Liposome encapsulated luteolin showed enhanced antitumor efficacy to colorectal carcinoma

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Liposome encapsulated luteolin showed enhanced antitumor efficacy to colorectal carcinoma

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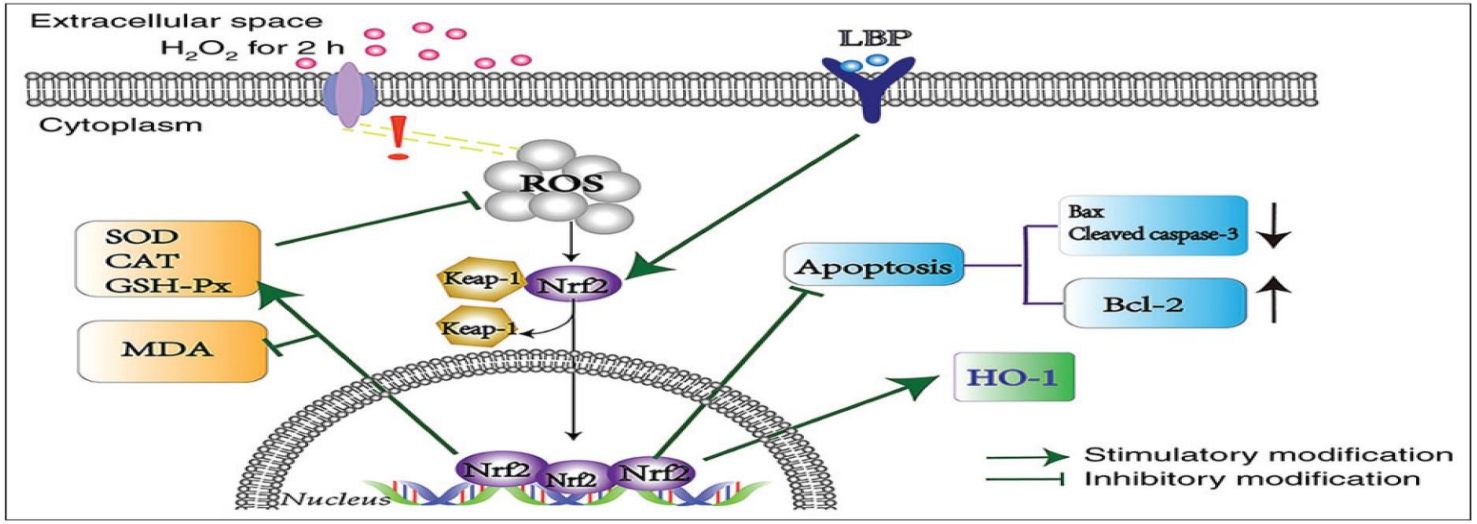
Abstract. Luteolin is a falconoid compound that is present in various types of plants and possesses remarkable potential as a chemopreventive agent. However, the poor aqueous solubility of luteolin limits its clinical application. In the present study, an approach towards chemoprevention was explored 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, Lipo-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 chemo-prevention in clinical settings.

Molecular Medicine Reports

ISSN 1791-2997
eISSN 1791-3004

An international journal devoted to Molecular Medicine

VOLUME 24, NUMBER 5, NOVEMBER 2021



Journal Metrics

Impact Factor: 2.952

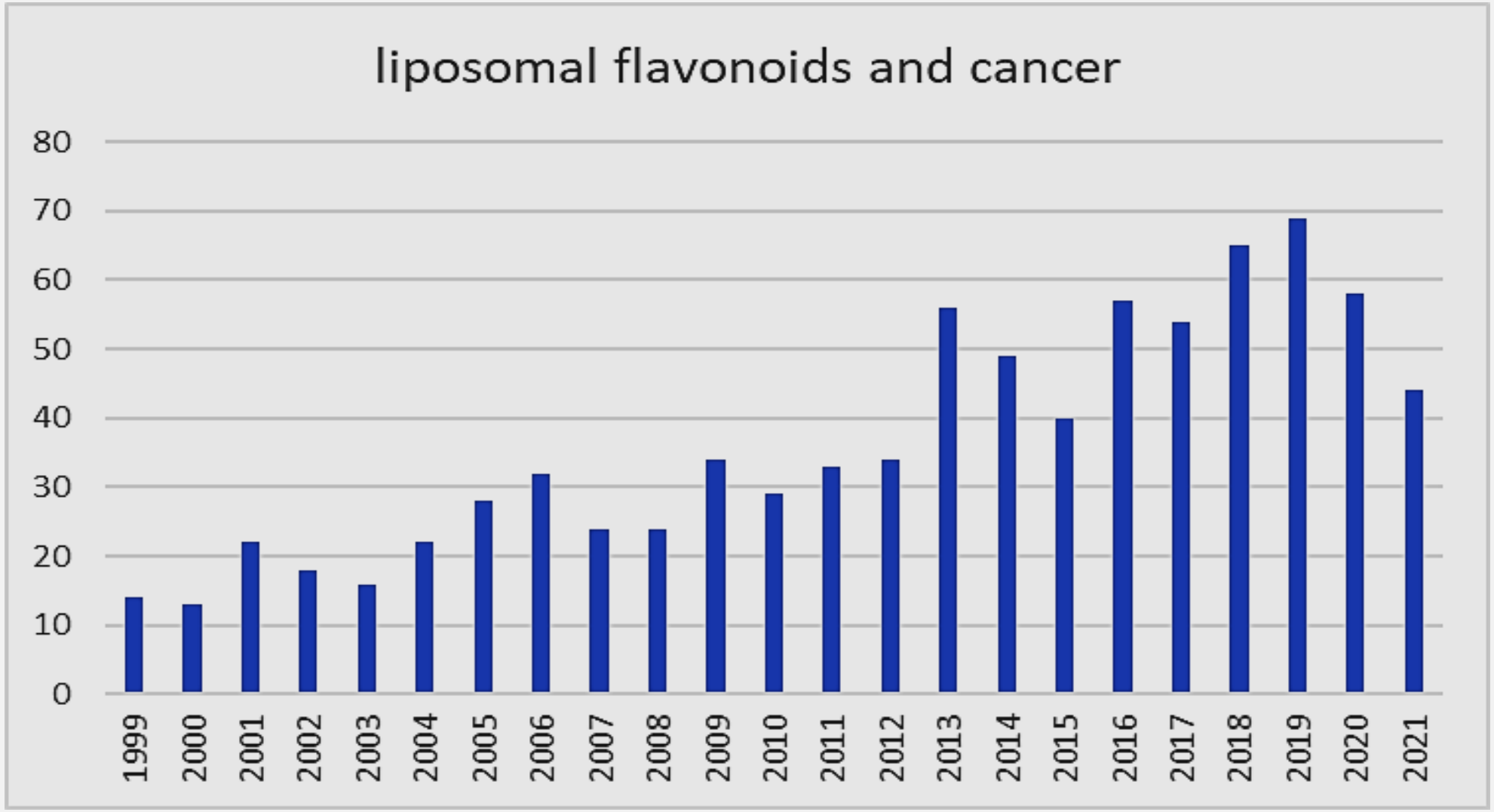
CiteScore: 4.5

CiteScore Rank: Q2: #142/325 Genetics

Source Normalized Impact per Paper (SNIP): 0.679

SCImago Journal Rank (SJR): 0.727

Research trend



Last update:
3/10/2021

introduction

- Colorectal carcinoma (CRC) :
  - third most common form of cancer
  - rectum exhibits common internal malignancies (1).
  - Treatment options :
    - radiotherapy, chemotherapy, surgery
    - outcomes are limited → cancer recurrence, toxicity on the human body, neurotoxicity, gastrointestinal reaction, kidney failure, and cardiotoxicity (2,3).
- Chemoprevention → to reverse, suppress, or prevent molecular or histologic premalignant lesions from progressing to invasive cancer by using natural or synthetic agents (4).

