Article

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Induction of Apoptosis and Inhibition of Angiogenesis by PEGylated Liposomal Quercetin in Both Cisplatin-Sensitive and Cisplatin-Resistant Ovarian Cancers

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The clinical efficiency of cisplatin against ovarian cancer is often limited by the development of drug resistance. In this work, we investigated PEGylated liposomal quercetin (Lipo-Que) on cisplatin-sensitive (A2780s) and cisplatin-resistant (A2780cp) human ovarian cancer models *in vitro* and *in vivo* to reveal whether a cisplatin-resistant ovarian cancer has susceptibility to quercetin (Que) and the mechanism of its antitumor activity. Lipo-Que was prepared using a solid dispersion method, and the obtained Lipo-Que is monodisperse with a mean diameter of 163 ± 10 nm. Besides, *in vitro* drug release assay showed a sustained release behavior of Lipo-Que. *In vitro* experiments suggested that Lipo-Que inhibited cell proliferation, induced apoptosis, and induced cell cycle arrest in both A2780s and A2780cp cells. Furthermore, antitumor activity of Lipo-Que was investigated in both cisplatin-sensitive and cisplatin-resistant human ovarian tumor xenograft models in nude mice. Lipo-Que significantly suppressed tumor growth in both models in comparison with free Que, blank liposomes (Lipo), or normal saline (NS). Furthermore, immunohistochemistry and immunofluorescence tests revealed that Lipo-Que induced apoptosis, decreased microvessel density, and inhibited proliferation of tumors in both A2780s and A2780cp tumor models. Therefore, our results suggest that Lipo-Que is an effective agent to inhibit tumor growth in both cisplatin-sensitive and cisplatin-resistant human ovarian cancers.

KEYWORDS: *Liposomes, Quercetin, Apoptosis, Antiangiogenesis, Cisplatin-Resistant Ovarian Cancer.*

INTRODUCTION

Ovarian cancer remains the leading cause of death in gynecologic malignancies. Ovarian carcinoma will develop in one of 70 women in their lifetime, and one woman in 100 will die of complications associated with this disease.[1] Treatment of human ovarian cancer with the DNA-damaging agents, such as cisplatin, is initially an effective means by which to arrest malignancy. However, following preliminary success in tumor regression, recurrence and resistance to further chemotherapeutic treatment often ensues. Resistance to cisplatin at the onset of treatment or at relapse is the most significant cause of treatment failure for ovarian cancers.[2] Therefore, development of compounds or drug delivery systems (DDSs) that are able to interact with their cellular targets in ways that will circumvent well-characterized resistant mechanisms would be instrumental in the ability to fight ovarian cancers.[3,4]

Quercetin (3,3′,4′,5,7-pentahydroxyflavone, Que) is a flavonoid molecule ubiquitous in nature that has been described as a potential anticancer agent[5] because of its

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ability to modulate cell proliferation, survival and differentiation and target key molecules responsible for tumor cell properties.[6,7] *In vitro* and preliminary animal and human data indicated that Que appeared to be associated with little toxicity when administered orally or intravenously.[8–12] Que exhibits anti-neoplastic activity through various mechanisms. The antioxidant effect can inhibit carcinogen activation as well as cellular damage due to free radical reactions.[13] Besides, it inhibits the growth of malignant cells by arresting cell cycle in the late G1 phase,[14] or it may cause apoptosis of tumor cells.[15] Que inhibits synthesis and expression of heat shock proteins,[16] and blocks signal transduction pathways by inhibiting protein tyrosine kinase, 1-phosphatidylinositol 4-kinase and 1-phosphatidylinositol 4-phosphate 5-kinase resulting in a reduction of inositol 1,4,5-trisphosphate concentration.[17] Que can also down-regulate the expression of oncogenes, e.g., c-myc and ki-ras[17] and induce wild-type p53.[18] All these data suggest that Que has the potential to be developed as an anticancer agent.

The extreme water insolubility of Que hampers its delivery to the tumor at an effective concentration. In order to prepare water-based formulation of Que, we encapsulate Que in the nonaqueous interior of the PEGylated liposomes, which is subsequently referred as Lipo-Que. In a previous study, we showed that Lipo-Que could significantly improve the water solubility of Que, prolong the circulation time in blood and enhance antitumor efficacy of Que.[19] On the other hand, we demonstrated that Lipo-Que was highly effective against mouse colorectal carcinoma, lung cancer and hepatomas in nude mice,[19] and we also demonstrated Lipo-Que could efficiently inhibited growth of human ovarian cancer cells *in vitro*.[17] Because Que and cisplatin act via separate pathways and the mechanisms of Lipo-Que is totally different from cisplatin,[20] the present study aimed to investigate its antitumor efficacy on cispatin-resistance ovarian cancer. Lipo-Que demonstrated a profound inhibitory effect on the growth of both cisplatin-sensitive (A2780s) and cisplatin-resistant (A2780cp) ovarian cancer cells *in vitro* and *in vivo*.

MATERIALS AND METHODS

Materials, Cells, and Animals

Lecithin (Sigma, USA), cholesterol (Sigma, USA), poly(ethylene glycol) (PEG, Mw = 4000, Aldrich, USA), quercetin (Sigma, USA), cisplatin (Sigma, USA), propidium iodide (PI, Sigma, USA), RNase A (Sigma, USA), dimethylsulfoxide (DMSO, Sigma, USA), Dulbecco's modified eagle medium (DMEM, Gibco, USA), L-glutamine (Gibco, USA), fetal bovine serum (FBS, Gibco, USA), trypsin (Invitrogen, USA), 3-(4,5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide (methyl thiazolyl tetrazolium, MTT, Sigma, USA), *in situ* cell death detection kits (Roche, Switzerland) were used as received. Primary antibodies were mouse anti-CD34 Class II (Gene Tech) and mouse anti-proliferating cell nuclear antigen (PCNA) clone PC 10 (Invitrogen, USA). Secondary antibodies for colorimetric immunohistochemical analysis were biotinylated goat anti-rat immunoglobulin and goat anti-mouse IgG (BD Biosciences Pharmingen, USA). The substrate buffer was 3-amino-9-ethylcarbazole (AEC) substrate kit. All other reagents used were all analytical reagent.

