydispersity, zeta potential and drug encapsulation efficiency. A549 cells were used as antitumour cell model to access the cellular uptake of the GCK and perform its antitumour function. The enhancement of in vivo antitumour efficacy of GCKT-liposomes was evaluated by nude mice bearing tumour model.

Key findings The results showed that GCKT-liposomes achieved a comparatively high drug loading efficiency and reasonable particle size at the ratio of 7:3 (phospholipid: TPGS). The in vitro release demonstrated that the dissolution of GCK was remarkably improved by entrapping it into liposomes. In addition, GCKT-liposomes exhibited a great hypersensitizing effect on A549 cells, and the cellular uptake was enhanced. Compared with free GCK, the IC\textsubscript{50} of GCKT-liposomes was significantly reduced (16.3 \pm 0.8 vs 24.9 \pm 1.0 \mu g/ml). In vivo antitumour assay also indicated that GCKT-liposomes achieved higher antitumour efficacy (67.5 \pm 0.5 vs 40.8 \pm 0.7\%).

Conclusion The novel GCKT-liposomes significantly improved the antitumour efficacy of GCK.

Introduction
Ginseng, one of most popular herbal has been widely used as an energy booster in China for thousands of years. Ginsenosides are one of the major active constituents in Ginseng. Until now, much effort has gone into the pharmacological activity of ginsenoside Rb\textsubscript{1}, Rb\textsubscript{2}, Rg\textsubscript{1}, Rg\textsubscript{2} and other active ingredients. However, ginsenoside compound K (GCK) was not taken seriously until Karikura M found that ginsenoside Rb\textsubscript{1}, Rb\textsubscript{2} were absorbed when converted into GCK\textsuperscript{[1]}

Ginsenoside compound K (20-O-beta-D-glucopyranosyl-20(S)-protopanaxadiol, Figure 1) is an intestinal bacterial metabolite of ginseng protopanaxadiol saponins.\textsuperscript{[2,3]} It has been reported that GCK has many pharmacological efficacies such as antitumour, anti-inflammation, anti-angiogenesis, antiallergic, antidiabetic, anti-ageing, cardiac protection and hepatoprotective effects.\textsuperscript{[4–11]} Among them, the anticancer activity of GCK has been deep studied, which indicated GCK could cause direct cytotoxic, growth-inhibitory and cellular apoptosis against tumour cells,\textsuperscript{[12–14]} suggesting the great potential of GCK for anticancer.

However, the poor aqueous solubility, serious efflux phenomenon and low membrane permeability limited the effective utilization and clinical application of GCK.\textsuperscript{[15]} In previous reports, various drug delivery systems have been developed for enhancing the antitumour effect of the active ingredients, such as micelles, liposomes and
Among these delivery systems, liposome is considered to be a stronger prospective alternative as several of these formulations have been under clinical assessment for anticancer. However, the traditional liposomes came with some serious problems, such as instability, insufficient drug loading, fast drug release and short blood circulation. Therefore, in this article, GCKT-liposomes was prepared to overcome these problems.

D-α-tocopheryl polyethylene glycol 1000 succinate (vitamin E TPGS or simply TPGS) is an amphiphilic structure derivative of natural vitamin E, which is formed by esterification of D-α-tocopheryl acid succinate with polyethylene glycol 1000. Its hydrophilic polar head portion (polyethylene glycol) and lipophilic alkyl tail (tocopherol succinate) make it to have good surfactivity. In addition, TPGS could increase drug encapsulation and solubility of hydrophobic drugs, extend the half-life of the drug and enhance the cellular uptake of the drug. Moreover, TPGS also could mediated multidrug resistance (MDR) by inhibiting the P-glycoprotein (P-gp) pump. Therefore, TPGS has been intensively applied as an ideal material in developing the various drug delivery systems, including TPGS-based pro-drugs, liposomes, micelles and nanoparticles.

Thus, this study aimed to increase the solubility and membrane permeability of GCK, as well as enhance its anticancer effect by inhibiting efflux phenomenon.

