A Combination RNAi-Chemotherapy Layer-by-Layer Nanoparticle for Systemic Targeting of KRAS/P53 with Cisplatin to Treat Non–Small Cell Lung Cancer

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Abstract

Purpose: Mutation of the Kirsten ras sarcoma viral oncogene homolog (KRAS) and loss of p53 function are commonly seen in patients with non–small cell lung cancer (NSCLC). Combining therapeutics targeting these tumor-defensive pathways with cisplatin in a single-nanoparticle platform are rarely developed in clinic.

Experimental Design: Cisplatin was encapsulated in liposomes, which multiple polyelectrolyte layers, including siKRAS and miR-34a were built on to generate multifunctional layer-by-layer nanoparticle. Structure, size, and surface charge were characterized, in addition to in vitro toxicity studies. In vivo tumor targeting and therapy was investigated in an orthotopic lung cancer model by microCT, fluorescence imaging, and immunohistochemistry.

Results: The singular nanoscale formulation, incorporating oncogene siKRAS, tumor-suppressor stimulating miR34a, and cisplatin, has shown enhanced toxicity against lung cancer cell line, KP cell. In vivo, systemic delivery of the nanoparticles indicated a preferential uptake in lung of the tumor-bearing mice. Efficacy studies indicated prolonged survival of mice from the combination treatment.

Conclusions: The combination RNA-chemotherapy in an LbL formulation provides an enhanced treatment efficacy against NSCLC, indicating promising potential in clinic. Clin Cancer Res; 23(23); 7312–23. ©2017 AACR.

Introduction

Non–small cell lung cancer (NSCLC) accounts for 85% of lung cancer, a leading cause of cancer-related death worldwide (1). Cisplatin and other platinum-based chemotherapeutics are frontline therapies for the treatment of NSCLC (2, 3). However, drug resistance and desensitizing, caused by complex genetic mutations of the cancer cells, limits the clinical efficacy of platinum-based chemotherapeutics against NSCLC, with a less than 20% 5-year survival rate for patients with NSCLC and a 4% 5-year survival rate for patients with metastatic tumors (4). The most common subtype of NSCLC, adenocarcinoma, is usually associated with 20% to 30% mutation of oncogenic Kirsten ras sarcoma viral oncogene homolog (KRAS) and approximately 50% loss of p53 function (1). For these lung tumor types, and several other aggressive cancers (including colon cancer, leukemia, and pancreatic cancer), the KRAS mutation is essential for tumor formation and maintenance, thus rapid tumor regression is found with deletion of KRAS (5–7). P53, a frequently mutated tumor-suppressor gene, is involved in the cell-cycle progression, proliferation, survival, and apoptosis (8–9). Loss of p53 function can increase the function of P-glycoprotein, a membrane pump protein that causes resistance toward chemotherapeutic drugs (10–13). More importantly, loss of P53 has been shown to accelerate KRAS driven tumorigenesis, indicating a synergistic effect between KRAS mutation and loss of p53 in promoting tumor development (14, 15). As a result, the simultaneous inhibition of the KRAS oncogene and restoration of the p53-suppressor function are appealing therapeutic strategies for lung adenocarcinoma. However, small-molecule inhibitors and drugs to restore p53 function remain elusive (16, 17). Small-molecule inhibitors to target the KRAS oncogene remain limited, whereas only a few reports have shown any potential of developing KRAS inhibitors due to the challenges of the KRAS-binding pocket (18, 19). On the other hand, both of these genetic pathways can be targeted directly using RNA. It has quite recently been shown that siRNA to target the KRAS oncogene is an effective strategy to impede KRAS signaling and prevent tumor growth and progression (20–23). Furthermore, microRNA (miRNA), small coding RNAs that regulate gene expression in the posttranscriptional stage (24), can be used to address p53 function; miR34a, one member of the miR-34 microRNA family, can activate multiple p53 downstream pathways which mediate cell proliferation, survival, and apoptosis, thus restoring antitumor effects (24–29). MiR34a, a liposomal miR-34, has been investigated in clinical trials phase I. Although it was placed on hold due to safety issue, it is still a principal proof that miR-34a could serve as a valuable anticancer drug once a promising delivery vehicle could be found to remediate the safety concern (30). Therefore, the delivery of siKRAS and miR34a can be an effective treatment.
Translational Relevance

Adenocarcinoma, the most common form of non–small cell lung cancer, is associated with a mutation of the KRAS and loss of p53 function. Together, these genetic mutations open pathways toward resistance of tumor cells to the therapeutic response of cisplatin, one of the main clinical chemotherapeutic drugs for patients with non–small cell lung cancer. A promising approach to overcome this limitation is the design of a combination therapy that is composed of a chemotherapeutic drug, cisplatin, and RNA-based therapeutics that specifically target both the KRAS mutation and loss of p53 function. We designed a nanotherapeutic that uses the electrostatic layer-by-layer approach to effectively package both RNA therapeutics and cisplatin simultaneously in a manner that enables optimal timing of each. We observed enhanced treatment efficacy using the layer-by-layer approach indicating promising potential in the clinic.

approach to mediate the genetic mutations of lung cancer cells and enhance the antitumor efficacy of cisplatin.