The derivation and source of established human ovarian cancer cell lines A2780s and A2780cp have been described previously.[21] Cells were cultured in a humidified atmosphere containing 5% CO_2 at 37 °C in DMEM supplemented with 10% FBS, 2 mM L-glutamine, 100 units/mL of penicillin and 100 mg/mL of streptomycin. Cisplatin was dissolved in DMSO and diluted in normal saline (NS) before use, and the final concentration of DMSO was 0.1%.

Female athymic nude mice (BALB/c), 6–8 weeks old, were purchased from the Vital River Lab Animal Technology Co., Ltd. (Beijing, China). Mice were housed and maintained in a specific sterile environment in the Laboratory Animal Center, National Key Laboratory of Biotherapy, Sichuan University, and were cared for in accordance with current guidelines. All the studies were approved and supervised by the Sichuan University Institutional Animal Care and Use Committee.

Preparation of PEGylated Liposomal Quercetin (Lipo-Que)

Lipo-Que was prepared using a solid dispersion method as previously described.[19,22] Briefly, the mixture of lecithin, cholesterol, PEG, and Que was dissolved in chloroform/methanol (3:1, v/v), and evaporated to dryness using a rotary evaporator under reduced pressure. The lipid films were dissolved in 5% glucose solution under ultrasonication, and subsequently concentrated and lyophilized. Blank PEGylated liposomes (Lipo) were prepared in the same way without Que. In the following studies, the dose of Lipo-Que was on a Que basis, and Lipo-Que or Lipo was dissolved in NS for *in vitro* and *in vivo* studies.

Characterization of Lipo-Que

Particle size and zeta potential of prepared Lipo-Que were determined by Malvern Nano-ZS 90 laser particle size analyzer (Malvern, UK). All results were the mean of three different simples, and all data were expressed as the mean ± SD.

The morphological characteristics of the prepared Lipo-Que were examined by transmission electron microscope (TEM, H-6009IV, Hitachi, Tokyo, Japan). Lipo-Que were diluted with distilled water and placed on a copper grid covered with nitrocellulose. The sample was negatively stained with phosphotungstic acid and dried at room temperature.

Drug loading (DL) and encapsulation efficiency (EE) of Lipo-Que were determined as follows. Briefly, 10 mg of lyophilized Lipo-Que were dissolved in 0.1 mL of methanol. The amount of Que in the solution was determined by high performance liquid chromatography (HPLC). DL and EE of Lipo-Que were calculated according to Eqs. (1) and (2):

$$\mathrm{DL} = \frac{\mathrm{Drug}}{\mathrm{Liposome} + \mathrm{Drug}} \times 100\% \quad (1)$$

$$\mathrm{EE} = \frac{\mathrm{Experimental\ drug\ loading}}{\mathrm{Theoretical\ drug\ loading}} \times 100\% \quad (2)$$

In Vitro Drug Release Assay

In vitro drug release assay of Lipo-Que or free Que was conducted using a modified dialysis method.[23,24] Lipo-Que or free Que was placed in a dialysis tube (molecular weight cutoff is 3.5 kDa and the dialysis area is about 1 cm^2), and the dialysis tubes were incubated in 10 mL of phosphate buffer solution (PBS, pH = 7.4, pre-warmed to 37 °C) containing Tween80 (0.5% wt) at 37 °C with gentle shaking (100 rpm). The media were displaced by pre-warmed fresh PBS at predetermined time. The supernatant of the removed release media were collected and stored at −20 °C until analysis. The released drug was quantified using HPLC. All results were the mean of three test runs, and all data were expressed as the mean ± SD.

In Vitro Cytotoxicity Evaluation

The growth-inhibitory activities of Lipo-Que or free Que on A2780s and A2780cp cells were evaluated by MTT assay. A2780s and A2780cp cells were cultured in DMEM containing 10% fetal bovine serum in a 96-well plate. Cells were exposed to various doses of Lipo-Que or free Que for 48 hours. The control cultures were left untreated. Then, MTT solution was added to each well, and cell viability was measured by the absorbance at 570 nm.

Induction of Apoptosis *In Vitro*

To quantitative assessment of apoptosis, A2780s cells and A2780cp cells were seeded into six-well plates at a concentration of 5×10^5 cells per well for 24 h. Cells in the exponential growth phase were then exposed to Lipo-Que (15 μg/mL), free Que (15 μg/mL), blank Lipo, or NS in 2 mL DMEM for additional 48 h. PI staining was used to determine modification in the cellular morphology.[25] Images of cells were taken using ZEISS AXIOVERT 200 microscope and Axio Cam MRm camera. Besides, the apoptosis of the cells was quantified by flow cytometric analysis as described elsewhere.[17] Briefly, cells were suspended in 1 mL of hypotonic fluorochrome solution containing 50 μg PI/mL in 0.1% sodium citrate plus 0.1% Triton X-100 and the cells were analyzed by the use of a flow cytometer (ESP Elite, Coulter). For semiquantitative determination of apoptosis, DNA laddering assays were performed as previously described.[26]

Effect of Lipo-Que and Free Que on Cell Cycle

Cell cycle assay of Lipo-Que or free Que on A2780s cells and A2780cp cells was performed using flow cytometry. A2780s or A2780cp cells cultured in 6-well plates were treated with Lipo-Que (15 μg/mL), free Que (15 μg/mL), or blank Lipo in 2 mL DMEM for 48h. Two milliliters of RPMI-1640 without treatment reagents were added as control. When cells were harvested, they were washed with PBS, fixed with pre-chilled 70% ethanol for 30min, pretreated with 20 μL RNase I (250 μg/mL) and stained with 200 μL PI (50 μg/mL) for 30 min. Cell cycle were analyzed by a flow cytometer.

In vivo Animal Models and Treatments

To establish *in vivo* cisplatin-sensitive or cisplatin-resistant ovarian cancer mouse model, A2780s or A2780cp (2×10^6 cells/0.1 mL PBS) were injected subcutaneously into the right flank of each mouse. Mice were assigned randomly to one of the four groups (five mice per group): Lipo-Que (50 mg/kg), free Que (50 mg/kg), blank Lipo, or NS. Treatment was initiated when tumor volume was approximately 100 mm^3. All these reagents were given every three-day for 27 days. Tumor size were measured every 3 days using the formula $A \times B^2 \times 0.5236$ (A, length; B, width; all were measured in millimeters). All mice were sacrificed on day 34, and tissue specimens were fixed in formalin for paraffin embedding.

