Liposome encapsulated luteolin showed enhanced antitumor efficacy to colorectal carcinoma

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Abstract. Luteolin is a falconoid compound that is present in various types of plants and possesses remarkable potential as a chemopreventive agent. However, the poor aqueous solubility of luteolin limits its clinical application. In the present study, an approach towards chemoprevention was explored using liposomes to deliver luteolin, and the antitumor efficacy was investigated in colorectal carcinoma. The present findings demonstrated that luteolin was efficiently encapsulated into liposomes with an encapsulation efficiency as high as 90%. The particle size of the liposomal luteolin (Lipo-Lut) and ζ-potential were optimized. In vitro studies demonstrated that Lipol-Lut had a significant inhibitory effect on the growth on the CT26 colorectal carcinoma cell line compared with free luteolin (Free-Lut). The in vivo study indicated that Lipo-Lut could achieve superior antitumor effects against CT26 tumor compared with luteolin alone. The present results suggested that liposome delivery of luteolin improved solubility, bioavailability and may have potential applications in chemoprevention in clinical settings.

Introduction

Colorectal carcinoma (CRC) is the third most common form of cancer in the world, and the rectum exhibits common internal malignancies (1). In China, CRC ranks 5th among cancer deaths, with increasing the incidence annually (2). Although there are treatment options including radiotherapeutic, chemotherapeutic regimes and surgical regimens available for the clinical management of CRC, outcomes of such strategies are limited by associated high probability of cancer recurrence, obvious toxicity on the human body, affecting neurotoxicity, gastrointestinal reaction, kidney failure, and cardiotoxicity (3,4). Chemoprevention is a strategy that was first proposed by Sporn et al (5). It is a way to reverse, suppress, or prevent molecular or histologic premalignant lesions from progressing to invasive cancer by using natural or synthetic agents (6). To date, many food/plant derived chemopreventive agents that exhibit strong efficacy against various cancers in vitro and preclinical models have been identified (7-10). Colorectal carcinoma is a rationale cancer target for chemoprevention studies due to high incidence of pre-neoplastic lesions and cancerous tumors (11). An ideal chemopreventive compound should be nontoxic, potent, highly effective, less expensive, and easily available (4).

Luteolin (3',4',5,7-tetrahydroxyflavone), a flavonoid polyphenolic compound found in many plant types such as fruits, vegetables, and medicinal herbs. It has been shown biological activities, such as anti-inflammatory, anti-allergy, and anticancer activities (12-14). Recent studies have reported the anticancer effects of luteolin against lung cancer, head and neck cancer, prostate, breast, colon, liver, cervical, and skin cancer was associated with inducing apoptosis, suppressing metastasis, and angiogenesis (15-22). These results of above studies warrant the further evaluation of the chemopreventive potential of luteolin in human subjects (4). However, it has very low bioavailability after oral administration and it is very difficult to make intravenous or intraperitoneal administration because of its poor aqueous solubility. Therefore, there is a clear need to increase its potential in clinical application (23).

During the past few years, liposomes have drawn much attention for cancer therapy because of longer blood circulation time, higher biocompatibility, excellent bioavailability, and higher tumor-specific delivery (24-27). Thus, in the present study, we investigated whether liposomes can be used as delivery system to improve the antitumor efficacy of luteolin in vitro and in vivo. We used CT26 cell and mouse tumor model to evaluate the activity of luteolin before and
after encapsulating into liposome. Meanwhile, we established a liposome-formulated luteolin that is capable of effective suppressing tumor growth through inducing apoptosis and inhibiting angiogenesis. It is our hope that understanding of these mechanisms in detail may provide a basis for novel targeting strategies for cancers.

Materials and methods

**Liposome preparation.** Lipo-Lut formulations were prepared by the thin film hydration method. Briefly, the mixtures of luteolin/cholesterol/lecithin (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) in 1:2:7 weight ratios were dissolved in ethanol and were transferred into a round bottom flask. The flask was then connected to a rotary evaporator at 50 rpm and water bath with temperature maintained at 40°C. Vacuum was applied to the flask to evaporate the ethanol and form a homogeneous lipid film on the flask wall. Then the lipid film was then hydrated in normal saline by rotating the flask at 37°C until the lipid film was completely hydrated. At last the luteolin liposome was sonicated with 50 watts of power for 10 min. The empty liposome without luteolin was prepared in the same as the Lipo-Lut.

