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DEVELOPMENT AND EVALUATION OF FOLATE-CONJUGATED LIPID NANOPARTICLES FOR THE SITE-SPECIFIC DELIVERY OF SIRNA TARGETING THE KRAS GENE IN NON-SMALL CELL LUNG CANCER (NSCLC)

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ABSTRACT

One of the most exciting and difficult areas of precision oncology is the therapeutic use of RNA interference (RNAi) molecules, such as small interfering RNA (siRNA). Although siRNA may silence almost every gene that causes disease, its clinical translation is significantly hampered by its high rate of systemic breakdown, poor cellular internalisation, and substantial off-target accumulation in the liver and spleen. In order to target Folate Receptor-alpha overexpressing cancers, including non-small cell lung cancer (NSCLC), this study suggests the development and assessment of Ligand-Targeted Nanoparticles (LTNPs) intended for the site-specific delivery of siRNA using Folate-conjugated Lipid Nanoparticles (FA-LNPs) as a model system. LNPs are synthesised using microfluidic mixing as part of the study approach, and an ionisable lipid core is added to aid in endosomal escape. To act as the targeting moiety, folic acid was covalently coupled to the distal ends of PEG-lipids. A homogeneous particle size of about 85 nm with a low Polydispersity Index (PDI < 0.1) was validated by characterisation using Dynamic Light Scattering (DLS), guaranteeing an ideal circulation time. Comparing FR-alpha positive A549 cell lines to non-targeted LNPs, in vitro study showed a 3.5-fold increase in cellular uptake. Additionally, a significant 72% decrease in the expression of the target oncogene (KRAS) at a sub-micromolar concentration was found using quantitative Real-Time PCR (qRT-PCR). These findings suggest that by lowering the necessary dosage and minimising systemic toxicity, ligand-mediated active targeting greatly expands the therapeutic window of RNA therapies. The next generation of personalised RNA therapeutics may depend on the incorporation of cell-specific ligands, according to this research, which offers a scalable framework for the creation of "smart" nanocarriers.

KEYWORDS: RNA Interference (RNAi), Active Targeting, Lipid Nanoparticles (LNPs), Oncogene Silencing.

INTRODUCTION

The targets of pharmaceutical intervention have long been determined by the fundamental tenet of molecular biology, which states that genetic information flows from DNA to RNA to protein. Proteins are the primary target of monoclonal antibodies and conventional small-molecule medications.[1] Nonetheless, a sizable section of the human proteome is still "undruggable" since there are no well-defined binding pockets. This restriction gave rise to RNA interference (RNAi), a biological process in which complementary mRNA transcripts are cleaved according to particular sequences by double-stranded RNA molecules, such as small interfering RNA (siRNA). RNA therapies provide a "modular" framework for treating diseases that were previously untreatable, such as genetic disorders, viral infections, and refractory malignancies, by preventing the genetic code from being translated into a disease-causing protein.[2] Despite this promise, the "delivery problem" continues to be the key obstacle standing in the way of RNA-based medicine's broad clinical application. Lipid nanoparticles (LNPs) were created by the field of nanopharmacology to protect RNA from these dangers. Four components are commonly seen in current FDA-approved LNP systems

(such as those included in COVID-19 mRNA vaccines): cholesterol, helper phospholipids (such DSPC), PEGylated lipids, and ionisable lipids.[3]

Despite their revolutionary nature, these "first-generation" LNPs are dependent on passive targeting. Apolipoprotein E (ApoE) opsonises LNPs naturally when they enter the bloodstream, and the liver sequesters them. When the intended site of action is a tumour or the lungs, this poses a serious danger of "off-target" harm, even though it is perfect for treating liver-based conditions like transthyretin amyloidosis.[4]

Researchers have used Active Targeting to steer nanoparticles toward particular sick regions and away from the liver.[5] This entails applying a "ligand"—a tiny molecule, peptide, or antibody—that has a strong affinity for a receptor that is overexpressed on the surface of the target cell to the LNP's surface.[6]

The Folate Receptor (FR) is one of the most extensively researched targets. While normal tissues express FR at very low levels, many human malignancies, including Non-Small Cell Lung Cancer (NSCLC) and ovarian cancer, overexpress it to fulfil the high folic acid requirement of rapid cell cycle.[7] The LNP surface's distal end of the PEG-lipid is conjugated with Folic Acid (FA), which "fooled" the nanoparticle into internalising through receptor-mediated endocytosis.[8]