Determination of Microvessel Density (MVD) and Tumor Cell Proliferation

The labeled streptavidin-biotin method was used to determine MVD of tumor tissues. Primary antibody (rat anti-mouse CD34) was applied overnight in a moist chamber at 4 °C. A standard avidin-biotin peroxidase technique (DAKO, Carpinteria, CA, USA) was applied. Briefly, biotinylated goat anti-rat immunoglobulin and avidin-biotin peroxidase complex were applied for 30 min each, with 30 min washes in PBS. The reaction was finally developed by DAKO Liquid DAB + Substrate-Chromogen System. The number of microvessels was assessed in five randomly selected fields in this area and viewed at 200 × magnification. The MVD of this section was obtained by counting the average number of vessels in the five fields. Images of tumor tissue and microvessels were taken using an OLYMPUS BX600 microscope and SPOT FIEX camera.

Immunohistochemical proliferating cell nuclear antigen (PCNA) staining of tumor tissues were used to evaluate proliferation activity of tumor cells. The slides for immunohistochemistry of PCNA were treated in the same way but the primary antibody was mouse anti-human PCNA (Invitrogen) and the secondary antibody, biotinylated goat anti-mouse IgG (BD Biosciences Pharmingen). To quantify PCNA expression, the PCNA labelling

index (PCNA LI) was calculated as number of PCNA-positive cells/total number of cells counted under 400 × magnification in five randomly selected areas in each tumor sample.

Terminal dUTP Nick-End Labeling (TUNEL) Detection of Apoptotic Tumor Cells

Tumor species embedded in paraffin were prepared as described above. TUNEL staining was performed using an *in situ* cell death detection kit following the manufacturer's recommended protocol. In tissue sections, five equal sized fields were randomly chosen and analyzed. The percentage of apoptotic cells was assessed in five randomly selected fields viewed at 200 × magnification, yielding the density of apoptotic cells (apoptosis index).

Toxicity Assays

To observe systemic toxicity of the treatments, we monitored gross measures such as huddling, weight loss, ruffling of fur, behavior and feeding characteristics. After the mice were sacrificed, the tissues of the liver, lung, kidney, spleens, heart, or intestines were fixed in 10% buffered formalin solution and embedded in paraffin. Sections of 3–5 μm were stained with hematoxylin and eosin (H&E) prior to microscopic analysis.

Statistical Analysis

For the *in vivo* experiments, differences in continuous variables (tumor volume, MVD and apoptotic index) were analyzed using the Student's t test for comparing two groups with $p < 0.05$ considered statistically significant. The results of the statistical analyses were presented as means ± SD. All statistical analysis was calculated by SPSS11.0 (SPSS, Chicago, IL, USA).

RESULTS

Characterization of Lipo-Que

Lipo-Que was prepared by solid dispersion method as previously described.[19, 22] DL and EE of the obtained Lipo-Que are 4.87 ± 0.05% and 92.53 ± 0.96%, respectively. Average particle size, polydispersity (PDI), and zeta potential of prepared Lipo-Que were 163 ± 10 nm, 0.085 ± 0.011, and −34.5 ± 2.3 mV, respectively (Fig. 1(A and B)). TEM image of Lipo-Que was showed in Figure 1(C), which indicated that Lipo-Que were monodisperse with spherical shape. Figure 1(D) presented the appearance of prepared blank Lipo and Lipo-Que, and a stable and homogeneous solution of Lipo-Que could be observed.

In Vitro Release Behavior of Lipo-Que

In vitro release assay of Lipo-Que was performed using a modified dialysis method,[23, 24] and the results were shown in Figure 2. As shown in Figure 2, a much slower and sustained release behavior of Lipo-Que was observed in comparison with rapid release of free Que. In the first 48 hours, 30.6 ± 4.7% of Que released from Lipo-Que, while 88.7 ± 5.8% of Que in free Que group released into the outside media. Furthermore, a two-week cumulative release rate of Lipo-Que (64.3 ± 5.7%) was much lower than that of free Que (95.2 ± 3.2%).

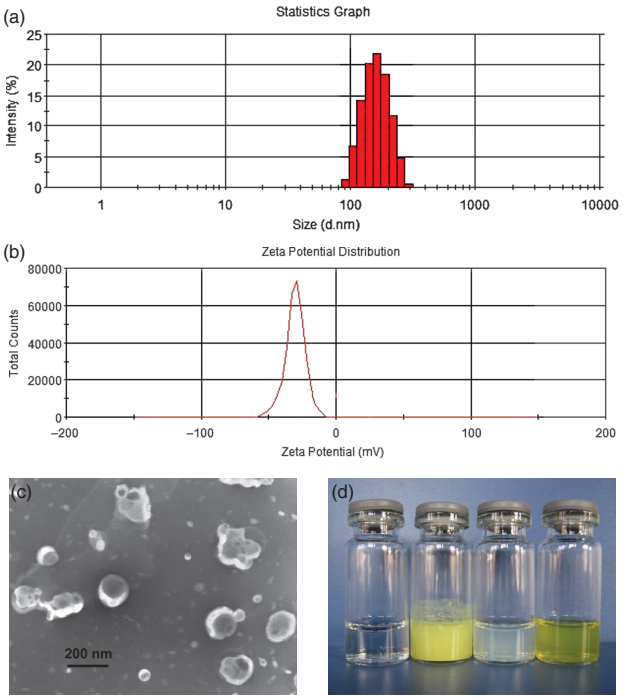

Figure 1. Preparation and characterization of Lipo-Que. (a) Particle size distribution of Lipo-Que, (b) Zeta potential of Lipo-Que, (c) TEM image of Lipo-Que, (d) Appearance of water, Que in water, blank Lipo, and Lipo-Que (from left to right).

Lipo-Que Inhibits Proliferation of A2780s and A2780cp Cells

The cisplatin resistance of A2780cp cells was confirmed by MTT assay. For A2780s cells, the IC50 of cisplatin

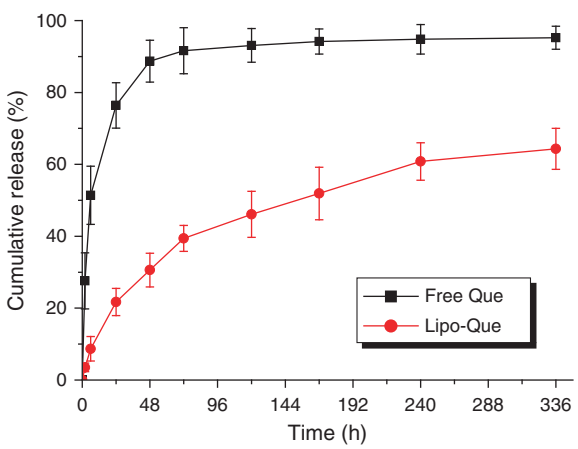

Figure 2. *In vitro* drug release behavior of Lipo-Que and free Que in PBS.

was 10 μM, whereas the IC50 of cisplatin was more than 100 μM for A2780cp cells. Therefore, A2780cp cells were at least ten times more resistant to cisplatin than A2780s cells (data not shown).

