RNAi therapeutics

SNALPing siRNAs in vivo

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The novel use of a lipid bilayer system to get fusogenic delivery of siRNAs to murine liver cells to inhibit replication of the Hepatitis B virus (HBV) is the latest in a series of studies that address the major problem that faces RNAi therapeutics: delivering the RNAi agents to where they can make a difference.

There have been many major breakthroughs in regulating the expression of deleterious genes, and one of the latest is RNA interference (RNAi). RNAi is an innate cellular process that can be harnessed to modulate sequence-specific gene expression via small double-stranded RNA triggers termed small interfering RNAs, or siRNAs.

Over four years ago, the first proof that siRNAs could trigger sequence-specific gene silencing in mammalian cells appeared.^{1,2} These reports set off a flurry of activity worldwide that involved thousands of studies that employed siRNAs to suppress

gene expression in cell culture. Despite the excitement of being able to knockdown the functional expression of virtually any endogenous or viral message in mammalian cell culture, it became instantly clear that RNAi applications using synthetic siRNAs *in vivo* were much more problematic due to instability of the small RNAs in plasma.

The stability problem was soon overcome via the use of selective backbone modifications and inverted bases at the ends of the molecules, but efficacy was only achieved with very large amounts of injected material.³ Also, there was no good method for the targeted delivery of siRNAs to specific tissues. When investigators in this field are asked what the major hurdle for therapeutic applications of RNAi are, the answer is usually 'delivery, delivery, delivery'.

Recently, the challenge of systemic *in vivo* delivery has been met head on by various groups using clever

designs of macromolecules to ferry the siRNAs through the bloodstream and into target tissues.^{4–6} These include conjugation of a cholesterol moiety to one of the strands of the siRNA duplex for systemic delivery to the liver and jejunum⁶, complexing of siRNAs to protamine fused with an antibody fragment for receptor-mediated targeting of siRNAs,⁵ and in this most recent study, by Morrissey *et al.*,⁴ the use of a novel lipid bilayer system.

What sets apart the lipid bilayer system from other methods of delivery is the fact that the delivery/siRNA combination facilitated relatively low dosing of siRNAs to maintain long-term, potent suppression of HBV replication in animals. How was this effective systemic delivery of siRNAs achieved? The answer lies in a novel lipid bilayer containing a mixture of cationic and fusogenic lipids coated with diffusible polyethylene glycol. These biopolymers are in the 120 nanometer diameter size range, and have been labeled as SNALPs, for Stable-Nucleic-Acid-Lipid-Particles. The lipid combination protects the siRNAs from serum nucleases and allows cellular endosomal uptake and subsequent cytoplasmic release of the siRNAs (Figure 1).

Prior studies from this same group of investigators utilized backbone-stabilized siRNAs minus a carrier to

Figure 1 *In vivo* delivery of siRNAs using SNALPs to achieve gene silencing. (a) The bilayer SNALP with encapsulated siRNAs fuses with cytoplasmic membrane. (b) SNALP and siRNAs are incorporated into an endosome. The modified siRNAs do not activate Toll-like receptors but escape from the endosome. (c) One of the two strands of the siRNA enters the RNA-induced silencing complex and targets degradation of a messenger RNA.

achieve effective inhibition of HBV replication in a murine model, but this approach required three doses daily of 30 mgs/kg for several days.³ When translated into human applications, for the average 70 kg adult this would require over 6 g/day of injected material. Not only would such huge doses be prohibitively expensive but also large amounts of injected nucleic acids could be quite toxic. Using the SNALP approach, the dosing regimen was reduced to 3 mg/kg three times daily. Following the initial routine of three daily injections, these investigators found that a single weekly injection was sufficient to maintain greater than a 10-fold inhibition of HBV antigen production out to 6 weeks.

Previous studies have shown that certain sequence motifs in siRNAs administered systemically lead to activation of interferons and inflammatory cytokines, particularly when they are delivered by lipid-based vehicles.^{7–10} Morrissey *et al.*⁴ investigated whether or not SNALP delivered, nonbackbone-modified siRNAs were immunostimulatory, and sure enough they were. The fact that the unmodified siRNAs were immunostimulatory indicates that they are subject to endosomal trafficking, where the RNA-activated Toll-like receptors 3 and 7 reside.

An important finding in this study is that backbone modifications of 2' fluoro-modified pyrimidines, 2' O-methyl purines and deoxyribose A, G and T's judiciously placed in both strands abrogate the immunostimulation. Not only did the backbone modifications prevent immunostimulation, they also had the added benefit of increasing the stability of the siRNAs from 2 min to 6.1 h in plasma, and 49 min to 15.1 h in the liver. The SNALP encapsulated, backbone-modified siRNAs were found to traffic primarily to the liver and spleen, with little accumulation in other tissues.

Overall, the SNALP delivery system has great potential for therapeutic applications of siRNAs for liver-based diseases. The application of

siRNAs for treating chronic HBV infection of course only serves as a proof of principle, as did the use of cholesterol-conjugated siRNAs for hypercholesterolemia⁶ in a previous study. Both of these conditions can be effectively treated much more cost effectively by other means. What is important though is the demonstration that non-toxic, therapeutically effective systemic delivery of siRNAs is a real possibility. It is clear from the Morrissey *et al.* studies^{3,4} that backbone-modified siRNAs on their own will find only limited applications therapeutically, perhaps only in non-systemic delivery situations.

The importance of the backbone modifications in the context of SNALP delivery resides more in the abrogation of immunostimulation than the increased siRNA half-lives in plasma and tissue. Since the SNALPs are apparently delivering the siRNAs through an endosomal mechanism, immunostimulation is a major concern. This may not be the case for cholesterol-conjugated siRNAs⁶ or the protamine-Fab-mediated targeted delivery of siRNAs⁵ which did not appear to result in immunostimulation, even when the siRNAs had minimal⁶ or no⁵ backbone modifications. In the end, it is always better to have choices in delivery, than to have to solely rely on one mode of delivery. Given the exciting new developments in approaches for systemic delivery of siRNAs, it should be possible to choose the one which best delivers the payload of siRNAs to the intended target. With these new and novel delivery approaches for siRNAs, we should expect to see a proliferation of *in vivo* studies using siRNAs to target disease-related transcripts in animal models. It is reasonable to believe that further experimenting with combinations of backbone modifications could lead to increased efficacy along with the benefit of reduced toxicity. Given the recent breakthroughs in safety and delivery, we can expect to see rapid movement of siRNAs from the lab to the clinic. ■

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