

Astragaloside IV Co-Loaded Osimertinib Liposomes Alleviate EGFR-TKI Resistance in Non-Small Cell Lung Cancer

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Introduction: Advances in tumor biology have established EGFR-tyrosine kinase inhibitors (EGFR-TKIs) as a key therapy for non-small cell lung cancer (NSCLC). Osimertinib (OSI), a third-generation EGFR-TKI, however, often leads to acquired resistance within about a year. Astragaloside IV (AS-IV), a bioactive saponin from *Astragalus membranaceus*, exhibits anti-tumor potential and may help reverse resistance-related epithelial–mesenchymal transition (EMT), a process mediated by TGF- β . While combining OSI with AS-IV is a promising strategy, its efficacy is limited by poor solubility and toxicity—challenges that can be addressed through liposomal co-delivery.

Methods: We prepared OSI and AS-IV co-loaded liposomes (LPs-OSI/AS) and characterized their physicochemical properties and drug release in vitro. Cellular uptake and anti-proliferative effects were evaluated in OSI-sensitive (NCI-H1975) and OSI-resistant (NCI-H1975/AR) NSCLC cells. EMT-related gene expression was analyzed by RT-qPCR. In vivo efficacy was assessed using a subcutaneous xenograft model in BALB/c-nu mice by monitoring tumor growth and body weight.

Results: LPs-OSI/AS liposomes were successfully prepared with uniform particle size (96.92 ± 12.04 nm, PDI 0.28 ± 0.01), high encapsulation efficiency (AS: $89.74 \pm 6.53\%$; OSI: $84.35 \pm 8.82\%$), and sustained release. In vitro, LPs-OSI/AS significantly inhibited proliferation of both NCI-H1975 and OSI-resistant NCI-H1975/OSIR cells, outperforming free drug controls without obvious cytotoxicity, and downregulated EMT-related genes (*Vimentin*, *TGF β 1*, *TGF β 2*). In vivo, LPs-OSI/AS (20 mg/kg OSI + 40 mg/kg AS) reduced tumor volume by approximately 70% compared to OSI monotherapy, with 100% survival and no significant body weight loss. The Ki-67 positive rate was substantially lower in the LPs-OSI/AS group (1.06%) than in the OSI monotherapy group (5.98%), and H&E staining confirmed superior pathological improvement. Safety evaluation demonstrated normal organ structures with no inflammation or necrosis.

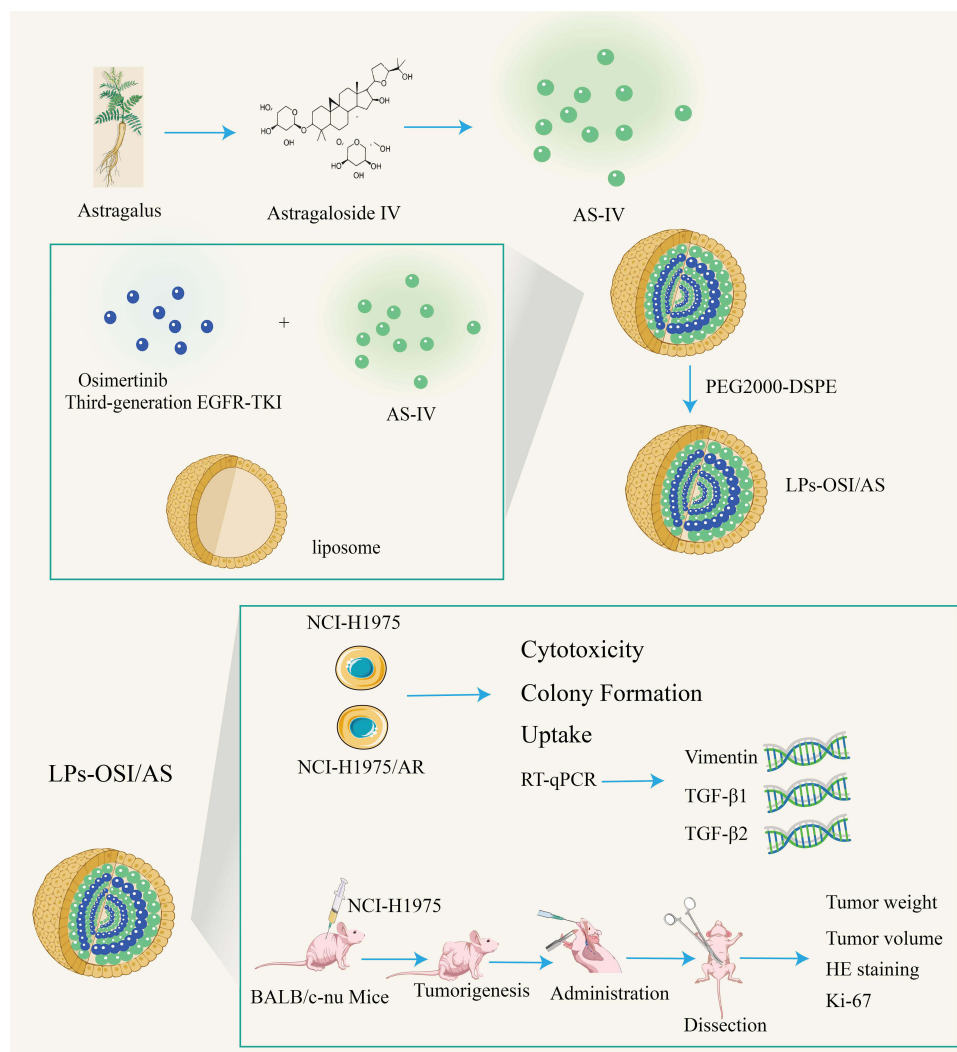
Conclusion: In summary, LPs-OSI/AS effectively inhibits tumor growth by regulating EMT, exhibits favorable biosafety, and represents a promising therapeutic strategy for NSCLC.

Keywords: NSCLC, EGFR-TKI, astragaloside IV, drug resistance

Introduction

Lung cancer, the type of cancer with the highest incidence and mortality rate in China,¹ is frequently diagnosed at an advanced stage for most patients due to nonspecific early symptoms, which often results in the loss of eligibility for surgical intervention. Non-Small Cell Lung Cancer (NSCLC) is the most common type of lung cancer, accounting for approximately 85% of all lung cancer cases and represents the predominant histological subtype, encompassing adenocarcinoma and squamous cell carcinoma.² In the course of clinical research on NSCLC, it has been established that a subset of patients' harbors activating mutations in the gene encoding the epidermal growth factor receptor (EGFR) tyrosine kinase. The discovery of activating EGFR mutations in a subset of NSCLC patients has driven the development

Graphical Abstract



of EGFR tyrosine kinase inhibitors (EGFR-TKIs), fundamentally reshaping the clinical management of this disease. This progression from oncogenic driver identification to molecularly targeted treatment marks a definitive advance in precision medicine. In advanced NSCLC, EGFR mutation frequency exhibits substantial ethnic variation, with approximately 90% of cases attributed to exon 19 deletions or the L858R point mutation in exon 21. From the perspective of molecular mechanisms,³ these genetic changes render EGFR to become an “oncogenic driver” by constitutively activating downstream signaling pathways independent of ligand binding, leading to uncontrolled tumor cell proliferation, survival, invasion, and migration, thereby promoting disease progression.

At present, three generations of EGFR-TKI have been approved for the treatment of NSCLC patients with EGFR mutations in various clinical settings. Gefitinib and erlotinib are the first-generation EGFR-TKI that can reversibly bind to wild-type and mutant EGFR.⁴ First-generation EGFR-TKI can reversibly inhibit wild-type EGFR and are primarily used as the first-line treatment of patients with EGFR exon 19 deletion (19del) or exon 21 point mutation (L858R).⁵ Afatinib and dasatinib are second-generation EGFR-TKI with a broader range of targets and have demonstrated significant clinical benefits in patients with advanced NSCLC with Ex19del and L858R mutations.⁶ Second-generation EGFR-TKI bind irreversibly to EGFR and also inhibit members of the HER family such as HER3⁷ and HER4, with

greater potency than the first-generation agents.⁸ They are primarily used as the first-line treatment for patients with sensitive EGFR mutations, and exhibit strong efficacy against some rare mutations like G719X, and L861Q.⁹ However, most patients develop acquired resistance during or within 9 to 14 months after EGFR-TKI treatment. Among them, the T790M mutation is the most common resistance mechanism in both the first- and second-generation EGFR-TKIs, a key drug resistance gene mutation in the EGFR pathway.¹⁰ This mutation occurs when methionine replaces threonine at position 790 in exon 20 of the EGFR gene.¹¹ Osimertinib (OSI) is a third-generation EGFR-TKI that effectively targets classic EGFR sensitizing mutations in exons 18, 19, and 21, and selectively inhibits the T790M resistance mutation.¹² Additionally, OSI's ability to penetrate the blood–brain barrier is more effective than that of other EGFR-TKI.¹³ Therefore, OSI is commonly used for patients with EGFR T790M-mediated resistance.¹⁴ However, similar to the first- and second-generation EGFR-TKI, patients receiving OSI still inevitably develop acquired resistance. Various acquired resistance mechanisms include EGFR-dependent and EGFR-independent resistance mechanisms. Compared with the first- and second-generation EGFR-TKI, the incidence of targeted resistance in OSI is lower. Only 10% to 20% of OSI patients will develop dependent resistance, while EGFR-independent resistance is more significant and frequent in OSI resistance.¹⁵ This might be attributed to the better dependency suppression effect of OSI. Nevertheless, preventing or delaying acquired resistance remains a critical challenge. Currently, the most prevalent strategy for addressing acquired resistance to EGFR-TKIs in current research is combination therapy. Among various approaches, the combined use of OSI and anti-cancer drugs is particularly widely adopted.

