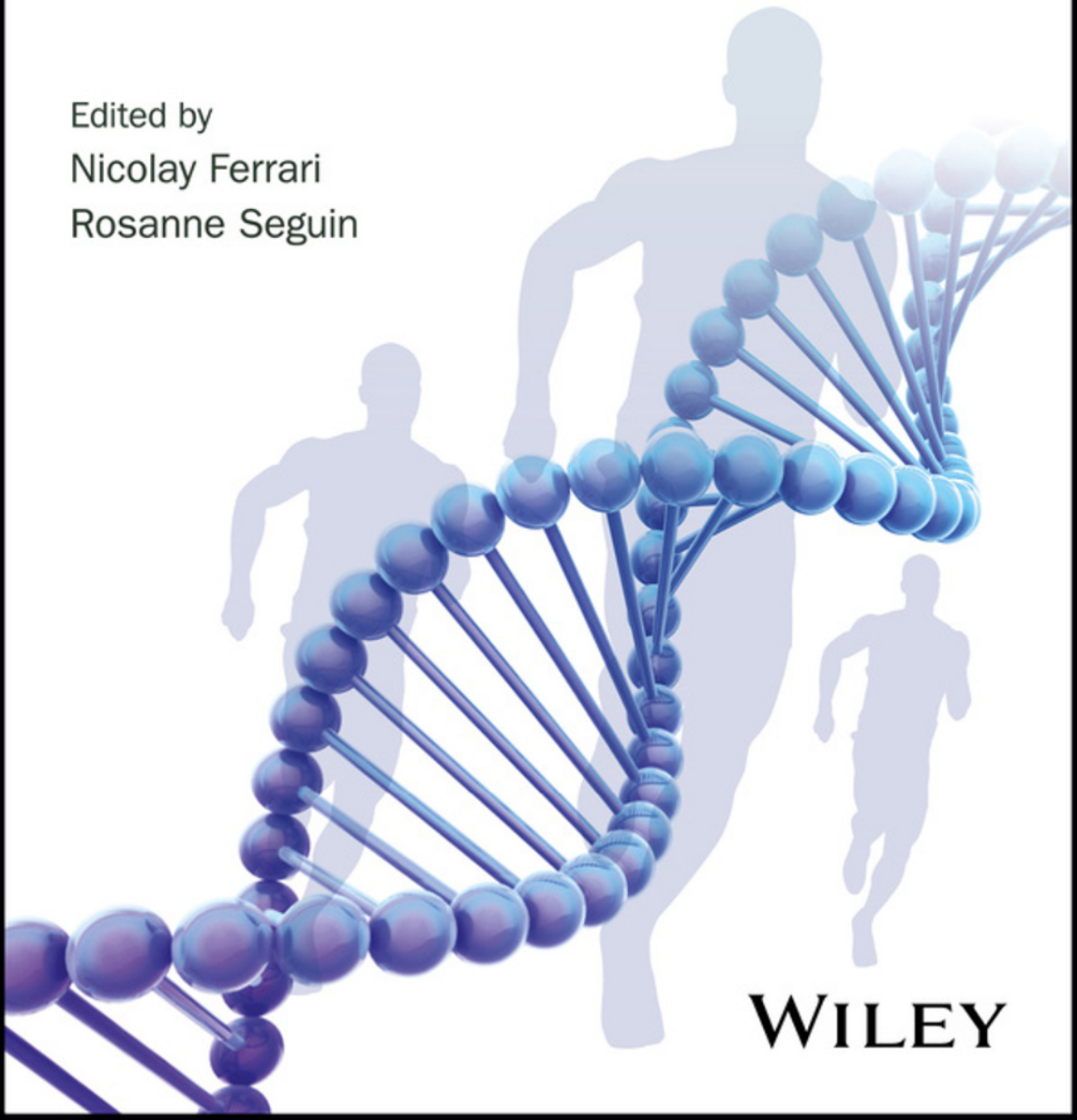
OLIGONUCLEOTIDE-BASED DRUGS AND THERAPEUTICS

PRECLINICAL AND CLINICAL CONSIDERATIONS FOR DEVELOPMENT

Edited by
Nicolay Ferrari
Rosanne Seguin



WILEY

Oligonucleotide-Based Drugs and Therapeutics

Oligonucleotide-Based Drugs and Therapeutics

Preclinical and Clinical Considerations for Development

Edited by

Nicolay Ferrari and Rosanne Seguin

WILEY

This edition first published 2018
© 2018 John Wiley & Sons, Inc.

All rights reserved. No part of this publication may be reproduced, stored in a retrieval system, or transmitted, in any form or by any means, electronic, mechanical, photocopying, recording or otherwise, except as permitted by law. Advice on how to obtain permission to reuse material from this title is available at http://www.wiley.com/go/permissions.

The right of Nicolay Ferrari and Rosanne Seguin to be identified as the Editors of this work has been asserted in accordance with law.

Registered Office
John Wiley & Sons, Inc., 111 River Street, Hoboken, NJ 07030, USA

Editorial Office
111 River Street, Hoboken, NJ 07030, USA

For details of our global editorial offices, customer services, and more information about Wiley products visit us at www.wiley.com.

Wiley also publishes its books in a variety of electronic formats and by print-on-demand. Some content that appears in standard print versions of this book may not be available in other formats.

Limit of Liability/Disclaimer of Warranty
In view of ongoing research, equipment modifications, changes in governmental regulations, and the constant flow of information relating to the use of experimental reagents, equipment, and devices, the reader is urged to review and evaluate the information provided in the package insert or instructions for each chemical, piece of equipment, reagent, or device for, among other things, any changes in the instructions or indication of usage and for added warnings and precautions. While the publisher and authors have used their best efforts in preparing this work, they make no representations or warranties with respect to the accuracy or completeness of the contents of this work and specifically disclaim all warranties, including without limitation any implied warranties of merchantability or fitness for a particular purpose. No warranty may be created or extended by sales representatives, written sales materials or promotional statements for this work. The fact that an organization, website, or product is referred to in this work as a citation and/or potential source of further information does not mean that the publisher and authors endorse the information or services the organization, website, or product may provide or recommendations it may make. This work is sold with the understanding that the publisher is not engaged in rendering professional services. The advice and strategies contained herein may not be suitable for your situation. You should consult with a specialist where appropriate. Further, readers should be aware that websites listed in this work may have changed or disappeared between when this work was written and when it is read. Neither the publisher nor authors shall be liable for any loss of profit or any other commercial damages, including but not limited to special, incidental, consequential, or other damages.

