Antisense Drug Technology

Principles, Strategies, and Applications

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Preface

For nearly two decades advances in molecular biology have resulted in extraordinary insights into normal and pathophysiological processes. Indeed we are in the midst of a process that will redefine diseases in molecular pathological terms. This process has already shown the central role that a relatively few complex multigene families play in physiological processes and pathophysiological events. In the next few years the pace at which new molecular targets will be identified will increase exponentially as the sequencing of the human and other genomes proceeds.

As exciting as the progress in the elucidation of molecular physiological processes is, identification of potential molecular targets is only the very first step in the discovery and development of improved therapeutic modalities for the vast array of diseases that remain undertreated. In fact, the identification of multigene families, the homologies between individual members of multigene families, and the likely roles of individual isotypes in disease states raise an important question: How will we create drugs of sufficient specificity to selectively interact with individual isotypes of multigene families?

That antisense technology may have the best potential for the rational design of isotype-selective inhibitors was obvious from its conception and led to the excitement about the technology, both as a tool to dissect the roles of various genes and to create new therapeutics. The question about antisense was: Would it work?

The chapters in this volume, recent publications, and recent symposia, such as the meeting sponsored by *Nature Biotechnology*, provide compelling answers to the questions about the technology. In the aggregate, the data provide ample justification for cautious optimism. It is clearly a remarkably valuable tool to dissect pharmacological processes and confirm the roles of various genes. Perhaps more importantly, even the first generation compounds—the phosphorothioates—may have sufficient properties to be of use as drugs for selected indications, and new generations of antisense drugs may broaden the therapeutic utility of drugs based on antisense technology.

Nevertheless, it is important to remember that we are less than a decade into the aggressive creation and evaluation of antisense technology. And we are attempting to create an entirely new branch of pharmacology: new chemical class, oligonucleotides; a new receptor, RNA; a new drug–receptor binding motif, hybridization; and new postreceptor binding mechanisms. Thus there are still many more questions than answers. Arguably, then, we are at the end of the beginning of this technology. There is a great deal more to do before we understand the true value and limits of antisense, but we are buoyed by the progress to date and look forward to the challenges ahead.

Stanley T. Crooke

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Basic Principles of Antisense Technology

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I. INTRODUCTION

Antisense technology continues to evolve rapidly. New insights into the technology, further appreciation of the characteristics of antisense drugs, and new applications of antisense technology have continued to develop.

The purposes of this chapter are to lay the foundation necessary to place the progress and issues in an appropriate pharmacological context and to summarize recent progress.

II. HISTORY

A. Polynucleotides

Prior to the evolution of effective transfection methods and an understanding of molecular biological techniques, DNA and RNA were administered as potential therapeutic agents. For example, DNA from several sources displayed antitumor activity and the activity was reported to vary as a function of size, base composition, and secondary structure (1–3). However, the molecular mechanisms by which DNA might induce antitumor effects were never defined and numerous other studies failed to demonstrate antitumor activities with DNA (4).

In contrast to studies on DNA as a therapeutic agent, substantially more work has been reported on RNA and polyribonucleotides. Much of the effort focused on the ability of polynucleotides to induce interferon (5) and the most thoroughly studied polynucleotide in this regard is double-stranded polyriboinos-

ine: polyribonocytidine (Poly rI: poly rC) (6). Poly rI: poly rC was shown to have potent antiviral and antitumor activities in vitro and in vivo and these activities were generally correlated with interferon induction (6).

Although poly rI: poly rC was shown to have antiviral and antitumor activities in animals, the compound produced substantial toxicities in animals and humans that limited its utility. For example, in mice, anti-RNA antibodies associated with glomerular nephritis and CNS toxicities were prominent. In rabbits, fever was dose limiting (for review, see Ref. 7). In rats, poly rI: poly rC was lethal at low doses and in mammals, including humans, poly rI: poly rC was immunotoxic resulting in fever, hypotension, antibody production, and T-cell activation. Pain, platelet depletion, and convulsions were also reported (7–15).

Methods to stabilize, enhance cellular uptake, and alter the in vivo pharmacokinetic properties of poly rI: poly rC were extensively studied. Polycationic substances such as DEAE-Dextran (diethylyaminoethyl Dextran), poly L-lysine, and histones were shown to complex with poly rI:poly rC and other polynucleotides and alter all of these properties (7). Poly rI: poly rC complexed with polylysine in the presence of carboxymethyl cellulose was studied in patients with cancer and found to be very toxic (12,13).

In short, poly rI: poly rC and other polynucleotides designed to induce interferon failed to demonstrate substantial antiviral or anticancer activities at doses that did not produce unacceptable toxicities. The mechanisms resulting in the toxicities are still not clearly understood. Complex formation with polycations altered pharmacokinetic properties, but did not enhance efficacy and probably exacerbated toxicities.

A second polynucleotide that has been studied extensively is ampligen, a mismatched poly rI:poly $rC_{12}U$ resulting in mispairing of the duplex (16). This compound has been shown to induce interferon and to activate 2′-5′ adenosine synthetase (17). Ampligen has similar properties to poly rI: poly rC, but has been reported to have broader activities and lower toxicities and is still in development.

Although the initial efforts that focused on polynucleotides that stimulate a variety of immunological events are not directly applicable to the more recent focus on specific effects of oligonucleotides, they have provided a base of toxicological information on the effects of polyanionic compounds and guidance in design of toxicological studies.

B. The Antisense Concept

Clearly the antisense concept derives from an understanding of nucleic acid structure and function and depends on Watson-Crick hybridization (18). Thus, arguably, the demonstration that nucleic acid hybridization is feasible (19) and the advances in in situ hybridization and diagnostic probe technology (20) lay the most basic elements of the foundation supporting the antisense concept.

However, the first clear enunciation of the concept of exploiting antisense oligonucleotides as therapeutic agents was in the work of Zamecnik and Stephenson in 1978. In this publication, these authors reported the synthesis of an oligodeoxyribonucleotide 13 nucleotides long that was complementary to a sequence in the respiratory syncytial virus genome. They suggested that this oligonucleotide could be stabilized by 3′- and 5′- terminal modifications and showed evidence of antiviral activity. More importantly, they discussed possible sites for binding in RNA and mechanisms of action of oligonucleotides. (For review see 128.)

Though less precisely focused on the therapeutic potential of antisense oligonucleotides, the work of Miller and Ts'o and their collaborators during the same period helped establish the foundation for antisense research and reestablish an interest in phosphate backbone modifications as approaches to improve the properties of oligonucleotides (21,22). Their focus on methyl phosphotriester–modified oligonucleotides as a potential medicinal chemical solution to pharmacokinetic limitations of oligonucleotides presages a good bit of the medicinal chemistry to be performed on oligonucleotides.

Despite the observations of Miller and Ts'o and Zamecnik and colleagues, interest in antisense research was quite limited until the late 1980s when advances in several areas provided technical solutions to a number of impediments. As antisense drug design requires an understanding of the sequence of the RNA target, the explosive growth in availability of viral and human genomic sequences provided the information form which ''receptor sequences'' could be selected. The development of methods for synthesis of research quantities of oligonucleotide drugs then supported antisense experiments on both phosphodiester and modified oligonucleotides (23,24). The inception of the third key component (medicinal chemistry) forming the foundation of oligonucleotide therapeutics, in fact, is the synthesis in 1969 of phosphorothioate poly rI; poly rC as a means of stabilizing the polynucleotide (25). Subsequently, Miller and Ts'o initiated studies on the neutral phosphate analogs, methylphosphonates (21), and groups at the National Institutes of Health and the Food and Drug Administration and the Worcester Foundation investigated phosphorothioate oligonucleotides (26–29). With these advances forming the foundation for oligonucleotide therapeutics and the initial studies suggesting in vitro activities against a number of viral and mammalian targets (28,30,31 Heikkila, 1987 #374, 32), interest in oligonucleotide therapeutics intensified.

C. Strategies to Induce Transcriptional Arrest

An alternative to the inhibition of RNA metabolism via an antisense mechanism is to inhibit transcription by interacting with double-stranded DNA in chromatin. Of the two most obvious binding strategies for oligonucleotides binding to double-stranded nucleic acids, strand invasion and triple-strand formation, triple-stranding strategies, until recently, attracted essentially all of the attention.

Polynucleotides were reported to form triple helices as early as 1957. Triple strands can form via non–Watson-Crick hydrogen bonds between the third strand and purines involved in Watson-Crick hydrogen bonding with the complementary strand of the duplex (for review see Ref. 33). Thus, triple-stranded structures can be formed between a third strand comprised of pyrimidines or purines that interact with a homopurine strand in a homopurine-homopyrimidine strand in a duplex DNA. With the demonstration that homopyrimidine oligonucleotides could indeed form triplex structures (34–36), interest in triple-strand approaches to inhibit transcription heightened.

Although there was initially considerable debate about the value of triple-stranding strategies versus antisense approaches (37), there was little debate that much work remained to be done to design oligonucleotides that could form triple-stranded structures with duplexes of mixed sequences. Pursuit of several strategies has resulted in significant progress (for review, see Ref. 33). Considerable progress in creating chemical motifs capable of binding to duplex DNA with high affinity and specificities-supportive binding to sequences other than polypyrimidine polypurine traits has been reported. For example, peptide nucleic acid (PNA) has been shown to focus on triple-stranded structures in isolated DNA and in mouse cell chromosomal DNA (38). Modifications such as 7-deazaxanthine and 2′-aminoethoxy were reported to enhance triplex formation (39,40). Additionally, 5′-propionyl-modified nucleosides have been shown to enhance triplex formation (41). Finally, substantial advances in sequence-specific binding in the minor groove of DNA have been reported (42).

In addition to advances in the chemistry of triplex formation, a number of studies have shown results in cells consistent with triplex formation in chromosomal DNA (38,43–45). Consequently, it can be concluded that progress in DNA triplex strategies has been impressive. Nevertheless much more progress is required before the approach is validated and the breadth of therapeutic potential defined.

Strand invasion, an alternative approach to obstructing transcription by formation of triple strands, has been shown to be feasible if analogs with sufficient affinity can be synthesized. PNAs have been shown to have very high affinity and be capable of strand invasion of double-stranded DNA under some conditions (46). Again, much remains to be learned about this analog class and other high-affinity analogs before the pharmacological potential of transcriptional arrest via strand invasion is defined.

D. Ribozymes and Oligonucleotide-Mediated RNA Cleavage

Ribozymes are RNA molecules that catalyze biochemical reactions (47). Ribozymes cleave single-stranded regions of RNA via transesterification or hydrolysis

reactions that cleave a phosphodiester bond (48). Clearly, a therapeutic oligonucleotide that could specifically bind to and cleave an RNA target should be attractive. To achieve potential therapeutic utility of ribozymes, two approaches have been taken. First, ribozyme-coding sequences have been incorporated into plasmids and administered, in effect ribozyme gene therapy (for example, see Ref. 49). Second, efforts to identify the minimum ribozyme structure and to introduce chemical modifications that retain ribozyme activity while enhancing stability to nucleases have been pursued (for example, see Ref. 50).

Ribozyme gene therapy has been shown to be feasible in vitro and there are suggestions that a ribozyme construct may be more potent than an antisense-containing vector. For example, the modification of a vector encoding a ribozyme designed to bind to the 5′-transactivator region (TAR) of human immunodeficiency virus (HIV) resulted in approximately 70% inhibition of TAR-dependent expression of a reporter gene while an antisense construct resulted in only 40–50% inhibition (51). More recently, a plasmid encoding a hammerhead ribozyme designed to cleave at the mRNA for a proto-oncogene was reported to reduce the mRNA of the proto-oncogene and growth of the cells (49). Thus, one ribozyme gene targeted to HIV has been reported to be administered, using a retroviral vector, to stem.

Cells removed from five HIV-positive patients and the stem cells were then returned to the patients. However, no data have yet been published concerning this study.

Substantial progress has also been reported with regard to the synthesis and testing of nuclease-resistant ribozyme drugs. Modifications including phosphorothioates and nucleoside analogs have been demonstrated to be incorporable in many sites in hammerhead ribozymes, to increase nuclease resistance and support retained ribozyme activity (50,52,53). In fact, modified relativity nuclease-resistant ribozymes were reported to decrease the target, stromelysin, mRNA levels in knee joints of rabbits after intra-articular injection (54). Further, the pharmacokinetics of a relativity nuclease-stable hammerhead ribozyme were determined after IV, SC, or IP administration to mice. The ribozyme was well absorbed after IP or SC dosing, distributed to liver, kidney, bone marrow, and other tissues, and displayed an elimination half-life of 33 min (55). This ribozyme designed to inhibit vascular endothelial growth factor (VEGF) receptor synthesis has been reported to be in clinical trials. Again, however, no data have been reported as yet.

Thus, substantial progress has been reported with regard to both ribozyme gene therapy and synthetic ribozyme drug therapy. Nevertheless, substantial hurdles remain before sufficient data derived from animal and human studies with multiple ribozymes confirm that the approach is validated. Clearly, for ribozyme gene therapy to be broadly applicable, the challenges gene therapy faces must be met and ribozymes must be shown to be of more value than simply expressing

antisense genes. To validate synthetic ribozymes, data in animals and humans for numerous ribozymes with careful evaluation of mechanism of action must be generated. Clearly, the 30-min elimination half-life must be lengthened with new modifications and again the value of a ribozyme versus a much simpler antisense approach must be defined.

E. Combinatorial Approaches to Oligonucleotide Therapeutics

At least two methods by which oligonucleotides can be created combinatorially have been published (56–58). The potential advantage of a combinatorial approach is that oligonucleotide-based molecules can be prepared to adopt various structures that support binding to non–nucleic acid targets as well as nucleic acid targets. These can then be screened for potential activities without knowledge about the cause of the disease or the structure of the target.

F. The Medicinal Chemistry of Oligonucleotides

Because it was apparent almost immediately that native phosphodiester oligodeoxy- or ribonucleotides are unsatisfactory as drugs because of rapid degradation (59), a variety of modifications were rapidly tested. As previously mentioned, perhaps the most interesting of the initial modifications were the phosphate analogs, the phosphorothioates (25), and the methylphosphonates (21). Both fully modified oligonucleotides and oligonucleotides "capped" at the 3′ and/or 5′ termini with phosphorothioate or methylphosphonate moieties were tested (60). However, studies from many laboratories demonstrated that capped oligonucleotides were relatively rapidly degraded in cells (61–63). Nor were point modifications with intercalators that enhanced binding to RNA (64,65), cholesterol (66), or poly L-lysine (67,68) sufficiently active or selective to warrant broad-based exploration.

Since the initial approaches to modifications of oligonucleotides, an enormous range of modifications, including novel bases, sugars, backbones, conjugates, and chimeric oligonucleotides, have been tested (for review see Ref. 69). Many of these modifications have proven to be quite useful and are progressing in testing leading to clinical trials.

III. PROOF OF MECHANISM

A. Factors that May Influence Experimental Interpretations

Clearly, the ultimate biological effect of an oligonucleotide will be influenced by the local concentration of the oligonucleotide at the target RNA, the concentra-

tion of the RNA, the rates of synthesis and degradation of the RNA, the type of terminating mechanism, and the rates of the events that result in termination of the RNA's activity. At present, we understand essentially nothing about the interplay of these factors.

1. Oligonucleotide Purity

Currently, phosphorothioate oligonucleotides can be prepared consistently and with excellent purity (70). However, this has only been the case for the past several years. Prior to that time, synthetic methods were evolving and analytical methods were inadequate. In fact, our laboratory reported that different synthetic and purification procedures resulted in oligonucleotides that varied in cellular toxicity (63) and that potency varied from batch to batch. Though there are no longer synthetic problems with phosphorothioates, they, undoubtedly, complicated earlier studies. More importantly, with each new analog class, new synthetic, purification, and analytical challenges are encountered.

2. Oligonucleotide Structure

Antisense oligonucleotides are designed to be single stranded. We now understand that certain sequences, e.g., stretches of guanosine residues, are prone to adopt more complex structures (71). The potential to form secondary and tertiary structures also varies as a function of the chemical class. For example, higher-affinity 2′-modified oligonucleotides have a greater tendency to self-hybridize resulting in more stable oligonucleotide duplexes than would be expected based on rules derived from oligodeoxynucleotides (Freier, unpublished results).

3. RNA Structure

RNA is structured. The structure of the RNA has a profound influence on the affinity of the oligonucleotide and on the rate of binding of the oligonucleotide to its RNA target (72,73). Moreover, RNA structure produces asymmetrical binding sites that then result in very divergent affinity constants depending on the position of the oligonucleotide in that structure (73–75). This in turn influences the optimal length of an oligonucleotide needed to achieve maximal affinity. We understand very little about how RNA structure and RNA protein interactions influence antisense drug action.

4. Variations in In Vitro Cellular Uptake and Distribution

Studies in several laboratories have clearly demonstrated that cells in tissue culture may take up phosphorothioate oligonucleotides via an active process, and that the uptake of these oligonucleotides is highly variable depending on many conditions (63,76). Cell type has a dramatic effect on total uptake, kinetics of

uptake, and pattern of subcellular distribution. At present, there is no unifying hypothesis to explain these differences. Tissue culture conditions, such as the type of medium, the degree of confluence, and the presence of serum, can all have enormous effects on uptake (76). Oligonucleotide chemical class obviously influences the characteristics of uptake as well as the mechanism of uptake. Within the phosphorothioate class of oligonucleotides, uptake varies as a function of length, but not linearly. Uptake varies as a function of sequence and stability in cells is also influenced by sequence (76,77).

Given the foregoing, it is obvious that conclusions about in vitro uptake must be very carefully made and generalizations are virtually impossible. Thus, before an oligonucleotide could be said to be inactive in vitro, it should be studied in several cell lines. Furthermore, while it may be absolutely correct that receptor-mediated endocytosis is a mechanism of uptake of phosphorothioate oligonucleotides (78), it is obvious that a generalization that all phosphorothioates are taken up by all cells in vitro primarily by receptor-mediated endocytosis is simply unwarranted.

Finally, extrapolations from in vitro uptake studies to predictions about in vivo pharmacokinetic behavior are entirely inappropriate and, in fact, there are now several lines of evidence in animals and humans that, even after careful consideration of all in vitro uptake data, one cannot predict in vivo pharmacokinetics of the compounds (76,79–81).

5. The Binding to and Effects of Binding to Non–Nucleic Acid Targets

Phosphorothioate oligonucleotides tend to bind to many proteins and those interactions are influenced by many factors. The effects of binding can influence cell uptake, distribution, metabolism, and excretion. They may induce nonantisense effects that can be mistakenly interpreted as antisense or complicate the identification of an antisense mechanism. By inhibiting RNase H, protein binding may inhibit the antisense activity of some oligonucleotides. Finally, binding to proteins can certainly have toxicological consequences.

In addition to proteins, oligonucleotides may interact with other biological molecules, such as lipids or carbohydrates, and such interactions, like those with proteins, will be influenced by the chemical class of oligonucleotide studied. Unfortunately, essentially no data bearing on such interactions are currently available.

An especially complicated experimental situation is encountered in many in vitro antiviral assays. In these assays, high concentrations of drugs, viruses, and cells are often coincubated. The sensitivity of each virus to nonantisense effects of oligonucleotides varies depending on the nature of the virion proteins and the characteristics of the oligonucleotides (82,83). This has resulted in con-

siderable confusion. In particular for HIV, herpes simplex viruses, cytomegaloviruses, and influenza virus, the nonantisense effects have been so dominant that identifying oligonucleotides that work via an antisense mechanism has been difficult. Given the artificial character of such assays, it is difficult to know whether nonantisense mechanisms would be as dominant in vivo or result in antiviral activity.

6. Terminating Mechanisms

It has been amply demonstrated that oligonucleotides may employ several terminating mechanisms. The dominant terminating mechanism is influenced by RNA receptor site, oligonucleotide chemical class, cell type, and probably many other factors (84). Obviously, as variations in terminating mechanism may result in significant changes in antisense potency and studies have shown significant variations from cell type to cell type in vitro, it is essential that the terminating mechanism be well understood. Unfortunately, at present, our understanding of terminating mechanisms remains rudimentary.

7. Effects of "Control Oligonucleotides"

A number of types of control oligonucleotides have been used including randomized oligonucleotides. Unfortunately, we know little to nothing about the potential biological effects of such ''controls'' and the more complicated a biological system and test the more likely that ''control'' oligonucleotides may have activities that complicate interpretations. Thus, when a control oligonucleotide displays a surprising activity, the mechanism of that activity should be explored carefully before concluding that the effects of the ''control oligonucleotide'' prove that the activity of the putative antisense oligonucleotide is not due to an antisense mechanism.

8. Kinetics of Effects

Many rate constants may affect the activities of antisense oligonucleotides, e.g., the rate of synthesis and degradation of the target RNA and its protein, the rates of uptake into cells, the rates of distribution, extrusion, and metabolism of an oligonucleotide in cells, and similar pharmacokinetic considerations in animals. Despite this, relatively few time courses have been reported and in vitro studies have been reported that range from a few hours to several days. In animals, we have a growing body of information on pharmacokinetics, but in most studies reported to date, the doses and schedules were chosen arbitrarily and, again, little information on duration of effect and onset of action has been presented.

Clearly, more careful kinetic studies are required and rational in vitro and in vivo dose schedules must be developed.

B. Recommendations

1. Positive Demonstration of Antisense Mechanism and Specificity

Until more is understood about how antisense drugs work, it is essential to positively demonstrate effects consistent with an antisense mechanism. For RNase H–activating oligonucleotides, northern blot analysis showing selective loss of the target RNA is the best choice and many laboratories are publishing reports in vitro and in vivo of such activities (85–88). Ideally, a demonstration that closely related isotypes are unaffected should be included. In brief, then, for proof of mechanism, the following steps are recommended.

1. Perform careful dose-response curves in vitro using several cell lines and methods of in vitro delivery.
2. Correlate the rank-order potency in vivo with that observed in vitro after thorough dose-response curves are generated in vivo.
3. Perform careful "gene walks" for all RNA species and oligonucleotide chemical classes.
4. Perform careful time courses before drawing conclusions about potency.
5. Directly demonstrate proposed mechanism of action by measuring the target RNA and/or protein.
6. Evaluate specificity and therapeutic indices via studies on closely related isotypes and with appropriate toxicological studies.
7. Use RNase H protection assays and transcriptional arrays to provide broader analyses of specificity where the assays have been validated.
8. Perform sufficient pharmacokinetics to define rational dosing schedules for pharmacological studies.
9. When control oligonucleotides display surprising activities, determine the mechanisms involved.

IV. MOLECULAR MECHANISMS OF ANTISENSE DRUGS

A. Occupancy-Only-Mediated Mechanisms

Classic competitive antagonists are thought to alter biological activities because they bind to receptors preventing natural agonists from binding the inducing normal biological processes. Binding of oligonucleotides to specific sequences may inhibit the interaction of the RNA with proteins, other nucleic acids, or other factors required for essential steps in the intermediary metabolism of the RNA or its utilization by the cell.

To create antisense inhibitors that clearly work through non-RNase H

mechanisms, the antisense agents must be modified sufficiently to not support RNase H cleavage. Fortunately, numerous analogs have been identified that do not support RNase H cleavage. These can be classified into modifications of sugar moiety, or the phosphate, or replacement of the sugar-phosphate backbone (89). Unfortunately, in a number of earlier publications, conclusions about mechanisms of action were drawn without using appropriately modified oligonucleotides.

1. Inhibition of 5′ Capping

Conceptually, inhibition of 5′ capping of mRNA could be an effective antisense mechanism. 5′ Capping is critical in stabilizing mRNA and in enabling the translation of mRNA (89). To date, however, no reports of antisense inhibitors of capping have appeared. This may be due to the inaccessibility of the 5′ end of mRNA prior to capping.

2. Inhibition of Splicing

A key step in the intermediary metabolism of most mRNA molecules is the excision of introns. These "splicing" reactions are sequence specific and require the concerted action of spliceosomes. Consequently, oligonucleotides that bind to sequences required for splicing may prevent binding of necessary factors or physically prevent the required cleavage reactions. This then would result in inhibition of the production of the mature mRNA. Although there are several examples of oligonucleotides directed to splice junctions, none of the studies present data showing inhibition of RNA processing, accumulation of splicing intermediates, or a reduction in mature mRNA. Nor are there published data in which the structure of the RNA at the splice junction was probed and the oligonucleotides demonstrated to hybridize to the sequences for which they were designed (31,90–92). Activities have been reported for anti-c-myc and antiviral oligonucleotides with phosphodiester, methylphosphonate, and phosphorothioate backbones.

Kole and colleagues (93–95) were the first to use modified oligonucleotides to inhibit splicing. They showed that 2′-oMe phosphorothioate oligonucleotides could correct aberrant beta-globin splicing in a cell-free system. Similar observations were in a cellular system (95).

In our laboratory, we have attempted to characterize the factors that determine whether splicing inhibition is effected by an antisense drug (96). To this end, a number of luciferase-reporter plasmids containing various introns were constructed and transfected into HeLa cells. Then the effects of antisense drugs designed to bind to various sites were characterized. The effects of RNase H-competent oligonucleotides were compared to those of oligonucleotides that do not serve as RNase H substrates. The major conclusions from this study were first that most of the earlier studies in which splicing inhibition was reported were probably due to nonspecific effects. Second, less effectively spliced introns

are better targets than those with strong consensus splicing signals. Third, the 3′-splice site and branchpoint are usually the best sites to which to target to the oligonucleotide to inhibit splicing. Fourth, RNase H–competent oligonucleotides are usually more potent than even higher-affinity oligonucleotides that inhibit by occupancy only.

To date, only a single study has demonstrated antisense-mediated redirection of splicing of an endogenous cellular mRNA (97). In this study, fully modified 2′-methoxyethyl (2′-MOE) oligonucleotides were designed to hybridize to sequences either within exon 9 or across various intron-exon junctions within IL-5 receptor-α pre-mRNA, and several were shown to redirect splicing of this pre-mRNA.

3. Translational Arrest

A mechanism for which many oligonucleotides have been designed is translational arrest by binding to the translation initiation codon. The positioning of the initiation codon within the area of complementarity of the oligonucleotide and the length of oligonucleotide used have varied considerably. Again, unfortunately, only in relatively few studies have the oligonucleotides, in fact, been shown to bind to the sites for which they were designed, and other data that support translation arrest as the mechanism have not been reported.

Target RNA species that have been reported to be inhibited include HIV (28), vesicular stomatitis virus (VSV) (67), n-myc (98), and a number of normal cellular genes (99–102). However, to demonstrate that RNase H is not involved in effects observed, it is necessary to use antisense drugs that do not form duplexes that are RNase H substrates, e.g., fully 2′ oligonucleotides.

Studies with peptide nucleic acid (PNA) analogs have shown that these analogs can inhibit translation in cell-free systems, but to date no data have been reported from cellular studies (103,104). For morpholono oligomers antisense activity has been reported in both cell-free and cellular assays (105,106). Numerous oligonucleotides with 2′-modifications have also been studied and have been shown to inhibit translation when targeted to 5′-UTR or the translation initiation codon (107). However, optimal inhibition is effected by binding at the 5′-cap in RNAs that have significant 5′-untranslated regions (108). In conclusion, translation arrest represents an important mechanism of action for antisense drugs. A number of examples purporting to employ this mechanism have been reported, and recent studies on several compounds have provided data that unambiguously demonstrate that this mechanism can result in potent antisense drugs.

4. Disruption of Necessary RNA Structure

RNA adopts a variety of three-dimensional structures induced by intramolecular hybridization, the most common of which is the stem loop. These structures play

crucial roles in a variety of functions. They are used to provide additional stability for RNA and as recognition motifs for a number of proteins, nucleic acids, and ribonucleoproteins that participate in the intermediary metabolism and activities of RNA species. Thus, given the potential general activity of the mechanism, it is surprising that occupancy-based disruption RNA has not been more extensively exploited.

As an example, we designed a series of oligonucleotides that bind to the important stem-loop present in all RNA species in HIV, the TAR element. We synthesized a number of oligonucleotides designed to disrupt TAR and showed that several indeed did bind to TAR, disrupt the structure, and inhibit TAR-mediated production of a reporter gene (58). Furthermore, general rules useful in disrupting stem-loop structures were developed as well (75).

Although designed to induce relatively nonspecific cytotoxic effects, two other examples are noteworthy. Oligonucleotides designed to bind to a 17 nucleotide loop in Xenopus 28 S RNA required for ribosome stability and protein synthesis inhibited protein synthesis when injected into *Xenopus* oocytes (109). Similarly, oligonucleotides designed to bind to highly conserved sequences in 5.8 S RNA inhibited protein synthesis in rabbit reticulocyte and wheat germ systems (110).

B. Occupancy-Activated Destabilization

RNA molecules regulate their own metabolism. A number of structural features of RNA are known to influence stability, various processing events, subcellular distribution, and transport. It is likely that, as RNA intermediary metabolism is better understood, many other regulatory features and mechanisms will be identified.

1. 5′-Capping

A key early step in RNA processing is 5′-capping (Fig. 1). This stabilizes pre-mRNA and is important for the stability of mature mRNA. It also is important in binding to the nuclear matrix and transport of mRNA out of the nucleus. As the structure of the cap is unique and understood, it presents an interesting target.

Several oligonucleotides that bind near the cap site have been shown to be active, presumably by inhibiting the binding of proteins required to cap the RNA. For example, the synthesis of SV40 T-antigen was reported to be most sensitive to an oligonucleotide linked to polylysine and targeted to the 5′-cap site of RNA (111). However, again, in no published study has this putative mechanism been rigorously demonstrated. In fact, in no published study have the oligonucleotides been shown to bind to the sequences for which they were designed.

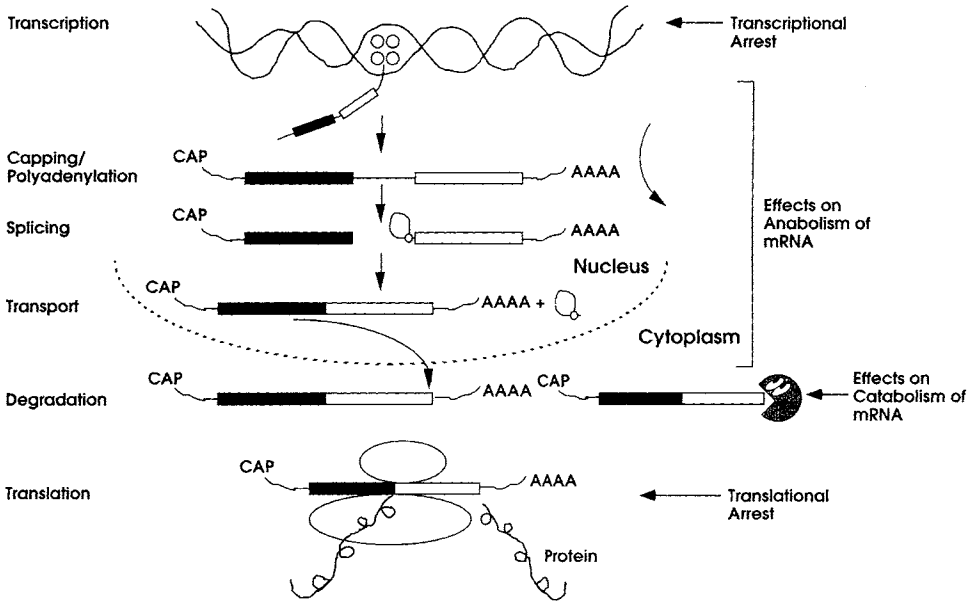

Figure 1 Pre mRNA is transcribed from a gene. It is then processed through a carefully choreographed set of steps to the mature mRNA which is exported to the cytoplasm. These steps include 5′ and 3′ modifications (5′ cap: 3′ poly A) splicing and specific transport activities. Numerous mechanisms of action for antisense drugs have been identified and they affect many steps post transcription and/or enhance cellular degradation of the RNA.

In our laboratory, we have designed oligonucleotides to bind to 5′-cap structures and reagents to specifically cleave the unique 5′-cap structure (112). These studies demonstrate that 5′-cap targeted oligonucleotides were capable of inhibiting the binding of the translation initiation factor eIF-4a (113).

2. Inhibition of 3′-Polyadenylation

In the 3′-untranslated region of pre-mRNA molecules are sequences that result in the posttranscriptional addition of long (hundreds of nucleotides) tracts of polyadenylate. Polyadenylation stabilizes mRNA and may play other roles in the intermediary metabolism of RNA species. Theoretically, interactions in the 3′-terminal region of pre-mRNA could inhibit polyadenylation and destabilize the RNA species. Although there are a number of oligonucleotides that interact in the 3′-untranslated region and display antisense activities, to date, no study has reported evidence for alterations in polyadenylation (85).

C. Other Mechanisms

In addition to 5′-capping and 3′-adenylation, there are clearly other sequences in the 5′- and 3′-untranslated regions of mRNA that affect the stability of the molecules. Again, there are a number of antisense drugs that may work by these mechanisms.

Zamecnik and Stephenson reported that 13 mer targeted to untranslated 3′- and 5′- terminal sequences in Rous sarcoma viruses was active (114). Oligonucleotides conjugated to an acridine derivative and targeted to a 3′-terminal sequence in type A influenza viruses were reported to be active. Against several RNA targets, studies in our laboratories have shown that sequences in the 3′-untranslated region of RNA molecules are often the most sensitive (115–117). For example, ISIS 1939, a 20-mer phosphorothioate that binds to and appears to disrupt a predicted stem-loop structure in the 3′-untranslated region of the mRNA for ICAM, is a potent antisense inhibitor. However, inasmuchas a 2′-methoxy analog of ISIS 1939 was much less active, it is likely that, in addition to destabilization to cellular nucleolytic activity, activation of RNase H (see below) is also involved in the activity of ISIS 1939 (85).

D. Activation of RNase H

RNase H is a ubiquitous enzyme that degrades the RNA strand of an RNA-DNA duplex. It has been identified in organisms as diverse as viruses and human cells (118). At least two classes of RNase H have been identified in eukaryotic cells. Multiple enzymes with RNase H activity have been observed in prokaryotes (118). Although RNase H is involved in DNA replication, it may play other roles in the cell and is found in the cytoplasm as well as the nucleus (119). However, the concentration of the enzyme in the nucleus is thought to be greater and some of the enzyme found in cytoplasmic preparations may be due to nuclear leakage.

The precise recognition elements for RNase H are not known. However, it has been shown that oligonucleotides with DNA-like properties as short as tetramers can activate RNase H (120). Changes in the sugar influence RNase H activation as sugar modifications that result in RNA-like oligonucleotides, e.g., 2′-fluoro or 2′-methoxy, do not appear to serve as substrates for RNase H (121,122). Alterations in the orientation of the sugar to the base can also affect RNase H activation as α-oligonucleotides are unable to induce RNase H or may require parallel annealing (123,124). Additionally, backbone modifications influence the ability of oligonucleotides to activate RNase H. Methylphosphonates do not activate RNase H (125,126). In contrast, phosphorothioates are excellent substrates (65,127,128). In addition, chimeric molecules have been studied as oligonucleotides that bind to RNA and activate RNase H (129,130). For example, oligonucleotides comprised of wings of 2′-methoxy phosphonates and a five-base

gap of deoxyoligonucleotides bind to their target RNA and activate RNase H (129,130). Furthermore, a single ribonucleotide in a sequence of deoxyribonucleotides was shown to be sufficient to serve as a substrate for RNase H when bound to its complementary deoxyoligonucleotide (131).

That it is possible to take advantage of chimeric oligonucleotides designed to activate RNase H and have greater affinity for their RNA receptors and to enhance specificity has also been demonstrated (132,133). In a recent study, RNase H–mediated cleavage of target transcript was much more selective when deoxyoligonucleotides comprised of methylphosphonate deoxyoligonucleotide wings and phosphodiester gaps were compared to full phosphodiester oligonucleotides (133).

Despite the information about RNase H and the demonstration that many oligonucleotides may activate RNase H in lysate and purified enzyme assays, relatively little is yet known about the role of structural features in RNA targets in activating RNase H (134–136). In fact, direct proof that RNase H activation is, in fact, the mechanism of action of oligonucleotides in cells has until very recently been lacking.

Recent studies in our laboratories provide additional, albeit indirect, insights into these questions. ISIS 1939 is a 20-mer phosphorothioate complementary to a sequence in the 3′-untranslated region of ICAM-1 RNA (85). It inhibits ICAM production in human umbilical vein endothelial cells and northern blots demonstrate that ICAM-1 mRNA is rapidly degraded. A 2′-methoxy analog of ISIS 1939 displays higher affinity for the RNA than the phosphorothioate, is stable in cells, but inhibits ICAM-1 protein production much less potently than ISIS 1939. It is likely that ISIS 1939 destabilizes the RNA and activates RNase H. In contrast, ISIS 1570, an 18-mer phosphorothioate that is complementary to the translation initiation codon of the ICAM-1 message, inhibited production of the protein but caused no degradation of the RNA. Thus, two oligonucleotides that are capable of activating RNase H had different effects depending on the site in the mRNA at which they bound (85). A more direct demonstration that RNase H is likely a key factor in the activity of many antisense oligonucleotides was provided by studies in which reverse-ligation PCR was used to identify cleavage products from bcr-abl mRNA in cells treated with phosphorothioate oligonucleotides (137).

Given the emerging role of chimeric oligonucleotides with modifications in the 3′- and 5′-wings designed to enhance affinity for the target RNA and nuclease stability and a DNA-type gap to serve as a substrate for RNase H, studies focused on understanding the effects of various modifications on the efficiency of the enzyme(s) are also of considerable importance. In one such study on *Escherichia coli* RNase H, we have recently reported that the enzyme displays minimal sequence specificity and is processive. When a chimeric oligonucleotide with 2′-modified sugars in the wings was hybridized to the RNA, the initial site of cleav-

age was the nucleotide adjacent to the methoxy-deoxy junction closest to the 3′-end of the RNA substrate. The initial rate of cleavage increased as the size of the DNA gap increased and the efficiency of the enzyme was considerably less against an RNA target duplexed with a chimeric antisense oligonucleotide than a full DNA-type oligonucleotide (138).

In subsequent studies, we have evaluated the interactions of antisense oligonucleotides with structured and unstructured targets, and the impacts of these interactions of RNase H in more detail (139). Using a series of noncleavable substrates and Michaelis-Menten analyses, we were able to evaluate both binding and cleavage. We showed that, in fact, *E. coli* RNase H1 is a double-strand RNA-binding protein. The Kd for our RNA duplex was 1.6 μM; the Kd for a DNA duplex was 176 μM; and the Kd for single-strand DNA was 942 μM. In contrast, the enzyme could only cleave RNA in an RNA-DNA duplex. Any 2′-modification in the antisense drug at the cleavage site inhibited cleavage, but significant charge reduction and 2′-modifications were tolerated at the binding site. Finally, placing a positive charge (e.g., 2′-propoxyamine) in the antisense drug reduced affinity and cleavage.

We have also examined the effects of antisense oligonucleotide-induced RNA structures on the activity of *E. coli* RNase H1 (140). Any structure in the duplex substrate was found to have a significant negative effect on the cleavage rate. Further, cleavage of selected sites was inhibited entirely, and this was explained by steric hindrance imposed by the RNA loop traversing either the minor or major grooves or the heteroduplex.

Recently we have cloned and expressed human RNase H1. The protein is homologous to *E. coli* RNase H1, but has properties similar to those described for human RNase H type 2 (141,142). The enzyme is stimulated by low concentrations of Mg^{2+}, inhibited by higher concentrations, and is inhibited by Mn^{2+} in the presence of Mg^{2+}. It is 33 kDa in molecular weight. It is double-strand RNA-binding protein and exhibits unique positional and sequence preferences for cleavage (143). Additionally, human RNase H2 has been cloned, but to date the expressed protein has not been shown to be active (144). Thus, we now have the necessary tools to begin to explore the roles of human RNase H's in biological and pharmacological processes and to begin to develop drugs designed to interact with them more effectively.

E. Activation of Double-Strand RNase

By using phosphorothioate oligonucleotides with 2′-modified wings and a ribonucleotide center, we have shown that mammalian cells contain enzymes that can cleave double-strand RNAs (145). This may be an important step forward because it adds to the repertoire of intracellular enzymes that may be used to cleave target RNAs, and because chimeric oligonucleotides 2′-modified wings and oligoribo-

nucleotide gaps have higher affinity for RNA targets than chimeras with oligodeoxynucleotide gaps.

F. Antisense Technology as a Gene-Functionalization Tool

Antisense technology has proven to be a very valuable tool with which to determine the function of genes, to evaluate their roles in various cellular pathways and pathophysiological processes and to determine whether they are attractive targets for drug discovery (146,147). In our laboratories, we have automated the in vitro processes to such an extent that antisense inhibitors to any gene can be identified within a few days and then can be employed in animal models.

V. CONCLUSIONS

Although many more questions about antisense remain to be answered than are answered, progress has continued to be gratifying. Clearly, as more is learned, we will be in the position to perform progressively more sophisticated studies and to understand more of the factors that determine whether an oligonucleotide actually works via an antisense mechanisms. We should also have the opportunity to learn a great deal more about this class of drugs as additional studies are completed in humans.

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2

Medicinal Chemistry of Antisense Oligonucleotides

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I. INTRODUCTION

A. Generic Nature of Antisense Oligonucleotides

Antisense oligonucleotide, like any other drug classes, have certain common biophysical and biochemical property requirements (pharmaokinetic and pharmacodymic properties) that must be met for them to serve as effective drugs. In the case of oligomeric materials such as antisense drugs, their relative large size, ionic charge, and structural relationship to natural nucleic acids present somewhat different problems than typical monomeric materials (small molecules). In fact, from the outset of antisense drug discovery, the prevailing perception was that the large, negatively charged oligonucleotides were not "drug-like" and thus, not likely to serve as drugs. Labeling a chemical as not drug-like suggests that there are definable physical chemical properties that describe a drug. It follows that monomeric drugs (small molecules) typically in the range of 150–600 atomic mass units with near neutral pKas are thought to "drug-like." The oligonucleotides that have entered clinical trials range from 15 to 35 mers (6000–12,000 amu) with a corresponding negative charges of 14–34 and clearly do not fit the "drug-like" label.

Although antisense oligonucleotides clearly have unfavorable pharmacokinetics that are related to their relative large size and ionic ester linkages, they represent the only chemical class that possesses significantly precise binding rules to allow the rapid discovery of leads for widely different targets. Antisense oligonucleotides possess the unique drug discovery property of encoded information (binding rules) in their pharmacophore. Chemicals of this nature have been re-

ferred to as "informational drugs" (1). This property also allows a level of specificity for their nucleic acid targets unsurpassed by any other drug class. Although the oligonucleotide sequence is changed for each new nucleic acid target, the pharmacokinetics, toxicity, and scale-up chemistries of each different antisense inhibitor are very similar (highly generic) for all sequences possessing the same chemical modifications. A comparable set of binding rules has not been established to allow rational targeting of nucleic acids or protein targets with any other class of chemicals. This is unfortunate, in that monomeric molecules (small molecules) being of much lower molecular weight than oligomeric materials (e.g., oligonucleotides), and "drug-like" would be expected to provide significant advantages in areas such as synthesis and pharmacokinetics.

B. Essential Properties for an Oligomeric Material to Serve as a Potential Antisense Drug

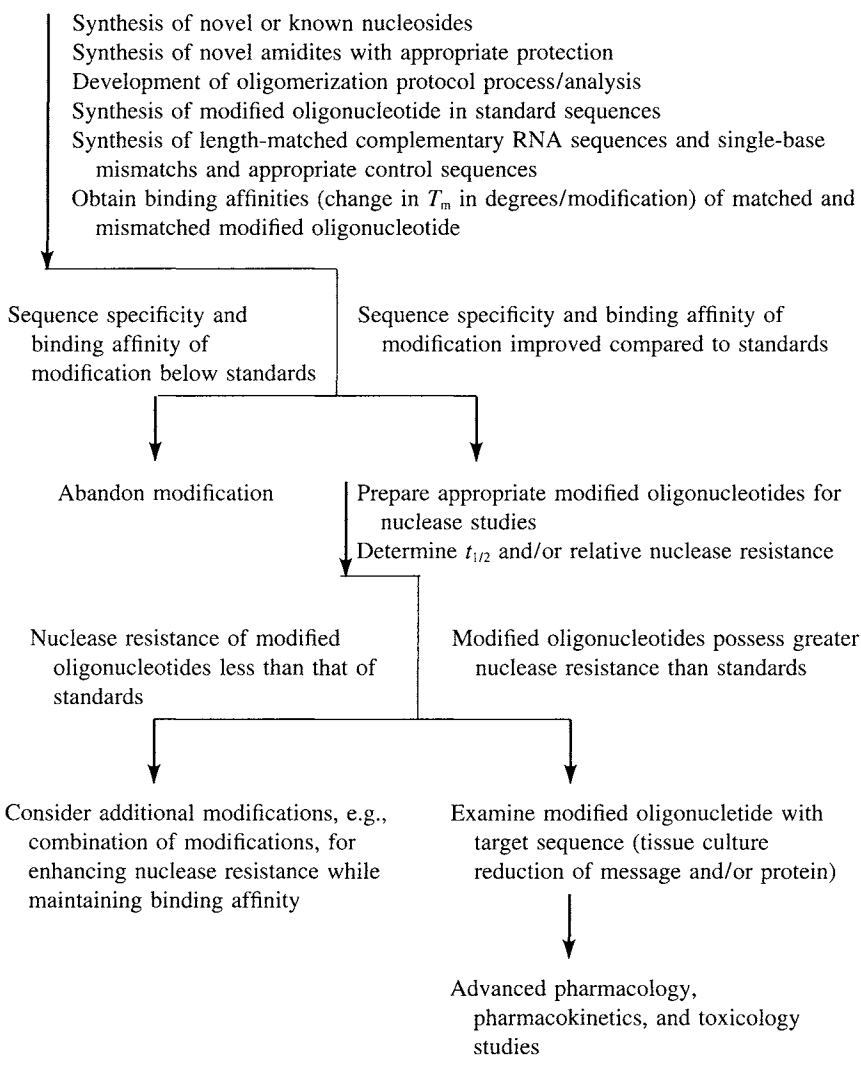
Antisense oligonucleotides, delivered parenterally or nonparenterally, must be sufficiently absorbed from the site of administration, distributed to various tissues, taken into target cells, and possess sufficient residence time and concentration at the site of action to elicit an effective biological response. The oligonucleotide must be resistant to chemical or enzymatic degradation throughout this process. At the intracellular site of action, the oligonucleotide must sequence-specifically bind (hybridize according to Watson-Crick rules) to the target RNA with an affinity that provides a sufficient residence time for inactivation of the function of the target RNA (mode of action). Inactivation of the targeted RNA may result from simply binding to the target sequence or more likely by endonucleolytic cleavage of the RNA target strand by RNase H. This is the process that a chemical material must pass through to potentially serve as an antisense drug. Not noted is the requirement of acceptable toxicity. Toxicity may arise from metabolites generated by nucleolytic degradation, particularly providing modified nucleotides, which may inhibit cellular nucleic acid metabolism. Oligonucleotides, particularly phosphorothioates, bind to a variety of proteins and this becomes another source of potential toxicity. Finally, toxicity may be derived from exaggerated pharmacology derived from reduction of the target message.

C. General Structure-Activity/Property Relationships Pathway to Enhance Oligonucleotide Drug Properties

New oligonucleotide modifications are initially examined for their ability to enhance binding affinities (T_m) while maintaining specificity and nuclease resistance ($t_{1/2}$). The experimental pathway for determining these properties, which is the

basis for the pharmacokinetic and pharmacology profile of an oligonuclotide, is depicted in Table 1. The excitement of oligonucleotides as drugs stems from the fact that, unlike other drug discovery approaches, they are informational materials (1), i.e., as noted, chemicals having a specific set of rules that clearly govern their binding to a specific nucleic acid receptor. These oligonucleotides bind to

Table 1 General Pathway for Structure-Activity/Property-Relationship Studies



their nucleic acid targets via Watson-Crick base-pairing rules. Thus, the first test to determine whether a modified oligonucleotide will be of interest is whether it maintains its sequence specificity according to Watson-Crick rules. A newly modified oligonucleotide not possessing this fundamental specificity property is an immediate failure in the SAR study. Having ascertained that a particular modified oligonucleotide has an acceptable level of base-pair specificity (level of P=S oligonucleotides), the next step in the SAR study is to determine how tightly the modified oligonucleotide binds to its target nucleic acid. This relates to the residence time that an oligonucleotide is bound to it receptor. As noted, the binding affinity of an oligomeric material is a physicochemical property, determined by measuring a modified oligomer sequence's specific interaction with its length-matched RNA complement. The hybridization or melting process is performed under a rather standard set of conditions designed to mimic an intracellular environment (2). As this is a calculation taken under artificial conditions, it may not accurately represent the binding of an oligomer to a native RNA inside a cell. The next essential property that an oligonucleotide must possess is sufficient resistance to degradation by exo- and endonucleolytic plasma and tissue nucleases. Nuclease resistance of an oligonucleotide is determined in various assays, such as, heat-inactivated fetal calf serum, cellular extracts, with purified exonucleases or endonucleases. These results are also likely to differ from the stability of an oligomer in an in vivo situation. The most meaningful method to determine the level of nuclease is the quantitation of full-length material isolated from animals dosed with the oligonucleotide (3). It is important to note that the measurement of these T_m and $t_{1/2}$ assays does allow structure-property/activity relationship studies to proceed, and thus provide reasonable methods to compare various oligonucleotide modifications at an early stage of the chemical synthesis process.

D. First-Generation Antisense Oligonucleotides

I consider the first-generation antisense oligonuclotides, initial modifications of DNA and RNA, as phosphoramidates, methyl phosphonates, phosphorothioate, phosphorodiamidate morpholinos, and α-oligonucleotides. Chemical, biochemical, and biophysical properties of the first generation oligonucleotides are listed in Table 2. In considering the requirements for oligonucleotides, the first obvious problem with unmodified DNA and RNA strands was that they were rapidly degraded by nucleases. Thus, first-generation modifications were primarily directed to stabilizing short strands of DNA and RNA to these enzymes. However, after considerable SAR studies in recent years, we now know that even if short strands of DNA or RNA were sufficiently stable to nucleases, they would be quite inferior to the properties of the current generation of modified antisense

Table 2 Chemical, Biochemical, and Biophysical Properties of First-Generation Oligonucleotides

High molecular weight (20- to 25-mers, 7000–9000 mw)
Highly negatively charged or neutral (19–24 charges)
Chirality in backbone (P=S, Me—P, N—P)
Biological and chemical instability (nucleases, pH, depurination, etc.)
Insoluble in common organic solvents depending on backbone modification (P=S vs. Me—P)
Oligomers not crystalline
Complex synthesis of reactive monomers
Unfamiliar with instrument synthesis of oligomers
Purifications by ion-exchange chromatography
Analysis and characterization not typical

oligonucleotides. This is because current modifications often not only provide enhanced nuclease resistance, but also address several other deficiencies exhibited by first-generation antisense molecules, including RNA and DNA. First-generation modifications, although not specifically designed for nuclease resistance, have a level of nuclease-resistance sufficient for parenteral applications. However, of these materials, only phosphorothioates were found to support an RNase H mechanism. Also, removal of negative charges had important implications concerning tissue distribution, particularly resulting in more rapid elimination. Phosphodiamidate morpholinos represent an interesting modification since, although not supporting an RNase H mode of action, a 20-mer form has progressed to clinical trials for restinosis (4). This oligomeric class is reviewed by P. Iverson in another chapter in this volume.

Obviously, the phosphodiester moiety of a nucleic acid is the primary recognition site for phosphodiesterases. This is verified in the case of methyl phosphonates and phosphoroamidates where removing an ionic oxygen atom provides a nuclease-resistant linkage. Also, modification of the prochiral phosphordiester provides a chiral mixture, which may provide individual isomers with differing levels of nuclease resistance. What other recognition sites are required for phosphordiesterase activity is not known. Phosphorodiamidate morpholinos are also chiral and lack charge, as well as having a morpholino ring instead of a ribofuranosyl ring. The heterocyclic orientation in α-oligonuclotides (connection site modification) apparently provides another sugar change that significantly differs from the natural substrate and provides nuclease resistance. An obvious challenge, at this stage of the drug discovery process, and based on the first-generation modifications, is to modify oligonucleotides to have high nuclease resistance and

support an RNase H mechanism. A further design feature is that the modifications should also provide increased binding affinities. Although one may focus SAR studies to enhance one property such as nuclease resistance, typically the modification will affect several properties.

E. Oligonucleotide Modifications and Drug Properties that May Be Altered

A diverse range of modifications, at all possible modification sites of an oligonucleotide, have been reported (reviewed in Refs. 5–8). A dimer of an oligonucleotide depicting subunits that may be modified to enhance oligonucleotide drug properties is depicted in Fig. 1. These subunits are composed of heterocycles, ribofuranosyl, phosphodiesters, and ribofuranosyl-phosphates (a four-atom linkage or backbone). Modifications can be performed on these subunits, as well as modifications that relate to how these units are connected (connection sites). Complete removal of the ribofuranosyl-phosphate backbone with appropriate re-

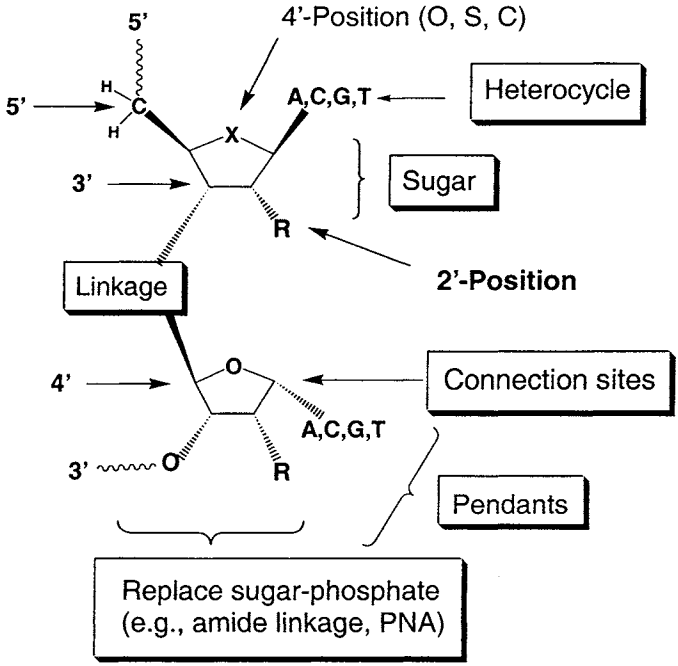


Figure 1 Oligonucleotide subunits.

placements and attaching, or conjugating, drug-enhancing moieties at various positions in the subunits are additional important modification areas. Finally, prodrug modifications may be employed to enhance drug properties. Most of the positions available in a G-C or A-T dimer (approximately 26 positions for each dimer), which do not directly interfere with Watson-Crick base pair–hydrogen bonding, have been modified.

Additional standards that one should be aware of are the impact modified oligonucleotide modifications may have on the cost of future antisense drugs. As a standard, the cost of P=S oligonucleotides should be considered. The cost of phosphorothioates has been dramatically reduced in the past 10 years owing to improvements in the process, cost reduction of key reagents, and the economics of larger-scale syntheses. Currently, the cost of goods for a 20-mer P=S oligonucleotide is less than $300/g and is projected to be less than $50/g as larger quantities are required. The 2′-*O*-(methoxyethyl)-modified oligonucleotides are derived from the ribonucleosides in RNA and should eventually be substantially less expensive than modified oligonucleotides derived from deoxyribofuranosyl nucleosides from DNA. P=S oligonucleotide antisense drugs are expected to be cost competitive when considering parenteral treatment, three times/week, with a 1 mg/kg dose. Modified oligonucleotides must also be cost effective and will need to be less expensive to synthesize (e.g., shorter), or have a dosing advantage (less often), or greater therapeutic index, or have other important advantages to offset a possible increase in cost of synthesis.

When considering research to modify oligonucleotides, it is important to know the extent that proprietary protection of a modification can be obtained. Important patent positions for many types of modifications have been established during the past 10 years as making drugs out of oligonucleotides has become of interest (9–11).

Modifications of oligonucleotides may be expected to address essentially every facet of antisense drug properties. Biophysical and biochemical properties that may be affected by modifications are listed in Table 3. Drug properties that will be affected by altering biophysical and biochemical parameters would include the general areas of pharmacokinetics, pharmacodynamics, and toxicology. In addition, certain modifications may represent cost and proprietary patent advantages.

II. THE MEDICINAL CHEMISTRY CHALLENGE

The medicinal chemistry challenge of optimizing oligonucleotides drug properties centers on the discovery of modifications that would be optimal for each stage of the pharmacokinetic process and appropriate pharmacology and toxicology as described above, and indeed, of more value, having the modification favorably

Table 3 Properties-Structure-Activity/Properties-Relationship Studies May Alter

Binding affinity
Base-pair specificity
Nuclease resistance
Support of endonucleolytic cleavage of the RNA of a heteroduplex
Chemical stability
Lipophilicity
Solubility
Protein-binding properties
Toxicological properties
Pharmacological properties

affect several drug properties. Most current modifications, in fact, do relate to more than one property. However, often the modification enhances one property while having a negative effect on other properties or a modification will have a positive effect on several drug properties. For example, most modifications of oligonucleotides that increase binding affinity and/or nuclease resistance will not support an RNase H mechanism. Modifications that allow removal of ionic phosphodiester linkages in an oligonucleotide, thus providing favorable physical chemistry properties, also have unfavorable elimination properties. Shorter oligonucleotides with reduced ionic charge may exhibit more favorable oral bioavailability than typically 20-mers, but they may bind to multiple complementary sequences, which may increase the potential for toxicity. On the other hand, certain 2′-*O*-modifications significantly increase binding affinities while providing significant nuclease resistance. The general relationships of various oligonucleotide subunits and oligonucleotide properties to important antisense drug properties are listed in Table 4. Although oligonucleotide protein interactions will certainly have an important effect on pharmacokinetics properties and potential toxicities, meaning relationships are not clear at this time.

In considering the relative importance of nuclease resistance of an antisense oligomer and its level of affinity to its RNA target, recent biological results suggest that stability of an oligonucleotide may be a more important property to enhance than binding affinity (12). Modifications that provide high-binding oligonucleotides but with low nuclease resistance have not provided significant biological activity. On the other hand, oligonucleotides such as phosphorothioates have provided significant biological activity. Although some modifications provide high binding affinities and high nuclease resistance, they may not exhibit useful antisense activities because they do not support an RNase H mechanism. A modification that will support an RNase H mode of action and possesses high T_m and $t_{1/2}$ values has not been reported. Thus, an ideal oligonucleotide modification

Table 4 General Effects of Oligonucleotide Subunits and Physical Chemical Properties on Antisense Drug Properties

Subunits/ properties	Binding affinity, T_m	Nuclease resistance, $t_{1/2}$	Rnase H support	Protein binding
Size (molecular weight)	Increase length, increase T_m	No known relationship	No known relationship	Increase length, increase binding
Ionic charge	Reduce negative charge, increase T_m?	Reduce negative charge, increase $t_{1/2}$	Reduce negative charge, reduce support	Decrease charge, decrease binding
Phosphorus modifications	Always reduce T_m vs. P=O	Always increase $t_{1/2}$	Negative charged modifications support, P=S>>> P—BH_3^-	Negative charged modifications increase, P=S << P—S_2
Ribofuranosyl conformation	3′-Endo corelates significantly with increase in T_m	Most ribofuranosyl modifications increase $t_{1/2}$	3′-Endo does not support	No known relationship
Phosphorothioate content	T_m decrease ~ 0.8°C/ P=S vs. P=O linkage	Increase P=S linkage, increase $t_{1/2}$	P=S backbone less active than P=O backbone	P=S linkage greatly enhanced
Heterocycle	Several heterocycle types increase; up to 3.0°C/ unit	Insignificant effect	Generally support	No known relationship
Linkage change	MMI, thioforacetal, amide-3 increase T_m up to 3.5°C/change	Complete resistance, linkage not an ester	Does not support	Less binding as charge is removed
$C_{2'}$-Ribofuranosyl	Typically increase T_m, wide range, up to 2.5°C/unit	Resistance correlated to size, positive charge highly resistant	Does not support	No known effect
$C_{3'}$-Ribofuranosyl	$C_{3'}$-Methyl and amino increase 3′-endo, increase T_m, up to 3.5°C/ unit	High level of nuclease resistance	Does not support	No known effect
Oligonucleotide sequence	G/C increase over A/T	Little effect	Little effect	Little effect

would provide an oligomer that hybridizes to target RNA with high binding affinity and specificity, be stable to nucleolytic degradation, allow a RNase H cleavage of the RNA target, and possess favorable protein-binding properties. This has led to the concept that to optimize the antisense activity of an oligomer, a combination of oligonucleotide modifications will be required (13,14). A single oligonucleotide modification simply will not effectively address all essential drug properties.

III. SCOPE OF REVIEW

We are now about 12 years from a period when several biotechnology companies formed with the primary focus of making drugs out of oligonucleotides. Since then, rather extensive medicinal chemistry efforts have been applied to this drug discovery paradigm. This has resulted in advances in all areas essential for registering drugs (15). Approximately 26 oligonucleotides have entered clinical trials; 16 of these are still in various phases of examination. The first antisense oligonucleotide drug, Vitravene, recently won approval from the Food and Drug Administration for treatment of cytomegalovirus retinitis in AIDS patients. Several oligonucleotides with anticancer and anti-inflammatory indications are in late phase II and III clinical studies. Most of the antisense preclinical and clinical areas are reviewed in other chapters in this volume. I have described the generic nature of antisense oligonucleotides, the essential requirements for an oligomeric material to serve as a potential antisense drug, first-generation antisense oligonucleotides, and the scope of oligonucleotide modifications achieved to date. In the following section, I will discuss the value of the most useful modifications within each oligomer subunit as they relate to enhancing antisense oligonucleotide drug properties. These are depicted in Figs. 2–7 along with their binding affinities (T_m).

IV. GENERAL COMMENTS ON THE VALUE OF CLASSES OF MODIFICATIONS WITHIN EACH SUBUNIT

From the first-generation modifications, we have learned of several types of structural changes that will provide nuclease resistance: removal of the phosphodiester negative charge (methyl phosphonates, phosphoramidates, alkyl phosphorotriesters), replacement of a nonbonding oxygen of the phosphordiester with sulfur (phosphorothioates), site of attachment of the nucleobase (α-oligonucleotides), the unnatural phosphoryl isomer (Rp), and the use of a combination of these changes as represented by a bis-amidate morpholino. Second-generation modifi-

cations, for the most part, represent entirely new approaches to achieving nuclease resistance. The key modifications will be discussed.

A. Heterocycle Modifications

The heterocycles (nucleobases) of nucleic acids provide the recognition points for the Watson-Crick base-pairing rules and thus any oligonucleotide modification must maintain these specific hydrogen-bonding interactions. The scope of heterocyclic modifications to enhance antisense drug properties is quite limited. This is verified by the fact that only four or five types of modified heterocycles have demonstrated useful oligonucleotide-binding properties (Fig. 2). The chemi-

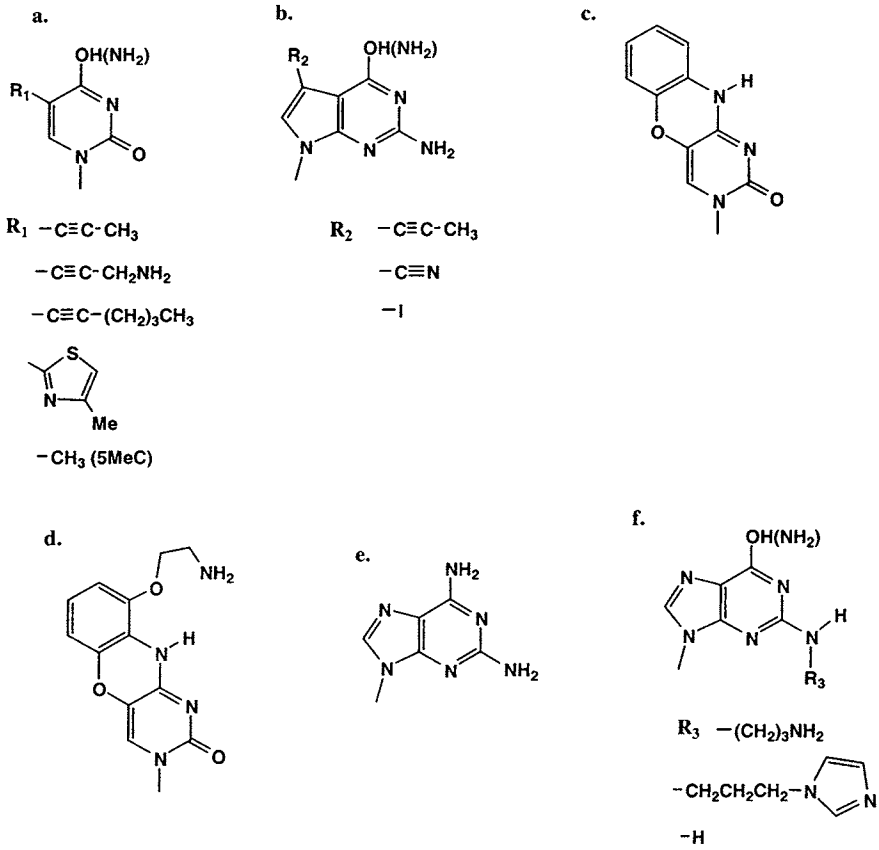


Figure 2 Heterocycle modifications.

cal changes are primarily placed on the backside of the Watson-Crick binding face, and thus, base-pair recognition is maintained. These heterocyclic modifications can be grouped into three structural classes: enhanced base stacking, additional hydrogen bonding, and the combination of these. Modifications that enhance base stacking by expanding the π-electron cloud of the planar systems are represented by the conjugated, lipophilic modifications in the 5-position of pyrimidines (16–19) and the 7-position of 7-deaza-purines (20–23). Substitutions in the 5-position of pyrimidines modifications include propynes, hexynes, thiazoles, and simply a methyl group (see Fig. 2a) and substituents in the 7-position of 7-deaza purines include iodo, propynyl, and cyano groups (see Fig. 2b). The Gilead research group, focusing on heterocyclic modifications, have modified out of the 5-position of cytosine by moving from the propynes to five-membered heterocycles to the most recently reported, tricyclic fused systems (Fig. 2c, d) emanating from the 4,5-positions of (cytosine clamps) (24–27). A second type of heterocycle modification is represented by the 2-amino-adenine (Fig. 2e) where the additional amino group provides another hydrogen bond in the A-T base pair, analogous to the three hydrogen bonds in a G-C base pair. Heterocycle modifications providing a combination of effects are represented by 2-amino-7-deaza-7-modified A (23) (Fig. 2b) and the tricyclic cytosine analog having an ethoxyamino functional group capable of an additional hydrogen bonding interaction in the major groove of heteroduplexes (27) (Fig. 2d). Furthermore, N2-modified 2-amino adenine (Fig. 2f) modified oligonucleotides have exhibited interesting binding properties (28,29). These heterocycle modifications (Fig. 2a–d) are positioned to lie in the major groove of the heteroduplex and may have cationic interactions that stablizes duplexes, do not greatly affect the sugar conformation of the heteroduplex (30), provide little nuclease resistance, but will generally support an RNase H cleavage mechanism (31). Although some sequence specificity of 3′-exonucleases and endonucleases has been reported (32), it is unlikely to be sufficient for in vivo applications. This suggests that modifying heterocycles to obtain nuclease resistance might not be a fruitful approach.

B. Pentofuranosyl Modifications

1. Introduction

Of the subunits of an oligonucleotide (Fig. 1), modifications in the ribofuranosyl moiety have received a great deal more attention than any other subunit. And possibly as a result of this effort, pentofuranosyl modifications have yielded the most value in the quest to enhance oligonucleotide drug properties. 2′-*O*-Ribofuranosyl modifications (Fig. 3) are of particular value as certain modifications have greatly increased nuclease resistance, have greatly increased binding affinity,

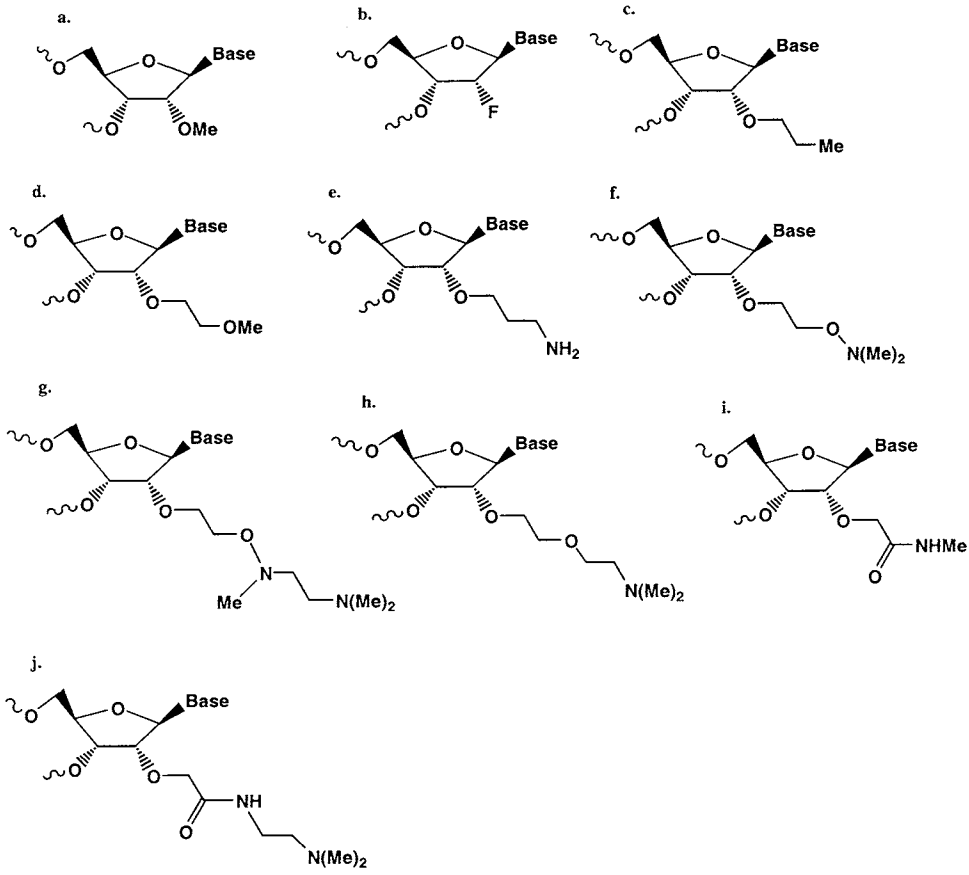


Figure 3 $C_{2'}$ Ribofuranosyl modifications.

have altered pharmacokinetics, and are potentially less toxic (reviewed in Ref. 5). Another advantage of ribofuranosyl modifications is that each nucleotide unit of an oligonucleotide has a ribofuranosyl unit that may or may not be modified. Thus, a 20-mer oligonucleotide has 19 ribofuranosyl units that may be modified individually or all the units modified uniformly to optimize antisense properties. The best example of this is the popular oligonucleotide gap strategy employed to support an RNase H mode of action while utilizing ribofuranosyl modifications to enhance binding affinities and nuclease resistance and alter protein binding and toxicology profiles (13,14). In this case, modified ribofuranosyl moieties are

Table 5 Modifications of the Ribofuranosyl Moiety

Modifications of nonconnecting carbon positions $C_{2'}$ and $C_{4'}$
Modifications of connecting carbons $C_{1'}$, $C_{3'}$, and $C_{5'}$
Replacement of sugar oxygen, $O_{4'}$
Anhydro sugar modifications (conformational restricted)
Cyclosugar modifications (conformational restricted)
Ribofuranosyl ring size change
Connection sites—sugar to sugar ($C_{3'}$ to $C_{5'}$/$C_{2'}$ to $C_{5'}$)
Hetero-atom ring–modified sugars
Combination of above modifications

typically placed contiguously in the flanks, whereas the ribofuranosyl units in the gap of the oligonucleotide are not modified so as to support RNase H binding and cleavage (see Section IV.E).

Preorganization of an oligonucleotide to mimic a canonical RNA strand (A form) will significantly enhance hybridization rates, which relates to enhanced binding affinity. The most important contributor to whether a nucleic strand is related to the A or B (DNA) form is the sugar conformation (33). Thus, increasing the stability of the 3′-endo ribofuranosyl conformation versus the 2′-endo conformation relates to greater stacking of the nucleobase and, thus, greater binding to its complementary RNA strand (33,34). Many ribofuranosyl modifications have been prepared and examined based mainly on an experimental approach (ease of synthesis). Because of this, many of the modifications have been examined that are not readily classified into rational hypotheses. Table 5 lists the various types of ribofuranosyl modifications that have been considered.

2. Cyclo-ribofuranosyl Modifications (Conformationally Constrained)

Owing to the potential value of constraining the ribofuranosyl moieties of antisense oligonucleotides to a 3′-endo conformation (A form), considerable research efforts in this area have been reported. Constraining the ribofuranosyl sugar by preparing bicyclo or tricyclo ribofuranosyls-modified oligonucleotides has been the most popular approach (34,35). Of these type ribofuranosyl modifications, locked nucleic acids (LNA), which bridge the $C_{4'}$ to the 2′-*O* with a methylene group, of the ribofuranosyl sugar are clearly the modification offering the most promise (Fig. 4c–e).

Restricting the conformational flexibility of the ribofuranosy moiety of uridine in an oligonucleotide by synthetically constructing an connecting bridge between the $C_{4'}$ and the $C_{2'}$ carbon atoms on the α-face of the ribofuranose (α-$C_{4'}$-CH_2O-$C_{2'}$), was communicated by the Imanishi laboratory in 1997 (36). Almost

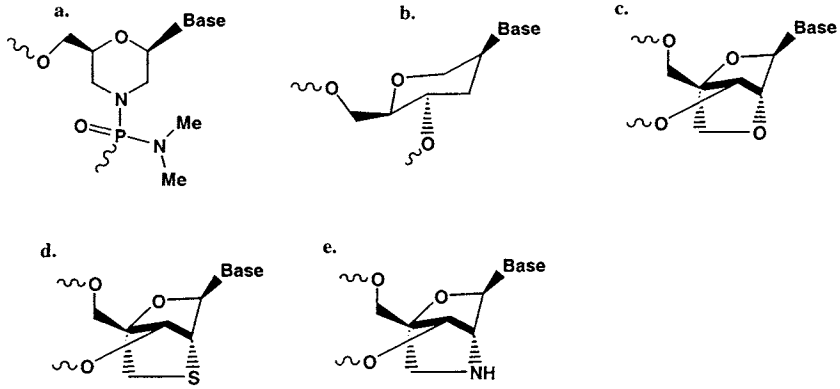


Figure 4 Extensive ribofuranosyl modifications.

simultaneously, the Wengel laboratory communicated the synthesis of oligonucleotides bearing ribothymidine and guanosines modified in the same manner (37). Both laboratories rapidly followed these initial communications with full reports of oligonucleotides bearing bridged uridine and cytidine (38) and bridged adenosine, cytidine, guanosine, 5-methylcytidine, ribo-thymidine, and uridine (39). The specific modification is an α-$C_{2'}$-$C_{4'}$ oxymethylene or α-$O_{2'}$-$C_{4'}$ methylene linker and has been labeled LNA (locked nucleic acids) by the Wengel lababoratory (37).

The LNA modification, because of the rigid bicyclo[2.2.1]heptane system, represents the first and only cyclo ribofuranosyl modification that truly imparts an inflexible conformation. Other modifications synthesized have *cis*-fused bicyclic ring systems or connections between the exo-$C_{5'}$ and an endo C atom, or longer linkers of *cis*-fusion, which all may still allow considerable conformational flexibility (34,35). X-ray crystallographology analysis of α-$O_{2'}$-$C_{4'}$ methylene-modified uridine indicated a sugar pucker pseudorotation phase *P* and X-angle of 17.4° and 195.7°, respectively, which is characteristic of a 3′-endo conformation of the sugar. In addition, the $C_{1'}$, $C_{2'}$, $C_{3'}$ sugar protons are found as single signals suggesting typical N-conformation (3′-endo conformation) (37).

LNAs have demonstrated unprecedented binding affinities of 4–8°C/unit in a variety of sequences. Slightly higher affinities are noted when binding to RNA complements versus DNA complements. The binding effects are additive and there is little dependence on the number of their location in the sequence. The Wengel laboratory has described the LNA modification as universal in its binding to nucleic acids (40). This is based on the hybridization studies of three types of LNA-modified 9-mer oligonucleotides: deoxy-LNA (a chimera of three

5-methyl uracil LNA units and six deoxynucleosides), ribo-LNA (a chimera of three 5-methyl uracil LNA units and six ribonucleoside units), and oligonucleotides uniformly modified with LNA units (nine 5-methyluracil LNA units). Absolute binding affinities of these types to their nine-mer RNA complement, as expected, yielded increases over their unmodified DNA parent in the order of uniform LNA (ΔT_m of 74°C, 5.1°C/unit), ribo-LNA (ΔT_m of 63°C, 8.3°C/unit), and deoxy-LNA (ΔT_m of 50°C, 7.3°C/unit). It was noted that the addition of six 5-methyluracils to the ribo-LNA to provide the uniform LNA nine-mer increased the T_m by 11°C, or about 1.8°C/added LNA unit. This suggested that an LNA unit inserted into a ribo- or deoxyoligonucleotide profoundly affects the neighboring ribofuranosy or deoxyfuanosyl units by inducing an overall preorganization, possibly by an increased intrastrand base stacking. It was noted that although only 5-methyl uracil was used in this particular binding affinity study, other pyrimidine and purine nucleobases provide similar data.

Studies of LNA units binding to mismatches and compared with natural DNA indicate that sequence specificity of the LNA units is as good as or better than DNA (compared to the natural DNA (39). A more exact determination of the specificity of LNA modified by studies of in vitro or in vivo biological systems has not been reported. The Wengel Laboratory has designed, synthesized, and collected conformation data on several other types of LNA nucleosides: α-LNA and xylo LNA (41), amino-LNA, and thio-LNA (42).

LNA-modified oligonucleotides are reported to be resistant to nuclease degradation (37,43). It is not known whether LNA oligomers will support an RNase H mode of action. Because of the potential value of this modification, it is reviewed more thoroughly by J. Wengel, in this volume.

3. $C_{2'}$-Ribofuranosyl Modifications

The primary purpose for modifying the $C_{2'}$ position of the ribofuranosyl subunit (Fig. 3) has been to enhance the binding affinity to target RNA and secondarily to enhance nuclease resistance. Again, the chemical principle driving this modification is to ''preorganize'' the antisense oligomer into an ''A''-form geometry by converting the ribofuranosyl moieties into a 3′-endo conformation having the torsion angles mimicking dsRNA (34,35). Several hundred 2′-*O*-modifications have been prepared and examined for their ability to increase T_m and $t_{1/2}$. A number of these have recently been reviewed (5,44–47). The most recent modifications include 2′-*O*-(dimethylaminooxyethyl) (48), 2′-*O*-acetamido (49), 2′-*O*-(dimethylaminooxyethoxyethyl), 2′-*O*-(dimethylaminoethoxyethyl), and 2′-*O*-(dimethylaminoethylacetamido) (50), which have shown a combination of binding affinity and nuclease resistance, superior at this stage to those of all other 2′-*O*-ribofuranosyl modifications. These modifications are depicted in (Fig. 3f–j).

4. Other Ribofuranosyl Modifications

More extensive sugar modifications are hexopyranosyl-like DNA (Fig. 4) (51) and the more recently reported C_3-ribofuranosyl modification represented by replacement of the connecting oxygen atom by a methylene (3′-methylene) (52) and by an amine (3′-amidates) (53). The 3′-amidates and 3′-methylenes provide a significant increase in binding affinity and are stable to nuclease degradation, but do not support an RNase H mode of action.

As noted, ribofuranosyl modifications that enhance binding affinity typically provide a higher level of 3′-endo ribofuranosyl conformation. Unfortunately, on hybridization to target RNA strands, an A-form duplex results, which does not support an RNase H mode of action. Thus, although a 3′-endo sugar conformation relates to increased binding affinity, the resulting duplexes do not support RNase H binding and/or cleavage. A ribofuranosyl modification that effectively supports an RNase H cleavage has not been reported. Thus, to utilize these important 2′-*O*-ribofuranosyl modifications, they are employed in a gap motif (gap technology, Section IV.E).

C. Linkage Change

A linkage change that removes the cleavable phosphodiester linking group would, of course, render an oligonucleotide stable to phosphodiesterases (nucleases). The more interesting examples of this class of modifications are methyl imino methyl (MMI) (54), thioformacetal (55), and amide-3 (56) (Fig. 5) in that these modifications, in addition to not being substrates for phosphodiesterases, remove one negative charge, are not chiral, and increase binding affinities compared to a

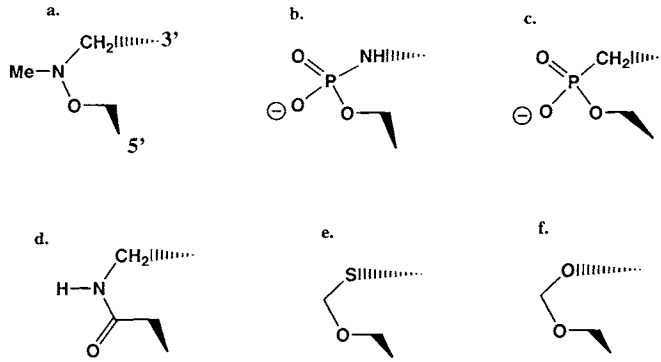


Figure 5 Linkage modifications.

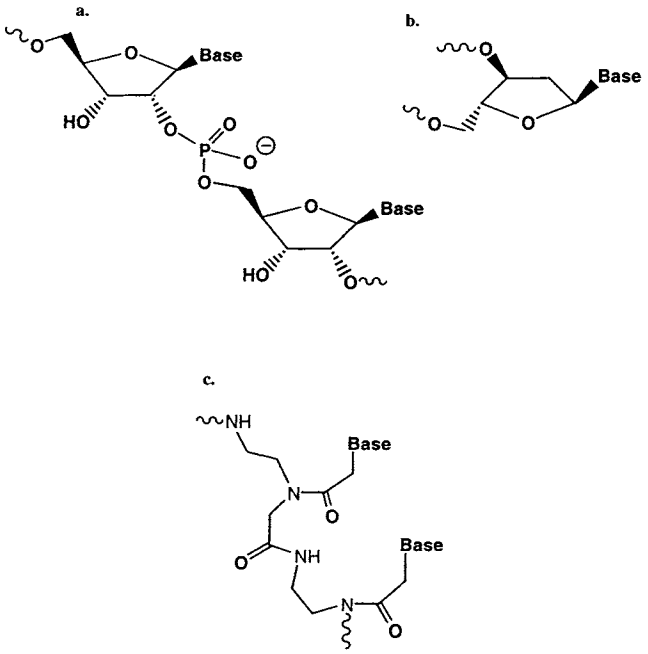


Figure 6 Connection and ribofuranosyl-phosphate replacement modifications.

phosphorothioate. General disadvantages of linkage change modifications are that they do not support an RNase H mode of action, they exhibit unfavorable pharmacokinetics (extensively concentrated in the kidneys and thus are rapidly eliminated), and they are considerably more expensive to synthesize.

Peptide nucleic acids (PNA) provide the best example of the complete removal of the ribofuranosyl-phosphate backbone. In this case the ribofuranosyl-phosphate is replaced with a peptide linkage (Fig. 6) (57). This oligomeric material is discussed by P. Nielson in another chapter in this volume.

D. Phosphorus Atom Modifications

Phosphors atom modifications typically involve changes to the nonbridging oxygen atom, such as the first-generation phosphorothioates (P=S), methylphosphonate (Me—P), and phosphoramidate (P—N) (Fig. 7). Later modifications of the phosphorus atom are phosphorodithioates (P—S_2, phosphoroselenoate (P=Se), and phosphotriesters (P—OR). More recent modifications include phosphoboranoates (58) and unsubstituted phosphoramidates (59). The phosphorothioates

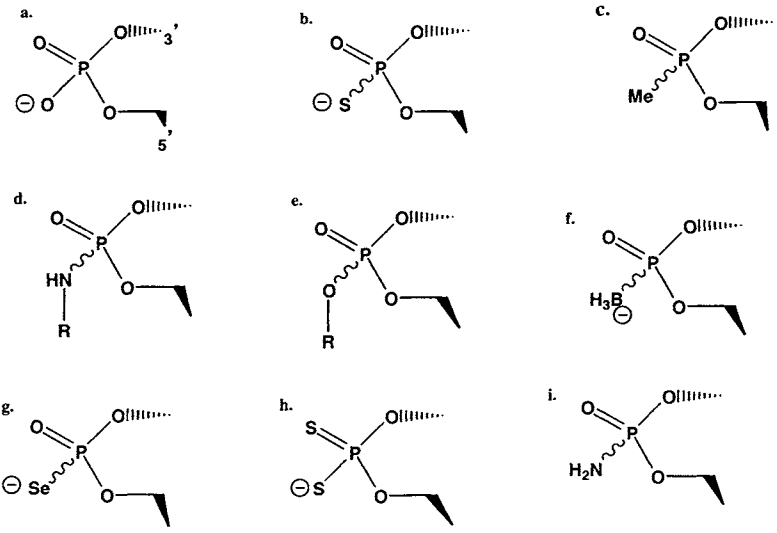


Figure 7 Phosphorus atom modifications.

have been the most useful modification of this class because they support an RNase H mode of action, are relatively easy to synthesize, and the chirality of the phosphorus atom does not greatly affect antisense properties. Borano oligonucleotides have been reported to support RNase H (58).

E. Oligonucleotide Motifs

As noted earlier, having developed high-binding and nuclease-resistant 2′-*O*-ribofuranosyl-modified oligonucleotides, it was rather disappointing that oligomers uniformly modified with these units were inactive or less active than their first-generation parent phosphorothioates. It is now well known that uniformly 2′-*O*-modified oligonucleotides do not support an RNase H mechanism. The 2′-*O*-modified oligonucleotide-RNA heteroduplex has been shown to present a structural conformation that is recognized by the enzyme but cleavage is not supported (60,61). The lack of activity of 2′-*O*-modified oligonucleotides has led to the development of a chimeric oligonucleotide strategy (gapmer technology) (14,62,63). This approach focuses on the design of high-binding, nuclease-resistant antisense oligonucleotides that are "gapped" with a contiguous sequence of 2′-deoxy phosphorothioates (Fig. 8). On hybridization to target RNA, a heteroduplex is presented that supports an RNase H–mediated cleavage of the RNA strand. The stretch of the modified oligonucleotide-RNA heteroduplex that is

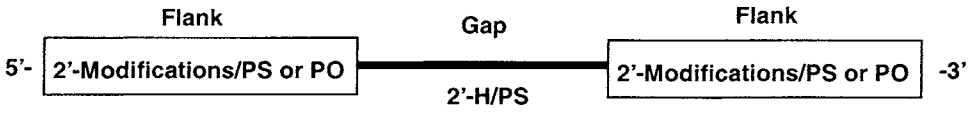


Figure 8 Gapmer technology.

recognized by RNase H may be placed anywhere within the modified oligonucleotide. The modifications in the flanking regions of the gap should not only provide nuclease resistance to exo- and endonucleases, but also not compromise binding affinity and base-pair specificity (64).

Modifications of the phosphorus atom of the natural phosphodiester linkage to provide methyl phosphonates, phosphorothioates, and phosphoramidates destabilize heteroduplexes −0.7 to −1.5°C for each modification (65–67) (Fig. 7). The decreased binding affinity of these modified oligonucleotides could be expected to reduce antisense effectiveness. In the case of chimeric 2′-*O*-methyl or 2′-fluoro-modified oligonucleotides, an enhancement in the binding affinity of about 1.5–2.3°C (compared to P=S phosphorothioates) for each modification obtained (67–69). However, it is now clear that 2′-*O*-methyl and 2′-F-modified DNA are not sufficiently nuclease resistant to have antisense value as P=O backbones (69–72). This problem can be circumvented by the use of 2′-*O*-methyl or 2′-F-modified phosphorothioates in the flanking regions to provide doubly modified oligonucleotides (68,69).

More recent research has focused on 2′-*O*-modifications, such as methoxyethyl (73), aminopropyl (74), and the newer cationic modifications (Fig. 3), which not only provide relatively high binding affinities but also a level of nuclease resistance that allows replacement of the thiophosphate with the natural phosphodiester linkage. 2′-*O*-Modifications, with a favorable combination of T_m and $t_{1/2}$, can be employed in the chimera strategy (Fig. 8), such that a significant portion of phosphorothioate linkages can be replaced with P=O linkages. Just how many sulfurs can be replaced depends on the length of the oligomer and the gap size or RNase H cleavage site. In a typical 21-mer with a 7-nucleoside gap, 65% of the P=S linkages are replaced with P=O linkages. Reduction of the sulfur content in a P=S oligonucleotide may have important implications for the pharmaokinetic and pharmacodynamic properties as well as the toxicity profile of oligonucleotides (73,75).

An important aspect of gapmer technology is that the gap or RNase H cleavage site must be protected from endonucleolytic cleavage. Phosphodiester linkages and even an alternating P=S/P=O motif did not provide a useful level of nuclease resistance for biological activity (64,65). A recent report of the lack of activity of "gapped" 3′-amidate phosphodiesters is likely due to endonuclease

degradation (52). Uniform phosphorothioates are the only useful modification to allow a reasonable combination of binding affinity and nuclease resistance and also support an RNase H mechanism. Hence, as noted earlier, most antisense activities require an RNase H mechanism, which in turn requires sulfur in the form of thiophosphate, somewhere in the chimeric molecule, for nuclease resistance. The biological activity of the most studied chimeras, 2′-*O*-methyl and 2′-*O*-methoxyethyl, is discussed by S. Agrawal and N. Dean in other chapters in this volume.

F. Pendant (Conjugated) Oligonucleotides

The sugar, heterocycles, and backbone (linkages) subunit modifications as depicted in Fig. 1 are core modifications of oligonucleotides, which have been modified to greatly enhance binding affinities and nuclease resistance of antisense oligonucleotides. To enhance additional antisense drug properties of an optimized, core-modified oligonucleotide, a variety of molecules (pendants) have been attached (conjugated) in a point modification motif (i.e., only one pendant in an antisense oligonucleotide). Pendant modifications have primarily been directed to enhancing oligonucleotide uptake. Other potential applications of pendants include increased solubility, lipophilicity, and means to attach synthetic cleavers, intercalaters (for improvements in binding affinity), and cross-linking and alkylating groups. Several reviews have discussed oligonucleotide pendants (76–78). Because of the potential value of this modification class, M. Manoharan reviews it in another chapter in this volume.

H. Prodrug Modifications

Oligonucleotides, particularly phosphorothioates, have considerable avidity for a variety of serum and tissue proteins. This is due to the highly negatively charged nature of the oligonucleotides as well as the lipophilicity of the sulfur atom of the thiophosphate linkages. Prodrug approaches, which are designed to mask the negative charge to allow altered pharmacokinetics, are of considerable interest. The most advanced approach is the pro-oligonucleotide described by the Imbach laboratory (79,80).

V. PERSPECTIVE

A P=S oligonucleotide (phosphorothioate) is now an available drug (Vitravene) and others will follow. However, to continually improve this novel and exciting drug class and to overcome first-generation limitations, structural changes are required. In the past 12 years a diverse range of modifications, at all possible

modification sites of an oligonucleotide (Fig. 1), have been reported. This application of traditional medicinal chemistry (structure-activity-relationship studies) to drug discovery in antisense oligonucleotides and oligonucleotides in general has answered many important questions. For example, as a result of this rather intense effort, we are now aware of modifications that stabilize oligonucleotides toward nucleolytic degradation [2′-*O*-(methoxyethyl, MOE); 2′-*O*-(aminopropyl, AP); 2′-*O*-propyl; 2′-*O*-(dimethylaminooxyethyl, DMAOE), 2′-*O*-(dimethylaminooxyethoxyethyl, DMAOEE), 2′-*O*-(dimethylaminoethoxyethyl, DMAEE), and 2′-*O*- (dimethylaminoethylacetamido, DMAEA) are examples], modifications that greatly enhance binding affinities while maintaining base-pair specificity (MOE, DMAOE, DMAEE, acetamido, and LNA are examples), and modifications that support endonucleolytic cleavage by RNase H (5-propynyl pyrimidines and G-clamps are examples). Although these are biochemical and biophysical properties, a large number of cellular and animal studies support the notion that enhancing these properties correlates with enhanced antisense biological activity in vivo. Unfortunately, a single modification that would provide high-binding, nuclease-resistant antisense oligonucleotides that would support an RNase H mechanism is not available. A modification of this type is of current interest in the antisense approach. We are also aware that changing the structure of phosphorothioate oligonucleotides provides an opportunity to alter their pharmacokinetic profile. In structural changes that remove sulfur (as thiophosphate) and/or change lipophilicity (e.g., by 2′-*O*-modifications), more favorable toxicity profiles have resulted (81). Although we are aware of these important antisense properties (and there may be many more), and we have learned how to control them, we are unaware of the optimum values at which to aim our modifications. In addition, antisense oligonucleotides that are orally available and/or penetrate the blood-brain barrier present the most current, important deficiency of antisense oligonucleotides. Recent reports of antisense oligonucleotides doubly modified at the 3′ and 5′ ends with 2′-*O*-methyl or 2′-*O*-methoxyethyl and P=S phosphorothioates, to provide a high level of nuclease resistance, have provided encouraging results that these pharmacokinetic deficiencies will soon be solved by appropriate chemical modifications (82). Furthermore, considerable formulation research is underway in several laboratories.

One should be aware of the level of accomplishments achieved in oligonucleotide medicinal chemistry research in the past 10 years. I have discussed these (binding affinities, nuclease resistance, support of RNase H, and cost of synthesis) and suggest that they be considered (as standards) before initiating or continuing certain oligonucleotide modification research (5). In addition, understanding the proprietary patent positions that have been established is an important research consideration. I believe that at this stage of oligonucleotide medicinal chemistry, it is highly unlikely that a single modification will be discovered that will significantly impact all of the important drug properties described above. The types of modified oligonucleotides currently being pursued (going beyond P=S oligonu-

cleotides) possess a combination of modifications and this trend will certainly continue as pendants will be conjugated to oligonucleotides with optimized core subunits to obtain a "completely" optimized oligonucleotide drug, which still may benefit from prodrug modifications.

I view the current "winners" or the first modifications most likely to be incorporated into antisense oligonucleotides that will undergo clinical trials as the RNA mimics, MOE, DMAOE, and AP, and the backbone modification, MMI *bis*-methoxys. These will likely be utilized in a gap strategy (gap technology). However, efforts to prepare uniform modifications, such as RNA mimics (2′-*O*-modifications), MMI, and PNA are of considerable interest, in that reliance on RNase H for a mode of action would not be required. In addition to these modifications, which act, either by direct binding (RNase H-independent) or RNase H, I believe modifications of the pendant subunit, such as cholesterol conjugates, folic acid conjugates, etc., and prodrug modifications, will become increasingly more important for optimizing multiple-modified oligonucleotides. It remains to be seen how valuable the newest, high-binding modifications such as LNA, tricyclic cytosine clamps, and DMAOE will be, in view of the current need for a gapmer strategy for useful biological activities.

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Analytical Methods for Antisense Drugs

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I. INTRODUCTION

Techniques for the analysis of nucleic acids have been available for many years (1). Analysis of oligodeoxyribonucleic acids by electrophoresis is the classic separation method that usually is performed using an entangled or crosslinked matrix. Separation is usually size-based, with smaller oligodeoxyribonucleic acids traveling faster than larger strands. Detection in these matrices usually relies on a stain (i.e., ethidium bromide), radioactivity, or may be performed by direct ultraviolet (UV) shadowing. High-performance liquid chromatography (HPLC) has also been a reliable method for separation and analysis of oligonucleotides. Quantitative methods for nucleic acids have used UV (which can provide good quantitation for pure samples) as well as derivatization of fluorescent dyes for samples that did not contain pure nucleic acid (2). HPLC analysis is generally quite good when the oligodeoxyribonucleic acid is short (~12–15 nucleotides in length), and when the phosphate-sugar backbone is unmodified. With the advent of the development of antisense oligonucleotides, specifically phosphorothioate oligodeoxynucleotides, as therapeutic agents, a new set of analytical challenges emerged. Classic electrophoretic methods generally still provide quite good separation of phosphorothioate oligodeoxyribonucleic acids, but HPLC methods often fail to provide single-nucleotide resolution. Additionally, as phosphorothioate oligodeoxynucleotides were examined as therapeutic agents, quantitation and identification of the parent oligonucleotide and its metabolites was required. To accomplish this, the development of more sensitive nonradiolabeled methods became imperative.

The ideal method for analysis of a xenobiotic moiety and its metabolites would be one with adequate sensitivity to detect the terminal elimination half-

life, would require little or no sample preparation, and would accurately separate, identify, and quantify all significant metabolites in addition to the parent compound. A short analysis time is also required to analyze the large number of samples required for a good understanding of the pharmacokinetics and potential toxicokinetics of a given compound. Determination of the levels of xenobiotics and their metabolites from in vivo samples has become routine for many classes of therapeutic compounds, especially low-molecular-weight therapeutics. Routes of metabolism, often by cytochrome P-450 monoxygenases, are readily delineated. Separation of the parent compound and concomitant metabolites from biological matrices is often accomplished using liquid-liquid or liquid-solid extraction followed by GC, LC, or LC-MS analysis. Alternatively derivatization techniques are also used to quantify the in vivo concentrations of xenobiotics.

The analysis of phosphorothioate oligodeoxynucleotides has progressed rapidly during the past decade. Initial analyses were highly dependent on following radiolabel and separations and used traditional polyacrylamide gel electrophoresis (PAGE) or HPLC methods. Although some of these methods provided adequate separation of the chain-shortened metabolites that result from exo- and endonucleolytic cleavage of the oligonucleotides (Fig. 1), they did not provide adequate sensitivity and complete structural elucidation of the metabolites. For example, an oligonucleotide one nucleotide smaller than the parent molecule might have lost that nucleotide from either the 5′ or the 3′ end of the molecule. Traditional length-based separation technologies cannot distinguish the two metabolites. Additionally, the loss of a sulfur, the xenobiotic moiety of the molecule, cannot be detected using length-based separation methods. More recently, newly applied methodologies have begun to address these limitations and provide more insight into the metabolites that are formed in vivo. In fact, more recent work has used LC-MS to characterize not only the disposition but the exact nature and character of the metabolites and there are initial indications that this methodology will be adapted to quantify the concentrations of the oligonucleotides. This review will critique the methods used for bioanalysis (Table 1) in terms of how close the method is to the putative ideal method as well as how the pharmacokinetic and metabolism information generated is consistent with generally accepted results in the field.

Figure 1 Length-based analysis of the metabolism of phosphorothioate oligodeoxynucleotides is consistent with exonucleolytic cleavage, from either the 3′ or the 5′ end of the molecule. Exonucleolytic cleavage from the 5′ end of the molecule would liberate a nucleoside (A), leaving behind an oligonucleotide with a 5′ phosphorothioate. A 5′ phosphatase would rapidly cleave the 5′ thiophosphate from the oligonucleotide (B). Exonucleolytic cleavage from the 3′ end of the molecule would liberate a 5′-nucleoside monothiophosphate (C).

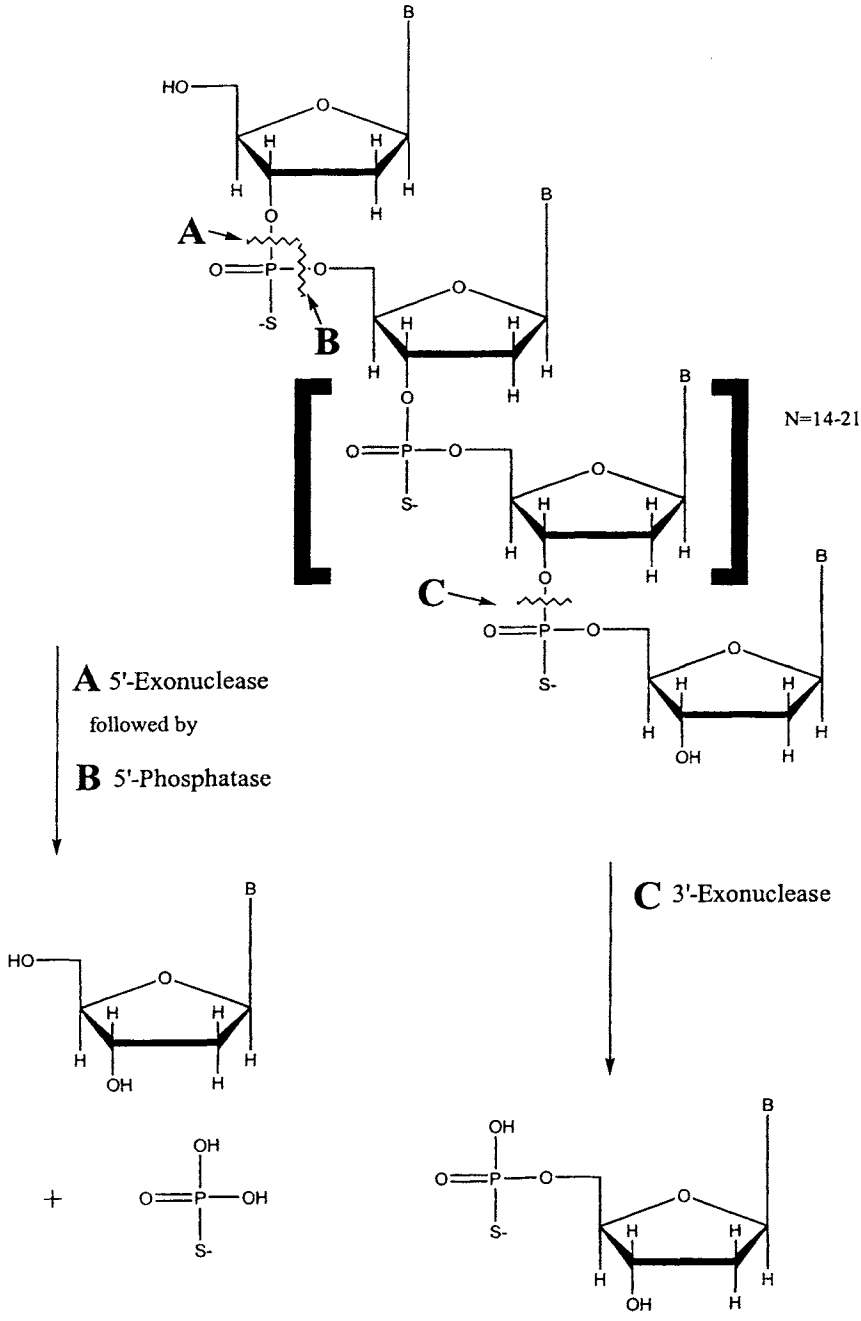

Table 1 Methods Used for Analysis of Phosphorothioate Oligodeoxynucleotides from Biological Matrices after In Vivo Studies

Method	Selectivity	Limit of detection	Comments	Ref.
HPLC-reverse phase	Not defined	~170 ng/mL	Resolution between oligonucleotides of different length not demonstrated; UV detection demonstrated	3
HPLC-anion exchange	N-2	16 ng/mL	Three-step liquid-liquid extraction, no selectivity for N-1; metabolites with oxidized backbones coeluted with chain-shortened metabolites	4
Radioactivity/PAGE gel	N-1	Not defined	Good selectivity for chain-shortened and elongated metabolites; no selectivity for oxidized backbones; no quantitation	6
Radioactivity/HPLC	N-2	Not defined	Radioactivity used in conjunction with HPLC gave general picture of metabolism; no quantitation	18
Capillary gel electrophoresis–UV	N-1	70 ng/mL	Good selectivity for chain-shortened metabolites; UV detection allowed direct determination of metabolites; no separation of oxidized metabolites from parent	33
Capillary gel electrophoresis–fluorescence	N-1	1 ng/ml	Selectivity and sensitivity good but not obtained simultaneously as fluorescent indicator was quenched in the presence of denaturants that separated full-length phosphorothioate oligodeoxynucleotide from N-1 metabolite	43
Cloning and sequencing	N-1	Not defined	Used to identify but not quantify chain-shortened metabolites; no demonstration of ability to identify all major metabolites	47
Hybridization	N-3	~20 ng/mL	Hybridization-based assays did not have selectivity for either chain-shortened metabolites or metabolites with oxidized backbones	48, 49
Mass spectrometry	N-1	Not defined	Used to identify chain-shortened metabolites as well as chain-elongated metabolites; no demonstration of ability to identify all major metabolites; currently qualitative rather than quantitative	54, 55, 62

II. HPLC ANALYSIS

Some of the earliest attempts to quantify oligonucleotides in biological fluids used HPLC after proteinase K digestion followed by liquid-liquid extraction. Bigelow et al. (3) used tetrabutylammonium dihydrogenphosphate as an ion-pairing agent with a C18 reverse-phase polymeric column. An acetonitrile gradient separated the oligonucleotide from impurities in the sample. However, the 28-mer and 4-mer oligonucleotides were not well resolved from a phenol peak generated during the phenol-chloroform extraction and, in fact, eluted on the tail of the large interfering peak. The authors showed the UV signal obtained from a 63-nM concentration sample injected out of water and suggested that by injecting a larger volume of sample they might obtain signals for samples with concentrations in the range of 20 nM. Although the separation was not optimal, the authors did demonstrate the possibility of adequate sensitivity by ultraviolet detection after extraction of in vivo samples.

Bourque and Cohen (4) digested extracts with proteinase K and then used three sequential extractions: the first with phenol-chloroform-isoamyl alcohol, the second with sec-butanol to concentrate the sample, and the third with diethyl ether to remove the sec-butanol. After initial examination of the extracted oligonucleotide, 2 mM DTT was added to the extraction solvents to minimize oxidation believed due to trace peroxides in the extraction solvents. To separate the oligonucleotides of different length they found that a nucleopak PA-100 strong anion exchange HPLC column (50 mm $\times$ 4 mm ID) gave the best resolution. A short linear gradient from low to high salt was used to separate the different length oligonucleotides. By adding a 10-mer phosphorothioate oligonucleotide as an internal standard they were able to normalize the results to account for variable recovery of the oligonucleotide and variation in the volumes of different extracts. Their estimated limit of detection was 4 ng of sample, more adequate for pharmacokinetic studies than the first-reported HPLC method. Although anion-exchange HPLC does not provide single nucleotide separation for oligonucleotides as the length approaches 20 nucleotides (5), the authors did have length-based separation.

Plasma oligonucleotide concentrations have been quantified directly without sample extraction, by using an anion-exchange chromatography. A HEMA anion-exchange column (50 mm $\times$ 4.6 mm ID) was equilibrated in 80 mM Tris-HCl, pH 8.0, containing 0.29 M NaCl with 5.8% acetonitrile. A combination of nonlinear gradients over 45 min to approximately 70 mM Tris-HCl, pH 8.0, with 1.3 M NaCl and 17% acetonitrile provided maximal separation between phosphorothioate oligonucleotides 6–18 nucleotides in length (Fig. 2, top) although longer oligonucleotides were not resolved. Because of the initial high salt concentration, nearly 0.3 M NaCl, nearly all UV-visible components in filtered plasma eluted quickly while the oligonucleotide eluted at 46 min (Fig. 2, bottom). How-

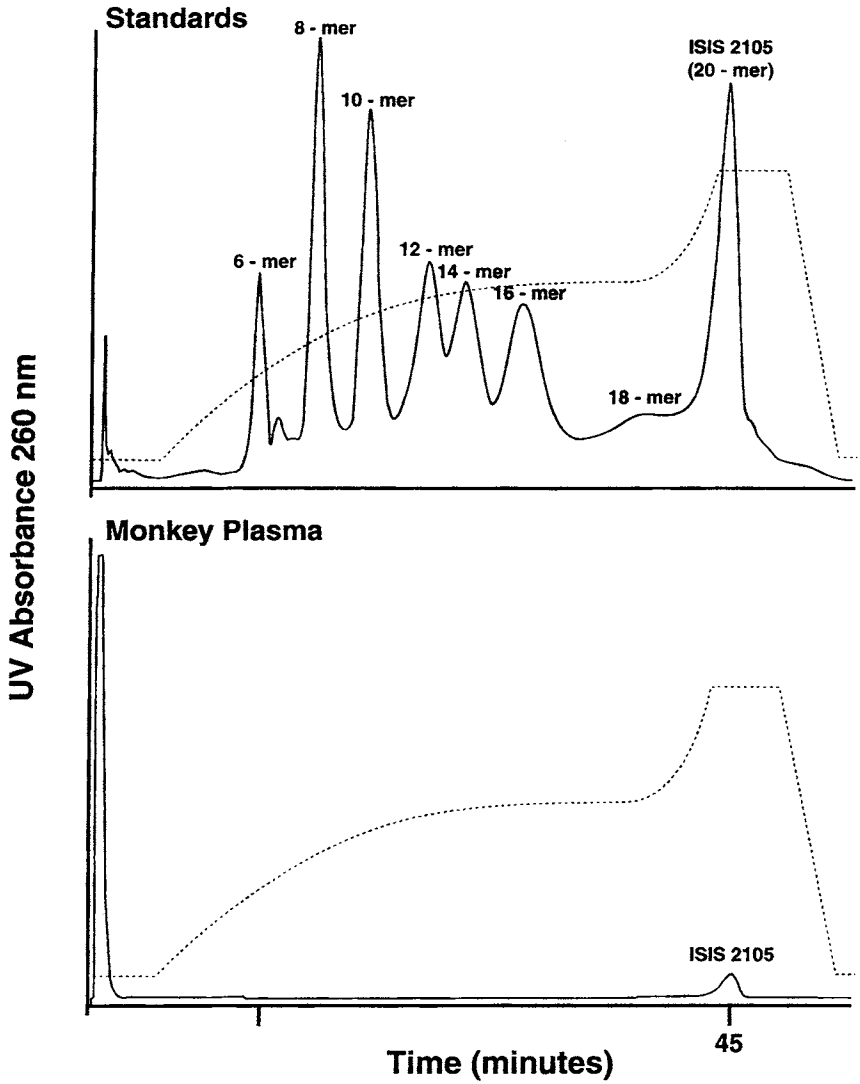

Figure 2 Using anion-exchange chromatography standards of phosphorothioate oligodeoxynucleotides ranging in length from 6 to 18 nucleotides are separated (top). The 18-mer and 20-mer phosphorothioate oligodeoxynucleotides are separated, but the 19-mer phosphorothioate (not shown) coelutes with the 20-mer phosphorothioate oligodeoxynucleotide. The phosphorothioate oligodeoxynucleotide in plasma is completely resolved from UV-absorbing components in plasma (bottom). The broad peaks seen using this separation method are the main reason that the sensitivity is poor.

ever, using a long gradient with a relatively large column resulted in significant peak broadening, limiting the sensitivity of this rather simple and convenient method. A standard curve showed that the method was reliable and linear for quantitation of samples of between 10 and 1000 μg/mL (r^2 = 0.9996). Although this might be adequate to determine the approximate concentration of oligonucleotide shortly after injection of relatively high amounts of oligonucleotide, it would not provide the sensitivity required for the analysis of samples used to define and understand the pharmacokinetics of the compounds, such as the terminal elimination half-life. Additionally, the method did not provide adequate resolution of metabolites from the parent compound and thus could not be used to study or understand the metabolism of phosphorothioate oligodeoxynucleotides.