In recent years, significant advancements have been achieved in the application of traditional Chinese medicine (TCM) in the field of tumor treatment. TCM has gained increasing recognition worldwide for its favorable safety profile, minimal side effects, and high patient tolerance.¹⁶ This therapeutic advantage is supported by high-level clinical evidence: a meta-analysis of 76 randomized controlled trials involving 9862 cancer patients showed that the combination of TCM injections with anti-tumor treatment reduced the risk of bone marrow suppression by 40% (RR 0.60, 95% CI 0.51–0.70) and the risk of gastrointestinal adverse reactions by 31% (RR 0.69, 95% CI 0.63–0.76). At the same time, it significantly reduced the incidence of various treatment-related adverse events such as liver damage, kidney damage, neurotoxicity, and oral mucositis. Among these, the GRADE ratings for evidence of bone marrow suppression and liver damage were of high certainty.¹⁷ In addition, TCM exerts anti-tumor effects through multiple mechanisms, significantly reduces chemotherapy-related adverse reactions, and simultaneously prolongs overall survival and progression-free survival in patients.^{18,19} The TCM approaches for lung cancer mainly focus on strengthening the body's resistance and tonifying deficiency. The addition of TCM to PD-1/PD-L1 inhibitors prolongs progression-free survival (PFS) and overall survival (OS) in patients with stage IIIB–IV NSCLC.^{20,21} Astragaloside IV (AS-IV), a key TCM compound known as the “best among all qing-tonifying herbs” Has been shown by modern pharmacology to possess anti-inflammation, immunomodulatory, antioxidation, anti-cancer, anti-diabetes, and heart protection.²² In terms of anti-tumor effects, AS-IV can enhance the therapeutic efficacy of chemotherapy drugs and reduce their toxicity through its extensive immune-boosting effects, and benefits to prevent recurrence and extend survival.²³ Numerous AS-IV-based formulations, such as Huang Qi Zhi Shi Tang, Huang Qi Fu Zheng Tang and Ku Shen Huang Qi Tang, are now used as adjuvant therapies to slow the progression of lung cancer and alleviate adverse reactions, thereby enhancing the immune function of patients.²⁴ AS-IV is a monomer extracted from *Astragalus membranaceus*, C₄₁H₆₈O₁₄, and AS-IV has been identified as a marker of phytochemicals in *Astragalus membranaceus*.²⁵ The anti-tumor effect of AS-IV has also been widely studied. Some researchers have demonstrated that AS-IV inhibits lung cancer progression and metastasis via the modulation of macrophage polarization, through the AMPK signaling pathway.²⁶ Transforming growth factor-β (TGF-β) is a key cytokine that regulates the tumor microenvironment and the biological behavior of tumor cells, which may attenuate or impair the cytotoxic efficacy of EGFR-TKIs against tumor cells. Notably, AS-IV has been shown to inhibit TGF-β1-induced epithelial–mesenchymal transition (EMT) in peritoneal mesothelial cells through the expression of Smad7.²⁷ EMT is crucial in the early progression of lung cancer cell metastasis and invasion, and most patients with acquired drug-resistant NSCLC also present with EMT characteristics.²⁸ Accumulating evidence indicates that the drug resistance generated by the first and second-generation EGFR-TKI is strongly associated with EMT, and reversing EMT can significantly increase the sensitivity of drug-resistant tumor cells and animal models.²⁹ Nevertheless, effective clinical interventions targeting EMT-mediated EGFR-TKI resistance remain lacking.

Current research on overcoming cancer drug resistance has led to the development of combination therapies utilizing multiple therapeutic agents. Although these combination treatments demonstrate synergistic antitumor effects and target multiple tumorigenic pathways, conventional drug co-delivery approaches are hampered by poor solubility, off-target organ toxicity, and suboptimal therapeutic efficacy. Therefore, it is crucial to develop novel drug delivery strategies capable of overcoming these limitations. Among various available delivery systems, nanoparticles and liposomes are the most widely employed. Compared to nanoparticles, liposomes offer distinct advantages, including the non-toxic and metabolizable nature of their lipid components, straightforward preparation processes that facilitate large-scale production, and high biocompatibility.³⁰ Given these favorable characteristics, this study selected liposomes as the preferred nanocarrier platform for drug delivery. In recent years, nanocarrier-based combination therapy has advanced towards intelligence and precision, breaking through traditional bottlenecks. Stimuli-responsive liposomes have become a hotspot, responding to tumor microenvironment factors (eg, acidic pH, high GSH) or external stimuli to achieve on-demand drug release, maximizing tumor-site efficacy and reducing systemic toxicity. Dual-responsive liposomes integrating multiple stimuli further enhance spatiotemporal control over drug release. Another frontier is the integration of nanocarriers with emerging technologies such as PROTACs, which degrade oncogenic proteins to overcome resistance. Liposomes improve the bioavailability of PROTACs and enable co-delivery with targeted drugs, generating synergistic effects, particularly promising for reversing EGFR-TKI resistance in NSCLC.³¹ Theranostic nanocarriers, loading both drugs and diagnostic agents, enable real-time monitoring of drug distribution and therapeutic effects, aiding efficacy evaluation.^{32,33} Drug combination strategies have shifted toward multi-mechanism combinations, such as targeted drugs with active components from traditional Chinese medicine, which aligns with the OSI-AS-IV combination used in this study. Liposomes co-loading such agents mitigate off-target toxicity and enhance synergy. Despite preclinical progress, clinical translation still faces challenges such as batch-to-batch consistency in large-scale production. Nevertheless, optimized liposome design provides a solid basis for overcoming NSCLC drug resistance. To date, no studies have explored the co-loading of OSI and AS-IV within a single nanocarrier specifically targeting EMT-mediated drug resistance, which provides the rationale for the present study.

In this study, we developed OSI co-loaded AS-IV liposomes (LPs-OSI/AS) and established a method of its preparation. The physicochemical characteristics and *in vitro* drug release profile of LPs-OSI/AS were systematically evaluated. Cellular uptake of the liposomes, as well as the inhibitory effects on proliferation, were assessed in NSCLC cell lines, including NCI-H1975 and OSI-resistant NCI-H1975 (NCI-H1975/AR). Changes in intracellular EMT-related gene expression were analyzed by RT-qPCR. Furthermore, a subcutaneous lung cancer xenograft model was established in BALB/c-nu mice to evaluate the *in vivo* therapeutic efficacy of LPs-OSI/AS based on tumor growth inhibition and body weight changes. This study represents the first attempt to co-load OSI and AS-IV into liposomes, thereby reducing OSI resistance and achieving therapeutic effects on NSCLC by mediating EMT and TGF- β . The findings provide a promising strategy for the treatment of NSCLC.

Materials and Methods

Cell Culture and Reagents

Fetal bovine serum (FBS) and Roswell Park Institute medium (RPMI-1640) were purchased from Gibco. 1× Dulbecco's phosphate buffer saline (D-PBS) and Trypsin-EDTA Solution were purchased from Biosharp. Memorial OSI were purchased from Shanghai Yuanye Bio-Technology. AS-IV was purchased from FEIYU Bio. Ethanol was purchased from Chengdu Xinhaixing chemical reagent factory. Acetonitrile, MTT and Carbinol were purchased from Sigma in USA. Lecithin High Potency, Lecithin and PEG-2000/DSPE were purchased from Lopoid in Germany. 4% Paraformaldehyde, trizol and diethyl pyrocarbonate water were purchased from Biosharp. Penicillin-streptomycin was purchased from Hyclone. Isopropanol and Trichloromethane were purchased from Chengdu Chron Chemicals Co., Ltd. NCI-H1975 cells were purchased from National Collection of Authenticated Cell Cultures. NCI-H1975/OSIR cells were a kind gift from Prof. Jinjian Chen (University of Macau). Specific pathogen-free BALB/c-nu mice were purchased from Home-SPF (Beijing) Biotechnology Co., Ltd.

Preparation of the LPs-OSI/AS

Liposomes co-loading OSI and AS-IV (LPs-OSI/AS) were prepared using the ethanol injection-sonication method and ammonium sulfate gradient method.³⁴ OSI (purity 99%), purchased from Shanghai Yuanye Biotechnology Co., Ltd.; AS-IV (purity 98%), purchased from Nantong Feiyu Biotechnology Co., Ltd. The dosages of the drugs were 2 mg of AS IV and 1 mg of OSI. Analytical grade ethanol was purchased from Chengdu Haixing Chemical Reagent Factory; chromatographic grade methanol and acetonitrile were purchased from Sigma-Aldrich (USA); analytical grade phosphoric acid and hydrochloric acid were both purchased from Sichuan Xilong Science Co., Ltd. Soy lecithin and egg yolk lecithin were purchased from Lopoid GmbH (Germany); cholesterol was purchased from Chengdu Kelong Chemical Reagent Factory; PEG-2000/DSPE was purchased from Lopoid GmbH (Germany); Tween 20 and propylene glycol were purchased from Chengdu Kelong Chemical Reagent Factory; PBS was purchased from Biosharp. In brief, EPC, PEG2000-DSPE and cholesterol were mixed at a mass ratio of 10:1:1. Subsequently, AS-IV was dissolved at 60°C and lipid: drug ratio of 10:1 in 2–3 mL of ethanol. 5 mL ammonium sulfate in the other beaker, heated in a water bath at 60 °C and stirred magnetically. Then added the AS-IV solution at the same temperature to the ammonium sulfate solution added dropwise using a syringe at a controlled rate, and continue to stir until the ethanol is completely volatilized. The suspension was filtered through a 0.45 µm membrane filter gets the liposomes of AS-IV. To obtain ammonium sulfate gradient, by using dialysis bag with a molecular weight cut-off 3.5 kDa to perform three consecutive dialysis exchanges in 10% sucrose solution. After preparing the ammonium sulfate gradient, added the ethanol solution of OSI to the AS-IV liposome dispersion and shaken 250rpm for 40 min in thermostat incubator. The loading was performed at 55°C above the phase transition temperature of EPC (45°C). Free OSI was removed by Ultrafiltration through an ultrafiltration tube with a molecular weight cut-off of 10 kDa. Liposomes co-loading OSI and AS-IV would be made by the above steps. Later, physicochemical properties (size, charge, lamellarity, drug encapsulation, drug loading, and in vitro-drug release) were measured for further experiments.