Cytotoxicity of Lipo-Que or free Que on A2780s and A2780cp cells was investigated using MTT method, and the results were presented in Figure 3. Both Lipo-Que and free Que at various concentrations significantly inhibited the growth of cisplatin-sensitive (A2780s) and cisplatin-resistant (A2780cp) cells in a dose-dependent manner. Furthermore, Lipo-Que showed a slight higher cytotoxicity on both cells, but no significant difference was observed. The result indicates that the cytotoxicity of the Lipo-Que is comparable to that of free Que even though Que was released in an extended behavior in Lipo-Que group.

Lipo-Que Induces Apoptosis of A2780s and A2780cp Cells *In Vitro*

DNA fragmentation assay, *in situ* PI staining of DNA, and flow cytometry assay were conducted to investigate the effect of Lipo-Que on cell apoptosis. In agarose gel electrophoresis of Lipo-Que-treated A2780s or A2780cp cells, there were ladder-like pattern of DNA fragments consisting of approximately 180 to 200 base pairs, which was consistent with internucleosomal DNA fragmentation (Fig. 4). No DNA ladder was detected in control cells.

Cisplatin-sensitive A2780s and cisplatin-resistant A2780cp ovarian cancer cells grown in six-well plates were treated with NS, blank Lipo, free Que, or Lipo-Que respectively for analysis of apoptosis. By fluorescence microscopy observation, both cells treated with free Que or Lipo-Que showed typical apoptotic morphology: reduction of cell volume and apoptotic body formation with brightly red fluorescent condensed (intact or fragmented) nuclei (Fig. 5). However, in NS-treated or blank Lipo-treated cells, these changes were much less.

Furthermore, flow cytometry assay of PI staining was applied to quantizate the apoptotic cells by observing sub-G1 (apoptotic) cells. As presented in Table I, sub-G1 cells in Lipo-Que-treated A2780s or A2780cp cells were significant higher (65$\pm$3% or 65$\pm$2%, respectively), compared with 54$\pm$3% or 55$\pm$3% in free Que group, 10$\pm$2% or 13$\pm$2% in blank Lipo group, and 2$\pm$1% or 3$\pm$1% in NS group. The results obtained from flow cytometry were in good agreement with DNA fragmentation in agarose gel electrophoresis and morphological changes in fluorescence microscopy of PI-staining.

Lipo-Que Induces Cell Cycle Arrest

Cell cycle analysis of A2780s or A2780cp cells treated with Lipo-Que, free Que, blank Lipo, or NS were performed using flow cytometric, and the results were summarized in Table I. Both cells treated with Lipo-Que or free Que resulted in G0/G1 and G2/M arrest in our experiments and decreased the S fraction significantly.

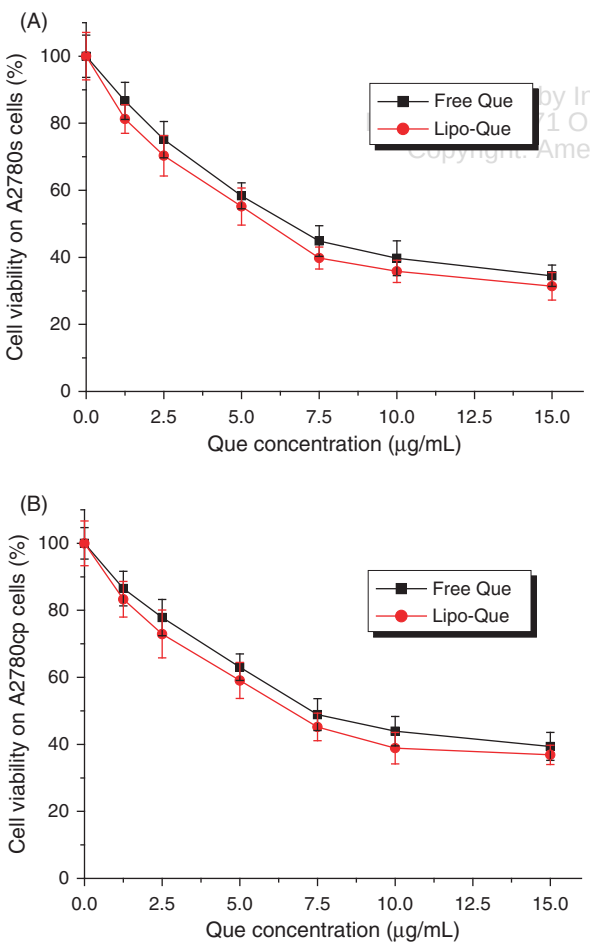

Figure 3. **Cytotoxicity assay of Lipo-Que and free Que on A2780s (A) and A2780cp (B) cells.**

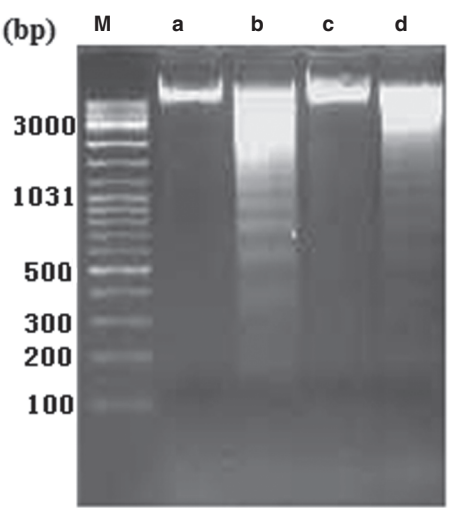

Figure 4. **DNA fragmentation of Lipo-Que treated cells (48 h). A2780s cells were treated with blank Lipo (lane a) or Lipo-Que (lane b); A2780cp cells were treated with blank Lipo (lane c) or Lipo-Que (lane d).**

