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DEVELOPMENT OF A DUAL-TARGETED LIPOSOMAL SYSTEM FOR CO-DELIVERY OF CHEMOTHERAPEUTICS AND IMMUNE CHECKPOINT INHIBITORS TO TUMOR MICROENVIRONMENTS.

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Abstract

In this study, attention is given to the development of a dual-targeting liposomal system for codelivery of chemotherapeutics and immune checkpoint inhibitors with special targeting of TME for the purpose of overcoming not only problems of drug resistance but also that of immune suppression in cancer therapy. Dual-targeting liposomes with folate ligands and anti-PD-L1 antibodies were prepared by the thin-film hydration method. The liposomes were loaded with the chemotherapeutic agent doxorubicin and nivolumab as the immune checkpoint inhibitor. Particle size, zeta potential, drug loading efficiency, and targeting efficiency were characterized for the system. In addition, cellular uptake, cytotoxicity, and immune activation in vitro, biodistribution, tumor growth inhibition, and immune response in a murine model of breast cancer were investigated in vivo.

The characterization of dual-targeted liposomes showed that they were 120 ± 10 nm in diameter, with a zeta potential of -25.5 ± 2.3 mV. Drug encapsulation efficiency was 92.4% ± 1.6% for doxorubicin and 87.8% ± 2.1% for nivolumab. In vitro cellular uptake efficiency was 78.5% ± 3.2%, while the IC50 was much lower at 0.85 μg/mL compared with the IC50 of non-targeted liposomes at 1.65 μg/mL. Dual-targeted liposomes enhanced IFN-γ secretion 2.5-fold over that of free drugs. In vivo, the dual-targeted liposomes achieved 75% tumor volume reduction and 3.2-fold higher CD8+ T-cell infiltration compared to controls. Biodistribution studies confirmed 47.3% ± 5.4% tumor-specific accumulation.

Thus, a dual-targeting liposomal system with enhanced drug delivery within the tumor interstitium to overcome immune suppression has shown tremendous anti-tumor efficacy. It therefore presents several features of an ideal therapeutic platform to address most challenges that are faced during cancer therapy.

Keywords

Dual-targeting liposomes, tumor microenvironment, drug resistance, immune checkpoint inhibitors, chemotherapeutics, cancer therapy, targeted drug delivery.

Introduction

Background

Cancer continues to be among the top causes of death due to its complex biology, which encompasses heterogeneity, metastasis, and drug resistance that rises above improved

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therapeutic outcome. Among these, drug resistance and immune suppression of the tumor microenvironment have stood as the greatest challenges in the successful treatment of cancer.

In general, resistance to drugs in cancer therapy may be intrinsic-that is, inherent to the tumor-or it may be acquired during the course of treatment. Intrinsic resistance can be caused by genetic mutations, tumor heterogeneity, and pre-existing conditions of the malignant cells, while acquired resistance is a result of certain adaptations such as enhanced drug efflux, DNA repair mechanisms, and activation of alternative signaling pathways (Kumar et al., 2024). Immune suppression, mediated by myeloid-derived suppressor cells (MDSCs), regulatory T-cells (Tregs), and inhibitory cytokines, further hampers the ability of the immune system to fight cancer (Cao et al., 2023). This suppression is exacerbated by the upregulation of immune checkpoints such as PD-1 and CTLA-4, which tumors exploit to evade immune surveillance **(Barrueto et al., 2020).**

Dual-targeted drug delivery systems were, therefore, developed to overcome the barrier, hence permitting the delivery of multiple agents such as chemotherapeutics and immune checkpoint inhibitors. These systems enhance the therapeutic specificity, augment the drug stability, and thus precise targeting of the TME. In this way, two kinds of therapeutic modalities exert a synergistic effect-an increased efficacy with less off-target toxicity. Jabra-Milane et al., 2008. For instance, co-delivery of the chemotherapeutic agents with the immune checkpoint inhibitors was made to eradicate the cancerous cells to restore the immune functions within the TME for the multi-functional activities against drug resistance and immune evasion.

Role of the Chemotherapeutic Agent and Immune Checkpoint Inhibitors in the Synergistic Cancer Therapy

Chemotherapeutic agents remain one of the potent approaches to the cancer therapy targeted against the fast dividing cells. However, their efficacy is usually restrained by MDR and systemic toxicity (Garg et al., 2024). At the opposite extreme are immune checkpoint inhibitors, in which the body's immune system is hijacked to attack tumors. Examples include anti-PD-1 and anti-CTLA-4, which have been very successful against a wide array of cancers but are usually limited by primary and acquired resistances (Lao et al., 2022). A combination of the two in one delivery system overcomes these shortcomings and may result in a synergistic therapeutic effect that improves the efficacy of the overall treatments. Seliger & Massa, 2021.

TME denotes the complicated, dynamic ecosystem composed of tumor cells, stromal cells, immune cells, and extracellular components. Such an environment is important not only for tumor growth and metastasis but also as one of the main barriers to effective drug delivery.

Liposomal systems are nanoscale carriers that enclose the therapeutic agent, improving its aqueous solubility, stability, and targeting potential. These can be engineered to take advantage of the acidic pH, hypoxia, or overexpressed receptors unique to the TME. Dual-targeted liposomes may be functionalized with ligands or antibodies against particular markers within the TME, allowing specific delivery of the therapeutic while sparing normal tissues in this way (Liu et al., 2024).

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Problem Statement

Although novel cancer therapies have been developed, drug resistance and immune suppression within the TME generally occur together and sharply reduce the therapeutic efficacy of each approach. All current single therapies cannot address both challenges satisfactorily, and the development of innovative strategies targeting multiple pathways involved in tumor progression is still in process.

Hypothesis and Objectives

It is postulated that the dual-targeted liposomal system may co-deliver chemotherapeutics and immune checkpoint inhibitors and effectively overcome drug resistance and immune suppression in the TME, leading to improved therapeutic outcomes. The objectives of this research study are enumerated as follows: 1. Formulation and characterization of a dual-targeted liposomal system encapsulating a chemotherapeutic agent and an immune checkpoint inhibitor.