Physiochemical Characterization of the LPs-OSI/AS

Particle Size, Morphology and Stability of Liposome

The particle size, polydispersity (PDI) and zeta potential of the liposomes, LPs-OSI/AS were measured by dynamic laser scattering through Malvern Zetasizer Nano ZS. In order to obtain the appropriate liposome concentration, each sample was diluted 10 times in distilled water before analysis. Samples of mono-loaded and co-loaded liposomes was lyophilized using freeze dryer. Powder sample of liposomes, AS-IV and OSI was subjected to differential scanning calorimeter (DSC; Germany) in the range of 0°C to 300°C at temperature rising of 10 °C per min to investigate thermal property. The morphology of LPs-OSI/AS was observed using transmission electron microscopy (TEM). After the liposome was diluted to an appropriate multiple, the solution was stained with 2% uranyl acetate and dropped onto a carbon-coated copper grid. Prepare three batches of LPs-OSI/AS, three samples of each batch were added in centrifugal tube and then centrifuged at 4000 rpm for 30 min, no delamination. At the same time, liposomes were stored at 4 °C, and evaluated particle size and PDI at days 0 and 30. To test the stability of liposomes, the samples centrifuged at 4000 rpm for 30 min, no delamination. This phenomenon shows that liposomes have good stability over 30 days. The encapsulation efficiency (EE%) of two drugs were determined using HPLC. Under optimized conditions, we synthesized LPs-OSI/AS. Finally, the EE of OSI and AS measured under this process were 90% and 80%, respectively. The dialysis bag is placed in the bottle with continuous stirring at 37°C, and determine the release of OSI and AS-IV from the liposome. The content of the two drugs released over a period of 72 h were measured by HPLC. Compared with two free liquid medicines, preparations of liposome show a certain degree of sustained release.

Encapsulation Efficiency

The encapsulation efficiency (EE, %) of AS-IV and OSI in the liposomes were evaluated using high-performance liquid chromatography (HPLC). The EE of AS-IV and OSI was calculated according to formula (1):

$$EE (\%) = (C1/C2) \times 100$$

Where C1 is the concentration of OSI or AS-IV loaded into liposomes, C2 is the concentration of OSI or AS-IV added during liposome preparation. Determined by the encapsulation efficiency of vesicles loaded with OSI via ammonium

sulfate gradient, liposomes were diluted ten times in 80% methanol (0.1% 1 M HCL) and then through 0.22 μm microporous membrane filter. Chromatographic separation was performed on a C18 column (250 mm $\times$ 4.6 mm, 5 μm) with the column temperature set at 30°C. The mobile phase was composed of 0.1% phosphoric acid water and acetonitrile (0–25 min, 10–95% acetonitrile). The flow velocity was 1.0 mL/min. The wavelength detection was set at 368 nm and the injection volume was 10 μL . Determining the encapsulation efficiency of vesicles loaded with AS-IV via ethanol injection method, 0.1 mL of the liposomes was first added in 0.9 mL methanol and then through 0.22 μm microporous membrane filter. Chromatographic column is same as that used to determine OSI. The mobile phase was composed of 65% pure water and 35% acetonitrile. The flow velocity was 1.0 mL/min. The wavelength detection was set at 205 nm and the injection volume was 20 μL .

Releasing Rate of AS-IV and OSI from Liposomes

The drug release profiles of LPs-OSI/AS was characterized using the dialysis method. Briefly, 5 mL of LPs-OSI/AS, LPs-OSI, LPs-AS, free AS-IV and free OSI was placed in the dialysis bag (MWCO 3.5 KDa) and then immersed into 30 mL of 10 mM PBS solution containing 0.5% Tween 80. All bottles containing dialysis bags were shaken at 100 rpm and 37 °C using a stable temperature horizontal shaker. At the predetermined time, 1 mL of samples were withdrawn and replaced with same volume fresh buffer solution. The collected samples were centrifuged at 12000 rpm for 15 min and then analyzed by HPLC. The amounts of AS-IV and OSI released from LPs-OSI/AS were determined using the above HPLC methods. Each experiment was repeated three times.

Biological Evaluation

MTT Assay

The cytotoxicity of LPs-OSI/AS for NCI-H1975 and NCI-H1975/OSIR were evaluated by MTT (Dimethyl thiazolyl-2,5-diphenyltetrazolium bromide) assay. NCI-H1975 and NCI-H1975/OSIR cells were seeded in 96-well plates at a density of 104 cells per well and culture until cells adhere. Next, the culture medium was replaced by fresh medium containing 10% FBS and a series of concentrations (the concentration of OSI were 1.25, 2.5, 5, 10, 20 μM) of LPs-OSI/AS, OSI+AS, OSI, or AS. After 48 hours, 20 μL of MTT (5 mg/mL) were added to each well and incubated for 4 hours. Finally, 100 μL of dimethyl sulfoxide (DMSO) was added to each well, and the plates were measured using a microplate reader (Guangzhou Darui Biotechnology Co., Ltd.) at 576 nm.

Clonogenic Assay

Clonogenic assay is a cell survival assay, which is based on a single cell's capability to grow into a colony. NCI-H1975 and NCI-H1975/AR cells in the logarithmic growth phase were seeded into 6-well plates at a density of 2500 cells per well. After incubation in an incubator for 24 hours, the drug was diluted in medium containing 0.5% FBS. The cells were divided into 6 groups in total, including the Control group, AS-IV group, OSI group, OSI+AS group, and LPs-OSI/AS group. With OSI as the reference, the drug was diluted to an OSI concentration of 5 μM , and each group had 3 replicate wells. The plates were then placed in a cell incubator for cultivation. After 24 hours of cultivation, the medium was replaced with complete medium; subsequent cultivation was carried out with the medium changed every 2 days. After 2 weeks of cultivation, the medium was discarded, and the wells were washed three times with PBS. Then, 400 μL of 4% paraformaldehyde was added to each well to fix the cells, followed by staining the fixed cells with crystal violet for 15 minutes. Finally, the staining solution was discarded, and residual staining solution in the wells was washed away with PBS. After air-drying at room temperature, cell clones were photographed to observe the clone formation. Generally, under a microscope, a colony containing more than 10 cells was defined as one clone.

Cellular Uptake Assay

To investigate the cellular uptake of LPs-OSI/AS, fluorescence microscopy was used for qualitative analysis of the cellular uptake of LPs-OSI/AS. NCI-H1975 and NCI-H1975/AR cells in the logarithmic growth phase were seeded into 6-well plates at a density of 10^6 cells per well. After 24 hours, LPs-OSI/AS containing Ce-6 was diluted in medium with 0.5% FBS (the concentration was 2.5 μM based on OSI, containing 100 ng/mL Ce-6). Then, 2 mL of the diluted solution was added to each well for a 4-hour incubation. Meanwhile, a control group with 100 ng/mL Ce-6 was set up. After

Table 1 The Primer of the Gene

Gene	Primer	Sequence
Vimentin	Forward	5'-TGACCTCTCTGAGGCTGCCAACCC-3'
Vimentin	Reverse	5'-TTCCATTTCACGCATCTGGCGTTC-3'
TGFBR1	Forward	5'-TGCTCGACGATGTTCCATTG-3'
TGFBR1	Reverse	5'-TACTCTCAAGGCTTCACAGCTC-3'
TGFβ2	Forward	5'-CCCCACATCTCCTGCTAATGT-3'
TGFβ2	Reverse	5'-AGGCAGCAATTATCCTGCAC-3'
GAPDH	Forward	5'-GGAGCGAGATCCCTCCAAAAT-3'
GAPDH	Reverse	5'-GGCTGTTGTCATACTTCTCATGG-3'

incubation, the medium was discarded, and the cells were washed 3 times with PBS. Subsequently, the cells were stained with 0.5 µg/mL DAPI for 20 minutes, followed by another 3 washes with PBS. The cellular uptake of LPs-OSI/AS was observed under a fluorescence microscope. In addition, flow cytometry was employed to quantitatively examine the uptake of LPs-OSI/AS by NCI-H1975 and NCI-H1975/AR cells at different incubation time points. After seeding and incubating the cells as described above for 24 hours, 100 ng/mL Ce-6 solution and Ce-6-containing LPs-OSI/AS solution were added at 1 hour, 2 hours, and 4 hours, respectively. At each corresponding time point, the cells were digested, and the obtained cells were fixed with 4% paraformaldehyde before being detected by flow cytometry.

Gene and Protein Analysis

The mRNA levels of TGF-β1, TGF-β2, Vimentin, E-Cadherin, ZEB1 and ZEB2 in the treated cells were quantitatively measured by RT-qPCR analysis. NCI-H1975 and NCI-H1975/OSIR cells were seeded into six-well plates at a density of 2×10^5 . After 24 hours, we treated NCI-H1975 and NCI-H1975/OSIR cells with blank medium, blank LPs-OSI/AS (equivalent to 10 nM OSI), OSI+AS (Contains the same concentration of OSI and AS as LPs-OSI/AS), OSI (10 nM), AS (Contains the same concentration of AS as LPs-OSI/AS) for 48 h and then harvested. Reagent of trizol was used to extract total RNA, 1 µL of RNAs were reverse-transcribed to cDNA using Revertaid first strand cDNA synthesis kit (Thermo scientific, USA). Gene expression analysis was performed using real-time PCR and measured by using the $2^{-\Delta\Delta C_t}$ method. GAPDH gene was used as an endogenous housekeeping gene. The sequences of each primer were as Table 1.

To further measure E-cadherin, P-smad2 and P-smad3 expression levels using Western blot. We lysed the harvested NCI-H1975 and NCI-H1975/OSI with RIPA buffer. The protein concentration was determined by BCA assay kit and loaded on SDS-PAGE electrophoresis. Then the gel was transferred to a PVDF membrane and incubated with rabbit polyclonal anti-E-cadherin antibody, rabbit polyclonal anti-P-smad2 antibody, rabbit polyclonal anti-P-smad3 antibody and mouse monoclonal anti-GAPDH antibody overnight at 4°C. The protein bands were washed and incubated with HRP-conjugated secondary antibody. Finally, signals were visualized by using ECL.