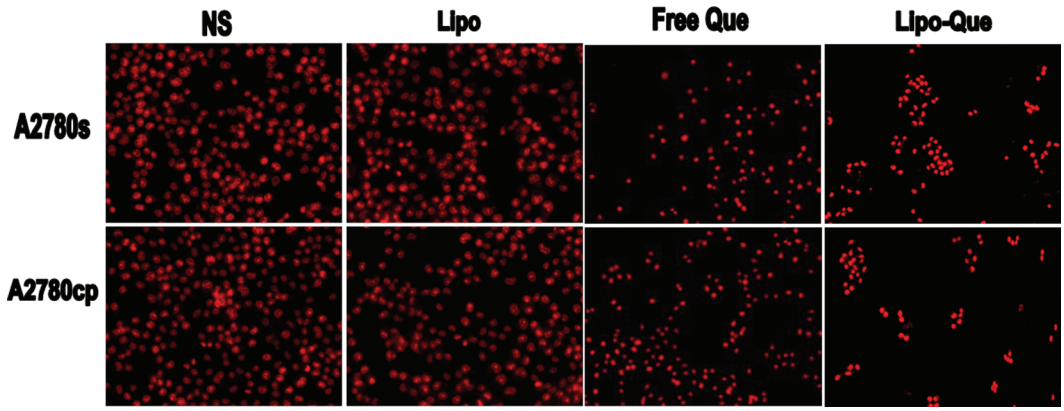

Figure 5. **Identification of apoptotic cells in A2780s and A2780cp cells. A2780s cells and A2780cp cells was treated with NS, blank Lipo, free Que, or Lipo-Que, respectively. Then, cells were labeled with PI as described, and examined for apoptosis by their ability to uptake PI.**

Furthermore, Lipo-Que showed a more remarkable effect on cell cycle arrest than free Que.

Lipo-Que Inhibit Tumor Growth *In Vivo*

The activity of Lipo-Que to inhibit tumor growth *in vivo* was evaluated in xenograft nude mouse model. To establish A2780s or A2780cp ovarian tumor model, mice were injected subcutaneously with A2780s or A2780cp ovarian cancer cells. When the bearing tumors were around 100 mm^3, mice were randomly assigned into the following four groups (5 mice per group): NS, blank Lipo, free Que (50 mg/kg), and Lipo-Que (50 mg/kg). Mice were treated once every three days via tail vein with above mentioned agents for 27 days. In both tumor models, Lipo-Que significantly inhibited tumor growth in comparison with control groups (Fig. 6). In cisplatin-sensitive A2780s tumor model, the mean tumor volume of Lipo-Que-treated mice on day 31 was 393 ± 155 mm^3, versus 1528 ± 356 mm^3 in free Que group ($P < 0.01$), 3592 ± 728 mm^3 in blank Lipo group ($P < 0.01$), and 4171 ± 1181 mm^3 in NS group ($P < 0.01$), respectively. Complete tumor regression occurred in one of the five mice that was administrated with Lipo-Que. In cisplatin-resistant A2780cp tumor

Table I. **Induction of apoptosis and cell cycle arrest by Lipo-Que or free Que in A2780s and A2780cp cells determined by flow cytometer.**

Cells	Group	Cell cycle			Apoptosis%
		G0/G1%	S%	G2/M%	
A2780s	NS	44 ± 4	40 ± 2	17 ± 2	2 ± 1
	Lipo	44 ± 2	38 ± 1	17 ± 2	10 ± 2
	Free Que	58 ± 5	27 ± 2	15 ± 2	54 ± 3
	Lipo-Que	63 ± 2	24 ± 2	14 ± 1	65 ± 3
A2780cp	NS	43 ± 5	39 ± 4	19 ± 2	3 ± 1
	Lipo	42 ± 2	37 ± 1	23 ± 3	13 ± 2
	Free Que	56 ± 6	31 ± 3	13 ± 2	55 ± 3
	Lipo-Que	59 ± 3	26 ± 1	15 ± 2	65 ± 2

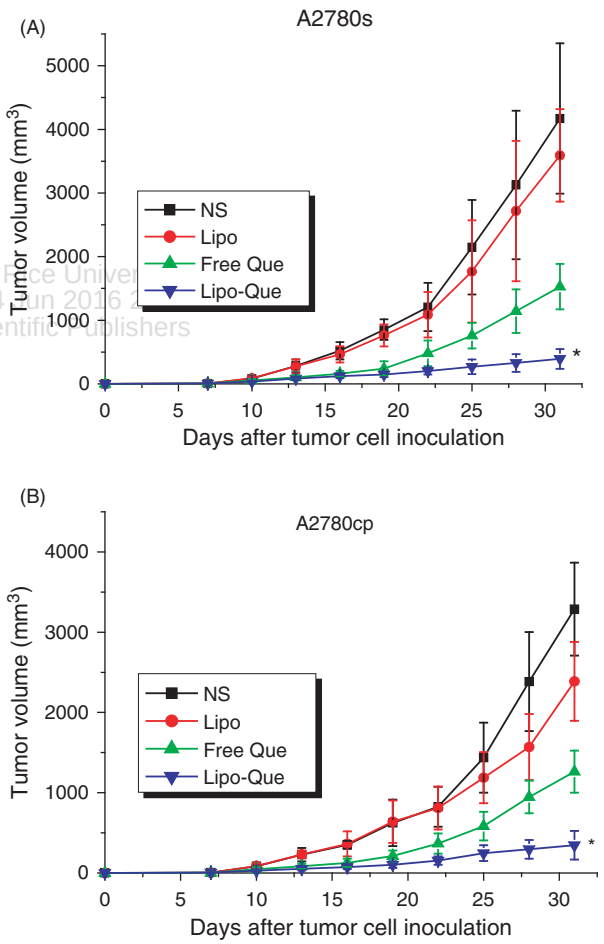

Figure 6. **Effect of Lipo-Que on tumor growth of tumor-bearing mice. A2780s cells (A) or A2780cp cells (B) were subcutaneously injected into mice, then mice were treated with intravenous injection of Lipo-Que, free Que, blank Lipo, or NS every three days for 27 days. Asterisks indicate systemic therapy with Lipo-Que resulting in significant tumor growth inhibition versus free Que ($p < 0.01$), blank Lipo ($p < 0.01$), or NS groups ($p < 0.01$); The points represent the average tumor volume ± SD.**

model, the mean tumor volume in Lipo-Que-treated mice was 346 ± 178 mm^3, versus 1263 ± 262 mm^3 in free Que-treated mice ($P < 0.01$), 2387 ± 491 mm^3 in blank Lipo-treated mice ($P < 0.01$), and 3287 ± 580 mm^3 ($P < 0.01$), respectively. The results indicated that Lipo-Que could effectively inhibit growth of both cisplatin-sensitive and cisplatin-resistant ovarian tumors *in vivo*, and Lipo-Que was more efficient than free Que in both tumor models.

