Ionizable lipid-assisted efficient hepatic delivery of gene editing elements for oncotherapy

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ABSTRACT

CRISPR/Cas9-based gene editing has emerged as a powerful biotechnological tool, that relies on Cas9 protein and single guided RNA (sgRNA) to edit target DNA. However, the lack of safe and efficient delivery carrier is one of the crucial factors restricting its clinical transformation. Here, we report an ionizable lipid nanoparticle (iLP181, pKa = 6.43) based on ILY1809 lipid enabling robust gene editing in vitro and in vivo. The ILP181 effectively encapsulate psgPLK1, the best-performing plasmid expressing for both Cas9 protein and sgRNA targeting Polo-like kinase 1 (PLK1). The ILP181/psgPLK1 nanoformulation showed uniformity in size, regular nanostructure and nearly neutral zeta potential at pH 7.4. The nanoformulation effectively triggered editing of PLK1 gene with more than 30% efficiency in HepG2-Luc cells. ILP181/psgPLK1 significantly accumulated in the tumor for more than 5 days after a single intravenous injection. In addition, it also achieved excellent tumor growth suppression compared to other nucleic acid modalities such as siRNA, without inducing adverse effects to the main organs including the liver and kidneys. This study not only provides a clinically-applicable lipid nanocarrier for delivering CRISPR/Cas system (even other bioactive molecules), but also constitutes a potential cancer treatment regimen based on DNA editing of oncogenes.

1. Introduction

The development of CRISPR/Cas gene editing technology has entered in a promising era, offering new treatments for diseases that are currently incurable. It has been applied to treat cancer [1], blister skin disease [2], Huntington’s disease [3], sickle cell disease (SCD) [4], HIV-1 infection [5], and to establish specific animal model carrying a modified genome [6], etc. It can also be used to detect certain nucleic acid molecules, facilitating disease diagnosis, pathogenic microorganism analysis and environmental monitoring [7,8]. To some extent, the emergence of CRISPR/Cas reflects the significant advances of human gene editing technology, and provides an effective and powerful platform to further understand gene functions, underlying biological or pathological mechanisms of various disease, and ultimately develop novel treatment regimens.

In order to transport CRISPR/Cas system into cells, various delivery technologies are used, such as electroporation, virus or non-viral carriers [9]. Viral carriers such as adeno-associated virus (AAV) and lentiviral vectors are frequently used to confer long-lasting effects, which helped in motivating the recent surge of successful gene therapies [10,11]. Two gene therapeutics based on AAV vectors, Luxturna and Zolgensma, have been approved by the U.S. Food and Drug Administration (FDA) in 2017.

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and 2019, respectively. Another therapeutic for the treatment of hemophilia A is expected to receive the FDA approval in 2021 [12]. However, safety concerns regarding AAV vectors have simmered for nearly 20 years. It was reported that newborn mice developed liver cancer after they were injected with a high dose of AAV vectors [13]. In 2018, James Wilson reported that a high dose of AAV vectors induced systemic and sensory neuron toxicities in juvenile nonhuman primates [14]. Recently, a study conducted on hemophilia dogs treated with AAV showed that the vector could insert payload into the host’s genome, close to genes that control cell growth. Therefore, the scientific researchers are concerned that, it is only a matter of time before such insertions causes cancers [15]. In addition, the use of AAV to deliver Cas9 mRNA may also causes sustained Cas9 protein expression, anti-Cas9 immune response and off-target editing. There is currently a lack of in vivo evidence for the biomedical efficacy of lentiviral vector-facilitated Cas9 delivery.

Comparatively, non-viral carriers represent a safe and efficacious approach that allows long-term administration [11]. Among the available diverse carriers, lipid nanoparticles (LNPs) are the most clinically-advanced as compared to other available options [16–21]. For example, Onpattro®, the siRNA-loaded LNP formulation, has been approved by the US FDA for the treatment of transthyretin mediated amyloidosis. Moreover, the three recently mRNA-based COVID-19 vaccines (mRNA-1273, BNT162b2, CVnCoV) are all LNP formulations [22, 23], and two of them (mRNA-1273 and BNT162b2) have been authorized the emergency use in December 2020 for the prevention of COVID-19. LNP is also the most potent delivery system for CRISPR/Cas and has been employed in delivering gene editing payloads to the cancer cells [1], hepatocytes [24–27], the lung [24] and the brain [28].