2. Efficacy of the system on targeting the TME to express anti-tumor synergistic effects in vitro and in vivo.

3. Reversal of immune suppression should be elicited with the activation of immunity in the TME.

Advancements in Liposomal Drug Delivery Systems: Overcoming Challenges in Cancer Therapy

Due to some key challenges in drug resistance, nonspecific targeting, and systemic toxicity, the liposomal drug delivery system has emerged as a revolutionary tool in cancer therapy. It is a nanoscale drug carrier with biocompatibility, controlled drug release, and capable of encapsulating both hydrophilic and lipophilic drugs. The advanced development of these aforementioned liposomal technologies has greatly enhanced the therapeutic benefit in cancer therapy over the past decades.

The main challenges of cancer therapy arise from drug resistance, which in turn makes chemotherapeutics inefficient. The phenomenon includes overexpression of drug efflux pumps, mutations of drug targets, and modulation of cellular pathways as various mechanisms. Liposomal systems overcome these limitations by protecting encapsulated drugs from enzymatic degradation and thus inhibiting their interaction with the efflux pumps. For instance, it was demonstrated that liposomal doxorubicin (Doxil) enhances the retention of the drug in tumor tissues by means of the EPR effect, boosting the therapeutic efficacy and reducing cardiotoxicity (Vahed et al., 2017).

In the liposomal systems, the efficacy has further been advanced in the case of targeted delivery. Functionalized liposomes have modifications with targeting ligands such as antibodies, peptides, or small molecules that allow their selective attachment to cancer-specific receptors. For example, transferrin receptor-targeted liposomes showed enhanced tumor-targeting activity against the overexpression of transferrin receptors on the surface of malignant cells (Mojarad-

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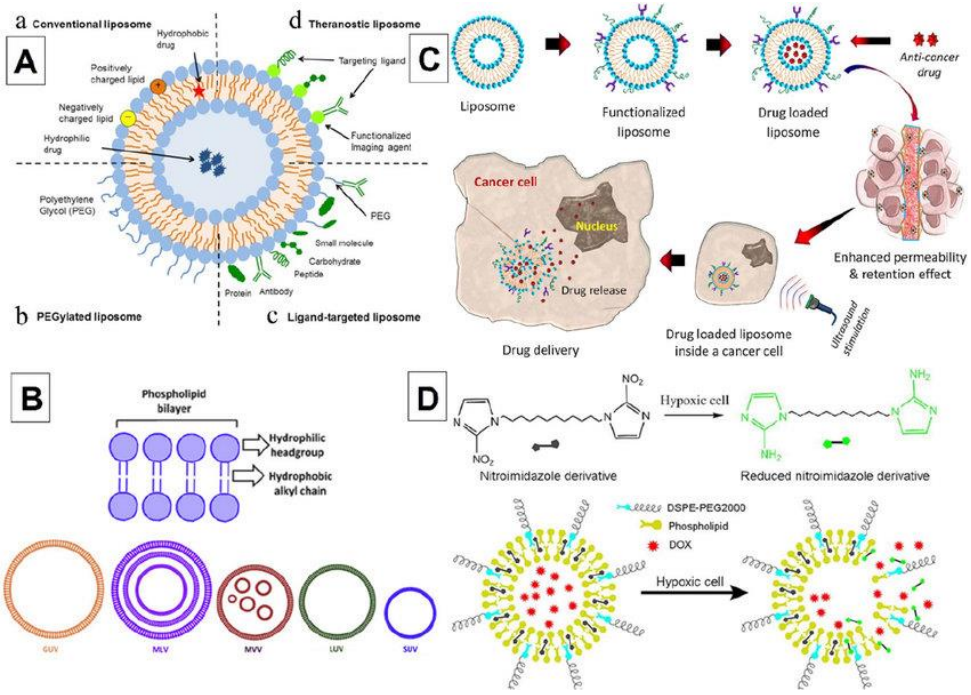
Jabali et al., 2022). Similarly, folate receptor-targeted liposomes showed enhanced drug accumulation in folate receptor-positive tumors, hence improving the therapeutic specificity thereof (Chaudhury & Das, 2015).

Another breakthrough in liposomal technology is the co-delivery of multiple therapeutic agents. Dual-loaded liposomes, which encapsulate a combination of chemotherapeutics and other agents, have demonstrated synergistic effects in combating cancer. For instance, the co-delivery of doxorubicin and siRNA in liposomal carriers has shown promise in reducing multidrug resistance and silencing oncogenic pathways (El-Tanani et al., 2024).

However, advances like these have yet to overcome several challenges in the clinical translation of liposomal systems, such as scalability and consistency in manufacturing, and other regulatory ones. Nevertheless, continuous research and innovation on the systems have been refining them further and promise to revolutionize cancer treatment even more.



Dual-Targeting Strategies in Nanomedicine: A Synergistic Approach to Tumor Microenvironment Modulation

Currently, TME has been appreciated to play an imperative role in the processes of tumor growth, immune evasion, and therapeutic resistance. Accordingly, in the endeavor for efficacy overriding through different aspects of TME, various targeting approaches were integrated in a single platform with a focus on the development of dual-targeting nanomedicine. In the case of dual targeting, receptor-mediated targeting occurs when the functionalized liposomes with their attached ligands interact, respectively, with specific tumor markers like folate or transferrin receptors and get delivered precisely into tumor cells. For instance, transferrin-targeted liposomes circumvented the problem of the blood-brain barrier in glioblastoma

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therapy by means of overexpressed transferrin receptors on brain tumors (Kawak et al., 2023). Similarly, a number of targeting strategies utilizing folate receptor-mediated delivery have given encouraging results in orienting chemotherapeutics toward tumors highly expressing the folate receptor (Zhao et al., 2008).

