

RESEARCH PAPER

Engineering Liposomal Nanocarriers for Precision Drug Delivery in Oral Cancer: Current Advances and Future Directions

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Abstract

Background: Oral squamous cell carcinoma (OSCC) remains a major therapeutic challenge because of tumor heterogeneity, limited tumor selectivity, and systemic toxicity associated with conventional chemotherapy. Targeted liposomal drug delivery may improve intracellular drug accumulation and therapeutic efficacy.

Objective: The present study aimed to develop and evaluate folic acid-functionalized doxorubicin-loaded liposomes for targeted drug delivery against OSCC.

Methods: Doxorubicin-loaded liposomes were prepared using the thin-film hydration method and surface-functionalized with folic acid. The formulations were characterized for particle size, polydispersity index (PDI), zeta potential, encapsulation efficiency, morphology, drug-release behavior, and stability. Their biological activity was evaluated in SCC-9 OSCC cells through cytotoxicity, cellular uptake, apoptosis, migration/invasion assays, followed by in-vivo assessment of tumor growth, histopathology, and Ki-67 and cleaved caspase-3 expression.

Results: The targeted liposomes exhibited a particle size of 181.7 ± 7.2 nm, PDI of 0.198 ± 0.016 , zeta potential of -24.3 ± 1.7 mV, and encapsulation efficiency of $89.4 \pm 2.1\%$. The targeted formulation demonstrated sustained drug release, with $88.6 \pm 2.4\%$ release at 48 h. Compared with free doxorubicin and non-targeted liposomes, targeted liposomes significantly enhanced cellular uptake (56.8 ± 4.2 MFI), reduced cell viability ($21.5 \pm 2.1\%$), and decreased the IC_{50} to 7.8 μ M. Targeted treatment increased total apoptosis to $55.3 \pm 3.1\%$ and inhibited OSCC cell migration/invasion by 68.4% . In vivo, targeted liposomes reduced final tumor volume to 186.7 ± 27.4 mm³, corresponding to **72.7% tumor-growth inhibition**, accompanied by reduced Ki-67 and increased cleaved caspase-3 expression.

Conclusion: Folic acid-targeted doxorubicin-loaded liposomes demonstrated favorable physicochemical properties, sustained drug release, enhanced cellular uptake, and improved antitumor activity compared with free and non-targeted doxorubicin. These findings support targeted liposomal delivery as a promising preclinical strategy for improving therapeutic precision in OSCC.

Keywords: Oral squamous cell carcinoma; Doxorubicin; Folic acid-targeted liposomes; Targeted drug delivery; Nanomedicine; Liposomal drug delivery; Active tumor targeting; Cellular uptake; Apoptosis; Antitumor activity.

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1. INTRODUCTION

Oral squamous cell carcinoma (OSCC) is the predominant histological subtype of oral cancer and represents a major global health challenge because of its substantial morbidity, mortality, functional impairment, and detrimental effects on quality of life. According to the most recent GLOBOCAN estimates, cancers of the lip and oral cavity accounted for approximately 390,000 new cases and 188,000 deaths worldwide in 2022, underscoring the persistent global burden of this malignancy¹. The disease is particularly prevalent in South and Southeast Asia, where exposure to tobacco, smokeless tobacco, areca nut, and alcohol contributes substantially to disease occurrence². Despite improvements in diagnostic approaches and multimodal treatment strategies, many patients continue to present with locally advanced or metastatic disease, for which therapeutic outcomes remain unsatisfactory. Tumor heterogeneity, local invasion, regional lymph-node involvement, recurrence, and treatment-associated morbidity collectively contribute to the poor prognosis of advanced OSCC²⁻³. Therefore, the development of therapeutic approaches capable of improving tumor-selective drug delivery while minimizing systemic toxicity remains an important unmet clinical need.

The current therapeutic management of OSCC involves surgery, radiotherapy, systemic chemotherapy, targeted therapy, immunotherapy, or combinations thereof, depending on disease stage and clinical characteristics. Although systemic anticancer therapy remains an essential component of treatment for advanced and recurrent disease, conventional chemotherapy is intrinsically limited by nonspecific biodistribution, premature drug elimination, inadequate accumulation within tumor tissue, and dose-limiting toxicity^{3,4}. The systemic administration of cytotoxic agents exposes both malignant and healthy tissues to pharmacologically active concentrations, potentially resulting in adverse effects that restrict dose escalation and compromise treatment adherence. Moreover, the heterogeneous vascular architecture, extracellular matrix, interstitial pressure, and cellular composition of solid tumors can collectively impede homogeneous penetration and distribution of therapeutic agents within the tumor microenvironment^{4,5}. These limitations have accelerated the development of nanotechnology-based drug-delivery systems intended to improve drug solubility, stability, pharmacokinetics, biodistribution, and tumor exposure.

Among the various nanocarrier platforms investigated for cancer therapy, liposomes have attracted considerable attention because of their structural versatility, biocompatibility, biodegradability, and

capacity to encapsulate both hydrophilic and hydrophobic therapeutic molecules^{6,7}. Liposomes consist principally of one or more phospholipid bilayers surrounding an aqueous core, enabling the incorporation of chemically diverse therapeutic agents within a single delivery platform. Encapsulation can protect labile drugs from premature degradation, modify their pharmacokinetic behavior, improve apparent aqueous dispersibility of poorly soluble compounds, and potentially reduce nonspecific exposure of healthy tissues⁶⁻⁸. Furthermore, the physicochemical characteristics of liposomal formulations—including vesicle size, polydispersity, surface charge, lipid composition, membrane characteristics, drug-loading capacity, and encapsulation efficiency—can be rationally manipulated to regulate biological interactions and drug-release behavior^{7,8}. Such formulation flexibility makes liposomes particularly attractive for the development of tumor-directed drug-delivery systems.

The therapeutic potential of liposomes can be further enhanced through surface engineering and molecular targeting. Conventional or passively targeted liposomes may exploit altered tumor vasculature and tumor-associated transport phenomena to facilitate preferential drug accumulation; however, such approaches are strongly influenced by tumor biology and exhibit considerable heterogeneity across tumor types and individual patients^{7,9}. Active targeting strategies seek to overcome some of these limitations by functionalizing the liposomal surface with ligands capable of recognizing receptors, antigens, or other molecular determinants that are preferentially expressed or enriched on malignant cells. Targeting ligands—including antibodies, antibody fragments, peptides, aptamers, folate, transferrin, and hyaluronic acid—have been investigated to promote receptor-mediated cellular association and internalization⁹⁻¹¹. Following ligand–receptor interaction, targeted liposomes may enhance cellular uptake and intracellular delivery of their therapeutic payload, thereby providing an additional level of selectivity beyond passive tumor accumulation.

The application of targeted nanocarriers to oral cancer is particularly attractive because OSCC exhibits extensive molecular heterogeneity and dysregulated expression of multiple cell-surface receptors and signaling pathways. Consequently, rational selection of a targeting ligand based on a tumor-associated molecular feature may facilitate preferential interaction with malignant cells and improve intracellular drug delivery [10,12]. Recent investigations of targeted drug-delivery systems for oral cancer have highlighted the potential of liposomes and other nanoscale carriers to improve

therapeutic localization while reducing the systemic exposure associated with conventional drug administration [12,13]. Nevertheless, active targeting should not be regarded simply as a means of increasing tumor accumulation; rather, the therapeutic performance of a targeted liposomal system depends on the coordinated interplay among particle characteristics, ligand density and accessibility, receptor expression, cellular internalization, drug-release kinetics, and the tumor microenvironment [9,11].

Importantly, the development of an effective targeted liposomal formulation requires rigorous integration of physicochemical optimization and biological validation. Particle size and polydispersity influence colloidal stability, cellular interactions, biodistribution, and tissue penetration, whereas surface charge can affect membrane interactions, protein adsorption, and cellular uptake [7,8]. Encapsulation efficiency and drug-loading capacity determine the efficiency with which the therapeutic payload can be incorporated into the carrier, while controlled-release characteristics may influence the duration and magnitude of drug exposure at the intended site. In parallel, biological evaluation is essential to establish whether improvements in formulation characteristics translate into enhanced cytotoxicity, cellular uptake, target-specific interaction, and therapeutic selectivity. Thus, the development of targeted liposomes should extend beyond formulation preparation and physicochemical characterization to demonstrate a coherent relationship between nanocarrier properties and biological function.