Induction of Tumor Cell Apoptosis

Tumor tissues were subjected to TUNEL assay for the determination of apoptotic index. Cell nuclei were stained with bright green which indicated apoptosis, and were recorded as TUNEL-positive nuclei. In both models, the Lipo-Que group has a significant higher apoptosis index of tumor cells compared with free Que, blank Lipo, or NS groups (Fig. 7). In A2780s tumors, the mean apoptotic index of cancer cells treated with Lipo-Que was $37.3 \pm 3.5\%$ versus $25.4 \pm 4.6\%$ in free Que group ($p < 0.05$), $5.6 \pm 2.1\%$ in blank Lipo group ($p < 0.05$), or $3.6 \pm 0.5\%$ in NS group ($p < 0.05$), respectively. In A2780cp tumors, the mean apoptotic index was $33.3 \pm 4.5\%$ in Lipo-Que-treated group versus $22.3 \pm 3.9\%$ in free Que group ($p < 0.05$), $6.8 \pm 0.8\%$ in blank Lipo group ($p < 0.05$), or $3.4 \pm 0.7\%$ in NS group ($p < 0.05$), respectively.

Inhibition of Tumor Angiogenesis

MVD was quantified in order to measure angiogenesis by immunolabeling of CD34 in paraffin embedded sections. The most highly vascularized areas of each tumor were identified at low power, and five high-power fields were counted in the area of greatest vessel density. In both A2780s and A2780cp models (Fig. 8), Lipo-Que apparently reduced the number of vessels (13.3 ± 6.3 and 16.5 ± 5.1, respectivel) compared with free Que group (26.3 ± 4.5, $p < 0.05$; 28.5 ± 5.3, $p < 0.05$), blank Lipo group (43.5 ± 12.7, $p < 0.05$; 47.3 ± 12.3, $p < 0.05$), or NS group (51.5 ± 14.3, $p < 0.05$; 58.5 ± 15.7, $p < 0.05$). There was no difference between NS and blank Lipo group in microvessel density.

Inhibition of Tumor Cell Proliferation

Tumor cell proliferation activity was detected using immunochemistry with PCNA. In both tumor models, Lipo-Que-treated group showed significant higher PCNA LI compared with free Que, blank Lipo, or NS group (Fig. 9). In A2780s tumors, PCNA LI in Lipo-Que

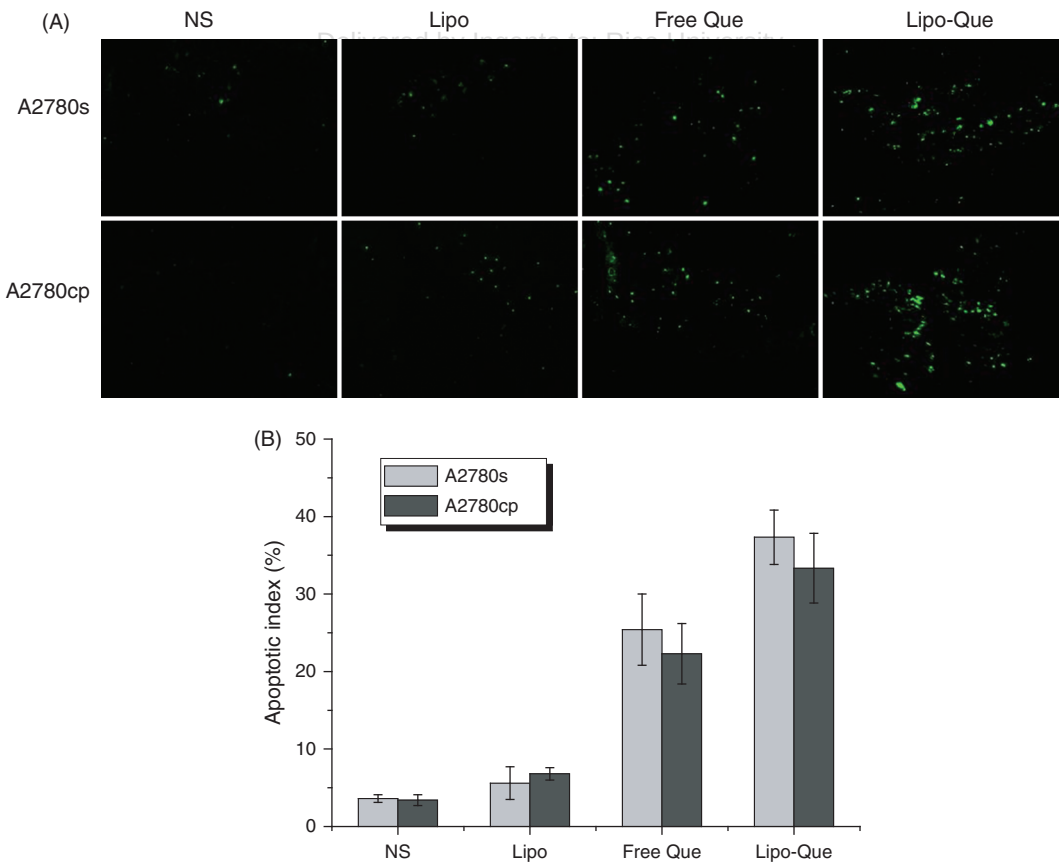

Figure 7. TUNEL immunofluorescent staining of tumors. A. Representative images of sections from A2780s and A2780cp tumors treated with NS, blank Lipo, free Que, or Lipo-Que; B. Apoptotic index of A2780s and A2780cp tumors were markedly increased in comparison with free Que ($p < 0.01$), blank Lipo ($p < 0.01$), or NS groups ($p < 0.01$).