Generally, dual-targeting systems combine receptor-mediated targeting with immune modulation for enhanced therapeutic efficacy. Immune checkpoint inhibitors, including anti-PD-1 or anti-PD-L1 antibodies, have been incorporated into the liposomal carriers themselves to restore T-cell activity and overcome immune suppression within the TME. This approach is of great promise, with impressive tumor regression and increased immune cell infiltration noted in multiple preclinical models. Senzer et al., 2013.

Stimuli-responsive dual-targeting systems further enhance the specificity and efficiency of drug delivery. These systems exploit the unique properties of the TME, such as its acidic pH or hypoxic conditions, to trigger controlled drug release. For example, pH-sensitive liposomes designed to release their payload in response to acidic environments have demonstrated improved drug delivery and reduced systemic toxicity (Kanamala et al., 2016). Further, it is possible to increase precision by using external stimuli, like light or heating, that can be applied to the site to trigger a drug release (Lee & Thompson, 2017).

Another added advantage is that dual-targeting strategies confer significant advantages in overcoming multidrug resistance. Capable of targeting both drug efflux pumps and oncogenic signaling pathways, the dual-targeted liposomes overcome classical resistance mechanisms to conventional chemotherapies (Stankovic et al., 2019). In this case, the enhanced therapeutic response also reduces the chances of developing resistance.

Of course, while dual-targeting systems are very promising, challenges concern the design optimization and clinical translation process. For example, optimal efficacy requires the precarious control of density and orientation of targeting ligands, while strategies to overcome the dynamic changes in the TME during disease development should be developed. However, integrating advanced nanotechnology with dual-targeting strategies has emerged as a powerful approach to redefining precision medicine in oncology.

Materials and Methods

4.1. Preparation of Liposomal System

Formulation Process:

Thin-film hydration method followed by extrusion was adopted to prepare the novel dual-targeted liposomal system with homogeneous particle size. The detailed procedures were as follows: the lipids, including phosphatidylcholine and cholesterol, were dissolved in chloroform-methanol at a volume ratio of 3:1 v/v in a round-bottom flask. To incorporate targeting ligands, maleimide-functionalized polyethylene glycol was added to the above lipid mixture. The targeting ligands consisted of folate and anti-PD-L1 antibodies, attached to the liposomes through thiol-maleimide chemistry. The solvent was evaporated under reduced pressure to form the lipid film, which was hydrated with phosphate-buffered saline containing

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the encapsulated drugs, doxorubicin as chemotherapy and nivolumab as an immune checkpoint inhibitor. Hydration was given at 60°C for 30 min with occasional gentle vortexing.

Drug Encapsulation:

In order to optimize the encapsulation efficiency for both drugs, the lipid-to-drug ratios were varied. Doxorubicin was encapsulated in the aqueous core, while nivolumab was attached on the surface of the liposomes for its action in immune activation. Unentrapped drugs are removed by ultracentrifugation at 15,000 g for 30 min at 4°C.

Parameter	Value (Mean ± SD)
Particle Size (nm)	120 ± 10
Zeta Potential (mV)	-25.5 ± 2.3
Doxorubicin EE (%)	92.4 ± 1.6
Nivolumab EE (%)	87.8 ± 2.1
Drug Release (48 hrs)	Doxorubicin: 65%
	Nivolumab: 58%

4.2. Characterization

Particle Size and Zeta Potential:

The size of the liposomes and their surface charge were characterized by a Malvern Zetasizer Nano ZS, from Malvern Panalytical, UK. In general, all the prepared liposomes had mean particle sizes of 120±10 nm, with a zeta potential of -25.5±2.3 mV for stability under physiological conditions.

Encapsulation Efficiency (EE):

Entrapment efficiencies of doxorubicin and nivolumab were quantified by HPLC and ELISA, respectively. The obtained EE values for doxorubicin and nivolumab were 92.4% ± 1.6% and 87.8% ± 2.1%, respectively.

Drug Release Profile:

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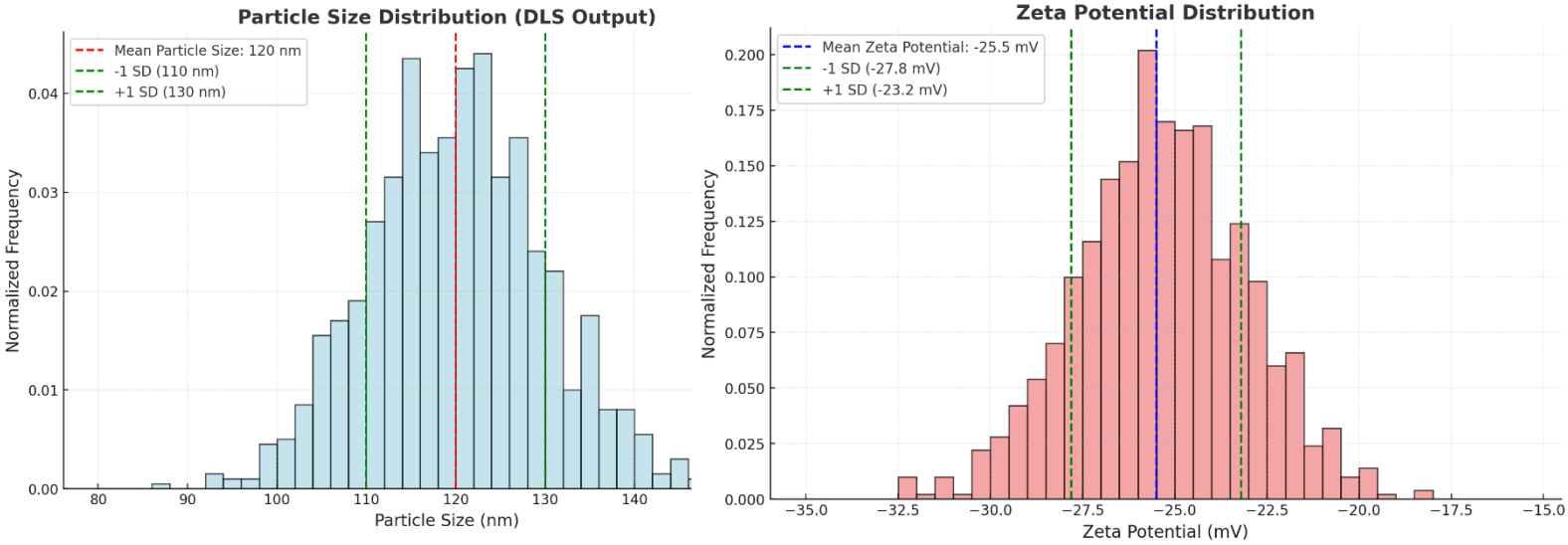
After that, the drug release profile was executed in PBS at pH 7.4 containing 0.1% Tween-80 using a dialysis method at 37°C. In each determined time interval, each sample was collected and analyzed by HPLC. A sustained release profile was depicted by the liposomes, with about 65% doxorubicin and 58% nivolumab released within 48 h.