Experimental Methods for in vivo Antitumor Studies

Male BALB/c-nu mice (weight 18 ± 2 g) were provided by SPF (Beijing) BIOTECHNOLOGY Co., Ltd. The tumor xenograft model was built by subcutaneous injection of 107 NCI-H1975 cells in the right armpit area. About two weeks, when the tumors reached enough volume, the BALB/c-nu mice were randomly assigned to five groups (n=6) and administered control (normal saline), AS (40 mg/kg), OSI (20 mg/kg), AS+OSI (40 mg/kg+20 mg/kg), LPs-OSI/AS (40 mg/kg+20 mg/kg) via gavage every day. The tumor volumes and body weights were measured on alternate days. Calculate the tumor volume according to the following formula: $V = (L \times W^2) / 2$, where W and L represented the smallest and largest diameter of the tumor, respectively. At three weeks after the first injection, the mice were anesthetized with isoflurane (Rivox, Shenzhen, China) and euthanized by decapitation, and tumors and organs such as heart, liver, spleen, lung, and kidney were dissected, weighed, and photographed. The tumors and organs were collected, weighed and photographed.

Paraffin-embedded tissue sections (4–5 μm) were deparaffinized and rehydrated through a graded ethanol series. Hematoxylin was applied for nuclear staining, followed by eosin for cytoplasmic counterstaining, in accordance with standard histological protocols. The sections were then dehydrated through increasing concentrations of ethanol, cleared in xylene, and mounted under coverslips for microscopic examination using a light microscope to enable histological assessment.

Statistical Analysis

All cellular assays were conducted in a minimum of three independent replicates. Results are presented as the mean $\pm$ standard error of the mean (S.E.M). Data analysis was performed using GraphPad Prism 8.0, with statistical significance assessed by One-way ANOVA and Student's *t*-test. A *p*-value of less than 0.05 was considered statistically significant.

Results

Physiochemical Characterization of the LPs-OSI/AS

LPs-OSI/AS was prepared and the effects of OSI incubation time on the size, PDI and encapsulation rate of LPs-OSI/AS were investigated. The results are shown in Table 2. When incubated for 40 minutes by OSI, the particle size was moderate and the PDI was consistent. We designed LPs-OSI/AS to release two-drug combinations in response to multidrug resistance in NSCLC cells. The liposomes form a stable liposomal structure and deliver two drugs into the tumor area.

The preparation of the LPs-OSI/AS sample is illustrated in Figure 1A. The resulting solution exhibits a pale blue, opalescent appearance. TEM of LPS reveals the appearance of liposomes, which are spherical (Figure 1B). The particle size of LPs-OSI/AS was determined by dynamic light scattering (DLS), with an average diameter as shown in Figure 1C. The zeta potential of LPs-OSI/AS was measured in millivolts (mV). For the free drugs, complete release was achieved within 10 hours. In contrast, approximately 80% of AS-IV and 60% of OSI were released from the liposomes after 72 hours. The *in vitro* drug release profile of LPs-OSI/AS was evaluated in phosphate-buffered saline (PBS) containing 0.5% Tween, and the results are presented in Figure 1D. The results demonstrated that the OSI solution and AS solution released rapidly, and the cumulative release rate reached more than 90% after 5 h, while the release of liposomes was significantly lagging behind the two free drug solutions, indicating that liposomes had sustained-release effect. Notably, the differential release rates (80% for AS-IV vs. 60% for OSI at 72 h) may be attributed to their distinct physicochemical properties and localization within the liposomal carrier. Specifically, AS-IV, being amphiphilic, likely partitions preferentially into the lipid bilayer, whereas OSI, which is more hydrophilic, resides predominantly in the aqueous core. Upon liposome destabilization, bilayer-associated AS-IV may be released more readily than core-loaded OSI, which requires more extensive lipid disruption or diffusion.

To further investigate the physical state of the encapsulated drugs, DSC analysis was performed on AS and OSI raw materials, blank liposomes, LPs-AS, LPs-OSI, and LPs-OSI/AS freeze-dried powder, with the resulting thermograms presented in Figure 1E. The phase transition temperature of free AS raw material ranged from 70°C to 90°C, while that of free OSI was observed between 50°C and 100°C. Notably, the phase transformation curves of blank liposomes, LPs-AS, LPs-OSI, and LPs-OSI/AS freeze-dried powder were nearly identical, suggesting successful encapsulation without significant drug leakage or crystallization. These DSC findings further support that both drugs are stably encapsulated in

Table 2 Effect of OSI Incubation Time on LPs-OSI/AS (n=3)

Time	Particle Size (nm)	PDI	Encapsulation Percentage (%)	
			AS (%)	OSI (%)
30 min	64.88 $\pm$ 13.85	0.30 $\pm$ 0.07	76.28 $\pm$ 5.79	70.56 $\pm$ 5.07
40 min	82.94 $\pm$ 8.46	0.26 $\pm$ 0.03	88.26 $\pm$ 8.40	88.08 $\pm$ 3.67
50 min	66.08 $\pm$ 4.07	0.33 $\pm$ 0.10	65.75 $\pm$ 12.33	71.46 $\pm$ 7.98

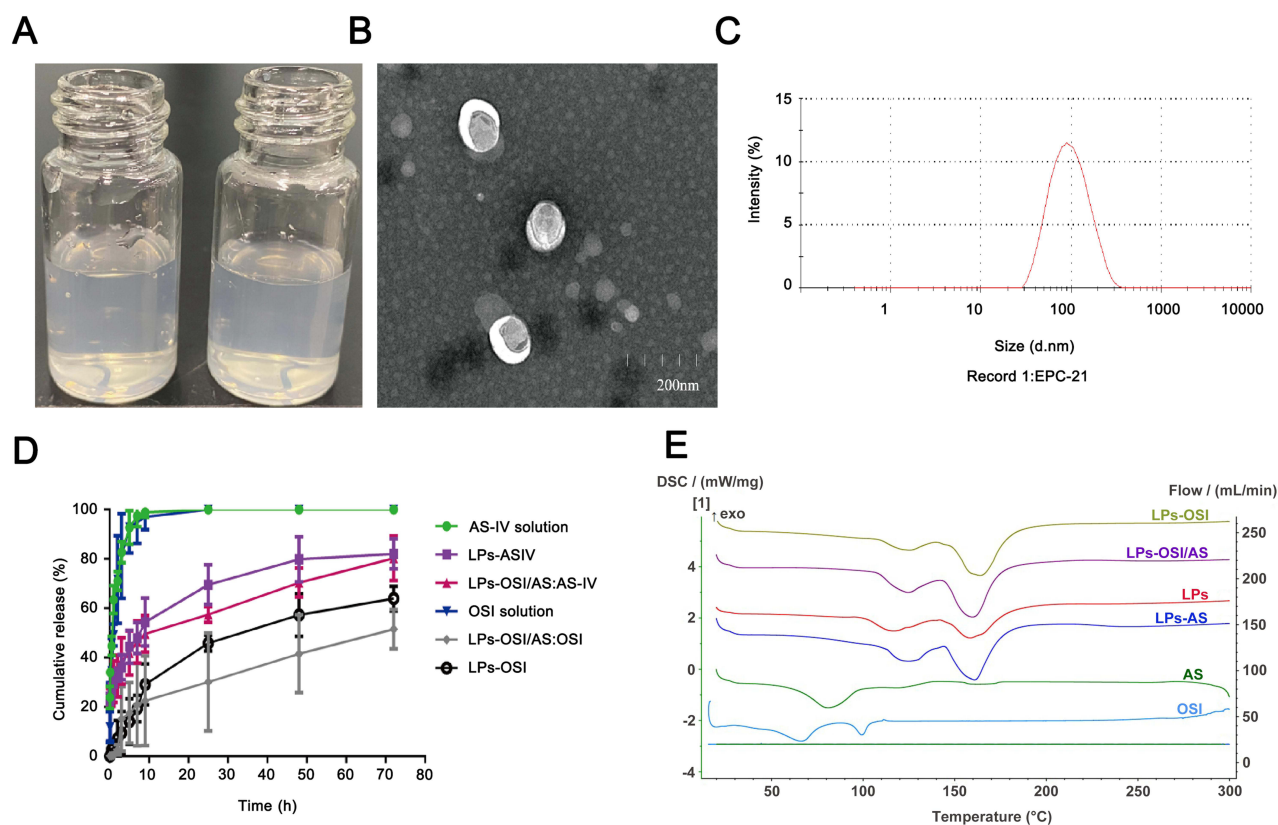


Figure 1 Physicochemical characterization of the LPs-OSI/AS. **(A)** LPs-OSI/AS sample diagram. **(B)** The morphology of liposomes was characterized by transmission electron microscopy (TEM). LPs-OSI/AS have a spherical structure and a smooth surface. Scale bar = 200 μ m. **(C)** The particle size of LPs-OSI/AS was determined using a Malvern ZS 90 laser particle size analyzer, and the particle size distribution of liposomes was investigated by dynamic light scattering (DLS). The particle size of LPs-OSI/AS is 96.92 ± 12.04 nm. **(D)** The release of AS-IV and OSI from LPs-OSI/AS was studied using the dialysis method. Samples were taken out for determination at time intervals of 5 min, 10 min, 20 min, 30 min, 45 min, 1 h, 1.5 h, 2 h, 3 h, 5 h, 7 h, 9 h, 24 h, 48 h, and 72 h, respectively. **(E)** The prepared blank liposomes (LPs), LPs-OSI, LPs-ASIV, and LPs-OSI/AS were lyophilized to prepare lyophilized powders. Subsequently, the free drugs of AS-IV and OSI, together with the prepared lyophilized powders, were subjected to DSC thermodynamic scanning.

an amorphous or molecularly dispersed state, and that the distinct-release behaviors likely reflect differences in intra-liposomal localization rather than physical instability.