**Size distribution and ζ-potential.** The mean particle sizes and ζ-potential of the obtained liposomes were measured by photon correlation spectroscopy using Malvern Zetasizer 3000 HS (Malvern Instruments, Malvern, UK) at 25°C. The form feature of the liposomes was determined by transmission electron microscopy (TEM) (H-600; Hitachi, Tokyo, Japan) using a negative staining method with 1% sodium phosphotungstate solution for 2 min at room temperature.

**Drug loading and encapsulation efficiency.** The prepared liposomes were solubilized in methanol [liposomes: methanol=1:9, volume/volume (v/v)]. After using a cyclomixer to completely extract the drug from lipid to methanol, the drug content was analyzed by an Agilent 1100 Series HPLC System (Agilent Technologies, Santa Clara, CA, USA). An octadeucylsilyl column (4.6x250.0 mm, 5 µm) was used for the analysis. The mobile phase was a mixture of acetonitrile and 0.1% formic acid (30:70, v/v), and the flow rate was 0.5 ml/min. The UV detection wavelength of 350 nm was used, the column temperature was 35°C. The amount of soluble unencapsulated drug was measured by ultrafiltration using centrifugal filter tubes with a molecular weight cut-off of 300 kDa (Millipore, Carrrigtowihll, Ireland). Drug loading and encapsulation efficiency were calculated using following equations 1 and 2, respectively. The data were obtained using three different batches of liposome preparations.

\[
\text{Drug loading (\%) = } \frac{C_s}{C_{\text{lipid}}} \times 100\% \quad (1)
\]

\[
\text{Encapsulation efficiency (\%) = } \frac{C_s}{C_{\text{total}}} \times 100\% \quad (2)
\]

Where \(C_s\) represents drug mass in liposome, \(C_{\text{lipid}}\) represents total liposome mass, and \(C_{\text{total}}\) represents total drug mass.

**Drug release.** The release of luteolin from liposomes in vitro was measured with a dialysis method. Lipo-Lut solution (0.5 ml) and Free-Lut (0.5 ml) in dimethyl sulphoxide (DMSO) solution were placed in a dialysis bag (MW cut off: 3500 Da). After that, dialysis bag was placed in a 50 ml PBS supplemented with 0.5% Tween-20 (PBST). This bottle was introduced in a shaking incubator with stirring speed of 100 rpm at 37°C. At specific time intervals (0, 30 min, 1, 2, 4, 6, 8, 12, 24, 48, 72, 96, 120 h), samples were withdrawn and replaced with an equal volume of medium. The amount of luteolin released at each time point was determined using HPLC, as described earlier. All assays were performed in triplicate.

To measure in vivo pharmacokinetics, BALB/c mice, weighting 18-20 g were randomly divided into two groups (six animals per time-point) for treatment with Free-Lut or Lipo-Lut at a dose of 50 mg/kg body weight via intravenous injection. After dosing, blood was immediately collected via cardiac puncture at 5, 15, 30, 45, 1, 2, 4, 6, 8, 10, 12, 24 h and centrifuged at 8,000 rpm for 10 min to separate the plasma. The diphenhydramine was added to plasma samples as the internal standard, and acetonitrile as protein precipitator. The drug was then extracted from plasma samples and processed for HPLC analysis to determine luteolin levels.

**MTT assay.** The murine CT26 colorectal carcinoma cell line was obtained from the ATCC, and it was maintained in RPMI-1640 (Sigma-Aldrich; Merck KGaA) supplemented with 10% fetal bovine serum (Invitrogen Life Technologies, Carlsbad, CA, USA) in a 5% CO₂ incubator at 37°C. The inhibition of luteolin to CT26 cell was determined by the MTT assay. Briefly, cells in the logarithmic growth phase (3x10⁵ or 5x10⁵/well) were placed in wells of a 96-well plate at 37°C overnight. Cells were then treated with various concentrations of Free-Lut or Lipo-Lut and cultured for 24. After an additional 3 h of culture with 0.5 mg/ml MTT at 37°C, 150 µl DMSO was added to each well to dissolve formazan crystals. The cells which received only the medium containing 150 µl DMSO served as the control group. All of samples were analyzed using a microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The test was repeated three times. The cell viability was assessed as a percentage of the absorbance present in the drug-treated cells compared to that in the control cells.