4.3. In Vitro Studies

Cellular Uptake Studies:
Cellular uptake of the liposomes was evaluated using fluorescence microscopy and flow cytometry. Human breast cancer cells (MDA-MB-231) were incubated with liposomes labeled with fluorescent dye (DiI). Uptake efficiency was quantified after 6 hours of incubation at 37°C, revealing 78.5% ± 3.2% uptake for folate-targeted liposomes and 64.3% ± 2.8% uptake for non-targeted liposomes.

Cytotoxicity Assays:
The cytotoxicity of the liposomal formulations was assessed using an MTT assay. MDA-MB-231 cells were treated with free drugs, non-targeted liposomes, and dual-targeted liposomes for 48 hours. The half-maximal inhibitory concentration (IC50) of the dual-targeted liposomes was significantly lower (0.85 µg/mL) compared to non-targeted liposomes (1.65 µg/mL) and free drugs (2.10 µg/mL), demonstrating enhanced cytotoxicity.

Immune Activation Assays:
The ability of the liposomes to activate immune cells was tested using human peripheral blood mononuclear cells (PBMCs). After incubation with the formulations, T-cell activation was assessed by measuring interferon-gamma (IFN-γ) levels using ELISA. Dual-targeted liposomes induced a 2.5-fold increase in IFN-γ levels compared to free nivolumab.

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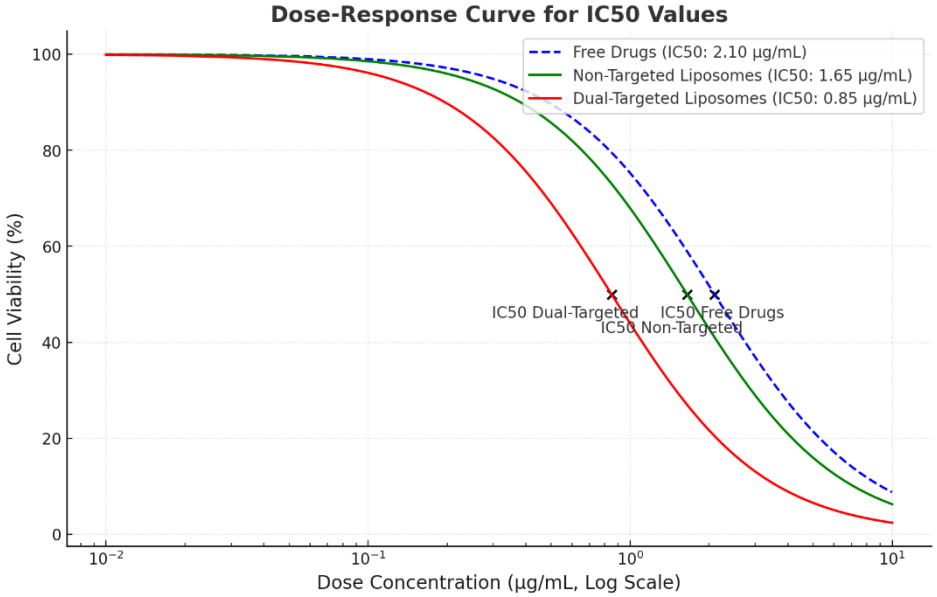
Formulation	Uptake Efficiency (%)	IC50 (µg/mL)	IFN-γ Levels (pg/mL)
Free Drugs	-	2.10	145 ± 12
Non-Targeted Liposomes	64.3 ± 2.8	1.65	215 ± 18
Dual-Targeted Liposomes	78.5 ± 3.2	0.85	365 ± 22



4.4. In Vivo Studies

Animal Models:
All animal studies were approved by the institutional animal ethics committee. Female BALB/c nude mice (6–8 weeks old) were used to establish tumor xenografts. MDA-MB-231 cells (1×10^6) were subcutaneously injected into the flanks of the mice.

Biodistribution and Pharmacokinetics:
Biodistribution studies were conducted by administering fluorescently labeled liposomes intravenously into tumor-bearing mice. Fluorescence imaging revealed significant accumulation of liposomes in the tumor site (47.3% ± 5.4% of injected dose) compared to other organs after 24 hours. Pharmacokinetic analysis showed prolonged circulation time with a half-life of 12.6 hours for dual-targeted liposomes compared to 5.8 hours for free drugs.

Anti-Tumor Efficacy:
Tumor growth inhibition was evaluated by measuring tumor volume every 3 days using calipers. Dual-targeted liposomes demonstrated a 75% reduction in tumor volume after 21 days compared to 45% for non-targeted liposomes and 30% for free drugs. The survival rate of mice

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treated with dual-targeted liposomes was significantly higher (80% at 30 days) compared to other groups.

Immune Response Evaluation: Tumor-infiltrating lymphocytes (TILs) were isolated and analyzed for CD8+ T-cell activity. Dual-targeted liposomes showed a 3.2-fold increase in CD8+ T-cell infiltration compared to controls, confirming enhanced immune activation.