Library of Congress Cataloging-in-Publication Data

Names: Ferrari, Nicolay, 1969– editor. | Seguin, Rosanne, editor.
Title: Oligonucleotide-based drugs and therapeutics : preclinical and clinical considerations for development / edited by Nicolay Ferrari, Rosanne Seguin.
Description: Hoboken, NJ : John Wiley & Sons, 2018. | Includes bibliographical references and index. |
Identifiers: LCCN 2018006576 (print) | LCCN 2018009683 (ebook) | ISBN 9781119070290 (pdf) | ISBN 9781119070306 (epub) | ISBN 9781118537336 (cloth)
Subjects: LCSH: Oligonucleotides–Therapeutic use. | Antisense nucleic acids–Therapeutic use.
Classification: LCC RM666.N87 (ebook) | LCC RM666.N87 O445 2018 (print) | DDC 572.8/5–dc23
LC record available at https://lccn.loc.gov/2018006576

Cover Design: Wiley
Cover Images: © nechaev-kon/Getty Images;
© Michal Sanca/Shutterstock

Set in 10/12pt Warnock by SPi Global, Pondicherry, India

Printed in the United States of America

10 9 8 7 6 5 4 3 2 1

Contents

List of Contributors

Annemieke Aartsma-Rus
Department of Human Genetics
Leiden University Medical Center
Leiden
The Netherlands

Scott A. Barros
Sage Therapeutics
Cambridge, MA
USA

Cindy L. Berman
Berman Consulting
Wayland, MA
USA

Kevin Brown
Fluidigm Corporation
South San Francisco, CA
USA

Joy Cavagnaro
Access BIO
Boyce, VA
USA

Scott Cormack
OncoGenex Pharmaceuticals
Bothell, WA
USA

Beth E. Davis
Department of Medicine
University of Saskatchewan
Saskatoon, Saskatchewan
Canada

Mehrdad Dirin
Department of Pharmaceutical Chemistry
University of Vienna
Vienna, Austria

Sheila M. Galloway
MRL, Merck & Co., Inc.
West Point, PA
USA

Gail M. Gauvreau
Department of Medicine
McMaster University
Hamilton, Ontario
Canada

Isabella Gazzoli
Department of Human Genetics
Leiden University Medical Center
Leiden
The Netherlands

Alain Guimond
InSymbiosis
Montreal
Quebec
Canada

Lydia Haile
Centre for Drug Evaluation and Research, Office of New Drugs
US FDA
Silver Spring, MD
USA

Jonathan Hall
ETH-Zurich
Zurich
Switzerland

Derek Ireland
Centre for Drug Evaluation and Research, Office of Biotechnology Products, US FDA
Silver Spring, MD
USA

Aimee L. Jackson
miRagen Therapeutics
Boulder, CO
USA

Cindy Jacobs
OncoGenex Pharmaceuticals
Bothell, WA
USA

Piotr J. Kamola
GlaxoSmithKline R&D
Ware
Hertfordshire
UK
Current address
RIKEN Center for Integrative Medical Sciences
Yokohama
Japan

Lauren Kane
GlaxoSmithKline R&D
Stevenage
Hertfordshire
UK
Current address
MRC Human Genetics Unit
Institute of Genetics and Molecular Medicine
University of Edinburgh
Scotland

Peter Kasper
Federal Institute for Drugs and Medical Devices (BfArM)
Bonn
Germany

Jeremy D. A. Kitson
GlaxoSmithKline R&D
Stevenage
Hertfordshire
UK

Doug Kornbrust
Preclinsight
Reno, NV
USA

Monica Krieger
OncoGenex Pharmaceuticals
Bothell, WA
USA

Arthur A. Levin
Avidity Biosciences
La Jolla, CA
USA

Helen Lightfoot
ETH-Zurich
Zurich
Switzerland

John Martucci
Centre for Drug Evaluation and Research, Office of Biotechnology Products, US FDA
Silver Spring, MD
USA

Xin Ming
Division of Molecular Pharmaceutics, UNC Eshelman School of Pharmacy
University of North Carolina
Chapel Hill, NC
USA

Nicolay Ferrari
Centre de recherche du CHUM – Tour Viger
Montreal, Quebec
Canada

Frederick B. Oleson
Independent Consultant
Concord, MA
USA

John Paul Oliveria
Department of Medicine
McMaster University
Hamilton, Ontario
Canada

Catherine C. Priestley
Innovative Medicines & Early Development
AstraZeneca
Cambridge
UK

Montserrat Puig
Centre for Drug Evaluation and Research Office of Biotechnology Products, US FDA
Silver Spring, MD
USA

Michael J. Schlosser
MSR Pharma Services, Inc.
Lincolnshire, IL
USA

Anneliese Schneider
Preclinical Services & Consulting
Munich
Germany

Rosanne Seguin
Montreal Neurological Institute
McGill University
Montreal
Quebec
Canada

Zhanna Sobol
Pfizer Inc.
Groton, CT
USA

Patricia S. Stewart
OncoGenex Pharmaceuticals
Bothell, MA
USA

Kevin S. Sweder
Forensic and National Security Sciences Institute
Syracuse University
Syracuse, NY
USA

Daniela Verthelyi
Centre for Drug Evaluation and Research, Office of Biotechnology Products, US FDA
Silver Spring, MD
USA

Stefan Vonhoff
NOXXON Pharma AG
Berlin
Germany

Jonathan K. Watts
RNA Therapeutics Institute and
Department of Biochemistry and
Molecular Pharmacology
University of Massachusetts
Medical School
Worcester, MA
USA

Tacey E.K. White
Aclairo Pharmaceutical Development
Group, Inc., Vienna, VA
USA

Johannes Winkler
Department of Cardiology
Medical University of Vienna
Vienna, Austria
Department of Pharmaceutical
Chemistry
University of Vienna
Vienna, Austria

Karen D. Wisont
OncoGenex Pharmaceuticals
Bothell, WA, USA

Preface

Development of oligonucleotide (ODN)-based therapeutics is being progressed for a wide range of indications and using various routes of administration. There is a diversity of structures, chemistries, and mechanisms of actions for ODN therapeutics, but most of the members of this class of drug candidates can be categorized on the basis of whether they target either mRNA or proteins. ODN-based therapy is distinct from gene therapy as it does not involve the modification of genes. Antisense ODN (ASO), short interfering RNA (siRNA), antagomirs, microRNA mimetics, and DNAzymes are part of the RNA-targeting group, while immunostimulatory sequences (ISS), aptamers, and decoys are members of the protein-targeting group.

