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NANOPARTICLES-BASED DELIVERY SYSTEM FOR CRISPR-MEDIATED GENE DISRUPTION AND TO DELIVER CRISPR CAS9 COMPONENTS TO CANCER CELLS

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ABSTRACT

Cancers are getting harder to treat because of poor editing efficiency within the tumors and the damage caused by conventional delivery methods. Our study details a system that comprises of lipid nanoparticles (LNP) with a newly designed amino ionizable lipid for enhanced delivery of CRISPR-Cas9, in so doing breathing life to the notion of gene editing. The direct use of CRISPR-LNPs targeting PLK1 (injecting sgPLK1-cLNPs) into the brain of an advanced glioblastoma led to more than 65% gene editing within the body, this also led to a 45% decrease in the rate of tumor proliferation along with a 25% increase in overall survival. For metastatic tumors, the LNPs were additionally modified by several means and made for antibody-mediated targeting. EGFR targeting led to significant accumulation of the clip PLK1 containing LNPs to disseminated ovarian tumors implants, resulting in approximate 75%. This greatly inhibited the rate of tumor

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growth and increased longevity by approximately 70%. This study paves the way of utilizing nanoparticles containing CRISPR to easily and effortlessly target and edit cancerous cells. This work will profoundly alter the landscape of cancer therapy and gene editing techniques.

KEYWORDS: Nanoparticles, Cancer cells, CRISPR, EGFR, CAS9

INTRODUCTION

Molecularly targeted inhibitors and immunotherapy have markedly enhanced cancer treatment outcomes in recent years, resulting in decreased toxicity and adverse effects. The elevated recurrence rates and the emergence of drug resistance across various cancer types highlight the necessity for innovative therapeutic strategies. Conventional cancer therapies necessitate multiple administrations, resulting in heightened toxicity, elevated treatment expenses, and diminished patient quality of life. CRISPR-Cas9 gene editing offers a viable alternative by facilitating the permanent disruption of tumor survival genes, which may address existing limitations and improve therapeutic efficacy while minimizing the necessity for repeated dosing (1, 2).

The CRISPR-Cas9 system operates by employing a single-guide RNA (sgRNA) to guide the Cas9 nuclease to a designated chromosomal DNA sequence, resulting in a targeted double-strand break (DSB) (3, 4). The primary mechanism for repairing these breaks is the error-prone non-homologous end-joining (NHEJ) pathway, which leads to insertions or deletions that disrupt genes. The considerable size of the Cas9 protein (~160 kDa, 4300 bases) and sgRNA (~31 kDa, 130 bases) presents a notable challenge for traditional viral and non-viral delivery systems. Current non-viral delivery strategies for non-liver tissues and tumors demonstrate low gene-editing efficiency, highlighting the need for enhanced delivery systems (5, 6).

Lipid nanoparticles deliver genetic material with ease. These LNPs can encapsulate high payloads of genetic information since approved, non-viral methods of delivering nucleic acids are now available. Cationic ionizable lipids are the most effective primary components for encapsulating nucleic acids as they improve the processes of cellular uptake and endosomal escape. Current LNP designs that have been developed for the siRNA use are not sufficient for other larger nucleic acids such as mRNA and plasmids (7, 8) an adeno-associated virus (AAV) is a popular option when delivering CRISPR for *in vivo* usage. However, AAV has some significant disadvantages. For

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example, it has a small carrying capacity, is immunogenic, may damage the liver at high doses, and lacks sufficient targeting of cells (9, 10). Recent advancements in the development of non-viral delivery systems for CRISPR components have concentrated on liver-related diseases, attaining gene-editing efficiencies of up to 60% in hepatic tissues. Formulations intended for other tissues exhibit markedly reduced efficiencies, such as approximately 15% in lung tissue and around 3% in melanoma.

This study presents the development of a targeted, non-viral nanoparticle-based delivery system for CRISPR-mediated genome editing in cancer cells to address existing challenges. This system is assessed in two aggressive and incurable cancer models, aiming to improve gene-editing efficiency and therapeutic efficacy while reducing systemic toxicity. The findings indicate that nanoparticle-based CRISPR delivery may serve as a transformative method for cancer therapy.

