Novel CRISPR-Cas9 Delivery for Chronic Myeloid Leukemia

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Introduction

In 2017, over 34,000 cases and over 24,000 deaths due to Chronic Myeloid Leukemia (CML) were reported worldwide (Lin et al., 2020). CML is caused by the BCR/ABL fusion gene, which produces constitutive tyrosine kinases (Faderl et al., 1999). Tyrosine kinase inhibitors (TKIs) are the accepted treatment for CML, yet up to 50% of CML patients develop TKI resistance due to leukemic stem cells (LSCs) (Rosti et al., 2017). Recent advances in genome engineering, particularly CRISPR-Cas9, are promising alternatives to TKIs. Extremely few studies employ CRISPR-Cas9 to treat CML, and studies that do solely focus on delivering Cas9 ribonucleoprotein, triggering adverse immune responses (Vuelta et al., 2021). Cas9 delivery in the form of mRNA potentially reduces off-target effects and minimizes genotoxicity in the cell nucleus compared to Cas9 in the form of DNA (Wu et al., 2014). To encapsulate Cas9 mRNA, the current gold standard is lipid nanoparticles; however, other nanoparticles—such as chitosan nanoparticles—have been proven to have gene editing efficiencies of over 90% when loading Cas9 RNPs and pDNA (Qiao et al., 2019; Liu et al., 2018), yet these nanoparticles have not been tested for Cas9 mRNA delivery. Other nanoparticles that remain unexplored for CML treatment include exosome-based and hybrid liposome-exosome nanoparticles (Vuelta et al., 2021). Therefore, these nanoparticles serve as promising mechanisms for Cas9 mRNA delivery to target the BCR/ABL gene in CML LSCs. Additionally, previous studies of Cas9 delivery utilize systemic delivery, yet local administration directly into the bone marrow may improve therapeutic efficacy (Ho et al., 2021). Therefore, combining an optimal nanoparticle encapsulation method of Cas9 mRNA with local administration may provide a novel CML treatment to target LSCs.

Question & Rationale

We propose a local CRISPR-Cas9 delivery system to target leukemic stem cells (LSCs) in the bone marrow and offer an alternative CML treatment for patients with TKI resistance. The system consists of injectable, nanoparticle-encapsulated Cas9 mRNA and sgRNA to knock out the BCR/ABL oncogene. This study optimizes the delivery system by testing the cytotoxicity of various methods of encapsulation in vitro, including Lipofectamine (LPF) 3000, LPF LTX, Chitosan (CS) nanoparticles, and hybrid exosome-liposome nanoparticles (HELNs). This study also optimizes HELN formulation by testing the cytotoxicity of different ratios of exosomes to liposomes (4:1, 1:1, and 1:4) and LPF 3000 vs LPF LTX for the liposome component.

Hypothesis

H_0: Chitosan nanoparticles have no effect on cell viability.

H_1: Chitosan nanoparticles will have significant impact on cell viability.

H_0: Exosome nanoparticles have no effect on cell viability.

H_1: Exosome nanoparticles will have significant impact on cell viability.

H_0: LPF LTX and LPF 3000 nanoparticles have no effect on cell viability.

H_1: LPF LTX and LPF 3000 nanoparticles will have significant impact on cell viability.

H_0: Exosome ratio of HELNs has no effect on cell viability.

H_1: Exosome ratio of HELNs is positively correlated with cell viability.

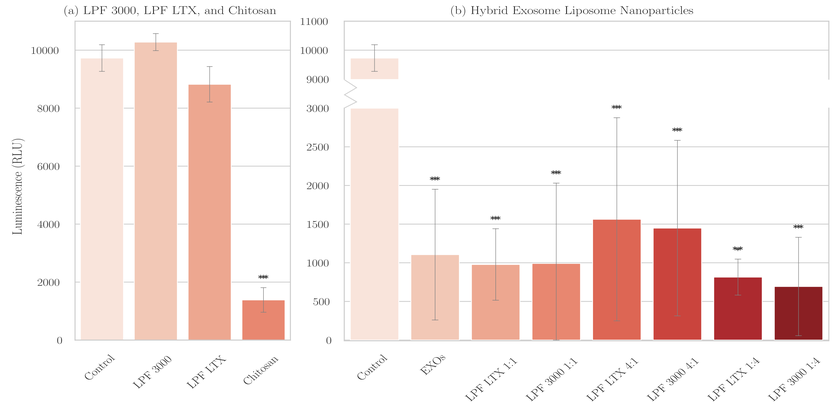
Methods

- K562 cells cultured in RPMI-1640 at 37 °C @ 0% CO_2
- Synthesized 7 of 10 groups: Chitosan (1) and exosome-LPF hybrid nanoparticles (6)
- Cas9 mRNA loaded into 10 of 10 groups: LPF 3000, LPF LTX, Chitosan, 4:1 & 1:1 & 1:4 exosome-liposome ratios with LPF 3000 and with LPF LTX
- Cells treated with the above nanoparticle-mRNA complexes
  - 1 additional group of cells treated with saline as control
- Cytotoxicity measured with luminescence viability assay

Results

Figure 1

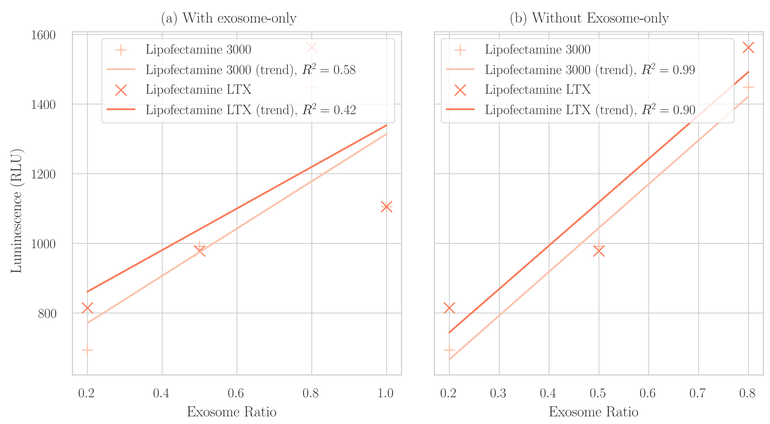
Luminescence Level (RLU) vs. Treatment to K562 Cells



Note. The error bars are calculated with 95% confidence interval. The luminesence level is proportional to cell viability.

