

参赛队员姓名： 李璐阳

中学： 四川省成都市第七中学国际部

省份： 四川

国家/地区： 中国

指导教师姓名： 李亦桐

指导教师单位： 四川省成都市第七中学国际部

论文题目： Doxorubicin-Loaded T-Cell-Derived
Engineered Membrane Nanovesicles for Enhanced
In Vitro Antitumor Activity

Doxorubicin-Loaded T-Cell-Derived Engineered Membrane Nanovesicles for Enhanced In Vitro Antitumor Activity

Luyang Li ¹

¹ *Chengdu No.7 High School, Chengdu 610041, Sichuan Province, China*

Abstract: Chemotherapy is widely used for cancer treatment, but its effectiveness is often limited by poor drug delivery and systemic toxicity. Cell-derived nanovesicles may provide a useful way to improve the delivery of chemotherapy drugs. In this study, we developed engineered membrane nanovesicles (EMNVs) from the cytotoxic T-cell line CTLL-2 and evaluated their potential for doxorubicin (DOX) delivery. CTLL-2-derived EMNVs were prepared by serial extrusion and retained several membrane-associated and cytotoxic proteins, including granzyme B and perforin-1. Notably, EMNVs were efficiently taken up by B16F10, Hepa1-6, GL261, and mEC25 tumor cells, but the cytotoxic effects of unloaded EMNVs were generally limited and varied among the cell lines. Therefore, DOX was loaded into EMNVs to generate doxorubicin-loaded EMNVs (EMNV_{sDOX}), which increased the delivery of DOX into the nuclei. EMNV_{sDOX} also significantly reduced tumor cell viability and increased apoptosis in tumor cell lines. Caspase-3 cleavage was detected after treatment with either free DOX or EMNV_{sDOX}, supporting the involvement of apoptosis. These findings suggest that EMNVs may serve as a nanocarrier for improving DOX delivery and cytotoxicity in vitro.

Keywords: CTLL-2 cells; engineered membrane nanovesicles; drug delivery; doxorubicin

Table of Contents

1. Introduction	1
2. Materials and methods	2
2.1 CTLL-2 cell culture	2
2.2 Preparation of CTLL-2-cell-derived engineered membrane nanovesicles	3
2.3 Transmission electron microscopy	3
2.4 Nanoparticle tracking analysis	3
2.5 Western blot analysis	4
2.6 Flow cytometry and confocal microscopy analysis of EMNV uptake	5
2.7 Flow cytometry and confocal microscopy analysis of DOX delivery	6
2.8 Optimization of electroporation conditions and measurement of DOX loading	6
2.9 CCK-8 cell viability assay	7
2.10 Cell apoptosis assay	8
2.11 Flow-cytometric analysis of apoptosis induced by DOX and EMNVs _{DOX}	9
2.12 Western blot analysis of apoptosis-related proteins	10
2.13 Statistical analysis	11
3. Results	
3.1 Characterization of CTLL-2 Cells and CTLL-2 Cell-Derived EMNVs	11
3.2 Construction of the EMNVs _{DOX} Delivery System and Evaluation of Its In Vitro Cytotoxic Effects	18
3.3 EMNVs _{DOX} Enhanced Caspase-3 Cleavage in Tumor Cells	27
4. Discussion	28
5. Limitations	30
6. Conclusion	30
Acknowledgements	31
References	33

1. Introduction

Cancer is a major global health problem and one of the leading causes of death worldwide (Sung et al., 2021). Currently, there are several cancer treatment methods including surgery, radiotherapy, chemotherapy, targeted therapy, and immunotherapy (Miller et al., 2022). However, many patients still suffer from tumor recurrence and drug resistance (Hanahan, 2022). Moreover, some treatments can also damage normal tissues and cause serious side effects (Miller et al., 2022). Therefore, new treatments and drug-delivery methods are still needed.

Cancer immunotherapy strengthens the immune system and helps immune cells recognize and eliminate cancer cells (Bagchi et al., 2021). Immune checkpoint inhibitors are now widely used to treat several types of cancer (Bagchi et al., 2021). However, they only work in some patients, and resistance may develop during treatment (Bagchi et al., 2021). The complex tumor environment and poor immune-cell infiltration can also reduce the effects of immunotherapy (Hegde & Chen, 2020). Therefore, safer and more effective approaches are needed, especially for solid tumors.

Exosomes are small extracellular vesicles with diameters of 30 to 150 nm which are formed through the endosomal system and released when multivesicular bodies fuse with the plasma membrane (Kalluri & LeBleu, 2020). Exosomes contain many bioactive molecules, including proteins, lipids, messenger RNAs (mRNAs), and microRNAs (miRNAs) (van Niel et al., 2022). They can transfer these molecules from one cell to another and therefore play an important role in communication between cells (van Niel et al., 2022). Exosomes are also involved in tumor growth, metastasis, and immune regulation (Kalluri & McAndrews, 2023).

Exosomes have several advantages as drug carriers. Their small size allows them to enter tissues and cross some biological barriers (Herrmann et al., 2021). They also show good biocompatibility and relatively low immunogenicity (Herrmann et al., 2021). Exosomes can carry chemotherapeutic drugs, nucleic acids, and therapeutic proteins (Liang et al., 2021). Their membrane structure can protect these molecules from degradation and help deliver them to recipient cells (Herrmann et al., 2021). However, natural exosomes are often produced in small amounts. Their isolation and purification can also be difficult and time-consuming (Herrmann et al., 2021). These problems may limit their large-scale use.

Engineered membrane nanovesicles may help overcome some of these limitations. They can be prepared by repeatedly passing cells through membrane filters which can produce more vesicles within a shorter time than natural exosome secretion (Jang et al., 2013). The resulting nanovesicles can retain some membrane proteins and other components from their parent cells

(Jang et al., 2013). They can also be loaded with chemotherapeutic drugs and used as drug-delivery carriers (Jang et al., 2013). Because these vesicles are produced artificially, they should be distinguished from naturally secreted exosomes (Welsh et al., 2024).

Immune-cell-derived vesicles can retain functional molecules from their parent cells and take part in antitumor immune responses (Kugeratski & Kalluri, 2021). CD8⁺ T cells are cytotoxic immune cells that can kill abnormal cells through molecules such as granzyme B and perforin (Kugeratski & Kalluri, 2021). Extracellular vesicles released by activated CD8⁺ T cells can also reduce tumor progression and affect the tumor environment (Seo et al., 2018). These findings provided the basis for using CTLL-2 cell membranes to prepare engineered membrane nanovesicles.

Doxorubicin is a widely used chemotherapeutic drug with strong antitumor activity (Rawat et al., 2021). However, its clinical use is limited by damage to normal tissues, especially the heart (Rawat et al., 2021). Loading doxorubicin into membrane nanovesicles may improve its delivery and reduce the exposure of normal tissues to free drugs (Herrmann et al., 2021). In this study, we prepared CTLL-2-derived engineered membrane nanovesicles (EMNVs) and loaded them with doxorubicin (DOX). We characterized their morphology, particle size, protein markers, and drug-loading capacity. We then studied their uptake by different tumor cells and evaluated their effects on cell viability and apoptosis.

2. Materials and methods

2.1 CTLL-2 cell culture

CTLL-2 cells were cultured in CTLL-2 complete medium containing 10% fetal bovine serum (FBS), 1% penicillin–streptomycin, and recombinant mouse interleukin-2 (IL-2) at a final concentration of 100 IU/mL. The cells were grown in suspension at 37°C in a humidified incubator with 5% CO₂. Frozen cells were rapidly thawed in a 37°C water bath and diluted with prewarmed complete medium. The cells were centrifuged at 800 rpm for 3 min to remove the freezing medium and resuspended in complete medium and seeded at a density of 3×10^5 cells/mL. Fresh medium and IL-2 were added according to cell growth. Approximately 3 days after seeding, the cell suspension was transferred to a 50-mL centrifuge tube and left at room temperature for 20–30 min to allow some cells to settle naturally. The upper suspension was collected and centrifuged at $300 \times g$ for 5 min. The naturally settled cells were combined with the cells collected by centrifugation and resuspended in complete medium. Cell concentration

and viability were measured using acridine orange/propidium iodide staining. The cells were then reseeded at 3×10^5 cells/mL for further culture or experiments.

2.2 Preparation of CTLL-2-cell-derived engineered membrane nanovesicles

Expanded CTLL-2 cells were collected by centrifugation at $300 \times g$ for 5 min and washed twice with phosphate-buffered saline (PBS) to remove residual culture medium and serum proteins. The cells were resuspended in PBS and transferred into a Mini Extruder. The cell suspension was passed back and forth through a 1- μ m polycarbonate membrane for 12 cycles to disrupt the cells and promote the formation of membrane-derived nanovesicles. The resulting suspension was centrifuged at $10,000 \times g$ for 10 min at 4°C to remove large vesicles and cell debris. The supernatant was collected and passed through a 0.22- μ m syringe filter to remove insoluble materials. The filtered suspension was transferred into ultracentrifuge tubes and centrifuged at $100,000 \times g$ for 70 min at 4°C using an SW32 Ti swinging-bucket rotor. After centrifugation, the supernatant was carefully removed, and the nanovesicle pellet was resuspended in a small volume of sterile PBS. The prepared engineered membrane nanovesicles were referred to as EMNVs. They were divided into aliquots and stored at -80°C, and repeated freeze-thaw cycles were avoided. Samples used for transmission electron microscopy and nanoparticle tracking analysis were stored at 4°C and analyzed within two days.

2.3 Transmission electron microscopy

The morphology of EMNVs and doxorubicin-loaded EMNVs (EMNV_{SDOX}) was characterized by transmission electron microscopy (TEM). Before analysis, the samples were diluted to a protein concentration of approximately 0.5 μ g/ μ L. A 10- μ L drop of each preparation was applied to a 200-mesh carbon support film grid (Zhongjingkeyi Technology Co., Ltd., China) and incubated at room temperature for 3 min to allow adsorption. The excess suspension was removed gently with filter paper. Each grid was then treated with 10 μ L of 2% phosphotungstic acid for 3 min for negative staining, after which the remaining stain was removed, and the grids were allowed to air-dry at room temperature. Images were acquired using a JEM-1400Flash transmission electron microscope (JEOL Ltd., Japan) operated at an accelerating voltage of 120 kV. Representative micrographs were collected for analysis.

2.4 Nanoparticle tracking analysis

The particle-size distribution, particle concentration, and zeta potential of EMNVs and EMNV_{SDOX} were analyzed with a ZetaView MONO nanoparticle tracking analyzer equipped

with a 488-nm laser (Particle Metrix, Germany). Before a sample was introduced, the measurement chamber was flushed with ultrapure water. Rinsing was continued until fewer than 10 particles were detected in the field of view. Each sample was first diluted 1,000-fold with ultrapure water, and the final volume was made up to at least 1.5 mL. Depending on the particle count obtained, the dilution was adjusted further to give approximately 50–400 particles at each measurement position. The diluted preparation was loaded slowly through a disposable syringe, while care was taken to prevent the introduction of air bubbles.

Particle size and concentration were measured with the particle-size mode selected in the instrument software. The measurements were carried out at 24°C, with recordings taken at 11 positions over two cycles. Camera sensitivity and shutter value were both set at 70. After image acquisition, the minimum brightness was set to 20, and the minimum and maximum particle areas were set to 5 and 1,000, respectively. The concentration measured by the instrument was then corrected according to the dilution used; the concentration of the original sample was calculated by multiplying the measured value by the corresponding dilution factor.

Zeta-potential measurements were performed on the same diluted preparations using the same instrument. For this analysis, the software was switched to zeta-potential mode, and measurements were conducted at 24°C under the instrument's designated settings. The zeta potential reported for each sample was derived from the readings obtained at the individual measurement positions. Between consecutive samples, the chamber was washed thoroughly with ultrapure water to prevent carryover.

2.5 Western blot analysis

Western blotting was used to compare the protein profiles of CTLL-2 cells and the derived engineered membrane nanovesicles (EMNVs). CTLL-2 cells were collected by centrifugation at $300 \times g$ for 5 min and washed twice with PBS. The cells were lysed in RIPA buffer on ice for 30–60 min. EMNVs suspended in PBS were mixed with RIPA buffer at a volume ratio of at least 1:1 and lysed under the same conditions. Both samples were sonicated on ice using 5-s sonication and 5-s pause cycles for a total sonication time of 1 min. The lysates were centrifuged at $10,000 \times g$ for 10 min at 4°C, and the supernatants were collected. Protein concentrations were measured using a BCA assay. BSA standards were prepared at concentrations of 0, 0.0156, 0.03125, 0.0625, 0.125, 0.25, 0.5, and 1 µg/mL. The BCA working solution was prepared by mixing reagents A and B at a ratio of 50:1. After 200 µL of the working solution was added, the samples were incubated at 37°C for 30 min. Absorbance was measured at 562 nm using a Tecan Infinite E Plex microplate reader (Tecan, Switzerland).