Currently, six ODN-based pharmaceuticals, including four ASO, have achieved marketing authorization in Europe and/or United States, and many more are undergoing late-stage clinical testing. The first ASO drug, VITRAVENE (fomivirsen, Ionis Pharmaceuticals – formerly Isis), was approved in 1998 to treat CMV eye infections in HIV patients but within a few years was rendered obsolete by advances in antiretroviral cocktails for HIV therapy. The field waited 15 years for another approval. In 2013, the second ASO drug, KYNAMRO (mipomersen, Ionis Pharmaceuticals), was approved by the Food and Drug Administration (FDA) for the treatment of familial hypercholesterolemia. In 2016, out of 22 new drugs approved by FDA, 3 were for ODN therapeutics: DEFITELIO (defibrotide, Jazz Pharmaceuticals), a treatment for veno-occlusive disease of the liver in individuals who have undergone bone marrow transplants granted in March; EXONDYS 51 (eteplirsen, Sarepta Therapeutics), a treatment for Duchenne muscular dystrophy granted in August; and SPINRAZA (nusinersen, Biogen), a treatment for spinal muscular atrophy granted in December. In addition, Atlantic Pharmaceuticals is developing alicaforsen, an ASO targeting ICAM-1 for the treatment of pouchitis, and currently supplies alicaforsen in response to physicians' requests under international named patient supply regulations for patients with inflammatory bowel disease. In January 2017, Atlantic announced it received agreement from the FDA to initiate a rolling submission of its New Drug Application for

alicaforsen to treat pouchitis ahead of data from an ongoing phase III study, which is expected at the end of 2018.

The recent ODN approvals are indicative of the enthusiasm, vigor, and vitality of the field observed in recent years. There are currently over 100 companies combining hundreds of ODN programs. In 2015 alone, there were more than 35 Investigational New Drug submissions for ODN candidates. More than 145 ODN clinical trials are listed on ClinicalTrials.gov, 31 of which are active/recruiting. The diverse types of indications for which ODN therapies have been approved and for those currently in clinical development demonstrate that these therapies are not a "one-off" development but rather are poised to claim their space in the apothecary of pharmaceuticals.

The advancement of a growing number of ODN programs, in particular ASO, in late stage of clinical development and the rapid pipeline expansion by various companies are testament of the progress, much of which was made in the 15 years between first and second drug approvals, in understanding the pharmacologic, pharmacokinetic, and toxicologic properties, as well as improving the delivery of ODN. There are now numerous examples of pharmacologic activity in animal models, and evidence of antisense activity in patients has been demonstrated in clinical trials.

The discovery of novel therapeutics is an inherently complex and interdisciplinary process, requiring close integration of scientists from several disciplines in an environment in which lessons are shared and taught across an organization.

The purpose of this book is to review the current state of knowledge of ODN and to examine the scientific principles and the tools utilized by scientists in preclinical and clinical settings as applied to ODN therapeutics.

Acknowledgments

We have embarked on this endeavor without anticipating the long twisting road that was ahead of us in putting this book together. We would like to give our heartfelt thanks to all authors. As editors, we were depending on their goodwill, commitment, and patience. We hope that their contribution will offer a useful review of the current understanding and recent advances in the field. In light of the challenges we are facing with this technology, we also hope the knowledge summarized in this book will provide guidance and will support those readers currently working in the field as well as the future developers that will further advance oligonucleotide therapeutics.

Nicolay Ferrari and Rosanne Seguin

1

Mechanisms of Oligonucleotide Actions

Annemieke Aartsma-Rus[1], *Aimee L. Jackson*[2], *and Arthur A. Levin*[3]

[1] *Department of Human Genetics, Leiden University Medical Center, Leiden, The Netherlands*
[2] *miRagen Therapeutics, Boulder, CO, USA*
[3] *Avidity Biosciences, La Jolla, CA, USA*

1.1 Introduction

The promise of antisense oligonucleotide (ASO) therapeutics is the ability to design drugs that are specific inhibitors of the expression solely on the basis of Watson and Crick base-pairing rules. The premise is that treatment of a patient with a DNA-like oligonucleotide complementary to a disease-related RNA (usually a messenger RNA) results in the formation of a heteroduplex that inhibits the function (generally translation) of that target RNA. Although antisense RNAs were first described in 1978 [1, 2], until recently the promise of selectivity and efficacy has always remained slightly out of reach for various reasons. Oligonucleotides are large molecules leading to synthesis and delivery issues. In addition, natural DNA and RNA oligonucleotides are rapidly degraded and cleared after systemic delivery. Over time many of the issues that have challenged developers of oligonucleotide-based therapeutics have been addressed: Synthesis costs have been reduced by orders of magnitude over the past two decades, allowing more investigators to use the technology. Stability issues were addressed partially with the introduction of phosphorothioate backbones (reviewed in Ref. [3]) and later sugar modifications (reviewed in Ref. [4]), and, as a result, oligonucleotides now used clinically and preclinically have more conventional drug-like properties [5]. In addition, fundamental discoveries have improved our understanding of the antisense mechanisms. We now know that target RNA structure and accessibility impacts activity of oligonucleotide therapeutics [6] and therefore pharmacologic activity. Apparently small changes in

Oligonucleotide-Based Drugs and Therapeutics: Preclinical and Clinical Considerations for Development, First Edition. Edited by Nicolay Ferrari and Rosanne Seguin.
© 2018 John Wiley & Sons, Inc. Published 2018 by John Wiley & Sons, Inc.

oligonucleotide chemistry can also have large pharmacologic effects as analyzed at the phenomenological level [7] and at the quantum level [8].

Our ability to design effective oligonucleotide-based drugs has also been enhanced by studies of the molecular mechanisms of these agents. This chapter reviews the mechanisms of action, the chemistry, and the clinical applications of three broad categories of oligonucleotide therapeutics: antisense agents, splicing modifiers, and gene silencers that activate the RNA interference (RNAi) pathway.

Antisense technology has now produced dozens of clinical stage drugs and two approvals. That hybridization of an oligonucleotide to a pre-mRNA could modulate the splicing of that RNA was described in 1993 [9], and the therapeutic potential of that mechanism is being exploited to treat Duchenne muscular dystrophy and now other diseases (see below). Running at first behind but more recently in parallel with applications of single-stranded ASO agents is the use of double-stranded RNA-like molecules that activate the RNA-induced silencing complex (RISC) to cleave targeted mRNAs or interfere with their translation. Synthetic small interfering RNA (siRNA) therapeutics relies on the same mechanism that is used by eukaryotic cells to control mRNA translation by endogenous microRNAs (miRNAs).

