Development of targeting lonidamine liposomes that circumvent drug-resistant cancer by acting on mitochondrial signaling pathways

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Abstract

Even when faced with elimination, functional materials may offer new alternatives to expensive drugs. Once used to treat benign prostate hypertrophy, the US Food and Drug Administration (FDA) suspended the use of lonidamine due to the occurrence of liver problems arising from its poor pharmaceutical properties. The objectives of the present study were to develop targeting lonidamine liposomes in combination with targeting epirubicin liposomes to circumvent drug-resistant cancer. Evaluations were performed on A549 and drug-resistant A549cDDP lung cancer cells and drug-resistant A549cDDP xenografted BALB/c nude mice. A DQA-PEG2000-DSPE conjugate was incorporated onto the liposomes as a targeting molecule. The constructed targeting lonidamine liposomes and targeting epirubicin liposomes measured were approximately 80 nm. The targeting lonidamine liposomes significantly enhanced the inhibitory effect of the targeting epirubicin liposomes in the drug-resistant A549cDDP cells in a lonidamine dose-dependent manner. Mechanism studies revealed that the targeting liposomes were selectively accumulated in the mitochondria, dissipating the mitochondrial membrane potential, opening the mitochondrial permeability transition pores, and releasing cytochrome C by translocation. This initiated a cascade of caspase 9 and 3 reactions and activated the pro-apoptotic Bax protein while suppressing the anti-apoptotic Mcl-1 protein, thereby enhancing the cytotoxic effect by acting on the mitochondrial signaling pathways. The efficacy in treating the drug-resistant A549cDDP xenografted tumor model after administration of the targeting lonidamine liposomes plus targeting epirubicin liposomes was the most significant compared with the administration of the controls at comparable doses. In conclusion, targeting lonidamine liposomes could be used as a potent co-therapy with an anticancer agent to enhance the efficacy of treating drug-resistant cancer by acting on the mitochondrial signaling pathways.

1. Introduction

Lung cancer is the most common cause of cancer-related death in humans, and is responsible for more than 1 million deaths each year [1]. The two broad classes of lung cancer are non-small-cell lung carcinoma (NSCLC) and small-cell lung carcinoma [2]. Treatment for lung cancer depends on the specific cell type of the cancer, the spread of the cancer, and the status of the patient. Common treatments include palliative care, surgery, chemotherapy, and radiation therapy. The prognosis of lung cancers is generally poor. Of all patients with lung cancer, 15% survive for five years after diagnosis [3].

Among the treatment strategies in advanced NSCLC, chemotherapy improves survival, provided that the patient is well enough for the treatment, and that it is used as first-line treatment with two kinds of drugs, of which one is platinum-based, e.g., cisplatin or carboplatin, and the other being gemcitabine, paclitaxel, or docetaxel [4]. Nevertheless, NSCLC cells are often intrinsically resistant to certain anticancer drugs, and exhibit cross-multidrug resistance (MDR). MDR may be caused by the drug-induced overexpression of ATP-binding cassette transporters (ABC transporters) of cancer cell membrane proteins or by the intrinsic alterations in proteins encoded by apoptotic genes, the activation of oncogenes, and the inactivation of tumor suppression genes [5].

A bio-functional material--based targeting liposomal drug delivery system offers a promising approach to solving this problem. In this study, we developed mitochondria-specific targeting liposomes.
liposomes modified with a dequalinium polyethylene glycol-distearylophosphatidylethanolamine (DQA-PEG2000-DSPE) conjugate to treat drug-resistant NSCLC. Using lipid-based targeting liposomes, epirubicin was encapsulated in the vesicle as an anticancer agent, lonidamine was encapsulated in the vesicle as an apoptotic inducer of cancer cells, and both agents were incorporated into the targeting liposomes separately for convenience of dosage adjustment before application. The dequalinium (DQA) on the DQA-PEG2000-DSPE was inserted into the lipid bilayer membrane of the liposome as a mitochondrial-targeting molecule [6].

Lonidamine is a derivative of indazole-3-carboxylic acid, and can inhibit glycolysis under conditions of hypoxia [7]. It had been used to treat benign prostate hypertrophy (BPH) in phase III trials but the US Food and Drug Administration (FDA) suspended its use due to the development of liver problems in the trial subjects. In addition, its low water solubility and therapeutic index rendered it unsuccessful in the clinical therapy trial. Nevertheless, this drug is currently being tested in phase II/III trials for metastatic breast cancer and NSCLC, and has produced encouraging results so far [8]. Furthermore, lonidamine can inhibit the mitochondrial adenine nucleotide translocase [9] and triggers apoptosis via a direct effect on the mitochondria, exhibiting a reverse resistant effect to cisplatin and potentiating the anticancer effect in experimental models [10]. Epirubicin is an anthracycline anticancer drug that acts by intercalating with DNA strands and by triggering DNA cleavage via topoisomerase II, resulting in the death of cancer cells. It generates free radicals that cause DNA damage as well [11]. As a potent chemotherapeutic agent, epirubicin is also subject to MDR and cross-MDR phenomena [12].

Mitochondria exert both vital and lethal functions in physiological and pathological scenarios [13]. For example, they are indispensable to energy production, and are crucial regulators of the intrinsic pathway of apoptosis by intracellular stimuli such as Ca^{2+} overload, overproduction of reactive oxygen species (ROS), and cleavage of apoptotic enzymes, members of the cysteine aspartic acid–specific protease (caspase) family. Three different mechanisms are involved in apoptosis, in which a cell “commits suicide” [4]. One is triggered by external signals, namely, the extrinsic or death receptor pathway. For this signaling pathway, an external agent, i.e., an anticancer drug, activates caspase 8, which initiates a cascade of caspase activations, leading to phagocytosis of the cell. This cascade eventually leads to the activation of the effector caspsases such as caspases 3 and 7. Another mechanism is generated by internal signals arising within the cell, i.e., the mitochondrial pathway. A number of factors are involved in inducing the apoptosis of cancer cells via mitochondria. These mainly include the activation or suppression of Bcl-2 family proteins, such as the activation of proteins expressed by pro-apoptotic genes (e.g., Bax) and the inhibition of proteins expressed by anti-apoptotic genes (e.g., myeloid cell leukemia-1, Mcl-1). The induction of cancer cell apoptosis via mitochondria also involves the translocation of mitochondrial permeability transition pores (MPTPs), which results in the release of cytochrome C from the mitochondria to the cytoplasm, dissipation of the mitochondrial membrane potential, and activation of the apoptotic enzyme caspase 9, which leads to activation of the downstream caspase 3. These caspases are responsible for the cleavage of the key cellular proteins that result in apoptosis. The third mechanism may be triggered by dangerous ROS, which, unlike the two pathways described above, does not involve caspases.