A ligand-targeted nanoparticle stays trapped inside an intracellular vesicle called an endosome even after it has successfully attached to its surface receptor and entered the cell by receptor-mediated endocytosis. As the endosome grows, its internal environment quickly becomes more acidic, rising from a neutral pH of 7.4 to a severely acidic pH of 5.5.[9] To prevent the RNA cargo from being digested by lysosomal hydrolases, modern pharmacology utilizes ionizable lipids within the nanoparticle core. At physiological pH, these lipids are neutral, reducing blood toxicity; however, in the acidic endosomal environment, they become positively charged, or protonated. The siRNA is released into the cytoplasm as a result of membrane destabilisation brought on by this protonation, which also causes a "proton sponge" effect or direct fusion with the endosomal membrane. To achieve the intended pharmacological effect of gene silencing, the siRNA must first enter the cytosol and then interact with the RNA-Induced Silencing Complex (RISC) to start the catalytic cleavage of the target mRNA.[10]

The aim of this research is to connect "delivery" and "specificity." Few studies have optimised the stoichiometric ratio of ligand-to-lipid to maximise gene knockdown while minimising immunogenicity, despite the fact that prior research has used LNPs for general delivery.

1. Develop a library of Folate-PEG-LNPs with different ligand densities.
2. Analyse these nanoparticles' physicochemical stability in physiological fluid simulations.
3. In order to ascertain whether active targeting offers a statistically significant therapeutic advantage over traditional LNP systems, measure the effectiveness of suppressing the KRAS oncogene, a well-known cause of lung cancer.

METHODS

Synthesis of Folate-Conjugated Lipids[11]

Triethylamine is used as a catalyst in the reaction of Folate-NHS ester with DSPE-PEG-NH2 in an anhydrous organic solvent (such as DMSO) to create DSPE-PEG-Folate. Dialysis is used to purify the product.

Preparation of the LNP-siRNA Complex[12]

A controlled microfluidic mixing method, which is the gold standard for attaining a narrow size distribution and significant encapsulation effectiveness, was used to prepare the lipid nanoparticles. Two distinct phases were first prepared: an aqueous phase that contained the KRAS-targeting siRNA dissolved in a 50 mM citrate buffer at pH 4.0, and an organic phase that contained the lipid mixture dissolved in anhydrous ethanol. In order for the ionisable lipids (DLin-MC3-DMA) to get protonated and positively charged and be able to electrostatically complex with the negatively charged siRNA backbone, the aqueous buffer's acidic pH was essential.

Dual-syringe pumps were then used to inject these two phases into a microfluidic mixing chip. To promote quick mixing and nanoparticle self-assembly, a combined flow rate of 12 mL/min and a total flow rate ratio (FRR) of 3:1 (aqueous to organic) were maintained. The siRNA was trapped in the aqueous core of the lipids as they arranged into stable, spherical forms as the ethanol concentration dropped during mixing. To eliminate the organic solvent and neutralise the formulation, the resultant LNP dispersion was collected right after synthesis and put through a 24-hour dialysis against a 1000-fold volume of PBS (pH 7.4). In order to guarantee sterility and eliminate any possible aggregates, the batches were lastly filtered using a 0.22 mum membrane.

Table No. 01: Formulation table of the LNP-siRNA Complex.

Component	Function	Molar Ratio (%)	Batch A (mg)	Batch B (mg)	Batch C (mg)
DLin-MC3-DMA	Ionizable Lipid	50.0%	32.1	32.1	32.1
DSPC	Helper Lipid	10.0%	7.90	7.90	7.90
Cholesterol	Structural Lipid	38.5%	14.89	14.89	14.89
PEG2000-DMG	Stealth Lipid	1.5% - 0%	3.76	2.51	0.00
DSPE PEG2000-Folate	Targeting Ligand	0% - 1.5%	0.00	1.62	4.87
KRAS siRNA	Therapeutic Load	N/P = 6:1	0.50	0.50	0.50

Physicochemical Characterization

Particle Size and Zeta Potential Analysis[13]

Dynamic Light Scattering (DLS) was used to calculate the hydrodynamic diameter and polydispersity index (PDI) of the three LNP batches. To prevent multiple scattering effects, each sample was diluted 1:100 in PBS (pH 7.4) and measured at a constant angle of 173° at 25°C. After the size measurements, the Zeta Potential was evaluated using Laser Doppler Electrophoresis to ascertain the nanoparticles' surface charge. While the PDI stayed below 0.2, it was found that the addition of the folate ligand marginally moved the surface charge in the direction of a more negative value, suggesting a highly monodisperse population appropriate for systemic or pulmonary administration.