Materials and Methods

Materials and animals

Ginsenoside compound K was prepared in our laboratory (Nanjing, China) and purity was >98%. D-α-tocopheryl polyethylene glycol 1000 succinate (TPGS) was purchased from Aladdin Industrial Co. Ltd. (Shanghai, China). Egg yolk lecithin (PC-98T) was acquired from Shanghai Advanced Vehicle Technology Pharmaceutical L.T.D Co Ltd. (Shanghai, China) Coumarin-6, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT), dimethyl sulfoxide (DMSO) were purchased from Nanjing SunShine Biotechnology Co. Ltd. (Nanjing, China) Coumarin-6 and DAPI were purchased from Sigma (Shanghai, China). DiR iodide (DiR) was purchased from Ganhua Trade Co. Ltd. (Shanghai, China). Human lung adenocarcinoma cell line A549 was purchased from Nanjing Key-GEN Biotech. Co. Ltd. (Nanjing, China). All reagents were of analytical grade except methanol, which was of chromatographic grade.

Male athymic nude mice were obtained from Changzhou Cavens Lab Animal Co. Ltd. (Changzhou, China). All animal experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) Jiangsu Provincial Academy of Chinese Medicine’s Experimental Animal Center.

Establishment of in vitro methodology for high-pressure liquid chromatography analysis

The concentration of GCK in the dissolution medium was determined by high-pressure liquid chromatography (HPLC, Agilent 1200; Agilent Co. Ltd, Waldbronn, Germany) equipped with a AgilentTM RP-C18 column (150 mm × 4.6 mm, 5 μm). The mobile phase of methanol and water (60 : 40, v : v) was used at a flow rate of 1.0 ml/min. The UV detector was set at 230 nm to analyse the column effluent, and the column temperature was set at 30 °C. The entire solution was filtered through a 0.45 μm membrane filter and degassed before use. The injection volume was 10 μl. The recovery rates for GCK were in the range of 98–101%, and the RSD were <2%. Intraday and interday precisions for GCK were below 2%.

Preparation of drug-loaded liposomes

TPGS-modified liposomes were prepared using the thin-film hydration method. In brief, GCK/coumarin-6/Dir, phospholipid and TPGS were dissolved in ethanol by ultrasonication. Subsequently, the solution was evaporated by rotary vacuum evaporation (RE-52AA; Shanghai Yarong Biochemical Co. Ltd, Shanghai, China) until a thin film was formed on the walls of the round-bottom flask. The film was vacuum-dried overnight at room temperature to remove the residual ethanol and then rehydrated in 0.9% NaCl solution to form GCK/coumarin-6/Dir-loaded liposome followed by lyophilization. Non-incorporated GCK/coumarin-6/Dir was removed from the solution by filtration with a 0.22 μm membrane before lyophilization.
TPGS-modified liposomes without drug (T-liposomes) were prepared as above.

**Characterization of GCKT-liposomes**

**Particle size, zeta potential and morphology analysis**

Mean size distribution (hydrodynamic diameter), polydispersity and zeta potential of the GCKT-liposomes were measured by dynamic light scattering (DLS) with a Zetasizer Nano ZS (Malvern Zetasizer 3000; Malvern Instruments Ltd, Worcestarshire, UK). The particle size and zeta potential measurements were carried out in triplicate. The morphology of GCKT-liposomes was evaluated by a transmission electron microscopy (TEM; JEM-1200EX, Tokyo, Japan) after negative staining with sodium phosphotungstate solution (2%, w/v).

**Drug loading and encapsulation efficiency**

The drug concentration of the GCK was measured by HPLC method. Briefly, 200 μl of GCKT-liposomes was dissolved and the phospholipid was disrupted by adding 800 μl methanol. The mixture was centrifuged at 13 000 g for 10 min, filtered with a 0.22 μm membrane filter and determined the amount of GCK in supernatant by HPLC method. All samples were analysed in triplicate. The encapsulation efficiency (EE%) and the drug loading efficiency (DL%) were calculated by the following equations:

\[
EE(\%) = \frac{\text{amount of GCK in liposomes}}{\text{amount of GCK fed initially}} \times 100\
\]

\[
DL(\%) = \frac{\text{amount of GCK in liposomes}}{\text{total amount of liposomes}} \times 100\
\]