In addition, ionizable lipid can be protonated at acidic (endosomal) environment, interact with endosomal membrane’s anionic lipids such as phosphatidylserine, facilitating membrane fusion, membrane disruption and endosomal escape [16, 29], thus, highlighting its overwhelming advantages for nucleic acid delivery. Herein, we developed a novel ionizable LNP (iLNPs), called iLP181, to evaluate its performances in delivering CRISPR/Cas system (Scheme 1). We constructed a plasmid containing the expression frame of Cas9 protein and sgRNA targeting PLK1 (polo-like kinase 1) gene, termed as Cas9-sgPLK1 plasmid or psgPLK1. The physicochemical properties of iLP181/psgPLK1 including the pKa value, size, morphology and zeta potential were characterized. The in vitro gene editing, intracellular delivery mechanism and in vivo biodistribution were explored. Finally, the therapeutic effects and toxicity of iLP181/psgPLK1 formulation on tumor-bearing mice were carefully evaluated.

2. Materials and methods

2.1. Materials

Cell culture reagents such as the Fetal bovine serum (FBS), trypsin, streptomyycin-penicilllin, Dulbecco’s modified Eagle’s medium (DMEM), Opti-MEM, Lipofectamine 2000 were purchased from Thermo Fisher. RNAlater® and TRIzol® were purchased from Sigma-Aldrich (St Louis, MO). Luciferase substrate was purchased from Promega Co.Ltd (Madison, USA). PCR Mix and RT-PCR Mix were purchased from YEASEN Co. (Shanghai, China). The DMG-PEG<sub>2000</sub> (R)-2,3-bis(Octadecyloxy)propyl-1-(methoxy polyethylene glycol 2000) carbamate and DSPC (1,2-Dioctadecanoyl-sn-glycero-3-phosphocholine) were purchased from Avanti Polar Lipids, Inc (Alabama, USA). The siPLK1, cholesterol and iLY1809 were provided by Suzhou Ribo Life Science Co. Ltd. (Suzhou, China). All the primers were provided by BioSune Co. (Shanghai, China).

2.2. Plasmid construction and preparation

Four sgRNAs targeting PLK1 gene were designed by using the online CRISPR design tool (http://crispr.mit.edu/). First, the expression cassette of sgPLK1 within sgRNAs lenti Guide-Puro was amplified by polymerase chain reaction (PCR) and cloned into the Bbs 1 restriction sites of PX458 plasmid. Then, the constructed Cas9-sgPLK1 plasmid (psgPLK1) was transfected into E. coli DH5α and cultivated at 37 °C in 400 mL LB broth (without ampicillin) for 4 h. Next, the E. coli DH5α solution was concentrated and streaked on solid LB culture (containing ampicillin) and incubated at 37 °C for 24 h. In the following days, the single colony of E. coli was added to LB culture (containing ampicillin) and was incubated for another 16 h at 37 °C. After cultivation, the E. coli was collected and the modified Cas9-sgPLK1 plasmid (psgPLK1) was purified with Endo Free Plasmid kits (TIANGEN).

2.3. Preparation and characterisation of iLP181/psgPLK1 formulation

The iLP181/psgPLK1 formulation is prepared according to previous reports with some modification [16,17,30]. Briefly, the lipids containing cholesterol, DSPC, iLY1809 and DMG-PEG<sub>2000</sub> were dissolved in ethanol solution and were added into sodium acetate solution to obtain the
pre-liposomes. Then the aqueous plasmid solution (300 ng/μL) was mixed with pre-liposomes at a volume ratio of 1:1. Subsequently, the mixture was incubated at 50 °C for 10–20 min, and dialyzed against PBS buffer to remove the organic phase. The final iLP181/psgPLK1 liposome formulation were used as prepared or kept at 4 °C for less than 3 months. The size, polydispersity index (PDI) and zeta potential of LNPs were determined by the Dynamic Light Scattering (DLS) equipment (Malvern, Zetasizer Nano ZS). The morphology was observed by transmission electron microscopy (TEM) (Hitachi, HT7700).