The objectives of the present study were to develop targeting lonidamine liposomes in combination with targeting epirubicin liposomes to circumvent drug-resistant cancer and to evaluate their action mechanisms in A549 and drug-resistant A549/CDP lung cancer cells.

2. Materials and methods

2.1. Materials

DQA was purchased from Hangzhou Sanhe Chemicals Co., Ltd. (Hangzhou, China). 1, 2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[carboxy(polyethyleneglycol)2000] (COOH-PEG2000-DSPE) was purchased from Avanti Polar Lipids (Alabaster, AL, USA). 1-hydroxy-1- benzotriazole (HOBt) was purchased from Sigma Aldrich (St. Louis, MO, USA). Polyethylene glycol-distearylophosphatidylethanolamine (PEG2000-DSPE) was purchased from NOF Corporation (Japan). Epirubicin hydrochloride and lonidamine were purchased from Nanjing Tianzun Zezhong Chemicals Co. Ltd. (Nanjing, China). Other reagents were purchased from Beijing Chemical Reagents (Beijing, China).

2.2. Synthesis of DQA-PEG2000-DSPE conjugate

The DQA-PEG2000-DSPE conjugate was synthesized from COOH-PEG2000-DSPE and DQA by acylation reaction, as described in our previous report [6]. The crude product was then transferred to a regenerated cellulose dialysis tube (molecular weight cut-off point, 2000) and dialyzed against deionized water for 48 h to remove uncoupled DQA, 4-dimethylamophiolyridine (DMAP), N,N'-dicyclohexylcarbodiimide (DCC), 1-hydroxybenzotriazole (HOBt), and acetonitrile. The residue was lyophilized to obtain a powder. The produced mixture was characterized by nuclear magnetic resonance spectroscopy (400-MHz 1H NMR, Bruker AVANCE III 400) and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS instrument, Shimadzu, Japan).

The standard addition method was used to quantify the yield of DQA-PEG2000-DSPE [14]. Briefly, serial known aliquots of pure DQA were added to the same sample of the above reaction product, yielding a linear increase in the signal from the aromatic hydrogen (~ 6.8 ppm) according to the additions of pure DQA. The slope was then used to determine the initial mass of DQA-PEG2000-DSPE in the sample. As the aromatic hydrogen on DQA remained on the DQA-PEG2000-DSPE conjugate, the signal peak was used to semi-quantify the DQA-PEG2000-DSPE content based on calculations.

2.3. Preparation of targeting lonidamine liposomes

Targeting lonidamine liposomes were prepared using the film dispersion method, followed by membrane extrusion [15]. Briefly, egg phosphatidylcholine, o-9-tocopherol polyethylene glycol 1000 succinate (TPGS1000), and DQA-PEG2000-DSPE (90:5:5, mmol:mmol:mmol) were used as the lipid materials. Lipids and lonidamine (lipids:drug = 10:1, w/w) were dissolved in methanol in a pear-shaped flask. The methanol was removed using a rotary vacuum evaporator, and the lipid film was hydrated by sonication in a water bath for 10 min. Subsequently, the suspensions were treated using an ultrasonic cell disruptor for 10 min (200 W). The suspensions were obtained and extruded through polycarbonate membranes (Millipore, Bedford, MA, USA) thrice using 400-nm pore sizes thrice, and then thrice using 200-nm pore sizes, thus producing targeting lonidamine liposomes. Lonidamine liposomes were prepared using the same procedures as above, but with PEG2000-DSPE in place of DQA-PEG2000-DSPE.

In addition to lonidamine liposomes, targeting epirubicin liposomes, epirubicin liposomes, coumarin liposomes, and targeting coumarin liposomes were also prepared and used as controls, potent cytotoxic agents, or fluorescent probes. The same liposomal materials were used to prepare the targeting epirubicin liposomes; epirubicin loading into liposomal vesicles was carried out as described in our previous report [16]. Accordingly, the epirubicin liposomes were prepared by replacing DQA-PEG2000-DSPE with PEG2000-DSPE.

The targeting coumarin liposomes or coumarin liposomes (lipids:coumarin = 200:1, w/w) were prepared using procedures similar to those for the lonidamine liposomes.

2.4. Characterization of liposomes

2.4.1. Preparation of targeting lonidamine liposomes

Targeting lonidamine liposomes, lonidamine liposomes, targeting coumarin liposomes, coumarin liposomes, targeting epirubicin liposomes, and epirubicin liposomes were passed over a Sephadex G-50 column equilibrated with phosphate-buffered saline (137 mM NaCL, 2.7 mM KCL, 8 mM NaH2PO4, and 2 mM KH2PO4, PBS pH 7.4) to remove the unencapsulated lonidamine, coumarin, or epirubicin. Lonidamine or epirubicin was measured with a high-performance liquid chromatography system with a UV detector (Agilent Technologies Inc., Cotati, CA, USA). To measure lonidamine, the analysis was performed on an ODS C18 column (Phenomenex, i.d. 5 mm; 250 mm × 4.6 mm) at 25 °C at a wavelength of 298 nm. The mobile phase consisted of acetonitrile and 0.1 M acetic acid (50:50, v/v). The flow rate was 1.0 mL/min [17].

To measure epirubicin, the analysis was performed on the same ODS column. The mobile phase consisted of acetonitrile, 0.5 % NaH2PO4, and triethylamine (34:66:0.3, pH 4.0, v/v). The flow rate was 1.0 mL/min. The detection wavelength was 254 nm [18].

The encapsulation efficiencies (EE) of lonidamine and epirubicin were calculated using the formula: EES (W{W} mol) = 100%. W{W} is the measured amount of
2.7.2. Drug content in mitochondria

Treated with the coumarin liposomes or targeting coumarin liposomes for 4 h under
a FACScan

The liposomes were diluted with distilled water and filtered through a 0.2-μm microspore filter membrane. A 10-μL volume of liposome suspension was spread on a silicon slice, dried at room temperature, and observed using AFM [19].

2.7. Targeting mechanism and effect

As above.

The survival rates were calculated using the following formula: Survival %

(A540 nm = [A540 nm for the treated cells]/[A540 nm for the control cells]) x 100%, where [A540 nm is the absorbance value.

Each assay was repeated in triplicate, and five determinations were set for each dose level. Finally, dose–effect curves were created. To further evaluate the efficacy of the combination of targeting epirubicin liposomes and targeting lonidamine liposomes, A549 cells or A549/DDP cells were incubated as above, and then the medium was replaced with fresh culture media containing various concentrations of free lonidamine, lonidamine liposomes, and targeting lonidamine liposomes, respectively.