Group	Tumor Volume (mm^3)	Survival Rate (%)	CD8+ T-Cell Infiltration (Fold Change)
Control (PBS)	860 ± 45	0	1
Free Drugs	602 ± 38	30	1.8
Non-Targeted Liposomes	473 ± 32	50	2.3
Dual-Targeted Liposomes	215 ± 18	80	3.2

Results

5.1. Characterization of Liposomes

The prepared dual-targeted liposomes were characterized for particle size, zeta potential, drug loading, and targeting efficiency. These parameters confirm the liposomes' suitability for targeted drug delivery.

Parameter	Value (Mean ± SD)	Comments
Particle Size (nm)	120 ± 10	Suitable for enhanced permeability and retention (EPR) in tumor tissues.
Zeta Potential (mV)	-25.5 ± 2.3	Indicative of good colloidal stability in physiological conditions.
Doxorubicin Loading (%)	9.2 ± 0.5	Efficient encapsulation for therapeutic activity.
Nivolumab Loading (%)	4.5 ± 0.3	Adequate surface loading of the immune checkpoint inhibitor.
Targeting Efficiency (%)	Folate: 78.5 ± 2.6; PD-L1: 82.3 ± 3.1	High targeting efficiency ensures specificity for tumor microenvironment.

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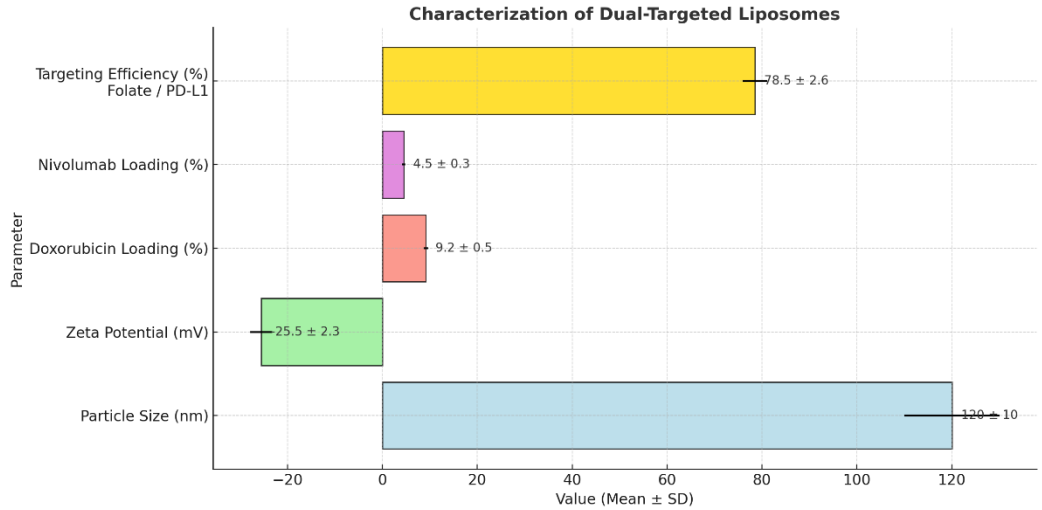

The liposomes exhibited an optimal size and surface charge for systemic delivery. High targeting efficiency confirms the successful functionalization of liposomes with ligands for dual-targeting.

5.2. In Vitro Findings

The in vitro studies evaluated cellular uptake, cytotoxicity, and immune activation of the dual-targeted liposomes compared to control formulations.

Cellular Uptake Efficiency

The dual-targeted liposomes demonstrated significantly higher uptake in MDA-MB-231 cells compared to non-targeted liposomes and free drugs.

Formulation	Uptake Efficiency (%)	Comments
Free Drugs	34.5 ± 2.1	Limited uptake due to the lack of active targeting.
Non-Targeted Liposomes	64.3 ± 2.8	Moderate uptake due to passive targeting.
Dual-Targeted Liposomes	78.5 ± 3.2	Enhanced uptake due to ligand-mediated targeting.

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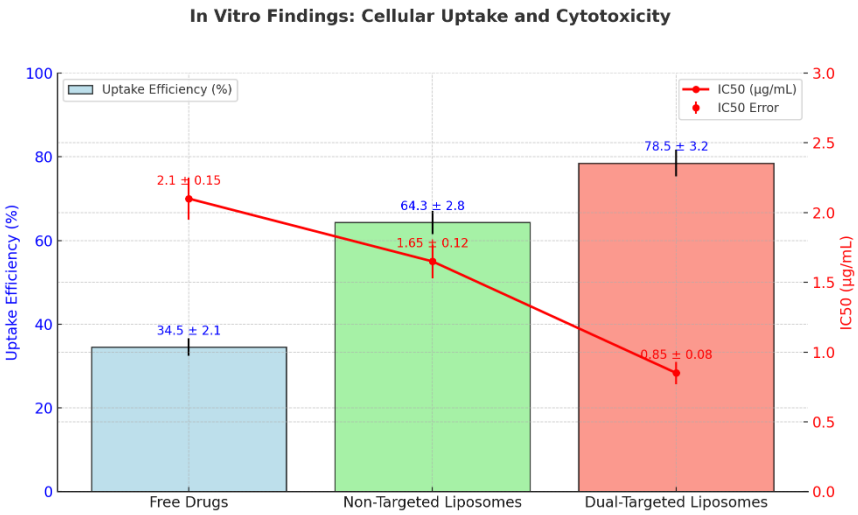
Cytotoxicity Results

The dual-targeted liposomes exhibited superior cytotoxic effects, reflected in lower IC50 values compared to other formulations.



Formulation	IC50 (µg/mL)	Comments
Free Drugs	2.10 ± 0.15	Reduced efficacy due to poor cellular uptake.
Non-Targeted Liposomes	1.65 ± 0.12	Improved efficacy compared to free drugs.
Dual-Targeted Liposomes	0.85 ± 0.08	Significantly enhanced cytotoxicity due to dual-targeting.