These three HPLC methods had different approaches for both sample preparation and analysis. The method with the least amount of sample preparation, filtering of plasma followed by direct injection on the anion-exchange HPLC column, had the highest limit of quantitation, making it extremely convenient but not particularly useful. Using proteinase K digestion followed by phenol-chloroform-isoamyl alcohol extraction might have proved more useful if either the HPLC method had provided better separation between the residual phenol peak and the oligonucleotide or if the residual phenol had been eliminated. The most sensitive and pharmacokinetically useful method (4) required the most extensive sample preparation, yet it still did not provide good separation between the parent compound and closely related metabolites, such as those smaller than the parent by only one nucleotide. Although these HPLC methods might be further optimized, there appear to be limitations inherent in the selectivity of the columns and buffer systems examined that would preclude accurate analysis of closely related metabolites (i.e., N-1 metabolites).

III. RADIOLABELED OLIGONUCLEOTIDES

The earliest pharmacokinetic studies of phosphorothioate oligonucleotides relied on radiolabel for following the disposition of the molecules (6,7). Although the distribution of total radioactivity can be measured by oxidation of the radiolabel, most investigators supplemented this basic analysis with either PAGE or HPLC analysis to derive some picture of the metabolism. However, this additional analysis of tissue samples required sample extraction. Agrawal et al. (6) published a method for the extraction of phosphorothioate oligodeoxynucleotides from tissue using 2 mg/mL proteinase K with 0.5% SDS in 20 mM Tris-HCl, pH 7.6, with 10 mM NaCl, and 10 mM EDTA. After a 2-h incubation at 37°C in the proteinase K digestion buffer the nucleic acids were separated from lipids and digested protein using phenol-chloroform extraction. After ethanol precipitation the radiolabeled oligonucleotide was analyzed using a 20% polyacrylamide gel with 7 M

urea. Although this would be a standard extraction for nucleic acids, no controls were shown or mentioned in regard to the recovery of the different lengths of phosphorothioate oligodeoxynucleotides through the ethanol precipitation. Ethanol precipitation might precipitate different lengths or sequences of phosphorothioates with differing efficiency and the apparent metabolism might inaccurately reflect the true metabolism. In addition, the apparent metabolites seen using slab gel analyses were highly dependent on the type and position of the radiolabel on the oligonucleotide. Goodarzi et al. (7) used ^{32}P-end-labeled phosphorothioate oligonucleotide in an in vivo study and found no evidence of intact oligonucleotide in liver 30 min after intravenous administration. In contrast Agrawal et al. (6) saw a substantial proportion of intact phosphorothioate oligonucleotide in both kidney and liver in mice 24 h after administration of the oligonucleotide. As that oligonucleotide was uniformly labeled with ^{35}S, the difference in the results between the two studies may be attributable to phosphatase cleavage of the end-labeled phosphate in the Goodarzi study. Using uniformly labeled ^{35}S-oligonucleotide Agrawal et al. (6) observed metabolites consistent with processive chain shortening as well as longer compounds, putatively due to nucleotide addition (6). Agrawal did not attempt to quantify the oligonucleotide or its metabolites using this methodology. Because of their lack of controls to demonstrate the relative recovery of different-length oligonucleotides after ethanol precipitation their results may indicate which metabolites are present in tissues without being indicative of the relative proportions.

Unfortunately not all researchers using radiolabel followed by slab gel analysis of metabolites used uniformly labeled oligonucleotide and their ambiguous results muddied the field. A comparison of the disposition of oligonucleotide postsynthetically labeled on either the 5′ or the 3′ end of the molecule consistently indicated a very short plasma $t_{1/2}$ for the oligonucleotide (8). However, the consistent results was very likely the result of two independent rapid degradations, a 5′ phosphatase removing the terminal 5′ phosphate, and a 3′ exonuclease removing the 3′ terminally labeled nucleotide (8). Unless determination of intact oligonucleotide is made independently, the results of studies with radiolabeled oligonucleotides often fail to enhance understanding of the disposition of these compounds.

Using uniformly radiolabeled oligonucleotide Oberbauer et al. (9) followed total radioactivity by counting samples, separated chain-shortened metabolites in tissues, plasma, and urine using slab gels, and examined the in vivo cellular distribution of the radioactivity using autoradiography. The goal of the study was to localize oligonucleotide within the kidney and determine the integrity of the oligonucleotide after cellular uptake. But neither measurements of total radioactivity nor autoradiography would distinguish low-molecular-weight metabolites from longer oligonucleotides, and the low-molecular-weight metabolites may have distributed differently than the parent molecule or the chain-shortened mole-

cules. Thus although autoradiography could pinpoint the subcellular location of radioactivity, this radioactivity was derived from the oligonucleotide, but was not necessarily currently associated with an oligonucleotide. Another label that has been frequently used in pharmacokinetic studies is ^{3}H-oligonucleotides that have been generated using a tritium-exchange method that labels the C8 position of purines (10). However, the label can exchange with water after extended incubation ($t_{1/2}$ approximately 2 months) at 37°C. In published studies using this label care has been taken to limit the duration of the study to the time before which tritium exchange with water becomes significant (11,12).

Radiolabel on the 5′ end of phosphorothioate oligonucleotides for in vivo pharmacokinetic studies (7,8) has resulted in data that are inconsistent with those generated using uniformly radiolabeled oligonucleotides (6,9,11,13). However, 5′-end labeling has been successfully used as a technique for analysis of oligonucleotides after extraction (14). After extraction the end-labeled oligonucleotides were separated on a 20% polyacrylamide slab gel and phosphorimager technology used to visualize the samples (14). Although this approach appeared to give a picture of metabolism consistent with the results of studies using uniformly radiolabeled oligonucleotide and end-labeling oligonucleotide is a standard molecular biology technique, there is no literature supporting its use for the quantitative analysis of phosphorothioate oligonucleotides. There are no data that show whether the efficiency of end-labeling is dependent on length or sequence, making the information attained regarding metabolism more qualitative than quantitative.

Researchers have also extracted nonradiolabeled oligonucleotides from biological fluids and tissues, separated metabolites from parent compound using slab gels, and then hybridized the oligonucleotides in the gels to radiolabeled complementary probes (15,16). Probing gels with radiolabeled complement avoids the problems associated with using radiolabeled oligonucleotides during in vivo studies. Standard curves done in the appropriate biological matrix suggest that this methodology can give good estimates of the amount as well as types of molecules remaining in a given biological matrix after in vivo administration (16). The apparent disadvantages of this method are the tedious extraction and the low throughput inherent to slab gel and hybridization methodologies.

Temsamani et al. (17) used complementary probes to hybridize with extracted oligonucleotides but did not initially separate the metabolites from the parent compound using polyacrylamide slab gels. Rather, all oligonucleotides present in the extracted sample were transferred to a nylon membrane using a standard blotting procedure. The amount of oligonucleotide bound to the membrane was then determined by hybridization with either ^{32}P-oligonucleotide or digoxigenin-11-ddUTP. The ^{32}P-oligonucleotide was easily detected using autoradiography while the digoxigenin-11-ddUTP was visualized using an antidigoxigenin alkaline phosphatase antibody conjugate with a chemiluminscent substrate

for the alkaline phosphatase (17). As with the previously discussed hybridization procedure, this method appeared to be quantitative for extracted samples. The authors recommended this procedure for oligonucleotides of 20 nucleotides in length or above because of the low efficiency of membrane binding by smaller oligonucleotides. Thus, this method would allow for combined concentrations of parent oligonucleotide together with an unknown proportion of smaller metabolites, but would not differentiate or identify the relative proportions of parent compound and multiple metabolites.

Slab gel analysis of radiolabel provides length-based separation of oligonucleotides of different length, and radioactivity can be used as a method of detection; however, the method is slow, tedious, and not easily automated. Alternatively, biological fluids and tissues have been analyzed for residual oligonucleotide levels after treatment with radiolabel oligonucleotides by HPLC. This, in fact, has probably been used more extensively for analysis than any methodology for the analysis of radiolabeled phosphorothioate oligonucleotides (13,18–21). Very likely this technique has been widely used because HPLC is relatively inexpensive and easily automated, widely available, and a well-established technology. Automation, which slab gels do not allow, has greatly enhanced the throughput possible for analysis of samples from pharmacokinetic studies. Again, as was discussed in the previous section, a critical factor affecting the reliability of the results obtained is the position of the radiolabel on the phosphorothioate oligonucleotide. That is, if the radiolabel on the oligonucleotide is not stable then the results derived from the radiolabel in combination with any other methodology are useless. Fortunately, most work done using the HPLC in combination with radiolabeled oligonucleotide has used radiolabel that is continuously associated with the oligonucleotide.

Anion-exchange HPLC methods have been used by a number of researchers (18,21–24). The columns separate phosphorothioate oligonucleotides based on length but do not provide baseline separation between oligomers of greater than 18 nucleotides in length (5). In addition anion-exchange columns separate oligomers based on the oxidation state of the backbone (4). That is, phosphorothioate compounds that have lost one or more of the sulfur substitutions from their backbones elute earlier on anion-exchange HPLC columns than fully thiated backbones. Thus, although using anion-exchange HPLC to follow radiolabeled phosphorothioate oligonucleotides is convenient, because the methodology can be automated, the lack of separation of different metabolites makes the methodology less than optimal. Nonetheless, anion-exchange HPLC analysis does provide a general picture of metabolism of phosphorothioate oligonucleotides. Ion-paired reverse-phase HPLC methods have also been combined with radiolabel in pharmacokinetic studies to generate a general picture of metabolism (13,19,25,26). As was observed with anion-exchange HPLC methods, reverse-phase HPLC methods do not resolve longer phosphorothioate oligonucleotides (13).

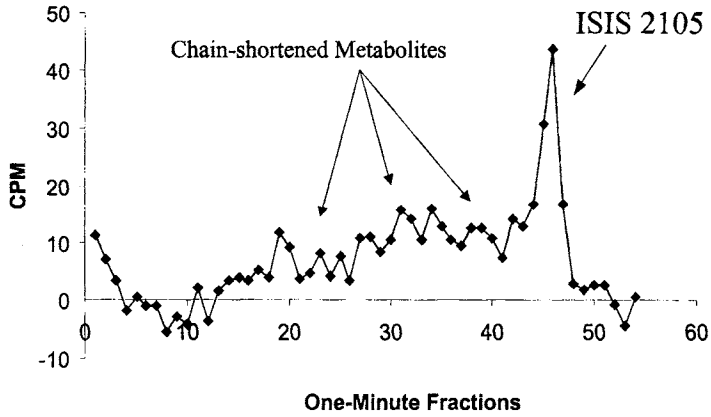

Figure 3 Using radiolabeled phosphorothioate oligodeoxynucleotide in combination with anion-exchange chromatography allows determination of the relative amounts of parent compound and chain-shortened metabolites, although good selective quantitation of each metabolite cannot be achieved. Forty-eight hours after intradermal administration of ^{14}C-labeled ISIS 2105 into the genital wart of a human, the parent 20-mer phosphorothioate oligodeoxynucleotide persists, although the radioactivity eluting earlier than the parent compound indicates that there is substantial metabolism. (Reprinted with permission of Mosby-Year Book, 1994).

With both types of HPLC column methods the analyses revealed that the radioactivity derived from the phosphorothioate oligonucleotide began to elute with smaller oligonucleotide pieces after in vivo administration (Fig. 3); that is, metabolism appears to generate chain-shortened oligonucleotides. Additionally, both methods for HPLC analysis are able to capture the monomeric units that are putatively liberated from the oligonucleotide during exonucleolytic cleavage (13,18,21,23). Because the resolution of different metabolites using these methods is limited, the utility of HPLC analysis of radiolabeled extracts is limited to a gross understanding of metabolism.

IV. DIRECT CELLULAR VISUALIZATION METHODS

A number of researchers have combined the information obtained from the gross disposition of the radiolabel, the slab gel, or HPLC analysis, which gives a general picture of the integrity of the oligonucleotide, with autoradiography, which provides information on the suborgan distribution of radiolabel derived from the oligonucleotide. If analysis of the metabolism by slab gel of HPLC analysis

shows that a substantial portion of the radioactivity remains associated with the parent compound or primarily longer oligomers, then the autoradiography can provide an indication of the suborgan disposition of the oligomers (13,27). However, in all cases where radiolabel has been analyzed the phosphorothioate oligodeoxynucleotide does not remain 100% intact; low-molecular-weight entities will also be seen in the autoradiography and may skew the apparent distribution (13). Sands et al. (13) showed a big difference in the distribution of radiolabel within the kidney between phosphorothioate and phosphodiester oligodeoxynucleotides, but by HPLC also showed that the phosphorothioate, but not the phosphodiester, was largely intact. Without the accompanying HPLC analysis to distinguish intact oligomer from highly degraded, the conclusions reached based on autoradiography alone would be misleading. Alternative methodologies such as using a fluorescently tagged oligonucleotide (28) might circumvent the probability that the signal observed is not a low-molecular-weight metabolite, but they do not demonstrate that the fluorescent signal is associated with the oligonucleotide. The additional unknown is whether or not the conjugated fluorescent molecule alters the subcellular distribution of the oligonucleotide. A third method for examining the suborgan and potentially subcellular distribution of phosphorothioate oligonucleotides is to do immunohistochemical staining (28). Overall staining sections of tissues can give only limited information regarding the amount of oligonucleotide in a given location, but may be complementary when used in conjunction with other methods.

V. CAPILLARY ELECTROPHORESIS

Despite early reports of success using HPLC for analysis of nonradiolabeled oligonucleotide concentrations in biological fluids, the early attempts to follow the disposition and metabolic fate used, almost exclusively, radiolabel as a method to detect the oligonucleotide. In addition to the difficulties of finding appropriate analytical tools to elucidate clear unambiguous information using these radiolabeled compounds, the cost of using radiolabel in larger animal and human studies was prohibitive. Thus, alternative methods were clearly needed. Capillary electrophoresis first emerged as an analytical tool for resolution of nucleic acids of different length in the late 1980s (29). However, the application of capillary electrophoresis to the separation and quantitation of phosphorothioate oligonucleotides, especially from biological matrices, required the resolution of several problems.

When scientists attempted to apply capillary electrophoresis technology to the separation of phosphorothioate oligonucleotides, they observed substantial peak broadening that decreased the resolution obtained. Improved resolution of

the phosphorothioate oligonucleotides by length was achieved by increasing the theoretical plates of the gel-filled capillary (30,31). Another issue preventing the use of capillary electrophoresis for quantitation was that electrokinetic injections can be strongly affected by the composition of the buffer precluding a linear response of sample concentration from a given voltage and length of injection. Srivatsa et al. (32) showed reproducible quantitation could be obtained by normalizing peak area of the unknown to an internal standard, an oligomer of similar length that was adequately separated from the parent compound. The ability to analyze samples from biological matrices, however, was dependent on finding a method to extensively purify the oligomer and metabolites from endogenous impurities that would interfere with the electrokinetic injection of the oligonucleotide onto the capillary as well as with the UV detection.

The method initially reported for analysis of phosphorothioate oligodeoxynucleotides from biological matrices (33) prepared samples for gel-filled capillary electrophoresis analysis using an extensive two-step solid-phase extraction method. The first solid-phase cartridge was an anion-exchange cartridge that purified the oligomer and metabolites from proteins and possibly lipids. However, elution from anion-exchange columns required extremely high concentrations of salt and thus a second solid-phase extraction column, a reverse-phase column, was required to desalt the material as eluted from the anion-exchange cartridge. Although not initially used, an additional membrane desalting step increased the eventual capillary electrophoresis signal so much that its use was incorporated into the method. Minor adjustments to this method have allowed purification of metabolites to different degrees such that oligonucleotides as small as 5 nucleotide units can be extracted equivalently with the internal standard and therefore quantified (11). Combining methods previously developed for the extraction of tissues for slab gel analysis (6) with solid-phase extraction methods (33) enabled researchers to analyze tissue in addition to biological fluids from pharmacokinetic studies using gel-filled capillary electrophoresis (34–40). The metabolism of phosphorothioate oligonucleotides seen using this method (Fig. 4) indicated continued exonucleolytic cleavage over time.

A good understanding of the pharmacokinetics and toxicokinetics of phosphorothioate oligonucleotides has been attained using solid-phase extraction followed by gel-filled capillary electrophoresis (37,41,42) but the method has limitations. The lower limit of UV quantitation, approximately 20 ng per sample, is too high to facilitate the determination of a terminal half-life. A terminal half-life was readily observed when following radiolabeled material (6,18), although the primary radioactivity remaining in blood at later time points may have been lower-molecular-weight metabolites and not parent compound. Additionally, the solid-phase extraction, even when automated, is time-consuming, requiring extensive resources for the analysis of a large number of samples.

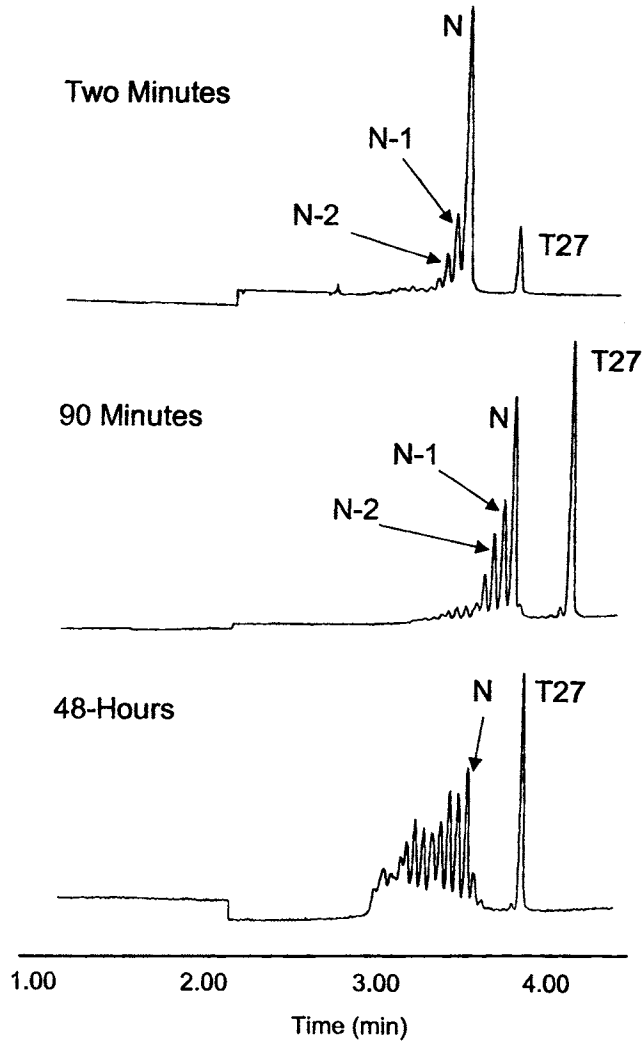

Figure 4 Using UV absorbance for detection of nonradiolabeled phosphorothioate oligodeoxynucleotides separated by gel-filled capillary electrophoresis allows nearly baseline resolution and detection of chain-shortened metabolites. Quantitation is achieved using an internal standard that extracts equivalently with the parent oligonucleotide (T_{27}, a 27-mer thymidine oligonucleotide in this instance). Using this methodology metabolism of ISIS 5132, a 20-mer phosphorothioate oligodeoxynucleotide was followed in kidney after intravenous administration. Metabolism was clearly evident even 2 min after administration and the metabolites observed increased in number and relative quantity for 48 h after administration.

VI. FLUORESCENT CAPILLARY GEL ELECTROPHORESIS

Reyderman and Stavchansky (43) attempted to address both issues of sensitivity and throughput that were problematic for gel-filled capillary electrophoresis analysis of phosphorothioate oligodeoxynucleotides by using fluorescence detection for quantifying the compounds in biological fluids. The authors introduced a dye that specifically fluoresced only when bound to single-stranded DNA, oligreen. Using this dye they attempted to inject plasma extracts containing phosphorothioate oligonucleotides after a simple phenol-chloroform for extraction. Their rationale was that although less oligonucleotide might be injected onto the capillary using an electrokinetic injection when the sample was less pure (because of impurities competing during the electrokinetic injection), the increased sensitivity due to the fluorescent detection might allow a net gain in sensitivity. However, the observed peaks of phosphorothioate oligonucleotides were too broad without the addition of denaturant to provide the advantageous single-base resolution obtained by CGE. Addition of denaturants such as urea, formamide, and guanidinium thiocyanate significantly quenched the fluorescent signal. Thus, the requirement of denaturants to provide single-base resolution of phosphorothioate oligonucleotides negated the sensitivity promise of this fluorescent method.

Gray and Wickstrom (44) added the oligreen dye directly to plasma and detected fluorescence using a 96-well-plate format with a fluorescent plate reader. Although the limit of quantitation was reasonably low, 15 nM, the method did not provide length selectivity. Smaller oligonucleotides were detected at a fraction of the levels of full-length oligonucleotide and thus the final measurement was an aggregate value that did not represent full-length oligonucleotide, nor did it represent total oligonucleotide. Rather, the derived value represented full-length phosphorothioate oligodeoxynucleotide as well as an unknown proportion of various smaller oligonucleotides. Assuming metabolism were consistent in different species, this method might be useful for toxicokinetic understanding, but would not seem to be the definitive method for defining the pharmacokinetics of the parent molecule.

Cohen and co-workers (45) developed a method that allowed for the sensitivity of fluorescence detection to be exploited. Extracted oligonucleotides were end-labeled with phosphate on the 5′ end, hybridized to a complementary oligonucleotide, and then ligated to an oligonucleotide with a fluorescent tag on the 5′ end of the molecule. Using this method these researchers were able to capture all metabolites that had intact 5′ ends of oligonucleotide, but would not capture any molecules in which the 5′ end had been cleaved. As a method to confirm the identity of the metabolites that they were able to ligate, they analyzed the molecules by MALDI-TOF. This confirmed that they could detect metabolites from the 3′ end of the molecule in an in vitro study of oligonucleotide stability. Using the same two techniques the researchers were able to distinguish metabo-

lites from the 3′ and 5′ ends of the molecule in plasma taken from monkeys chronically administered the phosphorothioate oligonucleotide. Although the fluorescent signal appeared to accurately reflect the UV signal, Cohen and coworkers did not discuss the potential use of this method for quantifying phosphorothioate oligonucleotides. The same method was also used by Gilar et al. (46) to characterize the kinetics of in vitro metabolism in biological fluids. Although the fluorescent labeling achieved by ligation may well provide a good and sensitive method for quantitation of phosphorothioate oligonucleotides, evidence for accurate and reproducible quantitation was not presented in either paper. The necessity of performing two enzymatic reactions to incorporate the fluorescent tag into the molecule may limit the number of samples that one could analyze using this methodology and may limit the ability of the method to accurately quantify oligonucleotides.

VII. CLONING AND SEQUENCING

Nucleic acid analysis is a well-developed field that continues to advance rapidly. Clearly, scientists exploring more robust and rapid methods for the analysis of phosphorothioate oligonucleotides have explored many standard nucleic acid analysis techniques as alternative methods for the analysis of phosphorothioate oligonucleotides. One of the more elegant, but time-consuming, methods involved insertion of extracted oligonucleotides into plasmids. Temsamani and coworkers (47) inserted the phosphorothioate oligodeoxynucleotides into the plasmids by first adding a 5′ phosphate to the molecules using T4 polynucleotide kinase and then adding a polyA tail using dATP and terminal deoxynucleotidyl transferase. The plasma pSL1190 was cleaved and a polyT tail added. The oligonucleotide and plasmid were annealed using the polyA-polyT tails, and the complementary strands synthesized using standard methodology. Cells were transformed with the plasmids to allow amplification, followed by sequencing of the region of each plasmid containing the extracted oligonucleotide metabolites using standard dideoxy chain-termination sequencing methods (47). Although this method might be useful in confirmation of metabolite identification obtained by other methods, the labor involved in this technique makes it extremely unlikely that the method would be useful for the high number of samples typically analyzed in a single pharmacokinetic study.

VIII. HYBRIDIZATION

A much simpler approach to quantitation of phosphorothioate oligonucleotides in biological fluids was the scintillation proximity assay developed by de Serres

et al. (48). The competitive hybridization method used a complementary oligonucleotide bound via a biotin linkage to a streptavidin-derivatized scintillation proximity bead. A constant concentration of the phosphorothioate oligodeoxynucleotide with ^{3}H radiolabel was added to the reaction mixture along with plasma containing an unknown concentration of phosphorothioate oligodeoxynucleotide. After an overnight incubation to allow equilibrium hybridization, the concentration of the phosphorothioate oligonucleotide in plasma was calculated from a standard curve. The greater the concentration of the nonradiolabeled phosphorothioate oligonucleotide, the lower the measured radioactivity. Because scintillation proximity assays are homogeneous systems and this assay required no sample preparation, the potential throughput for plasma samples was quite high. The lower limit of quantitation was set at 21 ng/mL, slightly lower than that obtained with solid-phase extraction followed by CGE analysis. The disadvantage of this method is a lack of specificity for full-length oligonucleotide, as chain-shortened metabolites also bind the complement, although with reduced efficiency. This competitive hybridization method would be advantageous as a high-throughput method for a rough estimate of total oligonucleotide in plasma but cannot be used to quantify full-length oligonucleotide in biological matrices.

A similar competitive hybridization assay was developed using an alternate detection system (49). The researchers used a 96-well-plate format with a complementary oligonucleotide immobilized on the plate. In lieu of a radioactive competitive oligonucleotide they used a biotinlyated oligonucleotide. As above, they generated a standard curve by the addition of increasing concentrations of nonbiotinylated phosphorothioate oligodeoxynucleotide in plasma. They detected the biotinylated oligonucleotide using a streptavidin-acetylcholinesterase conjugate followed by the addition of a substrate for acetylcholinesterase that formed color after cleavage. The lower limit of detection for this assay for phosphorothioate oligonucleotides was similar to that determined for the SPA, approximately 4 nM (approximately 20–30 ng). Both hybridization assays were limited in the limit of detection by the binding affinity (K_d) of the oligonucleotide for the complement, on the order of 10^{-8}, or low-nanomolar range. As was the case for the scintillation proximity assay, this hybridization assay did not have specificity for the parent compound alone and cross-reacted with chain-shortened oligonucleotides. However, both assays provided high throughput estimates of the concentration of the phosphorothioate oligonucleotides in biological fluids without extensive sample preparation.

IX. MASS SPECTROMETRY

Although mass spectrometry is perhaps the most commonly used analytical tool for the analysis of xenobiotics and metabolites, it took several years for the appli-

cation of this technique to analysis of phosphorothioate oligodeoxynucleotides isolated from biological fluids and tissues. Traditional electrophoretic, hybridization, and chromatographic methods provide quantitative data for measuring oligonucleotide drug levels in biological matrices. Some of these analytical methods also afford a reasonable separation and quantitation of parent oligonucleotide and metabolites. The identity of these species is inferred from their chromatographic, electrophoretic, or hybridization characteristics. However, a more detailed identification of the products of metabolism is not possible with most of these methods. For example, the slowest migrating peak in a polyacrylamide slab gel, or in a CGE electropherogram, is inferred to be the parent oligonucleotide, and the next slowest peak an N-1 species, and so on. An N-1 species could, however, be formed by removal of either the 3′- or the 5′-nucleotide by exonucleases. Both N-1 species would have similar electrophoretic migration characteristics. Assuming that the 3′- and 5′-terminal residues are different, unique masses would be detected. If these residues were the same, sequencing would be needed to differentiate between 3′-exo and 5′-exonuclease activity. The loss of a base or the replacement of a sulfur by oxygen in the internucleotide linkage is another potential metabolic product. Using traditional techniques, these species would not be readily identifiable. Oligonucleotide metabolites with slower electrophoretic migration times than that of the parent have been reported, but were not identified with traditional analytical techniques.

The development of mass spectrometry for the analysis of larger biomolecules (oligonucleotides and proteins) was dependent upon the advent of relatively mild ionization techniques. Two methods emerged that proved useful for oligonucleotide ionization: matrix-assisted laser desorption/ionization (MALDI) (50), and electrospray ionization (ESI). Production of ions by MALDI is accomplished by cocrystallizing a sample with a matrix, which absorbs energy from a laser. Upon laser irradiation, the crystalline matrix absorbs the laser's energy, which is translated into an ion plume composed of matrix and sample ions. Typically, a time-of-flight (TOF) detection system is then used to determine the mass. ESI, on the other hand, produces ions by spraying a solution containing oligonucleotide from the end of a capillary in the presence of a high voltage. Detection is commonly achieved by a quadrupole mass spectrometer. Mass measurement of phosphorothioate oligodeoxynucleotides typically is accurate to $\pm$ 0.01–0.1% at 10 kDa ($\pm$1–10 Da) using TOF or quadrupole detectors (51). Most expected metabolites are readily distinguished with this accuracy level.

Synthetic oligonucleotides were the subject of the early oligonucleotide mass spectrometry studies. The samples studied were generally normal, unmodified, oligodeoxynucleotides. The polyanionic character of the phosphate-sugar backbone of oligonucleotides was observed to have a high affinity for sodium ions. ESI and MALDI spectra of oligonucleotides were often composed of broad peaks and/or multiple peaks representing from one to many sodium adducts. The

presence of the cationic adducts made it difficult to determine the mass of the oligonucleotide, and the broad peaks resulted in a lower mass accuracy. Addressing this issue was paramount to making mass spectrometry a primary tool for the analysis of oligonucleotides.

Stults and Marsters (52) and Potier et al. (53) demonstrated that the use of ethanol precipitation after incubation with ammonium acetate minimized sodium adducts using ESI. The addition of various organic bases (triethylamine, trimethylamine, etc.) to the sample solution prior to spraying was shown to further reduce sodium adducts (53–56). Off-line dialysis using a floating membrane (57) and an on-line microdialysis device (58) was used for sample desalting prior to MALDI and ESI analysis, respectively. Huber and Buchmeiser (59) used an on-line cation-exchange cartridge, and demonstrated effective removal of sodium adducts from oligonucleotides. These methods enabled detection of oligonucleotide ions that were essentially free of cation adducts.

Unlike analysis of synthetic oligodeoxynucleotides where desalting is a major concern, mass spectrometry analysis of phosphorothioate oligonucleotides and metabolites from biological samples requires significant sample preparation. Sample preparation for mass spectrometry involves two crucial steps: (1) extraction from a biological matrix and (2) desalting of the sample to remove sodium and potassium ions that form adducts with the oligonucleotide. The two-step solid-phase extraction procedure used for CGE sample preparation (11,12,33) followed by more stringent desalting procedures as described earlier is typically used prior to mass spectrometry analysis. Early HPLC-ES/MS systems using neutral acetate buffers were able to reduce the level of sodium adducts and detect 5–7 nmol of a synthetic phosphorothioate oligonucleotide (56,60). Even with careful off-line desalting, metabolic oligonucleotide samples generally produce mass spectra containing numerous cation adducts, especially at low oligonucleotide concentrations.

Off-line desalting of phosphorothioate oligodeoxynucleotide metabolites was accomplished by Cohen et al. (61) by membrane dialysis followed by addition of cation-exchange resin to a MALDI matrix mixture. Phillips et al. (12) followed a two-step solid-phase extraction with membrane dialysis and addition of an organic base to displace cations prior to direct injection. To address this issue, Gaus et al. (55) developed an on-line HPLC-ES/MS method that produced nearly adduct-free spectra. This paper describes the use of a high-pH buffer containing an alkylamine to displace sodium and potassium adducts and to produce efficient electrospray ionization conditions. Because of the presence of many metabolites, these biologically derived samples produced quite complex ESI spectra (55,62). Although this method produced nearly adduct-free spectra, it did not separate individual oligonucleotide metabolites. It was found that better on-line metabolite separation could be attained when working at neutral pH, but at the cost of a concomitant loss in sensitivity (55,63). The mass separation afforded

by the quadrupole, however, was sufficient to identify 18 metabolites in one spectrum. This method improved detection sensitivity dramatically, as little as 10 pmol of metabolite was detected. These metabolites were found to be products of 3′-exo- and/or 5′-exonuclease activities. Off-line separation of oligonucleotide metabolites followed by on-line desalting enabled detection of a unique low-level metabolite not previously observed in more complex spectra (64).

Oligonucleotide separation and detection by ESI/MS was accomplished by Apffel et al. (63). They were able to overcome the sensitivity issue observed when using neutral buffers. Oligonucleotide separation at a neutral pH was demonstrated using triethylamine with 1,1,1-3,3,3-hexafluoro-2-propanol as the acid. They maintain that the volatile 1,1,1-3,3,3-hexafluoro-2-propanol evaporates rapidly during the electrospray ionization procedure, leaving a triethylamine/oligo solution. This is essentially the same condition that others had shown to be useful for reducing cation adducts (53–56). The resulting separations showed good oligonucleotide resolution and sensitivity and the spectra shown were relatively cation-free. A problem with interfering species from the 1,1,1-3,3,3-hexafluoro-2-propanol was observed, however. These background ions often appear at low m/z values and may or may not interfere with oligonucleotide analysis (63). This buffer system was first used on 2′-deoxyphosphorothioate metabolites by Griffey et al. (65). In this paper, phosphorothioate oligonucleotide metabolites were first fractionated off-line by anion-exchange chromatography. Each fraction contained several metabolites, which were further separated on-line by HPLC, then mass-analyzed using a quadrupole ion trap mass spectrometer. This paper describes the first mass spectrometry sequencing of phosphorothioate oligodeoxynucleotide metabolites, although Ni et al. demonstrated phosphorothioate sequencing using a triple-quadrupole mass spectrometer (66).

With adequate sample preparation, mass spectrometry is a powerful tool for the bioanalysis of phosphorothioate oligodeoxynucleotides. Mass spectrometry provides in-depth characterization of phosphorothioate metabolites that cannot be obtained from traditional methods. Further streamlining of sample preparation is needed to improve sample throughput, however. Mass spectrometry quantitation of phosphorothioate metabolites has yet to be accomplished, but should be an attainable goal. With improved sample preparation, on-line HPLC separation, and mass spectrometry identification and quantitation, mass spectrometry holds the promise of a high-throughput analytical tool that could supplant traditional phosphorothioate analytical methods.

X. NEEDED METHODS IMPROVEMENTS

Although substantial progress has been made in the bioanalysis of phosphorothioate oligodeoxynucleotides, improved methods are still needed. Many of the meth-

ods discussed in this review were clearly methods of necessity—an attempt to learn about the pharmacokinetics of the compound before an optimal method was available. None of the methods yet published can provide the sensitivity and selectivity required of an ideal method. Mass spectrometry is the only method to date that provides the desired selectivity, delineating chain-shortened metabolites from the 5′ and the 3′ end, as well as identifying molecules that have lost one or more sulfur molecules from the backbone or contain an apurinc site (55,62). As quantitation of smaller molecules using mass spectrometry has become routine, it is reasonable to assume that quantitation of phosphorothioate oligodeoxynucleotides using mass spectrometry will be possible in the near future. Although mass spectrometry methods may be developed, the current sample preparation procedures for mass spectrometry are extensive and time-consuming and hopefully improvements will be made in sample preparation methods as well.

Hybridization methods, which generally require little or no sample preparation, do not appear to have the needed selectivity; that is, they do not distinguish full-length from chain-shortened metabolites and certainly will not be able to distinguish oligodeoxynucleotides with backbones that have been partially oxidized. Currently none of the methods used for analysis have low enough limits of quantitation to define the terminal elimination half-life that can be observed when using radiolabeled phosphorothioate oligodeoxynucleotides. Automation of sample extraction prior to mass spectrometry or sensitivity and selectivity improvements in hybridization methods are methods development directions that might provide a closer realization of a method that would provide the sensitivity, selectivity, and simple sample preparation that would enable high throughput.

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4

A Role for Antisense Technology in the Discovery of Highly Specific and Versatile Signal Transduction Inhibitors

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I. INTRODUCTION

Defects in cell signaling pathways have been implicated in the etiology of most human diseases. Achieving an in-depth understanding of the complex mechanisms by which cells transduce extracellular signals into cellular responses in both normal and diseased systems is a critical step for the discovery of more effective drugs to treat human disease. Traditional approaches for studying cell signaling are severely limited for a variety of reasons. Antisense oligonucleotides (ASOs) represent a novel approach for studying signal transduction processes that offer significant advantages in terms of specificity and versatility. In this review, the advantages and opportunities that antisense technology offers for studying signal transduction pathways in mammalian cells and discovering signal transduction inhibitors for drug development are discussed.

Novel experimental approaches for determining the biological functions of cellular gene products have always been, and remain, one of the most intriguing and controversial subjects in biological research. Although significant progress has been achieved in the identification of new genes that are associated in one way or another with specific diseases, progress has generally been slow in determining the function of these genes in biological processes. This is largely due to the inherent difficulties associated with identifying relevant methods for determining gene function that offer acceptable specificity and do so in a time- and cost-effective manner. Furthermore, when success has been achieved in dem-

onstrating an essential role for a particular gene product in a disease process, the emergence of novel therapies that target the gene product and effectively treat the disease has been slow. This is largely due to the fact that most molecular or pharmacological approaches that are useful for determining gene function in preclinical models are not suitable as pharmaceutical agents for humans.

The need for novel methods to determine the function of specific gene products may be best exemplified in the field of signal transduction research. Virtually all diseases can be traced in some way to a defect in cell signaling. The number of regulatory proteins that have been shown or are believed to participate in the transduction of signals from the external environment into and through cells, resulting in cellular responses, is growing at a tremendous rate. Virtually every signal transduction protein that has been identified to date is just one member of a large multigene family that contains multiple isoforms, encoded by separate genes, which may possess similar but distinct cell signaling functions. Furthermore, additional isoforms are often produced by alternative splicing. The complexities of signal transduction pathways are further complicated by the discovery that individual signaling pathways, once thought of as having unique and exclusive functions in cells, often overlap and "cross-talk" with other pathways, resulting in modulation or redundancy of pathway function. Thus, due to the rate of discovery of new gene products involved in signal transduction, and the fact that cell signaling proteins are often structurally and functionally homologous, there is a great need for novel approaches to rapidly and selectively discriminate between highly related gene family members and modulate the activity of those gene products to determine biological function.

II. TRADITIONAL APPROACHES FOR STUDYING CELL SIGNALING

Historically, pharmacological inhibitors of cell signaling pathways have been identified serendipitously. In such cases, the putative mechanism of action of a drug underwent intense investigation for long periods of time to explain a well-documented pharmacological response. A good example of this is aspirin (salicylates), which was known to exert profound anti-inflammatory, antipyretic, and analgesic effects long before a basic understanding of its mechanism of action was attained (i.e., attenuation of prostaglandin biosynthesis and release through direct inhibition of cyclooxygenase-2) (1). Despite this generally accepted view, additional effects of aspirin on cell-signaling pathways continue to be identified including the recent discovery that aspirin may exert some of its pharmacological effects by modulating the NFκB pathway by inhibiting IKKβ function (2).

Although serendipitous discovery of cell-signaling inhibitors has contributed substantially to our current understanding of signaling pathways (not to men-

tion some very good drugs in the clinic), the real goal in signaling research is to identify all of the players that participate in signal transduction processes, and then to ascribe functions for each by selectively and efficiently modulating their activity. It is expected that this process, sometimes referred to as target validation, will result in the rational discovery of drugs with more attractive therapeutic indices, either through the identification of more relevant targets/pathways for drug screening or through the direct utilization of the inhibitor itself (or a derivative thereof) for therapeutics.

Numerous approaches have traditionally been taken over the years for the rational study of cell-signaling pathways (Fig. 1). These approaches can be classified into three major areas: (1) overexpression systems, in which the function of a particular protein, or a derivative thereof (i.e., a dominant negative mutant), is studied by expressing it at unusually high levels in a cell or whole animal; (2) gene knockout systems (typically in worms, flies, or mice), in which the function of a particular protein is studied by disrupting the function of the gene that encodes it; and (3) through the application of small-molecule, peptide, or antibody inhibitors in which inhibitor activity is measured against the function of a specific protein or cell pathway, or against a specific phenotype (e.g., apoptosis). All of these approaches have contributed significantly to our current understanding of cell-signaling pathways. However, each of these approaches also has significant disadvantages that limit their usefulness as tools for studying signal transduction pathways (Table 1).

A. Overexpression Systems

Overexpressing proteins, and mutants thereof, in cells is a commonly applied technique to determine the biological function of a protein. This technique gained significant popularity with the invention of epitope-tagging protein expression vectors (e.g., FLAG, c-myc) (3). When interpreting the results of overexpression systems, one has to take into consideration that protein overexpression creates a nonphysiological environment within cells that can contribute to artifactual results. In a situation where a particular protein is expressed at nonphysiologically high levels, abnormal protein-protein interactions may be forced to occur. This artifact can potentially occur through a number of mechanisms. For example, overexpression may result in altered intracellular protein localization, thereby creating an environment in which the proximity of proteins to one another is significantly altered. In addition, protein overexpression can directly affect protein-protein binding since these events are driven by both binding affinity and the concentration of the proteins involved. Since expression of proteins to nonphysiologically high levels can alter protein-protein interactions, overexpression approaches can produce results that do not reflect the normal cellular environment.

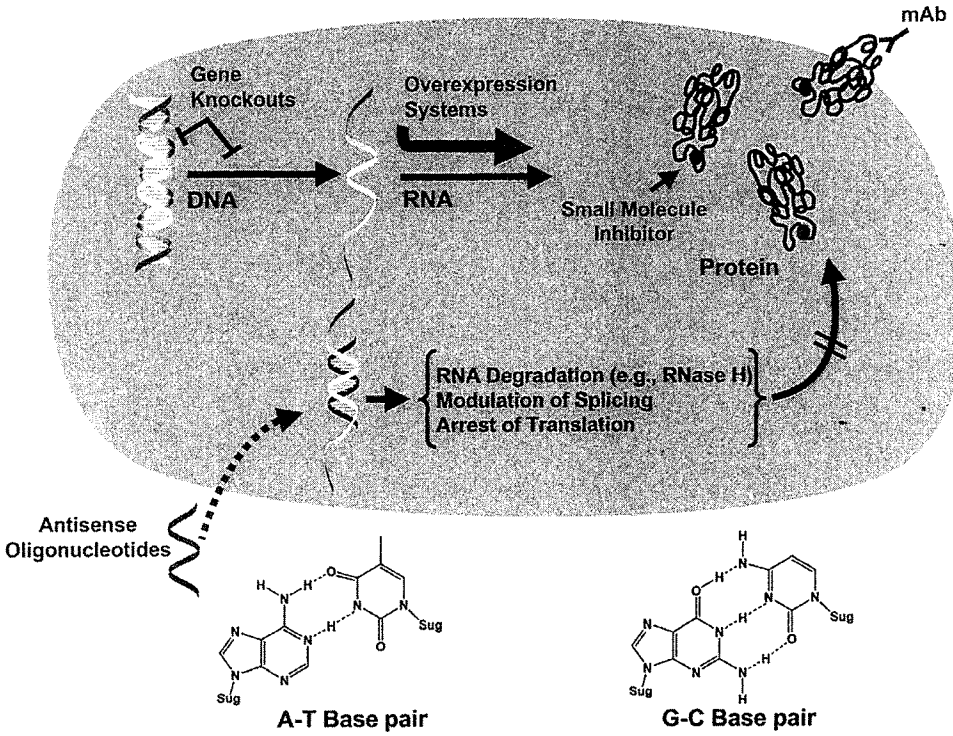

Figure 1 Experimental approaches for determining the function of cell-signaling molecules in cells. Shown are five approaches (gene knockouts, overexpression systems, small-molecule inhibitors, monoclonal antibodies (mAB), and antisense technology) that are currently used to modulate the function or the expression levels of a particular signaling molecule. The advantages and disadvantages of each approach are described in the text. Antisense technology utilizes chemically modified oligonucleotides that are designed to hybridize with target RNA sequences through a Watson-Crick hybridization mechanism that involves the formation of adenine-thymine (A-T) and guanosine-cytosine (G-C) base pairs.

Overexpression of dominant-negative mutants (e.g., kinase-dead or substrate-dead mutants) potentially creates an additional level of complexity for interpreting experimental results. These proteins often maintain their ability to interact with other proteins in the cell since the mutation site (usually the catalytic or activation domain) can be distinct from sites involving additional protein-protein interactions (4). As a result, dominant-negative approaches may not be capable of addressing additional functions for signaling molecules such

Table 1 A Comparison of Different Experimental Approaches for Modulating the Function of Cell Signaling Molecules

Method	Versatility	Specificity	Required resources	Cost	Probability of success	Potential for drug development
Overexpression systems	Low to moderate	Moderate	Moderate	Low	Moderate	Low
Gene knockouts (mammalian)	High	High	High	High	Moderate	None
Small-molecule inhibitors	Low	Low	High	High	Low	Yes
Monoclonal antibodies	Low	High	Moderate	Moderate	Moderate	Yes
Antisense oligonucleotides	High	High	Low to moderate	Low to moderate	High	Yes

as phosphorylation-independent activities (e.g., scafold functions). Since activities of this nature are well known to be critical for the normal function of a wide-range of signaling pathways (4), approaches that address only catalytic functions for signaling molecules may produce incomplete, or possibly inaccurate, descriptions of protein function. For example, transcription factors are usually comprised of multigene families that contain related isoforms that interact with one another, as well as with other transcription factor family members (e.g., fos/jun family and the NFκB family) (5,6). These interactions, which can be phosphorylation-independent, can take the form of homodimers (i.e., comprised of identical proteins) or heterodimers (i.e., comprised of different proteins), and the composition of the complexes can greatly affect the functional response (7). Thus, dominant-negative mutants of transcription factor transactivation domains, which cannot be phosphorylated, are still free to interact and dimerize with other transcription factor subunits, thereby exerting a titration effect on the subunit pool and potentially altering the biological response in a way that is not directly related to the protein's function.

B. Gene Knockouts

Disrupting gene function in mice, flies, and worms is a widely used approach for studying the role of proteins in signaling pathways. This method is tremendously valuable because it provides a wealth of information on the function of a particular protein under normal physiological conditions in animals and provides information that is not obtainable in cell culture systems. Furthermore, the growing number of ''knockout mice'' allows for crossbreeding, creating mice with multiple gene knockouts, which provides important information relating to differences and similarities in function of different gene products (8–10). The ability to create tissue-specific and temporally specific gene knockouts has provided further value for this approach (11–13).

Nevertheless, gene knockout approaches also possess distinct limitations. First, they can be very expensive to generate (i.e., mouse knockouts) and are very labor intensive. Furthermore, gene knockouts often lead to embryonic lethality, limiting their utility (14). In addition, a significant concern regarding gene knockout results is that the phenotype produced by the particular knockout may only reflect a function for that gene product during embryonic development, and may not be relevant to a mature, fully developed animal. Compensatory mechanisms may also exist during development to overcome a genetic deficiency that may be lacking in a fully developed animal. Finally, gene knockouts generated by different investigators sometimes produce different phenotypes, which is possibly related to the use of different strains of mice (15,16).

Not surprisingly, knockout animals often produce phenotypes that go beyond what one would expect from in vitro data, sometimes providing additional

information on the function of a particular protein or providing data that directly conflict with in vitro results. Examples of this can be found in studies of TNF-α signaling pathways. Mice lacking the signaling protein RIP1 (receptor-interacting protein 1) exhibit immune and adipose tissue defects that suggest a role for this protein in immune system and adipocyte biology (17). RIP −/− cells fail to activate NFκB in response to TNF-α, in agreement with earlier results from RIP1 overexpression studies (18,19). However, in contrast to overexpression results that support a role for RIP1 in TNF-α-mediated stimulation of the jun kinase (JNK) pathway, RIP1-deficient cells are competent in terms of stimulation of the JNK pathway by TNF-α (17,20).

TRAF2 (TNF-receptor-associated factor) is another example where overexpression systems produce results that are different than gene knockouts. Here, cells derived from TRAF2 knockout mice are competent for TNF-α-mediated induction of NFκB but not for activation of JNK (21). In contrast, overexpression systems support a role for TRAF2 in TNF-α-mediated induction of both JNK and NFκB pathways (22,23). Thus, overexpression systems and gene knockout systems can produce very different signaling responses, making it difficult to determine which is real and which is experimental artifact.

C. Small Molecules

Small-molecule inhibitors are the method of choice for many pharmaceutical companies for investigating the role of cell-signaling molecules in cellular responses and to validate these molecules as potential therapeutic targets. This class of inhibitors is attractive mainly owing to their ability to be more easily developed for clinical applications. They are generally inexpensive to make, often are orally bioavailable, and the pharmaceutical industry enjoys a wealth of experience in developing drugs of this class. Traditionally, small-molecule inhibitors have been discovered serendipitously as a result of semirandom screening procedures designed to impact a specific signaling reaction or biological phenotype. This approach has evolved into more sophisticated "high-throughput" screening procedures in which thousands of potential small-molecule inhibitors (i.e., chemical libraries) are screened for activity against a specific reaction or phenotype in a semiautomated manner. Active compounds (i.e., "hits") almost always require follow-up structure-activity studies for optimization of activity. More recently, the solving of protein crystal structures for specific signaling molecules has led to the initiation of more rational approaches for the design of potential small-molecule signal transduction inhibitors (24–26). However, the number of solved protein structures that are available for study is relatively small and the process that is required to obtain these structures is very labor intensive. Furthermore, this approach remains to be validated for the discovery of attractive inhibitors.

The routine discovery of suitable small-molecule signal transduction inhibitors is severely limited in practice owing, in part, to their lack of specificity. This conclusion stems from the fact that proteins that catalyze similar chemical reactions (e.g., phosphotransfer reactions for protein kinases) are highly conserved in areas that are normally most vulnerable for small-molecule intervention. However, there has been some success using this approach. Protein kinases and farnesyltransferases have experienced remarkable success as targets for small-molecule inhibitors. Examples of this include SB203580, which targets p38 mitogen–activated protein (MAP) kinase (27–32), PD098059 and U0120, which target the MAP kinase kinase family members, MEK-1 and MEK-2 (33–35), and substrate-based or microbially derived inhibitors of farnesyl:protein transferase, which were sought for the purpose of discovering small-molecule inhibitors of *ras* function (36). These inhibitors have proven to be extremely valuable tools for investigating the function of specific cell-signaling molecules in cell culture systems, in animal models, and, in some cases, have resulted in the initiation of clinical trials (36,37). However, despite this success, studies questioning the mechanism of action of these inhibitors have been reported. For example, in addition to p38 MAPK inhibition, SB203580 has also been shown to inhibit the activity of cyclooxygenases and other MAP kinases at physiologically relevant concentrations (37,38). Similarly, recent reports have suggested that *ras* is not the primary target underlying the antitumor activity displayed by farnesyltransferase inhibitors, not such a surprising conclusion since a wide variety of proteins are known to require farnesylation for their function (36,39,40). These examples illustrate the inherent difficulty in obtaining acceptable specificity for small-molecule inhibitors targeted against cell-signaling proteins.

A second major limitation of small-molecule inhibitors is their lack of versatility. Small-molecule inhibitors have most commonly demonstrated their utility as signal transduction inhibitors against receptor proteins and proteins that possess intrinsic enzymatic activity. Examples of this are β-adrenergic receptors and protein kinases (27–35,41,42). However, it is well known that signaling pathways rely heavily on a wide variety of proteins that do not function as receptors or possess intrinsic enzymatic activity. Examples of this include adaptor proteins, scaffold proteins, and transcription factors. These proteins typically function through specific protein-protein interactions that involve points of spatial contact over broad structural areas, making the possibility of small-molecule intervention virtually impossible (4).

D. Monoclonal Antibodies

The utility of monoclonal antibodies (mABs) as signal transduction inhibitors is primarily limited to cell surface proteins and secreted proteins owing to their inefficient delivery into cells. Cellular microinjection approaches using mABs

targeted against specific cellular proteins have been used to a limited extent for the study of signal transduction processes (43). However, this approach requires specialized skills, is labor intensive, and the information gained from these types of studies is severely limited owing to the constraints imposed by single-cell studies. Recent developments in antibody engineering techniques have led to the production of humanized mABs with improved affinity and reduced immunogenicity (44,45). These developments have greatly increased the value of mABs for therapeutic application and have resulted in the initiation of a number of mAB-based clinical trials and drug approvals (46–48). For example, Ritoxan, a mAB directed against the CD20 B-cell antigen, was approved for the treatment of non-Hodgkin's lymphoma in 1997, and Herceptin, a mAB directed against the Her2/neu receptor, was approved for the treatment of breast cancer in 1998 (49–51). The coupling of toxins, nuclides, or drugs to mABs is under investigation and may broaden the utility of mABs for therapeutics in the future.

III. ANTISENSE APPROACHES FOR STUDYING CELL SIGNALING

A. The Antisense Concept

Antisense oligonucleotides (ASOs) represent a new paradigm for the discovery of potent and specific drugs with fewer undesired side effects. The antisense concept is based on an understanding of nucleic acid structure and function and depends on Watson-Crick hybridization mechanisms (52). Unlike traditional pharmacological approaches that attempt to identify inhibitors of gene product function by targeting proteins, ASOs are designed to specifically modulate the information transfer of a particular gene into protein by hybridizing with and disrupting the function of pre-mRNA and mRNA, thereby preventing the mRNA from being translated (Fig. 1).

A number of mechanisms have been demonstrated by which ASOs exert their inhibitory effects on mRNA function. These mechanisms include inhibition of splicing, inhibition of protein translation by disrupting ribosome assembly, and most commonly, destruction of steady-state mRNA levels through the utilization of endogenous RNase H enzymes in cells (53–59). The exact mechanism by which a particular ASO acts is usually dependent on the chemical composition of the oligonucleotide and the hybridization site within the target RNA (53–59). ASOs display extremely high affinity and selectivity for their RNA targets, and therefore, have the potential to be much better tools for studying signaling pathways than classic, small-molecule drugs that bind to proteins with relatively low affinity and poor selectivity. In addition, ASOs have the added potential of being drug candidates for the clinic. Since virtually every step within cell-signaling cascades is governed not by a single protein with a unique structure, but instead,

by a family of highly homologous proteins encoded by multigene families, the specificity that antisense offers to selectively inhibit the expression of highly related regulatory proteins is an extremely valuable approach to investigate the detailed mechanisms of signal transduction processes. In fact, despite being a relatively new technology, antisense approaches have already been successfully employed for pathway analysis involving a wide range of signaling molecules. Some of these studies are listed in Table 2 for reference.

B. Specificity and Simplicity

Specificity and simplicity are two major advantages of the antisense approach for discovering novel inhibitors of protein expression. Since ASOs act by targeting virtually any region (including nontranslated sequences) within a pre-mRNA or mRNA, and due to the degeneracy of the genetic code, it is relatively easy to design an ASO that specifically inhibits a member of a multigene family regardless of the degree of amino acid conservation at the protein level (60–62). Properly designed ASO inhibitors have also been shown to be capable of redirecting specific pre-mRNA splicing events away from one protein isoform in favor of another, and this approach has been successfully employed to determine isoform-specific functions for alternatively spliced gene products in cells (59,63,64). Hence, isoform-specific targeting of cell-signaling molecules is not only limited to isoforms produced from different genes but can also be used to selectively target isoforms produced from a single gene. This is an important feature that antisense offers since many important cell signaling genes produce pre-mRNA transcripts that undergo alternative splicing to produce different isoform products (e.g., JNK1α1, α2, β1, β2; Bcl-X family members; cytokine receptors) (64–67). In addition, it is possible to obtain ASO inhibitors that target RNA sequences that are common to multiple family members and modulate expression of multiple isoforms simultaneously (68–70).

Results obtained using ASO approaches are experimentally easy to control for, which helps facilitate proof of mechanism. An antisense mechanism can be established through the use of multiple active ASO inhibitors for a given target (which should produce similar phenotypic effects) and mismatch control oligonucleotides that do not inhibit the expression of the cellular target, or that are intentionally designed to inhibit expression of the target, but to a lesser extent relative to the antisense molecule, thereby producing attenuated pharmacological responses (54,71,72). Furthermore, the design of inactive or partially active control oligonucleotides is fast and simple. Thus, depending on the needs of the investigator, ASOs can be designed to selectively inhibit the expression of individual isoforms produced by a single gene or separate genes, or can be used to inhibit expression of multiple isoforms simultaneously, and the tools for proving mechanism of action are readily available.

ASO approaches have been used successfully to suppress expression of multiple isoforms simultaneously (e.g., ERK1/2, JNK1/2) or individual isoforms separately (e.g., ERK1 or ERK2; JNK1 or JNK2) against a range of molecular target families for the purpose of addressing biological function within cell-signaling pathways (Table 2). An example of this is the jun kinase (JNK) family of MAP kinases. Small-molecule inhibitors that can selectively inhibit JNK activity have been aggressively pursued by pharmaceutical companies over the years with little success. However, ASOs have been successfully employed to selectively inhibit JNK protein expression in cells without affecting the expression of other kinases, and have been used to demonstrate unique functions for JNK isoforms in cell-signaling pathways (62,73–78). For example, ASOs targeting JNK2, but not JNK1, have been shown to suppress the expression of cell adhesion molecules in response to TNF-α in endothelial cells (62). In contrast JNK1, but not JNK2, antisense inhibition has been shown to prevent apoptosis in response to hypoxia/reoxygenation injury in kidney epithelial cells (79). Also using an antisense approach, Potapova et al. have shown that targeting either JNK1 or JNK2 inhibits growth of T98 glioblastoma cells, but that JNK2 inhibition (and not JNK1) induced cell death (77). These results suggest that JNK1 and JNK2 are necessary for basal growth of these cells, but that JNK2 is essential for survival.

In addition to isoform-specific functions for JNKs, ASOs have also been used to demonstrate functional redundancy between different JNK isoforms. In this study, ASO inhibition of JNK1 or JNK2 was not sufficient to induce hypertrophy in rat cardiac myocytes, as measured by atrial naturietic factor mRNA (ANF) expression. However, simultaneous inhibition of both JNK1 and JNK2 using a single ASO resulted in a profound induction of ANF transcript levels that was comparable to phenylephrine-mediated or endothelin-mediated induction of ANF (R. McKay and C. Glembotski, unpublished results). These results suggest that, in terms of suppressing ANF expression, JNK1 and JNK2 are functionally redundant in cardiac myocytes such that either one is capable of suppressing a hypertrophic response (e.g., ANF expression), and this suppression can be relaxed by simultaneous inhibition of JNK1 and JNK2.

C. Versatility

As discussed earlier, small-molecule inhibitors, mABs, and overexpression systems are severely limited in the types of protein targets that they are capable of impacting. However, because ASOs act by targeting RNA and not protein, they can inhibit the expression of virtually any class or type of protein regardless of function. This includes proteins that are sometimes easy to approach using traditional methods (e.g., enzymes and receptors) as well as proteins that are often difficult to obtain inhibitors against (e.g., adaptor proteins, structural proteins,

Table 2 Examples in Which Antisense Technology Has Been Employed to Determine Protein Function in Signal Transduction Pathways

ASO target	Cell type	Biological system/endpoints	Ref.
ERK1,2	Cardiac myocytes	ANF response, sarcomerogenesis	Glennon et al. (70)
	Vascular smooth muscle cells	DNA synthesis, p90 RSK activation	Robinson et al. (69)
JNK1,2	Erythroleukemia	Apoptosis	Seimiya et al. (74)
	Lung tumor	Proliferation	Bost et al. (75)
	Endothelial cells	CAM expression (TNF-α)	Xu et al. (62)
	Glioblastoma	Proliferation	Potapova et al. (77)
	Renal epithelial cells	Apoptosis	Cioffi and Monia (79)
RAS (Ha, Ki, N)	Endothelial cells	CAM expression (TNF-α)	Xu et al. (62)
	Bladder carcinoma, fibroblasts	Proliferation	Chen et al. (110)
	Colon epithelial	Regulation of CEA, β1 integrin expression	Yan et al. (111)
RAF	Endothelial cells	CAM expression (TNF)	Xu et al. (62)
	Smooth muscle cells	Proliferation	Cioffi et al. (112)
	Fibroblasts	ERK activation	Schulte et al. (113)
MAP kinase phosphatase	Vascular smooth muscle cells	ERK activation	Duff et al. (114)
PDE7	T cells	Proliferation	Li et al. (115)

PI3-kinase	Ovary cells/insulin responsive CHO (GRC-LR + 73)	DNA synthesis (insulin)	Siddhanta et al. (116)
PKCα; β; delta	Macrophage	NO release; INOS expression; NFκB activation	Chen et al. (117)
	Lung tumor	CAM expression	Dean et al. (118)
PKC-zeta	Vascular smooth muscle cells	ERK activation	Liao et al. (119)
PKC-epsilon	Endothelial cells	ERK activation	Traub et al. (61)
RhoA	Lung tumor	JNK activation	Roberts and Cowsert (120)
JAK2	Monocytes	15-LO activation	Roy et al. (121)
TyK2	Monocytes	15-LO activation	Roy et al. (121)
$P125^{FAK}$	Tumor (multiple)	Apoptosis	Xu et al. (122)
Gαi2	Neuronal	Ca^{2+} mobilization	Tang et al. (123)
Gαi3	Insulinoma	Adenylate cyclase activation	de Mazancourt et al. (124)
PLA_2	Rheumatoid synovial fibroblast	Prostaglandin biosynthesis	Roshak et al. (125)
Bcl-2	Lung tumor	Apoptosis	Ziegler et al. (126)
Al, Bcl-X	Endothelial cells	Apoptosis	Ackermann et al. (127)
PP5 phosphatase	Bladder carcinoma, fibroblasts	p53 activation	Zuo et al. (128)
MDM2	Tumor (multiple)	p53 activation	Chen et al. (129)

ASO, antisense oligonucleotide. Target indicates the intended RNA target (typically mRNA) that the ASO was designed to interfere with. See references for explanation of additional abbreviations and biological systems/endpoints.

transcription factors). Thus, from a practical standpoint, the versatility that antisense offers makes it the only available approach for certain molecular targets. In addition, the level of target inhibition can be easily titrated by altering ASO dose or concentration (unlike gene knockouts). This is an important property since different functional responses, including toxicity, will likely be affected by the level of inhibition that is produced. For example, one can imagine scenarios in which the level of inhibition of a key signaling protein may determine how many downstream pathways are impacted and to what extent.

Full-length gene sequence information is not required to obtain effective ASO inhibitors. This is especially important for determining the function of partial cDNA sequences (e.g., expressed sequence tags) found in genomic databases. With little more than a partial gene sequence, the practitioner of antisense drug discovery can rapidly design, synthesize, and test a series of ASOs in cell culture and determine whether the target gene is specifically inhibited. This process is relatively fast, making it possible to rapidly test hypotheses for addressing signal transduction mechanisms. The length of time and the resources required to identify lead ASOs by this approach is much less than by any other drug discovery method.

A good example of antisense versatility is illustrated in Fig. 2 in which highly selective and potent antisense inhibitors against most of the known signaling molecules within the NFκβ signaling pathway were identified. These targets include adaptor proteins, protein kinases, and transcription factors. Figure 3 illustrates the application of one of these antisense inhibitors, targeted against IKKβ, for determining the role of IKKβ in NFκβ signal transduction stimulated by TNF-α. In this study, HeLa cells were treated with a chimeric 2′-methoxyethyl phosphorothioate oligonucleotide targeted against IKKβ and the effects of IKKβ inhibition on TNF-α signaling at various steps in the pathway were determined. Antisense suppression of IKKβ protein levels resulted in diminished signaling responses to TNF-α at various levels of NFκβ signal transduction, including activation of IKβα (substrate for IKKβ), p65 NFκβ nuclear translocation, and induction of gene expression (E-selectin). This example demonstrates how antisense technology can be used to determine the role of a molecule in a signal transduction pathway in a highly specific manner.

D. Antisense Oligonucleotides as Drug Candidates

Although significant progress has been achieved in the identification of gene products implicated as causal factors in the onset or maintenance of human diseases, the emergence of novel therapies that specifically reverse the effects of these gene products has generally been slow. One of the major advantages that antisense technology offers for the study of cell-signaling pathways and for the clinical development of novel signal transduction inhibitors is the ability to di-

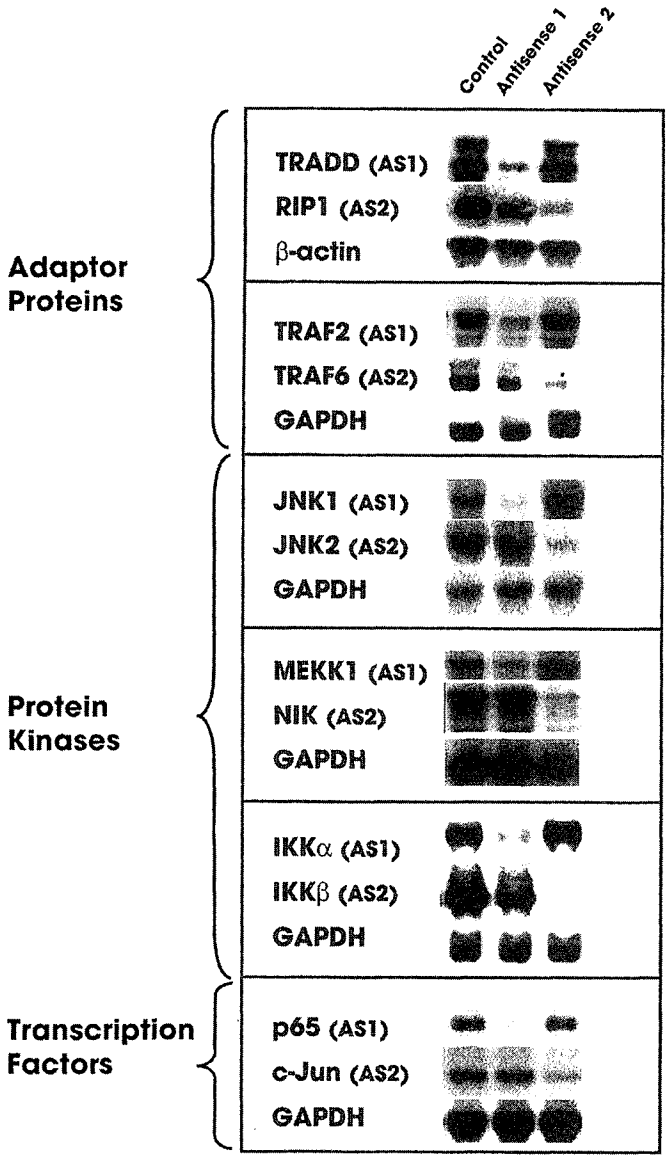

Figure 2 Antisense oligonucleotide-mediated suppression of proteins that participate in NFκβ signal transduction pathways. Cells were treated with antisense oligonucleotides in vitro using cationic lipid delivery methods. mRNA levels were determined for each target 20–24 h following oligonucleotide treatment by Northern blot analysis. All oligonucleotides were 2′-methoxyethyl-modified phosphorothioates.

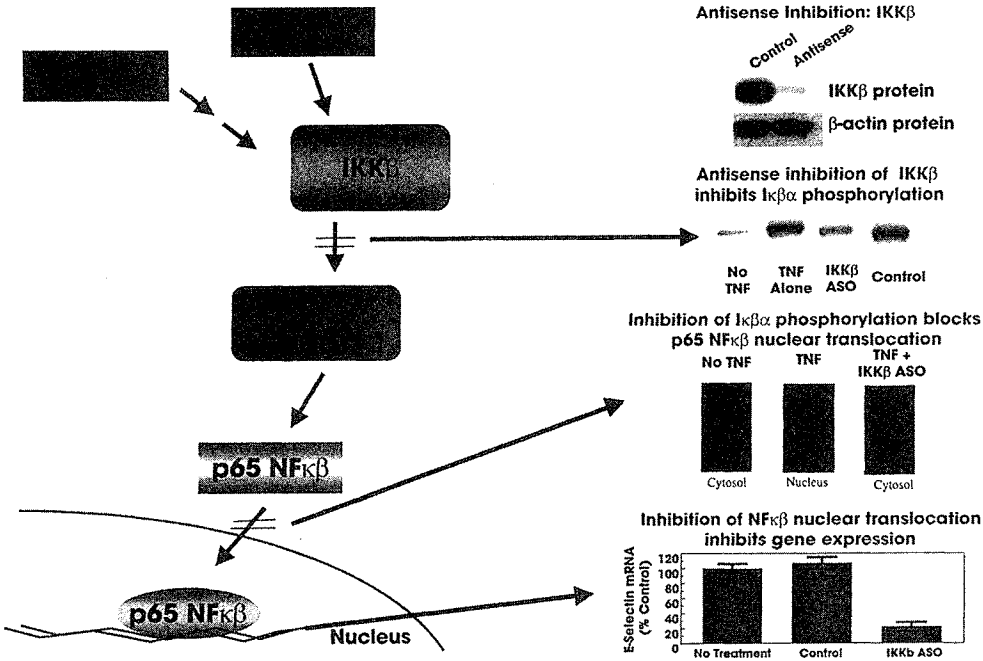

Figure 3 Antisense inhibition of IKKβ blocks NFκβ signaling in response to TNF-α. HeLa cells were treated with a 2′-methoxyethyl-modified antisense oligonucleotide targeted to human IKKβ and IKKβ protein levels were determined 48 h later. The effects of TNF-α treatment on IKBα phosphorylation, p65 NFκβ nuclear translocation, and E-selectin gene induction in cells treated with the IKKβ antisense oligonucleotide or a control oligonucleotide were determined. The results show that antisense inhibition of IKKβ blocks TNF-α-mediated induction of IKBα phosphorylation, NFκβ translocation, and induction of E-selectin mRNA levels.

rectly utilize the ASO that was selected from cell culture studies in animal models and, ultimately, in humans.

Recent publications have demonstrated that ASOs identified in cellular-based assays as inhibitors of gene expression are also effective in a wide range of animal disease models in a manner that is highly consistent with an in vivo antisense mechanism of action. This includes a wide range of in vivo therapeutic models including models of cardiovascular disease, inflammation, organ transplant, neurological disorders, and cancer (68,80–94). Based on results of this nature, the number of clinical trials that have been initiated to evaluate antisense drugs for human therapeutics is growing at an impressive rate (94–99) (Table 3). Moreover, the recent approval of

Table 3 Clinical Trials to Evaluate Antisense Drugs for Human Therapeutics

Compound	Target	Indications	Sponsoring company	Status
Vitravene (ISIS 2922)	CMV IE2	CMV retinitis	Isis/Ciba Vision	Approved
ISIS 3521	PKCα	Cancer	Isis	Phase III
G3139	bcl-2	Cancer	Genta	Phase III
ISIS 2302	ICAM-1	Crohn's disease Psoriasis (topical) Ulcerative colitis	Isis	Phase II
ISIS 5132	c-*raf*	Cancer	Isis	Phase II
ISIS 2503	Ha-*ras*	Cancer	Isis	Phase II
GEM 231	PKA	Cancer	Hybridon	Phase I
MG98	DNA methyltransferase	Cancer	MethylGene	Phase I
NEUGENE	c-myc	Restenosis	AVI	Phase I

CMV, cytomegalovirus; IE2, immediate early gene 2; ICAM-1, intercellular adhesion molecule 1; bcl-2, breakpoint cluster 2; Ha-*ras*, Harvey Rasheed rat sarcoma; c-*raf*, transforming gene of the murine sarcoma virus, sometimes referred to as *raf*-1; c-myc, cellular myelocytomatosis gene. Vitravene (Fomivirsen, ISIS 2922) has been approved for the second-line treatment of cytomegalovirus retinitis in patients with AIDS who are intolerant of or unresponsive to previous treatment(s) for the disease (100). All compounds are phosphorothioate oligodeoxynucleotides except GEM 231, which contains ''second-generation'' 2′ sugar modifications.

the first ASO therapeutic (Vitravene) by the Food and Drug Administration for the treatment of cytomegalovirus-induced retinitis demonstrates that ASOs have tremendous potential as therapeutic agents for treating human disease (100). Thus, antisense technology offers both highly selective and versatile tools for the functional validation of cell-signaling molecules, as well as a novel therapeutic approach for the treatment of human disease in the clinic.

E. Potential Limitations

All molecular and pharmacological approaches for determining the function of gene products in biological processes possess certain properties that may limit or prevent their utility as molecular probes under certain conditions. Despite the fact that antisense technology offers a variety of beneficial properties that make ASOs very attractive as tools for determining gene function relative to more traditional approaches, they do possess certain limitations. The first, and possibly most significant, limitation to the antisense approach is inherent to its mechanism of action. Since ASOs target RNA and not protein, biological consequences resulting from inhibition of a particular gene product are dependent on the normal decay rate (i.e., half-life) of the encoded protein product. In most cases, this is not a significant concern in that the majority of proteins in cells possess a normal half-life on the order of a few minutes (e.g., cell cycle proteins) to a day (e.g., *ras* proteins). However, some proteins are very long-lived and may require up to several days to sufficiently reduce steady-state levels of a particular protein product. Obviously, being forced to wait for such extended periods may have certain practical limitations and may impact the biological responses observed as a result of inhibiting gene expression. Therefore, the most attractive antisense targets for determining gene function are those that are either inducible or encode proteins with short to moderate half-lives.

One additional limitation to antisense technology that is inherent to its mechanism of action is the potential inability to inhibit gene function for proteins that are regulated posttranslationally. If a cell regulates the steady-state levels of a particular protein product by adjusting its protein half-life relative to mRNA production, approaches that inhibit RNA production may be unable to effectively reduce steady-state protein levels. Although such an outcome has yet to be reported for an antisense experiment, it demonstrates the importance of considering the possible mechanisms by which cells may compensate posttranslationally upon inhibition of mRNA function.

In addition to the potential limitations based on antisense mechanism of action, it is important to realize that certain other limitations may exist when using an antisense approach to determine gene function. The first generation of antisense analogs to be broadly examined for their properties as drugs are the phosphorothioates, where one of the nonbridging phosphoryl oxygens of DNA

is substituted with sulfur. This relatively simple modification results in dramatic improvements in nuclease stability and the in vitro and in vivo pharmacokinetics of oligonucleotides (101,102). However, phosphorothioates have been reported to possess certain nonspecific effects on protein function under certain conditions that are not based on antisense mechanism (54,57,103). To circumvent these problems, newer oligonucleotide modifications have been identified that reduce or eliminate the nonspecific activities associated with phosphorothioate modifications (104–109). Typically, this is accomplished using chemistries that leave the natural phosphodiester DNA backbone intact while modifying the 2′ sugar position, rendering a molecule with increased affinity for RNA and sufficient stability against nuclease degradation. However, backbone and heterocycle modifications are also attractive "second-generation" oligonucleotide chemistries that offer superior properties for gene functionalization studies. Thus, when possible, it is prudent to utilize newer oligonucleotide chemistries that display a level of specificity that is even greater than that of phosphorothioate ASOs.

Finally, one must realize that the selection of the optimal antisense sequence for inhibiting a particular mRNA still requires a fairly empirical approach in which multiple oligonucleotides must be evaluated for inhibition of target gene expression (60). This restriction is due to the inability to predict the optimal hybridization sites within a pre-mRNA sequence, which, in turn, is probably due to our inability to accurately predict RNA structure in cells. Thus, the practical aspects of synthesizing and testing a series of oligonucleotides should be considered prior to embarking on an antisense approach against a particular molecular target.

IV. FUTURE DIRECTIONS

Recent progress in antisense technology demonstrates that antisense inhibitors can be successfully employed for abrogating the function of specific gene products in vitro, in animal models, and in humans. The antisense approach offers many distinct and significant advantages over more traditional approaches for the study of cell-signaling pathways and the development of novel signal transduction inhibitors. Thus, this approach provides significant value both for studying the function of specific gene products in cells and for the discovery of novel therapeutic agents to treat human disease.

Nevertheless, it is clear that we are only at the earliest stages of understanding and exploiting antisense technology to serve both therapeutics and basic research. Some of the important issues that this technology has the potential to address relate to the functions of highly related members of multigene families within cell-signaling pathways in both normal and diseased settings. In-depth understanding of such pathways will undoubtedly lead to a much more profound

understanding of such critical processes as "cross-talk" between seemingly unrelated signaling pathways, tissue-specific and disease-specific functions for signaling proteins, and cellular compensation mechanisms to inhibitors of cell signaling, which will yield important insights to potential mechanisms of drug resistance for cell-signaling inhibitors. Thus, antisense technology has the potential to provide the tools for significantly advancing our current understanding of the molecular events that underlie signal transduction processes, which will very likely result in better therapeutic approaches for human disease.

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5
Methods of Selecting Sites in RNA for Antisense Targeting

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I. INTRODUCTION

The mechanism of action for antisense oligonucleotides requires that the oligonucleotide hybridize to its mRNA target. Therefore, in principle, design of an antisense oligonucleotide requires simply that the oligonucleotide be complementary to the mRNA. In practice, however, when several oligonucleotides complementary to an mRNA are screened, certain antisense oligonucleotides are more active and more potent than others in suppressing specific gene expression (1–12). In addition, some complementary oligonucleotides can show nonantisense effects (13–15). The most commonly used and most effective approach to discovery of antisense oligonucleotides involves synthesis of numerous oligonucleotides (up to several dozen) designed to hybridize to different regions of the targeted mRNA, followed by activity screening in cells (16).

Several attempts have been made to identify features of oligonucleotides that are associated with antisense activity. Development of successful methods for selection of active oligonucleotides prior to oligonucleotide synthesis and cell-based screening would have two benefits. First, the cost of antisense discovery would be reduced. If a computer algorithm could pick the most active compound for an antisense target, then synthesis and screening of multiple compounds could be eliminated. Second, identification of the features associated with specific and nonspecific effects of oligonucleotides would likely lead to a better understanding of the detailed mechanism of antisense activity and, potentially, to identification of compounds with even greater potency. This review will discuss methods that have been used to select antisense oligonucleotides, the

effectiveness of these methods, and the prospect for improved methods in the future.

II. RNA STRUCTURE CALCULATIONS

It has long been assumed that activity of an antisense oligonucleotide is directly related to the hybridization affinity of the oligonucleotide for its mRNA target. Support for this assumption comes from the observation that, at a given target site, longer oligonucleotides are more active than shorter ones (17). In addition, at a given site, oligonucleotide modifications that increase the melting temperature (T_m) of the oligonucleotide-RNA duplex, often increase antisense activity and/or potency (18–21). Mismatched oligonucleotides reduce the T_m and decrease the potency (22,23).

However, when comparing oligonucleotides targeted to different sites, T_m alone is not sufficient to ensure activity (3). It has long been believed that secondary structure in the mRNA target affects hybridization affinity differently at different sites and thus affects antisense efficacy (24–28). Therefore, methods for calculating RNA structure and calculating hybridization of the antisense oligonucleotide to the structured mRNA are useful for prediction of antisense activity.

Early attempts by Stull et al. (29) found moderate correlation (R = 0.66–0.99) between a predicted duplex score and antisense activity. Inclusion of an mRNA target secondary-structure score in the calculation actually worsened correlation between calculated hybridization affinity and antisense activity. Since Stull's publication, improvements have been made to the rules and parameters for prediction of RNA secondary structure. (30). Effective parameters for prediction of DNA:RNA duplex stability are available (31) and improved parameters for prediction of secondary structure in DNA oligonucleotides are also available (32–37). Mathews et al. (38) used these most up-to-date parameters to calculate equilibrium affinity of complementary DNA or RNA oligonucleotides to an RNA target taking into account the predicted stability of the oligonucleotide-target helix and the competition with predicted secondary structure of both the target and the oligonucleotide. When their predicted affinities were compared to antisense activity in one experiment (39), good correlation (R = 0.91) was found between duplex free energy and antisense activity. When oligonucleotide self-structure and/or target RNA structure were included in the calculation, antisense efficacy did not correlate with $\Delta G^\circ_{overall}$.

The reported correlations between predicted duplex stability and antisense activity do not extend broadly to additional targets. When a data set of 349 antisense oligonucleotides targeting 12 genes (Giddings and Matveeva, *http://antisense.genetics.utah.edu*) was evaluated for correlation between duplex stability

and antisense activity, the linear correlation coefficient was 0.22, suggesting that the strong correlations reported in earlier work do not extend to larger data sets.

There are several possible explanations for the lack of a strong correlation between calculated hybridization of an oligonucleotide to its mRNA target and observed antisense activity. One possibility is that the calculated binding energies do not represent true equilibrium affinities. Although current algorithms are good enough to correctly predict 73% of base pairs in structures determined from comparative sequence analysis (30), this level of accuracy may not be enough to allow prediction of good antisense-binding sites. In addition, current algorithms (38) use thermodynamic parameters for unmodified DNA or RNA when calculating free energies of antisense: RNA duplex formation or antisense oligonucleotide self-structure. Parameters determined from experiments using modified oligonucleotides could improve the predictions (40). Furthermore, parameters for predictions were measured in 1 M Na^+, 0.1 mM EDTA, and may not represent conditions of antisense binding. The large numbers of proteins involved in RNA synthesis, processing, transport, translation, and degradation almost certainly affect binding of the antisense oligonucleotide to its target.

A second possibility is that the antisense target is pre-mRNA and secondary structures predicted for mRNAs are not representative of structures in pre-mRNAs. It is known that pre-mRNA is the molecular target for many antisense oligonucleotides (41,42). The secondary structure of a pre-mRNA undergoing synthesis, processing, and transport is likely not fully predictable from simple thermodynamic consideration.

The third, and most likely, possibility is that equilibrium affinity is not the sole factor impacting antisense activity (43). Oligonucleotide sequence and structure may affect properties of the antisense compound such as its affinity for proteins, ability to support RNAse H cleavage of the target, delivery to the cellular site of activity, and metabolic stability. These factors will, in turn, affect antisense activity. On the other hand, equilibrium affinity is not unimportant. When oligonucleotide sequence is kept constant, mRNA secondary structure affects antisense activity in a predictable way; activity is lower in structured targets than in unstructured ones (44).

Although factors other than target structure clearly play a role in antisense activity, predictions of local secondary structure have proven effective in identifying oligonucleotides with greater activity than those found by simple oligonucleotide "walks." The strategy employed by Sczakiel and colleagues (45,46) searches for favorable local target elements, loops or bulges of ~10 nt, joints and terminal sequences. Although successful application of this strategy to other targets has not yet been reported, it is tempting to speculate that the success is due to the fact these favorable local target elements represent kinetically preferred sites. "Kissing" hairpins are known to be important for initiation of hybridization

of long antisense RNAs (47,48); these "favorable structures" may play a similar role for oligonucleotide hybridization.

III. OLIGONUCLEOTIDE MOTIF PREFERENCES

It has been suggested that active oligonucleotides contain certain sequence motifs. Tu et al. (49) report that TCCC is associated with antisense activity but no mechanism for this phenomenon was proposed. Smetsers et al. (50) previously reported that CCC is overrepresented in the antisense oligonucleotides in their data set but that TCC is underrepresented. They suggest that overrepresented motifs may be associated with protein-binding and nonantisense effects. Lesnik and Freier (51) offered a plausible explanation for the predominance of pyrimidines and especially C's in active oligonucleotides. They suggests that antisense activity is associated with high stability of the oligo: target hybrid *relative to the alternative RNA:RNA duplex*. Thus antisense oligodeoxyribonucleotides with high (70–80%) pyrimidine content and moderate (40–50%) (A + T) content are more likely to be active than oligonucleotides with different composition.

IV. CELL-FREE SCREENING AND COMBINATORIAL APPROACHES

Several groups have described combinatorial approaches for identification of optimal antisense sites in target mRNA using a cell-free assay. Typically, a library of randomized oligonucleotides is incubated with the target mRNA and RNase H. Mapping of the most favored RNase H cleavage sites results in identification of the most favored binding sites. This approach has been used to find sites for both antisense oligonucleotides (39,52–54) and ribozymes (55). It can, however, be complicated by interactions of library oligonucleotides with each other and by binding of multiple oligonucleotides to the mRNA target (56). Concerns over library complexity have limited oligonucleotide lengths in these studies to 10 nt. Optimal binding sites for short oligonucleotides may not predict those for longer, antisense oligonucleotides.

Matveeva et al. (57) were able to use longer oligonucleotides and reduce library complexity by restricting the oligonucleotide pool to oligonucleotides complementary to the mRNA target sequence. A similar, but less thorough screen was performed by Jarvis et al. (58), who used a cell-free RNase H assay with individual oligonucleotides to identify optimal sites for synthetic ribozymes.

Optimal binding sites have also been identified without using RNase H cleavage assays. Ecker et al. (13) screened randomized combinatorial libraries of 2′-*O*-methyl- and phosphorothioate-modified compounds and identified com-

pounds that bind to H-*ras* mRNA. Using oligonucleotide arrays on glass slides, Southern and colleagues (59,60) were able to identify compounds that bound tightly to c-raf mRNA and were able to select the site for ISIS 5132, the most potent c-raf antisense compound reported at that time. Their synthetic approach uses an elegant strategy that results in synthesis of only oligonucleotides complementary to the mRNA of interest.

The effectiveness of these cell-free approaches requires that the most favored site(s) for oligonucleotide binding to the mRNA in the cell-free system will be the target site for the most active antisense oligonucleotide. To test whether this was the case, Matveeva et al. (61) evaluated the correlation between activity in an RNase H mapping assay or a gel shift binding assay with antisense activity in cells. Moderate correlation with cellular activity ($R = 0.6$) was found for both cell-free assays. Similar correlation analysis of the randomized library data of Ho (39,52) and the array data of Mir (62) gave coefficients of correlation between activity in the cell-free assay and antisense activity ranging from 0.2 to 0.7 (O. Matveeva, J. Wyatt, and S. Freier, unpublished). Thus the correlation between activity in the cell-free assay and antisense activity is relatively weak.

Despite the relatively weak correlation observed between oligonucleotide binding in the cell-free assay and antisense activity, ribozymes (55) or antisense oligonucleotides (39,52,54,57) designed to sites identified by combinatorial selection were more likely to be active than those selected without initial cell-free screening. Thus these methods can improve the "hit rate" for antisense discovery. However, these methods are cumbersome and, at best, result in several leads that still need to be screened in a cell-based assay. Therefore, the benefit of improved hit rate may not make up for the substantial cost disadvantage associated with these cell-free combinatorial assays.

V. SPECIFICITY CONSIDERATIONS AND NONANTISENSE EFFECTS

A. Target Specificity

One attraction of antisense technology is that high specificity can be achieved. For example, inhibition of one isoform of a protein can be obtained without affecting another (11,63,64). Such specificity is hard to achieve with small-molecule drugs. To obtain such specificity, one must be careful to design antisense oligonucleotides that will not hybridize to related mRNA sequences (65). Since oligonucleotides with as few as three mismatches are reported to be inactive (23), three mismatches to related targets should be sufficient but more would be desirable.

Unfortunately, the most commonly used tool for identification of sequence

homology, BLAST (66), is ineffective at finding mismatched sites for oligonucleotides. This is because the default window size is 11, meaning that there must be 11 matches in a row for BLAST to find the homology. The window size can be set as small as 7, but even then 20-mers with two mismatches (for example, in positions 7 and 14) are not found. A more effective technique for finding mismatched sites is to use BLAST to identify other mRNA sequences with homology to the target of interest and then use a substring search to find mismatched sites in these mRNAs. Sites with zero or a few mismatches should be avoided.

B. Motifs that Support Nonantisense Effects

Nonantisense effects of G-rich phosphorothioate oligonucleotides are well known (13,14) and have been attributed to the tendency of these oligonucleotides to form G-quartet structures that then interfere with biological processes (67). The simplest way to avoid these effects is to avoid G-rich oligonucleotides. Restricting oligonucleotides to less than 50% G with no G_4 strings and at most one G_3 string usually does not detrimentally limit the number of oligonucleotides that can be selected from a target message.

Homopolymers of other sequences also form unusual structures (68). Although nonantisense effects of these structures are not well characterized, this should be considered when designing oligonucleotides rich in any single nucleotide or containing strings of any single nucleotide.

Other motifs are also reported to produce nonantisense effects. Krieg et al. (15) reported that oligonucleotides containing CG, especially those with RRCGYY, can stimulate murine B cells in vitro and in vivo. The active motif in human cells is GTCGTT (69). To avoid designing any oligonucleotides containing the dinucleotide CG is, however, an overly stringent requirement. It eliminates nearly half the possible oligonucleotides that hybridize to a typical message from consideration, many of which show no immune stimulation at all. Therefore, it may be more prudent to avoid oligomers with the consensus hexamer motifs or to restrict the number of CGs in the sequence to less than two. In addition, the immunostimutatory effects of CG motifs are easily eliminated by chemical modification (e.g., 5-methyl C) (70).

VI. OTHER CONSIDERATIONS

A. Cross-Species and Cross-Isoform Oligonucleotides

One feature of antisense inhibitors is that usually an active inhibitor of the human target is not an inhibitor of the same gene in mouse or another species. This is because mRNA sequences differ between species. It is sometimes possible, how-

ever, to select sites with high identity between two species and design oligonucleotides to those sites. If a sufficient number of such sites are tested, it may be possible to identify an antisense oligonucleotide with activity in both species. Similarly, if sufficient sequence identity exists between two isoforms, it may be possible to identify an antisense oligonucleotide with activity against both targets. Using this strategy an oligonucleotide with good activity against both JNK-1 and JNK-2 was identified (71).

B. Target Site Function

The preceding discussion has considered RNA secondary structure at the target site and oligonucleotide sequence but has not seriously addressed position of the target site on the mRNA relative to functional sites such as the coding region. This is because antisense oligonucleotides that operate by an RNAse H mechanism seem to be affected little by target site function. Potent oligonucleotides have been reported for the coding regions, untranslated regions, and even introns. On the other hand, antisense oligonucleotides that use a non-RNase H mechanism are typically restricted to specific functional sites. Morpholino oligonucleotides, for example, inhibit via translation arrest and are often located near or upstream of the AUG initiation codon (72). They can also inhibit splicing if placed at splice junctions (21). Thus target site function becomes more important if a ''steric blocking'' mechanism of action is employed.

VII. SUMMARY

Design of antisense oligonucleotides is, in principle, very simple. In practice, on the other hand, only a fraction of antisense oligonucleotides complementary to an RNA target are active. Computational predictions of hybridization affinity that take into account RNA target structure, oligonucleotide self-structure, and oligonucleotide-RNA hybridization have had limited success at identifying potent antisense sites. Cell-based screening of a number of compounds is still required. Combinatorial approaches offer the potential of finding the best antisense oligonucleotide for any target. These approaches have not, in general, identified compounds with substantially greater activity than those designed by more conventional methods. In addition, significant effort is required for the cell-free screen and several compounds must still be screened in cell-based assays. Although no single approach has yet provided a method for identifying the single best target site for an antisense oligonucleotide, several guidelines are listed here that may improve ''hit rates'' and avoid screening of compounds likely to have nonantisense activities.

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6
Pharmacokinetic Properties in Animals

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I. INTRODUCTION

Antisense technology is a fundamentally different approach to disease treatment both because it provides a simple and rational approach to drug discovery and because it targets a new biological template, mRNA. The mRNA target represents an earlier and, by definition of the antisense method, a more selective target for interrupting the translation of proteins that are involved in the cause or maintenance of disease.

The unique pharmacology of antisense oligonucleotides requires intense study of their in vivo pharmacokinetics. In addition, to aid in the characterization of the safety of antisense oligonucleotides, their exposure as a function of dose and ultimately the rates and mechanisms of clearance from the body must be defined. The development of these compounds as therapeutics has received increasing attention in recent years. As is often the case with new therapeutics and chemistries, the lack of sensitive and selective bioanalytical methods precludes the use of unlabeled materials. Therefore, many of the studies characterizing animal and human pharmacokinetics have relied on radiolabel tracer experiments. Over the past few years, however, methods that provide selective, sensitive, and reliable quantitation of oligonucleotides in biological fluids (1–6) and tissues (7) have allowed characterization of unlabeled material and provided information on metabolism of these compounds. These methods have greatly facilitated the advance of our understanding of antisense phosphorothioate oligonucleotide pharmacokinetics. The objectives of this review are to provide a summary of more recent progress in animal pharmacokinetics and to provide an integrated under-

thioate oligodeoxynucleotides (PS ODNs) in animals. Ultimately, pharmacokinetics of antisense oligonucleotides must include assessment of the kinetics of oligonucleotide delivery and clearance from the site of action coupled with ultimate pharmacological activity, antisense inhibition of specific target mRNA. This chapter will begin to address these issues by providing a review of the pharmacodynamics in the context of whole-animal pharmacokinetics to include tissue distribution and clearance kinetics of parent antisense oligonucleotide.

II. SEQUENCE-INDEPENDENT PHARMACOKINETICS

The existing data demonstrate that phosphorothioate oligodeoxynucleotide (PS oligonucleotide) pharmacokinetics are generally independent of sequence (8,9), suggesting that data from one sequence enhances our understanding of the pharmacokinetic characterization of other PS oligonucleotides. Antisense PS oligonucleotides are generally made up of 18–24 nucleotides linked with 17–23 phosphorothioate linkages each with a net negative charge. Considering their length, the number of negative charges, and their hydrophilicity, it is not surprising that there is little effect of sequence or the order of the nucleotides on the physical/chemical properties of these compounds. Given these similar properties, it should not be surprising that PS oligonucleotides as a class share many properties including pharmacokinetic properties. This hypothesis is supported by empirical evidence demonstrating that there are many similarities in pharmacokinetic properties of different oligonucleotide sequences. For example, monkeys infused with 1 mg/kg over 2 h with any of four different oligonucleotide sequences all have similar C_{max} values (Fig. 1).

The rates of plasma clearance of the oligonucleotides are also similar from sequence to sequence as can be seen from the similar slopes in Fig. 1. AUC values obtained in monkey studies also appear to be similar between sequences and then across species supporting the concept of sequence independence of the pharmacokinetics of these compounds (Table 1). Sequence-independent pharmacokinetics has been observed for a number of sequences in all of the animal species examined including human.

Tissue distribution also appears to be largely independent of sequence in mouse and monkey (Fig. 2). Kidney and liver always exhibit highest concentrations of PS oligonucleotides followed by spleen and lymph nodes. While subtle differences in tissue distribution have been observed in closely controlled studies presumably due to sequence-dependent differences in protein binding (10) or nuclease activity, these differences were not great enough to alter the relative order of distribution organs nor was there any measurable effect on plasma pharmacokinetics. Finally, mass balance excretion of two different sequences of radiolabeled PS oligonucleotide exhibited nearly equivalent excretion profiles 10 days after single-dose administration (Fig. 3).

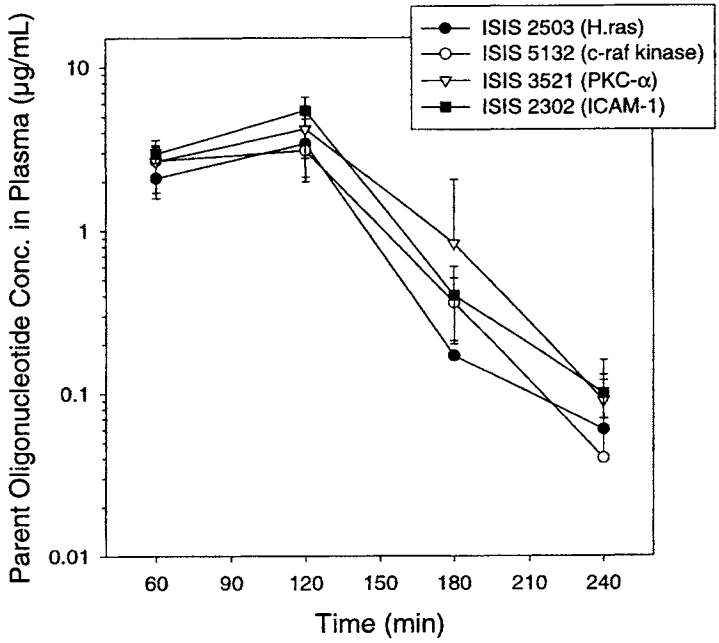

Figure 1 Plasma concentrations of intact oligonucleotides during and following a 2-h intravenous infusion of 1 mg/kg of various oligonucleotides. Plasma samples were extracted and analyzed by CGE. The data are expressed as the intact parent oliogonucleotides (each data point represents the average of three to five monkeys).

Table 1 Comparison of Monkey and Human Pharmacokinetic Parameters

Compound	Species	C_{max} (μg/mL)	AUC (μg/min/mL)
ISIS 2302	Monkey	4.59 ± 0.16	580 ± 11
	Human	3.96 ± 1.02	506 ± 56
ISIS 5132	Monkey	3.27 ± 0.89	396 ± 85
	Human	5.96 ± 2.85	720 ± 243
ISIS 3521	Monkey	4.30 ± 0.75	486 ± 63
	Human	5.45 ± 1.94	704 ± 158

Summary of maximum concentration in plasma (C_{max}) and area under plasma concentration-time curve (AUC) for both cynomolgus monkey and human given equivalent doses based on weight, 1 mg/kg, infused over a 2-h period, intravenously (average ± standard deviation, n = 3–6).

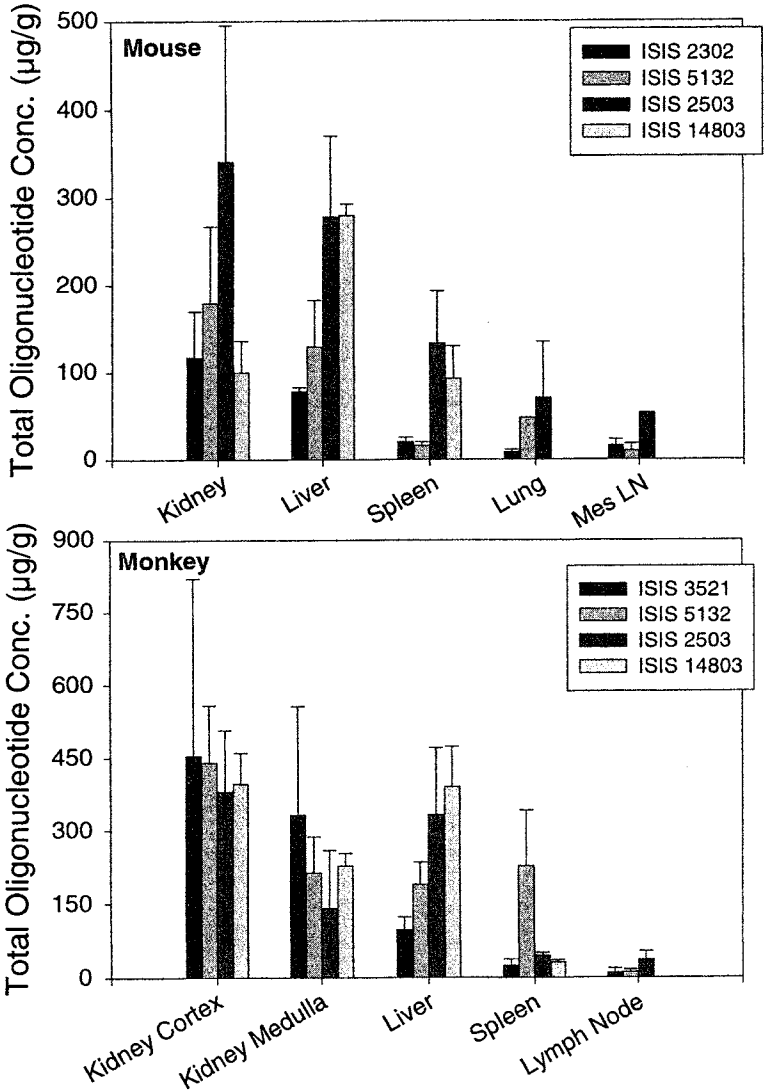

Figure 2 Phosphorothioate oligonucleotide concentrations in tissues from mouse and monkey. Mice were treated with ISIS 2302 for 2 weeks at 15 mg/kg every other day or 5132, 2503, or 14803 for 4 weeks at 20 mg/kg every other day and sacrificed 24 h after the last dose. Each bar represents the average of the concentration of total oligonucleotide in extracts from the respective tissues. Error bars are standard deviation of the mean. At least three animals are represented for each data point.

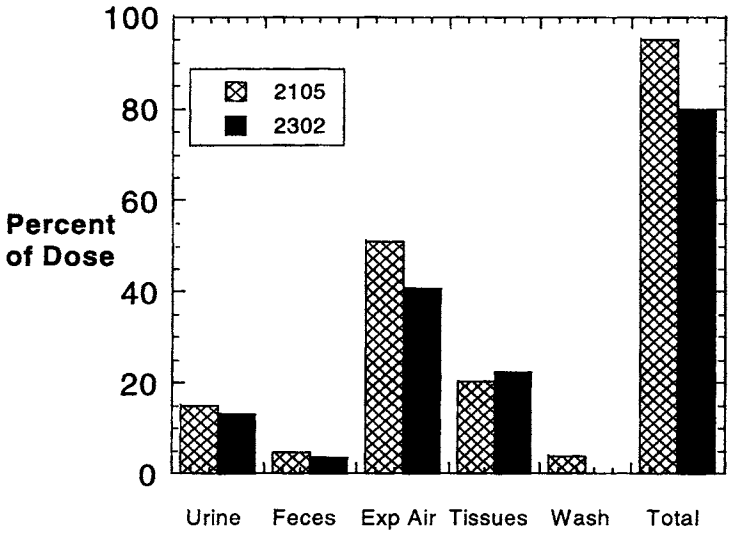

Figure 3 Percent of dose excreted in urine, feces, and expired air and remaining in tissue/carcass 10 days after single-dose administration of ^{14}C-labeled phosphorothioate oligodeoxynucleotides of two different sequences. ISIS 2105 is antisense to human papilloma virus mRNA and ISIS 2302 is antisense to human ICAM-1 mRNA. Both compounds are 20 mers. The radiolabel site is C-2 position of the thymidine nucleotides.

Thus, plasma pharmacokinetics and tissue distribution, clearance, and ultimately whole-body excretion of PS oligonucleotides have been shown to be sequence independent. This fundamental characteristic of these potentially useful therapeutic entities provides a clear path to rapid and safe development for multiple targets and varying diseases. It is likely that as we continue to explore the pharmacokinetics of this class of compound there will be sequences that exhibit some sequence-specific difference. Exonuclease metabolism rates examined in in vitro models, for example, differ in a sequence-specific manner (11). Additional research may provide evidence that such differences can also be seen in vivo.

III. PLASMA PHARMACOKINETICS

A. Intravenous Administration

After intravenous administration, phosphorothioate oligodeoxynucleotides are rapidly cleared from plasma. Plasma concentrations rapidly decrease following injection with distribution half-lives of 30–80 min (8,9,12). Radiolabel disposi-

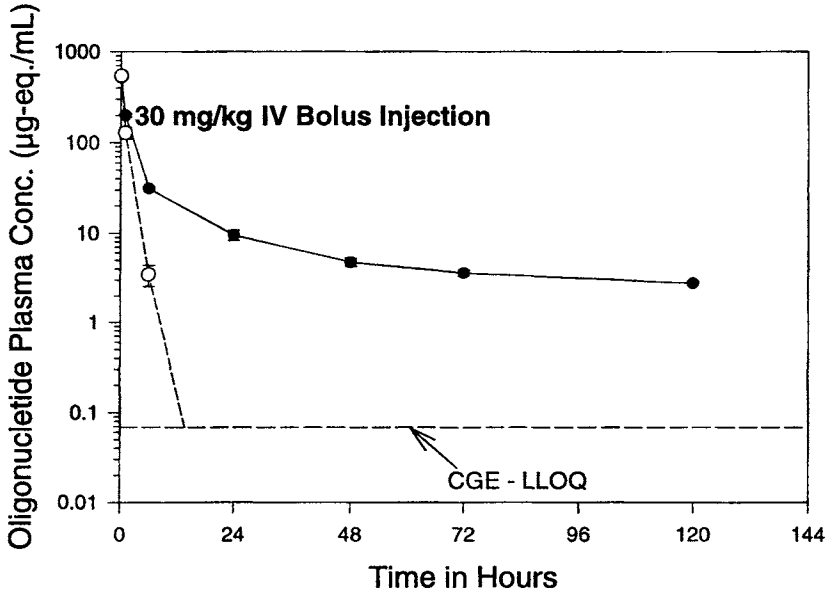

Figure 4 Radiolabel equivalent concentrations (filled circles) compared to parent oligonucleotide of ISIS 3521 (20-mer phosphorothioate oligodeoxynucleotide; assayed by CGE, open circles) concentrations following a bolus intravenous injection of ^{35}S-ISIS 3521 illustrates rapid distribution of parent oligonucleotide compared with prolonged circulation of presumed metabolites. Each time point represents the mean and standard deviation of three to six samples from separate animals.

tion is polyphasic with an additional much slower elimination phase with half-lives reported from 5 to as high as 75 h (Fig. 4), depending on the isotope used to label the oligonucleotide (13–18). The oligonucleotide-equivalent concentrations measured in plasma are several orders of magnitude higher than the intact drug concentrations measured by capillary gel electrophoresis (CGE) indicating that the radiolabel is no longer associated with the parent by later time points. Therefore, this much slower apparent terminal phase is likely a function of slow clearance of radiolabel from distributed tissue and as such appears to be associated with primarily low-molecular-weight metabolites of the original, parent PS oligonucleotide. For ^{14}C-labeled PS oligonucleotides, this slower elimination rate closely paralleled the clearance of oligonucleotide from tissue (17). The distribution portion of the plasma pharmacokinetic profile of intact oligonucleotide (parent) includes greater than 95% of the plasma AUC (internal data, unpublished). It can be deduced, therefore, that clearance of the parent oligonucleotide from

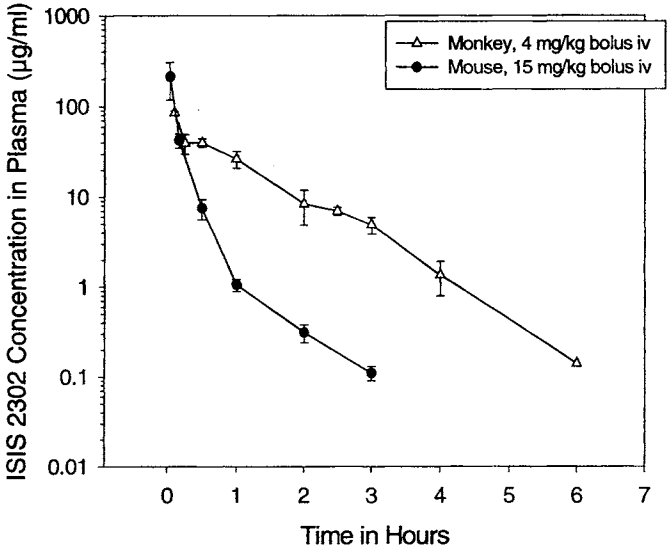

Figure 5 Plasma concentrations profiles for ISIS 2302 following bolus intravenous injections to cynomolgus monkeys and mice. Mice (three per time point) received a 15 mg/kg dose and monkeys (n = 3) received a 4 mg/kg dose.

plasma is a function of distribution to tissue and, to a lesser extent, exonuclease digestion/metabolism.

Plasma clearance rates are largely species independent in rat, rabbit, dog, and monkey and has been estimated to be between 1 and 3 mL/min/kg (8,9). Plasma clearance in mice has been shown to be somewhat faster than in other species (see Fig. 5), ranging from 8 to 14 mL/min/kg (19) (Yu et al., 2001, J Pharm Sci, in press). The more rapid plasma clearance in mice appears to be a function of more extensive exonuclease metabolism seen in mouse plasma (5) or more rapid tissue distribution due to more rapid circulation to tissues in mice. It is not clear which of these plays a dominant role, but it is clear that both of these mechanisms play a role in plasma clearance of PS oligonucleotides. Nevertheless, note that PS oligonucleotide pharmacokinetics scales well across species utilizing allometric correlations as a function of body weight (Fig. 6).

Intravenous infusion has been characterized in nonhuman primate toxicology studies to allow the study of higher doses while minimizing peak plasma concentrations (8,20). This intravenous dosing strategy has been employed in clinical studies for administration of PS oligonucleotides (21–24). After 2-h intravenous infusions in monkeys, maximum plasma concentrations are seen at or

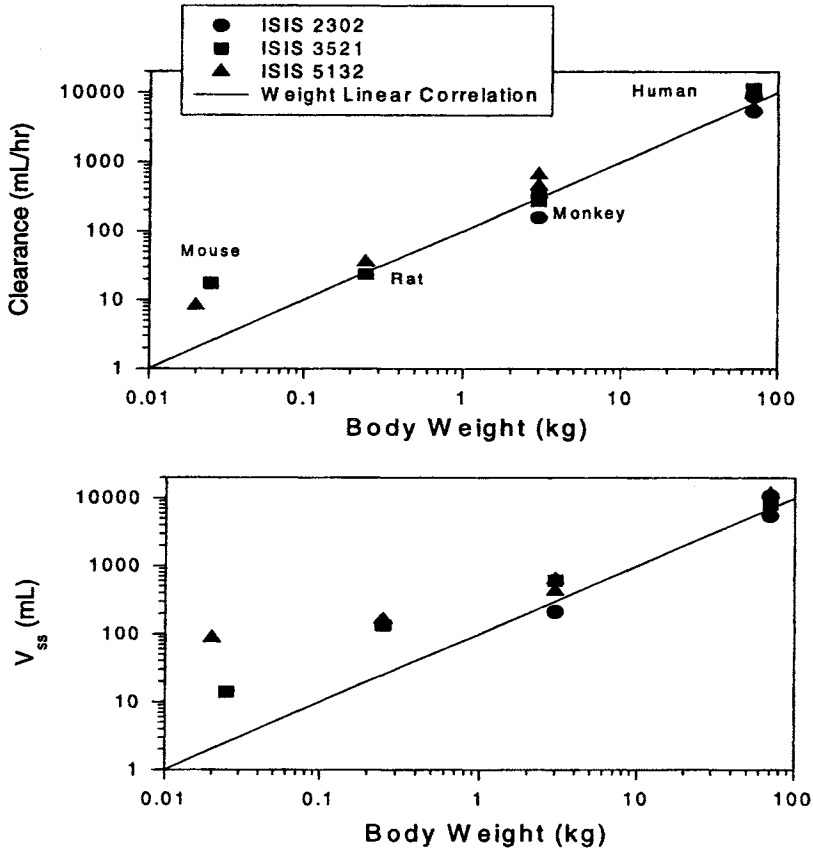

Figure 6 Allometric presentation of plasma pharmacokinetic data across species and different oligonucleotide sequences including ISIS 2302 for comparison. Each data point represents the mean of three to six animals or subjects. The doses used in the comparison were all ≤3 mg/kg. The slope of the line is 1 for both presentations, evidence that for all sequences the relationship is scalable directly by body weight.

near the end of the infusion period. The plasma concentrations at the end of the infusion are generally seen to be dose proportional. For example, in monkeys, plasma concentrations of 20 mer (parent oligonucleotide) were approximately 5, 15, and 50 μg/mL at the end of a 2-h infusion period for doses of 1, 3, and 10 mg/kg, respectively (8,9). After the infusion was stopped, PS oligonucleotide plasma concentrations decreased rapidly similar to that seen following intravenous bolus injection.

In mice, the plasma pharmacokinetics for doses ranging from 0.8 to 100 mg/kg appear to follow linear first-order pharmacokinetic principles with dose-proportional increases in maximum plasma concentrations and AUC (19). After repeated dose administration to mice every other day for 28 days, the plasma AUC and elimination half-lives increased at doses of 20 and 100 mg/kg, suggesting a change in clearance. After repeated administration at lower doses in mice and monkeys for up to 6 months, no change in plasma clearance was seen (internal data, unpublished). However, the area under the plasma concentration-time curves (AUC) increased greater than proportional for single doses administered to rats and monkeys at doses ranging from 1 to 30 mg/kg (Table 2). Collectively, these data suggest saturation of some disposition pathway at the higher doses that appears to occur only after repeated dosing in mice but is observed acutely in other animal species including humans (22). These nonlinear kinetics are characterized by slower apparent distribution half-lives at higher doses and a slowing in overall plasma clearance at higher doses. As will be described in metabolism sections in greater detail, it is now evident that the extent and rate of metabolism in vivo of PS oligonucleotides in plasma are not affected by increased dose (19) (Yu et al., 2001, J Pharm Sci, in press). However, distribution to organs that take up a large fraction of the oligonucleotide dose (liver and kidney) appears to saturate as dose increases (25–27). Taken together, these data suggest that the nonlinear behavior observed in plasma clearance may be related to saturation of distribution to major organs of distribution.