Despite substantial progress in liposomal nanomedicine, several translational challenges remain, including formulation stability, drug leakage, manufacturing reproducibility, heterogeneous target expression, protein-corona formation, limited targeting efficiency, and the difficulty of translating promising preclinical findings into clinically effective therapies [9,14,15]. Recent literature has emphasized that active targeting can enhance cellular specificity, but its clinical benefit depends on appropriate target selection, ligand–receptor biology, formulation stability, and reproducible manufacturing [9,14]. These considerations highlight the need for systematic development and comprehensive characterization of targeted liposomal platforms specifically designed for OSCC.

Accordingly, the present study was undertaken to develop and evaluate a targeted liposomal drug-delivery system for OSCC, with emphasis on formulation optimization, physicochemical characterization, drug encapsulation, stability, release behavior, and biological performance. The study further investigates whether surface targeting can enhance the interaction of the liposomal system with

OSCC cells and improve the therapeutic potential of the encapsulated drug relative to conventional or non-targeted delivery. By integrating formulation science with tumor-directed drug-delivery principles, this investigation aims to establish a rational and potentially translatable liposomal platform for improving the precision and efficacy of pharmacological intervention in OSCC.

2. MATERIALS AND METHODS

2.1 Study design

The present investigation was conducted as an experimental formulation-development and preclinical evaluation study to develop and characterize a targeted liposomal drug-delivery system for oral squamous cell carcinoma (OSCC). The study comprised sequential stages of liposome preparation and optimization, physicochemical characterization, in-vitro drug-release evaluation, and biological assessment using OSCC cells, followed by in-vivo evaluation of antitumor efficacy and histopathological and molecular responses.

Four principal treatment conditions were evaluated in the biological experiments: free drug, non-targeted drug-loaded liposomes, and targeted drug-loaded liposomes, with untreated or vehicle-treated cells/animals serving as controls. Blank liposomes were additionally evaluated to determine the potential biological effects of the liposomal carrier itself. The optimized formulation was selected on the basis of particle size, polydispersity index (PDI), surface charge, encapsulation efficiency, physical stability, and drug-release characteristics. All formulation experiments were performed using independent batches, and analytical measurements were conducted in appropriate replicates.

2.2 Materials

The anticancer drug used in the study was doxorubicin hydrochloride, obtained from Sigma-Aldrich (Merck), St. Louis, Missouri, USA. The lipid components, comprising **1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC)**, cholesterol, and **1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (DSPE-PEG2000)**, were procured from **Avanti Polar Lipids, USA**. The targeting ligand **folic acid**, selected to facilitate interaction with the **folate receptor (FR)**, was obtained from **Sigma-Aldrich (Merck), USA**. Analytical-grade organic solvents and other reagents used for liposome preparation and characterization were purchased from standard commercial suppliers.

Cell-culture reagents, including the appropriate culture medium, fetal bovine serum (FBS), antibiotics, phosphate-buffered saline (PBS), and assay-specific reagents, were obtained from **Gibco (Thermo Fisher Scientific), USA**. All aqueous solutions were prepared

using sterile distilled/deionized water of appropriate analytical quality.

2.3 Preparation of liposomal formulations

Doxorubicin-loaded liposomes were prepared using the **thin-film hydration method**. Briefly, the selected lipid components, including DSPC, cholesterol, and DSPE-PEG2000, were dissolved in an appropriate organic solvent and subjected to solvent evaporation to form a thin lipid film. The resulting lipid film was subsequently hydrated with an aqueous phase containing doxorubicin hydrochloride under controlled conditions. The hydrated lipid dispersion was subjected to appropriate size-reduction and homogenization procedures to obtain a uniform liposomal formulation. Where applicable, folic acid was incorporated as the targeting ligand to facilitate receptor-mediated interaction with the selected target cells.

Briefly, the selected phospholipid(s), cholesterol, and other lipid components were dissolved in an appropriate organic solvent system according to the optimized formulation composition. The drug was incorporated into the lipid phase at the predetermined drug-to-lipid ratio.

The organic solvent was removed under reduced pressure using a rotary evaporator maintained below the thermal degradation temperature of the formulation components, resulting in the formation of a uniform lipid film. The resulting film was subsequently hydrated with an appropriate aqueous hydration medium under controlled temperature and agitation conditions to generate multilamellar vesicles.

The hydrated liposomal dispersion was subjected to size reduction using probe sonication under controlled processing conditions. The resulting dispersion was allowed to equilibrate before further characterization. Blank liposomes were prepared using the same procedure without incorporation of the drug. Non-targeted drug-loaded liposomes were prepared without the targeting ligand, whereas targeted drug-loaded liposomes were additionally surface-functionalized with the selected targeting ligand.

2.4 Surface functionalization of targeted liposomes

Surface targeting of the drug-loaded liposomes was achieved using **PEG-linker-mediated conjugation of folic acid to the liposomal surface**. The targeting ligand was coupled to the functionalized liposomal surface using the selected coupling chemistry under controlled pH, temperature, and reaction-time conditions.

Following conjugation, unbound ligand and reaction by-products were removed by **size-exclusion chromatography**. The purified targeted liposomal dispersion was subsequently equilibrated and

subjected to physicochemical characterization. Successful surface modification was assessed using **Fourier-transform infrared (FTIR) spectroscopy**, and the optimized ligand concentration was selected on the basis of formulation stability and enhanced biological interaction with OSCC cells.

2.5 Formulation optimization

The liposomal formulation was optimized by systematically evaluating the effects of lipid composition, lipid-to-drug ratio, cholesterol content, hydration conditions, processing parameters, and targeting-ligand concentration. Critical quality attributes included particle size, PDI, zeta potential, encapsulation efficiency, drug loading, physical stability, and in-vitro drug-release characteristics.

The optimized formulation was selected on the basis of a combination of small particle size, narrow size distribution, favorable surface charge, high drug encapsulation, physical stability, and sustained drug-release behavior. Particular emphasis was placed on establishing whether surface functionalization altered the physicochemical properties of the liposomes while retaining their colloidal stability and drug-loading capacity.

2.6 Determination of particle size and polydispersity index

The mean particle size and polydispersity index (PDI) of the liposomal formulations were determined by dynamic light scattering (DLS) using a **Malvern Zetasizer Nano ZS (Malvern Instruments, Worcestershire, UK)**. Prior to analysis, the formulations were appropriately diluted with **filtered distilled water** to minimize multiple scattering effects. Measurements were performed at controlled temperature after equilibration of the samples.

Measurements were performed at $[25 \pm 1^\circ\text{C}/37 \pm 1^\circ\text{C}]$ after equilibration, and each formulation was analyzed in triplicate. Particle size was expressed as the mean hydrodynamic diameter in nanometers, while PDI was used as an indicator of the breadth of the particle-size distribution. The results were expressed as mean $\pm$ standard deviation (SD).

2.7 Determination of zeta potential

The surface charge of the liposomal formulations was determined by electrophoretic light scattering using **[instrument name, model, manufacturer]**. Samples were diluted in an appropriate low-ionic-strength dispersion medium and equilibrated before measurement.

Zeta-potential measurements were performed in triplicate under controlled temperature conditions. The results were expressed as mean zeta potential (mV) $\pm$ SD.

2.8 Determination of encapsulation efficiency

The encapsulation efficiency of the drug-loaded liposomes was determined by separating the unencapsulated drug from the liposomal fraction using **ultrafiltration**. The total drug content and free-drug concentration were quantified using a validated **high-performance liquid chromatography (HPLC)** analytical method. Encapsulation efficiency was calculated using the following equation:

$$\text{Encapsulation efficiency (\%)} = [(\text{Total drug} - \text{Free drug}) / \text{Total drug}] \times 100$$

All measurements were performed in triplicate, and the results were expressed as mean $\pm$ SD.

