Evaluations of Targeted Delivery Systems for RNA Therapeutics

Yurun Zhang[1], Dihang Lin[1], Gang Liu[1], Shunchuan Zhang[1], Andy Ng[1], Wenji Su[1]

[1]Early Discovery Platform, WuXi Biology, WuXi AppTec

WuXi Biology

Introduction

- RNA therapeutics can target or deliver protein-encoding mRNAs with high specificity, an advantage to treat diseases with known genetic mutations. The development of RNA therapies is hindered by a lack of efficient and specific delivery strategy. In recent years, significant progress has been made in conjugate-based and carrier-based delivery systems to improve safe and targeted delivery of oligonucleotides to a variety of tissues and organs.
- To effectively evaluate the capability of novel delivery systems, we have established a set of *in vitro* and *ex vivo* assays to assess the cellular uptake and functional delivery by novel delivery strategies. Here, we demonstrate our capability to assess the binding and internalization of GalNAc conjugates; and to visualize and quantify the cellular uptake, endosomal escape, and functional delivery of lipid nanoparticle (LNP) delivery.
- To overcome the liver tropism of LNPs and expand its therapeutic indications, active-targeted LNPs becomes a strategy for extrahepatic delivery. Here, we generated two types of active targeted LNPs through co-formulation with PDL1-targeting peptide and via surface conjugation with anti-CD22 antibody for specific tissue delivery.

Methods

Flow Cytometric Detection of binding and internalization of GalNAc conjugates

To measure the binding between GalNAc conjugates and ASGPR: Hepatic cells were incubated with GalNAc-Cy5 for 4 hours on ice followed by quantification of fluorescence intensities by flow cytometry. Regarding cellular uptake of GalNAc-Cy5 or GalNAc-conjugated siRNA compounds, hepatic cells were treated with test compounds for 4 hours at 37 ºC. Treated cells were then harvested for flow cytometric analysis. To evaluate the uptake efficiency of unlabeled GalNAc conjugates: Cy5-labeled GalNAc (EE) was mixed with GalNAc (L96) conjugates at different concentrations and then incubated with hepatocytes for 4 – 6 hours. Following incubation, cells were collected to measure the endocytosis of GalNAc (EE) -Cy5 via flow cytometry.

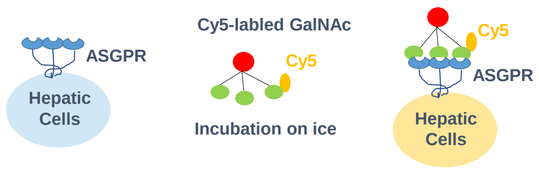
Evaluating the cellular uptake, endosomal escape and functional delivery of LNP

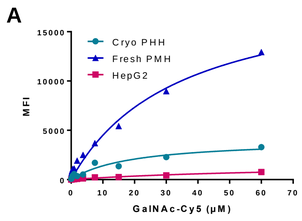
HeLa and HeLa-Gal9-GFP cells were treated with MC3-LNP containing Cy5-labeled, GFP-encoding mRNAs across a dose range of 0.1 – 1 µg/mL. Cellular uptake (Cy5) and functional delivery (GFP) were evaluated by measuring the fluorescence intensities. At 4, 6, and 16 hours post treatment, LNP-treated cells were harvested for flow cytometric analysis and confocal microscopy imaging. For live-cell imaging, MC3-LNP treated cells were imaged every 4 hours for 48 hours. To evaluate the endosomal escape of tested LNPs, HeLa-Gal9-GFP cells were imaged live using Yokogawa CQ1 high-content confocal imaging system. Foci formation by Gal9 was counted to quantify the level of endosomal escape followed by LNP treatment in vitro.

Results

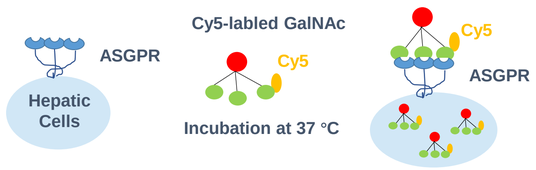
Binding and internalization of GalNAc conjugates