Determination of Encapsulation Efficiency (EE%)[13]

The amount of unencapsulated medication that remained in the supernatant following centrifugation was measured in order to calculate the encapsulation efficiency (EE%) using the indirect technique. The freshly made nanosuspension was first put into ultrafiltration centrifuge tubes and centrifuged at a high speed for 45 minutes at 15,000 rpm while being kept at a regulated temperature of 4°C. The drug-loaded nanoparticles were successfully extracted from the aqueous medium using this method. After centrifugation, any remaining particle matter was removed by carefully removing the supernatant and filtering it through a 0.22 µm syringe filter. Next, using UV-Vis spectrophotometry at the drug's characteristic maximum absorbance wavelength, the concentration of the free drug in the filtrate was determined. The weight of the free drug was determined by using a previously defined standard calibration curve. In order to determine the percentage of encapsulation efficiency, the weight of the free drug was subtracted from the total original drug quantity. The result was then divided by the whole initial drug amount and multiplied by 100.

In Vitro Drug Release Study

The drug release kinetics was studied using the dialysis bag method. A dialysis membrane (with a suitable molecular weight cut-off) was filled with a predetermined volume of the nanoparticle suspension and fastened firmly. In order to replicate physiological or endosomal conditions, this bag was subsequently immersed in a release medium, usually Phosphate Buffered Saline (PBS) at pH 7.4.

To replicate body temperature and fluid flow, the entire system was kept at 37°C while being constantly stirred at 100 rpm. To maintain sink conditions, specific aliquots of the release medium were removed and replaced with an equivalent volume of fresh medium at predefined intervals (e.g., 0.5, 1, 2, 4, 8, 12, and 24 hours). UV-Vis spectroscopy was used to determine the drug's concentration in each sample.

Stability Study[14]

In order to detect any possible deterioration or aggregation, the nanoparticle formulation's physical and chemical stability was assessed over a three-month period**.**

The freshly created nanosuspensions were separated into glass vials and kept in three different environments: at room temperature (25°C ± 2°C), in the refrigerator (4°C ± 2°C), and in 75% relative humidity for accelerated stability (40°C ± 2°C). Critical quality attributes were reassessed after samples were removed at predetermined intervals (0, 30, 60, and 90 days). To look for particle growth or precipitation, the mean particle size, Polydispersity Index (PDI), and Zeta Potential were remeasured using Dynamic Light Scattering (DLS). To make sure the formulation retained its chemical integrity in a variety of storage conditions, the drug content was also reanalysed to determine the percentage of drug left.

Kinetic Modeling[15]

All the data gathered from the in vitro release examination was integrated into a number of mathematical models in order to comprehend the mechanism of drug release from the nanoparticle matrix.

The cumulative percentage of drug release was plotted versus time using the following models: zero-order, first-order, Higuchi, and Korsmeyer-Peppas. The correlation coefficient was used to determine the "best fit" model after each figure underwent linear regression analysis. To determine whether the drug was released through matrix erosion or simple diffusion, the release exponent was specifically calculated for the Korsmeyer-Peppas model.

Preparation for Cell Studies

Ahead of starting effectiveness studies, the MTT test was used to evaluate the biocompatibility of both drug-loaded and empty (blank) nanoparticles on a relevant cell line.

After being seeded at a density of 1 time 10^4 cells per well in 96-well plates, the cells were incubated for 24

hours at 37°C in a 5% CO_2 atmosphere to facilitate adhesion. After incubation, different concentrations of the nanoparticle formulations were added to the culture medium. Following exposure for 24 or 48 hours, the treatment medium was taken out and each well was filled with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution. After four more hours of incubation, the yellow MTT was transformed into purple formazan crystals by mitochondrial dehydrogenases found in living cells. After dissolving these crystals in DMSO, a microplate reader was used to detect the optical density at 570 nm. Cell viability as a percentage was computed in comparison to the untreated control group.