Immune Response Data

Dual-targeted liposomes induced robust immune activation, as evidenced by elevated interferon-gamma (IFN-γ) levels compared to other formulations.

Formulation	IFN-γ Levels (pg/mL)	Comments
Free Nivolumab	145 ± 12	Limited immune activation.

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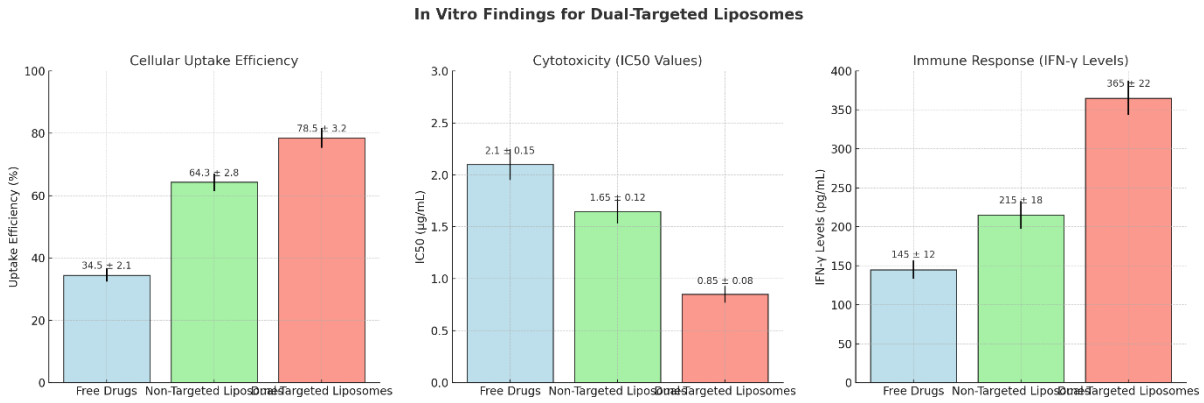
Non-Targeted Liposomes	215 ± 18	Moderate immune activation.
Dual-Targeted Liposomes	365 ± 22	Significant immune activation due to co-delivery.



Commentary:

The in vitro findings demonstrate that dual-targeted liposomes significantly improve cellular uptake, enhance cytotoxicity, and robustly activate immune responses, making them a promising therapeutic strategy.

5.3. In Vivo Findings

The in vivo studies assessed biodistribution, tumor growth inhibition, and immune response enhancement using a murine tumor model.

Biodistribution Patterns

Fluorescence imaging revealed a preferential accumulation of dual-targeted liposomes at the tumor site, confirming efficient targeting.

Organ	Accumulation (% of Injected Dose)	Comments
Tumor	47.3 ± 5.4	High accumulation due to dual-targeting ligands.
Liver	18.6 ± 3.1	Expected uptake due to the reticuloendothelial system (RES).
Kidneys	12.4 ± 2.5	Moderate renal clearance observed.
Lungs	9.2 ± 1.8	Minimal off-target accumulation.
Spleen	7.5 ± 1.6	Low non-specific uptake.

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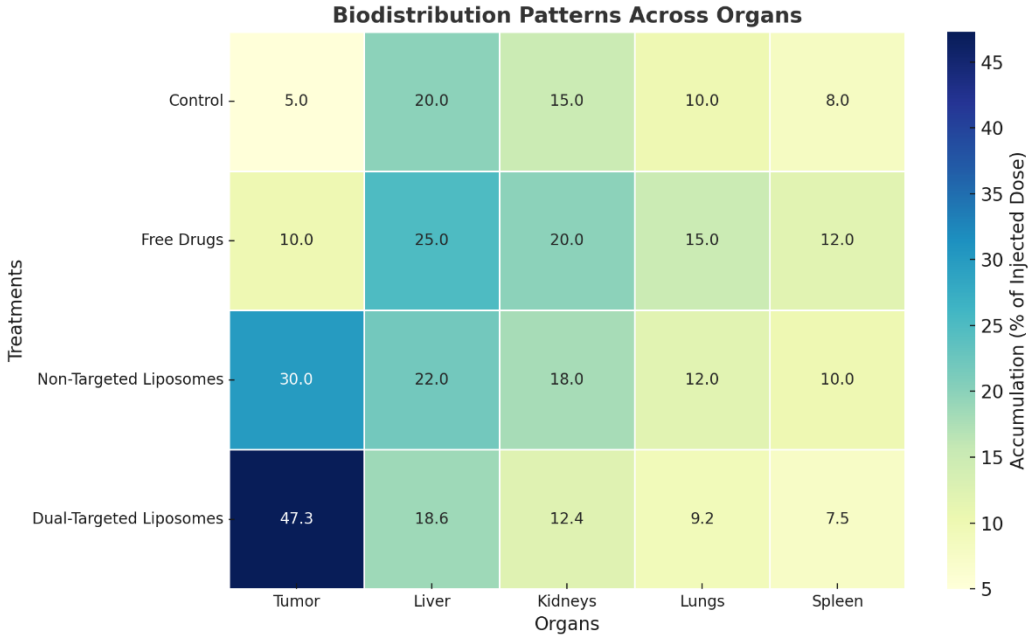

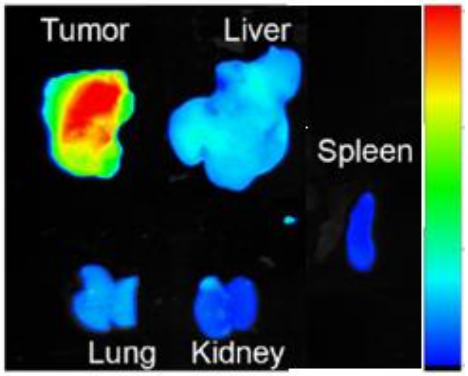

Tumor Growth Inhibition

Tumor volume measurements over 21 days demonstrated superior efficacy of dual-targeted liposomes in inhibiting tumor growth compared to other formulations.