Longer-term continuous infusion may have the advantage of providing prolonged exposure of the oligonucleotides to organs that do not readily take up oligonucleotide. Some initial evidence of this was observed in early continuous infusion studies reported by Iversen et al. (28). A direct comparison of rapid infusion and continuous infusion of ISIS 3521 in rats and monkeys has confirmed that higher tissue concentrations can be achieved (Geary et al., in preparation). These increases in tissue concentration are achieved in both major organs of distribution that exhibit saturable distribution and other tissues and organs that take up much smaller fractions of the oligonucleotide dose (Fig. 7). Upon continuous infusion, plasma concentrations of oligonucleotide achieve steady state by 8 h in rats and monkeys, consistent with rapid clearance from plasma. After 5 days of continuous infusion, the concentrations remain constant in plasma and then fall rapidly after the infusion is stopped. There does not appear to be any change in clearance even after 5 days of continuous infusion in rats (Fig. 8). These data taken together suggest that saturation of distribution may be an acute equilibrium-binding phenomenon on the surface of cells and tissues rather than true saturation of uptake into the organ or tissue. As will be discussed in more detail in the following sections, uptake in cells associated with tissues of distribution is believed to occur in sequential steps that require initial binding to tissue proteins followed by as yet poorly understood active or passive internalization into cells.

Table 2 Summary of Dose-Dependent Plasma Pharmacokinetics Observed for Two 20-mer PS Oligonucleotides in Rat and Monkey

Parameter	Rat ISIS 2302 (ICAM-1)		Rat ISIS 3521 (PKC-α)		Monkey ISIS 2302 (ICAM-1)		Monkey ISIS 3521 (PKC-α)		
dose (mg/kg)	4	20	3	30	1	4	1	3	10
C_{max} (μg/mL)	52	315	54	591	4.6	30	4.3	20.8	52.3
AUC (μg/h/mL)	39.2	290	45	676	9.67	77.5	8.10	37.2	110
$t_{1/2distribution}$ (min)	28	56	61	90	28	57	80	70	155
Cl_p (mL/min/kg)	1.70	1.15	1.10	0.74	1.72	0.86	2.06	1.34	1.51

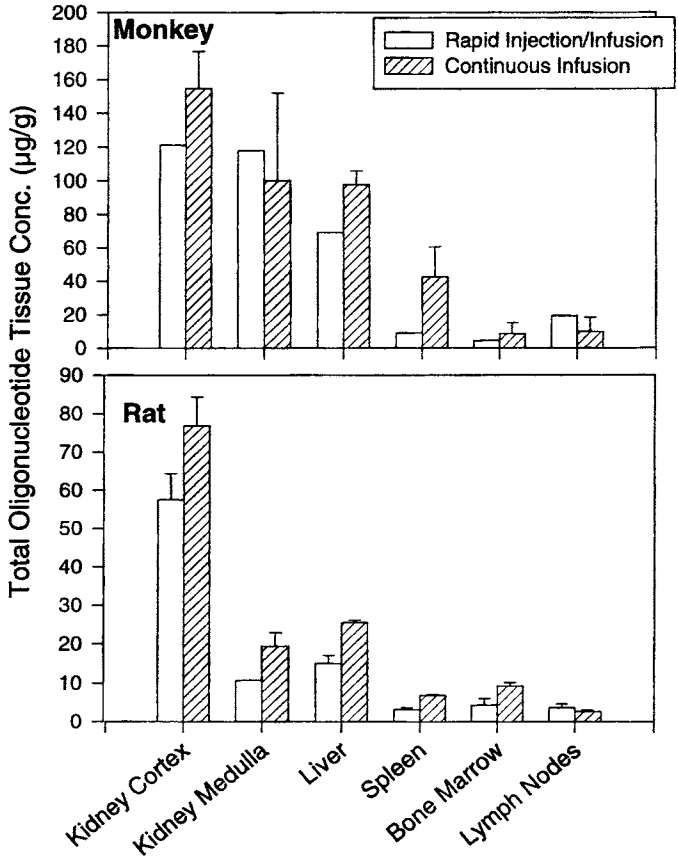

Figure 7 Increased distribution of oligonucleotide to tissues following continuous intravenous infusion of ISIS 3521 in monkeys and rats when compared with shorter-duration infusions (2-h infusion in the monkey) or bolus injection (rat). The tissues were collected at sacrifice 24 h after the dose was administered. Each bar represents the average of three to six animals [except for the 2-h infusion arm (open bar) of the monkey study, $n = 2$]. Error bar is the standard deviation. Dose: 3 mg/kg.

B. Absorption

Although oligonucleotides are hydrophilic, relatively large, and have multiple charges at physiological pH, antisense phosphorothioate oligodeoxynucleotides are remarkably well absorbed from intradermal and subcutaneous sites of injection (10,18,26,29,30). Low and dose-dependent systemic absorption is seen following intratracheal administration as well as via pulmonary delivery using aero-

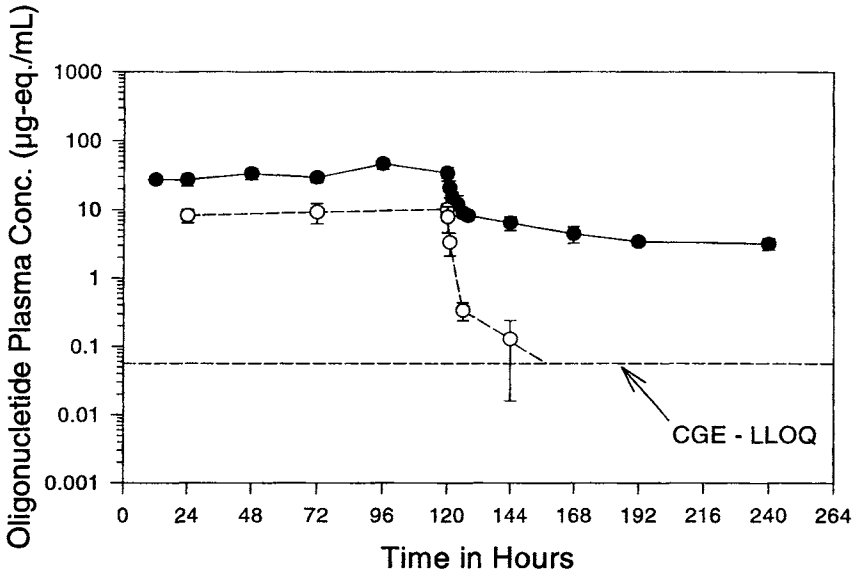

Figure 8 Plasma concentrations of radiolable equivalent oligonucleotide (filled circles) compared with intact ISIS 3521 (20-mer PS oligonucleotide, assayed by CGE, open circles) during a 5-day continuous infusion study in rats. Steady-state concentrations of parent compound as well as total radiolabel were achieved by 24 h consistent with the rapid disposition kinetics. Each time point represents the average and standard deviation of three to six samples from separate animals.

solized liquid containing oligonucleotide (30) (Templin et al., 2000, Antisense Nucleic Acid Drug Dev, in press). Nevertheless, oral absorption of this class of compounds is poor likely due to the extreme conditions encountered in the gastrointestinal tract resulting in poor stability coupled with poor permeability across the gastrointestinal epithelium (10,31,32) (Geary et al., 2001, in press).

1. Parenteral Administration

Treatment of systemic conditions by direct intravenous injection of antisense PS oligonucleotides bypasses absorption barriers for delivery of these compounds. However, peak plasma concentrations generated by rapid injection directly into venous blood is prohibited by hemodynamic effects that occur at high oligonucleotide plasma concentrations. Therefore, slow intravenous infusion (either 2-h or

continuous infusion over days) has been adopted for direct systemic delivery. These regimens have the disadvantage of being inconvenient for both the patients and the health-care professionals caring for the patients.

Intradermal injection of ^{14}C-labeled ISIS 2105 (human papilloma virus antisense) was explored as a potential route for local treatment of genital warts. However, ISIS 2105 was rapidly absorbed from the injection site (18) in rats with approximately 65% of the dose absorbed within the first hour. Peak plasma concentrations were observed within 30 min. This was later confirmed in clinical trials (29). While this represented a failed approach to localization of the oligonucleotide, it provided a clue that delivery into well-perfused regions in the body could result in nearly complete absorption of these compounds.

Subcutaneous administration of PS oligonucleotides likewise results in nearly complete absorption of these compounds over time (26,30). Subcutaneous injection also slows the input of oligonucleotide as compared to direct intravenous injection and thus results in lower maximum plasma concentrations (Fig. 9). This characteristic of subcutaneous administration is attractive for clinical purposes because it allows for rapid injection along with the convenience of self-administration without inducing the high plasma concentrations associated with established threshold for hemodynamic changes seen in monkeys (33,34). Although evidence of complement activation in the clinic has not materialized to date, the added convenience of self-administration should not be minimized. Furthermore, subcutaneous administration of PS oligonucleotides has been implemented in long-term safety studies 1–6 months in duration when repeated intravenous administration was not practical (internal data, unpublished).

2. Nonparenteral Administration

Pulmonary, oral, and rectal routes of administration for PS oligonucleotides have been explored. Intratracheal administration in rats resulted in significant, albeit dose-dependent bioavailability of ISIS 3521 (CGP 64128A) (30,35). The dose-dependent absorption may be the result of local irritation or inflammation caused by high localized concentrations of PS oligonucleotides. However, experiments with permeability markers appear to suggest that functional integrity of the tracheal or lung epithelia was not compromised. Thus, the increased absorption at higher doses may be related to a saturable absorption-limiting local tissue binding in the lung. Direct pulmonary delivery to mice using aerosolized ''naked'' PS oligonucleotide, ISIS 2105, administered at doses of 1.2–12 mg/kg, also resulted in dose-dependent systemic absorption. In this experiment, however, mild inflammatory response was observed at the 12-mg/kg dose. At doses at or below 3 mg/kg, toxicity of the lung was minimal to absent and resulted in excellent local exposure of most cell types in the lung (Templin et al., 2000, Antisense

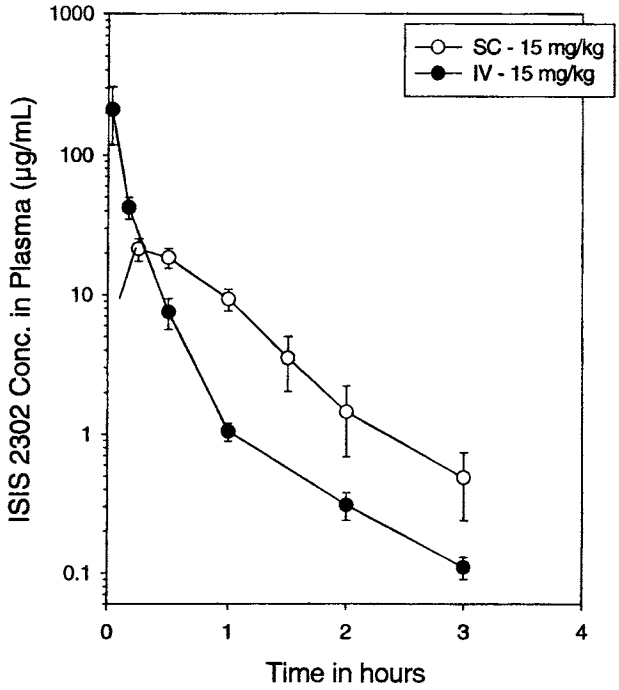

Figure 9 Plasma concentration profiles of intact ISIS 2302 (20 mer) as a function of time following single subcutaneous or intravenous bolus administration of 15 mg/kg in mice. Each time point represents the average of three animals. The error bars are standard deviation.

Nucleic Acid Drug Dev., in press). Other reports have shown impressive data supporting the utility of direct pulmonary delivery of antisense PS oligonucleotides for treatment of inflammatory diseases such as asthma (36,37).

Oral administration represents the most desirable route of administration for the convenience of treatment in the clinic, particularly for chronic diseases. However, the oral bioavailability of PS oligonucleotides is poor, with less than 1% of a radiolabeled dose absorbed (10,30). The permeability of PS oligonucleotides has been shown to be low using both in vitro methods (31) and an in situ rat intestinal method (32).

Improvement in permeability on the order of two- to fourfold was achieved by chemical modifications that will be common for the next generation of antisense oligonucleotides (38). We have also characterized the stability of PS oligonucleotides in the gastrointestinal tract and it is clear that they are susceptible to

rapid nuclease digestion. In the rat intestine, the half-life of oligonucleotide is approximately 1 h with no measurable intact oligonucleotide in the intestine by 8 h after administration (Geary et al., 2001, J PET, in press). These data suggest a substantial preabsorptive barrier to successful bioavailability of PS oligodeoxynucleotides. Once again, the second-generation modified oligonucleotide chemistries that afford much improved stability to nuclease digestion are likely to provide a more reasonable opportunity for success by this route of administration.

C. Plasma Protein Binding

Phosphorothioate oligodeoxynucleotides readily bind to plasma proteins. At clinically relevant doses, more than 96% of PS ODN in plasma is bound to plasma proteins in mice, rats, monkeys, and humans (Fig. 10). For all species tested, there is little change in binding over the concentration range from approximately 1 μg/mL to 68 μg/mL (Watanabe et al., 2000, manuscript in preparation). Albumin and α_2-macroglobulin are the major protein species that bind ISIS 2302 with relatively high capacity (39). Affinity of binding has been characterized for these two proteins using different analysis methods. Electrospray–mass spectrometry (ES-MS) provided estimates for dissociation constants of 3.1 μM (K_{D1}) and 11.9

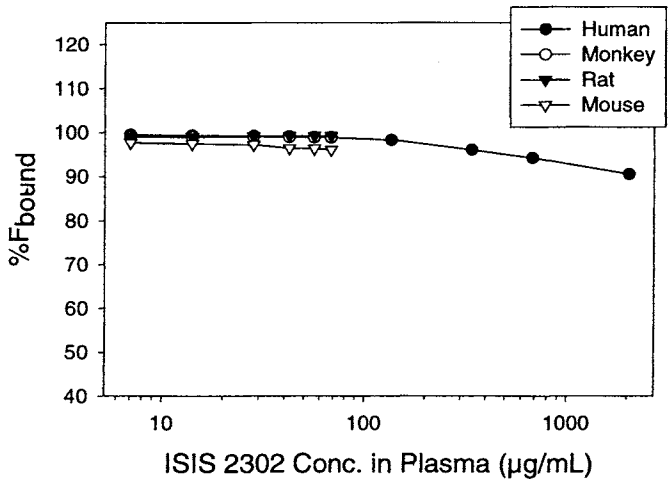

Figure 10 Comparison across species of fraction of ISIS 2302 in plasma that is in the bound form. Protein-binding experiments were conducted in fresh whole plasma. Each analysis involved five replicates and the error bars represent standard deviation of the mean.

μM (K_{D2}) for albumin (40). Binding to plasma proteins has been shown to be salt- and pH-dependent, suggesting that the binding is likely a nonspecific electrostatic interaction. The binding affinity of PS ODN to α_2-macroglobulin was found to be greater than albumin. Using surface plasma resonance (SPR), the dissociation constants were determined to be 4 nM (K_{D1}) and 72 nM (K_{D2}) for α_2-macroglobulin (internal data, unpublished) for another 20-mer phosphorothioate oligodeoxynucleotide.

In contrast, binding a 20-mer PS ODN, ISIS 2302, to α_1-acid glycoprotein (AAG), another high-capacity plasma protein, was negligible (internal data, unpublished). The levels of AAG in plasma of Crohn's disease patients is known to be increased (41) and these altered levels can affect the kinetics of drugs bound to AAG. Such is the case with the β blocker propanolol. The apparent lack of oligonucleotide binding to AAG makes it unlikely that AAG alterations in Crohn's patients will impact the pharmacokinetics of antisense oligonucleotides administered to this patient population.

Phosphorothioate oligonucleotides have been shown to bind to other less abundant plasma proteins, sometimes with high affinity. For example, SPR analysis demonstrated that PS ODN bind to thrombin-binding sites with relatively high affinities of 30 nM (K_{D1}) and 230 nM (K_{D2}) (internal data, unpublished). Factor H (33) and components of the intrinsic tenase complex (42) have also been shown to bind with PS ODN. The binding of PS oligonucleotides to these proteins in serum at high concentrations of oligodeoxynucleotides appears to disrupt normal enzymatic function and likely explains acute hematological effects observed in monkeys (33).

Protein binding may explain many other pharmacokinetic properties of ISIS 2302. The high degree of protein binding in circulation prevents any significant urinary excretion. Therefore, there is little glomerular filtration of the protein-bound oligonucleotide and the excretion of intact compound in urine is a minor pathway for its clearance from plasma (19). In mouse toxicology studies with doses ranging from 0.8 to 100 mg/kg, urine excretion of intact oligonucleotide was seen to be dose dependent as increased excretion was observed with increased dose. Thus, urine excretion occurred as concentrations of oligonucleotide exceeded the capacity of the plasma binding. Only after bolus intravenous injection of high doses (20 mg/kg) of ISIS 5132 in mice are plasma concentrations high enough to increase unbound fraction of oligonucleotide resulting in urinary excretion of intact oligonucleotide. However, saturation of plasma protein binding occurs only at plasma concentrations many fold higher than those achieved at therapeutic doses in the clinic (Watanabe et al., manuscript in preparation). The negligible renal excretion of oligonucleotide provides greater opportunity for PS oligonucleotide to distribute to peripheral tissues, the site of action.

Protein binding of PS oligonucleotide is reversible and exists as an equilibrium between high-affinity and low-affinity proteins. Some protein binding is

associated with plasma while other binding occurs in tissue as well. Tissue distribution is attributed to protein binding and the binding of higher-affinity proteins in organs and on the cell surface. Once bound, oligonucleotide is internalized in the cell. Thus, both whole-body distribution and cellular uptake of PS oligonucleotide and other oligonucleotides are highly dependent on protein binding (S. Crooke et al., JPET paper, in press). It is tempting to speculate that the observed saturable distribution characteristics of PS ODN may be associated with saturation of binding to proteins in specific tissues.

Note that extremely high concentrations of aspirin (2 mg/mL in plasma of treated rats) have been reported to displace another 25-mer PS oligonucleotide from whole-rat plasma proteins, increasing free fraction from approximately 6% to approximately 11% (43). The dose of aspirin required to achieve displacement of the PS oligonucleotide in vivo was 50 mg/kg. Increasing the free fraction of PS oligonucleotides resulted in decreased exposure of oligonucleotide in plasma and in tissue with concomitant increases in urinary and fecal excretion. Thus, displacement of oligonucleotide from plasma results in increased excretion and an overall decrease in exposure in plasma and tissue. However, the concentrations of aspirin required to accomplish this effect were many times higher than are clinically relevant. The mechanism for displacement was not examined. Nevertheless, these data point to the need for further understanding of the interaction of PS oligonucleotide binding with other drugs that bind to serum albumin or other relevant plasma proteins.

IV. TISSUE DISTRIBUTION AND CLEARANCE

Rapid clearance of PS oligonucleotides from plasma occurs with a concomitant appearance in tissues in mice, rats, and monkeys (13,16,19,26,44). The highest concentrations of PS oligonucleotides in all species studied were found in kidney, liver, spleen, and lymph nodes (Fig. 11), but PS oligonucleotide can be measured in almost every tissue, except brain, shortly after intravenous administration. Cell-specific distribution within kidney and liver has been extensively studied (45–52). Once again, the similarity between the distribution observed for PS oligonucleotides in various species provides evidence that the pharmacokinetics are largely driven by their chemical class and not by their specific sequence (9). The consistency of tissue distribution of PS oligonucleotide between species is thought to be the primary reason for the similarity in plasma kinetics between species.

Cellular distribution of PS oligonucleotides within various organs has been described using autoradiography (26,52–54), immunohistological methods (54–56), and suborgan as well as subcellular physical separations coupled with capillary gel electrophoresis (51).

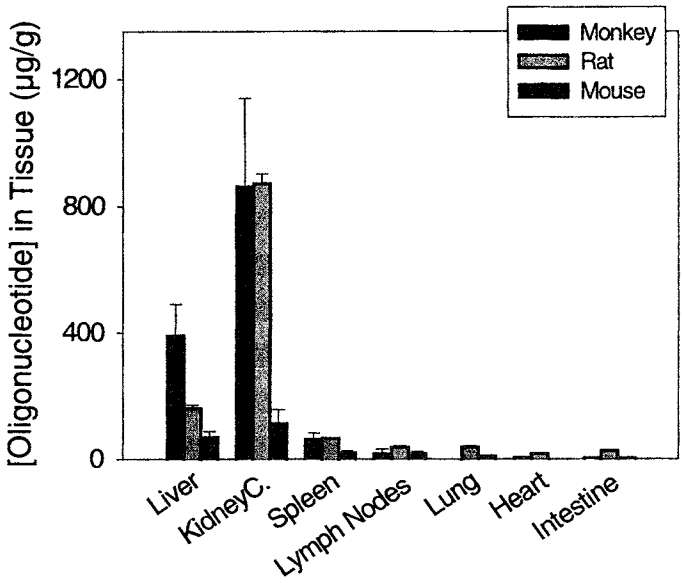

Figure 11 Comparison of the tissue concentration profiles for total oligonucleotide associated with ISIS 2302 across species. The monkey tissues were collected 48 h after the last dose following 28 days of dosing of alternate-day dosing of 4 mg/kg. The rat tissues were collected 48 h after the last of four doses administered on an alternate-day schedule for 1 week at a dose of 20 mg/kg. The mouse tissues were collected 24 h after the last of seven alternate-day doses of 15 mg/kg given over a 2-week period. Each bar represents at least three animals. Error bars are standard deviation.

Once PS oligonucleotides are distributed into tissues, their clearance is relatively slow (17,19,20). Initial distribution into tissues is associated with binding of the oligonucleotide to extracellular matrix, interstitium, or loosely bound to the cell membrane. However, by 4 h after intravenous administration in rats approximately half of the oligonucleotide measured in the liver was located intracellularly (51). By 24 h after injection very little oligonucleotide remains bound to extracellular components. Thus, it is likely that whole-organ pharmacokinetic evaluation after 24 h will represent intracellular exposure and whole-organ clearance will parallel cellular clearance. Tissue half-lives were comparable across species with the longest clearance half-lives seen in monkeys (Table 3). The relative rates of tissue elimination of ISIS 2302 in rats and mice and ISIS 3521 in monkeys are consistent with that reported for other PS ODN (17–19).

Thus, while PS oligonucleotides are cleared quickly from blood, their residence time in tissues in all species examined is relatively long. The relatively

Table 3 Comparison of Tissue Elimination Half-Lives (in Days) for Intact Oligonucleotide in Rodents and Monkeys

	Intact PS oligonucleotide half-life (in days)		
Tissue	Mouse[a]	Rat[a]	Monkey[b]
Kidney	2.8	4	5
Liver	1.8	2.6	2.8
Spleen	0.7	0.9	3.8
Lymph nodes	0.6	1.7	0.9

[a] ISIS 2302.
[b] ISIS 3521.

slow clearance from tissues is favorable for the intended pharmacological application, and alternate-day administration appears to maintain oligonucleotide concentrations sufficient to produce pharmacological activity. With half-lives estimated between 1 and 5 days, accumulation factors should range from 1.5- to approximately threefold depending on the tissue or organ, if the pharmacokinetics follows first-order, linear pharmacokinetic principles. In fact, the accumulation observed in mice and rat appears to be predicted based upon these principles.

A simulation exercise was undertaken based on the assumption that the tissue half-lives calculated for one sequence are relevant to another. The tissue concentrations for ISIS 2302 were simulated using tissue half-life data obtained for ISIS 3521 in monkey (25). Half-life in tissues determined on the basis of single-dose kinetics of ISIS 3521 accurately predicted the steady-state concentration for ISIS 2302 at the end of 1- or 6-month toxicity studies, and also predicted peak and trough concentrations produced by an intermittent dose schedule (Fig. 12). Slow clearance from tissues is also consistent with the apparent accumulation in target organs following every-other-day administration. The fact that accumulation in tissues occurs with repeated administration suggests that the saturation observed in liver as dose increased was a saturation of tissue distribution kinetics specifically, and not a saturation of the capacity of the organ to store oligonucleotide.

Although accumulation of ISIS 2302 and total oligonucleotide occurred with every-other-day administration, steady-state concentrations were achieved within 4 weeks of treatment (internal reports, unpublished). In subchronic and chronic treatment of monkeys, tissue concentrations of ISIS 2302 were the same at 28 days, 3 months, and 6 months of treatment, suggesting steady state was achieved within 4 weeks of treatment (internal data, unpublished). Furthermore,

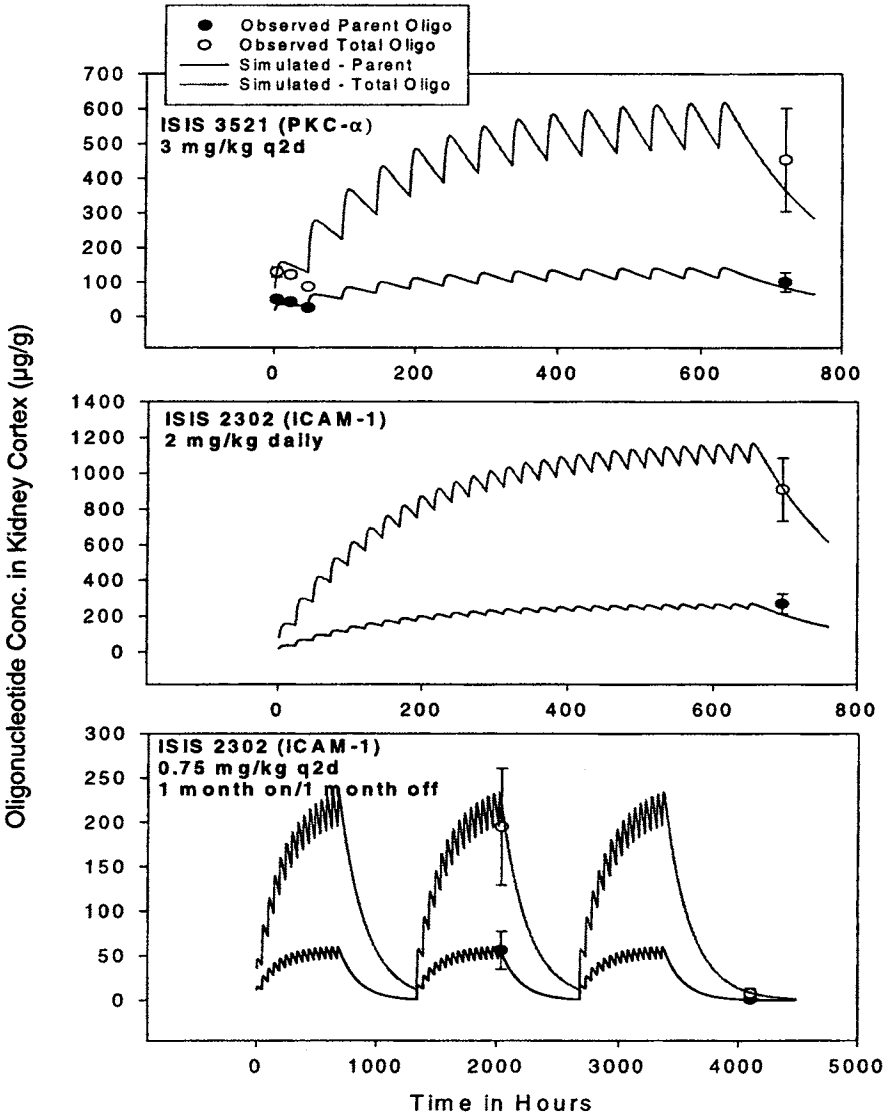

Figure 12 Simulated oligonucleotide concentrations compared with measured concentrations in monkey kidney cortex following repeated intravenous dosing by either alternate-day or daily dosing regimens. (Top) Data for ISIS 3521—28 days of dosing every other day; (middle) data for ISIS 2302—28 days of dosing daily; and (bottom) data for ISIS 2302 doses on alternate days for 1 month on and then 1 month off, repeated three times. Both total and parent oligonucleotide are represented in this simulation.

it is clear that steady-state concentrations were predicted on the basis of single-dose kinetics of a related 20-mer PS ODN.

Data from repeat-dose pharmacokinetic studies indicate that tissue concentrations of PS oligonucleotides can be maintained with every-other-day, or, by inference, 3-times-per-week treatment regimens. Thus, these dose regimens are appropriate for toxicity evaluation or therapeutic activity, because they maintain exposure to PS oligonucleotides in target organs for the duration of treatment and for some period of time thereafter. Complete clearance from tissues is predicted to occur by 2–4 weeks after cessation of dosing.

Although tissue exposure was dose-dependent, tissue concentrations in liver, and to some degree in kidney, increased less than proportional to dose (Fig. 13). For example, liver concentrations increased only fivefold as the dose increased from 2 to 20 mg/kg (22 ± 13 μg/g and 122 ± 23 μg/g, respectively). Nonlinear tissue distribution was observed for both the parent oligonucleotide and total measurable oligonucleotide (including chain-shortened metabolites). Since liver accounts for the greatest percentage of dose distributed to tissue, saturation of the oligonucleotide distribution correlates with greater than dose-proportional increases in plasma C_{max} and AUC values together with a decrease in plasma clearance described earlier.

V. METABOLISM AND EXCRETION

Metabolites of PS ODN are evident in both plasma and tissues almost immediately after dosing. Metabolism occurs predominantly through exonuclease-mediated cleavage of nucleotide residues from the parent oligonucleotide (3,5,6). There is no evidence that hepatic microsomal enzymes play a significant role in the elimination of this class of compounds (11). Exonucleases cleave single nucleotides from either the 3′ or 5′ end of the molecule, liberating mononucleotides and oligonucleotide metabolites that are shortened by one nucleotide. The first oligonucleotide metabolite is 19 nucleotides in length, and is referred to as a 19-mer or the N-1 metabolite. This metabolite continues to be shortened until it is completely degraded or the ''shortmers'' are excreted in the urine. Evidence for this progressive metabolism is found in electropherograms showing the profile of metabolites over time (Fig. 14). The metabolism is progressive such that an N-1 metabolite is the primary oligonucleotide metabolite followed by N-2, N-3, etc., as more nucleotides are sequentially removed.

The metabolic fate of PS ODN follows many, if not all, of the same pathways as endogenous nucleotides, nucleosides, and bases. Studies with PS oligonucleotides have exploited different radiolabeling schemes and a number of bioanalytical techniques to obtain a thorough understanding of the metabolic fate of the nucleotides and bases, as well as sulfur on the thioate linkage, the xenobiotic

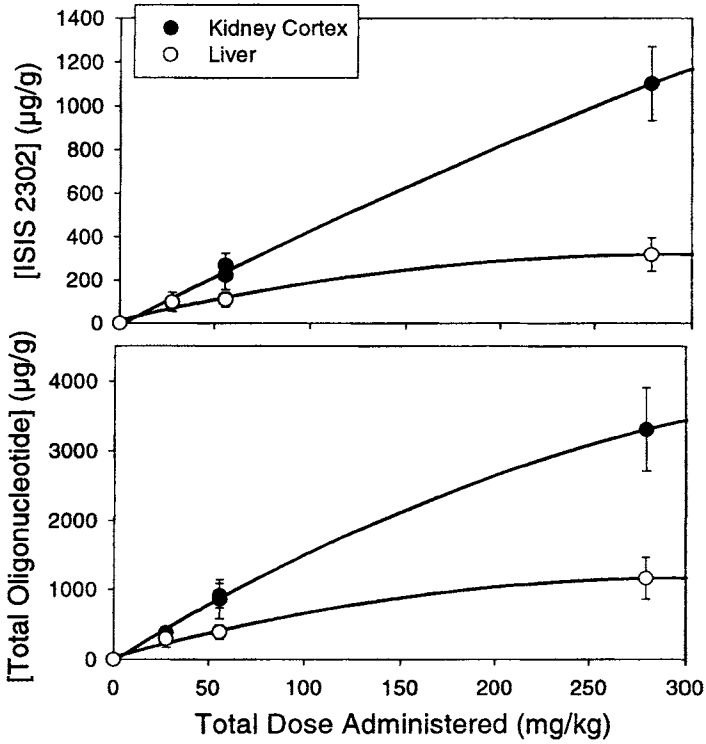

Figure 13 Distribution of oligonucleotide to liver and kidney as a function of total administered dose. ISIS 2302 and total oligonucleotide concentrations in liver and kidney from cynomolgus monkey are plotted against total dose administered. The doses were given as follows: 2 mg/kg given every other day for 28 days (total dose of 28 mg/kg), 4 mg/kg given every other day for 28 days (total dose of 56 mg/kg), and 20 mg/kg given every other day for the same period (total dose of 280 mg/kg). Approximately a 10-fold increase in dose resulted in less than a fourfold increase in ISIS 2302 concentrations in liver. Similar decrease in the amount of total measurable oligonucleotide taken up by liver tissue was seen over this dose range.

component of PS ODN. Mass balance studies using ISIS 2302 radiolabeled at the C-2 position of the thymines provided significant information on the metabolism of the oligonucleotide and the specific fate of the pyrimidine bases. In these studies, all excreta and expired air were collected from rats treated with ^{14}C-labeled ISIS 2105 (17,18). Liberation of $^{14}CO_2$ was the primary route of clearance of pyrimidine-derived radiolabel. Approximately 50% of the radiolabel is cleared over the course of 10 days as $^{14}CO_2$. Two other 20-mer PS ODN of different

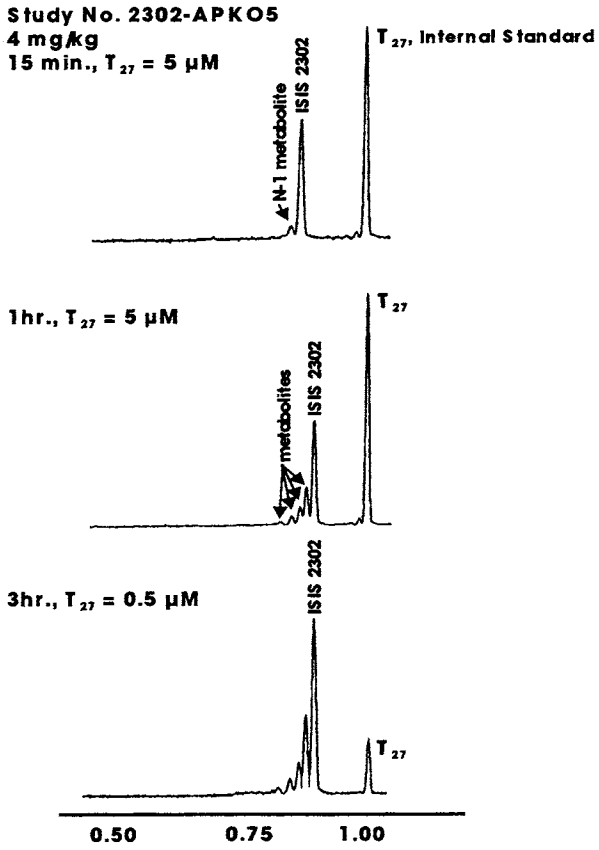

Figure 14 Capillary gel electropherograms of plasma taken from monkeys following a 4 mg/kg dose of ISIS 2302. T_{27} is a 27-mer deoxythymidine added as an internal standard at the concentration indicated.

sequence (ISIS 2922 and ISIS 2302) but equivalent label type and position have exhibited similar mass balance profiles of metabolic fate (internal data, not published). Taken together, these data suggest that the pattern of metabolism is common for the class.

A. Nuclease Metabolism

The successive removal of bases from the 3′ end of PS ODN is the major pathway for metabolic degradation in plasma (4,5), while both 5′ and 3′ exonuclease exci-

sion may occur in tissues (2,3,6). LC/MS and end-labeling techniques have been applied to allow differentiation and identification of the shortened metabolites.

Thus, exonuclease-mediated metabolism produces a metabolic pattern of progressively shorter metabolites each shortened by a single nucleotide. Metabolism by endonucleases would be expected to produce a metabolite pattern characterized by an irregular distribution of shortened metabolites and an enrichment of metabolites cleaved at specific intervals; for example, a 10-mer metabolite might be observed in the absence of the 11- or 12-mer. Phosphorothioate oligonucleotide metabolites consistent with endonuclease activity have not been observed in studies with PS ODN (5,11,19).

As PS ODNs are metabolized to shorter oligonucleotides after intravenous administration, the percent of oligonucleotide in plasma that is represented by full length (20 mer) drops from approximately 91% immediately following intravenous administration, to 65% by 10 min after injection in mice (19). Subsequent to this initial rapid shortening, however, the percentage of full-length ISIS 2302 slowly decreases over the next 3–4 h (Fig. 15). This apparent change in metabolic rate over time is observed in every species studied including human (21). The biphasic nature of the metabolism profile in plasma is poorly understood, but is thought to be related to the stereochemistry of a PS ODN.

The sulfurization process produces a random distribution of R and S chiral

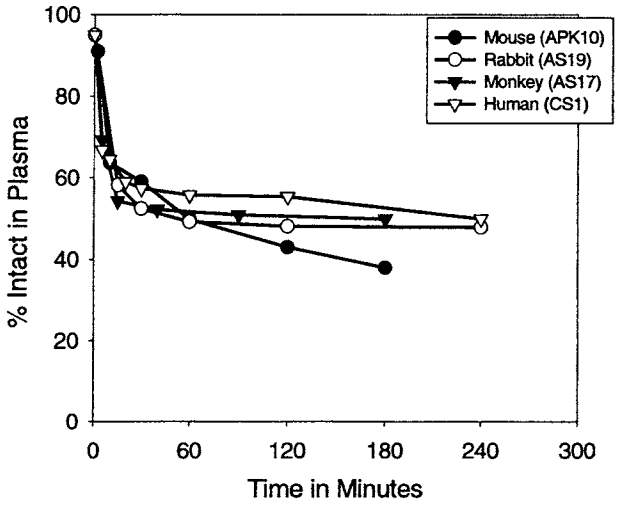

Figure 15 Rate and extent of metabolism seen in plasma and compared across species. Percent intact is the percent of total measurable oligonucleotide represented by parent ISIS 2302 (20 mer). Each point represents the average of three to six individuals.

centers at each phosphorothioate linkage. These are referred to as Rp and Sp centers. Therefore, phosphorothioate oligodeoxynucleotides are racemic mixtures of Rp- and Sp-phosphorothioate linkages, with approximately 2^{19} isomers in the mixture. The rate of exonuclease cleavage for the Rp stereoisomer is much more rapid than for the Sp isomer (57–59). The rate of metabolism of any single molecule of ISIS 2302 is expected to be relatively rapid until the exonuclease reaches an Sp linkage at which point it would slow. In plasma, where metabolism is almost exclusively from the 3′ terminus, this phenomenon would tend to favor ISIS 2302 metabolites that have a 3′ terminal Sp linkage. If approximately 50% of the linkages are Sp, then it would follow that metabolism would slow as the population of oligonucleotides became more and more enriched with oligomers that have the more slowly metabolized Sp linkage. Because approximately 50% of the oligonucleotide administered will have an Sp linkage at the 3′-most phosphorothioate linkage, metabolic slowing would be reached when approximately 50% of the parent compound remains.

In the absence of techniques to differentiate between stereoisomers it has not been possible to demonstrate that this enrichment actually occurs. However, kinetic modeling was performed to determine whether metabolic preferences might result in a pharmacokinetic profile like that observed in vivo. The pharmacokinetic model was built assuming a probability of 0.5 at each linkage for rapid (3-min Rp) or resistant (3-h Sp) cleavage (19). The kinetic pattern produced by this model is very similar to that observed in vivo, suggesting that stereochemical differences in metabolic rate could, at least theoretically, produce the metabolic slowing that is observed in plasma in all species. The biphasic profile of nuclease metabolism observed in the plasma of all species may, therefore, be explained by a differential rate of metabolism for the Rp and Sp diastereoisomers present at each phosphorothioate linkage of ISIS 2302. Note that this was observed in all species exposed to the drug, such that both toxicity studies and clinical trials evaluated the drug under conditions where there was the potential for this stereochemical enrichment.

Intact ISIS 2302 is always the most abundant oligonucleotide species observed in plasma or tissue following intravenous or subcutaneous administration in mouse or monkey. After repeated administration of PS oligonucleotide at doses ranging from 0.8 to 100 mg/kg in mice (19) and 1 to 10 mg/kg in monkeys (R. Yu et al., 2001, J Pharm Sci, in press), the metabolite pattern observed in plasma is unchanged suggesting that metabolism in the circulation is neither inhibited nor induced. Indeed the pharmacokinetic profile of oligonucleotide metabolites shortened by one or more nucleotides was similar to that of the parent oligonucleotide, ISIS 2302 (Fig. 16). Also, the pattern of metabolites in tissue was not altered after repeated every-other-day intravenous administration.

Intact PS ODN represented approximately 20–40% of the total oligodeoxynucleotide measured at 24 h or 72 h after dose administration in most mouse

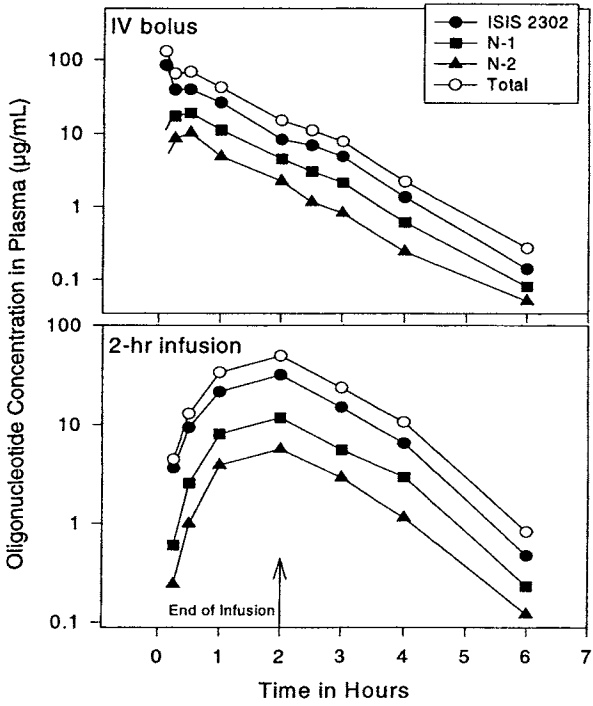

Figure 16 Plasma profiles for parent drug (ISIS 2302) and its shortened oligonucleotide metabolites in monkeys following bolus and 2-h intravenous infusion at a dose of 4 mg/kg (Study No. 2302-APK05). Each data point represents the average of three monkeys.

tissue (19,26). The remainder of the oligonucleotide that could be effectively measured was in the form of 19 to 16 mers. While oligonucleotides shorter than these have been observed, the levels detected were low and their contribution to the oligonucleotide burden was not great. The metabolites of PS oligonucleotides in tissue tended to diminish in parallel with one another such that the fraction detected intact diminished only slightly with time. In monkeys after intravenous administration of 4 mg/kg, intact ISIS 2302 represented approximately 45–60% of the total oligodeoxynucleotide measured in liver and kidney at 4 h and 35–45% after 48 h (internal data, not published).

Metabolites have been identified in tissues with elution profiles that could not be the result of single nucleotide excision. These metabolites, found exclusively in tissues, migrated more slowly than the parent oligonucleotide suggesting greater mass or lower charge-to-mass ratio. Mass spectral analysis of these metabolites demonstrated fragmentation patterns that were consistent with the addition

of one or more nucleotide bases to the parent oligodeoxynucleotide (60). Typically these slow-migrating metabolites comprised less than 10% of the total oligodeoxynucleotide measured at 24–48 h after administration. It is unknown whether these metabolites are pharmacologically active although at least one-fourth of the elongation forms should be a perfect match to the mRNA sequence, suggesting that there should be some pharmacological activity of these forms. These larger metabolic products clear from tissues along with ISIS 2302 and the other shortened oligonucleotide metabolites in mice at similar rates (internal data, unpublished).

B. Excretion

For each chain-shortening event one mononucleotide is release and some fraction of the oligonucleotide should be excreted as a low-molecular-weight metabolite in urine. In studies with radiolabeled PS oligonucleotide, there is evidence for low-molecular-weight metabolites in plasma, urine, and tissues that are poorly retained on anion-exchange HPLC and elute near the void volume (13). In studies utilizing ^{14}C-labeled ISIS 2302, more than 90% of the radiolabel recovered in urine is associated with these poorly retained metabolites (internal data, unpublished). Because the ^{14}C isotope is associated with thymine, it is likely that these poorly retained species are low-molecular-weight oligonucleotide fragments or released thymine nucleotides. That they are present in urine within 1 h of dosing further implicates their early excision from oligonucleotide at a time when the chain-shortened oligonucleotides are apparent in circulation and tissue. These early eluting peaks were present at early time points but did not accumulate over time. These data indicate that there is both the formation and the clearance of low-molecular-weight metabolites of ISIS 2302 in laboratory animals.

Urinary and fecal excretion represent minor pathways for elimination of parent oligonucleotide. Assay methods specific to parent compound indicate that urinary excretion is dose-dependent and only accounts for less than 0.5% of the dose at clinically relevant doses (19,21,61). ISIS 5132 administered to mice over a dose range of 0.8–100 mg/kg resulted in increasing amounts of parent oligonucleotide apparent in urine (19). At the lowest dose, no measurable parent drug was seen while at the highest dose of 100 mg/kg, approximately 6% of the dose was excreted in the urine over a 24-h period immediately following injection. The majority of the oligonucleotide observed in urine was consistent with chain-shortened oligonucleotide metabolites. Even with this, the total oligonucleotide measured in urine following a dose of 4 mg/kg in mice was 3.7% of the administered dose.

Regardless of radioisotope used, fecal excretion of radiolabel has been consistently less than 5% of the administered dose (13,17,26). Some evidence of parent oligonucleotide excretion via the bile has been shown in our laboratories,

although the extent of excretion is very low (less than 1% of the dose; internal data, unpublished). Thus, urine and fecal excretion combined contribute little to the clearance of intact parent oligonucleotide. Thus reports of high levels of radioactivity excreted in urine and feces are related to shorter oligonucleotide metabolites and to label itself (not associated with oligonucleotide).

VI. SECOND-GENERATION PHOSPHOROTHIOATE OLIGONUCLEOTIDES

Several investigators have described favorable pharmacokinetic characteristics associated with 2′-ribose alkoxy modifications of phosphorothioate oligonucleotides (62–64). The 2′-ribose sugar modification essentially converts the deoxynucleotide to a ribonucleotide and, depending on the chemistry applied to the 2′ position, has the ability to provide profound protection from exonuclease metabolism (38,65,66). Because the backbone of this new generation of oligonucleotides remains as the negatively charged phosphorothioate, plasma-protein-binding characteristics are not substantially altered (internal data, unpublished). Therefore, excretion in urine is low (less than 5% of the administered dose) and, like their oligodeoxynucleotide cousins, distribution to tissue is broad and rapid, nearly complete within a few hours after intravenous administration. Distribution to tissues is similar to that of the PS oligodeoxynucleotides with liver, kidney, spleen, and lymph nodes exhibiting highest concentrations of oligonucleotide.

Although many of the pharmacokinetic attributes assigned to phosphorothioate oligodeoxynucleotides also apply to 2′-ribose-modified PS oligonucleotides, nuclease metabolism is markedly reduced. Circulating chain-shortened metabolites are not detected in plasma. Furthermore, CGE profiles of oligonucleotide distributed to tissue exhibit very few shortened metabolites even several weeks after a single injection of these compounds (Fig. 17). This robust characteristic has resulted in prolonged elimination half-lives in the body (67). Half-lives of intact 2′-methoxyethyl ribose oligonucleotides ranged from 7 to 15 days, depending upon tissue assayed, in monkeys, suggesting that once-weekly dosing may be feasible.

As previously described, the improved stability of these compounds appears to result in improved absorption following oral or intraintestinal instillation in rats (32,68) (Geary et al., manuscript in preparation, 2000). Since permeability in the intestine is low and absorption rate slow, retaining the intactness of the compound for several hours should result in improved fraction of dose absorbed. Estimates as high as 5% oral bioavailability of intact 20-mer 2′-modified oligonucleotide has been reported (Geary et al., 2000, manuscript in preparation).

Because the binding affinity of these second-generation chemistries to their target mRNA is also improved (69), potency both in vitro and in vivo is expected

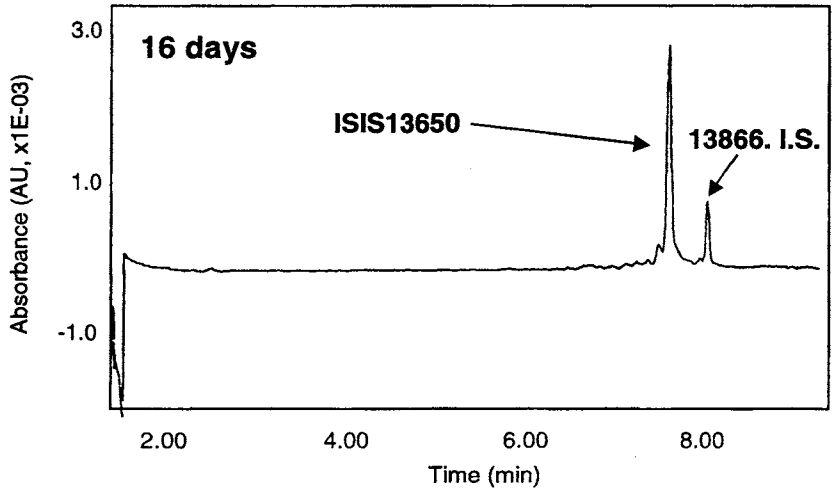

Figure 17 Representative electropherogram of extracted monkey kidney cortex collected 16 days after single dose administration of ISIS 13650, a 2′-methoxy ethyl-modified oligonucleotide targeting human c-*raf* mRNA. ISIS 13650 is modified on the six terminal nucleotides flanking both the 3′ and 5′ end of the 20-mer sequence.

to be improved compared to the first-generation PS ODN. The improved stability in vivo will further reduce the total doses required to elicit an antisense effect. Taken together, improved potency, bioavailability by nonparenteral routes of administration, and reduced dose schedule provide an attractive opportunity for treatment of unmet medical needs including chronic diseases such as inflammatory diseases, diabetes, and even cardiovascular diseases.

VII. PHARMACODYNAMICS CORRELATE WITH TISSUE PHARMACOKINETICS

The success of the antisense approach to therapy relies on the cellular distribution of the intact PS oligonucleotide. That is, antisense reduction of the expression of target mRNA relies upon suborgan distribution to the target cell type and, furthermore, subcellular distribution to the site of binding to its complement mRNA. The onset and duration of antisense therapeutic activity in the target cell type will provide the most reliable kinetic predictor for dose amount and frequency of administration.

The pharmacokinetics of PS oligonucleotides are characterized by rapid

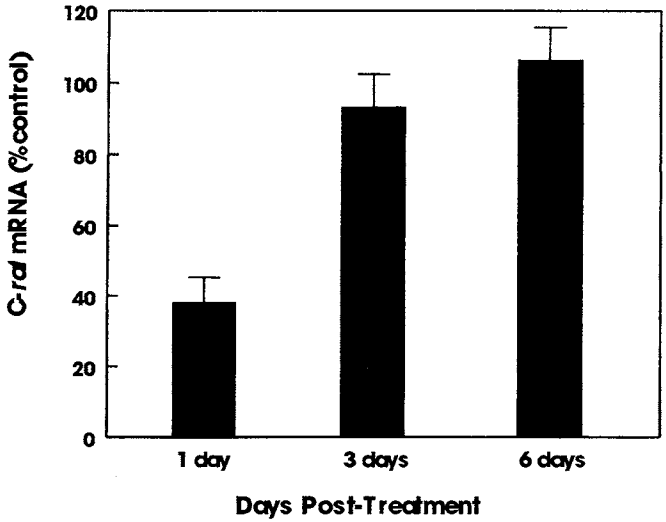

Figure 18 Northern analysis of c-*raf* kinase mRNA levels in whole-mouse liver measured as the percent of untreated control animals. By 3 days after antisense PS oligonucleotide treatment the mRNA levels had returned to baseline (100% of control). These pharmacodynamics are consistent with elimination kinetics of PS oligonucleotides from liver.

distribution from blood into tissues followed by prolonged residence in tissue. By 24 h after administration of a single dose of PS oligonucleotide, the oligonucleotide resides intracellularly in most tissues, excluding brain. Within tissues, the distribution of oligonucleotide varies by cell type but appears to be present in most cell types at some level (51). Intracellularly, more oligonucleotide appears to resides in the cytosol although higher concentrations are seen in the nucleus likely due to its smaller volume.

In preliminary in vivo pharmacodynamic experiments conducted in our laboratories, antisense pharmacodynamics in the liver closely parallel the pharmacokinetics of elimination of PS oligonucleotide from that organ (Fig. 18). The observed reduction in mRNA levels lasted approximately 1–2 days following intravenous administration of a mouse c-raf kinase antisense PS oligodeoxynucleotide while the half-life of elimination of PS ODN in the liver is approximately 2 days in mice. (Table 3). By 3 days after administration the mRNA had returned to baseline concentrations. Recently, investigators in our laboratories reported a single-dose pharmacology of ISIS 22023, a 2′-methoxy ethyl modified phosphorothioate oligonucleotide (second-generation) targeting mouse FAS mRNA. In this study, ISIS 22023 functionally down-regulated expression of the FAS mRNA in mouse liver for at least 10 days (70) following a single subcutane-

ous injection. By 14 days after a single dose of ISIS 22023 the mRNA levels had returned to baseline expression levels.

Thus, the pharmacokinetics and pharmacodynamics of PS oligonucleotides appear to be well correlated in vivo and support once-a-day or every-other-day systemic dosing for first-generation PS oligodeoxynucleotide chemistry. More convenient dosing schedules appear possible with once-weekly or once-every-other-week dosing of the second-generation 2′-ribose sugar–modified PS oligonucleotides indicated by initial evaluation of their pharmacokinetic and pharmacodynamic characteristics.

VIII. CONCLUSIONS

Pharmacokinetic studies of PS ODN demonstrate that they are well absorbed from parenteral sites, rapidly distributed broadly to all peripheral tissues, do not cross the blood-brain barrier, and are eliminated primarily by slow metabolism in tissues. Pharmacodynamics are well predicted by tissue uptake and elimination pharmacokinetics.

PS oligonucleotides are metabolized by exonuclease digestion. Further metabolism of the released mononucleotides follows normal endogenous metabolism of these compounds resulting in ultimate excretion of small-molecular-weight metabolites in urine and expired air. Excretion of the parent oligonucleotide in urine and feces is a minor pathway for elimination of these compounds with less than 1% of the dose excreted in the form of parent compound.

The pharmacokinetics of PS oligonucleotides in animals predict human pharmacokinetics and suggest similar distribution kinetics. In short, once-daily or alternating-day dosing should be feasible. This dosing schedule will result in continuous exposure of target tissues to the oligonucleotide and accumulation of oligonucleotide concentrations in tissues. Nevertheless, accumulation in plasma concentrations is not seen owing to rapid distribution into the tissue. This provides a favorable pharmacokinetic profile for this class of compounds. Improvements in the stability and ultimately in dosing routes for modifications of this chemical class (second-generation oligonucleotides) will provide an even greater breadth of use of antisense oligonucleotide therapy. Understanding the pharmacokinetics of PS oligonucleotides allows for rational improvements utilizing medicinal chemistry modifications. In general, the pharmacokinetic properties of this class of compounds appear to be largely driven by chemistry rather than sequence.

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7
Suborgan Pharmacokinetics

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I. INTRODUCTION

Antisense oligonucleotides are a promising new class of therapeutic agents designed to specifically and selectively alter the metabolism of RNA (1,2). The efficacy and safety of phosphorothioate oligodeoxynucleotides, the first generation of these compounds, in various animal models and in the clinical setting have been well documented in numerous publications (3–8). A great deal of information has also been gathered over the past 10 years concerning the basic pharmacokinetics of phosphorothioate oligodeoxynucleotides in vitro (9,10), in a variety of animal models and from human clinical trials (11–15).

As an earlier chapter in the book describes in vivo pharmacokinetics, we will only briefly summarize the pharmacokinetics of phosphorothioate oligodeoxynucleotides. After systemic administration, these drugs, which bind extensively to serum proteins, are rapidly cleared from blood, and distribute broadly to most peripheral tissues including liver, kidney, spleen, lung, lymph nodes, muscle, and bone. Liver and kidney represent the principal sites of accumulation (1,11–15). These compounds do not cross an intact blood-brain barrier. Although phosphorothioates are generally more stable in vitro and in vivo than their phosphodiester congeners, they are still labile and are cleared principally by metabolism through degradation by endo- and exonucleases present in serum and tissue (1,11,14,16). The aggregate data suggest that the absorption, tissue distribution, and metabolism of phosphorothioate oligodeoxynucleotides are similar across species, including mouse, rat, dog, and monkey, and independent of both route of systemic administration and oligonucleotide sequence (1,11,12,14).

II. SUBORGAN DISTRIBUTION: REVIEW OF THE LITERATURE

Over the past 6 years, in vivo experiments from ISIS and other laboratories (see Table 1) have studied whether systemically administered phosphorothioate oligodeoxynucleotides are localized within the cells of organs to which they distribute. The most commonly employed detection techniques, used separately or in combination, include (1) autoradiography with ^{3}H-, ^{35}S-, ^{125}I-, and ^{14}C-radiolabeled oligonucleotide (11,17–23), (2) indirect immunohistochemistry, with either monoclonal antibodies directed against a 20-mer phosphorothioate oligodeoxynucleotides (17) or antibodies to fluorescein or digoxigenin derivatized 15-mer phosphodiesters (20), (3) direct fluorescent microscopy with rhodamine (17) or fluoresceinated oligonucleotides (20), and (4) subcellular localization using ^{3}H oligonucleotide (24) or unlabeled oligonucleotide analyzed and quantitated directly by capillary gel electrophoresis (25).

A. Autoradiography

Nicklin and colleagues (11), Carome et al. (18), Oberbauer et al. (19), Rappaport et al. (21), Rifai et al. (22), and Sands and colleagues (23) used a variety of isotopically labeled phosphorothioate oligodeoxynucleotides of varying lengths, sequences, and directed against numerous targets, to detect oligonucleotide within the cells of various rodent organs.

Nicklin and colleagues (11) used light microscopy autoradiography to localize two 20-mer heterosequence phosphorothioate oligodeoxynucleotides, CGP-64128A-ISIS 3521, targeted to human protein kinase C-α, and CGP-69846A-ISIS 5132, which was designed to inhibit human c-raf-1 kinase (26,27) in rodent kidney, liver, and skin. Both compounds were labeled with ^{3}H throughout each of the molecules by tritium exchange at the C8 position of purine bases (28). Six hours after rats were administered an intravenous (iv) dose of 20 mg/kg of ^{3}H CGP-64128A, drug was readily detectable in the kidney, with highest concentrations found in the renal cortex and proximal tubules. Lower concentrations of radioactivity were localized in the renal medulla, distal tubules, and glomeruli. This compound was also found within the cells of rat liver. Similar experiments were performed using ^{3}H CGP-69846A. These studies indicated that radiolabeled oligonucleotide was detected in the Bowman's capsule of mice. The greatest concentrations of radioactivity were detected in the nonparenchymal cells, or the Kupffer and endothelial cells that line the hepatic sinusoids, while lower levels were localized in the cytoplasm of hepatocytes, or the parenchymal cells. In skin, oligonucleotide was diffusely distributed within the dermal layer, most likely associated with the extracellular matrix.

Carome et al. (18) studied the cellular uptake of a ^{35}S-labeled 20-mer phos-

Table 1 Subcellular Localization of Phosphorothioate Antisense Oligonucleotides

Author	Localization techniques	Organ (s)	Species	Ref.
Bijsterbosch et al.	Subcellular localization with ^{3}H-oligonucleotide	Liver	Rat	24
Butler et al.	Immunohistochemistry with monoclonal antibodies Direct fluorescent microscopy with rhodamine oligonucelotide Autoradiography with ^{14}C-oligonucleotide	Kidney Liver Connective tissue Intestine Spleen Bone marrow Skin	Rat Mouse	17
Carome et al.	Autoradiography with ^{35}S-oligonucelotide	Kidney	Rat	18
Graham et al.	Subcellular fractionation with CGE analysis	Liver	Rat	25
Nicklin et al.	Autoradiography with ^{3}H-oligonucleotide	Kidney Liver Skin	Rat Mouse	11
Oberbauer et al.	Autoradiography with ^{35}S-oligonucleotide	Kidney	Rat	19
Plenat et al.	Immunohistochemistry Direct fluorescent microscopy	Liver Kidney Connective tissue Spleen Exocrine glands Tumors	Nude mouse	20
Rappaport et al.	Autoradiography with ^{35}S-oligonucleotide	Kidney	Mouse	21
Rifai et al.	Autoradiography with ^{125}I-oligonucleotide	Liver Kidney Spleen	Mouse	22
Sands et al.	Autoradiography with ^{3}H-oligonucleotide	Kidney	Mouse	23

phorothioate oligodeoxynucleotides complementary to the HIV *tat* splice acceptor site in male Wistar rat kidneys after iv bolus injection. Kidneys were perfused with ice-cold PBS 30 min and 4 h after drug administration, removed, and placed in 10% phosphate-buffered formalin/glutaraldehyde before processing and autoradiography. At both time points, the highest amount of radioactivity was localized within the proximal tubules, with lower levels detected within the glomerulus, the epithelial cells of Bowman's space, and the distal tubular cells. No radiolabel was found in renal vasculature cells.

Oberbauer and colleagues (19) also used a ^{35}S-labeled phosphorothioate oligodeoxynucleotides (18-mer) to study renal uptake in rats after intravenous administration. Various pharmacokinetic parameters, including total organ accumulation, urinary excretion, and stability of the oligonucleotide, were evaluated 5, 24, and 96 h postinfusion. Using light microscopic autoradiography, they observed oligonucleotide in the early proximal tubules, while electron microscopic autoradiography indicated that compound was not simply localized to the endocytic-lysosomal pathway or the brush border of tubules.

A phosphorothioate oligodeoxynucleotide 20-mer targeted to the Tar element from HIV-1 transcripts internally labeled with ^{35}S was used by Rappaport et al. (21) to study suborgan distribution in kidney using FVB/N mice 15 and 30 min post iv injection. Kidneys were fixed in neutral buffered formalin, embedded in paraffin, and sections were coated with NTB2 emulsion and exposed at 4°C for 1 week. Tissue was counterstained with toluidine blue and photographed by dark-field microscopy. After 15 and 30 min, oligonucleotide was localized in both proximal and distal tubules, the Bowman's space, within glomerular epithelial cells, and along the brush border membrane. Because of the high concentration of radiolabel within Bowman's capsule, the authors suggested that oligonucleotide was filtered by the kidney, probably as a result of specific binding to renal epithelium.

Both light and electron microscopic autoradiography were employed by Rifai and colleagues (22) to localize two ^{125}I-radiolabled 15-mer phosphorothioate oligodeoxynucleotides (anti-TNF and randomer) in kidney and liver of female Balb/C mice 10 min after tail vein injection of drug. Within kidney, the predominant distribution of drug was in the lumen of proximal and distal tubules and within the glomerular urinary space. In liver, at this early time point, no radiolabel was detected within hepatocytes, the majority of drug being localized to the Kupffer cells in the sinusoidal spaces. Electron microscopic autoradiograpy indicated that the silver grains were seen within lysosomal structures and on cell surfaces, but not within the nuclei of cells. Data from 1 h autoradiograms showed that the amount of silver grains decreased relative to the earlier time point, although the pattern of distribution was similar to that seen at 10 min.

Sands et al. (23) used a 20-mer phosphodiester and its phosphorothioate congener, labeled internally with ^{3}H by ^{3}H-methylation of an internal deoxycyti-

dine with *Hha*l methylase and S-[^{3}H]adenosylmethionine, to study whole-organ distribution and kidney localization by autoradiography after injection of 6 mg/kg oligonucleotide into the tail vein of male CF1 mice. Radioactivity from the phosphorothioate oligodeoxynucleotide was detected in kidney cortex 2 h and 24 h postinjection. The pattern of radioactivity from the phosphodiester oligonucleotide differed from that of its phosphorothioate congener. The maximal concentration of drug could be detected in the medulla 5 min postinjection, but was also seen throughout the whole kidney. By 2 h, very little radiolabel derived from the phosphodiester remained anywhere in kidney.

B. Indirect Immunohistochemistry and Direct Fluorescent Microscopy

Plenat and colleagues (20) used three different 15-mer phosphodiesters (two heterosequences and one homo-β-deoxythymidine) targeted to the translation/initiation region of c-*myc* mRNA to perform localization studies on the organ, suborgan, and subcellular level after iv or ip injection into male athymic nude mice. Whole-animal autoradiography was only performed to determine overall tissue distribution using 15-mers that were 5′ end labeled by exchange with adenosine 5′-(γ[^{35}S]thio) triphosphate. Their data suggest, as did that of others (1,11,14), that drug was widely distributed in liver, kidney, spleen, lung, bone, and skeletal muscle.

The tissue cellular and subcellular distribution was determined by direct fluorescent microscopy or indirect immunohistochemistry using antibodies directed against fluorescein or digoxigen 15-mers that were functionalized on their 3′ ends with fluorescein or digoxigenin. The authors noted that the overall tissue and cellular distribution of the ^{35}S and both derivatized oligonucleotides were identical at 3 and 10 min postdose and appeared to be independent of oligomer sequence. Fluoresceinated or digoxigen-labeled compounds could be detected in most cells and tissues, the intervertebral discs, and the hard connective tissue matrix of bone. As shown before, very little oligonucleotide was found within the nervous system (11,14,15). In kidney, drug was localized in tubule cell nuclei, cytoplasm, and renal proximal tubules, while in liver, more intense staining was detected within the cytoplasm of nonparenchymal cells. Although hepatocytes displayed less intense staining than endothelial and Kupffer cells, some anti-c-*myc* oligonucleotide could be detected within hepatocyte nuclei using indirect fluorescent immunohistochemistry.

C. Localization by Subcellular Fractionation

Bijsterbosch et al. (24) determined the tissue distribution and specific liver suborgan localization in male Wistar rats of a parenterally administered ^{3}H 20-mer

phosporothioate antisense oligodeoxynucleotide specific for murine intercellular adhesion molecule-1. Liver, kidney, spleen, and bone marrow were organs of highest radiolabel accumulation. Suborgan distribution in liver was determined by subcellular fractionation 1 h after systemic administration of radiolabeled oligonucleotide. Based upon radioactivity measurements, the authors indicated that endothelial cells and hepatocytes accounted for the majority of liver cell uptake (56.1% vs. 39.6%, respectively), while Kupffer cells accumulated only 4.3% of the input dose of either 0.5 or 1 mg/kg of the ^{3}H compound. They also suggested that scavenger receptors on endothelial cells were the principal mechanism of oligonucleotide uptake in rat liver.

D. Summary of Literature Survey of Suborgan Distribution

As with any scientific procedure, there are caveats associated with all of the techniques described above that should be kept in mind while interpreting data derived from each of the experiments. Obviously, the histological procedures, i.e., autoradiography, indirect immunohistochemistry, and direct fluorescent microscopy, can provide only qualitative, not quantitative, information concerning the amount of oligonucleotide present within the cells of prepared tissue sections. More importantly, there are no methods to determine the actual integrity of the radiolabeled, fluoresceinated, or derivatized oligonucleotides or whether the conjugates remain attached to the oligonucleotides being studied. Determining the amount and extent of metabolism of the compounds being studied is obviously critical in any detailed pharmacokinetic analysis.

This last point highlights the issue concerning the backbone of the oligonucleotides used in various localization studies. As described in numerous publications, phosphorothioate oligodeoxynucleotides, where the nonbridging oxygen in the backbone is replaced by a sulfur atom, are more stable in vitro and in vivo than their unmodified phosphodiester congeners (1,16,29–31). While most laboratories used phosphorothioate oligodeoxynucleotides for the experiments described above (11,19,21–24), Plenat et al. (20) performed their studies with 15-mer phosphodiesters. Although their cell and subcellular localizations were performed with phosphodiesters derivatized on the 3′ ends of the molecules, these compounds were likely, even at the earliest time points, highly degraded by a variety of exonucleases, both 3′ and 5′, as well as endonucleases found in serum and tissues. Sands et al. (23), who compared various pharmacokinetic parameters of both phosphorothioate and phosphodiester congeners, determined, using paired-ion high-performance liquid chromatography (HPLC), that very little intact phosphodiester oligonucleotide was found in any mouse organ as early as 1 min after injection. These and other data comparing tissue metabolism of unmodified versus phosphorothioate oligonucleotides (16) make it unlikely that reliable suborgan localization data can be obtained from studies performed with phosphodiesters.

Concerns have also been expressed about the influence of chemical moities, including fluorochromes like fluorescein or acridine, on the uptake and distribution of oligonucleotides in vitro and in vivo (10). For example, acridine has been shown to alter a number of pharmacokinetic parameters, including intracellular distribution and stability (32,33). In vivo experiments in our laboratories also suggest that fluoresceinated phosphorothioate oligodeoxynucleotide tissue distribution is dramatically altered compared to the unmodified parent molecule (34).

A general problem relating to histological procedures involves diffusion or redistribution of oligonucleotides within tissues while they are being prepared for analysis. For example, Bennett et al. (35) reported that cells fixed with acetone or ethanol displayed a different pattern of fluorescein-labeled oligonucleotide distribution compared to unfixed cells. The authors suggested that aldehyde fixatives, because of their crosslinking capacity, might function better than organic solvents. They also observed that unfixed cells that had lost the integrity of their plasma membranes rapidly accumulated fluoresceinated oligonucleotides within the nucleus and nucleolus and suggested that this is a strong indication of nonviable or poorly fixed cells. Pichon and colleagues (36) confirmed the studies by Bennett and co-workers and also suggested that alcohol-containing fixatives, because of their lipid-dissolving abilities and protein-denaturing properties, lead to oligonucleotide relocalization. Paraformaldehyde fixation, on the other hand, efficiently crosslinks proteins and preserves cellular architecture, and therefore can be used without loss of oligonucleotide from cells and organelles. Plenat et al. (20) also noted, when preparing unfixed tissue for direct fluorescence and immunoenzymatic studies, that paraffin processing produced loss of oligonucleotide from tissue samples, necessitating snap freezing of tissues in liquid nitrogen.

The use of autoradiography has its difficulties, beginning with the fact that spatial resolution on the suborgan level is extremely limited (17,37). Direct autoradiography, the preferred method, provides limited sensitivity, which is highly dependent upon the choice of radioisotope, especially when fluorographic reagents or intensifying screens must be employed to increase detection when ^{32}P or weak β-emitters such as ^{3}H are used. Aside from the sensitivity issues, there are still the problems inherent in the preparation of tissue sections that were described above.

Subcellular fractionation with radiolabeled oligonucleotides has also been utilized to detect these compounds on the suborgan level (24,25). While this procedure is an established pharmacological technique, there are still limitations intrinsic to the differential centrifugation steps commonly used in that protocol. For this reason, appropriate controls are necessary to ensure that no significant redistribution or contamination between subcellular fractions has occurred during liver homogenization or organelle isolation (9,38).

As described above, Bijsterbosch et al. (24) used subcellular fractionation to localize a ^{3}H 20-mer phosphorothioate oligodeoxynucleotide within the liver

of rats. However, the authors never addressed oligonucleotide redistribution when studying phosphorothioate pharmacokinetics (9,25). Nor did the group determine the integrity of the radiolabeled material in rat liver or cellular compartments. Additionally, suggestions that phosphorothioate oligodeoxynucleotides are taken up in liver principally by scavenger receptors on endothelial cells, and that these compounds are not localized to liver Kupffer cells, are controversial. For example, Butler et al. (39), using Class A scavenger receptor knockout mice (SR-A-/-), demonstrated that phosphorothioate oligodeoxynucleotides distributed in a similar fashion in both knockout and wild-type mice. In other experiments Graham and co-workers (25) localized and directly quantitated, using capillary gel electrophoresis (CGE), significant amounts of phosphorothioate oligonucleotide in purified rat and mouse Kupffer cells.

III. SUBORGAN DISTRIBUTION: THE ISIS EXPERIENCE

At ISIS, we have studied the in vivo pharmacokinetics of phosphorothioate oligodeoxynucleotides in mouse, rat, monkey, and human (12–14,16). Two groups in particular, using a variety of localization techniques, have studied the suborgan distribution of these compounds in great detail over the past several years (17,25).

A. Histochemical Techniques

Butler and colleagues (17) studied the in vivo disposition of two 20-mer phosphorothioate oligodeoxynucleotide heterosequences after systemic injection into mice and rats. Tissue and cell localization were compared using direct fluorescence microscopy of rhodamine-conjugated ISIS 2105, a phosphorothioate oligodeoxynucleotide that inhibits the production of a gene product essential for the growth of human papillomavirus (40), immunohistochemistry with a monoclonal antibody that recognizes ISIS 2105, and finally, autoradiography of ^{14}C ISIS 2302.

The authors went to great lengths to ensure that the technical difficulties associated with tissue preparation and the issues concerning the influence of fluorochrome were addressed. For example, to prevent diffusion of phosphorothioates during the three histological procedures, all control and experimental animals were perfused with a fixative containing glutaraldehyde. This procedure was shown to effectively prevent oligonucleotide diffusion since (1) autoradiography and immunostaining worked equally well on cryostat or paraffin-embedded sections, indicating that tissues did not redistribute compounds during the parafin embedding; (2) the localization of rhodamine-labeled oligonucleotide was the same in fixed and nonfixed tissues; and (3) there was no observable redistribution

of the rhodamine-labeled oligonucleotide signal in tissue sections incubated in aqueous mounting media for over a week.

The effect of the fluorochrome rhodamine on oligonucleotide distribution was also experimentally addressed. The data indicated that all three histological methods showed the same pattern of oligonucleotide distribution in all tissues that were evaluated, suggesting that rhodamine conjugation did not alter tissue distribution. Additionally, it was demonstrated that tissues did not internalize free rhodamine, providing evidence that the signal was due to an oligonucleotide still conjugated to the fluorochrome. The best signal and spatial resolutions, in fact, were obtained with the fluorescently tagged compound.

Following validation of various experimental techniques, the distribution in kidney, liver, intestine, spleen, skin, skeletal muscle, bone marrow, and blood, of 5, 20, and 50 mg/kg of the two phosphorothioate oligodeoxynucleotides was studied in Balb/C mice and Sprague-Dawley rats. Figure 1 illustrates all three localization techniques in kidney and liver. In general, increasing the dose of drug increased the strength of the signals, but did not alter the pattern of distribution in most tissues. In kidney, using all three methods, drug was observed in the cortex, with strong localization in the epithelium of proximal tubules where diffuse cytoplasmic staining and large positive vesicles were detected, along with perinuclear and intranuclear staining (Fig. 1,b,d,f). Drug was also located within the glomerular capsule. These results are consistent with those previously described in this chapter (11,19–23) and suggest that phosphorothioate oligodeoxynucleotides undergo tubular reabsorption after glomerular filtration (41). Within liver, hepatocytes, Kupffer, and endothelial cells were shown to contain oligonucleotide. Drug was specifically localized in Kupffer cell, but not hepatocyte nuclei at these doses.

Localization experiments also demonstrated that phosphorothioate oligodeoxynucleotide accumulated in intestine and skin. In intestine, lamina propria and submucosa stained strongly for drug, with weak staining observed in columnar epithelial cells and none in smooth muscle cells of the muscularis mucosa and muscularis layers. In skin, only the dermis, subcutaneous tissue, and perichondrium were predominantly labeled. The data indicated that phosphorothioates accumulated in the loose and dense connective tissue of these organs, with macrophages and fibroblasts representing the most likely sites of drug accumulation. This information is consistent with reports in this chapter (11,20) and from other laboratories indicating that these compounds can bind to various proteins within connective tissue matrix such as laminin and fibronectin (42).

Finally, phosphorothioates were localized in large, nucleated cells, including megakaryocytes in the bone marrow, while erthrocytes were negative. In the spleen, lymphocytes in the white pulp were negative, whereas cells in the red pulp, possibly the phagocytic macrophages associating within the walls of the splenic sinuses, were strongly stained.

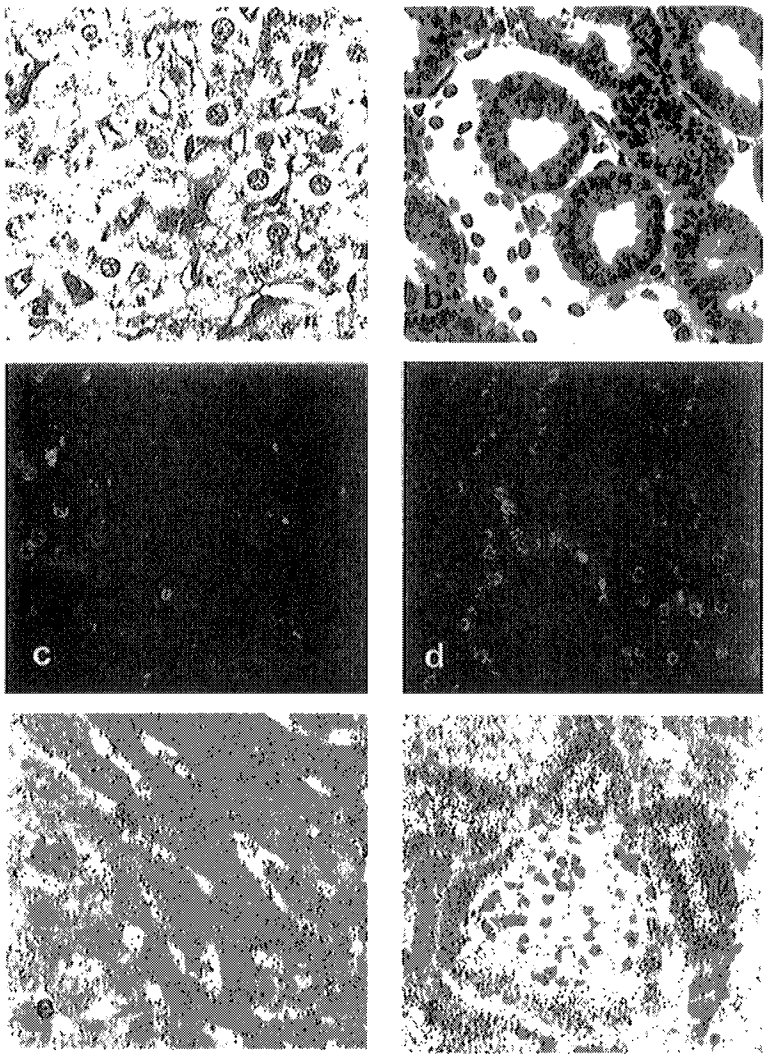

Figure 1 Comparison of histological methods in rodent livers (a, c, e) and kidneys (b, d, f) 2 h after injection of indicated phosphorothioate oligodeoxynucleotide. (a, b) Immunostaining of tissues from a rat injected with 20 mg/kg of ISIS 2105 with a mouse monoclonal antibody raised against ISIS 2105. Peroxidase activity was visualized with 3,3′-diamino-benzidine and sections are counterstained with hematoxylin. (×500.) (c, d) Fluorescence microscopy of tissues from a mouse injected with 5 mg/kg rhodamine-conjugated ISIS 2105. Sections are counterstained with DAPI. (×500.) (e, f) Autoradiographs of kidney and liver from a mouse injected with ^{14}C-radiolabeled ISIS 2302. Sections are counterstained with Nuclear Fast Red and Metanil Yellow. (×500.)

B. Subcellular Fractionation with CGE Analysis

1. Rat

Although the carefully controlled experiments described above provided useful organ and suborgan distribution information that was consistent with previously published reports, quantitative data concerning phosphorothioate oligodeoxynucleotide deposition and metabolism on the cellular and subcellular level were lacking. To provide more detailed quantitative localization and metabolic pharmacokinetic information, we used subcellular fractionation (9) and capillary gel electrophoresis (CGE) analysis (43,44) previously standardized in our laboratories to study the cellular and subcellular distribution and metabolic fate of a 21-mer phosphorothioate oligonucleotide, ISIS 1082, in rat liver over a 24 h period using a variety of doses (25).

CGE is an extremely sensitive analytical technique, with single-nucleotide resolution, which permits quantitation of phosphorothioate oligodeoxynucleotides, as well as newer chemistries, within specific cells and subcellular fractions in vitro and in vivo without the concerns associated with radiolabeling or conjugation. Some of those concerns include a loss of radio- or fluorescent signal due to nucleolytic cleavage of the reporter groups, nonspecific localization due to unincorporated label or fluorochrome, and aberrant oligonucleotide distribution in organs and cells due to the influence of fluorochomes (10,43,44). CGE and other analytical techniques will be discussed in greater detail by J. Leeds and L. Cummins in another chapter of this book.

Hepatocytes, Kupffer, and endothelial cells were isolated from whole liver using techniques previously described (25,45–47). The purity of the enriched cell populations, as assessed by flow cytometry and immunohistochemistry, was 90–95%. Viability, as determined by trypan blue exclusion, was generally greater than 85–90%.

To delineate the ratio of the systemic dose distributed between whole liver, parenchymal and nonparenchymal cells, and the extracellular matrix, in vivo uptake and fractionation experiments were performed using whole livers isolated 4 and 24 h (45) after iv bolus administration of a 10 mg/kg dose of ^{3}H- or ^{35}S-labeled ISIS 1082. Our results indicated that liver-associated radioactivity represented between 8.0% (4 h) and 10.0% (24 h) of the total dose, corresponding to an organ oligomer concentration of approximately 2.6 μM at 4 h, and 3.5 μM at 24 h, respectively, using either ^{3}H or ^{35}S.

Following collagenase treatment, radioactivity in the total unpurified cell isolates was measured and compared against the level determined previously in the whole liver. Results from these experiments indicated that approximately 54% of the total liver dose was cell associated after 4 h and by 24 h, 83% of the drug was associated within liver cell populations, the remaining amount of drug existing within the extracellular spaces and interstitium (45). These data are consis-

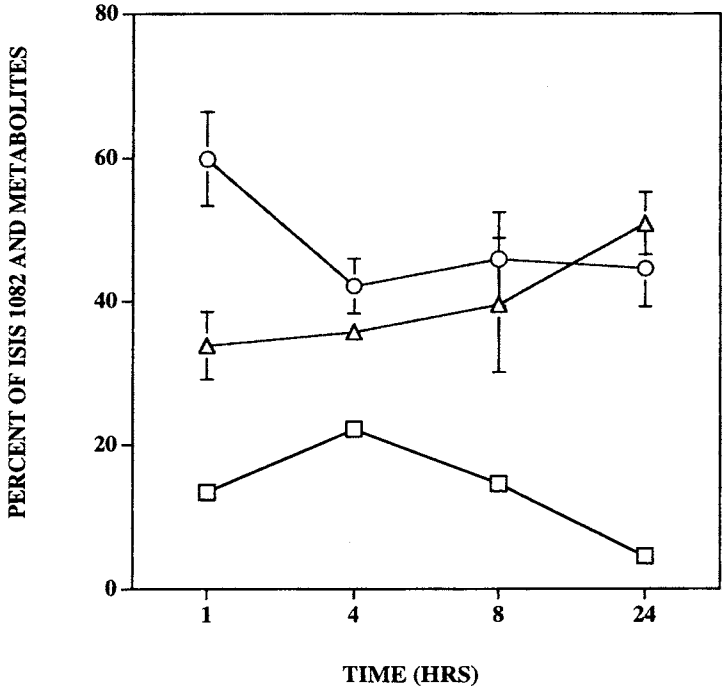

Figure 2 Effect of time on the proportion of ISIS 1082 and metabolites in hepatocytes (n = 9), Kupffer cells (n = 3), and endothelial cells (n = 6) 1, 4, 8, and 24 h after 10 mg/kg iv bolus. The mean and SEM for each cell type are plotted. -□-, hepatocytes; -△-, Kupffer cells; -○-, endothelial cells.

tent with reports from our laboratories (17) and others suggesting that these compounds accumulate in connective tissue and various matrix proteins (22,42).

Suborgan localization studies revealed dramatic differences in the amount of ISIS 1082 and metabolites among the liver cell types. As shown in Fig. 2, on a per-cell basis, Kupffer and endothelial cells contained approximately 80% of the total liver-cell-associated drug, equally distributed between the two nonparenchymal cell types. In contrast, hepatocytes accumulated the remaining 20% of the liver dose, with maximal levels being observed 4–8 h after systemic administration. The data in Fig. 2 also suggest that extensive cell-to-cell transfer of ISIS 1082 did not occur over a 24-h period.

Dose-response experiments suggest that liver cells display differential saturation kinetics (Fig. 3). At the 5 and 10 mg/kg doses, nonparenchymal cells contained the majority of the total liver-cell-associated drug, results consistent with the data presented in Fig. 2. After 24 h, at doses above 10 mg, Kupffer cell drug

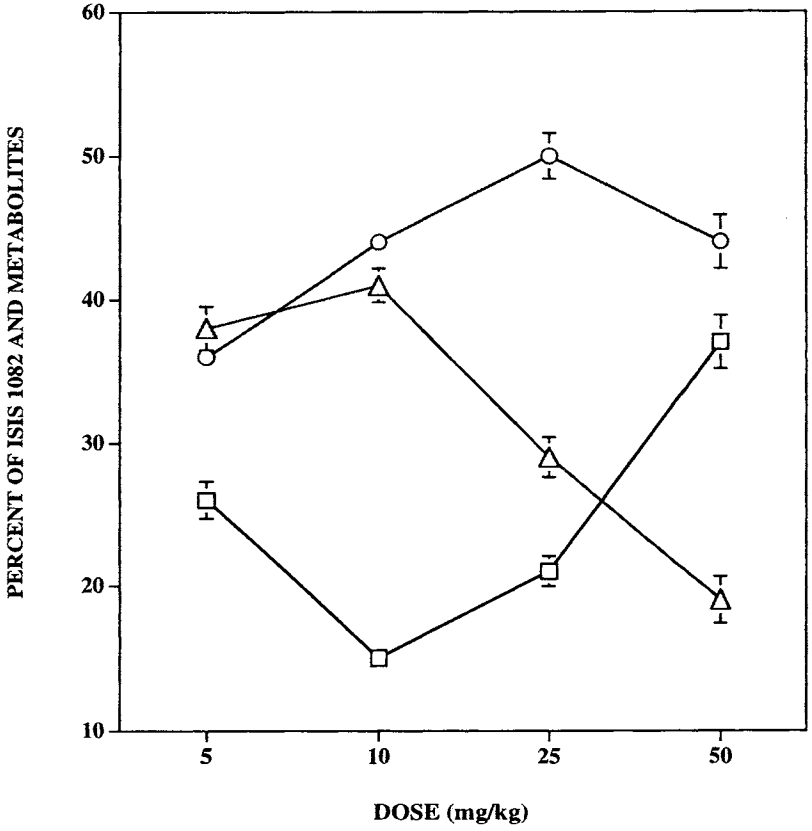

Figure 3 Effect of dose on the proportion of ISIS 1082 and metabolites in hepatocytes (n = 9), Kupffer cells (n = 3), and endothelial cells (n = 6) 24 h after iv bolus dosing at 5, 10, 25, and 50 mg/kg. The mean and SEM for each cell type are plotted. -□-, hepatocytes; -△-, Kupffer cells; -○-, endothelial cells.

levels appeared to saturate, while in endothelial cells, maximal drug levels were observed at the 25 mg/kg dose. In contrast, at doses above 25 mg/kg, hepatocyte drug levels continued to increase, with no evidence of saturation.

Subcellular fractionation to determine the intracellular localization and metabolism of ISIS 1082 as a function of time (Fig. 4) and dose (Fig. 5) also highlighted interesting hepatic cell differences. Various controls were performed to demonstrate that redistribution of oligonucleotide or cross-contamination did not occur when various subcellular fractions were isolated (9,38). As shown in Fig. 4, although hepatocytes contained both cytosolic and membrane-associated drug,

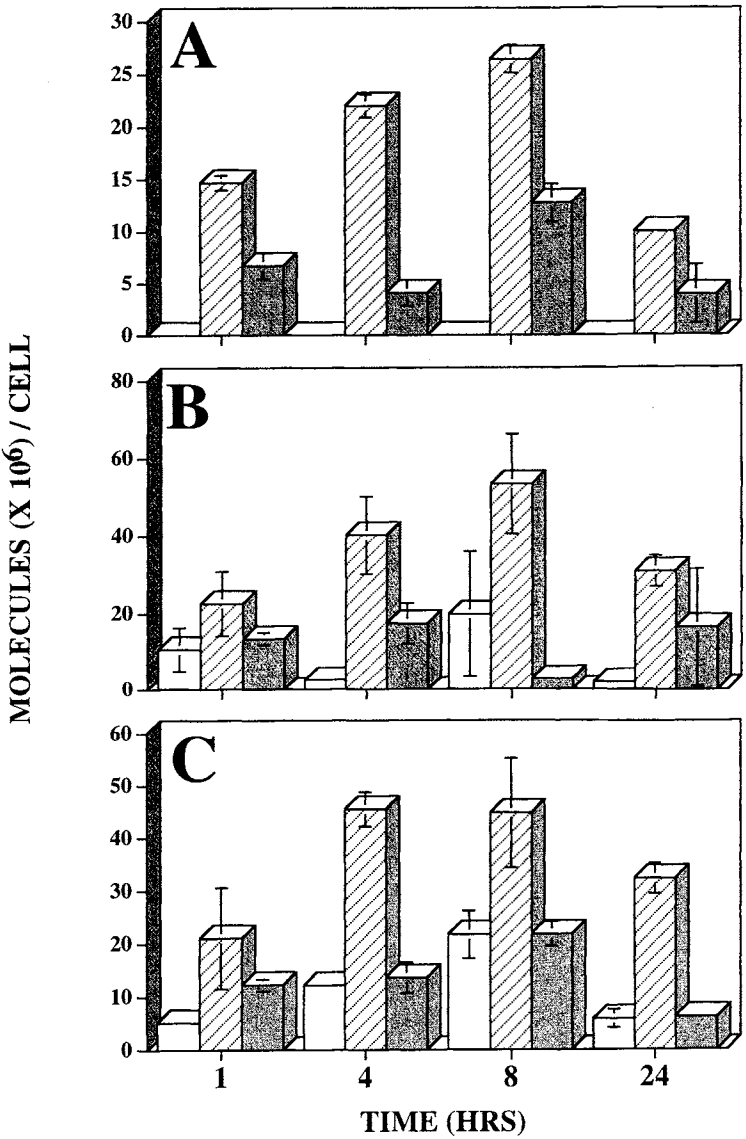

Figure 4 Subcellular localization of ISIS 1082 and metabolites over a 24-h period in rat hepatocytes (A), Kupffer cells (B), and endothelial cells (C). Values for each fraction represent the mean and SEM of intact drug and metabolites expressed in molecules per cell. Replicates are as follows: hepatocytes (n = 9), Kupffer values (n = 3), and endothelial cells (n = 9). Open bar, nuclear; crosshatched bar, cytosolic; black bar, membrane.

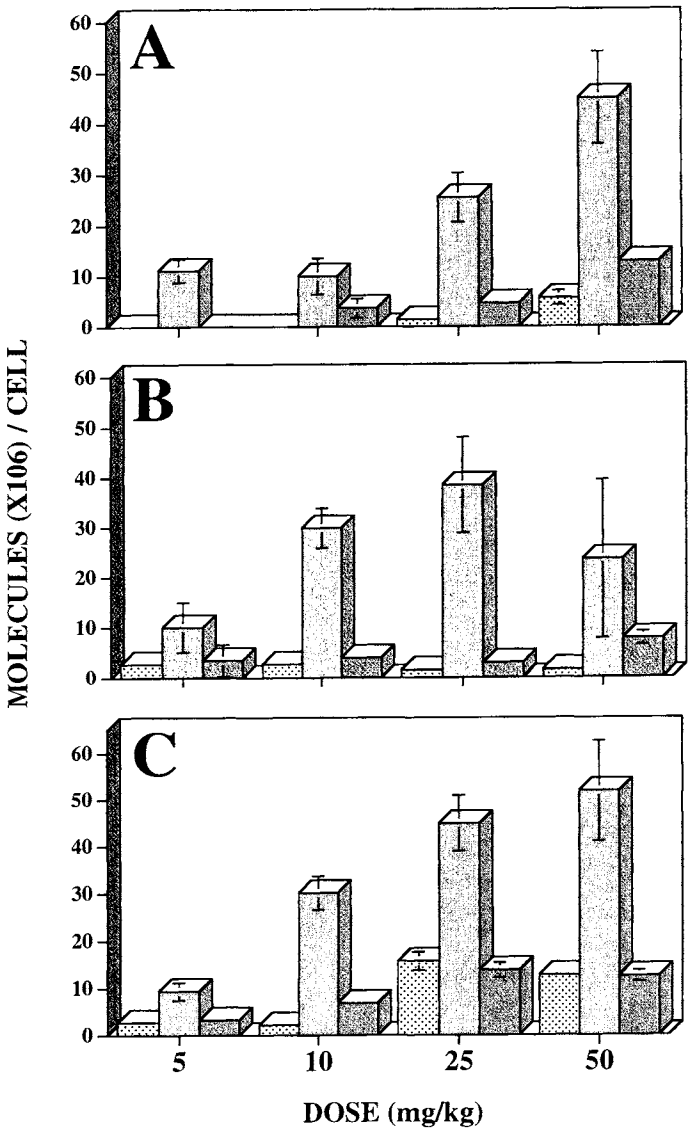

Figure 5 Subcellular distribution of ISIS 1082 and metabolites 24 h after 5, 10, 25, and 50 mg/kg iv bolus in hepatocytes (A), Kupffer cells (B), and endothelial cells (C). At doses below 25 mg/kg, hepatocytes possessed no nuclear-associated oligonucleotide. Values for each fraction represent the mean and SEM of intact drug and metabolites expressed in molecules per cell. Replicates are as follows: hepatocytes (n = 9), Kupffer values (n = 3), and endothelial cells (n = 9). Dotted bar, nuclear; light-gray bar, cytosolic; black bar, membrane.

no nuclear accumulation was observed over 24 h after 10 mg/kg dosing. In contrast, both Kupffer and endothelial cells accumulated substantial drug in all three subcellular fractions. When subcellular distribution was studied as a function of dose (Fig. 5), no nuclear-associated drug could be detected in hepatocyte nuclei at the 5 and 10 mg/kg dose levels. However, drug could be driven into this subcellular fraction by increasing the input dose to 25 mg/kg. Nonparenchymal cell nuclei, cytosol, and membrane fractions contained detectable drug at all concentrations. These data have also been corroborated by Butler et al. (17) using the three histological methods described above.

The stability of ISIS 1082 was also assessed in whole liver, in purified liver cells, and in hepatic subcellular fractions using CGE. Results from whole-liver drug stability studies evaluating the integrity of ISIS 1082 as a function of time are presented in Fig. 6, with panel A representing the percent of intact ISIS 1082 and panel B representing full-length drug, as well as the first two nucleotide elimination products (n-1, n-2), which also retain some pharmacological activity. One striking feature of these data is that a majority of ISIS 1082 metabolism occurred within the first hour following drug administration. In general, the amount of intraorgan metabolism appeared to be rather modest over the next 24-h period, with percent full length only decreasing by an additional 20%. As shown in Fig. 7, which presents drug stability in hepatocyte, Kupffer, and endothelial subcellular fractions, minimal intracellular metabolism occurred over a 24-h period.

In summary, a more complete picture of the complex suborgan and subcellular distribution of a phosphorothioate oligonucleotide within the rat liver has been achieved using subcellular fractionation and CGE analysis. It is clear, as has been described previously, in in vitro cellular uptake studies, early in vivo pharmacokinetic experiments (1,43), and the reports described in this chapter, that certain cell types in vivo have the capacity to internalize oligonucleotide without the requirement for cationic lipids (48,49). In disease states that require drug delivery to endothelial cells, for example, antisense drugs will likely prove highly effective, as these cells appear to actively take up drug. In other instances, drug delivery concentrations could be modulated to target less compliant cell types, such as hepatocytes.

Data derived from our studies have obvious pharmacological ramifications and suggest that to deliver phosphorothioate oligonucleotides to hepatocytes in rats at levels necessary for antisense inhibition, higher doses may be required relative to nonparenchymal cell types. This is consistent with studies in which effects on target RNA levels in whole liver and hepatic cell isolates have been measured after systemic administration (50; C.F. Bennett and V. Driver, personal communication). Specifically, when a 20-mer rodent-specific c-*raf* kinase antisense phosphorothioate oligodeoxynucleotide (ISIS 11061) was administered to rats, a dosing regiment of 100 mg/kg every 24 h for 4 days was required to demonstrate mRNA reduction in whole liver. Subsequent Northern blot analyses

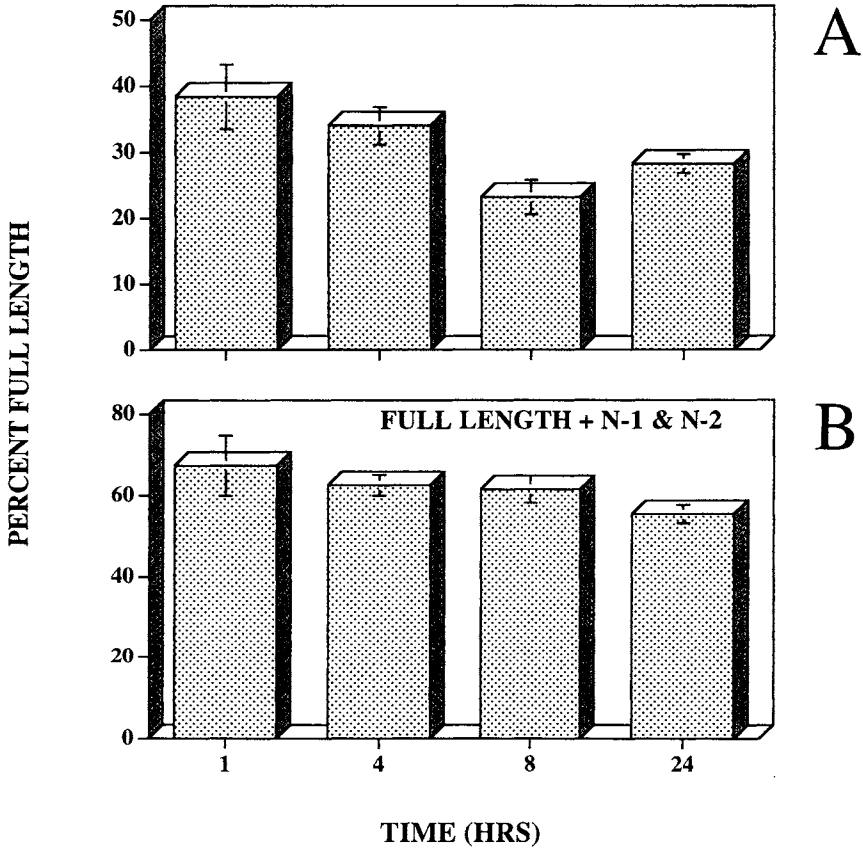

Figure 6 Effect of time on the stability of ISIS 1082 in whole rat liver (100 mg) after 10 mg/kg iv bolus. (A) Level of intact drug relative to metabolites; (B) intact and n-1 and n-2 metabolites as a measure of total pharmacologically active oligonucleotide present.

performed on parenchymal and nonparenchymal cellular isolates revealed that a majority of the c-*raf* message detected in whole-liver preparations was predominantly expressed in hepatocytes.

2. Mouse, Rat, and Monkey Comparison of Suborgan Distribution

In more recent experiments, we performed detailed, comparative pharmacokinetic studies between rat and mouse over a 24-h period after systemic dosing

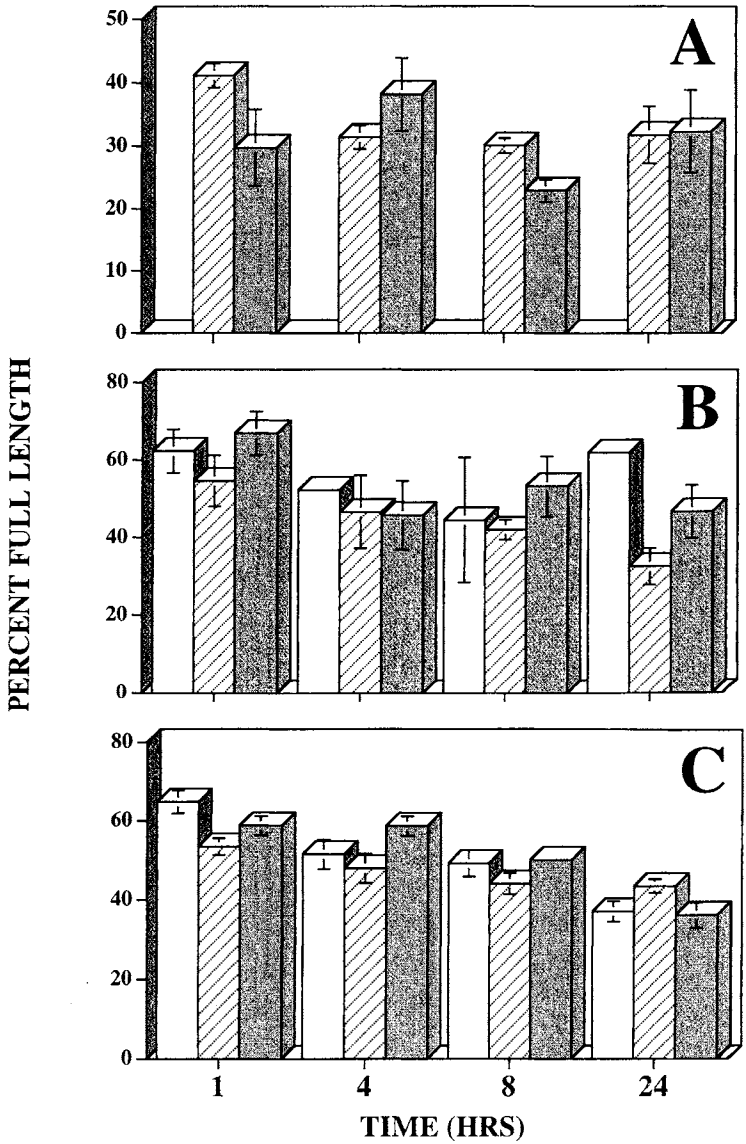

Figure 7 Effect of time on the stability of ISIS 1082 in hepatocyte (A), Kupffer cell (B), and endothelial cell (C) subcellular fractions following a 10 mg/kg IV bolus dose. In hepatocytes at this dose, no nuclear-associated oligonucleotide could be detected. Open bar, nuclear; crosshatched bar, cytosolic; black bar, membrane.

with 5–50 mg/kg ISIS 1082 (51). These studies revealed that there are detectable differences between Sprague-Dawley rats and CD-1 mice. While the overall disposition of drug was similar in whole-liver and purified-liver cells, there were species-specific differences in pharmacokinetics that became more pronounced on the suborgan level. In general, mice accumulated more drug in whole-liver and liver cells than rat. On the subcellular level, rat and mouse clearly differed in the amount of drug localized in hepatocyte nuclei; i.e., no ISIS 1082 was detected in rat hepatocyte nuclei at doses lower than 25 mg/kg, while mouse accumulated drug in all liver cell subcellular compartments above 5 mg/kg. Additionally, drug clearance from subcellular compartments in the mouse was faster and occurred to a greater extent than in the rat (51). Geary et al. (14), based upon experiments performed on the whole-organ level, also observed that mice cleared phosphorothioate oligodeoxynucleotides two- to threefold faster from target tissues, including liver, compared to rat and monkey.

The overall extent of metabolism in liver, including cells and subcellular fractions, was similar for mouse and rat. Also consistent between rat and mouse was the rapid and extensive degradation of ISIS 1082 by nucleases present in serum within the first hour after dosing. Additionally, high-performance liquid chromatography/electrospray mass spectrometry (HPLC/ES-MS) was performed to compare the types of nucleolytic activities involved in oligonucleotide metabolism. Our data demonstrated that 3′ exonucleases were responsible for the majority of degradation in rat hepatocytes, Kupffer and endothelial cells, and mouse hepatocytes. In contrast, 5′ exonucleases were the predominant enzymatic activity in murine nonparenchymal cells (51).

We extended the pharmacokinetic studies described in the last section and compared liver suborgan distribution of ISIS 1082 between the two rodent species and *Cynomolgus* monkeys 24 h after systemic administration (45,51). These experiments showed that general distribution of ISIS 1082 within whole liver and the cellular fractions of that organ were similar in the three species. However, as described earlier, there are remarkable differences in subcellular distribution of drug within the nuclei of hepatic cells in the rat when compared to the mouse or monkey. Like mouse, ISIS 1082 and metabolites accumulated in the nuclei of monkey liver cells. Although there were striking species-specific differences in distribution on the suborgan level, the extent of metabolism of ISIS 1082 after 24 h was roughly comparable in whole liver, in whole cells, and on the subcellular level. However, the rate of metabolism varied between species. ISIS 1082 was metabolized at a faster rate and to a greater extent in rat liver homogenates when compared to that observed for mouse or monkey (45).

3. Subcellular Distribution in Human Hepatocytes

A growing body of human pharmacokinetic information is being gathered from numerous clinical trials being conducted with phosphorothioate oligodeoxy-

nucleotides directed against a variety of disease and viral targets (6,8,12,52). Although data concerning absorption, plasma pharmacokinetics, and metabolism are available, it is obvious that studies examining suborgan distribution in humans are problematic. With these inherent limitations in mind, we compared the ex vivo uptake of 5′-fluorescein-coupled ISIS 1082 in primary human, monkey, and mouse hepatocytes following their attachment on collagen coated matrices (53–56). Over the 8-h experimental time period, cells consistently displayed greater than 90% viability, as assessed by trypan blue exclusion. Fluorescence microscopy (Fig. 8) revealed that primary hepatocytes of all three species internalized oligonucleotide in a similar fashion. In general, uniform cytoplasmic and nuclear staining was observed in all heptocytes. Interestingly, internalization of the conjugated oligonucleotide into these hepatocytes was accomplished without the need for cationic lipids, which are typically required to observe significant uptake of many oligonucleotides in some transformed tissue culture cell lines (10).

Experiments designed to quantitatively assess the proportion of oligonucleotide localized within subcellular compartments were also performed (Fig. 9). Following ex vivo treatment with 1 μM ISIS 1082, primary human hepatocyte nuclear, cytosolic, and membrane drug levels were measured over an 8-h period. Results from these experiments were compared against systemically administered drug from in vivo rat and mouse kinetic experiments. Oligonucleotide, once again, was extracted from subcellular isolates and subjected to CGE analysis (24). In general, human primary hepatocytes displayed a fourfold greater proportion of nuclear-associated oligonucleotide relative to mouse nuclei. At the 10 mg/kg dose level, no rat nuclear-associated drug was evident, as previously described. Another significant difference between human and rodent hepatocytes is the redistribution of oligonucleotide from the nuclear to cytosolic compartments in humans over 8 h.

Although no direct human-to-primate or rodent in vivo suborgan comparisons could be made, the indirect measures described above give some indication that human hepatocytes internalize phosphorothioate oligodeoxynucleotides and that a substantial portion of drug is distributed to the nuclear compartment. This could have a significant influence on the pharmacological effects of liver-specific antisense oligonucleotides if the target was localized within the nucleus.

IV. CONCLUSIONS

With the experimental caveats mentioned in Section II. D, and further complications in data analysis due to the different localization techniques, oligonucleotide backbones, lengths, sequences, doses, and rodent species and strains, a number of conclusions and generalizations are supported by the data. The most obvious conclusion derived from all of the studies is that phosphorothioate oligodeoxy-

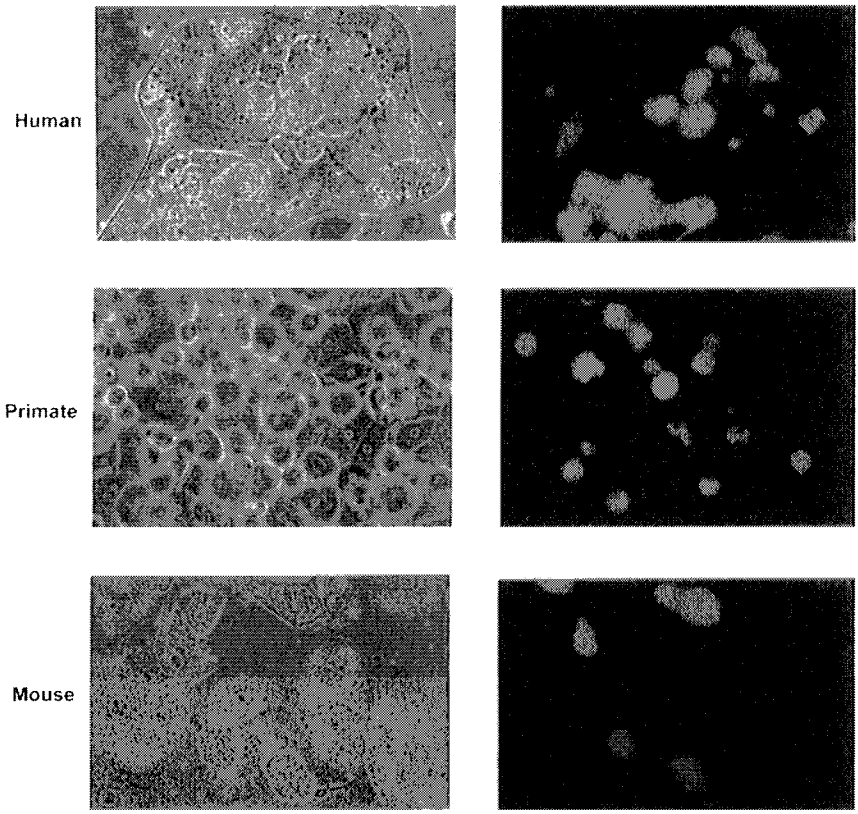

Figure 8 Fluorescent micrographs obtained following treatment with 5′-fluorescein-conjugated ISIS 1082. Primary human, monkey, mouse, and rat hepatocytes were isolated following collagenase perfusion and plated on collagen coated plates. Approximately 1 h after cell adherence, oligonucleotide was added at a 1 μM final concentration. This composite represents the fluorescence intensity observed after 8 h of treatment. (Left) Phase-contrast images of trypan-blue counterstained cells. (Right) Fluorescent micrographs obtained from the same field of cells.

nucleotides are unequivocally localized within the cells of organs, including liver, kidney, skin, spleen, and lymph nodes in a variety of animals after systemic administration of drug. Importantly, the broad distribution of these drugs in vivo has occurred without the need for special delivery systems involving complexation of oligonucleotides to substances like polycations or encapsulation within liposomes (57).