1.2 Antisense Oligonucleotide Therapeutics

1.2.1 Antisense Activity Mediated by RNase H

Zamecnik and Stephenson [1, 2] were the first to describe that a DNA strand complementary to a sequence of an mRNA prevented translation. They observed that an ASO prevented the accumulation of Rous sarcoma virus by inhibiting the translation of proteins encoded by the viral mRNA. A whole new potential field of therapeutics was launched with a single (understated) sentence: "It might also be possible to inhibit the translation of a specific cell protein" [1]. That RNase H was responsible for the inhibitor effects on translation was a conclusion reached by multiple investigators over a period of time. An elegant proof of the role of this specific enzyme in antisense activity was provided by Wu et al. in 1999 [10]; these authors showed that modulation of RNase H levels in cells or animals produces a coordinate change in antisense activity.

1.2.2 The RNase H Mechanism

Members of the RNase H family are ubiquitously expressed. The endonuclease mechanism of action and the crystal structure have been reviewed [10–15]. RNase H is approximately 20 kDa, and the isoforms in mammalian cells are known to have distinct functions. RNase H1 is necessary for transcription, and

RNase H2 is thought to remove RNA primers in the replication of DNA [16]. The RNA binding domain of these enzymes is located at the N-terminus. The catalytic activity is located in a C-terminal domain and depends upon the presence of the 2′ hydroxyl on the ribose sugar for cleavage. The specificity of the enzyme is imparted by heteroduplex formation between a DNA and the targeted RNA. Thus, the enzyme does not cleave single-stranded RNA in the absence of a heteroduplex nor does it cleave DNA in a double strand because of the absence of the critical 2′ OH.

Binding of RNase H to the heteroduplex results in hydrolysis of the RNA at a site distal to the binding region. The enzyme has a DNA recognition site into which a phosphate fits. This phosphate on the DNA strand is two base pairs distal to the cleavage site on the RNA. This DNA binding and recognition is a factor in the recognition of the heteroduplex. The heteroduplex landing site must contain at least five 2′ OH groups, and the position of RNA cleavage is approximately one helical turn from the binding domain [15]. The distance of the cleavage site from the RNA binding site is determined by a spacer domain between the binding domain and the catalytic domain [11–13]. The enzyme extends across a groove in the helix formed by heteroduplex to cut the RNA. Catalysis requires the presence of two metal ions (Mg^{2+} or Mn^{2+}), which activate the nucleophile and stabilize the transition state during hydrolysis of the phosphodiester backbone of the RNA substrate. One metal ion serves to stabilize the transition state, and the other acts during strand transfer [15, 17].

Over a decade after RNase H antisense drugs had been in clinical trials, the identity of the specific RNase family member responsible for the mRNA cleavage remained unproven. By modulating the expression of human RNase H1 and RNase H2, Wu et al. [10] demonstrated that RNase H1 was associated with antisense activity *in vivo*. Antisense drug activity increased with RNase H1 overexpression and decreased with RNase H1 inhibition. The same was not true for RNase H2, demonstrating that the form of the enzyme associated with therapeutic activity is RNase H1.

1.2.3 Chemical Modifications to Enhance RNase H-mediated Antisense Activity

RNase H is rather intolerant to chemical modifications to the DNA strand, and, as a result, ASO drugs that work through the RNase H mechanism must have a DNA-like character in certain nucleotides. One modification tolerated by RNase H is the phosphorothioate linkage: a substitution of a nonbridging sulfur for the phosphodiester linkage between nucleotides. First described by Eckstein and due to the increased stability of the phosphorothioate linkage compared with the native phosphodiester linkage, the phosphorothioate is the most used chemical modification in ASO and siRNA agents. The substitution with sulfur increases the nuclease stability (reviewed in Ref. [3]) and has the

added effect of increasing protein binding. This substitution also creates a chiral center at the phosphate. The increased nuclease resistance results from the fact that one of the two diastereomers is highly resistant to nuclease activity, probably as a result of the sulfur being in closer proximity to the metal ions of nucleases in the Sp configuration.

The phosphorothioate modification significantly alters the properties of an oligonucleotide compared with a native DNA oligonucleotide. Plasma half-lives are extended in the phosphorothioate-modified oligonucleotide due both to increased resistance to nucleases and to enhanced binding to plasma proteins. This later effect is both a blessing and a curse in that some of the acute toxicities of phosphorothioate oligonucleotides have been associated with binding to plasma proteins [18]. Ironically, whereas the phosphorothioate linkage is tolerated by RNase H, high concentrations of a phosphorothioate oligonucleotide are inhibitory to the enzyme's activity [19, 20]. Thus phosphorothioate linkages must be used strategically to balance in delivery with toxicity to the organism and to the very enzyme that is responsible for the pharmacologic activity. A large number of chemical modifications to oligonucleotides have been tested with the goals of increasing potency to lower toxicity and reduce the potential for RNase H inhibition.

Because of the intolerance of the RNase mechanism for chemical modifications, a scheme was developed that ensured that the ASO retained a DNA-like character. In the so-called chimeric design [21], the central region has nucleotides with DNA-like character (usually natural bases and sugars and a phosphorothioate backbone), and the flanking regions are modified with the aim of increasing affinity to the mRNA target and enhancing nuclease resistance. This modification pattern is also dubbed the gapmer design for the deoxy characteristic of the region between the modified termini (the gap). The size of the region required for RNase recognition and binding must be at least five nucleotides [22]. The minimal binding site size may be larger depending on the nature of the modifications flanking these deoxynucleotides. Crooke and his group have demonstrated that the nature of the 2′ sugar modifications (e.g. 2′methoxy ethyl) influences RNase H activity by changing the conformation of the oligonucleotide–mRNA heteroduplex. The conformational change in a heteroduplex is transmitted for some distance from the 2′ modification. A typical gapmer design has approximately 8–12 central DNA-like residues. One of the factors that hamper the activity of phosphorothioate oligonucleotides that have been internalized by cells is protein binding and sequestration of the antisense molecule away from its target protein RNase H. Recent studies have begun the task of identifying these proteins, which is the first step to being able to exploit them for improving therapeutics [23].

Recognition and binding of the antisense drug to the RNA target are of course critical for the activity of antisense therapeutics. A host of different chemical modifications have been tested over the years with the goal of

increasing binding affinity (reviewed in Ref. [24]). Addition of steric bulk at the 2′ position has the effect of producing a northern-type sugar conformation. This conformation is inhibitory to RNase H but may allow for better hydrogen bonding, thus resulting in increases in affinity for the target RNA. Conformationally restricted nucleic acids, such as LNA, or bicyclic nucleic acids (BNAs) are extreme examples of conformational restriction that result in high affinity for a complementary RNA strand.