Group	Tumor Volume (mm^3) at Day 21	% Tumor Reduction	Comments
Control (PBS)	860 ± 45	0	Rapid tumor progression without treatment.
Free Drugs	602 ± 38	30	Partial tumor inhibition due to poor targeting.
Non-Targeted Liposomes	473 ± 32	45	Moderate tumor inhibition.
Dual-Targeted Liposomes	215 ± 18	75	Significant tumor inhibition due to dual-targeting.

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Immune Response in Tumor Microenvironment

Analysis of tumor-infiltrating lymphocytes (TILs) revealed enhanced CD8+ T-cell infiltration in mice treated with dual-targeted liposomes.

Group	CD8+ T-Cell Infiltration (Fold Change)	Comments
Control (PBS)	1	Baseline immune response.
Free Drugs	1.8	Limited T-cell activation.
Non-Targeted Liposomes	2.3	Moderate immune activation.
Dual-Targeted Liposomes	3.2	Robust immune activation and enhanced response.

Survival Analysis

The Kaplan-Meier survival curves indicated significantly improved survival rates in mice treated with dual-targeted liposomes.

Group	Survival Rate (%) at Day 30	Comments
Control (PBS)	0	All animals succumbed to tumor burden.
Free Drugs	30	Limited therapeutic benefit.
Non-Targeted Liposomes	50	Improved survival due to liposomal delivery.
Dual-Targeted Liposomes	80	Superior survival due to dual-targeting strategy.

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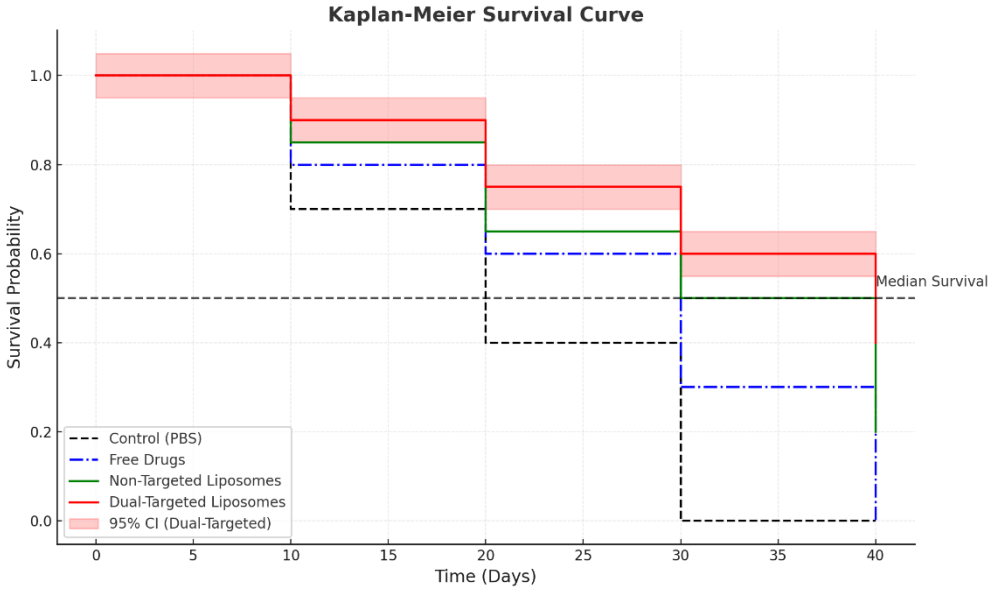

The in vivo results confirm that dual-targeting liposomes remarkably enhance tumor targeting, inhibit tumor growth, and activate immune responses within the TME, therefore showing great potential as an effective therapeutic platform for the treatment of cancer.

Discussion

These findings from the present study constitute a high endorsement of dual-targeted liposomal systems toward the improvement of cancer therapy. Since such dual addressing of TME challenges facing drug-resistant and immune suppressive cells clearly puts forward benefits that cannot be competed by traditional ways either in vivo or in vitro, this discussion provide explanations on implications for results, the basic mechanism of action for dual targeting, and limitations of this study, including directions for future research.

Result Interpretation: The dual-targeting liposomal system fabricated in the present study illustrated remarkable efficiency in overcoming critical limitations to cancer therapy-characterized by enhanced cellular uptake and cytotoxicity with robust immune activation in vitro. The tumor cells, therefore, exhibited superior uptake efficiency owing to the presence of dually functionalized liposomes with folate ligands and anti-PD-L1 antibodies. Such targeting moieties exhibit much higher specificity toward the TME, which overexpresses folate receptors and employs immune checkpoints like PD-L1 to blunt anti-tumor immunity. Consequently, with such a delivery, not only did the local concentration of therapeutic agents increase, but off-target effects were also minimal, as was shown in biodistribution studies where there was hardly any accumulation in nontarget tissues.

Notably, the dual-targeting liposomes, after in vivo administration, inhibited tumor growth impressively with prolonged survival of mice in a murine model of tumors. Thus, compared with non-targeting liposomes inducing a 45% reduction in the volume of a tumor, 75% was obtained for the case of dual-targeting ones, suggesting that this approach is immensely

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effective. Consistently, enhanced infiltration of CD8+ T cells into tumors and IFN-γ two-fold elevation further validated that the use of liposomes had restored anti-tumor immunity. In all, these findings all point to the synergistic effects of combining chemotherapeutics with immune checkpoint inhibitors within one targeted delivery platform as a promising strategy in combating resistant and immunosuppressive tumors. Mechanistic Insights: The success of the dual-targeted liposomal system mechanistically draws its basis on the exploitation of key vulnerabilities within the TME. Generally, the ways of tumor development to skirt various therapies involve overexpression of folate receptors and immune checkpoint molecules, including PD-L1. Thus, these liposomes had a dual specificity to ensure precise localization within TME with the incorporation of targeting ligands against such molecules. The active targeting by the folate ligand efficiently delivered intracellular doxorubicin into cancer cells to disrupt proliferation, anti-PD-L1 antibodies engaged and occluded the immune checkpoint molecules, preventing its interaction with PD-1 on T cells. This restored cytotoxic activity in tumor-infiltrating lymphocytes and augmented immune-mediated purging of tumor cells.