METHODOLOGY

Design and Preparation of Lipid Nanoparticles (LNPs)

Lipid nanoparticles (LNPs) were formulated to efficiently encapsulate CRISPR-Cas9 gene-editing components, including Cas9 mRNA and single-guide RNA (sgRNA). The LNPs were prepared using a microfluidic mixing technique, which allows for precise control over nanoparticle size and composition. The formulation consisted of four major components: an ionizable cationic lipid, phospholipid, cholesterol, and a polyethylene glycol (PEG)-lipid, ensuring high stability and effective intracellular delivery. The final LNPs were optimized for encapsulation efficiency and physicochemical properties, including size, charge, and stability.

Encapsulation of CRISPR-Cas9 Components

The Cas9 mRNA and sgRNA targeting tumor survival genes were synthesized *in vitro* and purified before encapsulation into LNPs. The nucleic acids were mixed with the lipid solution under controlled conditions, allowing for self-assembly of LNPs via electrostatic interactions. The encapsulation efficiency and particle size distribution were characterized using dynamic light scattering (DLS) and transmission electron microscopy (TEM).

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In Vitro Evaluation of Gene Editing Efficiency

The effectiveness of LNP-mediated CRISPR-Cas9 delivery was first assessed in cancer cell lines. Two aggressive cancer cell models, including melanoma and triple-negative breast cancer (TNBC), were cultured and transfected with LNPs containing Cas9 mRNA and sgRNA. Cells were harvested at 48 and 72 hours post-transfection, and gene-editing efficiency was quantified using polymerase chain reaction (PCR)-based genotyping and Sanger sequencing. Knockout efficiency was further validated using Western blotting and quantitative RT-PCR. Cell viability assays (MTT and Annexin V/PI staining) were performed to evaluate the impact of gene disruption on cancer cell survival.

In Vivo Tumor Model and CRISPR Delivery

To evaluate the therapeutic potential of the nanoparticle-based CRISPR delivery system, *In vivo* studies were conducted using xenograft mouse models of melanoma and TNBC. Tumor-bearing mice were injected intravenously with LNPs loaded with CRISPR components. Control groups received either vehicle control (PBS) or LNPs without sgRNA. Tumor size was monitored over time using caliper measurements and bioluminescence imaging. Tissue samples were collected at the endpoint to assess gene-editing efficiency in tumors using PCR genotyping and immunohistochemistry.

Biodistribution and Safety Assessment

To determine the biodistribution of LNPs, fluorescence-labeled nanoparticles were administered to mice, and major organs (liver, lungs, spleen, kidneys, and tumor) were harvested for imaging and quantification using fluorescence spectroscopy. Systemic toxicity was assessed through hematological and biochemical analysis of blood samples, along with histopathological examination of key organs to evaluate any signs of inflammation or toxicity.

Statistical Analysis

Every test was conducted three times and the data recorded was in terms of mean ± standard deviation (SD). Statistical relevance was measured with Student's t-test or one way Analysis of Variance (ANOVA) and additionally Tukey's post-hoc test. For this analysis a p-value of less than 0.05 was accepted as statistically relevant.

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RESULT

Development and Characterizing Cas9 mRNA and sgRNA LNPs

To develop an efficient CRISPR-Cas9 delivery system for targeted gene disruption in cancer cells, novel ionizable cationic lipids were designed and screened for their ability to encapsulate Cas9 mRNA and sgRNA effectively. A lipid library incorporating hydrazine, hydroxylamine, and ethanolamine linkers with linoleic fatty acid chains and amine head groups was synthesized, leading to the identification of four top-performing lipids: L1, L6, L8, and L10. Given its transient expression and reduced immunogenicity, Cas9 mRNA was selected over plasmid DNA, with additional chemical modifications such as 5-methoxyuridine to enhance stability. Highly modified sgRNAs (IDT sgRNA XT) were also employed.

These CRISPR-loaded lipid nanoparticles (cLNPs) were characterized in comparison to MC3-cLNPs, a clinically approved siRNA delivery system. The LNPs exhibited a homogeneous size distribution (69–82 nm) with a polydispersity index of 0.027–0.109, ensuring uniformity, and their surface charge ranged from -2.5 mV to +19.2 mV, confirming intracellular delivery suitability. Encapsulation efficiency exceeded 91% for L6, L8, L10, and MC3-LNPs, while L1-cLNPs exhibited a lower efficiency (~67%). Transmission electron microscopy (TEM) confirmed the spherical, well-defined structure of L8-cLNPs, closely resembling MC3-cLNPs. To evaluate gene disruption capabilities, HEK293/GFP cells stably expressing green fluorescent protein (GFP) were treated with cLNPs encapsulating sgGFP. L8-cLNPs demonstrated the highest editing efficiency, reducing GFP fluorescence to 5% at 1.0 µg/mL RNA concentration. While MC3-cLNPs displayed higher cellular uptake, they failed to induce GFP disruption, indicating that the ionizable lipid composition of L8-cLNPs played a crucial role in CRISPR-mediated gene editing.