**In vitro release of GCKT-liposomes**

The dialysis bag diffusion method was used to study the in vitro release behaviour of GCK from GCKT-liposomes. Briefly, 5 ml of GCKT-liposomes was placed into a dialysis bag (Mw = 3500 Da; Greenbird Inc, Shenzhen, China).\(^{[31]}\) hermetically sealed and immersed into 50 ml phosphate-buffered saline (PBS, pH 7.4) medium containing 0.5% (w/v) Tween 80 at 37 °C. The entire system was stirred at 100 rpm/min for 24 h. At predetermined time intervals, 1 ml release medium was withdrawn at 15, 30 and 60 min or 4, 8, 12 and 24 h and filtered through 0.45 μm membrane before transferred into HPLC vial. The same volume of fresh release medium then was added to the dialysis system. GCK released from the medium was conducted under the same conditions as the controls. The content of GCK in the samples was determined by HPLC as described above. All assays were performed in triplicate, and the drug release profiles were then calculated.

**In vitro cell cytotoxicity assay**

The human lung adenocarcinoma cell line A549 was cultivated in RPMI 1640 medium contained 10% foetal bovine serum (FBS; Gibco, Paisley, UK), 100 IU/ml penicillin and 100 μg/ml streptomycin at 37 °C in 5% CO₂.

The cytotoxicity of GCKT-liposomes against A549 cells was investigated using the MTT assay. A total of 100 μl A549 cells were seeded into 96-well plate at a density of 1 × 10⁵ single-cell suspension. After incubation for 24 h, the medium was discarded and the cells were treated with various GCK formulations at equivalent drug concentrations for 24 h. Then, the wells were washed twice with PBS, and 10 μl MTT (5 mg/ml) was added to each well of the plates. After incubation for 4 h, the medium was removed and 100 μl DMSO solution was added into each well dissolving the MTT formazan. The absorbance of each well was measured at the 570 nm wavelength by an iMark microplate reader (Bio-Rad Laboratories, California, USA). Each experiment was performed in triplicate. Cell viability was calculated by the followed equation:

\[
\text{cell viability (\%)} = \frac{A_{\text{sample}} - A_{\text{blank}}}{A_{\text{control}} - A_{\text{blank}}} \times 100\%
\]

Where \(A_{\text{sample}}\) is the absorbance of the cells incubated with the GCKT-liposomes, the \(A_{\text{control}}\) is the absorbance of the GCK and \(A_{\text{blank}}\) is the absorbance of cells incubated with culture medium only.

**In vitro cellular uptake**

For the in vitro qualitative study, the cellular uptake of the T-liposome was assessed by the fluorescence microscope. Confluent A549 cells were detached at a concentration of 1 × 10⁵ viable cells/chamber and seeded in a 24-well plate overnight. After that, the spent medium was discarded and was subsequently replaced by coumarin-6-loaded T-liposomes (C₆T-liposomes) solution (concentration of 0.2 mg/ml). After incubated for 2 h, the cell monolayer was washed three times with cold PBS (pH 7.4) and fixed with 95% ethanol for 20 min. After that, the cells were washed twice by PBS and the nuclei were marked by DAPI for another 30 min, washed triple using PBS and immediately observed by microscopic imaging.

The A549 cells were seeded into a 6-well plate. The cells were incubated for 24 h, and the original culture medium was fast discarded. The cells were subsequently treated with GCKT-liposomes (concentration of 26 μM) for 1-, 2- and
4-h incubation to estimate the influence of cellular uptake time to uptake. After the incubation, the suspension was removed, and the cells were washed three times using cold PBS 0.5 ml of trypsin–EDTA solution was added for 1 min and cells were scraped. The suspended fluid of cells was collected, and the supernatant was discarded after centrifugation at 3000 g for 2 min. The cell was placed in an ice bath under ultrasonic cell disruption for 30 min. After centrifuged, the GCK within supernatant was obtained. The solution was transferred to a clean centrifuge tube, dried with nitrogen, dissolved in methanol and filtrated with 0.45 μm millipore. The content of GCK was measured by HPLC determination.