In vitro Cellular Uptake of LPs-OSI/AS in NCI-H1975 and NCI-H1975/OSIR

To illustrate the intracellular uptake of LPs-OSI/AS in NCI-H1975 and NCI-H1975/OSIR cells (Figure 2), fluorescent images were acquired after incubation with coumarin-loaded LPs-OSI/AS (Figure 2A). Blue fluorescence represents DAPI-stained nuclei; Green represents the Ce-6 label, and the merged image displays the overlay of DAPI and Ce-6 channels within the same cellular distribution. In NCI-H1975 and NCI-H1975/OSIR cells, the green fluorescence of LPs-OSI/AS was significantly stronger than that of free Ce-6, indicating that the encapsulation of liposomes increased the uptake of LPs-OSI/AS. Fluorescent images demonstrate that coumarin-LPs provided a significantly higher uptake of coumarin in the NCI-H1975 and NCI-H1975/OSIR tumor cells, as compared to both Coumarin-LPS and plain coumarin. In addition, the effect of LPs-OSI/AS on drug uptake by cells was detected by flow cytometry. As shown in Figure 2C and D, both cell lines exhibited stronger uptake of Ce-6 when delivered via LPs-OSI/AS than in the form of free Ce-6. These findings suggest that liposomal formulation effectively enhances cellular internalization of the therapeutic agent.

Biological Evaluation

Cytotoxicity Study

The effects of different LPs-OSI/AS preparations on NCI-H1975 and NCI-H1975/OSIR tumor cells were detected by MTT and clonogenic assay (Figure 3). As shown in the MTT assay results (Figure 3A and B) that LPs-OSI/AS exhibited

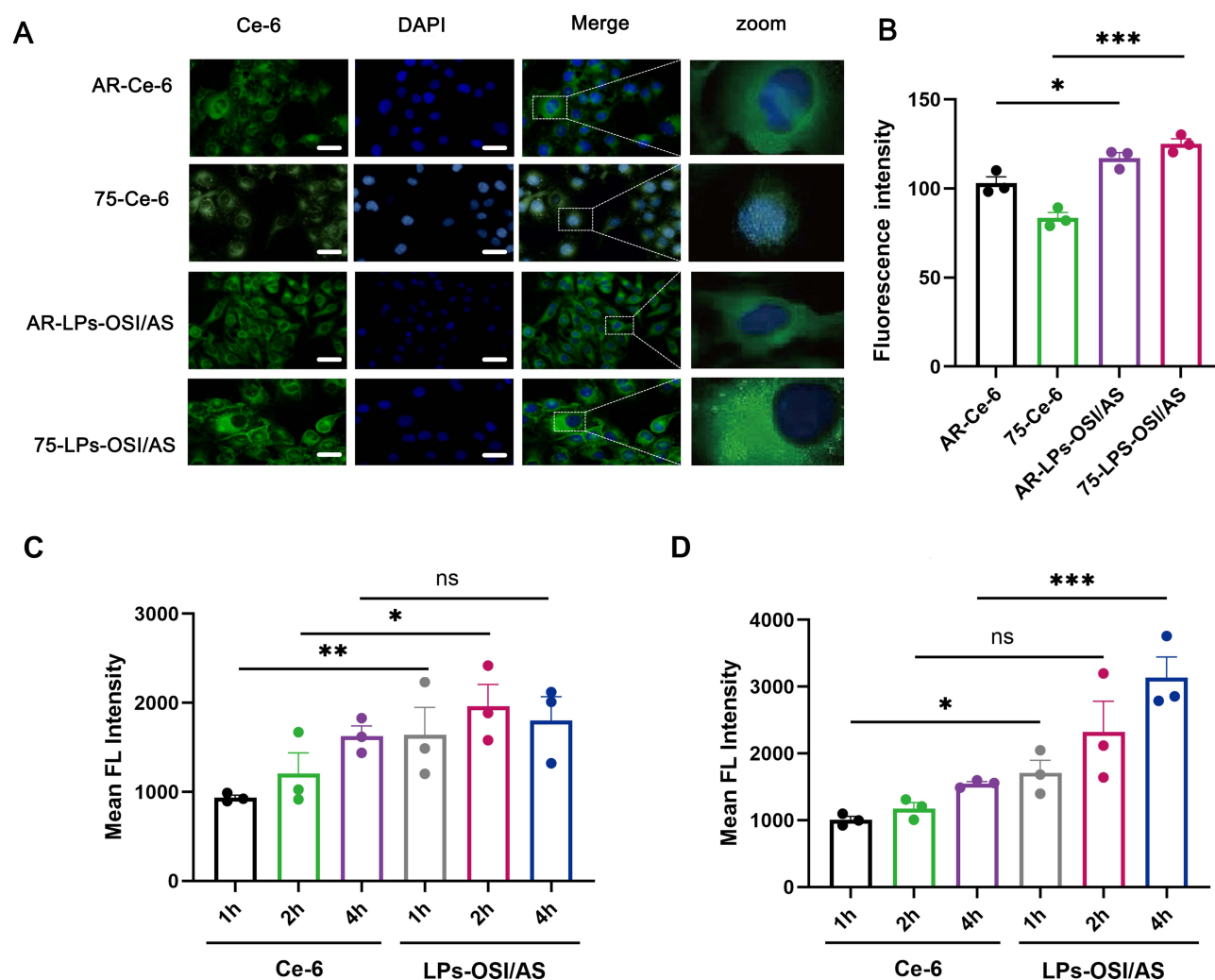


Figure 2 Ce-6-loaded LPs-OSI/AS was compared with free Ce-6 to investigate the cellular uptake of liposomes. (A) The uptake of LPs-OSI/AS by NCI-H1975 cells and NCI-H1975/OSIR cells was observed by fluorescence. (B) Image J software was used to analyze the uptake of liposomes by the two types of cells. (C and D) Cell uptake of LPs-OSI/AS by NCI-H1975 cells and NCI-H1975/OSIR cells was observed by flow cytometry. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$; ns, no significance. Scale bar = 25 μm .

a dose-dependent cytotoxicity in two cell lines. The combination of OSI and AS effectively enhanced the inhibitory effect on NCI-H1975 and NCI-H1975/OSIR cells compared to monotherapy, demonstrating superior antitumor activity. At a concentration of 20 μM , the cell viability of NCI-H1975 dropped to approximately 30%, while that of the drug-resistant NCI-H1975/OSIR cells remained below 50%, indicating that LPs-OSI/AS significantly enhances the therapeutic efficacy of the drugs. Co-encapsulation of OSI and AS into liposomes further increased cytotoxicity and achieved the strongest antitumor effect among all tested treatments. Although OSI alone and the physical mixture of OSI + AS exhibited notable antiproliferative effects, they failed to completely suppress cell proliferation, particularly in the resistant NCI-H1975/OSIR line. In contrast, LPs-OSI/AS markedly reduced cell proliferation. Notably, LPs-OSI/AS demonstrated a significant antiproliferative effect on NCI-H1975/OSIR cells, suggesting a potential mechanism involving the suppression of multidrug resistance. Furthermore, clonogenic assay results confirm that LPs-OSI/AS effectively inhibits cellular proliferation (Figure 3C). Compared with control groups, OSI alone, OSI + AS, and LPs-OSI/AS all suppressed colony formation in NCI-H1975 cells; however, only LPs-OSI/AS showed pronounced inhibition in NCI-H1975/OSIR cells. The colony formation rate was quantified and analyzed using Image J software, and the results are presented in Figure 3D and E). The addition of OSI alone led to a significant reduction in colony formation in NCI-H1975 cells, and treatment with LPs-OSI/AS resulted in significantly greater inhibition than the simple combination of