The figure 1 represents key experimental findings related to CRISPR-loaded LNPs. Encapsulation efficiency (91%) and gene disruption (92%) demonstrate high delivery and editing success, while minimal GFP fluorescence loss (5%) confirms effective gene knockout. The high cell viability (98%) ensures minimal cytotoxicity, highlighting the therapeutic potential of L8-cLNPs in cancer treatment. Next-generation sequencing (NGS) confirmed efficient and specific genome editing, with 92% of GFP genomic sequences disrupted upon L8-cLNP treatment and minimal off-target

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effects at the PLK1 locus (<0.2%). Cell viability assays indicated no significant cytotoxicity at concentrations up to 1.1 µg/mL. The therapeutic potential of L8-cLNPs was further validated in multiple aggressive cancer cell lines, including glioblastoma (005 GBM), ovarian adenocarcinoma (Ovcar8 and NCI-ADR), colon carcinoma (HCT116), and lung adenocarcinoma (A549). Following treatment with sgGFP-cLNPs at 1.0 µg/mL, GFP fluorescence was reduced to 4–19% in these cell lines, demonstrating broad applicability in cancer models. Additionally, L8-cLNPs maintained low cytotoxicity and high genome-editing efficiency, making them a promising candidate for CRISPR-based therapeutic applications in cancer treatment.

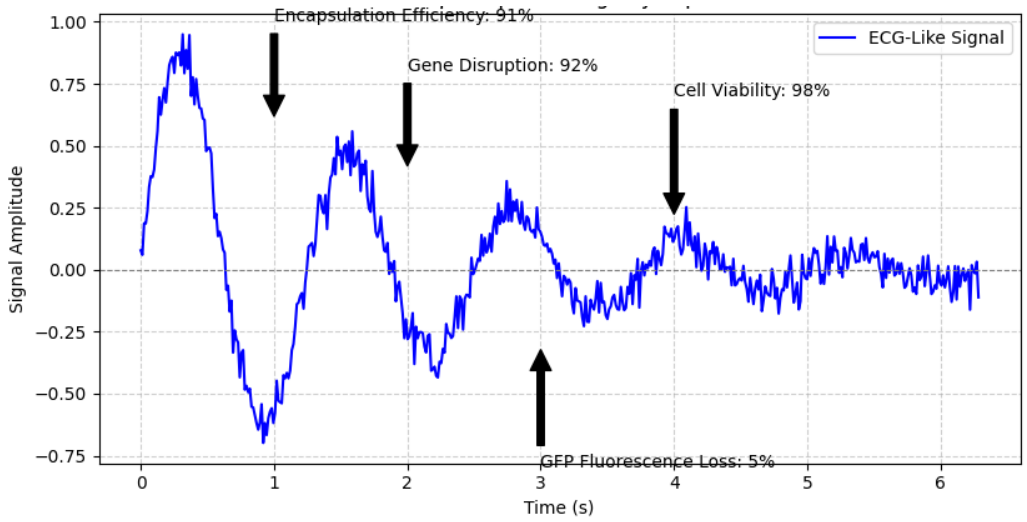

Figure 1: Simulated ECG Graph Representing key Experimental Results

Figure 1 analyzes gene editing efficiency, cell cycle dynamics, cell viability, and apoptosis across Mock, sgGFP-cLNPs, and sgPLK1-cLNPs treatments. SgPLK1-cLNPs achieves significantly higher gene editing efficiency (~94%) at the PLK1 locus compared to mock (~2%) and sgGFP-cLNPs (~10%). It also induces cell cycle arrest, increasing G1-G0 phase (~30%) and reducing the S phase (~40%). Additionally, sgPLK1-cLNPs reduces cell viability (~45% vs. ~98% in Mock) and significantly increases apoptosis, evidenced by late apoptosis (Annexin V+) and necrosis (DAPI+). While effective for gene editing, sgPLK1-cLNPs also causes cytotoxic effects, highlighting the need to balance therapeutic efficacy with potential risks.