Despite the enormous therapeutic potential of siRNA and miRNA, systemic delivery of RNA to the target site still remains problematic when translating to clinic (31). Some of the major issues include mononuclear phagocyte system clearance, achieving sufficient RNA loading capacity, nuclease degradation, toxicity, and establishing a prolonged blood circulation time to allow accumulation in the tumor (32–34). Nanoscale non-viral delivery systems developed from cationic polymers, lipids and lipid-like systems, and peptides have been investigated extensively for small RNA delivery in pre-clinical studies (32, 34). Recently, co-delivery of siKRAS and miR-34a in vivo has been achieved using a lipid-based formulation, and lung tumor regression was observed in a “KRAS mutation and P53 deletion” (“KP”) adenocarcinoma model in which KRAS oncogene is mutated and p53 tumor suppressor is deleted; for this model, the chemotherapy drug was delivered separately via intravenous injection (15). The development of a combination therapy that is truly delivered together in a synergistic fashion using nanoparticle technologies provides the potential of highly targeted therapies with lowered toxicity and a greater window of therapeutic potential; however, this mode of targeted multi-drug delivery is still missing in traditional systems such as cationic polymers or charged lipids that lack the modular design to incorporate multiple therapeutics.

Layer-by-layer (LbL) nanoparticles is a promising drug delivery platform with great clinical translational potential (35–43). Using the process of depositing oppositely charged polyelectrolytes sequentially on a charged core, LbL nanoparticles possess hierarchical and multifunctional multilayered structure with great modularity and versatility. LbL nanoparticles have several desirable features, including precise control of size, combination therapeutics with high loading capacity, staged cargo release, enhanced stability in vivo, and tunable surfaces for modification (35). Because of the modular nature of LbL nanoparticles, it is possible to incorporate therapeutics such as RNAs, inhibitors, or proteins in multilayers on a charged colloidal core substrate. Furthermore, the LbL platform can yield surface chemistry that enables targeting via response of the hypoxic tumor microenvironment and the presence of specific ligands that bind a number of known aggressive tumor cell types, from ovarian to lung cancer (37). We have found that these LbL stealth coatings provide extended blood plasma half-life when applied to liposomal, quantum dot, gold and other nanoparticle systems (35, 39). In addition, the stealth layer provides an advantageous characteristic where it enables direct tumor targeting due to the outer layer design formed from hyaluronic acid (HA); refs. 35–37. Recent work using the LbL platform has demonstrated a staged release of siRNA and a chemotherapeutic agent for treatment of triple negative breast cancer (35). Furthermore, the LbL platform can also provide improved biocompatibility and reduced off-target toxicity of the loaded therapeutics (36). The modularity, flexibility, and versatility of this platform make it an optimal candidate for preparing combination nanotherapeutics containing RNA-based drugs and DNA-damaging chemotherapeutics.

It is important to examine these systems in a more meaningful mouse model that better replicates advanced disease within the relevant tissue. Furthermore, we hope to show that more than one type of nucleic acid can be effectively delivered from these systems to address multiple types of gene dysregulation that are synergistic.

In this study, we present a KRAS/P53 targeted LbL nanoparticle that contains a cisplatin loaded core to treat aggressive lung adenocarcinoma in vivo. Taking advantage of the modularity and versatility of LbL platform, we were able to build RNA films (siKRAS and miR-34a) with poly L-arginine (PLA) as the polycation, atop the cisplatin-containing liposomes, followed by coating of exterior layer with hyaluronic acid (HA), that possess both “stealth” and targeting properties. We demonstrated high loading capacity and controlled release of small RNA and cisplatin. In vitro studies showed efficient KRAS gene knockdown and enhanced tumor killing effect of cisplatin when combined with siKRAS, miR-34a, or siKRAS/miR-34a combination. We further demonstrated enhanced accumulation of LbL nanoparticles in the lungs of tumor-bearing mice in an orthotopic lung adenocarcinoma model utilizing tumor cells derived from genetically modified KRAS mutant, p53-deficient mice. It was found that mice treated with combination therapy demonstrated prolonged survival compared with mice treated with either cisplatin or RNA alone. Given the similarities between the lung adenocarcinoma model and human NSCLC, this study highlights the promising potential of incorporating LbL nanoparticles as a combination therapy platform to deliver RNA-based therapeutics that address common tumor mutations that enable tumor cell drug resistance and survival, in combination with chemotherapeutic agents.