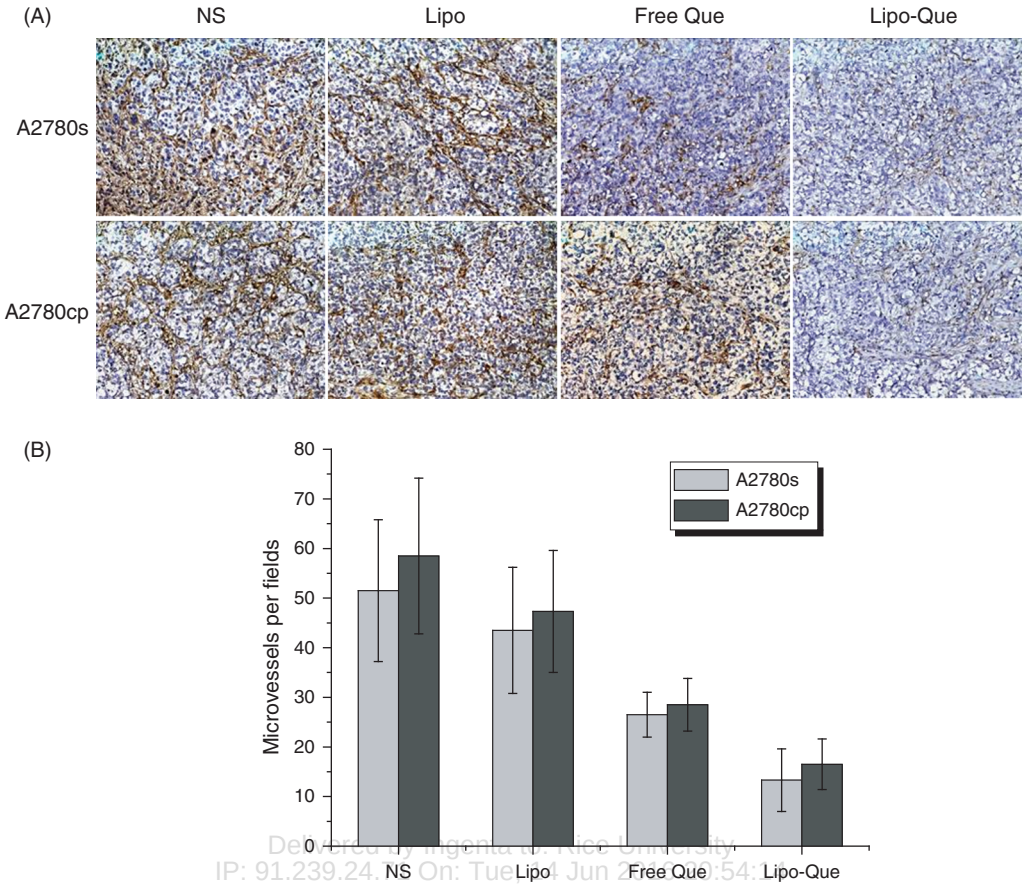

Figure 8. **CD34 immunohistochemical staining of tumors. A: Representative images of paraffin-embedded sections of A2780s and A2780cp tumors treated with NS, blank Lipo, free Que, or Lipo-Que; B. In A2780s tumors and A2780cp tumors, the number of microvessels was significantly smaller in Lipo-Que group compared with free Que ($p < 0.01$), blank Lipo ($p < 0.01$), or NS groups ($p < 0.01$).**

group was $14.8 \pm 4.4\%$ versus $34.2 \pm 5.2\%$ in free Que-treated group ($p < 0.05$), $74.4 \pm 6.6\%$ in blank Lipo-treated group ($p < 0.05$), and $71.6 \pm 8.2\%$ in NS-treated group respectively. Besides, in A2780cp tumors, PCNA LI in Lipo-Que group was $13.9 \pm 3.2\%$ versus $30.6 \pm 6.1\%$ in free Que-treated group ($p < 0.05$), $74.1 \pm 7.4\%$ in blank Lipo-treated group ($p < 0.05$), and $73.5 \pm 9.1\%$ in NS-treated group respectively. There was no significant difference between NS-treated and blank Lipo-treated groups ($p > 0.05$).

Toxicity Observation

During the experiment, animals treated with Lipo-Que did not show any signs of toxicity compared with control groups (NS, blank Lipo, and free Que groups), such as altered behavior and lost of weight, feeding, ruffling of fur and etc. Furthermore, no pathologic changes in the heart, liver, spleen, lung, kidney, and intestine were found with the Lipo-Que treatment and the control mice of both tumor models as assessed by hematoxylin-eosin (H&E) staining and viewed under the light microscope (data not shown).

DISCUSSION

Although cisplatin was considered to be a most important therapeutic advance in the treatment of ovarian cancer, its clinical effectiveness is frequently limited by the drug resistance and the recurrence of tumors after treatments.[27] It has been reported that drug resistance to currently used chemotherapeutics is thought to be partly mediated by the ability to circumvent apoptosis.[28–30] Inhibition of apoptosis is taken to be a major contributing factor to cisplatin-resistance in various ovarian cancer cell lines.[31, 32] Therefore, one strategy to combat drug resistance is to find a new treatment approach where there exist different mechanisms for apoptosis and less adverse effects to address the limited effectiveness of current treatment modalities for ovarian cancer.

Quercetin, a ubiquitous bioactive plant flavonoid, is known for its low toxicity and antitumor activity. It has been shown to have anti-oxidative, antiviral, anti-inflammatory and antitumor effects and other less well-defined effects on behavior.[33, 34] Zhi-ping Yuan et al. demonstrated that 50 mg/kg Lipo-Que administered via intravenous route in nude mice can effectively inhibit