Equal amounts of protein, ranging from 10 to 30 μg per lane, were mixed with 5 $\times$ loading buffer at a volume ratio of 4:1 and heated at 100°C for 5–10 min. The proteins were separated using FuturePAGE® 8% precast polyacrylamide gels (ACE Biotechnology, China) at 120 V. They were then transferred onto 0.45- μm PVDF membranes (Immobilon-P, Merck Millipore, IPVH00010) at 100 V for 60 min. The membranes were blocked with rapid blocking buffer according to the manufacturer's instructions. They were then incubated overnight at 4°C with primary antibodies against CD63 (Abways, CY5253), flotillin-1 (HuaAn Biotechnology, ET7107-82), calnexin (HuaAn Biotechnology), Na⁺/K⁺-ATPase (Abways), granzyme B (Abcam, ab255598), and Perforin-1. The antibodies against CD63, flotillin-1, calnexin, and granzyme B were diluted 1:1,000. The antibodies against Na⁺/K⁺-ATPase and Perforin-1 were diluted 1:2,000. After primary antibody incubation, the membranes were washed three times with TBST for 5–10 min each. The membranes were then incubated with an HRP-conjugated goat anti-rabbit IgG antibody (HuaAn Biotechnology, HA1001) at a dilution of 1:30,000 for 1 h at room temperature. After three further washes with TBST, the protein signals were detected using an enhanced chemiluminescence substrate and recorded using a chemiluminescence imaging system.

2.6 Flow cytometry and confocal microscopy analysis of EMNV uptake

The uptake of EMNVs by tumor cells was examined using flow cytometry and confocal laser scanning microscopy. Before fluorescence labeling, EMNVs suspended in PBS were diluted at least 1:7 with sterile ultrapure water to reduce the NaCl concentration to below 20 mM. A 5 mM DiR stock solution was added at a dilution of 1:1,000 to obtain a final DiR concentration of 5 μM . The samples were incubated at 37°C for 20 min in the dark and then centrifuged at 100,000 $\times g$ for 70 min to remove free DiR. The DiR-labeled EMNVs were resuspended in sterile PBS. B16F10, GL261, mEC25, and Hepa1-6 cells were seeded into 24-well plates at a density of 4×10^4 cells per well. Sterile coverslips were placed in the wells used for confocal microscopy. After 24 h, the cells were treated with DiR-labeled EMNVs at a final protein concentration of 10 $\mu\text{g}/\text{mL}$ and cultured for another 24 h. For flow cytometry, the cells were washed twice with PBS, detached using trypsin, washed twice again, and resuspended in 300 μL of PBS. DiR fluorescence was detected in the APC-Cy7-H channel using a NovoCyte Advanteon flow cytometer (Agilent Technologies, USA). Untreated cells were used as the control, and EMNV uptake was evaluated based on the percentage of DiR-positive cells. For confocal microscopy, the cells were washed twice with PBS and fixed with 200 μL of 4% paraformaldehyde for 15 min at room temperature. The cells were then washed twice with PBS

and permeabilized with 200 μL of 0.5% Triton X-100 for 5 min. After two further washes, the cells were stained with FITC-conjugated phalloidin diluted 1:200 in PBS for 30 min at room temperature. The nuclei were stained with DAPI for 10 min in the dark. The coverslips were mounted onto glass slides using an antifade mounting medium. DAPI, FITC, and DiR fluorescence signals were collected using a Zeiss LSM 900 confocal laser scanning microscope (Carl Zeiss, Germany).

2.7 Flow cytometry and confocal microscopy analysis of DOX delivery

The delivery of DOX into tumor cells was examined using a NovoCyt Advanteon flow cytometer (Agilent Technologies, USA) and confocal laser scanning microscopy. B16F10, Hepa1-6, GL261, and mEC25 cells were seeded into 24-well plates at a density of 4×10^4 cells per well. Sterile coverslips were placed in the wells used for confocal microscopy. After 24 h, the cells were treated with DOX or EMNV_{SDOX} at an equivalent DOX concentration of 1 μM and cultured for another 24 h. Untreated cells were used as the control.

For flow-cytometric analysis, the cells were collected and prepared according to the procedure described in the section “Flow cytometry and confocal microscopy analysis of EMNV uptake.” Briefly, the cells were washed twice with PBS, detached using trypsin, washed twice again, and resuspended in 300 μL of PBS. The intrinsic fluorescence of DOX was detected in the PE-H channel using a flow cytometer. Intracellular DOX accumulation was evaluated based on the mean fluorescence intensity of DOX.

For confocal microscopy, the cells were fixed, permeabilized, and stained according to the procedure described for the confocal analysis of EMNV uptake. Briefly, the cells were fixed with 4% paraformaldehyde, permeabilized with 0.5% Triton X-100, stained with FITC-conjugated phalloidin, and counterstained with DAPI. The coverslips were mounted using an antifade mounting medium. DAPI, FITC, and DOX fluorescence signals were collected using a Zeiss LSM 900 confocal laser scanning microscope (Carl Zeiss, Germany).

2.8 Optimization of electroporation conditions and measurement of doxorubicin loading

Doxorubicin was dissolved in sterile double-distilled water to prepare a 10 mM stock solution. The solution was filtered through a 0.22- μm sterile filter and protected from light. The EMNV stock solution had a protein concentration of 4.48 $\mu\text{g}/\mu\text{L}$. For each electroporation sample, 22.3 μL of the EMNV stock solution, corresponding to 100 μg of EMNV protein, was mixed with doxorubicin at final concentrations of 0.25, 0.5, 1.0, 2.0, or 3.0 mM. PBS

containing 400 mM sucrose was added to a final volume of 200 μ L. The mixtures were incubated at 4°C for 30 min and transferred into precooled 0.4-cm electroporation cuvettes. Electroporation was performed using a Gene Pulser Xcell system with a CE module (Bio-Rad, USA). Three conditions were tested: 125 V and 900 μ F, 250 V and 125 μ F, and 950 V and 50 μ F. A single pulse was applied to each sample. Samples containing the same amounts of EMNVs and doxorubicin without electroporation were used as controls. After electroporation, the samples were incubated at 37°C for 30 min to allow the nanovesicle membranes to recover. A total of 300 μ L of PBS was then added to each sample. The samples were transferred into 100-kDa centrifugal filter units (Solarbio, YA1869) and centrifuged at $4,000 \times g$ for 15 min. The retained EMNV_{SDOX} were washed twice with PBS and adjusted to a final volume of 200 μ L. The first filtrate and the two washing solutions were collected and combined to measure free doxorubicin. Doxorubicin standards were prepared at concentrations of 0, 0.019531, 0.0390625, 0.078125, 0.15625, 0.3125, 0.625, 1.25, 2.5, 5, and 10 μ M. A 200- μ L aliquot of each standard or diluted filtrate was added to a black 96-well plate. Fluorescence was measured at an excitation wavelength of 490 nm and an emission wavelength of 590 nm using a Tecan Infinite E Plex microplate reader (Tecan, Switzerland). The amount of free doxorubicin was calculated from the standard curve. The amount of loaded doxorubicin was calculated by subtracting the amount of free doxorubicin from the total amount initially added. Loading efficiency was calculated as the amount of loaded doxorubicin divided by the total amount of doxorubicin initially added and multiplied by 100%. The DOX loading content was calculated by normalizing the amount of loaded doxorubicin to the amount of EMNV protein and was expressed as μ mol/ μ g. In addition, the average amount of doxorubicin associated with each EMNV particle was estimated by dividing the total amount of loaded doxorubicin by the particle number measured using nanoparticle tracking analysis.

Based on the appearance of the DOX-containing EMNV mixtures and the overall loading performance, 950 V/50 μ F was selected as the final electroporation condition. EMNV_{SDOX} prepared using an initial doxorubicin concentration of 1.0 mM were used in the subsequent experiments.

2.9 CCK-8 cell viability assay

Cell viability following treatment with unloaded engineered membrane nanovesicles (EMNVs), doxorubicin (DOX), or DOX-loaded EMNVs (EMNV_{SDOX}) was assessed by a Cell Counting Kit-8 (CCK-8) assay. B16F10, Hepa1-6, GL261, and mEC25 cells were seeded in 96-well plates at 8×10^3 cells per well, with 100 μ L of complete culture medium added to each

well. After 24 h of culture, the medium was aspirated and replaced with 100 μ L of fresh medium containing the assigned treatment.

The first experiment was designed to examine the effects of treatment concentration and exposure time. DOX and EMNVs_{DOX} were added at matched DOX concentrations of 0, 0.1875, 0.375, 0.75, 1.5, or 3.0 μ M. Cells were exposed for either 24 or 48 h before viability was measured.

A separate experiment included four groups: Control, EMNVs, DOX, and EMNVs_{DOX}. The DOX and EMNVs_{DOX} groups were treated with 1 μ M DOX. For the EMNVs group, the amount of unloaded EMNVs was matched to that contained in the EMNVs_{DOX} treatment. Based on the measured DOX-loading content, the corresponding EMNV concentration was 2 μ g/mL. All treatments in this experiment lasted 24 h.

At the end of the incubation period, 10 μ L of CCK-8 reagent was added directly to each well. The plates were kept at 37°C for 2 h in the dark, after which absorbance at 450 nm was recorded with a Tecan Infinite E Plex microplate reader (Tecan, Switzerland). Wells containing no cells were included as blank controls. Untreated cells served as the control group, and five replicate wells were prepared for each group.

Cell viability was calculated using the following formula:

$$\text{Cell viability (\%)} = \frac{A_{\text{sample}} - A_{\text{blank}}}{A_{\text{control}} - A_{\text{blank}}} \times 100 \quad (1)$$

The half-maximal inhibitory concentration (IC₅₀) was estimated in GraphPad Prism by nonlinear regression. Before curve fitting, the DOX concentrations were log-transformed. The resulting concentration–response data were fitted with a four-parameter variable-slope model, and the fitted curve was used to determine the DOX concentration corresponding to 50% cell viability.

2.10 Cell apoptosis assay

The effect of unloaded EMNVs on tumor-cell apoptosis was measured using Annexin V–PE/7-AAD staining. B16F10, Hepa1-6, GL261, and mEC25 cells were seeded into 24-well plates at a density selected to reach approximately 70–80% confluence on the following day. Each group contained three replicate wells. When the cells reached the required confluence, the original medium was replaced with fresh medium containing EMNVs at final protein concentrations of 0, 50, 100, 200, or 300 μ g/mL.

After 24 h of treatment, the culture supernatant containing detached cells was collected. The remaining adherent cells were detached using EDTA-free trypsin and combined with the

cells collected from the supernatant. The cells were centrifuged at $300 \times g$ for 5 min at room temperature and washed twice with PBS. They were then resuspended in $1 \times$ binding buffer and adjusted to a concentration of 1×10^6 cells/mL.

A 100- μ L aliquot of the cell suspension was transferred into each tube and stained with 5 μ L of Annexin V-PE and 10 μ L of 7-AAD. The samples were incubated for 15 min at room temperature in the dark. Next, 400 μ L of $1 \times$ binding buffer was added to each tube. Unstained, Annexin V-PE single-stained, and 7-AAD single-stained controls were used to adjust the detector settings and fluorescence compensation.

The samples were analyzed using a NovoCyte Advanteon flow cytometer (Agilent Technologies, USA). Cellular debris and doublets were excluded using forward- and side-scatter parameters. At least 10,000 valid single-cell events were collected from each sample. Annexin V-PE-negative/7-AAD-negative cells were considered viable. Annexin V-PE-positive/7-AAD-negative cells were considered early apoptotic, whereas Annexin V-PE-positive/7-AAD-positive cells were considered late apoptotic or dead. The total apoptotic rate was calculated as the sum of the early and late apoptotic cell populations.

2.11 Flow-cytometric analysis of apoptosis induced by DOX and EMNVs_{DOX}

The apoptotic effects of DOX and EMNVs_{DOX} were compared using Annexin V-YSFluor™ 647/7-AAD staining. Annexin V-YSFluor™ 647 was used instead of Annexin V-PE because the intrinsic fluorescence of DOX could interfere with fluorescence detection in the PE channel.

B16F10, Hepa1-6, GL261, and mEC25 cells were seeded into 24-well plates at a density selected to reach approximately 70–80% confluence on the following day. Each treatment group contained three replicate wells. When the required confluence was reached, the cells were divided into Control, EMNVs, DOX, and EMNVs_{DOX} groups. The DOX and EMNVs_{DOX} groups received an equivalent DOX concentration of 1 μ M. The EMNVs group received 2 μ g/mL unloaded EMNVs, corresponding to the amount of EMNVs present in the EMNVs_{DOX} group. The cells were treated for 24 h.

After treatment, both the culture supernatant and adherent cells were collected using the procedure described in the section “Cell apoptosis assay.” The collected cells were washed twice with PBS and resuspended in binding buffer. The cells were then stained with Annexin V-YSFluor™ 647 and 7-AAD according to the manufacturer’s instructions and incubated at room temperature in the dark.