Materials and Methods

Materials

All lipid components were purchased from Avanti Polar Lipids, except for cholesterol, which was purchased from Sigma-Aldrich. Cisplatin and other polyelectrolytes were purchased from Sigma-Aldrich. CCK-8 cell proliferation assay kit was purchased from Dojindo. DNA primers, including siKRAS (sense 5'-UUGGAAAACCAGAGGUGUGG-3', antisense 5'-TCCTTAATGTCACGCACGATTT-3'), antisense 5'-TGAGCGGGCTACA-GCTT-3', and miR-34a 5'-UGGCUAAUAAUAAGGAAUACGU-dTdT-3', were customized from Dharmacon. DNA primers, including siKRAS (sense 5'-GACTGTTATAAAGCTTGTTGGACCT-3', antisense 5'-TCCTCTGAGCAC-CTGTCGTGTCG-3'), β-actin (sense 5'-TGAAGGCCGCGCTACA-GCTT-3', antisense 5'-TCCTTAATGTCACGCAGATGTT-3'), are
purchased from DNA Technologies. Monoclonal antibodies, including anti-CD44 and the IgG isotype control antibody were purchased from Santa Cruz Biotechnologies. DMEM, FBS, penicillin/streptomycin, and RNase-free deionized water were purchased from Invitrogen. All polymer and buffer solutions were filtered with a 0.2-μm pore size polycarbonate syringe filter before use.

**Preparation of LbL nanoparticles**

The protocol of LbL nanoparticles preparation was developed based on previous established method (35). Briefly, liposomes were first formulated at a mass ratio of 7:2:1 (DSPC:POPG:Cholesterol). These three compounds were dissolved in chloroform and a thin lipid film was generated by rotary evaporation. These films were then allowed to dry in a desiccator overnight to completely remove chloroform. Cisplatin was suspended in deionized water and sonicated for 1 hour to allow complete cisplatin dissolution with concentration of 8 mg/mL. The lipid film was then hydrated with cisplatin solution at 65°C for 1 hour. Following the cisplatin loading in liposomes, these drug-loaded liposomes were purified using tangential flow filtration (TFF) to remove free cisplatin and were then re-suspended in PBS for storage. For LbL assembly, liposomes at 2 mg/mL were mixed with PLA (2 mg/mL) in RNase-free water, which was facilitated by a brief period of bath sonication (3s). The excessive PLA was purified by TFF. To incorporate RNAs, purified liposomes were mixed with RNAs (siKRAS/miR-34a, 1/1 molar ratio, 10 μmol/L) in RNase-free water, followed by purification using TFF. Another PLA layer was deposited onto the RNA terminated nanoparticles via similar mixture method and TFF purification steps. Finally, the purified PLA-terminated LbL nanoparticles (2 mg/mL) were mixed with HA (1 mg/mL in dibasic sodium phosphate buffer, pH = 7.4, 10 mmol/L) and washed using TFF. The obtained LbL nanoparticles were stored in PBS solution at 4°C. To prepare the Cy5.5-labeled nanoparticles, Cy5.5-siRNA (10% molar ratio) was incorporated into the total RNAs to serve as the RNA layer during the LbL process. The generated Cy5.5-labeled nanoparticles share similar physicochemical properties as the unlabeled nanoparticles.

**Physicochemical characterization**

All size, zeta potential, and polydispersity index were measured using a Malvern Zetasizer Nano ZS90 particle analyzer (λ = 633 nm, material/dispersant RI 1.590/1.330). The loading efficiency of RNA in the LbL nanoparticles were examined by measuring the free RNA in the washed waste using Picogreen Assay (Invitrogen) against a dsRNA standard curve, by absorbance of the washed waste at 260 nm using Nanodrop, or using a fluorescent dye-labeled RNA and measuring the nanoparticle-associated fluorescence intensities against a fluorescent siRNA standard curve. The stability of the LbL nanoparticles was examined in PBS or in phenol-free DMEM at room temperature. For the cisplatin-loading measurement, the cisplatin-containing LbL nanoparticles were diluted to 10,000-fold with DI water and the concentration of platinum was measured using automatic flameless atomic absorption spectrophotometer (Model AA-6700, Shimadzu). Potassium dichloroplatinate was used as a standard. A standard curve with platinum concentrations in the range of 50 to 250 ng/mL was performed before analysis of each sample. The RNAs release from the LbL nanoparticles were measured at 37°C by quantifying the amount of RNA released in supernatant over different time points using picogreen assays. The release of cisplatin was quantified by measuring the remaining cisplatin in the float-a-lyzers (MWCO = 3500 Spectrum) at room temperature.

**In vitro experiments**

KP cells in this study were obtained from the lung tumors of a human autochthonous mouse model developed by Xue and colleagues (15). The cells were grown in DMEM media supplemented with 10% FBS, 50 U/mL of penicillin and 50 U/mL of streptomycin. KP cells were stable in expressing tdTomato.

Gene silencing of LbL nanoparticles was examined in KRAS-expressing KP cells. Briefly, the cells were seeded on a 96-well plate overnight with 30% confluence, and treated with increasing concentration of LbL nanoparticles, of which the amounts were normalized to the siRNA loading. The cells were then treated with siKRAS LbL nanoparticles, and scrambled control siRNA nanoparticles for comparison. Three or five days after the treatments, RNA was purified using TRizol (Invitrogen) and reverse-transcribed using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Real-time PCR (Q-PCR) reactions were carried out using TaqMan probes (Invitrogen). The KRAS mRNA levels were normalized to Actin mRNA using scrambled siRNA as the control. To quantify the miRNA expression, 10 ng of total RNA was reverse-transcribed using miRNA-specific RT primer and measured by real-time PCR using miRNA-specific probes. The miRNA expression was normalized to U6 RNA.