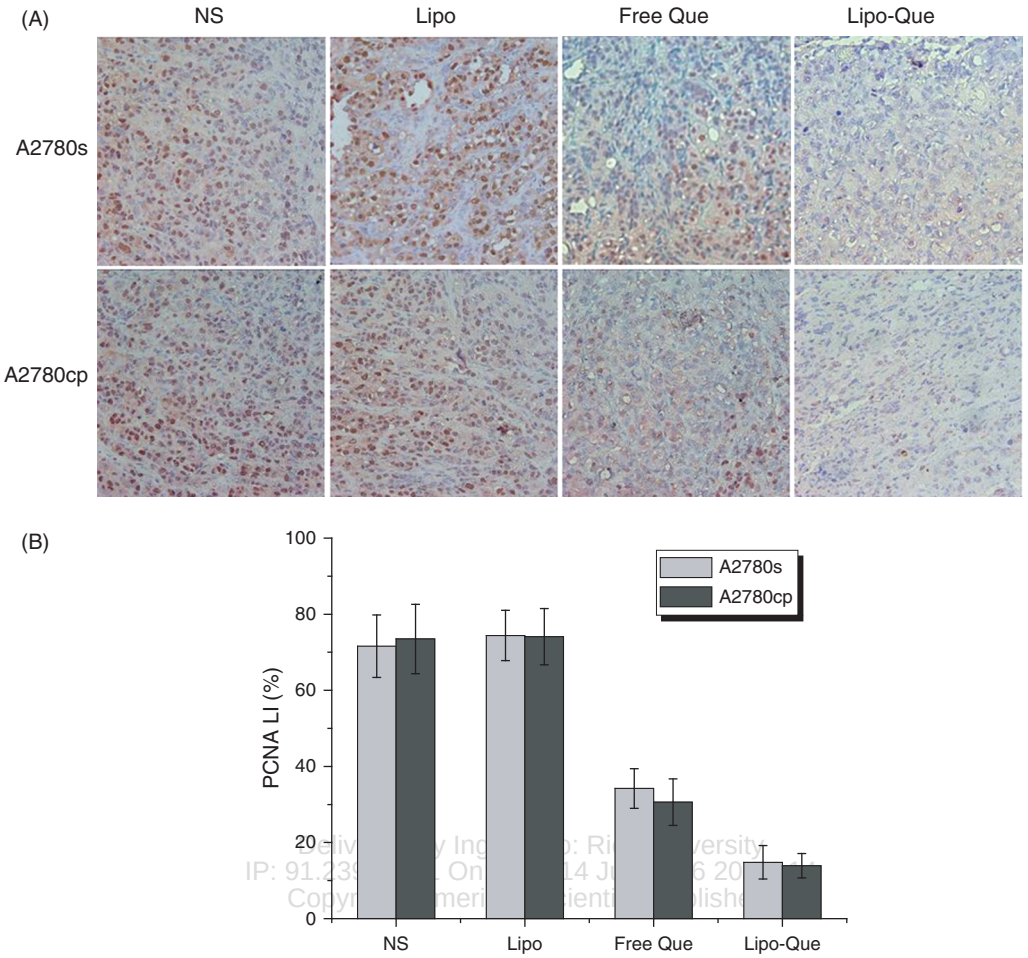

Figure 9. **PCNA immunohistochemical staining of tumors. A: Representative images of paraffin-embedded sections of A2780s and A2780cp tumors treated with NS, blank Lipo, free Que, or Lipo-Que; B. In A2780s tumors and A2780cp tumors, PCNA LI was significantly lower in Lipo-Que group compared with free Que ($p < 0.01$), blank Lipo ($p < 0.01$), or NS groups ($p < 0.01$).**

various types of tumors, and prolong the survival time of tumor-bearing mice without any systemic toxicities.[19] Other studies have also demonstrated the anti-cancer activity of quercetin both *in vivo* and *in vitro* on various tumors.[35–37]

Several observations were made in the current study concerning the antitumor activity of Lipo-Que on both cisplatin-sensitive and cisplatin-resistant human ovarian cancers. We have successfully prepared Lipo-Que with high EE ($92.5 \pm 0.96\%$), small particle size (163 ± 10 nm), and sustained release behavior, which supported by our results. The particle size and nanostructure also confirmed by TEM, which suggested Lipo-Que prepared in this work were stable and could be well-dispersed in aqueous solutions. We found that Lipo-Que showed a comparable *in vitro* cytotoxicity with free Que. The explanations may involve the two conflicting effects. After Que were manipulated to form nanoscale liposomes, the cellular uptake of Lipo-Que was enhanced, which would increase the cytotoxicity of Lipo-Que, whereas the sustained release behavior by liposomes would keep Que in a relative low concentration compared with free Que in the first 48 hours. Therefore, the cytotoxicity of Lipo-Que and free Que were comparable.

After Que was encapsulated into PEGylated liposomes, it could be well dispersed in water to form a homogeneous and stable solution for intravenous administration. Besides, nano-size and present of hydrophilic PEG shell of Lipo-Que prolonged their *in vivo* circulation time. Furthermore, the Lipo-Que could passively target to tumor site by the enhanced permeability and retention (EPR) effect, therefore improving their antitumor effects. We tested the Lipo-Que on cisplatin sensitive (A2780s) and-resistant (A2780cp) human ovarian cancer models. Lipo-Que effciently inhibited the growth of both A2780s and A2780cp cancer xenografts. For mice bearing A2780cp tumors, two of the five mice in the Lipo-Que-treated group demonstrated complete tumor regression. The histopathology of animal tissues revealed no systemic toxicity. These results strongly suggested that Lipo-Que may overcome cisplatin resistance in ovarian cancer. Although we did not examine the synergism of Lipo-Que with cisplatin, the data

obtained would suggest that this should be confirmed by future *in vivo* experiments.

In order to investigate the possible pathway that was involved with the antitumor effect of Lipo-Que *in vitro* and *in vivo*, the apoptosis of A2780s and A2780cp cells was analyzed *in vitro* and *in vivo* respectively. The experiments included PI staining fluorescence microscopy, DNA fragmentation assays and flow cytometric analysis of Lipo-Que-treated cancer cells. Our findings were consistent with the apoptosis-inducing ability of Que that was previously reported. The previously reported profound anti-tumor activity of Lipo-Que *in vitro*, prompted us to investigate TUNEL staining of Lipo-Que-treated tumor tissue to study the anti-tumor mechanisms *in vivo*. Our findings were also consistent with the apoptosis-inducing ability of quercetin mentioned above.

Another possible pathway that was involved with the antitumor effect of Lipo-Que *in vivo* was also studied. Angiogenesis plays a vital role in tumor growth and metastasis, and antiangiogenic therapy has been considered to be a new modality to treat human cancer.[38–41] One measure of angiogenic activity, MVD, is high in ovarian cancer tissue. Treatment with Lipo-Que resulted in the apparent reductions in intratumoral MVD of both A2780s and A2780cp tumors compared with free Lipo-treated or NS-treated mice.

CONCLUSIONS

In conclusion, the anti-tumor and anti-angiogenesis properties when integrated with the promising drug delivery system have made Lipo-Que the preferred choice not only for cisplatin-sensitive but also for cisplatin-resistant human ovarian cancers. Our findings collectively suggest that Lipo-Que is a promising agent that can be considered as a new treatment approach for human ovarian cancer, particularly for patients with cisplatin-resistance.

Declaration of Interest Statement

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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