The samples were analyzed using a CytoFLEX 5 flow cytometer (Beckman Coulter, USA). Annexin V–YSFluor™ 647 and 7-AAD fluorescence signals were detected using their corresponding fluorescence channels. Unstained and single-stained controls were used to adjust the detector settings and fluorescence compensation. Cellular debris and doublets were excluded before apoptosis analysis. Annexin V–YSFluor™ 647-negative/7-AAD-negative cells were considered viable. Annexin V–YSFluor™ 647-positive/7-AAD-negative cells were considered early apoptotic, and double-positive cells were considered late apoptotic or dead. Total apoptosis was calculated as the sum of the early and late apoptotic cell populations.

2.12 Western blot analysis of apoptosis-related proteins

Western blotting was used to examine Caspase-3 cleavage after treatment with DOX or EMNV_{SDOX}. B16F10, Hepa1-6, GL261, and mEC25 cells were seeded into 6-well plates at approximately 2.5×10^5 to 3.0×10^5 cells per well and cultured overnight. When the cells reached approximately 70–80% confluence, they were divided into Control, EMNVs, DOX, and EMNV_{SDOX} groups. The DOX and EMNV_{SDOX} groups received an equivalent DOX concentration of 1 μ M. The EMNVs group received unloaded EMNVs at a final protein concentration of 2 μ g/mL. The cells were treated for 24 h.

After treatment, the culture medium was removed, and the cells were washed twice with 1–2 mL of precooled PBS. The cells were lysed with 100–150 μ L of RIPA buffer per well and collected using a cell scraper. The samples were lysed on ice for 30–60 min and then sonicated on ice using 5-s sonication and 5-s pause cycles for a total sonication time of 1 min.

Protein concentrations were measured using a BCA assay. The BCA working solution was prepared by mixing reagents A and B at a ratio of 50:1. BSA standards were prepared at concentrations of 0, 0.0156, 0.03125, 0.0625, 0.125, 0.25, 0.5, and 1 μ g/ μ L. Protein samples and BSA standards were added to a 96-well plate, followed by 200 μ L of BCA working solution. The plate was incubated at 37°C for 30 min, and absorbance was measured at 562 nm. Protein concentrations were calculated using the BSA standard curve.

Equal amounts of protein, ranging from 20 to 30 μ g per lane, were separated using 4–20% ACE gradient polyacrylamide gels at 100 V for 70 min. The proteins were then transferred onto PVDF membranes at 100 V for 60 min. The membranes were blocked with rapid blocking buffer for 15 min and incubated overnight at 4°C with a SuperLepX Cleaved Caspase-3 (Asp175) Rabbit monoclonal antibody (Dakewe Biotech, 8600353; clone DR0617) at a dilution of 1:1,000 or a recombinant rabbit anti- β -Tubulin antibody (HUABIO, ET1602-4) at a dilution of 1:8,000. β -Tubulin was used as the loading control.

After primary-antibody incubation, the membranes were washed with TBST and incubated with an HRP-conjugated goat anti-rabbit secondary antibody at a dilution of 1:30,000 for 1 h at room temperature. After further washing, the protein signals were detected using an enhanced chemiluminescence substrate and recorded using a chemiluminescence imaging system.

2.13 Statistical analysis

Data processing, visualization, and statistical analysis were performed using GraphPad Prism version 8.0.2 (GraphPad Software, USA). Data are presented as the mean $\pm$ standard deviation (SD), unless otherwise stated in the figure legends. Data normality and homogeneity of variance were assessed before statistical comparisons. Comparisons between two independent groups were performed using a two-tailed unpaired Student's *t*-test when the data followed a normal distribution and showed equal variances. Comparisons among multiple groups were performed using one-way analysis of variance (one-way ANOVA), followed by Tukey's or Dunnett's multiple-comparisons test, as appropriate. Nonparametric tests were used when the data did not meet the assumptions of parametric analysis. Pearson's correlation analysis was used to examine correlations between normally distributed continuous variables. All statistical tests were two-sided. A *P* value of less than 0.05 was considered statistically significant, while nonsignificant differences were labeled as "ns."

3. Results

3.1 Characterization of CTLL-2 Cells and CTLL-2 Cell-Derived EMNVs

CTLL-2 cells were initially expanded in medium containing mouse IL-2. Under bright-field microscopy, they grew in suspension. At lower cell densities, most cells remained dispersed, whereas increasing cell density was accompanied by a closer, more compact distribution (Figure 1A). In the confocal images, green fluorescence was observed along the cell membranes, and the nuclei appeared blue after DAPI staining (Figure 1B).

Flow cytometry was subsequently used to examine the viability of the cultured cells and their expression of cytotoxic molecules. The predominant CTLL-2 population represented 85.56% of all collected events. After sequential gating, singlets accounted for 79.63% of the gated events, of which 98.86% were viable. Within the viable singlet population, 99.00% of cells were positive for both Granzyme B and Perforin-1 (Figure 1C). Thus, the cultured CTLL-

2 cells retained high viability together with a pronounced cytotoxic phenotype and were suitable as parental cells for subsequent EMNV preparation.

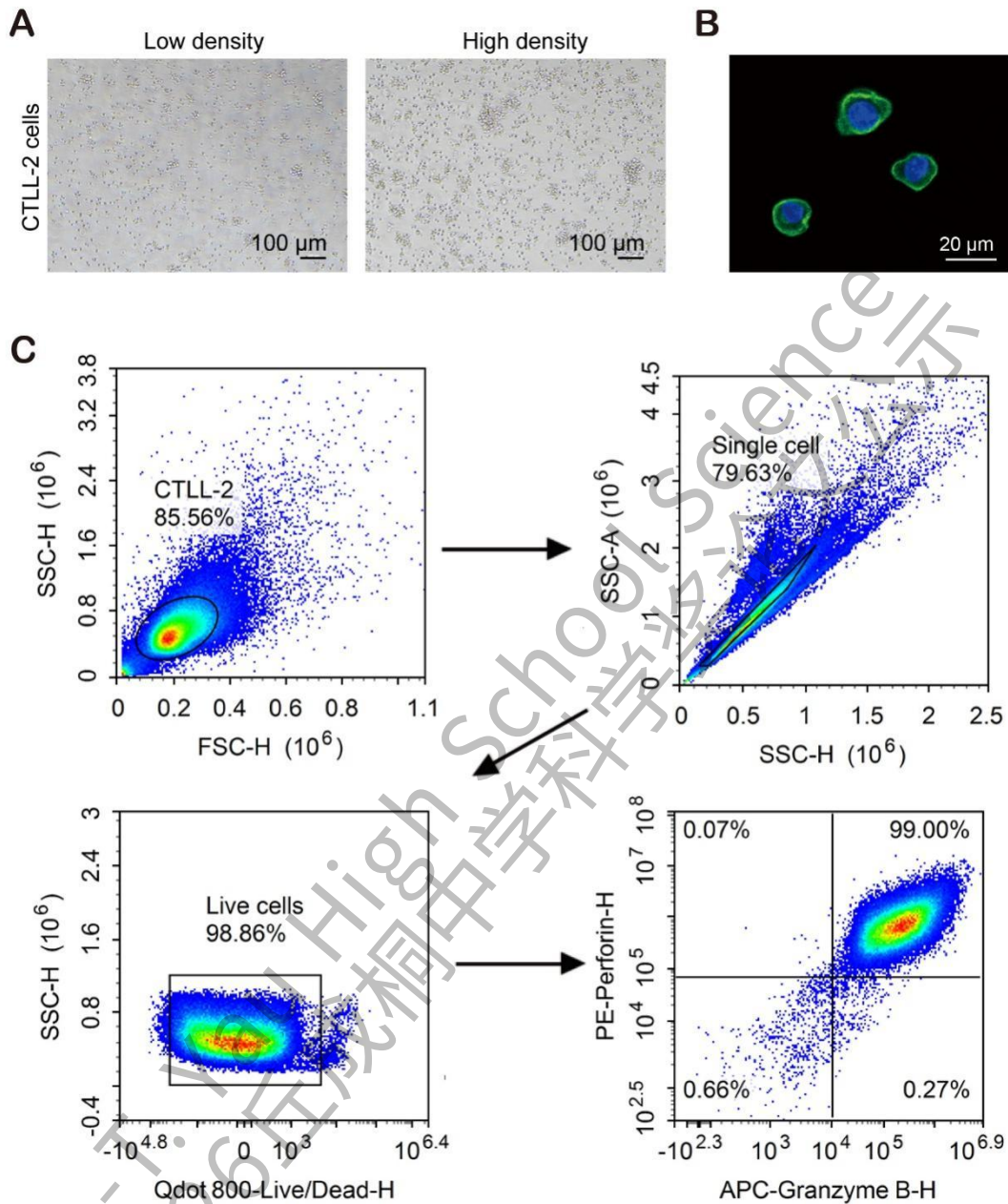


Figure 1. Culture and characterization of CTLL-2 cells. (A) Representative bright-field images of CTLL-2 cells cultured in medium containing mouse IL-2 at low and high cell densities. Scale bar, 100 μm . (B) Representative confocal microscopy image of CTLL-2 cells. Cell membranes are shown in green, and cell nuclei were stained with DAPI and are shown in blue. Scale bar, 20 μm . (C) Flow-cytometric analysis of Granzyme B and Perforin-1 expression in CTLL-2 cells. The main cell population, single cells, and live cells were selected by sequential gating before analyzing the percentage of Granzyme B/Perforin-1 double-positive cells.

Given the favorable cellular condition and retention of cytotoxic proteins, EMNVs were subsequently generated from CTLL-2 cells through serial extrusion. Nanoparticle tracking analysis revealed that the resulting CTLL-2-derived EMNVs exhibited a mean particle size of 211.2 nm (Figure 2A). Consistent with their nanoscale vesicular characteristics, the EMNVs displayed a negative surface charge, with a zeta potential of -24.04 ± 0.75 mV (Figure 2B). Transmission electron microscopy further revealed relatively intact vesicle-like structures, supporting the successful generation of CTLL-2-derived EMNVs (Figure 2C).

The protein composition of the EMNVs was then compared with that of parental CTLL-2 cells by Western blotting. The vesicle-associated proteins CD63 and Flotillin-1 were detected in the EMNV preparation, while Na^+/K^+ -ATPase and calnexin were detected in EMNVs, indicating that components from different cellular compartments were retained during EMNV generation. Of particular interest, Granzyme B and Perforin-1 were detected in both CTLL-2 cells and the resulting EMNVs (Figure 2D), suggesting that these cytotoxic proteins were retained during EMNV generation. Collectively, these findings demonstrate that CTLL-2-derived EMNVs possess characteristic nanoscale vesicular properties while preserving key cytotoxic proteins from their parental CTLL-2 cells.