In vivo pharmacodynamics study

Male athymic nude mice (22 ± 2 g) were randomly divided into several groups and maintained on a 12-h light–dark cycle with normal access to food and water. The GCK solution and GCKT-liposomes were prepared before use. A total of 5 mg of GCK was dissolved in 0.5 ml cremophor EL and ethanol mixed solution (1:1, v:v) [32] and then diluted to 1 mg/ml with distilled water used in group 2. GCKT-liposomes were prepared as section ‘preparation of GCKT-liposomes’.

For in vivo implantation, 200 μl of single-cell suspension containing 1 × 10^6 A549 cells in serum-free DMEM was prepared and was injected subcutaneously in the right flank of 6-week-old male nude mice under anaesthesia. After the tumour sizes reached about 60 mm³ (the day designated as Day 0), mice were randomly divided into three groups (n = 6), group 1 for saline solution, group 2 for GCK (15 mg/kg) and group 3 for GCKT-liposomes formulations (15 mg/kg), respectively. Mice were treated through the intravenous injections for five times every 3 days. Tumour volume and mouse weight were measured three times a week. Tumour volume was calculated using the formula of \( V = \frac{(a \times b^2)}{2} \), where \( a \) represents the longest diameter and \( b \) represents the shortest diameter perpendicular to length. At the end of the experiment, the animals were euthanized and the tumour were harvested, photographed and weighed.

In vivo imaging

When tumours reached suitable size, Dir-loaded T-liposomes (DirT-liposomes) were injected into the tail vein of the tumour-bearing mice at a dose of 5 mg/kg to investigate in vivo distribution. The mice were anaesthetized and imaged at the predetermined time (0.5, 2, 4, 8, 12 and 24 h) after intravenous injection using the Maestro in magnetic resonance imaging (NightOWLII LB983; Berthold, Bad Wildbad, Germany).

Statistical analysis

All experiments were repeated three times, and all data were expressed as mean ± SD. The in vivo release and MTT data were analysed using Mann–Whitney U-test. The nonparametric Kruskal–Wallis test was used to analyse the difference of tumour volume among the control group GCK and GCKT-liposomes group, followed by Dunn’s post hoc test. The value of \( P < 0.05 \) was considered significant.

Result

Characterization of GCKT-liposomes

The particle size distribution curves for the samples were unimodal, and the mean diameter is approximately 119.3 ± 1.4 nm (Figure 2a and b). The zeta potential of GCKT-liposomes was found to be negatively charged at minus 1.9 ± 0.4 mV. The drug encapsulation efficiency (EE%) of the GCK formulation was of above 98.4 ± 2.3%. The picture of TEM showed that the GCKT-liposomes were smooth surface and spherical nanoparticles.

Figure 2c showed the accumulated percentage release of GCK from GCKT-liposomes and the GCK solution in phosphate-buffered saline (PBS, pH 7.4) containing 0.1% (w/v) Tween 80. The GCKT-liposomes showed controlled release for more than 1 day without any burst release. The percentage of GCK released from GCKT-Liposome and free GCK was 32.1 ± 2.6% and 93.4 ± 1.5%, respectively (Figure 3). Compared with the free GCK, release behaviour from the GCKT-liposomes was significantly (\( P < 0.05 \)) sustained.

MTT assay

In vitro cytotoxicity of the GCKT-liposomes was examined with MTT assay on A549 cells. The results indicated that the GCKT-liposomes has smaller IC₅₀ (16.3 ± 0.8 μg/ml) values compared with GCK (24.9 ± 1.0 μg/ml), suggesting the GCKT-liposomes could significantly increase the cytotoxicity of GCK against A549 cell (\( P < 0.05 \)). The main reason probably is the enhancement of the ability of the drug into the cells. Additionally, T-liposomes without drug are non-cytotoxic to A549 cells.