The second observation is the consistency of oligonucleotide localization

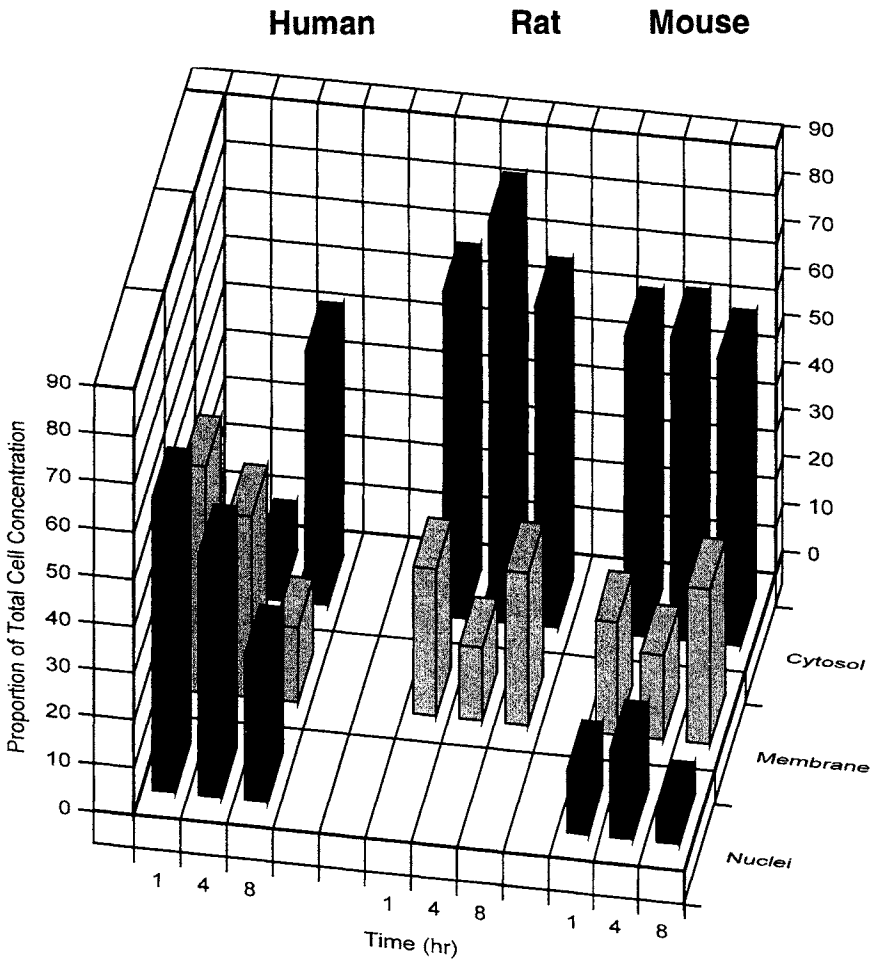

Figure 9 Effect of time on the proportion of ISIS 1082 found within nuclear, cytosolic, and membrane fractions isolated from human, rat, and mouse hepatocytes over an 8-h period after dosing. Results from human cells were obtained from ex vivo experiments whereas rodent results were produced following systemic delivery in vivo. Data are expressed as the proportion of the total cellular dose measured.

in those organs that accumulate the highest amounts of drug. For example Nicklin (11), Butler (17), Carome (18), Oberbauer (19), Plenat (20), Rappaport (21), and Rifai (22) all detected high concentrations of oligonucleotides in kidney in the renal proximal tubules, while some also localized drug to Bowman's capsule and the brush border (11,21,22). In liver, using a variety of indirect histochemical techniques, the highest concentrations of oligonucleotides were frequently detected within the cytoplasm of the nonparenchymal or Kupffer and endothelial cells (11,20,22). However, in some cases, drug was detected, albeit in lower concentrations, in hepatocytes (20,24).

In our laboratories, using a variety of detection methods, the suborgan and subcellular distribution and metabolism of phosphorothioate oligodeoxynucleotides has been carefully delineated and quantitated in mouse, rat, and monkey liver after systemic administration and primary human hepatocytes maintained in tissue culture. As described earlier, phosphorothioate oligodeoxynucleotides were localized within the nuclear, cytosolic and membrane compartments of all three liver cell types. Distribution varied as a function of time, dose, and species. HPLC/ES-MS analysis of liver samples also indicated the types of nucleolytic enzymes involved in the degradation of these compounds.

Based upon the aggregate data, it is also clear that localization data derived from a single technique, especially histological procedures, without validation with alternative methods, like CGE or HPLC/ES-MS for quantifying the amount and extent of metabolism, are incomplete and may provide misleading information. In our laboratories, we routinely perform both histological and subcellular fractionation and quantitative CGE to follow the organ and suborgan distribution of phosphorothioate oligodeoxynucleotides used in pharmacology and pharmacokinetic studies. We agree with Nicklin et al. (11) that multiple analytical techniques, including quantitative CGE, provide much more useful and complete pharmacokinetic information about the in vivo disposition of the phosphorothioates.

The in vivo suborgan localization experiments with phosphorothioate oligodeoxynucleotides, the first generation of antisense therapeutics in clinical trials, set the stage for answering a variety of questions concerning the in vivo pharmacokinetics of these exciting new drugs. Although we know these compounds enter cells and subcellular compartments of numerous organs, what is the mechanism of uptake? It was proposed that scavenger receptors were the primary mechanism of oligonucleotide uptake in liver and other tissues and cells like macrophages. As described earlier, Butler and colleagues (39) demonstrated, using Class A scavenger receptor knockout mice, that scavenger receptors alone cannot account for the bulk distribution of phosphorothioate oligodeoxynucleotides into tissues and cells and suggested multiple mechanisms of uptake probably exist. Current experiments in our laboratories are investigating the involvement of other molecules in in vivo uptake (M. Butler, personal communication; 58).

Other questions relating to the influence of oligonucleotide chemistry, sequence, species, and disease state on suborgan and subcellular distribution are currently being addressed in our laboratories. For example, at present, there are no explanations for the species-specific differences in subcellular distribution that we have observed in liver (45,51). Preliminary data derived from comparative pharmacological studies examining c-*raf* mRNA levels between mouse and rat suggest that the differences in subcellular distribution in hepatocytes may explain why antisense potency is greater in mouse than rat (48; J. Johnston, personal communication). Additionally, since only liver has been characterized in such detail, it is possible that differences in pharmacokinetics are present within other organs, such as kidney, spleen, and the gastrointestinal tract. We are currently developing analogous approaches for those organs as well. Given the complex interactions between cells and interstitium in vivo, the ability to examine and quantitate the distribution of antisense oligonucleotides using CGE after subcellular fractionation within a variety of organs represents a significant advance that has broad applicability for pharmaceutical research.

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8
Pharmacokinetic Properties in Humans

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I. INTRODUCTION

The use of antisense molecules to inhibit the expression of disease-causing proteins represents a new paradigm in disease treatment. The use of targeted inhibitors of protein translation is expected to become commonplace. Human diseases already being treated with antisense inhibitors of translation in clinical trials today include inflammatory bowel disease, hepatitis C, various cancers, and psoriasis. Important metabolic diseases such as diabetes are only minimally behind. Furthermore, the use of antisense inhibition of specific gene products is increasingly recognized as a valuable tool to help scientists understand the human genome and to elucidate new targets for therapeutic intervention.

The first antisense oligonucleotides to enter clinical trials have been phosphorothioate oligodeoxynucleotides, in which a single nonbridging oxygen of the internucleotide linkage was replaced by a sulfur to render nuclease resistance. The next generation of molecules will enter human trials by the year 2001 and will contain further modifications such as 2′-methoxyethyl modifications for improved potency and tissue elimination half-life, and methylated C residues for reduced immune stimulatory qualities. In fact, the tissue half-life of these newer agents is expected to be approximately 5× longer than that of the first-generation compounds (1).

Along with improved potency comes the potential for administration by nonparenteral routes. Indeed, work is already fairly advanced toward oral delivery of these later-generation oligonucleotides. A solid dosage form for oral delivery

of antisense oligonucleotides may already be available by the time of this publication. This was not thought possible just a short time ago.

Longer tissue half-lives mean the potential for less frequent dosing, which reduces patient cost and improves compliance by whatever route of administration available.

In this chapter we summarize currently available data on human pharmacokinetics of antisense oligonucleotides. As data in humans are not yet available for the newer modified oligonucleotides mentioned earlier, the focus of this chapter is on phosphorothioate oligodeoxynucleotides. The majority of our discussion will concern the compounds and references listed in Table 1.

II. PHARMACOKINETICS

A. General Pharmacokinetic Behavior in Humans

After intravenous administration of oligonucleotides, the plasma concentration-time profile is generally monophasic (<1 mg/kg). Use of radiolabeled material revealed a biphasic plasma profile with a much longer apparent elimination phase (2,3). Pharmacokinetics has been characterized for several oligonucleotides in a number of clinical studies (Table 1). The differences in the pharmacokinetics observed in these published clinical studies are mainly due to different assay methods used. The oligonucleotide assays used to date to describe the pharmacokinetic properties of various oligonucleotide preparations in humans and the associated shortcomings of each method are described below.

Capillary gel electrophoresis (CGE) provides excellent resolution for parent oligonucleotide and metabolites shortened by one or more nucleotides (N-1, N-2, etc.) with N-1 resolution, CGE is frequently used to quantitate parent oligonucleotide and metabolites (3–7,23). Another method, SAX-HPLC, measures primarily parent oligonucleotide; however, it does not provide adequate resolution to differentiate parent oligonucleotide and its N-1 metabolite. Similarly, measurement using radioactivity does not differentiate parent oligonucleotide from its metabolite, and very frequently, the radioactivity does not represent the intact oligonucleotide. High-performance electrophoretic chromatography (HPEC) was used for the analysis of a 20-mer phosphorothioate oligonucleotide complementary to p53 mRNA [OL(1)p53,a,b]. However, it is not clear from these reports whether the method is specific to parent oligonucleotide.

From the studies cited in Table 1, plasma concentrations of oligonucleotide increase during short infusion, and C_{max} is generally observed at or near the end of infusion. Parent oligonucleotide is cleared rapidly from the circulation and animal data demonstrate that it is distributed into tissues. Plasma distribution half-life is in the range of 30–90 min depending on dose. Thus the clearance from plasma measured in clinical trials is a distribution phase, not terminal elimination.

Table 1 Antisense Phosphorothioate Oligodeoxynucleotides Evaluated in Human Pharmacokinetic Studies

Compound (Ref.)	Length	Target	Indication	Dose	Route	Analytical methodology
OL(1)p53 (16)	20 mer	P53	Cancer	0.05 mg/kg/h for 10 days	10-day continuous IV infusion	HPEC
OL(1)p53 (14)	20 mer	P53	Cancer	0.05 mg/kg/h for 10 days	10-day continuous IV infusion	Postlabeling, ^{35}S-LSC, HPEC
ISIS 2105 (15)	20 mer	HPV-6/11	Antiviral	~0.06 mg/kg	Intradermal injection	^{14}C/HPLC
GEM 91 (2)	25 mer	HIV (gap/pol)	Antiviral	0.1 mg/kg	2-h IV infusion	^{35}S-PAGE
GEM 91 (12)	25 mer	HIV (gap/pol)	Antiviral	0.1–2 mg/kg	2-h IV infusion	HPLC
ISIS2302 (3)	20 mer	ICAM-1	Anti-inflammation	0.06–2 mg/kg	2-h IV infusion	CGE
G3139 (20)	18 mer	BCL-2	Cancer	2.0 mg/kg	14-day continuous subcutaneous infusion	HPLC
ISIS 5132 (4)	20 mer	c-raf-1	Cancer	0.5–6 mg/kg	2-h IV infusion	CGE
ISIS 5132 (23)	20 mer	c-raf	Cancer	0.5–5.0 mg/kg/day	21-day continuous infusion	CGE
ISIS 3521 (5)	20 mer	PKC-α	Cancer	0.5–3.0 mg/kg/day	21-day continuous infusion	CGE
ISIS 3521 (6)	20 mer	PKC-α	Cancer	0.15–6.0 mg/kg	2-h IV infusion	CGE
ISIS 2922[a]	21 mer	CMV	Antiviral	330 μg, induction and maintenance	Intra-vitreal injection	CGE

[a] Grillone (manuscript in preparation).

Plasma C_{max} and AUC generally increased greater than dose-proportional, associated with a slight increase in half-life and decrease in clearance as dose increased. These observations suggest that disposition of oligonucleotide follows nonlinear kinetics and distribution sites may be saturable. Clearance of oligonucleotide from plasma is predominantly by tissue distribution, and to a lesser extent, metabolism.

The analytical methods used to measure parent oligonucleotide (CGE or HPLC), although sensitive (limits of detection at 14–100 ng/mL), do not characterize the elimination rate of oligonucleotide from the whole body. In fact, the maximum plasma concentration of oligonucleotide is in the range of 5–15 μm/g at clinically relevant doses; therefore, plasma profile of five to six half-lives can be followed according to first-order principle using the current analytical techniques. True elimination rates are related to much slower clearance of oligonucleotide from tissue. Nonclinical pharmacokinetic studies have elucidated tissue clearance half-lives and they are generally greater than 24 h (8–11). Therefore, the plasma clearance measured using CGE or HPLC methods is a plasma distribution clearance, and does not represent total-body clearance.

When radiotracer techniques are used to characterize oligonucleotide pharmacokinetics, the radioactivity may be partly associated with parent oligonucleotide, partly associated with metabolites, and partly associated with the radiolabel of some unknown form (2). Therefore, the pharmacokinetic parameters generated from these studies are difficult to interpret. The prolonged elimination half-lives observed when measuring radiolabel are only partly reflective of parent compound clearance and thus are not useful for defining it.

The plasma pharmacokinetics as defined by cold assays such as CGE and SAX-HPLC provide excellent prediction of multiple dose behavior and, more importantly, accurately predict steady-state pharmacokinetics observed during continuous intravenous infusion (23,15). Therefore, it is clear that the distribution kinetics of oligonucleotides dominate the plasma pharmacokinetic behavior and clearance of parent oligonucleotide.

B. Individual Pharmacokinetic Parameters

1. Maximum Plasma Concentrations (C_{max})

During intravenous infusion, plasma concentrations of oligonucleotide increase and C_{max} is generally observed at or near the end of infusion. A number of studies reported that C_{max} increased with dose, and were observed to be greater-than dose-proportional (6,12). However, the nonlinear increase in C_{max} is not seen for all oligonucleotides studies (3). Figure 1 illustrates the relationship between C_{max} and dose following 2-h intravenous administration of various oligonucleotides, pooling together several studies from the literature.

Following continuous infusion, steady-state concentrations (C_{ss}) are

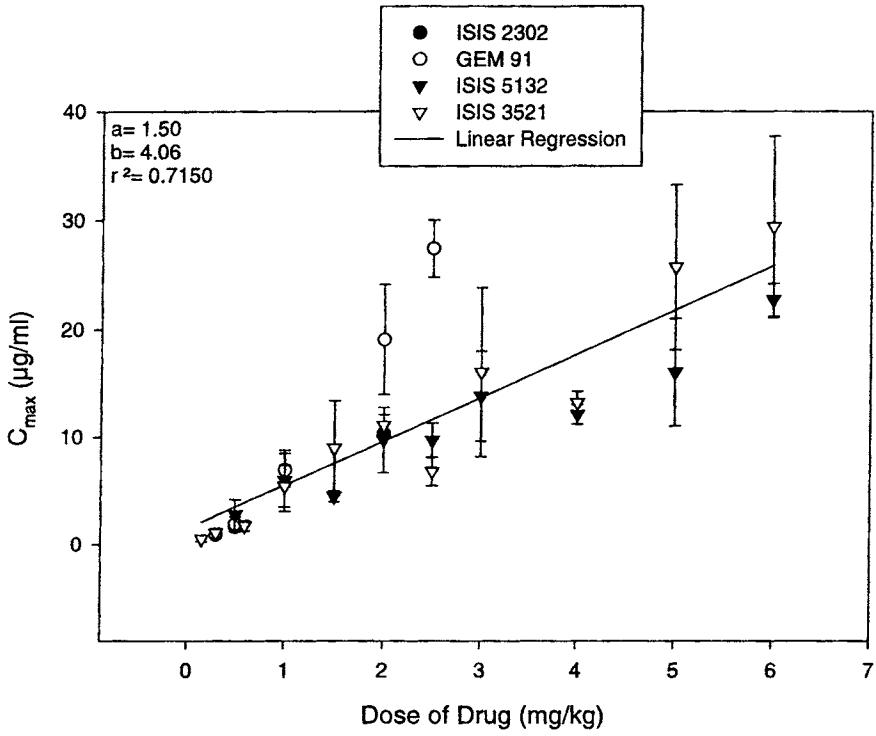

Figure 1 Plasma C_{max} as a function of dose after 2-h IV infusion of various phosphorothioate oligodeoxynucleotides.

achieved within 24 h. C_{ss} also increased in a manner consistent with nonlinear kinetics with saturable clearance following continuous intravenous infusion for a number of studies (5,23). However, this dose response is not observed for a 10-day continuous-infusion study for OL(1)p53 (13). Plasma concentrations of OL(1)p53 at steady state were reported as 3.03, 2.08, 3.73, 3.70, and 4.15 µg/mL at doses of 0.05, 0.10, 0.15, 0.20, and 0.25 mg/kg/h, respectively, which did not show a dose response as seen in other studies.

2. AUC

As shown in Fig. 2, AUC values reported are dose-dependent, and generally increased greater than proportional to dose, suggesting that there is a saturable component in the disposition of oligonucleotide (3,6,12).

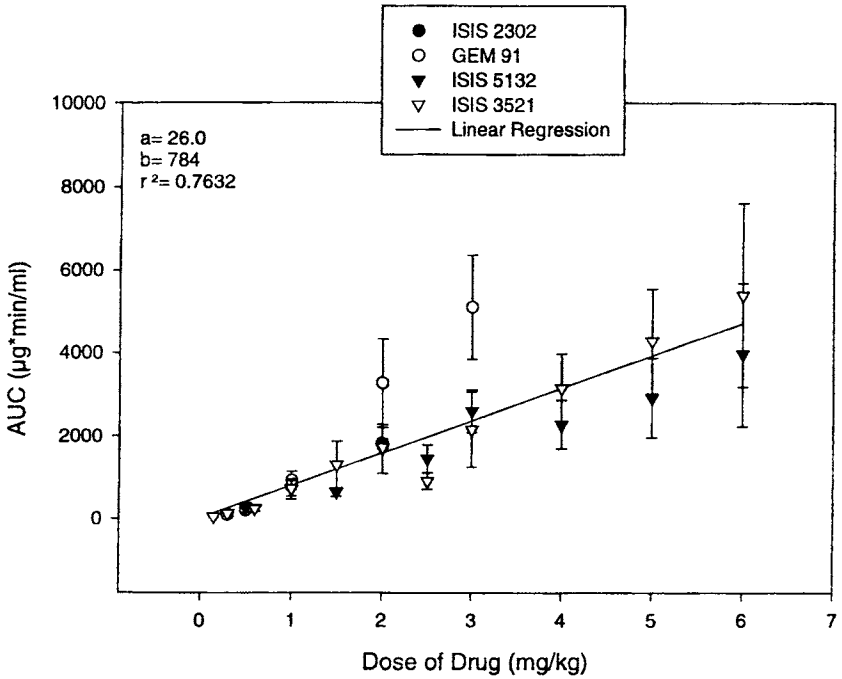

Figure 2 Plasma AUC as a function of dose after 2-h IV infusion of various phosphorothioate oligodeoxynucleotides.

3. Plasma Half-Life and Clearance

After intravenous administration, oligonucleotide concentrations decreased rapidly from plasma. Plasma half-life varied significantly, largely depending on the analytical method used for sample analysis. Plasma half-life is in the range of 30–90 min when CGE or AE-HPLC method is employed, which measures primarily intact oligonucleotide (3,12). Biphasic plasma profile of oligonucleotide is generally apparent when radiolabel analysis is used with a rapid α-$t_{1/2}$ in minutes and β-$t_{1/2}$ of 24–63 h (2,14). The measurement of radioactivity is the sum of intact oligonucleotide, chain-shortened metabolites, and small molecules associated with the radiolabel. Therefore, the half-life is an apparent half-life representing such a mixture.

Plasma half-life of parent oligonucleotide has been reported to increase with dose, which is associated with a decrease in plasma clearance (12). This dose dependency is not observed when total radioactivity measurement is used. Taken together with the fact that C_{max} and AUC increased greater than propor-

tional to dose alone, these data suggested that the disposition of oligonucleotide is saturable. A one-compartment model with parallel first-order and Michaelis-Menton elimination process was used to describe plasma concentration data following 2-h intravenous infusion, with K_m = 2.49–3.00 ng/mL and V_m = 0.03–0.14 ng/min/mL (12). Clearance from plasma is accomplished predominantly by distribution to tissues and, to a lesser extent, metabolism and urinary excretion. It has been proposed that there are two pathways for oligonucleotide clearance from plasma: a capacity-limited pathway that is saturable at low plasma concentrations and a second apparent first-order pathway (12).

C. Elimination

1. Metabolism

Phosphorothioate oligodeoxynucleotides in human plasma are rapidly metabolized to chain-shortened oligonucleotides by exonucleases in a processive fashion (Fig. 3) starting at the 3′ end. Parent oligonucleotide in plasma is the predominant oligonucleotide at all time points following intravenous administration (3–6; Nemunaitis et al., in press), which represents >50% of the total measurable oligonucleotide (Fig. 3, Table 2). Higher percentage of parent drug (>80%) observed at lower doses (≤0.3 mg/kg) may be overestimated owing to the plasma concentration of oligonucleotide being so low that the metabolites present were below the limit of quantitation of the analytical method.

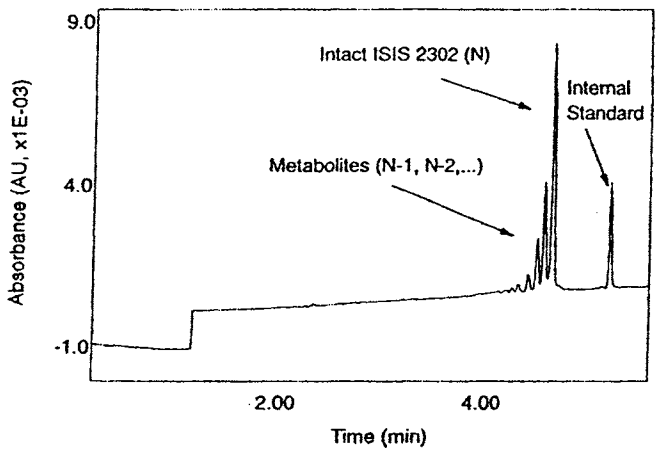

Figure 3 Representative electropherogram of ISIS 2302 and chain-shortened metabolites in human plasma at 1 h after 2-h IV infusion of 20 mg/kg ISIS 2302 (3).

Table 2 Metabolism of Oligonucleotide in Human Plasma at the End of 2-h IV Infusion Across Doses

Dose	Source	Compound	% Intact
0.15	Nemunaitis et al., 1999	ISIS 3521	91.2
0.3	Nemunaitis et al., 1999	ISIS 3521	83.2
0.5 mg/kg	Glover et al., 1997	ISIS 2302	69.6
0.6 mg/kg	Nemunaitis et al., 1999	ISIS 3521	83.7
1.0 mg/kg	Nemunaitis et al., 1999	ISIS 3521	64.3
2.0 mg/kg	Glover et al., 1997	ISIS 2302	66.5
2.0 mg/kg	Nemunaitis et al., 1999	ISIS 3521	55.8
2.5	Nemunaitis et al., 1999	ISIS 3521	60.9
3.0	Nemunaitis et al., 1999	ISIS 3521	58.2
4.0	Nemunaitis et al., 1999	ISIS 3521	57.6
5.0	Nemunaitis et al., 1999	ISIS 3521	54.4
6.0	Nemunaitis et al., 1999	ISIS 3521	54.6

The primary metabolite was the parent oligonucleotide shortened by one nucleotide (called N-1 in Fig. 3). Other chain-shortened metabolites were apparent in decreasing concentrations in order of the number of nucleotides deleted. The proportionality of metabolites over time remained consistent suggesting that metabolism in plasma is largely complete within 30 min following dose administration (3). The overall rate and pattern of metabolism in plasma does not appear to be altered greatly after repeated administration, with increasing dose, or with different oligonucleotide sequence (Table 2) (3,6). The eventual degree of metabolism to shortened oligonucleotide is independent of dose over the range of doses examined. Moreover, at all dose levels after repeated doses, there was no evidence of accumulation of oligonucleotide and metabolites in plasma with alternate-day dosing. The consistency in proportionality in metabolite pattern may be explained by (1) buildup of nuclease-inhibitory metabolites or (2) differential rate of metabolism for the Rp and Sp diasteroisomers that presents at each phosphorothioate linkage. The nuclease inhibition argument, however, appears in conflict to the data that neither changes with increased dose or with repeated administration. Therefore, the latter explanation relating to the stereochemistry of the phosphorothioate backbone appears to provide the most probable reason for the observed metabolism.

2. Excretion

Depending on the analytical method used, the reported urinary excretion varied significantly in the literature (Table 3). The majority of the excreted oligonucleo-

Table 3 Urinary Excretion of Oligonucleotide

Compound	Source	Method	Dose	% Excretion—parent	% Excretion—total
ISIS 2302	Glover et al., 1997	CGE	2 mg/kg 2 h IV	0.05 (6 h)	0.5
OL(1)p53	Bayever et al., 1993	HPEC	1.2 mg/kg/day for 10 days	>9–18	30–62 (10 day)
OL(1)p53	Bishop et al., 1996	HPEC	1.2–6 mg/kg/day for 10 days	17–59 (10 day)	NR
GEM 91	Zhang et al., 1995	LSC-^{35}S	0.1 mg/kg 2 h IV	NR	49.15 ± 6.80 (24 h)
ISIS 2105	Crooke et al., 1994	^{14}C-HPLC	~0.06 mg/kg intradermally	0	10

NR = not reported.

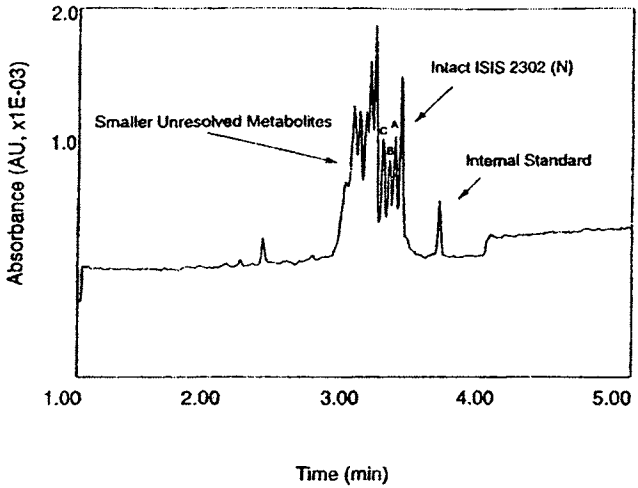

Figure 4 Representative electropherograms of ISIS 2302 and chain-shortened metabolites in human urine collected from 0 to 6 h after 2-h IV infusion (3).

tide species are chain-shortened metabolites (Fig. 4). Urinary excretion of total oligonucleotide is a minor route of elimination when analyzed using oligonucleotide-specific assay such as capillary gel electrophoresis (CGE), representing less than 1% of total administered dose (3). When ^{14}C-ISIS 2105 was administered by intradermal injection, urinary excretion was 10% of administered dose, and none of the radioactivity was related to the parent oligonucleotide (15). Higher urinary excretion (49.2 $\pm$ 6.8%) was reported when ^{35}S-labeled oligonucleotide was used. The majority of excreted radioactivity is degradative product that may not be associated with oligonucleotide (2). In contrast, cumulative urinary excretion of intact 20-mer phosporothioate oligonucleotide [OL(1)p53] was in the range of 9–59% (14,16) following 10-day continuous infusion, which is much higher than observed in other studies. This observation may be less reliable owing to the fact that the analytical method (HPEC) used was poorly characterized.

In summary, urinary excretion of oligonucleotide represents a minor route of elimination, regardless of route of administration.

D. Plasma Protein Binding

Many common drugs bind to circulating plasma proteins. Binding to plasma proteins can prevent filtration and elimination through urinary excretion. However, for conventional drugs, binding to plasma proteins can also prevent the drug from

passing through cell membranes, thus rendering it pharmacologically inactive. In addition, the degree of plasma protein binding also governs the volume of distribution of the drug, and is important in understanding the disposition of the drug.

Phosphorothioate oligodeoxynucleotides are highly bound to plasma proteins with whole plasma protein binding ranging from 91 to 99% for various sequence of oligonucleotide at clinically relevant doses (unpublished data). Binding to plasma proteins has been shown to be salt and pH dependent, suggesting that the binding is likely a nonspecific electrostatic interaction (17). Among the major protein species, phosphorothioate oligodeoxynucleotide primarily binds to albumin with high capacity and low affinity (K_{D1} = 2.8 ± 0.3 μM, K_{D2} = 10.0 ± 0.3 μM), and binds to α_2-macroglobulin with low capacity but high affinity (K_{D1} = 30 nM, K_{D2} = 1 nM) (17). The affinity for the α_2-macroglobulin protein was found to be higher than albumin. Binding of oligonucleotide to α_1-acid glycoprotein (AAG) is negligible (internal data, unpublished).

Previous reports reveal that phosphorothioate oligonucleotide also binds some less abundant plasma proteins, such as thrombin (unpublished data), Factor H (18), and components of the intrinsic tenase complex (19). Binding of oligonucleotide to these proteins at high oligonucleotide concentrations may have resulted in some acute toxicities such as increase in aPTT and complement activation, as observed in monkeys following intravenous dosing (18). In human studies, grade 1–2 elevations in aPTT have been reported, as well as elevations in C3a (4). These elevations have not been shown to be associated with clinical sequelae to date (data on file, ISIS Pharmaceuticals, Inc.).

Protein binding may explain many of the pharmacokinetic properties of phosphorothioates as a class of drug. The high degree of protein binding in the circulation prevents glomerular filtration and significant urinary excretion of the parent drug (full-length oligonucleotide); excretion generally represented less than 1% of administered dose following intravenous administration (3). Saturation of plasma protein binding generally does not occur in patients because plasma concentrations of oligonucleotide in patients at clinically employed doses are well below the plasma-protein-binding capacities.

If conventional pharmacokinetics were used to interpret oligonucleotide plasma binding, then one would predict that oligonucleotides would not distribute to tissues extensively owing to their high protein binding and would have a small volume of distribution. In reality, oligonucleotides distribute to tissues extensively as shown in preclinical studies (10). This tissue distribution may be explained by shuttling of oligonucleotide from low-affinity plasma proteins to high-affinity proteins residing in tissues. The reported volume of distribution of intact oligonucleotide at steady state is generally in the range of 90–300 mL/kg (Table 2), which is fairly small. The analytical method used for analysis of intact oligonucleotide is CGE or HPLC, neither of which is sensitive enough to characterize

the true elimination phase of oligonucleotide in plasma. The reported plasma concentration-time profile is generally the distribution phase of oligonucleotide disposition. In other words, the volume of distribution in these cases was calculated based on the data before steady state is reached and is an apparent volume of distribution.

E. Repeat Administration

After repeat administration, there is no measurable plasma accumulation following alternate-day 2-h intravenous administration, which is indicated by lack of measurable predose plasma concentrations and the absence of increase in C_{max} with repeated dosing. Moreover, there is no indication of change in the pattern of parent to drug metabolite in plasma over time (3,4,6). Taken together, repeated administration of oligonucleotides does not induce or inhibit its own metabolism.

F. Route of Administration

Plasma pharmacokinetics were not fully characterized following ophthalmic administration.

Plasma half-life and clearance following 2-h intravenous infusion did not change significantly compared to 21-day continuous infusion for a number of oligonucleotides when the same analytical method was used (Table 4). After continuous infusion, steady-state plasma concentrations were generally achieved within 24 h (5,14,23), which was consistent with the short plasma half-life (<91 min) seen. It was reported that the steady-state for OL(1)p53 was achieved within 24 h, which is contradictory to its long plasma half-life (24.4–62.5 h) (14). Based on the reported half-life of 24.4–62.5 h, steady-state concentrations should not be achieved until 122–313 h.

Although plasma pharmacokinetics was not characterized for G3139 following subcutaneous injection (20), plasma pharmacokinetics for another phosphorothioate oligonucleotide, ISIS 2302, was studied following subcutaneous injection and subcutaneous continuous infusion (unpublished internal data). Maximum plasma concentration was achieved 2–3 h after administration, suggesting rapid absorption from the dosing site. Both C_{max} and AUC increased as dose was increased and exhibited some trend toward greater than dose proportionality. However, plasma half-life (157–186 min) was not altered by increasing dose. These data suggest that the fraction absorbed into the systemic circulation may increase as dose increases. The prolonged half-life as compared to IV data suggests that absorption from the injection site is slow. The estimated bioavailability (F) following subcutaneous administration was 44–58% as compared to the IV data at equivalent dose.

Following intradermal administration, systemic absorption is rapid with

Table 4 Comparison of Pharmacokinetics Parameters of Different Route of Administration

Compound (Ref.)	Dose	Analytical method	Route	C_{max} (μg/ml)	Cl (mL/min/kg)	$t_{1/2}$
ISIS 2302[a]	2.23 mg/kg	CGE	Subcutaneous injection	6.05 ± 1.62		148 ± 27.9 min
ISIS 2105 (15)	~0.06 mg/kg	^{14}C-HPLC	Intradermal	<0.08	NR	$t_{1/2}$, α < 1 h $t_{1/2}$ β = 147 h
G3139 (20)	2 mg/kg/day	^{35}S-HPLC	14-day continuous subcutaneous infusion	2.7 ± 0.5	NR	NR
GEM 91 (12)	2.0 mg/kg	HPLC	2-h IV infusion	27.4 ± 2.62	0.68 ± 0.28	51 ± 8 min
ISIS 3521 (6)	2.0 mg/kg	CGE	2-h IV infusion	11.1 ± 0.98	1.16 ± 0.03	79.9 ± 26.5 min
ISIS 5132 (4)	2.0 mg/kg	CGE	2-h IV infusion	9.72 ± 3.03	1.27 ± 0.40	70.8 ± 5.9 min
OL(1)p53 (14)	2.4 mg/kg/day	^{35}S-LSC, post-labeling	10-day continuous IV infusion	2.08 ± 0.85	NR	42.6 ± 8.27 h
ISIS 3521 (5)	2.0 mg/kg/day	CGE	21-day continuous IV infusion	0.50 ± 0.13	2.88 ± 0.62	61 ± 14 min
ISIS 5132 (23)	2.0 mg/kg/day	CGE	21-day continuous IV infusion	0.76 ± 0.42	1.83 ± 1.01	91 ± 56 min

[a] Unpublished internal data.
NR = not reported.

plasma concentration peaking 1 h following administration. The β-$t_{1/2}$ is much longer (147 h) following intradermal administration compared to other routes of administration. One explanation for the long half-life is that the half-life represents total radioactivity (parent drug and metabolites) instead of the half-life for parent compound as was reported for the other studies. In addition, the terminal half-life may be overestimated because the plasma concentrations at the elimination phase are very close to the background levels (15).

Metabolism and metabolite pattern do not change with different length of infusion. Intact oligonucleotide is the principal oligonucleotide species and typically represented 50–65% of total detected oligonucleotide regardless of length of infusion (3–6,23).

G. Sequence Independence

Plasma pharmacokinetics of different sequences of oligonucleotide are similar. Following 2 mg/kg 2-h IV administration of the phosphorothioate oligonucleotides ISIS 2302, ISIS 3521, and ISIS 5132, plasma AUC is in the range of 1702–1825 μg/min/ml with C_{max} in the range of 9.72–11.1 μg/ml (Table 5). However, the pharmacokinetics for GEM 91 appears to differ from that of the other oligonucleotides. The AUC and C_{max} for GEM 91 are close to double those of the other oligonucleotides listed in Table 6, and are associated with a reduced clearance as compared with other oligonucleotides. This difference may be explained by (1) the difference in oligonucleotide length, and (2) the difference in analytical method used. GEM 91 is a 25-mer oligonucleotide, while the other oligonucleotides listed in Table 6 are 20-mer oligonucleotides. The rate of metabolism, distribution, and excretion of 25-mers may be slower than that of 20-mer oligonucleotides, resulting in a higher AUC and C_{max}, and less clearance. Moreover, HPLC was used to quantitate GEM 91 while CGE was used to quantitate the other

Table 5 Comparison of Pharmacokinetics of Different Sequences of Oligodeoxynucleotide in Humans Administered at 2 mg/kg by 2-h IV Infusion

Parameter	ISIS 2302 (3)	GEM 91 (12)	ISIS 5132 (4)	ISIS 3521 (6)
AUC (μg*mL/min)	1825 ± 111	3276 ± 1062	1702 ± 634	1728 ± 48
$t_{1/2}$ (min)	52.9 ± 6.0	51 ± 8	70.8 ± 5.9	79.9 ± 26.5
C_{max} (μg/mL)	10.3 ± 0.06	27.4 ± 2.62	9.72 ± 3.03	11.1 ± 0.98
Cl (mL/min/kg)	1.28 ± 0.12	0.68 ± 0.28	1.27 ± 0.40	1.16 ± 0.03
V_{ss} (mL/kg)	97.5 ± 7.1	960 ± 80	122 ± 37	NR

NR = not reported.

Table 6 Comparison of C_{max} and AUC of Parent Drug in Cynomolgus Monkeys and Humans After Administration of a 1 mg/kg dose As a 2-h Intravenous Infusion

Compound (Ref.)	Species	C_{max} (μg/mL)	AUC (μg*min/mL)
ISIS 2302 (21)	Monkey	4.59 ± 0.16	580 ± 11.4
	Human	3.96 ± 1.02	506 ± 56.4
ISIS 5132 (21)	Monkey	3.27 ± 0.82	369 ± 85.2
	Human	3.78 ± 0.61	612[a]
ISIS 3521 (21)	Monkey	4.30 ± 0.75	486 ± 63
	Human	4.78 ± 1.33	624[a]
GEM 91 (12)	Human	6.94 ± 1.56	927 ± 219

[a] $n = 2$.

oligonucleotides. The resolution of parent drug and metabolites with HPLC is not as good as with CGE; therefore, the observed concentrations for GEM 91 includes some of the major metabolites and may thus overestimate concentrations of GEM 91 in plasma. In summation, plasma pharmacokinetics of oligonucleotides are independent of oligonucleotide sequence.

H. Interspecies Scaling: From Mouse to Human

Interspecies scaling appears to be linear as a function of body weight rather than surface area using allometric analysis (7,21,22). Plasma clearance and volume of distribution at steady state at equivalent doses appear to be linearly correlated to body weight, with the exception of the mouse (Fig. 5, Chapter 4). The slope of the line is near unity for both pharmacokinetic parameters. The similarities in plasma pharmacokinetics across species strongly suggest that the underlying mechanisms for clearance are similar between species. Plasma pharmacokinetics for humans can be accurately extrapolated from rats to monkeys. In fact, the plasma concentrations and AUC in nonhuman primates and humans are nearly equivalent on a dose per unit body weight (mg/kg) basis (Table 6).

Therefore, the pharamcokinetic properies of phosphorothioate oligonucleotides are such that the data from preclinical models can easily be extrapolated to clinical situations such as designing dosing regimens. Because plasma kinetics are so highly dependent on tissue uptake and distribution, the similarities in plasma pharmacokinetics between the animal models and humans suggest that tissue kinetics may be similar, thus allowing for prediction of target organ concentrations of oligodeoxynucleotides in humans.

III. CONCLUSIONS AND SUMMARY

The pharmacokinetics of phosphorothioate oligonucleotides in humans are dose dependent, sequence independent, equivalent on a dose per body weight basis across nonhuman and human species, and characterized by high protein binding. Differing pharmacokinetic profiles observed in various clinical studies are due in large part to the analytical technique used, and dependent upon measuring parent versus parent plus chain-shortened metabolites.

C_{max} is generally observed at the end of infusion and clearance of oligonucleotide from plasma is predominantly by tissue distribution. After continuous infusion, steady-state concentration is generally achieved within 24 h. AUC is dose dependent and there appears to be a saturable component in the disposition of oligonucleotide. Plasma half-life is in the range of 30–90 min, depending upon dose. Phosphorothioate oligonucleotides in human plasma are rapidly metabolized to chain-shortened oligonucleotides by exonucleases in a processive fashion starting at the 3′ end (N-1, N-2, N-3, etc.). This metabolism is largely completed within 30 min and is independent of dose or sequence. Parent compound is the major component seen in plasma at all time points. Urinary excretion appears to be a very minor route of elimination (less than 1% of parent administered dose).

Approximately 91–99% of phosphorothioates are protein bound, with the major species being albumin and α_2-macroglobulin. Binding to thrombin, factor H, and components of the intrinsic tenase complex has also been shown to occur, and may account for some of the toxicities observed at high doses in nonclinical safety studies. Finally, plasma pharmacokinetics for humans can be accurately extrapolated from other species, and in nonhuman primates plasma concentration and AUC are nearly equivalent on a mg/kg basis.

Newer-generation oligonucleotides with additional modifications and enhancements are just now entering the clinic, and will likely exhibit different pharmacokinetic profiles. These modified oligonucleotides coupled with delivery systems and novel formulations will likely permit more convenient or flexible dosing, further enhancing the attractiveness of antisense as therapeutic agents for human disease.

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9
Toxicity of Antisense Oligonucleotides

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I. INTRODUCTION

There is tremendous therapeutic potential in being able to selectively down-regulate the expression of disease-inducing proteins. Antisense oligonucleotides hold this promise with their ability to specifically block mRNA translation and selectively knock down the expression of specific gene products. Hundreds of examples of sequence-specific reductions in target mRNA and/or protein expression can be found in the literature (reviewed in Crooke, *Handbook of Experimental Pharmacology*). Inhibiting expression in cell culture or in animal models is an important step toward developing drugs, but there are significant barriers when moving compounds from the laboratory to the clinic. For all drugs, including antisense oligonucleotides, these barriers include delivery to the active site, toxicity, and efficacy. This review will concentrate on toxicity, especially toxicity related to advances in antisense drug delivery.

In the antisense strategy, an oligonucleotide complementary to the mRNA of a target protein is administered, and the resulting mRNA-oligonucleotide hybrid is incapable of supporting translation either because of destabilization of the mRNA or physical interference with translation (1). Because activity of the antisense oligonucleotide is dependent on hybridization to the target mRNA, Watson and Crick base pairing determines the specificity. The longer the oligonucleotide, the lower the probability that a perfect match exists in the expressed genome.

Most of the oligonucleotides proposed for drug development have been between 20 and 30 nucleotides in length: long enough to specify a unique sequence in the expressed genome. Theoretically then, antisense oligonucleotides should be highly specific compounds with little chance of producing undesirable effects as a result of hybridization to unintended targets.

The theoretical benefits of antisense technology have been tempered by the reality of toxicities resulting from nonspecific interactions with proteins, quite independent of Watson and Crick base pairing. This review will focus on the toxicities associated with the administration of oligonucleotides and the dose-response relationships for those toxicities. For many of the toxic effects of oligonucleotides, considerable progress has been made in our understanding of the biochemical basis of the toxicities since the last major review (2). As this class of drugs has been in clinical trials for the past 7 years, a considerable amount of data on both the toxicity profile and the mechanistic basis for toxicities now exists. In addition, a review at this time integrates a large amount of new data on the effects of new routes of administration on the toxicity profile of antisense oligonucleotides. Table 1 presents the sequence for all oligonucleotides men-

Table 1 Sequences and Structures of Oligonucleotides

Name	Sequence/linkages
ISIS 3521	GsTsTsCsTsCsGsCsTsGsGsTsGsAsGsTsTsTsCsA
ISIS 2105	TsTsGsCsTsTsCsCsAsTsCsTsTsCsCsTsCsGsTsC
ISIS 2922	GsCsGsTsTsTsGsCsTsCsTsCsTsTsCsTsTsCsTsTsGsCsG
ISIS 13312	**gscsgststststsgs**CsTsCsTsCsTsTsCsT**stscststsgscs**G
ISIS 2302	GsCsCsCsAsAsGsCsTsGsGsCsAsTsCsCsGsTsCsA
ISIS 1082	GsCsCsGsAsGsGsTsCsCsAsTsGsTsCsGsTsAsCsGsC
ISIS 3082	TsGsCsAsTsCsCsCsCsCsAsGsGsCsCsAsCsCsAsT
ISIS 2503	TsCsCsGsTsCsAsTsCsGsCsTsCsCsTsCsAsGsGsG
ISIS 5132	TsCsCsCsGsCsCsTsGsTsGsAsCsAsTsGsCsAsTsT.
ISIS 13920	tsc′sc′sc′scsC′sC′sTsGsTsGsAsC′sAsTsgsc′sastst
ISIS 12449	AsCsCsGsAsTsAsAsCsGsTsTsGsCsCsGsGsTsGsAsCsG
ISIS 12450	AsCsCsGsAsTsAsCsCsGsGsTsGsCsCsGsGsTsGsAsCsG
rel a	GsAsGsGsGsGsAsAsAsCsAsGsAsTsCsGsTsCsCsAsTsGsGsT
Survivin	TsGsTsGsCsTsAsTsTsCsTsGsTsGsAsAsTsT
Bcl-X	CsTsAsCsGsCsTsTsTsCsCsAsCsGsCsAsCsAsGsT
GEM91	CsTCsTsCsGsCsACsCsCsAsTsCsTsCsTsCsTsCsCsTsTsCsCsT
GEM 91—C	***CTCTC***sGsCsAsCsCsCsAsTsCsTsCsTsCsTsCs***CTTCT***
En—1	TsTsAsGsCsTsTsCsCsTsGsGsTsGsCsGsTsGsGsA
AR177	GsToGoGoToGoGoGoToGoGoGoToGoGoGs

s, phosphorothioate linkage; o, phosphodiester linkage; c′, 5-methyl C; lower case bold, 2′ methoxyethoxy; upper case bold, 2′ methoxy; italics, methylphosphonate linkage.

tioned here. The options for routes of administration have expanded considerably since the last major review (3). It has now been possible to administer oligonucleotides by topical application to the skin, enema, inhalation, subcutaneous injection, and of course, intravenous injection and infusions. Research efforts are rapidly advancing oral formulations of oligonucleotides toward the clinic, and in this review data will be presented on the tolerability of oligonucleotides administered locally to the gut. The new information on mechanisms of toxicity and recent addition of more routes of administration for oligonucleotides provide a compelling reason to rereview the field.

It is important to consider the types of toxicity that might be induced by a class of compounds like antisense oligonucleotides (Table 1). There are a number of mechanisms whereby antisense oligonucleotides can produce toxicity. The most obvious mechanism is the actual reduction in target protein expression itself in a hybridization-dependent process. Other potential mechanisms include the antisense hybridization with related or nearly homologus sequences on nontarget mRNA, alterations of endogenous metabolic pathways induced by metabolites of oligonucleotides, and nonspecific interactions with proteins. Each of the above will be addressed in this review, but clearly the most prominent toxicological effects observed to date are all related to nonspecific binding of oligonucleotides to proteins.

A. Too Much of a Good Thing—Exaggerated Pharmacology

One potential mechanism of toxicity for any class of drug results from the pharmacological effect itself. For conventional drugs, it is expected that too much pharmacological activity will result in an adverse effect; e.g., antihypertensive drugs can produce syncope and collapse at suprapharmacological doses. For antisense drugs this type of adverse effect could be induced by the reduction in the target protein itself. In toxicity studies of oligonucleotides directed against a diverse range of gene products the profile of toxicity has been remarkably similar, and thus, independent of the targeted mRNA. To date, toxicity studies performed by Isis Pharmaceuticals or those published in the literature have targeted mRNAs such as c-raf kinase, protein kinase C alpha, intracellular adhesion molecule-1, and rel A (4–8). Because of differences in the target mRNA sequence between species, the characterization of the toxicity of many of these compounds has included both the human sequence and an oligonucleotide sequence specific for the test species run in parallel. Administering the murine-specific sequence allows us to determine whether knockdown of the target gene can produce exaggerated pharmacological activity. In all cases, the toxicity profiles of the human- and rodent-specific sequence have been qualitatively similar even in the presence of target mRNA reductions (4). In fact, the majority of oligonucleotides examined

in toxicity studies have been to mammalian targets, yet there has been no apparent consequence of the knockdown of protein expression. The apparent lack of toxicologically significant reductions in target protein expression is in part the result of careful selection of gene targets. For example, some antisense compounds inhibit specific isozymes of multigene families (e.g., PKC-α) that are important in a target disease, but the redundant function of other members of the gene family allows for normal cell function. Other antisense compounds have targeted inducible proteins that are up-regulated in certain disease conditions (e.g., ICAM-1), and thus have no effect on normal function. In the case of oligonucleotides designed to inhibit virus replication, obviously these have no expressed mammalian mRNA targets, and thus, no exaggerated pharmacology.

Had gene knockdown produced a toxic effect, the toxicity profile would differ from the well-characterized class effects of phosphorothioate oligonucleotides. To date, in in vivo studies, it has not. In vitro, a striking example of that type of toxicity has been described for an oligonucleotide designed to hybridize to the mRNA for human thymidylate synthetase. In vitro experiments with this oligonucleotide clearly demonstrate a reduction in cell growth rate that correlated with reductions in messenger RNA levels. A scrambled control oligonucleotide did not have the same growth-retarding effect (9). This effect demonstrates that selecting mRNA targets that are critical for cell survival will induce toxicities similar to those produced by small molecules that have the same intended target. In fact, when this oligonucleotide was used to treat cells in combination with 5-fluorouracil, another inhibitor of thymidylate synthetase, there was additive toxicity. Similarly, inhibition of Bcl-X_L sensitizes human keratinocytes to apoptotic stimuli such as UVB light, and inhibiting survivin causes cell-cycle- and caspase-dependent cell death (10,11). Although in vivo studies with these oligonucleotides have not been performed, it would be interesting to treat mice using rodent-specific analogs to characterize unique hybridization-dependent exaggerated pharmacology. Although exaggerated pharmacology has not been a major problem for drug development to date, in this laboratory, we continue to use species-specific oligonucleotides to test for this phenomenon.

B. The Luck of the Draw: Toxicity Resulting from Hybridization to Unintended mRNA Targets

The reported absence of toxicity resulting from exaggerated pharmacology is mostly the result of the initial selection of the mRNA target. While it is possible to select targets carefully, it is not possible to predict with 100% certainty that an antisense sequence will not have a target sequence match somewhere else in the expressed genome. As stated above, all of the oligonucleotides tested to date in subchronic toxicity assays have had remarkably similar patterns of toxicity indicating that there were no sequence-dependent toxicities. Thus, there are no

examples of toxicity resulting from unintended hybridizations, and the concept of unintended hybridization remains more a theoretical possibility rather than a reality.

Existing data support the hypothesis that such unintended hybridization is a remote possibility. The first piece of information to support this hypothesis is that the occurrence of an exact match for any given 20-base nucleotide is roughly 4^{20} (10^{12}) and the number of base pairs in the human genome is 4×10^9. Therefore, there is less than one chance in a thousand that another exact match would exist. Thus, the occurrence of an exact match is a relatively rare event. This calculation makes a relatively naïve assumption that bases are completely random in their distribution: an oversimplification, but it does point out the rarity of randomly finding an exact match for a 20-mer. As the oligonucleotide is metabolized and the length of the antisense compound decreases, there is an increasing probability that an exact match can be found. However, balancing this loss of uniqueness is a reduction in affinity as length decreases (12). In addition to the actual existence of a matching sequence, there are three additional conditions that all *must* be satisfied to have hybridization-dependent toxicity with an unintended target.

1. The oligonucleotide must hybridize with the target mRNA. The simple existence of a perfect match in the genome is not sufficient for an antisense effect. Messenger RNA is a highly structured oligomer, and structure inhibits the hybridization and ultimately the activity of antisense oligonucleotides (13,14). This was demonstrated recently by creating target sequences with differing levels of structure. When the target sequence was unstructured, the EC_{50} for inhibition of report gene construct containing the target was <75 nM. When the target sequence was completely embedded in a stem-loop structure, there was little or no inhibition of the reporter gene expression at concentrations up to 300 nM. When the target sequence was partially embedded in a stem (10 base pairs), the EC_{50} was in greater than 300 nM (15). These data demonstrate the sensitivity antisense oligonucleotides to structure. In practice, identification of oligonucleotides with antisense activity requires screening dozens of oligonucleotides, each with a perfect match to some portion of a target mRNA to identify an oligonucleotide that has activity (16). This empirical selection process typically yields only about 10–20% of selected oligonucleotides that have desired activity. The data from Vickers et al. provide support for the concept that mRNA is discriminating in its binding and that not all perfectly matched oligonucleotide sequences will bind and have activity.

2. The second criterion for antisense activity is that the mRNA must be expressed in the organism at the time of exposure to the antisense compound. Finding a matching or near-matching mRNA sequence in a sequence database is of theoretical interest, but for unintended hybridization to play a role in toxicity, its mRNA must be expressed. Each cell contains a full copy of the genetic material, but obviously there is differential expression of the genes based on the partic-

ular tissues or expressed in response to a specific stimuli. For there to be a toxicity resulting from knockdown of an inappropriate gene target, the gene target must be expressed as an mRNA, not just be a sequence in the genome.

3. Finally, if these conditions are met, to produce toxicity the nontarget mRNA needs to be a critical gene product for toxicity, for example, a gene that is critical for cell survival and not part of one of the many redundancies in biological systems.

It is going to be a rare set of circumstances when all these factors align, and thus, the chances of toxicity of an antisense oligonucleotide producing toxicity through hybridization to an unintended target are remote. The chances are even diminished further by factors such as the dose delivered to the cells. For example: cells expressing the unintended target may receive less of the dose than the intended target cells. Despite all these mitigating factors, toxicologists working on antisense compounds should continue to screen the existing sequence databanks for potential matches and continue to monitor for unexpected toxicological phenomena. In the future, as DNA arrays improve in their reproducibility and our ability to interpret the information from them improves, it may be possible to detect unintended reductions in mRNA expression even in the absence of gross biological consequences. For the present, detecting these events is next to impossible and toxicity from this phenomena appears to be an exceedingly rare event.

C. Bits and Pieces: Metabolism and Toxicity

The breakdown and release of metabolites is another proposed mechanism of toxicity for antisense oligonucleotides. Phosphorothioate oligonucleotides, like the endogenous oligonucleotides, are degraded by nucleases, which liberate mononucleotides (Fig. 1) (17–19). It appears that 3′-5′ exonucleases predominate in plasma and tissues, but in tissues there is evidence for 5′-3′ nucleases as well (20). For a phosphorothioate oligonucleotide, exonuclease activity at the 3′ end yields an oligonucleotide shortened by one unit, and a thiophosphate mononucleotide.

Cleavage from the 5′ end yields a shortened oligonucleotide and mononucleoside. In each of these reactions there is the release of a nucleotide or nucleoside that can potentially alter the nucleotide pools of target cells or, in the case of monothiophosphate nucleotides, compete with endogenous nucleotides in enzymatic processes like oligonucleotide synthesis, phosphorylation, or catabolic processes.

Assuming the newly released nucleotide follows the normal catabolic route, we can infer that the nucleotide liberated from the 3′ end would be catabolized by the removal of the thiophosphate leaving the nucleoside. The thiophosphate would then be diluted in the vast compartment of phosphate pools or spontaneously oxidized to phosphate with the liberation of sulfate. The nucleoside could

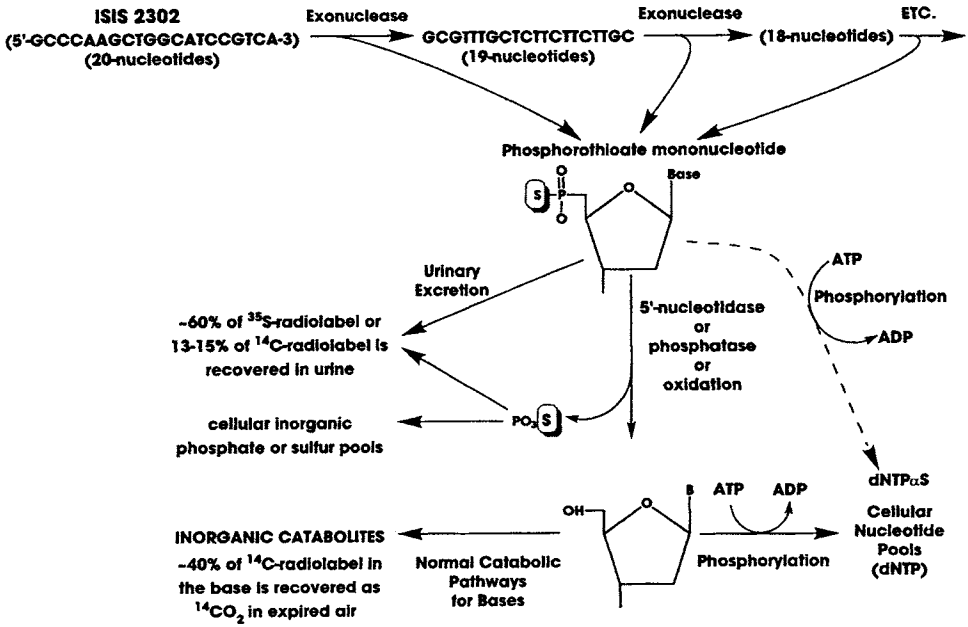

Figure 1 Scheme depicting the potential metabolic fate of a representative phosphorothioate oligodeoxynucleotide and its metabolites.

then be a substrate for a nucleoside phosphorylase yielding the ribose sugar and the base that can ultimately be metabolized to urea or CO_2 depending on whether it is a purine or pyrimidine. In a phosphorothioate oligonucleotide labeled at the C-2 position of thymidine, 50% or greater of the radiolabel is detected as CO_2, suggesting that at least half of the molecule is catabolized by normal pathways to yield innocuous inorganic metabolites (21,22). For each $^{14}CO_2$ liberated from a thymine there is the release of a thiophosphate moiety. When a phosphorothioate oligonucleotide is radiolabeled with ^{35}S in the thioate linkage, more than half of the radiolabel is detected in urine as small-molecular-weight metabolites. These data are consistent with the ^{14}C data, suggesting that the ultimate fate of the sulfur is in small-molecular-weight fractions like thiophosphates, sulfate, or mono- or dideoxynucleotides (Leeds and Levin, unpublished observation). These metabolites are innocuous and only through massive overdosing or very rapid metabolism could high levels of these innocuous metabolites produce chemical imbalances or toxicity. Given the slow rate of exonuclease metabolism of phosphorothioate oligodeoxynucleotides in tissues, accumulation of these metabolites is unlikely.

There are other possible metabolic pathways for the thiophosphate mononucleotides that are released by exonuclease cleavage. One pathway would be oxidation of the thiophosphate moiety to phosphate liberating sulfate and the normal nucleoside. We know that oxidation of the phosphorothioate is a common reaction that occurs even under storage conditions (Isis Pharmaceuticals, unpublished data). Alternatively, the thiophosphate mononucleotide could be dephosphorylated by the ubiquitous phosphatases, releasing thiophosphate whose fate was described above.

The last possible pathway for the thiophosphate mononucleotide could be into the nucleotide mono-, di-, and triphosphate pools. Thiophosphate mononucleotides are substrates for various kinases, and thus can be incorporated in these pools, but they are generally not as good a substrate as the phosphodiester. After additions of phosphates, the nucleotide thiotriphosphate (dNTPαS) could be incorporated in nucleic acids. However, the incorporation is stereospecific and only the S form of the thiotriphosphate is a substrate for the polymerases (23,24). There are at least two potential consequences of this incorporation. First, the thioate is oxidized and sulfate is released. Second, the molecule is stable and hybridizes in a sequence-dependent manner with the appropriate complementary nucleotide on the opposite strand. The basic premise behind antisense oligonucleotides is that they bind with high fidelity to their complementary sequences; thus endogenous DNA with the incorporation of thiophosphate containing nucleotide would bind with the appropriate high fidelity.

In vitro systems where cells can be exposed to relatively high concentrations of oligonucleotides (or their metabolites) and where it is possible to assess cell viability, energy charge, or nucleotide pools directly are useful for addressing potential consequences of metabolite exposure. In general, oligonucleotides are not cytotoxic either immediately or with prolonged incubations. Typically, the IC_{50}s for cell growth or viability have generally been >100 μM (25). Because the in vitro cytotoxicity of these compounds is low (25,26), it is highly unlikely that the (dNTPαS) are interfering with energy storage or production by altering ATP levels or regulation. To test for direct effects on DNA, RNA, and protein synthesis, incorporation of thymidine, uridine, and leucine was measured radiochemically in HeLa cells treated with concentrations of a phosphorothioate oligonucleotide at concentrations as high as 100 μM for 12–24 h. After 12 h, there was a slight reduction in thymidine and leucine incorporation at a concentration of 100 μM. After 24 h of incubation with the phosphorothioate oligonucleotide there were modest reductions (15–25%) in the incorporation of thymidine, uridine, and leucine at 100 μM, but only the thymidine incorporation showed any evidence of a concentration response relationship (27).

The only suggestion of toxicity resulting from mononucleotide metabolites was in a series of experiments performed in with BV133 cells or CD^{4+} bone marrow cells. In these experiments, there were marked reductions in cell growth

that appeared to correlate with the release of thymine, guanine, and adenine from the 3′ end of either phosphodiester or phosphorothioate oligonucleotides, and not an antisense effect. Notably cytosine was reportedly without growth-inhibitory effects. The hypothesis that release of mononucleotides was responsible for growth inhibition was supported by the fact that there was an increase in the inhibitory effects of oligonucleotides with increased concentrations of fetal calf serum or normal human serum. Heat treatment of the serum presumably to inactive nucleases diminished the effect. Inhibitory effects on cell growth could be reproduced by incubating with 10–100-μM concentrations of d-TMP, d-AMP, and d-GMP but not d-CMP (28). However, the hypothesis fails to explain why easily digested phosphodiester oligonucleotides (with a relatively equal distribution of bases) are so dependent on the identity of the first three nucleotides. These compounds are very labile in serum and complete degradation and release of all four nucleotides would have been expected over the time frame of the incubation. Thus, there are questions as to the nature of the observations reported.

Furthermore, this potential for inhibiting hematopoietic cell growth is not consistent with results from in vivo studies. Systemic administration of phosphorothioate oligonucleotides at the LD_{50} (>750 mg/kg) have not produced inhibitory effects on bone marrow cellularity nor have we observed changes in the polychromatic erythroblast to normochromatic erythroblast ratios in mouse micronucleus tests. After long-term exposures of mice or monkeys in general toxicity studies, we have not observed anemias or any indications in changes in bone marrow cellularity other than those induced by the immune stimulation associated with this class (vide infra). In vivo experiments have directly demonstrated that the marrow was exposed to both intact oligonucleotide and metabolites. Significant alterations in nucleotide pools, either increases or decreases, would also be associated with infidelities in DNA replication (29); however, no such changes have been detected in in vitro or in vivo genetic toxicity assays. Thus, the cytostatic effects of phosphorothioate oligonucleotides observed at high concentrations in vitro may be due to accumulation of nucleosides or nucleotides, but there is no evidence that nucleotide pools are altered and the effects are not seen in vivo.

One of the modifications that has been proposed for use in human clinical trials is the addition of a methoxyethoxy group (MOE) to the 2′ position of some or all of the nucleotides in an antisense oligonucleotide. This modification produces an extremely durable oligonucleotide that is very resistant to nuclease degradation. However, it was of interest to understand whether the release of mononucleotides with this modification was associated with toxicity. To assess the toxic potential of MOE-modified nucleosides, a mixture of mononucleosides was administered to mice in a rising-dose tolerance test with 300 mg/kg as the highest dose. Each step in the progression from 10, 30, 100, to 300 mg/kg was administered for 4 days. The distribution of the four nucleosides in the dose was equiva-

lent on the basis of weight. There were no significant findings. A second group of mice were treated for 28 days with 300 mg/kg of the mixture of nucleosides. No changes in clinical signs, body weight, histology, serum biochemistry, or hematology were associated with treatment (S. Courtney, Ciba Geigy, internal report). The doses used in these studies far exceed the concentrations of nucleotides or nucleosides that are released from the slow metabolism of this new class of antisense oligonucleotides. Thus, these data support the concept that mononucleosides released from the metabolism of modified oligonucleotides have a very low order of toxicity.

D. Nonspecific Protein Binding: Class-Related Toxicities

The toxicities that have been the most prominent in the development of antisense oligonucleotides appear to all have been related to the chemical class and have, for the most part, been independent of sequence and, therefore, independent of hybridization. The most compelling evidence that the toxicities observed with phosphorothioate oligonucleotides are related to chemical class, and not sequence, is the fact that the patterns of toxicity are remarkably similar from sequence to sequence. While there are differences in the responses of different species, within each species there is a remarkable consistency in the response of that species to oligonucleotide treatment. The qualitative features of the toxicities observed have been reproduced time and again for nearly a dozen phosphorothioate oligonucleotides described in the literature or submitted to regulatory agencies (2,8,30,31).

More quantitative assessments of the toxicity of some oligonucleotides have revealed differences between sequences, and it is possible to demonstrate that some of the characteristic toxicities of this class are exacerbated by particular sequence motifs (32). Most or all of these differences can be explained on the basis of differences in the immunostimulatory potential of these compounds in rodents, a well-known class effect that has been demonstrated to be dependent on sequence, but independent of hybridization. The phenomenon of immune stimulation, which is part of the characteristic response of rodents to exposure to phosphorothioate oligonucleotides, is discussed in Section I.D.2 where a more thorough explanation of the role of sequence in this particular biological activity of oligonucleotides is provided. Toxicities common to these compounds are organized by organ system, and the discussion is supplemented by results from mechanistic studies performed to help explain the relationship to exposure and species sensitivity.

The greatest amount of toxicity information available has been collected for the phosphorothioate oligonucleotides. There are "points to consider" documents from the Food and Drug Administration (33–35) that have recommended the design and content of toxicity studies intended to support therapeutic applica-

tion of phosphorothioate oligonucleotides, including evaluation in nonhuman primates and rodents. The reliance on nonhuman primates may be waning slightly, in light of the greater understanding of species differences in response, and as additional routes of administration or new disease indications are targeted.

The toxicity and pharmacokinetic evaluations of some of these newer therapeutic approaches are better evaluated in other species. For example, as we move toward topical therapeutics or orally administered formulations the reliance on primate data has shifted toward porcine and canine models. While there has yet to be a full characterization of the systemic toxicity of an antisense compound in dogs or pigs, it is useful to report that the plasma pharmacokinetics are very similar to those in other species, including humans, and tissue distribution appears to be similar as well. Because most of the toxicities of antisense oligonucleotides are observed in organs that accumulate the most oligonucleotide, we predict that the systemic toxicity profile in these species would resemble the toxicities already described, but proof awaits the first full-scale toxicity studies in these species.

Species specificity is also an important component in regard to understanding the relevance of toxicity study to therapeutic application. Rodents (and to a lesser extent lagomorphs) respond to treatment with phosphorothioate oligonucleotides with a constellation of effects related to immunostimulation. These effects have been reviewed elsewhere (2,36), but some review here is useful to fully outline what is known about species differences. The response of primates to treatment with phosphorothioate oligonucleotides differs from that of the rodent. Immunostimulation appears to play a minor role in toxicity and the dose-limiting toxicities are generally the acute toxicities as discussed below.

1. Hemostasis: Acute and Transient Toxicities

The acute toxicities of phosphorothioate oligonucleotides have been of considerable concern in the development of antisense compounds. While these compounds are not particularly potent acute toxicants in rodents, in nonhuman primates the situation is much different. Acute toxicities have been problematic and have influenced the manner in which these compounds are administered in clinical trials.

In rodents, doses of phosphorothioate oligonucleotides required to produce acute toxicity are in excess of 750 mg/kg. This figure was obtained for different sequences each targeting a different mRNA. There was only a small degree of variability in the estimated LD_{50} values from sequence to sequence, indicating that the LD_{50} in mice is independent of sequence. With relatively high LD_{50} values, these compounds appeared to have a low potential to induce acute toxicity in mice. There are no obvious causes of lethality in the mice in these studies.

In nonhuman primates there is a much different sensitivity to acute toxicity. Acute dose-limiting toxicities include a transient inhibition of the clotting cascade

and the activation of the complement cascade (37–41). Both of these toxicities are thought to be related to the polyanionic nature of the molecules and the binding of these compounds to specific protein factors in plasma. Inhibition of clotting times is not unique to monkeys, and has been observed in other species including rats (38). Inhibition of clotting times also likely occurs in mice, but has not been studied owing to the limited amount of blood available in mice. By contrast, complement activation appears unique to monkeys. No evidence of complement activation has been observed in mice, rats, or dogs.

Complement Activation. Rapid infusion or injection of phosphorothioate oligonucleotides in monkeys is associated with a low incidence of cardiovascular collapse and an anaphylactic-like response (37,39,42). Underlying the variable hemodynamic changes is a consistent pattern of alterations in hematology and serum biochemistry indicative of alternative-pathway complement activation (39). Hematological changes include transient reductions in neutrophil counts presumably due to margination, followed by neutrophilia and an increase in the number of band cells when immature cells are recruited. Such fluctuations in neutrophil counts are indicative of complement activation, which was confirmed by measurement of increases in complement split products. In fact, neutropenia coincided with peak levels of C5a split product. Hemodynamic changes characterized by transient hypertension during peak C5a exposure followed by hypotension and subsequently decreased cardiac output occurred infrequently, but in some animals, these hemodynamic changes can be lethal (37,39,42). Histological examination of these animals yielded no evidence of direct toxicity to the cardiovascular system, suggesting that the source of the toxicity was not a target organ effect, but rather some physiological phenomenon. Although the mediators of hemodynamic changes have not yet been identified, complement activation is associated with cellular activation and subsequent autocoid release could explain the observed changes in vascular permeability and tone. Further studies in primates have demonstrated that cardiovascular collapse was always associated with complement activation, such that treated monkeys demonstrating some degree of cardiovascular collapse or hemodynamic changes had markedly elevated levels of complement split products. Complement activation was necessary, but not sufficient to explain cardiovascular collapse; only a fraction of the animals with activated complement had cardiovascular functional changes. This finding suggested that there might be sensitive subpopulations or predisposing factors within individual animals that make them susceptible to the physiological sequelae of complement activation. Other mechanisms for observed hemodynamic changes have been proposed (43), but are not fully consistent with the clinical manifestations in monkeys and have not been borne out by the data.

As described previously, complement activation is independent of sequence and has been a consistent effect of several different phosphorothioate oligodeoxy-

nucleotides (Fig. 2). The monkey was assumed to be a good predictor for humans, and as a result, studies in primates have become commonplace for the assessment of toxicity with antisense oligonucleotides (33,34). Because of the life-threatening nature of this toxicity, considerable research has been performed to establish concentration-response relationships. This has led to characterization of a plasma threshold concentration required for complement activation, which has remained remarkably consistent over time and between different oligonucleotides. Complement is activated only at concentrations of phosphorothioate oligodeoxynucleotides that exceed a threshold value of 40–50 μg/mL. Bb levels remain unchanged from control values when plasma concentrations remain below the threshold. Similar if not identical threshold responses have been observed for five independent (Fig. 2), 20-mer phosphorothioate oligodeoxynucleotides and for an 8-mer phosphorothioate oligodeoxynucleotide that forms a tetrad complex (ISIS 5320, data not shown). For all of these compounds, when concentrations exceeded the threshold values there was a rapid and marked rise in Bb, indicative of complement activation. One compound, ISIS 14803, had a single data point that showed a rise before the typical threshold of 40–50 μg/mL concentration, but outside of that single point for all of the tested compounds the threshold has been constant.

This threshold information in monkeys has been used effectively to avoid complement activation in patients. Dose regimens used in clinical studies have extended the infusion duration to maintain a safety margin below the threshold for activation. Using these assumptions, there has been no meaningful increase in complement split products following 2-h intravenous infusions during clinical studies with phosphorothioate oligonucleotides performed by Isis Pharmaceuticals, although at low infusion rates there may be some increase in complement. The threshold determined is applicable for 2-h infusions. Longer and slower infusion may have different responses. In fact, in vitro data appear to predict these differences (see below).

Definitive proof that complement was the critical factor in the acute toxicities has been established using a recombinant inhibitor of complement activation (Table 2). Complement activation blocker-2 (CAB-2) is a chimeric protein comprised of Factor I cofactor protein and decay accelerating factor (44). Together these complement regulatory proteins inactivate the C3/C5 convertase, and thereby block production of the anaphylatoxins C3a and C5a. The ability of CAB-2 to block both alternative and classic pathway activation has been confirmed in vitro and in vivo, and the fusion protein is more potent than either of the complement regulatory proteins alone (44). Using a crossover design, three monkeys were treated with 20 mg/kg of ISIS 2302 in a 10-min infusion with or without pretreatment with CAB-2 at 13 mg/kg. Infusions of ISIS 2302 alone produced a pronounced activation of the alternative pathway of complement as indicated by the pattern of increases in complement split products with characteristic increases in Bb, C3a, and C5a. Mean plasma concentrations of Bb at the end of

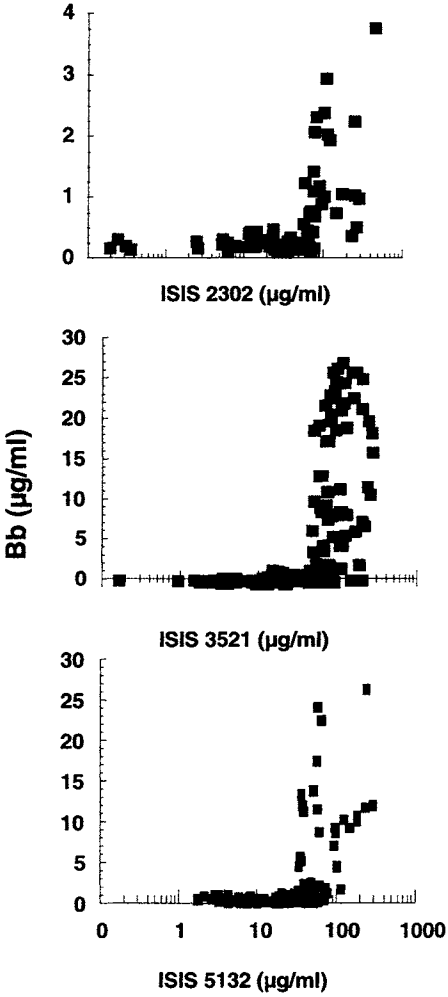

Figure 2 Plasma concentration of oligodeoxynucleotide versus Bb in cynomolgus monkeys treated with various regimens of ISIS 2302, ISIS 3521, or ISIS 5132. Concentrations in plasma samples collected during intravenous infusion were plotted against the Bb concentrations for each point. (Reproduced from Ref. 31 with the permission of *Anticancer Drug Design*.)

Table 2 Qualitative Representation of the Physiological Response to Rapid Intravenous Infusion of 20 mg/kg ISIS 2302 in Rhesus Monkeys in the Presence and Absence of an Inhibitor of Complement Activation

	ISIS 2302 (20 mg/kg)	
Parameter	None	+CAB-2
Lethargy	Moderate	NC
Emesis	Moderate	Mild
Periorbital edema	Moderate	NC
Hemodynamic change	Hyper/hypotension	NC
Neutrophil abnormality	Fluctuating count	NC
Complement split products	Bb, C3a, and C5a	Minimal
Cytokine	IL-6, MCP-1	NC

NC, no change.

infusion were 100-fold greater than baseline, and C3a and C5a were also markedly increased. Implanted telemetry devices, used for monitoring heart rate and mean arterial pressure, demonstrated that there were marked fluctuations in circulatory status consistent with the patterns seen in earlier experiments. One of three monkeys had acute cardiovascular collapse and died 3 h after treatment. Crossover treatment consisted of predosing with CAB-2 followed by the same ISIS 2302 treatment regimen. In the crossover group with CAB-2 treatment there was little or no liberation of complement split products and there were none of the typical hemodynamic or hematological changes or IL-6 elevations normally associated with administration of high doses of oligonucleotide (Fig. 3) (116). These data demonstrate that the anaphylactic-like response and the cardiovascular sequelae are all secondary to complement activation. These data also validate results from a previous study in which monkeys receiving a similar dose of oligonucleotide were largely protected from hemodynamic and hematological changes induced by complement activation by prior treatment with cobra venom factor to deplete the complement system.

The assumption that monkeys are representative of complement activation in humans is appropriately conservative for the purpose of safety assessments, but it should be noted that monkeys are uniquely sensitive to complement activation among the animal species examined. Dogs, guinea pigs, rabbits, rats, and mice have all been dosed at high enough doses to produce plasma levels in excess of the plasma threshold for complement. Never has there been observation of an anaphylactic-like response in any species other than monkey. This is particularly remarkable for species such as dogs and guinea pigs, which are traditionally sensitive to such effects. In dogs, CH_{50} measurement was directly assessed fol-

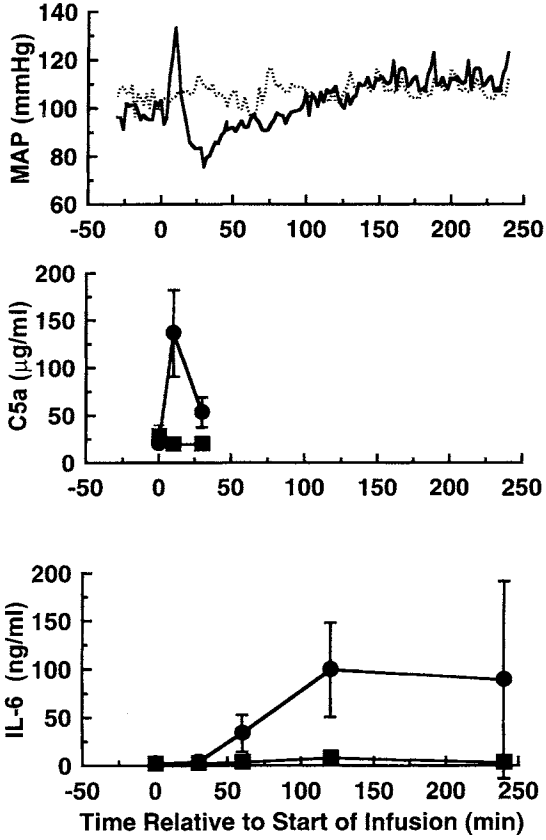

Figure 3 Effect of 20 mg/kg ISIS 2302 10-min infusion on mean arterial pressure (top), complement activation (middle), and cytokine production (bottom) in the presence or absence of CAB-2. Mean arterial pressure is presented as the group mean of three animals following ISIS 2302 only (solid line) or in the presence of CAB-2 (dotted line). Complement C5a and IL-6 cytokine effects are also presented as group mean values for ISIS 2302 only (●) or ISIS 2302 plus CAB-2 (■).

lowing intravenous 2-min infusion of 10 and 20 mg/kg ISIS 2105. While plasma oligonucleotide concentrations far exceeded the threshold for activation established in monkeys, no reduction in CH_{50} was observed (117). Since dose regimens have not exceeded activation threshold in patients, and no consistent evidence of complement activation has been observed, it is impossible to say whether humans would be equally sensitive or less sensitive than monkeys. (Data from

clinical studies obtained in rising-dose tolerance tests provided data to suggest that humans were not remarkably sensitive to complement activation.)

The characteristics of complement activation between monkey and human have been compared using an in vitro system for activation in serum. There were notable differences between the responses of monkey and human serum. These differences can be best characterized by comparing the concentration responses between monkey and human serum (Fig. 4). In human serum, there were minimal increases in C3a and Bb at concentrations of 25 or 50 μg/ml, but at concentrations ≥100 μg/ml, there was no consistent increase in split product formation, and often there was even a decrease relative to baseline values. In contrast, complement split products in monkey serum appeared to increase with increasing concentration and the response only began to taper off as concentrations increased >500 μg/ml. The concentration response profile in human serum could be explained by a biphasic response: very weak activation at low concentration and inhibition of complement activation at higher concentrations. This hypothesis was tested in human serum by adding exogenous complement activators like zymosan or cobra venom factor in the presence of varying concentrations of ISIS 2302. As predicted, the exogenous activation of the complement cascade was inhibited by ISIS 2302 in human serum, but there was no inhibition in monkey serum and in fact there was additive activation. Thus, it appears that in human serum, activation of complement differs significantly from that in monkey serum.

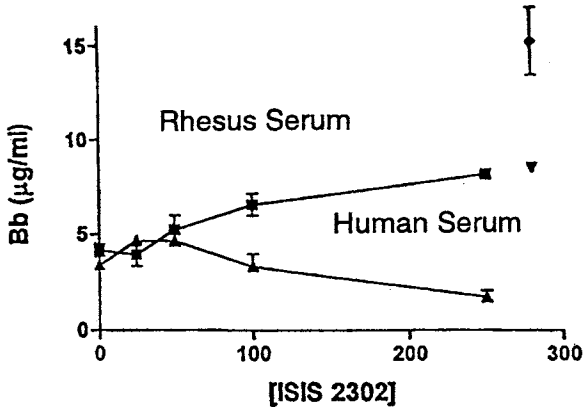

Figure 4 Comparison of oligonucleotide-induced complement activation in rhesus monkey and human serum. Bb is the complement split product specific to alternative pathway. The concentrations of Bb resulting from full activation of alternative pathway using zymosan are presented for monkey (▼) and human (■) serum.

The cascade in human serum is much more sensitive to inhibition compared to activation. These data appear to indicate that the extent of complement activation in humans may be reduced relative to monkeys. However, the study does provide some data suggesting that long low-dose exposure to phosphorothioate oligonucleotides might be able to produce a low level of activation that will over the course of days or weeks result in some elevation. Thus in vitro studies indicate that complement activation will *not* be completely absent in patients, and low levels of activation are possible. Although complement activation has not been observed in dose regimens using 2-h infusion, it is conceivable that activation might occur when low concentrations of oligonucleotide are sustained for longer periods, thus allowing for amplification of the cascade.

The ability to activate complement in monkey plasma in vitro has also facilitated a better understanding of the mechanism for activation. The mechanism of complement activation is now known to be related to an inhibition of Factor H, one of the factors that inhibits the constitutive activity of the alternative cascade (117). In this pathway a low level of constitutive activity is controlled by circulating negative regulatory proteins, Factor H and Factor I. Treatment of cynomolgus monkeys with ISIS 2302 results in an apparent reduction in circulating Factor H, which might allow uncontrolled amplification of this pathway (39). Other polyanions, such as heparin and dextran sulfate, have been reported to activate the alternative pathway in vitro through an interaction with Factor H (45). Increasing concentrations of oligonucleotide in isolated serum also appeared to reduce concentrations of Factor H. Based on the nature of the immunobinding assay, these results are now interpreted as an ability of the oligonucleotide to bind Factor H and prevent interaction with the antibody. There is high binding affinity between Factor H and the oligonucleotide, and mechanistic studies further implicated Factor H interaction in complement activation, by its ability to block activation at very low concentrations (117). Sequestering or reducing the activity of the regulatory factors like Factor H would allow the normally low constitutive activity of the alternative complement cascade to run unchecked.

One issue related to complement activation that is not fully understood is the variability in incidence and severity of the clinical manifestations in monkeys. We now have a very strong correlation between complement activation and secondary anaphylactic-like response, but it has been difficult to predict whether individual animals will experience clinical sign or cardiovascular collapse. Over the course of conducting many repeat-dose studies in monkeys with several different oligonucleotides, it is our experience that certain variability in the baseline physiological state of an animal can influence the susceptibility to an anaphylactic-like reaction. One of the contributing factors is stress.

There is a greater incidence of anaphylactic-like reactions in animals being restrained during a 2-h infusion as compared to animals treated with a slow bolus

administration or those infused remotely. Cynomolgus monkeys could easily tolerate intravenous administration of bolus injections of 10 and 50 mg/kg on alternate days for a month with no indication of cardiovascular collapse and only a few incidences of transient lethargy were noted after the first dose. The high dose would be expected to produce plasma concentrations that transiently exceed 500 μg/ml, yet no gross cardiovascular sequelae were observed (5). By comparison, lethargy and mortality were observed more frequently when conscious animals were restrained to receive longer infusion of 10 mg/kg (39). The stress effect in restrained monkeys is evident by a corticosteroid-mediated increase in neutrophil count during restraint (46). Presumably, the presence of an increased number of activated neutrophils at the time of complement activation can exacerbate downstream consequences. Obviously the stress effect can vary between animals, and thus contribute to the interanimal variability. Further evidence of a stress effect in restrained animals is that cardiovascular collapse has been observed in a control monkey after a 2-h constant infusion of saline in the (verified) absence of any drug exposure (Templin, Henry, and Levin, unpublished observations). It should also be mentioned that hemodynamic data derived from anesthetized animals should be interpreted with caution because of the potential influences on compensatory mechanisms. These observations are not intended to decrease the significance of complement activation, nor to suggest that eliminating outside influences will eliminate all of the complications of complement activation. However, more recent studies in monkeys have attempted to eliminate stress associated with infusions by employing self-contained wearable infusion pumps.

Ultimately, future generations of antisense oligonucleotides will attempt to avoid complement activation altogether. One way to avoid complement activation is to shield the oligonucleotide from plasma proteins, and thereby reduce the potential to interact with Factor H or other critical regulatory pathways. Using liposomal encapsulation, it was possible to give very rapid infusions (50 mg/kg over 30 min) of ISIS 2503 without significant complement activation. In contrast, unencapsulated ISIS 2503 at much lower doses and slower infusion rates produced clear increases in complement split products. Liposomal encapsulation of oligonucleotides seems to be one way to prolong plasma half-life and reduce toxicity. The acute toxicity of liposomal formulations may be improved over ''naked'' oligonucleotides, but their use in therapeutics is ultimately dependent on whether the disposition of the oligonucleotide is improved with respect to delivery to the target organs for therapeutic activity.

While modifying the delivery vehicle is one way to reduce toxicity, it is also possible to modify the oligonucleotide itself, and thereby, reduce the potential to activate complement. Chemical modifications of antisense oligonucleotides can be made that reduce their potential to activate complement. Reducing the number of thioate linkages is one way. Ar177 is a phosphodiester oligonucleotide that has phosphorothioate caps on the 3′ and 5′ termini (41,47). This oligodeoxy-

nucleotide is known to have a complex secondary or tertiary structure. Infusion over 10 min with up to 20 mg/kg produced only a very minor increase in plasma concentrations of Bb. The magnitude of the increase was less than a doubling of the values (41). A full phosphorothioate oligodeoxynucleotide of the same length would be expected to produce increases in Bb on the order of 10- or more fold over baseline values. Antisense oligonucleotides with morpholino modifications, which are uncharged, can also be infused at relatively high doses without apparent adverse effects or activation of the complement cascade (P. Iversen, personal communication).

To date, the data appear to support the hypothesis that modifications that tend to reduce plasma protein binding diminish the potential to activate complement. The establishment of an in vitro assay for complement activation has provided us with a tool for assessing the effects of chemical modifications on complement activation. Phosphorothioate oligonucleotides modified in the 2′ position with methoxyethyl groups appear to be less activating in human serum than the unmodified compounds of the first generation (117). Using compounds with a mixed phosphodiester/phosphorothioate backbone also confirms the hypothesis that reducing the number of thioate substitutions diminishes the potential for activating complement. When compounds modified at the 2′ position with fully thioate or mixed thioate/diester linkages were infused into monkeys, the results confirmed what had been observed in vitro: a reduction in complement activation.

Complement activation remains the most critical toxicity to avoid when administering polyanionic molecules like many of the antisense oligonucleotides. However, recent data from mechanistic studies, as well as the data from newer, more highly modified compounds, suggest that monkey data may overpredict the sensitivity of humans to some degree and that through modifications to the chemical nature of the oligonucleotide it is possible to reduce the liability of complement activation even further.

Inhibition of the Coagulation Cascade. The other acute toxicity of phosphorothioate oligonucleotides is prolongation of clotting times (2). Like complement this toxicity has been well characterized, but like complement activation, effects on clotting pathways are not species-specific, and are observed in all species studies, including humans. A number of different phosphorothioate oligodeoxynucleotides have been shown to alter the clotting cascade as indicated by a concentration-dependent prolongation of activated partial thromboplastin times (APTT) (38,41,48,49). In all studies in animals, APTT was prolonged more than prothrombin times (PT), indicating that the effect was more pronounced in the intrinsic pathway than the extrinsic pathway. Typically at the end of a 2-h constant IV infusion of 10 mg/kg in cynomolgus monkeys, there is roughly a 50% increase in APTT and a less than 20% increase in PT. Like the activation of the complement cascade, prolongation of the intrinsic arm of the clotting cascade is

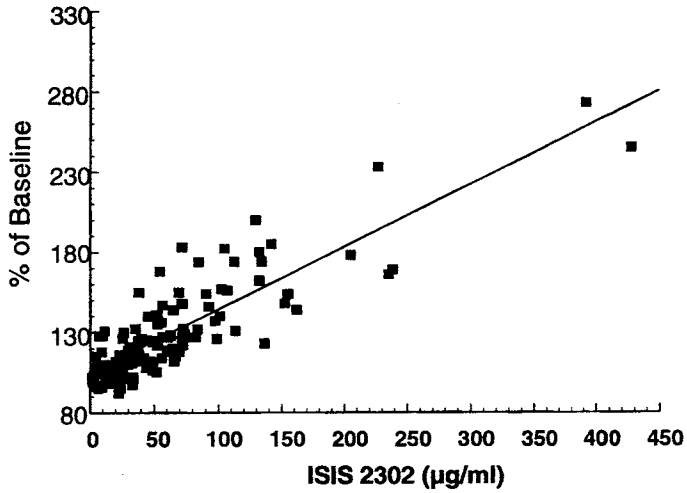

Figure 5 Concentrations of ISIS 2302 in monkey plasma versus APTT. APTT values are expressed as a percent change from baseline. Monkeys received infusions of various doses of ISIS 2302 and the concentration data obtained by CGE were plotted against the APTT values.

associated with peak plasma concentrations and is readily reversible. In both nonclinical and clinical studies with phosphorothioate oligonucleotides, the magnitude of the prolongation is directly and linearly proportional to the concentration of phosphorothioate oligonucleotide (38). When plasma concentration of phosphorothioate oligonucleotide is plotted versus prolongation of APTT, a straight line is obtained (Figs. 5 and 6) (50). The prolongation of APTT is also directly proportional to the length of the oligonucleotide. The longer the sequence, the greater the prolongation (2). There are some species differences in the inhibitory effects of phosphorothioate oligonucleotides. Monkeys appear to be slightly less sensitive to the inhibitory effects than humans (2). In both species (and probably others), these effects are transient and readily reversible. The readily reversible nature of the effects is apparent in the way that prolongations of APTT parallel the plasma concentrations of the drug, rising and falling in concert.

The similarities between phosphorothioate oligonucleotides, and other polyanions like heparin, with respect to protein binding are striking. The physicochemical nature of the antisense oligonucleotides suggested that interactions with one of the binding sites of thrombin could be a mechanism for clotting inhibition (48). However, analysis of the concentration-response relationship demonstrates that the concentration required to produce thrombin inhibition was inconsistent with other data. The actions on thrombin occur largely at a concentration range

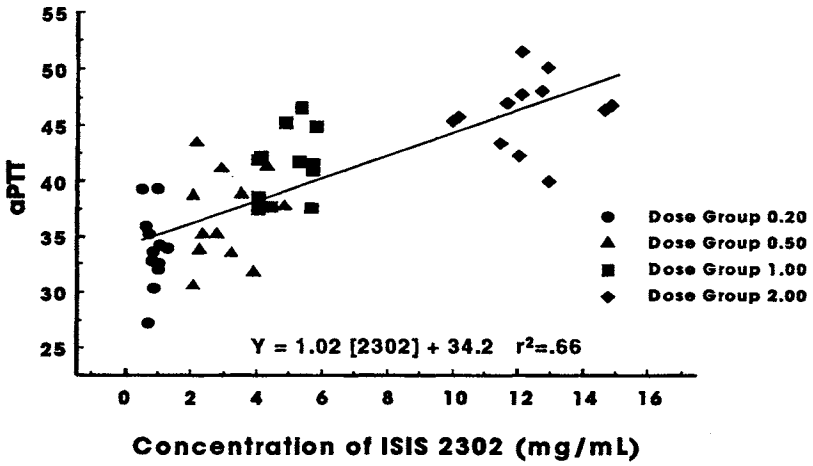

Figure 6 Concentrations of ISIS 2302 in human plasma versus APTT. Volunteers were treated with 2-h infusions of ISIS 2302 at 0.2, 0.5, 1.0, and 2.0 mg/kg. Plasma concentrations of ISIS 2302 are plotted against absolute values for APTT in seconds. The equation represents the best-fit line to the data.

in excess of the concentrations known to prolong APTT. By performing reconstitution experiments with plasma lacking specific intrinsic clotting factors in the presence of a phosphorothioate oligonucleotide, it was possible to determine which step in the cascade was inhibited by phosphorothioate oligonucleotide (40). Addition of Factor XIa, IXa, VIIIa, or Va failed to restore APTT to control levels when a phosphorothioate oligonucleotide was present. However, it was possible to reverse the inhibitory effects on APTT by adding activated Factor X, suggesting that the intrinsic tenase complex was the target for inhibition. Direct effects on the tenase complex were confirmed using direct enzymatic assays of tenase activity with a chromogenic substrate. In this assay a 50% inhibition in tenase activity was achieved at a concentration in the range of 5–10 μg/ml oligonucleotide. These results are highly consistent with magnitude and sensitivity of the inhibition observed in a plasma in vitro and results from clinical trials.

The nature of the interaction between phosphorothioate oligonucleotides and intrinsic tenase complex was further explored using enzyme kinetics. ISIS 2302 was demonstrated to be a noncompetitive inhibitor of Factor X activation. Factor X assembly, cofactor interactions, and catalytic activity were examined. There were no alterations in binding or activation of Factor X by Factor IXa–phospholipid complex. There was some inhibitory effect of ISIS 2302 on the Factor IXa–Factor VIIIa interaction, but the magnitude of the inhibition was not

sufficient to explain the inhibition in vivo. Factor X generation by Factor IXa was substantially inhibited when the Factor IXa complex was activated by ethylene glycol (118). The effect of APTT is probably the result of multiple interactions of the oligonucleotide with the cascade. There is the interaction directly with thrombin and there are interactions with Factor IXa and Factor VIIIa as well as the generation of active Factor Xa. Despite the potential multiplicity of interactions with the clotting cascade, the clinical consequence of the effect is easily limited by limiting plasma concentrations.

Infusion of various phosphorothioate oligonucleotides at 10 mg/kg or administration of slow bolus injections of 50 mg/kg have resulted in transient prolongation of APTT, but there has been no evidence of internal organ hemorrhage in any of these studies (5). Superficial bruising was observed at blood collection sites and areas of restraint in monkeys treated with 50 mg/kg. Even at subcutaneous injection sites where local concentrations can be expected to reach concentration of >500 μg/g, there have been no reports of local hemorrhage.

In clinical trials phosphorothioate oligonucleotides have been administered by intravenous infusions either over 40–120 min or continuously for 24 h. Transient elevations in APTT have been observed (51). As predicted from animal studies, the prolongation was most prominent in short infusions when plasma concentrations were highest (Shanahan, Isis Pharmaceuticals, unpublished observations). We have taken data from a Phase I study with human volunteers and plotted the increase in APTT as a function of plasma concentration. In this study, the relationship between plasma concentration (μg/ml) and APTT was linear with a slope of approximately 1 s increase in APTT per μg/mL of oligonucleotide in plasma. Thus, maintaining plasma concentrations below 30 μg/ml will insure that APTT is not doubled over baseline. Importantly, because the inhibitory effect is transient and plasma is cleared with a half-life of less than an hour, any prolongation of APTT will be short-lived. Thus, the clinical relevance of this potential toxicity is limited even for unmodified phosphorothioate oligonucleotides.

The potential for significant prolongation of coagulation cascade is reduced even further with modifications to the chemistry of the oligonucleotides. A modified version of the anti-HIV compound GEM91 with eight methyl phosphonate linkages substituted for phosphorothioate linkages had reduced potency for prolonging APTT compared to the fully thiated version (52). Morpholino linkages because of the reduction in overall charge would also be expected to produce fewer effects on the clotting cascade and this appears to be the case (Iversen, this book). As described above, reducing the number of thioate linkages either by truncating the oligonucleotide or by chemical modification ameliorates the inhibition of clotting.

From the point of view of reducing toxicity, it appears that reducing the number of phosphorothioate linkages is beneficial, but our present understanding of the pharmacokinetics of phosphorothioate oligonucleotides indicates that there

are also positive aspects to this chemistry, the most important of which is the affinity of this class of drugs for binding to plasma proteins. It is this protein binding that deters glomerular filtration and allows these compounds to bind to cellular proteins and transit cell membranes. Thus, while it is easy to replace phosphorothioate linkages with modifications, the key to these substitutions is to create new chemistries that bind to mRNA with high affinity, bind to plasma proteins with some affinity, and support the action of RNase H. To attain these criteria in a single chemistry while keeping toxicity low remains the challenge of chemists, and toxicologists.

2. Subchronic Toxicity: Organ System

The subchronic effects of oligonucleotides, particularly phosphorothioate oligodeoxynucleotides, have been similar for all of the compounds examined. These class-related effects are the result of accumulations of oligonucleotides in specific tissues like kidney and liver (Table 3). The one toxicity that varies with sequence and is not strictly correlated with organ accumulations is immune stimulation in rodents.

Immune System. Immune stimulation is the most extensively studied toxicity associated with administration of phosphorothioate oligodeoxynucleotides. As reviewed previously (2), exposure of rodents to phosphorothioate oligodeoxynucleotides produces a constellation of effects characterized by splenomegaly, lymphoid hyperplasia, and diffuse multiorgan mixed mononuclear cell infiltrates (Fig. 7). At doses that exceed 10 mg/kg there is a prominent infiltration of mixed mononuclear cells in liver, kidney, heart, lung, thymus, pancreas, and salivary glands among others (4,6,8,31). The lesions are reversible upon removal of treatment although full resolution of the cellular infiltrates can take weeks. These cellular infiltrates are not associated with any fibrosis nor architectural changes in the organs where they occur (6,8). Qualitatively the effects of immune stimulation are common from sequence to sequence, but potency varies considerably with sequence. For reasons described below, it is clear that immune stimulation is not an antisense effect and is categorized as a hybridization-independent effect.

Phosphorothioate oligodeoxynucleotides also induce immunostimulatory effects in isolated splenocytes as well. These experiments were performed using the classical mitogenic assay, measuring tritiated-thymidine incorporation in splenocytes. The availability of an in vitro model of immune stimulation has facilitated the characterization of structure-related activity, the species differences, as well as the delineation of mechanisms of the stimulatory effects.

From early experiments it was noted that incorporation of thymidine in splenocytes by oligonucleotides occurred in much the same way as that observed for bacterial DNA or sulfated polyanions (53–55). This was the first indication that immune stimulation was a polyanion effect as opposed to an antisense effect.

T cells proved to be nonresponsive to stimulation by phosphorothioate oligodeoxynucleotides, but T-cell-depleted or B-cell-enriched splenocyte fractions were responsive (56,57). This in vitro finding is consistent with the fact that B cells are a target for expansion as determined by histopathological examination of lymphoid tissue. Microscopically there are areas of apparent proliferation in B-cell centers (follicular hyperplasia) in lymphoid tissues from treated mice. Increases in histiocytes and stromal cells were equally prominent (58). Further characterization of the mitogenic response of B cells or splenocytes to treatment with stimulatory oligonucleotides revealed an increase in the secretion of IgG, IgM, and IgA, as would be expected in a mitogenic response. This stimulation of immunoglobulin production is polyclonal and has been observed both in vitro and in vivo with certain oligonucleotides (56,57,59,60). Similar immunoglobulinemia has been reported in treated mice for stimulatory sequences. There is no indication that the immunoglobulins secreted are specifically directed toward the oligodeoxynucleotide.

In vitro splenocyte cultures have also proved to be representative of treated animals with respect to stimulation of cytokine production. Both splenocytes and treated mice respond to oligonucleotide exposure with a Th1-type cytokine response.

Isolated splenocytes have also proved useful in the study of species sensitivity and sequence-dependent differences in potency as described below. However, the immunological reaction to phosphorothioate oligonucleotides is complex, and oversimplified in the context of this in vitro model. For example, quantitation of lymphocyte populations in spleens from treated mice using flow cytometry reveal an increase in B cells, but it appears that the greatest change in cell number is associated with increases in monocytic cells and endothelial cells (58,61). While not surprising, this observation is important to understand immune stimulation as more than a simple B-cell proliferative effect, but more accurately as a mitogenic effect toward a number of cell types and a change in cell migration.

While immune stimulation is a common property of phosphorothioate oligodeoxynucleotides, the dose-response relationship for this toxicity varies depending on the sequence (36) or the chemistry of the oligonucleotide (32,62). Using spleen weight as a marker of immunostimulation in mice, we have demonstrated that there is greater than a fivefold difference in the immunostimulatory potential of unmodified phosphorothioate oligonucleotides (Fig. 8) (36). Thus, it is inappropriate to generalize about dose-response relationships for these effects. The quantitative response rather than the qualitative response characterizes the sequence dependence. The observation of sequence-dependent potency of immunostimulatory effects of phosphorothioate oligodeoxynucleotides was reported independently from a number of different laboratories (59,63,64). Even for compounds like ISIS 2302 that are relatively weak immunostimulators the full spec-

Table 3 Organ/System Summary of Phosphorothioate Oligonucleotide Toxicities

Organ/system	Species	Finding
Bone/connective tissue	Mouse, rat, monkey	No significant findings
CNS/nervous	Mouse, rat, monkey	No significant findings
Cardiovascular	Mouse, rat	No significant findings except for mononuclear cell infiltrates
	Monkey	Cardiovascular collapse related to complement activation
		Associated with plasma levels that exceed 40–50 μg/mL; no histological evidence of direct cardiotoxicity
Endocrine	Mouse, rat, monkey	No treatment of related lesions except mononuclear cell infiltrates in rodents
Gastrointestinal	Mouse, rat	Presence of mononuclear cell infiltrates
	Monkey	No significant effects
Hematopoiesis/blood	Mouse	Reductions in platelets after repeated administration of 50 mg/kg; extramedullary hematopoiesis in spleen and liver
		Increases in WBCs
	Monkey	Occasional transient acute reductions in platelet numbers during infusion; transient increases in APTT
Hepatic	Mouse	Single-cell necrosis, hepatocytomegaly with no evidence of hepatic dysfunction at doses greater than 50 mg/kg and accompanied by increases in transaminases; mononuclear cell infiltrates
	Monkey	Slight increases in transaminases, little or no evidence of direct hepatoxicity

Immune	Monkey, mouse, rat	Dose-related increases in B cells in lymphoid organs; in mice and rats increased spleen and lymph node size and diffuse multiorgan infiltrates of mononuclear cells
Integument	Monkey, mouse, rat, rabbit, pig	Local inflammatory responses to intradermal or SC injections, and topical administration
Kidney	Mouse	No significant alterations at clinically relevant doses; minimal to mild reductions in proximal tubular brush border height and appearance of enlarged active nuclei; at doses of 100 mg/kg tubular degeneration, mononuclear cell infiltrates present
	Rat	No significant alterations at clinically relevant doses; tubular degenerative changes at doses of 40 mg/kg or greater; functional changes only in the presence of significant histopathogical changes; mononuclear cell infiltrates
	Monkey	No significant alteration at clinically relevant doses; dose-related tubular degenerative changes at higher doses; minimal to mild changes in the proximal tubular epithelium at 10 mg/kg and more prominent lesions at 40 and 80 mg/kg with alteration in functional markers observed at higher doses
Reproductive	Mouse, rat, rabbit, monkey	No treatment-related lesions observed; presence of mononuclear cell infiltrates in uterus and ovaries in rodents; no changes in testes; dose-related increase in spontaneous abortions associated with maternal toxicity; reductions in fetal weight and fetal effect also associated with significant maternal toxicity
Pulmonary	Mouse, rat	Presence of mononuclear cell infiltrates
	Monkey	No treatment-related lesions

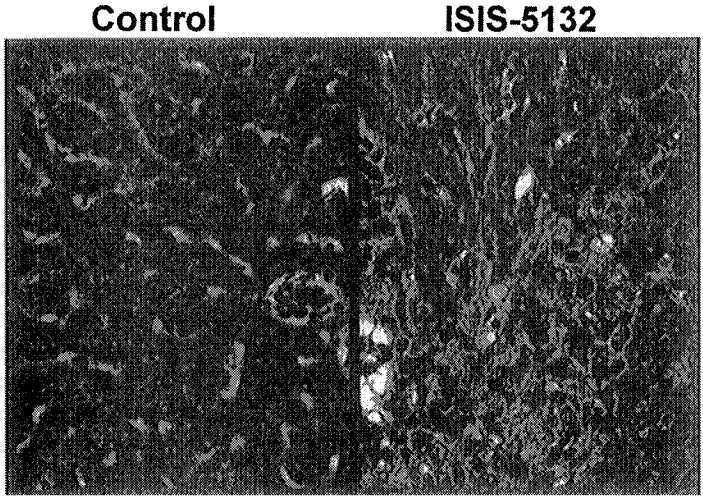

Figure 7 Hematoxylin-and-eosin-stained sections from mice treated with either saline or 50 mg/kg of ISIS 5132. The accumulation of mononuclear cells is apparent in the section from treated mice.

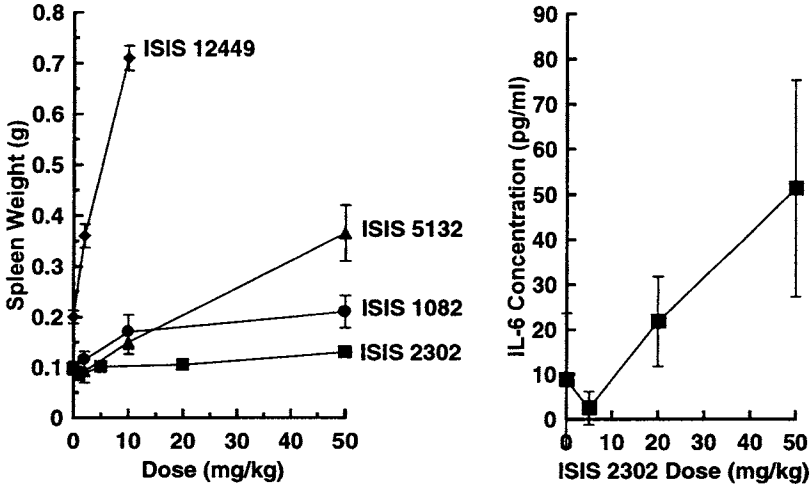

Figure 8 Dose-response effects for spleen weight and IL-6 production in mice. Data on spleen weight are presented for several different oligonucleotide doses for 2–4 weeks. Cytokine levels were measured in mice treated 3 h after the last dose of ISIS 2302 in a 4-week study.

trum of immunostimulatory effects is observed. This fact does not imply that immunostimulatory effects are hybridization-dependent; rather, it has been suggested that the most potent immunostimulatory motifs mimic bacterial DNA, and are, therefore, recognized as a danger signal (65). Immune stimulatory motifs optimized for rodents have been described in the literature, and include palidromic sequences and CG (i.e., cytosine-guanosine) dinucleotide motifs (63,64). The potency of CG motifs is further dependent on the flanking sequences. Optimal immune stimulation of the CG dinucleotide in mice is achieved with two 5′-purines and two 3′-pyrimidines (66).

It is clear that while specific sequence motifs are critical for optimal immunostimulatory activity in rodents, they do not necessarily explain all of the immunostimulatory activity. We investigated the sequence specificity of immune stimulatory effects using spleen weight as an index of in vivo immune stimulation (36). Fifteen different 20- or 21-mer phosphorothioate oligodeoxynucleotides were administered to mice at doses up to 100 mg/kg for 2–4 weeks. Within the series, there are marked differences in the immunostimulatory effects. One oligodeoxynucleotide, ISIS 12449, had the optimal six-nucleotide palindromic element (AACGTT), as well as three other CG dinucleotides also thought to be favorable to stimulation (63,64). ISIS 12449 was more potent than a series of other oligodeoxynucleotides that contained from 0 to 3 CG dinucleotides in random sequence context (Fig. 8). Although potency does differ between sequences, the histological examination of the spleens demonstrated that qualitatively the effects in the spleens were similar (36,67). However, even oligonucleotides that did not contain the CG motif were stimulatory. Thus, there is no clearly defined rule for establishing the potency of different sequences in mice. In mice the degree of B-cell stimulation follows a set of rules that have not been fully defined. Mice appear to be more promiscuous in that many different CG motifs will stimulate and it is possible to get immune stimulation without CG motifs. Clearly CG motifs and palindromic elements containing CG are important in the potent stimulation of both B-cell proliferation and cytokine release, but it is clear (36) that there are other yet-to-be-defined elements whose presence is immunostimulatory. These rules need to be factored into decisions about the design of sequences for antisense use.

Immune stimulation can be viewed as a nonspecific toxicological effect, or it can be used as an intended pharmacological effect. It is important for the reader to make this distinction when reviewing the literature. In the latter case the mitogenic effects of highly stimulatory oligonucleotides can be used as adjuvants. Immunostimulatory oligodeoxynucleotides have been shown to enhance the immunological response to both viral and tumor antigens in mice (60,68,69). In the application of oligonucleotides as adjuvants, most stimulatory sequences available are administered in conjunction with the specified antigen.

The remainder of this review will focus on advances in our understanding of the safety implications of immune stimulation since the last review. The single

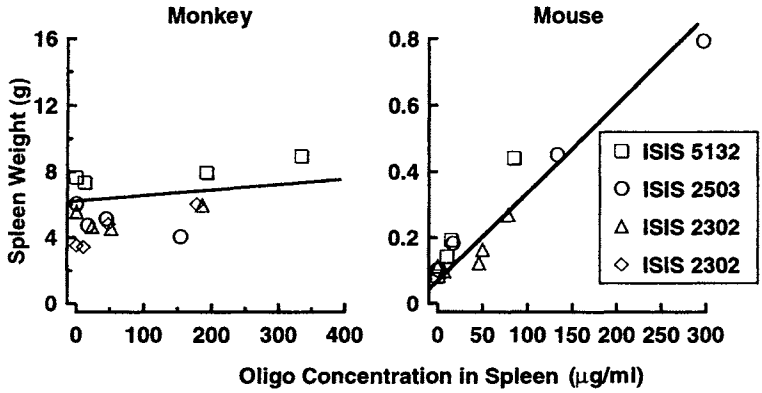

Figure 9 Correlation between oligonucleotide concentration in spleen and spleen weight in mice and monkeys. Data were collected from animals treated for 4 weeks with the exception of spleen weight from the ISIS 2302 6-month monkey study.

most important advancement in the understanding of the implication of immune stimulation antisense therapy since the last review pertains to the species sensitivity. We have studied up to seven different oligonucleotides in monkeys without observing significant increases in spleen weight or multiorgan mixed mononuclear cell infiltrate that are the hallmarks of this effect in rodents (Fig. 9). The only manifestation of immune stimulation in monkeys has been occasional and mild follicular hyperplasia in spleen and irritation associated with irritation at subcutaneous dose sites. Similar species sensitivity was observed in isolated splenocytes. Increases in ^{3}H-thymidine incorporation were observed in splenocytes from all species studied, but there sensitivity decreased with mice > rats > monkeys (70). While these differences are reflective of the in vivo data, it is unclear whether the proliferative effects measured in splenocytes are completely predictive of what is occurring in the whole animal. We have also demonstrated that the species differences in the immunostimulatory effects are unrelated to pharmacokinetics. In fact, the lymphoid organs of primates often have higher concentrations than those in rodents dosed with similar or even higher doses (Fig. 9). Thus, differences in response are independent of pharmacokinetics. It is not yet clear that the reduced sensitivity of splenocytes from primates completely explains the differences in responsiveness in vivo.