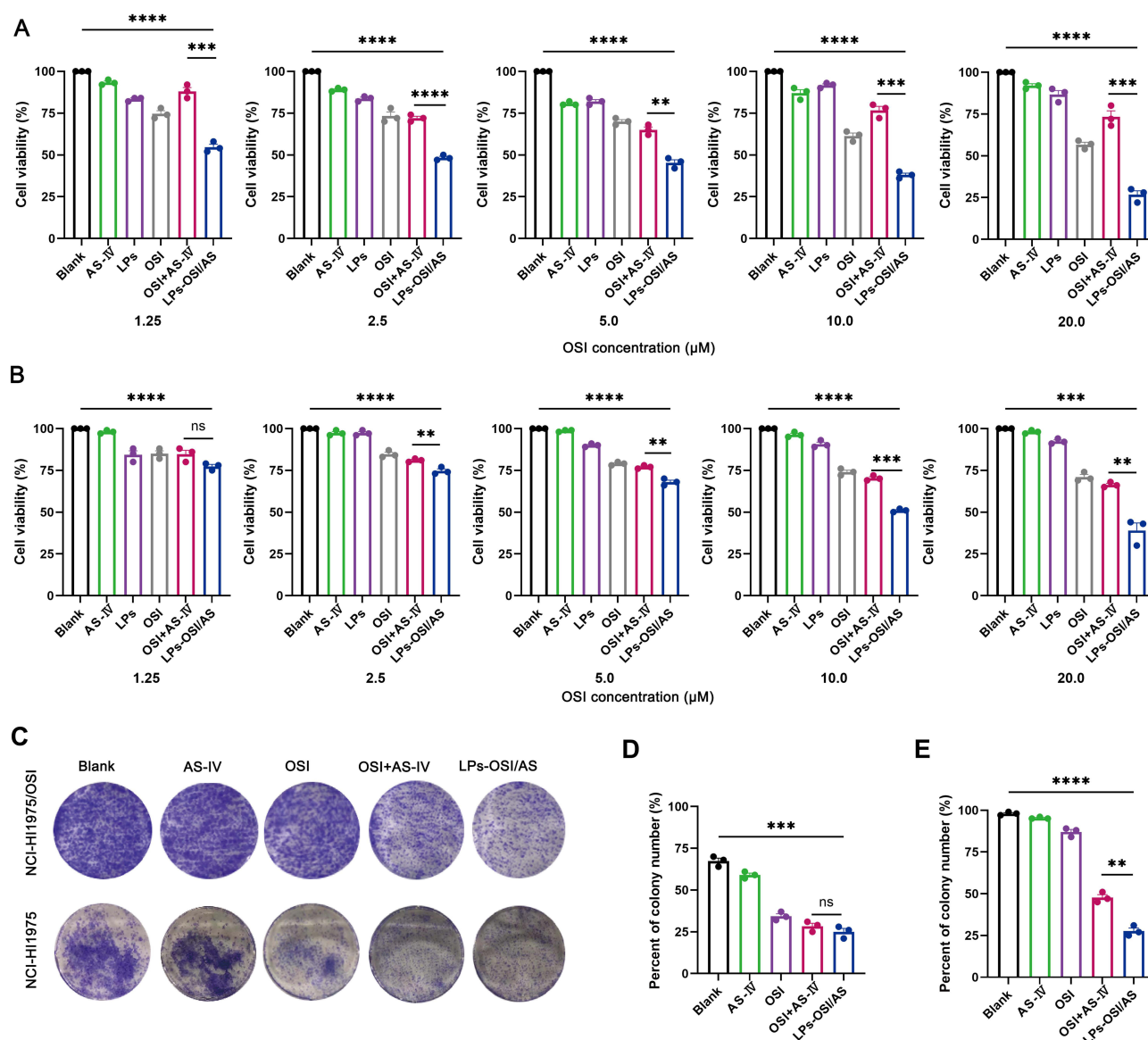


Figure 3 The effects of different LPS-OSI/AS preparations on NCI-H1975 and NCI-H1975/OSIR tumor cells. **(A and B)** The survival rate of NCI-H1975 cells and NCI-H1975/OSIR cells was determined by MTT method, five concentrations were set for each group, which were 1.25, 2.5, 5, 10, and 20 μM respectively, calculated as OSI. **(C)** The effect of drugs on the proliferation of NCI-H1975 cells and NCI-H1975/OSIR cells in each group was determined by colony formation assay. **(D and E)** Count the number of clones as mentioned above under a microscope. The clone formation rate (%) is calculated by the formula: Clone Formation Rate (%) = (Average Amount of Clones / Number of Inoculated Single Cells) × 100%. ***p* < 0.01, ****p* < 0.001, *****p* < 0.0001; ns, no significance.

OSI and AS. In drug-resistant cells, OSI monotherapy had minimal impact on clonogenic potential, whereas the co-delivery of OSI and AS—particularly via liposomal co-encapsulation.

Gene and Protein Analysis

The mRNA expression of TGF-β1, TGF-β2, Vimentin were analyzed in the NCI-H1975 and NCI-H1975/OSIR tumor cells using real-time PCR. As shown in Figure 4, OSI group exhibited the higher mRNA expression of TGFβ1, TGFβ2 and Vimentin, indicating that OSI promotes the induction of EMT in NCI-H1975 cells. LPs-OSI/AS exhibited the lowest expression levels of these markers. LPs-OSI/AS treatment had a greater suppressive effect on TGFβ1, TGFβ2, Vimentin mRNA expression compared to OSI+AS group. Results indicated that LPs-OSI/AS showed significant suppression of the EMT in NCI-H1975 cells, and reduced the EMT phenomenon observed in NCI-H1975/OSIR. Although OSI monotherapy inhibited tumor cell viability in NCI-H1975 cells, it concurrently upregulated EMT-associated genes, including

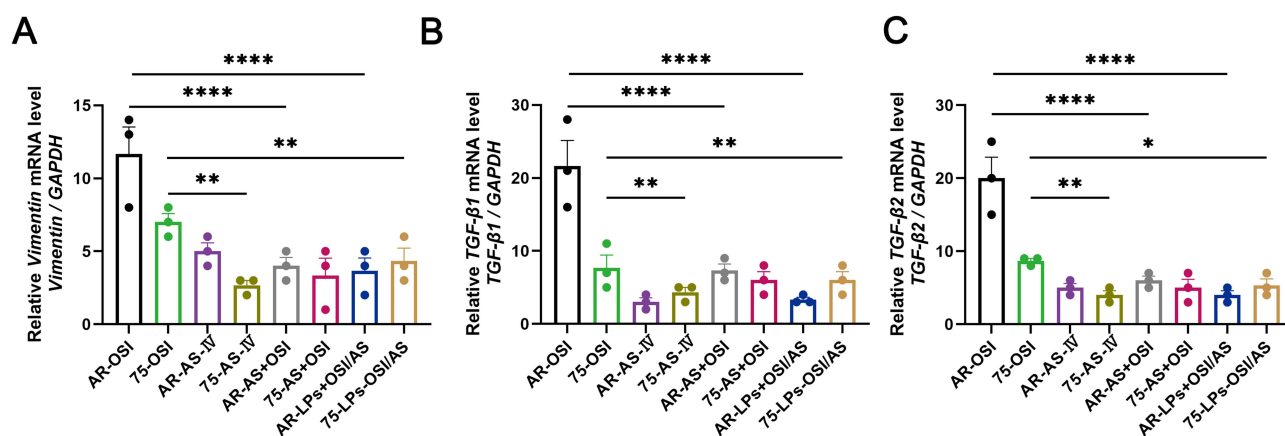


Figure 4 The expression of EMT-related genes in NCI-H1975 and NCI-H1975/OSIR cells was detected by RT-qPCR. ((A): Vimentin; (B): TGFβ1; (C): TGFβ2). * $p < 0.05$, ** $p < 0.01$, and **** $p < 0.0001$.

TGF-β1, TGF-β2, and Vimentin. By contrast, the co-loaded liposomal delivery of AS with OSI effectively inhibited the expression of these EMT markers, with the liposomal formulation exhibiting stronger inhibitory effects than the free drug combination. In resistant NCI-H1975/OSIR cells, OSI alone significantly increased the expression of EMT-related genes, whereas both the combination therapy and the liposome group showed reduced expression of these genes. This suggests that the liposomal formulation may modulate or partially influence the regulation of EMT.

In vivo Antitumor Efficacy

Effect on Tumor Growth

The BALB/c-nu mice subcutaneous transplanted tumor model was established using human NCI-H1975 cells and treated with AS, OSI, OSI+AS, and LPs-OSI/AS respectively. To ensure experimental parallelism, all groups were given a dosage of 20 mg/kg OSI and 40 mg/kg AS. The drugs were administered daily for 21 days. Body weight and tumor volume were assessed one day after the initiation of treatment. As shown in Figure 5A, LPs-OSI/AS inhibited growth of the NCI-H1975 cells and reduced the volume of the tumors compared with control group. Tumor volume measurements are presented in Figure 5B. In the control group, tumors exhibited rapid growth, with volumes reaching 510 mm³ by day 15. AS monotherapy exhibited limited efficacy in inhibiting tumor growth. Both the OSI group and the OSI+AS group showed certain inhibitory effects on tumor growth; however, the combination therapy (OSI+AS) demonstrated stronger inhibition compared to the single drug treatment (OSI alone), indicating enhanced anti-tumor effects through drug combination therapy (Figure 5B). Notably, LPs-OSI/AS exhibited the most pronounced antitumor effects among all treatment groups, suggesting that co-loaded liposomes significantly enhance the therapeutic efficacy of these drugs compared to their combined administration. Prior to and following administration, the weight trajectory of the BALB/c-nu mice was depicted (Figure 5C), revealing no significant decrease in their body weight. Following a 21-day experimental period, all the BALB/c-nu mice exhibited a survival rate of 100%. There was a significant decrease in tumor volume in the treatment group with OSI, OSI+AS and LPs-OSI/AS group, when compared with the control and AS group. The average weight and volume of the tumors are shown in Figure 5D. Compared with the control and AS groups, all treatment groups exhibited statistically significant reductions ($P < 0.05$), with the most pronounced effect observed in the LPs-OSI/AS group (Figure 5D). Overall, these results demonstrate that LPs-OSI/AS exerts a stronger antitumor effect in vivo than OSI+AS.

Following euthanasia of the BALB/c-nu mice, the expression of Ki-67 protein in tumor tissues was assessed using immunohistochemical staining with a Ki-67-specific antibody, and representative results are presented in Figure 5E. Positive cells in the immunohistochemical sections were quantified using Image-Pro Plus 6.0 software. Furthermore, Graphpad Prism data analysis software was used to perform statistical analysis on the positive rate of KI67-positive cells in each group, and the results are presented in Figure 5F. The percentage of positively stained area was determined as 11.32% in the Control group, 7.10% in the AS group, 5.98% in the OSI group, and 3.85% in the OSI+AS group, while the LPs-OSI/AS group