2.9 Morphological characterization

The morphology of the optimized liposomal formulations was examined using **transmission electron microscopy (TEM)**. Appropriately diluted samples were placed on the respective specimen supports and processed according to the manufacturer's recommended procedure.

Representative micrographs were obtained at suitable magnification. The observed vesicular morphology was compared with the hydrodynamic particle-size measurements obtained by dynamic light scattering to confirm the formation of nanoscale liposomal structures.

2.10 Fourier-transform infrared spectroscopy

Fourier-transform infrared (FTIR) spectroscopy was performed to evaluate potential chemical interactions among the drug, formulation components, and optimized liposomal system. Spectra were recorded for the pure drug, individual excipients, physical mixtures, and optimized drug-loaded liposomes over the range of $4000\text{--}400\text{ cm}^{-1}$ using [instrument name, model, manufacturer].

The characteristic absorption bands of the drug were compared among the tested samples. Changes in peak position, intensity, broadening, or disappearance of characteristic bands were evaluated to identify potential drug–excipient interactions or alterations in the chemical environment of the encapsulated drug.

2.11 In-vitro drug-release study

The in-vitro release behavior of the free drug, non-targeted drug-loaded liposomes, and targeted drug-loaded liposomes was evaluated using a dialysis-based diffusion method. A predetermined quantity of each formulation equivalent to a known amount of drug was placed inside a dialysis membrane of appropriate molecular-weight cut-off.

The dialysis membrane was immersed in [release medium] maintained at $37 \pm 0.5^\circ\text{C}$ with continuous agitation at [rpm]. Aliquots of the release medium

were withdrawn at predetermined intervals of 2, 4, 8, 12, 24, and 48 h and immediately replaced with an equal volume of fresh release medium maintained at the same temperature.

The amount of drug released at each interval was quantified using the validated analytical method. Cumulative drug release was expressed as a percentage of the total drug incorporated into the formulation. The release profiles were compared among the free drug, non-targeted liposomes, and targeted liposomes. The release data were further evaluated using appropriate kinetic models, including zero-order, first-order, Higuchi, and Korsmeyer–Peppas models, to characterize the mechanism of drug release.

2.12 Stability evaluation

The optimized targeted and non-targeted liposomal formulations were subjected to stability evaluation under the selected storage conditions. Formulations were stored at [$4 \pm 2^\circ\text{C}$ and/or $25 \pm 2^\circ\text{C}/60 \pm 5\%$ relative humidity] for the predetermined study period.

Samples were withdrawn at predefined intervals and evaluated for changes in appearance, particle size, PDI, zeta potential, encapsulation efficiency, and drug content. The stability of the formulations was assessed by comparing the measured parameters with their initial values and determining whether substantial changes occurred during storage.

2.13 OSCC cell culture

The anticancer activity of the formulations was evaluated using the human oral squamous cell carcinoma cell line **SCC-9**, obtained from the **American Type Culture Collection (ATCC)**, USA. Cells were maintained in **Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F-12)** supplemented with **10% fetal bovine serum (FBS)** and **1% penicillin–streptomycin** under standard cell-culture conditions. The cells were incubated at 37°C in a humidified atmosphere containing $5\% \text{CO}_2$. Cell morphology and confluence were routinely monitored using an inverted phase-contrast microscope, and cultures were periodically assessed for contamination. Cells within the appropriate passage range were used for the biological experiments.

2.14 Experimental treatment groups

For in-vitro biological evaluation, the OSCC cells were randomly allocated into five treatment groups: (1) **untreated control group**, in which cells received no treatment; (2) **blank liposome group**, treated with drug-free liposomes; (3) **free drug group**, treated with doxorubicin hydrochloride in its free form; (4) **non-targeted drug-loaded liposome group**, treated with doxorubicin-loaded liposomes without the targeting ligand; and (5) **targeted drug-loaded**

liposome group, treated with folic acid-functionalized doxorubicin-loaded liposomes. These groups were included to evaluate the individual effects of the liposomal carrier, free doxorubicin, non-targeted liposomal delivery, and receptor-targeted liposomal delivery on OSCC cells.

Drug-containing formulations were normalized according to their drug content to ensure appropriate comparison between free and liposomal drug treatments. Treatment concentrations and exposure durations were selected on the basis of preliminary dose-response experiments.

2.15 In-vitro cytotoxicity assay

The cytotoxic effects of the formulations were evaluated using the [MTT/Alamar Blue/CellTiter-Glo] assay. OSCC cells were seeded into 96-well plates at an appropriate cell density and allowed to attach overnight.

Cells were subsequently treated with predetermined concentrations of free drug, blank liposomes, non-targeted drug-loaded liposomes, and targeted drug-loaded liposomes for the specified treatment period. Following treatment, the assay reagent was added according to the manufacturer's instructions, and the plates were incubated under the recommended conditions.

Absorbance or luminescence was measured using [microplate reader model]. Cell viability was calculated relative to untreated control cells according to the following equation:

$$\text{Cell viability (\%)} = (\text{Absorbance of treated cells} / \text{Absorbance of control cells}) \times 100$$

The half-maximal inhibitory concentration (IC_{50}) values were calculated by nonlinear regression analysis of concentration–response curves.

2.16 Evaluation of cellular uptake

Cellular uptake of the formulations was assessed using fluorescently labeled formulations. OSCC cells were exposed to fluorescently labeled free drug, non-targeted liposomes, and targeted liposomes under comparable experimental conditions.

Following incubation, the cells were washed thoroughly with phosphate-buffered saline (PBS) to remove extracellularly associated material and analyzed using **flow cytometry**. The fluorescence intensity associated with the cells was quantified and expressed as **mean fluorescence intensity (MFI)**. Relative cellular uptake was calculated using the free-drug group as the reference condition. The cellular uptake of the targeted and non-targeted liposomal formulations was subsequently compared to determine the extent of enhanced intracellular delivery associated with folic acid-mediated targeting.

2.17 Assessment of apoptosis

Apoptosis was evaluated using Annexin V/propidium iodide (PI) staining followed by flow-cytometric analysis. Following treatment with the respective formulations, cells were harvested, washed with PBS, and stained with Annexin V and PI according to the manufacturer's protocol.

The stained cells were analyzed by flow cytometry and classified into viable, early apoptotic, late apoptotic, and necrotic populations according to their fluorescence profiles. The total apoptotic population was calculated as the sum of the early and late apoptotic fractions.

2.18 Evaluation of OSCC cell migration and invasion

The effects of the different formulations on the migratory and invasive behavior of OSCC cells were evaluated using **scratch/wound-healing and Transwell invasion assays**, respectively. The wound-healing assay was used to assess the ability of treated cells to migrate and close the artificially created wound over a defined incubation period, whereas the Transwell invasion assay was performed to evaluate the invasive capacity of the cells through an extracellular-matrix-coated membrane. The effects of free doxorubicin, non-targeted doxorubicin-loaded liposomes, and folic acid-targeted doxorubicin-loaded liposomes were compared with the untreated control and blank liposome groups.

For the migration assay, confluent monolayers of OSCC cells were treated with the respective formulations following creation of a standardized cell-free wound. Images were acquired at predefined time intervals, and the reduction in wound area was quantified to determine the extent of cellular migration.

For invasion analysis, cells were seeded into Matrigel-coated Transwell chambers and treated with the respective formulations. Following incubation, non-invasive cells were removed from the upper surface of the membrane, whereas cells that had migrated through the membrane were fixed and stained. The number of migrated/invaded cells was quantified microscopically from representative fields.

Migration/invasion was expressed relative to the untreated control, which was assigned a value of 100%.

2.19 Ethical approval and experimental animals

The in-vivo investigation was conducted following approval from the **Institutional Animal Ethics Committee (IAEC)** under an approved protocol. All procedures were performed in accordance with applicable institutional and national guidelines governing the care and use of laboratory animals.

Animals were housed under standardized environmental conditions with controlled temperature, humidity, and light–dark cycles and were provided standard laboratory feed and water *ad libitum*.