The reason for differences in species sensitivity to immune stimulation has become more clear with the recent description of the optimal stimulatory sequences in primates (71). Whereas the rodent responds to the relatively simple six-nucleotide AACGTT motif, and is rather promiscuously stimulated, the optimal primate motif is a more complex element. The differences in stimulating

elements demonstrate that the nature of the response is very much different in primates than in rodents. In primates, including humans, for an oligonucleotide to have stimulatory activity the 5′ end must have a TC and there must be multiple (>2) repeats of GTCGTT with separation between the repeats. Note that there are species differences, such that active sequences can be found for stimulating human B cells that are relatively inactive for other primates, and vice versa (72). It is this sequence, then, that is effective as an adjuvant in chimpanzee or monkey. In practice, then, this more stringent requirement for optimal stimulatory sequence motifs by primates confirms the impression from toxicity studies in monkeys that suggests immune stimulation is less of a concern than observed in rodents. Knowing the optimal motif for primates, these sequences can be avoided in design of future antisense therapeutics.

Although immune stimulation is primarily a rodent effect, a full understanding of the nature and impact of these effects is still desirable. It now appears that in addition to differences in species sensitivity, there are significant strain differences in mice, with CD-1 mice being more sensitive than C57BL/6 or C3H (62). We now know that these changes can occur quite quickly. In CD-1 mice, changes associated with immunostimulation can be observed within hours of a single administration of a potent immunostimulatory oligonucleotide and effects can persist for weeks afterward. A single dose of 10 nmol/mouse of a phosphorothioate oligonucleotide produced peak increases in spleen weights 6 days after dosing (58). With repeated dosing for 2–4 weeks, the cellular infiltrate and splenomegaly progress slightly, but increases are small following the initial burst observed in the first week or two of dosing. As a result of this slow progression, with time the appearance of the infiltrate and the parenchyma of the affected organ does not markedly change, such that samples from mice treated from 2 weeks through 6 months appear very similar (119). Thus, contrary to what one might expect of a proliferative effect, immune stimulation appears to achieve some steady-state equilibrium with repeated chronic administration. More research into the kinetics of immune stimulation is needed.

The complexity of immunostimulatory effects of phosphorothioate oligodeoxynucleotides in rodents has been revealed by the investigation of cytokine activation. What was once considered a B-cell proliferative effect is now better characterized as a Th1-type immune activation. Incubation of mouse splenocytes with oligodeoxynucleotides in vitro stimulated the secretion of IL-6, IL-12, and interferon gamma (73–76). Release of cytokines may then stimulate B-cell proliferation. Using MACS sorting it was possible to demonstrate that it was B cells that were the source of IL-6 and IL-12 and that CD^{4+} T cells were also sources of IL-6 and IFN-γ (73). NK cells were further identified as a source of IFN-γ. These results could be reproduced in vivo. While it is likely that IL-6 is responsible for B-cell proliferation and differentiation, it is not clear what the initiating event is. It is also possible that oligodeoxynucleotide treatment can stimulate

cytokine production by other cells types that could modulate proliferative activity and have a profound influence on the nature of the responses to this class of compounds. For example, primary cultures of human keratinocytes secrete IL-1α in response to treatment with a phosphorothioate oligodeoxynucleotide (77). The release of cytokines by non-lymphoid-derived cells following exposure to oligodeoxynucleotides is a possible explanation for the cellular infiltrate observed in many organs and tissues in rodent toxicity studies and may be a factor in the increased sensitivity of rodent to the immunostimulatory effects of phosphorothioate oligonucleotides.

The immunostimulatory effect of phosphorothioate oligonucleotides is in fact more than just a proliferative response. Clearly the most striking finding in phosphorothioate oligonucleotide–treated mice is the multiorgan infiltrate of mononuclear cells. So in addition to proliferative effects, other processes are occurring, such as, for example, local changes in cytokine release or changes in the expression patterns of adhesion molecules that attract inflammatory cell populations to particular organs. Immunohistochemical analysis of the nature of the infiltrating cells in the liver or kidney of treated mice confirms that a large percentage of the infiltrating cells in tissues consists of monocytes, cells that stain positive for LFA-1, CD18, and Mac-1 (62). The presence of large numbers of monocytes in tissues is associated with circulating cell populations and reflects an increase in monocytes as well (4,6,8). The liver and kidney of treated mice appeared to be primed for an infiltration of mononuclear cells in that expression of intracellular adhesion molecule 1 (ICAM-1), vascular endothelial cell adhesion molecule-1, and IL-6 were up-regulated on endothelium of local blood vessels (62). These data reveal that the multiorgan mixed mononuclear cell infiltrate is related to local changes in the affected tissues.

The precise mechanism leading to immune stimulation is not yet known. High-affinity cellular receptors for deoxyoligonucleotides have been hypothesized and these may control these immunostimulatory effects (78). While there is one report suggesting that immobilized oligonucleotides are immunostimulatory (57), other studies appeared to have demonstrated internalization of the oligodeoxynucleotide is required for immunostimulatory effects (79). The exact nature of the binding sites and whether internalization of oligonucleotide motifs provides signals for cytokine release remain to be elucidated.

Oligonucleotides that are stimulatory reduce the concentration of IκBα and IκBβ resulting in an activation of NFκB. It has been hypothesized that following internalization of the stimulatory oligonucleotides, acidification of the endosomes and release of reactive oxygen species is the initiating event in the immunostimulation. Secondary changes to IκB results in activation of NFκB ultimately resulting in the transcriptional changes associated with proliferation and the induction of cytokines known to play a role in the overall pattern of responses to immunostimulatory oligonucleotides (80).

What are the functional consequences of immune stimulation? A number of studies have been performed recently to further understand the effect of immune stimulation on cellular and humoral immune function. Obviously since the potency for immune stimulation is sequence dependent, the functional effects on the immune system will also likely change with sequence. The first compound we have experience with in immune function assays is ISIS 2302, which happens to have low potency for immune stimulation relative to other phosphorothioate oligodeoxynucleotides (Table 3). Studies were performed in mice treated every other day for 4 weeks with doses ranging from 1 to 50 mg/kg. Humoral immune function was assessed by measuring B-cell number, B-cell response to LPS mitogenic activation, serum cytokine, and serum immunoglobulin concentrations (IgG and IgM). The response to sheep RBC challenge was also measured, but this response actually involves a combination of cellular and humoral immune response. While spleen weights and total number of B cells were increased at 20 and 50 mg/kg, the effects on humoral immunity were limited. Note that increases in IL-6 are at least as sensitive a marker of immunostimulation as spleen weights (Fig. 8). The mitogenic response to LPS was increased, but there was no increase in serum immunoglobulin concentrations and no change in the response to sheep RBC. There was also no production of ISIS 2302–specific or anti-dsDNA antibodies, indicating immune stimulation was consistent with a mitogenic effect, but was not due to an antigenic response of the oligonucleotide. With respect to cellular immune function, T-cell proliferation and differentiation were not affected by ISIS 2302 treatment, but NK-cell activity was increased at the higher doses (Table 3). Increases in the concentrations of IL-6, MCP-1, and IL-12 accompanied these changes in immune cell function. The effects of ISIS 2302 on immune function are limited and were observed at the relatively high doses required to produce increases in spleen weight. While ISIS 2302 is considered representative of the class of oligonucleotides, these results should be interpreted with an understanding of the low potency for this oligonucleotide. Thus, the effects on immune function could be different for more potent immune stimulatory oligonucleotides. From this characterization it is evident that the immunostimulatory effects do not markedly alter immune function.

One of the remarkable features of the immune stimulation induced by phosphorothioate oligonucleotides is the absence of an antibody response directed against the oligonucleotides. The absence of an antigenic response to phosphorothioate oligodeoxynucleotides was further confirmed in chronic and anaphylactic studies. Mice and monkeys treated repeatedly with ISIS 2302 for up to 6 months have not shown any evidence of IgG or IgM directed against the oligonucleotide (Isis, unpublished). In another study, monkeys received a classic sensitization and booster-dose regimen of ISIS 2302 in the presence or absence of Freund's adjuvant, and were then challenged with intradermal doses of ISIS 2302. The site of challenge was monitored for wheal-and-flare type reaction. After a relatively

aggressive immunization protocol, there was no evidence for immune reactivity against ISIS 2302, but monkeys sensitized to the positive control ovalbumin demonstrated the appropriate wheal-and-flare reaction in response to the intradermal challenge with ovalbumin. These compounds are poor antigens.

Because reduction in ICAM-1 expression may inherently alter immune function we examined the effects of a murine specific analog of ISIS 2302 in a battery of immunotoxicology assays. The difficulty in these types of experiments is separating potential intended pharmacological effects from the more nonspecific mitogenic effects of oligonucleotides described above. In the case of the murine-specific ICAM-1 inhibitor, ISIS 3082, the potency for nonspecific immune stimulation is low, comparable to ISIS 2302 (i.e., human specific inhibitor of ICAM-1). The best pharmacological activity has been obtained at doses ranging from 0.5 to 10 mg/kg. Using the lower range of the doses, the specific effects resulting from the antisense activity can be divorced from potential nonspecific activity produced by immune stimulation. It was demonstrated that ISIS 3082 produced a slight decrease in the ability of splenocytes to act as antigen-presenting cells and inhibited dinitrofluorobenzene (DNFB) contact hypersensitivity at doses ranging from 1 to 20 mg/kg (Fig. 10 and Table 4). However, there was no other evidence of impaired cellular or humoral immune function. This pattern of change in immune function is consistent with that reported for ICAM-1-deficient mice, and is explained by the effect of ISIS 3082 on ICAM-1 expression.

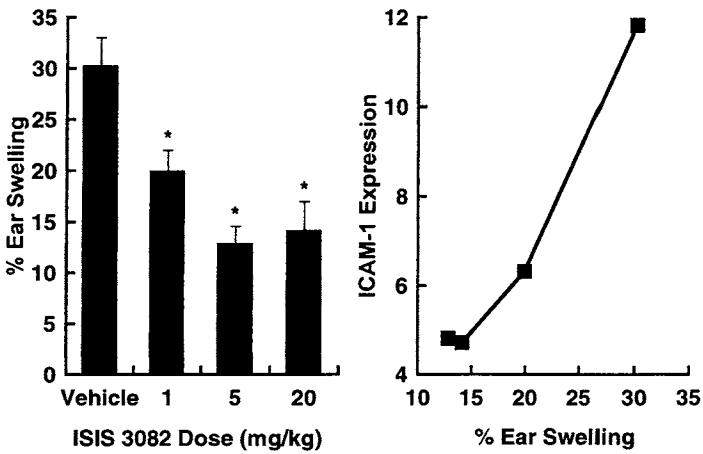

Figure 10 Correlation between decreased sensitivity to DNFB and ICAM-1 expression in mice treated with ISIS 3082. DNFB-induced inflammation was determined by measuring ear thickness following sensitization and challenge with the DNFB. ICAM-1 expression was determined using immunohistochemistry and image analysis.

Table 4 Immunotoxicity Evaluation of ISIS 2302 in Mice

Parameter	Dose of ISIS 2302			
	1 mg/kg	5 mg/kg	20 mg/kg	50 mg/kg
General immune stimulation				
Spleen weight	NC	NC	Trace	+1
B-cell number	NC	NC	NC	+1
Humoral immune function				
LPS response	nt	NC	+1	+1
Sheep RBC response	NC	NC	NC	NC
Total IgG/IgM	nt	NC	NC	NC
2302-specific IgG	nt	NC	NC	NC
Cellular immune function				
Cytotoxic T-cell activity	nt	NC	NC	NC
NK-cell activity	nt	NC	+2	+2
Cytokine levels				
IL-6, IL-12, MCP-1	nt	NC	Trace	+1

nt, not tested; NC, no change; +1 = mild; +2 = moderate.

Flow cytometry indicated that ICAM-1 expression on lymphocytes of mice treated with ISIS 3082 was largely unchanged, explaining why cellular immunity was relatively unaffected. (There was a slight reduction in the two-way mixed lymphocyte response.) The inhibition of contact hypersensitivity to DNFB was associated with decreases in ICAM-1 staining at the challenge site, as well as decreases within systemic tissues such as liver. Thus, the anti-inflammatory effect (Fig. 10) could be explained by an inhibition of ICAM-1 expression on the vascular endothelium, thereby preventing the inflammatory reaction. These data provide an additional example of how the nonspecific effects of phosphorothioate oligonucleotide do not markedly alter immune function. In addition, they demonstrate that this particular target, ICAM-1, can be knocked down and yet immune competence is maintained.

Structural modifications to oligodeoxynucleotides have been shown to reduce the potency of immune stimulation. The replacement of cytosine with 5-methyl cytosine is very effective for reducing the immunostimulatory potential of oligonucleotides in mice particularly when the cytosine is part of the CG motif (66). In mice, when sequences with 5-methyl cytosine are compared with the same sequence without methylation, the methylated sequence has a lower potency for inducing immune stimulation as determined by spleen weight (62).

Reducing the anionic nature of the oligonucleotide by replacing phosphorothioate linkages with methyl phosphonate linkages on each of the 3′ and 5′ termini reduced the proliferative effects and the secretion of IgG and IgM, suggesting

that this modification can also be used to ameliorate immune stimulation (81). Presumably morpholino modified oligonucleotides should also have reduced immunostimulatory effects (see Iversen, this volume).

Immunostimulation and the presence of the inflammatory cells in tissues may influence the toxicity profile of oligonucleotides. There is a clear relationship between potency for immunostimulation, spleen weight, and reductions in circulating platelets in mice (32,82). Likewise the hepatoxicity of phosphorothioate oligonucleotides may be exacerbated by the presence of immunostimulatory cells or by local release of cytokines. Mice treated with a highly stimulatory oligonucleotide had more pronounced elevations of ALT and AST than mice that received equivalent doses of the same sequence except that the CG had been reversed to GCs (32). The consequences of chronic B-cell proliferation are not yet known, but a 2-year bioassay is in progress in CD-1 mice. It is possible that immunostimulation may have promoter-like effects within the immune system or that the presence of mononuclear infiltrates in tissues may be promotional. In either case these types of effects could complicate the interpretation of rodent carcinogenicity studies. In light of the species differences in response, the relevance of the sequelae of immunostimulation may not be relevant.

Hematopoietic. Acute and subchronic exposures to phosphorothioate oligonucleotides have not produced alterations in hematopoiesis. Treatment of mice with some oligonucleotides has been associated with increases in circulating mononuclear cells, consistent with the increase in this cell type in peripheral tissues as part of immunostimulation. Other than this exception, hematology studies have not demonstrated marked changes in circulating cell populations. Analysis of red cell progenitor cells in mice treated with near lethal doses of a number of oligonucleotides failed to demonstrate any changes in cell numbers or distribution (see section on genotoxicity below). Sarmiento et al. reported that treatment (100–150 mg/kg/day $\times$ 14) of a phosphorothioate oligonucleotide produced some changes that were interpreted as reduction in megakaryocytes (8). However, no quantitation was performed and other studies have not confirmed this effect.

Immune stimulation in rodents has been associated with reductions in platelet numbers, but is not associated with changes in platelet production in the marrow. It has been possible to correlate the potency of immune stimulation with reductions in platelets (82), suggesting that sequestration or destruction in the hypertrophied spleen may be a factor. There is no evidence for a direct effect on marrow. Megakaryocyte number and appearance in bone marrow are normal as is the morphology of megakaryocytes in extramedullary sites.

Treating monkeys with up to 10 mg/kg on alternate days for 6 months produced no changes in bone marrow morphology or circulating cells. In isolated instances during infusions some monkeys have shown transient thrombocytopenia. Platelets return to normal values when the infusion is stopped and plasma clears of oligonucleotides. The thrombocytopenia is short-lived and occurs in

individual monkeys but the susceptibility to this effect is not reproducible. That is, on particular days some individuals might be sensitive, but on another day the same individuals might not be affected. There is no apparent pattern in susceptibility. This thrombocytopenia differs from the effect seen in mice. In mice there is a transient thrombocytopenia that is dependent on immunostimulation. In patients treated with the anti-HIV antisense compound GEM91, there were reductions in platelet numbers that were readily reversible with cessation of treatment (83). A similar reduction in platelets has been observed in patients in cancer trials given other phosphorothioate oligonucleotides (see Dorr, this volume). The pattern of reductions in platelets in monkeys is reminiscent of the effect in humans but is much more rapidly reversible. In both nonhuman primates and humans there does not appear to be a deficit of production of platelets, but rather some sequestration or trapping of platelets that results from administration of oligonucleotides.

Hepatic. The liver is one of the sites of accumulation for phosphorothioate oligonucleotides (17,18,21). The most commonly observed histopathological change in livers from treated mice and monkeys is the presence of basophilic granules in the cytoplasm of Kupffer cells and Kupffer cell hypertrophy. These granules or inclusions are now known to represent oligonucleotide taken up by Kupffer cells, and stain positively by immunohistochemistry using an antibody directed toward oligonucleotide. The amount and extent of Kupffer cell enlargement is directly proportional to dose. These effects are independent of sequence and are considered to represent the uptake of oligonucleotide and oligonucleotide metabolites by these phagocytic cells. Kupffer cell hypertrophy and basophilic granules are reversible, but full resolution may take weeks consistent with the rate of clearance of oligonucleotide from liver.

In primates, hepatotoxicity has generally not been observed after repeated dosing with phosphorothioate oligonucleotides. Occasional small increases in transaminases have been seen in studies, but are limited to high doses and are without morphological consequences. At a dose of 50 mg/kg of ISIS 2302 every other day for 4 weeks there was a slight elevation of AST in female cynomolgus monkeys, but there was no evidence of hepatocellular changes and the values for the monkeys in this group were within the normal range. Following subcutaneous doses of ISIS 3521 and ISIS 5132 of up to 80 mg/kg every 2 days for four doses, there was Kupffer-cell hypertrophy and periportal-cell vacuolation, but no indication of necrosis and only a very slight increase in ALT (31). These minor effects occur at doses manyfold higher than intended clinical doses. Treatment of monkeys with 10 mg/kg ISIS 2302 for 6 months produced no other significant changes in the liver. Considering the relatively small change observed and the high multiple dose over clinically used doses in humans, it does not appear as if the slight increase in liver function tests observed in monkeys after 4 weeks of treatment with 50 mg/kg is the result of an important toxicity. Note

that transient increases in transaminases have been observed in clinical trials with GEM91, but these reversed spontaneously during the course of treatment (83).

Hepatic changes are more common in rodents treated with phosphorothioate oligodeoxynucleotides. As with the monkey, the most common morphological effect of treatment with phosphorothioate oligonucleotide was Kupffer-cell hypertrophy with basophilic granules. In addition, immune stimulation in mice was evident in liver as indicated by the infiltration of mixed mononuclear cell infiltrates (Fig. 7). Histological examination also revealed multifocal hepatocellular degeneration or single-cell necrosis, which was associated with increases in serum transaminases (ALT and AST) and decreased levels of albumin and cholesterol consistent with some degree of mild hepatic dysfunction (4,6,8). These changes were dose-related; for example, slight increases in ALT and AST were observed in mice treated by intradermal injection with ISIS 2105 for 4 weeks at 21.7 mg/kg/day, but no changes were found at lower doses (30). Recent studies have suggested that the hepatocellular effects are exacerbated by immunostimulation (32). A correlation between the severity of immunostimulatory effects and hepatotoxicity seems to support this hypothesis. With repeated doses of oligonucleotides of 100–200 mg/kg in rodents, single-cell necrosis and hepatocytomegaly become more prominent and the effects can lead to increased mortality.

A cellular basis for the hepatocellular effects in rodents has not been established. The species differences in sensitivity to the hepatocellular effects do not correlate with species differences in pharmacokinetics. While the mouse is more sensitive to the toxicity, the concentrations in mouse liver are lower than those achieved in the primate liver. These data suggest that there could be an inherent species difference in sensitivity or perhaps the differences are related to the exacerbation of toxicity by the immunostimulatory effects, which are more prominent in rodents.

Gastrointestinal. Subchronic intravenous or subcutaneous administration of oligonucleotides to monkey or mice has not been associated with significant alterations in the gastrointestinal tract. The only observation occasionally seen in mice is mononuclear cell infiltrates in the lamina propria of mice that is considered part of the immunostimulatory effects. Gastric motility and gastric acid secretion have also been studied following single dose of up to 100 mg/kg ISIS 2302 iv in rodents as part of a safety pharmacology battery, with no apparent effect on function in this organ system. To further explore the tolerability of the gastrointestinal tract to local concentrations of oligonucleotides, a series of studies was performed with an enema formulation of ISIS 2302 in mice and dogs. Enemas were administered and retention was maintained (in dogs) for approximately 4 h. Gastrointestinal tissues were collected at 4 or 24 h. No alterations were observed in intestinal tissues, even at concentrations as high as 100 mg/mL. Plasma levels of ISIS 2302 were minimal or below limits of detection in dogs following enema administration, indicating that plasma exposure would not

be a significant concern with this route of administration. Oligonucleotide could be detected in the mucosa and in the lamina propria by either CGE or immunohistochemistry at 4 h, but by 24 h there was minimal oligonucleotide remaining. Systemic absorption of <5% of the administered dose was estimated by the detection of ISIS 2302 and metabolites in liver and kidney. The low concentrations in these tissues were dose-dependent and were not associated with alterations in clinical chemistry parameters or morphological changes. Thus, local tolerability of oligonucleotides was demonstrated. These findings have potential utility for future studies employing local delivery of antisense drugs to the gastrointestinal tract and have implications for future oral formulations of antisense drugs that will need to expose the gut to locally high concentrations of oligonucleotide.

Renal. The kidney is also a major site of deposition of phosphorothioate oligodeoxynucleotide with the proximal tubule epithelial cells being a major site of storage (84–86). As described for Kupffer cells, accumulation of oligonucleotide can be visualized in the form of basophilic granules in renal proximal tubular epithelium (primarily S1 and S2 segments) (4,5). In plasma, phosphorothioate oligonucleotides are generally bound to plasma proteins, which reduces or eliminates most glomerular filtration. Micropuncture studies demonstrate that tubular fluid has a much reduced concentration of oligonucleotide compared to plasma, further supporting the concept that glomerular sieving of plasma prevents significant excretion of phosphorothioate oligonucleotides (84). However, these studies demonstrated that oligonucleotide that is filtered can be resorbed by proximal tubular epithelium. Studies with nonfiltering kidneys have demonstrated that accumulation of oligonucleotide by proximal tubular cells can also result from nonluminal uptake. Thus, the uptake by proximal tubular cells is the result of both basolateral uptake from the circulation and tubular reabsorption (84,87).

Approximately 20% of an intravenous dose accumulates in the kidney (21). When the renal effects in monkeys at various doses were examined, it was clear that below 10 mg/kg there are no meaningful histological changes in the kidneys of primates. At a dose of 10 mg/kg administered every other day for 4 weeks, minimal to moderate changes in the height of the brush border and some evidence for regenerative changes in the proximal tubular epithelium were observed. At doses from 40 to 80 mg/kg, there is mild focal tubular degeneration (4,31,88). Even in the presence of histopathological changes, there is no increase in serum urea nitrogen or creatinine in either species, nor were there alterations in markers of renal function. Urinary levels of *N*-acetylglucosaminidase, total protein, and retinol-binding-protein levels were unaffected until significant morphological alterations were present (89). These data suggest there is a clear dose-response relationship in the renal effects of phosphorothioate oligodeoxynucleotides. The data also demonstrate that the minimal changes observed at 10 mg/kg are not toxicologically significant and at clinically relevant doses there was no indication of renal dysfunction. When treatment was extended to 6 months, there was no

increase in the morphological alterations observed at the 10 mg/kg dose level, and thus, no exacerbation of renal toxicity with chronic administration despite the maintenance of steady-state kidney levels of oligonucleotide. Renal function studies have been performed in dogs at doses up to 20 mg/kg. No changes in inulin or PAH clearance were observed in these studies, indicating normal renal function.

In mice, renal morphological changes were observed only at nearly lethal doses of 100 or 150 mg/kg/day. The higher doses required to elicit renal toxicity are attributed to differences in target organ exposure between mice and monkeys. The concentrations in the renal cortex associated with minimal to mild (and not clinically relevant) renal tubular atrophy or regenerative changes in monkeys are approximately 1000 μg/g of tissues. The cortex concentrations of total oligodeoxynucleotide that are associated with moderate degenerative changes after subcutaneous doses of 40–80 mg/kg in the monkey are greater than 2000 μg/g. Since mice clear oligonucleotide more quickly than monkeys, higher doses are required to achieve the same kidney concentrations in mice.

In clinical usage, doses of 3 mg/kg every 2 days produce steady-state cortex concentrations of total oligodeoxynucleotide in the range of 400–500 μg/g, demonstrating significant margin of safety from clinical doses to those doses associated with even the most minimal renal morphological changes.

Application of clearance and steady-state pharmacokinetic models suggests that continued administration of oligodeoxynucleotide at a dose of 3 mg/kg should never achieve the renal concentrations associated with dysfunction or even minimal morphological changes (17,90). Recently we have shown that phosphorothioate oligonucleotides do in fact follow steady-state kinetics in rats. Repeated subcutaneous injections of doses of ISIS 2105 to rats resulted in attainment of steady-state concentrations by 14–31 days, consistent with known organ half-lives for phosphorothioate oligonucleotides (91).

Because the kidney is a key organ in the distribution of phosphorothioate oligonucleotides, it was of interest to determine what effects renal dysfunction might have on the disposition of this class of compounds. This is particularly important because of the numbers of patients with impaired renal function, including patients with renal transplants who have received these drugs. Models of glomerular and proximal tubular dysfunction were employed. Glomerular nephritis was induced in rats by treating them with an anti-Thy1.2 antibody, and proximal tubular damage was produced by treatment with cisplatin. Both treatments produced the expected morphological changes. Glomerular functional deficits were confirmed by measuring urinary protein excretion, and proximal tubular damage was confirmed in cisplatin-treated rats by an increase in BUN. Untreated animals or animals with experimental renal damage were then administered 10 mg/kg ISIS 3521 as a single intravenous injection.

Despite the high concentrations of oligonucleotide in the kidney, urinary excretion of oligonucleotide is a relatively minor component of the clearance

process. In rats with glomerular and proximal tubular damage, this was still the case, although there was an increase in the urinary excretion of oligonucleotide. The total oligonucleotide excreted was still less than 2.5% of the administered dose.

With changes in renal function present, it was of interest to determine whether there were alterations in the disposition of oligonucleotide in plasma as well as kidney and other organs. Plasma pharmacokinetics of either total oligonucleotide or parent drug were unchanged with respect to AUC, C_{max}, or half-life. In kidneys of rats with glomerular damage there were no differences in cortex or medullary concentrations of oligonucleotide. In contrast, proximal tubular damage reduced AUCs for both cortex and medulla 40–50% from control values. There were no changes in liver concentrations for either damage model. There also were no significant differences in spleen concentrations, although in the proximal tubular damage model there was a trend toward increased spleen concentrations. That spleen would be a site of accumulation of oligonucleotide when other systemic stores became saturated is supported by previous observations in rats (19). However, it is clear from these data that even in the presence of measurable changes in renal function, there are no gross changes in tissue pharmacokinetics (except in the kidney) and that other organs must uniformly be taking up the oligonucleotide that is no longer being accumulated by the kidney. As a result, the changes in pharmacokinetics are modest or undetectable. Thus, in renal compromised animals, there appear to be no significant changes in pharmacokinetics that should impact on safety (120).

Cardiopulmonary. Toxicity studies in which the oligonucleotide was administered by subcutaneous or intravenous administration have not produced any direct treatment-related cardiac effects in monkeys or mice. Hemodynamic changes in monkeys described above are secondary to complement activation, and the occasional observation of mononuclear cell infiltrates is associated with the immunostimulatory effects in rodents. However, no direct toxic effects have been noted in the heart or cardiac muscle that could be attributed to exposure with phosphorothioate oligonucleotides. Concentrations of oligonucleotide in the heart and lung are relatively low percentages of the total dose (17,19,21). In fact, to produce pharmacological activity in models of pulmonary inflammation relatively high doses of oligonucleotide must be administered (92).

To more efficiently treat pulmonary diseases with antisense therapeutics, inhalation exposure to phosphorothioate oligonucleotide was assessed in mice using nose-only exposure (119). Both the tissue kinetics and tolerability of an inhaled aerosol formulation of a phosphorothioate oligonucleotide were characterized following single or repeated exposures on alternate days for four doses. To maximize the potential for deposition into the bronchiolar and alveolar regions of lung, mean particle size was targeted for 1–5 μm. The single-dose study characterized the response to nebulized solutions of 10 or 100 mg/mL. Multiple-dose

studies employed a 12 mg/ml solution and dose was varied by altering the exposure time from 37.5 to 150 min.

In the single-dose-exposure groups, assuming that 10% of the inhaled dose was deposited, the dose at the lungs was approximately 1.2 and 12 mg/kg. Mice responded to the presence of oligonucleotide aerosol by reducing their respiratory rate slightly, a well-known response to many aerosols. There was significant deposition of oligonucleotide in the lungs with pulmonary concentrations of 50–150 μg/g found in the low- and high-dose groups. This oligonucleotide was cleared from the lung, but significant concentrations remained 24 h after the end of exposure. Oligonucleotide was detected by immunohistochemistry in type I and II pneumocytes, as well as endothelium and alveolar macrophages. The high concentration of oligonucleotide used in this study produced some local edema, and a general appearance of greater density of lung tissue. At 1.2 mg/kg there were no significant changes in morphology.

When mice were exposed to an aerosol of 12 mg/mL, the estimated pulmonary doses resulting from timed exposures were 0.9, 1.6, and 3.1 mg/kg/exposure. There was a minimal increase in cellular infiltrates in the mid- and high-dose groups, but no edema or change in cell density was apparent in lungs of treated animals at these doses. The appearance of cell infiltrates in lung following pulmonary exposure is consistent with the observation of cell infiltrates in the lung following high doses administered intravenously. Thus, this reaction may be a component of the immunostimulatory properties in rodents.

Twenty-four hours after the fourth exposure, pulmonary concentrations of total oligonucleotide measured by CGE were in the range of 50–100 μg/g of tissue for the dose range, demonstrating significant local accumulation. (Systemic exposure was detectable, but limited by this route of administration.) These preliminary experiments indicate that with inhalation exposure it is possible to produce significant concentrations of oligonucleotide in the lung in the absence of toxicity. It will be of interest to determine the role of immunostimulation in the response of the lung and to examine whether oligonucleotides with different chemistries or sequences produce quantitatively different responses.

Integumentary. Following intravenous administration of phosphorothioate oligonucleotides, skin accumulates low concentrations of oligonucleotide, although with its large size, the total burden in skin is a significant proportion of the total administered dose (19,21). Intravenous administration has not been associated with any significant toxicological effects. Immunohistochemical localization of oligonucleotide in skin is possible following intravenous administration, but the concentrations are relatively low. Systemic administration of oligonucleotides may not yield high enough local concentrations of oligonucleotide to produce therapeutic activity.

To attain higher concentrations of active oligonucleotide in skin lesions, one strategy was to inject oligonucleotide intradermally in close proximity to a

lesion. For example, ISIS 2105 was tested for activity against human papilloma virus in genital warts. Toxicity studies performed with intradermal injections yielded local areas of mononuclear infiltrates and other evidence of immunostimulatory effects. These were prominent in mice and rabbits. The limitation of this approach was that the local concentrations of oligonucleotide were relatively short-lived and the oligonucleotide was absorbed effectively from this intradermal site. Note that the species differences in the immunostimulatory effects were demonstrated quite remarkably in these studies in that the response of the rodent species to ISIS 2105 was much more marked than the responses of human patient. The data support the concept that the erosions and local inflammatory effects observed in rodents were part of the generalized phenomena of immunostimulation and not some corrosive or dermatoxic effect. (This hypothesis was tested directly—see below.)

Local irritation from subcutaneous injections of phosphorothioate oligonucleotides has been observed in both mice and monkeys. The nature of the reaction at the injection site is typical of local irritation. The principal alterations in primates consisted of subcutaneous fibrosis and perivascular lymphoid infiltration. The latter morphological alteration was suggestive of a local inflammatory response to the injection of ISIS 2302. There was an increased incidence of histiocytic infiltrates and hemorrhage after 26 weeks of subcutaneous injections to mice. There was not a clear dose-dependence; however, the changes were considered to be minimal to slight in the lowest-dose groups and moderate to moderately severe in the high-dose group (15 mg/kg every 2 days).

More therapeutic utility for dermatological diseases could be obtained by having topically applied formulations. Recently, we have developed a topical formulation that allows for the permeation of significant concentrations of oligonucleotide into skin (121). While it is difficult to localize oligonucleotide in skin using CGE, oligonucleotide can be visualized by immunohistochemistry in various layers of the skin following application. In mouse, rat, or pig skin, and in human skin transplanted onto the backs of SCID mice, it is possible to detect oligonucleotide in the dermis, the granular layer, and of course in the epidermis. The concentrations of oligonucleotide attained using topical formulations are sufficient to reduce gene expression locally (see Hardee et al., this volume). Uptake by skin is dose-dependent and concentration-dependent responses in skin have been reported for both reductions in gene expression and toxicological responses. Repeated application of the cream formulation of ISIS 2302 (10% w:w) to the dorsal skin of rats or minipigs produced mild to minimal edema and erythema that resolved over the course of continued dosing. At least part of the edema and erythema is thought to be physical effects of repeated application and washing. In rats treated with the 10% ISIS 2302 cream there was minimal evidence for mononuclear cell infiltrates.

Using a compound with greater immunostimulatory potential (ISIS 2105), we found that more irritation resulted from treatment with ISIS 2105 than was

seen with ISIS 2302. There was a much more pronounced mononuclear cell infiltrate in histological sections of rat skin. In pig skin, the local irritation produced by ISIS 2105 was less pronounced than in the rat. We reasoned that this inflammatory effect observed in rodent skin was secondary to the immunostimulatory potential of ISIS 2105 and not some other sequence-dependent effect.

To test this hypothesis, a study was carried out that compared the topical tolerability of three phosphorothioate oligonucleotides applied topically. The compounds examined included ISIS 12449, a highly potent immunostimulatory sequence, with the optimized rodent CG motif; 12450, a version of ISIS 12449, without the optimal CG motifs; and ISIS 2105, a moderately potent immunostimulatory compound. The potential to produce local irritation mirrored the immunostimulatory potential of the sequence such that topical irritation decreased in order as follows ISIS 12449 > ISIS 2105 > ISIS 12450 (Fig. 11). The immunostimulatory potential of each of these compounds was confirmed by administering them systemically; intravenous injections to rats yielded spleen weights that followed the similar pattern with ISIS 12449 > ISIS 2105 > ISIS 12450. Thus, the irritation observed with some phosphorothioate oligonucleotides following application to rodent skin is yet another example of immunostimulation. Note that the topical tolerability of all the phosphorothioate oligonucleotides, even the most potent,

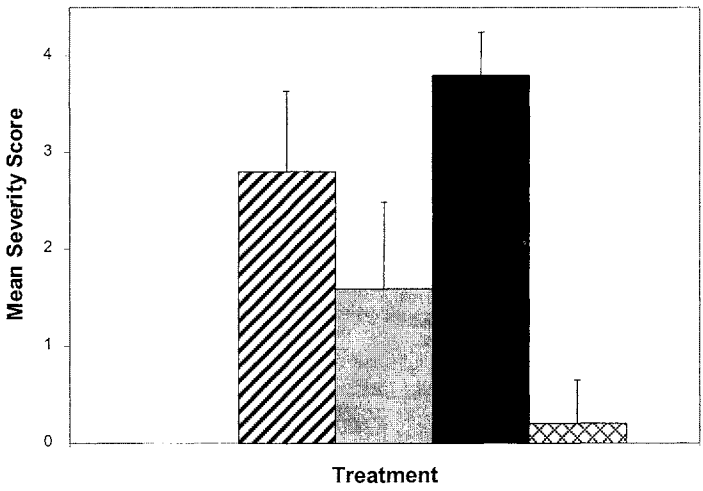

Figure 11 Mean dermal response scores from rats treated with cream formulations containing 10% ISIS 2105 (hatched bars), ISIS 12450 (gray bar), and ISIS 12449 (cross-hatched bar). Rats were treated daily with one of the creams and dermal irritation was assessed using a four-point scoring system. The results represent means and standard deviations.

was excellent. Only when concentrations reached 5% or 10% did the most potent oligonucleotides begin to show moderate local erythema and edema in the rat. Consistent with our experience with relative species sensitivity, the pig was generally more tolerant. With a number of test oligonucleotides at concentrations lower than 5%, the effects on skin were minimal (122).

Pharmacokinetic analyses of the skin demonstrated that substantial concentrations were present in skin and that there was minimal systemic exposure, indicating that topical application has the potential to localize treatment to an effected area of skin without significant systemic effects. With studies of 13 weeks in rats and pigs completed with ISIS 2302 in this topical formulation and with minimal changes observed at formulated concentrations up to 10%, there is a great deal of optimism for good tolerability in patients.

Musculoskeletal. Oligonucleotide is distributed to skeletal muscle and bone, although neither accumulates high concentrations of phosphorothioate oligonucleotides. There are no known toxicities in these systems as a result of the class effects of phosphorothioate oligonucleotides. Oligonucleotide is known to accumulate in bone marrow and in mice at doses near the LD_{50} there are only minor changes in bone marrow cellularity. In mouse micronucleus tests there have been no marked changes in red cell precursor cell populations suggesting that even at doses of >750 mg/kg, the effects on marrow are not marked. Like many other tissues in rodent, skeletal muscle occasionally has been observed with mononuclear cell infiltrates, secondary to the proinflammatory effects.

Nervous System. The high molecular weight and the charged nature of phosphorothioate oligonucleotides prevents them from crossing the blood-brain barrier and concentrations in brain are exceedingly low (19,21) and have perhaps been overestimated using radiochemical assays because of residual blood. As a result of the absence of distribution to the brain, we have not observed any direct effects on the central nervous system in toxicity studies. The indications of decreased motor activity or collapse that were observed in primates have all been secondary to complement activation and associated cardiovascular effects.

Despite the inability for oligonucleotides to cross the blood-brain barrier, antisense techniques are among the most well-accepted tools for modulating gene expression in the central nervous system (CNS) (93–95). These studies generally employ direct injections into specific regions of the brain. There have been reports that repeated injections of phosphorothioate oligonucleotides directly into the brain will result in cellular toxicity and gliosis (96). The mechanism of this toxicity has not been thoroughly explored, but local injections of oligonucleotides (2 nmol) might be expected to produce local concentrations that could be in the range of 1000 μg/g of tissue, depending on how far (and how fast) the oligonucleotide diffuses from the site of application. These high local concentrations might produce nonspecific local toxicities and cell death.

The first marketed antisense product, fomivirsen, is a phosphorothioate oligonucleotide that is used for treating human cytomegalovirus infections of the retina. To produce adequate local concentrations of the oligonucleotide, the compound is injected into the vitreous and then concentrates at the site of action, the retina. Fomivirsen has been shown to effectively inhibit virus replication, thus saving the sight of patients with active CMV retinitis.

In nonclinical studies the distribution of oligonucleotide from vitreous to retina could be observed over time. The vitreous cleared fomivirsen by a combination of metabolism and distribution to surrounding tissues including retina. The clearance rate in rabbit vitreous was approximately 60 h and retinal clearance rates were approximately 96 h. In monkeys, the half-life was dose-dependent ranging from 44 to 74 h as dose increased from 57 to 115 μg (97). These relatively long half-lives in the retina indicated that the dosing interval could be rather long. In fact, the drug can be administered every other week. Besides the significant advantage of locally high concentrations, intravitreal injection also results in minimal systemic exposure, and thus, no systemic toxicities. When clinically relevant doses of fomivirsen were administered to monkeys there was little or no systemic exposure.

The toxicity profile after intravitreal injection was dominated by a local proinflammatory effect in both the rabbit and the monkey. In rabbits, ocular inflammation was dose-dependent in severity and observed at all doses examined, with 100% incidence at concentrations greater than or equal to 4 μM. Inflammation was largely reversible at concentrations up to 10 μM, peaking around day 8 and resolving by day 15 after a single injection. Ocular inflammation was observed in monkeys, but the incidence was lower (generally 15–40% of treated eyes), was observed equally at all dose levels, and was also occasionally observed in control eyes, suggesting that the injection technique or procedure was a factor in the response. In toxicity studies in monkey multiple injections of up to 10 μM were performed weekly or on alternate weeks. This dose of fomivirsen is roughly equivalent to the clinically used dose. Higher doses were not possible because of the exaggerated responses observed in these laboratory animals. In the monkey, inflammation was associated with a number of physiological responses including vasculitis, posterior synechia, neovascularization of iris, hypopion, and closure of the drainage angle resulting in increased ocular pressure, and changes in the pigmentation pattern of retinal pigment epithelium (RPE) cells. In both the rabbits and monkeys coadministration of corticosteroids could ameliorate the inflammatory effects and in the absence of inflammation there was no indication of ocular toxicity. In particular, there was no indication of retinal toxicity. These data demonstrate that the proinflammatory response in these studies is the primary toxic effect and the primary cause of ocular changes.

This hypothesis was also supported by experiments where fomivirsen was modified with 5-methyl cytosine and 2′-methoxyethoxy groups in the 3′- and 5′-

terminal regions. This compound, ISIS 13312, was much less immunostimulatory in vitro and was markedly less proinflammatory in rabbits and monkeys. In addition to its lower immunostimulatory potential, the 2′-MOE modification increases the stability of the molecule, and its half-life in monkey retina was on the order of months rather than days. This provides a more attractive dosing interval as well as lowered toxic potential.

The CNS including the retina is not generally exposed to oligonucleotides in the course of systemic dosing. However, in specialized studies when these have been exposed locally there is only evidence for toxicity when local concentrations are extraordinarily high (e.g., after direct CNS injections) or when secondary effects like inflammation are induced by the antisense oligonucleotide.

E. Reproductive

Like the brain, the male germ cells in the tests are sequestered behind tight vascular junctions, and the blood-testes barrier, like the blood-brain barrier, appears to prevent the accumulation of systemically administered phosphorothioate oligonucleotides. Little or no accumulation of oligonucleotide can be detected in testes and the small amounts of radiolabel sometimes found at early times after administration are probably the result of trapped blood. There have been no observations of adverse effects in the male reproductive tract following treatment with phosphorothioate oligonucleotides either in the testes or in the secondary sex organs in subchronic toxicity studies or in reproductive function studies. Thus, the class effects of phosphorothioate oligonucleotides do not appear to affect male reproductive function. However, it is possible that there could be molecular targets that produce male reproductive toxicity indirectly by reducing the expression of specific targets on the systemic side of the blood-testes barrier. To date no such targets have been identified.

Reproductive function in female mice and rabbits treated with phosphorothioate oligonucleotides has been characterized in typical reproductive toxicity tests. In mice and rats there is sufficient oligonucleotide present such that at high doses in toxicity studies the typical mononuclear cell infiltrates have been observed in ovaries with more potent immunostimulatory compounds. Mononuclear cell infiltrates can be seen in uterus as well. These organs are known to accumulate oligonucleotide following systemic exposure. In fact, immunohistochemistry of the ovary clearly demonstrates oligonucleotide uptake and suggests that the ovary could be a possible target for antisense activity, if the appropriate target were selected (Fig. 12). In detailed studies of reproductive function no effects on ovarian function have been observed in mice or rabbits.

In segment 1, 2, and 3 reproductive toxicity tests with ISIS 2302, there were no effects in mice on reproductive function in females or malformations in the offspring and in segment 3 studies. There were no observed effects in the F1

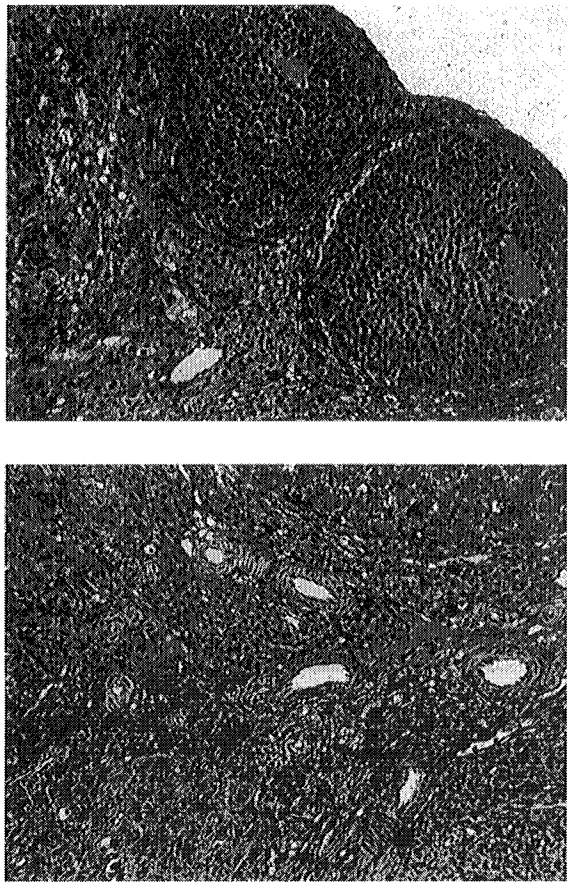

Figure 12 Paraffin-embedded sections of ovaries from a rat injected with 50 mg/kg of ISIS 13920 were sequentially immunostained with a primary mouse monoclonal antibody raised against phosphorothioate oligonucleotides (114) and an HRP-linked second antibody. Peroxidase activity was visualized with 3,3′-diamino-benzidine and sections are counterstained with hematoxylin.

development, behavior, or mating performance (123). Because ISIS 2302 does not have pharmacological activity in mice, parallel studies examining the toxicity of a murine analog, ISIS 3082, were performed and were also negative. Rabbits treated under a segment 2 protocol also had no effects on litter parameters or fetal development (124). The only alteration observed was a reduction in fetal weight in rabbits that was associated with a reduction in maternal weight and

evidence of slight maternal toxicity. These data suggest that at the doses administered, there was no evidence of class-related alterations in development or fertility. The results of teratology experiments are consistent with the absence of significant exposure to oligonucleotide. Documentation of oligonucleotide concentration revealed that at doses that produce significant accumulation in maternal target organs, and mild maternal toxicity, concentrations in placenta or fetal tissues were at or below limits of detection. The negligible concentrations in developing fetus indicate that the oligonucleotide does not efficiently cross the placental vasculature.

However, when oligonucleotides with more potent immunostimulatory effects were administered to pregnant rabbits, there were alterations in reproductive outcome resulting from cytokine-induced spontaneous abortions. Excessive systemic maternal toxicity (and immunostimulation) complicated assessment of teratogenic effects of fomivirsen (ISIS 2922) at the doses selected. Profound maternal toxicity was observed. Treatment with 3 and 9 mg/kg/day of fomivirsen decreased body weight (10 and 20%, respectively), decreased body weight gain, decreased food consumption, and produced a condition where there were scant feces—all indicative of maternal toxicity. There were also changes in serum chemistry and histopathology indicative of dose-dependent renal and hepatic toxicity. Toxicity at 3 and 9 mg/kg was associated with increased incidence of spontaneous abortions (4/20 and 14/20, respectively). The increased rate of abortion is considered secondary to maternal toxicities. Treatment with high systemic doses of fomivirsen induced immune stimulation, and this class of compounds is known to induce the production of inflammatory cytokines. Cytokine release is associated with increases in the rate of abortions (98,99). In addition to abortions, maternal toxicity was associated with increased number of resorptions at 9 mg/kg/day, decreased fetal body weight at 3 and 9 mg/kg, decreased percentage of male fetuses at 3 and 9 mg/kg, and dilated ventricles of the brain in four fetuses at 9 mg/kg. No dilated ventricles in the brain were observed at a dose level of 3 mg/kg where there was moderate maternal toxicity. Exposure was assessed by analyzing tissues collected at the time of sacrifice 11 days after the last dose. Maternal target organ exposure was dose dependent, but no oligonucleotide was detected in placenta, fetal liver, or fetal kidney. Therefore, the increased frequency of abortion and other fetal alterations was considered secondary to maternal toxicity.

Data from mice support the concept that the effects on reproduction in the rabbit were secondary to severe maternal toxicity. In mice, no maternal toxicities were observed and there were also no effects on fertility or developmental toxicities. Estrus cycling, mating performance, and fertility were unaffected by treatment. Litter parameters were normal in all treatment groups and fetal abnormalities not observed. Evidence of maternal toxicity in mice treated with 9 mg/kg included changes in hematology parameters (decreased red blood cell and platelet

counts), increased organ weight (liver and spleen), extramedullary hematopoiesis in liver and spleen, and morphological changes in renal proximal tubular epithelium. Maternal toxicity at 9 mg/kg was consistent with dose-dependent exposure in liver and kidney that achieved concentrations as high as 25 and 70 μg/g, respectively. Oligonucleotide was measured in placenta, but uptake was low relative to other maternal tissues. Placental concentrations were approximately 0.6 and 2 μg/g at doses of 3 and 9 mg/kg, respectively. Minimal uptake of oligonucleotide by placenta limited fetal exposure. No oligonucleotide was detected in fetal kidney and concentrations were below 1 μg/g in fetal liver. Taken together, the lack of fetal exposure and the relationship between the degree of maternal toxicity and fetal outcome in mice and rabbits indicate that maternal toxicity is responsible for the increased incidence of abortion and fetal resorption and fetal toxicity. In the absence of severe maternal toxicity there were no effects on reproductive performance or development.

When a phosphorothioate oligodeoxynucleotide directed toward the developmentally significant gene, Sry, or control oligodeoxynucleotides were administered to pregnant mice at various stages of gestation at a dose of approximately 18 mg/kg/dose no abnormalities were observed (100). Part of the reason for a lack of direct effects of phosphorothioate oligonucleotides on the development appears related to pharmacokinetics. The pharmacokinetic analyses that accompanied the reproductive toxicity studies demonstrated that there was negligible exposure of the fetus to oligonucleotides. In fact, placental concentrations of oligonucleotide were also relatively low compared to maternal organs, a finding that was somewhat surprising considering the degree of vascularization and the pinocytotic activity of some cell types.

One hypothesis for this low level of exposure of the placenta and fetus was that the antisense oligonucleotide was being administered as a bolus dose and the rapid clearance from plasma of a bolus dose may minimize the opportunity for placental and subsequently fetal exposure. To maximize the potential for fetal exposure, ^{3}H-ISIS 2105 was administered either as a 3-h infusion of 6.6 mg/kg/h, total dose 20 mg/kg, or as a continuous 7-day infusion at 0.3 mg/kg/h, total dose 59 mg/kg. After a single 3-h infusion of 20 mg/kg the fetal plasma levels of radioactivity were less than 5 μg equivalents/mL compared to maternal >100 μg equivalents/mL of radioactivity. After 7 days of constant infusion fetal concentrations of radiolabel were 15–20% of those of maternal levels. Despite the fact that there was detectable radiolabel and measurable levels of oligonucleotide in the fetal circulation, the fetal organ levels were very low compared to maternal organ levels (Fig. 13). These data suggest that the exposure of the conceptus to oligonucleotide is very limited with this class of oligonucleotide.

Similar results were obtained in a series of experiments in which the efficacy and pharmacokinetics of a first-generation and second-generation oligonucleotide were compared. For the first-generation compounds (phosphorothioate

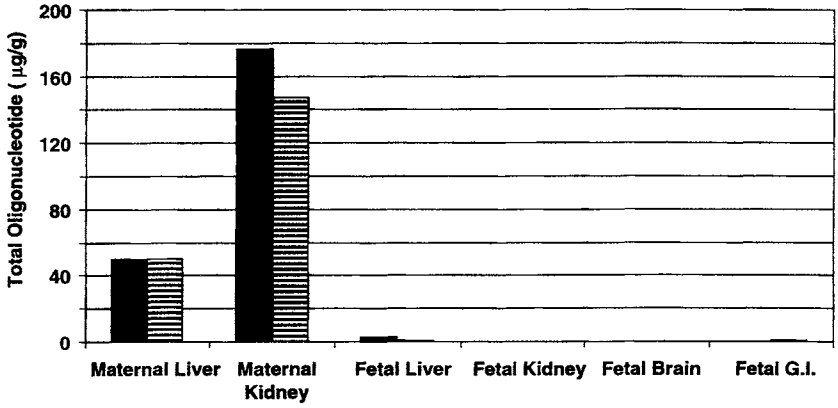

Figure 13 Maternal and fetal organ concentrations of total oligonucleotide after a 7-day infusion of radiolabeled ISIS 2105. Pregnant rats were implanted with an osmotic minipump and infused from gestation day 12–19. At the end of infusion the animals ($n = 5$) were sacrificed immediately (filled bars) or sacrificed 4.5 h after the removal of the pumps (hatched bars) to allow oligonucleotide to clear from the blood. The bars represent total oligonucleotide in µg/g determined by CGE. Fetal organs represent pooled organs.

oligodeoxynucleotides) targeted to VEGF there was no evidence of placental transfer and the oligonucleotide was essentially inactive, producing no developmental alterations. However, a single dose (18 mg/kg) of a second-generation oligonucleotide, modified by the addition of four 2′-*O*-methyl groups at the 3′ and 5′ termini, reduced target-gene expression in a developing mouse embryo using an antisense oligonucleotide targeted to VEGF (101). The down-regulation of gene expression was correlated with detection of 2′-*O*-modified oligonucleotide in embryonic tissues. The developmental abnormalities induced by treatment on gestational days 7.5–8.5 with this antisense compound to VEGF were reminiscent of the alterations in morphology observed in homozygous VEGF knockout mice. A five-base mismatch control 2′-*O*-methyl oligonucleotide was without effect at the same dose. For the active oligonucleotide, a dose-response relationship was apparent. At a dose of 20 mg/kg there was a reduction of 54% in VEGF expression.

Antisense technology can also be used as a tool to help understand the developmental process. After administration of an antisense oligonucleotide to E-cadherin, it was possible to knock down the expression and demonstrate a role for this gene in development in developing mice, something that was not possible because homozygous knockouts are lethal at the blastocoele stage (101). These

results demonstrate that one of the real key roles for antisense compounds outside of the therapeutic arena is as a tool to modulate gene expression for research purposes. The advantage of antisense is shown in these studies in which the ability to down-regulate genes at specific time periods is what differentiates antisense from knockout technologies. Thus, using antisense it is possible to examine the specific role of a gene at a specific time in development.

Antisense oligonucleotides have been employed by developmental biologists (102,103) in vitro to examine the role of specific genes in development (see section on research applications of antisense).

The absence of significant transplacental exposure with first-generation oligonucleotides and the apparent ability of second-generation oligonucleotides to cross the placenta and affect gene expression suggest that improvements in oligonucleotide design may markedly change the utility of these compounds. The extra durability of the second-generation compounds produces a more potent antisense oligonucleotide and this may allow antisense compound to be used in novel ways, for example to alter fetal gene expression for therapeutic utility.

F. Genetic Toxicology

A number of phosphorothioate oligonucleotides have been examined in a number of genotoxicity assays (Table 6). These studies have demonstrated that they appear not to be mutagenic or clastogenic in prokaryotic or eukaryotic cells. Additionally, in vivo assays have confirmed these reports.

1. *Salmonella–Escherichia coli*/Microsome Plate Incorporation Assay

The purpose of these studies was to evaluate the potential for phosphorothioate oligodeoxynucleotides to induce reverse mutations in bacteria as an indicator of genotoxicity. The protocol was similar to the standard Ames assay, where the test material is incubated with several different histidine-dependent strains of *S. typhimurium* (TA1535, TA1537, TA98, and TA100), and mutagenicity is assessed by the frequency of reversion to histidine independence. In addition, five of the six compounds were tested using a single *E. coli* tester strain, WP2 (tryptophan auxotroph). All incubations were performed with and without the addition of a mammalian metabolic activation fraction derived from rat liver (S9). Appropriate positive control mutagens were run for each tester strain. The six phosphorothioate oligodeoxynucleotides have been evaluated at concentrations ranging up to 5 mg/plate, which is the standard upper limit dose in this assay for a soluble and noncytotoxic compound. No increase from background mutation frequency was observed in any of the tester strains incubated with any of the six phosphorothioate oligodeoxynucleotides, and all were considered negative in this series of assays.

Table 5 Assessing the Impact of ICAM-1 Suppression by ISIS 3082 on Immune Function in Mice

	Dose of ISIS 3082		
Parameter	1 mg/kg	5 mg/kg	20 mg/kg
General immune stimulation			
Spleen weight	NC	NC	NC
Splenocyte population	NC	NC	NC
Humoral immune function			
Sheep RBC response	NC	NC	NC
Cellular immune function			
One-way MLR	NC	NC	NC
Two-way MLR[a]	+1	+1	+1
Cytotoxic T-cell activity	NC	NC	NC
DNFB contact hypersensitivity[a]	+1	+2	+2
ICAM-1 expression			
B cell, T cell, Neutrophil	NC	NC	NC
DNFB challenge site (ear)[a]	+1	+2	+2
Liver[a]	+2	+1	NC

NC, no change; +1 = mild; +2 = moderate.

[a] Score represents the relative magnitude change from baseline. The effect of ISIS 3082 in these cases was a decrease in measured response from baseline.

2. In Vitro Mammalian Cytogenetic Assay

The ability of phosphorothioate oligodeoxynucleotides to induce chromosomal damage in cultured Chinese hamster ovary (CHO) cells was evaluated as an indicator of potential clastogenicity in a mammalian cell line. Four oligonucleotides were tested at concentrations up to the standard limit of 5000 μg/mL. Both initial and independent repeat assays were performed. CHO cells were exposed to phosphorothioate oligodeoxynucleotides for periods of 18–44 h in the absence of liver S9. Exposure time with metabolic activation was 2–4 h, followed by growth periods of 12–40 h. A decrease of >25% in mitotic index (relative number of cells in mitosis versus number counted) was observed with cells incubated at concentrations > 1250 μg/mL in the absence of liver S9 compared to vehicle controls. No decrease in mitotic index was observed in the presence of liver S9 because of the shorter duration of exposure. Cell growth (percent of cells in the treatment flasks versus the control flasks) was assessed as an indicator of cytotoxicity in the most recent study (fomivirsen) and concentrations of 5000 μg/mL of phosphorothioate oligodeoxynucleotides without liver S9 typically decreased cell growth. However, no effect on cell viability as indicated by trypan blue exclusion was evident. In the presence of liver S9, no inhibition of growth occurred.

3. L5178Y/TK$^{+/-}$ Mouse Lymphoma Mutagenesis Assay

The mutation frequency following exposure of L5178Y/TK$^{+/-}$ mouse lymphoma cells to phosphorothioate oligodeoxynucleotides was evaluated in the presence and absence of liver S9. The concentrations evaluated in this assay ranged up to 5000 μg/mL. Three oligonucleotides were assayed and both compounds were relatively noncytotoxic (i.e., ~50% inhibition of cell growth relative to vehicle control). At the high concentrations (4000 and 5000 μg/mL) of phosphorothioate oligodeoxynucleotides, cell growth relative to controls was 56–143% and the presence or absence of a metabolic activation system (rat liver S9 homogenate) did not consistently alter cell growth. In the initial or the confirmatory trials with or without metabolic activation no increases in mutation frequency occurred in cultures incubated with phosphorothioate oligonucleotides ODNs relative to vehicle controls, while those cultures incubated with the appropriate positive control had significant increases in mutation frequencies. Under the conditions of this assay, both phosphorothioate oligodeoxynucleotides tested, fomivirsen and ISIS 2302, were concluded to be negative in the L5178Y/TK$^{+/-}$ mouse lymphoma mutagenesis assay.

4. In Vitro Rat Hepatocyte UDS Assay

One phosphorothioate oligodeoxynucleotides, ISIS 2105, was evaluated for its potential to induce unscheduled DNA synthesis (DNA repair) as an indicator of genotoxicity. The protocol was similar to the standard Williams assay, where the test material is incubated with freshly isolated rat hepatocytes for 18–20 h, and DNA damage is assessed by the net number of silver grains present (nuclear grains/cytoplasmic grains). Dimethyl benzanthracene was the positive control mutagen and significantly induced DNA repair. In a preliminary screen for cytotoxicity, relative cytotoxicity of 59% occurred at 5000 μg/mL of ISIS 2105 as indicated by leakage of LDH from cells. Microscopic observations of hepatocytes incubated with 5000 μg/mL also indicated a moderately high degree of toxicity. No cytotoxicity was observed at the lower concentrations (~1500 μg/mL). Concentrations of 150, 500, 1500, 3000, and 5000 μg/mL were subsequently evaluated for genotoxicity. Cytotoxicity was evident at 3000 and 5000 μg/mL. No induction of DNA repair was observed at any concentration of ISIS 2105, and this compound is considered *negative* in this assay.

5. Micronucleus Cytogenetic Assay in Mice

Four phosphorothioate oligodeoxynucleotides were evaluated in a murine model for the potential increase in micronucleated polychromatic erythrocytes. Male and female mice received a bolus dose of 175, 350, and 700 mg/kg ISIS 2105 or 145, 290, and 580 mg/kg ISIS 2922 by intravenous injection. With ISIS 2302,

male and female mice received 250, 500, and 1000 mg/kg (117). The third compound, ISIS 5132, had a pilot acute toxicity experiment using doses up to 2000 mg/kg. In this experiment, toxicity was determined at doses of 2000 mg/kg and greater and the definitive experiment used doses of 600, 800, and 1000 mg/kg. High doses were based on 80% of a determined $LD_{50/7}$ for the respective compounds. Bone marrow in all studies was collected from five mice/sex/time point at 24, 48, and 72 h following treatment with a single dose of phosphorothioate oligonucleotides ODNs. No significant increase in micronucleated polychromatic erythrocytes occurred from administration of phosphorothioate oligodeoxynucleotides. No effect on PCE or total erythrocytes were noted, which indicated no bone marrow toxicity. The positive control, cyclophosphamide, increased micronucleated PCEs in the bone marrow without a decrease in the PCE to total erythrocyte ratio. These results indicated that the four phosphorothioate oligodeoxynucleotides tested, fomivirsen, ISIS 2105, ISIS 2302, and ISIS 5132, were negative in an in vivo micronucleus assay.

6. Exposure of Cells to Oligonucleotides in Genotoxicity Assays

Although cytotoxicity or cytostasis was observed in vitro, because of the molecular weight of phosphorothioate oligonucleotides there has always been some question as to whether these compounds actually got into the test cells. To directly answer that question we performed a parallel series of experiments with ISIS 2302. The cellular uptake of ISIS 2302 was confirmed in separate experiments using the same cell lines and culture conditions. Using capillary gel electrophoresis, ISIS 2302 was measured in extracts of CHO cells, L5187Y cells, and *S. typhimurium* TA98 (117). Intracellular uptake was dependent on incubation concentration and time. In vivo, uptake in bone marrow of treated mice was both time- and dose-dependent. Intracellular localization in cell cultures and treated mice was further confirmed by immunohistochemical staining.

The absence of genotoxicity for this class of compounds is not surprising considering their intended mechanism of action. Antisense oligodeoxynucleotides are designed to interact with mRNA and are not able to interact with DNA. This selectivity is based on the binding affinity of phosphorothioate oligodeoxynucleotides for DNA, which is approximately 10-fold less than the affinity of single DNA strands for their complementary strand. Thus, phosphorothioate oligodeoxynucleotides are not likely to interact with genomic DNA. The pharmacological effect of antisense oligonucleotides is posttranscriptional. Binding of the oligonucleotide to its mRNA target results in inhibition of protein translation. Posttranscriptional effects should not alter the genome, and thus, would not be detected by the assays used. One remaining issue surrounding the genotoxicity testing pertains to the species specificity of antisense effects. The phosphorothio-

ate oligodeoxynucleotides tested are all specific for human or viral sequences, and it is therefore highly unlikely that complementary binding of these antisense compounds would occur in the rodent or bacterial models. Thus, in the genotoxicity assays performed to date, the genotoxic potential evaluated is that of the chemical class and should not vary with specific sequence.

The phosphorothioate oligonucleotides tested to date have possessed unmodified nucleoside and ribose constituents; therefore, unlike nucleoside or nucleotide analogs, incorporation of mononucleotides from the antisense compounds would not result in mutations or mismatches because the nucleoside/nucleotides are the same as the endogenous nucleosides and nucleotides. Phosphorothioate oligodeoxynucleotides are chemically similar to endogenous deoxynucleic acids (DNA), except for the substitution of a nonbridging oxygen in the phosphodiester linkage with sulfur. The sulfur substitution imparts nuclease resistance and allows for sufficient longevity in tissues for pharmacological efficacy. While the sulfur in these oligonucleotides may appear similar to phosphorothioate ester pesticides, the molecules in which they are contained are chemically very different. The primary metabolic pathway for degradation of phosphorothioate oligonucleotides appears to be exonuclease degradation (17,20,104). The sequential metabolism from the 3′ end results in successively shorter oligodeoxynucleotides with the released mononucleotides. These mononucleotides are likely reprocessed or further metabolized to CO_2 as indicated by Cossum et al. (21,22). Imbalances in nucleotide pools have been studied as a potential mechanism for affecting mutations. Several studies have addressed the issue of imbalances in nucleotide pools (105–107). Each has demonstrated that biasing nucleotide pools with an increase in an individual nucleotide at extraordinary concentrations, typically in the millimolar range, will alter the repair of induced mutations or DNA damage. While administration of phosphorothioate oligonucleotides may increase intracellular pools of nucleotides, the increase in pools will not be biased to a single nucleotide, but likely represent increases in two or more nucleotides. In addition, assessment of intracellular quantities of phosphorothioate oligonucleotides in the liver after administration of a dose to rodents demonstrates levels or concentrations estimated in an individual cell are in the femtomole range (108), which are substantially lower than doses required of a single nucleotide (millimole) to induce an effect of DNA fidelity (105,107). Thus, no bias in nucleotide pools is considered likely, and therefore, subsequent effects on DNA are considered even more remote.

The primary metabolic pathway for degradation of phosphorothioate oligonucleotides in vivo appears to be exonuclease degradation (17,104). This is the sequential metabolism from the ends of the oligodeoxynucleotide that results in successively shorter oligodeoxynucleotides with the release of mononucleotides. Both cells and liver homogenates have demonstrated metabolism similar to that observed in vivo (108,109). The mononucleotides produced from nuclease metab-

olism are likely reprocessed or further metabolized to CO_2 (21,22). The use of rat liver homogenate as the activation system in in vitro assays did not result in the induction of genotoxicity.

In this array of assays, phosphorothioate oligonucleotides or their metabolites exhibited no mutagenic activity; from these data it appears that as a class, phosphorothioate oligonucleotides pose no risk of genotoxicity. Note as well that ISIS 13312 (Table 1) represents a second-generation compound with 2′ modifications and it too was not genotoxic in the Ames test. This result bodes well for the second generation of modified oligonucleotides.

G. Research Applications of Antisense Oligonucleotides in Toxicology

The toxicology community-at-large is beginning to employ antisense oligonucleotides as tools to probe mechanisms of toxicity. As tools for toxicological research, antisense agents are proving to be of great utility for dissecting out important enzymes in activation or detoxification pathways, as well as modulating resistance or sensitivity to various toxicants. There are numerous examples in which investigators have applied this technology to understanding mechanisms: a few examples are summarized below.

A number of studies have been performed to modulate toxicity with antisense agents. In these cases it is not the toxicity of the oligonucleotide that is being examined, but oligonucleotides are being used in these experiments to reduce a target protein concentration to modulate the sensitivity of cells to other toxic insults. For example, inhibiting the stress protein Grp78 sensitized renal epithelial cells to oxidative stress induced by tert-butyl hydroperoxide (110), thus defining a role for this protein in cytoprotection to oxidative stress. Similarly, sensitivity to NO-induced toxicity was increased in PC-12 cells treated with an antisense inhibitor to Mn superoxide dismutase, suggesting a role for SOD in protection from NO toxicity. Taking this experiment one step further, cortical cells treated with the same inhibitor of MnSOD expression were more susceptible to NMDA-induced toxicity. Taken together, these experiments suggest a role for MnSOD and NO excitatory neurotoxicity (111).

In contrast to the above experiments, others have used antisense oligonucleotides to protect against toxicity. Didier et al. used antisense inhibitors to different zeta or epsilon subunits of the NMDA receptor in an attempt to define the subunits associated with excitatory neurotoxicity in cerebellar cultures (112). The requirement for uridine phosphorylase and thymidine phosphorylase in the cytotoxicity of 5-FU in colon tumor cell lines was also demonstrated with antisense oligonucleotide directed toward these targets (9).

Developmental biologists need tools for altering gene expression at specific times during gestation to understand the temporal importance of specific gene

Table 6 Summary of Genetic Toxicology Assays for the Various Phosphorothioate Oligodeoxynucleotides

Assay and intended target	ISIS 2105 HPV	ISIS 2302 ICAM-1	ISIS 2922 (fomivirsen) CMV	ISIS 3521 PKC-a	ISIS 5132 c-raf	ISIS 13312 CMV
Bacterial mutagenesis						
Salmonella	√	√	√	√	√	√
E. coli		√	√	√	√	√
In vitro assays						
In vitro cytogenetics	√	√	√		√	
Mouse lymphoma mammalian mutagenesis		√	√			
CHO/HGPRT mammalian mutagenesis	√					
In vitro rat hepatocyte UDS	√					
In vivo assay						
In vivo mouse micronucleus	√	√	√		√	

products. Sadler et al. used antisense control oligodeoxynucleotides as tools to knock out the expression of specific gene product, Engrailed-1 (En-1), in cultured mouse embryos (102,103). These experiments demonstrated that sequence-dependent alterations in developing mice embryos could be produced by a single intra–yolk sac injection of 15–25 μM oligodeoxynucleotide. At these concentrations no malformations were observed in embryos treated with control oligodeoxynucleotide. The malformations produced by this direct injection technique were reminiscent of the malformations observed in transgenic mice lacking En-1. Yolk sac injections in mouse embryos of an antisense phosphorothioate oligodeoxynucleotide (final concentration 25 μM) directed to retinol-binding protein produced apparent reductions in retinol, while a rearranged control oligodeoxynucleotide did not (113). These data suggest that phosphorothioate oligodeoxynucleotides can have activity in embryos when they are exposed directly at high concentrations and at the critical times of gestation. The effects on developing embryos were sequence specific.

In each of the examples mentioned above scientists have employed antisense modulation of gene expression as a tool to explore mechanisms of toxicity. As we begin to try and unravel the intricacies of gene–gene interactions and to employ microarray technology to dissect out pathways associated with toxic phenomena, it will become increasingly important to be able to modulate the expression of specific genes in a temporally controlled way. It appears that antisense technology is the most facile way to achieve this end.

II. SUMMARY

Significant progress in understanding the mechanisms of toxicity of phosphorothioate oligonucleotides makes these compounds even more useful as therapeutic agents. We now have a more complete understanding of the acute effects of this class of compound and with recent data we have developed a more thorough understanding of the nature of species differences and the biochemical or molecular basis for some of these differences. This understanding is making it possible to not only use the first-generation of compounds more safely and effectively, but our understanding of the limitations of the first generation of compounds is providing a basis for designing the next generations of antisense agents.

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Clinical Safety of Phosphorothioate Oligodeoxynucleotides

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I. INTRODUCTION

Antisense drugs consisting of modified DNA sequences have been under clinical evaluation for most of the past decade. More than 20 synthetic oligonucleotides have been administered to humans (1). These investigational drugs, the vast majority being phosphorothioate oligodeoxynucleotides, have been studied in patients with a variety of inflammatory, infectious, cardiovascular, and malignant diseases. Isis Pharmaceuticals, Inc. has performed clinical studies of nine oligonucleotides, eight of which are phosphorothioate oligodeoxynucleotides. Approximately 1800 subjects have participated in these clinical trials, including 1100 who received the drugs systemically as intravenous infusions. To date, drugs with phosphorothioate backbones have displayed very similar, benign clinical safety profiles. The high degree of similarity in both clinical safety findings and preclinical toxicology studies of the dozen or so phosphorothioate oligonucleotide sequences that have been evaluated in humans suggests the major toxicities of these compounds are due to their common chemical structure rather than their specific nucleotide sequences. Toxicities related to inhibition of messenger RNA (mRNA) expression, either of the specific targets of the oligonucleotides or of nontarget mRNA, have not been described for any of these drugs to date.

Preclinical toxicology and pharmacokinetic studies of phosphorothioate oligodeoxynucleotides administered systemically, primarily to mice and monkeys, identified several potential toxicities for these compounds (2). Administration of these negatively charged, modified DNA sequences was shown to produce changes in coagulation parameters, specifically inhibition of the intrinsic tenase

complex (3,4), and to have the potential to activate the alternative pathway of complement (5–7). The potential for cardiovascular effects secondary to the latter was of concern in early clinical studies of phosphorothioate oligodeoxynucleotides. In addition to these acute effects, administration of phosphorothioate oligodeoxynucleotides produced two subchronic effects in the preclinical studies. First, the compounds distributed highly to liver and kidney in animal models and the accumulation of high concentrations of drug in these organs was associated with minor histological changes. These preclinical findings suggested the potential for hepatic or renal toxicity in humans. Second, nonspecific stimulation of the immune system was observed, primarily in rodents, with little to no effect in monkeys. The immune stimulation resulted in splenomegaly and the enlargement of liver and lymph nodes accompanied by immune cell infiltration of those organs.

These four toxicological effects occur following systemic administration of all phosphorothioate oligodeoxynucleotides examined to date regardless of the nucleotide sequence of the compounds indicating the effects are predominantly due to the sugar-phosphorothioate backbone of the oligonucleotide. Minor quantitative differences in these effects between different members of the chemical class have been observed. Longer oligonucleotides, i.e., those containing more phosphorothioate linkages, are more potent in producing some of the effects than shorter ones (2). Minor differences in plasma protein-binding affinity have also been observed between different oligonucleotides of the same length. These differences suggest that oligonucleotide sequence may quantitatively influence the toxicity profile of individual class members. Sequence differences associated with immune stimulation have been observed (8). Characterization of optimal nucleotide sequences for immune stimulation demonstrates that sequence-dependent effects are possible (9,10).

However, sequence-dependent qualitative differences in the toxicity profiles between different phosphorothioate oligonucleotides have not been observed. Targeting of mRNA for gene products essential for the survival of normal cells has been avoided in the development of antisense drugs. In addition, there have long been concerns about the potential for oligonucleotide binding to nontarget mRNA sequences or DNA sequences in the cell genome that are similar or identical to the target sequence. To date, toxicity of phosphorothioate oligodeoxynucleotides, which would suggest the occurrence of hybridization of the antisense oligonucleotide to nontarget sequences, has not been observed in preclinical or clinical studies for any of the drugs that have been studied.

Of the four toxicological effects identified in preclinical studies, only the effect on coagulation as evidenced by transient prolongation of activated partial thromboplastin time (aPTT) has been routinely observed in clinical studies of phosphorothioate oligodeoxynucleotides. The aPTT abnormalities have been modest, dependent upon dose and schedule of administration, have resolved

within a few hours of the end of intravenous infusion, and have not been associated with clinical sequelae. The main side effects seen in the clinical studies of phosphorothioate oligodeoxynucleotides include fever, chills, nausea, vomiting, facial flushing, flu symptoms, and fatigue. Changes in complement values and thrombocytopenia have been affected by both dose and schedule.

II. VOLUNTEER STUDIES

Volunteer studies with phosphorothioate oligodeoxynucleotides have been limited in number. Most antisense drugs have been studied initially in patients with the disease for which the drug is being developed. Isis Pharmaceuticals has conducted placebo-controlled, volunteer studies with ISIS 2105, an inhibitor of human papilloma virus, and ISIS 2302, an inhibitor of intercellular adhesion molecule-1 (ICAM-1).

A. ISIS 2105—An Inhibitor of Human Papilloma Virus

An early Phase I study in healthy subjects was conducted with ISIS 2105. In this study, drug was injected intradermally three times on alternate days into forearm skin. This drug and its route of administration were intended as a treatment for genital warts. However, intradermal administration of ^{14}C-labeled drug resulted in rapid absorption and distribution from skin into the systemic circulation (11,12). The studied dose levels caused no systemic toxicities but local side effects were seen at injection sites in all drug-treated subjects. None of the placebo-treated subjects exhibited the local side effects. Lower doses resulted in erythema but intradermal doses of greater than 5 mg/kg of ISIS 2105 per skin site caused desquamation, erosion, and ulceration, all of which healed spontaneously. Biopsy of the affected injection sites in two subjects revealed a dense inflammatory reaction, with T- and B-lymphocyte involvement. These findings are indicative of the nonspecific immune stimulation caused by this class of drugs, which is probably mediated by cytokine release. This unintended effect of oligonucleotides is being exploited for its potential immunoadjuvant role in a variety of indications.

B. ISIS 2302—An Inhibitor of Intercellular Adhesion Molecule 1

Placebo-controlled, healthy volunteer studies were conducted for the initial evaluations of both intravenous and subcutaneous administration of ISIS 2302. In the intravenous dose-escalation trial, ISIS 2302 was initially administered as a single 2-h infusion to three of four subjects per cohort at dose levels ranging from 0.06 mg/kg, based on the ISIS 2105 experience, to 2 mg/kg. The tolerability of four

doses given every other day was then evaluated at dose levels ranging from 0.2 to 2 mg/kg (13). Preclinical studies of ISIS 2302 had shown consistent effects on aPTT, complement activation leading to cardiovascular effects and proximal tubule epithelial necrosis at high doses (7,14). Thus, intensive monitoring of vital signs, electrocardiogram, clotting function, neutrophil count, complement split products C3a and C5a, and urinary microproteins was performed.

The study showed a clean safety profile. A consistent and dose-related increase in aPTT was seen in subjects who received single and multiple doses of ISIS 2302 from 0.5 mg/kg to 2.0 mg/kg. The maximum increase in aPTT was generally seen at or near the end of the 2-h infusion, coinciding with peak plasma drug concentrations. Activated PTT values returned to baseline levels between 2 and 4 h following the end of the infusions. The maximum median percentage increase in aPTT, expressed as percent of control, was 47% in the 2 mg/kg dose group. There was no evidence of exaggeration or attenuation of this effect with repeated dosing of up to four doses. Smaller increases in prothrombin time and thrombin time were seen in subjects who received 1.5 and 2 mg/kg ISIS 2302, but these were more variable and less clearly dose-related. The drug did not affect bleeding times. These anticoagulant effects were mild and transient and thus do not pose significant risk to future trial patients with normal clotting function. The potential risk for patients with abnormal baseline clotting function exists, however, and these patients have generally been excluded from clinical trials of drugs with phosphorothioate backbones.

The observation of complement activation in nonhuman primate studies led to careful monitoring of complement values in this trial. Complement split product C5a values remained unchanged by ISIS 2302 administration. The levels of complement split product C3a were unaffected by single doses of ISIS 2302. However, a small increase in the median C3a level was seen after the fourth dose of 1 mg/kg and the third and fourth doses of 2 mg/kg, occurring 1–2 h after the beginning of infusion, and recovering spontaneously within a few hours thereafter. These laboratory findings were not considered to have clinical importance. Continuous electrocardiogram monitoring and frequent measurements of blood pressure, heart rate, and neutrophil count during and for 2 h following each infusion did not detect any of the changes associated with complement activation in preclinical studies.

Some drug-treated subjects showed increases in liver enzymes above the normal range. These were matched by increases in the same enzymes (ALT and AST) in placebo-treated subjects. Both drug- and placebo-treated subjects showed decreases in red blood cell indices, as expected, due to intensive blood sampling, but there was no indication of drug-related hematological toxicity. No local side effects were seen at injection sites and the only symptom that occurred with a higher frequency in drug-treated than placebo-treated subjects was headache.

In contrast, when single and multiple subcutaneous injections or continuous subcutaneous infusion of ISIS 2302 was administered at dose levels up to 2 mg/kg in healthy male volunteers, there were significant local toxicities. The major problems experienced were inflammation at the injection/infusion site and marked lymphadenopathy. Injection site inflammation was observed in all drug-treated subjects at all doses. This was similar to that seen with intradermal administration of ISIS 2105. Lymphadenopathy in draining nodes of injection sites occurred at the 1 mg/kg and 2 mg/kg doses and was painful in some patients. It was also associated with peaks in plasma C3a levels after multiple doses and was considered dose limiting (15).

The amount of inflammation at injection sites appeared to be related to dose level and schedule. Continuous infusion of drug exacerbated the inflammatory effect compared to bolus injection of the same dose level. In the worst cases, painful erythema and induration reached 7 cm in diameter and lasted up to 2 weeks. No local inflammatory effects were seen when placebo was infused using the same technique.

Warm, tender lymphadenopathy was seen in the inguinal nodes that drain thigh injection sites in subjects who received subcutaneous injections of 1 and 2 mg/kg ISIS 2302 repeated every other day and in those who received 0.5 mg/kg/day by 6-day continuous subcutaneous infusion. It was postulated that this was caused by high local concentrations of cytokines, related to injection site inflammation. It is also possible that locally high concentrations of drug caused complement activation. Higher peaks in C3a were seen after multiple subcutaneous doses than by the same doses administered intravenously. After subcutaneous dosing, low-grade fever was experienced by some subjects. A higher incidence of flu-like symptoms (myalgia, malaise, and anorexia), headache, nausea, and vomiting was also observed in the drug-treated groups compared to the placebo-treated group.

III. DOSE- AND SCHEDULE-RELATED SAFETY PROFILE OF OLIGONUCLEOTIDES

The most aggressive assessment of phosphorothioate oligodeoxynucleotide dose and schedule of administration has been in studies of patients with cancer. In these studies, Isis Pharmaceuticals has conducted dose-escalation trials of three antisense drugs using three different schedules. To avoid the toxicities predicted to occur with high plasma concentrations of these drugs, intermittent and continuous intravenous infusions have been studied. ISIS 3521, an inhibitor of protein kinase C-α expression, and ISIS 5132, an inhibitor of C-Raf kinase expression, have each been studied using 2-h infusions given three times per week for 3 weeks at doses ranging from 0.15 to 6 mg/kg/infusion (16–18). These two drugs

have also been evaluated at doses ranging from 0.5 to 3 mg/kg/day for ISIS 3521 and 0.5 to 5 mg/kg/day for ISIS 5132 by 3-week continuous intravenous infusion (19,20). ISIS 2503, an inhibitor of H-Ras expression, has been evaluated as a 14-day continuous intravenous infusion at doses ranging from 1 to 10 mg/kg/day (21). Finally, weekly 24-h infusions at doses ranging from 3 to 30 mg/kg/day of dosing have been studied for all three drugs (20,22,23). In all of these studies, drug treatment was repeated as long as the treatment was tolerated, the patient's disease did not progress, and the patient chose to continue to participate in the study. Aside from the studies evaluating the weekly 24-h infusions, patients were given a 1-week rest period between regimens. A number of patients have been treated for three or more cycles of dosing. The greatest number of treatment cycles given was 17 to a patient in the study of ISIS 3521 administered as a thrice-weekly 2-h infusion for 3 weeks, repeated every 4 weeks (16).

A. Complement

As previously described, preclinical studies showed that the administration of phosphorothioate oligodeoxynucleotides activates the alternative pathway of complement when the plasma concentration of these drugs exceeds a threshold concentration. Above the threshold concentration, about 40–50 μg/mL for intact oligonucleotide and 60–70 μg/mL for total oligonucleotide, complement split products are detected in plasma during infusion and for 2–4 h after infusion. The occurrence of complement split products was generally transient with peak levels at the time of peak plasma concentrations of the drugs. Split-product levels rapidly decline as the plasma concentration of oligonucleotide declines following the end of infusion. Bolus intravenous injections of phosphorothioate oligodeoxynucleotides that result in high peak plasma concentrations of these drugs have, in some animals, been associated with severe hypotension and cardiovascular collapse. For this reason, clinical studies of phosphorothioate oligonucleotides have employed routes and schedules of administration that avoid peak plasma concentrations greater than the threshold concentrations identified in monkeys.

When ISIS 5132 was given by 2-h infusions at 0.5 to 6.0 mg/kg there was no effect on the C5a anaphylatoxin or Bb complement split product levels. There was a minor trend toward an increase in C3a levels with increasing dose level (Fig. 1). Dose escalation in this trial was discontinued, despite the absence of dose-limiting toxicity, based on the likelihood of exposing patients to drug plasma concentrations associated with complement activation in monkeys (18). Similarly, administration of ISIS 3521 by 2-h infusion at doses of 0.15–6.0 mg/kg produced no significant effect on C5a levels and only minor effects on C3a levels (16).

In the continuous-infusion studies of ISIS 3521 administered for 21 days at doses ranging from 0.5 to 3.0 mg/kg/day, of ISIS 5132 given for 21 days at

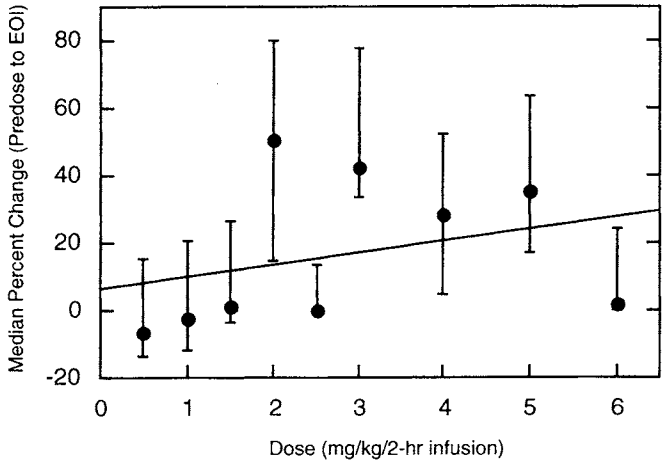

Figure 1 Effect of ISIS 5132 administered as 2-h intravenous infusions on the level of complement split product C3a. For each patient, C3a levels were determined before and at the end of each infusion during the first week of the first treatment cycle and the first infusion of the second treatment cycle and the percent change from the predose value was calculated for each infusion. Since no evidence enhanced or diminished response following repeated administration was observed, the results from all infusions were pooled for calculation of the median value. The bars indicate the 25th to 75th quartile ranges.

doses ranging from 0.5 to 5 mg/kg/day, and of ISIS 2503 infused for 14 days at doses ranging from 1.0 to 10 mg/kg/day, no consistent or dose-related changes in complement split-product levels were noted (20,24,25). Further dose escalation in the study of ISIS 3521 administered by continuous infusion was halted because of dose-limiting fatigue and thrombocytopenia at 3 mg/kg/day; subsequent studies of ISIS 3521 by this schedule have been conducted using a dose of 2 mg/kg/day. In contrast, further dose escalation was terminated in the trials of ISIS 5132 and ISIS 2503 in the absence of dose-limiting toxicities. These studies were discontinued because the doses reached were in excess of those predicted by preclinical pharmacology studies to distribute effective drug concentrations in tumors. No changes in complement were observed in the trials evaluating continuous infusion for 2–3 weeks of ISIS 3521, 5132, or 2503.

Substantially larger increases in the levels of C3a and Bb were observed in the studies of these three drugs given by 24-h infusion (21–23). ISIS 3521 was administered at doses ranging from 6 mg/kg to 24 mg/kg, ISIS 5132 at doses from 6 mg/kg to 30 mg/kg, and ISIS 2503 at doses from 3 mg/kg to 24 mg/kg. The results for ISIS 5132 are representative of those obtained for the other two

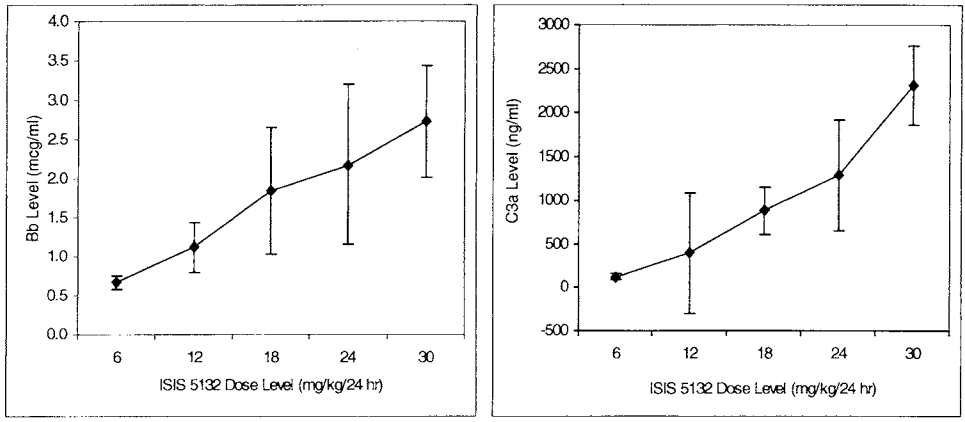

Figure 2 Effect of administration of ISIS 5132 by 24-h infusion on complement split products Bb and C3a.

phosphorothioate oligodeoxynucleotides (Fig. 2). Of the two complement split products, C3a showed the greater increase, from a mean end-of-infusion level of 122 ng/mL for the 6 mg/kg infusions to 2316 ng/mL for the 30 mg/kg dose. The mean predose levels for the different dose groups ranged from 109 to 278 ng/mL. For Bb, the mean end-of-infusion level rose from 0.67 μg/mL at 6 mg/kg to 2.73 μg/mL at 30 mg/kg while the mean predose levels varied from 0.68 to 1.22 μg/mL.

Despite these relatively large increases in the levels of complement split products, clinical evidence of complement activation in these studies was limited and was primarily observed in patients treated with the higher doses. One patient treated with ISIS 5132 at 30 mg/kg developed a fever of 39.5°C and a Coombs positive hemolytic anemia during her first infusion. She had no evidence of cardiovascular or renal compromise and the event resolved following cessation of treatment and packed red blood cell transfusion. Another patient, also treated with ISIS 5132 at 30 mg/kg, developed acute renal failure and anasarca. This patient also had rapidly progressive colon cancer so that a clear attribution of the patient's toxicity to drug could not be ascertained.

The results from these 24-h infusion studies are not consistent with a simple direct correlation between threshold plasma concentration and complement activation. Steady-state plasma concentrations were reached by 4 h after the start of the 24-h infusions and these concentrations were well below the peak plasma concentrations achieved by a 2-h infusion of the same drug at a dose of 6 mg/

Table 1 Comparison of C_{max}/C_{ss} of Intact ISIS 3521 in μg/mL Given by Three Different Schedules

2-hr infusion		21-day infusion		24-hr infusion	
Dose level	C_{max}	Dose level	C_{ss}	Dose level	C_{ss}
3	16.0 ± 7.81	0.5	0.18 ± 0.09	6	2.24 ± 1.01
4	13.2 ± 1.04	1	0.24 ± 0.02	12	5.66 ± 1.74
5	25.7 ± 7.58	2	0.50 ± 0.13	18	9.12 ± 2.49
6	29.4 ± 8.33	3	1.26 ± 0.15	24	11.8 ± 0.30

Values are expressed as mean ± standard deviation. Dose level is in mg/kg.

kg (Table 1) (16,19). Yet, no clear effect on complement split-product levels was observed when either ISIS 3521 or ISIS 5132 was given by 2-h infusion.

These findings have led to a reassessment of the relationship between peak plasma concentration of phosphorothioate oligodeoxynucleotides and activation of the alternative complement pathway. The mechanism of complement activation by phosphorothioate oligodeoxynucleotides is complex. Complement activation by phosphorothioate oligodeoxynucleotides is species specific and occurs in humans and monkeys, but not in dogs, mice, rats, or rabbits. In vitro experiments suggest humans are also less sensitive to complement activation than monkeys. The differential effects among species may be explained by a differential biphasic concentration response of these drugs on complement. The mechanism for complement activation in monkey and human serum appears to involve the interaction of the oligonucleotide with Factor H, which interferes with the regulatory function of Factor H. However, the mechanism for this apparent biphasic effect in humans has not yet been elucidated (26).

A biphasic response to phosphorothioate oligonucleotides is unlikely sufficient, however, to explain the observed differences in complement activation among the various doses and schedules studied since comparable plasma concentrations can be achieved between the 2-h infusion and 24-h infusion schedules using different dose levels (Table 1). Yet, increased complement split products are observed with the 24-h infusion and not with the 2-h infusion. Further evaluation of dose and infusion schedules will be required to help clarify the relationship between pharmacokinetics and complement activation.

An alternative explanation, such as cytokine stimulation, may explain the inconsistent results on complement noted among the various schedules (27–29). As shown in Table 2, three patients treated with ISIS 3521 at 24 mg/kg/24 h had substantial increases in IL-6, IL-1Rα, and IL-1β with an apparently smaller increase in TNF-α. Patients treated with this schedule frequently had symptoms

Table 2 ISIS 3521 Serum Cytokine Levels When Given by 24-hr Infusion at 24 mg/kg[a]

Cytokine	Predose (range)	Postdose (range)
TNF-α (pg/mL)	4 (0–12)	15 (0–44)
IL-6 (pg/mL)	45 (0.6–121)	210 (169–239)
IL-1Rα (pg/mL)	42 (0–127)	892 (205–1854)
IL-1β (pg/mL)	335 (300–394)	5719 (5391–5971)

[a] The limit of detection is 0 pg/mL for IL-1β, TNF-α, and IL-6. The limit of detection for IL-1Rα is 4 pg/mL. The values represent the mean for three patients based on the maximum value at 4, 8, or 24 h following the start of the infusion.

consistent with cytokine release with flu symptoms including fever, myalgias, fatigue, headache, and chills, particularly at the higher dose levels studied. Thus, although the plasma concentration threshold relationship may still hold, other factors may be involved when these drugs are given at higher dose. Very similar changes in these cytokines were observed in patients treated with ISIS 2503 at 18 mg/kg and 24 mg/kg and with ISIS 5132 at 24 mg/kg and 30 mg/kg. Cytokine values were not determined at lower doses.

B. Coagulation

As previously described, these studies showed that the prolongation of aPTT was proportional to the concentration of oligonucleotide in plasma. The greatest prolongation in these studies occurred at the end of the 2-h infusion and aPTT rapidly returned to normal following cessation of dosing. Repeated dosing did not affect the magnitude of this effect.

Similar effects of phosphorothioate oligodeoxynucleotide administration on aPTT have been observed in clinical studies of these drugs. As with the preclinical studies, the increases in aPTT have been proportional to the plasma concentration of oligonucleotide. Activated aPTT values return to normal following cessation of drug administration. Figure 3 shows the dose dependence of this effect for ISIS 5132 administered as a 2-h infusion. In contrast, prothrombin time is minimally affected at the 6 mg/kg dose. Similar results have been obtained with all other phosphorothioate oligodeoxynucleotides examined. The change in aPTT has not been clinically significant. Clinical signs of impaired coagulation such as bruising or hemorrhaging have not been observed in conjunction with the transient increases in aPTT. There were no changes in bleeding times in volunteers during treatment with ISIS 2302 (13).

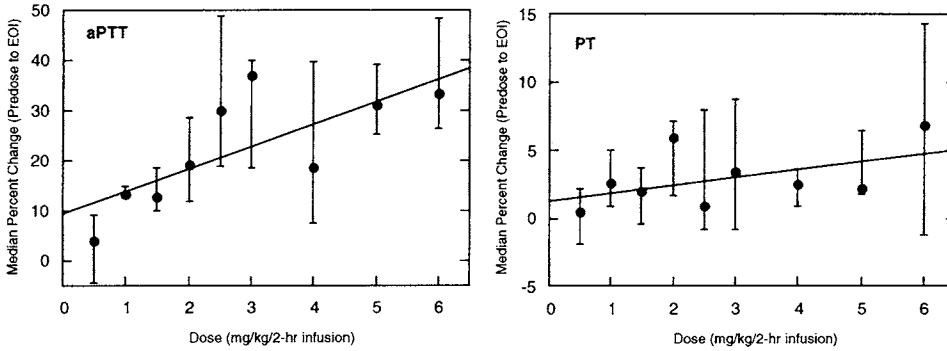

Figure 3 Effect of ISIS 5132 administration of activated partial thromboplastin time and prothrombin time. For each patient, aPTT and PT values were determined before and at the end of each infusion during the first week of the first treatment cycle and the first infusion of the second treatment cycle and the percent change from the predose value was calculated for each infusion. Since no evidence enhanced or diminished response following repeated administration was observed, the results from all infusions were pooled for calculation of the median value. The bars indicate the 25th to 75th quartile ranges.

C. Thrombocytopenia

Thrombocytopenia was the most prominent toxic effect, occurring in about one-third of the patients, in those studies where phosphorothioate oligodeoxynucleotide was administered as a 21-day continuous infusion (Table 3), although it was rarely to a sufficient degree to necessitate dose reduction or require medical intervention. In the phase I, dose-escalation trial of ISIS 3521 by this schedule, two of the six patients treated at 3 mg/kg/day experienced thrombocytopenia. One had grade 4 thrombocytopenia ($<25{,}000/mm^3$) with bruising and one had

Table 3 ISIS 3521: Thrombocytopenia for Patients in Single-Agent Continuous-Infusion Trials (n = 143)

Dose (mg/kg/day)	# Patients treated	Number of patients by highest grade				
		0	I	II	III	IV
≤1.5	9	4	3	2	0	0
2.0	122	76	25	13	6	2
3.0	12	6	3	1	1	1

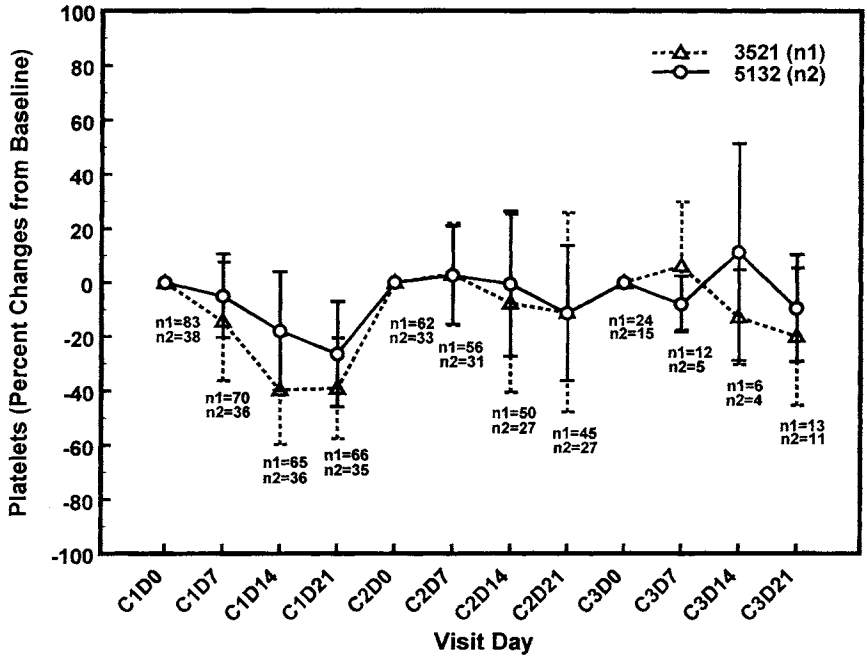

Figure 4 Mean percent changes of platelets from baseline during three cycles of 21-day continuous infusion of 3521 and 5132.

grade 4 bleeding associated with grade 2 thrombocytopenia ($<75{,}000/mm^3$). Grade 2 or grade 3 ($<50{,}000/mm^3$) thrombocytopenia was also observed at lower dose levels, including 0.5 mg/kg/day, the lowest dose level studied. Patients typically reached their platelet nadir during the second or third week of the infusion (Fig. 4), and in several cases platelet counts improved in the face of continued infusion (19). In the analogous trial of ISIS 5132, mild to moderate thrombocytopenia was observed at all dose levels above 0.5 mg/kg/day and was not clearly dose-related. Two episodes of severe thrombocytopenia were attributable to bacteremia or sepsis syndrome during treatment with ISIS 5132. One of the four patients treated at 4 mg/kg/day had moderate thrombocytopenia (grade 2; 50,000 to $75{,}000/mm^3$); no other drug-related toxicity was observed among the four patients treated at this dose level (16). As with ISIS 3521, thrombocytopenia observed with ISIS 5132 was transient, sometimes recovering during continued therapy or in most cases after the scheduled week off treatment. Clinically significant bleeding was minimal and, with the exception of a patient with a nosebleed, did not appear to be drug-related.

The occurrence of thrombocytopenia was substantially less frequent yet more clearly dose related in the 14-day continuous infusion studies of ISIS 2503 and the weekly 24-h infusion studies of the three drugs. The episodes observed were largely mild to moderate in intensity and mainly occurred at the highest dose levels in these studies, 8 and 10 mg/kg/day for the 14-day infusion study and 24 and 30 mg/kg for the 24-h infusion studies. In the 24-h infusion studies, the thrombocytopenia was observed at the end of infusion and was transient; platelet counts returned to baseline prior to the next scheduled infusion. Thrombocytopenia in the 2-h infusion studies was infrequent, sporadic, and not clearly dose related. These results suggest that thrombocytopenia is schedule dependent but may be dose dependent as well.

The mechanism for the thrombocytopenia observed in the 21-day continuous-infusion studies remains unclear. This side effect appears to be related to the phosphorothioate linkages in the oligonucleotide backbone rather than inhibition of a specific target. The observed thrombocytopenia has been fairly quickly reversible following cessation of therapy, and thus is unlikely to be antibody mediated. Unfortunately, only one bone marrow biopsy (in a patient receiving ISIS 3521) has been obtained to further evaluate this toxicity. Decreased megakaryocytes were noted. However, this patient had received extensive prior marrow toxic chemotherapy and pretreatment megakaryocyte numbers were not available for comparison purposes. In general, thrombocytopenia occurred primarily in patients with low normal baseline values, presumably as a consequence of prior chemotherapy. Limited platelet aggregation studies have been performed in patients treated with ISIS 3521. The results of these studies did not suggest a mechanism for this side effect.

Nonclinical studies in mice and monkeys have not provided significant insight into the mechanism for the thrombocytopenia observed in humans. Although thrombocytopenia accompanying administration of phosphorothioate oligodeoxynucleotides is observed in both of these species, the genesis of the effect appears to be different. In mice, thrombocytopenia appears to be due to sequestration of platelets secondary to immune stimulation-induced enlargement of the spleen, liver, and other organs. Rodents are particularly sensitive to the immunostimulatory effects of phosphorothioate oligodeoxynucleotides. Primates do not have an immunostimulatory response similar to mice. Thus, thrombocytopenia in monkeys appears to have a different basis. In monkeys, platelet levels decline gradually during the infusion of oligonucleotide and then rebound to normal levels quickly after the end of infusion. Neither the mouse nor the monkey effects provide simple explanations for the thrombocytopenia observed in the 21-day continuous-infusion studies performed in cancer patients. Furthermore, it is possible that the mechanism for changes in platelet count in patients treated by 21-day continuous infusion may differ from that in patients treated by weekly 24-h infusion. Thrombocytopenia has been reported to be associated with admin-

istration of recombinant TNF-α/IFN-γ in association with complement activation (28). G3139, an inhibitor of bcl-2, given by continuous subcutaneous infusion for 14 days appeared to have a clear relationship between plasma concentration and risk of thrombocytopenia. Five of five patients with plasma steady-state concentrations of 4 μg/ml or greater had platelet count nadirs of fewer than 50,000/mm^3. One of 12 patients with a steady-state concentration under 4 μg/ml developed thrombocytopenia of similar severity (30).

D. Renal and Hepatic Toxicity

In preclinical studies, the kidneys were found to retain the highest concentration of phosphorothioate oligodeoxynucleotides and the liver contained the highest amount of oligonucleotide. In addition to the appearance of basophilic granules, shown to be drug deposits, in Kupffer cells and renal tubular epithelial cells, histopathological changes have been observed in these two organs. Although these changes occur only at dose levels well in excess of those employed in clinical trials, patients in the initial clinical trials of phosphorothioate oligodeoxynucleotides were intensively monitored for signs of renal and hepatic toxicities. For signs of kidney impairment, standard laboratory tests as well as more esoteric parameters such as urinary retinol-binding protein have not detected any acute injury.

Clinical renal toxicity related to drug accumulation has not been observed in the trials of ISIS 2302, 3521, 2503, or 5132. Mild changes in renal function, as determined by slight increases in urea and creatinine, were reported for G3139. These biochemical changes were more common at doses of 147.2 mg/m^2 or higher but resolved after the completion of treatment (30).

The risk of hepatic toxicity in patients with normal liver function at the onset of treatment appears to be quite small for doses below 6–10 mg/kg. Reports of apparent drug-related hepatic toxicity have emerged for two antisense drugs. A phosphorothioate, 2′-methoxyoligonucleotide gapmer, that targets the regulatory subunit α of type I protein kinase A resulted in dose-related increases in liver function tests when given as a 2-h infusion twice per week at doses of 240 mg/m^2 and 360 mg/m^2. Doses $\leq$160 mg/m^2 produced no changes in alanine aminotransferase levels while Grade 3 toxicity was observed in three of six and three of three patients treated at the two higher doses, respectively. This suggests that this novel chemistry or the target may have greater potential for hepatic toxicity (31). In a Phase I study of MG98, also a phosphorothioate 2′-methoxyoligodeoxynucleotide gapmer that targets DNA methyltransferase, two of two patients treated at 240 mg/m^2 developed Grade 3 hepatic toxicity but not at lower doses when given by 21-day continuous infusion (32). In a study of G3139, an 18-base phosphorothioate oligodeoxynucleotide, no liver toxicity was reported at doses up to 195.8 mg/m^2 by continuous subcutaneous infusion for 14 days (30). How-

ever, when given as a 14–21-day continuous intravenous infusion in combination with paclitaxel, transaminitis was reported to be dose limiting at 6.9 mg/kg/day (33). Thus, it is difficult to conclude that target- or chemistry-related toxicity is responsible for these observations.

The potential for increased hepatic toxicity in patients with baseline abnormalities of serum ALT has also been examined. A double-blind, placebo-controlled study of ISIS 2302 was recently completed for the treatment of Crohn's disease in which patients were randomized to receive placebo for 4 weeks, a 2-week schedule of ISIS 2302 followed by 2 weeks of placebo, or a 4-week schedule of drug. The dose given was 2 mg/kg in both the treatment arms. For the purposes of this analysis, patients receiving 4 weeks of ISIS 2302 are compared to those receiving 4 weeks of placebo. A total of 101 patients received placebo and 99 received ISIS 2302. In this study, patients were eligible with normal ALT values or values up to 2.5 times normal. Thirteen placebo and 10 treated patients had elevated ALT values at baseline. Of these, none of 13 placebo patients and one of 10 treated patients had an increase in ALT to above 2.5 times normal. Among the remaining patients with normal baseline ALT values, nine of 88 placebo patients had increases in ALT to above-normal values while 15 of 89 treated patients developed ALT values to above-normal values. One of these 15 patients had an increase in ALT between 2.5 and 5 times normal during treatment (Grade 2 toxicity).

Liver and other potential toxicities have also been evaluated in 154 cancer patients treated with ISIS 3521 or ISIS 5132 according to the schedules described above. Eligibility for these studies allowed patient entry with baseline ALT values up to 2.5 times normal (Grade 1 value by NCI Common Toxicity Criteria) or between 2.5 and 5 times normal (Grade 2 value by NCI Common Toxicity Criteria). Of 119 patients with normal ALT at baseline, 28 had Grade 1 values during the first cycle of treatment and one had an increase to Grade 2. Thirty-one patients had Grade 1 values at baseline. Nine and two of these patients had Grade 2 and Grade 3 toxicity, respectively. Both patients with Grade 3 toxicity (ALT $>$ 5–10 times normal) had progressive liver metastases. Zero of four patients with Grade 2 ALT at baseline developed more severe liver toxicity. There was not a control group for comparison of the frequency of liver function test abnormalities in the cancer studies.

Thus, the evidence that liver toxicity may be associated with oligonucleotide therapy is not straightforward. Hepatic toxicity may be dose related although hepatic toxicity has not been seen with ISIS 2503 when given at doses as high as 10 mg/kg/day for 14 consecutive days; this dose is comparable to a dose of approximately 360 mg/m^2 (25).

Other parameters of toxicity, such as prolongation of aPTT or thrombocytopenia, do not appear to be more frequent or severe in patients with abnormal

ALT at baseline. Similar analyses were conducted for patients with elevated AST at baseline and for patients with either transaminase abnormal. These drugs did not appear to have greater toxicity in patients with abnormal liver function tests.

The pharmacokinetic behavior of ISIS 3521 and ISIS 5132 for patients with elevations in serum transaminases at study entry was compared to those with normal enzyme levels. Two patients treated with ISIS 3521 in the Phase I study of the 2-h infusion schedule had elevated serum transaminase concentrations (ALT $>$ 75 or AST $>$ 83 prior to drug administration) at study entry. The pharmacokinetic data from these patients were compared with those of patients with normal ALT and AST levels. Maximum plasma concentrations measured at or near the end of the 2-h infusion and the AUC were not different in patients with altered hepatic function (Fig. 5) with the exception of one patient. This patient had low body weight and exhibited substantially lower-than-expected plasma concentrations with a relatively high plasma clearance.

In the Phase I study of ISIS 5132 administered by 2-h intravenous infusion, three patients had altered hepatic function at baseline, defined as ALT $>$ 75 or AST $>$ 83. Once again, neither maximum plasma concentrations measured at or near the end of the 2-h infusion or AUC were altered in patients with elevated serum transaminases when compared with normal patients receiving equivalent

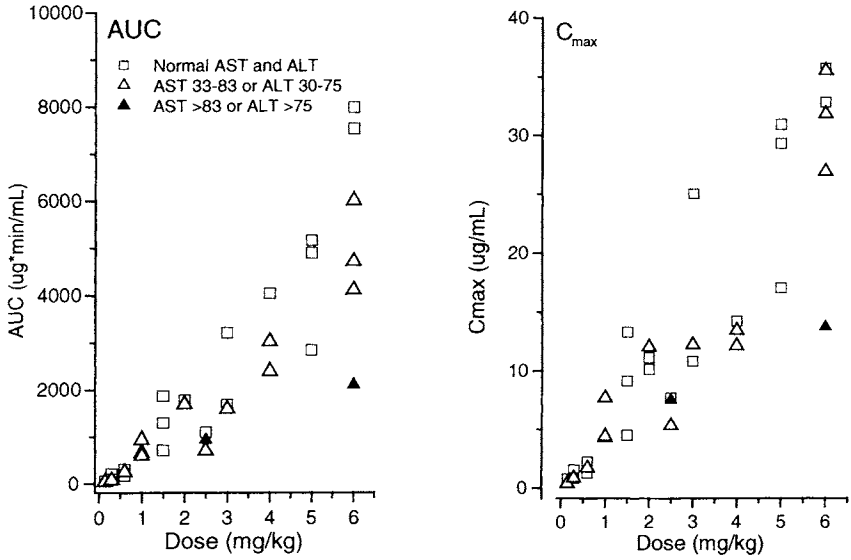

Figure 5 Maximum plasma concentrations (C_{max}) and plasma AUC of ISIS 3521 in patients with altered liver function.

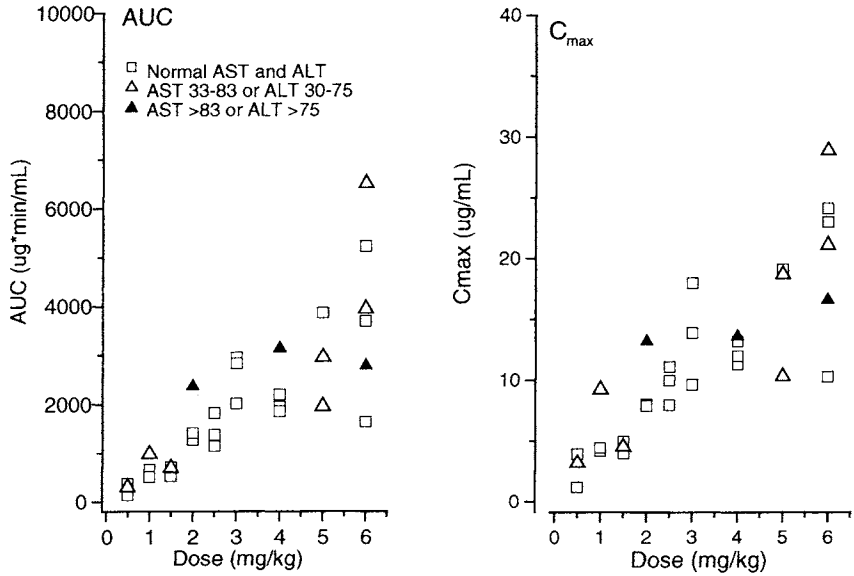

Figure 6 Maximum plasma concentrations (C_{max}) and plasma AUC of ISIS 5132 in patients with altered hepatic function.

doses (Fig. 6). Analysis of variance (ANOVA) conducted comparing total plasma clearance in patients with altered hepatic function with those exhibiting normal hepatic function showed no statistical differences ($p = 0.532$).