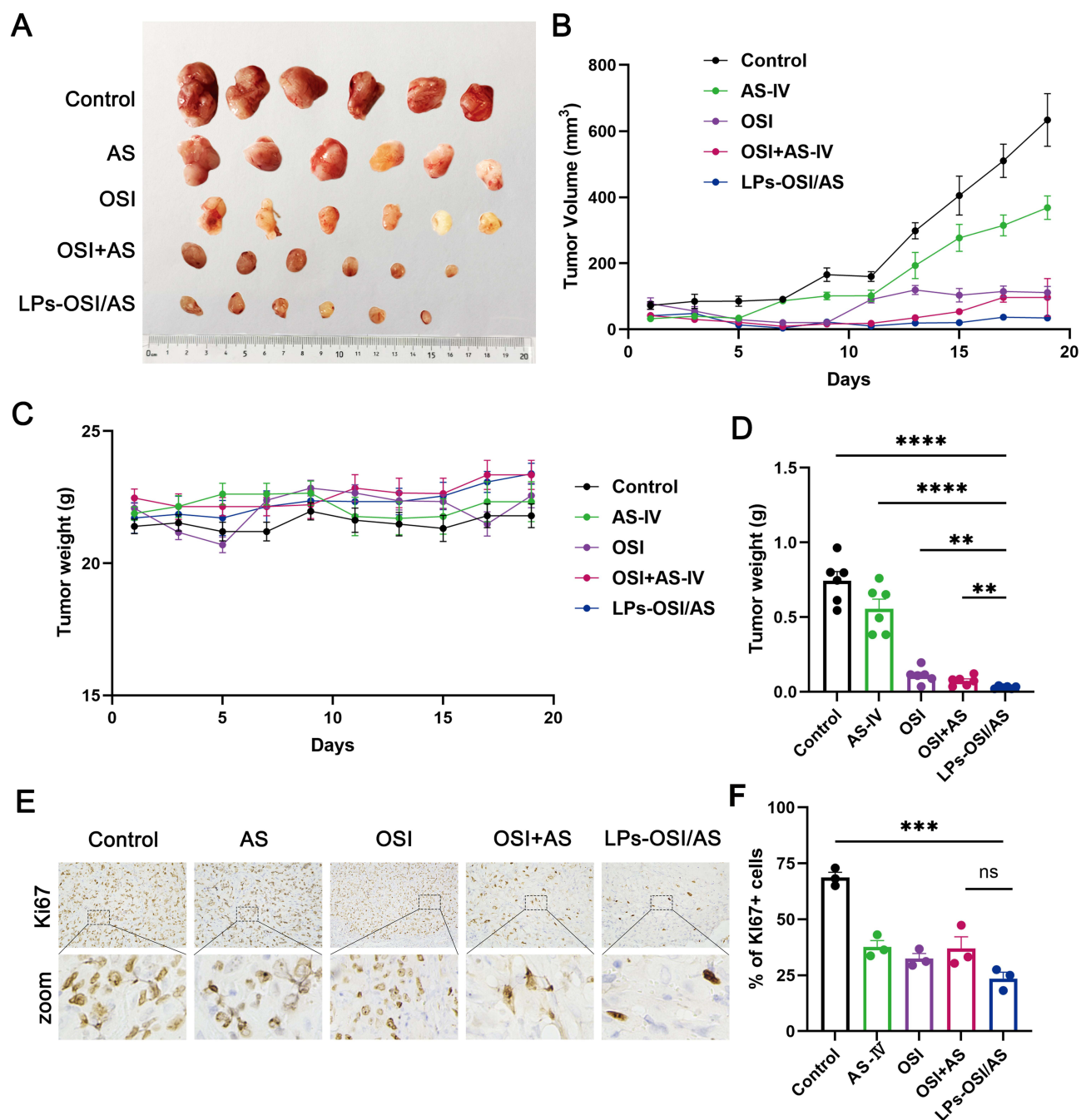


Figure 5 Evaluate the anti-tumor effect of co-loaded liposomes. **(A)** The results showed that LPs-OSI/AS had better anti-NCI-H1975 tumor activity compared with OSI+AS alone and combined. **(B)** Tumor growth curve. **(C)** Weight change curve of BALB/c-nu. **(D)** Tumor weight of different groups. **(E)** The expression of Ki-67 in tumor tissues of each group was detected by immunohistochemistry. **(F)** Image J software was used to analyze the percent of Ki67+ cells, The positive rate of Ki67 = (Number of positive cells / Total number of cells) × 100%. ***p* < 0.01, ****p* < 0.001, and *****p* < 0.0001; ns, no significance.

exhibited a markedly reduced positive area of approximately 1.06%. As Ki-67 is a well-established marker of cellular proliferation, a higher Ki-67 positivity rate correlates with increased tumor cell proliferation. The significantly lower Ki-67 expression in the LPs-OSI/AS group indicates that this treatment effectively suppresses tumor cell proliferation.

H&E Staining of Tumor Tissue and Immunohistochemistry

H&E staining was used to observe the pathological changes of tumor tissue in each group. The results of H&E staining were similar to those of BALB/c-nu mice, further supporting the superior efficacy of the LPs-OSI/AS group in

suppressing tumor growth. As shown in **Figure 6A**, tumor cells in the Control group exhibited normal morphology, with most cells appearing intact and densely packed, clearly visible nuclei, and only minimal evidence of cellular condensation. In the AS group, tumor cells remained largely confluent and tightly interconnected; however, a more pronounced reduction in cell diameter was observed compared to the Control group. In contrast, extensive cellular damage characterized by marked cell shrinkage and disrupted intercellular connections was evident in the OSI, OSI+AS, and LPs-OSI/AS groups. Notably, the LPs-OSI/AS group demonstrated the most dramatic effects: the majority of cells within the visual field appeared collapsed and clustered, with markedly diminished or absent nuclei. These findings collectively indicate that the LPs-OSI/AS formulation exerts the strongest inhibitory effect on tumor progression.

In addition, the heart, liver, spleen, lung, kidney and other tissues of nude mice were observed by H&E staining to observe the pathological changes, and the results were shown in **Figure 6B**. H&E staining of cardiac tissue showed that cardiomyocytes in each group had a normal morphology, with no obvious necrosis, fibrosis, or inflammatory cell infiltration. In lung tissues, the AS-IV group exhibited local thickening of the pulmonary interstitium with inflammatory cell infiltration, while the LPs-OSI/AS group showed intact alveolar structures and a normal interstitial cell density. Liver tissues were normal in all groups, with hepatocytes arranged regularly and no edema, steatosis, or necrosis. Regarding the spleen, the LPs-OSI/AS group was dominated by lymphocytes without definite inflammatory or neoplastic lesions; the OSI group showed clonal proliferation with replacement of the normal structure, and both the AS-IV group and the OSI +AS group exhibited destruction of the normal structure and invasive growth. In renal tissues, some areas of the renal tubules in the AS-IV group and the OSI group showed structural disorders, while the LPs-OSI/AS group had neatly

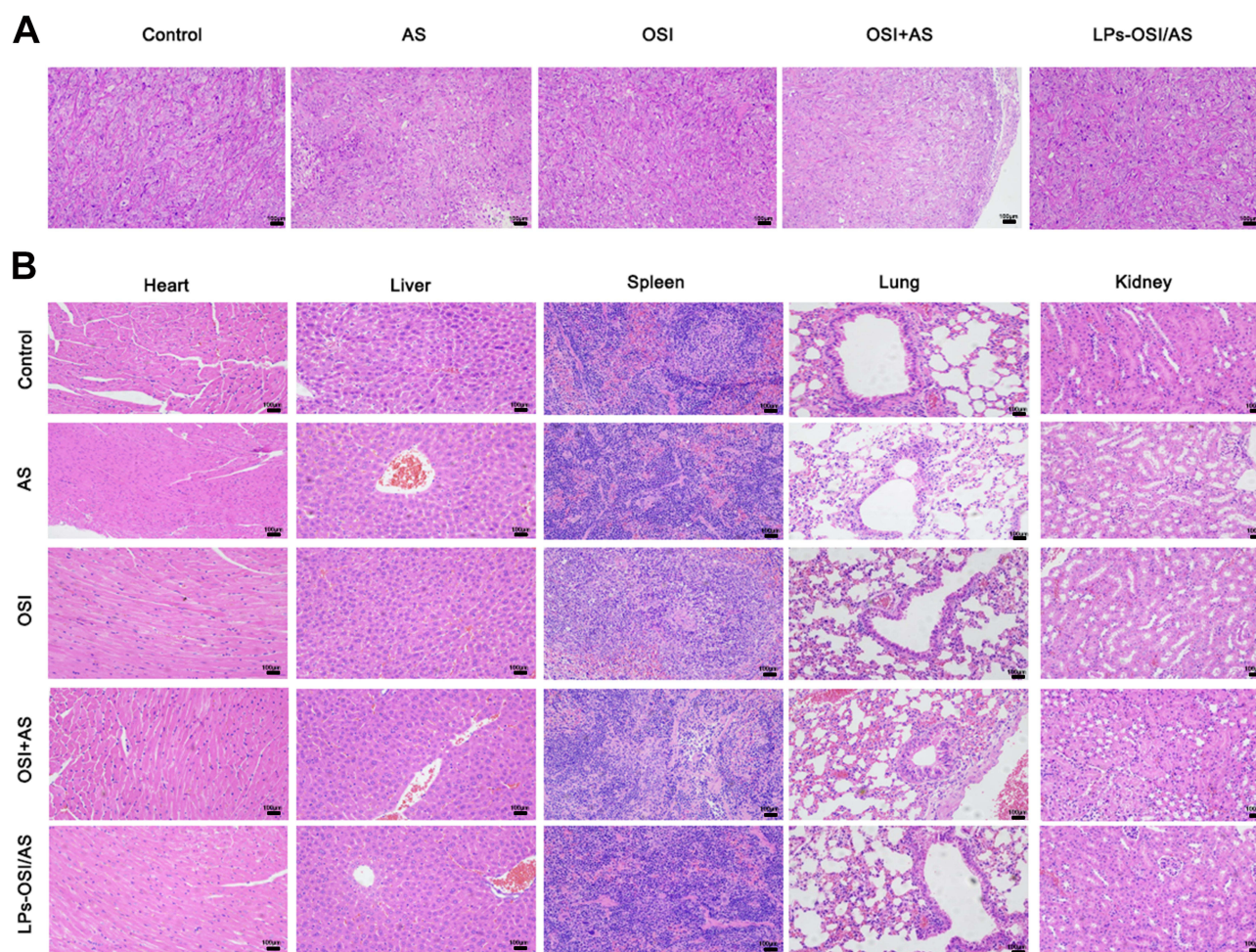


Figure 6 The staining status of the tissues. **(A)** H&E staining was used to observe the pathological changes of tumor tissue in each group. **(B)** Pathological changes of heart, liver, spleen, lung and kidney were detected by H&E staining. Scale bar = 100 μm.

arranged renal tubules with regular lumens and no obvious aggregation of inflammatory cells. In summary, LPS-OSI/AS exhibited good safety under the experimental conditions.