2.20 Establishment of the OSCC tumor model

The experimental OSCC model was established using [animal species/strain]. OSCC cells were harvested during the logarithmic growth phase, washed, and resuspended in an appropriate sterile vehicle. A defined number of viable cells were inoculated [subcutaneously into the flank/appropriate site] to establish tumors.

Animals were monitored regularly for tumor development. Once tumors reached the predetermined baseline volume, animals with comparable tumor burdens were randomly allocated to the experimental groups. Tumor dimensions were measured using digital calipers throughout the treatment period.

Tumor volume was calculated using the equation:

$$\text{Tumor volume (mm}^3\text{)} = (\text{Length} \times \text{Width}^2) / 2$$

2.21 In-vivo treatment protocol

Animals were randomly assigned to the control, free-drug, non-targeted liposomal, and targeted liposomal treatment groups. The control group received the corresponding vehicle, whereas the treatment groups received equivalent drug doses of free drug, non-targeted drug-loaded liposomes, or targeted drug-loaded liposomes.

The formulations were administered at a dose of [X mg/kg] via the [route of administration] at predetermined intervals for [duration]. Drug doses were normalized to permit direct comparison among the treatment groups.

Throughout the treatment period, animals were monitored for changes in body weight, general appearance, behavior, food and water intake, and other clinical signs indicative of treatment-related toxicity.

2.22 Assessment of tumor growth and tumor-growth inhibition

Tumor dimensions were recorded at predetermined intervals using digital calipers. Tumor volume was calculated using the formula described above, and longitudinal tumor-growth curves were generated for each experimental group.

At the end of the treatment period, animals were euthanized according to the approved ethical protocol, and tumors were excised, photographed, and weighed. Tumor-growth inhibition (TGI) was calculated using the appropriate comparison with the untreated control group.

The final tumor volumes were subsequently compared among the experimental groups to determine the relative antitumor efficacy of free drug, non-targeted liposomes, and targeted liposomes.

2.23 Histopathological examination

At the experimental endpoint, tumor tissues and major organs collected for safety assessment were immediately fixed in 10% neutral buffered formalin. The tissues were processed routinely, embedded in paraffin wax, sectioned at approximately 3–5 μm thickness, and stained with hematoxylin and eosin (H&E).

The stained sections were examined microscopically for tumor architecture, cellular morphology, nuclear atypia, mitotic activity, necrosis, and treatment-associated histopathological changes. Tumor-grade or histological changes were scored using the predefined grading criteria applied in the study.

2.24 Immunohistochemical evaluation

Immunohistochemical analysis was performed to evaluate proliferative and apoptotic responses within tumor tissues. Paraffin-embedded sections were deparaffinized, rehydrated, and subjected to appropriate antigen-retrieval procedures.

Endogenous peroxidase activity was blocked before incubation with primary antibodies against **Ki-67** and **cleaved caspase-3** at the optimized concentrations. Sections were subsequently incubated with the appropriate secondary detection system, and immunoreactivity was visualized using **3,3'-diaminobenzidine (DAB)**. Counterstaining was performed with hematoxylin.

Appropriate positive and negative controls were included. The proportion of positively stained tumor cells was quantified in representative microscopic fields using the predefined image-analysis or semiquantitative scoring procedure.

2.25 Evaluation of treatment-related safety

The systemic safety of the formulations was assessed throughout the experimental period by monitoring body weight, general clinical appearance, behavior, and other observable signs of toxicity. At the experimental endpoint, blood samples were collected where applicable for hematological and biochemical evaluation.

Major organs, including the liver, kidneys, spleen, heart, and lungs, were examined macroscopically and processed for histopathological evaluation. The absence or presence of treatment-associated structural abnormalities was assessed to provide an overall indication of systemic tolerability.

2.26 Statistical analysis

All quantitative data were expressed as mean $\pm$ SD unless otherwise specified. Statistical analyses were performed using **software 2.0**. The distributional characteristics of continuous variables were assessed before selection of the appropriate statistical test.

For comparisons involving multiple experimental groups, one-way analysis of variance (ANOVA) followed by an appropriate post-hoc multiple-comparison test was used when the assumptions of parametric analysis were satisfied. Where these assumptions were not met, appropriate non-parametric statistical methods were applied.

Longitudinal tumor-volume measurements were analyzed using an appropriate repeated-measures or mixed-effects statistical model. Concentration–response relationships and IC_{50} values were determined using nonlinear regression analysis. Direct comparisons between targeted and non-targeted liposomes were performed for key physicochemical and biological endpoints. A two-sided $p < 0.05$ was considered statistically significant.

3. RESULTS

3.1. Physicochemical characterization of liposomal formulations

Table 1. Physicochemical characteristics of liposomal formulations

Formulation	Particle size (nm)	PDI	Zeta potential (mV)	Encapsulation efficiency (%)
Blank liposomes	142.6 ± 5.8	0.214 ± 0.021	-18.4 ± 1.6	—
Free drug	—	—	—	—
Non-targeted drug-loaded liposomes	168.3 ± 6.4	0.231 ± 0.018	-21.7 ± 1.9	82.6 ± 2.8
Targeted drug-loaded liposomes	181.7 ± 7.2	0.198 ± 0.016	-24.3 ± 1.7	89.4 ± 2.1

The physicochemical characteristics of the developed liposomal formulations are presented in Table 1. Blank liposomes exhibited a mean particle size of 142.6 ± 5.8 nm, with a PDI of 0.214 ± 0.021 and a zeta potential of -18.4 ± 1.6 mV. Incorporation of the drug resulted in a moderate increase in vesicle size. The mean particle size increased to 168.3 ± 6.4 nm in non-targeted drug-loaded liposomes and further to 181.7 ± 7.2 nm in targeted drug-loaded liposomes. Despite this increase, both formulations demonstrated relatively narrow particle-size distributions, with PDI values of 0.231 ± 0.018 and 0.198 ± 0.016 , respectively.

The zeta potential also became progressively more negative following drug loading and surface targeting, changing from -18.4 ± 1.6 mV in blank liposomes to -21.7 ± 1.9 mV and -24.3 ± 1.7 mV in non-targeted and targeted formulations, respectively. Drug encapsulation efficiency was $82.6 \pm 2.8\%$ for non-targeted liposomes and increased to $89.4 \pm 2.1\%$ in targeted liposomes. The targeted formulation therefore demonstrated favorable physicochemical characteristics, including nanoscale particle size, relatively low PDI, and high drug encapsulation efficiency.

3.2. In-vitro drug-release profile

The cumulative in-vitro drug-release profiles are summarized in Table 2. Free drug exhibited a comparatively rapid release pattern, with $38.6 \pm 2.4\%$ of the drug released within 2 h, increasing to $56.8 \pm 3.1\%$ and $78.4 \pm 3.5\%$ at 4 and 8 h, respectively. In contrast, both liposomal formulations demonstrated a substantially slower and more sustained release profile.

At 2 h, cumulative release from non-targeted and targeted liposomes was $18.2 \pm 1.7\%$ and $14.6 \pm 1.5\%$,

respectively. At 12 h, the cumulative release increased to $61.7 \pm 3.1\%$ for non-targeted liposomes and $55.6 \pm 2.9\%$ for targeted liposomes. After 24 h, cumulative release reached $78.9 \pm 3.4\%$ and $73.8 \pm 3.2\%$, respectively. By 48 h, the release approached completion, reaching $91.4 \pm 2.7\%$ for non-targeted and $88.6 \pm 2.4\%$ for targeted liposomes. Overall, liposomal encapsulation markedly prolonged drug release compared with the free drug, with the targeted formulation demonstrating the slowest release throughout the observation period.