introduction

- CRC → rationale cancer for chemoprevention studies, due to high incidence of pre-neoplastic lesions and cancerous tumors (5).
- ideal chemopreventive compound → potent, highly effective, less expensive, easily available (3).
- Luteolin (3',4',5,7-tetrahydroxyflavone) → flavonoid poly-phenolic compound found in many plant types
- Biological activities → anti-inflammatory, anti-allergy, anti-cancer against lung, head and neck, prostate, breast, colon, skin cancers via inducing apoptosis, suppressing metastasis, and angiogenesis (6,7,8).

introduction

- Luteolin → poor aqueous solubility
  - low bioavailability after oral administration
  - difficult to make intravenous or intraperitoneal administration

- Liposomes:
  - longer blood circulation time
  - higher biocompatibility and excellent bioavailability
  - higher tumor-specific delivery (9,10,11).

Materials and methods

Liposome preparation

- thin film hydration method :

1. mixtures of luteolin/cholesterol/lecithin in 1:2:7 weight ratios
2. dissolved in ethanol, were transferred into a round bottom flask
3. The flask connected to a rotary evaporator at 50 rpm and water bath at 40˚C
4. Vacuum → evaporate the ethanol, form a homogeneous lipid film on the flask wall
5. the lipid film hydrated in normal saline by rotating the flask at 37˚C
6. luteolin liposome was sonicated with 50 watts of power for 10 min

Size distribution and ζ-potential

- mean particle sizes and ζ-potential:
  - photon correlation spectroscopy using Malvern Zeta sizer 3000 HS at 25˚C

- form feature of the liposomes:
  - transmission electron microscope (TEM)
  - negative staining method with 1% sodium phosphotungstate solution for 2 min at 25˚C

Drug loading and encapsulation efficiency

1. prepared liposomes were solubilized in methanol
2. cyclomixer ⟶ extract the drug from lipid to methanol
3. HPLC ⟶ octadecylsilyl column, mixture of acetonitrile and 0.1% formic acid as mobile phase, flow rate at 0.5 ml/min
4. UV detection wavelength at 350 nm

- soluble unencapsulated drug was measured by ultrafiltration using centrifugal filter tubes with a molecular weight cut-off of 300 kDa
- Drug loading and encapsulation efficiency:

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

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

Drug release

- dialysis method : for in vitro
  1. Lipo-Lut and Free-Lut in dimethyl sulphoxide (DMSO) solution placed in a dialysis bag
  2. dialysis bag placed in a 50ml PBS supplemented with 0.5% Tween-20
  3. shaking incubator with stirring speed of 100 rpm at 37˚C
  4. The amount of luteolin released at each time-point was determined using HPLC
- in vitro:
  1. BALB/c mice, treated with Free-Lut or Lipo-Lut, dose of 50 mg/kg body weight via IV injection
  2. Blood collected, centrifuged at 8,000 rpm for 10 min, diphenhydramine added,
  3. drug extracted from plasma samples, HPLC analysis to determine luteolin levels

MTT assay

- CT26 colorectal carcinoma cell line, RPMI-1640, 10% fetal bovine serum, 5% CO2 incubator at 37˚C
- Cells at logarithmic growth phase placed in a 96-well plate at 37˚C overnight
- treated with various concentrations of Free-Lut or Lipo-Lut and cultured for 24, additional 3 h of culture with 0.5 mg/ml MTT at 37˚C
- samples analyzed using a microplate reader

Assessment of apoptosis

- flow cytometry → Annexin V-fluorescein sothiocyanate (FITC) and propidium iodide (PI)
  1. Treated cells incubated for 24 h at 37˚C in 6-well plates
  2. cells were trypsinized, washed and resuspended in PBS
  3. treated with Annexin V-FITC and PI in the dark for 15 min.
  4. Flow cytometry analysis was performed with an EpicsXL Coulter flow cytometer

Mouse tumor model

- Female BALB/c mice, 6 weeks old and weighting 18-20 g
- The murine tumor models established by subcutaneous inoculation in the right flanks of female BALB/c mice with CT26 cells
- The drug treatments were initiated when tumors reached the volume of 100 mm3, day 7 post-cell inoculation, dose of 50 mg/kg
- Groups of normal saline (NS), empty liposome (EM-Lipo), Free-Lut, and Lipo-Lut via the tail vein every 2 days a total of five times
- 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 fixed in 4% paraformaldehyde for 12 h and embedded in paraffin
- Sections (5-μm thick) were cut, dewaxed, rehydrated, and stained with H&E
- inhibitory effect on neovascularization → frozen tissues sectioned (5 μm), fixed in acetone
- tissues sections incubated with monoclonal anti-CD31 antibody, flowing stained with a secondary goat antibody (FITC) , number of microvessels counted
- immunofluorescence of Ki-67 expression → monoclonal anti-Ki67 antibody, FITC labelled goat anti-mouse secondary antibody
- apoptotic cells detected with TUNEL Detection kit

Statistical analysis

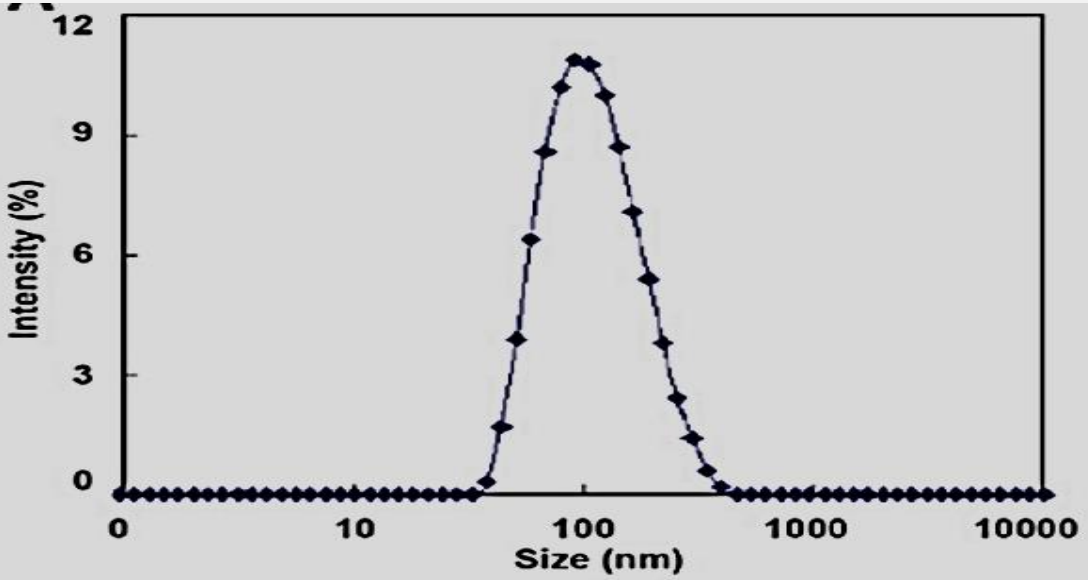
- SPSS statistics 17.0
- Quantitative data expressed as the mean ± standard deviation of three independent experiments and analyzed by one-way ANOVA
- Comparison between the groups was made by analyzing data with S-N-K method
- A P-value <0.05 was considered to indicate a statistically significant difference