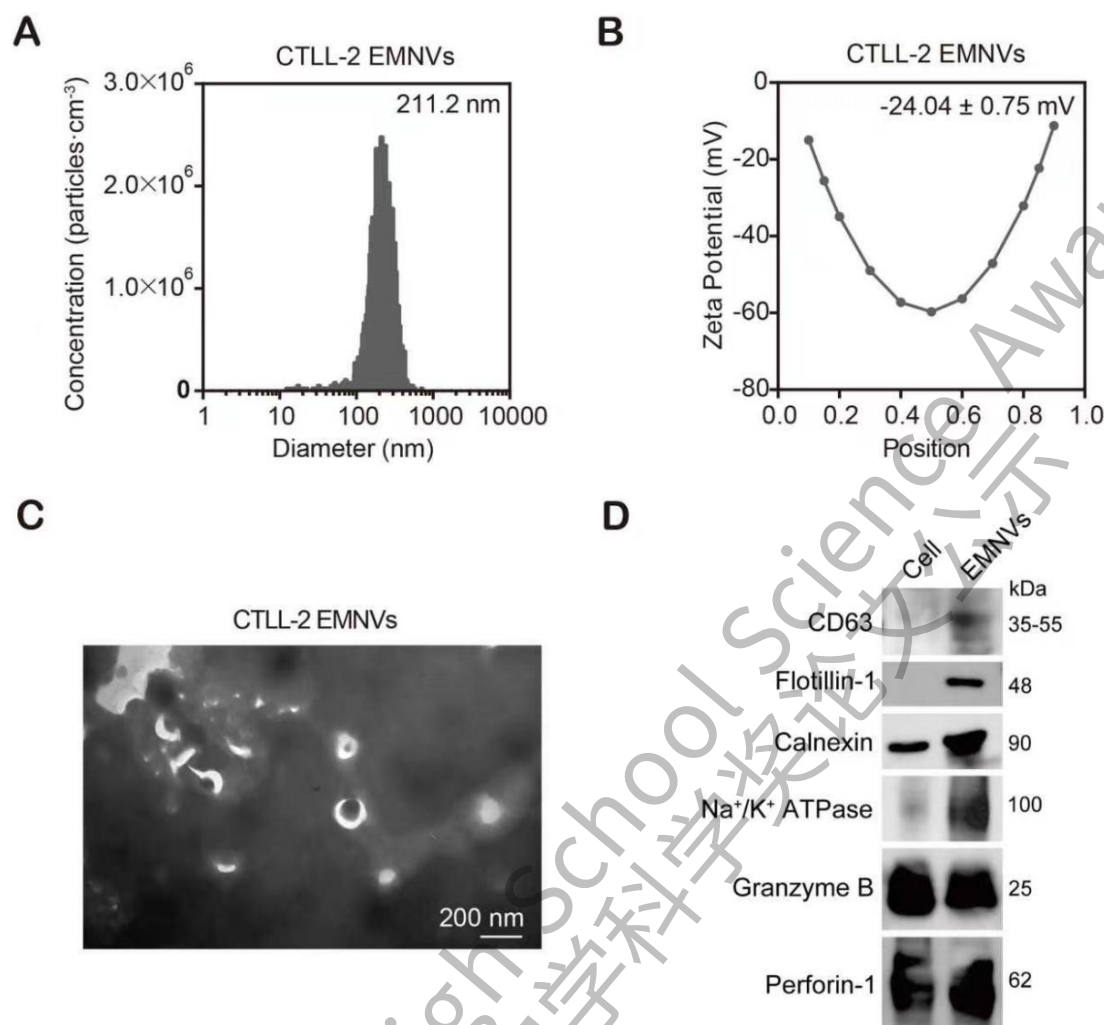


Figure 2. Preparation and characterization of CTLL-2-cell-derived engineered membrane nanovesicles. (A) Particle-size distribution of CTLL-2-cell-derived engineered membrane nanovesicles (EMNVs) measured by nanoparticle tracking analysis (NTA). (B) Zeta potential of EMNVs measured by NTA. (C) Representative transmission electron microscopy (TEM) image showing the morphology of EMNVs. Scale bar, 200 nm. (D) Western blot analysis of vesicle-associated proteins (CD63 and Flotillin-1), cytotoxic molecules (Granzyme B and Perforin-1), the plasma membrane-associated protein Na⁺/K⁺-ATPase, and the endoplasmic reticulum-associated protein Calnexin in CTLL-2 cells and EMNVs.

3.2 EMNVs were efficiently internalized by multiple tumor-cell lines but exhibited limited cytotoxic activity

Because EMNVs contained Granzyme B and Perforin-1, we next examined whether they could be taken up by tumor cells. EMNVs were labeled with DiR and incubated with B16F10,

Hepa1-6, GL261, and mEC25 cells for 24 h. Flow cytometry showed a clear increase in DiR fluorescence in all four cell lines compared with the Control groups. The percentages of DiR-positive cells were 99.78% in B16F10 cells, 100.00% in Hepa1-6 cells, 100.00% in GL261 cells, and 99.99% in mEC25 cells (Figure 3A). Thus, nearly all cells in each tested cell line were positive for DiR after incubation with DiR-EMNVs.

Furthermore, confocal microscopy was used to observe the distribution of DiR fluorescence. Little or no red fluorescence was detected in the Control groups. In contrast, strong red signals were observed within the cellular regions of all four tumor-cell lines treated with DiR-EMNVs. The red fluorescence was mainly found around the nuclei and within the regions marked by Phalloidin staining (Figure 3B). These results supported the efficient uptake of DiR-EMNVs by different tumor-cell types.

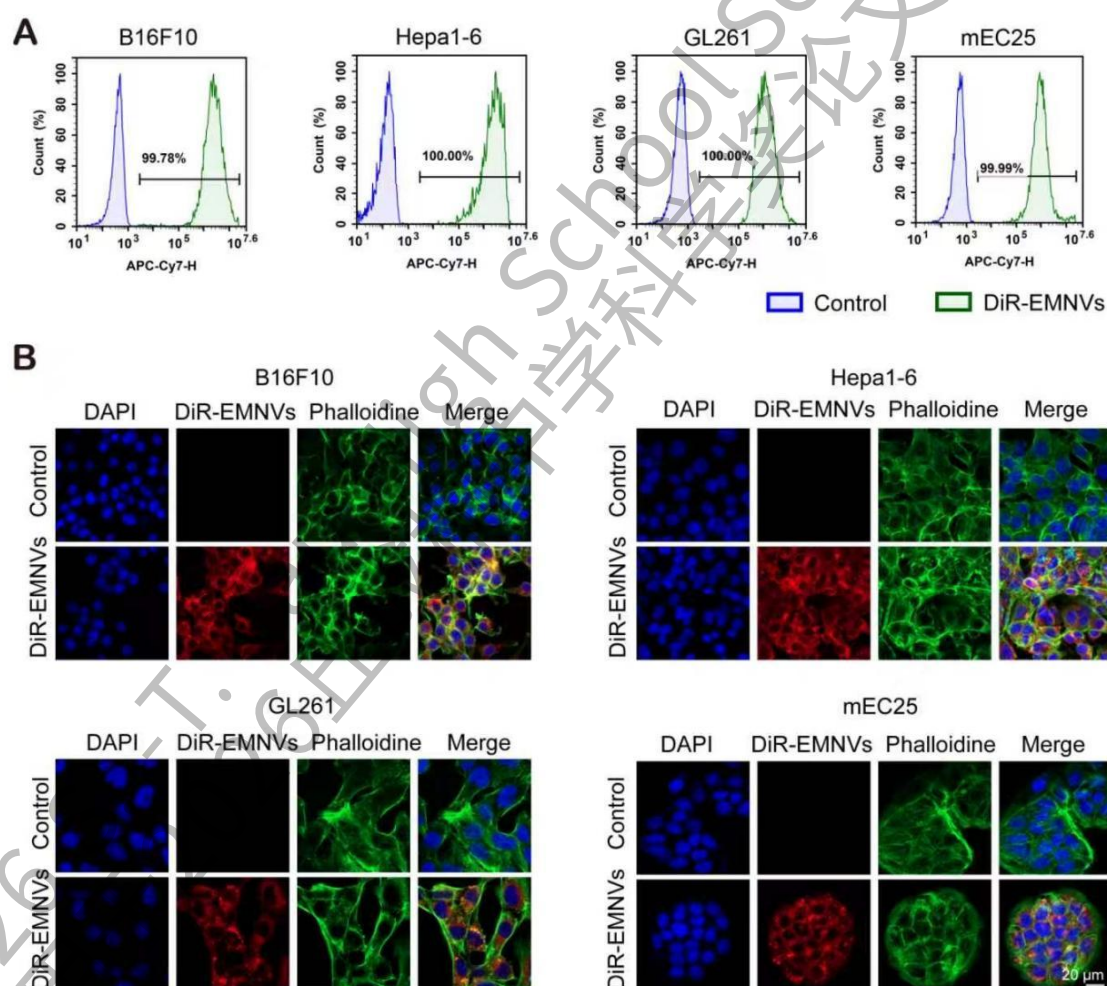


Figure 3. Analysis of tumor-cell uptake and intracellular fluorescence distribution of EMNVs. (A) After B16F10, Hepa1-6, GL261, and mEC25 cells were incubated with DiR-labeled EMNVs for 24 h, the percentages of DiR-positive cells were measured by flow cytometry to evaluate cellular uptake of DiR-EMNVs. Untreated cells were used as controls. (B) Confocal laser scanning microscopy was used

to examine the distribution of DiR-EMNV-associated fluorescence signals in B16F10, Hepa1-6, GL261, and mEC25 cells. Cell nuclei were stained with DAPI (blue), the actin cytoskeleton was stained with Phalloidin (green), and DiR-EMNVs are shown in red. Scale bar, 20 μm .

Next, we evaluated whether unloaded EMNVs could directly induce tumor-cell apoptosis. B16F10, Hepa1-6, GL261, and mEC25 cells were treated with 0, 50, 100, 200, or 300 $\mu\text{g/mL}$ EMNVs for 24 h. Cell apoptosis was then measured using Annexin V-PE/7-AAD staining (Figure 4A). The four cell lines showed clearly different responses to EMNV treatment.

B16F10 cells showed the lowest sensitivity. No significant increase in apoptosis was observed at any tested EMNV concentration, including 300 $\mu\text{g/mL}$ (Figure 4B). In contrast, Hepa1-6 cells were more sensitive. Treatment with 50 $\mu\text{g/mL}$ EMNVs significantly increased apoptosis, and the apoptotic rate continued to rise as the EMNV concentration increased. Therefore, EMNVs induced apoptosis in Hepa1-6 cells in a concentration-dependent manner.

GL261 cells showed an intermediate response. Treatment with 50 $\mu\text{g/mL}$ EMNVs did not cause a significant change. However, apoptosis increased significantly when the concentration reached 100 $\mu\text{g/mL}$, and a further increase was observed at 300 $\mu\text{g/mL}$. Meanwhile, mEC25 cells showed no significant response at concentrations from 50 to 200 $\mu\text{g/mL}$. A significant increase in apoptosis was only observed at 300 $\mu\text{g/mL}$ (Figure 4B).

Overall, the direct cytotoxic effect of unloaded EMNVs depended strongly on the tumor-cell line. EMNVs induced clear apoptosis in Hepa1-6 cells and a smaller response in GL261 cells. However, their effect was limited in mEC25 cells and was not significant in B16F10 cells within the tested concentration range. Therefore, the cytotoxic proteins naturally carried by EMNVs were not sufficient to produce a strong and broad cytotoxic effect against all tumor-cell types. To address this limitation, DOX was loaded into EMNVs to construct the EMNVs-DOX delivery system.

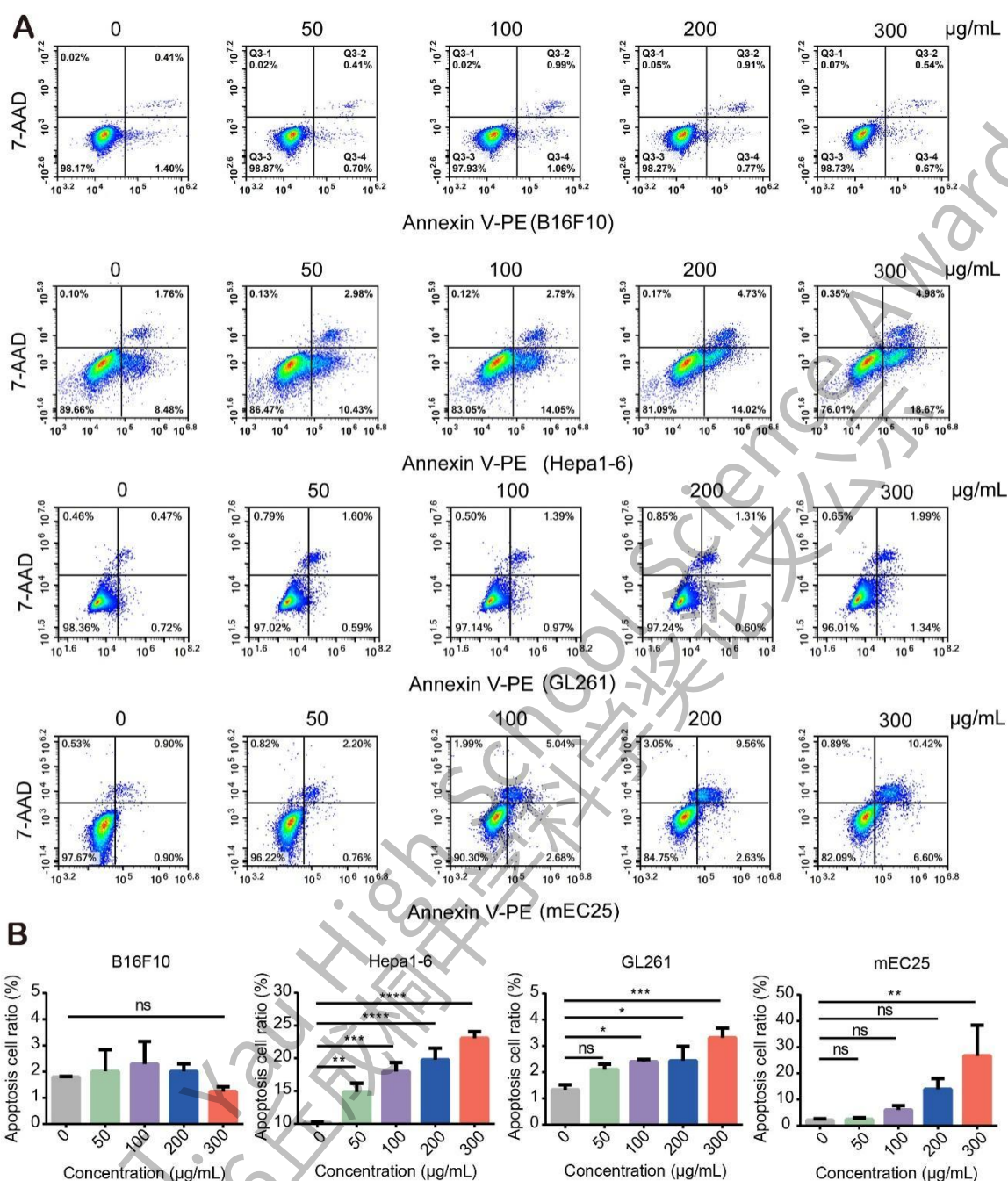


Figure 4. Effects of EMNVs on apoptosis in different tumor-cell lines. (A) B16F10, Hepa1-6, GL261, and mEC25 cells were treated with EMNVs at different concentrations (0, 50, 100, 200, and 300 $\mu\text{g/mL}$) for 24 h. Cell apoptosis was then examined by flow cytometry using Annexin V-PE/7-AAD staining. (B) Quantitative analysis of the total apoptotic rates of the four tumor-cell lines after treatment with different concentrations of EMNVs. The total apoptotic rate was calculated as the sum of the early and late apoptotic cell populations. Data are presented as the mean $\pm$ SD. ns, not significant; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$.