**Assessment of apoptosis.** The apoptotic rate of CT26 cells were detected by flow cytometry using the Annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). CT26 cells cultured in 6-well plates were treated with various concentrations of Free-Lut or Lipo-Lut, along with no drug treatment as control. After incubation for 24 h at 37°C, cells were trypsinized, washed and resuspended in PBS. The cells were subsequently treated with Annexin V-FITC and PI in the dark for 15 min. Flow cytometry analysis was performed with an EpicsXL Coulter flow cytometer (Beckman Coulter, Fullerton, CA, USA) and repeated thrice.

**Mouse tumor model.** Female BALB/c mice, 6 weeks old and weighting 18-20 g, was obtained from Beijing HFK Bioscience Co., Ltd. (Beijing, China). The mice were kept in specific pathogen free (SPF) conditions for 1 week prior to start of experimental procedures. All the animal experiments were evaluated and approved by the Animal and Ethics Review
The murine tumor models were established by subcutaneous inoculation in the right flanks of female BALB/c mice with CT26 cells (1x10^6/mouse). The growth of the tumor was monitored, and tumor volumes were calculated from vernier calipers every 3 days, following the formula of 0.52 x length x width^2. The drug treatments were initiated when tumors had reached an average volume of 100 mm^3, which occurred around day 7 post-cell inoculation. The luteolin dose administered to the mice was 50 mg/kg. The mice were randomly assigned into four groups (six animals per group). Each group was respectively treated with normal saline (NS), empty liposome (EM-Lipo), Free-Lut, and Lipo-Lut via the tail vein every 2 days a total of five times. After the last treatment, the mice were sacrificed on the day 22, and the tumors were excised, weighed and fixed in 10% neutral buffered formalin solution or frozen at ‑80˚C.

Hematoxylin and eosin (H&E) staining and immunofluorescence staining. CT26 xenograft specimens were fixed in 4% paraformaldehyde for 12 h and embedded in paraffin. Sections (5-µm thick) were cut, dewaxed, rehydrated, and stained with H&E. To observe the inhibitory effect on neovascularization, the frozen tissues were sectioned (5 µm) and fixed in acetone. The tissues sections were incubated with monoclonal anti-CD31 antibody (Santa Cruz Biotechnology, Inc.) at 4˚C overnight, flowing stained with a secondary goat antibody (FITC). The number of microvessels per high-power field was counted in sections. The immunofluorescence of Ki-67 expression in tumor was done as follows: Tissue sections were incubated with monoclonal anti-Ki67 antibody followed by incubation with FITC labelled goat anti-mouse secondary antibody (Santa Cruz Biotechnology, Inc.). Tissue apoptotic cells were detected with TUNEL Detection kit (Promega, Madison, WI, USA), according to the manufacturer’s instructions. All the slides were evaluated by fluorescence microscope. Five areas were randomly selected from each slide for analysis.

Statistical analysis. The SPSS statistics 17.0 (SPSS, Inc., Chicago, IL, USA) were used for statistical analysis. Quantitative data were expressed as the mean ± standard deviation of three independent experiments and analyzed by one-way ANOVA. Comparison between the groups was made by analyzing data with S-N-K method. A P-value <0.05 was considered to indicate a statistically significant difference.

Results

Characterization of Lipo-Lut. Our results demonstrated the successful application of the thin-film hydration method to formulate the water soluble Lipo-Lut. As shown in Fig. 1, dynamic light scattering results showed that the diameter of Lipo-Lut was around 105 nm. The ζ-potential of Lipo-Lut was 0.12 mv. The morphology of Lipo-Lut was observed using TEM, most Lipo-Lut was spherical and had a regular shape. Free-Lut appeared stratified in water, had apparent precipitation in the bottom of the bottle. Lipo-Lut can be stably suspended in water solution. Consequently, the drug loading and encapsulation efficiency were found to be 10 and 90%, for the Lipo-Lut formulation.
Drug release and Pharmacokinetics of Lipo-Lut. The *in vitro* release (Fig. 2A) results showed that the luteolin can be released slowly from the liposomes and then Free-Lut was released very quickly. The cumulative percentage release demonstrated that the amount of drug released from liposomes was gradually increased over time, and after 120 h there was an increase of over 80%. The free drug exhibited high level (80%) at 8 h.

To assess whether the liposome improved bioavailability of poor water soluble drugs, the mean plasma concentration-time profiles of luteolin after intravenous administration of free and liposome drug were presented in Fig. 2B. Free-Lut was rapidly cleared and the plasma levels of luteolin were less than 50% of the injected dose within 5 min of injection. Compared to free drug, luteolin concentration in plasma was almost 10-fold higher for Lipo-Lut at 2 h after drug injection, a result that was most evident at time-points beyond 1 h. The results demonstrated that liposomal encapsulation reduced drug elimination.