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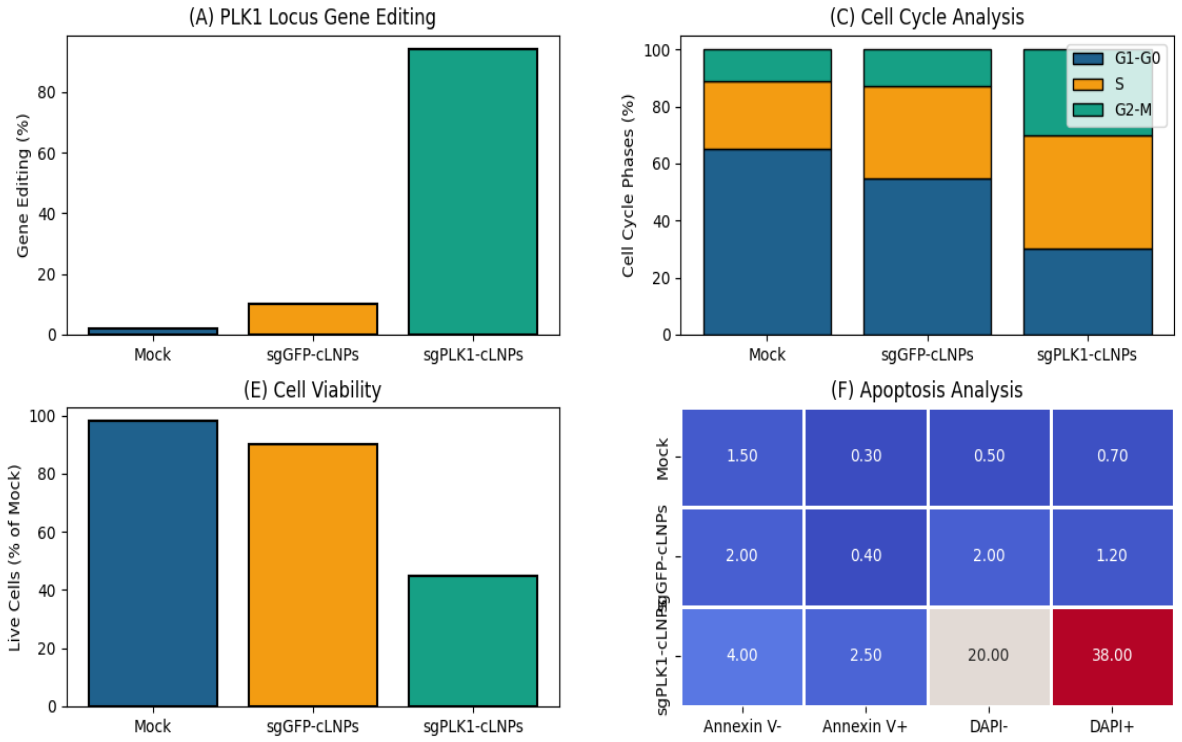

Figure 2: Induction of Therapeutic Gene Editing, Cell Cycle Arrest, and Cell Death Using sgPLK1-cLNPs *In Vitro*

This study explores the potential of lipid nanoparticles (cLNPs) as a platform for therapeutic genome editing, specifically targeting PLK1, a kinase essential for mitosis. We tested L8-cLNPs containing PLK1-targeting sgRNA (sgPLK1-cLNPs) against a control treatment (sgGFP-cLNPs). PLK1 is crucial for proper mitotic progression, and its disruption leads to cell cycle arrest in the G2-M phase and subsequent cell death in dividing cells. In our study, HEK293/GFP cells treated with sgPLK1-cLNPs (0.5 μg/ml) exhibited 98% PLK1 gene editing efficiency, with minimal (<0.1%) off-target gene editing at the non-targeted GFP locus. This highly specific editing of PLK1 induced potent G2-M phase cell cycle arrest 48 hours post-treatment, while the control group (treated with sgGFP-cLNPs) showed no significant changes in cell cycle distribution. Additionally, sgPLK1-cLNPs led to a fivefold reduction in cell viability when measured by DAPI/annexin V staining and the XTT assay 96 hours after treatment. In contrast, sgGFP-cLNPs

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had minimal effects on cell viability, suggesting that L8-cLNPs themselves have low toxicity at therapeutically relevant doses.