3.2 Construction of the EMNVs-DOX Delivery System and Evaluation of Its In Vitro Cytotoxic Effects

3.2.1 Electroporation Loading of DOX and Optimization of Loading Conditions

An EMNV_{SDOX} delivery system was prepared by introducing DOX into EMNVs through electroporation. Three electrical settings—125 V/900 μ F, 250 V/125 μ F, and 950 V/50 μ F—were first evaluated. After electroporation, the DOX-containing suspensions treated at 125 V/900 μ F or 250 V/125 μ F showed obvious color changes, suggesting that the physicochemical state of DOX might have been altered under these conditions. Little visible change occurred in the samples treated at 950 V/50 μ F; their original orange-red color was largely retained (Figure 5A). This setting was therefore used for the subsequent optimization experiments.

The effect of the initial DOX concentration was then examined while the amount of EMNV protein was kept constant. DOX was added at concentrations ranging from 0.25 to 3.0 mM, after which the mixtures were electroporated and unencapsulated DOX was removed. The purified EMNV_{SDOX} preparations became progressively more orange red as the initial DOX concentration increased (Figure 5B). This visual change was consistent with a greater amount of DOX remaining associated with the purified vesicles.

A fluorescence-based standard curve was established to quantify the loaded DOX. Across the concentration range tested, fluorescence intensity was strongly and linearly correlated with DOX concentration. The regression equation was $y = 1025.9x - 63.411$, with an R^2 of 0.999 (Figure 5C). This equation was used to determine the amount of DOX associated with the EMNVs and to calculate the loading efficiency and loading content.

Loading efficiency and loading content did not show the same concentration-dependent behavior. The loading efficiency rose from approximately 36% at 0.25 mM to about 41% at 0.5 mM. When the initial DOX concentration was increased further, however, the efficiency declined, reaching approximately 31%, 17%, and 12% at 1.0, 2.0, and 3.0 mM, respectively (Figure 5D). Increasing the input DOX concentration above 0.5 mM therefore did not increase the proportion of drug incorporated into the EMNVs.

The amount of DOX loaded per unit of EMNV protein followed a different pattern. A significant increase was observed between 0.25 and 0.5 mM and again between 0.5 and 1.0 mM. No significant difference was detected between 1.0 and 2.0 mM or between 2.0 and 3.0 mM (Figure 5E). The loading capacity thus appeared to approach saturation at an initial DOX concentration of approximately 1.0 mM. Since both loading efficiency and loading content were taken into consideration, 1.0 mM was selected for the following experiments.

The optimized EMNV_{sDOX} preparation was also examined for its physicochemical properties. Nanoparticle tracking analysis gave a mean particle size of 133.4 nm (Figure 5F). A negative surface charge was preserved, with a zeta potential of -17.53 ± 0.62 mV (Figure 5G). TEM images showed that the vesicles remained relatively intact and retained a vesicle-like structure after electroporation and DOX loading (Figure 5H). These results provided an optimized electroporation procedure for DOX incorporation while showing that the EMNVs maintained their nanoscale vesicular properties after drug loading.

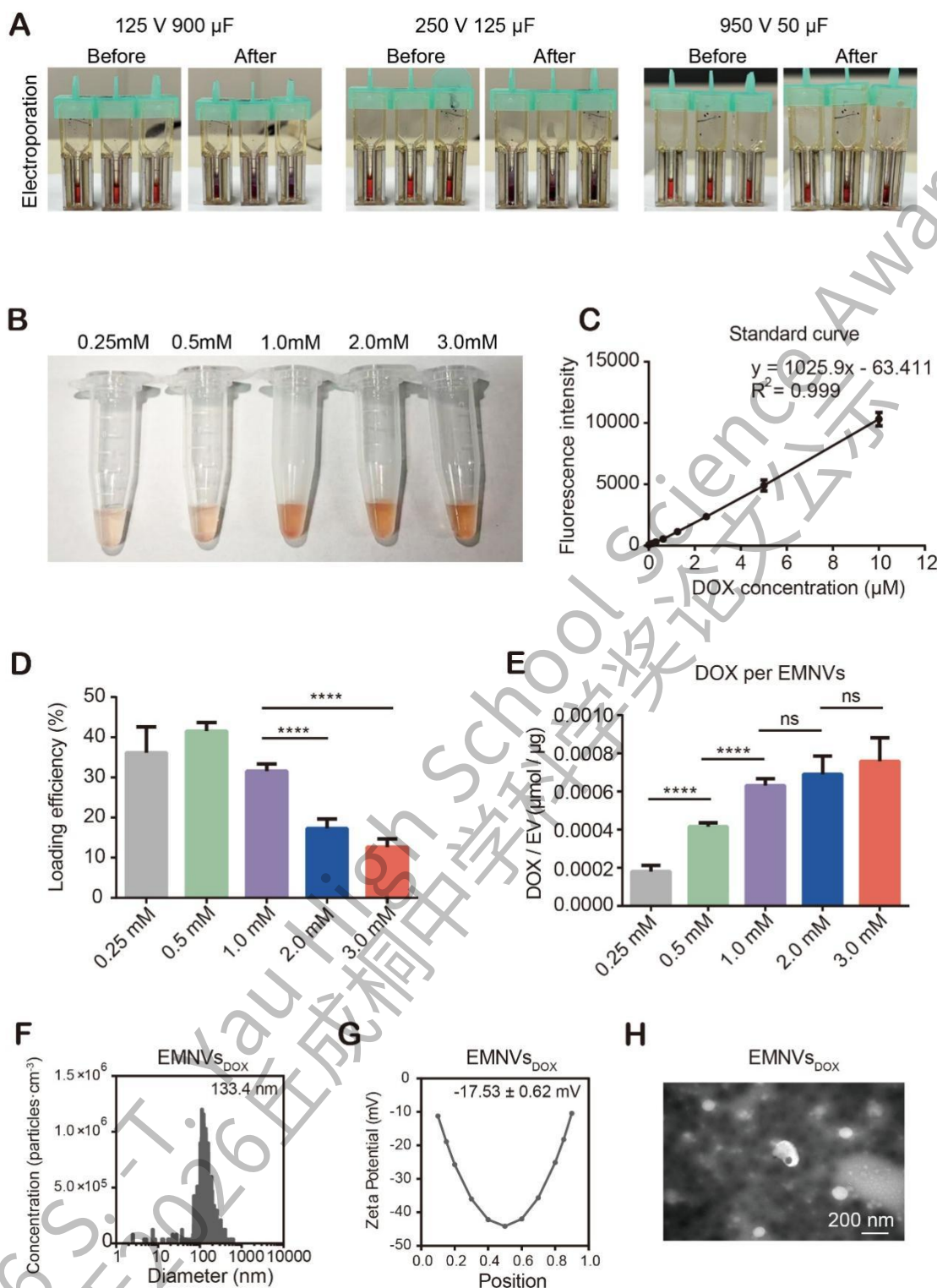


Figure 5. Optimization of electroporation conditions for DOX loading and preparation and characterization of EMNVs_{DOX}. (A) Representative photographs showing the color of DOX-containing EMNV mixtures before and after electroporation under different conditions (125 V/900 μ F, 250 V/125 μ F, and 950 V/50 μ F). (B) Representative photographs of purified EMNVs_{DOX} prepared using different initial DOX concentrations (0.25, 0.5, 1.0, 2.0, and 3.0 mM). (C) Fluorescence intensities

of DOX standard solutions at different concentrations and the corresponding standard curve. (D) DOX loading efficiency at different initial DOX concentrations. (E) DOX loading content normalized to EMNV protein at different initial DOX concentrations. (F) Particle-size distribution of EMNV_{SDOX} measured by nanoparticle tracking analysis (NTA). (G) Zeta potential of EMNV_{SDOX} measured using the zeta-potential mode of the nanoparticle tracking analyzer. (H) Representative transmission electron microscopy (TEM) image showing the morphology of EMNV_{SDOX}. Scale bar, 200 nm. Data are presented as the mean $\pm$ SD. ns, not significant; **** $P < 0.0001$.

3.2.2 EMNV_{SDOX} Enhanced Intracellular DOX Delivery and Reduced Tumor-Cell Viability

Given the limited cytotoxicity of unloaded EMNVs, we next investigated whether DOX loading could enhance the intracellular delivery and antitumor activity of EMNVs. B16F10, Hepa1-6, GL261, and mEC25 cells were treated with free DOX or EMNV_{SDOX} at an equivalent DOX concentration of 1 μ M. Flow cytometric analysis revealed a marked increase in intracellular DOX fluorescence following EMNV_{SDOX} treatment compared with free DOX. Consistently, the mean fluorescence intensity of DOX was significantly higher in the EMNV_{SDOX} group across all four tumor-cell lines (Figure 6A), indicating that EMNV encapsulation enhanced the intracellular accumulation of DOX.

The intracellular distribution of DOX was further examined by confocal microscopy. As expected, little or no red fluorescence was detected in the Control groups, whereas distinct DOX fluorescence was observed following treatment with either free DOX or EMNV_{SDOX}. Notably, the overall fluorescence signal was stronger in cells treated with EMNV_{SDOX}, and a substantial proportion of the DOX signal overlapped with the DAPI-stained nuclear regions (Figure 6B). These findings indicated that EMNV-mediated delivery not only increased intracellular DOX accumulation but also preserved its access to the nucleus, a major site of DOX action.