2.4. pKa determination

A fluorescent probe of 2-(p-toluidino)-6-naphthalene sulfonic acid (TNS) was used to determine the pKa value of iLP181 formulation. First, iLP181 (10 mM) solution was prepared in PBS. Then a series of iLP181 solutions with a pH range of 3.00–10.00 were prepared, containing 1 mM TNS, 10 mM ammonium acetate, 10 mM 4-morpholineethanesulfonic acid (MES), 10 mM HEPES and 130 mM NaCl. The fluorescence intensity of each solution was measured with a spectrophotometer at the excitation wavelength of 321 nm and emission wavelength of 445 nm. The sigmoidal best fit analysis was performed to analyze the fluorescence data. The pKa value was calculated and defined as the pH giving rise to the semi-maximal fluorescence intensity [29].

2.5. Cell culture

HEK293A, human embryonic kidney cell line, and HepG2-Luc, human hepatocellular carcinoma cell line with stable luciferase expression, were cultured with DMEM containing 10% FBS, 100 IU/mL penicillin, and 100 g/mL streptomycin at 37 °C in a humidified atmosphere with 5% CO2.

2.6. Plasmid validation using fluorescence microscopy

Because the expression frame of green fluorescence protein (GFP) was inserted to the backbone of the commercial PX458 plasmid, we used as controls. When the cells were transfected for 24 h, the GFP fluorescence intensity of each solution was measured with a spectrophotometer at the excitation wavelength of 488 nm and emission wavelength of 509 nm. Meanwhile, the GFP fluorescence intensity was quantified using Image J software.

To validate the delivery efficiency of the LNP, the HEK293A cells were transfected with iLP181/PX458-E and iLP181/psgPLK1 LNPs using the same transfection procedure as mentioned above. Cells without transfection or transfected with Lipofectamine 2000-formulated psgPLK1 were used as controls. When the cells were transfected for 24 h, the GFP expression and cellular location were observed using confocal laser scanning microscope (CLSM). Meanwhile, another parallel test was conducted for the flow cytometry analysis (BD, USA).

2.11. Endosomal escape efficiency

The HepG2-Luc cells were sed in 6-well plates with 2 × 105 cells per well and were cultured overnight with complete medium. The cytoplasmic GFP signal was observed using a confocal laser scanning microscope (CLSM). The results showed that the GFP signal was efficiently delivered into the cytoplasm, indicating effective endosomal escape efficiency.
2.12. Animals

All animals were purchased from SPF (Beijing) biotechnology co., LTD. and maintained in Laboratory Animal Center. All the animals associated experimental procedures were performed in accordance with the principles of the Institutional Animal Care and Use Committee (IACUC) of Beijing Institute of Technology.

2.13. In vivo biodistribution

The 6–8 weeks old, weighing 18–22 g C57BL/6 mice were used for in vivo biodistribution study. The iLP181-formulated Cy5-nucleic acid (iLP181/Cy5-NA) or red fluorescent protein (RFP)-expressed plasmid (iLP181/plasmid) were prepared according to aforementioned protocol and were injected into mice via tail vein at the dose of 1 mg/kg. The concentration of Cy5-NA and RFP-expressed plasmid all are 600 ng/μL and were injected into mice via tail vein at the dose of 1 mg/kg. The flanks of BALB/c nude mouse. When the volume of tumor reached 8 times. Body weights were monitored during the entire experiment and injected every 2 days at a dose of 0.5 mg/kg (for siRNA or plasmid). Animal sacrifice was defined as *P < 0.05 and **P < 0.01.