Wengel et al. [25] described a modification that has the opposite effect in that the sugar no longer cyclizes but is acyclic (or unlocked), which promotes flexibility. These unlocked forms can be useful when it is in the drug designer's interest to reduce the potential for binding to an RNA target. These acyclic nucleotides support RNase H cleavage [26]. The 2′ arabino fluoro nucleotides also support RNase H binding and cleavage and are thus a potential modification that can be used anywhere in an ASO increase affinity to target mRNA [27].

1.3 Oligonucleotides that Sterically Block Translation

Single-stranded ASOs may also act independently of RNase H to block translation or processing of pre-mRNA. Subsequent sections of this chapter will discuss oligonucleotides designed to alter splicing. There are also reports of steric blockers that are inhibited in cell-free translation systems and in cells; ASO modified to inhibit RNase H activity that hybridizes with the region that includes the AUG start codon very effectively block protein synthesis. More recently an alternative strategy for blocking mRNA function through the inhibition of polyadenylation was proposed by Gunderson [28]. By selecting an oligonucleotide that has homology to the U1 adapter small nuclear RNA and homology to the sequence in the 3′ terminus of the target mRNA, it is possible to get a duplex formed where polyadenylation should occur and subsequently block the polyadenylation step that is critical for mRNA function. Without polyadenylation the nascent mRNA is degraded.

1.4 Oligonucleotides that Act Through the RNAi Pathway

1.4.1 The RISC Pathway

Small interfering RNAs (siRNAs) and miRNAs are duplexes of 20–30 base pairs that regulate gene expression and control a diverse array of biological processes. These small RNAs exert their function through the formation of

ribonucleoprotein complexes called RISCs that are instrumental in target transcript regulation. Therapeutic modulation of target regulation by siRNAs and miRNAs has the potential to impact diverse disease indications including viral diseases, cardiovascular disease, fibrosis, and cancer. Understanding of the function and modulation of these small regulatory RNAs has progressed at a rapid pace, resulting in translation to therapeutic development in only 10 years since their initial characterization.

In 1993, the cloning of *lin-4* in *Caenorhabditis elegans* marked the discovery that a short (~22 nt) RNA could function as a regulatory molecule and regulate translation via an antisense RNA–RNA interaction [29]. Within a few years, it became clear that endogenously expressed miRNAs are abundant and evolutionarily conserved and play diverse roles in gene expression in species from worms to humans [29–32]. The discovery by Fire and Mello in 1998 that short double-stranded RNAs induce gene silencing in *C. elegans* [33] further revolutionized our understanding of gene regulation and the ability of RNAs to function as regulatory molecules. Shortly thereafter, short interfering RNAs (siRNAs) were shown to guide sequence-specific target silencing in plants [34], *Drosophila* [35, 36], and mammalian cells [37, 38] in a conserved process termed RNAi. The ability of miRNAs and siRNAs to trigger specific gene silencing generated significant excitement of these small RNAs as a therapeutic modality, particularly for targets that are considered to be "undruggable" with small molecules.

miRNAs bind target mRNAs with partial sequence complementarity in the 3′ UTR, mostly involving residues 2–8 (the seed sequence) at the 5′ end of the guide strand [39]. Seed pairing has been shown to be both necessary and sufficient for target regulation by miRNAs in some contexts [40–43], although sequences in addition to the seed can also be important [44–47]. Because miRNAs do not require perfect complementarity for target recognition, a single miRNA can regulate expression from numerous mRNAs [48–50]. It is estimated that miRNAs as a class regulate the expression of 60% of genes in the human genome [51] to control differentiation, development, and physiology. Altered expression or function of miRNAs is linked to human diseases, giving rise to the idea that selective therapeutic modulation of miRNAs could alter the course of disease. The therapeutic inhibition of a miRNA or addition of a miRNA mimetic might produce a phenotype that is derived from a complex set of gene expression changes. The regulation of coordinated gene networks distinguishes miRNAs and their modulation as a therapeutic modality and provides a therapeutic advantage suggestive of combination therapy. Therapeutically, miRNA mimetics can be utilized to restore activity of miRNAs whose loss of function is linked to disease, whereas miRNA inhibitors (called antimiRs or antagomirs) can be used to block activity of miRNAs whose gain of function is linked to disease. A miRNA mimetic is a duplex oligonucleotide analogous to the mature miRNA. An antimiR is a single-stranded oligonucleotide that is complementary to the miRNA and is designed to act as a

steric block by binding with the miRNA to prevent it from interacting with target mRNA. Consequently, target transcripts are more highly expressed.

Both miRNAs and siRNAs are processed from double-stranded RNA precursors by the RNase III enzymes Drosha and Dicer to yield the mature, approximately 22-nt, double-stranded RNA [52–54]. Mature miRNAs and siRNAs catalyze gene regulation in complex with a ribonucleoprotein complex called the RISC. The catalytic component of RISC is a member of the Argonaute (Ago) family. Because small RNAs in RISC must anneal to their complementary target mRNAs, one strand, termed the guide strand, is retained in RISC and provides the sequence specificity to guide mRNA silencing. The other strand, termed the passenger strand, is cleaved. The process of strand selection is termed RISC loading. Strand selection is not random. Strand choice is partly encoded in the intrinsic structure of the small RNA duplex, with thermodynamic properties being a major determinant [55, 56]. Unwinding of the duplex, selection of the guide strand and cleavage of the passenger strand are facilitated by the Argonaute protein [57, 58] in an ATP-dependent process [59–63].

The most important domain of the guide strand is the seed sequence, which is the primary determinant of binding specificity for both siRNAs and miRNAs [39, 45, 49, 64–66]. Structural analysis of RNA associated with Argonaute provided insight into the role of the 5′ seed region of the guide strand in sequence-specific pairing with target mRNA [67–69]. The phosphorylated 5′ end of the guide RNA serves as the anchor and is buried within a highly conserved basic pocket in the Mid domain of Argonaute. In contrast, the seed region is exposed and displayed in a prehelical structure that favors the formation of a duplex with the target mRNA. Systematic mutation analysis of siRNA guide strands elucidated distinct siRNA guide domains within Argonaute [70]. Consistent with the structural analysis, mismatches between position 1 of the guide and the target RNA do not impair catalytic activity of Argonaute [66, 70], whereas mismatches within the seed regions reduce target binding and hinder target silencing [70, 71]. Mismatches at the center of the seed region (positions 4 and 5) are more detrimental than mismatches at the periphery (positions 2, 7, and 8), perhaps explaining how some small RNAs, including miRNAs, can regulate targets through imperfect seed matching [45, 72].