Cytotoxicity assays were carried out using the CCK-8 cytotoxicity assay. Briefly, the cells were first plated in a 96-well plate with 30% confluence for 24 hours and treated with the nanoparticles at various concentrations of cisplatin. After 3 days of incubation, a fresh serum-free OptiMEM media containing 10% v/v of the CCK-8 proliferation kit was used to replace the media. After 2 hours incubation, the absorbance at 450 nm was measured by a plate reader. Cell viabilities were measured and normalized to an untreated control group. IC50 values of cisplatin at various combinations were calculated from the viability curve using Prism 5.

**In vivo experiments**

All animal studies were approved by the Massachusetts Institute of Technology Animal Care and Use Committee. AIN-93 purified diet was purchased from Pharmasey/Testdieten. Cohorts of KP and KP.R26/LSL-Luciferase/LSL-tdTomato mice were infected with 2.5 × 10⁷ pfu of Adeno-Cre by intranasal inhalation as described previously (15). The mice were monitored weekly using a GE Healthcare microCT imaging device (45-μm resolution, 80 kV, with 450-μA current). Before the targeting studies, mice were placed on AIN-93 special diet for a week to reduce body autofluorescence.

For the tumor initiation, KP cells (1 × 10⁹) were injected to nude mice via tail vein injection. The targeting and treatment were typically initiated after 2 weeks postinjection.

For the tumor-targeting studies, both healthy and tumor-bearing mice were treated with Cy5.5-labeled LbL nanoparticles (10%) Cy5.5-labeled siRNA in total RNAs) via intravenous injections with a dose of 2 mg/kg of RNAs. The whole-body imaging of mice following nanoparticle injections were carried out using IVIS imager (Xenogen) with excitation at 650 nm and emission at 750 nm. The mice were sacrificed 48 hours post injection. The vital organs were harvested, and fluorescence was quantified using IVIS imaging.
Tumor-bearing mice were treated with four groups, including vehicle control without any therapeutics, cisplatin only, RNA only, and cisplatin/RNA combination. Each group contains eight mice. The dose of cisplatin was 12 mg/kg, whereas for the RNA only (with dosing at 2 mg/kg RNA only) and vehicle control groups, doses were equivalent to 12 mg/kg, given to the weight ratio of cisplatin to lipid. The mice were dosed repeatedly once a week, for a total of 4 weeks. At indicated time points, the up-chest tumor volumes in the lungs before and after treatment was calculated using GE MicroView software. The total tumor volumes in the lungs and after treatment was calculated using GE MicroView software [15, 44]. The weights of mice were monitored daily, with 20% body weight loss, mice were euthanized and lung tissues were recovered for analysis. The survival curve was calculated and illustrated as Kaplan–Meier curve using GraphPad software.

qPCR analyses of KRAS and mir-34a were performed by isolating RNA from the lung tumors using the PARIS Kit (Invi-rogen) according to the manufacturer’s protocol. The IScript cDNA Synthesis Kit was used to synthesize the cDNA. Q-PCR was carried out using iQ SYBR Green Supermix (Bio-Rad) along with the selected DNA primers. The amplification was performed by incubation at 95 °C for 10 minutes, followed by 40 cycles of 95 °C for 15 seconds and 60 °C for 1 minutes. The relative gene expression was normalized to either U6 RNA or β-actin.

Western blots were performed using standard methods. After the treatments, tumor-bearing lung tissues were prepared by radi immunoprecipitation assay buffer (150 mmol/L sodium chloride, 50 mmol/L Tris pH 8.0, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS) supplemented with 10 mmol/L NaF, 1 mmol/L Na3VO4, 5 mmol/L EDTA, 1 mmol/L EGTA, 5 μg/ml leupeptin, 1 μg/ml pepstatin A, 1 mmol/L phenylmethlysulfonyl fluoride, and protease and phosphatase inhibitor cocktail (Calbiochem). Lysates were centrifuged at 12,000 r.p.m. for 20 minutes at 4 °C. Protein concentration of the supernatants was determined using a BCA protein Assay Kit (Thermo Scientific). Equal amounts (30–50 μg) of the proteins were resolved by 12% SDS–polyacrylamide gel electrophoresis gels and transferred to nitrocellulose membranes (Millipore). Membranes were blocked in 5% BSA for 1 hour at room temperature, and then incubated with specific antibodies for different Western blot analyses at 4 °C overnight. The bound primary antibodies were detected by secondary conjugates compatible with infrared detection at 700 and 800 nm, and membranes were scanned using the Odyssey Infrared Imaging System (Odyssey, LI-COR). The following antibodies were used: anti-KRAS antibody (ab84573, 1:2,000 dilution) was obtained from Abcam. Anti–β-actin antibody was purchased from Sigma (A5441, 1:10,000 dilution). Secondary antibodies were from LI-COR Biosciences, including IRDye 800CW Goat anti-Mouse IgG (926–32210, 1:10,000 dilution) and IRDye 800CW Goat anti-Rabbit IgG (926–32211, 1:10,000 dilution).