In vitro cellular uptake assay

The quantitative cellular uptake of A549 cells after 1, 2 and 4-h incubation with the C₆⁻T-liposomes was shown in Figure 4. It displayed that there was a trend of increase for C₆⁻T-liposomes uptake by the increase of incubation time. Among the different time periods, it was clearly noticed that the C₆⁻T-liposomes achieved the appreciably higher cellular uptake than free drug. In addition, the cellular uptake
of C₆T-liposomes was higher than free coumarin-6 during the same time. The microscopic imaging of A549 cells after 2-h incubation with the free coumarin-6 and C₆T-liposomes was shown in Figure 5. It can be observed that the green fluorescence intensity of C₆T-Liposome in the A549 cells was stronger than that of the free coumarin-6. This demonstrated that the cell uptake of the coumarin-6 was enhanced by C₆T-liposomes.

**In vivo antitumour efficacy**

The *in vivo* antitumour effect of GCK delivered by GCKT-liposomes was been demonstrated in athymic nude mice bearing tumour. The tumour volume time curve and weight analysis are shown in Figure 6. The tumour volume kept increasing treated with saline, and the tumours of free GCK group grew up slowly compared with control group, signifying GCK was efficient at inhibiting tumour growth. Moreover, the mean tumour size of GCKT-liposomes group was 219.0 ± 17.0 mm³ at initial and 45.8 ± 3.2 mm³ at the end of the study, which was extremely lower than that of the control group (Figure 6a). It was indicated that the GCKT-liposomes was shown extremely effective suppression of tumour volume compared to the saline group (*P* < 0.05).

To estimate the adverse effects of the GCKT-liposomes formulations, the weight of mice was also recorded during the treatment and the results are shown in Figure 6b.
It is observed that the average body weight of the GCKT-liposomes group was shown slow increase in the initial days and unchanged afterwards, indicating the GCKT-liposomes had a low toxicity on mice. In addition, the body weights of mice in the control group slowly increased in the initial 9 days, which could be related to the increasing tumour weight, and slightly decreased afterwards, which should be attribute to the unhealthy caused by tumour growth.

In vivo targeting imaging of GCKT-liposomes

To investigate whether GCKT-liposomes have indeed specifically targeting ability in vivo, the T-liposomes were labelled with DiR dye and then injected into the tail vein of the tumour-bearing nude mice. As shown in Figure 7, the DiRT-liposomes were concentrated in the liver at the first 0.5 h. The fluorescence intensity in tumour tissue was steadily enhanced at 2 h and reached the maximum at 8 h. Thereafter, fluorescence signals at the tumour site were clearly observed and maintained for 24 h. Therefore, GCKT-liposomes could delivery GCK competently into tumour site, enhanced its permeability and retained it in the tumours cells in vivo.

Discussion

Among antitumour drugs, the difficulties reaching into the tumour site are one of the major restrictions of antitumour effect. In this article, the pharmacological efficacies of GCK have been limited due to the poor tumour-targeting, thus limiting the clinical treatment.

This study was intended to investigate whether GCKT-liposomes can enhance antitumour activity of GCK. Here, several missions have been completed: (1) the cellular uptake of the fluorescent coumarin-6 was enhanced by C6T-liposomes in A549 cells; (2) the GCKT-liposomes strategy can enhance the cytotoxicity of GCK on A549 cells; (3) a significant tumour inhibition effect of GCKT-liposomes was observed in tumour-bearing nude mice; (4) GCKT-liposomes showed a low toxicity in tumour-bearing nude mice.

Membrane bound active transport systems play an important role in the transport of drugs across biological barriers. TPGS could mediated multidrug resistance (MDR) by modulating the P-glycoprotein (P-gp) pump, which is energy-independent processes of drug transport.[25] Phospholipid in the liposomes as an important component of the cell membrane can sustain cell membrane fluidity with a great biocompatible and non-cytotoxic, which can be used to improve the permeability and enhanced ADME (adsorption, distribution, metabolism...).
and excretion) process.\textsuperscript{[33]} It has been proved that GCK underwent strong P-gp-mediated efflux.\textsuperscript{[15]} In lighting of these findings, TPGS and phospholipid were firstly be used as a carrier for GCK to increase the antitumour activity in the present study. As expected, data obtained from MTT study imply that the GCKT-liposomes is 34.6\%\%\% more efficient than GCK, and the cellular uptake of GCK was enhanced by the GCKT-liposomes. In addition,