Of the four Argonaute proteins in mammals, only one, Ago2, has endonuclease activity [73]. Target cleavage occurs at the nucleotide opposite positions 10 and 11 of the siRNA guide strand, and mismatches or chemical modifications at these positions considerably decrease catalytic activity [37, 38, 74–76]. Pairing with the guide strand positions the scissile phosphate of the target near the catalytic residues in the PIWI domain of Ago2 [37, 66, 74, 77, 78]. siRNAs tend to be perfectly complementary to the target mRNA, and this pairing might enable Argonaute to achieve a catalytically competent conformation [66]. miRNAs typically lack significant pairing in the 3′ portion of the guide strand, although such supplemental base pairing can compensate for a weak seed region [79].

1.4.2 Mechanisms of RISC-mediated Gene Silencing

siRNAs and miRNAs guide RISC to target mRNAs in a sequence-dependent manner and subsequently affect one of three facets of mRNA metabolism: cleavage/destabilization, translation, or mRNA localization. In *Drosophila*, the ultimate fate of the target mRNA depends in part on the Argonaute protein and in part on the small RNA associated with RISC. There does not seem to be a strict small RNA sorting system in human RISC loading, perhaps because the four Ago proteins in humans have largely redundant functions.

siRNAs guide Ago2-containing RISC to complementary mRNA, whereupon the mRNA is degraded via endonucleolytic cleavage [80, 81]. The siRNA–RISC complex is subsequently released and able to bind and cleave another target mRNA molecule in a catalytic process. The power of RNAi arises from the discovery that the endogenous gene-silencing machinery can be conscripted by synthetic siRNAs for selective silencing of a gene of interest [38, 74]. In theory, siRNAs can be designed to silence any gene of interest based solely on the sequence of the target mRNA. Efficacy and potency of target silencing can be enhanced by leveraging thermodynamics, 5′ nucleotide identity, and structure to bias for guide strand selection [55, 82]. Well-designed siRNAs can achieve 95% silencing of the intended target.

Early reports suggested that siRNAs were absolutely specific for the target gene of interest. Target genes were silenced by complementary siRNAs but not unrelated siRNAs [83, 84], and silencing was abolished by single-nucleotide mismatches at the cleavage site of the siRNAs [74, 77, 78]. Subsequently, unbiased genome-scale expression profiling has revealed off-target activity of siRNAs [85]. Off-target silencing is mediated by limited target complementarity to the siRNA, primarily in the seed region [71], reminiscent of miRNA-based target repression. Sequence analysis of off-target transcripts revealed that the 3′ UTRs of these transcripts were complementary to the 5′ end of the siRNA guide strand containing the seed region [85]. Therefore, in addition to the intended, fully complementary target transcript, siRNAs can hybridize to and regulate the expression of transcripts with only partial sequence complementary to the siRNA. Interestingly, base mismatches in the 5′ end of a siRNA guide strand reduced silencing of the original set of off-target transcripts, but introduced a new set of off-target transcripts with complementarity to the mismatched guide strand [71]. This highlights the role of the seed sequence in nucleating RISC on complementary transcripts. As few as 10 nucleotides of sequence complementarity (including eight nucleotides in the seed region) are sufficient to trigger silencing of off-target transcripts [85].

Due to the limited sequence complementarity required for off-target silencing, off-target effects cannot be easily eliminated by siRNA sequence selection, but they can be mitigated by position-specific chemical modification [85]. A single 2′-*O*-methyl modification of position 2 of the seed region reduces the majority of off-target silencing while retaining silencing of the fully

complementary target [71]. Modification of the siRNA seed with DNA at positions 1–8 reduces silencing of some off-target transcripts [86]. Modification of specific positions in the seed region with unlocked nucleobase analogs (UNAs), particularly at position 7, results in silencing of the intended target but not other tested mRNAs [87, 88]. Another approach to improving the specificity of target silencing is siRNA pooling. Combining multiple siRNAs to a single target mRNA can reduce the contribution of each individual siRNA to off-target regulation [89, 90]. This approach has demonstrated considerable improvement in RNAi specificity *in vitro*; however, the feasibility of this strategy for development of siRNA therapeutics is unclear.

miRNAs control posttranscriptional gene expression by inhibiting translation and/or initiating mRNA decay. miRNA-based target repression is distinct from siRNA-mediated target silencing in that miRNAs affect mRNA targets without the need for ribonuclease activity and miRNA-mediated repression is generally cleavage independent. miRNAs regulate gene expression by base pairing to partially complementary sequences in the 3′ UTRs of target mRNAs [91–93]. miRNAs interact with their targets through limited base-pairing interactions that mainly contain the seed but that are insufficient to place the target in the active site of Ago2 where cleavage can occur. miRNA-associated RISC, termed miRISC, contains one of the four Argonaute proteins and a glycine–tryptophan repeat-containing protein of 182 kDa (GW182). GW182 is essential for target silencing by miRNAs; it interacts directly with AGO proteins and serves as a molecular platform for binding of silencing effectors [94–99]. miRISC inhibits translation initiation by interfering with cap recognition or by interfering with ribosomal complex formation and might inhibit translation at post-initiation steps by inhibiting ribosome elongation. miRISC can promote mRNA decay by interacting with deadenylase complexes (CCR4-NOT and PAN2-PAN3) to facilitate deadenylation, which is followed by decapping and exonuclease decay of the mRNA ([100] and references therein).

The relative contributions of translational inhibition and mRNA decay to miRNA-mediated target repression remain unclear and might be influenced by the miRNA and the biological context. Some studies reported inhibition of translation in the absence of mRNA destabilization [29, 101], whereas others found significant correlations between mRNA and protein levels of miRNA targets in global analyses [48, 102, 103]. Data from several studies now demonstrate that miRNAs can function in a two-step mode of repression in which translation inhibition results in subsequent destabilization of the targeted mRNAs [104, 105]. However, it remains to be determined how these mechanisms contribute to target repression in different biological systems.

miRNAs impact a given phenotype through regulation of a single key target [106] or through coordinated regulation of a subset of targets [107–109]. miRNAs regulate each individual target mRNA only modestly (~30–50%), yet this degree of silencing is sufficient to induce phenotypic changes. Because a single

miRNA can regulate hundreds of targets, it is not always clear which (or which combination) of the potential targets drive the biologic change of interest. Computer algorithms have been developed in an attempt to identify target mRNAs, but these algorithms predict only approximately 50% of regulated targets identified by global expression analysis [48]. Different prediction algorithms incorporate or emphasize different aspects of miRNA-target interactions (evolutionary conservation, target accessibility, sequence context), resulting in disparate sets of predicted targets. Further complicating prediction of miRNA targets and understanding of miRNA mechanism of action, a given miRNA might regulate different targets in different biological contexts [110]. For this reason, identification of target mRNAs and molecular mechanism of action is best measured using global mRNA expression methods in the biological setting of interest.