E. Constitutional Effects of Oligonucleotide Administration

Administration of phosphorothioate oligodeoxynucleotides is associated with the appearance of a variety of constitutional symptoms. These, too, appear to be somewhat schedule and dose dependent. More severe flu symptoms including headache, fever, fatigue, myalgias, chills, and facial flushing occurred in studies that evaluated 24-h weekly infusions with ISIS 3521, ISIS 5132, and ISIS 2503, particularly at doses of 18 mg/kg and above (21–23). This is likely related to cytokine release observed on this schedule (Table 2). The frequency and severity of constitutional symptoms in patients treated with ISIS 3521 when given by continuous 3-week infusion are shown in Table 4. Flu syndrome has been infrequently seen when these drugs are given on this schedule. Occasional patients have experienced fever at the higher doses on the intermittent schedule (2-h infusions, three times per week) for each of the antisense drugs that has been evalu-

Table 4 ISIS 3521—Safety Summary in Single-Agent Continuous-Infusion Trials (n = 146)

AE	Number of pts	Number of events	Number of events of grade			
			1	2	3	4
Asthenia	47	81	43	32	5	1
Nausea	21	36	26	8	2	0
Anorexia	18	30	24	6	0	0
Constipation	10	14	10	4	0	0
Fever	8	10	4	5	1	0
Vomiting	6	7	4	3	0	0

ated on this schedule. The fatigue reported with continuous-infusion schedules appears to be drug-related but may also be disease-related. Table 5 summarizes the predominant side effects for each schedule.

IV. SUBCUTANEOUS DELIVERY

Clinical trials evaluating subcutaneous delivery of phosphorothioate oligodeoxynucleotides have found that local skin irritation and regional, painful lymphadenopathy makes it difficult to use this route of administration at doses greater than 0.5 mg/kg (30,34). A chemical modification that appears to be less proinflammatory than the chemistries studied to date uses 5′-methyl-cytosine heterocycles rather than unmodified cytosine (35). The first drug with this chemistry, ISIS

Table 5 Constitutional Symptoms Associated with Different Schedules of Phosphorothioate Oligodeoxynucleotides

Schedule	Clinical toxicities
2-h infusion	Gr. 1–2 elevation of aPTT
	Low-grade fevers
Continuous infusion	Fatigue (asthenia)
	Gr. 1–2 thrombocytopenia (ISIS 3521)
24-h weekly infusion	Fever/chills/flu syndrome
(≥18 mg/kg)	Elevation of C3a, Bb, no clear clinical significance
	Gr. 1–2 elevation of PT/aPTT
	Gr. 1–2 thrombocytopenia

14803, is currently in early development. Both intravenous and subcutaneous administration are to be evaluated in this initial study.

V. SUMMARY

In this chapter, the focus has been primarily on assessment of toxicity of oligonucleotides in patients with cancer who have been treated with dose-intense regimens. Phosphorthioate oligodeoxynucleotides have very modest toxicity. Both dose and schedule appear to contribute to the quality and quantity of toxicity seen. The most clearly and consistently dose-related effect of this class of drugs is prolongation of the activated partial thromboplastin time. No effect on aPTT is observed when drug is given by continuous infusion up to 5 mg/kg/day. On other schedules, this effect is transient and does not appear to be associated with a significant risk of bleeding or prolonged bleeding times. The relationship between plasma concentration of drug and complement activation appears to be more complex than previously described (6,26). However, it may well be multifactorial in origin such that the plasma concentration relationship with complement activation is in fact correct though on certain schedules cytokine release may be a secondary cause. The observation that there may be a biphasic effect on complement activation in human serum will be difficult to determine in the clinic.

Thrombocytopenia appears to be a more common problem with continuous-infusion schedules but is generally not severe and may improve in the face of continued treatment. At the high doses studied for cancer drugs, the severity of thrombocytopenia is acceptable. It is unlikely that these drugs will be given by continuous infusion for diseases other than cancer. The doses and schedules used for ISIS 2302 for inflammatory diseases have not resulted in any meaningful decreases in platelet counts nor has this problem been seen with other drugs at lower doses using intermittent schedules. The rapidity of improvement when drug is stopped and the rapidity of its onset in animal models without changes in bone marrow cellularity suggest that this effect is not secondary to hematopoietic stem cell toxicity. Rather it is more consistent with peripheral sequestration although a mechanism for this has not been elucidated.

Organ toxicity does not appear to be a significant safety issue at this time. Controlled clinical trials have not demonstrated a significant difference in the frequency of elevated liver function tests compared to placebo-treated patients. Mild changes in renal function have been reported in one study but have not been a consistent finding. Nevertheless, it will be important to continue to be vigilant for the possibility of these or other organ toxicities as greater experience is gained with this class of drugs. Constitutional symptoms that have been observed in the studies reported to date seem likely to be related to cytokine release, though further work is necessary to characterize this observation.

Many of the toxicities are likely related to the negatively charged, sulfur-substituted backbone that is common to all of the drugs described in this chapter. As chemistries evolve that allow different backbones to be used, the spectrum of toxicity may well change. Nevertheless, this generation of oligonucleotide chemistry has been very well tolerated in the hundreds of patients studied to date.

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General Pharmacology of Phosphorothioate Oligodeoxynucleotides

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I. INTRODUCTION

Pharmacology has historically been defined as the science of drugs, which includes their chemical constitution, biological effects, therapeutic applications, toxicological effects, and pharmacokinetics. Phosphorothioate oligodeoxynucleotides, like any other chemical class of drugs, exhibit complex pharmacological behavior. During the past 10 years there have been tremendous advances concerning our understanding of the pharmacology of phosphorothioate oligodeoxynucleotides. In fact, more is known regarding the pharmacology of phosphorothioate oligodeoxynucleotides than that of many marketed drugs. Other chapters in this volume summarize what is known concerning the chemistry of phosphorothioate oligodeoxynucleotides, their pharmacokinetics, toxicology, and therapeutic potential. Therefore, this chapter will focus on their biological effects or pharmacodynamics.

II. THE RECEPTOR

There are multiple mechanisms by which phosphorothioate oligodeoxynucleotides can produce pharmacodynamic effects in cultured cells or in living organisms. Many of these mechanisms are currently being exploited as potential therapeutics, including antisense mechanism of action, immune stimulation, and aptameric ef-

fects. These effects can be attributed to an interaction between the phosphorothioate oligodeoxynucleotide and functionally important molecule(s) in the biological system. As the pharmacodynamic effects are varied, it is likely that there are multiple receptors with which the oligonucleotides can potentially interact, some designed for and others by accident. In some cases, the receptor responsible for the pharmacodynamic effects has not been well characterized, as is the case for the immunostimulatory effect of phosphorothioate oligodeoxynucleotides.

The best-characterized mechanism by which a phosphorothioate oligodeoxynucleotide produces a pharmacodynamic effect is through an antisense mechanism of action, i.e., a sequence specific reduction in target gene expression resulting from an interaction of the oligonucleotide with the RNA encoding the protein of interest. In this case the receptor for the oligonucleotide is the RNA inside the cell. The oligonucleotide can interact with various classes of RNA such as mRNA, pre-mRNA, ribosomal RNA, snRNA, tRNA, etc. The interaction with the receptor RNA is through hydrogen bonding as described by Watson and Crick (1). Because Watson-Crick base pairing has been so well characterized it is possible to design a phosphorothioate oligodeoxynucleotide capable of binding to the target RNA; however, not all oligonucleotides designed to hybridize to a target RNA produce the desired pharmacodynamic effect (2–6). At this time we do not understand why only a limited number of oligonucleotides designed to hybridize to RNA produce an effect. One of the most likely explanations for this observation is that the antisense oligonucleotide binding sites are already occupied through formation of higher-order secondary and tertiary structures in the RNA or by a protein bound to the RNA. Alternatively, the oligonucleotide-binding site may be sterically inaccessible due to RNA structure or through a protein interaction. Several studies have addressed the influence of RNA secondary structure on the ability of an oligonucleotide to bind (7–10). These studies demonstrate that antisense oligonucleotides do not bind well to sites in the RNA that are in a duplex structure. This is especially true of phosphorothioate oligodeoxynucleotides as they exhibit decreased binding affinity for the target RNA compared to natural nucleic acids (11,12). Oligonucleotides can bind to short single-strand regions, followed by propagation of the heteroduplex into structured regions (7,13). Most studies have addressed the influence of RNA structure on oligonucleotide binding by designing a series of oligonucleotides to hybridize to various regions of a target RNA predicted to exhibit secondary structure. One of the main limitations of this approach is that the sequence, base composition, and structure of each oligonucleotide examined are unique. Vickers et al. recently addressed this issue by varying the structure of the oligonucleotide-binding site, while the oligonucleotide was kept constant (10). This study utilizing three different oligonucleotides demonstrate that structure in the RNA does influence oligonucleotide binding and subsequent antisense activity. However, it should be noted that anti-

sense activity was observed even when the target site on the RNA was predicted to be completely within a stem-loop structure, albeit less activity compared to a region predicted to be unstructured. These results suggest that antisense oligonucleotides are capable of binding to structured regions of RNA; alternatively one cannot rule out the possibility that a structure different from that predicted formed in the cell.

Currently, algorithms for prediction RNA secondary structure are not capable of predicting the structure of a large mRNA. These algorithms may be useful for identifying local structure in small segments of the RNA. They are also useful for predicting equilibrium affinity of complementary DNA oligonucleotides to an RNA oligonucleotide, as a tool for selecting higher-affinity oligonucleotides (14). It should be kept in mind that the structure of a mRNA is dynamic. The local structure in a pre-mRNA would be predicted to be different than the local structure in a mature, fully processed mRNA due to the presence of intervening sequences in the pre-mRNA. Thus the structure of the RNA should change during RNA processing events. Second, during translation, local structure is disrupted as the ribosome moves down a transcript. Based upon these dynamics, there may be a finite time during the lifetime of an RNA in which an oligonucleotide has access to the binding site. Again this will be very difficult to predict, but may partially explain why in some instances antisense oligonucleotides fail to completely suppress target mRNA levels in treated cells.

Because of limitations in our understanding of RNA and oligonucleotide structure in cells, the most direct approach for the identification of an effective antisense oligonucleotide inhibitor is through screening a series of oligonucleotides designed to hybridize to the target RNA. Ideally the screen would test for direct effects on target RNA or protein to avoid some of the pitfalls described below. We have successfully identified effective oligonucleotides for hundreds of targets by screening a series of oligonucleotides designed to hybridize to a target RNA (for example, see Refs. 4–6,15–18). The results from these screens clearly demonstrate that some oligonucleotides designed to hybridize to specific regions on a target RNA are more effective than others are. As an example we have found some oligonucleotides designed to hybridize to the target RNA that inhibit expression of the target protein by 90% or better while others completely fail to inhibit expression at the same concentration. In general, we have found screening between 10 and 20 phosphorothioate oligodeoxynucleotides will yield at least one effective antisense inhibitor. Optimizing for the most potent and effective oligonucleotide allows one to use the oligonucleotides at lower concentrations, avoiding some of the nonspecific effects observed at high oligonucleotide concentrations. In addition, screening for direct effects on target RNA or protein expression builds one's confidence that the lead identified is working by an antisense mechanism of action.

III. MECHANISMS OF PHOSPHOROTHIOATE OLIGODEOXYNUCLEOTIDES

Phosphorothioate oligodeoxynucleotides like any other drug, produce both desirable and nondesirable effects in the test tube, in cells, and in vivo. Unfortunately, much attention has focused on the undesirable effects of phosphorothioate oligodeoxynucleotides. As discussed below, phosphorothioate oligodeoxynucleotides will bind to proteins and other macromolecules producing biological effects. However, if properly optimized and controlled for, it is possible to demonstrate robust antisense effects with phosphorothioate oligodeoxynucleotides.

A. Antisense Mechanism of Action

Upon binding to the appropriate RNA in the cell, phosphorothioate oligodeoxynucleotides can interfere with the function of the RNA by multiple mechanisms. The best-characterized mechanism by which phosphorothioate oligodeoxynucleotides inhibit expression of the targeted gene product in cells is through RNase H–mediated hydrolysis of the target RNA (4,19–23). RNase H is a ubiquitous RNase that degrades the RNA strand of an RNA-DNA heteroduplex. Thus the phosphorothioate oligodeoxynucleotide binds to the target RNA, RNase H is recruited to the heteroduplex, and the target RNA is hydrolyzed resulting in the generation of two RNA fragments and release of the intact phosphorothioate oligodeoxynucleotide. The fates of the RNA fragments are varied. In most cases the RNA fragments are rapidly degraded by cellular RNases. However, in some instances the RNA fragment or fragments are stable thus enabling detection of the cleavage products (21,22,24). As RNase H does not degrade the phosphorothioate oligodeoxynucleotide, it may recycle and bind a second RNA. Thus the most common mechanism of action for phosphorothioate oligodeoxynucleotides appears to be catalytic cleavage of the targeted RNA by recruitment of a cellular nuclease.

Phosphorothioate oligodeoxynucleotides also have the potential to inhibit gene expression by binding to and occupying sites on the target RNA critical for function of the RNA. In cell-free systems, phosphorothioate oligodeoxynucleotides will block translation in the absence of RNase H, demonstrating that binding to specific sites on the target RNA can inhibit protein translation. However, if RNase H is present in the lysates, the activity of the oligodeoxynucleotides is greatly enhanced. In cell culture models phosphorothioate oligodeoxynucleotides have been identified that selectively inhibit the expression of the targeted gene product without promoting a loss of the targeted RNA (4,25,26). In each instance the oligonucleotide bound to the 5′-untranslated region of the target RNA, which would block translation. It cannot be unequivocally ruled out that the oligonucleotides promote RNase H–mediated cleavage of the target RNA, but the resulting

3′-cleavage product is stable. In the case of ICAM-1 and E-selectin, the oligonucleotides bound to sites within 75 bases from the 5′-terminus of the transcript (15). Loss of 50–75 nucleotides from a 3000+ nucleotide transcript is too small a difference to resolve by agarose gel electrophoresis. The finding that high-affinity 2′-modified oligonucleotides, which do not support RNase H activity, also inhibited expression of the respective gene products supports that at least part of the mechanism of action for the phosphorothioate oligodeoxynucleotides is independent of RNase H (4,15,27).

B. Proof of Antisense Mechanisms

As discussed below, phosphorothioate oligodeoxynucleotides are capable of interacting not only with RNA, but with a number of other biological molecules. Interaction with molecules other than the targeted RNA potentially can contribute to pharmacological activity. Therefore, it is important to be able to provide evidence that the observed biological effect is due to the desired mechanism of action. For phosphorothioate oligodeoxynucleotides used as antisense agents a number of controls can be employed to help support an antisense mechanism (28–30). Briefly these include direct demonstration that either target RNA or protein product is reduced following treatment with the oligonucleotide, demonstration of a dose-response relationship, use of several control oligonucleotides, and demonstration that the oligonucleotide is selective for the targeted gene product. Ideally all four types of controls should be employed in the experimental model. If the observed biological effect is unexpected, then more extensive demonstration that the effect is due to an antisense effect is warranted.

Ideally, changes in targeted RNA and protein should be observed following treatment with phosphorothioate oligodeoxynucleotides. A reduction in target RNA is not always observed following treatment with phosphorothioate oligodeoxynucleotides, yet the oligonucleotides produce a marked reduction in protein expression. Therefore, if a phosphorothioate oligodeoxynucleotide failed to produce a reduction in target RNA it does not mean that it is not producing the pharmacological effect through an antisense mechanism of action. However, without demonstration that the oligonucleotide is producing a reduction on either target RNA or protein, the argument that the compound is working by an antisense mechanism of action is much less compelling. The advantage of measuring reductions in target RNA is that in general, it is possible to easily do so for every cellular target by either Northern blot analysis, ribonuclease protection assays, quantitative RT-PCR, or nucleotide arrays, whereas quantitating changes in cellular proteins can be challenging depending on the availability and quality of antibody reagents. Since antisense oligonucleotides do not directly inhibit the function of the targeted protein, the protein product must turn over before a biological effect is observed. Protein half-life is highly variable so this should be taken into

account when examining effects on protein expression and also when examining for phenotypic changes in cells following oligonucleotide treatment.

There has been some discussion in the literature as to what should be used as a control oligonucleotide (29–32). Unfortunately there are no universal rules that can be applied, as each oligonucleotide can form a unique structure and therefore it is not possible to completely control for all potential activities with a single oligonucleotide. Therefore, under ideal situations multiple control oligonucleotides should be used. One of the more compelling pieces of evidence that an oligonucleotide is producing an effect by an antisense mechanism of action is through the use of a series of mismatch control oligonucleotides, establishing a rank-order potency. As an example, Monia et al. evaluated the effects of seven oligonucleotides containing between one and seven mismatches for effects on C-raf expression (33). They demonstrated a very good correlation between the destabilizing effects on hybridization affinity and potency in a cell culture model. A single mismatch resulted in 7°C loss in T_m and a twofold loss in potency. An oligonucleotide with five mismatches resulted in a 19°C loss in T_m and no activity in a cell culture model. Similar studies have been performed for a fas antisense oligonucleotide, in which a decrease in fas expression in liver was correlated with protection against fas antibody-induced hepatitis (34).

A single oligonucleotide that contains between five and 10 mismatches compared to the antisense oligonucleotide or a scrambled control oligonucleotide in which the base composition of the oligonucleotide is maintained, but the oligonucleotide sequence is randomized (scrambled), is most commonly used as control. Alternatively, a sense control oligonucleotide is used that has the same sequence as the targeted region of the RNA. Both approaches have merits and limitations. Finally, some investigators use a randomized pool of oligonucleotides. The latter controls for a chemical class effect of the oligonucleotides but is a poor control for possible sequence-specific interactions with macromolecules other than the intended target RNA, as the concentration of any single oligonucleotide in the pool is extremely low. As discussed earlier, use of more than a single control oligonucleotide in an experimental model helps make a more compelling case that the oligonucleotide is producing an effect by an antisense mechanism of action.

Demonstration that the antisense oligonucleotide does not affect expression of closely related gene products also helps to establish an antisense mechanism. Numerous papers have been published demonstrating selectivity for the targeted gene product using phosphorothioate oligodeoxynucleotides and no effect on related gene products (for example, Refs. 5,18,25,35–38). One of the strongest arguments demonstrating that phosphorothioate oligodeoxynucleotides can selectively inhibit expression of a given target was provided by a study by Monia et al. in which they demonstrate selectivity for the mutated version of ha-ras compared to the wild-type form (37). The two forms of ras differ by a single

nucleotide. A 17-mer phosphorothioate oligodeoxynucleotide exhibited approximately 10-fold selectivity for the mutated form of ha-ras. The known biology of the targeted gene product should be considered when designing control experiments. Inhibition of the expression of a protein can have global effects on the expression of other gene products in cells as a result of a true antisense effect. This is especially true of transcription factors or signal transduction molecules. One way to examine for sequence specificity is to perform a kinetic experiment in which the effects on target expression are examined early after treatment with the antisense oligonucleotide. This is especially useful for proteins that have a half-life of 24 h or more. Using cationic lipids or electroporation to deliver oligonucleotides, one can measure a reduction in target RNA by 4 h, with maximal reductions occurring by 6–8 h after oligonucleotide treatment (39). This time is short enough that a decrease in target RNA can be detected with minimal effect on target protein. Therefore, there should be minimal effects on other gene products as a result of the protein product regulating their expression.

C. Nonantisense Effects of Phosphorothioate Oligodeoxynucleotides

Numerous papers have been published regarding the nonantisense effects of phosphorothioate oligodeoxynucleotides. These papers have been extremely useful in raising awareness as to the potential pitfalls associated with use of phosphorothioate oligodeoxynucleotides in biological systems. However, generalizations about their effects have created many misconceptions regarding their value as antisense agents. While it is true that phosphorothioate oligodeoxynucleotides interact with a variety of biological molecules, if properly designed and optimized, phosphorothioate oligodeoxynucleotides can be very effective antisense agents. In interpreting results obtained using phosphorothioate oligodeoxynucleotides it is helpful to be aware of the numerous nonantisense effects described for these agents. The interactions with proteins can be divided into sequence-independent, sequence-specific, and structure-specific binding events, each of which may have different characteristics and effects. Examples of the types of nonantisense effects reported for oligonucleotides are provided in Table 1. Nonspecific binding to a wide variety of proteins has been demonstrated. Exemplary of this type of binding is the interaction of phosphorothioate oligonucleotides with serum albumin (40–42). The affinity of such interactions is low. The K_d for albumin is approximately 150–400 μM, thus in a range similar to aspirin or penicillin (43).

Phosphorothioate oligodeoxynucleotides have been shown to be competitive inhibitors of DNA polymerase α and β with respect to the DNA template and noncompetitive inhibitors of DNA polymerases γ and δ (44). Despite this inhibition, several studies have suggested that phosphorothioate oligonucleotides might serve as primers for polymerases and be extended (45–49). In our labora-

Table 1 Examples of Nonantisense Activities of Oligonucleotides

Type of activity	Sequence specific	Ref.
Inhibition of thrombin activity	Yes	178
Inhibition of gp120-CD4 interaction	Yes	65
Binding to CD4	No	57
Binding to bFGF	No	60
Binding to fibronectin	No	179
Binding to laminin	No	179
Binding to nucleolin	No	56
Inhibit binding of interferon-γ to IFN-γ receptor	Yes	67
Inhibition of PDGF binding to PDGF receptor	No	60
Inhibition of EGF binding to EGF receptor	No	180
Inhibition of type II phospholipase A_2	Yes	66
Binding to NF-κB	No	69
Induction of Sp1 binding activity	No	54
Inhibition of protein kinase C activity	No	61
Inhibition of DNA polymerases	No	44, 181
Inhibition of RNase H	No	44, 51, 52
Binding to Mac-1 (CD11b/CD18)	No	182
Inhibition of CD28 expression	Yes	72, 73
B-cell mitogen	No, Yes	75, 79–81
Activation of NK cells	Yes	84Ballas, 1996 #9045, 183
Activation of dendritic cells	Yes	184
Inhibition of *Plasmodium* proliferation	No	185
Inhibition of epithelial cell proliferation	Yes	70
Inhibition of smooth muscle cell proliferation	Yes	71
Inhibition of herpes simplex virus growth	Dose dependent	74, 186
Inhibition of cell adhesion to substratum	yes	68, 69, 187
Inhibition of in vitro translation reactions	No	188
Inhibition of transferrin receptor expression	No	189

tories, we have shown extensions of two to three nucleotides only, that appear to be due to addition of a ribonucleotide to the phosphorothioate oligodeoxynucleotide (48). At present, a full explanation as to why longer extensions are not observed is not available. Various viral polymerases have also been shown to be inhibited by phosphorothioate oligonucleotides (45). Phosphorothioate oli-

godeoxynucleotides bind to RNase H when in an RNA-DNA duplex and the duplex serves as a substrate for RNase H (44). At higher concentrations, presumably by binding as a single strand to RNase H, phosphorothioate oligonucleotides inhibit the enzyme (44,50–52). Again, the oligonucleotides appear to be competitive antagonists for the DNA-RNA substrate. Phosphorothioate oligonucleotides have been reported to be competitive inhibitors for HIV-reverse transcriptase (53) and inhibit RT-associated RNase H activity (44).

In addition to inhibition of various polymerases, phosphorothioate oligodeoxynucleotides have been reported to interact with nucleic-acid-binding protein such as transcription factors and single-strand nucleic-acid-binding proteins (54–56). However, very little is known about these binding events. Other proteins that phosphorothioate oligodeoxynucleotides have been demonstrated to interact with include the cell surface protein, CD4 (57), HIV gp120 protein (58,59), basic fibroblast growth factor (60), and protein kinase C (61). Additionally, we have shown nonsequence specific inhibition of RNA splicing by phosphorothioate oligonucleotides (62).

In several cases phosphorothioate oligonucleotides have demonstrated apparent sequence-specific effects that have been attributed to interaction with cellular proteins. These sequence-specific effects were due to the presence of three or more consecutive guanine residues that can form a guanine quartet (G quartet) structure (63,64). The G quartet is a cyclical planar array of four guanine bases of hydrogen bonded by Hoogsteen G-G base pairs. Several G quartets may stack upon one another to form a four-stranded helical structure stabilized by monovalent cations. Such structures are thought to occur naturally in telomeric DNA and immunoglobulin switch region sequence. Phosphorothioate oligodeoxynucleotides capable of forming G-quartet structures have been demonstrated to bind the gp120 protein of HIV (65), type IIa phospholipase A2 (66), interferon-γ receptors (67), the nucleolar protein nucleolin (Bates, 1999), to inhibit cell attachment to plastic substrate (68,69), inhibit smooth muscle cell or epithelial cell proliferation (70,71), inhibit CD28 synthesis (72,73), and inhibit herpes virus infection of cells (74). On the basis of these results, it is best to avoid oligonucleotides that have the capability to form G-quartet structures for use in antisense experiments.

Another example of sequence-specific effects of phosphorothioate oligodeoxynucleotides is immune cell activation attributable to the presence of an unmethylated cytosine followed by guanine (CpG motif) (75–77). Most phosphorothioate oligodeoxynucleotides produce some activation of cells of the immune system in a sequence-independent manner (78–81); however, the presence of a CpG motif flanked by the appropriate sequences results in at least a 500-fold increase in the potency for immune stimulation (75). All immunostimulatory sequences have in common a CpG motif with a preference for two purines on the 5′ side of the motif and two pyrimidines on the 3′ side. Methylation of the

cytosine results in a marked decrease in immune stimulation. Immune stimulatory phosphorothioate oligodeoxynucleotides activate many different types of immunocompetent cells including B lymphocytes, NK cells, macrophages, monocytes, and dendritic cells, but not T lymphocytes (82). The structure-activity relationship for activation of NK cells is different than that for B lymphocytes suggesting that different mechanisms may be responsible for activation of the different cell types (76,83,84). At this time the mechanisms responsible for cellular activation are not well characterized. Internalization of CpG oligonucleotides appears to be required for activation of B lymphocytes, as oligonucleotides bound to a solid substrate are not immunostimulatory (75). Further evidence that CpG oligonucleotides activate cells following internalization is provided by studies demonstrating that inhibition of acidification of endosomal vesicles prevents cell activation (85,86). Previous data suggesting that CpG oligodeoxynucleotides would activate B lymphocytes if immobilized on a solid support (87) may be explained by apparent leaching of the oligodeoxynucleotide from the agarose beads (88). Immunostimulatory oligodeoxynucleotides containing the CpG motif have been shown to activate the MAP kinase and NFκB pathways in B lymphocytes and induce expression of the prosurvival gene Bcl-X_L (85,89–91). Preliminary studies suggest that CpG oligodeoxynucleotides may bind to specific cellular proteins; however, the identity of the proteins is unknown at this time (82).

Owing to the potential for phosphorothioate oligodeoxynucleotides with certain sequence motifs to produce nonantisense effects, it would be best to avoid these motifs when the oligonucleotides are initially designed. Thus oligonucleotides with more than three guanines in a row should be avoided as should oligodeoxynucleotides with CpG motif preceded by purines on the 5′ side and pyrimidines on the 3′ side. Alternatively one could use 5-methylcytosine and avoid most of the immunostimulatory effects. With proper design, selection, and use of appropriate controls it is possible to develop compelling evidence that phosphorothioate oligodeoxynucleotides can produce pharmacological effects by an antisense mechanism of action.

IV. IN VITRO PHARMACOLOGICAL EFFECTS OF PHOSPHOROTHIOATE OLIGODEOXYNUCLEOTIDES

Numerous reviews have been published describing the pharmacological activity of phosphorothioate oligodeoxynucleotides in cell culture model systems (28,45,92–99). Phosphorothioate oligodeoxynucleotides have been studied in a variety of cellular phenotypic assays including viral replication, cell proliferation, immune cell activation, apoptosis, secretion, cell mobility, etc. In addition, phosphorothioate oligodeoxynucleotides have been used to help define biochemical pathways in cells by targeting ion channels, protein kinases, protein phosphatases,

transcription factors, etc. Their effects in cellular systems are as diverse as the molecular targets they were designed to hybridize.

Many of the early studies explored the use of phosphorothioate oligodeoxynucleotides as antiviral agents. Phosphorothioate oligodeoxynucleotides were identified that inhibited replication of human immunodeficiency virus, herpes simplex virus, human cytomegalovirus, human papillomavirus, human and duck hepatitis B, etc. (100–109). In most cases, it was possible to demonstrate a sequence-specific reduction of viral replication. However, studies that examined the mechanism of action of the phosphorothioate oligodeoxynucleotides in detail suggested that inhibition of viral replication may not be entirely due to an antisense mechanism of action (58,102,103,110,111). Part, but not all, of the nonantisense effects may be attributable to inhibition of viral infection of host cells. However, sequence-specific reduction in targeted viral transcript has been demonstrated in several nonreplication model systems in which either the viral transcript was transfected into host cells or virus has integrated into the genome, demonstrating that phosphorothioate oligodeoxynucleotides are capable of inhibiting viral gene expression by a mechanism consistent with an antisense mechanism of action (102,104,112–115). What component of the antiviral effects observed with first-generation phosphorothioate oligonucleotides in vivo is due to a specific antisense effect or to a nonantisense effect is difficult to determine today. As described elsewhere in this volume, there is one phosphorothioate oligodeoxynucleotide drug product on the market for the treatment of CMV retinitis, Vitravene (116,117).

Another area of intense investigation has been the use of phosphorothioate oligodeoxynucleotides for the treatment of cancer. Numerous studies have demonstrated sequence-specific inhibition of gene products thought to be critical for the survival or function of the cancer cell (reviewed in Refs. 118–120). These studies have examined the antiproliferative effects of phosphorothioate oligodeoxynucleotides designed to inhibit the expression of various gene products such as c-*myc*, c-*myb*, ha-*ras*, ki-*ras*, *bcl*-2, p120, c-*raf*kinase, MDM-2, *her*-2, etc. (6,38,121–130). It should be cautioned that some of the early studies used phosphorothioate oligodeoxynucleotides that have the potential to form a G-quartet structure. As described above, phosphorothioate oligodeoxynucleotides that have the potential to form G-quartet structures have been documented to inhibit cell proliferation by a nonantisense mechanism of action. Furthermore, phosphorothioate oligodeoxynucleotides have also been shown to bind to several growth factor receptors that could also contribute to inhibition of cell proliferation by a nonantisense mechanism (Table 1). Thus caution should be used when examining phosphorothioate oligodeoxynucleotides in cellular proliferation assays. It is important that other corroborative data such as additional biochemical data and rank-order potency be obtained before concluding that the observed phenotypic effect is due to a selective inhibition of target gene expression. Despite these concerns

several studies have been published demonstrating convincing evidence of phosphorothioate oligodeoxynucleotides inhibiting proliferation of cancer cells by a mechanism consistent with an antisense mechanism of action (38,129–135). One of the best examples that phosphorothioate oligodeoxynucleotides can inhibit gene expression by an antisense mechanism of action was a study published by Monia et al., in which they selectively inhibited the expression of the mutated form of ha-ras, but not the wild-type form (124). Conversely an oligonucleotide designed to bind to the wild-type form of ha-ras selectively inhibited wild-type expression, but not mutant ha-ras expression. In that these variants of ha-ras differ by a single base substitution, it is difficult to ascribe these results other than through an antisense mechanism of action.

Phosphorothioate oligodeoxynucleotides have been used in a wide variety of additional cell culture model systems. Similar to the antiproliferative effects of the oligonucleotides, caution should be used in interpreting some of the early papers describing physiological/pharmacological consequences of inhibiting expression of a given gene product if documentation of a reduction gene expression by either a quantitative RNA or protein analysis was not provided.

V. IN VIVO PHARMACOLOGICAL EFFECTS OF PHOSPHOROTHIOATE OLIGODEOXYNUCLEOTIDES

A. Neuropharmacological Studies

Antisense oligonucleotides have emerged as a very useful tool for neuropharmacological studies (136–138). Delivery of phosphorothioate oligodeoxynucleotides to CNS tissue has largely been accomplished by intracerebral, intraventricular, or intrathecal injections, as minimal oligonucleotide crosses an intact blood-brain barrier. Contributing to the successful application of antisense oligonucleotides may be functional differences between neural tissues and many peripheral tissues. As an example, it has been documented that nuclease activity in the CNS is reduced compared to that in serum and other peripheral tissues. Second, neural tissues generally do not proliferate in contrast to most peripheral tissues. Both attributes would contribute to maintenance of oligonucleotide levels within the neural cells for prolonged periods (139–141). Several studies have documented that cells in the CNS will accumulate oligonucleotide when delivered centrally, with infusions of oligonucleotide distributing more broadly than bolus injections (140,142–144). A gradient of oligonucleotide can be detected, with highest concentrations of oligonucleotide found in cells adjacent to injection site. There have been numerous studies published using antisense oligonucleotides to examine the role specific genes play in behavioral models, neurotransmission, neural development, and cell survival (reviewed in Refs. 137,138). In many cases these studies have documented a selective down-regulation of targeted gene ex-

pression by the oligonucleotide, supporting an antisense mechanism of action. As an example, Porreca et al. recently published that an antisense oligonucleotide targeting the tetrodotoxin-insensitive sodium channel PN3/SNS delivered by intrathecal injection decreased the expression of the channel in dorsal root ganglion and blocked a hyperalgesic response and reduced tactile allodynia in rats with spinal nerve ligation (145). These studies demonstrate that the oligonucleotide can diffuse to dorsal root ganglion cells following intrathecal administration and that the PN3/SNS sodium channel may be a therapeutic target for the treatment of neuropathic pain.

B. Hepatic Pharmacology

The highest percentage of the total dose of a phosphorothioate oligodeoxynucleotide accumulates in the liver after parenteral administration (40,47). One of the first studies that clearly demonstrated that phosphorothioate oligodeoxynucleotides were capable of selectively reducing gene expression in rodent tissues was a study by McKay and Dean in which they demonstrate a dose-dependent reduction in protein kinase C-α (PKC-α) expression in the liver of mice (35). The oligonucleotide reduced expression of PKC-α mRNA but not other PKC isoenzymes in a sequence-specific manner. The oligonucleotide was administered without any formulation, demonstrating that phosphorothioate oligodeoxynucleotides distribute to liver cells in a functional manner. These initial observations have been extended to cytochrome P-450 enzymes and cholesterol ester transfer protein (146–149). In the former case the authors conjugated the oligonucleotide to cholesterol to increase distribution to the liver and in the latter case the authors complexed the oligonucleotide to asiologlycoprotein poly-L-lysine complex to increase delivery to the liver. Doses as low as 30 μg/kg were sufficient to reduce CETP in the circulation and plasma cholesterol levels after 16 weeks of twice-weekly treatment. These studies demonstrate the feasibility of antisense oligonucleotide therapy for hepatic diseases.

C. Cardiovascular Pharmacology

Several reports have been published demonstrating that phosphorothioate oligodeoxynucleotides targeting genes involved in cell proliferation and prevention of apoptosis are effective in preventing neointimal hyperplasia of injured arteries, inducing regression of atherosclerotic lesions, and preventing sclerosis of vein grafts. Although the initial work describing effects of a c-*myb* and c-*myc* antisense oligonucleotides (150,151) appear to be due, in part, to the G-quartet structure in the oligonucleotides (71), results obtained with other oligonucleotides cannot be attributable to such structures. In particular, work by Dzau and colleagues has broadly demonstrated that oligonucleotides can be delivered to vascular tissue

and inhibit gene expression by a mechanism consistent with an antisense mechanism (152,157). Their initial studies utilized liposomes derivatized with hemagglutinating virus of Japan (HVJ) to deliver the oligonucleotides to animals (158). This approach can be used acutely; however, antigenicity of such liposome preparations precludes their use chronically. More recently, Dzau and colleagues have demonstrated that pressure is an effective means for delivering oligonucleotides to vascular tissues (154,159). This work has been extended to cardiac tissue, demonstrating sequence-specific down-regulation of gene expression following ex vivo delivery of an ICAM-1 antisense oligonucleotide under pressure (160). Pollman et al. also used a cationic liposome formulation to deliver an antisense oligonucleotide targeting the antiapoptotic gene *Bcl-X_L* to artheromatous tissue (155). They demonstrated that inhibition of Bcl-X_L induces apoptosis in vascular smooth muscle cells and reduces atherosclerotic lesions (155). These results demonstrate that delivery of phosphorothioate oligodeoxynucleotides to vascular tissue can be enhanced by liposomal preparations or by pressure to easily accessible sites such as large vessels or transplanted tissue.

Studies examining the effects of antisense oligonucleotides targeting the β_1 adrenergic receptor on blood pressure and cardiac contractility have recently been published (161,162). A single intravenous injection of a phosphorothioate oligodeoxynucleotide targeting the rat β_1 adrenergic receptor mixed with a cationic lipid mixture decreased β_1, but not β_2 receptor expression in cardiac tissue and blood pressure for up to 18 days. The oligonucleotide failed to reduce blood pressure in the absence of cationic lipid formulation, suggesting that the formulation either enhances delivery to cardiac and renal tissues or, alternatively, enhances the stability of the oligonucleotide. In contrast to atenolol, a traditional β_1 adrenergic receptor antagonist, the antisense oligonucleotide lowered blood pressure without decreasing heart rate. The oligonucleotide/lipid complex reduced receptor expression in cardiac and renal tissues but not CNS tissues, possibly avoiding some of the CNS toxicities of small molecule β blockers. These results combined with earlier studies examining angiotensin receptor (163) warrant continued evaluation of antisense oligodeoxynucleotides for the treatment of hypertension.

D. Cancer Pharmacology

Cancer researchers have devoted enormous resources attempting to identify genes that are selectively expressed in cancer cells but not normal cells. In most instances, genes that were originally thought to be expressed exclusively in cancer cells were shown with more extensive analysis to also be expressed in normal tissues. Possible exceptions are genes that arise as a result of a specific translocation such as *bcr/abl* in acute lymphocytic leukemia (164). One of the attractive features of antisense technology is the ability to selectively reduce the expression

of a single gene product. As a result, antisense oligonucleotides have been broadly exploited in the cancer research field. Some of the first reports on in vivo effects of antisense oligonucleotide were papers demonstrating that a phosphorothioate oligodeoxynucleotide targeting the c-*myb* gene inhibited growth of melanoma cells in nude mice (165) and an oligonucleotide targeting *BCR-ABL* suppressed growth of a human leukemic cell in mice (166). Subsequently numerous papers have been published demonstrating inhibition of human tumor xenograft growth by antisense phosphorothioate oligodeoxynucleotides targeting various gene products (123,131,132,167–172). As an example several manuscripts have been published demonstrating the effectiveness of a *bcl*-2 antisense oligodeoxynucleotide in lymphoma, melanoma, and prostate cancer xenografts (123,173,174) and in patients (175). The encouraging preclinical studies with antisense phosphorothioate oligodeoxynucleotides have resulted in several oligodeoxynucleotides being evaluated in clinical trials (176).

VI. CONCLUSIONS

Phosphorothioate oligodeoxynucleotides remain the most commonly used oligonucleotide modification. Although much attention has focused on the undesirable activity observed when they are used in cellular and in vivo studies, when properly optimized they can produce true antisense effects. Information gained by use of phosphorothioate oligodeoxynucleotides in preclinical and clinical applications has helped focus on true issues facing the technology rather than perceived issues (177). Based upon these findings, newer chemical modifications and formulations have been identified that enhance the properties of antisense oligonucleotides. Although much is still to be learned regarding oligonucleotide pharmacology, tremendous progress has been made over the past decade.

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Pharmacology of 2′-*O*-(2-Methoxy)ethyl-Modified Antisense Oligonucleotides

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I. INTRODUCTION

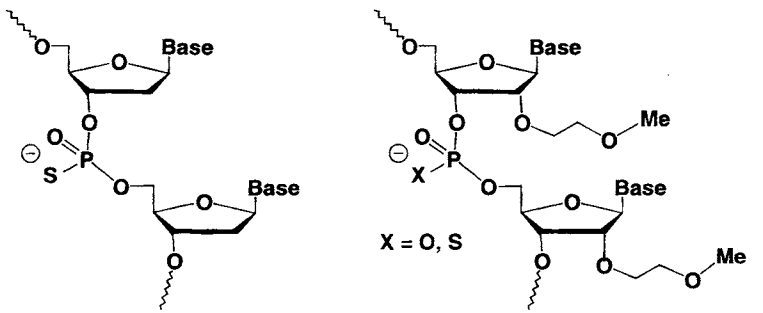
The idea of using antisense oligonucleotides with sequences complementary to specific mRNA transcripts to reduce encoded protein expression was first proposed by Zamecnik in 1978 (1). Conceptually this approach is elegant in its simplicity, as the interaction between drug and receptor is through the well-characterized process of Watson-Crick base pairing, and inhibitor design is therefore dictated by the base sequence of the target mRNA. Consequently, unlike small-molecule- and antibody-based approaches to inhibitor identification, antisense drug design is entirely independent of protein structure, function, or localization. As with most drugs, the chemistry of the first antisense inhibitors developed was not optimal for maximum biological activity. For example, early investigators used unmodified phosphodiester DNA as antisense molecules; however, the activity of these compounds is severely limited by their relative instability toward nucleases. As our understanding of antisense oligonucleotide pharmacokinetics and pharmacology has advanced, this has lead to an increased appreciation of the characteristics and requirements of oligonucleotides for improved biological activity. Guided by this knowledge, research in oligonucleotide medicinal chemistry has sought to develop rationally chemically modified oligonucleotides, capable of greatly improved characteristics compared to these early compounds. In the present review, we will focus on one such chemistry, the 2′-*O*-(2-methoxy)ethyl (2′-MOE) modification.

II. PHOSPHOROTHIOATE OLIGODEOXYNUCLEOTIDES

Irrespective of the mechanisms of action of an oligonucleotide, to demonstrate optimum biological activity and therapeutic utility, certain criteria must be met. It is the quest to improve these oligonucleotide characteristics that drives much of the research in oligonucleotide medicinal chemistry. These oligonucleotide characteristics include:

1. The ability to bind to target mRNA sequences with high affinity and specificity.
2. Sufficient resistance to both 3′- and 5′-exonucleases, as well as endonucleases present in serum and tissues, to avoid rapid cleavage and degradation.
3. The ability to be internalized into cells in sufficient quantities to evoke a biological response.
4. The ability to evoke a mechanism of action that leads to inactivation or destruction of the targeted mRNA.
5. Demonstration of appropriate pharmacokinetics and acceptable toxicity in vivo.

The appreciation of the nuclease instability issues surrounding the use of phosphodiester DNA led to the development of the widely used, first-generation chemistry of the phosphorothioate oligodeoxynucleotide. In this modification, an equatorial oxygen atom in the phosphate backbone of the oligonucleotide is replaced by a sulfur atom. The resulting compound has considerably more nuclease resistance than unmodified phosphodiester DNA and retains the ability to support RNase H–mediated cleavage of hybridized mRNA sequences. When carefully



2'-deoxy-phosphorothioate-DNA (2'-H/P=S) **2'-*O*-MOE: 2'-*O*-(2-Methoxyethyl)-RNA**

Figure 1 Structures of important first- and second-generation antisense chemistries.

designed and used, phosphorothioate oligodeoxynucleotides are effective and specific inhibitors of gene expression, both in tissue culture and in animals (2–4). Consequently, over the last 10 years phosphorothioate oligodeoxynucleotides have become the workhorse of antisense research and drug development. In addition to the publication of many articles describing their use as research tools to dissect gene function, the first phosphorothioate antisense drug was recently approved for use in humans by the Food and Drug Administration.

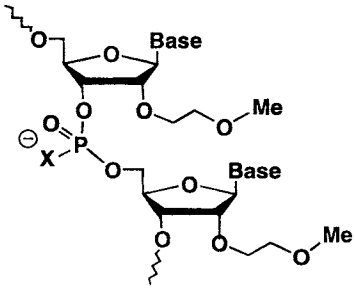
Unfortunately, as with most other first-generation drugs, phosphorothioate oligodeoxynucleotides do have some characteristics that limit their effectiveness. Each incorporation of a phosphorothioate residue into the oligonucleotide sequence generates a chiral center, and reduces the binding affinity for target mRNA by 1–1.5°C. In addition, although relatively stable toward nucleases, phosphorothioate oligodeoxynucleotides will degrade with time. As this class of compound has been evaluated in animals and humans, a number of hybridization-independent toxicities have also been identified that limit their effectiveness (see later section). As a result of this recognition of the limitations of phosphorothioate oligodeoxynucleotides, considerable effort has gone into identifying chemical modifications that will minimize these effects. However, no single modification should be thought of as ''the best'' modification. Different modifications will be optimal for different antisense applications, and this must be taken into consideration when designing experiments and considering which combinations of modifications to use.

III. CARBOHYDRATE 2′-MODIFIED OLIGONUCLEOTIDES

Among the different sites available for chemical modification in a nucleoside building block, the 2′-position of the carbohydrate moiety (5) has proven to be valuable for a number of reasons:

1. 2′-Modifications with an electronegative substituent can confer an RNA-like $C_{3'}$-endo conformation to the antisense oligonucleotide. Such a preorganization favors an RNA like conformation (6–8) and greatly improves the binding affinity to the target RNA.
2. 2′-Modifications have the potential to provide nuclease resistance to oligonucleotides, even as a PO internucleotide linkage (9).
3. 2′-Modifications provide chemical stability against potential depurination conditions either during synthesis or after administration of the drug.
4. 2′-Modifications can alter the lipophilicity of the oligonucleotides, which may influence their pharmacokinetic properties.

Among the possible 2′-modifications, 2′-*O*-alkyls, 2′-*O*-alkyls with glycol ether linkages, 2′-F, and 2′-*O*-aminoalkyls have been studied for their pharmaco-



MOE: 2'-*O*-(2-Methoxyethyl)-RNA

- **ΔTm=2.0°C**
  - **Relative to P=S**
- **Gauche Effect**
- **MOE (P=O)**
  - **Nuclease resistance similar to 2'-H P=S**

Figure 2 Highly nuclease resistant, high-receptor-affinity RNA mimetics with P=O or P=S 2′-*O*-MOE.

kinetic, pharmacodynamic, and pharmacological properties. Within 2′-*O*-alkyls, with increase in size of the alkyl chain, the binding affinity (10) drops from 2.0 to −2.0°C while the nuclease resistance increases with increase in alkyl chain (9), the *O*-pentyl group (as PO) exhibiting nuclease resistance similar to that of 2′-deoxyphosphorothioates. The 2′-F modification, which locks the sugar conformation in a very high $C_{3'}$-endo conformation ($C_{3'}$-endo > 90%), offers the greatest increase in binding affinity (+3.0/nucleotide linkage) (11). Unfortunately, this high-affinity modification does not offer any resistance to nucleases as a phosphodiester (PO). It requires the phosphorothioate backbone (PS) to exhibit sufficient nuclease resistance.

IV. 2′-*O*-(2-METHOXY)ETHYL CHEMISTRY AND BIOPHYSICAL CHARACTERISTICS

Among the 2′-modifications well studied at Isis Pharmaceuticals, one modification stands out in terms of improved binding affinity to target RNA, increased

nuclease resistance, and enhanced pharmacological activity. This is the 2′-*O*-(2-methoxy)ethyl or 2′-MOE modification (12–14). 2′-MOE forms duplexes with RNA that are 2°C more stable on average per modification than the corresponding 2′-H/PS-DNA·RNA (12–15). The higher RNA affinity is accompanied by a significantly enhanced protection against nuclease degradation, comparable to that of 2′-H/PS-DNA (5,12–14). Egli et al. have rationalized (16,17) the extraordinary nuclease stability and binding affinity of 2′-MOE-RNA by studying crystal structures of 2′-MOE-RNA molecules. The origins of the promising antisense properties displayed by 2′-MOE-RNA have been determined by studying high-resolution crystal structures of

1. A palindromic DNA oligomer GCGTAT$_{MOE}$ACGC having a single 2′-MOE modification (16).
2. A completely 2′-MOE-modified RNA duplex, the first for an RNA molecule carrying a chemical modification on every residue (17).

In the first structure, the stabilizing 2′-MOE substituent as well as the modified furanose ring ($C_{3'}$-endo pucker) are conformationally preorganized for an A-form duplex. The conformation of the torsion angles around the ethyl C-C bonds in the side chain fall into the synclinal conformation. The resulting conformation is compatible with the minor groove topology in an A-form RNA duplex. The orientation of the side chain is further constrained by the coordination of water molecules involving O2′, O3′, and the ether oxygen. The water molecule lies within 3 angstroms of these three atoms. Such a hydrogen bonding would significantly contribute to preorganization of the modified nucleoside into an A form. This complexation will also inhibit nucleases from cleaving the phosphate below the O3′ atom.

The recent structural (17) results with a fully modified 2-MOE dodecamer CGCGAAUUCGCG are consistent with the higher RNA affinity of 2′-MOE-RNA compared with DNA. The sugars are locked in the $C_{3'}$-endo conformation, as expected for any RNA mimic. As mentioned earlier, in the case of the 2′-*O*-alkyl modifications, longer substituents destabilize the duplex formed between the modified strand and the RNA target (10). However, conformational preorganization with 2′-MOE-RNA includes both sugar moiety and the substituent and the gauche effect in the 2-*O*- modification is extended. The observed conformational preferences of the 2-*O*- moiety also prevail in the single-stranded state, providing an entropic advantage for base pairing.

A further feature of 2′-MOE-RNA established by the crystal structures is the extensive hydration of the minor groove and backbones. Up to three water molecules can be stabilized by the 2-*O*- moiety, assisted by bridging and nonbridging phosphate oxygens. The chelate-like trapping of water molecules between oxygen acceptors of substituent and backbone suggests an important role of water in the overall stability of 2′-MOE-RNA. In A-RNA, the 2′-hydroxyl

groups can also stabilize water bridges between sugar and base and sugar and phosphate moieties in the minor groove (18). By comparison, the hydration shells of phosphate groups, sugars, and bases overlap only weakly in B-DNA (19).

The precise origins of nuclease resistance of 2′-*O*-MOE oligomers are not fully borne out even by this crystallographic study. Steric hindrance is a likely cause of the improved resistance observed with 2′-MOE-RNA. Thus, the 2′-MOE is similar to 2′-*O*-butyl in steric bulk. The intricate water network between the 2-*O*- substituent and bridging and nonbridging phosphate oxygens may enhance steric hindrance. Stably bound water molecules could also interfere with phosphoryl transfer or could alter the reactivity of the phosphodiester moiety.

Interestingly, molecular dynamics simulations of an 2′-MOE-RNA single strand furnished a rigid structure that closely resembles the conformation of the 2-*O*- strand in the duplex state (20). The structure and physical properties of 2′-sugar-substituted *O*-(2-methoxyethyl) nucleic acids have been studied using molecular dynamics simulations (20). Nanosecond simulations on the duplex 2′-MOE-[CCAACGTTGG], α-[CCAACGUUGG] in aqueous solution have been carried out using the particle mesh Ewald method. Parameters for the simulation have been developed from ab initio calculations on methoxyethyl fragments in a manner consistent with the AMBER 4.1 force field database. The simulated duplex is compared with the crystal structure of the self-complementary duplex d[GCGTAT$_{MOE}$ACGC]2, which contains a single modification in each strand. Structural details from each sequence were analyzed to rationalize the stability imparted by substitution with 2′-MOE side chains. Both duplexes have an A-form structure, as indicated by several parameters, most notably a $C_{3'}$-endo sugar pucker in all residues. The simulated structure maintains a stable A-form geometry throughout the duration of the simulation. The presence of the 2′ substitution appears to lock the sugars in the $C_{3'}$-endo conformation, causing the duplex to adopt a stable A-form geometry. The side chains themselves have a fairly rigid geometry with trans, trans, gauche+/−, and trans rotations about the C2′-O2′, O2′-Cα′, Cα′-Cβ′, and Cβ′-OCH_3 bonds, respectively.

V. 2′-*O*-(2-METHOXY)ETHYL-MODIFIED OLIGONUCLEOTIDES: IN VITRO PHARMACOLOGY

An ever-increasing number of studies have been published demonstrating the utility of oligonucleotides containing this chemical modification as effective inhibitors of gene expression in tissue culture. Binding to a targeted mRNA sequence by 2′-MOE-modified oligonucleotides can reduce expression of the encoded protein by a number of different mechanisms. The precise mechanism used will depend upon the placement of the 2′-MOE-containing residues, the target sites within the mRNA transcript, and the incorporation of additional modifica-

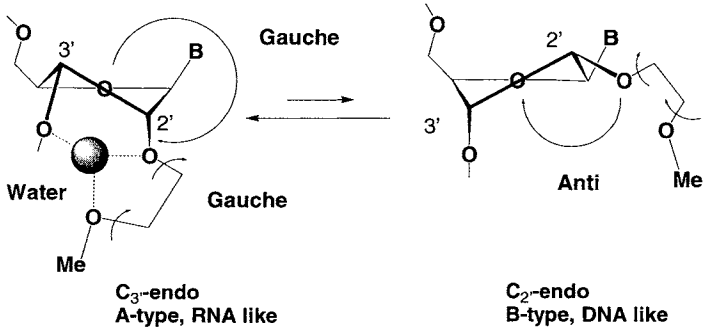


Figure 3 Conformational equilibrium in 2′-*O*-(MOE) RNA.

tions into the oligonucleotide sequence. Although multiple mechanisms of action for oligonucleotides are known, or have been postulated (21), to date four different mechanisms of action have been reported for 2′-MOE-containing oligonucleotides.

A. RNase H–Dependent mRNA Cleavage

Oligonucleotides containing exclusively the 2′-MOE modification fail to support RNase H cleavage of hybridized RNA transcripts (22). This is presumably due to the formation of RNA-RNA-like hybrids rather than RNA-DNA hybrids, which are required for RNase H cleavage of the RNA strand of the duplex. This limitation has been overcome by the development of gapmer or chimeric oligonucleotides. Oligonucleotides in this class contain both 2′-MOE modifications (which enhance hybridizing affinity and increase nuclease resistance) combined with a series of consecutive ''gaps'' of oligodeoxynucleotide residues to support RNase H cleavage of hybridized mRNA (23).

A comprehensive comparison of the cellular pharmacology of chimeric 2′-MOE/oligodeoxynucleotides with 2′-*O*-methyl and oligodeoxy phosphorothioate compounds was recently reported (23). In these studies, a series of oligonucleotides targeting human PKC-α were evaluated. It was found that the optimum number of consecutive oligodeoxy residues required for activity in this sequence

was eight; however, activity was seen with as few as four. The incorporation of 2′-MOE chemistry resulted in a number of significant improvements in oligonucleotide biophysical characteristics and cellular pharmacology. These include an increase in hybridization affinity toward a complementary RNA of 1.5°C per modification, and an increase in resistance toward both 3′-exonucleases and intracellular nucleases. An in vitro nuclease assay was used to examine the ability of a series of oligonucleotides to withstand digestion by snake venom phosphodiesterase (a 3′-exonuclease). Under conditions that resulted in 50% digestion of a full-length phosphorothioate oligodeoxynucleotide, approximately 75% of a chimeric 2′-*O*-methyl/phosphodiester oligonucleotide was degraded. In contrast, only approximately 40% of the 2′-MOE/phosphodiester oligonucleotide was degraded, demonstrating that even combined with a phosphodiester backbone, the 2′-MOE modification provides greater nuclease resistance than that obtained with a phosphorothioate oligodeoxynucleotide. When the two 2′-*O*-modified sugar residues were evaluated in the context of a phosphorothioate backbone, the 2′-MOE modification was again superior to the 2′-*O*-methyl-containing sequence, and demonstrated very little degradation. The increased nuclease resistance demonstrated by the 2′-MOE-modified oligonucleotides also resulted in a significantly increased duration of action for this class of compound when compared to phosphorothioate oligodeoxynucleotides. When transfected into cells, 2′-MOE-modified oligonucleotides were able to suppress target mRNA (PKC-α) expression for at least 72 h. In contrast, mRNA expression levels in the cells treated with the phosphorothioate oligodeoxynucleotide had returned to control. The ability to maintain reduced target gene expression for extended periods will be particularly valuable when targeting mRNA transcripts that encode proteins with particularly long half-lives.

The superior ability of 2′-MOE-containing chimeric oligonucleotides to produce highly specific and potent reductions in targeted gene expression has led to their increased use as tools to dissect gene functions in tissue culture. Some recent examples of publications using 2′-MOE chimeric oligonucleotides for gene functionalization studies are listed in Table 1. It is clear from these studies, using oligonucleotides targeting members of large, multigene families, that 2′-MOE modified chimeric oligonucleotides are highly effective inhibitors of gene expression. As a consequence, this class of compound has superseded traditional phosphorothioate oligodeoxynucleotides as the tool of choice for target validation studies.

B. Modulation of Pre-mRNA Splicing

Splicing of pre-mRNA transcripts is a highly regulated event relying on the precise recognition of splice sites by splicing machinery. Through RNA splicing, it is possible to generate functionally distinct proteins from a single transcript. Be-

Table 1 Published Examples of the Use of 2′-MOE-Containing Chimeric Oligonucleotides to Define Gene Function in Tissue Culture

Target gene	Functional consequences of target protein knockout	Ref.
Protein kinase C-α	Reduced phorbol ester–mediated c-*jun* induction in A549 lung carcinoma cells	23
Protein kinase C-α	Reduced phorbol ester–mediated responses in fibroblasts	31
Protein kinase C-ε	Reduced phorbol ester–mediated activation of Na/Pi uptake in fibroblasts	32
Protein phosphatase type 5	Regulation of p53 and p21 expression in multiple cells	33
Protein phosphatase type 5	Regulation of glucocorticoid receptor–mediated growth arrest in multiple cell types	34
Protein phosphatase type 1γ1	Cytokinesis in A549 lung carcinoma cells	35
Survivin	Prevention of mitotic progression in multiple cell types	36
Bcl-xL and A1	Potentiation of TNF-α-mediated apoptosis in endothelial cells	37
Bcl-xL	Potentiation of UV- and drug-induced apoptosis in epithelial cells	38
c-*raf*	Cytokine signaling in endothelial cells	39
Ki-ras	Reduced proliferation of renal fibroblasts	40
JNK/p38	Apoptosis and survival of blood cells	41
E2A-Pbx1	Regulation of WNT gene expression	42
P-glycoprotein	Potentiation of drug-induced apoptosis	43
Thymidylate synthase	Potentiation of drug-induced apoptosis	44

cause aberrant splicing results in a number of human diseases, the ability to either correct RNA splicing or drive the generation of a specific transcript could have enormous therapeutic potential. A number of studies have been performed demonstrating that oligonucleotides can be used to direct splicing of pre-mRNA transcripts (reviewed in Ref. 24). A general requirement for successful modulation of splicing appears to be a very high-affinity interaction between the oligonucleotide and the targeted transcript. In addition, the oligonucleotide should not support RNase H cleavage of the hybridized mRNA. Some earlier studies have successfully used 2-*O*-methyl oligonucleotides to accomplish this (24); however, 2′-MOE oligonucleotides would appear to be better suited to this strategy owing to their enhanced binding affinity when compared to 2-*O*-methyl oligonucleotides. Indeed, the first example of regulation of pre-mRNA splicing by a 2′-MOE-modified oligonucleotide was recently reported in a series of experiments designed to modulate RNA splicing of the Bcl-x gene product (25). The Bcl-x gene is an example of how alternative RNA splicing can generate proteins with antagonistic functions. Alternative splicing of the Bcl-x pre-mRNA gives rise to two transcripts, coding for either a long (Bcl-xL) form, which is antiapoptotic, or a short (Bcl-xS) form, which is proapoptotic. In this study, an oligonucleotide (containing all 2′-MOE and no deoxy residues) was identified that shifted the production of two spliced transcripts from a long to a short form. This resulted in cells becoming more sensitive to induction of apoptosis by chemotherapeutic drugs. Curiously, in this study the optimum mRNA sequence targeted that gave maximal ''switching'' of splicing did not overlap a splice junction, but was just upstream of these sequences. Future studies will determine the general applicability of this finding and better define the mRNA sites to target to obtain optimal modulation of splicing. Clearly however, fully 2′-MOE-modified oligonucleotides represent an exciting and attractive novel approach to manipulating mRNA splicing.

C. Inhibition of Translation

Translation of mRNA into encoded proteins is a complex and multistep process, requiring the interaction of multiple proteins with the mRNA to be translated. The three steps required are recognized as initiation, elongation, and termination. Prevention of binding of the protein translational machinery to mRNA by an oligonucleotide at any one of these steps has the potential to interrupt protein synthesis. Therefore, multiple opportunities exist for blockade of translation by a direct interaction of an antisense molecule with a targeted mRNA sequence. The characteristics of an oligonucleotide capable of functioning by such a mechanism would seem to be similar to those required to manipulate RNA splicing, namely a very high-affinity interaction between the oligonucleotide and the targeted transcript and an inability to support RNase H cleavage of the hybridized mRNA.

Some examples of this approach have been reviewed recently (21) with oligonucleotide containing peptide nucleic acid, morpholino, α-anomeric sugar, and 2′-sugar modifications.

To date, there are two reports of 2′-MOE-modified oligonucleotides inhibiting protein synthesis by an RNase H–independent blockade of translation. In the first of these studies a series of 2′-modified antisense oligonucleotides that differ in binding affinity to the target RNA and nuclease resistance, targeting the 5′ cap of the human ICAM-1 transcript, were evaluated for their effect on ICAM-1 protein expression in human umbilical vein endothelial cells (22). Two 20-mer 2′-MOE-modified oligonucleotides, in the context of either P=O or P=S backbones, proved to be the most potent, reducing ICAM-1 protein expression with IC_{50} values of 2 and 6 nM, respectively. In contrast, a phosphorothioate oligodeoxynucleotide demonstrated an IC_{50} of 50 nM. The mechanism of action of the 2′-MOE-modified oligonucleotides was characterized and was demonstrated to be due to selective interference with the formation of the 80-S translation initiation complex. Other RNA metabolic processes such as splicing and transport out of the nucleus were not affected by the oligonucleotide. Curiously, the reduction in ICAM-1 protein expression was accompanied by an increase in ICAM-1 mRNA abundance in the cytoplasm. A second example of this approach was recently reported with human HCV as the molecular target (26). A uniformly modified 2′-MOE oligonucleotide, this time complementary to the AUG initiation of protein synthesis codon, reduced HCV core protein expression levels without reducing HCV RNA levels. These two studies demonstrate the utility of ''fully modified'' 2′-MOE oligonucleotide to reduce protein synthesis directly. With so few studies reported, the optimal sites to target for this approach have not been fully characterized—however, the work of Baker et al. (27) strongly suggests that the most 5′ sequences of a transcript seem a good place to start.

D. Decapping Strategies

Alternatives to RNase H–mediated mRNA cleavage strategies have been described (21). One strategy takes advantage of the unique structure of the 5′ cap of mRNA, a structure that is required for mRNA stability in cells. Removal of the 5′-cap structure through chemical cleavage by complexes covalently attached to an oligonucleotide has been proposed as a mechanism to increase the activity of oligonucleotide compared to noncomplexed oligonucleotides. One example of the successful utility of this approach has been reported for 2′-MOE-modified oligonucleotides (27). Attachment of a lanthanide macrocycle complex to the 3′-terminus of an antisense oligonucleotide, which targets the 5′-terminus of ICAM-1 mRNA, potentiated the inhibitory activity of this antisense oligonucleotide.

VI. 2′-*O*-(2-METHOXY)ETHYL-MODIFIED OLIGONUCLEOTIDES: IN VIVO CHARACTERISTICS

A. Toxicology

The toxicological characteristics of phosphorothioate oligodeoxynucleotides have been very well characterized (see Levin et al., this volume), and the major dose-limiting toxicities to date appear to be hybridization-independent effects. These include complement activation and a series of responses collectively regarded as a form of immune stimulation. The severity of responses is species specific, with rodents appearing to be more sensitive to immune stimulation induced by phosphorothioates and primates much less so. The immune stimulatory effects include splenomegaly, lymphoid hyperplasia, and mixed mononuclear cell infiltrate in numerous tissues. The development of chemical modifications, which reduce the severity of these oligonucleotide toxicities, would therefore be of considerable value. The ability of a series of chemically modified oligonucleotides (including 2′-MOE-modified compounds) to elicit such an immune response in mice was recently examined in an effort to characterize toxicities of novel oligonucleotides (28). Male mice were dosed with increasing amounts of oligonucleotides (up to 50 mg/kg every other day for 14 days) and effects on liver, kidney, and spleen determined. Immune stimulation was greatly decreased when mice were dosed with oligonucleotides containing 5-methyl cytosine, and further decreased by the 2′-MOE modification. Administration of these chemically modified oligonucleotides to mice did not produce splenomegaly even at 50 mg/kg doses. These data clearly demonstrate that 2′-MOE-modified oligonucleotides are less toxic than the widely used phosphorothioate oligodeoxynucleotides.

B. Pharmacokinetics

The tissue distribution of phosphorothioate oligodeoxynucleotides after systemic administration is well known. Compound accumulates in multiple sites, including liver, kidney, and bone marrow (see Geary et al., this volume). The distribution of 2′-MOE/phosphorothioate chimeric oligonucleotides is very similar overall. However, the increase in nuclease resistance of 2′-MOE-containing sequences compared to oligodeoxynucleotides greatly increases the durability of the former class of compound in tissue. Phosphorothioate oligodeoxynucleotides are cleared from tissue by the action of nucleases, primarily 3′-exonuclease activity. The ability of 2′-MOE-containing sequences to withstand degradation largely prevents this occurrence and results in sustained levels of intact oligonucleotide for significantly prolonged periods after cessation of animal dosing. Therefore, elimination of 2′-MOE oligonucleotides appears to be by cellular efflux rather than metabolism as for phosphorothioate oligodeoxynucleotides. The latter is a much

slower process, and the different rates of elimination of the two classes of compound can be clearly seen in Fig. 4. In this study, mice were dosed with either a phosphorothioate oligodeoxynucleotide (ISIS 2503) or a 2′-MOE phosphorothioate chimeric sequence (ISIS 13920). Animals were sacrificed either 1 or 7 days after the last oligonucleotide dose and the distribution of oligonucleotide determined by immunohistochemistry using an antibody that recognizes these oligonucleotide sequences. At 24 h after dosing, the phosphorothioate oligodeoxynucleotide and 2′-MOE compound appear to distribute similarly in terms of the cellular localization within organs. Immunoreactivity is primarily localized to the proximal tubules in the kidneys, and to endothelium, Kupffer cells, and hepatocytes in the livers from mice injected with either form of oligonucleotide. However, at 7 days after injection, little immunostaining of the phosphorothioate oligodeoxynucleotide can be detected in liver, while immunostaining for the MOE compound is still clearly visible. This is due to the sustained levels of intact drug for the latter class of compound, and elimination of the former.

To date, the majority of in vivo pharmacokinetic and pharmacological data available for antisense oligonucleotides comes from parenteral dosing. This is because phosphorothioate oligodeoxynucleotides demonstrate poor oral bioavailability. The lack of oral bioavailability is most likely due to the relative instability of phosphorothioate oligodeoxynucleotides in the gastrointestinal tract, as well as poor absorption resulting from the size and charge of this class of compound. The significant increases in nuclease resistance obtained by incorporating 2′-MOE modifications into an oligonucleotide sequence offer the potential for increasing the amount of compound absorbed subsequent to oral dosing. This has been examined recently, with 2′-MOE compounds in the context of both a phosphorothioate and a phosphodiester backbone in an in situ single-pass perfusion model in rat intestine (29). It was found that modifying some, or all, of the oligonucleotide with 2′-MOE groups substantially increased intestinal permeability of the oligonucleotides. Both partially and fully 2′-MOE-modified oligonucleotides showed a two- to fourfold increase in permeability compared to unmodified phosphorothioate oligodeoxynucleotides. The presence of a phosphodiester backbone in the 2′-MOE compounds increased intestinal permeability still further. Paracellular absorption, believed to be the dominant route for uptake of oligonucleotides, is influenced by the size, charge, and hydrophilicity of an antisense compound and correlates strongly with water flux and intercellular tight junction diameter. The highly improved uptake of 2′-MOE oligonucleotides relative to phosphorothioate oligodeoxynucleotides is likely a consequence of the different backbone polarities and is consistent with the extensive hydration of the 2-*O*- substituents. These data raise the exciting possibility that 2′-MOE modified oligonucleotides may be developed as orally bioavailable drugs in the not-too-distant future.

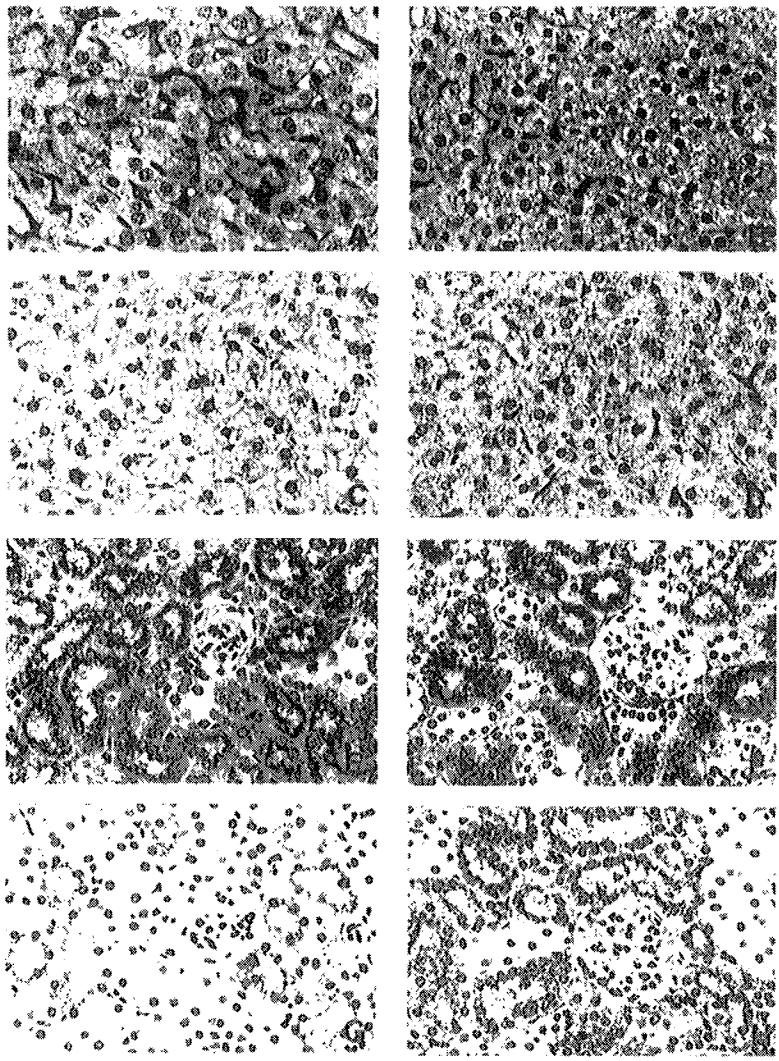


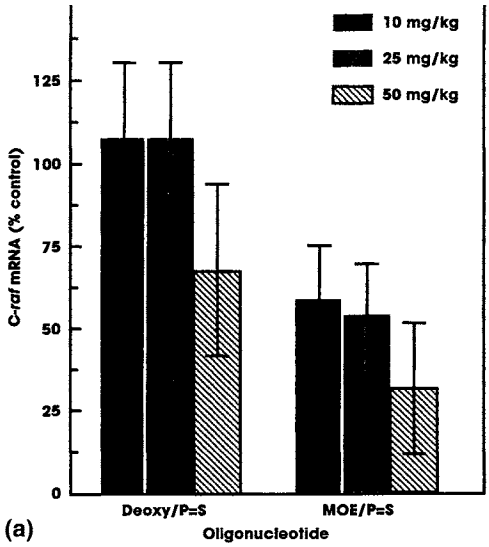
Figure 4 Phosphorothioate oligodeoxynucleotide and 2′-MOE localization in mouse liver and kidney after systemic administration. 10 mg/kg of the phosphorothioate oligodeoxynucleotide (A, C, E, G) or the 2′-MOE phosphorothioate chimeric oligonucleotide (B, D, F, H) was injected into mice (i.v.), and the mice were sacrificed either 24 h (A, B, E, F) or 7 days (C, D, G, H) later. Sections of liver (upper four panels) or kidney (lower four panels) sections were immunostained with a mouse monoclonal antibody that recognizes both oligonucleotides (45). Peroxidase activity, and hence oligonucleotide concentration was visualized with 3,3′-diamino-benzidine and sections are counterstained with hematoxylin.

C. Pharmacology

Phosphorothioate oligodeoxynucleotides have demonstrated pharmacological activity in multiple animal models and in humans. To date, no published examples of such activities for 2′-MOE-containing oligonucleotides have been reported. The significant increases in both oligonucleotide potency and durability demonstrated for this class of compound in tissue culture are anticipated to lead to much improved activity in vivo. We have studied whether this prediction is true in a simple animal model system. Oligonucleotides accumulate in liver tissue and have been used to reduce target gene expression in this tissue previously (30). Therefore, we have evaluated the ability of two oligonucleotides designed to inhibit expression of c-raf kinase; one is a phosphorothioate oligodeoxynucleotide, the second is a 2′-MOE phosphorothioate oligodeoxynucleotide. In the first series of experiments mice were dosed with one of each of the two compounds daily at either 10, 25, or 50 mg/kg (i.p.), and then the expression of c-raf mRNA was determined by Northern blotting. Under these dosing conditions, only at 50 mg/kg does the phosphorothioate oligodeoxynucleotide show any reduction in c-raf mRNA expression (Fig. 5a). The 25 and 10 mg/kg dose levels are without effect. In contrast, all three doses of the 2′-MOE-modified oligonucleotide reduce c-raf mRNA expression in a dose-dependent manner. In a second series of experiments designed to examine the duration of action of the oligonucleotides, mice were dosed with either of the two antisense compounds daily for 3 days at 100 mg/kg (i.p.). At this dose, both compounds are equally active at reducing c-raf mRNA expression 24 h after the last oligonucleotide dose. However, a clear difference in the activity of the two compounds is apparent at later times. By 3 days after termination of dosing the phosphorothioate oligodeoxynucleotide is no longer active, and c-raf levels have returned to control. In contrast, the 2′-MOE compound is able to maintain reduced target gene expression for at least 6 days after the last oligonucleotide dose. This is entirely consistent with the pharmacokinetics for this class of compound described above, and demonstrate that 2′-MOE-modified oligonucleotides are more potent and exhibit a more prolonged duration of action than first-generation phosphorothioate oligodeoxynucleotides. We have found that this type of prolonged inhibition of target mRNA expression by 2′-MOE compounds is typical for a large number of genes, and appears to be independent of target sequence (data not shown).

VII. CONCLUSIONS AND PERSPECTIVES

Although they represent a specific and active class of antisense molecule, ''first generation'' phosphorothioate oligodeoxynucleotides can be improved upon. Considerable effort has gone into the systematic synthesis and evaluation of a



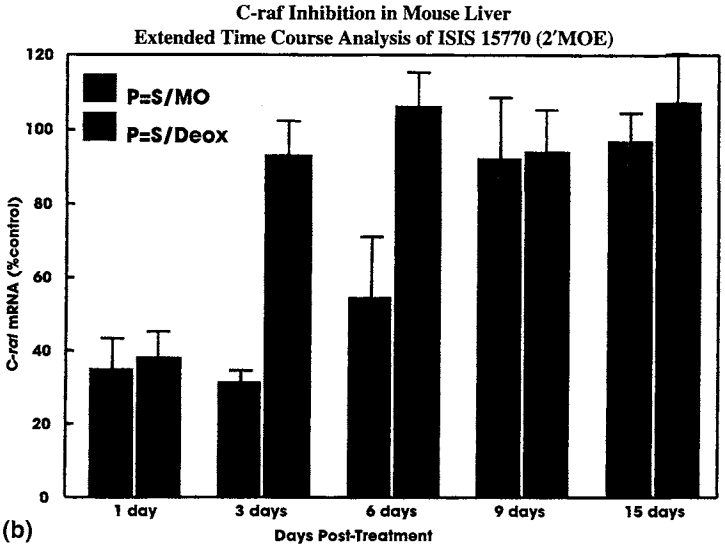


Figure 5 Inhibition of c-raf mRNA expression in mouse liver after systemic administration of either a phosphorothioate oligodeoxynucleotide or a 2′-MOE phosphorothioate chimeric oligonucleotide. (a) Mice were administered the indicated dose of oligonucleotide (i.p.) once a day for 3 days. Twenty-four hours later animals were sacrificed and c-raf mRNA expression was determined by Northern blotting. (b) Mice were administered either of the two indicated oligonucleotides once a day for three days (i.p.) at 100 mg/kg. At the indicated time after the last dose, animals were sacrificed and c-raf mRNA expression was determined by Northern blotting.

large number of oligonucleotide chemical modifications in attempts to accomplish this goal. With the characterization of the 2′-MOE modification a new and improved ''second generation'' class of compound appears to have been identified, which is able to overcome many of the limitations of the first-generation class. These include increased potency, increased nuclease resistance and duration of action, as well as reduced toxicity. As research tools to dissect gene function 2′-MOE compounds are clearly superior to phosphorothioate oligodeoxynucleotides, and they offer the opportunity to develop a class of highly potent and prolonged inhibitors of gene expression in both tissue culture and animal models. As antisense drugs, 2′-MOE oligonucleotides offer the potential to demonstrate an increased therapeutic window compared to phosphorothioate oligodeoxynucleotides. In addition, the significant increase in nuclease resistance may offer the potential for oral administration. Such an accomplishment would increase greatly the number of disease indications that become practical to treat with antisense drugs, and therefore significantly enhance the value of this therapeutic approach.

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13
LNA (Locked Nucleic Acid)

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I. INTRODUCTION

A large number of chemically modified analogs of the natural oligonucleotides (ONs) have been synthesized in recent years (1–5), in part stimulated by promising therapeutic and diagnostic applications. Researchers have synthesized fully modified ON analogs or have incorporated a number of modified monomeric or dimeric building blocks into otherwise unmodified ONs with the main aim of increasing the affinity and selectivity of binding toward complementary unmodified ONs. Recently we have been evaluating the binding properties of a number of ON analogs containing bicyclic furanose moieties with focus on derivatives with the natural pentofuranose 3′-*O*- to 5′-*O*-linked phosphordiester backbone. We were hoping that conformational restriction of the furanose ring would lead to entropically favorable duplex formation. Thus, whereas the ribofuranose rings of the natural DNA (2′-deoxy-β-D-ribofuranosyl) and RNA (β-D-ribofuranosyl) monomers are flexible, the conformation of the furanose ring of the monomeric nucleotides when present in a double helix is stirred into either an *S*-type conformation (2′-*endo* type conformation, B-form duplexes) or an *N*-type conformation (3′-*endo* type conformation, A-form duplexes) (Fig. 1).

A number of ON analogs containing nucleoside monomers with bi- or tricyclic furanose moieties have been reported, *e.g.*, bicyclic furanose nucleosides with an additional 3′-*C*,5′-C-ethylene bridge (6), bicyclic carbocyclic nucleosides (7,8), tricyclic furanose nucleosides (9), and bicyclic furanose nucleosides with an additional 2′-*O*,3′-*C*-linkage (10–12). Molecular modeling and simple model building indicated to us that LNA (locked nucleic acid) containing 2′-*O*,4′-*C*-methylene-linked ribonucleotide LNA monomers would be essentially locked in an *N*-type conformation (^3E conformation, see Fig. 1) and we therefore decided

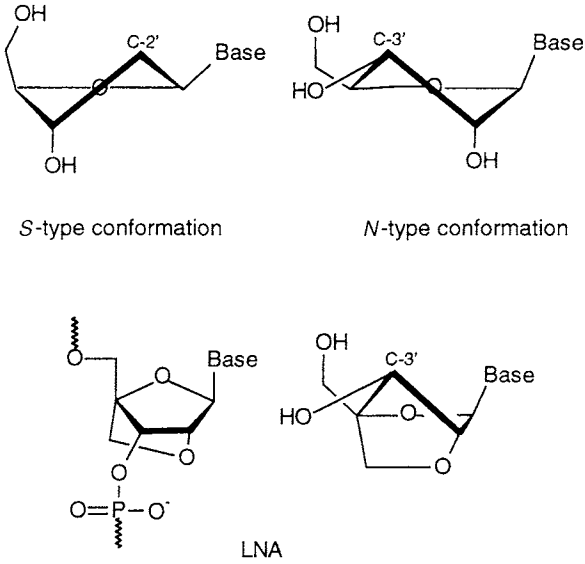


Figure 1 Nucleoside conformations. Shown are *S*- and *N*-type furanose conformations of the natural DNA (2′-deoxy-β-D-ribofuranosyl) and RNA (β-D-ribofuranosyl) nucleoside monomers and the locked *N*-type furanose conformation of an LNA monomer.

to approach their synthesis and to evaluate the thermal stability of duplexes involving LNA (13–17). It should be mentioned here that we have defined an LNA as an ON containing one or more 2′-*O*,4′-*C*-methylene-linked ribonucleoside monomers, and that Imanishi et al. independently have synthesized the uracil and cytosine LNA nucleosides and confirmed their locked *N*-type conformation by X-ray crystallography (18) as we have using NMR (13,14,19).

II. SYNTHESIS OF LNA AND LNA ANALOGS

A. Synthesis of LNA

We have synthesized LNA monomers containing six different nucleobases, namely adenine, cytosine, guanine, 5-methylcytosine, thymine, and uracil, using a convergent strategy starting from the known 4′-*C*-hydroxymethyl pentofuranose derivative **1** (20) (Fig. 2). Initially, the tri-*O*-acetyl furanose **2** (55% yield from **1**) was obtained by regioselective 5-*O*-benzylation (BnBr, NaH, DMF) followed by acetolysis (80% AcOH) and acetylation (Ac_2O, pyridine). Stereoselective reaction of the coupling sugar **2** with silylated thymine and TMS-triflate followed

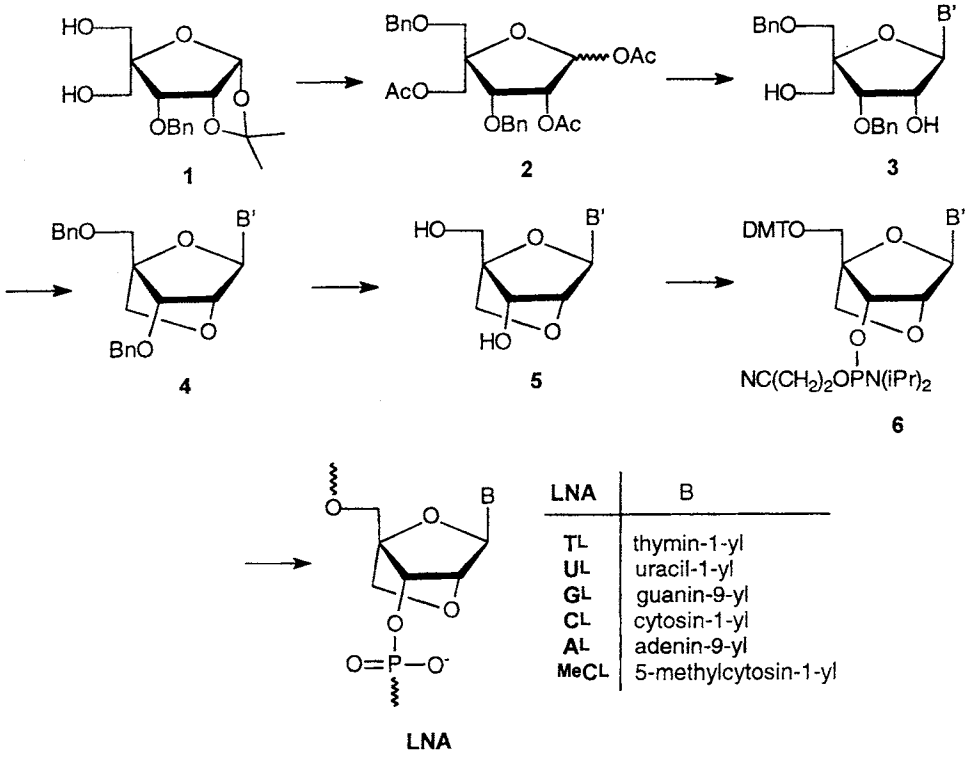


Figure 2 Synthesis of LNA nucleosides and LNA amidites.

by deacetylation furnished the nucleoside diol **3** (B′ = thymin-1-yl, 74% yield). Subsequent cyclization (4′-*O*-tosylation followed by treatment with NaH in anhydrous DMF) to give the bicyclo[2.2.1]nucleoside derivative **4** (B′ = thymin-1-yl, 42% yield) and debenzylation (H_2, 20% $Pd(OH)_2$, EtOH, 98%) afforded the parent LNA-T nucleoside (1*S*,3*R*,4*R*,7*S*)-7-hydroxy-1-hydroxymethyl-3-(thymin-1-yl)-2,5-dioxabicyclo[2.2.1]heptane (**5**, B′ = thymin-1-yl). To prepare for automated synthesis of LNA, the desired phosphoramidite derivative **6** (B′ = thymin-1-yl) was prepared by 5′-*O*-DMT protection followed by 3′-*O*-phosphitylation (65% combined yield from **5**). Syntheses of LNA nucleosides with other nucleobases were accomplished using an identical approach (Fig. 2) (14).

We have developed an alternative strategy for convergent synthesis of LNA nucleosides based on glycosylation reactions with a 4-*C*-tosyloxymethyl furanose derivative (Fig. 3) (21). Thus, tosylation of 4-*C*-hydroxymethyl furanose **7** (14,22) followed by acetolysis afforded 1,2-di-*O*-acetyl furanose **8** (21) in 59%

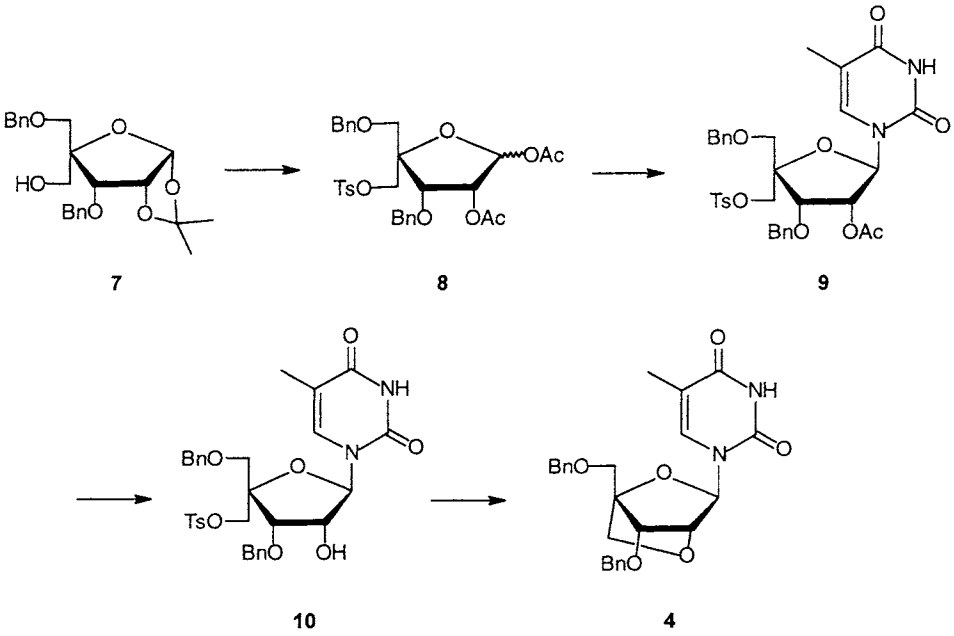


Figure 3 Synthesis of the LNA thymine nucleoside using the 4-*C*-tosyloxymethyl furanose derivative **8**.

yield. Furanose **8** proved efficient as an intermediate for nucleoside coupling reactions as, *e.g.*, coupling with thymine stereoselectively afforded the desired β-configurated 4′-*C*-tosyloxymethyl nucleoside **9** in 92% yield. Chemoselective cleavage of the acetyl ester with half-concentrated methanolic ammonia (4 h, RT, 87% yield) afforded nucleoside **10**, which by treatment with sodium hydride in anhydrous DMF (37 h, O°C) was cyclized to give the known bicyclic nucleoside **4** (14) in 92% yield. Overall, this method results in improved yields compared to the method depicted in Fig. 2.

In an alternative approach, we have used a linear strategy to synthesize the LNA adenine nucleoside **14** (Fig. 4) (21). Selective tosylation (1.5 eqv. TsCl, 4 h, O°C, 37% yield) of 4′-*C*-hydroxymethyl-2′,3′-*O*-isopropylidene-6-*N*-benzoyladenosine (**11**) (23) followed by removal of the isopropylidene group (CF_3COOH, 30 min, RT, 94% yield) afforded nucleoside **12**, which was converted to the 3′-*O*,5′-*O*-protected derivative **13** [1.2 eqv. 1,3-dichloro-1,1,3,3-tetraisopropyldisiloxane ($TIPDSCl_2$), 2 h at O°C, heating to RT, 78% yield] to prevent undesired oxetane ring formation in the next step. Cyclization of **13** (NaH, 30 min, O°C, 84%) yielded smoothly the tricyclic nucleoside intermediate,

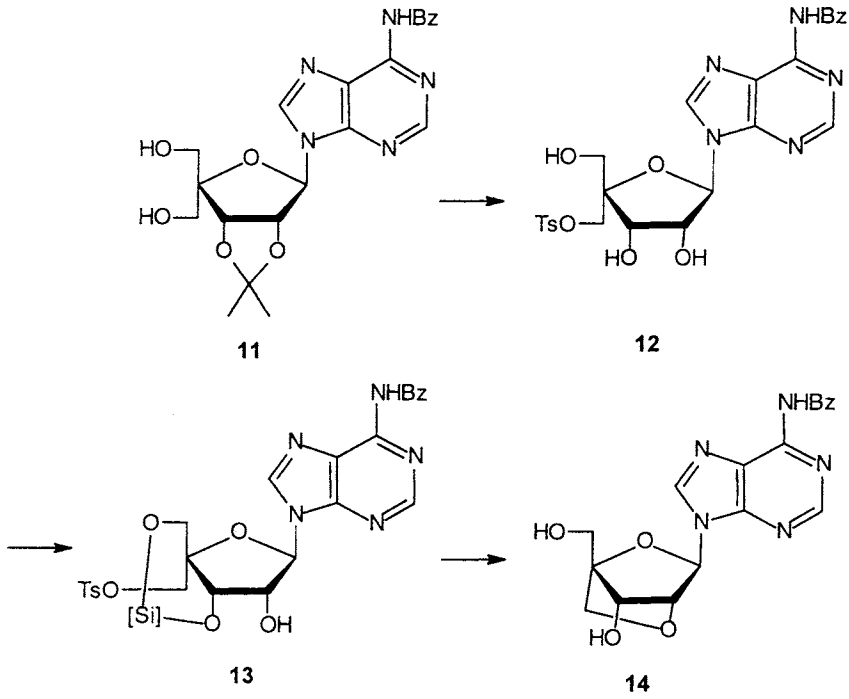
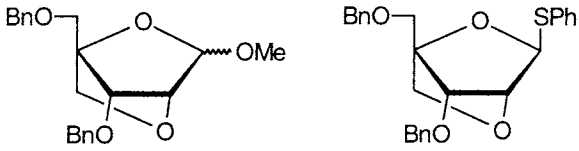


Figure 4 Linear synthesis of LNA adenine nucleoside **14**. [Si] = 1,1,3,3-tetraisopropyldisiloxane-1,3-diyl.

which after desilylation (1 h, RT) furnished the known LNA adenine nucleoside **14** (14).

As will be apparent below, we are interested in evaluating the properties of diasteroisomeric forms of LNA and thus in developing synthetic methods for their preparation. With the aim of obtaining α-anomeric LNA (α-LNA) a coupling reaction between the bicyclic methyl furanosides **15** (Fig. 5) and silylated thymine failed to give the desired anomeric mixture of LNA nucleosides. A ring-opened product was isolated in 59% yield (24) probably resulting from TMS-triflate-mediated ring opening favored by the considerable strain in the bicyclic structure. Instead, the bicyclic phenyl thiofuranoside **16** (Fig. 5) proved useful as an intermediate for condensation (silylated thymine; NBS as thiophilic activator, 61% yield, α:β ~ 2:1) to give after debenzylation the known β-LNA thymine nucleoside **5** and the anomeric α-LNA thymine nucleoside, although in low yields. Chemoselective cleavage of the anomeric bond by NBS explains that no ring opening was observed using thiofuranoside **16** (24).



15 16

Figure 5 Structures of bicyclic furanosides evaluated as coupling intermediates.

B. Synthesis of 2′-Thio-LNA and 2′-Amino-LNA

We identified a number of LNA analogs as attractive, among these 2′-amino- and 2′-thio-LNA, which we have synthesized in the pyrimidine series (Figs. 6 and 7) (25–27). We find the possibility of utilizing the 2′-amino functionality of 2′-amino-LNA monomers as a structurally well-defined conjugation site especially appealing. 4′-*C*-Hydroxymethyl nucleoside diols **3** (with thymine or uracil nucleobase) were transformed into the di-*O*-tosylated nucleosides **17** (Fig. 6, only the thymine derivative $\mathbf{17}^{T}$ is shown) using *p*-toluenesulfonyl chloride and 4-*N*, *N*-dimethylaminopyridine (DMAP) in dichloromethane (80% and 78% yield, respectively). Treatment of nucleoside $\mathbf{17}^{T}$ with neat benzylamine afforded in 30% yield the desired bicyclic 2′-*C*,4′-*C*-aminomethylene-linked 2′-deoxyribonucleoside **18**. A similar cyclization was effectively performed on the uracil analog of **17** (potassium thioacetate in DMF, 75% yield) but the concomitant debenzylation proved impossible in our hands. The cyclization reactions involving both an intermolecular and an intramolecular nucleophilic substitution step with the heteroatom nucleophiles via an initial 2,2′-anhydro intermediate (26) thus proved effective. However, the necessity of an anhydro intermediate for stereochemical inversion at the 2′-carbon atom limits the utility of this strategy to the pyrimidine series. The most convenient procedure for debenzylation of 2′-benzylamino nucleoside **18** turned out to involve treatment with ammonium formate and 10% palladium on carbon yielding in 54% yield the desired 2′-amino-LNA nucleoside **19** (Fig. 6). To obtain the corresponding 2′-thio-LNA nucleoside **24**, the route depicted in Fig. 7 was followed (25) as a consequence of the failed debenzylation described above. Thus, debenzylation of the di-*O*-tosylated uracil derivative of nucleoside **17** in 49% yield afforded nucleoside **21** [20% $Pd(OH)_2/C$, H_2], which was TIPDS-protected in 97% yield with $TIPDSCl_2$ in anhydrous pyridine to give the bicyclic derivative **22**. Reaction with potassium thioacetate in DMF to give a tricyclic intermediate (77% yield) followed by desilylation (TBAF in THF, 69% yield) afforded the desired 2′-thio-LNA nucleoside **24**. Importantly, the furanose rings of the 2′-amino-LNA nucleoside **19** and the 2′-thio-LNA nucleoside **24** were by ^{1}H NMR experiments shown to adopt locked 3′-*endo* (^{3}E) conformations very similar to the furanose conformation of the parent LNA monomers.

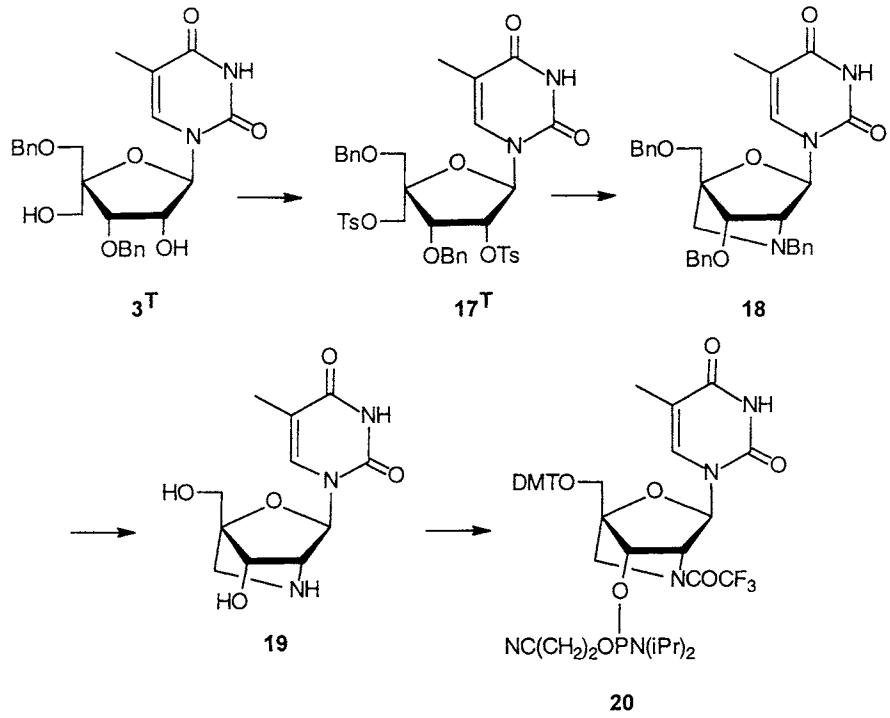


Figure 6 Synthesis of the 2′-amino-LNA nucleoside **19** and the phosphoramidite derivative **20**.

Using standard procedures (5′-*O*-DMT protection and 3′-*O*-phosphitylation), the nucleosides **19** (after chemoselective trifluoroacetylation in 81% yield of the 2′-amino functionality using ehthyl trifluoroacetate and DMAP in methanol) and **24** were converted into the phosphoramidite derivatives **20** and **25**, respectively (26,27).

C. Synthesis of α-L-LNA (α-L-Ribo-Configured LNA)

Recently, we have initiated a program focused on diastereoisomeric forms of LNA, *i.e.*, "α-L-LNA" (α-L-ribo isomer) (28–31), "xylo-LNA" (β-D-xylo isomer) (28,29), and "α-L-xylo-LNA" (α-L-xylo isomer) (30,31). Preliminary results have shown very appealing properties of α-L-LNA (*vide infra*) for which our first synthetic methodology follows (Fig. 8) (31). The known 4′-*C*-hydroxymethyl nucleoside **26** (32) obtainable from 1,2:5,6-di-*O*-isopropylidene-α-D-glucofuranose was mono-protected (1.1 eqv. DMTCl, anhydrous pyridine; 31% yield) to

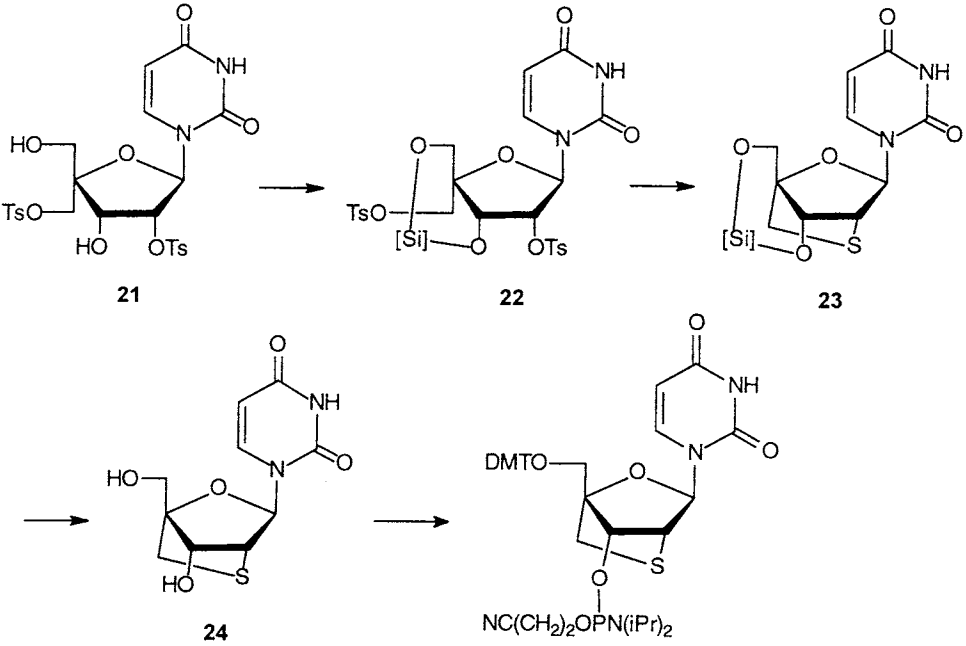


Figure 7 Synthesis of the 2′-thio-LNA nucleoside **24** and the phosphoramidite derivative **25**. [Si] = 1,1,3,3-tetraisopropyldisiloxane-1,3-diyl.

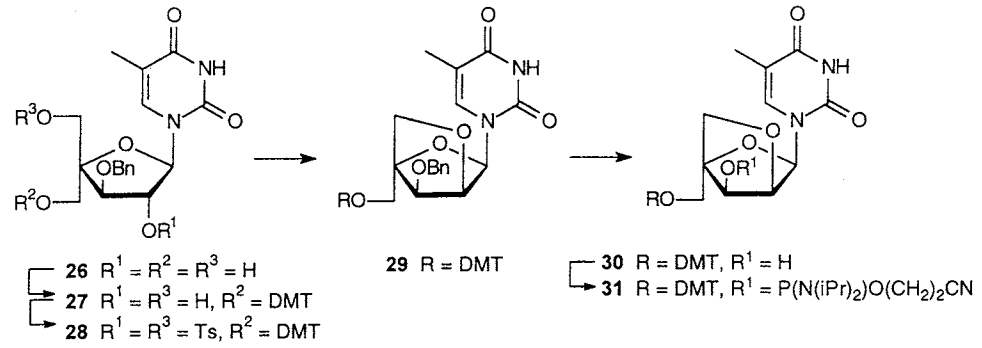


Figure 8 Synthesis of α-L-LNA derivatives.

give nucleoside diol **27**, which was converted into the di-*O*-tosylated derivative **28** in 63% yield by treatment with excess TsCl and a catalytic amount of DMAP in anhydrous pyridine. Reaction of nucleoside **28** in 2 M aqueous NaOH in a 1:1 mixture of H_2O:EtOH directly furnished the desired bicyclic 2,5-dioxabicyclo[-2.2.1]heptane derivative **29** in 81% yield. A reaction cascade involving 2,2′-anhydro nucleoside formation, hydrolysis of this intermediate, and an intramolecular nucleophilic attack by the inverted 2′-OH group at the 5′-tosyloxy group explains the efficient conversion of compound **28** to **29**. Nucleoside **30** was obtained from nucleoside **29** in 80% yield by chemoselective debenzylation, which as a minor product also furnished the corresponding α-L-LNA thymine nucleoside diol (31). Eventually, 3′-*O*-phosphitylation of **30** yielded the desired α-L-LNA phosphoramidite derivative **31** (28).

D. Synthesis of Abasic LNA

The abasic LNA derivative **35** (33) was synthesized with the purpose of investigating the effect of backbone preorganization in relation to the thermal stability of nucleic acid duplexes in general, and duplexes involving LNA in particular. As the first step toward the synthesis of abasic LNA (Fig. 9), the 4-*C*-hydroxymethyl furanose **1** (14) was first mesylated (MsCl, anhydrous pyridine) and then transacetalized (20% HCl, MeOH, H_2O) to give the anomeric mixture of methyl furanosides **32**, which subsequently was cyclized (NaH, DMF) furnishing methyl furanosides **33** (24). Hydrolysis by treatment with 80% AcOH followed by

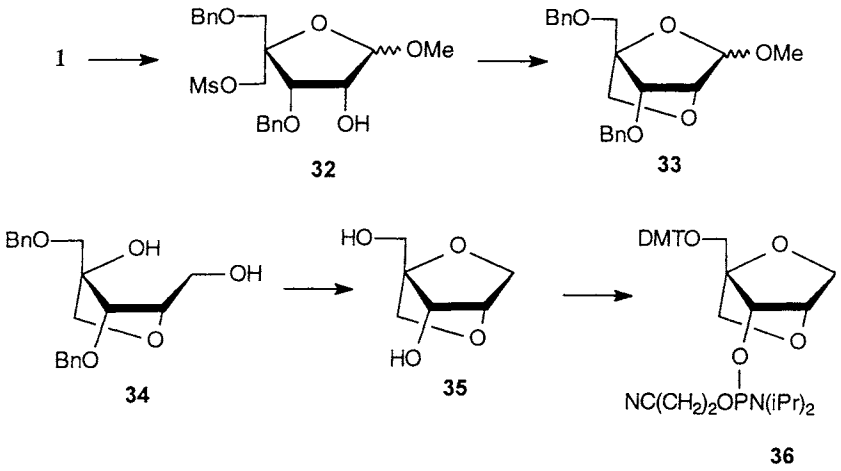
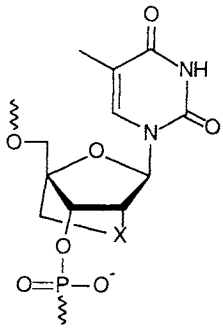


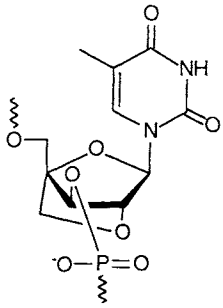
Figure 9 Synthesis of abasic LNA derivative **35** and amidite **36**.



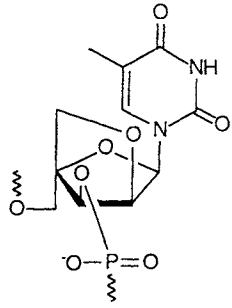
LNA (T^{L}, X = O)

2'-Thio-LNA (U^{LS}, X = S, uracil base)

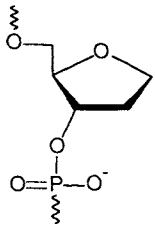
2'-Amino-LNA (T^{LNH}, X = NH)



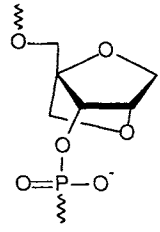
xylo-LNA (xT^{L})



α-L-LNA ($^{\alpha L}T^{L}$)



X



X^{L}

Figure 10 Monomeric structures of selected LNA monomers.

reduction of the resulting aldehyde ($NaBH_4$ MeOH) generated diol **34** in 86% yield. To obtain derivative **35** corresponding to an LNA nucleoside without the base, selective tosylation of compound **34** (TsCl, pyridine; 71% yield), cyclization (NaH, DMF; 82% yield), and debenzylation (H_2, 10% Pd/C, EtOH; 80% yield) was performed. The necessary phosphoramidite derivative for preparation of abasic LNA, namely compound **36**, was obtained from **35** by selective 5′-*O*-DMT protection (DMTCl, pyridine; 73% yield) followed by 3′-*O*-phosphitylation in 62% yield (33).

E. Automated Synthesis of LNA and LNA Analogs

One appealing feature of LNA is its close structural resemblance with DNA (and RNA). Thus, the chemical constitutions of the sugar moiety are the same except for the additional (oxy)methylene linkage, and phosphate groups constitute the internucleoside linkages. This has a number of consequences, *e.g.*, that the aqueous solubility of LNA is like that of DNA, that no additional protecting groups (compared to DNA synthesis) are needed, that the standard coupling chemistry and synthesis programs of commercial DNA synthesizers can be used, and that LNA monomers routinely can be mixed with other standard or modified phosphoramidite building blocks on a synthesizer. In the next section, the binding properties of LNA and LNA analogs containing the monomers depicted in Fig. 10 are discussed. The incorporation of the parent LNA monomers as well as the modified ones shown have been described earlier (see the relevant references), but as mentioned earlier, essentially standard coupling conditions have been applied successfully. However, in our research programs, to ensure maximum stepwise coupling yields (>98%), prolonged coupling times (5 min or longer) have in general been used with the modified LNA-type phosphoramidites. LNA and LNA analogs are compatible with standard cleavage from the solid support and removal of protecting groups (*e.g.*, conc. aqueous ammonia, 55°C, 16h). After satisfactory analysis of purity (capillary gel electrophoresis) and composition (MALDI-MS), the melting temperatures (T_m values) were measured toward complementary single-stranded DNA and RNA strands and compared with the T_m values of the corresponding unmodified reference duplexes (Tables 1, 2, and 3).

III. HYBRIDIZATION STUDIES WITH LNA AND LNA ANALOGS

A. 9-Mer Mixed Sequences

The ability of LNA and LNA analogs to recognize complementary nucleic acids has been evaluated mainly in a 9-mer mixed-sequence context, and representative results are shown in Table 1 (13–15,26,27,30). In entries 3–5, results for parent

Table 1 Hybridization Studies with 9-Mer LNAs and LNA Analogs Toward Complementary DNA and RNA

		DNA complement	RNA complement
Entry	Sequence	T_m/°C	T_m/°C
1	5′-d(GTGATATGC)	28	28
2	5′-r(GUGAUAUGC)	27	38
	Deoxy-LNA		
3	5′-d(GT^{L}GAT^{L}AT^{L}GC)	44	50
	Ribo-LNA		
4	5′-r(GT^{L}GAT^{L}AT^{L}GC)	55	63
	Homo-LNA		
5	5′-(G^{L}T^{L}G^{L}A^{L}T^{L}A^{L}T^{L}G$^{L\ Me}$C^{L})	64	74
	2′-Thio-LNA		
6	5′-d(GU^{LS}GAU^{LS}AU^{LS}GC)	42	52
	2′-Amino-LNA		
7	5′-d(GT^{LNH}GAT^{LNH}AT^{LNH}GC)	39	47
	α-L-LNA		
8	5′-d(G$^{\alpha L}$T^{L}GA$^{\alpha L}$T^{L}A$^{\alpha L}$T^{L}GC)	37	45

A = adenosine monomer, C = cytidine monomer, G = guanosine monomer, U = uridine monomer, T = thymidine monomer, ^{Me}C = 5-methylcytidine monomer. Oligo-2′-deoxyribonucleotide sequences are depicted as d(sequence) and oligoribonucleotide sequences as r(sequence). The melting temperatures (T_m values; medium salt buffer consisting of 10 mM sodium phosphate, pH 7.0, 100 mM sodium chloride) were obtained as the maxima of the first derivatives of the melting curves (A_{260} vs. temperature) using 1.5 μM concentrations of the two complementary strands (assuming identical extinction coefficients for all thymine nucleotides). All transitions were monophasic.

LNAs are depicted. It should be mentioned here that "deoxy-LNA" denotes an LNA consisting of LNA monomers and 2′-deoxyribonucleotide monomers, "ribo-LNA" an LNA consisting of LNA monomers and ribonucleotide monomers, and "homo-LNA" a fully modified LNA consisting entirely of LNA monomers. The results reveal that LNA is able to recognize both complementary DNA and RNA with remarkable thermal affinities. Thus, significantly increased melting temperatures (T_m values) were observed for the duplexes involving LNA compared to the corresponding unmodified reference duplexes (entries 3–5 compared to entries 1 and 2; ΔT_m/modification = +4.0 to +9.3°C toward DNA; +4.0 to +8.3°C toward RNA). Especially incorporation of the three LNA monomers into an oligoribonucleotide sequence (entry 4; ribo-LNA) leads to significantly enhanced duplex stabilities, which might be explained by the conformational fit between the ribonucleotide and LNA monomers (*N*-type conformations). It is important to note that LNA-mediated hybridization has proven very selective

toward base-pair mismatches (13–16). Imanishi and co-workers have independently published similar T_m values for other mixed-sequence deoxy-LNAs (34), and recently some studies on triple-helix formation using an LNA-type *C*-nucleoside monomer (35).

