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Systemic delivery

The last hurdle?

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Recent successes in effectively delivering new siRNA antisense agents systemically to produce therapeutic outcomes in mice[1,2] have been extremely impressive, but how close are we to developing a general approach for systemic delivery in humans? Have we finally cleared the daunting hurdle that systemic delivery presents?

Various setbacks have dogged the progress of antisense drug discovery in the last 10 years, not least being the recent failures of Affinitak™ and Genasense® in phase III clinical trials. As a result, the antisense approach has been losing credence as an effective route to novel drugs. However, a considerable rejuvenation is occurring through RNA interference technology, which is essentially a recently discovered, new biological version of the antisense approach. However, it is becoming clear that a major unmet challenge for the field is to develop methods that allow effective and simple cellular, and especially systemic, delivery of genetic therapy agents in general and antisense agents in particular. Many 'solutions' to this problem have been published on this subject during the last decade, but we yet have to see an effective delivery technology. Also, it is most likely that *in vivo* delivery methods that are effective for some type of drugs, such as siRNA- or other phosphate-based (anionic) antisense agents, will not be applicable for chemically unrelated agents, for example, those based on morpholino or peptide nucleic acid (PNA) (charge neutral) chemistry.

Nonetheless, the antisense principle is at least as appealing and attractive now as it was when originally conceived a quarter of a century ago.[3] Discoveries in basic molecular biology and medicine have steadily turned up new mechanisms for silencing genes, such as RNase H and most recently the RNA interference cellular machinery, as well as a large collection of novel medically relevant gene targets. Especially exciting is the discovery that more than two-thirds of all human genes are also regulated and diversified through alternative splicing, and that many diseases are rooted in splicing defects or alterations. Several recent reports have shown that antisense agents are quite powerful tools and hopefully eventually drugs for redirecting and/or correcting incorrect splicing of pre-mRNA.[4] Most recently, this principle was elegantly demonstrated in a mouse muscular dystrophy model, to partly restore muscle function in these animals, thereby giving promise for actual drug development.[2]

These many *in vitro* and animal study success stories are encouraging. However, effective, safe and general principles for systemic delivery of these large (>3000 Da) hydrophilic and most often polyanionic molecules would be an even bigger boost for drug discovery and drug development efforts in the field.

Recent large-scale efforts to develop siRNA-based therapeutics have also had to confront the hurdle of systemic delivery.[5] While siRNA technology has proven extremely powerful and robust for cell culture work, transferring this success to animals or humans is proving very difficult, due to insufficient bioavailability of the compounds. Thus, it is very encouraging that Soutschek *et al*[1] have recently been able to demonstrate siRNA-mediated downregulation of apolipoprotein B in the liver (and jejenum) of mice using cholesterol conjugates.

The authors of this new study have made an important step in the right direction, but one should be cautious in one's enthusiasm. The effects were preferentially seen in the liver, which is a relatively easy organ to target, and also quite high dosages (three injections each of 50 mg/kg) were required for the effect. In view of the extremely high potency of the siRNA in *in vitro* cell cultures, one must conclude that only a very small fraction of the injected siRNA actually reaches its molecular mRNA target in the liver cells. Thus, it is unfortunately not likely that the simple cholesterol conjugation will solve the general delivery problem of siRNA.

Some years ago, cationic cell penetrating peptides such as penetratin, Tat and later transportan,[6] and not least oligo arginine,[7] were introduced as general *trans*-membrane carriers of a variety of cargoes including oligonucleotides and PNA. Somewhat disappointingly, however, the initial euphoria has been dampened as it is being realized that the main uptake route for most – if not all – of these peptides is endosomal,[8] and that the reagents thus have to escape the endosomal compartment, in order to enter the cellular compartments of action: the cytoplasm and/or the nucleus. Adding yet another level of complexity to the problem of cellular delivery is the fact that the real challenge is to deliver the compounds to cells in an organ in an animal (human).

Therefore, novel ideas and principles for systemic delivery are still extremely welcome and should be carefully considered and evaluated. Unfortunately, however, in the past initial promising findings have often been over-interpreted, or unduly generalized. The cell is an extremely complex, well-ordered, compartmentalized, and dynamic structure, not just a nucleus and a cytoplasm surrounded by a bilayer lipid membrane. Therefore, the question of cellular uptake, especially of gene-targeted drugs, is not simply a question of getting access to the interior of the cell. In the most rudimentary form, it is a question of delivering the agent to its genetic target at effective concentrations. Therefore, an uptake study without biological efficacy data is close to useless for evaluating the effectiveness of a particular approach to this problem.

A recent paper in which Ly *et al*[9] studied backbone-modified PNA is a good example of a study that suffers from the lack of solid biological efficacy data, but also suffers other

shortcomings. Standard fluorescence microscopy data presented are on fixed cells, which usually produce serious artifacts (redistribution within the cell upon fixation). Furthermore, it is close to impossible to make any credible/reliable conclusions concerning intracellular distribution without confocal microscopy data. Finally, fluorescence microscopy by itself does not address the crucial issue of efficiency of the delivery. Therefore, on the basis of the results presented by Ly *et al*, it is unfortunately not possible to assess the importance of their finding and their claim that previously described arginine backbone-modified PNA oligomers[10] are effectively taken up by human cells and therefore are promising antisense agents.[9]

There is sufficient evidence to show that multiple guanidinium groups on a molecule do indeed promote 'cellular uptake' to some extent.[7,11] However, the absolute efficacy and exact cellular/molecular mechanism(s) for this are still very uncertain. Nonetheless, the arginine PNAs[9,10] are indeed worthy of further studies, and it would be very interesting to see how they perform (relative to other delivery protocols) in very simple efficacy model systems both *ex vivo* in HeLa cells[12] as well as *in vivo* in a mouse model[13] based on luciferase or GFP reporter genes. It is also worth noting that 'guanidinium DNA' has been synthesized,[14] but not thoroughly characterized in terms of 'cellular' behavior, and that polycations in general are adhering to and internalized by eukaryotic cells. Thus, as in most cases of new claims in science, caution is advised as we await further documentation.

The question therefore remains: can effective, robust (and simple) *in vivo* (and *in vitro*) delivery methods for antisense agents and other gene therapeutic drugs be discovered? They are eagerly awaited by both the academic researchers, as wells as by the biotech and the pharmaceutical industry. ■

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