3. Results and discussion

3.1. Construction of Cas9-sgPLK1 plasmid (psgPLK1)

PLK1 (polo-like kinase 1) is a serine/threonine kinase, that is highly expressed during the G2 phase of the cell cycle, where it control the metaphase-anaphase transition and mitotic exit [31]. It is often overexpressed in many tumor cells [32,33]. Knockout or knockdown of PLK1 gene by gene editing or other technologies is a promising approach for cancer treatment [1,34–37]. Before the construction of CRISPR/Cas9 plasmid, we designed four single chain sgRNA sequences targeting the PLK1 gene using an online design tool (http://crispr.mit.edu), and prepared double stranded sgRNAs by gradient annealing method. We then modified the commercial PX458 plasmid (PX458-E) by inserting sgRNA sequences into the sgRNA scaffold using T4 DNA ligase (Fig. S1). The modified plasmids were expanded by transfection and cultivation of E. coli. The sequencing results demonstrated that there are no mismatch base pairs, which verified that the recombinant Cas9-sgPLK1 plasmid (psgPLK1) was successfully constructed (Fig. S1).

3.2. Preparation and characterization of iLY181 LNPs

To date, several ionizable lipids have been successfully developed for the delivery of RNAs and DNAs, such as Dlin-MC3-DMA and C12-200. Conventionally, an ionizable lipid consists of hydrophobic amino head and hydrophobic alkyl chain tails. Lipid containing ionizable amines has been reported to deliver small RNAs more effectively in vivo than non-ionized lipids which contain permanently charged quaternary amine moieties in their hydrophilic head group. Excellent intracellular delivery relies on an optimal balance of ionized amines to facilitate the binding and release of nucleic acids by LNPs. pKa value is an important molecular characteristic of ionizable lipids, and lipids with pKa value between 6.2 and 6.5 showed better siRNA delivery in vivo. With such a sharp pKa, ionizable LNPs have minimum charge in neutral physiological environment to maximize hepatocyte uptake by ApoE-mediated endocytosis and maximum positive charge in acidic endosomal compartment to promote membrane disruption. As a result, lipids with pKa value between 6.2 and 6.5 achieve a balance between the two opposing delivery requirements, improving the resulting functionality. The pKa of clinically-applied Dlin-MC3-DMA was 6.44, and the ED50 (median effective dose for SUV gene silencing in female C57BL/6 mice by i.v. administration) of LNPs prepared based on Dlin-MC3-DMA and C12-200 is 0.03 mg/kg (before composition optimization) or 0.005 mg/kg (after composition optimization) [38], and 0.02 mg/kg [39], respectively, which were two of the lipids with the best delivery efficiency reported at present. In addition to pKa, appropriate hydrophobic tail numbers, length and lipid chain unsaturation also served as important considerations for lipid design. Based on these lipid design principles, we synthesized and screened an ionizable lipid, named iLY1809. The chemical structures of iLY1809, DSPC, DMG-PEG2000 and cholesterol were shown in Fig. S2.

The iLY1809 based LNPs (iLP181) containing various lipids were easily prepared by pumping the lipid solution (the organic phase) into the citrate buffer to form classic bilayer-structured vesicles (Fig. 1A). The CRISPR/Cas9 plasmids were then encapsulated into the LNPs through an additional incubation step with iLP181. This incubation step is essential for reassembling of LNPs and stabilizing the system. The lipid iLY1809 employed in this study is a proprietary ionizable lipid, which exhibits positive charges and entraps nucleic acids at acidic pH but becomes neutral charge at physiological pH. The PEG lipid affords a diffusible outer PEG layer to increase the half-life of LNPs in the bloodstream [40]. The DSPC influence the morphology and size of the nanoparticles, thereby affecting the delivery efficiency [41]. The last...
component cholesterol is the most common lipid used in preparation of LNPs, which helps to stabilize the LNP [41,42]. The pKa value of iLP181 formulation determined via TNS assay was approximately 6.43 (Fig. 1B), which was in line with our designing concept and met the requirements of ionizable lipid nanoparticle enabling efficient endosomal escape of the nucleic acid payloads in cells [38,43]. In addition, the formulated iLP181/Crispr LNPs showed uniform spherical shapes with clear bilayer membranes (Fig. 1C). The size was approximately 100 nm (Fig. 1C, D and E). The polydispersity index (PDI) was lower than 0.2, indicating a narrow distribution of particle size (Fig. 1E). The zeta potential was close to neutral at physiological pH. More than 85% cell viability was observed in the iLP181 formulation-treated cells, indicating the ideal biocompatibility of iLP181 LNPs in vitro (Fig. 1F).

