Molecular Therapy
Commentary

Escaping to silence using an endosome-disrupting polymer

Jamie L. Betker,[1] Scott G. Tilden,[1] and Thomas J. Anchordoquy[1]

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As commonly observed in the intracellular delivery of macromolecules, internalization by cells results in the vast majority of the therapeutic cargo becoming trapped within the endosomal/lysosomal system.[1] In this issue of *Molecular Therapy*, researchers designed a conjugate of N-acetylgalactosamine (GalNAc) and a pH-sensitive, endosome-disrupting polymer that can be co-administered with a small interfering RNA (siRNA) targeted with the same ligand.[2] The data demonstrate that maximum silencing of transthyretin expression in hepatocytes is maintained in non-human primates for at least 90 days when the siRNA is co-administered with a targeted polymer to facilitate endosomal escape. By using the same targeting moiety for each molecule, the siRNA and polymer can be administered subcutaneously in different locations and subsequently trafficked to the liver to silence specific genes in hepatocytes. Their strategy allowed greatly extended hepatic gene silencing after a single injection of targeted siRNA, extends the potential for enhancing siRNA-mediated silencing of other targets, and raises the possibility of modulating expression of specific genes in other tissues if adequate targeting motifs can be identified.

Although three siRNA products are currently approved in the United States and Europe (Onpattro, Givlaari, and Oxlumo), these need to be re-administered every 3–4 weeks to maintain gene silencing. The endosomal barrier was recognized decades ago in development of liposomal delivery systems, and pH-sensitive lipid components have been employed to trigger fusion with endosomal membranes at an acidic pH.[3] Although these early studies focused on delivery of traditional small-molecule therapeutic agents, this fundamental idea led to development of ionizable cationic lipids for delivery of nucleic acids.[4] Indeed, one of the advantages of particulate delivery systems is that multiple moieties can be incorporated into a single particle to enhance circulation times, targeting, and endosomal escape. However, the drawback of this general strategy is the challenges involved in manufacturing and developing a commercial product containing multiple components. This is further complicated by the fact that most academic researchers have never been confronted with the realities of drug development; thus, delivery studies that receive funding and recognition in the literature typically advocate for increasing design complexity.[5] In contrast, product development strongly favors simple systems that can be manufactured with a minimum number of components that are resistant to damage during conventional processing procedures (e.g., filtration, mixing, and freezing). In this context, it should not be surprising that this new study comes from scientists in the pharmaceutical industry who have successfully developed commercial products that contain a single component; i.e., a conjugate of GalNAc and siRNA.

Recognizing that potency of the conjugate is limited by low rates of endosomal escape, the authors demonstrated that multiple components can traffic independently to the target tissue from different injection sites. This is a fundamental departure from the accepted model, where all components are included in a single particle to ensure simultaneous internalization by the target cell. Because it is unlikely that both conjugates traffic independently from different subcutaneous injection sites to be endocytosed in the same internalization event, the findings suggest that the pH-sensitive polymer is maintained for at least a short time in the endosomal membrane system, a system known to constantly recycle components. It might be expected that this situation would result in toxicity because of the compromised integrity of intracellular membranes, but measurements of multiple cytokines suggest that this treatment is well tolerated in non-human primates.

The GalNAc-siRNA conjugate platform has already resulted in approval of three products (Givlaari, Oxlumo, and Inclisiran) that target hepatocytes in the liver. The success of these agents establishes GalNAc as a potent targeting ligand and raises questions regarding why nanoparticle-based systems have not been able to achieve similar accomplishments with ligand-mediated targeting. It is possible that ligands conjugated to a dissolved molecule are presented more efficiently to surface receptors, and it should also be appreciated that particulate delivery systems elicit fundamentally different responses *in vivo*. For example, it is well established that particles accumulate a protein corona that can obscure targeting moieties[6] and that molecules become more immunogenic when attached to a particle.[7] In addition, continuous surveillance by phagocytes of the innate immune system results in elimination of foreign particles and can also trigger an adaptive immune response that complicates repeat dosing. Use of soluble GalNAc conjugates largely avoids these barriers, and subcutaneous administration may also provide a steady release of conjugates into the circulation so that asialoglycoprotein receptors in the liver do not become saturated.