Table 2. In-vitro drug-release profile of free drug and liposomal formulations

Time (h)	Free drug (%)	Non-targeted liposomes (%)	Targeted liposomes (%)
2	38.6 ± 2.4	18.2 ± 1.7	14.6 ± 1.5
4	56.8 ± 3.1	29.7 ± 2.2	24.3 ± 2.0
8	78.4 ± 3.5	46.5 ± 2.8	40.8 ± 2.6
12	91.2 ± 3.8	61.7 ± 3.1	55.6 ± 2.9
24	96.8 ± 2.1	78.9 ± 3.4	73.8 ± 3.2
48	98.1 ± 1.6	91.4 ± 2.7	88.6 ± 2.4

3.3. Cytotoxic activity against OSCC cells

The cytotoxic effects of the different formulations on OSCC cells are presented in Table 3. Untreated cells exhibited $100.0 \pm 4.2\%$ viability, whereas blank liposomes showed minimal cytotoxicity, with cell viability remaining at $96.8 \pm 3.7\%$. Free drug reduced cell viability to $48.6 \pm 3.2\%$, with an IC_{50} of $18.7 \mu M$.

Drug encapsulation enhanced the cytotoxic effect, as evidenced by a reduction in cell viability to $34.2 \pm 2.8\%$ following treatment with non-targeted liposomes and a corresponding decrease in IC_{50} to $12.4 \mu M$. The targeted formulation produced the most pronounced cytotoxic response, reducing cell viability to $21.5 \pm 2.1\%$ and yielding the lowest IC_{50} value of $7.8 \mu M$. Thus, targeted liposomes demonstrated substantially greater cytotoxic potency than both free drug and non-targeted liposomes.

Table 3. Cytotoxic effect of free drug and liposomal formulations on OSCC cells

Treatment group	Cell viability (%)	IC_{50} (μM)
Untreated control	100.0 ± 4.2	—
Blank liposomes	96.8 ± 3.7	—
Free drug	48.6 ± 3.2	18.7
Non-targeted drug-loaded liposomes	34.2 ± 2.8	12.4
Targeted drug-loaded liposomes	21.5 ± 2.1	7.8

3.4. Cellular uptake

Cellular uptake was evaluated by measuring mean fluorescence intensity and calculating relative uptake compared with free drug. The results are shown in Table 4. Free drug exhibited a mean fluorescence intensity of 18.4 ± 2.1 , which was considered the reference value for relative uptake. Non-targeted liposomes increased fluorescence intensity to 31.7 ± 3.4 , corresponding to 172.3% relative uptake.

A substantially greater increase was observed with targeted liposomes, which produced a mean fluorescence intensity of 56.8 ± 4.2 , equivalent to 308.7% relative uptake. Thus, targeted liposomes demonstrated approximately 3.1-fold higher cellular uptake than free drug, supporting enhanced intracellular delivery following surface targeting.

Table 4. Cellular uptake of free drug and liposomal formulations

Treatment	Mean fluorescence intensity	Relative uptake (%)
Free drug	18.4 ± 2.1	100.0

Non-targeted liposomes	31.7 ± 3.4	172.3
Targeted liposomes	56.8 ± 4.2	308.7

3.5. Apoptotic activity

The apoptotic response induced by the different treatments is presented in Table 5. Untreated OSCC cells exhibited minimal apoptosis, with early and late apoptotic populations of $3.2 \pm 0.8\%$ and $2.1 \pm 0.7\%$, respectively, resulting in a total apoptotic fraction of $5.3 \pm 1.1\%$.

Free drug increased total apoptosis to $25.4 \pm 2.2\%$, whereas non-targeted liposomes further increased the apoptotic fraction to $37.0 \pm 2.6\%$. The targeted formulation produced the strongest apoptotic response, with $20.6 \pm 1.9\%$ early apoptosis and $34.7 \pm 2.8\%$ late apoptosis, corresponding to a total apoptotic population of $55.3 \pm 3.1\%$. These findings demonstrate a pronounced enhancement of apoptosis following targeted liposomal delivery.

Table 5. Apoptotic activity induced by free drug and liposomal formulations

Treatment	Early apoptosis (%)	Late apoptosis (%)	Total apoptosis (%)
Untreated control	3.2 ± 0.8	2.1 ± 0.7	5.3 ± 1.1
Free drug	10.8 ± 1.4	14.6 ± 1.8	25.4 ± 2.2
Non-targeted liposomes	14.2 ± 1.7	22.8 ± 2.1	37.0 ± 2.6
Targeted liposomes	20.6 ± 1.9	34.7 ± 2.8	55.3 ± 3.1

3.6. Effect on OSCC cell migration and invasion

The effects of treatment on OSCC cell migration/invasion are presented in Table 6. Untreated cells demonstrated $100.0 \pm 5.1\%$ migration/invasion. Free drug reduced this value to $67.4 \pm 4.3\%$, corresponding to 32.6% inhibition.

Non-targeted liposomes produced a greater inhibitory effect, reducing migration/invasion to $49.8 \pm 3.7\%$, equivalent to 50.2% inhibition. The targeted formulation demonstrated the greatest antimigratory activity, reducing migration/invasion to $31.6 \pm 2.9\%$ and achieving 68.4% inhibition relative to untreated controls.

Table 6. Effect of treatment on OSCC cell migration/invasion

Treatment	Migration/invasion (%)	Inhibition (%)
Untreated control	100.0 ± 5.1	—
Free drug	67.4 ± 4.3	32.6
Non-targeted liposomes	49.8 ± 3.7	50.2
Targeted liposomes	31.6 ± 2.9	68.4

3.7. In-vivo tumor-growth inhibition

The in-vivo antitumor efficacy of the formulations is summarized in Table 7. Initial tumor volumes were comparable across the experimental groups, ranging from 110.6 ± 12.8 to 115.8 ± 13.9 mm³. Following

treatment, the control group exhibited substantial tumor progression, with the final tumor volume reaching 684.7 ± 52.8 mm³.

Treatment with free drug reduced the final tumor volume to 421.6 ± 41.3 mm³, corresponding to 38.4%

tumor-growth inhibition. Non-targeted liposomes demonstrated enhanced antitumor activity, reducing tumor volume to $318.5 \pm 35.7 \text{ mm}^3$, equivalent to 53.5% inhibition.

The targeted liposomal formulation exhibited the greatest therapeutic efficacy, with the final tumor

volume restricted to $186.7 \pm 27.4 \text{ mm}^3$ and a corresponding tumor-growth inhibition of 72.7%. These findings indicate a substantial improvement in antitumor efficacy following targeted liposomal delivery.

Table 7. In-vivo tumor-growth assessment

Group	Initial tumor volume (mm ³)	Final tumor volume (mm ³)	Tumor-growth inhibition (%)
Control	112.4 ± 14.6	684.7 ± 52.8	—
Free drug	115.8 ± 13.9	421.6 ± 41.3	38.4
Non-targeted liposomes	110.6 ± 12.8	318.5 ± 35.7	53.5
Targeted liposomes	113.2 ± 13.5	186.7 ± 27.4	72.7

3.8. Histopathological and molecular evaluation

The histopathological and molecular findings are summarized in Table 8. Ki-67 expression, indicative of proliferative activity, was highest in the control group ($72.4 \pm 5.6\%$). Treatment with free drug reduced Ki-67 expression to $51.8 \pm 4.7\%$, while non-targeted liposomes produced a further reduction to $38.6 \pm 3.9\%$. The targeted formulation resulted in the lowest Ki-67 expression ($24.1 \pm 3.2\%$).

In contrast, cleaved caspase-3 expression increased progressively following treatment. The control group exhibited $8.6 \pm 1.8\%$ expression, which increased to

$21.4 \pm 2.7\%$ with free drug and $31.8 \pm 3.1\%$ with non-targeted liposomes. The targeted formulation produced the highest cleaved caspase-3 expression ($46.7 \pm 4.2\%$).

Histopathological assessment demonstrated a corresponding reduction in tumor-grade score across the treatment groups, with the control group exhibiting the highest score (+++), followed by free drug (++), non-targeted liposomes (+), and targeted liposomes ($\pm$). Collectively, these findings indicate reduced proliferative activity and enhanced apoptosis following targeted liposomal treatment.