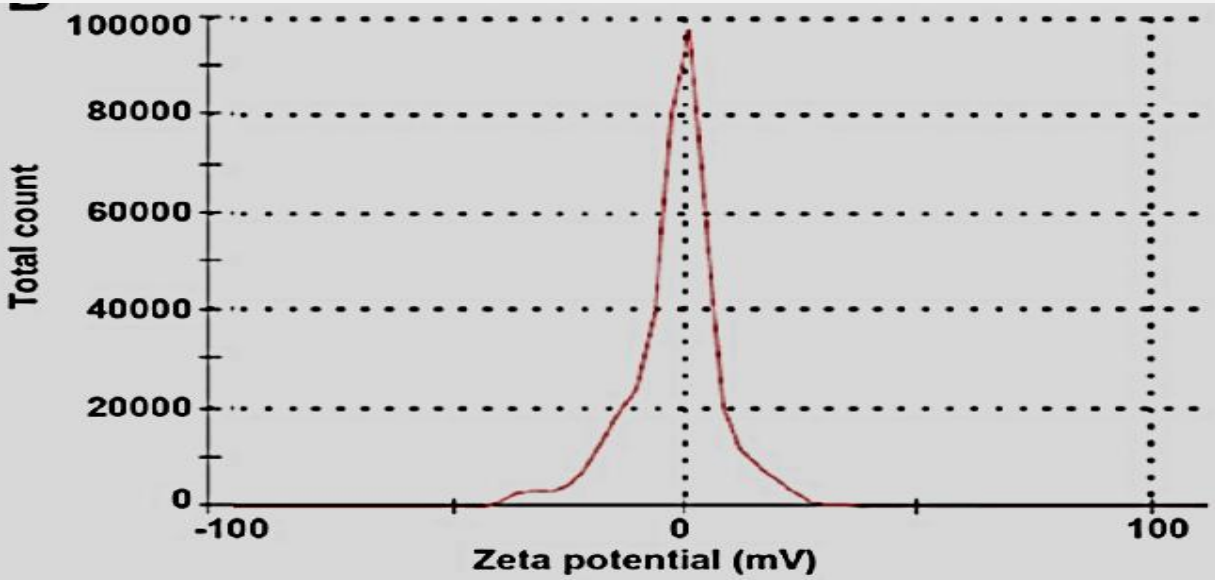
Results

Characterization of Lipo-Lut

- dynamic light scattering results:
  - diameter of Lipo-Lut was around 105 nm
  - ζ-potential of Lipo-Lut was 0.12 mv
- TEM results :
  - Lipo-Lut was spherical and had a regular shape
- Free-Lut ⟶ stratified in water, precipitated in the bottom of the bottle
- Lipo-Lut can be stably suspended in water solution
- drug loading and encapsulation efficiency ⟶ 10 and 90%, for the Lipo-Lut formulation

Characterization of Lipo-Lut





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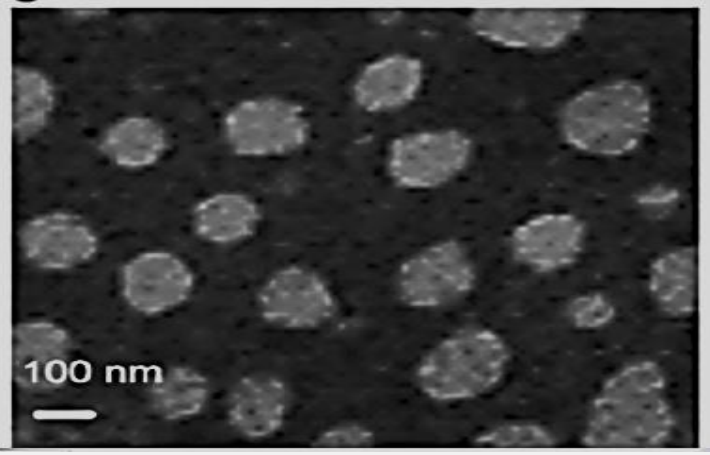

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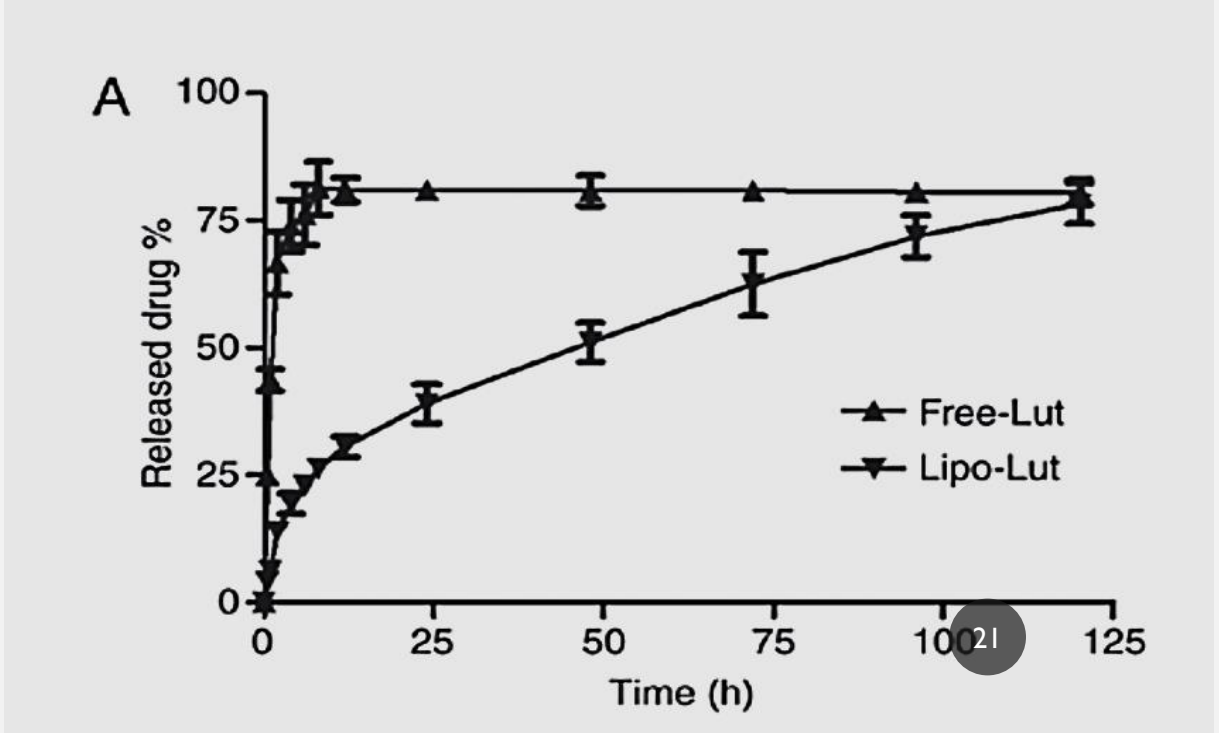
Drug release and Pharmacokinetics of Lipo-Lut