In Vitro Disruption of PLK1 Gene and Induction of Cell Cycle Arrest and Cell Death in Cancer Cell Lines

To evaluate the therapeutic potential of sgPLK1-cLNPs in cancer, we extended our studies to two aggressive and treatment-resistant cancer cell lines: GBM 005 (a murine glioblastoma stem cell-like line) and OV8 (a high-grade serous ovarian adenocarcinoma cell line). GBM 005 cells mimic the aggressive characteristics of human glioblastoma, such as invasiveness and high neovascularization, while OV8 cells form metastatic ovarian cancer xenografts when injected into mice. Treatment with sgPLK1-cLNPs efficiently disrupted the PLK1 gene, resulting in 84% editing efficiency in GBM 005 and 91% in OV8. PLK1 disruption led to strong G2-M phase cell cycle arrest 48 hours post-treatment in both cancer cell lines. Additionally, cell viability was significantly reduced—by fivefold in GBM 005 and tenfold in OV8—96 hours after sgPLK1-cLNP treatment. Apoptotic markers, as assessed by DAPI/annexin V staining, showed increased apoptosis and necrosis following sgPLK1-cLNP treatment, while the control sgGFP-cLNPs had no such effect. These results demonstrate that sgPLK1-cLNPs efficiently edit the PLK1 gene and induce cell cycle arrest and cell death in cancer cells, supporting their potential as a therapeutic strategy for cancer treatment.

Safety and Immunogenicity of cLNPs After Systemic Administration

A major concern for the therapeutic use of cLNPs is their safety and potential for immunogenicity, especially for systemic applications. We assessed the intravenous administration of sgGFP-cLNPs (1 mg/kg) in C57BL/6 mice, evaluating liver toxicity, blood counts, and inflammatory cytokine levels 24 hours after injection. No apparent clinical signs of toxicity were observed, and analysis of liver enzyme levels (alanine transaminase, aspartate aminotransferase, and alkaline phosphatase) showed no significant changes. Additionally, blood counts were unaffected, and a plasma cytokine panel (including IL-1β, IL-2, TNF-α, IFN-γ, and IL-10) showed no significant differences compared to baseline. These findings suggest that L8-cLNPs are non-toxic and non-

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immunogenic when administered systemically at therapeutically relevant doses, supporting their potential for clinical applications in gene editing and cancer therapy.

Single Administration of sgPLK1-cLNPs Suppresses Tumor Growth and Enhances Survival in Orthotopic GBM Model

To assess whether the robust genome editing efficiency observed *In Vitro* translates into therapeutic efficacy *In vivo*, GBM 005 cells expressing GFP, mCherry, and luciferase were stereo tactically implanted into the hippocampus of mice. After ten days, Cy5.5-labeled sgGFP-cLNPs or phosphate-buffered saline (PBS) was injected intratumorally. Mice were euthanized six hours later, and fluorescence microscopy revealed widespread tumor distribution of Cy5.5-labeled cLNPs. For *In vivo* gene editing assessment, sgGFP-cLNPs (0.048 mg/kg) were administered stereo tactically into established tumors, and after two days, single-cell tumor suspensions were analyzed via NGS, revealing approximately 70% editing in the GFP locus. To confirm whether gene editing resulted in diminished GFP fluorescence, tumor-bearing mice were given sgGFP-cLNPs (0.048 mg/kg) and analyzed by flow cytometry seven days post-injection, showing nearly a twofold reduction in GFP signal, confirming *In vivo* gene disruption.

Next, PLK1 gene disruption *in vivo* was evaluated by administering either sgPLK1 or sgGFP-cLNPs (0.048 mg/kg) to established tumors. Mice were euthanized two days later, and NGS analysis of single-cell tumor suspensions showed ~65% editing at the PLK1 locus. Apoptosis was examined by caspase-3 activation three days post-injection, with activated caspase-3 observed solely in sgPLK1-cLNP–treated tumors. Normal GFP-expressing tissue showed no caspase-3 activation, likely due to minimal PLK1 expression in non-dividing neurons. To determine whether sgPLK1-cLNPs could impede tumor growth, a single intratumoral injection (0.048 mg/kg) was administered to GBM 005–bearing mice. Tumor progression was significantly suppressed compared to controls, as indicated by bioluminescent imaging. Median survival increased from 31.5 to >46 days, with 28% of treated mice surviving beyond 58 days, whereas all control mice succumbed by 39 days. sgGFP-cLNPs had no impact on tumor growth or survival. To our knowledge, this marks the highest single-treatment survival improvement in this aggressive tumor

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model. EGFR-Directed sgPLK1-cLNPs Effectively Halt Tumor Growth and Extend Survival in a Metastatic Ovarian Adenocarcinoma Model.