**Lipo-Lut demonstrated better tumoricidal effect on colorectal cancer cells than the Free-Lut.** Lipo-Lut and Free-Lut were evaluated for inhibition against CT26 cells using MTT assay. The results (Fig. 3) clearly established that Lipo-Lut exhibited potent inhibitory effect, as similar to Free-Lut. Further, both drugs showed dose-dependent inhibition of cell growth. After the incubation of the cells with Lipo-Lut or Free-Lut for 24 h, the Lipo-Lut showed significantly higher inhibition compared to the Free-Lut at all the concentrations tested. In contrast, the empty liposomes did not show any toxicity to the cells (data not shown). These data indicated that Lipo-Lut inhibited tumor proliferation *in vitro*.

Next, we determined whether the inhibitory effect of Lipo-Lut involved the initiation of apoptosis. A flow cytometry analysis of Annexin V staining for phosphatidylserine, an early apoptosis marker, was performed for evaluation of apoptotic cells after incubating CT26 cells with Lipo-Lut or Free-Lut at various concentrations. The cells were collected and stained with Annexin V-FITC and PI. When the cells undergoing apoptosis, the phosphatidylserine normally located in the inner leaflet of the cellular membrane translocates to the outer leaflet of the plasma membrane at the early stages of apoptosis, which can be labeled with Annexin V-FITC. Viable cells with intact membranes exclude PI whereas the membranes of dead and damaged cells are permeable to PI. CT26 cells were divided into four groups, ie, necrotic cells (upper left quadrant, Annexin V+/PI+), healthy viable cells (lower left quadrant, Annexin V-/PI-), cells in the early apoptosis stage (lower right quadrant, Annexin V+/PI-), and cells that are in late apoptosis or already dead (upper right quadrant, Annexin V+/PI+). As shown in Fig. 4, more than 95% of control CT26 cells (control) were viable whereas all the cells incubated with Lipo-Lut or Free-Lut displayed evidence of apoptosis. The percentage of early apoptotic cells and late apoptotic cells in Lipo-Lut or Free-Lut were showed dose dependence. Meanwhile, the extent of apoptosis in the Lipo-Lut group was significantly higher than Free-Lut group.

**Lipo-Lut demonstrated better tumoricidal effect on the mouse tumor model than the Free-Lut.** To validate antitumor efficiency of Lipo-Lut *in vivo*, we used a CT26 colorectal carcinoma graft model in BALB/c mice (Fig. 5). Fig. 5A displayed the tumor volume of each group during the 22-day treatment. Compared to the NS and EM-Lipo, the final tumor volume of mice treated with luteolin was notably reduced. The Lipo-Lut group exhibited the most significant inhibitory efficacy compared with the other groups. There was no significant difference between NS group and EM-Lipo group. At the end
of the treatment, the tumor weights were obtained (Fig. 5D), the results were consistent with the tumor volumes at the end of the treatment. The smallest tumor weights were observed in the Lipo-Lut group among the four groups. These results indicated that the treatment of Lipo-Lut resulted in a robust efficacy in reducing tumor volume. Body weights were not significantly different in the four groups throughout the whole experiment (Fig. 5B).

**Lipo-Lut induced apoptosis.** To further investigated whether the *in vivo* antitumor effects of the Lipo-Lut were associated with enhanced induction of the apoptotic cells, TUNEL assay was applied. As shown in Fig. 6, a significant number of apoptotic cells appeared with green fluorescence in the tumor tissue of luteolin-treated mice. Treatment with Lipo-Lut clearly produced more pronounced apoptotic cells than treatment with the Free-Lut. These results suggested that Lipo-Lut inhibited tumor growth probably through induction of tumor cellular apoptosis.

**Lipo-Lut inhibited tumor vascularization.** We performed immunofluorescence analysis with anti-CD31 monoclonal antibody to observe the new vasculature content in frozen tumor sections (Fig. 7). As shown in Fig. 7D, CD31-positive endothelial cells in luteolin treated groups had weaker fluorescence than those of NS group and EM-Lipo group (Fig. 7A and B). The results from the determination of microvessel numbers (Fig. 7E) revealed that there were significantly less number of microvessels present in the Free-Lut group and Lipo-Lut group compared to the control group (P<0.01). In addition, Lipo-Lut group reduced number of microvessels more remarkably compared with Free-Lut groups (P<0.01). No significant difference was observed between NS group and EM-Lipo group (P>0.05).