- luteolin released slowly from the liposomes, Free-Lut released very quickly

- cumulative percentage release ⟶ the amount of drug released from liposomes gradually increased over time, after 120 h increased over 80%

- free drug exhibited high level (80%) at 8 h



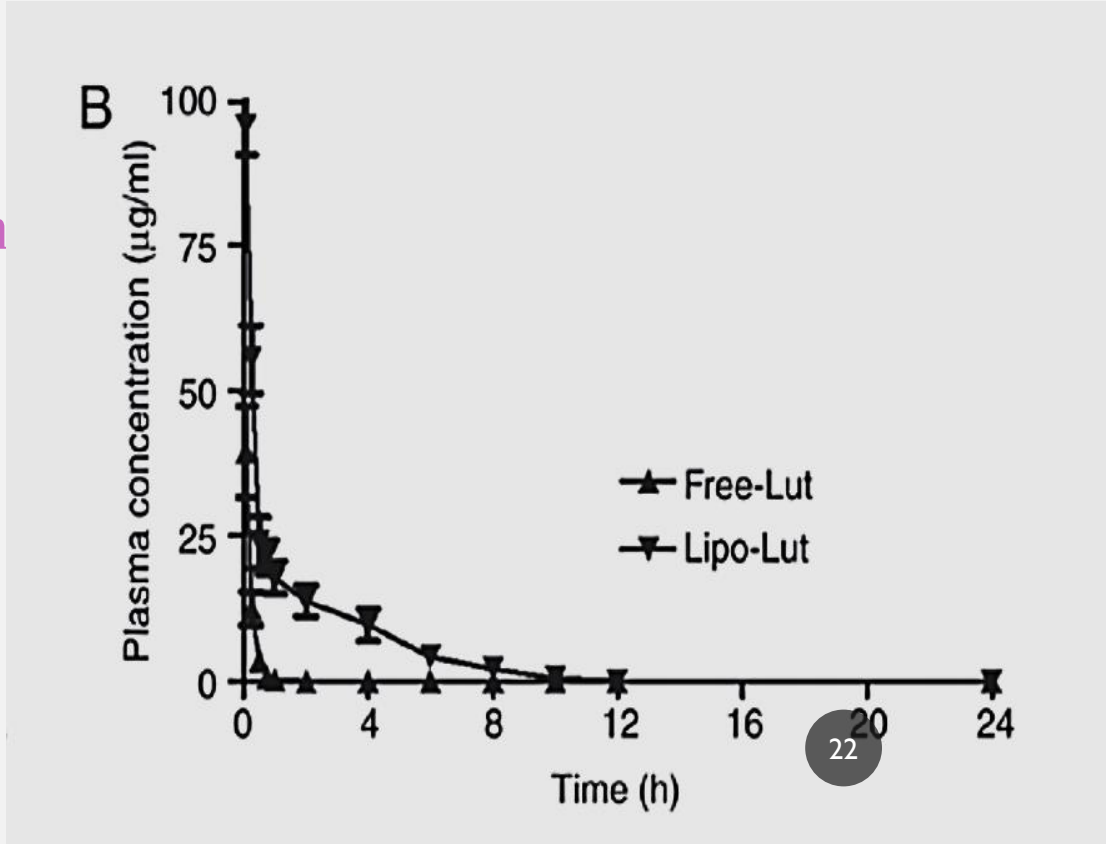
Drug release and Pharmacokinetics of Lipo-Lut

- the liposome improved bioavailability of poor water soluble drugs ⟶ mean plasma concentration-time profiles of luteolin after IV administration of free and liposome drug

- Free-Lut rapidly cleared, plasma levels were less than 50% of the injected dose within 5 min of injection

- luteolin concentration in plasma was almost 10-fold higher for Lipo-Lut at 2 h after drug injection

- liposomal encapsulation reduced drug elimination



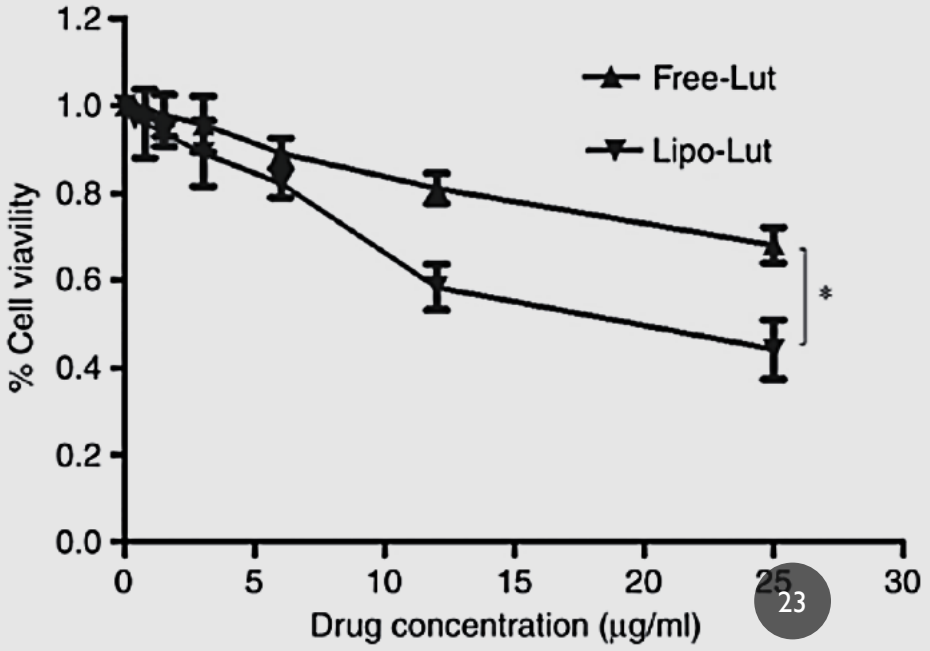
Tumoricidal effects on colorectal cancer cells

- Lipo-Lut exhibited inhibitory effect, as similar to Free-Lut, both drugs showed dose-dependent inhibition of cell growth