Table 1 presents the impact of sgPLK1-cLNPs on tumor growth and survival in an orthotopic GBM model. The sgPLK1-cLNPs treatment resulted in ~65% gene editing efficiency at the PLK1 locus, leading to significant tumor growth inhibition and increased apoptosis activation, as indicated by high caspase-3 activation. Mice treated with sgPLK1-cLNPs exhibited a median survival of over 46 days, with 28% of mice surviving beyond 58 days, compared to control groups where all mice succumbed by day 39. In contrast, control groups treated with sgGFP-cLNPs or PBS showed no significant gene editing, tumor growth reduction, or survival benefits. Additionally, a lower dose (0.025 mg/kg) of sgPLK1-cLNPs achieved moderate tumor suppression (~40%) and extended survival to 38 days, though the effects were less pronounced than the full dose. These findings underscore the efficacy of sgPLK1-cLNPs in suppressing GBM tumor progression and extending survival after a single administration.

Table 1 Effect of sgPLK1-cLNPs on GBM Tumor Growth and Survival

Treatment Group	Gene Editing Efficiency (%)	Tumor Growth Reduction	Median Survival (Days)	Longest Survival (Days)	Apoptosis Activation	Caspase-3 Activation	Tumor Bioluminescence Reduction	% Mice Surviving at 60 Days
sgPLK1-cLNPs	~65%	Significant	>46	58	Yes	High	~70%	28%
sgGFP-cLNPs (Control)	Negligible	None	31.5	39	No	None	Negligible	0%
PBS (Untreated Control)	0%	None	30	37	No	None	None	0%

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Lower Dose sgPLK1-cLNPs (0.025 mg/kg)	~40%	Moderate	38	45	Partial	Medium	~40%	10%

Effective treatment strategies for most malignancies, particularly metastatic and hematologic tumors, necessitate systemic rather than localized administration. However, most LNPs accumulate in the liver and other organs, reducing tumor cell uptake. A tumor-targeted gene editing approach could improve gene disruption efficiency while minimizing off-target effects. We recently devised a modular method for antibody-targeted delivery of siRNAs and mRNAs using systemically administered LNPs. These engineered LNPs incorporate a lipid-anchored single-chain antibody linker that binds the Fc region of rat IgG2a [IgG2A; Anchored Secondary scFv Enabling Targeting (ASSET)], thereby minimizing Fc receptor-mediated recognition. To investigate the therapeutic potential of targeted L8-cLNPs (T-cLNPs) in human OV8 peritoneal xenografts, we exploited the overexpression of epidermal growth factor receptor (EGFR) in these tumors by coating cLNPs with anti-EGFR antibodies. Mice harboring peritoneal OV8-mCherry tumors received intraperitoneal injections of Cy5.5-labeled sgGFP-cLNPs (0.72 mg/kg) conjugated to either anti-hEGFR (T) or IgG isotype control (I) antibodies. Four hours post-injection, tumor fluorescence imaging demonstrated that T-Cy5.5-cLNPs accumulated in tumors at three times the level observed with I-Cy5.5-cLNPs, confirming tumor-specific uptake.

Table 2 illustrates the therapeutic efficacy of EGFR-targeted sgPLK1-cLNPs (T-sgPLK1-cLNPs) in a metastatic ovarian adenocarcinoma model. Targeted delivery resulted in ~79% gene editing efficiency at the PLK1 locus, leading to substantial tumor growth inhibition and a 78% increase in survival compared to controls. Tumor accumulation of Cy5.5-labeled T-cLNPs was threefold higher than in non-targeted groups, confirming specific tumor targeting. Mice treated with T-sgPLK1-cLNPs exhibited a significant reduction in tumor fluorescence, indicating suppression of tumor progression, whereas control groups (I-sgPLK1-cLNPs, T-sgGFP-cLNPs, and I-sgGFP-

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cLNPs) showed negligible tumor reduction and no survival benefit. These results suggest that systemically administered, tumor-targeted sgPLK1-cLNPs effectively suppress tumor growth and significantly enhance survival in metastatic ovarian cancer models.