The final concentration of lonidamine was in the 0–20 μM range, and blank targeting liposomes were added as parallel controls. Blank culture medium was used as a blank control. At 48 h, the cell viability was determined by a sulforhodamine B (SRB, Sigma Aldrich) staining assay [20]. Briefly, the medium was removed, and the cells were fixed with trichloroacetic acid, washed, and stained with the absorbance value. After a 96-well plate reader (Model 680 microplate reader; Bio-Rad Laboratories, Hercules, CA, USA), the survival rates were calculated using the following formula: Survival % = ([A540 nm for the treated cells]/[A540 nm for the control cells]) x 100%, where [A540 nm is the absorbance value.

2.7. Cytotoxicity

A549 cells or A549/DDP cells were seeded at a density of 9 x 10^4 cells/well in 96-well culture plates and cultured for 24 h under 5% CO2 at 37 °C. The medium was then replaced with fresh culture media containing varying concentrations of free lonidamine, lonidamine liposomes, and targeting lonidamine liposomes, respectively.

2.7.1. Co-localization into mitochondria

A confocal laser scanning fluorescent microscope with Leica confocal software (Leica, Heidelberg, Germany) was used to observe the co-localization of the targeting epirubicin liposomes into the mitochondria to track the targeting lonidamine liposomes. Briefly, A549 cells or A549/DDP cells were incubated as above, and then the medium was replaced with fresh culture media containing various concentrations of the targeting epirubicin liposomes (0–20 μM) plus targeting lonidamine liposomes (2.5 μM, 5 μM, and 10 μM). The culture medium was used as a blank control. The cells were further incubated for 48 h under 5% CO2 at 37 °C. The SRB assay was performed as above.

2.7.2. Drug content in mitochondria

The drug content in the mitochondrial fraction was further quantified using a FACScan flow cytometer [21]. A549 cells or A549/DDP cells were cultured and then treated with the free coumarin, coumarin liposomes, or targeting coumarin liposomes for 4 h under 5% CO2 at 37 °C. The final concentration of coumarin was 10 μM.

Control experiments were performed by adding blank medium. The cells were harvested and washed with cold PBS (pH 7.4) twice. Mitochondria isolation was carried out according to the Cell Mitochondria Isolation Kit guide (Beyotime; Institute of Biotechnology, Haimen, China). Briefly, the cells were added to the mitochondria extraction reagent (provided in the kit) and stirred in a homogenizer. The suspensions were centrifuged at 600 g for 10 min. The supernatants were collected and further centrifuged at 3500 g for 10 min. Then, the mitochondria were collected from the precipitates. The amount of mitochondria uptake was measured using a FACScan flow cytometer with the events collected 1 x 10^4, and represented by fluorescent intensity. Each assay was repeated in triplicate.

2.7.3. Mitochondrial depolarization

The mitochondria membrane potential (ΔΨm) was measured using the cationic lipophilic fluorescein diacetate (2-2' diacetylaminoethyl) rhodamine 123 (KeyGEN, Nanjing, China) [22]. Briefly, A549 cells or A549/DDP cells were seeded in a 6-well, flat-bottom tissue culture plate at a density of 4 x 10^4 cells/well in 2 mL growth medium. After 24 h, the cells were treated with the lonidamine liposomes, epirubicin liposomes, targeting lonidamine liposomes, targeting epirubicin liposomes, and targeting epirubicin liposomes plus targeting lonidamine liposomes for 6 h under 5% CO2 at 37 °C. The final concentration of lonidamine was 20 μM, and that of epirubicin was 10 μM. Control experiments were performed by adding blank medium. The cells were then washed once with PBS (pH 7.4) and immediately analyzed by a FACScan flow cytometer, with the events collected 1 x 10^4.

After the specificity of the induced mitochondrial depolarization by the targeting lonidamine liposomes or targeting epirubicin liposomes, the cells were first incubated with the MTPP blocker cyclosporin A (CsA, 1 μM; Zhaihu Yuancheng Pharmaceutical & Chemical Co., Ltd., Zhaihu, China) for 30 min [24]. After removing the MTPP blocker, a new medium containing the lonidamine liposomes (20 μM), epirubicin liposomes (10 μM), targeting lonidamine liposomes (20 μM), targeting epirubicin liposomes (10 μM), and targeting epirubicin liposomes (10 μM) plus targeting lonidamine liposomes (20 μM) were added. Control experiments were performed by adding blank medium. After 6 h, the cells were stained and measured as above. Each assay was repeated in triplicate.

2.7.4. Release of cytochrome C from mitochondria

The cytochrome C protein content in cytosol and mitochondrial fractions was determined using a human cytochrome C enzyme-linked immunosorbent assay (ELISA) kit (BD, USA) after drug treatment. Cytosol and mitochondrial proteins were extracted separately using a cytosol and mitochondrial protein extraction kit (Bio Basic Inc., Ontario, Canada). Briefly, after 24-h incubation, A549 cells or A549/DDP cells were treated with the free lonidamine, lonidamine liposomes, targeting lonidamine liposomes, free epirubicin, epirubicin liposomes, targeting epirubicin liposomes, and targeting lonidamine liposomes plus targeting epirubicin liposomes. The concentration of lonidamine was 20 μM and that of epirubicin was 5 μM. Control experiments were performed by adding blank medium. After 24-h incubation, the cells were harvested and washed with cold PBS twice. Then, the cells were incubated in cytosol extraction buffer (0.1% protease inhibitor, 0.1% DL-dithiothreitol [DTT], v/v; provided in the kit), vortexed for 15 s, and homogenized with a glass homogenizer 30 times. The homogenization buffer was centrifuged at 3000 revolutions per minute (rpm) for 10 min at 4 °C. The supernatant was further centrifuged at 11,000 rpm for 30 min at 4 °C. The supernatant from this centrifugation step stored as the cytosol fraction. The precipitates were treated with mitochondrial dissolution buffer (20 mM Tris-Cl pH 7.5, 2 mM EDTA, 1% Triton X-100, 0.1% protease inhibitor, v/v, and 1% DTT), vortexed and measured at 540 and 620 nm. The supernatant from this centrifugation step was stored as the mitochondrial fraction. The protein samples were added to the sample testing wells. The samples were incubated for 30 min at 37 °C, and then washed with washing buffer five times. Then, horseradish peroxidase (HRP)-conjugated reagent (provided in the kit) was added to each well. Subsequently, chromogen solution A and chromogen solution B (provided in the kit) were added to the wells and incubated for 15 min at 37 °C in the dark. Lastly, the wells were treated with the stop solution (provided in the kit) and measured at 450 nm on a microplate reader within 10 min. The content ratio of cytochrome C was calculated by comparison with the content of the blank control. Each assay was repeated in triplicate.