The corresponding T_m values for the structually similar LNA analogs, 2′-thio-LNA (27) and 2′-amino-LNA (26) (entries 6 and 7) are comparable to those obtained for the corresponding LNA (entry 3). As the 2′-thio-LNA monomer is the uracil and not the thymine derivative, it should be noted that we earlier observed identical melting temperatures for the LNA thymine and uracil monomers (14). These results show that the ribo-configured LNA-type bicyclo[2.2.1]heptane skeleton is generally applicable as a structural element in high-affinity ONs. The 2′-amino functionality of 2′-amino-LNA is a potentially useful a conjugation site, and we have synthesized the *N*-methyl dervative as the first step in this direction. The T_m values were also in this case strongly increased compared to the reference value (26).

The melting temperatures for a highly interesting diastereoisomeric form of LNA, namely α-L-LNA [in this example consisting of three 2′-*O*,4′-*C*-methylene-α-L-ribofuranosyl thymine monomer(s) and six unmodified DNA monomers] are also shown in Table 1 (entries 8 and 30). Though not quite as thermally stable as duplexes involving the corresponding LNA (entry 3), remarkably increased T_m values are observed (ΔT_m/modification = +3.0 toward DNA and +5.3°C toward RNA). These results appear surprising considering the very different configurations of an α-L-LNA and a DNA monomer. However, modeling studies performed with the α-L-LNA monomer $^{\alpha L}\mathbf{T}^{L}$ and the LNA monomer $\mathbf{T}^{L}$ show convincingly a close three-dimensional proximity of the thymine moieties and of the respective 5′- and 3′-oxygen atoms. This may explain the high binding affinities observed for the stereoirregular α-L-LNA (entry 8). Experiments involving singly mismatched sequences clearly established the hybridization of at least this α-L-LNA to be very specific obeying the Watson-Crick base-pairing rules (30).

Results from LNA:LNA base-pairing experiments are not shown here. We have, however, reported results indicating that LNA:LNA hybridization constitutes the most stable nucleic acid type duplex system hitherto discovered with increases in the T_m value of approximately 12°C for each LNA:LNA base pair compared to the corresponding unmodified duplex (16).

B. 10- and 14-Mer Homothymine Sequences

To allow further evaluation of diastereoisomeric forms of LNA, the 10- and 14-mer homothymine sequences depicted in Table 2 were synthesized and their melting behavior toward complementary DNA (dA_{i4}) or complementary RNA (rA_{14}) studied (13,28,29). The unprecedented binding affinities of fully modified LNA

Table 2 Hybridization Studies with Homothymine LNA and Homothymine Diastereoisomeric LNAs

Entry	Sequence	dA_{14} complement T_m/°C	rA_{14} complement T_m/°C
1	T_{14}	36/32[a]	32/28[a]
2	T_{10}	24/20[a]	18
3	$T_5(\mathbf{T}^L)_4T_5$	42	52
4	$T_5(^{\alpha L}\mathbf{T}^L)_4T_5$	36	46
5	$T_5(\mathbf{xT}^L)_4T_5$	n.t.	n.t.
6	$5'$-$(\mathbf{T}^L)_9T$	80	71
7	$5'$-$(\mathbf{xT}^L)_9T$	48	57
8	$5'$-$(^{\alpha L}\mathbf{T}^L)_9T$	63	66

See Footnote to Table 1.

[a] The latter T_m value was recorded for the reference duplex in the experimental series involving entries 4, 5, 7, and 8. "n.t." indicates that no melting transition was observed above 20°C.

(entry 6), as well as the affinity-enhancing properties (especially toward complementary RNA) of LNA monomers as well as α-L-LNA monomers when incorporated into otherwise unmodified DNA strands (entries 3 and 4), are clearly seen. On the contrary, the incorporation of four xylo-LNA monomers (entry 5) has a detrimental effect on the binding properties. However, in a fully modified context (entry 7) xylo-LNA is able to efficiently bind toward both DNA and RNA (ΔT_m = +3.1 and +4.3°C, respectively, compared to the unmodified duplexes in entry 2) (29). The fully modified α-L-LNA (entry 8) display binding affinities (ΔT_m = +4.8°C toward DNA and +5.3°C toward RNA) approaching those of the corresponding LNA (entry 6) (29).

IV. TOWARD AN EXPLANATION OF THE UNPRECEDENTED HYBRIDIZATION PROPERTIES OF LNA

A. NMR and CD Studies

To study the structural basis for the unique hybridization properties of LNA, a collaborative project with the NMR group of J. P. Jacobsen (Department of Chemistry, University of Southern Denmark) has been intensified. Initially, a singly modified LNA [5′-d(CCGC$\mathbf{T}^L$AGCG)] was synthesized and annealed to its complementary oligodeoxynucleotide (19). The found structure of the duplex

reveals as expected that the LNA monomer adopts a 3′-*endo* furanose conformation. The overall structure of the duplex closely resembles that of the corresponding unmodified B-form duplex. However, significant alterations around the LNA monomer were detected, *e.g.*, changes associated with more efficient base stacking. NMR experiments on duplexes involving LNAs with a larger number of LNA monomers have shown that an LNA monomer is a partly modified LNA induces *S*-type to *N*-type conformational shifts in neighboring unmodified monomers (36). These shifts are associated with the overall conformation of the duplexes approaching that of an A-type duplex leading to improved base stacking. In fact, preorganization of the corresponding single-stranded LNAs was observed (36).

The observations described in the preceding paragraph are in accordance with our results from CD spectra of duplexes involving LNA (16). Thus, CD spectra of an LNA:RNA duplex showed this to structurally strongly resemble the A-form duplex of the RNA:RNA reference, whereas an LNA:DNA duplex displayed a CD spectrum characteristic of neither genuine B-type nor A-type duplex. Returning to the remarkable hybridization properties of α-L-LNA it is noteworthy that CD spectra have shown the overall conformations of an LNA:RNA duplex and the corresponding α-L-LNA:RNA duplex to be comparable (30). This strongly indicates an antiparallel binding mode for not only LNA but also for α-L-LNA.

B. Hybridization Properties of Abasic LNA

In Table 3, the T_m values toward complementary single-stranded DNA for four 13-mer mixed sequences are shown. These experiments were conducted to evaluate whether an abasic LNA monomer $\mathbf{X}^L$ induces an increased duplex stability relative to the abasic DNA monomer **X** (33). Like the parent LNA monomers, the abasic LNA monomer $\mathbf{X}^L$ display a locked furanose conformation and restricted backbone conformations. From the data of Table 3 it is clear that also the intro-

Table 3 Hybridization Studies with Abasic LNA

		DNA complement
Entry	Sequence	T_m/°C
1	5′-d(CAGTGA-T-ATGCGA)	48
2	5′-d(CAGTGA-$\mathbf{T}^L$-ATGCGA)	53
3	5′-d(CAGTGA-**X**-ATGCGA)	27
4	5′-d(CAGTGA-$\mathbf{X}^L$-ATGCGA)	27

See Footnote to Table 1.

duction of a single LNA monomer induces a significant increase in the thermal stability (entry 2 compared with entry 1; $\Delta T_m = +5°C$). As expected from earlier data (37), the introduction of the abasic DNA monomer **X** had a detrimental effect on the T_m, value (entry 3; $T_m = 27°C$; $\Delta T_m = -21°C$ compared to the reference value of entry 1). The T_m value depicted in entry 4 for the abasic LNA monomer $\mathbf{X}^L$ was the same as that obtained for **X** (33).

Quenched backbone torsions and/or improved nucleobase stacking are possible explanations for the significant conformational shifts observed (NMR experiments) (36) for partly modified LNA. The melting studies with abasic LNA, excluding base-stacking interactions with monomer $\mathbf{X}^L$, demonstrate that there is no stabilizing effect in locking the furanose and backbone conformations of the abasic DNA monomer **X**. This indicates that backbone preorganization plays only a minor role for the increased duplex stabilities with LNA and, in combination with the NMR experiments, leads to the suggestion that mainly improved nucleobase stacking induces preorganization (conformational changes) in an LNA. The duplex-stabilizing effect of this favorable nucleobase stacking could be of both enthalpic and entropic origin. It is at this point impossible to draw any firm conclusions on this, but we have earlier reported the hybridization of a fully modified LNA to be enthalpically driven and the hybridization of partly modified LNA to be entropically driven (16).

V. LNA AS DIAGNOSTIC PROBE OR ANTISENSE AGENT

So far this chapter has been focused on summarizing the synthesis and hybridization properties of LNA and LNA analogs. However, the uniquely efficient and selective binding of LNA and LNA analogs toward complementary DNA and RNA opens a plethora of opportunities for diagnostic and therapeutic applications using LNA. Based on the first experiments, the use of LNA as a capture probe in diagnostics looks very promising. As one example, sequence-selective capture of digoxygenin (DIG)-labeled double-stranded PCR products has been accomplished (17). A DNA capture probe [5′-biotin-d(GGTGGTTTGTTG)] or an LNA capture probe [5′-biotin-d($\mathbf{G^LG^LT^LG^LG^LT^LT^LT^LG^LT^LT^L}$dG)] was used in experiments both with and without prior denaturation. After immobilization (streptavidin-coated microtiter plates), the amount of captured DIG-labeled PCR product was quantified (peroxidase activity; peroxidase-conjugated anti-DIG antibodies). Under denaturing conditions (5 min, 95°C; then 15 min, 25°C) both the DNA and the LNA capture probe were effective. Without denaturation (15 min, 25°C), capture was possible exclusively with the LNA capture probe. The mechanism for the latter capture is presently unclear, but capture of a noncomplementary PCR product was negative in control experiments (17). Another convincing demonstration of the usefulness of LNA in diagnostics was detection of the Factor

V Leiden mutation by direct allele-specific hybridization of PCR amplicons to immobilized LNAs (38). Also in this setup, LNA demonstrated excellent specificity and reproducibility.

A prerequisite for efficient antisense action of LNA is satisfactory membrane permeability. In a preliminary experiment, fluorescein-labeled LNA [5′-(**T^{L}G^{L}C^{L}C^{L}T^{L}G^{L}C^{L}A^{L}G^{L}G^{L}T^{L}C^{L}G^{L}A ^{L}C^{L}T**)] was transfected into living MCF-7 breast cancer cells (250 mM LNA, 0.8 μg/mL lipofectin, 35°C, 24 h) (17,39). The fluorescence was found in the nuclei of approximately 35% of the cells. It appeared from these experiments that the LNA was stable *in vivo* during the first 3 days after transfection. This substantiates our earlier experiments showing stability toward the 3′-exonuclease snake venom phosphordiesterase (13). The first antisense experiments in animals have been performed with success. Thus, direct injection of 15-mer LNAs targeted against the delta opioid receptor into the cerebrospinal fluid of living rats produced a sequence-dependent antisense effect (39). In addition, LNAs containing as much as 60% LNA monomers [5′-d(**GT^{L}GT^{L}C^{L}C^{L}GA^{L}GA^{L}CGT^{L}T^{L}G^L**)] were shown to activate RNase H–mediated degradation when hybridized to their RNA complements (39).

VI. CONCLUSION

Preparation of the LNA nucleosides requires a number of synthetic steps, but from the point when the bicyclic nucleoside diols have been obtained, standard conversions furnish the desired phosphoramidite buiding blocks. The universality of LNA mediated hybridization has been clearly demonstrated by the unprecedented hybridization properties of deoxy-LNA, ribo-LNA, homo-LNA, 2′-amino-LNA, 2′-thio-LNA, and α-L-LNA toward both complementary DNA and RNA. Other attractive characteristics of LNA (*e.g.*, standard oligomerization conditions, straightforward compatibility with the natural nucleotide monomers, and good aqueous solubility) originate from its close structural resembeance with the natural nucleic acids. This fact may also be advantageous in connection with the development of LNA-based technologies as many principles and systems already well established with DNA are directly applicable with LNA. Preliminary studies with LNA in relation to diagnostic as well as therapeutic applications have shown promising results.

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14

Antisense Properties of Peptide Nucleic Acid (PNA)

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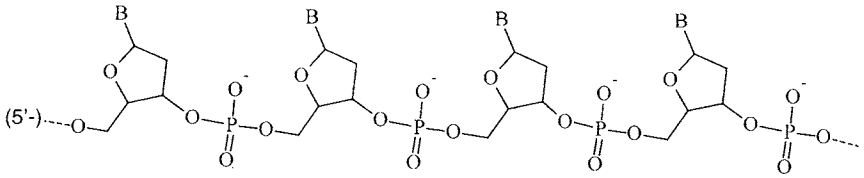
I. INTRODUCTION

Peptide nucleic acid (PNA) was conceived as a pseudopeptide mimic of a triplex-forming oligonucleotide (Nielsen et al., 1991). Despite the fact that PNAs do not readily bind to targets in double-stranded DNA via major groove triplex-helix formation (but rather by strand displacement), they are powerful structural mimics of DNA (or RNA) (Eriksson and Nielsen, 1996a, b; Brown et al., 1994; Betts et al., 1995; Rasmussen et al., 1997). Thus PNA oligomers form very stable duplexes with sequence-complementary RNA or DNA. In most cases such PNA-RNA and PNA-DNA exhibit higher thermal and thermodynamic stability as compared to the corresponding DNA or RNA complexes without sacrificing sequence specificity (Egholm et al., 1993; Ratilainen et al., 1998; Jensen et al., 1997; Giesen et al., 1998; Schwarz et al., 1999, Chakrabarti et al., 1999).

II. CHEMISTRY

Since the introduction of aminoethylglycine PNAs (aegPNAs) (Fig. 1) in 1991, a multitude of derivatives and analogs have been synthesized, but only a few appear to have properties that represent advantages for antisense and antigene applications. Substitution of the glycine of the backbone with other α-substituted amino acids can be accomplished without major penalty in DNA and RNA hybridization properties (Haaima et al., 1996; Püschl et al., 1998) and in the case of D-lysine, PNA oligomers (Fig. 2) with slightly improved binding are obtained



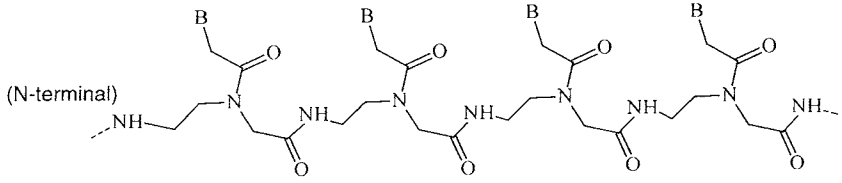


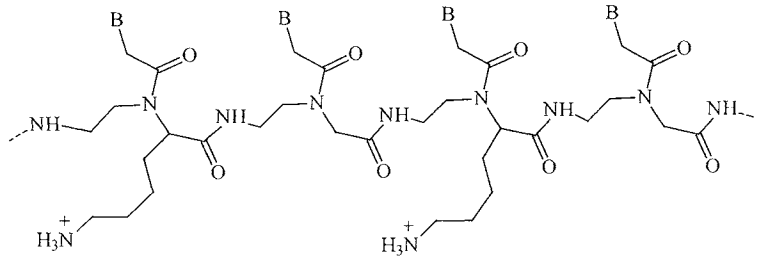
Figure 1 Chemical structures of aminoethyl glycin PNA (aeg-PNA) as compared to DNA. B is a nucleobase (adenine, cytosine, guanine, thymine).

(Haaima et al., 1996). Such PNA oligomers containing a few lysine backbone substitutions even exhibit improved uptake by eukaryotic cells (Ljungstrøm et al., 1999). PNA-DNA chimeric oligomers (Fig. 2) have also been prepared to provide RNase H recruitment as well as to allow cellular uptake mediated by cationic liposomes (Uhlmann et al., 1996).

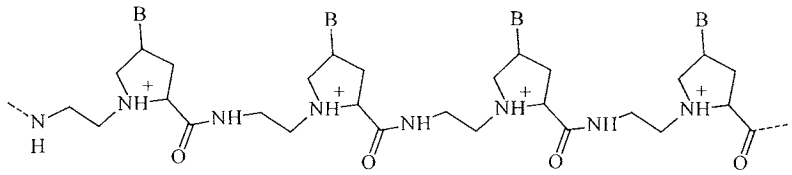
Recently, a PNA analog with a constrained cyclic backbone, the aminoethylprolyl (aep) PNA (Fig. 2), was prepared, and at least in the triplex-binding mode this modification shows significant hybridization stabilization (D'Costa et al., 1999). It will be of great interest to learn whether a similar stabilization of PNA-DNA (and RNA) duplexes results upon incorporation of aepPNA units.

III. MODIFIED NUCLEOBASES

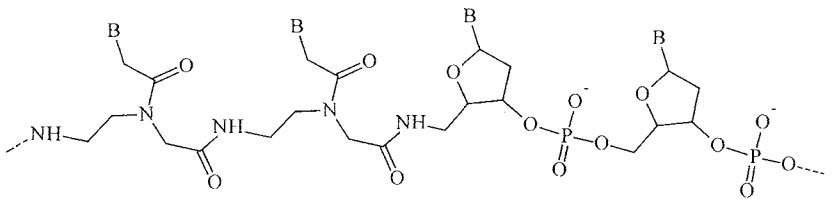
A few nonnatural nucleobases have also been reported in a PNA context. Of these the pseudoisocytosine is routinely used as a protonated cytosine mimic for PNA_2-DNA triplex formation (Egholm et al., 1995). Diaminopurine as a substitute for adenine results in a 2–4°C stabilization per substitution in PNA-DNA



aeg/ael-PNA



aep-PNA



PNA-DNA chimera

Figure 2 Chemical structures of aminoethyl glycin PNA containing units with aminoethyl lysine (ael) backbone, aminoethyl prolyl (aep)-PNA, and a 5′ amino carboxamide PNA-DNA chimera.

(or RNA) complexes (Haaima et al., 1997), and in combination with 2-thiouracil, it constitutes a pseudocomplementary base pair that allows targeting of mixed purine-pyrimidine sequences (containing >50% AT) in double stranded DNA by a double-duplex invasion mechanism (Lohse et al., 1999).

IV. ANTISENSE AND ANTIGENE EFFECT OF PNA IN VITRO

Several in vitro studies have established PNA as a strong inhibitor of transcriptional and translational processes. Antigene effect (i.e., sequence-specific inhibition of transcription) can be obtained by triplex binding of PNA to the template strand (Nielsen et al., 1994; Praseuth et al., 1966). The very stable PNA_2-DNA complex then constitutes a sterical hindrance for RNA polymerases resulting in a complete arrest of further mRNA elongation at the targeted site. In contrast to the triplex complexes, double binding of PNA to both the template and the nontemplate strand by double-duplex invasion does not result in inhibition of transcription, as the RNA polymerase seems capable of breaking the PNA-DNA binding (Lohse et al., 1999). Binding of triplex-forming PNAs to a regulatory motif, a NF-κB transcription factor site, resulted in a strong inhibition of transcription due to the sterical blockage of NF-κB binding (Vickers et al., 1995).

Like transcription, translation is generally arrested at sites of PNA triplex formation with the target. Furthermore, if PNA are targeted to positions near the start codon, translation initiation can be almost completely inhibited using duplex binding PNA hetero-oligomers (Hanvey et al., 1992; Knudsen and Nielsen, 1996; Gambacorti-Passerini et al., 1996). A single study has demonstrated that also PNA bound by duplex formation to a downstream target of the messenger (HA-*ras*) can inhibit translation elongation, but rather than a general quality of PNA this may well be a special case dependent on the specific target (Dias et al., 1999). Also, it has been shown that PNA targeted to the 5′-untranslated region of a gene (*bcl-2*) can inhibit translation, and that PNAs targeted to different regions of the messenger can give an additive effect resulting in a stronger translation block than obtainable with either of the applied PNAs alone (Mologni et al., 1998, 1999). As neither PNA-RNA duplexes nor PNA_2-RNA triplexes trigger RNase H activity, the sequence-specific inhibition of the translational processes exerted by PNA is probably caused by steric blockage of ribosomal assembly and/or action.

A special case of antisense application of PNA has been the use of PNA to inhibit telomerase activity (Norton et al., 1996; Hamilton et al., 1999). In these studies PNAs complementary to the RNA component (especially the 11-base template sequence) of the telomerase holoenzyme efficiently inhibited telomerase activity in a cell-free system with IC_{50} in the picomolar-to-nanomolar range. Fur-

thermore, a similar study has shown that the conjugation of an antitelomerase PNA to a 7-mer peptide consisting of mainly cationic amino acids resulted in increased inhibition of telomerase activity compared to the unconjugated PNA (Harrison et al., 1999). This finding gives hope that conjugation of PNA to certain peptides, which may be important to confer cell permeability to PNA as discussed in the next section, will not influence negatively on the antisense potency of PNA.

Also the elongation process of reverse transcription can be inhibited specifically by PNA. Using, among others, HIV-1 reverse transcriptase and different targets in the HIV-1 RNA genome, it has been convincingly demonstrated that both triplex-bound and duplex-bound PNA can arrest elongation of growing cDNA at the specific site binding (Koppelhus et al., 1997; Lee et al., 1998; Boulme et al., 1998). In particular it was found that while a triplex-forming bis-PNA was capable of completely inhibiting HIV-1 RT activity at a molar ratio of PNA/RNA of only 6, the molar ratio of PNA/RNA needed using a duplex forming PNA hetero-oligomer was approximately 10. Furthermore, using the bis-PNA and the HIV-1 template RNA for both in vitro reverse transcription and in vitro translation analysis, inhibition of translation was approximately 10 times less sensitive than the HIV-RT. This was demonstrated by the finding that a molar ratio of the bis-PNA to the RNA of approximately 50 was necessary for complete inhibition of translation (Koppelhus et al., 1997).

In conclusion, firm evidence has been established that PNA bound to DNA or RNA can inhibit the initiation and/or elongation processes of RNA polymerase, ribosomes, and reverse transcriptase, respectively. Also, it had been established that the inhibition is due to a steric blockage at the targeted site of the DNA or RNA. RNA polymerase seems least vulnerable to PNA inhibition and invasive triplex formation of PNA with the template strand is necessary. Reverse transcription (including telomerase activity) seems most sensitive to PNA inhibition as this is observed at considerably lower molar ratios of PNA to RNA than found for ribosomes.

V. CELLULAR UPTAKE OF PNA

PNA is very stable under in vivo conditions as demonstrated by the findings that practically no degradation of PNA takes place in human serum or in cellular extracts of prokaryotic or eukaryotic cells (Demidov et el., 1994). This finding, taken together with the promising in vitro antisense and antigene properties of PNA, has given high promise to PNA as a lead antisense and antigene drug candidate. Unfortunately, like other largely hydrophilic macromolecules, PNA shows only limited passive diffusion across phospholipid membranes (Wittung et al., 1995). Furthermore, the uncharged backbone of PNA has so far excluded use of the common DNA transfection methods as these are taking advantage of

the negative charge of the DNA phosphate backbone. However, some protocols for cell delivery of PNA have been reported.

Scarfi et al. (1997) have reported that a small PNA ([adenine]$_3$-biotin) conjugated to a four-residue hydrophobic peptide moiety (Phe-Leu-Phe-Leu) diffuses over the cell membrane in erythrocytes and Namalwa cells (B-cell-derived lymphoma cells). The diffusion into erythrocytes was determined by separating the cytoplasmatic fraction from the cell membrane by simple centrifugation, followed by methanol extraction and subsequent HPLC-MS analysis. The result showed a significant and time-dependent accumulation of the PNA-peptide chimera in the cytoplasmatic fraction of hemolysed erythrocytes. However, no experiments were done to further elucidate the localization of the PNA-peptide chimera in this system, and entrapment in cytosolic microvesicles cannot be ruled out from the simple centrifugation protocol used. Also, the very high concentration of 1 mM used might indicate a very inefficient uptake mechanism. In the Namalwa cells the uptake of the PNA-peptide chimera was done by simple fluorescence microscopy, which seems to show mainly membrane-associated fluorescence upon fixation and staining with FITC-streptavidin. Also in these experiments the authors chose a very high concentration (0.1 mM) of the PNA-peptide chimera. Taken together it is by no means evident that the PNA-peptide chimera is delivered to the cytosol in any of the two cellular test systems. In a later study (Scarfi et al., 1999) the same authors used a similar approach to evaluate the cellular uptake of radioactively labeled anti-NOS PNAs conjugated to similar strongly lipophilic peptide moieties and saw a three- to 25-fold increase in the uptake of conjugated PNAs compared to non-peptide-conjugated PNAs. Although the authors in this study use ultracentrifugation to separate the cytosol and membrane fractions of the lysed cells, they do not present data to corroborate that the PNA is freely dissolved in the cytosol and not embedded in endocytotically derived microvesicles or just small membrane fragments. Rather than a crude separation protocol, precise electron microscopy or confocal fluorescence microscopy should be applied for conclusive determination of cell compartmentalization.

Other attempts to increase membrane permeability of PNA by conjugation to a peptide have been reported. Basu and Wickstrom (1997) conjugated a PNA to a 12-residue D-peptide analog of insulin-like growth factor 1 known to bind insulin-like growth factor 1 receptor (IGF-1 R), and studied the cellular uptake of this PNA-peptide conjugate. The uptake and compartmentalization of the flouresceinated PNA was studied in BALB/c 3T3 and p6 cells (BALB/c 3T3 cells overexpressing a transfected IGF-1 R gene) by laser confocal microscopy, and revealed extensive granulate uptake consistent with receptor-mediated endocytosis.

Yet another system involving conjugation of PNA to a short peptide has been reported. Simmons et al. (1997) conjugated an 11-mer PNA to the third

helix of the homeodomain of antennapedia, which have been reported to have receptor-independent cell penetration capability (Derossi et al., 1998). The authors found that only if the full-length homeodomain (16 residues) was used did intracellular localization of the conjugate take place. Analysis of the uptake was performed by laser-scanning confocal microscopy and showed mainly granulate cytoplasmatic fluorescence. The cells used were the human prostate tumor–derived DU145 cells. No sign of cytotoxicity was observed at concentrations of conjugate as high as 10 μM, and intracellular localization of the conjugate was observed at concentrations as low as 500 nM.

Also Pooga et al. (1998) have used PNA conjugated to the antennapedia peptide and another oligopeptide, transportan, to mediate cellular uptake of PNA in human Bowes melanoma cells. They found that PNA coupled to antennapedia or transportan by a disufide bridge was taken up by the cells and subsequently distributed rather uniformly in the cells with moderate accumulation in the nuclei. Uptake was visualized by fluorescence microscopy in fixed cells stained for the biotinylated PNAs with steptavidin-FITC. As discussed in the next section, also antisense activity of the PNA was reported in this study.

A quite different approach was performed by Faruqi et al. (1998), who used streptolycin-O for permeabilization of the mouse fibroblast cell line 3340 before applying unmodified PNA. This procedure resulted in a somewhat diffuse, mainly cytoplasmatic,uptake of PNA as observed by scanning confocal microscopy.

Another attempt to increase the cellular uptake of PNA has been the conjugation of PNA to a lipophilic group, an adamantyl moiety. This conjugation was found to increase the passive diffusion rate of PNA across phospholipid membranes, and increase the transfection efficiency of PNA when used in conjunction with cationic lipids (Ardhammar et al., 1999; Ljungstrøm et al., 1999). Unfortunately, when a range of different PNA oligomers and cell lines was studied, it was evident that the cationic-mediated uptake of adamantylated PNAs is very dependent on the specific PNA sequence and also the specific cell type used, in a yet-undefined way. Thus, in laser confocal microscopy an unpredictable uptake pattern ranging from clear endosomal localization to a somewhat diffuse cytoplasmatic and sometimes nuclear localization was observed depending on the specific combination of PNA sequence and cell type (Ljungstrøm et al., 1999).

An interesting method for transferring PNA to cell in cultures has been presented by Corey's group (Hamilton et al., 1999). Taking advantage of an established DNA transfection protocol, PNA is hybridized to a partially complementary DNA oligonucleotide and then subsequently transfected into cells using cationic lipids. Fluorescence microscopy and FACS analysis consistently showed a markedly increased uptake of PNA as a result of this procedure. Further efforts to visualize the intracellular fate of the PNA were not made, but as discussed in

the next section, biological effects seemed to verify a significant cellular uptake of the PNAs.

Furthermore, a somewhat similar transfection protocol described by Brandén et al. (1999) seems to validate the principle that DNA can be used as a vehicle for intracellular delivery of PNA. In this study fluorochrome-labeled 15-mer oligonucleotides were hybridized to complementary PNAs covalently linked to the SV40 core nuclear localization signal (NLS) and subsequent transfection into different cells using the nonlipid transfection reagent polyethylineimine. Confocal scanning microscopy clearly showed that the highest nuclear localization rate was obtained using oligonucleotides hybridized to a PNA containing the functional NLS. Thus it seems PNA-NLS chimera can act even as carrier for DNA once in the cytosol.

Covalently linked PNA-DNA chimera have also been developed with the aim of facilitating uptake cationic liposomes as well as to provide RNase H–mediated degradation of the target mRNA (Uhlmann et al., 1996).

Finally, it has been claimed that neuronal cells readily take up unmodified PNA even without the use of any kind of transfection procedure. The first report on this unexpected finding was based on indirect evidence for uptake using the biological response to PNA microinjected into the periaquductal gray of male Sprague-Dawley rats (Tyler et al., 1998). Later a more direct detection of PNA was performed using a gel shift assay to show the presence of very minute amounts of PNA in brain tissue following intraperitoneal injection. (Tyler et al., 1999). The biological impact of PNA in these systems is discussed in the next section.

A more direct study of PNA and neural cells was performed by Aldrian-Herrada et al. (1998), who used magnocellular neurons from the hypothalamic supraoptic nucleus to study the uptake and effect of PNA. In short, they found that unmodified (naked) PNA was taken up within hours while conjugation of PNA to a retroinverso derivative of the antennapedia peptide dramatically improved uptake kinetics. In both cases, PNA was first seen in the apical part of the neuronal cytoplasm, then followed by a spread over the entire cell, including nucleus and neurites. The labeling of the cells was punctuated at high magnifications, but uptake kinetics and the independence of temperature observed for the uptake of this peptide-conjugated PNA seem consistent with a receptor-independent mechanism.

In conclusion, different approaches have been tried to improve cellular uptake of PNA. These studies have shown that uptake can indeed be improved by conjugating PNA to small peptides or a lipophilic moiety. Furthermore, it seems evident that a strong relation between uptake kinetics, compartmentalization, and cell type exists for any of the PNA chimeras that have been investigated in more than one cell type. In general, though, the studies done so far on the intracellular compartmentalization of PNA and PNA-peptide chimeras seem to show a ten-

dency for PNA accumulation in cytosolic vesicles. Most interestingly, this is not the case for the PNA-peptide conjugates studied in human Bowes melanoma cells. Also puzzling are the reports on neuronal uptake of unmodified PNA. Although it seems unlikely, it cannot be excluded that special transport receptors with PNA-binding capacity exist in some specific cell types. Nevertheless, we find that a comprehensive and comparative study on the uptake of PNA and PNA-peptide conjugates in different cell types is needed, a study that also takes into account the differences on the microscopic appearance of intracellular compartmentalization known to arise from different fixation protocols (Pichon et al., 1999).

VI. EX VIVO AND IN VIVO EFFECTS OF PNA

The limited bioavailability of PNA has excluded a comprehensive study of the ex vivo and in vivo effects of PNA. Nevertheless, the improved cellular uptake resulting from the protocols described above has led to a number of observations, which will be summarized in this section. Also, it is important to realize that the very strong antigene and antisense effects of PNA observed in vitro give hope that a true antigene or antisense effect can be obtained even with a very limited uptake of PNA resulting from, e.g., endosomal escape. Thus, a strong diffuse cytosolic or nuclear labeling may not necessarily be a prerequisite for a strong cellular effect of PNA. In fact, it is possible that even a visually undetectable uptake can result in a strong effect. On the other hand, no such relation has been established for other antisense agents like phosphodiester or phosphorothioate oligonucleotides.

The first report on intracellular effects of PNA was contributed by Hanvey et al. (1992), who showed that microinjected PNA resulted in a strong and sequence-specific down-regulation of the SV40 large T antigen in a cell line constitutively expressing this gene (tsa 8). However, microinjection of PNA is too laborious to offer a broadly applicable system for the ex vivo studies of PNA.

Scarfi et al. (1999) found that hydrophobic peptide-PNA chimeras targeting a homopurine stretch of the inducible nitric oxide synthetase (NOS) gene were capable of reducing the activity of this enzyme in lysates of stimulated macrophages RAW 264.7. The cells were incubated for 4 days with 1 μM of the PNA-peptide chimera. A 14-mer PNA gave rise to a more than 40% reduction in NOS activity while shorter PNAs as well as a 14-mer-scrambled control sequence had no effect. No internal standard was tested to evaluate any unspecific effect of the active PNAs, and thus a sequence-specific, nonantisense effect on the NOS activity cannot be excluded from these experiments.

Stronger proof for a specific intracellular (site-specific mutagenic) effect of PNA has been provided by Faruqi et al. (1998). Permeabilizing the cells with

streptolysin-O, they were able to show that PNAs specific for clamping an 8- or a 10-bp site in the *supFG1* mutation reporter gene carried within a chromosomally integrated, recoverable λ phage shuttle vector in mouse fibroblast induced mutation frequencies 10 times higher than the background. PNAs with three or four mismatches were ineffective in the mutagenesis assay, and no increased mutagenesis was detected with any of the PNAs in the nontargeted *cII* gene. Thus, meeting the two basic criteria for a specific antigene effect, this study strongly indicates that PNA is indeed capable of performing in vivo as predicted from the in vitro studies.

Also strongly in favor of a true intracellular ''antisense'' effect of PNA is the report that specific PNAs targeted at template regions of the essential RNA components of telomerase are capable of a strong reduction in functional telomerase activity (Hamilton et al., 1999). Thus, a reduction of more than 85% of telomerase activity was obtained in both DU145 (prostate-tumor-derived) and 293 (adenovirus-transformed human) cells. The efficient reduction seen as a result of targeting PNA to the template region of the holoenzyme seems to reflect the high accessibility of this region that is needed to bind and extend telomeric DNA during normal functioning of this ribonucleoenzyme. A scrambled PNA showed no inhibition. The PNA was introduced to the cells by the use of partially complementary DNA oligonucleotides in combination with cationic lipids as described in the previous section. A concentration of 1 μM of each oligomer was used.

PNA conjugated to either the antennapedia or the transportan peptide and targeted to the galanin receptor type 1 (Ga1R1) is apparently taken up in Bowes melanoma cells in a nonendocytotic way as described earlier. In accordance with the uptake pattern, a strong down-regulation of the ^{125}I galanin–binding capacity was seen in these cells in response to addition of PNA-peptide antisense chimeras. Surprisingly, a PNA complementary to the 18–38 position of the coding region was three times more efficient in inhibiting ^{125}I galanin binding than a PNA targeted to the 1–21 position (EC_{50} of 0.20 μM and 0.60 μM, respectively). Efficient down-regulation of Ga1R1 was seen by immunoprecipitation of the galanin-1 receptor 36 h after addition of 3 μM of PNA. A scrambled PNA sequence showed no effect on galanin receptor expression, and neither of the PNAs influenced on the presence of actin as measured by subsequent immunoprecipitation of the galanin depleted supernatants (Pooga et al., 1998). However, viability of the cells was not assessed thoroughly in this study, and a nonantisense, but sequence-specific, interference with Ga1R1 expression/galanin binding can formally not be completely excluded from the presented results. Thus, further studies in the Bowes cells/PNA-peptide chimera system are needed for the final proof of concept. Nevertheless, intrathecal treatment of the rats with micromolar concentrations of the PNA constructs seemed to corroborate the findings in Bowes cells, as binding of ^{125}I galanin was reduced in the dorsal horn of the spinal chord.

Furthermore, electrophysiological testing of the flexor reflex showed significant changes in animals treated with the active constructs as compared to animals treated with the scrambled controls.

Very peculiar are the reports from Richelson's group that unmodified PNA injected intraperitoneally or to the periaqueductal gray gives a specific and efficient antisense and antigene effect in the brains of rats. (Tyler et al., 1998, 1999). In these studies, PNAs were targeted to either the rat neurotensin receptor or the opioid mu receptor, and sequence-specific effects were observed in response to subsequent treatment with neurotensin (reduced antinociception and hypothermia) or morphine (reduced antinociception) indicating significant down-regulation of the targeted receptors. Furthermore, binding experiments in neuronal homogenates from the PNA-treated rats corroborated the conclusion that the observed effects were indeed due to PNA-mediated antisense and antigene effect on their specific targets. However, although it is generally accepted that the in vivo situation can differ markedly from the conditions ex vivo, the potency of PNA to cross not only cell membranes but also the blood-brain barrier in vivo is indeed surprising, and contrasts a previous study (Pardridge et al., 1995). Moreover, the finding in these studies that efficient antisense effect can be obtained with PNA targeted to a downstream region of the mRNA (+ 103 from start codon of neurotensin receptor type 1; NTR 1) is surprising (but plausible). Finally, the suggested antigene effect of a hetero purine-pyrimidine PNA targeted to the codon region of the DNA is highly controversial, as such PNAs do not bind to double-stranded targets in vitro. Even double-duplex invasion complexes are not capable of arresting (phage) RNA polymerase elongation in vitro (Lohse et al., 1999). Therefore, more studies are required to understand and explain these results.

VII. ANTIMICROBIAL PNAs

Promising results have recently been obtained in bacteria by taking advantage of an *E. coli* mutant strain (AS19) that has ca. 10-fold increased sensitivity to antibiotics in general due to a cell wall/membrane defect. It was shown that bis-PNAs directed against homopurine targets in the peptidyl transferease center or the α-sarcin loop of the 23S ribosomal RNA of *E. coli* very efficiently inhibited translation in a cell-free system at concentrations similar to tetracycline (Good and Nielsen, 1998a). Furthermore, these PNAs were able to inhibit the growth of AS19 at low micromolar concentrations, with a potency that is ca. 10% that of tetracycline. In another study these results were extended to antisense inhibition of β-glactosidase and β-lactamase using PNAs targeted to the translation initiation regions of the mRNA of these these enzymes (Good and Nielsen, 1998b). Most encouragingly, it was shown that treatment of formerly penicillin-

resistant (caused by β-lactamase) AS19 with anti-β-lactamase PNA resensitized the bacteria to penicillin. These results provide proof of the concept that PNAs can be used to develop gene-targeted, designed antibiotics against (multidrug resistant) bacteria, although much more work obviously is needed to obtain PNAs with greatly improved uptake in pathogenic bacteria. The antisense technique may, however, also be employed as a target validation technology for finding novel targets for traditional small-molecule antimicrobial drugs.

VIII. CONCLUDING REMARKS

A wide variety of recent results have clearly provided significantly improved methods for delivery of PNAs to eukaryotic cells, and several of these methods have already yielded exciting antisense responses. The prospects of at least assessing the antisense (and antigene) potential, and hopefully also of developing new gene therapeutic drugs, look brighter than ever. Clearly many obstacles are still ahead, but the next few years should prove whether the optimism and enthusiasm is to be rewarded.

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Phosphorodiamidate Morpholino Oligomers

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I. INTRODUCTION

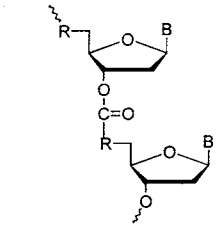
A. History

Antisense technology is now applied not only to immediate therapeutic applications but also to gene functional analysis, validation of molecular medicine, and confirmation of therapeutic approaches. The most fundamental aspects of antisense technology rely upon: (1) hybridization of nucleic acid oligomer to target mRNA sequences, (2) sufficient biological stability of the antisense oligomer, and (3) the capacity of the oligomer:mRNA complex to inhibit gene expression. Hybridization is most frequently assured through the use of unmodified bases. Oligomer stability requires modifications of either the sugar or backbone linkage. Finally, the oligomer:mRNA complex inhibits gene expression by either mediating the cutting of target mRNA or mechanisms that result in steric interference with translation of mRNA. Sentiment summarized by Zamecnik (1996) and others conclude the inability to activate RNase H represents a disadvantage for some oligomer compositions. The exceptional in vivo efficacy of the phosphorodiamidate morpholino oligomers (PMO), which do not activate RNase H, suggest this issue should be reevaluated.

B. Chemistry

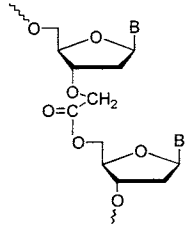
Non-ionic internucleoside linkages were specifically designed for anticipated advantages in formation of stable hybrid duplexes with RNA, increased resistance to nucleases, and enhanced cellular uptake. A variety of non-ionic oligomer struc-

Table 1 Representative structures of non-ionic oligomer chemistries



a R= -O-
b R= -NH

1



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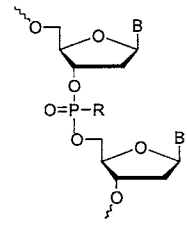
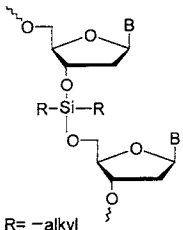


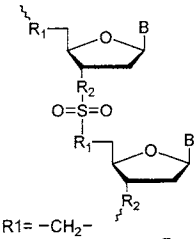
a R= -O-CH_3
b R= -O-alkyl
c R= -CH_3

3



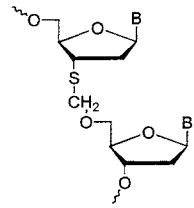
a R= –alkyl
b R= –phenyl

4



a R1= –CH_2–
b R1= –NH–
c R2= –CH_2–
d R2= –NH–

5



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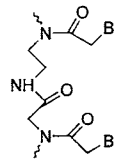

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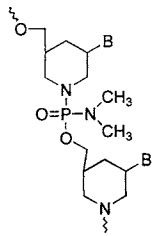

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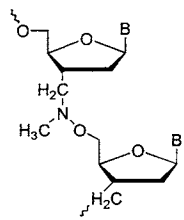

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tures are shown in Table 1. Very early work exploring the non-ionic chemistries described the preparation of carbonate-**1a** (Mertes and Coates, 1969) and carbamate-linked **1b** (Gait et al., 1974) oligonucleotides. Little biohemical data have been reported for these structures. The phosphate backbone was also replaced in the carboxymethyl-**2** (Jones et al., 1973) and the silyl-linkages **4** (Olgive and

Cormier, 1986), which represent attempts resulting in oligomers with relatively low T_m and sensitivity to acid hydrolysis. Substitutions of the PO linkage with SO_2 as sulfones **5a,c** (Roughton et al., 1995), sulfonamides **5b,c** (McElroy et al., 1994), and sulfamates **5b,d** (Huie et al., 1992) were prepared but little antisense activity has been reported. The methylphosphonates **3c** (Miller et al., 1989) are the most extensively studied neutral oligomers. Enthusiasm for methylphosphonates has been blunted owing to limited aqueous solubility in the range of 2–8 mg/mL (Miller et al., 1993) and complex hybridization properties due to the differences in Rp and Sp configurations (Jaworska and Stec, 1990). These problems led other investigators to avoid chiral phosphate linkages in the thioformacetal **6** (Matteucci et al., 1991), the peptide nucleic acids **7** (Nielsen et al., 1991), and the MMI neutral linkages **9** (Vasseur et al., 1992). However, a phosphorodiamidate linkage **8** (Kang et al., 1992) combined with a morphoino ring demonstrate exceptionally favorable base stacking, relatively high duplex stability, relatively good aqueous solubility, and no complexity in hybridization due to Rp and Sp configurations.

II. PHARMACOLOGY

A. Biological Advantages

The neutral character of our PMO chemistry for antisense oligomers avoids a variety of potentially significant limitations observed with PSO chemistry. The PMOs are resistant to the nucleases found in serum and liver extracts (Hudziak et al., 1996) and exhibit a high degree of specificity and efficacy both in vitro and in cell culture (Stein et al., 1997; Summerton et al., 1997; Taylor et al., 1996, 1997). The antisense mechanism of action appears to be through the PMO hybrid duplex with mRNA to inhibit translation (Stein et al., 1997; Iversen et al., 1999a). Finally, PMOs have demonstrated antisense activity against the c-*myc* pre-mRNA in living human cells (Giles et al., 1999). The combination of efficacy, potency, and lack of nonspecific activities of the PMO chemistry support the potential development.

The internucleoside charges of the phosphorothioate analogs are responsible for nonspecific effects through binding to cellular and extracellular proteins (Stein and Cheng, 1993; Stein 1995; Shoeman et al., 1997). Nonspecific interactions can lead to detrimental physiological effects in vivo including inhibition of the clotting cascade, activation of the complement cascade, and severe hypotension (Abraham et al., 1997; Cornish et al., 1993; Galbraith et al., 1994; Henry et al., 1997; Levin et al., 1998; Wallace et al., 1996). Coadministration of polyanions such as dextran sulfate or polyinosinic acid changes the distribution and pharmacokinetics of PSOs owing to apparent competition for transport of polyanions (Sawai et al., 1996; Steward et al., 1998). Further, bolus injection of PSO can

lead to blockade of sympathetic tone due to antagonism of alpha-1-adrenergic receptors (Iversen et al., 1999a).

A second disadvantage of the PSO chemistry involves the RNase H mechanism of action. RNase H is a ubiquitous enzyme present in the cell nucleus and cytoplasm in concentrations sufficient to mediate the degradation of the RNA in an RNA:DNA heteroduplex (Walder, 1992). Hence, RNase H–competent oligonucleotide chemistries such as PSO lack specificity, in part, because duplexes as short as five base pairs in length can be cleaved by this enzyme (Crouch and Dirksen, 1982). Cleavage of the RNA in such short duplexes may occur once in every 1000 bases, or approximately once in every transcript for every gene. The PMO chemistry is not RNase H–competent and its mechanisms of action rely on steric blockade of ribosomal assembly and translation arrest.

B. Mechanisms of Action

1. Hybrid Arrest of Translation/Interference with Ribosomal Assembly

The PMO:mRNA duplex-mediated hybrid arrest of translation may be quantified using fusion of the PMO target mRNA with the luciferase reporter gene. The inhibition of the reporter gene may be evaluated by classic methods in enzymology such as Lineweaver-Burke plots. When the PMO is directed at the 5′-UTR, the inhibition is noncompetitive but when the PMO is directed at the AUG translation initiation start site, then biphasic, mixed inhibition is observed. These observations are described in detail in Ghosh and Iversen (1999).

Detailed analysis of a PMO with sequence antisense to c-*myc* (AVI-4126) was evaluated with the c-*myc* transcript fused to the luciferase reporter gene (see Table 2). When the AUG translation start site of c-*myc* is cloned out of frame with the luciferase AUG site, binding of AVI-4126 to the mRNA acts as an inhibitor in the 5′-UTR. This is because the only way to translate luciferase in frame utilizes the luciferase AUG start site. The inhibition observed from AVI-4126 is noncompetitive, reflected in the diminished V_{max} (V_{max} = 16,655 in the absence of AVI-4126 and 13,175 in the presence of AVI-4126) with no change in K_m (K_m = 2.47 in the absence of AVI-4126 and 2.60 nM in the presence of AVI-4126). However, deletion of the A in the luciferase AUG site inactivates the luciferase translation start site forcing translation to begin at the c-*myc* AUG start site and brings the c-*myc* AUG into frame for translation of luciferase. AVI-4126 is now a biphasic, mixed inhibitor with V_{max} decrease from 52,493 in the absence of AVI-4126 to 23,403 in the presence of AVI-4126. The K_m reduces from 1.18 nM in the absence of AVI-4126 to 0.62 nM in the presence of AVI-4126.

Table 2 AVI-4126 Is a Biphasic Mixed Inhibitor of Translation

c-*myc* start site	Luciferase start site
mRNA 5′-G CGA CGA UGC CCC UCA ACG UUA GUC GAC AUG GAA GA-3′	
: : : : : : : : : :	
AVI-4126 3′-C GCT GCT ACG GGG AGT TGC A-5′	
Control (water)	100 nM AVI-4126
V_{max} = 16,655	V_{max} = 13,175*
K_m = 2.47 nM	K_m = 2.60 nM
c-*myc* start site	δ Luciferase start site
mRNA 5′GCG ACG AUG CCC CUC AAC GUU AGU CGA CUG GAA GA-3′	
: : : : : : : : : :	
AVI-4126 3′-CGC TGC TAC GGG GAG TTG CA-5′	
Control (water)	100 nM AVI-4126
V_{max} = 52,493	V_{max} = 23,403*
K_m = 1.18 nM	K_m = 0.62 nM*

* Indicates significantly different from control.

2. Interference with Pre-mRNA Splicing

Inhibition of pre-mRNA splicing has been demonstrated with PMOs in vitro and in vivo (Schmajuk et al., 1999). Recently, c-*myc* mRNA splicing was inhibited with a 28-mer PMO, which overlaps with much of the sequence in AVI-4126 and is directed at the intron-1 exon-2 junction, including the translation initiation AUG start site (Giles et al., 1999). They also showed that a 15-mer PMO directed at the translation initiation start site but not the intron-1 exon-2 region inhibited c-*myc* mRNA translation but did not interfere with pre-mRNA splicing. These studies demonstrated a PMO could be designed with at least two antisense mechanisms of action: (1) inhibition of mRNA translation and (2) inhibition of pre-mRNA splicing.

C. Specificity Studies

A plasmid containing the c-*myc* AUG translation start site cloned upstream of the luciferase reporter gene as described earlier was employed to investigate the specificity of AVI-4126. The binding of the oligomer sequence congeners was determined by monitoring the hyperchromic shift of the PMO:RNA hybrid duplex. The transition temperature is referred to as the melting temperature, or T_m. Initially, the efficacy (maximal % inhibition) and potency (concentration at which a 50% inhibition was observed) were determined in an in vitro translation assay

Table 3 PMO Specificity Targeting RNA

Sequence (5′-3′)	T_m (°C)	Cell free		Cell culture	
		Efficacy	Potency	Efficacy	Potency
ACGTTGAGGGGCATCGTCGC	87.8	97	141.6	60.2	290.0
ACG-TGAGGGGCATCGTCGC	79.8	87	136.2	43.0	1856.0
ACGTTGAGGGGC-TCGTCGC	78.1	86	153.2	44.7	1315.0
ACGTTGAGGGGCATCGTC-C	82.7	68	238.5	51.2	822.7
ACGTTGAGGGGCATCGTgcC		61	2717	Nd	Nd
ACGTTGAGGGGCATCGTCcg		73	1435	Nd	Nd
ACGTTcAGGGcCATCcTCGC	58	38	342.8	Nd	Nd
GGCAT		0	—	Nd	Nd
GAGGGGCATC		0	—	Nd	Nd
TTGAGGGGCATCGTC		44	175.5	Nd	Nd

(see Table 3). The studies also utilized the same plasmid vector stably integrated into HeLa cells to further investigate the same parameters of efficacy and potency in cell culture. A single deletion reduces efficacy by 5–32% in the in vitro translation assay and 4–25% in cell culture. Truncation of the sequence to less than 15 bases or sequences with greater than four mispairs in a 20-mer result in inactive oligomers with respect to c-*myc* inhibition.

D. In Vivo Efficacy Studies

PMOs have been evaluated in a number of in vivo models to confirm the antisense efficacy (Table 4). The studies involve different organs ranging from liver and kidney to tumors and the vascular wall. The dose that is determined to be effective varies with the organ or system that is targeted. The reason for these differences may be due to the different PMO distribution or it may be due to dynamic considerations presented by the cell physiology.

1. Mouse In Vivo Efficacy Studies; Lewis Lung Carcinoma

Mice bearing subcutaneous, syngeneic Lewis lung carcinomas (LLC) have been treated with AVI-4126. The LLC cell line showed sequence-specific growth inhibition with AVI-4126 in vitro. C57BL/6J mice were injected with 200,000 viable LLC cells into the right rear flank. Two days later, the mice were treated with saline, a scrambled PMO, 5′-ACT GTG AGG GCG ATC GCT GC-3′, or AVI-4126. Western blots of tumors removed 4 h after 0.1 mg/mouse dose of AVI-4126 injected i.p. show a reduction in c-*myc* protein, which was not observed after injection of either saline or the scrambled control. The AVI-4126 oligomer

Table 4 Phosphorodiamidate Morpholino Oligomer Activity in Animal Models

Target	Route	Species	Model	Ref.
p53	Intraperitoneal	Rat	Liver regeneration	Arora and Iversen, 2000
c-*myc*	Intraperitoneal	Mouse	Polycystic kidney	Rickers et al. 2001
c-*myc*	Intraperitoneal	Mouse	Cancer	Knapp (in prep.)
c-*myc*	Intraperitoneal	Rat	Liver regeneration	Arora et al., 2000
c-*myc*	Endoluminal catheter	Rabbit	Restenosis	Kipshidze et al. (submitted)
c-*myc*	Endoluminal catheter	Pig	Restenosis	Kipshidze (in prep.)
c-*myc*	Endoluminal microbubbles	Pig	Restenosis	Hiser et al., 1998; Porter et al., 1999
VLA4	Intraperitoneal	Mouse	Stem cell mobilization	J. Jackson (in prep.)
TNF-α	Pulmonary	Mouse	Pulmonary inflammation	Qin et al., 2000
TNF-α	Intravenous	Rat	Septic shock	Oettinger (in prep.)
CYP3A2	Intraperitoneal Intravenous Oral Transdermal	Rat	Drug metabolism	Iversen (in prep.)

was also recovered from the tumor and HLPC analysis demonstrated the oligomer remained intact. No treatment-related deaths occurred in 84 mice treated. The antitumor activity of AVI-4126 is dose dependent from 10 to 200 μg/mouse/day daily for 7 days.

2. Mouse In Vivo Efficacy Studies in a Polycystic Kidney Disease Model

Polycystic kidney disease (PKD) is one of the the most common genetic diseases in himans, affecting 500,000 adult Americans. The correlation between high c-*myc* expression and PKD is well established and rodent models are available to evaluate antisense PMOs directed at c-*myc* mRNA. These observations show AVI-4126 treatment of neonatal mice had no effect on total body weight or hematocrit, indicating no detrimental effect on normal cell proliferation in developing organs. The AVI-4126 treatment of cystic mice significantly reduced the degree of renal enlargement and renal function was improved based on decreased serum urea nitrogen. There is no significant difference between the scrambled

control sequence and vehicle control treatments, indicating AVI-4126 effects are sequence-specific.

3. Rat In Vivo Efficacy Studies; Liver Regeneration Studies

A rat liver regeneration model demonstrated the in vivo efficacy and potency of AVI-4126 (Arora et al., 2000). A single i.p. injection immediately after partial hepatectomy showed a sequence-specific and dose-dependent inhibition of c-*myc* expression and liver regeneration. The inhibition of c-*myc* resulted in reduction of proliferating cell nuclear antigen (PCNA), and arrested cells in the G0/G1 phase of the cell cycle. Further, the cell cycle checkpoint protein, p53, was inhibited in a dose-dependent manner but p21 (waf-1) was unaffected.

The rat liver regeneration model provided an opportunity to evaluate the in vivo efficacy of AVI-4126 after oral administration. A single dose of 2.5 mg/kg was administered in a volume of 0.5 mL at the time of hepatectomy. The rats were sacrificed 24 h after partial hepatectomy and flow cytometric analysis of hepatocytes indicated arrest in the cell cycle. Further, the intact AVI-4126 was recovered from plasma 30 min after oral administration and from the regenerating liver 24 h after oral administration. More detailed analysis of PMO oral availability is currently in progress.

4. Rabbit In Vivo Efficacy Studies; Endoluminal Delivery

Studies were conducted that indicate AVI-4126 is effective in preventing restenosis in rabbits (Iversen et al., 1998; Iversen et al., 1999b, c, d, e; Kipshidze et al., 1999). Twenty-five New Zealand, white, atherosclerotic rabbits maintained on a diet of 0.25% cholesterol were anesthetized, a transport catheter was inserted into the iliac artery, and PTCA was performed (8 atm for 30 s 3 times). The endoluminal delivery of saline or 0.5 mg of AVI-4126 to the PTCA site was at 2 atm via the outer balloon for 2 min. Angiography was performed at harvest and vessels were pressure-fixed in formalin, processed, and stained with hematoxylin and eosin, Morvat's, smooth muscle actin, and PCNA. The area of the intima and media was determined by planimetry. Morphometric analysis demonstrated significant improvement in the lumen area. The intimal area was also significantly improved in the AVI-4126-treated animals.

A long-term follow-up study was also conducted. The objective of this study was to determine the long-term impact of antisense therapy using AVI-4126 to evaluate the possibility of aneurysm formation and determine long-term efficacy. Ten New Zealand white rabbits were treated as above with four control animals receiving endoluminal delivery of 2.0 mL of saline and six animals receiving endoluminal delivery of 2.0 mL of AVI-4126 solution containing 0.5 mg.

The 6 month follow-up study indicates AVI-4126 significantly reduces

myointimal thickening from 1.63 ± 0.25 mm^2 in controls to 0.63 ± 0.36 mm^2 in treated vessels ($p < 0.05$). Further AVI-4126 significantly preserves the lumen area from 0.30 ± 0.14 mm^2 in control rabbits to 1.32 ± 0.41 in treated rabbits ($p < 0.05$). Finally, the vessels do not continue to thin resulting in potential aneurysm formation.

5. Pig In Vivo Efficacy Studies; Perfluorocarbon Bubble Delivery

An alternative to catheter delivery for AVI-4126 may reduce the extent of vascular injury. AVI-4126 will adsorb to the surface of perfluorocarbon (C_4F_{12})-exposed sonicated dextrose and albumin (PESDA) microbubbles. These microbubbles can be destroyed in an ultrasound fields, thus delivering AVI-4126 to the site of microbubble destruction. Twenty-eight pigs underwent mid-left anterior descending coronary artery balloon injury and were randomized into either (1) 20-kHz ultrasound with i.v. delivery of 1 mg AVI-4126 on PESDA, (2) 1 mg AVI-4126 alone, and (3) saline control groups. At 30 days the pigs underwent intravascular ultrasound (IVUS) to determine total vessel area (TVA). Vessels were recovered and plaminetry determination of maximal intimal thickness (MIT) and lumen area (LA) were calculated. The observations indicate AVI-4126 is effective after PESDA delivery in the pig. Abstracts describing these observations have been presented at the American Heart Association meetings (Hiser et al., 1998; Porter et al., 1998, 1999).

III. TOXICOLOGY STUDIES

A. Single-Dose Acute Toxicity Study in Rats

An acute single-dose toxicity study was conducted in rats according to the U.S. FDA Good Laboratory Practice Regulations. AVI-4126 was well tolerated at doses up to 150 mg/kg administered by intravenous injection. No mortality, clinical signs, or gross lesions were evident. The dose of 150 mg/kg is expected to be nearly 100 times the anticipated therapeutic dose for initial clinical applications.

B. Multidose Toxicity Study in Rats

A multidose toxicity study was conducted in rats according to the U.S. FDA Good Laboratory Practice Regulations. Daily intravenous administration of AVI-4126 for 14 days at dose levels up to 30 mg/kg/day were well tolerated by Sprague-Dawley rats. No animals died or exhibited any clinical signs of adverse

effect. There were no effects on body weight, food consumption, clinical pathology parameters, organ weights, or gross morphology. The only AVI-4126-related alterations observed in this study were minor histopathological alterations in the kidneys, liver, and lymph nodes. The changes in these organs were all of the same nature, and consisted of the appearance of basophilic granules within the cytoplasm of renal tubular epithelial cells, hepatic Kupffer cells, and lymph node macrophages. The granulation was observed in the kidneys of animals from all dose groups, but the incidence and intensity of the granulation were dose dependent. At the highest dose level of 30 mg/kg/day, the granulation was graded from minimal to moderate, while at the 0.3 and 3.0 mg/kg/day dose levels, the granulation was minimal in all affected animals. Basophilic granulation in hepatic Kupffer cells and lymph nodes was seen only in high-dose (30 mg/kg/day) animals, and was graded as minimal in all animals. Based on published information on other oligonucleotides (Monteith et al., 1999), the granular material is believed to reflect deposition of the oligonucleotide (and/or metabolites) and uptake by intracellular lysozymes. As such, it represents a relatively benign uptake-and-clearance process. In support of this interpretation, the basophilic granulation observed in this study was not accompanied by any degenerative changes, and there were no associated alterations in organ weight or clinical pathology parameters. Also, the granularity is reversible, although clearance of the material was not complete within the 2-week recovery period in the present study. This observation is consistent with the tissue stability of the oligonucleotide as well as with studies of other oligonucleotides. Thus, there were findings in this study that were considered toxicologically significant at dose levels up to 30 mg/kg/day. These results contrast with the previously reported effects of another structural class of oligonucleotides, i.e., those with a phosphorothioate modification, which typically elicit significant histophathological alterations in the spleen and kidneys in this dose range (Levin et al., 1998).

C. Single-Dose Toxicity Study in Primates

A single-dose toxicology study was conducted in primates according to the U.S. FDA Good Laboratory Practice Regulations. Intravenous administration of a single 10 mg/kg dose of AVI-4126 was well tolerated by cynomologus monkeys. There were no clinical signs that were considered test-article-related, and no effects on body weight, clinical pathology indices, or cardiovascular parameters. It is noteworthy that AVI-4126 did not produce either of the primary blood-level-related toxicities that have been seen in monkeys with the phosphorothioate class of oligonucleotides, i.e., activation of the alternate complement pathway and prolongation of blood clotting. The absence of these effects with AVI-4126 attests to the qualitatively different safety profile of this phosphorodiamidate morpholino-oligonucleotide, relative to the phosphorothioate class.

D. In Vitro Toxicology Studies

1. Ames Prokaryotic Mutagenicity Studies

Studies were conducted in three separate strains of *S. typhimurium* (1535, 1537, and 1538) for evaluation of mutagenic potential of AVI-4126 and the monomeric base analogs. This assay has been of questionable value for polymeric drugs because many polymeric drugs are unable to enter the bacteria. The phosphorodiamidate morpholino oligomers (PMO) do enter bacteria such as *E. coli* and *S. typhimurium*. Novel positive controls may be designed, which include sequences complementary to the bacterial 16s rRNA that prevent bacterial growth and induce bacterial death. AVI-4126 is not toxic to *S. typhimurium* while the 16s rRNA antisense sequences reduce bacterial growth by 80% with incubations of 1 μM. The three strains were selected to evaluate alternative mutagenic events; 1535 is more sensitive to point mutations induced by direct-acting mutagens such as sodium azide, and 1537 and 1538 are more sensitive to frameshift mutations such as those caused by metabolic activation of polycyclic hydrocarbons (e.g., 2-aminofluorene). The assay was conducted with the S9 metabolic activation fraction. Background mutation frequencies are in the range of two to five colonies, the 2-aminoflourine-positive controls produce approximately 200 colonies in the presence of liver S9 fraction in TA 1538 at 1 μg/culture, and sodium azide produced over 400 colonies in TA1535 at 10 ng/culture. The full-length AVI-4126 did not demonstrate any mutagenic potential (one to four colonies) in any of the three strains at either 1 or 10 μM concentration with or in the absence of metabolic activation provided by the S9.

2. V79 Eukaryotic Mutagenicity Studies

Studies were conducted in a Chinese hamster fibroblast V79 (eukaryotic) assay of mutagenesis. Mutagenesis was not observed with AVI 4126 (Ghosh and Iversen, 1999).

3. Zebrafish Teratogenicity Studies

An evaluation of teratogenesis in zebrafish embryos was conducted with 60 zebrafish blastula-stage embryos incubated for 24 h in 0, 0.1, 1, 10, 30 and 100 μM of AVI-4126. There were no significant differences in lethality in these groups.

IV. PHARMACOKINETIC STUDIES

Pharmacokinetic studies were performed for AVI-4126 in the rat using three different approaches. A carboxyfluorescein was conjugated to AVI-4126 by two different linkage chemistries, unmodified AVI-4126 were evaluated by reverse-

Table 5 Organ Distribution of Two Different Conjugates

	Tether	No tether
Liver	46 ± 10.0	31 ± 0.3
Kidneys	15 ± 1.0[a]	2.8 ± 0.2
Spleen	1.0 ± 0.1	1.8 ± 1.3
Brain	0.0 ± 0.0	0.0 ± 0.0
Heart	0.1 ± 0.1	0.2 ± 0.2
Lungs	0.2 ± 0.2	0.8 ± 0.5
Testes	0.0 ± 0.0	1.3 ± 0.3
Urine	35 ± 4.7[a]	4.6 ± 0.6
Total recovered	97	43

[a] Significantly different from the no-tether conjugate AVI-4126.

phase HPLC, and a radioactive congener of AVI-4126 is currently being evaluated. A primate study utilized the unmodified AVI-4126 and was monitored by binding to a complementary fluorescein-labeled DNA (FDNA) and evaluation of this duplex by HPLC.

A. Studies in Rat

Initial studies employed fluorescein conjugated to the phosphorodiamidate linkage at the 5′ end of AVI-4126 either at the end of a short alkyl "tether" or via a piperazine conjugate (no tether). The organ distribution 48 h after injection, as a percent of the injected dose, is summarized in Table 5. A trend toward greatest accumulation in liver, kidney, and spleen is observed.

B. Study in Primates

The studies in the primates utilized a more sophisticated method of analysis that combines much greater sensitivity of 0.01 μg/mL with extraction efficiency of nearly 100% and is capable of resolving N-1 mers from full-length AVI-4126. A summary of the plasma kinetics, is presented in Table 6. The AVI-4126 detected in these studies was full length with no evidence of N-1 metabolites for up to 24 h.

V. SUMMARY

PMOs represent a non-ionic and non-RNase H–mediated approach to antisense inhibition of gene expression. The mechanisms of action involve both interfer-

Table 6 Summary of Plasma Pharmacokinetics

Study	Rat	Primate
Dose (mg/kg)	30	10
Number of animals	4	3
Intercept A (μg/mL)	23	70 ± 6.3
Intercept B (μg/mL)	53	45 ± 2.0
Cp0 (μg/mL)	76	115 ± 4.3
Distribution half-life (h)	0.39	0.70
Elimination half-life (h)	5.9 ± 0.33	10 ± 1.0
Volume of distribution (L/kg)	0.39	0.09
Steady-state volume of distribution (mL)	330 ± 190	430 ± 59
Area under curve (μg/min/ml)	330 ± 57	990 ± 130
Mean residence time (h)	6.1 ± 1.2	16 ± 1.8
Plasma clearance (mL/min)	20 ± 4.1	31 ± 2.3

ence with ribosomal assembly, thus preventing translation, and interference with intron-exon splicing of pre-mRNA preventing appropriate translation of selected mRNA. PMOs have demonstrated in vivo efficacy in multiple animal models of cancer, polycystic kidney disease, liver regeneration, and vascular restenosis following balloon injury associated with angioplasty. In vivo efficacy has been observed in mouse, rat, rabbit, and pig models. Further, the in vivo efficacy has involved both single-dose administration and multiple daily doses for up to 14 days. Finally, efficacy has been observed following intravenous, intraperitoneal, subcutaneous, transdermal, and oral administration.

PMOs have been evaluated in single- and multiple-dose GLP toxicity studies involving rats and primates. PMOs were well tolerated following single i.v. doses up to 150 mg/kg in the rat. Daily i.v. doses for 14 consecutive days were well tolerated with the exception of a benign basophilic granulation in hepatic Kupffer cells and lymph nodes, which were nearly completely reversible in a 2-week recovery period. Bolus i.v. administration of 10 mg/kg of PMOs to cynomologus monkeys was very well tolerated with no alterations in blood pressure, clotting time, or complement activation frequently associated with bolus injection of other oligonucleotide chemistries. PMOs were nonmutagenic in both the Ames bacterial mutagenesis assays and the V79 eukaryotic mutagenesis assays. Finally, PMOs were evaluated in a zebrafish embryogenesis model to investigate potential teratogenesis and no significant decrease in embryo survival was noted for 11 days of development at concentrations of up to 100 μM.

Pharmacokinetic studies have been conducted in rat, rabbit, and monkey. The plasma kinetics are best fit with a two-compartment model with a short distribution half-life of 12–40 min and an elimination half-life of approximately 6–

12 h. The plasma clearance ranged from 15 to 30 mL/min. The liver and kidneys are organs of highest accumulation after i.v. doses to rats. No detectable metabolism has been observed in blood plasma or urine to date. Excretion into urine appears to be the primary route of elimination.

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16

Oligonucleotide Conjugates in Antisense Technology

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I. INTRODUCTION

Antisense oligonucleotide therapeutics originate from the specific molecular recognition event between the mRNA of the gene to be inhibited and the synthetic oligonucleotide drug. In addition to this key pharmacodynamic process, many pharmacokinetic processes have to be successful for the desired pharmacological endpoint. Between delivery into the patient's body and degradation or inhibition of translation of the target mRNA, success of this drug development technology relies on many receptor-ligand recognition processes.

Critical to the entire process is entry of the antisense oligonucleotide into the target cell. Certain cell surface receptors can be employed for cell-specific and tissue-specific delivery of oligonucleotides (using, for example, oligonucleotide-folate conjugates and oligonucleotide-carbohydrate cluster conjugates). The lifetime of oligonucleotides within the body is controlled by their interaction with proteins such as human serum albumin (HSA); pharmacokinetic properties can be modulated by conjugating oligonucleotides to small molecules that have a specific recognition site in HSA. Third, the biostability of oligonucleotides is governed by their interaction with exo- and endonucleases. The biostability of the oligonucleotides can be improved by introducing ligands and appropriate cationic tethers, which can interfere with interactions involving nucleolytic enzymes, oligonucleotides and metal-ion cofactor ternary complexes. The key event of inactivation of target mRNA, either by cleavage of target mRNA in mRNA-antisense oligonucleotide duplexes by RNase H or by non-RNase H mechanisms, can also be influenced by small-molecule ligands attached to the oligonucleotide.

The first generation of antisense oligonucleotides, with the 2′-deoxyphosphorothioate modification, has favorable pharmacokinetic properties. However, these oligodeoxynucleotides have toxicities correlated with the phosphorothioate content in the backbone. Overcoming the toxicity without sacrificing the favorable pharmacokinetic or protein binding properities is a challenge and one could envision achieving this with suitable oligonucleotide conjugates with appropriate chemical modifications. In this review we will summarize the different chemical conjugation modifications that have been employed to synthesize designer antisense oligonucleotides that maximize the favorable recognition events involved in delivery of antisense molecules to target mRNA.

The desired properties of antisense compounds are summarized in Fig. 1. A potent antisense drug should ideally have resistance to nucleases, high affinity for the target mRNA, favorable pharmacokinetics, and should cause inactivation of the target mRNA either by RNase H–mediated cleavage or by non-RNase H mechanisms (1–3). Many of these properties can be realized by suitable choice of oligonucleotide modifications. Certain tethered ligands may improve the cellular delivery of oligomers and increase their affinity for the target gene and resistance to nucleases. Other modifications can play the role of synthetic nucleases. Modifications to sugar or backbone or ligand conjugation can modulate the extent of protein binding, either to enhance biodistribution or to lessen side effects due to

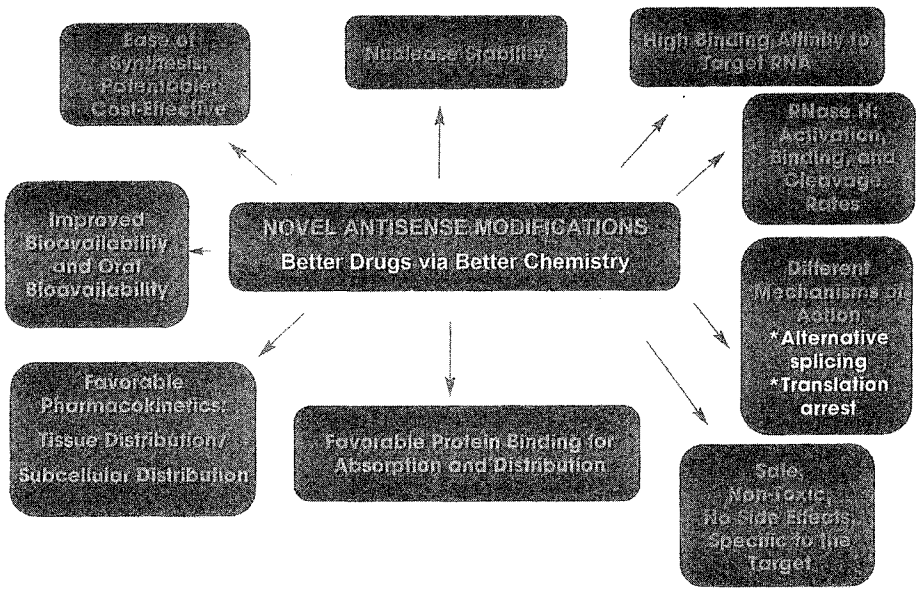


Figure 1 Desired properties for antisense drugs.

nonspecific protein binding. Some ligands are capable of improving oral-absorption properties of antisense oligonucleotides.

A few earlier reviews (4,5) addressed the chemical methodology and applications of oligonucleotide conjugates up to the year 1993. Owing to space limitations, it is impossible to provide a comprehensive review in this report. Here we will present a summary of highlights of this area by discussing some recent key advances.

II. BASIC CHEMISTRIES MODIFIED BY LIGAND CONJUGATION

Conjugations have been made to antisense oligonucleotides with the chemistries shown in Fig. 2. These oligonucleotides include 2′-deoxyphosphorothioates (2′-H/P=S) and 2′-*O*-(2-methoxyethyl) (2′-*O*-MOE) and 2′-*O*-methyl (2′-OMe) as both phosphorothioate and phosphodiester.

Oligonucleotides possessing the 2′-*O*-alkyl modifications with and without conjugation have been well studied (6). Oligonucleotides with 2′-modifications exhibit high binding affinity to target RNA, enhanced chemical stability (relevant to depurination during synthesis and biostability needed during passage in the gut), nuclease resistance to both endo- and exonucleases, and increased lipophilicity. All high-binding-affinity 2′-modifications have C3′-endo sugar pucker. Both gauche effects and charge are important in determining extent of nuclease resistance. Pharmacokinetic properties of oligonucleotides are also altered by 2′-modifications. As oligonucleotides with 2′-modifications form stable triple helices as well as duplexes, they promise to be versatile compounds in controlling gene expression by antigene and antisense technologies.

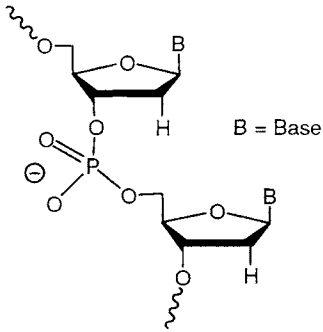
In certain reports, phosphodiester 2′-deoxyoligonucleotides (2′-H/P=O) conjugated to ligands have been studied. In addition, peptide nucleic acid (PNA) (7–9) oligomers and methylphosphonate oligomers with ligand conjugates have been characterized. Surprisingly, other classes of modified antisense oligonucleotides (10) (e.g., morpholinos, MMI, or phophoramidates) have not been further conjugated and studied.

III. APPLICATIONS

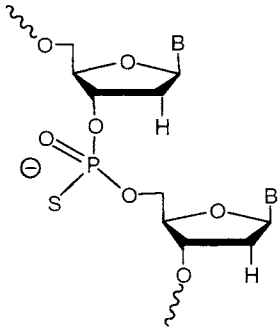
A. Lipophilic Molecular Conjugates

1. Rationale: Conjugates to Improve Cellular Uptake and Alter Pharmacokinetics

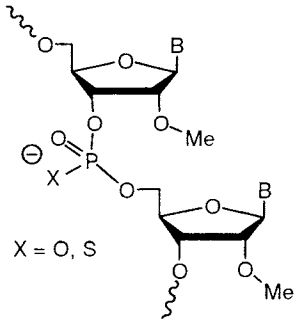
Oligonucleotides are hydrophilic molecules by virtue of their phosphate (or modified phosphate) and sugar backbone. While the nucleobase is hydrophobic, hydro-



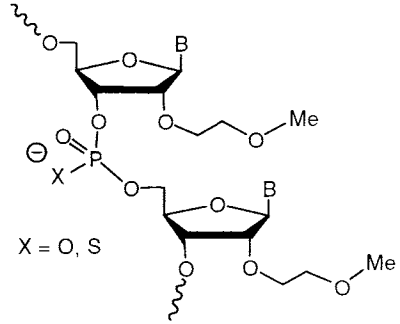
2'-deoxyoligonucleotide



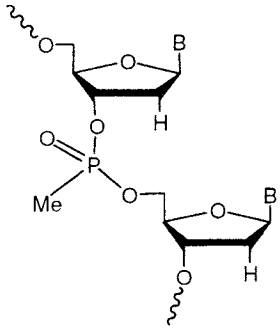
2'-deoxyoligonucleotide phosphorothioate



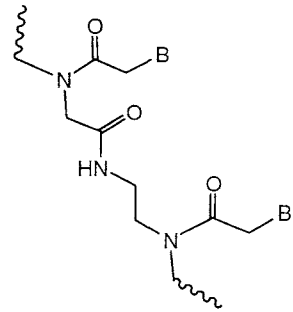
2'-*O*-Me: 2'-*O*-Methyl RNA



2'-*O*-MOE: 2'-*O*-(2-Methoxyethyl) RNA



2'-deoxyoligonucleotide methylphosphonate



PNA: Peptide Nucleic Acid

Figure 2 First-generation and second-generation chemistries to which ligands have been conjugated.

philicity dominates due to the extensive hydrogen bonding possible with the phosphate and sugar residues. This intrinsic hydrophilicity is augmented by the anionic nature of the backbone. The hydrophilic character and the anionic backbone of the drug reduces cellular permeation. Conjugation of lipophilic molecules is the obvious way to solve the cellular permeation problem.

Various lipophilic molecules have been conjugated to antisense oligonucleotides and Fig. 3 shows the structures of the compounds. Among them, cholesterol is perhaps the best characterized. It has been studied by various groups for the past 11 years (11) and has been reported to enhance binding of oligonucleotides to lipoproteins and, thereby, enhance cellular association and transport (12,13). The majority of this section will concentrate on the considerable data available on cholesterol-conjugated oligonucleotides. Data available on other lipophilic ligands will also be summarized.

2. Uridine-Conjugated Lipophilic Phosphoramidites and Solid Supports

Synthesis of 5′-*O*-dimethoxytrityl-2′-*O*-(6-aminohexyl)uridine and the 3′-isomer, 5′-*O*-dimethoxytrityl-3′-*O*-(6-aminohexyl)uridine, has been described by Manoharan et al. (14,15). Derivitization of these amines (Fig. 4) with cholesteryl chloroformate yielded cholesterol carbamate derivatives. Adamantane acetic acid, eicosenoic acid, and pyrene butyric acid were converted to their pentafluorophenol esters and condensed with these amines. 1,2-Di-*O*-hexadecyl-rac-glycerol was converted to the corresponding carbonate using disuccinimidyl carbonate. The carbonate was condensed with the amines to yield the modified nucleosides containing linkages. The nucleoside conjugates, after purification on a silica gel column, were phosphitylated to yield the corresponding phosphoramidites and then incorporated into oligomers. Each nucleoside was then condensed with long-chain alkylamino controlled pore glass (CPG).

3. Cholesterol-Conjugated ICAM-1 Antisense Oligonucleotides

An antisense oligonucleotide targeting the 3′ untranslated region of mouse intercellular adhesion molecule-1 (ICAM-1) was used for characterization of lipophilic conjugates. ISIS-3082 (see Table 1 for oligonucleotide sequences), a phosphorothioate oligonucleotide, shows antisense inhibition in cell culture with an IC_{50} of 100 nM when formulated with a cationic lipid for delivery. Uridine nucleoside synthons containing cholesterol at the 2′ or 3′ position were synthesized and incorporated at the 5′ end of the ISIS-3082 resulting in the oligonucleotide-cholesterol conjugate ISIS-8005 (14).

Cell culture experiments were used to evaluate the effect of ISIS-3082 and ISIS-8005 on ICAM-1 expression without any cationic lipid. ISIS-8005 inhibited ICAM-1 in a dose-dependent manner with an IC_{50} of 2.5 μM, while ISIS-3082

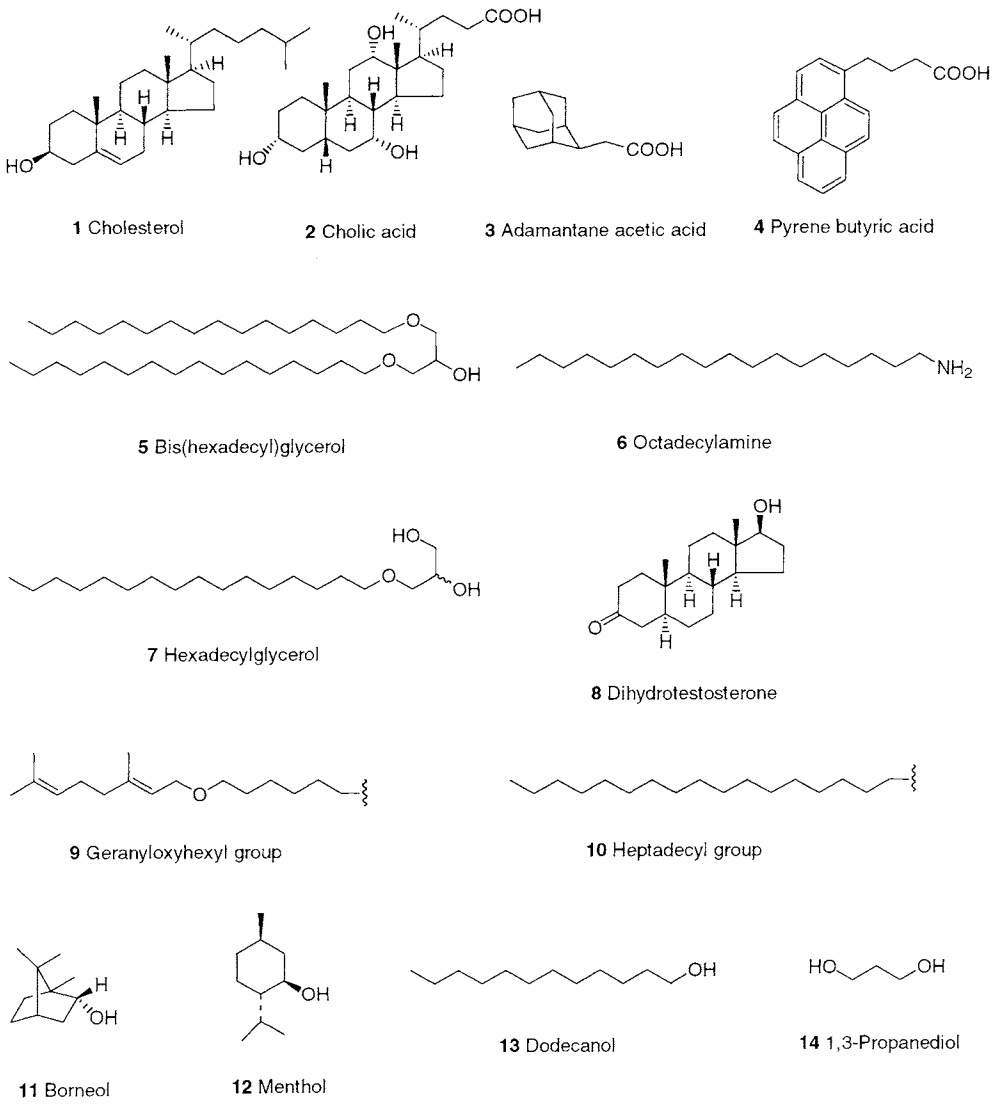
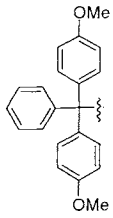
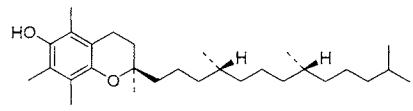


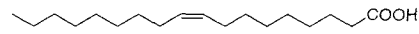
Figure 3 Lipophilic molecules.



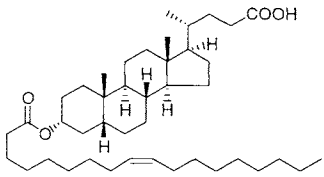
15 DMT group



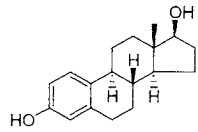
16 Vitamin E



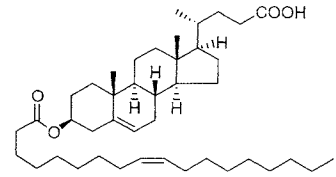
17 Oleic acid



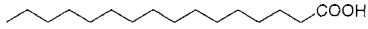
18 O^3-(Oleoyl)lithocholic acid



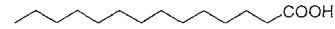
19 Estradiol



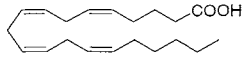
20 O^3-(Oleoyl)cholenic acid



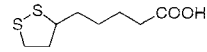
21 Palmitic acid



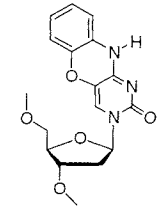
22 Myristic acid



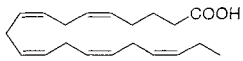
23 Arachidonic acid



24 Lipoic acid



25 Phenoxazine analog



26 Eicosenoic acid

Figure 3 Continued

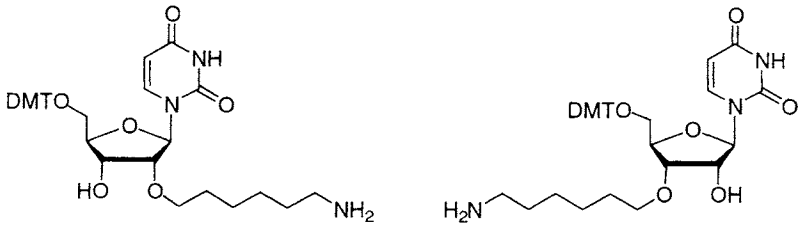


Figure 4 2′- and 3′-*O*-(6-aminohexyl) uridine derivatives.

did not show any activity, even when high concentrations of oligonucleotide were used. Furthermore, the inhibition of protein expression appears to be target specific. Neither molecule showed significant inhibition of VCAM-1 expression.

To understand the role of cholesterol in improving the function of ISIS-3082, we asked whether this molecule works merely because it is more hydrophobic than an unconjugated oligonucleotide or through specific protein-mediated (e.g., apo-ε) binding and entry into cells. The answer was obtained by synthesizing and analyzing other lipophilic conjugates of ISIS-3082. Adamantane, pyrene, eicosenoic acid, and C_{16}-glyceride lipid nucleoside conjugates were synthesized and incorporated into ISIS-3082 in the same fashion as cholesterol. Similar lipophilic molecules have been conjugated to oligonucleotide and studied in an HIV system (16).

A reverse-phase HPLC assay was used to measure the relative lipophilicities of these conjugates as a model for the interaction between the cell membrane and the antisense oligonucleotide. The retention time of the oligonucleotide (and

Table 1 ICAM-1 Oligonucleotides with Lipophilic Modifications

Compound	Composition
ISIS-3082	5′-Ts G s C s A s T s sC s C s C s C s C s A s G s G s C s C s A s C s C s A s T
ISIS-9047	5′-T*s G s C s A s T s C s C s C s C s C s A s G s G s C s C s A s C s C s A s T (T* = 5′-octadecylaminothymidine)
ISIS-8005	5′-U* s G s C s A s T s C s C s C s C s C s A s G s G s C s C s A s C s C s A s T (U* = 5′-(2′-*O*-hexylamino-carbonyl-oxycholesterol)-uridine
ISIS-9388	5′-T s G s C s A s T s C s C s C s C s C s A s G s G s C s C s A s C s C s A s U* (U* = 5′-(3′-*O*-hexylamino-carbonyl-oxycholesterol)-uridine

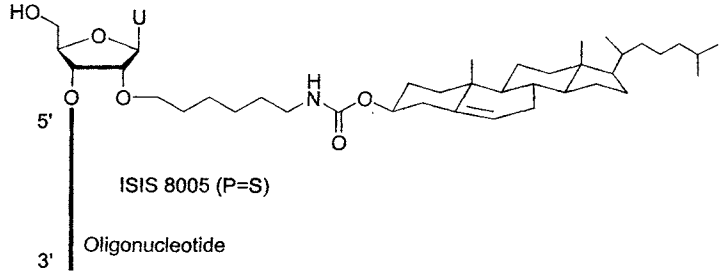
presumably the lipophilicity) increases with the number of carbon atoms in the pendant group. There is a linear correlation between the percentage of acetonitrile needed for elution and the total number of carbons. The two compounds having the same number of carbons (pyrene and eicosenoic acid) elute at the same time, while the group having the greatest number of carbons (glyceride lipid) has the longest retention time. Thus a wide spectrum of lipophilicites was observed from the least lipophilic, unconjugated ISIS 3082, to the glyceride lipid conjugate, ISIS 11826. In the antisense efficacy assays, without any added cationic lipid formulation, relative order of lipophilicity was not reflected in efficacy. While the cholesterol conjugate does inhibit ICAM-1 expression, other conjugates failed to inhibit ICAM-1 expression within the concentration range of 1–10 μM of oligonucleotides. The cholesterol-conjugated oligonucleotide shows a linear dose-dependent response in controlling the ICAM-1 expression. This experiment suggests that a receptor-mediated process may be operating in the case of cholesterol-conjugated oligonucleotides.

4. Pharmacokinetics of Cholesterol Conjugates and Other Lipophilic Conjugates

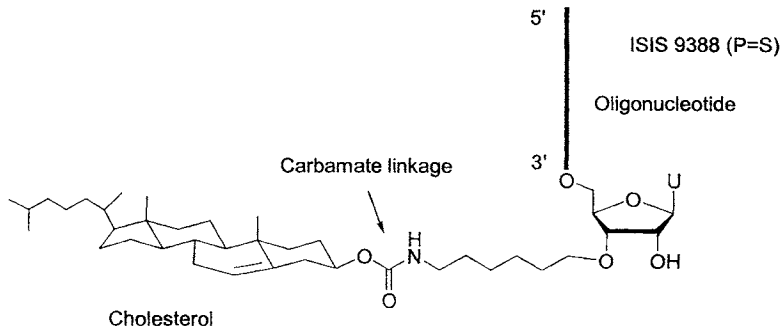
Biophysical and pharmacokinetic properties of lipophilic analogs of ISIS-3082 listed in Table 1 have been evaluated and reported (17). Compared to the parent compound, ISIS-3082, the three analogs (Fig. 5) with lipophilic conjugates, ISIS-9047 (5′-octadecylamine), ISIS-8005[5′-(2′-*O*-hexylamino-carbonyl-oxycholesterol)], and ISIS-9388 [3′-(3′-*O*-hexylamino-carbonyl-oxycholesterol)] weremore lipophilic than ISIS-3082 (three- and sevenfold, respectively, for the first two compounds as measured by reverse-phase HPLC retention times) but had similar binding affinity for complementary RNA (measured by thermal melting analysis, T_m).

Tissue distribution and half-life in mice were analyzed using radioatively labeled phosphorothioate ISIS-3082 and cholesterol and C_{18} amine analogs. After bolus intraveneous injection, the initial volumes of distribution of these more lipophilic phosphorothioate analogs, ISIS-9047 and ISIS-8005, were less and the initial clearance from plasma was slower than was that of ISIS-3082. ISIS-3082 distributes mainly to liver and kidney. Conjugation to cholesterol (ISIS-8005) or to C_{18} amine (ISIS-9047) increased substantially the fraction of the dose accumulated by the liver. Both also had a somewhat longer retention in plasma than ISIS-3082. However, neither lipophilic conjugate had an effect on metabolite patterns in plasma, liver, or kidney compared to ISIS-3082.

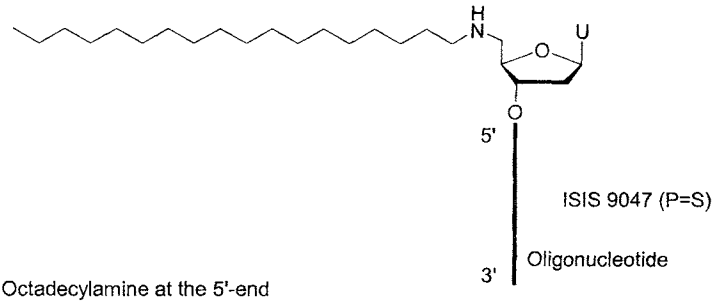
As a model to relative protein binding to human serum albumin, binding constants to bovin serum albumin (BSA) were measured. Binding to serum proteins plays a key role in the pharmacokinetics of oligonucleotides and, in view of the effects of phosphorothioates on clotting and complement activation, their



Cholesterol at the 5'-end



Cholesterol at the 3'-end



Octadecylamine at the 5'-end

Figure 5 Isis lipophilic conjugates described in Table 1.

toxicological properties as well. As a model for protein binding to human serum albumin in plasma, binding constants to BSA were measued. Binding of ISIS-3082 to BSA was comparable to that observed for other phosphorothioate oligodeoxynucleotides (17). Binding was salt-dependent and, at physiological salt concentrations, the K_d was approximately 140 μM. The affinities of the lipophlic conjugates, ISIS-8005 and ISIS-9047, were greater at physiological salt concentrations than the affinity of ISIS-3082. Experiments in which ISIS-3082, ISIS-9047, and ISIS-8005 were coincubated confirmed the lack of salt dependency of binding of the two analogs and the salt dependency of binding ISIS-3082 to BSA. These data and other data suggest that phosphorothioate linkages are necessary for binding to BSA under physological conditions, and that increased lipophilicity, either throughout the molecule or at the 5′-terminus, increased binding at physiological salt concentrations. Thus, more lipophilic phosphorothioate-containing analogs may bind to more than one type of site in BSA or more tightly to the phosphorothioate site.

The differences in serum protein binding are reflected in the pharmacokinetics of the analogs. The 5′-cholesterol adduct (ISIS-8005) and the C_{18} amine conjugate (ISIS-9047) both showed increased retention in plasma relative to ISIS-302. Both also increased the proportion of dose in the liver substantially compared to ISIS-3082. It is not clear whether this change is due to an active transport of the lipophilic conjugates into the liver or whether the effects observed were simply due to the changes in lipophilicity. However, there was no improvement in distribution to central nervous system.

Neither the 5′-cholesterol nor C_{18} amine modification enhanced resistance to metabolism significantly compared to ISIS-3082 when oligonucleotide was analyzed after extraction from liver of treated mice. However, the 3′-cholesterol conjugate of ISIS 3082 (ISIS-9388) was much more stable than the 5′-conjugate. The 3′-hydroxyl group, which is involved in the nucleophilic attack of the adjacent phosphate bond when the exonuclease enzyme makes a complex with the nucleic acid, is unavailable in ISIS-9388.

The 3′-cholesterol analog (ISIS-9388) was evaluated for its binding to lipoproteins and its biodistribution (18). ISIS-9388 associated with lipoproteins and had an altered metabolic fate compared with the nonconjugated phosphorothioate oligonucleotide ISIS-3082. The lipoprotein-associated oligonucleotide is not rapidly filtered by the kidneys and probably does not leak as rapidly in peripheral tissues as the underivatized oligonucleotide. As a result, ISIS-9388 circulates longer, which allows a longer exposure to its target.

5. In Vivo Therapeutic Efficacy of Cholesterol-Conjugated ICAM-1 Oligonucleotides

The greater concentration in liver was correlated with the therapeutic effect of the ISIS-8005 as measured by ICAM-1 mRNA levels in mouse liver in vivo. In

lipopolysaccharide-induced expression of ICAM-1 mRNA by intravenous treatment of the mouse with ISIS 8005 at a dose of 10 mg/kg 24 h and 2 h prior to polysaccharide treatment, improved efficacy of the drug was observed presumably due to cholesterol conjugation, as indicated by mouse ICAM-1 RNA levels in the liver. At this concentration, the unmodified oligonucleotide ISIS-3082 does not have any effect.

6. Evaluation of Cholesterol-Conjugated Antisense Oligonucleotides in Other Biological Targets

Following the methods used to synthesize 2′- and 3′-cholesterol-uridine conjugates (15,19), the chemistry was extended to other nucleosides (adenosine and cytosine) and antisense oligonucleotide conjugates for several disease targets were synthesized. Synthesis of these cholesterol nucleosides was carried out by condensing cholesterol chloroformate with 2′-*O*-alkylamine or 3′-*O*-alkylamine of the appropriate nucleoside. The 2′-*O*-alkylamines were derived from direct alkylation procedure (20).

The 3′-cholesterol conjugated cytosine CPG was incorporated into an Ha-*ras* antisense oligonucleotide ISIS-13748 (the conjugate is the analog of 2′-deoxy-oligonucleotide phosphorothioate ISIS-2570). This compound was evaluated to determine the effect of cholesterol conjugation on RNase H activity in a cell-free assay. The cholesterol conjugate did not affect the RNase H cleavage rates or the extent of cleavage of the target RNA (Lima and Crooke, unpublished results, Isis Pharmaceuticals).

Activity of cholesterol-conjugated 2′-deoxy and 2′-*O*-MOE gapmer phosphorothioate oligonucleotides targeted against PKC-α and C-*raf* mRNAs has been reported (21). ISIS-8006, the cholesterol conjugate, was as active as the phosphorothioate oligonucleotide, ISIS-5132, in the presence of cationic lipids. In cultured T24 cells, in the absence of cationic lipids, ISIS-8006 was able to inhibit C-*raf* kinase mRNA expression while ISIS-5132 was inactive at 5-μM concentration. In the same experiment, cholesterol-conjugated ICAM-1 antisense oligonucleotide was inactive in inhibiting C-*raf* kinase, supporting an antisense mechanism of action.

Cholesterol analogs of an antisense oligonucleotide targeting PKC-α have also been evaluated. ISIS-3521 is a potent, selective inhibitor of PKC-α gene expression in cell culture, has been shown to inhibit tumor growth in mice (22), and is currently in Phase III clinical trials. Three cholesterol analogs listed in Table 2 were tested in A549 cells and in T24 cells at 10-μM concentration without cationic lipids. The cholesterol analogs were able to reduce PKC-α mRNA levels in both cell lines while ISIS-3521 was inactive.

Table 2 Human C-*raf*, PKC-α and H-*ras* Oligonucleotides and Gapmers and Their Cholesterol Conjugates

Compound	Sequence	Target	Chemistry
ISIS-2570	CCACACCGACGGCGCCC	Human H-ras	2′-H/P=S
ISIS-13748	CCACACCGACGGCGCCC*	Human H-*ras*	2′-H/P=S with 3′-cholesterol
ISIS-5132	TCCCGCCTGTGACATGCATT	Human c-*raf*	2′-H/P=S
ISIS-8006	U*CCCGCCTGTGACATGCATT	Human c-*raf*	2′-H/P=S and 5′-cholesterol
ISIS-3521	GTT CTC GCT GGT GAG TTT CA	Human PKC-α	2′-H/P=S
ISIS-8007	GU*T CTC GCT GGT GAG TTT CA	Human PKC-α	2′-H/P=S
ISIS-9520	U*GTT CTC GCT GGT GAG TTT CA	Human PKC-α	2′-H/P=S and 5′-Cholesterol
ISIS-12373	GTT CTC GCT GGT GAG TTT CA U*	Human PKC-α	2′-H/P=S and 3′-Cholesterol
ISIS-9531	*GUU CUC GCT GGT GA GUU UCA U*	Human PKC-α	P=S gapmer; 2′-F in wings
ISIS-9533	*GUU CUC GCT GGT GA GUU UCA U**	Human PKC-α	P=S gapmer; 2′-F in wings and 3′-cholesterol

7. Effect of Cholesterol Conjugation: Reports from Other Laboratories

Inhibition of expression of the multidrug resistance-associated P-glycoprotein by phosphorothioate and 5′ cholesterol-conjugated phosphorothioate antisense oligonucleotides has been reported (23). Multiple drug resistance (MDR) is a result of overexpression of the P-glycoprotein drug transporter, a product of the MDR1 gene, and is a significant problem in cancer therapeutics. It was shown that 2′-deoxy phosphorothioate antisense oligonucleotides reduce levels of MDR1 message, inhibit expression of P-glycoprotein, and affect drug uptake in MDR mouse 3T3 fibroblasts. An oligonucleotide (ISIS-5995) directed against a sequence overlapping the AUG start codon was effective in reducing MDR1 transcript and protein levels when used at submicromolar concentrations in conjunction with cationic lipids, whereas a scrambled control oligonucleotide (ISIS-10221) was ineffective. Substantial and specific antisense effects could also be attained with a 5′ cholesterol conjugate of the ISIS-5995 sequence without the need for cationic lipids. The 5′ cholesterol ISIS-5995, but the not 5′ cholesterol ISIS-10221, reduced MDR1 message and P-glycoprotein levels by 50–60% when used at 1-μM concentrations. In parallel, treatment with 5′ cholesterol ISIS-5995 also enhanced cellular accumulation of rhodamine 123, a well-known substrate of the P-glycoprotein transporter. The effectiveness of the cholesterol-conjugated ISIS-5995 appears to be due to its rapid and increased cellular uptake as compared to unconjugated oligonucleotide, as indicated in flow cytometry and confocal microscopy studies.

The pharmacokinetics of cholesterol conjugated oligonucleotides with unconjugated phosphorothioate oligonucleotides in female mice has been reported also by the researchers at Genta. They also observed that conjugation of cholesterol to phosphorothioate oligonucleotides increased the plasma half-life (24). Sixty minutes after injection, the levels of 3′-cholesterol conjugates are 3.8 times higher than those of unconjugated oligonucleotide, while the levels of 5′- and 5′,3′-cholesterol conjugated oligonucleotides are 7.4 times higher.

Cholesterol conjugation has also been studied by Iverson et al. (25). 5′-Cholesteryl-conjugated phosphorothioate oligodeoxynucleotides with sequence complementary to the rat CYP2B1 mRNA were evaluated in adult male Sprague-Dawley rats for their pharmacokinetic properties and ability to modulate CYP2B1 expression in vivo. After intraperitoneal administration of ^{35}S-labeled oligodeoxynucleotides, volume of distribution for the phosphorothioate was reduced to 33% for the 5′-cholesteryl-conjugate oligodeoxynucleotide and the elimination half-life was reduced 50% for the cholesteryl-modified oligodeoxynucleotide relative to unconjugated controls. Hexobarbital sleep times, a measure of CYP2B1 enzyme activity in vivo, increased nearly 30% in cholesterol oligodeoxynucleotide–treated animals.

Alefelder et al. reported the introduction of 3′- and 5′-terminal phosphorothioates into oligonucleotides and their postsynthetic modification with α-(bromoacetamido)-3-cholesterol and 2-(5′-nitropyridyl)-3-cholesterol disulfide to give cholesterol conjugates (26). A similar approach was used by Zhang et al. based on a phosphoramidite intermediate (27). The phosphorothioate derivatives with cholesterol at the 3′-end exhibit potent anti-HCMV activity, enhanced nuclease resistance and cellular association. An H-phosphonothioate solid-phase synthesis method facilitated the synthesis of oligonucleotide conjugates, as demonstrated by the example of attachment of 5′-cholesterol oligonucleotides to phophorodithioates (28). Acetal-mediated cholesterol conjugation has been reported by Pfleiderer's group (29). The 5′-*O*- or 2′-*O*-position of appropriately protected thymidine or uridine was subjected to acid-catalyzed reaction with cholesterylvinylether (29). The corresponding cholesteryl-acetals were derivatized to the phosphoramidites or succinates attached to polystyrene as solid support.

The effects of conjugating cholesterol to either or both ends of a phosphorothioate oligonucleotide were analyzed in terms of cellular uptake and antisense efficacy against the p75 nerve growth factor receptor (p75) in differentiated PC12 cells, which express high levels of this protein (30). The addition of a single cholesteryl group to the 5′ end significantly increased cellular uptake and improved p75 mRNA down-regulation compared with the unmodified oligonucleotide. The 3′-cholesterol analog was more active still. Bis-cholesteryl (5′- and 3′-) conjugated oligonucleotide was even more potent and at 1 μM as effective as high concentrations of cycloheximide at decreasing synthesis of p75. Inhibitory effects on the multiplication of mouse hepatitis virus by cholesterol-modified oligonucleotides complementary to the leader RNA have also been reported (31).

Cellular uptake of 3′-cholesterol-conjugated oligonucleotides has been examined with a real-time confocal laser microscopy (32). Cytosolic uptake of cholesterol conjugate was five times as rapid as that of phosphorothioate oligonucleotides and nuclear uptake of cholesterol conjugate was twice as fast as that of unmodified oligonucleotide. In this study, oligonucleotides were also labeled with 5′-fluorescein and the effect of fluorescein on uptake has not been separated from the effect of cholesterol.

Inhibition of transactivation of human immunodeficiency virus type-1 (HIV-1)-LTR by cholesterol-conjugated antisense oligonucleotides was compared to that of their unconjugated analogs in vitro (33) to study the efficiency of antisense oligonucleotides in inhibiting LTR-(HIV-1)-directed CAT expression catalyzed by tat protein. Antisense oligonucleotides modified by conjugation of cholesterol at the 3′ end have a severalfold higher inhibitory response, and the inhibition by antisense oligonucleotides is sequence-specific.

In addition to effects on cellular uptake, cholesterol modulates oligonucleotide-mRNA hybrid stability via hydrophobic interactions (34). Two series of 3′-cholesterol- and/or 5′-cholesterol-conjugated oligonucleotides have been synthe-

sized. In the first group of compounds, the cholesteryl group was tethered at opposite ends of two oligonucleotides complementary to adjacent regions on the target. The cholesterols were adjacent to each other when the oligonucleotides were hybridized. When both oligonucleotides were hybridized to complementary RNA, an increase in the T_m of up to 13.3°C was observed in comparison to the T_m of two unmodified oligonucleotides of the same sequence. The authors observed a higher level of mismatch discrimination when the two adjacent cholesterol conjugates were compared to one continuous oligomer of the same overall length. The second set of compounds were 5′,3′-bis-cholesterol-containing oligomers, capable of forming "clamp-shaped" triple-stranded complexes, where cholesterol groups were attached to the termini of duplex- and triplex-forming domains. Stabilization of triplexes by up to 30° due to intercholesteryl interaction was observed. The authors detected no triplex formation with a mismatched target. These data suggest that significant stabilization of complexes of nucleic acids could be achieved through intercholesteryl hydrophobic interaction.

LeDoan et al. reported on interactions of phosphodiester oligonucleotides (with the sequence of Hybridon GEM-91) linked to the cholesterol group at internal position (3, 7, or 22 positions of cholesterol) (35). The conjugates were assessed for their capacity to bind, penetrate, and partition in the cytoplasmic compartment of murine macrophages. The results showed that lipophilic conjugates bind to cells much faster ($t_{1/2} \leq 10$ min) than do underivatized oligonucleotides. The fraction of oligomers that can freely diffuse from the cytosol was comparable for the phosphorthioate GEM-91 and for phosphodiester with cholesterol conjugated at position 7 (50–60% of the internalized oligomers), while the fraction of phosphodiester with cholesterol conjugated at position 3 was less (30% of the internalized oligomers). The cytosolic fraction of internalized oligomers was studied by membrane permeabilization with digitonin. Membrane binding and cell internalization correlated well with the hydrophobicity of the conjugates as characterized by their partition coefficients in a water-octanol system. However, no pharmacological end points are provided.

Srinivasan et al. (36) evaluated interactions of deoxyphosphorothioate and cholesterol-conjugated oligonucleotides with BSA and human serum albumin (HSA) in vitro. The equilibrium dissociation constant K_m for the binding of a 20 mer with BSA and HSA range between 1.1 and 5.2×10^{-5} and 2.4 and 3.1×10^{-4} M, respectively. HSA or BSA linked to Sepharose was incubated with a 15-, 20-, or 24-mer phosphorothioates and selected drugs known to be highly protein bound (nifedipine, warfarin, midazolam, probenecid, indomethacin, and mitoxantrone) were added. Up to 30% of S-ODN was displaced by warfarin in competition binding assays. Conversely, when HSA-bound warfarin was incubated with a variety of oligonucleotides, a 5′-cholesterol-conjugated 20-mer phosphorothioate displaced warfarin to a greater extent than unconugated oligonucleotide. This experiment explains, in an indirect way, observations that choles-

terol-conjugated oligonucleotides have longer plasma circulation than unmodified oligonucleotides.

8. Mechanism of Action of Cholesterol-Conjugated Oligonucleotides

The mechanism of action of cholesterol-conjugated oligonucleotides in uptake enhancement and hence improving the efficacy has not been clearly established, although a receptor-mediated process involving lipoproteins has been implicated (12,13). Second, the lipophilicity of the hydrophobic steroid skeleton of cholesterol may be optimum to enhance cellular association. Third, cholesterol conjugates may form micellar structures that facilitate the transport of the conjugate within the cell. More mechanistic studies are needed to establish the exact mode of action.

9. Toxicity of Cholesterol Conjugates and Other Lipophilic Conjugates

The toxicological properties of 2′-deoxyphosphorothioate ISIS-3082 and cholesterol analogs ISIS-9047 and ISIS-8005 (Fig. 5) were examined in Balb/c mice (37). Oligonucleotides were administered at a high dose of 50 mg/kg by i.v. bolus injection into the tail vein every other day for 14 days. In general, the properties exhibited for ISIS-3082 and the analogs were similar. Mice treated with ISIS-3082 were observed to have increases in liver transaminases and a decrease in triglycerides consistent with results from previous studies performed in CD-1 mice. Spleen weights were also increased in ISIS-3082-treated mice, but no histopathological alterations were noted. Alterations induced by ISIS-9047 and ISIS-8005 were qualitatively similar to those seen after treatment with ISIS-3082, but in general were more pronounced. Greater reductions in cholesterol and platelet counts and increases in blood urea nitrogen were observed relative to ISIS-3082. Red blood cell (RBC) counts and hematocrit were also reduced in mice treated with ISIS-9047 and ISIS-8005 relative to the ISIS-3082 treatment group. Kupffer cell hypertrophy and basophilic inclusions in Kupffer cells were observed in mice treated with ISIS-9047 and ISIS-8005, but not in ISIS-3082-treated mice. Iverson (25) observed some toxicity in his CYP12 study with cholesterol conjugates and ascribed them to the linkers used.

In a different study, a 20-mg/kg dose of ISIS-122726, a 2′-*O*-MOE gapmer oligonucleotide phosphorothioate with 3′-cholesterol, was administered to mice and biodistribtion and toxicity were evaluated after 24 h. No visible signs of toxicity were observed. This dose is lower than that reported by Henry et al. (37) and also involves a different chemistry (M. Butler, unpublished results). Nevertheless, it is an encouraging finding to further evaluate cholesterol conjugates for in vivo applications.