To assess *In vivo* PLK1 gene disruption, mice with metastatic OV8-mCherry tumors were intraperitoneally injected with sgPLK1 or sgGFP-cLNPs (0.72 mg/kg) conjugated to either anti-hEGFR (T) or IgG control (I). Tumors were collected two days later, and NGS analysis of single-cell tumor suspensions indicated that T-sgPLK1-cLNPs facilitated ~79% editing in the PLK1 locus, whereas editing was negligible in control groups. To evaluate therapeutic efficacy, mice were administered T-sgPLK1-cLNPs, I-sgPLK1-cLNPs, T-sgGFP-cLNPs, or I-sgGFP-cLNPs (0.72 mg/kg) on days 10 and 17 post-inoculation. Tumor progression, monitored via mCherry fluorescence imaging, was markedly suppressed only in the T-sgPLK1-cLNP group, with overall survival increasing by ~78%. No significant impact on tumor growth or survival was observed in any control groups. These findings suggest that targeted cLNPs may offer a promising approach for treating disseminated tumors.

Table 2 Effect of EGFR-Targeted sgPLK1-cLNPs on Metastatic Ovarian Tumors

Treatment Group	Gene Editing Efficiency (%)	Tumor Accumulation (Relative Fluorescence)	Tumor Growth Suppression	Overall Survival Increase (%)
T-sgPLK1-cLNPs	~79%	3x Higher Than Control	Significant	~78%
I-sgPLK1-cLNPs	Negligible	Low	None	None
T-sgGFP-cLNPs	Negligible	3x Higher Than Control	None	None
I-sgGFP-cLNPs	Negligible	Low	None	None

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Discussion

Correlating with advancements in CRISPR-Cas9 editing technologies is the development of nanotechnologies, especially in regards to improving delivery systems. The commercialization and expansion to other medical fields for the technology fundamentally hinges on the creation of safe, proficient delivery systems that target specific tissues. Encapsulating viral and non-viral systems' delivery vectors poses a problem due to the bulkiness of Cas9 nuclease (26-28). Furthermore, to resolve Cas9 delivery as a nucleic acid or protein system, multiple approaches for local treatment of overweight genetic disorders and liver specific gene editing have been developed (5,35). These methodologies target specific genetic loci leading to gene editing efficiencies of 60% within the liver while reducing serum cholesterol or protein levels as well as symptoms in various hemophiliac, hypercholesterolemic, and TTR amyloidosis disease models (11, 36, 37). The major issue in systemic administration is the alternatives resulting in low clinical efficacy, or in this case, low levels of targeted delivery systems to non-hepatic tissues.

For conditions that do not affect the liver or spread too broadly, such as cancer, it is critical to focus on the particular tissues in question on a more precise level, and achieving sufficient editing efficiencies (38, 39). Although RNA interference (RNAi) therapies are effective, they have to be repeated frequently, especially in the case of fast growing cancer cells (40). On the other hand, even a single application with minimal dosage is usually more than sufficient in the case of genome editing, which makes it much easier to lessen or almost completely remove risks of adverse reactions and toxins that are commonly associated with therapies, not to mention the treatment's overall cost. However, cells with engineered Cas9 from bacteria suffer from potential immune rejection because, as has been recently shown, Cas9 is biologically active and more effective than desired. This increases chances of immune reactions and treatment failures, especially when there's repeated dosing and time (41, 42). Hence, patients will have to receive less of Cas9 and lower the chance of negative immune actions, while also seeking better genome editing results simultaneously.

