ELSEVIER

Teaser Locked nucleic acid-modified antisense oligonucleotides (LNAs) are widely used. The structural diversity of LNAs affects most drug properties. Exploiting this diversity offers new opportunities for discovering LNA-based drugs.

Locked nucleic acid: modality, diversity, and drug discovery

Q1 **Peter H. Hagedorn[1], Robert Persson[1], Erik D. Funder[1], Nanna Albæk[1], Sanna L. Diemer[1], Dennis J. Hansen[1], Marianne R. Møller[1], Natalia Papargyri[2], Helle Christiansen[1], Bo R. Hansen[1], Henrik F. Hansen[1], Mads A. Jensen[1] and Troels Koch[1]**

[1] Roche Pharmaceutical Discovery and Early Development, Therapeutic Modalities, Roche Innovation Center Copenhagen, 2970 Hørsholm, Denmark
[2] Department of Biotechnology and Biomedicine, Technical University of Denmark, 2800 Kgs. Lyngby, Denmark

Over the past 20 years, the field of RNA-targeted therapeutics has advanced based on discoveries of modified oligonucleotide chemistries, and an ever-increasing understanding of how to apply cellular assays to identify oligonucleotides with pharmacological properties *in vivo*. Locked nucleic acid (LNA), which exhibits high binding affinity and potency, is widely used. Our understanding of RNA biology has also expanded tremendously, resulting in new approaches to engage RNA as a therapeutic target. Recent observations indicate that each oligonucleotide compound is a unique entity, and small structural differences between oligonucleotides can often lead to substantial differences in their pharmacological properties. Here, we outline new principles for drug discovery exploiting oligonucleotide diversity to identify rare molecules with unique pharmacological properties.

Introduction

The flow of genetic information is a highly complex process. Of the approximately 43 000 human genes currently annotated by the Ensembl project (release 89), approximately 47% encodes proteins, and the rest are all transcribed into different types of noncoding RNA (ncRNA) [1]. It is clear that these ncRNAs represent a long and growing list of different types of RNA with various functional roles and regulatory interactions [2,3]. This knowledge has provided a stronger basis for the specific annotation of the relationships between structure, type, and function of RNAs and human disease [4]. This one reason for the increased interest in RNA therapeutics, which focuses not only on classic protein-coding mRNA intervention, but also on how the manipulation of Q2 noncoding regulatory RNA can provide novel drugs for previously untreatable diseases.

Corresponding author: Koch, T. (troels.koch@roche.com)

Peter Hagedorn has a MSc in theoretical physics and biophysics from the Niels Bohr Institute at the University of Copenhagen, and a PhD in molecular biology and bioinformatics from Ris½ National Laboratory, Denmark. For the past 6 years, Peter has been exploring the structural diversity of LNA-modified antisense oligonucleotides and how this impacts measured properties, with a particular focus on developing predictive mathematical models that capture this diversity.

Robert Persson has a PhD in microbiology and biochemistry from the University of Lund, Sweden. He has worked with the intracellular transport of secretory proteins and viral membrane proteins and with the pharmacokinetics of proteins and oligonucleotides for more than 25 years. During the past 8 years, Robert has focused on the bioanalysis, biodistribution, and pharmacokinetics of LNA oligonucleotides.

Troels Koch has a PhD in bioorganic chemistry from the University of Copenhagen, Denmark. He has worked in the area of nucleic acid chemistry and biology for 20 years. He co-founded Santaris Pharma A/S, which pioneered the locked nucleic acid (LNA) platform and LNA therapeutics. Santaris was acquired by Roche in August 2014, and he is presently vice president and head of research at the Roche Innovation Centre in Copenhagen. His main responsibilities are to develop the chemical and biological properties of LNA, improve and upgrade the LNA platform, refine LNA antisense drug discovery processes, and establish a RNA therapeutics drug pipeline in Roche.

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A rational strategy to target RNA is by synthetic oligonucleotides. Although RNA exists in a complex biological matrix, endogenous enzymatic RNA-processing mechanisms can be exploited and recruited by the heteroduplex formation between RNA and synthetic oligonucleotides. RNA exhibits high turnover rates and is more labile than DNA [5]; thus, inhibitory effects induced by the oligonucleotide binding complement can be executed rapidly and effectively. Zamecnik and Stevenson [6] were the first to show that a single-stranded synthetic DNA oligonucleotide could inhibit the expression of RNA, resulting in the stimulation of substantial research and development activity [7–13].

The past four decades of research and development in oligonucleotide-based RNA therapeutics has produced groundbreaking results. The recent successes are based on improvements in three essential parameters for antisense technologies: (i) medicinal chemistry efforts have devised much-improved nucleic acid modifications for antisense oligonucleotides (AONs), of which the high-affinity LNA [14–17] is among the most widely used; (ii) the knowledge revolution of the functions and biological molecular mechanisms of RNA; and (iii) the translational drug discovery part (i.e., discovery processes and models used in drug discovery). The latter relates to bioinformatics drug design, prediction algorithms, *in vitro/vivo* assays and models, better pharmacokinetics/pharmacodynamics (PK/PD), absorption, distribution, metabolism, and excretion (ADME), and toxicology study designs.