- After 24h of incubation, Lipo-Lut showed higher inhibition compared to the Free-Lut at all the concentrations

- empty liposomes did not show any toxicity

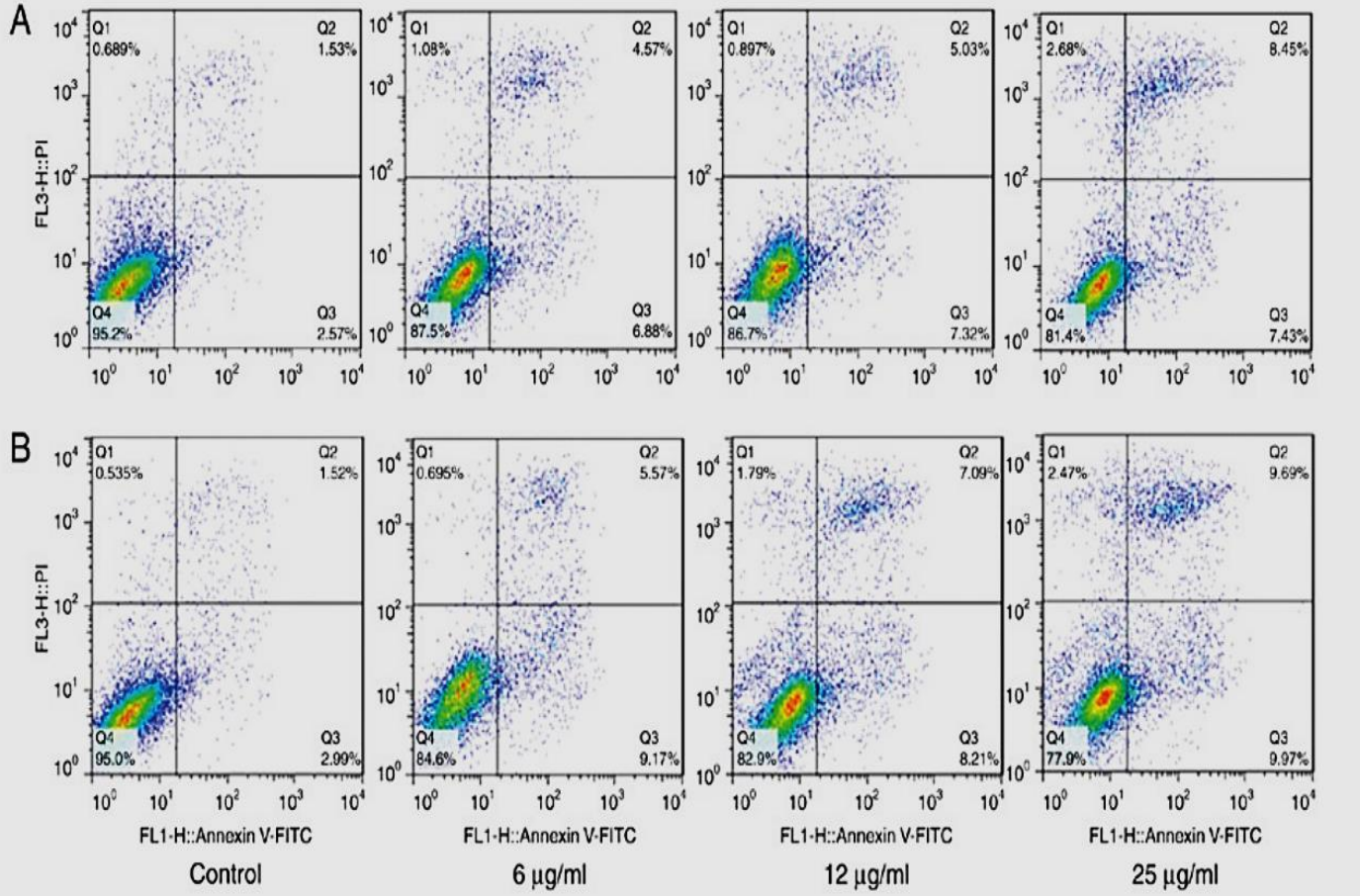
- Lipo-Lut inhibited tumor proliferation in vitro