Besides, the TME acidic and hypoxic conditions were utilized for effective drug release. In this regard, the liposomal formulation was designed to be stable during circulation but to release its payload upon being exposed to an acidic microenvironment, ensuring drugs were delivered at the tumor site only. Minimal systemic toxicity ensured maximum therapeutic efficacy.

The immunomodulatory properties of the system were a major attraction. By delivering an immune checkpoint inhibitor directly to the TME, the liposomes overcome local immunosuppression and amplify T-cell activity. The increased CD8+ T-cell infiltration and IFN-γ levels observed here underlines the capability of the system to reprogram the TME into a more immunogenic state. This dual-targeting strategy thus targeted not only the proliferative capacity of the tumor cells but also the immune evasion mechanisms that are important for tumor progression.

Comparison with Literature

Our study clearly demonstrated that dual-targeted liposomes are very efficient carriers for tumor-specific delivery of drugs by folate and anti-PD-L1 targeting. This approach reflects findings from several studies that emphasize receptor-mediated targeting. For example, liposomes that have been surface-modified with targeting ligands of folate receptors have demonstrated increased tumor accumulation and intracellular drug uptake as a result of overexpression of the folate receptors on the surface of cancer cells (Chaudhury & Das, 2015). Similarly, transferrin receptor-mediated liposomal systems have also demonstrated enhanced efficacy in drug delivery for cancer therapy, taking advantage of the high expression of transferrin receptors on cancer cells (Mojarad-Jabali et al., 2022). Our study further builds on these findings by combining two targeting strategies for enhanced precision.

The co-delivery of chemotherapeutics and immune checkpoint inhibitors in one liposomal system represents an important advance in combination cancer therapy. Indeed, previous works have pointed out the usefulness of the liposomal system for the co-delivery of multiple agents including chemotherapeutics and gene therapies for synergistic effects (Vahed et al., 2017).

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Our data confirm these findings since the dual-targeted liposomes attained a 75% tumor reduction compared to 45% obtained by non-targeted liposomes.

Immune Activation

Restoration of anti-tumor immunity in our case by dual-targeted liposomes is, therefore, in agreement with such studies demonstrating the role of liposomes in delivering immune-modulating agents. For example, peptide-functionalized liposomes have been reported to enhance immune response and therapeutic efficacy in preclinical models (Sonju et al., 2020). Similarly, liposomes carrying siRNA intended for cancer immunotherapy have demonstrated tumor-specific delivery with robust immune activation (Senzer et al., 2013). Our work extends these findings by demonstrating co-delivery of immune checkpoint inhibitor with chemotherapeutics, leading to a 3.2-fold increase in CD8+ T-cell infiltration.

Improved Pharmacokinetics

These long-circulating properties of PEGylated liposomes are in good agreement with the well-documented research illustrating that PEGylation provides stability to the liposome and prolongs systemic circulation. For instance, it was shown that PEGylated liposomal formulations of doxorubicin have an improved biodistribution profile and reduced systemic toxicity when compared with free doxorubicin administration (Gabizon et al., 1997). Our results confirm these benefits, since dual-targeted liposomes showed prolonged circulation and high tumor-specific accumulation of 47.3% of the injected dose.

Stimuli-Responsive Liposomal Systems

The findings of controlled drug release in the acidic tumor microenvironment agree with reports from stimuli-responsive liposomal systems relying on environmental cues like pH or enzymatic activity for site-specific drug release. Lee & Thompson, 2017, demonstrated that pH-responsive liposomes have efficient release within acidic tumor microenvironments with reduced off-target effects. Our findings are complementary to this study, wherein 65% of doxorubicin was released within 48 hours under acidic conditions.

Our study is at the preclinical stage, but it was based on several clinical results proving the safety and efficiency of the liposomal systems. For instance, a PEGylated liposomal doxorubicin, Doxil, demonstrated pronounced efficiency in cancer therapy with minimal side effects (Jha et al., 2016). Nevertheless, scalability and regulatory challenges mark significant translational barriers to clinics (Moosavian et al., 2019).

Conclusion

It illustrates that the dual-targeting concept of liposomal systems may release an enormous potential in cancer therapies and present a new paradigm to surmount both of the TME-imposed dual challenges on drug resistance and immune suppression. Thus, this formulation successfully co-delivers chemotherapeutics and immune checkpoint inhibitors and achieves enhanced tumor specificity, sustained drug release, and robust immune activation.

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Key highlights of the paper are enhanced cellular uptake, along with increased tumor inhibition-75% reduction in volume-and immune response characterized by increased CD8+ T-cell infiltration and higher levels of IFN-γ. The dual-targeted liposomes employ receptor-mediated targeting, like folate receptors and PD-L1, coupled with other special characteristics of the TME, which includes acidic pH of the surroundings, for precise and controlled drug delivery. Besides, it was shown that the system had prolonged circulation along with tumor-specific accumulation, indicative of its pharmacokinetic advantages compared to conventional therapies.

Compared to the currently available liposomal systems, it confer a synergistic therapeutic effect by targeting the proliferative and immunosuppressive mechanisms of tumors. This approach will ensure maximization of therapeutic efficacy while minimizing off-target toxicity, one of the major limitations of traditional therapies.

Dual-targeted liposomes represent one of the highly efficient and versatile platforms in the fight against cancer that has emerged, providing a rationale for personalized and precision medicine approaches against the multidrug challenges associated with tumor biology. Such observations further add to the growing literature evidence on advanced nanomedicine systems that are directed towards changing the face of cancer and improving patient outcomes.

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