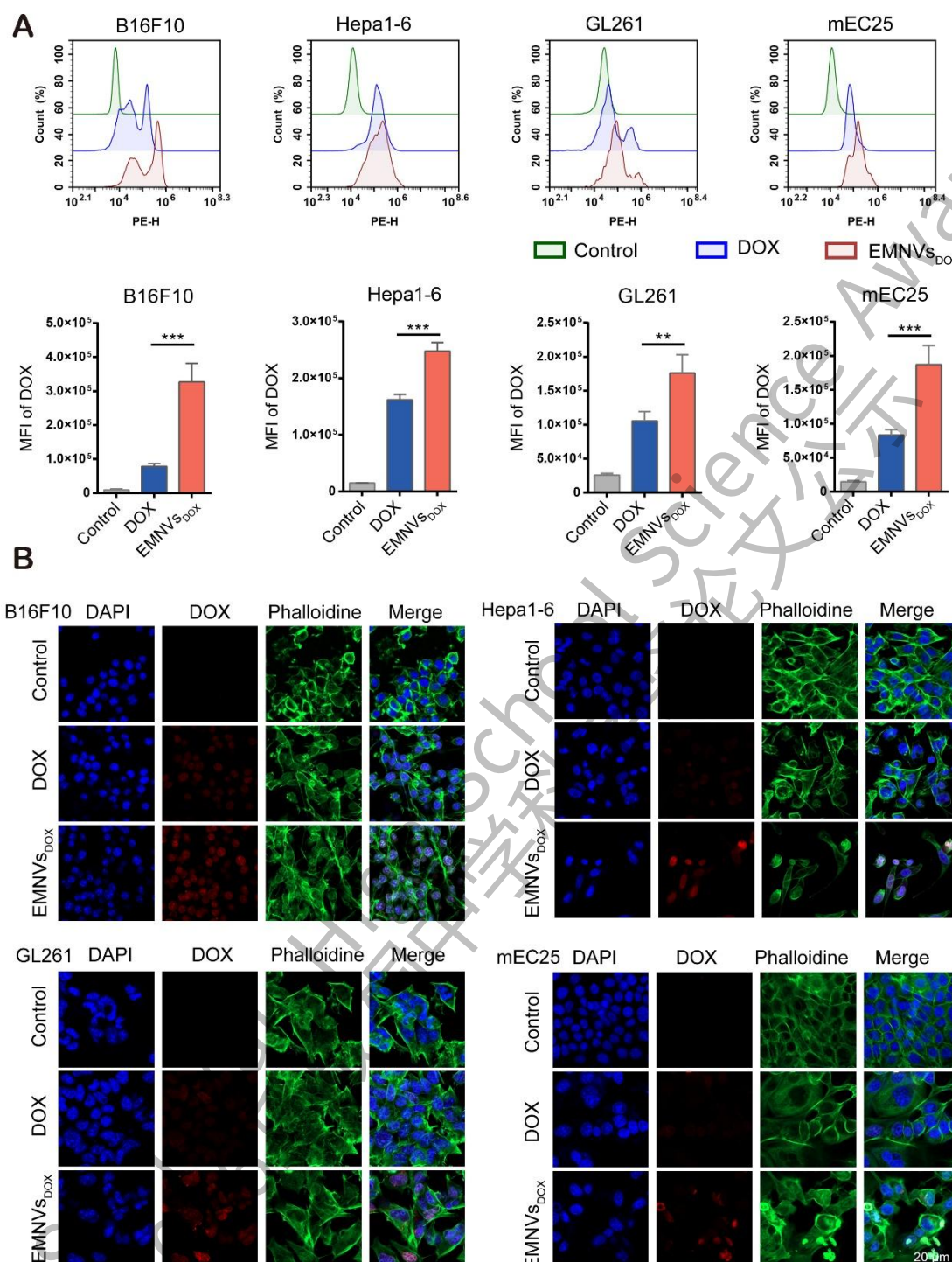


Figure 6. EMNVs_{DOX}-mediated cellular uptake and intracellular distribution of DOX. (A) B16F10, Hepa1-6, GL261, and mEC25 cells were incubated with DOX or EMNVs_{DOX} at an equivalent DOX concentration of 1 μM for 24 h. The mean fluorescence intensity (MFI) of intracellular DOX was measured and quantified by flow cytometry. Untreated cells were used as the Control. (B) Confocal laser scanning microscopy was used to examine the distribution and subcellular localization of DOX fluorescence in cells treated with DOX or EMNVs_{DOX}. Cell nuclei were stained with DAPI, and the actin cytoskeleton was stained with Phalloidin. DOX is shown in red. Scale bar, 20 μm.

We next determined whether the enhanced intracellular DOX delivery translated into greater antitumor activity. B16F10, Hepa1-6, GL261, and mEC25 cells were exposed to free DOX or EMNV_{SDOX} at equivalent DOX concentrations ranging from 0 to 3 μ M for 24 or 48 h, followed by CCK-8 analysis. Both treatments reduced cell viability in a concentration- and time-dependent manner; however, EMNV_{SDOX} generally produced a stronger inhibitory effect than free DOX (Figure 7A).

The increased potency of EMNV_{SDOX} was reflected in the IC₅₀ values across the different tumor-cell lines. In B16F10 cells, EMNV_{SDOX} reduced the IC₅₀ from 1.384 to 0.524 μ M at 24 h and from 1.075 to 0.623 μ M at 48 h, corresponding to approximately 2.6-fold and 1.7-fold reductions, respectively. A similar trend was observed in Hepa1-6 cells, where the IC₅₀ decreased from 1.719 to 0.801 μ M at 24 h and from 0.681 to 0.537 μ M at 48 h, representing approximately 2.1-fold and 1.3-fold reductions.

The enhanced potency was also evident in GL261 cells, although the magnitude of improvement was comparatively smaller. The IC₅₀ values of EMNV_{SDOX} were 0.487 and 0.360 μ M at 24 and 48 h, respectively, compared with 0.586 and 0.537 μ M for free DOX. Thus, EMNV-mediated delivery further increased the sensitivity of GL261 cells to DOX, particularly at 48 h. Notably, the difference was most pronounced in mEC25 cells at 24 h. The IC₅₀ of free DOX was not reached within the tested concentration range of up to 3 μ M, whereas EMNV_{SDOX} exhibited an IC₅₀ of 1.095 μ M under the same conditions. These results demonstrated that EMNV-mediated DOX delivery could substantially enhance drug potency even in tumor cells with relatively low sensitivity to free DOX.

To further distinguish the contribution of EMNVs from that of DOX itself, B16F10, Hepa1-6, GL261, and mEC25 cells were treated for 24 h with Control, unloaded EMNVs, free DOX, or EMNV_{SDOX}. The DOX and EMNV_{SDOX} groups received an equivalent DOX concentration of 1 μ M. Unloaded EMNVs alone did not significantly affect cell viability compared with the Control group. In contrast, EMNV_{SDOX} consistently produced a significantly greater reduction in cell viability than free DOX in all four tumor-cell lines (Figure 7B). Collectively, these findings demonstrated that EMNV loading substantially enhanced intracellular DOX accumulation and potentiated its cytotoxic activity across diverse tumor-cell lines, supporting EMNVs as an effective vehicle for improving DOX delivery.

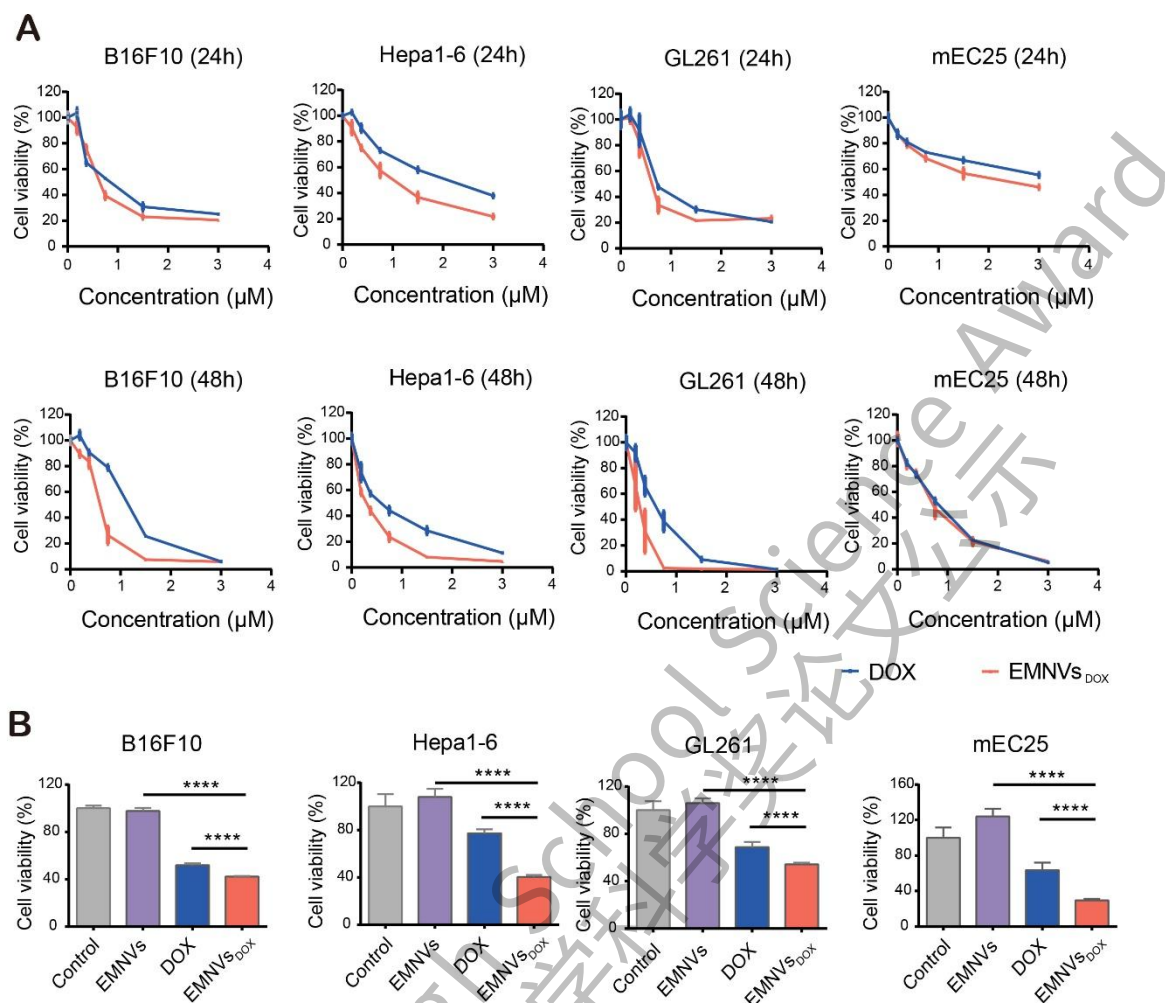


Figure 7. EMNVs_{DOX} enhanced DOX-mediated tumor-cell killing. (A) B16F10, Hepa1-6, GL261, and mEC25 cells were treated with DOX or EMNVs_{DOX} at different concentrations ranging from 0 to 3 μM for 24 or 48 h. Cell viability was measured using the CCK-8 assay, and the half-maximal inhibitory concentration (IC_{50}) was calculated from the dose–response curves. (B) B16F10, Hepa1-6, GL261, and mEC25 cells were treated with Control, EMNVs, DOX, or EMNVs_{DOX} for 24 h, and cell viability was measured using the CCK-8 assay. The equivalent DOX concentration in the DOX and EMNVs_{DOX} groups was 1 μM . The EMNVs group received an amount of EMNVs matched to that present in the EMNVs_{DOX} group. Data are presented as the mean $\pm$ SD. **** P <0.0001.

3.2.3 EMNVs_{DOX} Increased Apoptosis in Tumor Cells

Because EMNVs_{DOX} caused a greater loss of cell viability, we next examined whether apoptosis contributed to this effect. B16F10, Hepa1-6, GL261, and mEC25 cells were exposed for 24 h to Control, unloaded EMNVs, free DOX, or EMNVs_{DOX}. The DOX and EMNVs_{DOX} treatments contained the same DOX concentration (1 μM). Apoptosis was then analyzed by flow cytometry.

Unloaded EMNVs had little effect on the apoptotic fraction in any of the four cell lines relative to the Control group. The response to free DOX differed among the cell types. In each case, however, the apoptotic fraction was higher after EMNV_{SDOX} treatment than after exposure to free DOX (Figure 8). This pattern was consistent with the stronger cytotoxic effect of EMNV_{SDOX} detected in the CCK-8 assay.

The extent of the increase was not identical across the cell lines. In B16F10 cells, free DOX increased apoptosis compared with the Control treatment, whereas EMNV_{SDOX} produced a more pronounced increase. The apoptotic rate was significantly higher in the EMNV_{SDOX} group than in the free-DOX group. Hepa1-6 cells showed only a limited apoptotic response to free DOX, but this response increased markedly when DOX was delivered by EMNVs. GL261 cells displayed a similar trend, with EMNV_{SDOX} inducing significantly more apoptosis than free DOX.

mEC25 cells also responded to both DOX treatments. Free DOX clearly increased the apoptotic fraction, and a further increase was observed after EMNV_{SDOX} treatment. The apoptotic rate in the EMNV_{SDOX} group was significantly higher than that in the free-DOX group (Figure 8D). Taken together, these results suggest that EMNV_{SDOX} enhanced DOX-induced apoptosis in all four cell lines, which may account, at least in part, for its greater cytotoxicity.