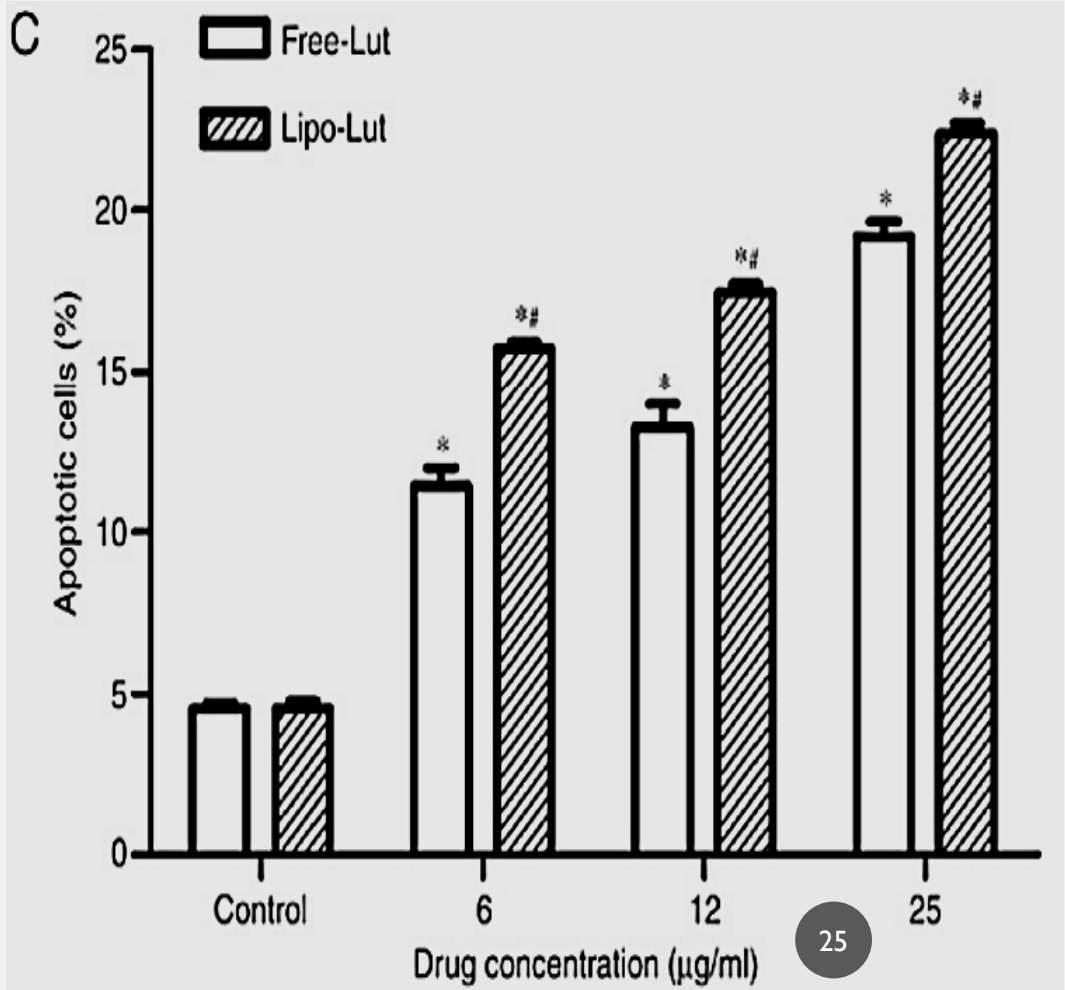


Tumoricidal effects on colorectal cancer cells

- the inhibitory effect of Lipo-Lut involved the initiation of apoptosis
  - flow cytometry analysis of Annexin V staining for phosphatidylserine
- CT26 cells were divided into four groups:
  - 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-)
  - cells that are in late apoptosis or already dead (upper right quadrant, Annexin V+/PI+)
- more than 95% of control CT26 cells were viable, all the cells incubated with Lipo-Lut or Free-Lut displayed evidence of apoptosis