Figure 2

Regression Plot of Exosome Concentration vs. Viability



Statistical Analysis

1. Statistically significant cell viability reduction in CS, exosomes, and HELNs (Figure 1) through T-test and Mann-Whitney-U test
   a. Only control, Chitosan, and Lipofectamine 3000/LTX groups' data follow a normal distribution, determined through the Shapiro-Wilk test
2. Strong positive linear relationship between exosome concentration (excluding 1.0 exosome ratio) and cell viability
   a. Weak positive linear relationship between exosome concentration (including 1.0 exosome ratio) and cell viability

Table 1

Probability Value of Statistical Tests

Comparison	P-value
Control vs. Chitosan for luminescence	$p < 0.001$
Control vs. Exosome-liposome hybrids for luminescence	0.0143
Linear relationship betweeen exosome concentration (excluding exosome-only) and luminescence	$p < 0.001$
Linear relationship between exosome concentration (including exosome-only) and luminescence	0.349

Discussion & Future Work

- Chitosan is significantly more cytotoxic compared to industry standard (LPF)
- 0% CO2 incubation possible explanation for low viability
  - Especially for exosome-liposome hybrid lipid nanoparticles (data collected 24 hours after Chitosan & Lipofectamine 3000/LTX groups)
  - Will conduct future trials with 5% CO2 incubation to reproduce low cytotoxicity based on established research
- Positive linear relationship between exosome concentration and cell viability warrants future investigation of high exosome concentrations for optimization
  - The sudden viability reduction at 1.0 exosome ratio suggests a cutoff where exosomes become excessive and cytotoxic
  - Comparison of high exosome concentrations to LPF to determine optimal encapsulation method of Cas9 system
- Future experimentation:
  - Loading full Cas9 mRNA and sgRNA complex to assess gene editing efficiency
  - Optimize Cas9 mRNA/sgRNA to nanoparticle ratio for efficient loading and gene editing
  - Optimize liposome formulations against LPF for exosome-liposome hybrid nanoparticles
  - Further explore local administration of treatment via intra-bone marrow injection with chemoattraction

References

Faderl, S., Talpaz, M., Estrov, Z., O'Brien, S., Kurzrock, R., & Kantarjian, H. M. (1999). The biology of chronic myeloid leukemia. *New England Journal of Medicine*, *341*(3), 164–172. https://doi.org/10.1056/NEJM199907153410306

Ho, T.-C., Kim, H. S., Chen, Y., Li, Y., LaMere, M. W., Chen, C., Wang, H., Gong, J., Palumbo, C. D., Ashton, J. M., Kim, H.-W., Xu, Q., Becker, M. W., & Leong, K. W. (2021). Scaffold-mediated CRISPR-Cas9 delivery system for acute myeloid leukemia therapy. *Science Advances*, *7*(21), eabg3217. https://doi.org/10.1126/sciadv.abg3217

Lin, Q., Mao, L., Shao, L., Zhu, L., Han, Q., Zhu, H., Jin, J., & You, L. (2020). Global, regional, and national burden of chronic myeloid leukemia, 1990–2017: A systematic analysis for the global burden of disease study 2017. *Frontiers in Oncology*, *10*, 580759. https://doi.org/10.3389/fonc.2020.580759

Qiao, J., Sun, W., Lin, S., Jin, R., Ma, L., & Liu, Y. (2019). Cytosolic delivery of CRISPR/Cas9 ribonucleoproteins for genome editing using chitosan-coated red fluorescent protein. *Chemical Communications*, *55*(32), 4707–4710. https://doi.org/10.1039/C9CC00010K

Rosti, G., Castagnetti, F., Gugliotta, G., & Baccarani, M. (2017). Tyrosine kinase inhibitors in chronic myeloid leukaemia: Which, when, for whom? *Nature Reviews Clinical Oncology*, *14*(3), Article 3. https://doi.org/10.1038/nrclinonc.2016.139

Vuelta, E., Ordoñez, J. L., Alonso-Pérez, V., Méndez, L., Hernández-Carabias, P., Saldaña, R., Sevilla, J., Sebastián, E., Muntión, S., Sánchez-Guijo, F., Hernández-Rivas, J. M., García-Tuñón, I., & Sánchez-Martín, M. (2021). CRISPR-Cas9 Technology as a Tool to Target Gene Drivers in Cancer: Proof of Concept and New Opportunities to Treat Chronic Myeloid Leukemia. *The CRISPR Journal*, *4*(4), 519–535. https://doi.org/10.1089/crispr.2021.0009

Wu, X., Kriz, A. J., & Sharp, P. A. (2014). Target specificity of the CRISPR-Cas9 system. *Quantitative Biology*, *2*(2), 59–70. https://doi.org/10.1007/s40484-014-0030-x

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