Antisense Therapeutics

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METHODS IN MOLECULAR MEDICINE™

Antisense Therapeutics

Second Edition

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Foreword

We are now more than 15 years into a large-scale experiment to determine the viability of antisense technology. The challenges of creating a new pharmacological drug discovery platform are prodigious, requiring sizeable investments, long-term commitment, insight, and perseverance. For antisense technology to progress, advances in understanding the behavior of the receptor, RNA, and the behavior of the drugs, oligonucleotide analogs, were necessary. A new medicinal industry, the medicinal industry of oligonucleotides, had to be invented, and numerous drug development challenges—such as creating efficient manufacturing and analytical processes and formulations—had to be overcome. All of those advances then needed to be focused in drug candidates designed to interact with specific targets and to be effective in patients with specific diseases. This has taken time and a good bit of money and although the progress in the technology has been gratifying, there have, of course, been failures of individual clinical trials and individual drugs along the way.

What have we learned? Antisense technology works. Oligonucleotide analogs with a reasonable drug-dependent property can be synthesized and used to inhibit gene function through a variety of antisense mechanisms. Antisense drugs distribute to a wide range of tissues and reduce the expression of targets in a dose fashion consistent with the pharmaceutics of the drugs. First-generation antisense drugs are sufficient for relatively severe indications and second-generation drugs are performing significantly better. Moreover, these drugs are effective by a wide variety of routes including intravenous, subcutaneous, intradermal, rectal, and aerosol, and progress in oral delivery has been reported. Today numerous clinical trials in a wide range of diseases using a variety of oligonucleotide chemistries and antisense mechanisms are in progress.

In this year alone, positive clinical data in rheumatoid arthritis, diabetes, hyperlipidemia, cancer, and other diseases have been reported.

In this edition of *Antisense Therapeutics*, a number of approaches to antisense and therapeutic areas are discussed, as well as specific diagnostic opportunities. That the breadth of activities presented in this volume is as impressive as it is and yet does not begin to cover all of the work in progress, underscores the range of utility and potential value of antisense technology.

Nevertheless, despite antisense being an accepted tool that has facilitated better understanding of biological systems, much remains to be done before the true potential of the technology for therapeutic purposes can be defined. What this volume emphasizes, however, is that exponential progress in defining the long-term roles and value of antisense-based therapeutics is being made.

We look forward to the continued evolution of the technology.

Stanley T. Crooke, MD, PhD

Preface

This is the second edition of *Antisense Therapeutics*. The first edition was edited by Sudhir Agrawal and published in 1996. At that time there was no therapy based on antisense, but plenty of promise for the highly specific targeting of genes that cause disease. Antisense oligonucleotides were first reported as viral replication inhibitors by Paul Zamecnik and Mary Stephenson in 1978. Although this was excellent work, nothing much happened until new procedures for synthesizing DNA sequences were developed. Once oligonucleotides were easy to make, more and more studies were published in the 1980s, most of which were directed to cells in culture. In the early 1990s antisense oligonucleotides were increasingly tested in vivo. There were many controversies and a great deal of concern about backbone modification of the phosphodiester bridges that link the DNA bases. To protect against breakdown by nucleases in cells or blood, phosphorothioate oligonucleotides were adopted. In 1998 a phosphorothioated antisense agent was the first FDA-approved antisense therapy. Vitravene™, developed by Isis Pharmaceuticals, made antisense therapeutics a reality.

Since then, the complete sequencing of the human genome in April, 2003 has demonstrated the presence of a vast number of targets for antisense oligonucleotides. So we now have thousands of targets, hundreds of preclinical animal studies, and some 20 clinical trials ongoing. Any successful trial with an antisense compound will open a floodgate of new therapies for a panoply of diseases.

This second edition of *Antisense Therapeutics* deals less with the basic science of antisense and more with the actual therapeutic applications. For that reason it is organized into disease states.

I thank the authors for their patience and their strong contributions. Since this book was being edited at a time when I moved from the University of Florida to the University of South Florida, I ended up with two secretaries. I would like to thank Ms. Gayle Butters at the University of Florida and Mr. Eric J. Wheeler at the University of South Florida for their essential help. I am also grateful to Craig Adams at Humana Press for his patience.