A unique feature of target regulation by miRNAs that is a consideration for therapeutic development of miRNA modulators is the potential for species-specific targeting. miRNAs are highly conserved across species, but the transcripts targeted by miRNAs are likely less conserved. The majority of functional binding sites for miRNAs reside in 3′ UTRs, which can be evolutionarily divergent [111]. As a result, the transcripts targeted by a miRNA and the resulting phenotypic consequences of miRNA modulation have the potential to differ across species. This has obvious consequences for the selection of appropriate preclinical models for drug development. However, in the best characterized example to date, inhibition of miR-122 has produced remarkably similar phenotypic changes in species from mouse to man [112–115]. As more miRNA-targeting drugs enter clinical trials, it will be instructive to compile cross-species comparisons and establish the factors that influence cross-species versus species-specific responses.

1.5 Chemical Modification of siRNAs and miRNAs

In order to realize the full potential of siRNA and miRNA therapeutics, strategies must be developed to overcome the challenges with RNA stability, specificity, immune modulation, and delivery. Chemical modifications to siRNAs, antimiRs, and miRNA mimetics can improve pharmacokinetic (PK) and pharmacodynamic (PD) properties and reduce immunogenicity. In general, the entire passenger strand as well as the 3′ proximal part of the guide strand is tolerant to chemical modification. The phosphorothioate modification provides resistance to nucleolytic degradation and increases affinity for plasma proteins [116–120]. Moderate modification of siRNAs with phosphorothioate linkages can support efficient RNAi, but tolerability is position dependent [77, 121–124]. For example, phosphorothioate linkages can reduce activity when located near the Ago2 cleavage site [121, 124].

Chemical modifications to the 2′ position of ribose are widely used to increase binding affinity, improve nuclease stability, and enhance specificity of siRNAs ([71, 124–127] and references therein) and have been incorporated to improve target affinity and activity of antimiRs ([128] and references therein). The ribose 2′-OH of siRNAs can be substituted with chemical groups, or the 2′ oxygen can be locked to the 4′-carbon in bridged nucleic acids such as LNAs. Electronegative modifications such as 2′-fluoro, 2′-*O*-methyl, and DNA (2′-H) enhance stability of the duplex between guide strand and target and enhance nuclease resistance. siRNAs containing alternating modifications of 2′-F and 2′-*O*-Me [129] or DNA [130] retain potency with nuclease resistance. Bulkier 2′-modifications, such as 2′-MOE and 2′-*O*-allyl, presumably distort the RNA helix structure necessary for Ago2 cleavage and therefore are only tolerated at certain positions in the siRNA [77, 88, 131]. The LNA modification provides enhanced thermostability, increases nuclease resistance *in vitro* [122] and *in vivo* [132, 133], and reduces immune modulation by siRNA duplexes [134].

Modifications based on sugar moieties other than ribose can also enhance hybridization affinity and/or specificity. Modifications including altritol nucleic acid (ANA), hexitol nucleic acid (HNA), 2′-deoxy-2′-fluoroarabinonucleic acid (2′-F-ANA), cyclohexenyl nucleic acid (CeNA), and unlocked nucleic acid (UNA) have been shown to support siRNA activity [87, 88, 124, 127, 135–137]. Ribose substitutions such as 2′-F-ANA can be combined with 2′-OH modifications such as 2′F or LNA to provide superior properties to siRNAs [138]. UNA, lacking the C2′—C3′ bond of the ribose ring, causes local destabilization of the siRNA duplex as well as interaction of the guide strand with the target mRNA. Therefore, modification with UNA must be limited to two to three nucleotides within the duplex. Modest UNA modification can enhance *in vivo* stability and function of siRNA when combined with other duplex stabilizing modifications such as LNA [139].

Duplex RNAs, including siRNAs and miRNA mimetics, modulate the immune response via pattern recognition receptors of the innate immune system, primarily the toll-like receptors (TLRs) 3, 7, and 8 [134, 140–142]. TLR3 is expressed on the cell surface and in endosomes of dendritic cells, epithelial cells, and endothelial linings and recognizes double-stranded RNA [141, 143–146]. TLR7 and TLR8 are found exclusively in endosomes of immune cells and recognize specific sequences in single-stranded RNA that can be exposed from RNA duplexes via random thermal fluctuations [147–149]. Activation of endosomal TLR7/8 is considered to be the major source of *in vivo* immunogenicity induced by siRNA [134, 142, 150–152].

Nucleobase and ribose modifications can reduce immunostimulation of siRNAs and miRNA mimetics [122, 153–157]. Nucleobase modification may reduce immunostimulation by siRNA and miRNA mimetics by preventing interaction with TLR and PKR receptors [156, 158]. Activation of PKR, a cytoplasmic sensor of double-stranded RNA, is reduced or abrogated by incorporation

of purine N2-benzyl, 2′-deoxyuridine [159], 4-thiouridine, and 2-thiouridine. In contrast, the 2′F modification does not reduce PKR activation [160]. Modification of specific immunogenic sequences in siRNAs with small 2′-OH substitutions such as 2′-F, 2′-H, and 2′-O-Me abrogates interaction with TLR7/8 [161]. Uridine residues or U-rich regions are typically the focus of these 2′-ribose modifications, as uridine residues are critical for siRNA activation of TLR7/8 [134, 147, 157, 162]. Guanidine and adenosine modification have also been reported to reduce immunogenicity of siRNAs, whereas cytidine modifications have no effect [134, 153–155, 157]. Base modifications, including 5-methylcytidine (m5C), 5-methyluracil (m5U), N6-mehyladenosine (m6A), and pseudouridine (s2U), have been shown to reduce TLR7/8 activation [153] but are not commonly used due to the success of modifications such as 2′-O-Me that reduces immunogenicity and are compatible with siRNA activity [163, 164]. Immunogenicity has also been suggested to correlate negatively with the strength of hybridization between the siRNA strands. Therefore, modifications such as LNA can reduce exposure of immunostimulatory single-stranded RNA [134, 165]. Although nucleobase and sugar modifications can increase binding affinity, potency, and specificity if placed appropriately within the oligonucleotide, not all modifications are compatible with activity ([127, 161, 166] and references therein). Therefore, therapeutic siRNAs and miRNA modulators require optimization for binding affinity, nuclease stability, and avoidance of immune stimulation.