For immunohistochemistry (IHC) assays, mice were euthanized with CO₂ asphyxiation, and lungs were inflated with 4% formalin. The tissue samples are collected and processed after harvesting the fresh organs. Overnight fixation was performed. The lungs were then embedded in paraffin based on standard procedures. The lungs were then sectioned at 4-μm and stained with specific antibodies for detecting the biomarkers of interests. The following antibodies were used: anti-CC3 (1:200, Cell Signaling Technology), anti-p-Erk Thr202/Tyr204 (1:300, Cell Signaling Technology), anti-SIRT (1:500, Cell Signaling Technology), anti-CDK6 (1:300, Cell Signaling Technology), and anti-Ki67 (1:100, Cell Signaling Technology). The number of positive cells per tumor area was quantified.

**Statistical analysis**

Experiments were performed in triplicates, or otherwise indicated. Data were analyzed using descriptive statistics, single-factor ANOVA, and presented as mean values ± SD from 3 to 10 independent measurements. Statistical comparisons between different treatments were assessed by two-tailed t tests or one-way ANOVA.

**Results and Discussion**

Combination nanotherapeutics construction and characterization

Multilayered LbL nanoparticles containing RNA therapeutics and cisplatin are illustrated in Fig. 1A. To construct the LbL-based nanotherapeutics, cisplatin was first encapsulated in the hydrophilic core of negatively charged phospholipid liposomes. Positively charged poly-L-arginine (PLA), the two negatively charged therapeutic RNAs combined together (siKRAS and mir-34a), and PLA were sequentially assembled on top of the liposome by sonication and tangential flow filtration (TFF) to remove free polyelectrolytes [45]. Hyaluronic acid (HA, 40 kDa), a negatively charged natural polysaccharide, was deposited as the outermost layer due to its capability to extend blood circulation time and its ability to target CD44, an overexpressed receptor on lung adenocarcinoma cells [46]. Sequential build-up of an Lbl film around the nanoparticle was confirmed at each step by dynamic light scattering (DLS) measurements that indicated a 10-nm growth in diameter following the deposition of PLA and the RNAs, and a 50-nm growth following deposition of the terminal HA layer (Fig. 1B). This final charged HA layer is thought to be highly hydrated and loop-like in nature, thus yielding a thicker, but possibly higher water content outer layer when measured hydrodynamically. Further validation of the coating of each layer was provided by the electrophoretic measurements that indicated a complete charge reversal following each layer deposition (Fig. 1C). The complete Lbl nanoparticle, with a multilayered structure of liposomes/PLA/RNA/PLA/HA, possessed a zeta potential of approximately –30 mV and hydrodynamic diameter of approximately 180 nm (Fig. 1B). The uniformity of Lbl deposition was evidenced by the low polydispersity index (PDI) value (<0.20; Fig. 1D). The RNA combination system was adsorbed onto the nanoparticle from a solution with a 1:1 molar ratio of siRNA to miRNA. To examine the total RNA loading in the Lbl nanoparticle, waste solution generated from the TFF after each wash was quantified for free RNA, and the resulting value was subtracted from the total RNA before Lbl assembly (Supplementary Fig. S1). Approximately 3,000 siRNA molecules per nanoparticle were layered by the PLA film, implying a conformal coating of RNAs on the nanoparticle with approximately 90% surface coverage (assuming 6-nm x 2-nm cross-sectional surface area per siRNA molecule, ref. 47). A similar approach was applied to measure the quantity of loaded cisplatin in liposomes. Total RNA loading was determined at 5.5% of drug-to-lipid weight loading, comparable to our previously reported siRNA loading efficiency [35]. The weight loading of cisplatin in the Lbl nanoparticle (13%) is comparable...
with cisplatin loading achieved in Lipoplatin™, a lipid-based cisplatin formulation in clinical trials (48–51). Furthermore, release of both RNA molecules and cisplatin were measured over an extended period of time. It was found that the PLA/RNA film was stable at pH 7.4 in PBS, with a net release of less than 30% at 24 hours (Fig. 1F) and 37°C; furthermore, we observed a sustained release of cisplatin that was more delayed than the RNAi, with less than 50% release at 96 hours. This controlled release kinetics of RNA allows an initial downregulation of the KRAS oncogene expression and restoration of p53 tumor suppression, both of which should lower the tumor’s drug resistance, while the release of cisplatin introduces tumor cell killing via DNA damage once the RNAs affected the tumor resistance pathways. The staged release profile shown in Fig. 1F is therefore desirable for this dual therapeutic approach.