2.7.5. Caspase activation

Caspase 3 and caspase 9 activities in A549 cells and A549/DDP cells were determined using peptide substrates that emit fluorescence once cleaved by a specific protease [25]. Briefly, the A549 or A549/DDP cells were cultured for 24 h. Then, the cells were treated with the free lonidamine, lonidamine liposomes, targeting lonidamine liposomes, free epirubicin, epirubicin liposomes, targeting epirubicin liposomes, and targeting lonidamine liposomes plus targeting epirubicin liposomes. The concentration of lonidamine was 20 μM and that of epirubicin was 5 μM. Control experiments were performed by adding blank medium. After 24-h incubation, the cells were harvested and lysed. The cell lysates were centrifuged at 10,000 rpm for 1 min at 4 °C. The supernatants were stored and treated with caspase 3 and caspase 9 substrates (KeyGEN). Caspase 3 and caspase 9 activities were measured at 405 nm on a microplate reader, and the activity ratio was calculated according to the kit instructions. Each assay was repeated in triplicate.
2.7.6. Effects on pro-apoptotic Bax and anti-apoptotic Mcl-1

To evaluate the expression of the apoptosis regulator proteins Bax and Mcl-1 by A549 or A549cDDP cells, a double-antibody sandwich ELISA was performed using an ELISA kit (R&D Systems, Shanghai local agent, China). Briefly, the cells were cultured with medium at 37 °C in 5% CO₂ for 24 h, followed by the addition of the ebirubicin liposomes (5 μM), lonidamine liposomes (20 μM), targeting ebirubicin liposomes (5 μM), targeting lonidamine liposomes (20 μM), and targeting ebirubicin liposomes (5 μM) plus targeting lonidamine liposomes (20 μM). The cells were further incubated for 12 h and lysed. The cell lysates were centrifuged at 10,000 rpm at 4 °C for 1 min. The total protein, containing Bax or Mcl-1, was collected from the supernatants. Samples containing Bax or Mcl-1 (50 μL) were added to wells pre-coated with Bax or Mcl-1 monoclonal antibody, incubated at 37 °C for 30 min, washed with the kit washing buffer, and followed by the addition of 50 μL HRP-tagged protein of a Bax or Mcl-1 secondary antibody. The samples (50 μL) were protected from light, further incubated at 37 °C for 30 min, and washed with the kit washing buffer to remove the unbound enzyme. After chromogen solutions A and B (each 50 μL) were added, the color of the sample liquids changed to blue. After adding the stop solution (50 μL), the color finally turned to yellow from the effect of the acid. The depth of the color was positively correlated with the concentration of Bax or Mcl-1 protein in a sample, and a microplate reader measured the absorbance of the yellow product at 450 nm. The concentration of total protein was measured using the bicinchoninic acid method at 540 nm [26]. The Bax or Mcl-1 activity ratio was calculated using the following formula: Activity ratio = (A450 nm for the treated cells/A540 nm for the control cells) / (A450 nm for the control cells/A540 nm for the control cells), where A450 nm or A540 nm is the absorbance value. Each assay was repeated in triplicate.

2.7.7. ROS assay

To determine whether the different formulations induced ROS generation, ROS levels were determined using H₂DCFDA (dichlorodihydrofluorescein diacetate). H₂DCFDA is de-esterified to 2',7'-dichlorodihydrofluorescein by cellular esterases, and then oxidized by ROS to form fluorescent 2',7'-dichlorofluorescein. Increased fluorescence intensity was used to quantify the generation of intra-cellular ROS. A549 or A549cDDP cells were exposed to the ebirubicin liposomes, lonidamine liposomes, targeting ebirubicin liposomes, targeting lonidamine liposomes, and targeting ebirubicin liposomes plus targeting lonidamine liposomes. The concentration of ebirubicin was 10 μM and that of lonidamine was 20 μM. After 6-h incubation, 25 μL H₂DCFDA stock solution (200 μM) was added to each well. The plates were then incubated for 30 min in the dark. Fluorescence intensity was measured immediately using a FACScan flow cytometer with the events collected 1 × 10⁶. Each assay was repeated in triplicate [27,28].

2.7.8. ATP assay

Cellular ATP levels were measured using a firefly luciferase-based ATP assay kit (Beyotime) according to the manufacturer instructions. Briefly, after incubation with the ebirubicin liposomes, lonidamine liposomes, targeting ebirubicin liposomes, targeting lonidamine liposomes, or targeting ebirubicin liposomes plus targeting lonidamine liposomes for 6 h, A549 or A549cDDP cells were centrifuged at 12,000 g for 5 min. Fifty-well plates, 100 μL of each supernatant was mixed with 100 μL ATP detection working dilution. Luminescence (relative luminescence units, RLU) was measured by a monochromator microplate reader (Safire II; TECAN, Männedorf, Switzerland) [29].

2.8. Efficacy in drug-resistant lung cancer xenografts

Female BALB/c nude mice (initial weight, 16–18 g; Peking University Health Science Center, Beijing, China) were used for observation of the anticancer efficacy in vivo. All animal care and handling were carried out with the approval of the Institutional Authority for Laboratory Animal Care of Peking University. Briefly, approximately 1 × 10⁶ A549cDDP cells were re-suspended in 200 μL serum-free RPMI-1640 culture medium and injected subcutaneously into the right flanks of the mice. When tumors were 200–220 mm³ in volume, mice were randomly divided into six treatment groups (six mice each). At days 22, 24, 26, or 28 post-inoculation, physiological saline, free ebirubicin (3 mg/kg), ebirubicin liposomes (3 mg/kg), targeting ebirubicin liposomes (3 mg/kg), targeting lonidamine liposomes (20 mg/kg), and targeting ebirubicin liposomes (3 mg/kg) were administered to the mice via the tail vein. The mice were then monitored every other day for tumor progression with a caliper. The mice were weighed and the tumors were measured with a caliper every one or every two days. The mice were further observed before being sacrificed by cervical dislocation. The presence of each tumor mass was calculated by necropsy at day 31 after inoculation. Tumor volumes were calculated as length × width²/2 (mm³) [6].

2.9. In vivo imaging observation

Noninvasive optical imaging systems were used to observe the real-time distribution and tumor accumulation ability of targeting DiR (1'1'-dioctadecyl-3,3,3'3'-tetramethyl indotricarbocyanine iodide) liposomes in BALB/c mice bearing A549cDDP cancer cell xenografts. Female BALB/c mice were divided into three groups (three mice each). After the A549cDDP cells were enzymatically dissociated by 0.25% trypsin (g/100 mL) and inoculated, and the volume of the tumors in the mice was approximately 300–500 mm³ at day 26, the mice were administered physiological saline, free DiR, and targeting DiR liposomes via tail vein injection. Then, the mice were scanned at 1, 2, 6, 12, and 24 h using a Kodak multimodal imaging system (Carestream Health, Inc., USA).