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I

Introduction

1

Antisense Therapeutics

A Promise Waiting to Be Fulfilled

M. Ian Phillips

1. Introduction

During the past decade, only one antisense-based therapy has received full Food and Drug Administration (FDA) approval. Vitravene™, developed by Isis Pharmaceuticals, was the first drug based on antisense technology to be successfully commercialized and used in treatment ***(1)***. The therapeutic area it is used in is a small niche related to the treatment of preventing blindness in acquired immunodeficiency syndrome (AIDS) patients by inhibiting cytomegalovirus-induced retinitis. The success of Vitravene, however, showed that antisense could be taken all the way through the FDA approval process and provide those patients taking it with a vitally important effect. With Vitravene we saw the first breakthrough in antisense therapy, and, yet, euphoria has turned to disappointment without a second breakthrough. Subsequent trials of Affinitak (Isis), an antisense inhibitor of protein kinase C α, failed to show statistically significant benefits as an antisense therapy for the treatment of non–small cell carcinoma of the lung better than the median survival with control treatments. The results nevertheless proved that antisense was well tolerated and tended toward greater benefit to the survival of patients ($p < 0.054$). The promise of antisense therapy is so attractive that some 20 trials continue.

The appeal of antisense is that it potentially provides highly specific, nontoxic effects for safe and effective therapeutics of an enormous number of diseases including AIDS, Crohn's disease, pouchitis, psoriasis, cancers, diabetes, mulitiple sclerosis, muscular dystrophy, restenosis, asthma, rheumatoid arthritis, hepatitis, skin diseases, polycystic kidney disease, and chronic cardiovas-

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cular disease, such as hypertension, restenosis, and heart failure. Successes in phase I have shown that antisense therapy consistently has excellent safety results. With each trial we learn more, and this makes each new antisense drug candidate more easy to test. We are hampered by a lack of understanding of the theoretical considerations for optimal antisense inhibition. Failures in the past have been the result of incorrect design and use of unmodified backbones causing instability, overly long oligonucleotides leading to unpredictable targeting, and aptermeric or nonantisense effects. However, with each experiment we learned more. For example, high doses of antisense in monkeys triggered cardiovascular collapse ***(2)***. This result was a setback until it was found that the reaction could be accounted for by the extremely high doses and a sensitivity to complement activation unique to nonhuman primates ***(3)***. Human trials, by contrast, have shown how well antisense is tolerated and how few side effects are encountered. The number of trials is increasing, and more than 2000 patients have received antisense. Isis is the leader with 11 phase I, 7 phase II, and 3 phase III trials. Genta is active with Genasense, and antisense to Bcl 2 for antitumor cell treatment is in phase III. AVI Biopharm has a third generation antisense platform, and around this it is testing four phase I, five phase II, and two phase III trials. Hybridon has conducted two phase I and has two phase II trials planned.

2. Mechanism of Antisense Inhibition

Antisense oligonucleotides (AS-ODNs) are designed to bind and inactivate specific mRNA sequences inside cells. The potential uses for AS-ODNs is vast because RNA is so ubiquitous and abundant. With the publication of the human genome sequence, we now have such a wide open access to the sequences of genes that antisense can in theory be applied to almost every known gene to inhibit its mRNA. Inhibiting mRNA prevents specific proteins from being produced. Although routine human therapy may have been difficult to achieve, at a scientific level, antisense gene knockdown has become one of the fastest ways to study new therapeutic targets.

AS-ODNs are synthetically made, single-stranded short sequences of DNA bases designed to hybridize to specific sequences of mRNA forming a duplex. This DNA-RNA coupling attracts an endogenous nuclease, RNase H, that destroys the bound RNA and frees the DNA antisense to rehybridize with another copy of mRNA ***(2)***. In this way, the effect is not only highly specific but prolonged because of the recycling of the antisense DNA sequence. The reduction in mRNA reduces the total amount of protein specified by mRNA. It is also theorized that hybridization sterically prevents ribosomes from translating the message of the mRNA into protein. Therefore, there are at least two