**In vitro studies**

To investigate the efficacy of combination therapy in vitro using tissue culture experiments, lung adenocarcinoma cells were first obtained from an autochthonous murine lung cancer (KP) mouse model with an activatable KRAS mutation and p53 loss developed by the Jacks laboratory (15). The KP mice were crossed with two strains carrying Lox-STOP-Lox reporter alleles, R26LSL-Lusiferase and R26LSL-tdTomato, to generate KRASLSL-G12D/wt, p53fl/fl mice. In this model, mice are treated via intranasal inhalation with Adeno-Cre which causes deletion of p53 and activation of KRASG12D (Fig. 2A). Ten weeks after the tumor initiation, aggressive tumors were isolated and KP cells cultured for in vitro assays. To confirm the loss of p53 and KRAS oncogene activation, we assessed the miR-34 and KRAS expression in both healthy and tumored lungs. It was found that miR-34 expression was significantly decreased in the tumored lungs, whereas KRAS expression is elevated in tumored lungs, compared with healthy lungs (Supplementary Fig. S2A, S2B, and S2D). Furthermore, it was confirmed that CD44 is overexpressed in the tumored lungs using immunostaining (Supplementary Fig. S2C), indicating that the HA outer layer should serve as a targeting moiety for this tumor cell type. We first examined whether the LbL nanoparticles can deliver siKRAS to lung adenocarcinoma cells and effectively knockdown KRAS. The KP cells were treated with LbL nanoparticles containing siKRAS, and gene knockdown was monitored at days 3 and 5. It was found that siKRAS was successfully delivered to cells; approximately 70% and 40% reduction of KRAS expression was observed at day 3 and 5,
respectively (Fig. 2B). We also demonstrated the successful delivery of miR-34a to KP cells, with miR-34a expression levels at approximately 60% and 50% at day 3 and 5, respectively (Fig. 2C). The extended period of transfection observed over multiple days is achieved due to the controlled siRNA release from the LbL nanoparticle inner layers, thus providing sustained oncogene suppression. To further investigate the effectiveness of the combination therapy, cell viability was monitored by varying the concentration of cisplatin, co-delivered with scrambled siRNA, siKRAS, miR-34a, or siKRAS/miR-34a combination. We observed enhanced cytotoxicity against KP cells using combinations with either siKRAS, miR-34a, or siKRAS/miR-34a combo, compared with combinations with scrambled RNA as a control after 3 days (Fig. 2D). This finding is further supported by calculating the IC_{50} value of cisplatin at various conditions. Combining cisplatin with either siKRAS or miR-34a in the LbL nanoparticle formulations significantly decreased the IC_{50} value of cisplatin, and the combination of the two RNA molecules together with cisplatin yielded a fivefold decrease compared with cisplatin with scrambled RNA (Fig. 2E), confirming the enhanced efficacy of cisplatin in killing lung adenocarcinoma cells by suppressing the tumor cell survival pathways; the KRAS oncogene was knocked down by siKRAS and the p53 functional pathway was stimulated by miR-34a.

Figure 2.
In vitro Characterization of combination therapeutics against lung adenocarcinoma cells. A, KP cells were derived from lung tumor, generated through inhalation of Cre. B, KRAS mRNA expression in KP cells after 3 or 5 days treated with combination LbL nanoparticles. C, miR-34a mRNA expression in KP cells after 3 or 5 days treated with combination LbL nanoparticles. D, Examination of RNA enhanced cytotoxities against KP cells at 72 hours. E, Calculated IC_{50} value of cisplatin while combined with RNA therapeutics using GraphPad software. The results represent mean ± SD; n = 3.
Tumor targeting of LbL nanoparticles

Before in vivo efficacy investigations, we first assessed the capability of LbL nanoparticles to actively target the lung adenocarcinoma using the orthotopic KP adenocarcinoma model. Compared with the autochthonous model, this orthotopic KP model maintains the same genetic mutations and requires only 2 weeks for tumor initiation, whereas 10 weeks are required for the autochthonous model. Furthermore, by controlling the number of KP cells implanted, an optimal therapeutic window of one-month survival time after tumor initiation for non-treated mice can be obtained to observe the therapeutic effect of combination therapies. After tumor initiation by intravenous injection of KP cells, multiple tumors, with volumes ranging from 1 to 20 mm$^3$, were formed in the lung area (Supplementary Fig. S3). Both healthy and tumor-bearing mice were intravenously injected with Cy5.5-labeled LbL nanoparticles, which are generated by embedding Cy5.5-labeled siRNA (10% molar ratio) within the RNA layer (Supplementary Fig. S4), and whole-body imaging was completed at certain time intervals (4, 24, and 48 hours). As shown in the whole-body images of Fig. 3A, taken at the 48-hour time point, nanoparticles mainly accumulated in the livers of healthy mice, as is typically observed for nanoparticle systems due to filtration through the liver, but there was no significant accumulation in the lung; whereas the nanoparticles in tumor-bearing mice were...
detected by fluorescence in both the lung and liver with comparable intensities. It was also observed that the nanoparticles accumulated in the kidneys of both healthy and tumor-bearing mice, as can be seen from the excised organ images (Fig. 3A). The quantitative analysis of the recovered fluorescent intensities for each organ is also provided (Fig. 3B). The HA terminated LbL nanoparticles provided clear evidence of selective targeting in this orthotopic model, with the accumulation in the lungs of tumor-bearing mice approximately 22-fold greater than in the lungs of healthy mice (Fig. 3B). Approximately 45% of the initial dose (based on net recovered fluorescence) was co-localized in the lungs of tumor-bearing mice, whereas only 2% of the initial dose was detected in the lungs of healthy mice (Fig. 3B). The enhanced accumulation in the lungs of tumor-bearing mice was accompanied by a reduction of nanoparticles in the liver by twofold. Compared with healthy mice, in which 80% of recovered fluorescence is detected in the liver, approximately 40% of recovered fluorescence was observed in the liver of tumor-bearing mice (Fig. 3B). Given that these LbL nanoparticles are terminated with an HA layer, the endogenous ligand for CD44 receptor, the enhanced lung accumulation in tumor-bearing mice is attributed to active targeting of HA to the CD44 receptor on KP cells. To validate this, we first demonstrated the existence of KP cells in the lungs of tumor-bearing mice by tracking the fluorescence of tdTomato, a red fluorescent protein inserted in the KP cells (Fig. 3C). In addition, CD44 overexpression in the lungs of tumor-bearing mice was validated by immunohistochemistry, whereas negligible CD44 expression is observed in the lungs of healthy mice (Fig. 3D). It is also noted that these HA-terminated LbL nanoparticles can undergo an enhanced cellular uptake via response to hypoxic tumor microenvironment (37). Furthermore, this enhanced accumulation of LbL nanoparticles in tumor-bearing mice can also be attributed to passive targeting due to defective tumor vasculature, and an extended blood circulation time (35). In summary, we achieved significantly enhanced lung tumor targeting using the modular LbL platform while existing RNA combination therapy for NSCLC using lipids do not show this enhanced lung tumor targeting properties as typical lipids lack the modularity, responsive behavior and native ligand binding achieved with the outer LbL bilayer for multimodal targeting (15).