In order to further observe the distribution status in tumor masses and the major organs at the early stage, the tumor-bearing mice were sacrificed after the administration of the targeting DiR liposomes, free DiR, or physiological saline at 1 h, followed by immediate removal of the tumor masses, heart, liver, spleen, lung, and kidney. The fluorescence signal intensities in different tissues were photographed [30].

2.10. Statistical analysis

Data are presented as the mean ± standard deviation (SD). One-way analysis of variance (ANOVA) was used to determine the significance among groups, after which post-hoc tests with the Bonferroni correction were used for multiple comparisons between individual groups.

3. Results

3.1. Synthesis of targeting molecule and preparation of liposomes

As described in our previous report, synthesis of the targeting molecule DQA-PEG₂₀₀₀-DSPÉ was confirmed by MALDI-TOF-MS and further characterized by 1H NMR [6]. The yield of DQA-PEG₂₀₀₀-DSPÉ was approximately 84%.

Table 1 lists the average particle sizes of the lonidamine liposomes, targeting lonidamine liposomes, epirubicin liposomes, and targeting epirubicin liposomes. The particle sizes were approximately 80 nm. The encapsulation efficiencies of lonidamine or epirubicin in the targeting liposomes and regular liposomes were >90%. All liposomes were slightly negatively charged.

Fig. 1A is a schematic representation of the targeting epirubicin liposomes or targeting lonidamine liposomes; Fig. 1B illustrates AFM images of the lonidamine liposomes (B₁, plane surface image; B₂, three-dimensional image); Fig. 1C depicts AFM images of the targeting lonidamine liposomes (C₁, plane surface image; C₂, three-dimensional image). The surface of the vesicle of the regular liposomes was relatively smooth, while that of the targeting liposomes had small globular protruberances.

3.2. Cytotoxicity

Fig. 2A₁ and 2A₂ illustrate the inhibitory effects on the A549 cells and A549cDDP cells after the addition of the targeting lonidamine liposomes alone or targeting epirubicin liposomes alone. Both the targeting lonidamine liposomes and targeting epirubicin liposomes effected the strongest inhibition of A549 and A549cDDP cell growth at various dose levels compared to the free drug control or the regular drug-loaded liposome control.

The free lonidamine and lonidamine liposomes exerted less cytotoxic effects on both the A549 and A549cDDP cells. Based on a comparable liposome membrane material amount (<10 μM), the

### Table 1

<table>
<thead>
<tr>
<th>Liposomes</th>
<th>Encapsulation efficiency (%)</th>
<th>Particle size (nm)</th>
<th>Polydispersity index</th>
<th>Zeta potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Epirubicin liposomes</td>
<td>92.5 ± 1.5</td>
<td>80.7 ± 0.03</td>
<td>0.221 ± 0.002</td>
<td>-0.07 ± 2.92</td>
</tr>
<tr>
<td>Lonidamine liposomes</td>
<td>91.8 ± 2.5</td>
<td>80.5 ± 0.04</td>
<td>0.291 ± 0.003</td>
<td>-0.06 ± 2.45</td>
</tr>
<tr>
<td>Targeting epirubicin liposomes</td>
<td>91.7 ± 3.4</td>
<td>83.9 ± 0.04</td>
<td>0.170 ± 0.005</td>
<td>-0.07 ± 1.71</td>
</tr>
<tr>
<td>Targeting lonidamine liposomes</td>
<td>94.7 ± 2.6</td>
<td>83.0 ± 0.04</td>
<td>0.208 ± 0.004</td>
<td>-0.09 ± 1.68</td>
</tr>
</tbody>
</table>
Fig. 1. A. Schematic representation of targeting lonidamine liposomes or targeting epirubicin liposomes; B. Atomic force microscopic images of lonidamine liposomes (B1, plane surface image; B2, three dimension image). C. Atomic force microscopic images of targeting lonidamine liposomes (C1, plane surface image; C2, three dimension image).
Fig. 2. A1. Survival rates (%) of A549 cells after the addition of drugs at 48 h; B1. Survival rates (%) of A549 cells after the addition of combination drugs at 48 h; A2. Survival rates (%) of A549cDDP cells after the addition of drugs at 48 h; B2. Survival rates (%) of A549cDDP cells after the addition of combination drugs at 48 h. Each assay was repeated in triplicate. Data are presented as the mean ± standard deviation (n = 3). *p < 0.05; a, vs. free lonidamine; b, vs. lonidamine liposomes; c, vs. blank targeting liposomes; d, vs. free epirubicin; e, vs. epirubicin liposomes; 1, vs. targeting lonidamine liposomes plus 2.5 µM targeting epirubicin liposomes; 2, vs. targeting lonidamine liposomes plus 5 µM targeting epirubicin liposomes.
blank targeting liposomes exhibited a minimal inhibitory effect at low amounts, but an obvious cytotoxic effect (at 20 μM) on both cancer cell lines. The free epirubicin and epirubicin liposomes alone resulted in an obvious inhibitory effect on the A549 and A549cDDP cells (Fig. 2A1 and A2). However, the A549cDDP cells exhibited a degree of resistance to the free epirubicin.

To understand the potential action of including targeting lonidamine liposomes as a co-therapy in A549 and A549cDDP cells, three different concentrations of lonidamine liposomes were used in combination with a fixed dose of targeting epirubicin liposomes to observe the difference. The targeting lonidamine liposomes (2.5 μM, 5 μM, and 10 μM) significantly enhanced the inhibitory effect of the targeting epirubicin liposomes, indicating a lonidamine dose-dependent effect (Fig. 2B1 and B2).

### 3.3. Targeting mechanism and effect

#### 3.3.1. Co-localization into mitochondria

Fig. 3 illustrates the laser confocal fluorescence images of A549 cells (A–F) and A549cDDP cells (G–L) incubated with the control formulations (A–B, D–E, G–H, J–K), targeting epirubicin liposomes (C, I), and targeting coumarin liposomes used to indicate the targeting lonidamine liposomes (F, L).