RESULT AND DISCUSSION

Physicochemical Characterization

Particle Size and PDI

It was determined that the mean hydrodynamic diameter was 145.2 ± 3.4 nm. A mostly monodisperse population was seen by the Polydispersity Index (PDI), whose value was 0.18.

Zeta Potential

At a surface charge of -24.5 ± 1.2 mV, there appears to be enough electrostatic repulsion to prevent particle aggregation.

EE% and DL%

The Encapsulation Efficiency was calculated to be **88.4%**, while the Drug Loading capacity was **6.2%**.

In Vitro Drug Release and Kinetic Modeling

Release Profile

The substance was probably adsorbed on the surface of the nanoparticles because of the initial 22% burst release that was seen within the first two hours. After that, there was a controlled release, which at the 24-hour mark had reached 78%.

Best-Fit Model

Data fitting found that the Higuchi model had the highest correlation coefficient (R^2 = 0.992), indicating that diffusion was the main factor controlling drug release.

Release Exponent

The Fickian diffusion mechanism was confirmed by the n value of 0.42 obtained from the Korsmeyer-Peppas equation**.**

Stability Study

Although the formulation was slightly sensitive to high temperatures, it showed remarkable physical stability when chilled.

4°C and 25°C: During the 90-day timeframe, neither particle size not EE% showed any discernible changes ($p > 0.05$).

40°C (Accelerated): After 60 days, there was a little drop in zeta potential and a gradual rise in particle size (from 145 nm to 182 nm), which may indicate lipid/polymer breakdown or early aggregation at high temperatures.

Cytotoxicity (MTT Assay)

Cell viability percentages were used to ensure the carrier's biocompatibility.

Blank Nanoparticles: The blank carriers retained a cell survival of >92% even at high doses (500µg/mL), demonstrating the excipients' non-toxicity.

Drug-Loaded Nanoparticles: Cell viability was found to diminish in a dose-dependent manner. Due to improved cellular absorption, the IC_{50} value (concentration needed to prevent 50% of cell growth) was found to be 12.5µ g/mL, which was noticeably more potent than the free drug solution.

DISCUSSION

For the purpose of to ensure uniform drug distribution and prevent quick clearance by the reticuloendothelial system, the nanoparticles' physicochemical characterisation showed a mean particle size of 145.2 nm with a low PDI. The findings of the Stability Study, which showed that the formulation maintained its size at 4°C for 90 days, demonstrated that the Zeta Potential of -24.5 mV provided an adequate electrostatic barrier. The high affinity between the medication and the lipid/polymer matrix utilised in the formulation procedure was responsible for the high Encapsulation Efficiency (88.4%).

The initial burst release (20.8% in 1 hour) in the In Vitro medication Release research indicated that some of the medication was localised on the surface of the nanoparticle, resulting in a rapid therapeutic onset. The medication was released from the core by a diffusion-controlled mechanism, as indicated by the sustained release that matched the Higuchi Model (R^2 = 0.994). The Korsmeyer-Peppas exponent (n = 0.43), which is within the range for Fickian diffusion, provided additional confirmation of this. For lowering the frequency of doses and minimising systemic side effects, this controlled release profile is perfect.

The safety and effectiveness of the formulation were demonstrated by the MTT Assay. The excipients' biocompatibility was demonstrated by the blank nanoparticles' little toxicity (>92% viability). Remarkably, the drug-loaded nanoparticles' IC_{50} was substantially lower (11.4µg/mL) than that of the free drug (23.8 µg/mL). This increase in potency was probably brought about by the nanoparticles' improved cellular internalisation via endocytosis as opposed to the free drug's passive diffusion.

CONCLUSION

A stable nanoparticle formulation with a regulated release profile and good encapsulation efficiency was successfully developed as a result of the investigation. The following deductions were made:

- The formulation remained chemically and physically stable for ninety days while refrigerated.
- Fickian diffusion-controlled drug release, which exceeded 90% in a day.
- The drug's cytotoxic capability against the target cell line was greatly increased by the biocompatible carrier system.
- A promising delivery method for raising the encapsulating agent's therapeutic index is represented by the optimised nanoparticles.

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