Furthermore, the stability of iLP181/plasmid LNPs was measured in body fluid-mimicking system. The size and zeta potential of LNPs were stable and uniform after they were incubated in PBS or in 10% human serum for two weeks, verifying the outstanding stability and relative safe character of iLP181 LNPs (Fig. 1G and H).

3.3. In vitro gene editing efficiency of iLP181/plasmid LNPs

To validate the gene editing efficiency, we transfected plasmid into the HEK293A cells using Lipofectamine 2000. The expression of GFP, a tag protein in PX458 plasmid, was observed by fluorescence microscope at 24 h and 48 h post transfection, respectively (Figs. S3A and S3B). It was illustrated that both PX458-E and psgPLK1 effectively expressed GFP in HEK293A cells. In addition, the delivery efficiency of iLP181 LNPs in cells were also evaluated by laser confocal microscopy. As shown in Figs. S3C and 3D, the GFP expression pattern was the same with that shown in Figs. S3A and S3B. Therefore, the iLP181 displayed comparable intracellular delivery efficiency with Lipofectamine 2000 for Crispr/Cas plasmid.

In addition, based on the self-designed series of sgRNA sequences targeting PLK1 gene, we also constructed a series of Crispr/Cas9 plasmids and evaluated their gene editing efficiency in HEK293A cells using Lipofectamine 2000. It was shown that the sgPLK1-1 sequence exhibited the best activity and the gene editing efficiency reached 33% (Fig. 2A). We then formulated the plasmid containing sgPLK1-1 sequence with iLP181 and detected the long-term mRNA knockdown performance in HepG2-Luc cells. Cells were transfected for 3 d and 7 d, respectively, and were collected to analyze the mRNA level. It was revealed that iLP181 mediated remarkable mRNA knockdown efficiency in HepG2-Luc cells, especially 32% knockdown efficiency on 7 d, indicating that the plasmid-mediated gene editing effect was long-term (Fig. 2B).

It is reported that ionizable LNP (iLNPs) can deliver the payloads to hepatocytes via an active liver-targeting mechanism. When iLNPs are systematically administered, they may bind with apolipoprotein E (ApoE) in circulation, then interact with low-density lipoprotein receptor (LDLR), a highly-expressed protein on hepatocytes, and finally being...
internalized by hepatocytes via LDLR-mediated endocytosis [43, 44]. In this study, the LDLR over-expressed HepG2-Luc cells were used to evaluate whether ApoE was an important factor for cellular uptake of iLNPs. As shown in Fig. 2C and D, it can be clearly found that when ApoE was added into the culture medium, the amounts of cellular uptake of iLNPs were significant more than that without ApoE, demonstrating that ApoE significantly boosted the cellular uptake of iLNPs. In addition, subcellular localization of the payload nucleic acids also verified that ApoE promoted the internalization of iLP181 LNPs (Fig. 2E and F).