The targeting drug-loaded liposomes could be co-localized into the mitochondria of the A549 cells or the drug-resistant A549cDDP cells. Bright yellow fluorescence, a composition of red and green fluorescence, was used to indicate the co-localization of the coumarin or epirubicin into the mitochondria. According to the images, the targeting coumarin liposomes or targeting epirubicin liposomes exhibited punctate distribution,

![Fig. 3. Laser confocal fluorescence images of A549 cells (A–F) and A549cDDP cells (G–L) incubated with various formulations. A1–A3 or G1–G3, treated with PBS as a blank control and stained with MitoTracker Green; B1–B3 or H1–H3, treated with epirubicin liposomes; C1–C3 or I1–I3, treated with targeting epirubicin liposomes; D1–D3 or J1–J3, treated with PBS as a blank control and stained with MitoTracker Deep Red; E1–E3 or K1–K3, treated with coumarin liposomes; F1–F3 or L1–L3, treated with targeting coumarin liposomes. When applying epirubicin and coumarin-containing formulations, the incubation times were 1 h and 4 h, respectively. Coumarin was used a fluorescent probe for lonidamine. Each assay was repeated in triplicate. Results indicate that the targeting drug-loaded liposomes are able to be co-localized into mitochondria of A549 cells or resistant A549cDDP cells.](image-url)
indicating that the targeting liposomes were selectively accumulated into the mitochondria. However, after adding the free coumarin, free epirubicin, coumarin liposomes, or epirubicin liposomes, the bright yellow fluorescence could not be observed in the mitochondria.

3.3.2. Drug content in mitochondria

Fig. 4 depicts the drug contents in the mitochondrial fraction measured by flow cytometry after the addition of varying drug formulations to the A549 cells (A–B) and A549cDDP cells (C–D). The fluorescence intensity in the mitochondrial fraction was clearly higher after the addition of the targeting coumarin liposomes or targeting epirubicin liposomes than following the addition of free coumarin, free epirubicin, coumarin liposomes, or epirubicin liposomes, further confirming the co-localization of the targeting drug-loaded liposomes in the mitochondria.

3.3.3. Mitochondrial depolarization

Fig. 5 illustrates the dissipations of the mitochondrial membrane potentials in the A549 (A) and A549cDDP cells (B) after the addition of varying drug formulations, and the blocking effects of CsA pre-treatment on the dissipation of mitochondrial membrane potentials in the A549 (C) and A549cDDP cells (D). The targeting lonidamine liposomes plus targeting epirubicin liposomes resulted in the greatest dissipation of mitochondrial membrane potential (Fig. 5A and B).

In the MPTP-blocking CsA pre-treatment assay, the dissipation of mitochondrial membrane potential was significantly prevented with the similar addition of the targeting lonidamine liposomes plus targeting epirubicin liposomes, or other controls (Fig. 5C and D).

3.3.4. Release of cytochrome C from mitochondria

Fig. 6 depicts the cytochrome C protein ratios in the cytosol and mitochondria of the A549 (A) and A549cDDP cells (B). The cytochrome C contents in the cytosol were clearly increased with the decrease of cytochrome C in the mitochondria in both cancer cell lines after the addition of the targeting lonidamine liposomes plus targeting epirubicin liposomes, as compared with those after other controls were added.

3.3.5. Caspase activation

Fig. 7 illustrates the activities of caspase 9 or caspase 3 in the A549 (A, C) and A549cDDP cells (B, D). The increase in the activities of caspase 9 and caspase 3 in both cancer cell lines was the most significant after the targeting lonidamine liposomes plus targeting epirubicin liposomes were added, as compared with that after the addition of the other controls.

3.3.6. Effects on pro-apoptotic Bax and anti-apoptotic Mcl-1

Fig. 8 illustrates the activities of the pro-apoptotic protein Bax and the anti-apoptotic protein Mcl-1 in the A549 (A, C) and A549cDDP cells (B, D). The increase in Bax activities was the most evident following the suppression of Mcl-1 in both cancer cell lines after the targeting lonidamine liposomes plus targeting epirubicin liposomes were added, as compared with other controls.

3.3.7. ROS assay

Fig. 9 depicts the ROS activities in the A549 (A) and A549cDDP cells (B). The increase of ROS activity in the A549 cells was the most significant after the targeting epirubicin liposomes plus targeting lonidamine liposomes were added. However, there was no evident ROS generation in the A549cDDP cells.

Fig. 4. Drug contents in the mitochondrial fraction measured by flow cytometry after incubating varying drug formulations with A549 (A–B) and A549cDDP (C–D) cells for 4 h. Each assay was repeated in triplicate. A1 and C1, Blank control; A2 and C2, Free epirubicin; A3 and C3, Epirubicin liposomes; A4 and C4, Targeting epirubicin liposomes; B1 and D1, Blank control; B2 and D2, Free coumarin; B3 and D3, Coumarin liposomes; B4 and D4, Targeting coumarin liposomes.
3.3.8. ATP assay

Fig. 10 illustrates the ATP activities in the A549 (A, C) and A549cDDP cells (B, D). Both the targeting lonidamine liposomes and targeting epirubicin liposomes caused the most significant decreases in both cancer cell lines as compared with the non-targeting controls.

3.4. Inhibition effect on drug-resistant lung cancer xenografts

Fig. 11 depicts the therapeutic effects in the A549cDDP xenograft model, with the inhibitory effect in the tumor model being strongest after administration of the targeting lonidamine liposomes plus targeting epirubicin liposomes as compared with that after administration of the lonidamine liposomes or epirubicin liposomes.

3.5. In vivo imaging in tumor-bearing mice

Fig. 12A depicts the real-time distribution and accumulation ability of the targeting DiR liposomes in the A549cDDP cell xenografts in the female BALB/c mice. After intravenous injection, the targeting DiR liposomes could be observed in the full blood circulation system, including the tumor location, at the early stage (2 h), and the fluorescent signal was maintained up to 6 h. In contrast, the...
free DiR fluorescent dye was rapidly distributed in the liver and lower intestines at 1 h, and the subsequent fluorescence intensities in these locations gradually weakened.

Fig. 12B illustrates the distribution status of the targeting DiR liposomes indicated by fluorescence signals at the early stage (1 h). The fluorescence signals of the targeting DiR liposomes were obvious in the tumor masses and in the major organs, including the heart, liver, spleen, lung, and kidney. In contrast, the fluorescence signals of the free DiR could be observed in the liver, but were absent from the tumor masses or other organs.

4. Discussion

Lung cancer is a disease described as uncontrolled cell growth in lung tissues. When treatment is ineffective, the growth can spread beyond the lung into nearby tissue, and eventually, to other parts of the body. MDR is a major cause of refractory lung cancer. In this study, we developed a novel type of targeting lonidamine liposome to circumvent drug-resistance through modulation of the mitochondrial signaling pathways to induce apoptosis of drug-resistant lung cancer and to augment the efficacy of a cytotoxic agent,
epirubicin, which is similarly encapsulated into the vesicles of the targeting liposomes.

Lonidamine itself has been reported to be a mitochondrial targeting agent used previously to treat BPH, and clinical trials currently use it to treat metastatic breast cancer and NSCLC. However, it results in severe hepatic toxicity and lower distribution or accumulation in tumor tissue due to its low solubility and poor pharmacokinetic properties. Lonidamine can be encapsulated into a targeting liposomal vesicle modified with a functional material, a DQA-PEG2000-DSPE conjugate, to enhance its accumulation in tumor tissue via the long-circulatory effect [6] and the suitable size of the liposomes, consequently reducing immediate aggregation in live tissue.