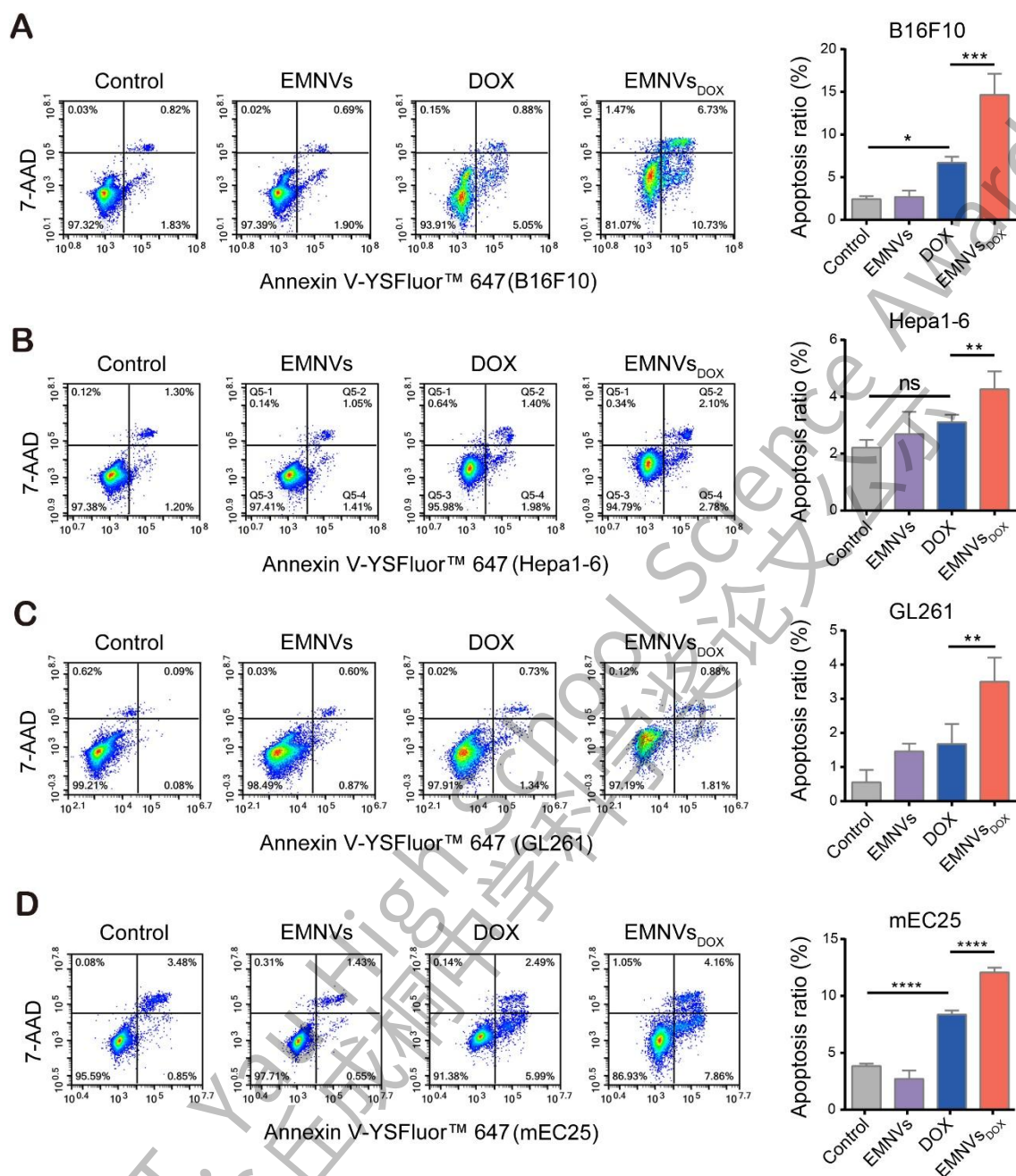


Figure 8. Flow-cytometric analysis of EMNVs_{DOX}-induced apoptosis in tumor cells. (A) B16F10, (B) Hepa1-6, (C) GL261, and (D) mEC25 cells were treated with Control, EMNVs, DOX, or EMNVs_{DOX} for 24 h. Apoptosis was detected by flow cytometry using Annexin V–YSFluor™ 647/7-AAD staining. The final DOX concentration was 1 μ M in both the DOX and EMNVs_{DOX} groups. The EMNVs group received an amount of EMNVs matched to that in the EMNVs_{DOX} group.

3.3 EMNVs_{DOX} Enhanced Caspase-3 Cleavage in Tumor Cells

The extent of Caspase-3 activation varied among the different tumor-cell lines. In B16F10 cells, both DOX and EMNVs_{DOX} induced strong Cleaved Caspase-3 signals. The band intensities appeared generally comparable between the two treatment groups (Figure 9A).

In Hepa1-6 cells, Cleaved Caspase-3 was detected in both treatment groups. However, the signal was more evident in the EMNVs_{DOX} group than in the DOX group (Figure 9B). Similarly, in GL261 cells, EMNVs_{DOX} treatment produced a stronger Cleaved Caspase-3 signal than DOX treatment (Figure 9C).

In mEC25 cells, both DOX and EMNVs_{DOX} also induced clear Caspase-3 cleavage. The band intensities appeared generally similar between the two treatment groups (Figure 9D).

Overall, compared with DOX, EMNVs_{DOX} produced more evident Caspase-3 cleavage in Hepa1-6 and GL261 cells, whereas similar cleavage patterns were observed between the two treatments in B16F10 and mEC25 cells. These findings were consistent with the apoptosis results and suggested that the enhanced cytotoxicity of EMNVs_{DOX} was associated with activation of the Caspase-3-related apoptotic pathway. However, the difference in Caspase-3 cleavage between DOX and EMNVs_{DOX} varied among the tumor-cell lines. Therefore, the improved antitumor activity of EMNVs_{DOX} may involve both enhanced intracellular DOX delivery and cell-line-dependent apoptotic responses.

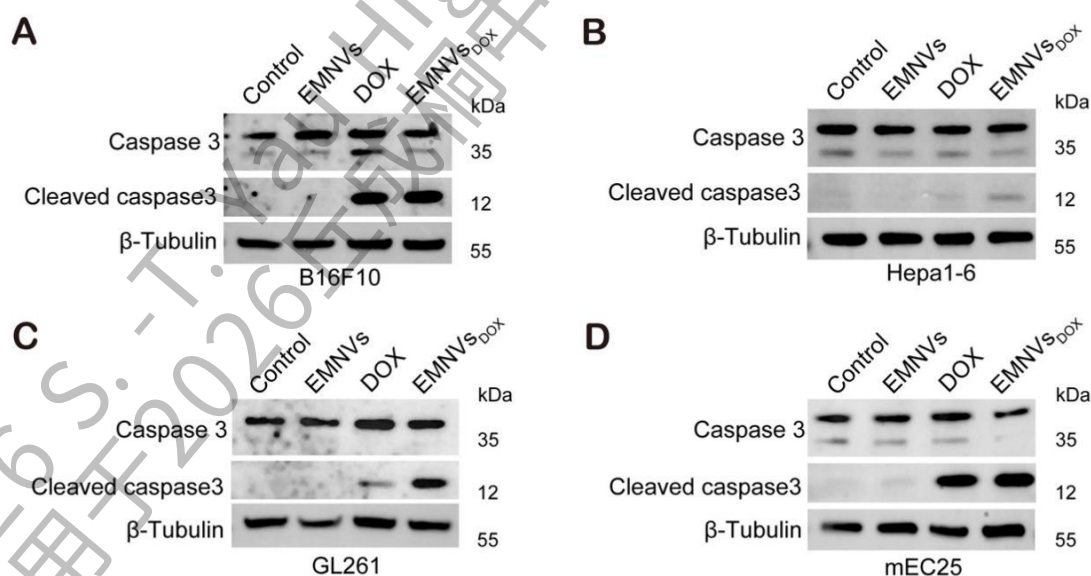


Figure 9. Analysis of EMNVs_{DOX}-induced activation of the Caspase-3-related apoptotic pathway in tumor cells. (A) B16F10, (B) Hepa1-6, (C) GL261, and (D) mEC25 cells were treated with Control, EMNVs, DOX, or EMNVs_{DOX} for 24 h. The expression levels of Caspase-3 and Cleaved Caspase-3

were examined by Western blotting. β -Tubulin was used as the loading control. The final DOX concentration was 1 μ M in both the DOX and EMNV_{SDOX} groups, and the EMNVs group received an amount of EMNVs matched to that in the EMNV_{SDOX} group.

4. Discussion

In this study, we developed engineered membrane nanovesicles (EMNVs) from the cytotoxic T-cell line CTLL-2 and investigated their potential as carriers for doxorubicin (DOX). Previous studies have shown that extracellular vesicles from activated CD8⁺ T cells can inhibit tumor progression by targeting tumor-associated mesenchymal cells, whereas CAR-T-cell-derived exosomes can retain cytotoxic proteins and exert direct antitumor effects (Fu et al., 2019; Seo et al., 2018). In contrast, we explored whether CTLL-2-derived EMNVs could be used as drug delivery vehicles. Our results showed that EMNVs were efficiently taken up by multiple tumor-cell lines, although their intrinsic cytotoxicity varied among cell types. Importantly, loading DOX into EMNVs enhanced intracellular DOX accumulation, reduced tumor-cell viability, and increased apoptosis compared with free DOX. These findings demonstrate that cytotoxic T-cell-derived EMNVs can serve not only as carriers of endogenous cytotoxic proteins but also as vehicles for delivering exogenous chemotherapeutic drugs.

Another interesting finding in this study is that efficient EMNV uptake did not necessarily result in strong cytotoxicity. Nearly all B16F10, Hepa1-6, GL261, and mEC25 cells took up EMNVs, whereas their responses to unloaded EMNVs differed substantially. Hepa1-6 cells were relatively sensitive, GL261 and mEC25 cells showed weaker responses, and B16F10 cells were largely resistant. This discrepancy suggests that vesicle internalization alone does not determine the biological activity of EMNVs. Cell-derived nanovesicles retain membrane components from their parental cells, which may facilitate interactions with recipient cells (Jang et al., 2013; Yoon et al., 2015). However, mechanically generated EMNVs cannot fully reproduce the functions of intact cytotoxic T cells, including immune-synapse formation and directional release of cytotoxic granules. Their antitumor activity may therefore depend on the abundance, localization, release, and activity of cytotoxic cargos, together with the intrinsic susceptibility of recipient tumor cells. These findings suggest that EMNVs may be more advantageous as drug carriers than as substitutes for intact cytotoxic T cells.

The enhanced activity observed after DOX loading further supports this concept. Mechanically produced cell-derived nanovesicles have previously been explored for chemotherapy and biomolecule delivery (Jang et al., 2013; Yoon et al., 2015). In the present

study, CTLL-2-derived EMNV_{SDOX} increased intracellular DOX accumulation in all four tumor-cell lines, and the delivered DOX reached the nucleus. Consistently, EMNV_{SDOX} generally showed lower IC₅₀ values and stronger cytotoxicity than free DOX, with the most pronounced improvement observed in mEC25 cells, which showed relatively limited sensitivity to free DOX. These results suggest that EMNVs can improve the cellular delivery of DOX and thereby enhance its antitumor activity. The increased effect may result from more efficient cellular uptake and subsequent intracellular trafficking of DOX. Because DOX exerts its antitumor effects through mechanisms including DNA damage and topoisomerase II inhibition, the enhanced intracellular delivery provided by EMNVs may increase the effective exposure of tumor cells to the drug (Minotti et al., 2004). Thus, DOX loading may help address the limited and cell-dependent cytotoxicity of unloaded CTLL-2-derived EMNVs and enhance their use as chemotherapy nanocarriers.

The loading experiments also provide useful information for the development of EMNVs as a drug delivery platform. As the initial DOX concentration increased, the DOX loading content gradually approached a plateau. This finding suggests that EMNVs have a limited loading capacity and that increasing the input drug concentration does not necessarily improve loading efficiency. Previous studies have reported that electroporation can cause loading artifacts, such as siRNA precipitation, during extracellular vesicle loading (Kooijmans et al., 2013; Vader et al., 2016). In our experiments, visible color differences were observed in the DOX-containing EMNV mixtures under some electroporation conditions, possibly because DOX entered or became associated with the EMNVs during the loading process.

We also found that CTLL-2-derived EMNV_{SDOX} induced higher apoptotic rates than free DOX in all four tumor-cell lines, while Western blotting confirmed caspase-3 cleavage after treatment with free DOX or CTLL-2-derived EMNV_{SDOX}. Interestingly, the extent of caspase-3 cleavage did not completely parallel the difference in apoptotic rates among the cell lines. In Hepa1-6 and GL261 cells, EMNV_{SDOX} produced more pronounced caspase-3 cleavage than free DOX, whereas B16F10 and mEC25 cells showed relatively similar caspase-3 cleavage between the two treatments. This finding suggests that the enhanced in vitro cytotoxic activity of EMNV_{SDOX} may involve apoptosis but does not solely depend on caspase-3 activation. Differences in apoptotic kinetics or the involvement of additional cell-death pathways may contribute to the distinct responses of tumor cells. Further studies should examine whether the cytotoxic molecules in EMNVs enhance the effect of DOX.

Overall, our findings establish a potential strategy for repurposing cytotoxic T-cell-derived membrane nanovesicles as chemotherapy delivery vehicles. Instead of relying on the

limited cytotoxicity of unloaded EMNVs, we used CTLL-2-derived EMNVs as carriers for DOX. This strategy combines the efficient cellular uptake of EMNVs with the established cytotoxic activity of DOX. It also reduces the dependence on the limited and cell-line-dependent cytotoxicity of unloaded EMNVs. This approach combines the membrane characteristics of cell-derived nanovesicles with the established cytotoxic activity of DOX and provides a basis for further development of immune cell-derived nanovesicles for therapeutic drug delivery.

5. Limitations

While our study indicates that CTLL-2-derived EMNVs could enhance DOX delivery and cytotoxicity against tumor cells, several issues require further investigation. First, EMNVs_{DOX} was evaluated only *in vitro*, and its pharmacokinetics, biodistribution, therapeutic efficacy, and systemic toxicity *in vivo* remain unknown. Therefore, further *in vivo* studies are needed to assess its antitumor activity and safety. Cell-derived vesicles may show different biodistribution patterns depending on their cell source, administration route, and surface properties (Wiklander et al., 2015). Therefore, efficient uptake by cultured tumor cells does not necessarily indicate tumor-selective delivery *in vivo*.