Recent developments have also provided insights into the fundamental properties of the chemical modality. Small chemical modifications of AONs have been shown to produce large property differences even among AONs belonging to the same chemical or structural class. The old concept of the 'portability of chemistry' for AONs, whereby nearly all properties are shared for a given chemical class, has turned out not to be true for many important drug properties (e.g., activity and toxicity). In other words, we now know that the structure–activity relations (SARs) for AONs are more subtle. The fact that AONs belonging to a given structural class based on only a few parameters share the same properties and act more as individual unique compounds diversifies and complicates drug discovery [13,18–20]. However, the fact that small chemical modifications, even the configuration of a single bond, can have profound property impacts creates a basis for SAR studies comparable to classic small-molecule drug discovery. This differentiated property diversity has functional consequences that result from compound and/or sequence libraries, making it possible to identify compounds that meet a range of specific and strict selection criteria. Therefore, during the discovery phase, it is possible to produce more unique compounds for nearly any RNA target with larger therapeutic indexes (TI).

In this review, we focus on three aspects of LNA: (i) state of the art of the modality, including structure, fundamental properties, preferred designs, mechanisms of action, and cellular uptake; (ii) Illustrate how subtle structural modifications have profound impacts and, in some cases, produce 'all or none' phenotypic effects; and (iii) how the insight or 'Erkenntnis' of this property diversity can be used to transform and improve the classic oligonucleotide drug discovery process and lead generation.

Chemistry sets boundaries for success

Three generations of modification chemistries so far

In the first generation of single-stranded therapeutic oligonucleotides, the phosphorothioate (PS) internucleoside linkage was the only chemical modification [8,21–24]. The PS modification replaces one of the nonbridging oxygen atoms with sulfur in the internucleoside phosphate (Fig. 1ai). This modification creates a chiral center at phosphorous producing two isomers: Rp and Sp. Thus, for every PS linkage introduced in an oligonucleotide, two diastereoisomers are formed. Given that conventional solid-phase PS synthesis is not stereoselective, an *n*-mer PS oligonucleotide contains random mixtures of 2^{n-1} diastereoisomers [25–30]. However, recent developments have now made it possible to synthesize individual stereoisomers with high stereoselectivity [31–34], and with good yields [35–37]. Diastereoisomers are different chemical compounds and the diversity of properties for a given PS oligonucleotide will be a gradient spanning from a little to a very large difference in property. Generally, oligonucleotides with a Sp configuration provided better exonuclease resistance compared with oligonucleotides with a Rp configuration, whereas Rp isomers provided better endonuclease resistance and were better substrates for DNA-dependent RNA polymerases, RNase H, and stimulated immune responses [38–40]. Compared with the random mixtures, Rp oligonucleotides exhibited higher melting temperature (T_m) against RNA, whereas Sp analogs had lower T_m. In the first-generation oligonucleotide drugs, the PS modification was normally not stereocontrolled. Except for rare examples [41], this class of drugs has not provided an adequate TI for AONs [42]. Two reasons for this were that the PS modification decreases the T_m of the hybrid duplex and, although it protects against nuclease degradation to some extent, this protection was still not adequate on its own for most therapeutic purposes [43]. Still, the PS modification, stereocontrolled or not, turned out to be a central modification for later generations. Combined with the increased nuclease stability, PSs also contribute to improved PK and cellular uptake properties of AONs. Unless otherwise stipulated, all antisense data described here come from fully PS-modified AONs.

A preferred strategy to improve the binding affinity of AONs to RNA was to introduce electronegative substituents in the 2′-position of the furanose (Fig. 1aii) [7,8,44–47]. For nucleic acid analogs of this structural class, melting temperatures increased by approximately 0.5–1.5 °C/modification against RNA. The most significant members of this class are the 2′-F, 2′-O-CH_3 and 2′-O-CH_2CH_2-O-CH_3 (MOE) substituent groups [44,46]. This second generation of drugs shows clear improvements compared with first-generation PS drugs, and two drugs of this type, mipomersen [48] and nusinersen [49], have now been approved by the US Food and Drug Administration (FDA).

A high point for synthetic nucleic acid analogs was reached in 1997 with LNA [15,50] (Fig. 1aiii). LNA exhibited unprecedented high RNA-binding affinity [51], providing melting temperature increases of 3–9 °C/modification depending on the oligonucleotide position and design complements [52]. The affinity increase per LNA nucleoside substitution is a reproducible ('portable') property [14,51–55] and, combined with the increased nuclease stability, the driver behind the many therapeutic and diagnostic life-science applications that have been demonstrated with, and published on, LNAs [56–63]. Given these significant

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