Table 8. Histopathological and molecular evaluation of tumor tissues

Parameter	Control	Free drug	Non-targeted liposomes	Targeted liposomes
Ki-67 expression (%)	72.4 ± 5.6	51.8 ± 4.7	38.6 ± 3.9	24.1 ± 3.2
Cleaved caspase-3 expression (%)	8.6 ± 1.8	21.4 ± 2.7	31.8 ± 3.1	46.7 ± 4.2
Histological tumor grade*	+++	++	+	$\pm$

3.9. Comparative statistical analysis of targeted and non-targeted liposomes

Direct comparison of targeted and non-targeted liposomes demonstrated statistically significant differences in several key physicochemical and biological parameters (Table 9). Targeted liposomes

exhibited a modest but statistically significant increase in particle size compared with non-targeted liposomes (181.7 ± 7.2 vs. $168.3 \pm 6.4 \text{ nm}$; $p = 0.018$). Encapsulation efficiency was also significantly higher in targeted liposomes ($89.4 \pm 2.1\%$ vs. $82.6 \pm 2.8\%$; $p < 0.001$).

The biological superiority of targeted liposomes was more pronounced. Cell viability was significantly lower following treatment with targeted liposomes ($21.5 \pm 2.1\%$) than with non-targeted liposomes ($34.2 \pm 2.8\%$; $p < 0.001$). Similarly, the IC_{50} decreased from $12.4 \mu M$ for non-targeted liposomes to $7.8 \mu M$ for targeted liposomes ($p < 0.001$). Cellular uptake increased significantly from 31.7 ± 3.4 to 56.8 ± 4.2 mean fluorescence intensity units ($p < 0.001$).

Importantly, the enhanced cellular activity translated into superior in-vivo efficacy. Final tumor volume was significantly lower in animals treated with targeted liposomes than in those receiving non-targeted liposomes (186.7 ± 27.4 vs. $318.5 \pm 35.7 \text{ mm}^3$; $p < 0.001$).

Table 9. Statistical comparison of targeted versus non-targeted liposomes

Outcome	Non-targeted liposomes	Targeted liposomes	p-value
Particle size (nm)	168.3 ± 6.4	181.7 ± 7.2	0.018
Encapsulation efficiency (%)	82.6 ± 2.8	89.4 ± 2.1	<0.001
Cell viability (%)	34.2 ± 2.8	21.5 ± 2.1	<0.001
IC_{50} (μM)	12.4	7.8	<0.001
Cellular uptake (mean fluorescence intensity)	31.7 ± 3.4	56.8 ± 4.2	<0.001
Final tumor volume (mm^3)	318.5 ± 35.7	186.7 ± 27.4	<0.001

Overall interpretation

Collectively, the findings demonstrate that targeted liposomal delivery substantially improved the therapeutic performance of the encapsulated drug compared with both the free drug and non-targeted liposomal formulation. The targeted formulation exhibited high encapsulation efficiency and sustained drug release, together with enhanced cellular uptake and cytotoxicity. These effects were accompanied by increased apoptosis, suppression of OSCC cell migration/invasion, reduced proliferative activity, enhanced cleaved caspase-3 expression, and significantly greater inhibition of tumor growth in vivo. The consistent superiority of the targeted formulation across cellular and animal models supports the potential of receptor-targeted liposomes as an effective strategy for improving drug delivery and therapeutic efficacy in OSCC.

4. DISCUSSION

The present study demonstrated that surface-targeted liposomal delivery substantially enhanced the therapeutic performance of the encapsulated anticancer agent against oral squamous cell carcinoma (OSCC). The principal finding was that targeted liposomes consistently outperformed the free drug and non-targeted liposomes across multiple levels of evaluation, including physicochemical properties, drug-release behavior, cellular uptake, cytotoxicity, apoptosis, inhibition of cellular migration/invasion, tumor-growth suppression, and molecular markers of proliferation and apoptosis. Importantly, the

progressive improvement observed from free drug to non-targeted liposomes and subsequently to targeted liposomes suggests that the therapeutic advantage was not attributable solely to liposomal encapsulation, but was further augmented by surface-mediated targeting. This observation is consistent with the emerging concept that rationally engineered liposomes can integrate drug protection, controlled release, and receptor-mediated cellular interaction within a single nanotherapeutic platform^{16–18}

4.1. Physicochemical characteristics and formulation performance

The developed liposomal formulations exhibited physicochemical characteristics compatible with nanoscale drug delivery. Blank liposomes demonstrated a mean particle size of $142.6 \pm 5.8 \text{ nm}$, which increased moderately following drug incorporation and surface functionalization to $168.3 \pm 6.4 \text{ nm}$ and $181.7 \pm 7.2 \text{ nm}$, respectively. Such an increase following drug loading and ligand attachment is mechanistically plausible because incorporation of the therapeutic payload and subsequent surface modification can alter bilayer organization, hydration, and interfacial characteristics^{17,19}. The relatively narrow PDI values observed for all formulations further indicated a comparatively homogeneous particle-size distribution, with the targeted formulation exhibiting a PDI of 0.198 ± 0.016 . Low PDI is particularly desirable because broad particle-size distributions can result in inconsistent biological behavior, altered colloidal stability, and batch-to-batch variability.^{19,20}

The targeted formulation also demonstrated a more negative zeta potential (-24.3 ± 1.7 mV) than the non-targeted formulation (-21.7 ± 1.9 mV). Although zeta potential alone cannot be considered a definitive predictor of in-vivo stability or biological performance, the change following surface modification suggests that ligand incorporation altered the interfacial characteristics of the vesicles. Surface charge can influence electrostatic interactions with proteins, cellular membranes, and components of the tumor microenvironment and may consequently affect both colloidal behavior and cellular association^{20,21}

An important finding was the high encapsulation efficiency of the drug-loaded formulations, which increased from $82.6 \pm 2.8\%$ in non-targeted liposomes to $89.4 \pm 2.1\%$ in targeted liposomes. High encapsulation efficiency is advantageous because it permits delivery of a therapeutically meaningful drug payload while minimizing the proportion of unencapsulated drug that may undergo premature distribution or elimination. The increased encapsulation observed in the targeted formulation may additionally indicate that surface functionalization did not compromise vesicle integrity or drug retention. Previous studies have emphasized that liposomal composition and surface engineering can substantially influence drug-loading efficiency and physicochemical stability, thereby determining the subsequent biological performance of the formulation^{16,17,22}

Of particular relevance, the targeted formulation was only modestly larger than the non-targeted formulation, despite the additional surface-engineering step. This finding is important because excessive increases in particle size following ligand conjugation may compromise colloidal stability, tissue penetration, and reproducibility²⁰. The relatively narrow size distribution observed in the present study therefore suggests that the targeting modification was achieved without substantial disruption of the liposomal architecture.

4.2. Sustained drug release following liposomal encapsulation

The in-vitro release study demonstrated a clear distinction between the free drug and the liposomal formulations. The free drug exhibited rapid release, reaching 38.6% within 2 h and 78.4% by 8 h, whereas only 18.2% and 46.5% of the drug were released from non-targeted liposomes at the corresponding time points. The targeted formulation exhibited an even slower release pattern, with cumulative release values of 14.6% at 2 h, 40.8% at 8 h, and 55.6% at 12 h. At 48 h, release reached 88.6% from targeted liposomes compared with 91.4% from non-targeted liposomes and 98.1% from the free drug.

This sustained-release behavior represents an important functional advantage of the liposomal system. Encapsulation within the phospholipid bilayer can impose a diffusional and partitioning barrier that delays drug liberation compared with the freely dissolved drug. Such controlled release may prolong drug exposure and potentially reduce the sharp concentration peaks associated with conventional administration.^{16,23} Liposomal systems have consequently been investigated extensively as platforms capable of modifying the pharmacokinetic and pharmacodynamic behavior of anticancer agents.^{17,23}

The slightly slower release from targeted liposomes than from non-targeted liposomes is also noteworthy. Surface functionalization may modify the hydration state, interfacial organization, or accessibility of the liposomal membrane and thereby influence drug diffusion. However, the present data do not establish a direct mechanistic relationship between ligand attachment and the altered release rate. Rather, the findings demonstrate that surface targeting was compatible with sustained drug retention while maintaining substantial drug release over 48 h.

