**Oligonucleotide therapies for the lung: ready to return to the clinic?**

The most promising targets for treating lung diseases are often intracellular molecules recalcitrant to conventional pharmacotherapy. In this respect, antisense oligonucleotides (ASO) would be valuable tools to treat airways disease, as the plurality of their potential mechanisms of action is well-established, their efficiency has been extensively validated *in-vitro*,and their potential clinical value has been demonstrated with numerous regulatory approvals. Following the two eye-injectable formulations fomivirsen and pegaptanib, the recent successes of the systemically active ASOs mipomersen, defibrotide, and eteplirsen have been underscored by the remarkable clinical outcomes of the intrathecally dosed, RNA splicing modulator nusinersen, a 2nd generation 2’-methoxy ethyl ASO indicated for spinal muscular atrophy (*1*). For lung disease, it is also clear that relatively facile topical administration, i.e. inhaled or intranasal dosing, results in efficient and uniform distribution across the lumen of large and small airways, accessing both epithelial and sub-epithelial compartments.

Yet in spite of high confidence in the treatment rationale and mechanism of action, it appears that oligonucleotides delivered topically to the lung either rapidly access circulation via epithelial transcytosis or are removed by alveolar macrophages, exerting minimal if any action in the cytosol of cells relevant to lung disease. Moreover, use of cell penetrating peptides, liposomes or nanoparticle delivery systems has not so far been shown to eliminate circulatory clearance, may activate immune responses, or drive macrophage phenotypic changes that, together or in isolation, may present risks to patients (*2*). Importantly, the recently published study by Crosby *et al.* (*3*) signifies a new addition to the evidence that airways disease, and cystic fibrosis (CF) patients in particular, may stand to benefit greatly from splicing modulator or RNase H-active ASO therapeutics, when 3rd generation, so-called bi-cyclic or bridged (2’-4’ constrained) nucleoside chemistry is used. Specifically, the authors use constrained ethyl (cEt) as opposed to the simpler and more widely available constrained methyl, or “locked” nucleic acid (LNA) chemistry (*4*). Representing continued efforts from Ionis Pharmaceuticals to exploit the affinity and stability advantages of bridged nucleic acids and their long-standing interest in the airways disease space (*2*), Crosby *et al.* (*3*) report on the potentially therapeutic effect of an inhaled, 16-mer RNase H-active ASO against *Scnn1a*, a subunit of the amiloride-sensitive luminal sodium uptake channel (ENaC) for the treatment of CF.

Cystic Fibrosis is an autosomal recessive disorder characterized by mucous over-secretion in epitheliated tissue, such as the respiratory, intestinal and pancreatic duct lumens. In the airways, additional physiological changes include reduction of airway surface liquid, mucus dehydration and mucus accumulation: the resulting mucous plugs cause inflammation, promote infection, and tissue damage, resulting in 80% mortality and a median life expectancy of 37 years of age (*5*). The majority of patients suffer loss of function mutations in the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene, but the precise site of its expression across the respiratory tree remains unclear due to the very low expression levels of the transcript. However, as CFTR encodes for a luminal chloride secretion channel, extensive research (*6*) points to solute imbalance in the airway lumen as the mechanism that underlies disease. Elimination of ENaC hyperactivity in CF, which is thought to contribute to luminal solute imbalance and airway surface liquid volumes, has been long proposed as a promising treatment approach, if persistent rather than transient ENaC activity downregulation can be achieved (*6*). This is supported by complementary data, including knockout of Nedd4L, a suppressor of ENaC, that induces a CF-like phenotype in mice and is lethal by three weeks of age. Thus, using two different mouse models of CF, an adult mouse βENaC overexpression model and a Nedd4L knock-out neonate mouse model, the authorsshowed that ENaC-specific ASOs resulted in ~50% (neonate) to ~85% (adult) reduction of ENaC mRNA levels. Physiologically, the treatment also reduced mucus metaplasia, inflammation and airway hyper-responsiveness (AHR) in an experimental model of cystic fibrosis, and reduced mucous hypersecretion and inflammation in neonate airways. Taken together, these results indeed suggest that treating CF by oligonucleotide therapies might be within clinical reach. These results follow the recent publication of relevant 3rd party patents (US20150211010) and data (*7*) on the *in vitro* utility of exon skipping antisense for known CFTR mutations, and join the continued debate around whether ENaC is indeed a suitable target for CF treatment (*6*).