Tumoricidal effects on colorectal cancer cells

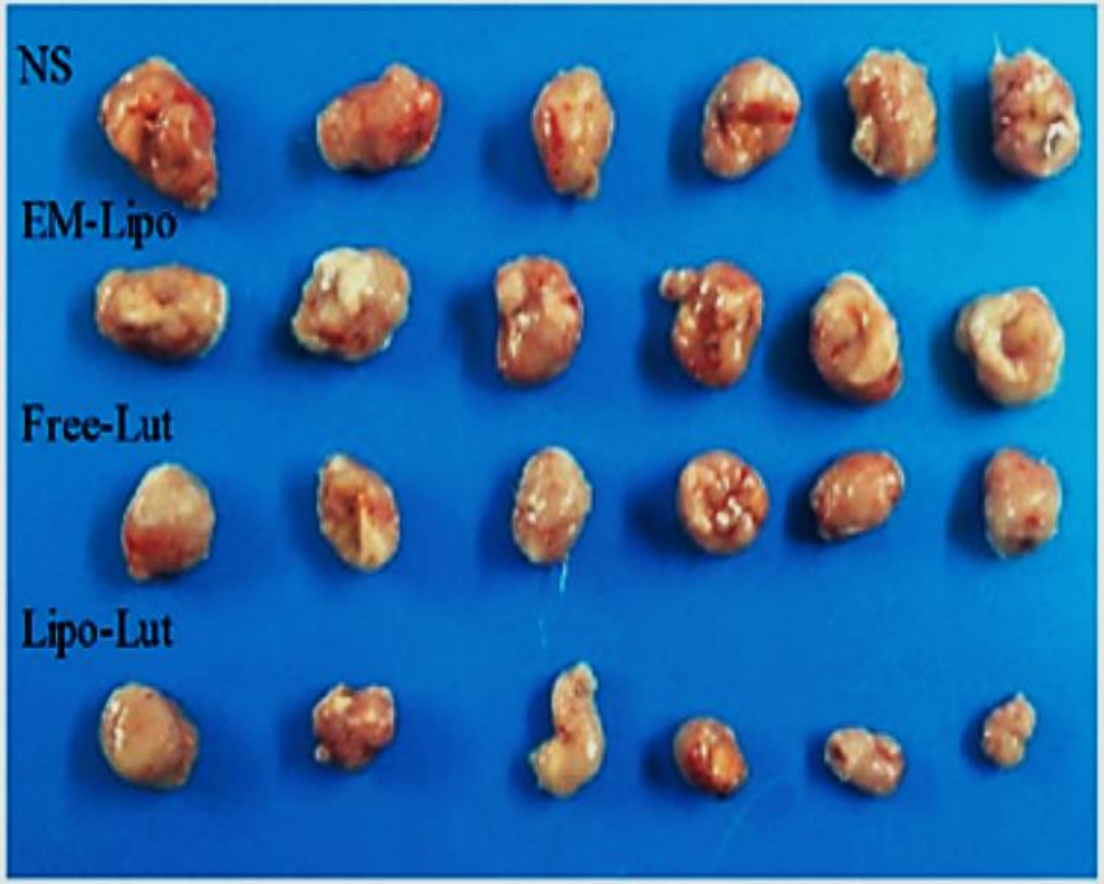


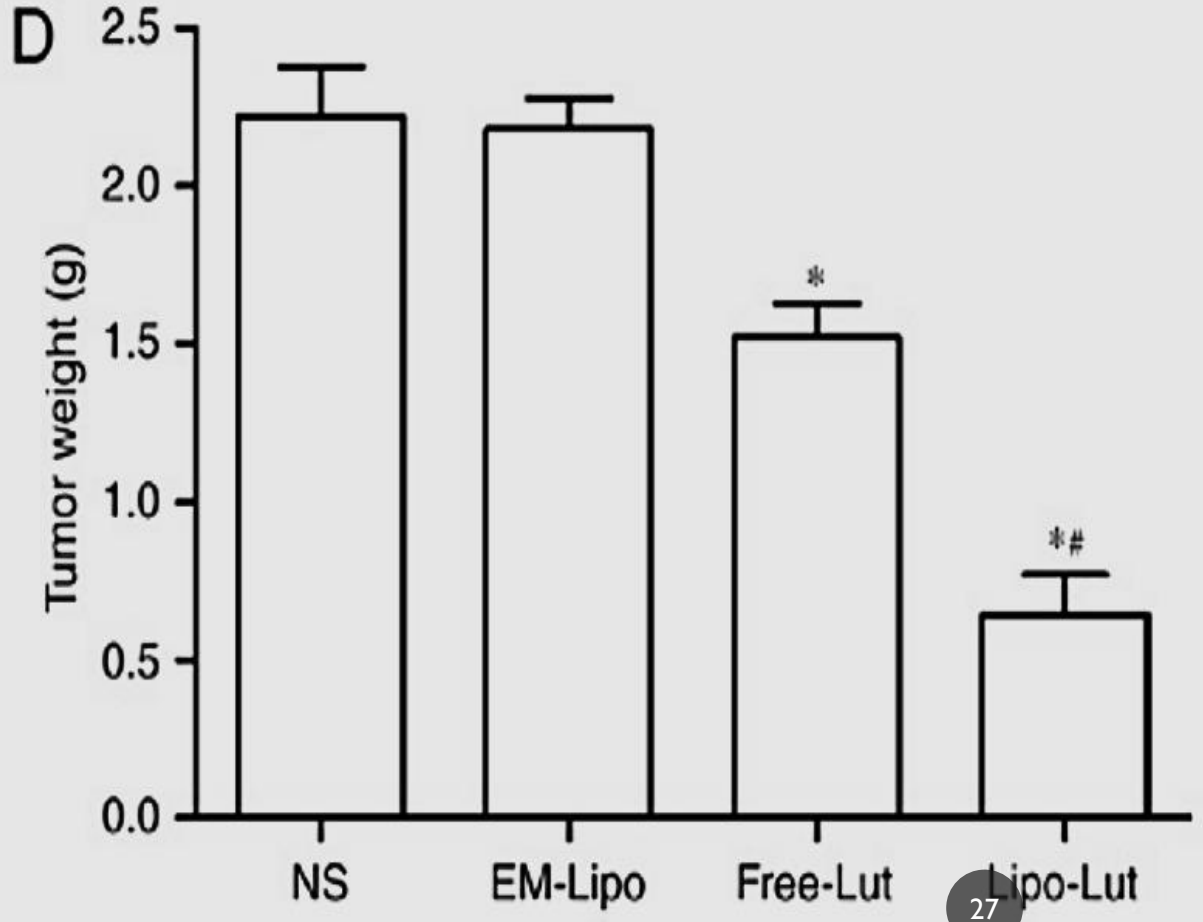


Tumoricidal effects on the mouse tumor model

- CT26 colorectal carcinoma graft model in BALB/c mice, 22-days of treatment
- Four groups of NS, EM-Lipo, Free-Lut, Lipo-Lut
- the final tumor volume of mice treated with luteolin was notably reduced
- Lipo-Lut group exhibited the most significant inhibitory efficacy
- no significant difference between NS group and EM-Lipo group
- The smallest tumor weights were observed in the Lipo-Lut group
- the treatment of Lipo-Lut resulted in a robust efficacy in reducing tumor volume

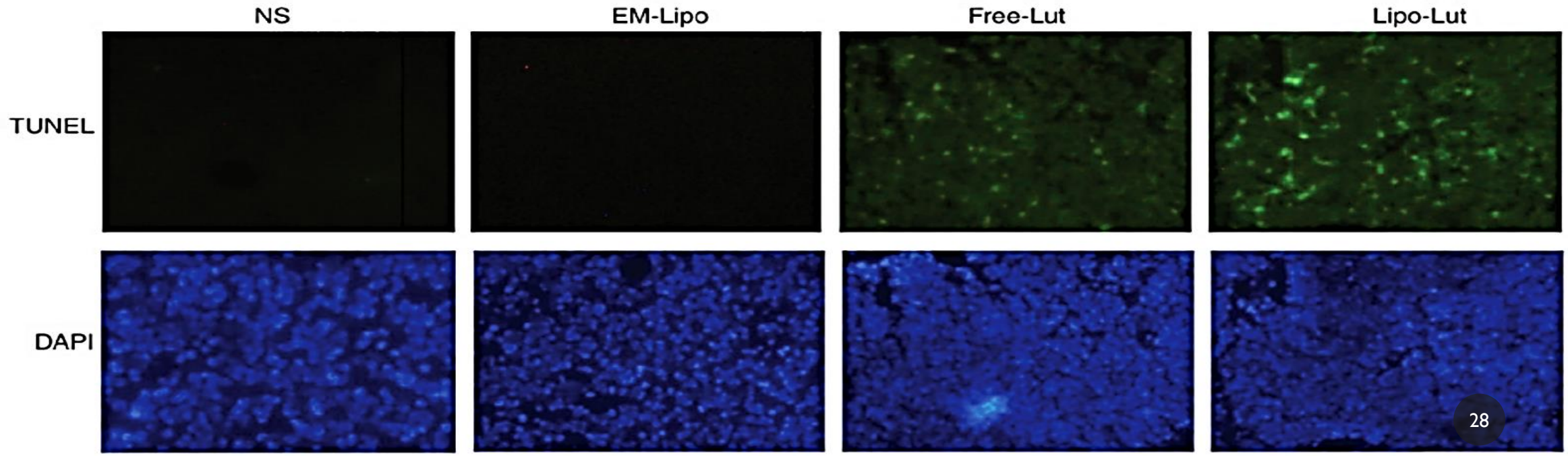
Tumoricidal effects on the mouse tumor model





Lipo-Lut induced apoptosis

- TUNEL assay
- a significant number of apoptotic cells appeared with green fluorescence in the tumor tissue of luteolin-treated mice
- Treatment with Lipo-Lut produced more apoptotic cells than treatment with the Free-Lut



Lipo-Lut inhibited tumor vascularization

- immunofluorescence analysis with anti-CD31 monoclonal antibody
- CD31-positive endothelial cells in luteolin treated groups had weaker fluorescence than those of NS group and EM-Lipo group
- less number of microvessels in the Free-Lut group and Lipo-Lut group compared to the control group ($P<0.01$)
- 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 inhibited tumor vascularization