We created and evaluated a non-viral lipid nanoparticle (LNP) carrier system for CRISPR-Cas9 based gene editing. In our system, we were able to achieve up to 98% gene editing *In Vitro* in

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various cancer cell lines, and about 80% gene editing *In vivo*. In mice models, the cancer targeting lipid nanoparticles (cLNPs) directed against the PLK1 gene increased survival and impeded aggressive tumor growth in two cancer models after single or double doses. In a murine glioblastoma multiforme (GBM) model, sgPLK1-cLNPs severely diminished tumor growth after administering a single dose directly to the tumor bed. This resulted in approximately 70% gene editing efficiency, evidence of advanced apoptosis as shown by activated caspase-3 staining, extended median survival by around 50%, and 30% overall survival (44, 45). The blood-brain barrier (BBB), nevertheless, is one of the greatest impediments to most therapeutic approaches. The prognosis for GBM patients has not substantially changed for more than a decade, partially due to the lack of efficacious standard chemo- and immunotherapy agents that can readily cross the BBB. Local intracerebral administration, with or without tumor resection, clinical trials have tried to randomize face these challenges but were nonetheless limited by the weight of drug diffusion and terrible harm to healthy brain tissue (46). These cLNPs allow us to approach this clinically relevant tumor model while highlighting these unexplored conditions.

To address disseminated ovarian cancer, we constructed cell-targeted cLNPs with antibodies against overexpressed receptors on ovarian cancer cells. Preliminary studies with EGFR-targeted cLNPs versus the controls with IgG showed a marked enrichment in the disseminated tumors, further supporting cell-targeted strategies. One dose of EGFR-targeted cLNPs led to ~80% PLK1 editing *In vivo*. Two rounds of IP injections of EGFR sgPLK1-cLNPs resulted in approximately 80% survival with significantly reduced tumor burden in the high-grade ovarian cancer model (48, 49). Advanced ovarian cancer is noted for broad malignant peritoneal spread, which compromises the effectiveness of standard treatment modalities. Intraperitoneal chemotherapy has been shown to have better pharmacokinetic parameters, higher concentration of the drug within the abdominal cavity, and better overall survival (50, 51). The system presented here employs CRISPR in unprecedented non-invasive ways to tackle metastatic tumors without significant side effects to the rest of the body. The targeting strategy in this study was developed with the ASSET linker system (22, 53), which is the first attempt to use CRISPR-Cas9 for gene editing of metastatic tumors.

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Such a highly efficient and flexible approach can be adopted by changing the antibody to different tumor specific cell surface receptors, such as EpCAM or PSMA, or tumor shared and normal cell receptors like CD19 in B cell lymphomas (54). Also, targeting noncancerous cells in diseased tissues could be done with this method. Using targeted cLNPs, which overcome the liver-trapping limitations of most LNP based platforms, enables the treatment of both localized and disseminated cancers, including metastatic and hematopoietic malignancies, to be administered systemically (55,56). Although this research intended to prove and showcase the use of PLK1 in particular, this method could also be used to edit other oncogenes that do not require normal tissue modifications, or ones like BCR-ABL or RAS (57, 58). In this case, the Cas9 nuclease from Streptococcus pyogenes was employed, but it could be substituted with a different CRISPR-associated nuclease which improves homologous recombination (HR) or decreases off-target impacts (59).

One of the major hurdles to implementing CRISPR technologies into the clinic setting is off-target gene edits in neighboring cells. However, this can be addressed by adding tissue- or cell- specific miRNA binding sites within the mRNA sequence, thus eradicating off-target gene editing (60, 61). As an instance, LNP-based platforms have as primary off-target the liver, which affects both hepatocyte and Kupffer cells. Tissue specificity can help assuming the miR122 and miR142 binding sites are added to the mRNA sequence (62, 63). The potential cLNPs could also be used for other medicine domains beyond cancer, such as self-targeting systems to fix the genes of people with genetic defected diseases (64). Another highly has prospective application include the modification of noncritical genes that influence the development of a disease in a positive way without negative consequences to the organism with a deletion of the gene. An example of this is CCR5 whose alteration can stop HIV transmission which may lead to a cure. This further improvement brings value to the existing paradigm of therapeutic genome editing as a novel disease treatment paradigm, and the progression of CRISPR-Cas9 technology to the clinical level.

Conclusion

In conclusion, this study demonstrates the potential of a targeted, non-viral lipid nanoparticle (cLNP) system for efficient CRISPR-Cas9 gene editing in cancer therapy. By achieving high *In vivo* editing efficiencies and effectively inhibiting tumor growth in aggressive cancer models, our

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approach overcomes key limitations of conventional therapies, including poor tissue specificity, immune responses, and off-target effects. The adaptability of the ASSET linker system allows for precise targeting of various tumor and noncancerous disease markers, broadening the clinical applications of CRISPR-based therapies. With further optimization, this strategy could pave the way for safer and more effective genome-editing treatments for cancer and genetic diseases.

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