On the other hand, despite similarly exciting preclinical disease biomarker data with viral infection, asthma and emphysema models, all clinical efforts to date in respiratory disease oligonucleotide therapeutics have been poor (*2*). Thus, studies with three different 2nd generation ASO compounds developed originally by Ionis (previously known as ISIS), AIR645 against IL13/IL4 (Altair Therapeutics Inc.), ASM8 dual ASO formulation against CCR3 (Topigen / Pharmaxis Pharma), as well as the Alnylam siRNA compound ALN-RSV01 against RSV1 (Alnylam) have failed in phase II clinical trials (*2*). Across these studies, despite high confidence in the treatment rationale and drug mechanism of action, it appeared that oligonucleotides delivered topically to the airways either rapidly entered circulation or were removed by alveolar macrophages, perhaps too efficiently, offering limited (<50%) benefit at the tissue level, and inducing a foamy macrophage phenotype (*8-10*). Notably, Ionis published in 1998 on the rapid systemic access of 30-mer, 2nd generation ASO delivered topically to the lung (*11*) and evidence of systemic access and even urinary elimination at the lower limit of assay detection for this compound class was reported even in man (*12*). More recently (2011), 16-mer, 3rd generation LNA ASO, chemically comparable to the 3rd generation cEt chemistry (*4*) employed by Crosby *et al.*,were also shown to rapidly reach circulation and end up in the urine as early as 15 min after intratracheal dosing in mice. Crucially no on-target effect was detected in lung cells after *in vivo* dosing using a high precision, high power bioanalytical approach involving comparisons of fixed numbers of pneumonocytes obtained by tissue disruption and cell sorting by cell type: epithelial cells, macrophage cells, or mixed cell populations representative of the entire lung cell type diversity (6). As 16-mer oligos have a lower molecular weight than 30-mer oligos, the observation of circulatory access was not entirely unexpected. Indeed, many biopharmaceutical peptides and proteins are very efficient at crossing the pulmonary epithelia into circulation (*13*). However, as imaging showed ASO association with cells across the entire lung, lack of any appreciable effect was surprising. Furthermore, building on the predicate airways work with 30-mer 2nd generation phosphorothioate ASO, the 2011 study demonstrated that the phosphorothioate backbone drove alveolar macrophage loading; in addition, after exit from the airways it allowed for retention in circulation, followed by biological activity in the liver and kidney (*9*). More importantly from a patient safety perspective, a separate clinical expert panel on the safety of inhaled drugs recommended chronic studies of the foamy macrophage phenotype induced by oligonucleotides upon topical dosing to the lung, to rule out long-term immune risks. (*10*) This matter is acutely relevant to airways disease when such phenotypes have been link with deterioration of respiratory status, at least in asthma. (*14*)

