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Oligonucleotides make sensible strides

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The 4th annual IBC *EuroTIDES 2003* conference (11–12 November 2003, Berlin, Germany) drew together many influential figures from academic and commercial backgrounds to showcase recent advances in antisense therapies and the emerging clinical prospects for RNA interference (RNAi). The main emphasis of the conference was to address the difficulties that have thus far prevented antisense techniques from fulfilling their long believed potential, namely instability and non-specificity, and to offer solutions that might deliver a new generation of antisense products to the market.

A well-attended pre-conference workshop paid particular attention to improvements in the synthesis of RNA molecules for *in vivo* studies. Quality control procedures and methods to ensure the removal of impurities are of great relevance to the degree of scaling up of RNA production required to satisfy the recent explosion in demand for RNAi reagents. In particular, it will be important to reassure members of the pharmaceutical industry that antisense DNA and RNA technology is amenable to the large-scale production that is necessary to resolve the current issues of purity and yield, which potentially limit the commercial application of these drugs in the treatment of human disease.

Nucleotide analog chemistry

Locked nucleic acids (LNAs) were the popular theme of the first morning, with interesting applications reported from many groups. Key among these was the application of LNAs to the problem of serum stability of oligonucleotides. Troels Kock (Santaris Pharma A/S; http://www.santaris.com) described 'gapmers', oligonucleotides that recruit RNase H through a traditional antisense mechanism to cleave across a phosphodiester (PO) or mixed phosphodiester/phosphorothioate (PO/PS) oligonucleotide central region but are modified at either end to enhance stability and longevity (up to 72 h in cell culture). Favorable, sub-nanomolar IC_{50} values were demonstrated in cell culture and, when applied to animal models, gapmers inhibited xenograft prostate and pancreatic tumors at doses of 5 mg/kg/day.

The enhanced binding affinity of LNA modified bases was exploited by Jesper Wengel (University of Southern Denmark; http://www.sdu.dk) to enhance the activity of DNAzymes (catalytic DNA moieties). However, it was not clear whether increased target affinity of the LNA modified bases [which is affected by melting temperature (Tm)] or improved resistance to nuclease degradation contributed most to the elevated overall activity.

LNAs have also proved their versatility through their application to RNAi. Kock reported that end-incorporation of LNA enhances the nuclease resistance of siRNA duplexes without affecting their activity in cell culture, whereas Jens Kurreck (Free University of Berlin; http://www.fu-berlin.de) provided evidence that the 5′ end of the antisense strand of an siRNA might allow greater scope for modification than previously thought, presenting 5′ modified siRNAs that showed moderate activity *in vivo*. These studies suggest that siRNAs could be responsive to other stabilizing modifications that would potentially enable their application as therapeutic molecules.

Modifications to improve delivery

The keynote presentation, delivered by Michael Gait (University of Cambridge; http://www.cam.ac.uk), provided an overview of the delivery problem that is central to the use of oligonucleotides as therapeutics. Predominantly electronegative oligonucelotides must penetrate cells and reach the cellular location of their target, while avoiding sequestration and/or degradation by the endosomal pathway. Oligonucleotide–peptide conjugates (e.g. Transportan–TP10) that could be delivered into cells in the absence of a transfection agent, as well as their application to LNA-stabilized antisense gapmers and siRNAs, were described. Gait illustrated the importance of correct cellular localization of siRNA duplexes through their conjugation to peptide sequences, and demonstrated that the activity of the duplexes could be enhanced when coupled to the 2-N-(2′-s-triphenylmethylacetyl) amino-(N′-acetylglycine) phenylpropamide (MPG2) nuclear localization sequence peptide (NLS) (–) linker relative to a NLS (+) linker (the proteins thought to mediate RNA interference are localized in the cytoplasm).

In addition, Gait argued for the exploitation of endosomal uptake pathways as an efficient means for cellular uptake, suggesting the use of a Tat peptide with a scissile linker that might enable a conjugated antisense reagent to be released from the endosome within a cell [1]. This proved

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an issue of contention, however, for researchers who have long studied the role of the endosome in sequestering foreign agents. Cy Stein (Albert Einstein College of Medicine; http://www.aecom.yu.edu) argued that the endosome was a far more effective screening organelle than is often appreciated, suggesting that escape from the endosome might prove too great a challenge for the current generations of oligonucleotides.

Design of siRNAs

The importance of understanding the global nature of a system, rather than just the effecter unit, was emphasized in presentations on the application of RNAi to biological problems. Several speakers provided evidence that the selection of siRNA targets is complicated and poorly understood. The consensus success rate in designing effective siRNA was suggested to be around 40%. 'Highly active' siRNAs (i.e. those that display >70% target reduction concentrations below 20 nM) are considered to be the most favorable reagents for gene-knockdown, because duplexes that can be used at low concentrations limit the potential for 'off-target' effects. Although the subject was broached by some speakers, it has not been comprehensively investigated and thus remains an important area of research that must be explored further before potential siRNAs are committed to expensive clinical trial regimes. Several speakers suggested methods to improve sequence selection, including RNase H mapping, antisense array hybridization and computer modeling [2], which showed some correlation between predicted target mRNA structure and siRNA activity. However, it was also noted that binding of the siRNA duplex to the RNA induced silencing complex (RISC) might also impose some sequence selection in determining highly active siRNAs.