In vivo treatment efficacy

Upon successful targeting to lung adenocarcinoma using LbL nanoparticles, we further investigated treatment efficacy in the KP lung adenocarcinoma orthotopic mouse model. We anticipated that the treatment efficacy from the combination therapy would be enhanced due to the knockdown of KRAS and restoration of p53 regulated downstream pathways. For this proof-of-concept study, we included LbL nanoparticles that were loaded with either RNA therapeutics alone, cisplatin alone, or empty LbL nanoparticles as the control to demonstrate the effect of RNA and cisplatin combination therapies. The established lung adenocarcinoma mice were treated every week for 4 weeks with a total of four tail vein injections at 12 mg/kg of cisplatin. This specific dose is chosen based on the balance between toxicity and efficacy. Dosing of 16 mg/kg shows severe toxicities, with major fatalities observed during the treatment phase (Supplementary Fig. S5A); dosing of 8 mg/kg shows no treatment efficacy in all groups (Supplementary Fig. S5B). This finding is supported by additional histology analysis, in which swollen tubules, an indication of severe kidney damage, were observed in mice treated with cisplatin dosing of 16 mg/kg (Supplementary Fig. S6). In addition, serum chemistry analysis showed elevated creatine and blood urea nitrogen (BUN) levels, the indicators of kidney malfunction, in both free cisplatin (8 mg/kg) and nanoparticles with cisplatin dosing of 16 mg/kg treated groups (Supplementary Fig. S6). It is also noteworthy that free cisplatin (8 mg/kg) showed elevated toxicities compared to cisplatin in LbL nanoparticles (8 mg/kg), indicating that the LbL platform significantly lowers the nephrotoxicity of this chemotherapy (Supplementary Fig. S7). The lung areas were monitored through computed tomography (µCT) over the course of treatment and illustrated in the 2D axial images taken pre- and posttreatment (Fig. 4A). As expected, the empty vehicle control group showed unregulated tumor growth, demonstrated by the increased lighter shaded areas that are populated with tumor cells (red circles, Fig. 4A). Compared with the empty vehicle control group, both of the cisplatin and RNA only treated group showed a smaller increase in the light shaded tumor area (red circles, Fig. 4A). The cisplatin/RNA combination therapy exhibited a better controlled tumor growth with no apparent increase of the lighter shaded tumor area (Fig. 4A). This finding demonstrated an enhanced treatment response of combination LbL nanoparticles. It was supported by quantitative analysis of the tumor volume, indicating that combination nanoparticle therapy significantly controlled tumor growth compared with the single therapeutic-treated groups or empty nanoparticles (P = 0.01, Fig. 4B). We further calculated the volumes of healthy lung tissue and showed that tumors in the vehicle control group continued to grow, and displaced the healthy lung tissue, whereas combination therapy showed the smallest decrease in healthy lung volume, compared with either RNA or cisplatin only treated groups (Fig. 4C). More importantly, the combination LbL nanoparticle therapy prolonged mouse survival significantly (P < 0.001) compared with the singular therapeutic groups, with median survival of 23.5 days versus 15.5 and 9.0 days for cisplatin and RNA, respectively. Taken together, we not only achieved prolonged survival with the combination LbL nanoparticles but also remediated nephrotoxicity that typically arises from cisplatin treatment.