Despite these reports, Crosby *et al.* claim lack of systemic access or activity in the kidney following inhaled administration of their 3rd generation ENaC ASO. Interestingly, ENaC is expressed only in the collecting ducts and distal convoluted tubules of the kidney- sites acknowledged by the authors as refractory to ASO treatment. Thus, with no effort undertaken to quantify the ASO localization after inhalation in the kidney, these results perhaps are more relevant to showing which kidney cells are not accessible by 3rd generation ASO, rather than confirming lack of access to this tissue. The authors also pursue a conceptually smart approach in anticipating the efficacy of their ASO chemistry in the lung. Thus, they replicate the Nedd4L knockout effect on inducing CF-like phenotype by administering a Nedd4L ASO to wild type mice. Unfortunately, looking at the pro- and anti-inflammatory effects of the CF-inducing Nedd4L ASO and the CF symptom alleviating ENaC ASO, respectively, it is difficult to disentangle the extent of on-target mechanism of action for both of these oligonucleotides. Whilst molecular evidence of ENaC mRNA cleavage is taken for granted by the authors, at the same time, it is well established that minimal motifs may activate immune responses where others may indeed unexpectedly suppress them (*15*). Yet despite the hard lessons in the field, such as the phase III abandonment of Quark’s TLR3-activating siRNA that, administered subcutaneously could arrest neovascularization in the eye (*16*), no immune profiling appears to have been attempted by Crosby *et al.* for either of these ASOs. Thus, with the explicit absence of an on-target mechanism of action in the lung cells and the absence of evidence ruling out unexpected immunomodulatory effects, it remains to be proven whether the fascinating CF biomarker changes reported are indeed on account of the expected mechanism of action. Whilst these pre-clinical results are as convincing as those reported in previous studies evaluating RNase H-active antisense for asthma, the outcomes in Crosby *et al.* could be due to agonist/antagonist effects in inducing and inhibiting, respectively, severe inflammation. After all, the TLR4 agonist lipopolysaccharide (LPS) is a well-established tool for the induction of mucous hypersecretion as well as the host of associated osmoregulatory gene expression changes reported in the proposed Nedd4L ASO CF model (*17*). Thus, as with previous efforts, interpretation of the reported data as a mechanistically-relevant, on target and effective treatment of CF might be premature.

At the peak of excitement over the potential of RNAi and subsequently 3rd generation antisense for treating lung diseases, many sought to drug the undruggable targetome with this therapeutic class. Large pharma which actively engaged in evaluating these modalities in airways disease eventually concluded that intracellular delivery in the airways remained an outstanding issue, as much as local immunomodulation was a risk. Both of these issues remain unaddressed in Crosby *et al.* and the few other reports since 2011. Notably, emerging alternative approaches, such as *in vitro* transcribed RNA therapy tested in the airways, have proactively explored these aspects (*18*)*,* with CureVac GmbH being a positive example of exploratory safety practice. Such efforts are commendable and set a new standard in qualifying nucleic acid therapy data, aligned to large pharma calls for the pursuit of exploratory safety studies confirming the proposed mechanisms of action and limiting assumptions around the translation of drug function across the development pipeline (*19*).

Irrespective of historical data on what is and is not immunomodulatory in oligonucleotides vs. the intricacies of nucleoside chemistry, a clinically proven and pharmaceutically relevant solution to achieving intracellular delivery, as indeed recognized by Ionis for hepatic indications, involves the use of receptor-specific ligands. Thus, conjugation of N-acetyl-glucosamine (GalNAc) to oligonucleotide drugs has been shown to target the hepatocyte-specific asialglycoprotein receptor (ASGPR) and reduce drug doses by up to 60-fold in phase II clinical trials (*20*). Unfortunately, a prototype GalNAc-ASGPR pair suitable for the oligonucleotide delivery into airways cells remains undiscovered, but appears necessary. We believe that with rational, tractable and systematic design and validation of oligonucleotide-conjugated ligands to well-studied respiratory cell receptors, antisense and other oligonucleotide therapeutics may yet still offer clinically and commercially attractive solutions for the treatment of airways disease. Success is likely to largely depend on focusing treatment to cell types with functions relevant to the disease being treated, considering carefully the need for the phosphorothioate backbone which drives alveolar macrophage and systemic tissue loading, and producing evidence of exclusively on-target molecular pharmacology, in a statistically robust and appropriate manner. We thus urge the community to capitalize on the GalNAc paradigm and, in seeking to extend the clinical potential of oligonucleotide drugs, intensify the exploration of targeted delivery, both into airways cells and disease-relevant tissues beyond.

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