3.4. The endosomal escape

Increasing evidences demonstrated that only 3.5% (even less than 1%) internalized nucleic acid can escape from endosome and be released into the cytosol to mediate gene silencing [45-48], necessitating the development of delivery vehicles with the ability of rapid endosomal escape. Hence, we conducted the experiments to explore the endosomal escape of iLP181 and compare the advantages between iLP181 and commercial transfection reagents Lipo2000. In Fig. S4A, the internalization and intracellular trafficking of iLP181/Cy5-labelled nucleic acids (iLP181/Cy5-NA) into HepG2-Luc cells were carefully investigated to elucidate the underlying mechanism. It was observed that, 1–3 h after transfection, the iLP181/Cy5-NA escaped from the endosome/lysosome (Fig. S4C), while there was no significant escape from endosomes in the groups of Lipo2000/Cy5-NA. The MFI of iLP181/Cy5-NA and Lipo2000/Cy5-NA increased with the extension time of transfection, indicating more and more nucleic acids accumulated in HepG2-Luc cells (Figs. S4B and 4E).

We further examined the internalization and subcellular localization
in the presence of chloroquine (50 μM, a lysosomal disrupting agent) and bafilomycin A1 (200 nM, a proton pump inhibitor that selectively inhibits vacuolar H+ - ATPase) to evaluate whether the iLP181/nucleic acid induce effective transfection enhancement and whether iLP181 utilize lysosome/endosome for intracellular trafficking, respectively [49]. The transfection of iLP181 LNPs and Lipo2000 formulation was conducted in complete medium (DMEM) containing serum. The MFIs of iLP181 LNPs was significantly stronger than the group Lipo2000 (Fig. 3B). Meanwhile, the colocalization ratio between nucleic acid and lysosome/endosome was calculated and applied to evaluated the endosome escape efficiency. Addition of chloroquine to the cell led to a significant decrease in the colocalization ratio value of the iLP181 LNPs (~58.33%) and Lipo2000 formulation (~17.26%), whereas addition of bafilomycin A1 to the cell led to a significant increase in the colocalization ratio value of the iLP181 LNPs (~87.00%) and Lipo2000 formulation (~43.32%) (Fig. 3C). The MFIs of the Lipo2000/Cy5-NA were generally weak, which results in a smaller colocalization value compared to the iLP181/nucleic acid complexes. The results showed that chloroquine slightly increased transfection of iLP181 LNPs and Lipo2000, while bafilomycin A1 decreased their transfection (Fig. 3C).

3.5. In vivo biodistribution

We then evaluated the biodistribution of iLP181 LNPs in tumor-bearing mice. The Cy5-NA and red fluorescent protein (RFP)-expressed plasmid were formulated with iLP181 to prepare uniform LNPs, which were administered via tail vein into C57BL/6 mice at a dose of 1 mg/kg (Fig. 4). The Cy5 and RFP fluorescence were assessed by an in vivo imaging system at different time points. As shown in Fig. 4A and B, the Cy5 fluorescence in group of naked Cy5-NA disappeared at 24 h post administration, whereas iLP181 LNPs exhibited significant strong fluorescence for at least 24 h in mice and mainly accumulated in the liver and tumor. The quantitative analysis of mean fluorescence intensities confirmed the above results (Fig. 4C-F). Furthermore, the expression of RFP was also detected. In Fig. 4G, it was found that the LNPs group had the strongest RFP fluorescence signal in tumors within the examined hours. It is extremely difficult for naked nucleic acids to accumulate in tumors. Moreover, the RFP fluorescence intensities in tumors did not significantly reduce within 5 d. This phenomenon was consistent with
the fluorescence quantitative analysis of various organs (Fig. S5). These results suggested that iLP181 was an excellent systemic delivery system for CRISPR/Cas system.