Promising results from animal models

Exciting reports of both antisense and siRNA applications in animals and humans provoked much interest and some controversy. Jens Kurreck reported that traditional antisense and siRNA induced responses in rats that were injected intrathecally with reagents targeted against the VR1 ion-channel, which is involved in the sensing of multiple pain stimuli (e.g. heat, acid and vanilloids). Fluorescently labeled siRNAs were located in brain slices, and animals showed a marked reduction in pain response for 5–6 days after a single injection of 1 μg of siRNA. Further research indicated that a marked effect could be observed with an injection of only 1 ng of siRNA, and that control animals briefly showed some non-specific behavioral change – prompting some experienced antisense researchers to reminisce about the early *in vivo* promise of first generation antisense molecules. Cy Stein urged stricter controls and more detailed proof that siRNAs are acting solely through an RNA interference pathway in whole animals.

Stein himself presented a study of G3139, a promising oligonucleotide purported to act in prostate tumor cell lines against bcl-2 (a key mediator of cellular apoptosis, and hence a promising target for anticancer therapies). This presentation provided evidence that G3139 was not acting against bcl-2 through a traditional antisense mechanism, but via a concerted and complex manner that warranted further study [3]. However, it must be appreciated that the traditional 'β-tubulin control' of a western blot does not provide rigorous evidence of the specificity of an antisense reagent, and the increasing availability of 'whole genome' mRNA chips should provide researchers with a ready method of validating their oligonucleotides in culture before further (and probably expensive) animal trials.

Specific antisense effects were reported with a class of telomerase inhibitors from Sergei Gryazanov (Geron Corporation; http://www.geron.com). This study also provided a further example of improved oligonucleotide activity through conjugation. In the case of the anti-telomerase, antisense reagent GRN163, lipid conjugation was shown to improve the IC_{50} from 2.5 μM to ~0.1 μM. The modified oligonucleotide showed significant, and telomerase specific, tumor inhibition in a mouse xenograft model through shortening of telomeres and induction of cellular senescence [4].

Perspectives and challenges ahead

There is some hope of overcoming the maligned non-specific effects of antisense reagents. Several speakers presented human clinical trials of immune stimulatory non-methylated CpG oligonucleotides. Eugen Uhlmann (Coley Pharmaceutical group; http://coleypharma.com) presented a category of Toll-like receptor 9 (TLR9) agonists that use the innate immune response pathway to induce interferon-α (IFN-α) secretion to combat tumor growth. The TLR9 agonist ProMune™ (Coley Pharmaceutical Group; http://www.coleypharma.com) was shown to act synergistically with Taxol (a common chemotherapy agent) to block tumor outgrowth in animal models. Further clinical trials showed ProMune to have antitumor activity in five human cancers, while demonstrating an excellent safety profile and tolerability.

Defibrotide, a polydisperse (22 kD) oligonucleotide with aptameric activity, was described by Paul Richardson (Dana–Farber Cancer Institute; http://www.dfci.harvard.edu), as a multifunctional agent against hepatic venoocclusive disease (VOD), which occurs in approximately one in four patients that undergo stem cell

transplants in the US each year. Clinical trials of patients treated at the onset of symptoms post-transplant with Defibrotide (either 25 or 40 mg/kg/day), showed a 46% increase in the number of patients surviving for two years following the start of treratment, while the toxicity profile was low.

Concluding remarks

The advancement of antisense drugs to market, and the hope that they might begin to return on the significant investment that has been devoted to the field, continues to suffer from the weight of expectation. Setbacks, such as the case of Isis 2302 (Isis Pharmaceuticals; http://www.isispharm.com) used in the treatment of Crohn's disease, only serve to increase the pressure on companies to justify funding for antisense technologies and development. Volker Wacheck (University of Vienna; http://www.univie.ac.at) cautioned against the traditional assumptions of clinical trials (e.g. that dose-related toxicity can be a surrogate for efficacy). In addition, he emphasized that the use of biomarkers and surrogate endpoints as indicators of therapeutic intervention must be limited to the degree that they can predict clinical benefit, and that effects on these markers must be validated as early as possible (i.e. preclinical phase). The ultimate goal is not a maximal effect on the surrogate endpoint, but is to determine the optimal risk–benefit ratio from a clinical standpoint.

The conference itself was well-attended and effectively scheduled, providing a lively forum for attendees to discuss the necessary advancement of a mature field. There was a clear progression in presentations – from addressing the technical issues and solutions to discussing the synthesis and application of anti-mRNA reagents, the results from *in vivo* trials and culminating in the discussion of the progress made to date, and problems that still remain. Although there are still challenges to be overcome, the commercial promise of antisense technologies could still be achieved.

References

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