Figure 6  \textit{In vivo} antitumor study of GCKT-liposomes in nude mice implanted with A549 cells. Tumor volumes (a) and body weight (b) were monitored daily. Kruskal–Wallis test revealed that there was a significant difference of tumor volume among the three groups (\(P < 0.05\)). Post hoc Dunn's test showed that the tumor volume between the ‘Control’ and ‘GCKT-liposomes’ are different at 15 day (\(P < 0.05\)). The results were presented as the mean ± SD (n = 6).

Figure 7  Fluorescence images of the mice bearing A549 cells on right sides at different time points after intravenous injection of DiRT-liposomes.
time-dependent behaviour existed in the process of cellular uptake of GCK.

Furthermore, the poor solubility of GCK is another limitation for the enhancement of efficacy. The TPGS–phospholipid complex ratio was changed to 3 : 7 (w/w) and the solubility of GCK increased to 1.0 mg/ml. This might be attributed to the increase in hydrophobic interactions between the aromatic ring of TPGS and phospholipid, which can be beneficial to the stability of hybrid nanoparticles.[30] The size of particles in drug delivery is essential for antineoplastic agents, and particle with appropriate size can accessed the target site accurately. Particles ranging from approximately 10 to 200 nm can selectively leave the tumour vessels and accumulate in the interstitial space and tissues of the tumour based on the EPR effect, which did not existed in normal tissues.[34] The diameter of GCKT-liposomes was about 119.3 ± 1.4 nm, and microscopy of GCKT-liposomes revealed that its surface was spherical and smooth. This obvious stable structure may be due to the steric hindrance between TPGS and phospholipid, which has confirmed by Cheow’s report. The stability of T-liposomes was improved by the fact that the hydrophilic PEG1000 head of TPGS extend outwards into the aqueous phase and the lipophilic end embedded into the nanoparticles (Figure 8).[35] Such suitable and stable characteristic can extend the residence time in tumour tissues. Thus, the in vivo activity of GCKT-liposomes may also be improved by the augmented accumulation in tumour tissues. In vivo imaging study was designed to demonstrate the above viewpoint, and the results indicated that the DirT-liposomes could maintain longer time at the tumour site and GCKT-liposomes could extremely enhance the antitumour efficacy of GCK.

In summary, combined effect of the enhancement of absorption and the passive targeting by phospholipid as well as the enhanced sensitization of MDR tumour cells by TPGS to GCK could be the major reasons for the significant repression of tumour growth for GCKT-Liposome.

Conclusion

In this study, GCKT-Liposome composed of phospholipid and TPGS was prepared to entrap the poorly soluble drug of GCK. The GCKT-liposomes formulated with higher encapsulation efficiency of 98.4 ± 2.3% and an
uniform particle size around 119.3 ± 1.4 nm. In vitro release of GCKT-liposomes can achieve a relatively controlled release. The GCKT-liposomes also could significantly enhance the cellular uptake and its cell cytotoxicity in A549 cells, as well as exhibited higher antitumour efficacy in tumour-bearing athymic nude mice compared with free GCK. In conclusion, this study has identified that GCKT-liposomes could considerably enhance antitumour and targeting efficacy, which may be apotential that GCKT-liposomes could considerably enhance anticancer activity in Hepato cells, as well as exhibited higher antitumour efficacy in tumour-bearing athymic nude mice compared with free GCK. In conclusion, this study has identified that GCKT-liposomes could considerably enhance antitumour and targeting efficacy, which may be apotential.

**Declarations**

**Conflict of interest**

The Authors declare that they have no conflict of interests to disclose.

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**References**


24. Prashant C *et al.* Super paramagnetic iron oxide – loaded poly (lactic acid)-D-alpha-tocopherol polyethylene glycol 1000 succinate copolymer
nanoparticles as MRI contrast agent. *Biomaterials* 2010; 31: 5588–5597.