3.6. Anti-tumor activity in vivo

PLK1 is considered a proto-oncogene involved in cell cycle regulation, neoplastic transformation and tumor suppressor p53 related pathways. Overexpression of PLK1 is a common event in tumor cells. Liposomal siPLK1 formulation, TKM-080301, previously was investigated in phase II clinical trial for treating solid tumors [50]. Thus, anti-tumor effect of iLP181/psgPLK1 was assessed using a xenograft tumor-bearing murine model. The mice were given different formulations every other day, their tumor volume and weight were recorded (Fig. 5A). At the end of experiment, the expression of luciferase in tumors were detected by intraperitoneal injection of luciferase substrate. Compared with other groups, the luciferase activity in iLP181/psgPLK1-treated mice was significantly reduced, which should be attributed to the shrinkage of the tumor and decrease of cancer cell population induced by gene abolishment of PLK1 (Fig. 5B). In addition, the changes of tumor volume were recorded (Fig. 5C), indicating that the tumor growth rate of iLP181/psgPLK1 group was significantly slower than that of other groups. At the end of the experiment, tumors were isolated and the total RNA was extracted. It was observed that
PLK1 mRNA expression was obviously inhibited by iLP181/psgPLK1 (Fig. 5D), suggesting gene editing happened in this group. The iLP181/siPLK1 group showed comparable inhibition efficiency with respect to PLK1 mRNA, because no difference was observed between iLP181/psgPLK1 and iLP181/siPLK1. Moreover, no significant changes of body weight (Fig. 5E) and liver/body weight ratio (Fig. 5F) were recorded, which confirmed that iLP181 formulation did not trigger significant adverse effects in mice. The H&E staining of tumor sections further manifested that significant cell apoptosis and death occurred in animals treated with iLP181/psgPLK1 or iLP181/siPLK1 (Fig. 5G). These results illustrated that gene editing system of psgPLK1 was successfully delivered into the tumor cells by iLP181, and significant inhibition of tumor growth in vivo was achieved. Considering the PLK1 is a widely expressed and overexpressed oncogene, the iLP181/psgPLK1 can be applied to treat other tumors with high expression of PLK1 gene, such as lung cancer, breast cancer and so on.

3.7. In vivo safety evaluation of iLP181 LNPs

The hemolysis assay was applied to evaluate the iLP181 LNPs safety in blood circulation system (Fig. 6A). There was no hemolysis occurred in the groups of iLP181-formulated PX458-E, siPLK1 and psgPLK1 LNPs at pH7.4, whereas a little hemolysis appeared in these groups at pH5.5, which further manifested that significant cell apoptosis and death occurred in animals treated with iLP181/psgPLK1 or iLP181/siPLK1 (Fig. 5G). Besides, no significant changes of body weight (Fig. 5E) and liver/body weight ratio (Fig. 5F) were recorded, which confirmed that iLP181 formulation did not trigger significant adverse effects in mice. The H&E staining of tumor sections further manifested that significant cell apoptosis and death occurred in animals treated with iLP181/psgPLK1 or iLP181/siPLK1 (Fig. 5G). These results illustrated that gene editing system of psgPLK1 was successfully delivered into the tumor cells by iLP181, and significant inhibition of tumor growth in vivo was achieved. Considering the PLK1 is a widely expressed and overexpressed oncogene, the iLP181/psgPLK1 can be applied to treat other tumors with high expression of PLK1 gene, such as lung cancer, breast cancer and so on.

4. Conclusion

CRISPR/Cas technology has emerged as a powerful genome editing tool for biomedical applications. However, development of safe and efficient non-viral delivery technology for CRISPR/Cas system is urgently required and challenging. We here reported a proprietary ionizable lipid-based LNP, termed iLP181, and thoroughly evaluated its performance of delivering anti-cancer CRISPR/Cas9 system both in vitro and in vivo. Four plasmids containing the expression frame of Cas9 protein and sgRNA targeting PLK1 were designed and screened. One of them with the best activity (psgPLK1) was selected, and loaded with iLP181. Our study proved that iLP181/psgPLK1 was effectively internalized by hepatoma carcinoma cells by binding with ApoE. Long-term gene editing was achieved both in vitro and in vivo. Moreover, iLP181 LNPs exhibited robust endosomal escape in delivering nucleic acids compared with commercial Lipo2000, thus leading to significant tumor growth inhibition in tumor-bearing mice. In addition, proposed lipid formulation was well tolerated by the animals, and it can be used for safe and effective delivery of CRISPR/Cas system in vitro as well as in vivo.
Methodology, Formal analysis, Data curation, Writing – review & editing, Visualization, Supervision, Funding acquisition.

Declaration of competing interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bioactmat.2021.05.051.

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