Discussion

NSCLC comprises adenocarcinoma, squamous cell carcinoma, and large cell carcinoma.³⁵ Although NSCLC typically progresses slowly with delayed metastasis, the majority of patients are diagnosed at advanced stages, precluding curative surgery. Current standard treatments include chemotherapy, targeted therapy, and immunotherapy, with precision therapies forming the cornerstone of management for advanced disease. EGFR, a tyrosine kinase receptor, normally regulates cellular growth and differentiation. In NSCLC, EGFR mutations lead to constitutive activation, driving uncontrolled tumor proliferation and functioning as an oncogenic driver.³⁶ EGFR-TKIs competitively bind the EGFR tyrosine kinase domain, blocking downstream signaling, suppressing tumor growth, and promoting apoptosis. With superior specificity and reduced toxicity compared to chemotherapy, EGFR-TKIs represent first-line treatment for EGFR-mutant NSCLC. Following iterative development through three generations, these agents have seen progressive improvements in selectivity, efficacy, and safety. OSI, a widely used third-generation EGFR-TKI, demonstrates enhanced clinical benefits over earlier generations. However, resistance typically emerges after 1–2 years of treatment, spurring investigation into combination therapies aimed at delaying resistance and improving outcomes, particularly in high-risk advanced NSCLC.

OSI resistance involves multiple mechanisms, broadly categorized as EGFR-dependent or EGFR-independent. TGF- β activates both Smad-dependent and non-Smad pathways (eg, PI3K/AKT, MAPK), which can sustain tumor proliferation even under EGFR inhibition.³⁷ By regulating transcription factors such as Snail³⁸ and Twist, it promotes the transformation of tumor cells from an epithelial phenotype to a mesenchymal phenotype.³⁹ TGF- β also recruits cancer-associated fibroblasts and immunosuppressive cells such as Tregs,⁴⁰ fostering a protective tumor microenvironment that attenuates OSI efficacy. EMT downregulates EGFR expression and reduces dependency on EGFR signaling, limiting OSI-mediated pathway inhibition.⁴¹ It also activates anti-apoptotic pathways (eg, Bcl-2 upregulation) and enhances migratory capacity, promoting metastasis and further contributing to drug resistance.⁴² The mechanisms by which OSI induces EMT primarily involve the activation of the TGF β 2/SMAD and NF- κ B signaling pathways,⁴³ accompanied by Golgi remodeling and alterations in secretory programs.⁴⁴ However, this effect exhibits significant cell-type dependence. In PC-9 (EGFR exon 19 deletion) and H1975 (L858R/T790M mutation) cells, OSI treatment stably induces the upregulation of mesenchymal markers and a classic EMT phenotype.⁴⁵ In contrast, in HCC827 cells, EMT manifestations are heterogeneous among different resistant sublines, with some sublines displaying spindle-shaped morphological changes while others do not show obvious alterations. HCC4006 cells also demonstrate dynamic plasticity of the EMT phenotype during the stepwise acquisition of resistance.⁴⁶ Even among different resistant clones with the same genetic background, the occurrence and extent of EMT vary. Furthermore, this effect is modulated by multiple factors, including the stage of drug exposure (upregulation of EMT-related genes occurs at the early tolerant stage, whereas a more stable phenotype is formed after long-term exposure),⁴⁷ the tumor microenvironment (resistant cells induce phenotypic changes in surrounding sensitive cells through paracrine TGF β 2 signaling),⁴⁴ and genetic background (the relative contribution of EMT as a resistance mechanism differs between T790M-positive and T790M-negative cells). Together, these observations fully reflect the high plasticity and context dependence of EMT regulation.⁴³ Thus, TGF- β and EMT represent critical biomarkers and therapeutic targets in overcoming OSI resistance.

Astragalus membranaceus, a traditional Chinese medicinal herb used to replenish qi, contains the active component AS-IV. Modern pharmacological studies reveal that AS-IV not only modulates immune function—enhancing T-cell and macrophage activity—but also exhibits antitumor, anti-inflammatory, and cardioprotective properties.⁴² These activities provide a rationale for integrating traditional Chinese medicine with Western oncology, particularly in mitigating treatment-related adverse effects and improving quality of life during adjuvant anticancer therapy. AS-IV exerts multimodal antitumor activity through pleiotropic mechanisms. It directly suppresses tumor cell proliferation and induces apoptosis,⁴⁸ while downregulating MMP-2 and MMP-9 expression and inhibiting EMT, thereby attenuating tumor cell migration and invasion.⁴⁹ AS-IV also modulates antitumor immunity by enhancing the activity of CD4⁺ T cells,⁵⁰ CD8⁺ T cells, and natural killer (NK) cells, while reducing the infiltration of regulatory T (Treg) cells and M2-type

macrophages, thereby alleviating the immunosuppressive microenvironment. Moreover, AS-IV can reverse drug resistance by downregulating drug-efflux proteins, thereby increasing intracellular concentrations of chemotherapeutic or targeted agents.⁵¹ Despite these promising properties, the clinical translation of AS-IV is hampered by its poor aqueous solubility, low lipophilicity, and extensive hepatic metabolism, leading to subtherapeutic plasma levels and limited efficacy as monotherapy. Consequently, AS-IV is primarily used as an adjuvant agent. Strategies to overcome these limitations include developing novel drug delivery systems to improve its bioavailability and combining AS-IV with targeted therapies such as OSI to enhance antitumor responses and reduce on-target toxicity.

Based on the established role of AS-IV in overcoming OSI resistance, its multimodal antitumor effects, and current pharmacological limitations, we established validated in vitro analytical methods for OSI and AS-IV, and developed LPs-OSI/AS using ethanol injection combined with an ammonium sulfate gradient approach. The resulting liposomes exhibited a uniform size distribution (96.92 ± 12.04 nm; PDI 0.28 ± 0.01), high drug encapsulation (AS: $89.74 \pm 6.53\%$; OSI: $84.35 \pm 8.82\%$), excellent stability, and sustained-release profiles. LPs-OSI/AS significantly suppressed proliferation in both NCI-H1975 and OSI-resistant NCI-H1975/OSIR cells in vitro and demonstrated potent antitumor efficacy in vivo. With combination therapies becoming increasingly central to oncology treatment, LPs-OSI/AS represent a promising strategy for NSCLC. Furthermore, a reliable HPLC method was established for the simultaneous quantification of OSI and AS, enabling precise characterization of the optimized liposomal formulation.

In vitro cytotoxicity assay demonstrated that LPs-OSI/AS significantly inhibited proliferation of both NCI-H1975 and OSI-resistant NCI-H1975/OSIR cells, with superior efficacy compared to single-agent or free drug combinations. No notable cytotoxicity was observed within the tested lipid concentration range. Furthermore, RT-qPCR analysis indicated that LPs-OSI/AS downregulated expression of EMT-related genes, including *Vimentin*, *TGF β 1*, and *TGF β 2*. A reduction in vimentin expression levels is often accompanied by an attenuated mesenchymal phenotype, and under such conditions, improved sensitivity to OSI can be observed. This suggests that downregulation of vimentin may partially contribute to alleviating the resistant state, although this process likely involves a more complex EMT-related regulatory network, indicating a role in reversing TGF- β -mediated EMT in resistant cells.

In vivo, a BALB/c nude mouse subcutaneous xenograft model was established using human NCI-H1975 cells. AS monotherapy showed limited antitumor efficacy, while the OSI and OSI+AS groups exhibited gradually enhanced inhibitory effects, with LPs-OSI/AS demonstrating the most significant tumor suppression. No obvious body weight loss was observed in any group, and the 21-day survival rate was 100%. Compared with the control and AS groups, tumor weight and volume were significantly reduced in all treatment groups ($P < 0.05$), especially in the LPs-OSI/AS group. Immunohistochemistry revealed Ki-67 positive rates of 11.32% in the control group, 7.10% in the AS group, 5.98% in the OSI group, 3.85% in the OSI+AS group, and only 1.06% in the LPs-OSI/AS group, indicating the strongest inhibition of tumor proliferation. H&E staining showed that cellular collapse and nuclear loss were most prominent in the LPs-OSI/AS group, with pathological changes superior to those in other groups. H&E staining of major organs demonstrated that the LPs-OSI/AS group maintained normal structure of the heart, liver, lung, and kidney without obvious inflammation, necrosis, or fibrosis. Slight pathological alterations were observed in the lung, spleen, and kidney in the AS, OSI, and OSI+AS groups. In conclusion, LPs-OSI/AS exhibits significantly stronger in vivo antitumor efficacy than the free drug combination, with favorable biosafety.

Together, we developed an OSI-AS co-loaded liposomal delivery system and systematically evaluated its pharmacodynamic profile through formulation screening, physicochemical characterization, in vitro release kinetics, cytotoxicity and uptake assays, and in vivo antitumor efficacy studies.

Conclusion

This study developed an innovative liposomal nanoplatform for the co-delivery of OSI and AS-IV. This optimized formulation, characterized by its spherical morphology, high stability, and excellent drug-loading capacity, provided sustained drug release. LPs-OSI/AS synergistically enhanced cellular uptake and potently suppressed the proliferation of both NCI-H1975 and OSI-resistant (NCI-H1975/AR) NSCLC cells, in part by modulating EMT pathways. Consequently, this combination strategy demonstrated marked antitumor efficacy in vivo with a favorable safety profile, providing a potentially valuable therapeutic strategy and direction for overcoming EGFR-TKI resistance. Notably, lecithin,

cholesterol, and OSI—the key components of this formulation—have already received FDA approval for clinical use. This pre-existing regulatory approval is expected to substantially lower the barriers associated with future human trials and formulation development, thereby facilitating the clinical translation of this liposomal platform.

Data Sharing Statement

The datasets used and/or analyzed during the current study are available from the corresponding author (Le-Le Zhang - zhanglele@cdu.edu.cn) on reasonable request.

Ethics Approval

All animal experiments were performed in compliance with the Guidelines for the Care and Use Ethics Committee of Chengdu University (Approval No. 2019-0306) and were conducted in accordance with the Laboratory Animal Environment and Facilities (GB 14925-2010).

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Disclosure

The authors report no conflicts of interest in this work.

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