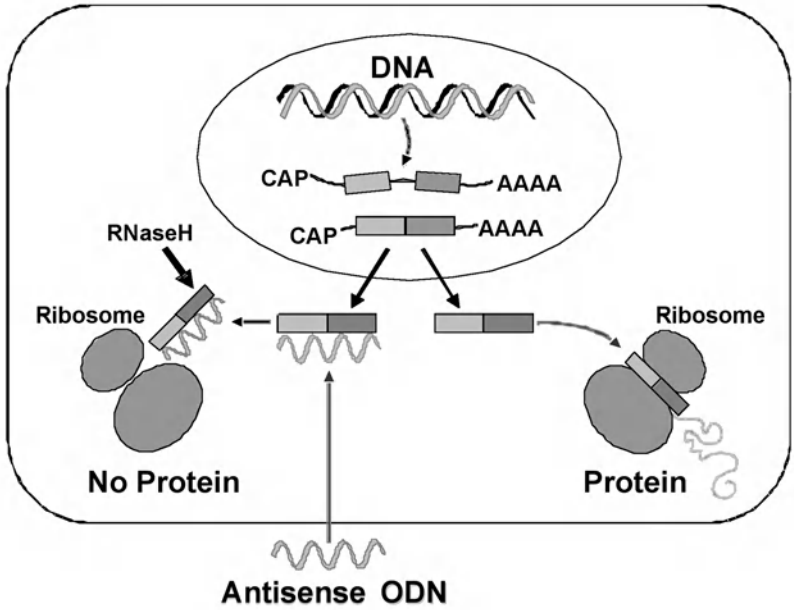

Fig. 1. Mechanism of AS-ODN posttranscriptional inhibition. AS-ODN enters the cell by an unknown uptake mechanism and hybridizes with a copy of a specific mRNA. The ODN-RNA duplex then prevents protein translation by (1) attracting RNase H to degrade the RNA and (2) steric hindrance of the ribosomal access and/or assembly. Note that the extent of inhibition depends on the AS-ODN competing with endogenous copies of RNA.

ways in which antisenses can work to effectively reduce the amount of protein being elaborated: RNase H degradation of RNA and hindering of ribosomal assembly and translation (**Fig. 1**). However, unless the antisense is designed to inhibit transcription, antisense would rarely be 100% inhibitory because the antisense inhibition of RNA does not shut down the transcription of endogenous copies of mRNA. It competes with the RNA being produced by the cell, and the effect is a gene knockdown rather than knockout. This has the advantage of being more physiological as a therapeutic agent, since antisense does not cause a mutation and does not prevent a protein that is involved in normal physiology from assuming its role. What antisense therapy does very effectively is reduce overexpression of proteins, and it is the overexpression of proteins that can cause disease states.

3. Stability

One of the problems that dogged early attempts to achieve a therapy with antisense was the question of stability. This is largely being answered by numerous ways to modify backbones of the DNA sequence in an AS-ODN. Native DNA has a phosphodiester bridge between each successive base of the DNA sequence. It was quickly learned that unmodified AS-ODNs were very short lasting, because they were unprotected from breakdown by nucleases, which break apart the nuclear acids. A very successful modification was phosphorothioate in which a sulfur atom replaces one oxygen atom in the phosphorate group of the phosphodiester bond. Phosphorothioate oligonucleotides are resistant to nucleases and are stable. This extends the life of the AS-ODN to several days instead of a few hours. Many variations on this theme have been tested and patented so that there is now a range of second- and even third-generation backbone modifications available ***(2–4)***. Each company appears to favor its own particular modification. Isis uses phosphorothivates with 2'-*O*-methyl modification. Hybridon favors its IMO™ backbone modification, which can increase or decrease immunomodulation. AVI Biopharm has used NeuGene® as a platform of third-generation antisense for its nine clinical trials. A factor in developing backbone modifications such as these and others, including peptide nucleic acid, is the cost.

4. Cellular Uptake

Another area that has required time (and money) to investigate is the optimal conditions for uptake and distribution. This is particularly important when it comes to systemic injection as opposed to the early experiments in which antisenses were simply applied to cells in culture. There is both uptake and efflux of intact AS-ODNs in cells ***(5)***.The backbone modifications become extremely important when systemic injections are used because of nucleases and the binding of oligonucleotides to proteins. The backbone modification can alter cell uptake, distribution, metabolism, and excretion. Nonantisense effects are a concern because they may alter the interpretation of whether the antisense effect is truly through an antisense mechanism or not. Mechanisms for the uptake of oligonucleotides into cells are still not clearly understood. The lack of a theory of the uptake and kinetic effects on oligonucleotides has required a lot of trial-and-error studies. This affects how to determine the optimal length of the oligonucleotide, the optimal concentration for effective treatment, and the frequency of treatments to maintain constant therapy. Despite these complications and holes in the study of antisense, phosphorothioated oligonucleotides are surprisingly easy to work with. In our own studies, which were in vivo applications of AS-ODN, we aimed injections into the brain and