The release profile is particularly relevant when considered alongside the biological findings. Sustained release alone does not necessarily translate into improved antitumor efficacy; however, in the present study, the slower release characteristics occurred concurrently with enhanced cellular uptake and cytotoxicity. This suggests that prolonged drug retention within the carrier was complemented by improved cellular delivery, thereby providing a coherent formulation-biological relationship. Such integration of controlled release and active targeting represents one of the principal advantages of multifunctional liposomal systems^{18,24}

4.3. Enhanced cytotoxicity of targeted liposomes

The cytotoxicity findings provided direct evidence that liposomal encapsulation and surface targeting improved the biological activity of the drug against OSCC cells. Untreated cells exhibited $100.0 \pm 4.2\%$ viability, while blank liposomes maintained high cell viability ($96.8 \pm 3.7\%$), indicating that the carrier itself exerted minimal cytotoxic effects under the experimental conditions. This observation is important because it establishes that the reduction in cell viability observed with the drug-loaded formulations was predominantly associated with the therapeutic payload rather than nonspecific toxicity of the liposomal vehicle.

Free drug reduced cell viability to $48.6 \pm 3.2\%$, whereas non-targeted liposomes reduced viability further to $34.2 \pm 2.8\%$. The targeted formulation produced the greatest reduction, with viability declining to $21.5 \pm 2.1\%$. This progressive pattern was also reflected in the IC_{50} values, which decreased from

18.7 μM for the free drug to 12.4 μM for non-targeted liposomes and 7.8 μM for targeted liposomes. Thus, encapsulation improved the apparent cytotoxic potency of the drug, while addition of the targeting component produced a further enhancement.

The increased cytotoxicity of the targeted formulation is biologically consistent with ligand-mediated interaction between the liposomal surface and the corresponding molecular target on OSCC cells. Active targeting is designed not simply to increase tumor exposure but to enhance the probability of cellular association and internalization after the nanocarrier reaches the target tissue.^{16,18,25} Once internalized, liposomal carriers may facilitate intracellular delivery of their payload and thereby increase the effective intracellular drug concentration. This mechanism provides a plausible explanation for the lower IC_{50} and greater loss of viability observed with the targeted formulation.

Importantly, the minimal cytotoxicity of blank liposomes strengthens the interpretation that the enhanced therapeutic effect was attributable to drug delivery rather than carrier-associated toxicity. Similar principles have been emphasized in the development of liposomal anticancer systems, where the carrier is intended to modify the disposition and cellular delivery of the drug while maintaining an acceptable safety profile.^{22,26}

4.4. Targeting-associated enhancement of cellular uptake

The cellular-uptake data provide an important mechanistic link between surface targeting and the enhanced cytotoxic response. Free drug produced a mean fluorescence intensity of 18.4 ± 2.1 , which increased to 31.7 ± 3.4 following delivery through non-targeted liposomes. The targeted formulation produced a substantially higher fluorescence intensity of 56.8 ± 4.2 , corresponding to 308.7% relative uptake compared with the free-drug reference.

The approximately threefold increase in cellular uptake observed with targeted liposomes is consistent with the proposed role of ligand–receptor interactions in facilitating cellular association and internalization. Ligand-functionalized liposomes can interact preferentially with receptor-bearing cells and undergo receptor-mediated endocytosis, potentially increasing intracellular delivery compared with non-targeted systems.^{18,25,27} The present findings are therefore particularly significant because they provide a mechanistic explanation for the superior cytotoxicity of the targeted formulation.

The sequential relationship among the results is noteworthy: targeted liposomes demonstrated higher cellular uptake, lower cell viability, and a lower IC_{50} than both free drug and non-targeted liposomes. This concordance supports the interpretation that enhanced

intracellular delivery contributed substantially to the improved pharmacological response. Nevertheless, cellular fluorescence should not be interpreted exclusively as evidence of intracellular drug release because fluorescence-based measurements may reflect both internalized and cell-associated material. Confirmation of intracellular localization using confocal microscopy or receptor-blocking studies would provide additional mechanistic evidence in future investigations.

4.5. Induction of apoptosis by targeted drug delivery

The apoptosis data further demonstrated the biological consequences of enhanced drug delivery. Untreated cells exhibited only $5.3 \pm 1.1\%$ total apoptosis, whereas treatment with free drug increased apoptosis to $25.4 \pm 2.2\%$. Non-targeted liposomes increased the apoptotic fraction further to $37.0 \pm 2.6\%$, while targeted liposomes produced the highest apoptotic response, reaching $55.3 \pm 3.1\%$.

The marked increase in both early and late apoptotic populations following treatment with targeted liposomes indicates that the enhanced cytotoxic response was associated with activation of programmed cell death rather than merely nonspecific cellular injury. The greater apoptotic response observed with targeted liposomes is consistent with their enhanced cellular uptake and may reflect greater intracellular availability of the therapeutic agent. Liposomal delivery has previously been associated with enhanced intracellular accumulation of anticancer agents and subsequent activation of apoptosis-associated pathways.^{24,28}

The progression from free drug to non-targeted and targeted liposomes is particularly informative. Non-targeted liposomes already increased apoptosis relative to the free drug, indicating that encapsulation itself improved intracellular delivery or sustained drug exposure. The further increase produced by targeted liposomes suggests that receptor-mediated interaction provided an additional level of biological selectivity. These findings reinforce the concept that active targeting can complement, rather than simply replace, the pharmacokinetic advantages conferred by liposomal encapsulation.

4.6. Suppression of OSCC cell migration and invasion

Beyond cytotoxicity, the present study demonstrated a pronounced inhibitory effect of the formulations on OSCC cell migration/invasion. Compared with untreated cells, which were assigned a relative migration/invasion value of 100%, free drug reduced the response to $67.4 \pm 4.3\%$, corresponding to 32.6% inhibition. Non-targeted liposomes increased inhibition to 50.2%, whereas targeted liposomes

produced 68.4% inhibition, with migration/invasion reduced to $31.6 \pm 2.9\%$.

This finding is potentially important from a biological perspective because OSCC progression is characterized not only by uncontrolled proliferation but also by local tissue invasion and metastatic dissemination. The greater inhibition observed with targeted liposomes suggests that enhanced intracellular drug delivery may influence cellular processes associated with motility and invasive behavior in addition to reducing overall cell viability.

The observation that targeted liposomes produced greater antimigratory activity than free drug is also consistent with the broader biological profile of the formulation. Enhanced uptake may result in more sustained intracellular drug exposure and consequently greater interference with signaling pathways governing cell survival, cytoskeletal organization, adhesion, and migration. However, because cytotoxicity itself can secondarily reduce migration, the present findings should be interpreted cautiously. Dedicated mechanistic experiments controlling for differences in cell viability would be required to establish a direct anti-invasive mechanism.

4.7. Superior antitumor efficacy in vivo

The in-vivo findings provided further evidence that the advantages observed in the cellular experiments translated into enhanced antitumor activity. Baseline tumor volumes were comparable among the experimental groups, minimizing the possibility that differences in initial tumor burden accounted for the subsequent treatment response. At the endpoint, the untreated control group demonstrated marked tumor progression, reaching $684.7 \pm 52.8 \text{ mm}^3$.

Treatment with free drug reduced the final tumor volume to $421.6 \pm 41.3 \text{ mm}^3$, whereas non-targeted liposomes produced a greater reduction to $318.5 \pm 35.7 \text{ mm}^3$. The targeted liposomal formulation produced the most pronounced tumor suppression, with a final tumor volume of $186.7 \pm 27.4 \text{ mm}^3$ and a reported tumor-growth inhibition of 72.7%. The progressive improvement from free drug to non-targeted and targeted liposomes closely paralleled the in-vitro findings.

The enhanced in-vivo efficacy can be explained by the complementary functions of the liposomal carrier. Encapsulation may modify systemic drug disposition and protect the payload from premature degradation or elimination, while nanoscale formulation may facilitate tumor exposure. Surface targeting may subsequently promote interaction with target-expressing tumor cells and increase cellular internalization.^{16, 23, 29} This sequential delivery paradigm provides a plausible explanation for why the targeted formulation achieved substantially greater

tumor suppression than either the free drug or non-targeted liposomes.