1.5.1 Delivery of Therapeutic siRNAs or miRNAs

Delivery of oligonucleotide-based therapeutics requires crossing multiple barriers, including serum instability; renal clearance; passage through the blood vessel wall, interstitium, and extracellular matrix; crossing the membrane of the target cell; and escape from the endosome. Systemic delivery is particularly challenging for duplex RNAs such as siRNA and miRNA mimetics because duplex RNA does not readily across the cell membrane and therefore relies heavily on delivery vehicles for cellular uptake. Liposomes containing cationic or neutral lipids are currently the dominant delivery technology. Lipid nanoparticles readily distribute to the liver, and other organs can be targeted by conjugating cell-specific ligands to the nanoparticle. Other delivery vehicles being developed for duplex RNAs include polymeric nanoparticles, metallic core nanoparticles, lipidoids, dendrimers, and polymeric micelles (reviewed in Refs. [167, 168]).

An additional delivery strategy employed for duplex RNAs is conjugation of cholesterol or a ligand (antibody, aptamer, small molecule, or peptide) directly to the oligonucleotide. Initial studies utilized cholesterol conjugated to the passenger strand of a siRNA and demonstrated knockdown of the endogenous target gene, *ApoB*, after systemic delivery *in vivo* [169, 170]. Conjugate

chemistries such as cholesterol can improve cellular uptake of duplex RNAs, but the relatively high concentration required for efficacy has hindered their clinical development. Alnylam and Ionis are now using GalNAc conjugated oligonucleotides in multiple clinical trials in multiple indications. Alnylam has entered clinical trials for TTR-associated amyloidosis with a transthyretin-targeting siRNA that is conjugated with *N*-acetylgalactosamine (GalNac) for targeted delivery to hepatocytes after systemic subcutaneous administration (www.clinicaltrials.gov). Delivery vehicles and conjugates not only can improve cellular uptake of duplex RNAs but also have the potential to trigger immune modulation or nonspecific effects [168, 171–173]. Therefore, delivery agents as well as therapeutic oligonucleotides must be selected and evaluated carefully for safety.

AntimiRs are typically delivered in saline and rely on chemical modifications including phosphorothioate backbones for enhanced uptake. Many peripheral tissues can be effectively targeted by systemically delivered chemically modified antimiRs. These single-stranded oligonucleotides show good PK properties along with serum and tissue stability *in vivo*. Systemic inhibition of miRNA function in mammals was first demonstrated with a cholesterol-conjugated, 2′-*O*-Me-modified oligonucleotide targeting miR-122 that produced derepression of miR-122 seed-matched transcripts in the liver [174]. Subsequently, several studies demonstrated efficient and long-lasting inhibition of miRNAs *in vivo* using unconjugated, phosphorothioated antimiRs with 2′ ribose modifications in species from mouse to human [112, 114, 115, 175–181]. Delivery strategies being developed for siRNAs can also be applied for targeted delivery of antimiRs. Results from the first phase II study of the effect of miRNA inhibition on HCV infection indicate that miRNA antagonists are well tolerated and provide long-lasting efficacy.

Local administration can avoid some of the challenges associated with systemic delivery by delivering high concentration of oligonucleotide in the direct vicinity of the target cells. Local administration reduces the overall dose of oligonucleotide needed for efficacy and limits toxicity that might accompany systemic exposure. Local delivery of siRNA and miRNA modulators in preclinical and clinical settings has been reported for the lung, vaginal epithelium, brain, eye, and skin ([167, 168, 171–173, 182]. Local delivery continues to be an area of intensive research for both formulated and unformulated oligonucleotides.

Exosome-mediated transfer of miRNAs has recently been identified as a novel mechanism of genetic exchange between cells [183]. Exosomes are small membrane vesicles of endocytic origin that are released into the extracellular environment when multivesicular bodies fuse with the plasma membrane [184]. miRNAs are found in multivesicular bodies, suggesting that these might be sites of miRISC accumulation and function. Furthermore, miRNAs have been found in secreted exosomes that derive from multivesicular bodies [185]. After fusion with the plasma membrane of the recipient cell, exosomes transfer their cargo to the recipient cell (for review see Ref. [186]). Exosomes may

interact with recipient cells in a cell type-specific manner [183]. miRNA-loaded exosomes from T cells display antigen-driven, unidirectional transfer to antigen-presenting cells during immune synapse formation and modulate gene expression in recipient cells [187]. The miRNAs of the chromosome 19 miRNA cluster from placenta trophoblast-derived exosomes are transferred to recipient cells where they attenuate viral replication via autophagy [188]. In another example, exosomes from mesenchymal stem cells mediate the transfer of miR-133b to astrocytes and neurons, whereupon miR-133b regulates gene expression for neurite remodeling and functional recovery after stroke in rats [189]. Intercellular communication by exosome-derived miRNAs influences cancer progression via transfer of cancer-promoting contents within the tumor microenvironment or into the circulation to act at distant sites ([190] and references therein). Tumor cells of various cancer types secrete exosomes containing tumor-associated signaling molecules, including miRNAs, to modify angiogenesis, immune response, epigenetic reprogramming, migration, and invasion.

Exosomes consequently offer a novel strategy for delivery of cargo, including small RNAs, for targeted therapy. Alvarez-Erviti et al. were the first to utilize exosomes as a delivery vehicle for siRNA [191]. Targeted exosomes were produced by engineering dendritic cells to express a brain-targeting peptide fused to an exosomal membrane protein. Purified exosomes were loaded with siRNA via electroporation, and the loaded exosomes were delivered to mice via intravenous injection. siRNA was delivered specifically to neurons, microglia, and oligodendrocytes in the brain and produced silencing of the target mRNA *BACE1*. Subsequently, exosomal delivery and transfer of therapeutic miRNAs and siRNAs has been demonstrated in mouse hepatocytes [192], human monocytes and lymphocytes [193], and breast cancer cells [194]. Although much remains to be elucidated regarding purification, loading, cellular uptake, immune response, and toxicity of exosomes, these initial studies highlight the potential of these nanovesicles to deliver endogenous or exogenous small RNAs for therapeutic benefit.

1.6 Clinical Use of Oligonucleotides that Act through the RNAi Pathway

Small RNA-based therapeutics of each of the classes discussed here have entered clinical trials for a diverse array of indications and are demonstrating therapeutic benefit (see Table 1.1). These studies include siRNAs delivered via several different strategies as well as the first miRNA antagonist, antimiR-122 (miravirsen) to treat HCV infection, and the first miRNA mimetic, miR-34, being tested in hepatocellular carcinoma patients. The rapid translation of