into the blood at receptor targets involved in cardiovascular disease. We found highly significant effects using AS-ODNs of 15–18 bases in length delivered in the brain without any vehicle ***(6)*** and in the blood delivered with liposomes ***(7)***. Call it science or dumb luck, we nevertheless were able to show significant physiological effects of antisense delivery in models of hypertension. Because hypertension is a chronic disease, the findings were remarkable because of the long-lasting efficacy of a single antisense treatment. Reductions in blood pressure lasted weeks with a single systemic injection of antisense targeting β-1 receptors ***(8)***.

The distribution of AS-ODNs injected systemically is to all parts of the body except the brain. The lipophobicity and/or negative change appear to prevent AS-ODNs from crossing the blood-brain barrier. However, the oligonucleotides accumulate in liver, kidney, and spleen. The lack of entry into the brain probably translates into few side effects. With the antisense to β-1 receptors, this could be a definite advantage ***(8)***. For treating liver or kidney disease, however, AS-ODNs might have a built-in advantage in terms of delivery.

5. The Target

Clearly, the target protein for antisense inhibition is crucially important for a therapeutic effect. To reach the target, the antisense therapy must enter the cell through an uptake mechanism and escape from endosomes and lysosomes within the cell in sufficient amounts to avoid intracellular degradation. If the target mRNA is shielded or coiled, it may be difficult for AS-ODNs to hybridize. DNA and RNA are folded and studded with regulated proteins. Predicting how RNA folds and its secondary structures in a living cell is still very difficult. Once again, trial and error must be used. The stability of the oligos also depends on the interactions of the G-C proportions because of the three hydrogen bonds instead of the two hydrogen bonds that are in the A-T interaction. Having sufficient length of bases is necessary to make a specific match, but having too long a sequence can overlap the coding regions and inhibit more than single-target RNA.

Even when everything is successful and there is good uptake—good inhibition of the target—it does not necessarily lead to a therapeutic effect, because the target may not be the only player in the disease. If knocking down one gene leads to an increase in a compensatory gene, there may be little or no effect. Alternatively, a target gene may have been involved in starting the disease, but once the disease is present that target is no longer necessary, and, therefore, inhibiting it does not alter the disease state. Targeting transcription factors or signaling pathway proteins important in regulating cells may not be specific enough. If the target protein is overexpressed only in the disease state, then

antisense should be efficacious, but if the target is similarly expressed in both normal and malignant cells, antisense treatment may cause both types of cells to undergo apoptosis. Then the therapy becomes a question of benefit vs risk. Because of the competition for RNA inhibition with antisense vs endogenous production of copies of mRNA in a cell, antisense for cancer is not a cell killer and, therefore, will not destroy all cancerous cells. However, it can be used with other treatments for cancer, and that is the protocol proposed for Affinitak and for Genasense.

6. Alternative to Oligonucleotides

In recent years, there has been a tremendous increase in interest in morpholinos ***(9)***, small inhibitory RNA (siRNA) ***(10)***, as well as ribozymes ***(11)***. Morpho-linos are assembled from four different morpholino subunits each of which contains one of the four genetic bases linked to a six-sided morpholine ring. Morpholinos are supposed to have complete resistance to nucleases, high sequence specificity, and predictable targeting because they invade the RNA secondary structure and are fast and easy to deliver to the nucleus without liposome delivery systems. siRNAs are double-stranded RNA (dsRNA) molecules of 21–25 bp in length. They mediate RNA interference, an antiviral response initially identified in *Caenorhabditis elegans* and subsequently found active in specific gene silencing in many other organisms including mammalian cells. The sense and antisense strands of an siRNA first unwind, and the antisense strand binds to the target mRNA and recruits RNA-induced silencing complex (RISC) (**Fig. 2**). The sense strand is released from RISC, and RISC catalyzes the mRNA cleavage. The gene silencing efficiency of siRNA has reportedly been greater than antisense in general, typically reaching 80–90%. However, the maximal effects of optimal AS-ODNs and siRNAs targeting the same mRNA sequence are comparable. siRNAs are being used because of their stability and specificity, but it is not clear how effective they will be in systemic injections or oral delivery. Vickers et al. ***(12)*** conducted a comparative study of single-stranded AS-ODNs vs siRNA. Examination of 80 siRNA oligonucleotide duplexes designed to bind human RNA showed that both strategies are valid in terms of potency, maximal effects, specificity, and duration of action, at least in vitro.