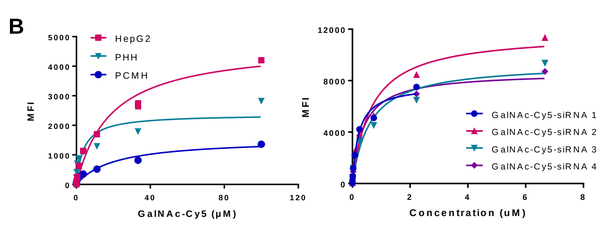
Binding of GalNAc-ASGPR



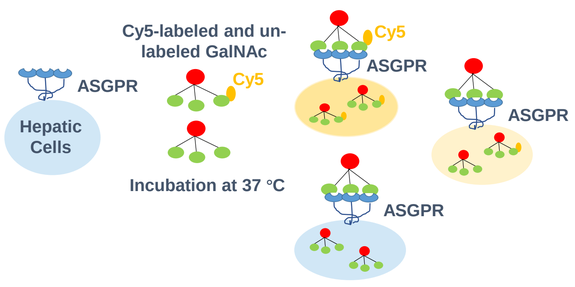


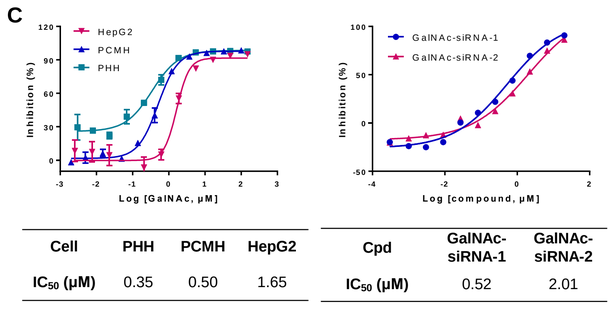
Internalization of GalNAc conjugates





Internalization of GalNAc conjugates (Competitive)





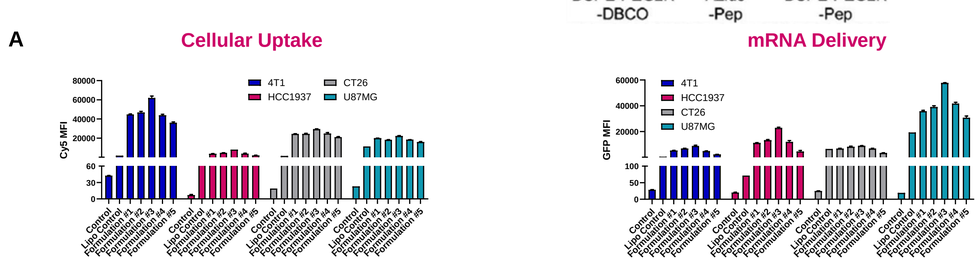
Cell	PHH	PCMH	HepG2
IC_{50} (µM)	0.35	0.50	1.65

Cpd	GalNAc-siRNA-1	GalNAc-siRNA-2
IC_{50} (µM)	0.52	2.01

Figure 1. Characterization of the binding and internalization of GalNAc-Cy5 and GalNAc-siRNA conjugates in hepatocytes. A. Cryopreserved primary human hepatocytes (PHHs), freshly-isolated primary mouse hepatocytes (PMHs), and HepG2 cells were incubated with GalNAc-Cy5 on ice for 4 hours to evaluate the binding by flow cytometry. **B.** PHHs, PCMH, and HepG2 cells were incubated with GalNAc-Cy5 or GalNAc-siRNA conjugates at 37 ºC followed by flow cytometric analysis of Cy5 fluorescence intensities to evaluate uptake of GalNAc conjugates. **C.** GalNAc (EE) - Cy5 was mixed with different concentrations of GalNAc (L96), and then incubated with hepatocytes for 4 hours. The uptake of GalNAc (EE) - Cy5 was analyzed by flow cytometry. PCMH: Primary C. monkey hepatocytes.

Active-targeted LNP as a strategy for extrahepatic delivery

Co-formulation Strategy (PDL1-peptide active targeted LNP)



Post-modification Strategy (CD22 antibody active targeted LNP)