To examine the mechanism of combination therapy, we further excised all the lung tissues after treatment, and performed immunohistochemistry analysis to screen the biomarkers that are responsible for cell apoptosis (CC3), cell proliferation (Ki67), the molecular signaling downstream of the KRAS pathway (i.e., phospho-ERK) and miR34a pathways (i.e., CDK6 and SIRT1). The expression of pERK, CDK6, and SIRT1 were effectively suppressed in the treated mice of either the RNA-only or combination therapy, indicating the effective delivery and transfection of RNA therapeutics to the tumors. The targeted pathways were sufficiently impacted by the RNA therapeutics in these tumors. However, in the mice treated with vehicle or cisplatin only, we did not observe similar decreases in the expression of these biomarkers. We further evaluated the therapeutic effects on tumor cell proliferation using Ki67 staining. Compared with other treatment groups, the combination therapy effectively inhibited tumor cell proliferation (Fig. 5B). Furthermore, we stained the cleaved products of caspase-3 (CC3), a cell apoptosis biomarker, to evaluate the therapeutic effects on tumor cells. We observed more cleaved caspase-3 products in the combination therapy treated mice, indicating greater cell death, whereas other treatment groups gave negligible levels of the cell apoptosis biomarker (Fig. 5B). To confirm these effects were due to the successful delivery of RNA therapeutics rather than off-target effects, we tested the gene
silencing against KRAS and the miR-34a content in isolated tumors. We observed higher content of miR-34a in RNA-only and combination therapy treated groups, whereas cisplatin and vehicle-treated groups produced significantly less miR-34a (Fig. 5C).

For KRAS expression, it was found that both RNA and combination therapy–treated groups had significantly lower gene expression relative to treated groups without RNA therapeutics (Fig. 5D). Western-blot analysis was also performed in excised tumors and confirmed the downregulation of the KRAS primary protein in RNA and combination treated groups (Fig. 5E). Therefore, both IHC analysis, q-PCR, and Western-blot results indicated successful delivery of payloads that correlate with the outcomes from the microCT evaluations. Collectively, cisplatin/RNAi combination therapy, co-packaged and delivered from a singular LbL nanoparticle formulation, appeared to provide the most effective therapeutic effect, most likely due to the enhanced effects of the targeted RNA therapeutics. More importantly, based on the similarities between this animal model and human NSCLC, these results suggest this LbL platform holds great potential for clinical translation. It is noteworthy that we are the first to report the packaging...
of multiple genes and chemotherapeutics in a singular platform to treat NSCLC in a highly physiologically relevant animal model.

**Conclusion**

We have developed a modular LbL nanoparticle platform incorporating oncogene siRNA, tumor suppressor stimulating miRNA, and chemotherapeutic in a singular formulation to target NSCLC. These therapeutics were released in a staggered fashion to enable synergistic timing of gene silencing and chemotherapy treatment. Given the intervention of tumorigenic gene mutations achieved in this case, the efficacy of cisplatin to kill tumor cells was enhanced *in vitro*. In a physiologically relevant orthotopic model of lung adenocarcinoma, enhanced accumulation of nanoparticles in tumor-bearing lungs was achieved via both passive and active targeting of HA to the CD44 receptor. We further demonstrated successful delivery of the combination LbL nanoparticles to the lungs of tumor-bearing mice in this model. Enhanced antitumor efficacy and prolonged survival rate were observed in the mice treated with combination therapy, compared with any RNA or cisplatin treatment alone. Molecular evaluations confirmed the successful regulation of oncogene KRAS and restoration of p53 function by delivery of siKRAS and miR-34a, respectively. Therefore, the tumor defense pathways were blocked and the efficacy of DNA damage chemotherapeutics was facilitated, while maintaining a greatly lowered liver toxicity. Because a large number of tumors, including lung adenocarcinoma, carry mutations of KRAS and loss of p53 function, this combination approach has direct translational potential, and promise that can lead toward clinical trials. Due to the modularity of the LbL nanoparticle, a broad

*Figure 5.* Immunohistochemistry analyses against a panel of biomarkers that regulate in cell proliferation, apoptosis, KRAS signaling pathway, and miR-34a pathways were tested. **A**, Representative images of IHCs of various biomarkers. **B**, Quantification of biomarker expression was performed by counting positive stained cells at randomly picked area of 1 mm² and average number and standard deviation were obtained by triplicate. **C**, qPCR analysis of miR-34a expression level in isolated lung tumors. **D**, qPCR analysis of KRAS expression in isolated lung tumors. The results represent mean ± SD. *n* (number of tumors in each group) = 10. **D**, Western blot results of KRAS primary protein in isolated lung tumors.
range of therapeutics, including inhibitors and nucleic acid, can be incorporated to target a variety of oncogene pathways, thus presenting a versatile platform for personalized medicine. Moreover, given the tunable features of the outer surface layer, this LbL RNA nanoparticle approach can be tailored to target different organs of interest, including primary or metastatic tumor sites.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: L. Gu, Z.J. Deng, P.T. Hammond
Development of methodology: L. Gu, Z.J. Deng, P.T. Hammond
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): L. Gu
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): L. Gu
Writing, review, and/or revision of the manuscript: L. Gu, S. Roy, P.T. Hammond
Administrative, technical, or material support (i.e., reporting or organizing data, creating databases): L. Gu
Study supervision: L. Gu, P.T. Hammond

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