The design of AS-ODNs and siRNAs follows different rules. Unlike AS-ODNs, the selection of an effective siRNA does not depend on the secondary mRNA structure or sequence accessibility. Instead, nucleotide composition and the release rate of the sense strand from RISC seem to play major roles. Several siRNA molecules targeting the same mRNA can be used in combination to achieve greater effects and to avoid cellular resistance to siRNA. An independent combinatorial effect of AS-ODNs and siRNAs has also been observed

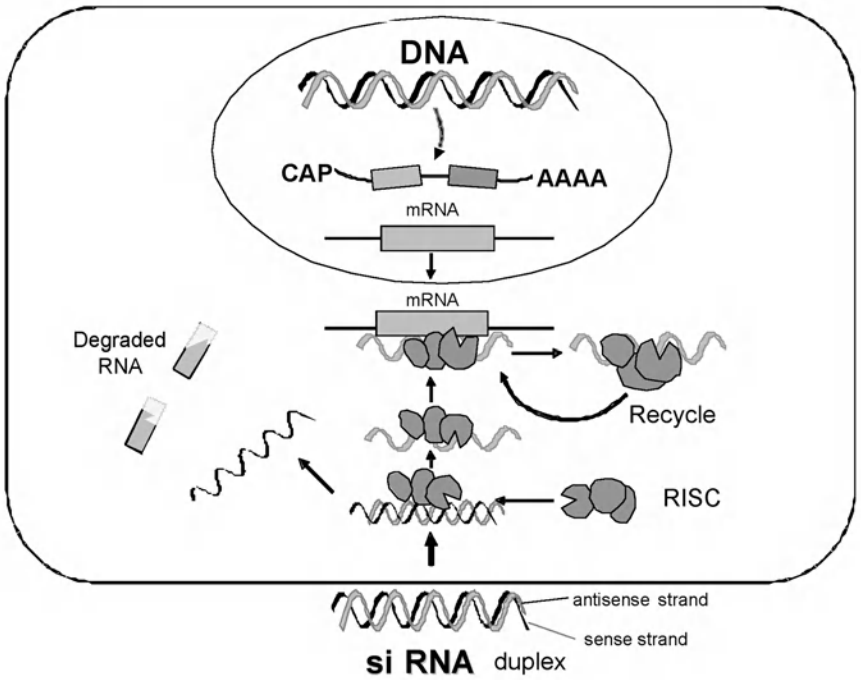

Fig. 2. Mechanism of siRNA. Synthetic siRNA enters the cell as a dsRNA with sense and antisense strands. RISC multiprotein made up of helicase, RNase III, and an activating protein unwinds the two strands of RNA and uses the antisense to recognize the chosen sequence of RNA. The RNase cleaves the sequence of mRNA, which is degraded by cellular nucleases. The RISC-antisense complex can then recycle and silence more copies of mRNA.

when siRNA was coadministered with nonhomologous AS-ODNs, targeting distant regions of the same mRNA. As alternative therapeutics, development of siRNA has covered a wide variety of disease models in a short time. The most studied fields of siRNA application are cancer and infectious diseases. siRNA has been administered in vivo in unmodified states. Following iv injection into mice, the highest inhibition of target mRNA was found in liver, kidney, spleen, lung, and pancreas. If both strategies are equally effective, then the deciding factor in choosing one over the other would depend on the price of production. In addition, experience with AS-ODNs will count for some time against the newness of siRNA molecules. However, a lot will depend on whether there are side effects that are not due to the antisense mechanism, or if one approach is associated with more side effects than the other.

7. Conclusion

The brief history of antisense therapeutics has been characterized by cycles of success and disappointment. However, through it all, the promise of antisense therapy has been so appealing that hope remains for that blockbuster breakthrough that will open the doors for so many potential treatments. There are now thousands of targets available with known genomic sequences. There are hundreds of preclinical studies pointing to new treatments with antisense. And there are a score of human trials that are paving the way. Once one major treatment is accepted, each new antisense therapy will be more easily and quickly brought to those who suffer from diseases that are not yet satisfactorily treated with drugs.

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