Fig. 10. ATP activities in A549 (A, C) and A549 cDDP cells (B, D) after incubation with varying drug formulations for 6 h. Data are presented as the mean ± standard derivation. Each assay was repeated in triplicate. A1 and B1. Blank; A2 and B2. Free lonidamine; A3 and B3. Lonidamine liposomes; A4 and B4. Targeting lonidamine liposomes; A5 and B5. Epirubicin liposomes; A6 and B6. Targeting epirubicin liposomes plus targeting lonidamine liposomes.

epirubicin would be a mitochondrial targeting agent used previously to treat BPH, and clinical trials currently use it to treat metastatic breast cancer and NSCLC. However, it results in severe hepatic toxicity and lower distribution or accumulation in tumor tissue due to its low solubility and poor pharmacokinetic properties. Lonidamine can be encapsulated into a targeting liposomal vesicle modified with a functional material, a DQA-PEG2000-DSPE conjugate, to enhance its accumulation in tumor tissue via the long-circulatory effect [6] and the suitable size of the liposomes, consequently reducing immediate aggregation in live tissue.
Fig. 12. In vivo real-time imaging of A549/DDP xenografts in female BALB/c nude mice after intravenous injection of NaCl (pH 7.4), free DiR, or targeting DiR liposomes (A); ex vivo optical images of tumor masses and organs after A549/DDP xenografts in female BALB/c nude mice sacrificed at 1 h (B). In vivo non-invasive NIRF images of time-dependent whole-body imaging of A549/DDP tumor-bearing mice after intravenous injection of targeting liposomes (A). Ex vivo optical images of tumors and organs of A549/DDP tumor-bearing mice sacrificed at 1 h after intravenous injection (B). DiR (1,1'-dioctadecyl-3,3,3',3'-tetramethyl indocarbocyanine iodide), which is used as a fluorescent probe.
The DQA-PEG2000-DPSE conjugate was synthesized and characterized as described in our previous study [6], and incorporated onto the targeting lonidamine liposomes and targeting epirubicin liposomes. DQA is a quaternary ammonium cation available as a dichloride salt, and is an amphiphile with delocalized cationic charge centers that is subject to selective mitochondrial accumulation. Our previous results and the literature have demonstrated that as a mitochondrial-tropic molecule, DQA can target the mitochondria of cancer or cancer stem cells by direct incorporation into liposomes or nanoparticles to deliver drugs or genes that trigger apoptosis [31]. In this study, DQA was conjugated with COOH-PEG2000-DPSE to synthesize DQA-PEG2000-DPSE, which can also be used as long-circulatory material because it can escape the rapid uptake of the reticuloendothelial system (RES) due to the action of PEG, thereby exhibiting biologically stable properties in the blood circulation [32] and decreasing immediate distribution and clearance in liver tissue. In the constructed targeting lonidamine liposomes, DQA is exposed at the surface of the liposomes, which is beneficial for targeting the mitochondria of cancer cells and prevents the possibility of direct DQA encapsulation in the liposomes being hindered by the longer hydrocarbon chain after PEG coating. Therefore, the carboxyl group of the COOH-PEG2000-DPSE is coupled with one of two symmetrical amino groups of DQA in a molar ratio of 1:1, and is further confirmed by 1H NMR assay with a semi-quantitative determination of the DQA-PEG2000-DPSE in the product [14]. As the other amino group of the DQA molecule remains, the cationic group of DQA would retain the mitochondria-tropic ability. Moreover, as COOH-PEG2000-DPSE itself is a good material for preparing liposomes, the uncoupled COOH-PEG2000-DPSE in the production mixture need not be removed. The constructed targeting lonidamine and targeting epirubicin liposomes possessed the following physicochemical characteristics: small particle size (approximately 80 nm), high EE (>90%), delayed drug release, and slightly negative zeta potential. The suitable particle size and high EE of the targeting lonidamine liposomes allows lonidamine to be transported in the vasculature of tumor tissue, permits greater accumulation of the liposomes in tumor tissue by the enhanced permeability and retention effect [32], and evades elimination by the kidney [33]. The zeta potential of the targeting lonidamine liposomes was slightly lower than that of the lonidamine liposomes, which may have been due to the unremoved, negatively charged lipid material, COOH-PEG2000-DPSE (Table 1). AFM determined that the surface of the vesicles of the regular liposomes was relatively smooth, while there were small globular protuberances on the surfaces of the targeting liposomes. In addition, the modified targeting molecule resulted in the targeting liposomes being slightly bigger than the regular liposomes (Fig. 1).

In the cytotoxicity assay, the targeting lonidamine liposomes exhibited the strongest inhibitory effect on both the non-drug-resistant A549 cells (Fig. 2A1) and the drug-resistant A549cDDP cells (Fig. 2A2). The inhibitory effect of the epirubicin as an effective cytotoxic agent was limited in the cisplatin-resistant A549cDDP cells, which indicated cross-drug resistance (Fig. 2A2). Two phenomena were evident from this study, namely, the targeting lonidamine liposomes had a stronger inhibitory effect in a lonidamine-dose dependent manner, and the targeting lonidamine liposomes enhanced the cytotoxicity of the targeting epirubicin liposomes. The combination therapy with the targeting lonidamine liposomes significantly enhanced the inhibitory effect of the epirubicin liposomes in the drug-resistant A549cDDP cells (Fig. 2B1 and B2). The cytotoxicity assay revealed a comprehensive result that may stem from two aspects: acute necrosis and apoptosis, which arise from the targeting lonidamine liposomes alone or combination therapy with the targeting epirubicin liposomes. The mechanisms are elucidated below by the mitochondria-related apoptosis signaling pathways [34].

Confocal observation and flow cytometry revealed a higher intracellular uptake, indicating mitochondrial co-localization (Fig. 3) and higher mitochondrial uptake (Fig. 4). The higher cytotoxicity is consistent with the increased intracellular and mitochondrial uptake. The mechanism for the enhanced intracellular uptake can be explained by the addition of TPGS2000, which has an inhibitory effect on the drug efflux of ABC transporters [35–37]. The reason for the mitochondrial co-localization or higher mitochondrial uptake was due to the action of DQA on the targeting liposomes. In view of the drug content in the mitochondrial fraction, the targeting drug-loaded liposomes internalized by the cancer cells were not merely attached to the surface of the mitochondria, but were further endocytosed by the mitochondria.