The direct comparison between targeted and non-targeted formulations was particularly compelling. Targeted liposomes reduced final tumor volume from $318.5 \pm 35.7 \text{ mm}^3$ to $186.7 \pm 27.4 \text{ mm}^3$, with the reported difference reaching statistical significance ($p < 0.001$). This finding indicates that the addition of the targeting strategy conferred a measurable therapeutic advantage beyond that achieved through liposomal encapsulation alone.

Nevertheless, the interpretation of targeted delivery in vivo requires appropriate qualification. Ligand-mediated targeting does not necessarily guarantee selective accumulation exclusively within tumor tissue. Protein-corona formation, receptor heterogeneity, ligand accessibility, reticuloendothelial clearance, and tumor penetration can all influence the ultimate performance of targeted liposomes.^{20, 30} Consequently, the current findings should be regarded as strong preclinical evidence of enhanced therapeutic performance rather than definitive proof of exclusive tumor-specific localization.

4.8. Histopathological and molecular evidence supports the antitumor effect

The histopathological and molecular findings provided additional biological validation of the observed reduction in tumor growth. Ki-67 expression, a widely used marker of proliferative activity, was highest in the control group ($72.4 \pm 5.6\%$) and progressively decreased following treatment with free drug, non-targeted liposomes, and targeted liposomes, reaching $24.1 \pm 3.2\%$ in the targeted group.

The reduction in Ki-67 expression was accompanied by a reciprocal increase in cleaved caspase-3, an established marker of execution-phase apoptosis. Cleaved caspase-3 expression increased from $8.6 \pm 1.8\%$ in controls to $21.4 \pm 2.7\%$ with free drug, $31.8 \pm 3.1\%$ with non-targeted liposomes, and $46.7 \pm 4.2\%$ with targeted liposomes. The inverse relationship between proliferative activity and apoptotic signaling provides molecular support for the superior antitumor effect of the targeted formulation.

The histopathological findings were concordant with these molecular observations, with the most pronounced treatment-associated reduction in tumor-grade/severity score observed in the targeted liposome group. Collectively, the reduction in Ki-67, elevation of cleaved caspase-3, and improvement in histological appearance indicate that targeted liposomal treatment affected both proliferative and apoptotic components of tumor biology.

Importantly, these findings also demonstrate that the therapeutic benefit of the targeted formulation was not

restricted to an isolated cytotoxicity assay. Instead, the biological response was reproduced across multiple experimental levels—from increased cellular uptake and apoptosis *in vitro* to suppression of tumor proliferation and enhancement of apoptotic signaling *in vivo*. Such concordance strengthens the overall mechanistic interpretation of the study.

4.9. Integrated interpretation of targeted versus non-targeted liposomes

The comparative analysis presented in Table 9 provides perhaps the clearest evidence for the added value of active targeting. Targeted liposomes were modestly larger than non-targeted liposomes but exhibited significantly higher encapsulation efficiency. More importantly, the targeted formulation produced significantly lower cell viability, greater cellular uptake, and substantially lower final tumor volume than the non-targeted formulation.

The particle-size difference between the two formulations was statistically significant, although its biological magnitude was relatively modest. In contrast, the differences in cellular uptake and therapeutic efficacy were considerably larger. This distinction is important because it suggests that the biological superiority of the targeted formulation was unlikely to be explained simply by the approximately 13-nm increase in particle diameter. Rather, the substantially enhanced cellular interaction associated with surface targeting provides a more plausible explanation for the observed improvement in biological activity.

The overall pattern therefore supports a mechanistic sequence in which liposomal encapsulation improved drug retention and release characteristics, while surface targeting further increased cellular association and intracellular delivery. The increased intracellular exposure was associated with enhanced cytotoxicity and apoptosis, reduced migratory/invasive behavior, and ultimately greater tumor-growth suppression. This formulation-to-function relationship is consistent with contemporary models of active liposomal targeting in which physicochemical optimization and biological recognition operate synergistically rather than independently.^{18, 25, 31}

4.10. Translational significance and study limitations

The findings have potential significance for the development of precision drug-delivery strategies for OSCC, particularly because conventional systemic therapy remains constrained by limited tumor selectivity and treatment-associated toxicity. Targeted liposomes offer the possibility of integrating controlled drug release with molecular recognition, potentially improving the therapeutic index of established anticancer agents rather than requiring the development of entirely new drugs. Recent literature

has continued to identify liposomes as one of the most mature nanocarrier platforms for tumor-directed therapy, while emphasizing the importance of formulation reproducibility, target validation, stability, and manufacturing scalability for clinical translation.^{16, 18, 32}

Several limitations should nevertheless be acknowledged. First, the present investigation demonstrated enhanced therapeutic performance but did not establish the complete molecular mechanism responsible for ligand-mediated targeting. Receptor-expression analysis, receptor-blocking experiments, competitive ligand studies, and quantitative assessment of ligand density would provide stronger evidence of receptor-specific internalization. Second, although the *in-vitro* uptake results strongly support enhanced cellular association, additional intracellular-localization studies would help distinguish receptor-mediated internalization from nonspecific surface association. Third, the *in-vivo* model does not completely reproduce the anatomical, immunological, vascular, and stromal complexity of human OSCC. Fourth, the present study did not comprehensively establish pharmacokinetic and biodistribution profiles, which are essential for determining whether enhanced antitumor activity is accompanied by improved tumor-to-normal-tissue drug exposure. Finally, long-term toxicity, immunogenicity, formulation scalability, and manufacturing reproducibility require further investigation before clinical translation can be contemplated.

These limitations do not diminish the central finding of the study but instead define the next stages of translational development. Future investigations should incorporate quantitative biodistribution studies, pharmacokinetic profiling, receptor-specific validation, comprehensive toxicological evaluation, and orthotopic or patient-derived OSCC models. Such studies would determine whether the enhanced therapeutic response observed in the present work can be reproduced under clinically more representative conditions.

4.11. Overall significance

Taken together, the present findings demonstrate that targeted liposomal delivery substantially improved the therapeutic performance of the encapsulated drug against OSCC. The formulation exhibited favorable nanoscale characteristics and high drug encapsulation while providing sustained drug release. More importantly, surface targeting was associated with a marked increase in cellular uptake, enhanced cytotoxicity, greater apoptotic induction, suppression of migration/invasion, reduced tumor-cell proliferation, increased cleaved caspase-3 expression, and superior inhibition of tumor growth *in vivo*.

The consistency of these effects across physicochemical, cellular, molecular, and animal-

level endpoints supports the hypothesis that targeted liposomes can provide a more effective delivery platform than free drug or non-targeted liposomes. The present study therefore provides a rational preclinical foundation for the further development of receptor-targeted liposomal systems as precision drug-delivery platforms for OSCC. At the same time, successful clinical translation will require rigorous validation of target specificity, pharmacokinetics, biodistribution, safety, manufacturing reproducibility, and performance in clinically representative tumor models.^{30, 32}

5. CONCLUSION

The present study demonstrates that folic acid-targeted doxorubicin-loaded liposomes provide a promising drug-delivery strategy for oral squamous cell carcinoma (OSCC). The optimized targeted formulation exhibited favorable physicochemical characteristics, high drug encapsulation, sustained drug release, and enhanced cellular uptake compared with free doxorubicin and non-targeted liposomes. These advantages were associated with greater cytotoxicity, increased apoptosis, inhibition of OSCC cell migration and invasion, reduced tumor-cell proliferation, enhanced cleaved caspase-3 expression, and significantly greater inhibition of tumor growth in vivo.

Overall, the findings indicate that surface functionalization of liposomes can provide an additional therapeutic advantage beyond conventional liposomal drug delivery. The targeted formulation therefore represents a promising preclinical platform for improving the precision and therapeutic efficacy of doxorubicin in OSCC. Further studies involving receptor-specific validation, pharmacokinetic and biodistribution assessment, long-term toxicity evaluation, and clinically relevant OSCC models are warranted to establish its translational potential.

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