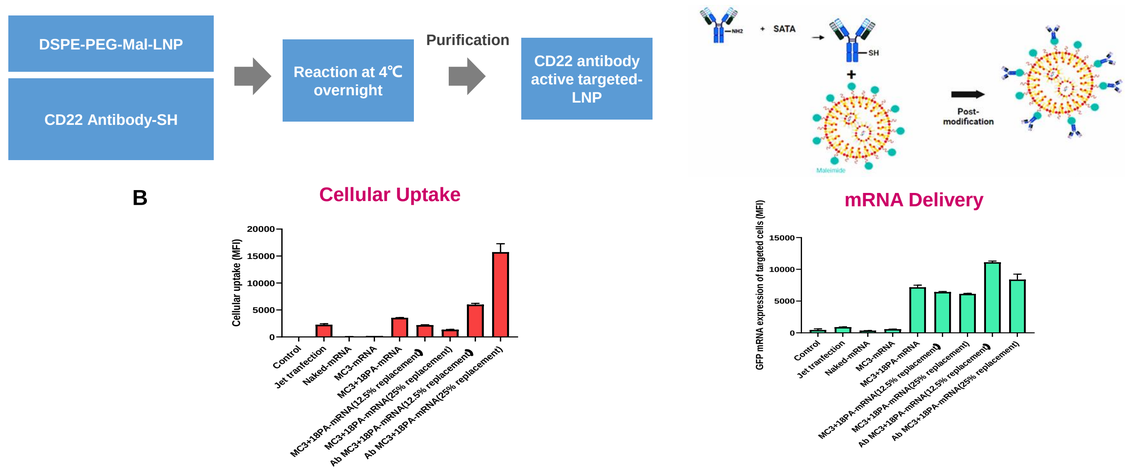


Figure 3. In vitro evaluation of cellular uptake and mRNA delivery of active-targeted LNPs. A and B. Fluorescence intensities of Cy5 and GFP signals by flow cytometry, following treatment with PDL1-peptide active targeted LNPs and CD22 antibody active targeted LNPs.

Visualization and quantification of cellular uptake and delivery of LNP via live-cell imaging, confocal microscopy, and flow cytometry

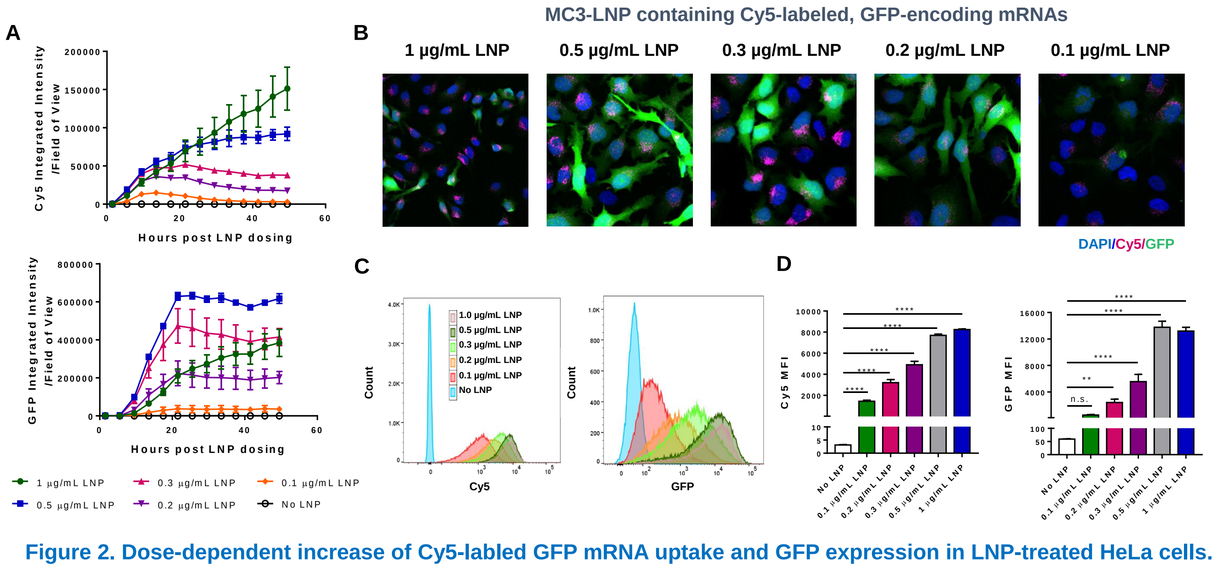


Figure 2. Dose-dependent increase of Cy5-labled GFP mRNA uptake and GFP expression in LNP-treated HeLa cells. A. Fluorescence intensities of Cy5 and GFP signals by live-cell imaging up to 48 hours post-LNP dosing. **B.** Confocal microscopy of LNP-treated Hela cells at 16 hours post treatment. **C and D.** Representative flow plots (**C**) and flow cytometric analysis (**D**) of the mean fluorescence intensities of Cy5 and GFP in LNP-treated Hela cells at 16 hours post-dosing.