**Lipo-Lut decreased Ki-67 expression.** The expression of Ki-67 is strictly associated with tumor cell proliferation and growth, and is widely used in routine pathological investigation as a proliferation marker and a diagnosis tool (28).
Immunofluorescence examination of Ki-67 staining revealed a greater inhibition of proliferating cells in the Lipo-Lut-treated mice than other groups (Fig. 8).

Discussion

The present study demonstrated that liposome-encapsulated luteolin exerted stronger tumor growth-inhibition than free luteolin both in vitro and in vivo. Liposome have been widely used as potential drug delivery systems (DDS) for delivery of anti-cancer agents (24,29). Over the past few decades, several liposome-encapsulated drugs have been approved for clinic applications. The best-known formulation is liposome-encapsulated doxorubicin, marketed as Doxil or Caelyx (30-32), which produced less cardiotoxicity than free doxorubicin while providing comparable antitumor activity (33,34). In our study, we have prepared water soluble Lipo-Lut, sized ~105 nm. According to the cancer type, the size of the gaps between the endothelial cells of the tumor capillaries ranges from 100 to 780 nm, as opposed to that in a typical normal endothelium of 5-10 nm (35,36). Liposomes of about 100 nm in diameter have been demonstrated to be optimal for the delivery of anticancer drugs to tumors (24,37), which readily translocate across the capillary endothelium. Liposomes are known to be safe and well tolerated delivery system. Many researchers demonstrated that the actions of...
some poor water soluble drugs were evidently enhanced after they were encapsulated into liposome (38,39). This study showed that Lipo-Lut could prolong the drug release. Meanwhile, the concentration in plasma-time profiles indicated that the bioavailability of Lipo-Lut has been improved.

In order to study the antitumor efficacy of Lipo-Lut in vitro, we initially compared the efficacy of Free-Lut and Lipo-Lut on CT26 cell growth inhibition by MTT assay and apoptosis by flow cytometry. We observed that Lipo-Lut exhibited more effectively than Free-Lut. This prompted us to further evaluate the antitumor activities of Lipo-Lut on CT26 cells grafts in BALB/c mice. The results suggested that luteolin encapsulated into liposome exerted stronger tumor growth-inhibiting effects, suppressed angiogenesis, increased apoptosis than Free-Lut. Angiogenesis plays a important role in the progress of tumor growth and invasion. The most essential angiogenic factors is vascular endothelial growth factor (VEGF). It have been reported that luteolin could significantly inhibit VEGF-stimulated endothelial cell proliferation, migration, invasion and tumor angiogenesis by targeting VEGF receptor 2-regulated AKT/ERK/mTOR/P70S6K/MMPs pathway, leading to the inhibition of tumor growth and tumor angiogenesis. A study from Norhaizan suggested that luteolin induced apoptosis in colon cancer by modulating the expressions of bax, Bcl-2 and caspase 3 in vitro and in vivo. On the other hand luteolin acted against DNA damage and activated DNA repair mechanism in Caco-2 colon cancer cells (40–42). Moreover, Owing to the leaky vasculature of the tumor tissue,
these may provide a channel allowing liposome to more easily target tumor tissue (1). Meanwhile, solid tumors usually lack effective lymphatic drainage. All of these factors lead to the accumulation of liposome in the tumor microenvironment much more than they do in normal tissues (43,44). Most likely because of a combination of these advantages, Lipo-Lut was a marked inhibition of tumor growth over time when compared to Free-Lut in a mouse tumor model. In fact, the present study also indicated that Nano-luteolin showed higher efficacy compared to Free-Lut against lung cancer and head and neck cancer (4). However, Lipo-Lut was unable to completely inhibit tumor growth. In order to gain better therapeutic efficacy, it is necessary to optimize liposome formations and therapeutic scheme.

We prepared a novel antitumor agent using luteoside as the delivery system to encapsulate luteolin. The Lipo-Lut inhibited activity of tumor growth more effectively than the Free-Lut in both CT26 cells and mouse tumor model of colorectal carcinoma. The mechanisms of action of Lipo-Lut appear multifaceted. Firstly, it could directly induce apoptosis of tumor cells. Secondly, tumor angiogenesis were reduced, blocking the nutrition supply into tumor tissue, which promoted apoptosis of tumor cells. Third, Lipo-Lut inhibited tumor proliferation. The results in this study could contribute to the development of chemotherapy for patients with colorectal carcinoma in future clinical applications.

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