Second, the mechanisms underlying EMNV uptake by tumor cells were not determined. Comparative studies with normal cells to investigate the cytotoxicity are needed to clarify the specificity of EMNV delivery.

Third, the stability of DOX after electroporation and its release from EMNVs were not fully characterized. Further quantitative analyses are needed to evaluate DOX loading, stability, and release in the EMNVs_{DOX} system.

6. Conclusion

In this study, we prepared engineered membrane nanovesicles (EMNVs) from the cytotoxic T-cell line CTLL-2. These EMNVs contained granzyme and perforin. Although they were taken up by almost all the tested tumor cells, their direct killing effect varied greatly among different cell lines. To improve their antitumor effect, we loaded doxorubicin (DOX) into the EMNVs, which efficiently delivered DOX into tumor cells and their nuclei. In all four tumor cell lines, EMNVs_{DOX} generally showed lower IC₅₀ values than free DOX under the tested conditions. EMNVs_{DOX} also caused stronger tumor-cell apoptosis at the same DOX concentration. Further studies showed that Apoptosis contributed to its cytotoxic effect and was accompanied by caspase-3 cleavage. Collectively, T-cell-derived EMNVs may serve as

effective carriers for chemotherapy drugs, which can improve drug delivery efficiency and enhance the antitumor effect. This study provides a new strategy for using immune cell-derived vesicles in cancer treatment.

Acknowledgements

The idea for this project grew from my interest in cancer treatment. My mother works in cancer-related research, and her influence led me to pay attention to this field throughout middle and high school. When the Life Science Club asked me to give a presentation, I once chose a cancer-related topic to share with other students. My mother also sometimes talked with me about cancer treatment and the problems associated with chemotherapy drugs. During one of these conversations, she mentioned that doxorubicin (DOX) is widely used in cancer treatment but can also affect normal tissues. This made me wonder whether drug delivery could be improved. I then began learning more about DOX, cell-derived nanovesicles, and the possibility of using immune-cell membranes as drug carriers.

After that, I discussed my ideas in detail with Ms. Li, my biology teacher. At the beginning, my research question was still quite broad. Ms. Li helped me organize my thoughts and gradually develop a clearer direction. Together, we focused on whether engineered membrane nanovesicles made from the cytotoxic T-cell line CTLL-2 could be used to deliver DOX to tumor cells. With her guidance, we developed the main experimental plan and decided to examine EMNV uptake, DOX loading, intracellular drug accumulation, cell viability, apoptosis, and caspase-3 cleavage.

I took part in the main experimental work of this project. This included CTLL-2 cell culture, preparation of CTLL-2-derived engineered membrane nanovesicles, DOX loading by electroporation, and treatment of different tumor cell lines. I also participated in the experiments used to examine EMNV uptake and DOX delivery, including flow cytometry and confocal microscopy. In addition, I carried out or participated in the CCK-8 cell viability assay, apoptosis analysis, and Western blotting for apoptosis-related proteins. I organized the experimental results, calculated the IC_{50} values, prepared the figures, and wrote the main parts of the research report.

During the project, I faced many difficulties in the laboratory. At first, I was not familiar with the layout of the laboratory or the storage conditions for different samples. I also did not know how to use some of the instruments, adjust their settings, or export the results correctly. Yuling Yang, a senior student in the laboratory, helped me with these problems. She showed

me where different samples should be stored, explained the basic steps for using the instruments, and reminded me of important safety precautions. When I had problems with instrument settings or data export, she helped me check the operation step by step.

Her help was especially important when I was learning to work more independently in the laboratory. She did not simply complete the experiments for me. Instead, she explained what I needed to pay attention to and allowed me to practice the procedures myself. She also helped me become familiar with daily life in the laboratory. I am very grateful for her patience and practical support.

Some of the experimental results also required careful discussion. For example, EMNVs were taken up efficiently by almost all of the tested tumor cells, but the direct cytotoxic effects of unloaded EMNVs differed among the cell lines. This result was not what I first expected. I discussed it with Ms. Li and gradually understood that efficient uptake did not always lead to strong cytotoxicity. This helped us focus more on the use of EMNVs as carriers for DOX rather than relying only on their natural cytotoxic molecules.

The DOX-loading experiments also required optimization. Different electroporation conditions produced different results, so we compared several settings and examined both loading efficiency and loading content. After the experiments, I discussed the results with Ms. Li and learned how to compare the data and choose suitable conditions for later experiments. These experiences taught me to look at more than one result and to avoid making conclusions based on a single observation.

After collecting the experimental data, I worked with Ms. Li to analyze the results. She answered my questions, helped me understand the meaning of the data, and taught me how to organize the figures and explain the findings. She also guided me in writing the report. She explained the basic structure of a research paper and helped me revise, shorten, and reorganize several sections. Her advice was especially helpful when I needed to connect the results with the research question and avoid making conclusions that were too strong. With her help, I completed a more organized and complete report.

I would also like to thank Professor Deng for allowing me to use the laboratory space and equipment. His permission made it possible for me to complete the practical part of this project. I am grateful to all the laboratory members who helped create a safe and supportive working environment. The guidance and practical assistance described above were provided without payment.

I am also deeply grateful to my mother. Her work in oncology-related research first encouraged me to think about cancer treatment and drug delivery. She helped me understand

that biology is not only a subject in a textbook. It is closely related to real medical problems and everyday life. Her influence encouraged me to stay curious and to ask why a treatment works, why it sometimes fails, and how it might be improved.

Finally, I would like to thank the teachers and school administrators who supported my participation in this competition. They helped review my materials, complete the required forms and signatures, and finish the submission process. Their support made it possible for me to present this project.

AI was not used to select the research topic, design the experimental plan, conduct the experiments, collect or analyze raw data, generate experimental results, or make the final scientific decisions. I completed the experimental work under the guidance of Yuling Yang and wrote the manuscript under the guidance of Ms. Li.

This project was not always easy, but it taught me to be more patient, careful, and responsible. I am thankful to everyone who answered my questions, encouraged me, and helped me continue when I faced difficulties. I will remember their support as I continue to learn and explore biology in the future.

References

- Bagchi, S., Yuan, R., & Engleman, E. G. (2021). Immune Checkpoint Inhibitors for the Treatment of Cancer: Clinical Impact and Mechanisms of Response and Resistance. *Annu Rev Pathol*, 16, 223–249. <https://doi.org/10.1146/annurev-pathol-042020-042741>
- Fu, W., Lei, C., Liu, S., Cui, Y., Wang, C., Qian, K., Li, T., Shen, Y., Fan, X., Lin, F., Ding, M., Pan, M., Ye, X., Yang, Y., & Hu, S. (2019). CAR exosomes derived from effector CAR-T cells have potent antitumour effects and low toxicity. *Nat Commun*, 10(1), 4355. <https://doi.org/10.1038/s41467-019-12321-3>
- Hanahan, D. (2022). Hallmarks of Cancer: New Dimensions. *Cancer Discov*, 12(1), 31–46. <https://doi.org/10.1158/2159-8290.Cd-21-1059>
- Hegde, P. S., & Chen, D. S. (2020). Top 10 Challenges in Cancer Immunotherapy. *Immunity*, 52(1), 17–35. <https://doi.org/10.1016/j.immuni.2019.12.011>
- Herrmann, I. K., Wood, M. J. A., & Fuhrmann, G. (2021). Extracellular vesicles as a next-generation drug delivery platform. *Nat Nanotechnol*, 16(7), 748–759. <https://doi.org/10.1038/s41565-021-00931-2>
- Jang, S. C., Kim, O. Y., Yoon, C. M., Choi, D. S., Roh, T. Y., Park, J., Nilsson, J., Lötvall, J., Kim, Y. K., & Gho, Y. S. (2013). Bioinspired exosome-mimetic nanovesicles for targeted delivery of chemotherapeutics to malignant tumors. *ACS Nano*, 7(9), 7698–7710. <https://doi.org/10.1021/nn402232g>

- Kalluri, R., & LeBleu, V. S. (2020). The biology, function, and biomedical applications of exosomes. *Science*, 367(6478). <https://doi.org/10.1126/science.aau6977>
- Kalluri, R., & McAndrews, K. M. (2023). The role of extracellular vesicles in cancer. *Cell*, 186(8), 1610–1626. <https://doi.org/10.1016/j.cell.2023.03.010>
- Kooijmans, S. A. A., Stremersch, S., Braeckmans, K., de Smedt, S. C., Hendrix, A., Wood, M. J. A., Schiffelers, R. M., Raemdonck, K., & Vader, P. (2013). Electroporation-induced siRNA precipitation obscures the efficiency of siRNA loading into extracellular vesicles. *J Control Release*, 172(1), 229–238. <https://doi.org/10.1016/j.jconrel.2013.08.014>
- Kugeratski, F. G., & Kalluri, R. (2021). Exosomes as mediators of immune regulation and immunotherapy in cancer. *Febs j*, 288(1), 10–35. <https://doi.org/10.1111/febs.15558>
- Liang, Y., Duan, L., Lu, J., & Xia, J. (2021). Engineering exosomes for targeted drug delivery. *Theranostics*, 11(7), 3183–3195. <https://doi.org/10.7150/thno.52570>
- Miller, K. D., Nogueira, L., Devasia, T., Mariotto, A. B., Yabroff, K. R., Jemal, A., Kramer, J., & Siegel, R. L. (2022). Cancer treatment and survivorship statistics, 2022. *CA Cancer J Clin*, 72(5), 409–436. <https://doi.org/10.3322/caac.21731>
- Minotti, G., Menna, P., Salvatorelli, E., Cairo, G., & Gianni, L. (2004). Anthracyclines: molecular advances and pharmacologic developments in antitumor activity and cardiotoxicity. *Pharmacol Rev*, 56(2), 185–229. <https://doi.org/10.1124/pr.56.2.6>
- Rawat, P. S., Jaiswal, A., Khurana, A., Bhatti, J. S., & Navik, U. (2021). Doxorubicin-induced cardiotoxicity: An update on the molecular mechanism and novel therapeutic strategies for effective management. *Biomed Pharmacother*, 139, 111708. <https://doi.org/10.1016/j.biopha.2021.111708>
- Seo, N., Shirakura, Y., Tahara, Y., Momose, F., Harada, N., Ikeda, H., Akiyoshi, K., & Shiku, H. (2018). Activated CD8(+) T cell extracellular vesicles prevent tumour progression by targeting of lesional mesenchymal cells. *Nat Commun*, 9(1), 435. <https://doi.org/10.1038/s41467-018-02865-1>
- Sung, H., Ferlay, J., Siegel, R. L., Laversanne, M., Soerjomataram, I., Jemal, A., & Bray, F. (2021). Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries. *CA Cancer J Clin*, 71(3), 209–249. <https://doi.org/10.3322/caac.21660>
- Vader, P., Mol, E. A., Pasterkamp, G., & Schiffelers, R. M. (2016). Extracellular vesicles for drug delivery. *Adv Drug Deliv Rev*, 106(Pt A), 148–156. <https://doi.org/10.1016/j.addr.2016.02.006>
- van Niel, G., Carter, D. R. F., Clayton, A., Lambert, D. W., Raposo, G., & Vader, P. (2022). Challenges and directions in studying cell-cell communication by extracellular vesicles. *Nat Rev Mol Cell Biol*, 23(5), 369–382. <https://doi.org/10.1038/s41580-022-00460-3>

- Welsh, J. A., Goberdhan, D. C. I., O'Driscoll, L., Buzas, E. I., Blenkiron, C., Bussolati, B., Cai, H., Di Vizio, D., Driedonks, T. A. P., Erdbrügger, U., Falcon-Perez, J. M., Fu, Q. L., Hill, A. F., Lenassi, M., Lim, S. K., Mahoney, M. G., Mohanty, S., Möller, A., Nieuwland, R.,...Witwer, K. W. (2024). Minimal information for studies of extracellular vesicles (MISEV2023): From basic to advanced approaches. *J Extracell Vesicles*, 13(2), e12404. <https://doi.org/10.1002/jev2.12404>
- Wiklander, O. P., Nordin, J. Z., O'Loughlin, A., Gustafsson, Y., Corso, G., Mäger, I., Vader, P., Lee, Y., Sork, H., Seow, Y., Heldring, N., Alvarez-Erviti, L., Smith, C. I., Le Blanc, K., Macchiarelli, P., Jungebluth, P., Wood, M. J., & Andaloussi, S. E. (2015). Extracellular vesicle in vivo biodistribution is determined by cell source, route of administration and targeting. *J Extracell Vesicles*, 4, 26316. <https://doi.org/10.3402/jev.v4.26316>
- Yoon, J., Jo, W., Jeong, D., Kim, J., Jeong, H., & Park, J. (2015). Generation of nanovesicles with sliced cellular membrane fragments for exogenous material delivery. *Biomaterials*, 59, 12–20. <https://doi.org/10.1016/j.biomaterials.2015.04.028>