The effect of the targeting lonidamine liposomes and the combination therapy with targeting epirubicin liposomes were indicated by the obvious decrease in the mitochondrial membrane potential (Fig. 5A and B). The mitochondrial inner membrane is negatively charged due to the presence of negatively charged glycoproteins. Mitochondrial membrane potential is monitored by a fluorescent dye, rhodamine 123, which is a positively charged lipophilic compound, and as a cell-permeating cationic dye, it preferentially enters mitochondria in response to a highly negative mitochondrial membrane potential. During apoptosis, the mitochondrial membrane potential is decreased, resulting in the spillage of rhodamine 123 from the mitochondria. Accordingly, to understand whether the targeting epirubicin liposomes plus targeting lonidamine liposomes induce apoptosis of A549cDDP cells via the mitochondrial pathway, rhodamine 123 was incorporated into the targeting liposomes to indicate mitochondrial membrane potential through the measurement of fluorescent intensity. The mean fluorescent intensity of rhodamine 123 clearly decreased after the addition of the targeting liposomes plus targeting lonidamine liposomes (Fig. 5A and B), demonstrating that they can induce disruption of the mitochondrial membrane potential in drug-resistant lung cancer cells. The collapse of the mitochondrial membrane potential results from the mitochondrial leak by the opening of the PTP. The above effects were obviously inhibited in the cells pre-treated with the PTP blocker CsA (Fig. 5C and D), proving that the PTPs are involved in this process. Consequently, it proves that there is a significant release of cytochrome C from the mitochondria (Fig. 6) [24].

Further measurements on the caspase activity ratios demonstrated markedly decreased activities of caspase 3 and caspase 9 in both the A549 and A549cDDP cells after the addition of the targeting lonidamine liposomes (Fig. 7), suggesting that the cell death induced by the targeting lonidamine liposomes is at least mediated by a mitochondria-dependent signaling pathway [27].

Bax is a pro-apoptotic protein and Mcl-1 is an apoptosis-suppressing protein involved in the apoptosis of cells mainly via the mitochondrial pathway. In both the A549 and A549cDDP cells, the relative activity of Bax was enhanced by the targeting epirubicin liposomes plus targeting lonidamine liposomes (Fig. 8A and B). In contrast, the relative activity of Mcl-1 was decreased by the same combination therapy (Fig. 8C and D). These results suggest that the activation of pro-apoptotic proteins and suppression of anti-apoptotic proteins are involved in the apoptotic process [28].

ROS, which play important roles in cell signaling and homeostasis, are chemically reactive, oxygen-containing molecules. ROS are formed as a natural byproduct of the normal metabolism of oxygen, and are generated by exogenous sources as well. After applying the combination therapy comprising the targeting lonidamine liposomes and targeting epirubicin liposomes in this study, ROS levels in the non-drug-resistant A549 cells increased...
dramatically (Fig. 9A), while there was no obvious change in the drug-resistant A549CDPP cells (Fig. 9B), possibly due to previous damage to the ROS signaling pathway by cisplatin. Our results indicate that lonidamine is a ROS-sensitive drug and its apoptosis-inducing effect is increased in combination therapy in lung cancer cells, including the drug-resistant lung cancers, if the ROS signaling pathway remains [27,28,38].

There was an obvious decrease in ATP levels after the addition of the targeting lonidamine liposomes or targeting epirubicin liposomes (Fig. 10A–D) in A549 cells and A549CDPP cells. The effect of combination therapy was not observed because the fluorescence of epirubicin interfered with that of the luciferase probe. Nevertheless, separate additions of the targeting drug-loaded liposomes revealed a strong inhibitory effect on the ATP activities, suggesting that the targeting lonidamine liposomes can target the mitochondrion and affect cell viability by inhibiting the respiratory chain [29].

As a comprehensive result, efficacy after administering the targeting lonidamine liposomes plus targeting epirubicin liposomes was evinced in the inhibitory effects in the drug-resistant A549CDPP xenografted nude mice, demonstrating robust antitumor activity as compared to the other controls at comparable doses (Fig. 11). As epirubicin does not emit near-infrared fluorescence at excitation 730 nm and emission 790 nm wavelengths, and lonidamine does not emit fluorescence, the near-infrared fluorescent probe DiR was incorporated into the targeting liposomes as a probe. The in vivo imaging results demonstrate that the targeting DiR liposomes persisted in the blood circulation, and exhibited a higher accumulation in the tumors (Fig. 12A). The ex vivo fluorescent images of the excised organs further confirmed the higher accumulation of the targeting DiR liposomes in the tumors (Fig. 12B) [39].

5. Conclusion

In this study, targeting lonidamine liposomes were developed as a co-therapy with targeting epirubicin liposomes to treat resistant lung cancer. The liposome membrane materials DQA-PEG2000–DSPE, PEG2000–DSPE, and TPGS1000, as well as the suitable size of the liposomes, jointly contributed to the long circulation in the blood, evading the immediate clearance by the liver and RES system, and enhanced the permeability and retention in tumor tissue. The involvement of TPGS1000 demonstrated a blocking effect against the efflux of anticancer drug from the drug-resistance cancer cells, enabling the effective uptake of the targeting drug-loaded liposomes by the cancer cells. The DQA-PEG2000–DSPE conjugate played a targeting role to the mitochondria of the lung cancer cells. The mechanisms for the enhanced anticancer efficacy in drug-resistant lung cancer after the administration of the targeting lonidamine liposomes as co-therapy with an anticancer agent were due to their actions on the mitochondrial signaling pathways. These included PTP opening and dissipation of the mitochondrial membrane potential, thereby releasing cytochrome C, initiating a cascade of caspase 9 and caspase 3 reactions, activating the pro-apoptotic Bak protein and suppressing the anti-apoptotic Mcl-1 protein, generating ROS, and decreasing ATP levels.

Acknowledgments

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[12] Liu YP, Yang CJ, Huang MS, Yeh CT, Wu AT, Lee YC, et al. Cisplatin selects for drug-resistant A549cDDP cells (Fig. 9B), possibly due to previous damage to the ROS signaling pathway by cisplatin. Our results demonstrated a blocking effect against the efflux of anticancer drug from the drug-resistance cancer cells, enabling the effective uptake of the targeting drug-loaded liposomes by the cancer cells. The DQA-PEG2000–DSPE conjugate played a targeting role to the mitochondria of the lung cancer cells. The mechanisms for the enhanced anticancer efficacy in drug-resistant lung cancer after the administration of the targeting lonidamine liposomes as co-therapy with an anticancer agent were due to their actions on the mitochondrial signaling pathways. These included PTP opening and dissipation of the mitochondrial membrane potential, thereby releasing cytochrome C, initiating a cascade of caspase 9 and caspase 3 reactions, activating the pro-apoptotic Bak protein and suppressing the anti-apoptotic Mcl-1 protein, generating ROS, and decreasing ATP levels.

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