Utilizing Galectin-9 foci formation to monitor endosomal escape of LNP

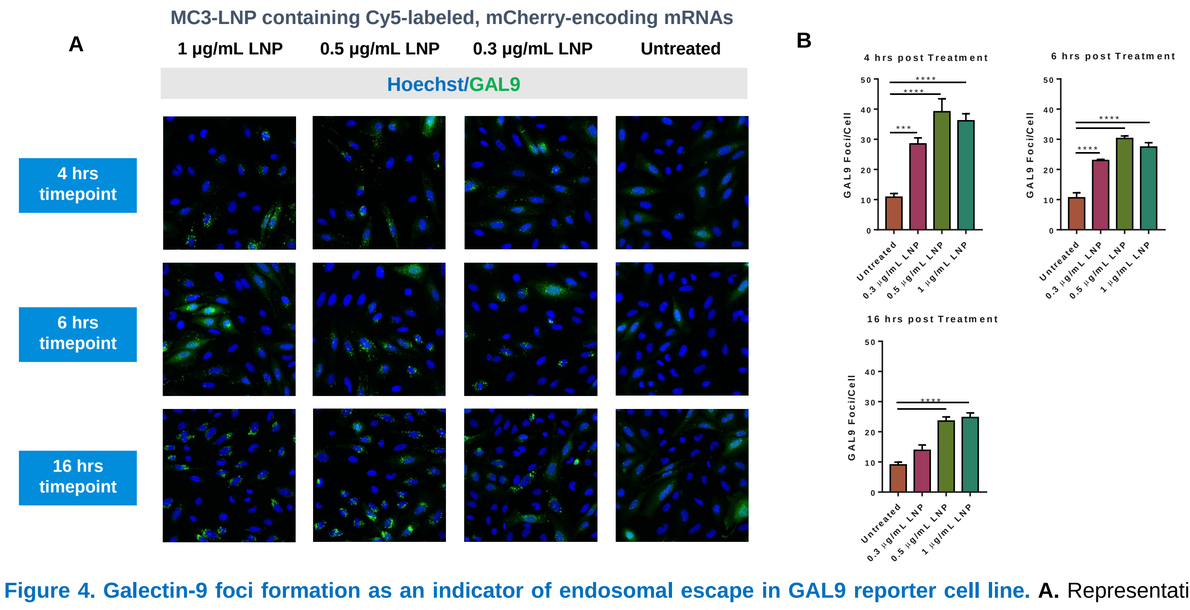


Figure 4. Galectin-9 foci formation as an indicator of endosomal escape in GAL9 reporter cell line. A. Representative images of GAL9 foci formation in HELA-GAL9-GFP cell line at 4, 6, and 16 hrs timepoints following LNP treatment at different concentrations. Blue = Hoechst; Green = GAL9. Original magnification was 20X objective. **B.** Quantification of GAL9 foci formation at 4, 6, and 16 hrs following LNP treatment at different concentrations.

Conclusions

We have established a set of assays to evaluate the uptake and delivery of conjugate-based and carrier-based delivery systems, including: 1) The binding and uptake of GalNAc conjugates into primary hepatocytes; 2) The cellular uptake and delivery of mRNA by LNP; 3) the endosomal escape of LNP in Galectin-9 cell line. In addition, we also demonstrated two strategies to generate active targeted LNPs via 1) co-formulation of PDL1 targeting peptide and 2) conjugation of anti-CD22 antibody onto LNPs for specific organ targeting. In summary, our team has accumulated extensive experiences in assessing in vitro delivery of oligonucleotides conjugated with various delivery molecules. Our results also demonstrate our capability to generate active targeted LNPs via post-modification and co-formulation. The platform we have established will further empower potential clients to accelerate discovery and development process of RNA therapeutics.

References

[1] Paunovska K, Loughrey D, Dahlman J E. Drug delivery systems for RNA therapeutics[J]. Nature Reviews Genetics, 2022, 23(5): 265-280.
[2] Munson M J, O'Driscoll G, Silva A M, et al. A high-throughput Galectin-9 imaging assay for quantifying nanoparticle uptake, endosomal escape and functional RNA delivery[J]. Communications biology, 2021, 4(1): 211.
[3] Kornilova, Anna Y , et al. Development of a fluorescence polarization binding assay for asialoglycoprotein receptor. Analytical Biochemistry 425.1(2012):43-46
[4] Michael T , Lisa H , Alexey R ,et al. Asialoglycoprotein receptor 1 mediates productive uptake of N-acetylgalactosamine-conjugated and unconjugated phosphorothioate antisense oligonucleotides into liver hepatocytes[J].Nucleic Acids Research, 2017(21):12388.DOI:10.1093/nar/gkx960.

药明康德 WuXi AppTec

Contact Us

400-820-0985

www.wuxibiology.com

BD USA: mahnaz_arjomand@wuxiapptec.com

BD EU/UK/Israel: dave_madge@wuxiapptec.com

BD China: xu_longji@wuxiapptec.com

BD Japan: fumio_itoh@wuxiapptec.com

BD Korea: sycho@wuxiapptec.com

WuXi Biology Website | LinkedIn | WeChat