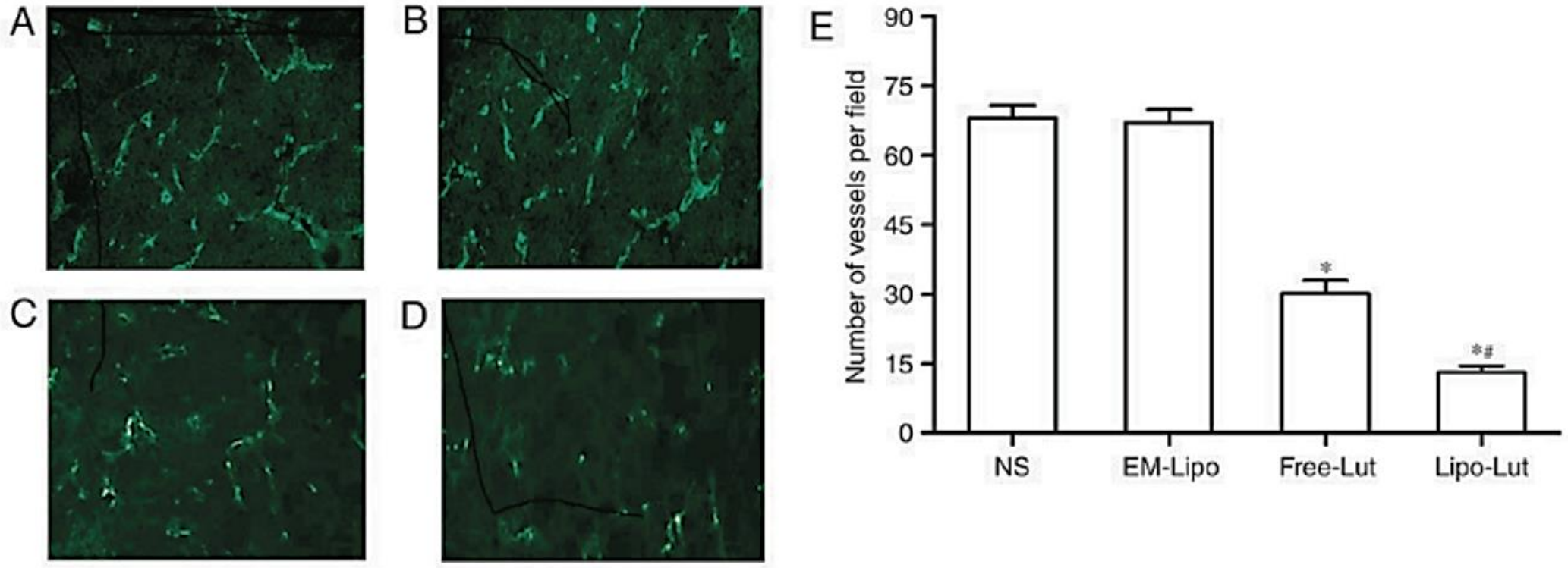
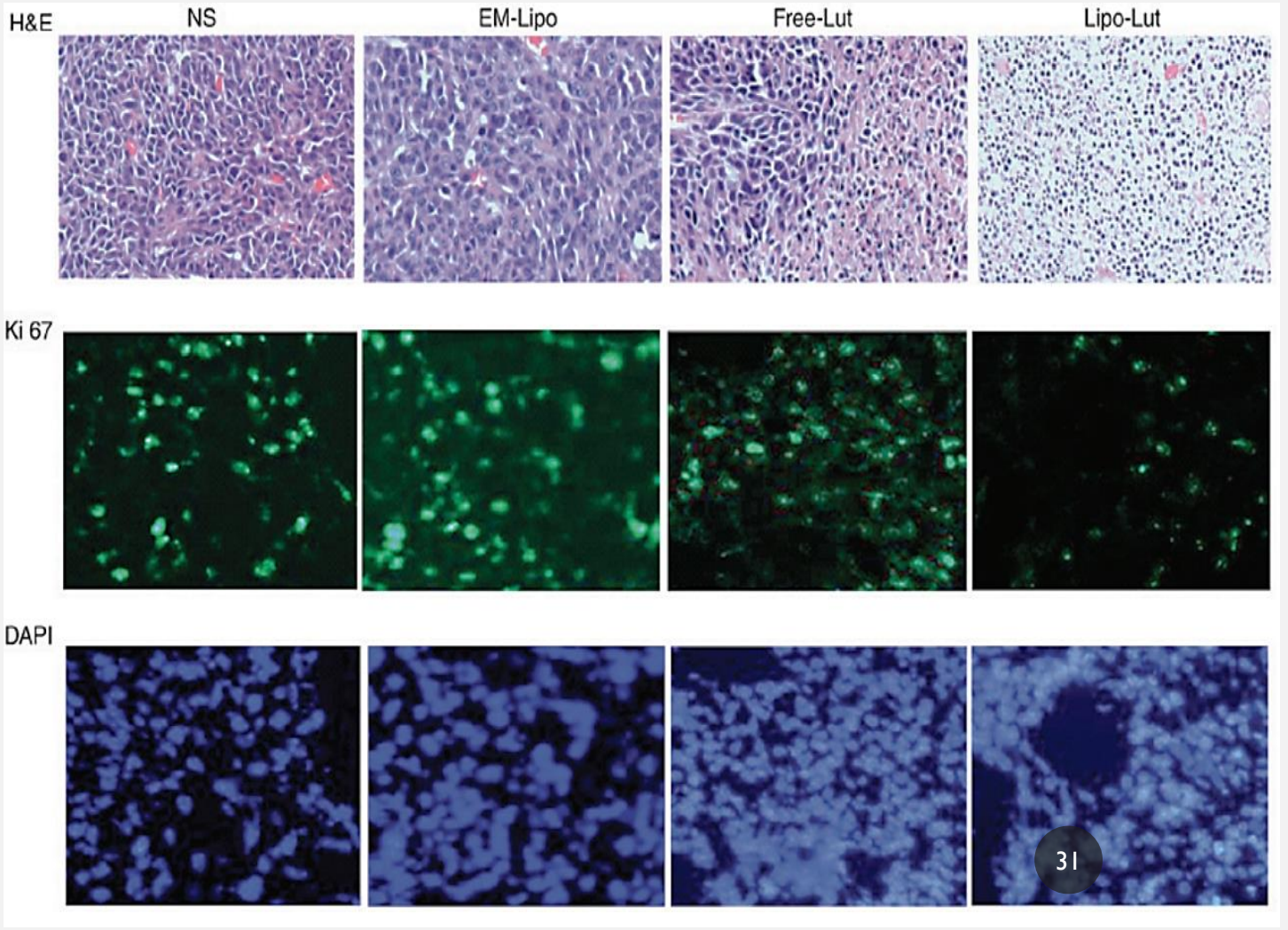


Figure 7. Inhibition of tumor angiogenesis. Frozen tumor tissue sections were stained for blood vessels by incubation with anti-CD31 Ab followed by incubation with secondary Ab conjugated with FITC and the tumor blood vessels were visualized by fluorescence microscopy. (A) NS; (B) EM-Lipo; (C) Free-Lut; (D) Lipo-Lut; and (E) the number of vessels per x100 field were counted, five fields per slide and at least three slides per group were detected. Data are presented as the mean ± standard deviation. $^{*}P<0.01$ vs. control; $^{\#}P<0.01$ vs. Free-Lut. FITC, fluorescein isothiocyanate.

Lipo-Lut decreased Ki-67 expression

- The expression of Ki-67 is associated with tumor cell proliferation and growth, widely used in routine pathological investigation as a proliferation marker
- Immunofluorescence examination of Ki-67 staining revealed an inhibition of proliferating cells in the Lipo-Lut-treated mice



Discussion

- liposomes → safe, well tolerated and potential drug delivery system
  - best-known formulation is liposome-encapsulated doxorubicin (Doxil or Caelyx) (12,13).
  - less cardiotoxicity, providing comparable antitumor activity (14,15).
- the actions of some poor water soluble drugs enhanced after encapsulation into liposome
- normal endothelium gaps → 5-10 nm
- tumor capillaries gaps → 100 to 780 nm (16)
- Water soluble lipo-lut size → ~105 nm
- Lipo-Lut could prolong the drug release, bioavailability of Lipo-Lut improved
- Lipo-Lut have more tumor growth inhibition activities than Free-Lut on CT26 cells

Discussion

- the antitumor activities of Lipo-Lut on CT26 cells grafts in BALB/c mice
  - stronger tumor growth-inhibiting effects
  - suppressed angiogenesis, increased apoptosis
- Angiogenesis plays a important role in tumor growth and invasion, The most essential factor is vascular endothelial growth factor (VEGF)
- luteolin could inhibit VEGF-stimulated endothelial cell proliferation, migration, invasion and tumor angiogenesis
- A study from Norhaizan ⟶ luteolin induced apoptosis in colon cancer by modulating the expressions of bax, Bcl-2 and caspase 3 in vitro and in vivo
- luteolin acted against DNA damage and activated DNA repair mechanism in Caco-2 colon cancer cells (17,18).

Discussion

- accumulation of liposome in the tumor microenvironment is more than normal tissues (19,20)
  - leaky vasculature of the tumor tissue ⟶ provide a channel allowing liposome to more easily target tumor tissue (1).
  - solid tumors usually lack effective lymphatic drainage

- 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

- Mechanism of action :
  - Induction of apoptosis in tumor cells
  - reduced tumor angiogenesis, blocking the nutrition supply into tumor tissue
  - Lipo-Lut inhibited tumor proliferation

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