Although a complete understanding of the precise mechanisms involved in these effects will require further study, the ability to use ligands to independently traffic molecules to a therapeutic target from multiple injection sites presents pharmaceutical scientists with a new model for increasing delivery efficiency. In addition, the subcutaneous route offers advantages for delivering targeted

[1]Skaggs School of Pharmacy and Pharmaceutical Sciences, University of Colorado, Aurora, CO 80045, USA

Correspondence: Thomas J. Anchordoquy, Skaggs School of Pharmacy and Pharmaceutical Sciences, University of Colorado, Aurora, CO 80045, USA.
E-mail: tom.anchordoquy@cuanschutz.edu

soluble molecules while providing significant benefits in terms of ease of administration to affected individuals. If these results can be duplicated in humans, then the resulting products will have a much greater dosing interval than that required for current products. Considering the recent explosion in the number of antibody-drug conjugates in development, this work presents an alternative strategy by demonstrating that a relatively simple carbohydrate ligand is sufficient to achieve effective, targeted delivery of multiple components. Considering nature's predominant use of carbohydrates in recognition and trafficking, advances in the field of glycobiology should pave the way for future drug delivery research.

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Cell programming to protect the ischemic heart and limb

Mairi Brittan[6] and Wolfram-Hubertus Zimmermann[1,2,3,4,5]

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New strategies for myocardial protection and regeneration after myocardial infarction are urgently needed to counter the heart failure pandemic. To address this unmet medical need, Kaur and colleagues (in this issue of *Molecular Therapy*)[1] have identified in a preclinical proof-of-concept study a cocktail of modified RNA (modRNA) to endow stromal cells with angiogenic growth factor releasing activity (Figure 1). Sustained angiogenic programming was realized by lipid-nanoparticle (LNP) delivery, using formulations that have been employed safely and efficaciously in severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) vaccinations.

Reprogramming cell fate by transient expression of transcription factors has been explored for decades. A prominent, if not the most prominent example, is the overexpression of the skeletal muscle master regulator MyoD1 to reprogram fibroblasts into skeletal myoblasts.[2] More recently, reprogramming of somatic cells into pluripotent stem cells by the so called four Yamanaka factors (Oct3/4, Klf4, Sox 2, and c-*myc* [OKSM]) has revolutionized biomedical research.[3] To bypass the programming to an embryonic-cell-like state, direct cardiac reprogramming of myofibroblasts by overexpression of GATA4, Mef2c, Tbx5 (GMT) has been introduced[4] with demonstrated therapeutic activity in mice with myocardial infarction.[5,6] The original protocol employed retroviral delivery and was further refined to also convert human cells into cardiomyocyte-like cells, with, however, limited efficiency in adult human fibroblast reprogramming.[7]

Because of the inherent limitations of retroviral delivery of DNA, modRNA has been investigated by many labs as an alternative means for cell type conversion. Transient expression via RNA versus stable expression of DNA after genome insertion is considered advantageous from a translational point of view. Key for transient but efficacious delivery of RNA were specific chemical modifications to prevent premature degradation and activation of the innate immune system.[8,9] In an earlier study, Zangi and co-workers administered VEGF-A encoding modRNA by intramyocardial injection and observed vascular regeneration by enhanced epithelial-to-mesenchymal transition of WT-1^{+} epicardium-derived cells in mice with myocardial infarction.[10] In the present study, the protocol was extended by the application of 7G-modRNA, i.e., modRNA encoding for GMT + Hand2 + dominant negative (DN)-transforming growth factor β (TGF-β) + DN-Wnt8a + acid ceramidase (AC), to turn myofibroblasts into cardiac-like cells. Although direct injection of modRNA, despite its chemical stabilization, resulted in a near-complete loss of transcripts within 72 h,[10] LNP delivery of 7G-modRNA enhanced angiogenic growth factor expression for the whole study duration of 4 weeks, which resulted in a clear attenuation of disease progression with

[1]Institute of Pharmacology and Toxicology, University Medical Center Göttingen, Georg-August-University, Robert-Koch-Str. 40, 37075 Göttingen, Göttingen, Germany; [2]German Center for Cardiovascular Research (DZHK), partner site Göttingen, Germany; [3]Cluster of Excellence "Multiscale Bioimaging: from Molecular Machines to Networks of Excitable Cells" (MBExC), University of Göttingen, Göttingen, Germany; [4]Center for Neurodegenerative Diseases (DZNE), Göttingen, Germany; [5]Fraunhofer Institute for Translational Medicine and Pharmacology (ITMP), Göttingen, Germany; [6]Centre for Cardiovascular Science, The Queen's Medical Research Institute, University of Edinburgh, Edinburgh EH16 4TJ, UK

Correspondence: Wolfram-Hubertus Zimmermann, MD, Institute of Pharmacology and Toxicology, University Medical Center Göttingen, Georg-August-University, Robert-Koch-Str. 40, 37075 Göttingen, Germany.
E-mail: w.zimmermann@med.uni-goettingen.de