10. Antisense Applications of Other Lipophilic Molecules

Among steroid analogs, androstan-3-one (dihydrotestosterone) has been covalently linked to PNA to target c-*myc* DNA in prostate cancer cell nuclei (38). LNCaP cells, which express the androgen receptor gene, and DU145 cells, in which the androgen receptor gene is silent, were used in this study. Androstan-3-one was covalently linked to the NH_2-terminal position of a PNA complementary to a sequence of c-*myc* oncogene via a short polyethylene glycol linker. To allow visualization of the antisense compounds, both the PNA conjugate and a control PNA were modified with a rhodamine reporter group attached at the COOH-terminal position; the cellular uptake was monitored by confocal fluorescence microscopy. PNA-rhodamine was detected only in the cytoplasm of both cell lines. The rhodamine signal of androstan-3-one-conjugated PNA was localized in nuclei as well as in cytoplasm of LNCaP cells, which express the receptor. In contrast, uptake of the conjugated PNA was minimal in DU145 cells and exclusively cytoplasmic. In LNCaP cells, *myc* protein level remained unchanged by exposure to the free PNA whereas a significant and persistent decrease was induced by the PNA-steroid conjugate. In DU145 cells, *myc* expression was unaltered by the PNA with or without the steroid. These results were obtained despite poor solubility of the steroid-PNA-rhodamine conjugates. Despite use of short PEG linkers, with two hydrophobic molecules, rhodamine and testosterone, no better than a 2-μM concentration of steroid-PNA could be obtained, while the free PNA was soluble to 500 μM solution. It remains a possibility that the observed pharmacology may simply be due to the increased lipophilicity rather than a receptor-mediated process. Unfortunately, this study did not use other lipophilic conjugates as controls to prove that efficacy was indeed a result of an androgen receptor–mediated process.

To minimize the side effects associated with phosphorothioate oligonucleotides and to improve cellular uptake, Uhlmann et al. used partially phosphorothioate-modified oligodeoxynucleotides having a 3′ hydrophobic tail derived from 1-*O*-hexadecylglycerol (39). The oligodeoxynucleotides are protected from nucleases by phosphorothioate internucleotide linkages at both the 3′ and 5′ ends and at internal pyrimidine sites. A 12-mer conjugate targeting the H-*ras* gene retained the high sequence specificity of oligodeoxynucleotides and provided inhibition of *Ras* p21 synthesis with minimal toxicity, even without the use of a cellular uptake enhancer such as cationic lipids. Moreover, treatment of T24 cells, a radiation-resistant human tumor cell line that carry a mutant *ras* gene, with the conjugated oligonucleotide resulted in a reduction in the radiation resistance of the cells in vitro. The growth of RS504 (a human c-H-*ras*-transformed NIH/3T3 cell line) mouse tumors was significantly inhibited by the combination of intratumoral injection of the anti-*ras* conjugate and radiation treatment. Longer oligonucleotide conjugates (15 mers) did not show the free permeability sug-

gesting that the hydrophilicity/lipophilicity balance is dependent on the length of the oligonucleotide.

In the same H-*ras* system, Herdewijn's group (40) observed phosphodiester oligodeoxynucleotides conjugated with 1,3-propanediol reduced the growth of cells expressing mutated H-*ras*. These conjugates also had improved 3′-exonucleolytic stability relative to unmodified phosphodiester oligonucleotides when treated with snake venom phosphodiesterase. It is not clear whether this phosphodiester oligonucleotide is capable of forming any secondary structures that may have enhanced its nuclease resistance.

A series of conjugates of lipophilic alkyl groups at the 5′ end of oligodeoxyribonucleotides targeted against human plasminogen activator inhibitor (PAI-1) mRNA were synthesized via the oxathiaphospholane approach (41). The most active conjugates in cell culture contained menthyl or heptadecanyl groups. When conjugated to lipophilic alkyl residues, phosphodiester antisense oligonucleotides, which otherwise exhibit only limited anti-PAI-1 activity, were found to be more active than phosphorothioate oligonucleotides. The cytotoxicity of anti-PAI-I oligonucleotides and their conjugates was also evaluated in EA.hy 926 hybrid endothelial cells (42). Some cytotoxicity was found for cholesteryl and bornyl conjugates at concentrations higher than those used for antisense inhibition experiments.

Enhancement of antiherpetic activity of antisense phosphorothioate oligonucleotides targeting the immediate early (IE) pre-mRNA of HSV-1 has been observed with another terpene alcohol, geraniol (43). Geraniol enhanced the antiherpetic activity of the oligonucleotide with less cytotoxicity than the unmodified phosphorothioate and in a sequence-specific manner. Terminal modification with geraniol did not affect binding affinity with complimentary DNA. Cytoplasmic distribution of geranyl-oligonucleotide was confirmed by confocal microscope. While some of the geranyl conjugate was seen in the nucleus, the unmodified compound had a punctate distribution in the cytoplasm with little in the nucleus. These results suggested that the geranyl modification enhanced antiherpetic activity by changing the subcellular distribution of the oligonucleotide.

Synthesis, biochemical, and biological studies on oligonucleotide phosphodiesters bearing a lipophilic dimethoxytrityl group have been reported (44). These conjugates are totally resistant to nucleases present in human serum and do not affect cleavage of a complementary oligoribonucleotide by RNase H. These modified oligonucleotides exhibited better antisense inhibition of expression of plasminogen activator inhibitor type-1 within endothelial cells than unconjugated oligonucleotides.

Phenoxazine (45), a tricyclic 2′-deoxycytidine analog, was designed to improve stacking interactions between heterocycles of oligonucleotide/RNA hybrids and to enhance cellular uptake. Incorporation of four phenoxazine bases into a 7-mer phosphorothioate oligonucleotide targeting SV40 large T antigen

enhanced in vitro binding affinity for the RNA target compared to unmodified phosphorothioate oligonucleotide. In the absence of cationic lipid, the phenoxazine oligonucleotide exhibited unaided cellular penetration, nuclear accumulation, and antisense activity at 3-μM concentration in a variety of tissue culture cells. However, as the length of the oligonucleotide was increased, the extent of uptake in the absence of cationic lipid decreased.

Interaction of oligonucleotide conjugates with the dipeptide transporter system (DTS) in Caco-2 cells has been evaluated (46). While conjugation of oligonucleotides to lipophilic groups can improve delivery to cells, the enhanced cellular binding may also facilitate undesired nonspecific interactions. It has been shown that phosphorothioate oligonucleotides conjugated to either vitamin E or 2-di-*O*-hexadecyl-3-glycerol inhibit the dipeptide transporter system in cultured Caco-2 intestinal cells. Because the DTS mediates the binding and absorption of nutrient peptides and important drugs, such as the cephalosporin and penicillin antibiotics, this finding has possible implications in relation to the potential toxicity of lipophilic conjugates in vivo. It also suggests a potential drug interaction with lipophilic oligonucleotide conjugates if they were to be delivered orally.

Cellular effects of lipid soluble groups conjugated and parent antisense c-*myc* oligodeoxynucleotides depend on the mode of delivery (47). Such lipophile conjugated phosphodiesters antisense c-*myc* oligodeoxynucleotides inhibited cultured human aortic smooth muscle cell growth by 57.5% in the 4 days following a 24-h exposure to the conjugated antisense phosphodiester oligonucleotides. Liposome-mediated, but unconjugated, phosphodiester and phosphorothioate oligonucleotides showed less growth inhibition (24.4% and 29.5% inhibition, respectively). Smooth muscle cell growth inhibition by antisense c-*myc* oligodeoxynucleotides correlated with the suppression of nuclear c-*myc* protein expression. Thus, antisense c-*myc* oligodeoxynucleotides conjugated to lipidic molecules enhanced cellular incorporation as well as intracellular retention of oligodeoxynucleotides, resulting in rapid and sustained inhibition of c-*myc* expression of smooth muscle cells.

The effect of streptolysin-O permeabilization, electroporation, lipofectin and lipophilic conjugation on intracellular delivery and efficacy of phosphodiester/methylphosphonate oligonucleotides targeting BCR-ABL in chronic myeloid leukemia cells has been determined (48). The 5′ end of the oligonucleotides was modified with cholesterol, vitamin E, polyethylene glycol, *N*-octyl-oligooxyethylene, and dodecanol. The authors concluded that lipophilic conjugation or cationic lipid treatment does not improve either intracellular delivery or antisense efficacy. In contrast, both electroporation and streptolysin-O permeabilization–enhanced uptake unmodified phosphodiester/methylphosphonate oligonucleotides and achieved significant suppression of target mRNA levels. It must be emphasized that this study is limited to a single class of antisense molecule.

Perhaps more important, the in vivo consequences of this conclusion are not clear. Obviously, electroporation and streptolysin-O permeabilization are not practical methods of antisense drug administration in vivo.

11. Complexes Derived from Lipophilic Molecule–Conjugated Oligonucleotides and Liposomes and Other Oligonucleotide Carriers

Lipophilic molecules help oligonucleotides cross the cell membranes and may also enhance association with formulations and carriers of antisense drugs. Letsinger employed oligonucleotides with cholesteryl groups tethered near one or both termini to increase association with liposomes (49). Liposomes tagged with such oligonucleotides are recognized by complementary oligonucleotides free in solution or bound to a membrane.

Preparation of lipophilic conjugates of oligodeoxynucleotides and their interaction with low-density lipoprotein (LDL) has been described (50). The high expression level of receptors for LDL on tumor cells makes LDL an attractive carrier for selective delivery of drugs to these cells. Rump et al. incorporated oncogene-directed antisense oligodeoxynucleotides into the lipid moiety of LDL. Oligodeoxynucleotides conjugated to oleic acid, cholesterol, and several other steroid lipids were tested. The oligodeoxynucleotides conjugated with cholesterol and the oleoyl esters of lithocholic and cholenic acid associated readily and nearly completely with LDL. Unmodified oligodeoxynucleotides did not associate with LDL at all while oligodeoxynucleotides conjugated with the dioleoyl ester of chenodeoxycholic acid associated incompletely. Free lithocholic acid and oleic acid are probably not sufficiently lipophilic to induce association with LDL whereas the dioleoyl ester structure is probably too bulky and extended to allow partitioning into the lipid moiety of LDL.

Mishra et al. studied improved leishmanicidal effect of phosphorotioate antisense oligonucleotides by LDL-mediated delivery (51). Phosphorothioate oligonucleotides were linked at the 5′ end to a palmityl group. This modification enabled the oligonucleotide to form a stable noncovalent complex with LDL through hydrophobic interactions. The antisense effect of LDL-oligonucleotide complexes was assayed by targeting the miniexon sequence of *Leishmania amazonensis* in infected mouse peritoneal macrophages. A 16-mer palmityl-oligonucleotide/LDL complex exerted a more pronounced sequence-specific effect than the free oligomer; about 25% and 10% of infected macrophages were cured by a 48-h incubation in the presence of 2.5 μM of the complexed and the free oligomer, respectively. When oxidized LDL was used for complexation, a further twofold increase in the antisense effect was observed suggesting that alternative (unregulated) scavenger receptor can be used for even more efficient

delivery of antisense oligonucleotides into macrophages. In addition, a significant reduction of the parasitic load was observed in those cells that were not fully cured.

Antisense effects of cholesterol-oligodeoxynucleotide conjugates associated with poly(alkylcyanoacrylate) nanoparticles were evaluated (52). The cholesteryl residue was conjugated via disulfide bond to either the 5′ or 3′-terminal phosphate group of oligodeoxynucleotide dodecamers complementary to the mutated region of H-*ras* oncogene mRNA. Each of the conjugates was able to form a complementary duplex with a 27-base fragment of mRNA with the H-*ras* mutation but not with the wild-type sequence. Efficient sequence-specific RNase H cleavage of complementary mRNA was induced with low ($\leq$500 nM) concentrations of the conjugates. At higher concentrations, this cleavage was progressively inhibited, probably due to inhibition of enzyme by the cholesterol residue. The hydrophobic conjugates could be adsorbed onto poly(isohexylcyanoacrylate) nanoparticles via their cholesteryl moieties and delivered to eukaryotic cells (52). Cholesterol-conjugated oligonucleotides were able to sequence-specifically inhibit the proliferation of T24 human bladder carcinoma cells in culture.

Cellular uptake of adamantyl-conjugated peptide nucleic acids has been reported (53). PNA 15-mers conjugated to adamantyl acetic acid and labeled with fluorescein have been prepared, and their liposome-mediated uptake in human cells in culture (HeLa, IMR-90, and MDA-MB-453) has been studied by confocal fluorescence microscopy. It was found that adamantyl-PNAs show greatly improved endosomal cellular uptake, but that this uptake is dependent on the cell line. Cellular uptake of such lipophilic PNAs is further mediated by cationic liposomes, and in some cases, the intracellular localization is diffuse cytoplasmic or nuclear, again cell-type-dependent. The results show that this lipophilic PNA modification can greatly improve cellular uptake, but the effect appears strongly cell-type- as well as PNA-sequence-dependent. The role of adamantane is obviously augmented by the fluorescein tag.

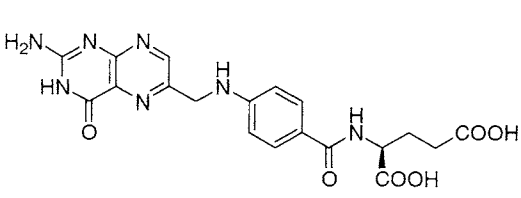
12. Novel Polyaminolipids Based on Cholesterol

Two new amphipathic polyaminolipids (54) have been synthesized by conjugating spermidine or spermine linked through a carbamate linkage to cholesterol. The polyaminolipids are relatively nontoxic to mammalian cells. In tissue culture assays, using fluorescent-tagged or radiolabeled triple-helix-forming oligonucleotides, spermine-cholesterol and spermidine-cholesterol significantly enhance cellular uptake of the oligomers in the presence of serum. Uptake into cells of the spermine-cholesterol oligonucleotide is comparable to uptake of unmodified oligonucleotide formulated with DOTMA/DOPE [a 1 : 1 (w/w) formulation of the

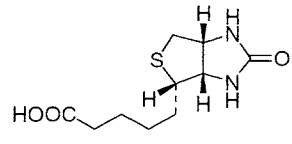
cationic lipid *N*-{1-(2,3-dioleyloxy)-propyl}-*N*,*N*,*N*-trimethylammonium chloride (DOTMA) and the neutral lipid dioleylphosphatidylethanolamine (DOPE)]. Uptake of the spermidine-cholesterol conjugate was most effective in increasing cellular uptake of oligonucleotides. The internalized oligonucleotides are routed to the nucleus as early as 20 min after treatment, suggesting that the polyaminolipids increase the permeability of cellular membranes to oligonucleotides. At later times, much of the incoming oligonucleotides are sequestered within punctate cytoplasmic granules, presumably compartments of endosomal origin. Coadministration with polyaminolipids markedly improves the cellular stability of the oligonucleotides; more than 80% of the material can be recovered intact up to 24 h after addition to cells. In the absence of the polyaminolipids, nearly all of the material is degraded within 6 h.

B. Vitamin Conjugates

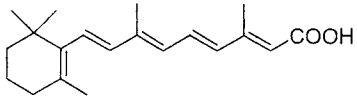
Cells and tissues have specific transport systems for nutrients such as vitamins. Thus conjugation of antisense oligonucleotides to vitamins is expected to improve transport into cells. Figure 6 shows a number of vitamins that have been conjugated to oligonucleotides. This section will summarize results of experiments with this type of conjugates.



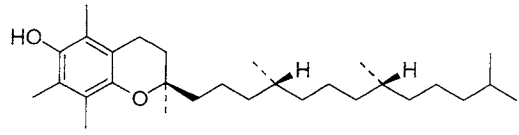
1 Folic acid



2 Biotin



3 Retinoic acid



4 Vitamin E

Figure 6 Vitamins conjugated to oligonucleotides.

1. Folic Acid Conjugates

Folate-mediated targeting of therapeutic and imaging agents to cancer cells has been recently reviewed (55). The vitamin folic acid enters cells either through a carrier protein, termed the reduced folate carrier, or via receptor-mediated endocytosis facilitated by the folate receptor. Because folate-drug conjugates are substrates of the folate receptor, they penetrate cells exclusively via folate receptor-mediated endocytosis. When folic acid is covalently linked via its γ-carboxyl to a drug or the binding affinity for the folate receptor (K_d without modification is approximately 10^{-10} M) is not measurably compromised, and endocytosis proceeds relatively unhindered, promoting uptake of the attached drug by the folate receptor-expressing cell. Because folate receptors are significantly overexpressed on a large fraction of human cancer cells (e.g., ovarian, lung, breast, endometrial, renal, colon, and cancers of myeloid hematopoietic cells), this methodology may allow for the selective delivery of a wide range of therapeutic agents to tumor tissue. Several recent reports have identified the folate receptor (folate binding protein) (56–62) as a tumor marker that is overexpressed on many cancer cell surfaces, but is highly restricted in most normal tissues.

Folate-mediated targeting of antisense oligodeoxynucleotides to epithelial ovarian cancer cells has been reported (61). A conjugate was prepared by directly coupling folic acid to the 3′ terminus of an anti-c-fos oligonucleotide and its cellular uptake and tumor inhibitory effect were evaluated in FD2008 cells that overexpress folate receptors. When an oligodeoxynucleotide with mixed phosphorothioate/phosphodiester (PS/PO) backbone was conjugated with folic acid, its uptake by FD2008 cells was increased by about eightfold. In contrast, conjugation of folate to the oligonucleotide did not increase its uptake by CHO cells that do not express folate receptors. The unmodified PS/PO antisense oligonucleotide had some inhibitory effect on the growth of FD2008 cells. However, its activity was significantly increased following conjugation with folic acid. Oligonucleotide of scrambled sequences, with and without conjugation with folic acid failed to inhibit the growth of FD2008 cells. As further indication that increased uptake and antisense activity were due to the folic acid conjugation, the increase in the uptake and growth inhibition could be blocked by adding an excessive amount of folic acid. Thus, direct derivatization of oligodeoxynucleotide with folate significantly improves their targeting efficiency to tumor cells in vitro and these conjugates have the potential for treating solid tumors that overexpress folate receptors.

Synthesis of fully protected folic acid–conjugated nucleoside phosphoramidites and their incorporation into antisense oligonucleotides have been reported (63). Synthesis of some nucleoside and nonnucleoside folic acid analogs has also been described (63,64). A modified synthetic method for synthesis of multigram

quantities of pterin aldehyde has been developed and multigram quantities of protected pteroic acid have been produced using a reductive coupling method. A synthetic methodology, which makes it possible to synthesize α- as well as γ-folic acid–conjugated oligonucleotides, has been developed from the pteroic acid. This led to the first synthesis of phosphoramidite derivatives of folic acid–nucleoside conjugates with defined regiochemistry. This method allows incorporation of folic acid–modified nucleoside monomers to oligonucleotides in large-scale synthesis. Using this phosphoramidite, ISIS-32274, a phosphorothioate oligonucleotide was synthesized in large-scale quantities (Fig. 7).

A method for solid-support synthesis of oligonucleotide conjugates of oligodeoxyribonucleotide phosphorothioate with folic acid, retinoic acid, arachidonic acid, and methoxypoly(ethylene glycol)propionic acid has been reported (65). The 5′-amino-functionalized oligonucleotide was synthesized on solid support using *N*-pent-4-enoyl-derived nucleoside phosphoramidites followed by conjugation either to the ligand acids, using a carbodiimide as a coupling reagent, or to their corresponding succinimidyl derivatives. No biological data have been reported.

A convenient methodology that involves initial synthesis of cysteinyl derivatives of the carboxylic acids on solid phase has been used to conjugate oligodeoxynucleotide 13-mers to cholic, folic, lipoic, and pantothenic acids through thioether and disulfide bonds (66).

In another study, a trimeric oligonucleotide has been conjugated to folic acid to improve antiviral activity (67). To improve cell permeability, monomeric 3′-deoxyadenosine (cordycepin) and antivirally active trimeric 3′-deoxyadenylyl-(2′-5′)-3′-deoxyadenylyl-(2′-5′)-3′-deoxyadenosine [(2′-5′)d3′(A-A-A); cordycepin-trimer core] were modified at the 2′-*O* or 5′-*O* position by myristic, cholic, and folic acid linked by a 6-aminohexanoyl spacer.

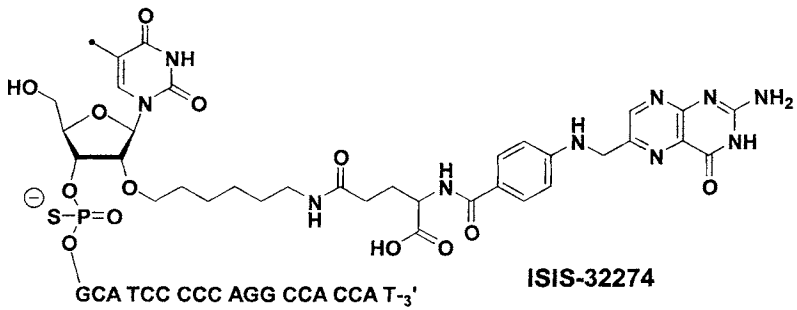


Figure 7 Folic acid–conjugated ICAM-1 oligonucleotide.

2. Folate-Conjugated Oligonucleotide Carriers

Polylysine covalently linked to folic acid was complexed to phosphorothioate 15-mer antisense c-*myc* oligodeoxynucleotides for delivery into human melanoma (M-14) cells (68). The complexed oligodeoxynucleotides showed enhanced cellular uptake compared with free oligodeoxynucleotide. There was also a significant decrease in the intracellular c-*myc* protein level and a reduction of the growth rate and colony-forming capacity of the cells after treatment with complexed oligonucleotide. No such effect was observed when scrambled sequences were administered. The efficacy of the uptake of the complex is receptor-related since a transferrin-polylysine carrier produced no significant effects (in melanoma cells the iron uptake is mediated by an oxidoreductase present in the cell membrane and not by the transferrin receptor pathway). Similarly Citro et al. (69) have covalently linked a polylysine chain (10,000–20,000 mw) to compounds such as folic acid, retinoic acid, transferrin, insulin, and estradiol, to deliver c-*myc* antisense oligonucleotide into tumor cells.

Antisense oligodeoxynucleotides targeted to the human epidermal growth factor (EGF) receptor were encapsulated into liposomes linked to folate via a polyethylene glycol spacer (folate-PEG liposomes) and delivered into cultured KB cells via folate receptor–mediated endocytosis (70). Cellular uptake of a phosphodiester 15-mer antisense oligonucleotide encapsulated in folate-PEG-liposomes was nine times higher than that of oligonucleotide encapsulated in nontargeted liposomes and 16 times higher than that of unencapsulated oligonucleotide. Treatment of KB cells with the antisense oligonucleotide in folate-PEG liposomes resulted in growth inhibition and significant morphological changes. An oligonucleotide with identical sequence, but three 3′-terminal phosphorothioate linkages, encapsulated in folate-PEG liposomes exhibited virtually identical growth inhibitory effects, reducing KB cell proliferation by >90% 48 h after the cells were treated for 4 h with 3 μM oligonucleotide. Unencapsulated phosphodiester caused almost no growth inhibition, whereas the PO/PS oligonucleotide was only one-fifth as potent as the folate-PEG-liposome-encapsulated oligonucleotide. Growth inhibition was probably due to reduced EGFR expression because indirect immunofluorescence staining of the cells with a monoclonal antibody against the EGFR showed an almost quantitative reduction of the EGFR in cells treated with folate-PEG-liposome-entrapped phosphodiester antisense oligonucleotide. Antisense oligonucleotide encapsulation in folate-PEG liposomes promises efficient and tumor-specific delivery and phosphorothioate oligonucleotides appear to offer no major advantage over native phosphodiester oligonucleotides when delivered by this route. However, no pharmacokinetic study has been performed to substantiate this conclusion.

3. Other Vitamins

The vitamin biotin plays an important role in biological diagnostic assays owing to its unusually high affinity ($K_D = 10^{-15}$M) for streptavidin and avidin. Biotin conjugation to macromolecules can also be used as a vitamin-mediated delivery system for macromolecules into cells (71–74). Complexation of avidin to biotin-oligonucleotides (phosphodiesters and PNA) has been used to enhance the uptake of oligonucleotides. Appropriate placement of biotin in oligonucleotides could also provide increased nuclease resistance.

We reported two biotin-conjugated nucleoside building blocks using 2′- or 3′-*O*-(6-aminohexyl)-5′-*O*-dimethoxytrityl-5-methyluridine and the *O*-nitrophenyl ester of N^1-(4-*t*-butyl benzoyl)-D-(+)-biotin (19). The 2′-biotin conjugate was further converted to a phosphoramidite and the 3′-conjugate was subsequently converted to the CPG derivative. Oligonucleotides with uniform phosphodiester or phosphorothioate backbones and one or more biotin molecules have been synthesized using these building blocks.

Preparation of vitamin B_6-peptide and vitamin B_6-peptide-oligonucleotide conjugates has been carried out (75,76). However, no biological studies have been reported. Similarly, no biological studies have been reported for retinoic acid, pantothenic acid, or vitamin E conjugates.

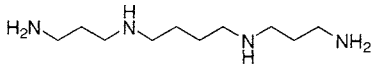
C. Polyamines and Cationic Groups

1. Polyamine Conjugates

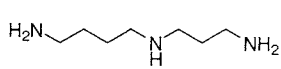
Polyamine-conjugated oligonucleotides form amphipathic molecules and reduce the net negative charge on oligonucleotides. As these modified oligonucleotides may serve as ligands for polyamine receptors present on certain cells, polyamine conjugation may improve the antisense activity of oligonucleotides. Some of the cationic groups that have been conjugated to oligonucleotides are shown in Fig. 8. Since the monoalkylamines and at least some of the amino groups of the polyamines are protonated at the physiological conditions, they can also improve the hybridization rates of antisense oligonucleotides to the target RNA. Corey demonstrated 48,000-fold acceleration of hybridization by oligonucleotides conjugated to cationic peptides derived from lysines (77).

2. Cationic and Zwitterionic Oligonucleotides

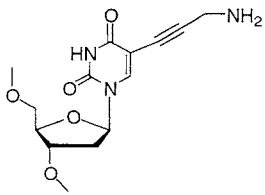
Cationic oligonucleotides (78) (oligonucleotides with positively charged backbones) and zwitterionic oligonucleotides (20) (oligonucleotides with positively charged tethers) are interesting classes of nucleic acids because of their intrinsically favorable binding properties toward RNA and single-stranded as well as



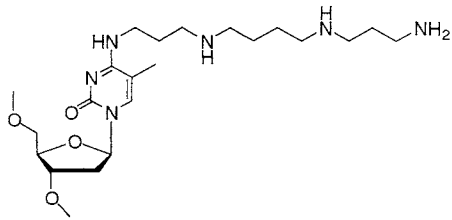
1 Spermine



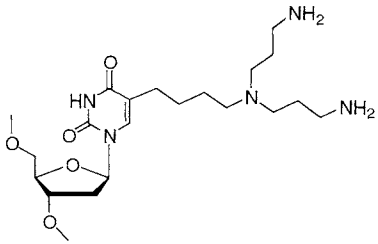
2 Spermidine



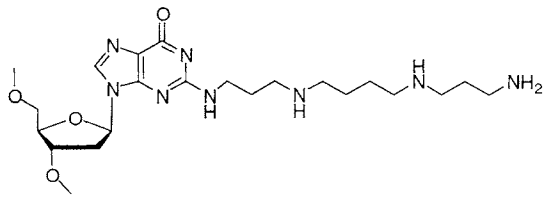
3 5-(3-Amino-1-propynyl)-2'-deoxyuridine residue



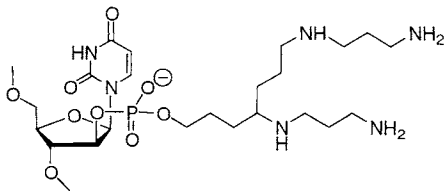
4 5-Methyl-2-oxo-4-spermine-1-(2-deoxyribose)pyrimidine residue



5 5-(4-*[N,N*-bis-(3-aminopropyl)amino]butyl]-2'-deoxyuridine residue



6 2-Spermine-2'-deoxyinosine residue



7 *Ara*-Uridine-2'-(sperminepropyl)phosphate residue

Figure 8 Representative cationic groups conjugated to oligonucleotides.

double-stranded DNA. High-affinity binding is due to charge neutralization and fast on-rates of hybridization. In addition, these oligonucleotides are expected to have good cellular permeation properties.

Synthesis of 5-Me-dC-amidite regiospecifically tethered at C4 with triethylenetetramine and spermine and their site-specific incorporation into DNA to obtain oligonucleotide-polyamine conjugates has been reported (79,80) by Prakash et al. Markiewicz et al. (81,82) described synthesis of a polyaminooligonucleotide derived from spermine based on a similar approach. Nara et al. (83) reported conjugation of synnorspermidine at C-5 position od 2′-deoxyuridine while Behr's group conjugated spermine at the C-2 position of purines (84–86). Sund et al. (87,88) described conjugation of a new C-branched spermine derivative to 5′ or 2′ position via a phosphate group. Guzaev and Manoharan reported (89) polyamine conjugation via a chloacetyl linker. None of these reports have any RNA hybridization or antisense activity. Against single-stranded DNA a small destabilization in hybridization was observed while against double-stranded DNA some stabilization was observed. No RNA hybridization data are available. It is worthwhile to revisit these compounds and evaluate for antisense properties.

Several novel 2′-cationic modifications giving rise to zwitterionic oligonucleotides have been developed and incorporated into oligonucleotides and evaluated for antisense properties (Fig. 9). The modified oligonucleotides exhibit very high nuclease resistance due to the "charge effect" and maintain high binding affinity to target RNA when the modifications are dispersed throughout the oligonucleotide. Among the 2′ modifications reported in the literature, the 2′-*O*-

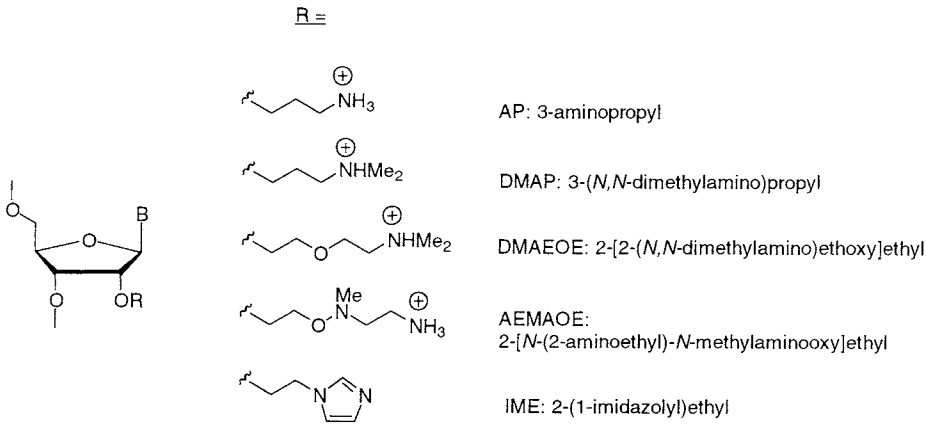


Figure 9 Cationic 2′-modifications in the protonated form at the physiological pH.

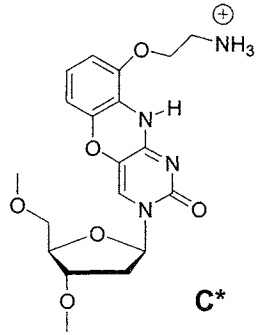
(3-aminopropyl) modification (2′-*O*-AP) (3), offers a 1.0°C increase in binding affinity/modification as a diester (2′-*O*-AP/P=O) compared to 2′-deoxyphosphorothioate compounds. This modification as a phosphodiester linkage exhibits nuclease resistance (measured as the half-life of the full-length oligonucleotide, $t_{1/2}$) approximately 6–8 times better than a 2′- deoxyphosphorothioate modification. Thus, 2′-*O*-AP/P=O oligonucleotides have shown the greatest nuclease resistance of any modification tested thus far ($t_{1/2} > 24$ h in snake venom phosphodiesterase assays compared with 4 min for unmodified phosphodiester oligonucleotide). The superior nuclease resistance of the 2′-*O*-AP modification was attributed to the cationic side chain, which interacts competitively with the metal ions involved in the phosphodiesterase activity of the nucleolytic enzymes.

The 2′-*O*-dimethylaminopropyl (2′-*O*-DMAP) oligonucleotide, the dimethyl analog of 2′-*O*-AP, is expected to be more lipophilic than the 2′-*O*-AP-modified oligonucleotides, a characteristic that should improve the cellular and protein-binding properties and hence the biodistribution of the 2′-*O*-DMAP oligonucleotides compared to 2′-*O*-AP oligonucleotides. Many other 2′-modified zwitterionic oligonucleotides have been made and await pharmacokinetic and pharmacological evaluation (90,91). Cationic modifications in oligonucleotides with phosphorothioate backbones will be extremely valuable, as this class of compounds will have different protein-binding properties from unmodified phosphorothioate oligonucleotides.

The ''G-clamp'' is a heterocycle modification (92,93), a cytosine analog derived from phenoxazine that forms an additional hydrogen bond to guanine (Fig. 10). This modification was designed to enhance oligonucleotide/RNA hybrid affinity and 6–18°/modification increase in T_m have been reported. The data suggest that the additional hydrogen bond is formed between the ammonium group on the G-clamp and the Hoogsteen face of guanine. Model building suggests the interaction is with the *O*-6 group on guanine. The tether arm appears not to be long enough to reach the *N*-7.

Dose-dependent, sequence-specific antisense inhibition was observed at nanomolar concentrations of the G-clamp phosphorothioate oligonucleotides against two different mRNA targets (92,93). Incorporation of a single G-clamp modification into a c-*raf* 20-mer phosphorothioate oligonucleotide targeting c-*raf* increased the potency 25-fold relative to the parent 2′-deoxyphosphorothioate. In cell-free assays, the G-clamp oligonucleotides activated RNase H–mediated cleavage. In experiments targeting $p27^{kip1}$, a single nucleotide mismatch between the oligonucleotide containing the G-clamp and the target mRNA reduced the potency of the oligonucleotides by fivefold. Further evaluation of in vivo pharmacokinetics and pharmacology of oligonucleotides with this modification is needed.

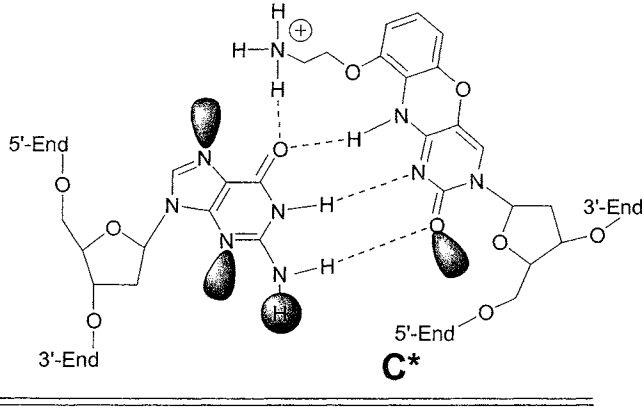
A.



G clamp

B.

major groove



minor groove

Hydrogen bonding in G:G clamp base pair

Figure 10 (A) G-clamp and (B) the hypothetical four-bond base pairing.

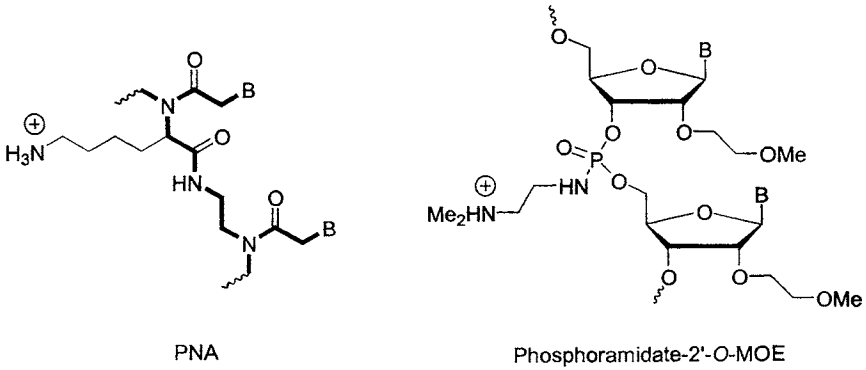


Figure 11 Cationic backbone modifications.

Another interesting base modification is at the 5 position of uridine. Oligomers with 5-(3-aminopropyn-1-yl)-2′-deoxyuridine substitutions (94) show a marked increase in duplex DNA and RNA-DNA stability but no loss in base-pairing specificity.

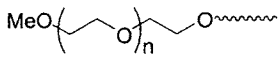
Cationic groups have also been introduced into the backbone of oligonucleotides and into the backbone of PNA (Fig. 11). Modification of the PNA backbone with lysine alleviates some of the synthesis, purification, and aggregation problems associated with neutral PNAs, especially those rich in purine. Lysine residues also improve the binding affinity by 1°/modification. In some targets (e.g., IL-5Rα, J. Karras, unpublished results) and cell types (e.g., HeLa cells, R. Kole, unpublished results) improved uptake and antisense activities have been observed.

Some phosphoramidate backbone derivatives provide methods of introducing cationic backbones into oligonucleotides. These are easily synthesized from H-phosphonate precursors and can have either 2′-deoxy or 2′-modified substituents. 2′-*O*-MOE containing cationic dimethylaminoethyl phosphoramidates have been synthesized and found to have excellent binding affinity and nuclease-resistance properties (95).

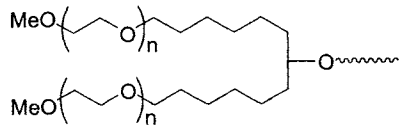
D. PEG Conjugates

1. Biological Activity

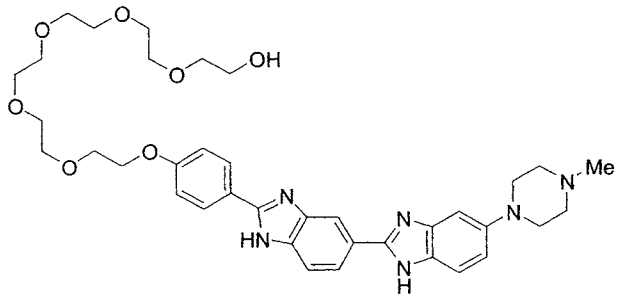
Polyethylene glycols (PEG) are known to play an important role in the pharmacokinetic behavior of therapeutic proteins. In addition to serving as ligands themselves, they also serve as linkers for conjugating other ligands (Fig. 12).



MPEG (M.W. = 10000)



$[MPEG]_2$ (M.W. = 10000)



Hoechst 33258 with PEG linker

Figure 12 PEG conjugates.

An oligonucleotide that targets human ICAM-1 has been conjugated to a series of PEG esters of average molecular weight 550, 2000, and 5000 (corresponding to 11, 44, and 110 ethylene glycol residues, respectively). Our results indicate that PEGs interfere with the cellular permeation of oligonucleotides in vitro (14). This result is in conflict with positive data observed from other laboratories summarized below.

The effect of the different structures of high-molecular-weight polyethylene glycol chains on the biological properties of the conjugated antisense oligonucleotides has been investigated by Bonora et al. (96). Two different conjugates of an anti-HIV 12-mer oligonucleotide have been tested for antisense activity in MT-4 cells (97). Only the oligonucleotide conjugated to the linear monomethoxy polyethylene glycol (MPEG) showed anti-HIV activity. The 12-mer, when conjugated to a branched (MPEG)2, was inactive, as was the unmodified oligonucleotide.

A 20-mer oligonucleotide targeting mouse β-globin mRNA has been conjugated at the 5′-terminus to *bis*-aminoalkyl PEG using imidazole-activated carbox-

ylic acid esters (98). At 15 μM the conjugate selectively inhibited Hb synthesis in cultured Friend murine erythroleukemia cells by 95%.

Synthesis of oligonucleotides conjugated to PEG at the 5′, the 3′, or both 5′ and 3′ termini has been reported (99,100). In one study, pools of oligonucleotide conjugates consisting of 10–400 different PEG units and attachment points (3′-terminal, 5′-terminal, and internal positions) were synthesized (101). Conjugate synthesis was performed using phosphoramidite solid-phase chemistry and suitably protected polyethylene glycol phosphoramidites and PEG-derivatized solid supports containing polydispersed PEGs of various molecular weights. The number and attachment sites of coupled ethylene glycol units greatly influence the hydrophobicity of the conjugates, as well as their electrophoretic mobilities. Conjugation had little effect on the hybridization behavior of oligonucleotide conjugates with unmodified complementary oligonucleotide strands. Conjugates with PEG coupled to both 3′- and 5′-terminal positions showed a more than 10-fold increase in exonuclease stability.

2. PEG Copolymers

Conjugates of double-stranded oligonucleotides with PEG and keyhole limpet hemocyanin (KLH) have been developed as a model for treating systemic lupus erythematosus (102). Two types of oligonucleotides were synthesized with linker groups attached at the 5′ end. Both were repeating dimers of deoxycytidine and deoxyadenosine. A 20-mer was prepared with a thiol-containing linker, masked as a disulfide, and a 50-mer was prepared with a vicinal diol-containing linker. A tetraiodo-acetylated PEG derivative was synthesized and reacted with the thiol-containing 20-mer to provide an oligonucleotide PEG conjugate with precisely four oligonucleotides on each PEG carrier. The vicinal diol on the 50-mer was oxidized to an aldehyde and conjugated to KLH by reductive alkylation. The conjugates were annealed with complementary $(TG)_n$ strands. The double-stranded oligonucleotide-KLH conjugate is an immunogen, eliciting the synthesis of antibodies against oligonucleotides. In contrast, the PEG conjugate has the biological property of specifically suppressing the B cells that make antibodies against the immunizing oligonucleotide.

Cationic copolymers for DNA delivery have been synthesized by conjugating PEG and polyspermine (PSP) and polyethyleneimine (PEI) (103). These molecules spontaneously form electrostatic complexes with a 24-mer phosphorothioate oligonucleotide. The copolymer complexes are water soluble. This is in marked contrast with the complexes formed by nonmodified PSP and PEI, which immediately precipitate out of solution. The potentiometric titration study suggests that the amino groups of the copolymers form a cooperative system of salt bonds with the thiophosphate groups of the phosphorothioate oligonucleotide. The PEG-PEI complexes are stable at physiological pH and ionic strengths. As

a result of formulation with the PEG-PEI, the interactions of PS oligodeoxynucleotides with the serum protein BSA are decreased and the oligonucleotide is protected against nuclease degradation. The simplicity of preparation and long shelf life make these systems attractive as potential pharmaceutical formulations for oligonucleotides. In the absence of any in vivo pharmacokinetics study, it is not clear whether the reduced interaction with serum albumin is advantageous or not.

3. PEGS as Linkers

The PEG group has been used as a simple linker in some types of conjugates, e.g., intercalators, steroids, small-molecule drugs, and electron-transfer agents, where solubility is an issue. For example, Wiederholt and McLaughlin have used PEGs to conjugate Hoechst 33258 and Ruthenium complexes to oligonucleotides (104,105).

E. Multivalent Carbohydrate Clusters

1. Rationale

An in vivo study of distribution of a phosphorothioate oligonucleotide within rat liver after intravenous administration has revealed an important aspect of distribution of antisense drugs: distribution of the drug to a tissue does not mean that the drug has been localized to target cells within that tissue. Graham et al. (106) have shown that after a 10-mg/kg dose of phosphorothioate oligonucleotide, nonparenchymal (i.e., Kupffer and endothelial) cells contained approximately 80% of the total organ dose, equivalently distributed between the two cell types. Only 20% of the oligonucleotide was associated with hepatocytes. Nonparenchymal cells contained abundant nuclear, cytosolic, and membrane drug levels over a wide dose range, while hepatocytes had no detectable nuclear association. These results suggest that efficient methods must be devised to deliver oligonucleotide to functionally important parenchymal cells. Carbohydrate cluster conjugates (Fig. 13) may offer a solution to this problem.

In their pioneering work, Wu and Wu demonstrated (107) that oligonucleotides complexed to polylysine that is covalently linked to asialoorosomucoid were easily taken up into human hepatocellular carcinoma (HepG2) cells by the asialoglycoprotein (ASGP) receptor.

2. Conjugates of YEE(ahGalNAc)$_3$

Hangeland et al. reported (108) enhanced 20- to 40-fold cellular uptake in HepG2 cells when antisense oligonucleotides (methylphosphonates) were conjugated to the triantetennary, *N*-acetylgalactosamine neoglycopeptide, Tyr-Glu-Glu-

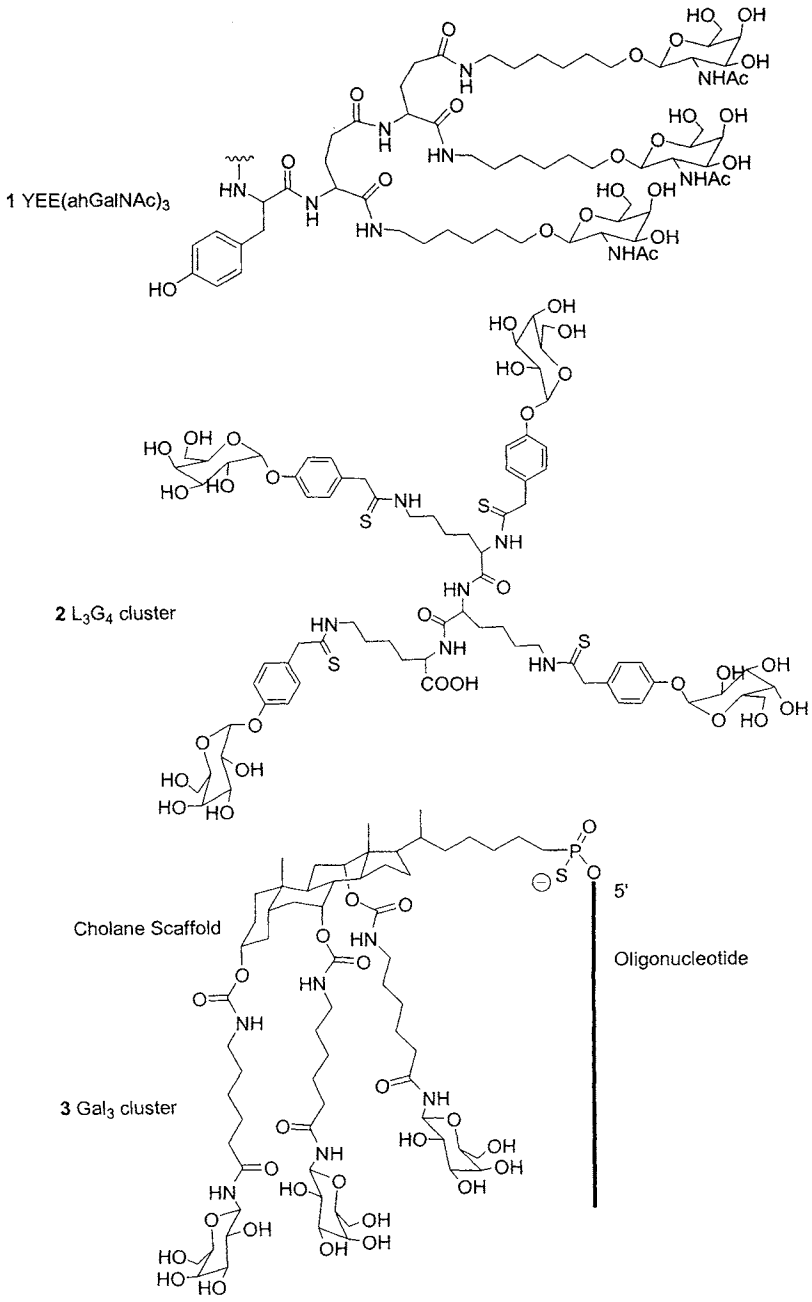


Figure 13 Carbohydrate clusters for parenchymal cell–specific targeting.

(aminohexyl GalNAc)$_3$, YEE(ahGalNAc)$_3$. This is a glycotripeptide known to bind to Gal/GalNAc receptor sites on hepatocytes with an affinity of K_d = 7 nM. More recently, Duff et al. reported (109) that phosphorothioate oligomers conjugated to the above glycotripeptide showed a sequence-specific suppression (greater than 90% inhibition) of the integrated HBV viral expression in hepatoma cells in a dose-dependent concentration range of 1–20 μM of oligonucleotides.

3. Lysine-Based Galactose Clusters (L3G4)

Improved targeted delivery of oligodeoxynucleotides to parenchymal liver cells in vivo has been demonstrated by Biessen et al. (110–112). They have conjugated oligonucleotide to the ligand for the asialoglycoprotein receptor, a receptor found on parenchymal liver cells. A 20-mer phosphodiester oligonucleotide 3′-capped with an amine was derivatized with a ligand with high affinity for this receptor: *N*2-(*N*2-(*N*2,*N*6-bis[*N*-[p-(β-D-galactopyranosyloxy)-anilino)thiocarbamyl]-L-lysyl)-*N*6-(*N*-[p-(β-D-galactopyranosyloxy)-anilino]thiocarbamyl)-L-lysyl)-*N*6-[-*N*-(p-[β-D-galactopyranosyloxy]anilino)thiocarbamyl)-L-lysine (L3G4). The ligand has a K_d of 6.5 nM. Both uptake studies in vitro and confocal laser scan microscopy studies demonstrated that the L3G4-conjugated oligonucleotide was far more efficiently bound to and taken up by parenchymal liver cells than underivatized oligonucleotide. Studies in rats showed that hepatic uptake was greatly enhanced from 19 ± 1% for unconjugated oligonucleotide to 77 ± 6% of the injected dose after glycoconjugation. Importantly, accumulation into parenchymal liver cells was improved almost 60-fold after derivatization with L3G4, and could be attributed to the asialoglycoprotein receptor.

4. Cholane-Based Galactose Clusters

At Isis, a multivalent carbohydrate recognition motif for the asialoglycoprotein receptor has been designed for parenchymal liver cellul targeting of antisense drugs (113). The basis for this approach lies in the fact that membrane lectins recognize and bind to carbohydrate clusters and facilitate receptor-mediated endocytosis. The K_D for this recognition process increases with the number of carbohydrate ligands present. For monovalent ligands, it is 10^{-3} M; for divalent ligands it is 10^{-6} M, and for triantennary cluster conjugates it is 10^{-9} M. The asialoglycoprotein receptor (ASGP-R) is expressed in parechymal liver cells. The receptor spans the membrane and presents its carbohydrate recognition domain (CRD) to the extracellular space. The ligands are internalized via coated pits and vesicles. The receptor is recycled once the ligand is transferred to lysosomes. Some of the recent studies from other laboratories (109) demonstrated 20–40-fold increases in intracellular concentration of carbohydrate-conjugated oligonucleotides relative to unconjugated molecules. The majority of the oligonu-

Table 3 Cholane-Based Galactose Cluster–Conjugated Antisense Oligonucleotides

Sequence	Modification	Target
$(Galactose)_3$-chol-ATC CAT TCT GCC CCC AAG GA	P=S, 2′-H	Mouse c-*raf*
$(Galactose)_3$-chol-*TCCAG* CACT TTC TTT *TCCGG*	P=S, —— = 2′-*O*-MOE	Mouse FAS antigen
$(Galactose)_3$-chol-*GTGCT* A GCC TCT GGA *TTT GA*	P=S, —— = 2′-*O*-MOE	Human PTEN

chol = cholane scaffold.

cleotide concentrates in vesicular compartments and about 25% is released intact into the cytosol.

We synthesized carbohydrate clusters by attaching three carbohydrate ligands to a cholane-based backbone via ε-aminocaproic acid linkers. Conjugation of the bulky carbohydrate clusters to antisense oligonucleotides was achieved by solid-phase synthesis using low-loading macroporous resins (113). Antisense oligonucleotide conjugates have been synthesized that target c-*raf*, FAS antigen, and human PTEN mRNAs (Table 3). Initial molecular modeling studies aided in positioning of galactose residues with an interunit distance of 13–15 Å. The galactose cluster-cholane scaffold (Gal_3Chol) was conjugated to oligonucleotide using phosphoramidite chemistry with >25% coupling efficiency. As the conjugates were hydrophobic they were readily purified by reverse-phase HPLC.

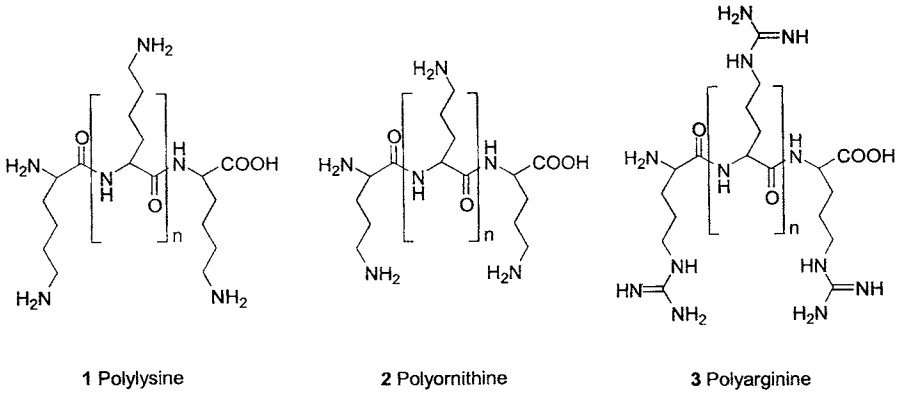
F. Peptide Conjugates

1. Rationale

Peptide conjugates of antisense agents have been made with several major goals. To change pharmacokinetic distribution, cationic groups (polylysine, polyornithine, polyhistidine, and polyarginine) and hydrophobic groups (aromatic amino acid–containing peptides) have been attached to oligonucleotides. Peptide ligands with cellular receptors have been conjugated to oligonucleotides to enhance cellular recognition and absorption. Finally, oligonucleotides have been modified with peptides that may function as synthetic nucleases.

2. Peptides as Polycations

Thermodynamic melting studies of a small library of 49 peptide-oligonucleotide conjugates have been analyzed to explore the influence of various peptide side chains on the hybridization properties of the DNA (114,115). An invariant 8-



1 Polylysine 2 Polyornithine 3 Polyarginine

Figure 14 Cationic peptides.

mer oligonucleotide was coupled to a five-residue variable peptide region composed of the cationic amino acids lysine, ornithine, histidine, and arginine, the hydrophobic amino acid tryptophan, and alanine as a spacer. Melting temperature analysis indicated nearly 1°C increase for each cationic residue present and T_m depended principally on the number of cationic residues. Thus the free energies of binding for polycationic peptide-oligonucleotides were significantly enhanced compared with the unmodified 8-mer. The origin of this stabilizing effect was derived from a more exothermic enthalpic term. A study of pH dependence showed that the polyhistidine conjugate was particularly sensitive to pH changes near neutrality, as indicated by a significant rise in T_m from 19.5° at pH 8.0 to 28.5° at pH 6.0.

In another study, the hybridization properties of a series of oligomers, based on two different 9-mer oligodeoxynucleotide sequences with an appended oligoarginine chain, were investigated (116). The oligomers were either peptide-oligonucleotide conjugates (116) or peptide-bridged oligonucleotide pairs (e.g., $(Arg)_n$-oligonucleotide or oligonucleotide-$(Arg)_n$-oligonucleotide). For the double-linked oglionucleotides, it was found that the peptide bridge induces the two 9-mers to bind complementary single-stranded DNA or RNA targets with substantially enhanced thermal stability. Single or double-linked oglionucleotides complexed to complementary RNA were able to activate RNase H.

A synthetic 12-mer oligodeoxyribonucleotide has been coupled at its 5′ terminus to a series of positively charged $(\delta$-ornithine$)_n$-cysteine peptides (117). Site-directed cleavage with RNase H demonstrated that the peptide-modified oligomer hybridized with its RNA target sequence. Increased affinity for target mRNA was also observed.

Melting studies of the complex between an H-*ras* antisense oligonucleotide carrying nuclear localization peptide sequences (NLS) (Table 4) and target mRNA showed that the conjugated oligonucleotide formed a more stable than duplex than did unmodified oligonucleotides (118). Despite the presence of the peptide, good mismatch discrimination was maintained when the conjugated oligonucleotide bound target RNA.

3. Peptides for Endosomal Release: Fusogenic Peptide Conjugation

A fusogenic peptide (119) derived from the influenza hemagglutinin envelope protein has been conjugated to antisense oligonucleotide. This peptide changes conformation at acidic pH and destabilizes the endosomal membrane resulting in an increased cytoplasmic delivery of the antisense drug. The use of a similar fusogenic peptide conjugated to an anti-TAT antisense oligodeoxynucleotide via a disulfide or thioether bond resulted in five- to 10-fold improvement of the anti HIV activity of the phosphodiester antisense oligonucleotide on de novo infected CEM-SS lymphocytes in serum free media. No toxicities were observed at the effective doses (0.1–1 μM). However, no sequence specificity was observed and the fusogenic peptide possessed some antiviral activities on its own (IC50: 6 μM). A $(\text{deoxyphosphorothioatecytidine})_{28}$-peptide (S-dC28) conjugate and a streptavidin-peptide-biotinylated S-dC28 adduct showed activity similar to the unconjugated S-dC28 oligonucleotide (IC_{50}: 0.1–1 nM).

Enhanced cellular uptake of oligonucleotides by epidermal growth factor (EGF) receptor–mediated endocytosis in A549 cells in epithelial cancer cells has been demonstrated (120). To overcome the problem of endosomal entrapment associated with receptor-mediated delivery, the authors evaluated the effects of two fusogenic peptides, polymyxin B and influenza HA2 peptide, for their capability to promote cytoplasmic delivery of oligonucleotides. A conjugate consisting of EGF and poly-L-lysine (PL) was synthesized and complexed with 5′ fluorescently labeled oligonucleotide. Cellular uptake of this complex in the presence or absence of the fusogenic peptides was monitored fluorometrically and intracellular distribution of the oligonucleotide was determined. Cells treated with the complex exhibited intracellular fluorescence intensity significantly enhanced over that of controls treated with the oligonucleotide alone. Fluorescence microscopic studies revealed, however, that the complex appeared to be accumulated in endocytic vesicles. Exposure of the cells to complex in presence of HA2 peptide and polymyxin B resulted in a more diffused intracellular fluorescence pattern and an increase in fluorescence intensity. These results are consistent with the known fusion and destabilizing activities of the peptides. The uptake of the complex was shown to occur via the EGF receptor–mediated pathway. Since EGF receptors are overexpressed in many cancer cell types, the EGF-PL conju-

gate may potentially be used as an effective and selective delivery system to enhance uptake of oligonucleotides into cancer cells.

4. Cell Permeation Peptides: Ant Peptide and Tat Peptide

Another approach to the intracellular delivery of oligonucleotides is based on the use of several types of "delivery peptides" that seem to have the ability to carry large, polar molecules including peptides, oligonucleotides, and even proteins across cell membranes. Two examples of delivery peptides are a 35-amino-acid sequence ("Tat") from the HIV Tat protein, and a 16-amino-acid sequence ("Ant") from the *Drosophila* antennapedia protein (Fig. 15). Antennapedia-type peptides have been used to deliver oligonucleotides, including peptide nucleic acids, into neuronal cells, but their general applicability is yet to be completely studied. Other types of peptides, containing hydrophobic motifs and special recognition motifs, have also been used for antisense delivery (Table 4).

Astriab-Fisher et al. prepared (121) Ant and Tat peptide-oligonucleotide conjugates for the MDR-1 system. The phosphorothioate oligonucleotide component of the conjugates was complementary to a site flanking the AUG of the message for P-glycoprotein, a membrane ATPase associated with multidrug resistance in tumor cells (ISIS-5995). Both types of peptide-antisense oligonucleotide conjugates, but not mismatched control conjugates, provided substantial inhibition (34%) of cell surface expression of P-glycoprotein. The peptide-oligonucleotide conjugates were more potent in the presence of serum than when used under serum-free conditions; this is in contrast to cationic lipid–based approaches for intracellular delivery of nucleic acids. Effective inhibition of P-glycoprotein expression was attained with submicromolar concentrations of antisense conjugates under serum-replete conditions. Flow cytometry profiles indicated the conjugates accumulated in cells to a much greater degree than the free oligonucleotide. The conjugate reached the nucleus while the free oligonucleotide had virtually no intracellular fluorescence.

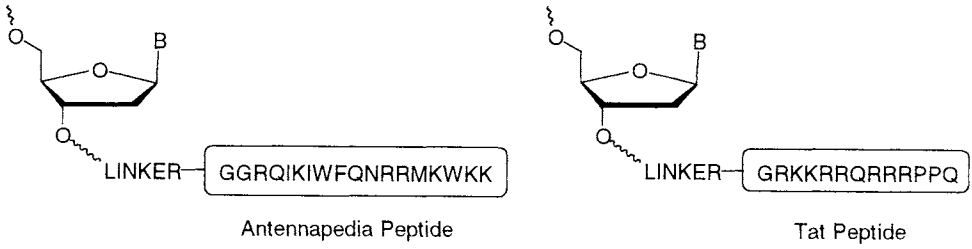


Figure 15 Cell permeation peptide conjugates.

Table 4 Peptides Used for Cellular Delivery/Improved Uptake of Antisense Oligonucleotides

No.	Peptide sequence	Source
1	Arg-Gln-Ile-Lys-Ile-Trp-Phe-Gln-Asn-Arg-Arg-Met-Lys-Trp-Lys-Lys RQIKIWFQNRRMKWKK	Antennapodia helix 3 (43–58) Antp-HD
2	Gly-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg-Pro-Pro-Gln GRKKRRQRRRPPQ	HIV Tat fragment (48–60)
3	Gly-Trp-Thr-Leu-Asn-Ser-Ala-Gly-Tyr-Leu-Leu-Gly-Pro-Ile-Asn-Leu-Lys-Ala-Leu-Ala-Ala-Leu-Ala-Lys-Lys-Ile-Leu GWTLNSAGYLLGPINLKALAA-LAKKIL	Transporton: chimeric galanin and mastoporan
4	Asp-Ala-Ala-Thr-Ala-Thr-Arg-Gly-Arg-Ser-Ala-Ala-Ser-Arg-Pro-Thr-Glu-Arg-Pro-Arg-Ala-Pro-Ala-Arg-Ser-Ala-Ser-Arg-Pro-Arg-Arg-Pro-Val-Glu DAATATRGRSAASRPTERPRAPAR-SASRPRRPVE	HSV VP22
5	Lys-Leu-Ala-Leu-Lys-Leu-Ala-Leu-Lys-Ala-Leu-Lys-Ala-Ala-Leu-Lys-Leu-Ala KLALKLALKALKAALKLA	Amphiphilic model peptide
6	Gly-Ala-Leu-Phe-Leu-Gly-Trp-Leu-Gly-Ala-Ala-Gly-Ser-Thr-Met-Gly-Ala-Trp-Ser-Gln-Pro-Lys-Lys-Lys-Arg-Lys-Val GALFLGWLGAAGSTM-GAWSQPKKKRKV	Signal sequence based peptide I
7	Ala-Ala-Val-Ala-Leu-Leu-Pro-Ala-Val-Leu-Leu-Ala-Leu-Leu-Ala-Pro AAVALLPAVLLALLAP	Signal sequence based peptide II
8	Pro-Lys-Lys-Lys-Arg-Lys-Val PKKKRKV	SV40 antigen T nuclear localization signal
9	Form Met-Leu-Phe-Tyr fMLFY	Platelet activating factor receptor of the neutrophils
10	*N*, *N*-dipalmitylglycyl-apolipoprotein E (129–149)	
11	Lys-Asp-Glu-Leu KDEL	
12	Fusogenic peptides	
13	Leu-Ala-Glu-Leu-Leu-Ala-Glu-Leu-Leu-Ala-Glu-Leu-LAEL-LAEL-LAEL; 4_3E	

Nuclear delivery of antisense oligodeoxynucleotides and selective inhibition of cholesteryl ester transfer protein (CETP) expression by an antisense oligonucleotide complexed to *N,N*-dipalmitylglycyl-apo E peptide has been shown in a Chinese hamster ovary (CHO) cell line stably tranfected with human CETP (122). *N,N*-Dipalmitylglycyl-apolipoprotein E (129–169) peptide (dpGapoE) is an efficient gene delivery system for both plasmids and antisense oligodeoxynucleotides. dpGapoE contains the minimum determinants for binding to both lipid surfaces and the LDL receptor. This could be used to target oligonucleotides to liver. After transfection of oligodeoxynucleotides by dpGapoE, translocation of oligonucleotide to the nuclei and concentration in nuclear structures was observed in >95% of the cells at 6 and 12 h by fluorescence microscopy with oligonucleotide observed for >48 h. No membrane disruption was observed after transfection. Cellular CETP mRNA levels gradually declined, and the maximum reduction in the mRNA level (>50%) was observed at 36 h, after which the mRNA level started to recover. CETP activity in the culture medium declined over 72 h, with maximum reduction observed at 36 h (54% of control). Neither CETP mRNA levels nor CETP activity changed after the transfection of sense phosphorothioate oligodeoxynucleotides delivered by dpGapoE complex or naked antisense oligodeoxynucleotides. This is the first demonstration of the use of an LDL receptor-binding peptide for the delivery of antisense oligonucleotides. This approach may enable gene regulation in vivo and development of antiatherosclerotic agents to alter high-density lipoprotein metabolism.

Eighteen conjugates of phosphorothioate oligonucleotides to membrane translocation and nuclear localization peptides were prepared in good yield and were thoroughly characterized with electrospray ionization mass spectra (123). When applied to cells, conjugates exhibiting membrane translocation and nuclear localization properties displayed efficient intracellular penetration but failed to show improved antisense effects. Studies on the intracellular distribution of the fluorescein-labeled conjugates revealed their trapping in endosomes.

Langel et al. (124–127) demonstrated that conjugates of transporter peptides to PNA showed improved delivery and were able to regulate galanin receptor levels and modify pain transmission in vivo. A PNA antisense 21-mer to the human type 1 galanin receptor was linked via a labile cysteine disulfide bond to biotin-labeled peptides known to impart cell membrane permeant properties. These peptides were transportan [galanin(1–12)-Lys-mastoparan(1–14)amide] and antennapedia [Antp (43–58), the third helix of atennapedia homeodomain]. The resulting conjugates improved internalization and down-regulated the human galanin receptor in Bowes cell line and in rat spinal cord in vivo. Intrathecal administration of the peptide-PNA construct resulted in a decrease in galanin binding in the dorsal horn. Owing to decreased binding, galanin could not inhibit the C fibers stimulation-induced facilitation of the rat flexor reflex, demonstrating

that peptide-PNA constructs acted in vivo to suppress expression of functional galanin receptors. These peptides have been demonstrated to translocate across the plasma membrane of eukaryotic cells by an energy-independent pathway (128).

Nine different peptides containing a hydrophobic motif associated with a nuclear localization signal (PKKKRKV) (129) derived from SV40 antigen T separated by various linkers were synthesized on solid phase. The hydrophobic sequence corresponded either to a signal peptide sequence of *Caiman crocodylus* (MGLGLHLLVLAAALQGA) or to a fragment of the fusion peptide of gp41N (GALFLGWLGAAGSTMGA) while the hydrophilic sequence was that of a nuclear localization signal. The C-termini of these peptides bear a cysteamide group linked to a fluorescent probe to allow the cellular localization to be determined. The MGLGLHLLVLAAALQGA-PKKKRKV peptide conjugate was successfully synthesized using a disulfide bridge and then used to target fluorescent antisense phosphorothioate oligodeoxynucleotides into fibroblasts. The presence of a linker appears to play a role in the cellular localization. In 5-min incubation time more than 90% cells were targeted. It appeared that the membrane-associated conformational state of the peptides is crucial for the internalization process and endocytosis can be ruled out since no temperature (4° or 37°C) effect on the internalization was observed.

The signal peptide Lys-Asp-Glu-Leu (KDEL) was shown to carry oligonucleotides to the endoplasmic reticulum and from there to the cytosol and the nucleus where their targets are located (130). A 5′,3′-modified pentacosanucleotide, complementary to the translation initiation region of the gag mRNA of HIV, was coupled to a (bromoacetyl)dodecapeptide containing a KDEL signal sequence. The anti-HIV activity of the pentacosanucleotide was compared with that of pentacosanucleotide-dodecapeptide conjugates linked through either a thioether bond or a disulfide bridge. The conjugate with a thioether bond has a higher antiviral activity than the peptide-free oligonucleotide or the conjugate linked via a disulfide bond.

In another approach, an oligonucleotide-Tat peptide conjugate, having dual binding capability for a designated RNA, was designed (131). The peptide portion of the conjugate interacts with a folded domain in the RNA, whereas the oligonucleotide portion hybridizes with a nearby single-stranded region in the RNA. The dual specificity was proven in a model HIV-1 TAR RNA system using an RNase H–cleavage assay to assess antisense binding to this RNA. The peptide portion of the conjugate was shown to confer increased specificity on the oligonucleotide.

Antisense oligonucleotides targeting human immunodeficiency virus type I (HIV) have been linked to fusion peptides derived from the HIV transmembrane glycoprotein gp41 (132). Thermal denaturation studies showed that the interaction of the conjugate with its complementary strand was similar to that of unmodi-

fied oligonucleotides. Thus, in this example, the peptide does not confer additional stability to the oligonucleotide-mRNA complex.

5. Reports on Synthesis and Purification of Peptide Conjugates

A number of methods have been used to synthesize and purify oligonucleotide-peptide conjugates. Gait et al. (133) have developed a method of fragment coupling of presynthesized peptides to the 2′ position of a selected nucleotide within an otherwise protected oligonucleotide chain attached to a solid support. Synthesis of nucleopeptide-oligonucleotide conjugates has been carried out on δ-ornithine peptides by modification of the α-amino ornithine functional group with pyrimidyl-1- and purinyl-9-acetic acids or with pyrimidyl-1- and purinyl-9-alanines (134). Nucleopeptides were prepared on solid polymer bearing a photoactivatable linker. Conjugates with the 16-mer oligonucleotide complementary to the env AUG codon region of the Friend murine leukemia virus were prepared in this manner.

Solid-phase synthesis of several peptide-oligonucleotide conjugates has been achieved using a peptide-fragment coupling strategy on a controlled pore glass support (135,136). The conjugates contained either a hydrophobic tetrapeptide (Leu-Gly-Ile-Gly) or an eight-residue basic peptide of the HIV-1 Tat protein coupled to one of two oligodeoxyribonucleotides (an oligoribonucleotide or a mixed ribo/2′-*O*-Me oligonucleotide). Improved yields were obtained when internucleotide β-cyanoethyl groups were removed from the support-bound oligonucleotide prior to peptide-fragment coupling, and by use of a long alkyl spacer in the linkage between peptide and oligonucleotide.

Another study describes synthesis of DNA-peptide conjugate molecules on oxime resin (137). The oligonucleotide and peptide are covalently linked by cleaving the DNA fragment synthesized on modified oxime resin in the presence of independently prepared peptide fragment bearing free terminal α-amino group and protected side-chain residues. This method affords DNA conjugate molecules in moderate to good yields. A different solid-phase synthesis of oligonucleotides conjugated at the 3′ termini to a peptide has been developed (138). A 17-mer antisense oligonucleotide against HIV-1, linked at the 3′ terminus to the tripeptide Gly-Gly-His,was prepared in good yields and characterized by MALDI-TOF mass spectrometry.

A highly basic peptide (net charge +8) derived from the HIV-1 Tat protein was conjugated with quantitative yield to a 19-mer rhodamine-labeled phosphodiester oligonucleotide activated by the pyridinesulfenyl group (139). To avoid precipitation due to antagonist charges of the oligonucleotide and the peptide, the conjugation was performed in high salt concentration (400 mM) and acetonitrile (40%).

Synthesis and characterization of very short peptide-oligonucleotide conjugates used for the transport of antisense oligonucleotides (140) and stepwise solid-phase synthesis of peptide-oligonucleotide conjugates on new solid supports have been described (140,141). These supports are designed to link the 3′ terminus of an oligonucleotide to the C-terminal end of a peptide via a phosphodiester or phosphorothioate bond in the process of stepwise solid-phase assembly.

G. Cleaving Agents: Synthetic Ribonucleases

1. Overview

In an attempt to identify alternatives to the antisense oligonucleotide-RNase H–catalyzed degradation of target RNA, several laboratories continue to search for efficient sequence-specific cleavage agents. Unlike 2′-deoxyphosphorothioates, most second-generation oligonucleotide modifications do not induce cleavage of mRNA by an RNase H–based mechanism. This necessitates design and synthesis of new RNA-cleaving agents. A wide range of small molecules capable of hydrolytically cleaving RNA under various conditions have been studied and reviewed (142–144). The cleavage effectiveness of these reagents can be enhanced by coupling them to molecules that bind to nucleic acids, such as intercalators and polycations (Fig. 16). Attachment of reactive groups to oligonucleotides complementary to segments of the target RNA directs the hydrolytically active groups to the desired RNA sites. Hydrolytically active groups tested include metallocomplexes, oligopeptides, amines, and constructs containing constituents of the active centers of nucleases (imidazole, guanidinium, carboxyl, and amino groups). Design of small molecules capable of effective catalytic cleavage of RNA under physiological conditions may enable development of conjugates of antisense oligonucleotides that are active in cells. Cleaving molecule-intercalator (e.g., imidazole-acridine conjugates) conjugates have been synthesized and studied as a t-RNA cleaving agent (145).

Recently, a diimidazole construction mimicking the active center of RNase H has been conjugated to oligonucleotides complementary to *Leishmania amazonensis* miniexon and preminiexon sequences (146). The conjugates were shown to cleave the target RNAs at specific positions in cell-based assays. In another report, the sequence-specific, hydrolytic cleavage of mRNA from the HIV gag gene has been reported (147). A terpyridyl Cu(II) complex was used for cleavage. An oligonucleotide bearing a histamine group at the 3′-end was able to cleave complementary RNA in a sequence-specific manner in the presence of Zn^{2+} ions in about 5% efficiency (148).

2. Lanthanide Complexes

Hall et al. (149) have shown the sequence-specific cleavage of a synthetic RNA using europium [Eu(III)] complexes linked to complementary oligonucleotide.

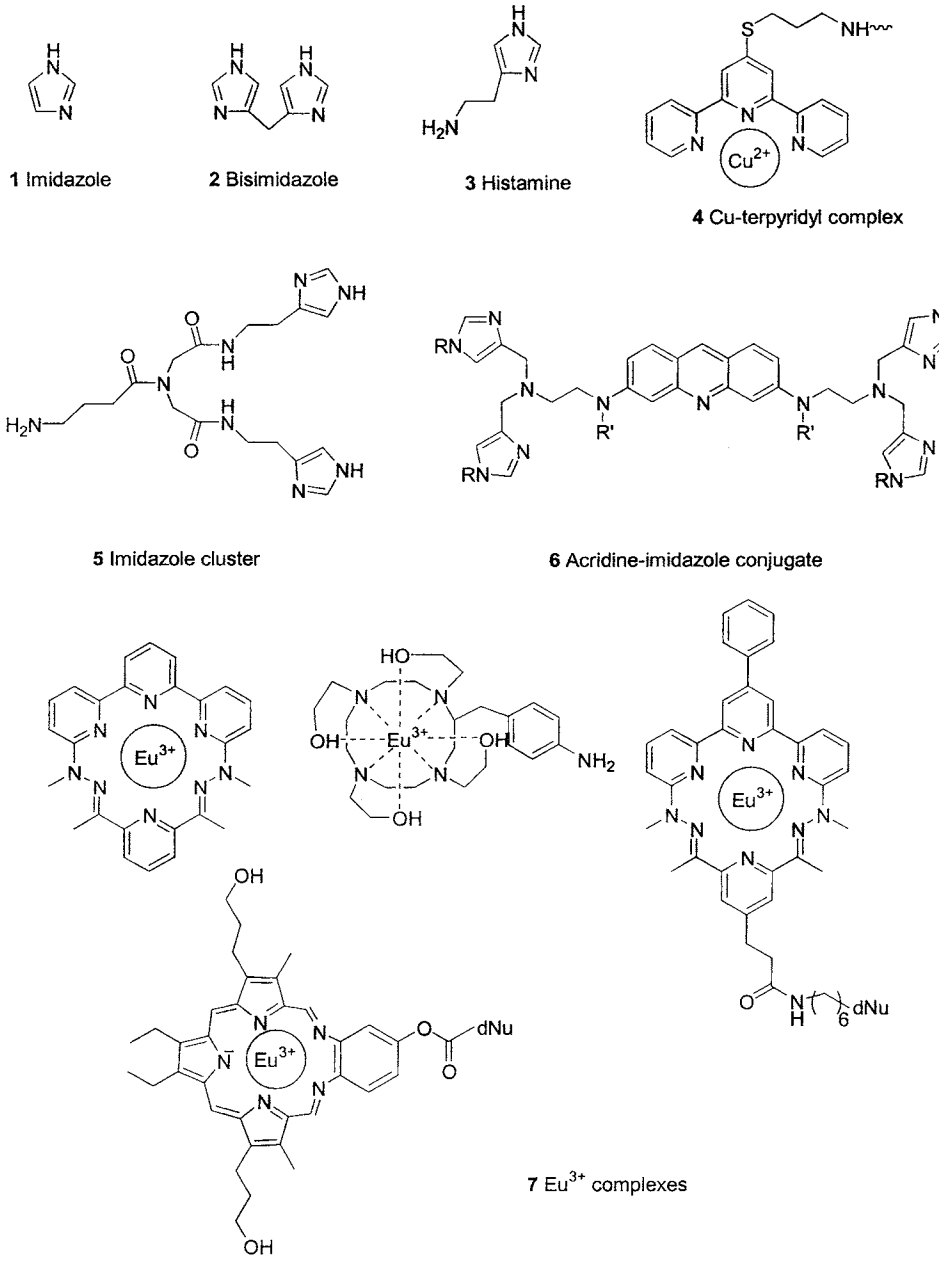


Figure 16 Cleaver molecule functionalities.

The cleavage efficiency of the conjugate strongly depend on the nature of the linker between the oligonucleotide and the complex. Almost complete cleavage of the RNA target was achieved within 16 h at 37°C with eight to 40 equivalents of the oligonucleotide conjugate.

Oligonucleotide conjugates of Eu(III) tetra-azamacrocycles with pendant alcohol and amide groups promoted sequence-specific RNA cleavage (150). Eu(III) complexes of two neutral bifunctional tetra-azamacrocyclic ligands [1-(1-carboxamido-3-(4-nitrophenyl)propyl)-4,7,10-tris(2-hydroxyethyl)-1,4,7, 10-tetraazacyclododecane and 2-(4-nitrobenzyl)-1,4,7,10-tetrakis(2-hydroxyethyl)-1,4,7,10-tetraazacyclododecane] were synthesized. Eu(III) complexes of the isothiocyanate derivatives of these macrocycles were treated with oligonucleotides containing 2′-*O*-propylamine linkers to form conjugates. Twofold excess concentration of the conjugate compared to RNA concentration for 16 h at 37°C produced 10–15% hydrolytic cleavage of a complementary oligoribonucleotide promoted by Eu(III) macrocyclic-oligonucleotide conjugate. Cleavage is not observed in the presence of Eu(III) conjugates containing scrambled sequences nor by free complex. The extent of cleavage observed is similar for conjugates containing either Eu(III) macrocyclic complex, despite the fact that one of the free macrocyclic complexes is more reactive than the other.

An oligonucleotide-Eu(III) complex conjugate designed to cleave the 5′ cap structure of the ICAM-1 transcript improved the antisense activity in cells (151). The 5′ cap structure of cellular and viral mRNAs synthesized by RNA polymerase II is a N7-methylated guanosine residue that is linked by a 5′-5′ triphosphate linkage to the 5′ terminus of the mRNA. Attachment of a Eu $(THED)^{3+}$ analog to the 3′ terminus of an antisense oligonucleotide (uniform 2′-*O*-MOE oligomer) that targets the 5′ cap region of ICAM-1 mRNA improved the inhibitory activity of the antisense oligonucleotide in cytokine-treated endothelial cells.

3. Peptides as RNA Cleavers

Oligonucleotide-peptide complexes offer another non-RNase H mechanism of sequence-specific, hydrolytic cleavage of mRNA. Oligonucleotide-peptide conjugates designed for mRNA cleavage have been obtained using several methods. By appending a maleimide group to an oligonucleotide, selective coupling to the thiol side chain of a cysteine residue in a peptide has been performed in 53% overall yield (152). Two oligonucleotide conjugates with peptide moieties at the active site mimic of RNase A (HGH motif) and the Cu(II) complexing metallopeptide (GGH motif) have been synthesized by solid-phase synthesis methods with pentafluorophenyl active esters of amino acids and Boc-His(Tos)-OH (153).

Highly efficient endonucleolytic cleavage of single-stranded RNA by a 30-

amino-acid zinc-finger peptide (Fig. 17) was reported by Lima and Crooke (154). The peptide sequence corresponds to a single zinc finger of the human-male-associated ZFY protein, a transcription factor belonging to the Cys_2His_2 family of zinc-finger proteins. Interestingly, RNA cleavage was observed only in the absence of zinc. Coordination with zinc resulted in complete loss of RNase activity. The active structure was found to be a homodimeric form of the peptide. Dimerization occurred through a single intermolecular disulfide between two of the four cystines. The catalytic activity was single-stranded RNA-specific; single-stranded DNA, double-stranded RNA and DNA, and 2′-*O*-methyl-modified oligonucleotides were not degraded by the peptide. The peptide specifically cleaved after pyrimidines with a preference for the dinucleotide sequence 5′-pyr-A-3′. The RNA cleavage products consisted of a 3′ phosphate and 5′ hydroxyl. The initial rates of cleavage (V_0) observed for the finger peptide were comparable to rates observed for human RNases, and the catalytic rate (k_{cat}) was comparable to rates observed for the group II intron ribozymes. The pH profile exhibited by the peptide is characteristic of general acid-base catalytic mechanisms observed with other RNases. Different chemical methods have been proposed to conjugate this peptide to antisense oligonucleotides (155).

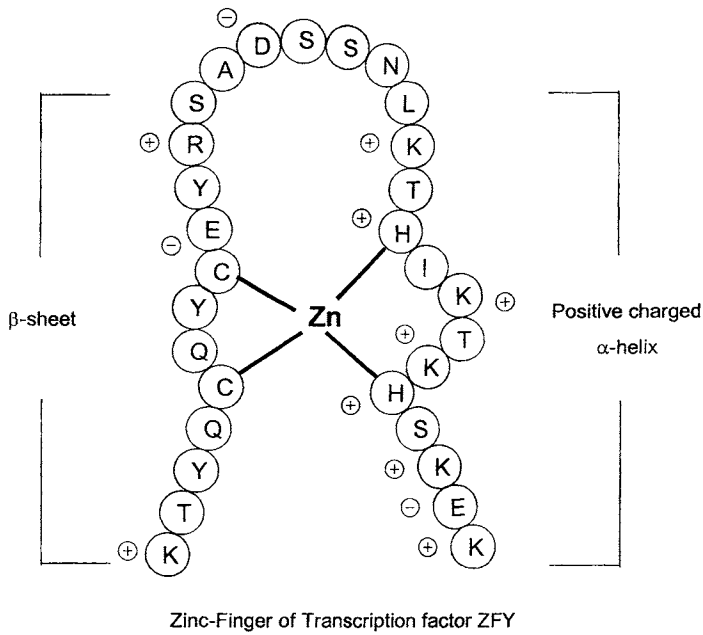


Figure 17 Zinc-finger RNA cleaver peptide.

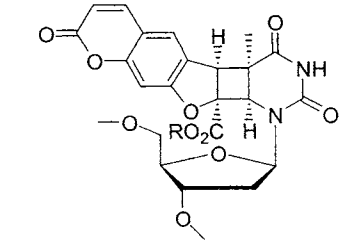
Design of a synthetic nuclease using a zinc-binding peptide tethered to a rhodium intercalator has been reported to hydrolyze DNA (156). A 16-amino-acid peptide, DPDGLGHAAKHEAAAK, which binds stoichiometric zinc ion, has been tethered to the DNA-intercalating metal complex $Rh(phi)_2bpy'$ (phi = phenanthrenequinone diimine, bpy′ = 4-butyric acid-4-methyl-2,2′-bipyridine) to construct a synthetic DNase. In this combination of DNA-binding and reactive moieties, the rhodium intercalator delivers the appended peptide for reaction with DNA. In the presence of Zn^{2+}, the $Rh(phi)_2bpy'$-peptide conjugate at μM cleaves supercoiled pBR322 DNA and a 17-base pair oligonucleotide duplex under mild conditions. The rate constant for the cleavage of pBR322 DNA by $Rh(phi)_2bpy'$-peptide at pH 6.0 is $2.5 \pm 0.2 \times 10^{-5}$/s. Product analysis of cleaved oligonucleotide fragments shows 3′-hydroxyl termini exclusively. These results demonstrate a stereospecific, hydrolytic DNA cleavage reaction by a synthetic complex; similar experiments with RNA have not been reported.

H. Crosslinking Agents

1. Psoralens

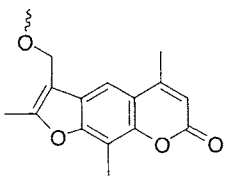
Psoralens are naturally occurring aromatic compounds consisting of a furan ring fused to a coumarin. They can be isolated from plants or from microorganisms including fungi. Psoralens are very effective interstrand DNA crosslinking agents by a (2 + 2) cycloaddition involving furan ring of the psoralen and the 5,6-double bond of a thymidine. Psoralens have demonstrated clinical value in the treatment of psoriasis, vitilago, cutaneaous T-cell lymphoma, and certain inflammatory processes.

Inhibition of collagenase type I expression by psoralen antisense oligonucleotides in dermal fibroblasts has been documented (157) by Lin et al. Type I collagenase plays an important role in both tumor metastasis and the remodeling of connective tissue in normal human skin, during wound healing, and may participate in the pathophysiology of some dermatological diseases such as skin cancer and a chronic blistering disease called recessive dystrophic epidermolysis bullosa. In an effort to specifically inhibit collagenase expression, phosphorothioate antisense oligonucleotides, linked at the 5′ ends with photoreactive 4′-(hydroxyethoxymethyl)-4,5′,8-trimethylpsoralen (HMT), were directed against the 5′ end of the collagenase mRNA (Fig. 18). Two antisense-HMT molecules targeting a region overlapping the initiation codon were compared. Only one oligonucleotide targeted the HMT moiety to a region with a 5′TpA. This oligonucleotide-HMT conjugate was 50-fold better than the other at crosslinking to its target sequence after UVA irradiation. Tissue culture experiments, conducted by incubation of collagenase-specific antisense-HMT oligonucleotides with fibroblasts in mono-

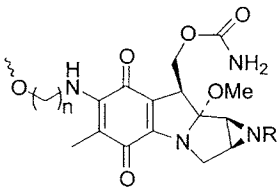


R = H, Me

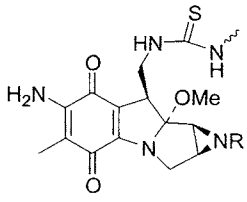
Psoralen containing oligonucleotides



HMT Psoralen



Mitomycin C



Porfiromycin C

Figure 18 Crosslinking agents.

layer or in three-dimensional dermal equivalent, showed lowered collagenase levels 24 h after UVA irradiation as compared to controls.

Kobertz and Essigmann reported (158) the synthesis of a derivatized cis-syn furan-side psoralen thymidine monoadduct that was incorporated into a DNA segment including a prototypical human TATA box sequence. In >90% yield, this oligonucleotide formed a crosslink with a complementary DNA strand by irradiation at 366 nm. The crosslinked duplex was reversible either with 254 nm light or by heating in aqueous base. The psoralen oligodeoxyribonucleotides (Fig. 18) possess the same crosslinking abilities as those produced by prior methods, but the method affords the flexibility to place the adduct in any sequence context. This makes it feasible to utilize the valuable crosslinking properties of furan-side psoralen monoadducts in hybridization assays where site-specific crosslinking is desired.

To selectively mutate and inactivate specific genes in human cells Glazer (159) has developed a triplex strategy for gene knockout. Triplex-forming pso-

ralen-conjugated oligonucleotides can direct DNA damage and consequently mutations to selected sites within mammalian cells.

2. Mitomycin C and Analogs

Mitomycin C (MC, Fig. 18), a pyrrolo (1,2-a) indole derivative, is a bioreductive alkylating agent and a clinically used natural anticancer drug. Tomasz et al. (160,161) have shown mitomycin derivatives selectively alkylate guanine residues at N2 position within duplex DNA with a remarkable preference for 5′-d(CG) sequences. Oligodeoxyribonucleotides conjugated at the 5′-terminal phosphate with mitomycin C via $(-CH_2-)_n$ tethers of different lengths (n = 6, 12) were synthesized and their interaction with complementary single-stranded DNA oligonucleotides was investigated (160). The usual enzymic bioreductive techniques known to trigger mitomycin C alkylation of DNA were employed in the reaction between the mitomycin-oligonucleotide conjugates and their targets. The DNA target is alkylated by the mitomycin C moiety of the ODN conjugate at the 2-amino group of a guanine base.

Conjugates of mitomycin C and 15-mer oligodeoxyribonucleotides have been synthesized by tethering the 7-amino group of mitomycin C by either a $(-CH_2-)_6$ or a $(-CH_2-)_{12}$ linker to the 5′-terminal phosphate of an oligodeoxynucleotide (161). The conjugates were selectively crosslinked to complementary 18-mer RNAs as shown by RNase H digestion. The crosslinking was dependent on reductive activation of the mitomycin C moiety of the conjugates by NADPH-cytochrome c reductase/NADPH. Crosslinking efficiencies of the conjugates were 50 and 25% in the case of the $(-CH_2-)_{12}$ tether and the $(-CH_2-)_6$ tether, respectively. These results point out the feasibility of sequence-targeted alkylation of RNA by mitomycin via antisense recognition.

Another report from the laboratory of Kohn (162) demonstrated conjugation via 10 position of mitomycin c using isothiocyanate chemistry to form thiourea linkages. A hexylamino spacer at the 5′ terminus of a phosphorothioate oligodeoxynucleotide was conjugated to a C(10)-activated mitomycin [10-des(carbamoyloxy)-10-isothiocyanatoporfiromycin]. Four antisense agents were designed to target a 30-base-long region from the coding region of the human FGFR 1 gene. The mitomycin c conjugates at μM concentration reduced the number of FGFR1 receptors in human aortic smooth cells, suggesting down-regulation of FGFR1 gene expression. Further, this conjugate inhibited cultured human aortic smooth muscle cell proliferation and was less cytotoxic than free porfiromycin. The mechanism of action of the conjugate has yet to be completely established, but these results suggest that antisense targeting of the mitomycin C plays a role in activity.

I. Porphyrin Conjugates

Porphyrin-conjugation (Fig. 19) can have a number of positive effects on antisense activity. They may function as hydrolytic cleaving agents like the lanthanide complexes described earlier or as oxidative or photoactive cleaving agents. The modification may also protect the oligonucleotides from nuclease digestion. These ligands are hydrophobic and thus may alter oligonucleotide distribution in vivo. Finally, as model heme ligands, they may enhance uptake into certain cell types to target liver cells.

1. Porphyrin Conjugates as Cleaving Agents

Magda et al. reported (163,164) a synthetic approach in which a dysprosium(III) texaphyrin (''porphyrin from Texas'') (DyTx) metal complex was attached to the 5′ end of oligodeoxynucleotides in the course of solid-phase synthesis using phosphoramidite synthesis. This synthetic methodology has been extended to the preparation of analogs in which the complex is attached at an internal position within the DNA oligomer. Under conditions of 25-fold conjugate excess, such 5′-derivatized constructs were shown to effect the site-specific cleavage of a cognate RNA sequence at 37°C in 6 h. A constrained short linker was more efficient than longer linkers. This mode of complex attachment affords enhancement of product release as the possibility of RNA fragment release because the number of base pairs joining the RNA to the DNA conjugate is reduced by a factor of ca. 2 upon cleavage. In an effort to further improve the efficacy of this system, kinetic and thermodynamic measurements were carried out using the Gd(III), Eu(III), Dy(III), and Lu(III) texaphyrin derivatives with single-stranded RNA (165). Through titration and kinetic experiments evidence for metal-porphyrin binding through a templated-stacking mode as well as a binding affinity trend (Dy>Gd>Eu>Lu) was observed and the dominant factor in reactivity is the coordination capability of the central lanthanide cation.

Sessler et al. reported (166) synthesis of sapphyrin-oligonucleotide conjugates. The sapphyrin-oligonucleotide conjugate produces photodamage on the complementary oligonucleotide target when irradiated at wavelengths above 620 nm. Upon piperidine treatment, guanine residues on the target strand in the vicinity of the sapphyrin macrocycle are found to be cleaved more effectively than guanines remote from the sapphyrin subunit. No sequence-specific photomodification was observed when noncomplementary oligonucleotides were used as a target. The duplexes formed between the sapphyrin conjugate and complementary nucleic acids were found to have higher T_m than analogous control systems consisting of unmodified oligonucleotides of the same sequence. T_m studies using variable salt concentrations and oligonucleotide targets indicate that the binding enhancement is due to hydrophobic interactions. The sapphyrin unit attached to

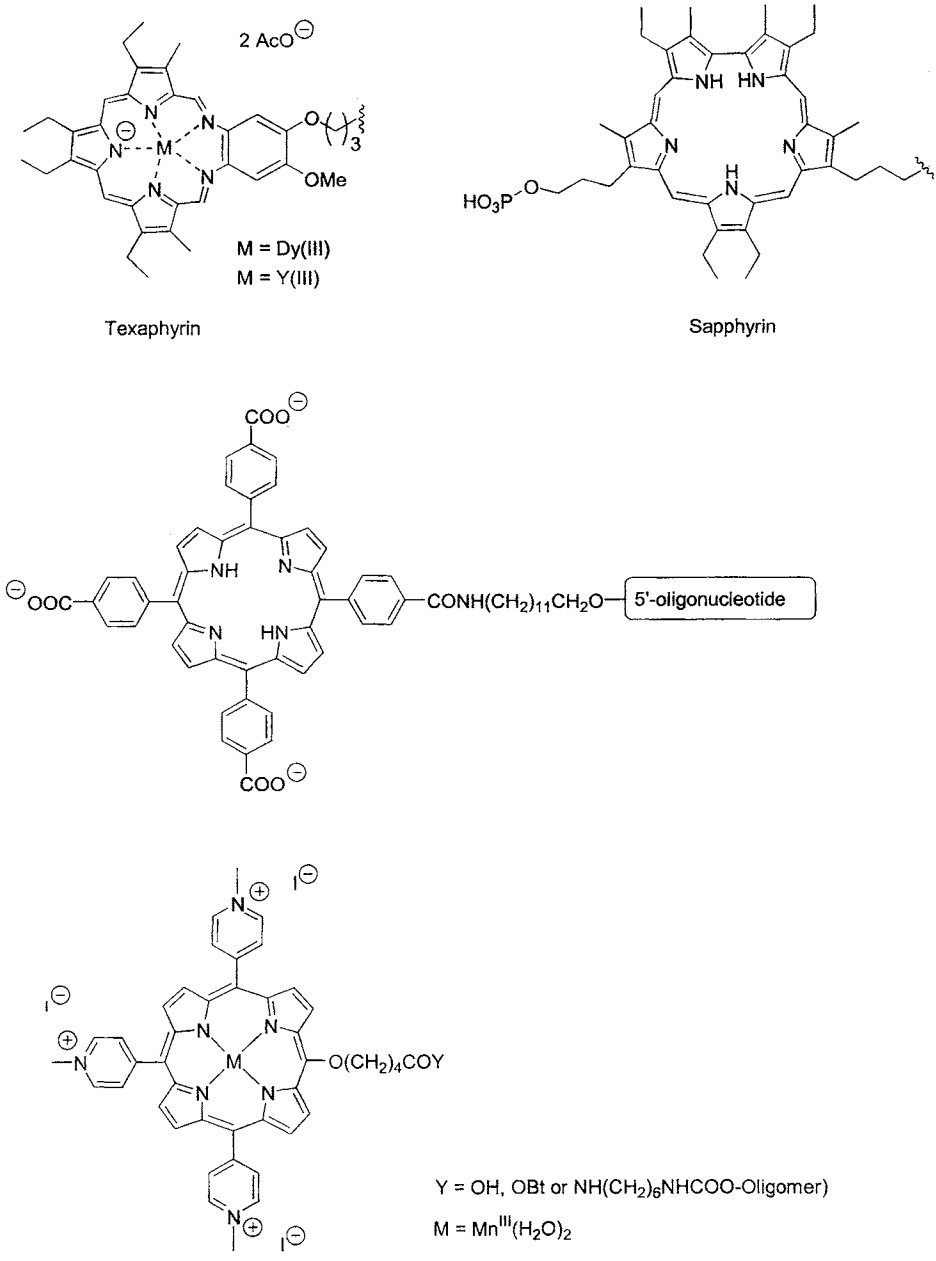


Figure 19 Porphyrins and their oligonucleotide conjugation sites.

an oligonucleotide can thus serve a dual role; it can act as a sequence-specific photomodification agent at irradiation at wavelengths > 620 nm, and it can increase the affinity of a sapphyrin-bearing oligonucleotide to a complementary sequence. The mechanism of the degradation involves direct electron transfer between purine bases (e.g., guanine residues) and the sapphyrin in either a triplet or single excited state. The second possibility is that a photoexcited sapphyrin in its triplet state reacting with molecular oxygen to form singlet oxygen, which reacts readily with guanine (167).

Cleavage of double-stranded DNA was performed with cationic manganese porphyrin complexes linked via a spermine tether to the 3′ or 5′ side of triple-helix-forming oligonucleotides (cleaver-TFO conjugates) (169). The targeted sequence was a 15-polypurine sequence present in the env gene of HIV-1 (positions 7301–7315). The oligonucleotide contained only thymine and 5-methylcytosine residues and one adenine at the 3′ end so that a 3′-polyamine linker could be readily introduced by reductive amination of the 3′-apurinic polypyrimidine oligonucleotides. TFO-cleaver conjugates were prepared in moderately good yield with only two equivalents of the Mn-TrisMPyP-COOH precursor. These new metalloporphyrin-TFO conjugates in 10-fold excess were able to cleave a complementary 45-mer duplex present at 10 nM concentration. Synthesis of cationic nonmetalated or zinc-porphyrin-oligonucleotide conjugates has been described and compared to the synthesis of the corresponding cationic manganese analogs (170). A manganese cationic porphyrin covalently linked to the 5′ end of an antisense oligonucleotide was shown to mediate sequence-specific oxidative lesions on a mRNA target when activated by potassium monopersulfate $KHSO_5$ (171). This manganese porphyrin cleaver did not induce direct RNA strand breaks but promoted the arrest of reverse transcription due to oxidative damage on bases of the RNA template. Using the same chemistry, to determine the most efficient compound for in vitro DNA cleavage (172), the nature and the length of the tether were modulated, the metalloporphyrin entity was modified (metal, ligand), and different ways of activation of the metalloporphyrin were assayed. The location of the peptidic bond within the linker greatly affected the cleavage efficiency of the different conjugates. The authors claim that the most efficient conjugate for oxidative DNA cleavage was a manganese tetrapyridiniumylporphyrin-oligonucleotide compound. When the metalloporphyrin moiety was activated by a reducing agent in the presence of molecular oxygen, DNA cleavage was efficient at a concentration of the reducing agent that avoided reduction of the activated DNA cleaver.

Synthesis and in vitro nuclease efficiency toward a single-stranded DNA target of cationic, anionic, and hydrophobic manganese-porphyrins covalently linked to the oligonucleotides has been reported (174). The most effective nuclease activity was obtained with the cationic metalloporphyrins, suggesting that the affinity of the cleaver to the DNA target is a key factor in cleavage of the nucleic acid.

2. Porphyrin Conjugates as Targeting Agents

Delivery of oligoribonucleotides to human hepatoma cells using cationic lipid particles conjugated to ferric protoporphyrin IX (heme) has been attempted (173). The receptor-ligand interaction between hepatocyte heme receptors and heme was evaluated as a basis for developing a targeted cationic lipid delivery reagent for nucleic acids. Heme (ferric protoporphyrin IX) was conjugated to the aminolipid dioleoyl phosphatidylethanolamine (DOPE) and used to form cationic lipid particles with dioleoyl trimethylammonium propane (DOTAP). These lipid particles protect oligoribonucleotides from degradation in human serum and increase oligoribonucleotide uptake into human hepatoma cells when compared with the same lipid particles prepared without heme. The heme level in lipid particles that was optimal for oligonucleotide delivery was also optimal for maximum expression of plasmid-encoded luciferase. The enhancing effect of heme was evident only when the particles had a net negative charge. Fluorescence microscopy showed that the heme-lipid complex delivered oligoribonucleotides into both the hepatoma cell cytoplasm and nucleus. The carrier system may thus be a potentially useful delivery vehicle for oligonucleotide-based therapeutics and transgenes, appropriate for use in such liver diseases as viral hepatitis, hepatoma, and hypercholesterolemia.

Conjugates of meso-(tetra-4-carboxyphenyl)porphine (TPPC4) (176) as a photosensitizer to oligonucleotides with chain lengths of 27, 20, 17, and 11 bases complementary to rat actin mRNA were prepared by the solid-phase method. Internalization and phototoxicity of these conjugates were investigated in cultures of RR1022 cells. The conjugates were shown to accumulate in the cytoplasm and this accumulation clearly depended on the length and sequence of the oligonucleotide moiety. Thus, the internalization of the phototoxic porphyrin was dependent upon the binding affinity of the oligonucleotide substituent to the target mRNA.

3. Other Applications of Porphyrin Conjugates

The biological activity of porphyrin-oligodeoxynucleotides targeting the initiation sites of bcl-2 and c-myb mRNAs was investigated in human lymphoma and leukemia cells (177). Porphyrin conjugation enhanced cellular uptake, retarded enzymic degradation, and provided site-directed chemical reaction with the targeted nucleic acid in vitro. The cell culture results showed that bcl-2 and c-myb antisense porphyrin conjugates were more efficient in inhibiting bcl-2 and c-myb than unconjugated oligodeoxynucleotides and conjugated controls.

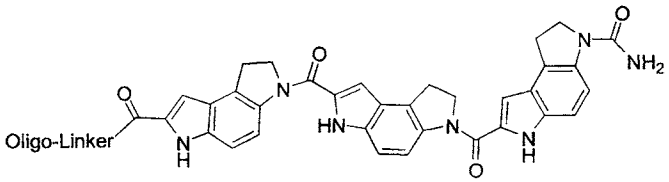
A 5′-GCGAAAGC minihairpin structure was added to the 3′ end of an oligonucleotide substituted at the 5′ end by a manganese cationic porphyrin to enhance the 3′-exonuclease resistance of these cleaver-antisense molecules (168). The influence of this minihairpin on the 3′-exonuclease resistance, the binding

affinity to a complementary single-stranded DNA, and the cleaving efficiency of Mn-cationic porphyrin oligonucleotide conjugates were compared to those of the parent molecule without the 3′-hairpin. The results showed that the 3′-hairpin slightly decreased the binding affinity and consequently the cleaving efficiency of the conjugated molecule toward a target sequence, but the much higher nuclease resistance makes 3′-minihairpin-protected metalloporphyrin oligonucleotides good candidates as reactive antisense oligonucleotides for studies on cells.

A convenient solid-phase synthesis of oligonucleotides conjugated at the 3′ end with a tetraphenylporphyrin residue, by means of a new polymeric support bearing a lysine derivative linker, has been developed (175). A porphyrin-linked 17-mer, designed for antisense experiments, has been prepared in good yields, and its hybridization properties with a complementary DNA fragment evaluated by UV thermal analysis.

J. Minor Groove Binder Conjugates

A new controlled pore glass support was described that allows for the direct synthesis of oligonucleotide derivatives carrying a minor groove binding (MGB) agent at the 3′ terminus (178). The MGB consisted of three repeating 1,2-dihydro-3H-pyrrolo (2,3-e)indole-7-carboxylate (DPI) subunits (Fig. 20). The DPI trimer (DPI3) was prepared directly on the CPG support using repeated addition of the DPI subunit. The subunit was protected at the N-3-position with tert-butyloxycarbonyl residue and activated at the 7-carboxy residue by esterification with the 2,3,5,6-tetrafluorophenyl group. A linker, which provided the starting point for oligonucleotide synthesis, was introduced by reaction of the terminal N-3 with *p*-nitrophenyl 4-[bis(4-methoxyphenyl)phenylmethoxy] butyrate. When used as a support for oligonucleotide synthesis, this modified CPG gave the desired 3′-DPI3-oligonucleotide conjugate in good yield. This conjugate formed stabilized complexes with complementary polyribo- and hyperstabilized complexes with



5′-Linker = -$(CH_2)_6NH$-

Minor groove binders

3′-Linker = -$CH_2CH(OH)CH_2NHCO(CH_2)_2NH$-

Figure 20 Minor groove binder ligands.

complementary polydeoxyribonucleic acids compared with unconjugated oligonucleotide. Melting transitions were cooperative.

An MGB derivative [*N*-3-carbamoyl-1,2-dihydro-3H-pyrrolo(3,2-e)-indole-7-carboxylate tripeptide; CDPI3] was covalently linked to the 5′ or 3′ end of oligodeoxynucleotides complementary or possessing a single mismatch to M13mp19 single-stranded DNA (179). Absorption thermal denaturation and slot-blot hybridization studies showed that conjugation of CDPI3 increased both the specificity and the strength of hybridization. Primer extension of the same phage DNA by phage T7 DNA polymerase (Sequenase) was blocked when a complementary 16-mer-CDPI3 conjugate was hybridized to a downstream site. Approximately 50% of the replicating complexes were arrested when the blocking oligodeoxynucleotide was equimolar to the phage DNA. Inhibition was unaffected by 3′ capping of the oligodeoxynucleotides with a hexanol group or by elimination of a preannealing step. Blockage was abolished when a single mismatch was introduced into the oligonucleotide or when the MGB was either removed or replaced by a 5′-acridine group. A 16-mer with a 3′-CDPI3 moiety failed to arrest primer extension, as did an unmodified 32-mer. The exceptional stability of hybrids formed by CDPI3-oligodeoxynucleotides is attributed to binding of the tethered tripeptide in the minor groove of the hybrid. When that group is linked to the 5′ end of a hybridized oligodeoxynucleotides, it probably blocks DNA synthesis by inhibiting strand displacement. These oligodeoxynucleotides conjugated to CDPI3 offer attractive features as diagnostic probes and antigene agents.

K. Polymer Conjugates

Synthesis and characterization of an hydroxypropyl methacrylate (HPMA)-based polymer as a carrier for the delivery of antisense oligonucleotides has been studied (180). The polymer contains active-sulfhydryl groups for coupling of ligands through a disulfide linkage. A sulfhydryl-terminated antisense oligonucleotide can be efficiently conjugated to the polymer. Cultured cells efficiently take up the polymer-oligonucleotide conjugate.

Fullerene-oligonucleotide conjugates have been synthesized and evaluated for photoinduced sequence-specific DNA cleavage, interaction with poly(alkylcyanoacrylate) nanoparticles, and biological activity (181). C60-Buckminsterfullerene bearing an alkylating or carboxyl group was attached to the modified oligonucleotide via alkylation or acylation of nucleophilic residue. For three different fullerene-oligonucleotide conjugates, light-induced guanine-specific cleavage of targets was observed in the expected location of the fullerene moiety. The conjugate is adsorbed on the surface of poly(alkylcyanoacrylate) nanoparticles without cationic lipids due to hydrophobic interactions between fullerene moiety and poly(alkylcyanoacrylate) matrix. The biological activity of a fullerene conjugated

to an oligonucleotide complementary to luciferase was studied in Jurkat cells expressing luciferase. The biological activity of the conjugate did not exceed that of the nonconjugated oligonucleotide, even when the conjugate was adsorbed on the hydrophobic nanoparticles.

L. Antibody Conjugates

Specific recognition of antibody-oligonucleotide conjugates by radiolabeled antisense nucleotides has been developed as a novel approach for two-step radioimmunotherapy of cancer (182). One of the major challenges in radioimmunotherapy is the specific delivery of radioisotopes to tumor cells while minimizing normal tissue radiation. In this respect, the application of two-step pretargeting schemes generally leads to more favorable tumor-to-normal-tissue uptake ratios than direct administration of radioimmunoconjugates. In this strategy, the antibody-DNA conjugate is administered first, followed by targeting with radiolabeled antisense DNA. The oligodeoxynucleotides used were modified in part with methylphosphonate (to ensure stability against nucleases). Both a murine IgG (anti-hCG) and the human antitumor IgM 16.88 were conjugated with one to three oligonucleotides via the heterobifunctional crosslinkers. Incubation of these immunoconjugates with the radiolabeled antisense DNA revealed specific hybridization with the antibody-linked oligonucleotides. Antigen-binding studies performed with antigen-coated matrices showed that the immunoreactivity of the antibody-DNA conjugate was preserved. The radiolabeled DNA was hybrided selectively to the oligonucleotides of the immunoconjugate, even when the antibody-DNA conjugate was bound to its antigen. Targeted cellular delivery of ^{125}I-oligonucleotide conjugated to H 17E2 antibody (a murine antibody of the IgG1 isotype), for cancer therapy, has also been described (183). This antibody binds to placental alkaline phosphatase (PLAP) expressed on the surface of many neoplasms including testicular and ovarian tumors.

Improved cellular delivery of antisense oligonucleotides using transferrin receptor antibody-oligonucleotide conjugates (184) has been described by Akhtar. A maleimide-derivatized IgG was reacted with a 5′-thiol-modified oligodeoxyribonucleotide. Human glioblastoma cell line U87-MG and the human endothelial cell line ECV304, which both express the transferrin receptor on their surfaces, bound and internalized the antibody-oligonucleotide conjugates threefold more effectively than free oligonucleotides.

The same group examined uptake of rat transferrin receptor antibody (OX-26) conjugates into an immortalized cell line called RBE4 as an in vitro model of the blood-brain barrier (185). Uptake of the conjugate into this cell model was twofold higher than the free oligonucleotide and its uptake mechanism was consistent with transferrin receptor–mediated endocytosis. Exocytosis profiles of the OX-26-oligonucleotide conjugates were different from profiles of either free

oligonucleotide or a nonspecific IgG-oligonucleotide conjugate. The OX-26 conjugates showed altered subcellular distribution, possibly involving "deeper" cellular compartments. Treatment of cells with monensin further increased the intracellular accumulation of the OX-26-oligonucleotide conjugates suggesting that trafficking of the conjugate may involve the trans-Golgi network.

Monobiotinylated and nonbiotinylated PNA antisense molecules complementary to the region around the methionine initiation codon of HIV-1 rev mRNA were synthesized (186). Both biotinylated and nonbiotinylated PNAs were incubated in the presence or absence of the OX26-SA delivery system. The PNAs were conjugated to OX26 via biotin as a complex. The antisense molecules efficiently recognized and inhibited the translation of the *rev* gene in a translation arrest assay in rabbit reticulocyte lysate. OX26-SA conjugates are transported into the brain at levels comparable to that of morphine, suggesting that anti-*rev*-OX26-SA conjugates may be an optimal formulation for antisense delivery to the brain. However, it is not clear how these large molecules are able to cross the blood-brain barrier.

M. Protein Conjugates

Antisense oligonucleotides have been covalently attached to asialoglycoprotein (ASGP) via disulfide bond conjugation (187). Multiple (approximately six) oligonucleotides can be conjugated to each ASGP molecule. The ASGP was used to deliver antisense oligonucleotides complementary to the mRNA of the interleukin-6 signal transduction protein (gp 130) to hepatoma (HepG2) cells in vitro. These conjugates inhibited the cytokine-stimulated up-regulation of the acute-phase protein haptoglobin.

Targeted delivery of antisense oligonucleotides by asialooromucoid (AsOR) protein conjugates has also been described (188). Binding of poly(L-lysine)-AsOR protein conjugates with phosphorothioate antisense oligonucleotides to chloramphenicol acetyltransferase (CAT) led to the formation of 50- to 150-nm toroids. Exposure of the antisense oligonucleotide complexes (3 μM oligonucleotide) to NIH 3T3 cells, genetically modified to express both the AsOR receptor and CAT, inhibited CAT expression by 54%. The CAT inhibition was completely blocked by excess AsOR. Equivalent inhibition of CAT activity with purified oligonucleotide alone was not observed until 30 μM concentration. Furthermore, examination of the cells using indirect immunofluorescence for the presence of CAT protein showed 28% of cells exposed to the conjugates lacked any detectable CAT enzyme. Cells exposed to oligonucleotide alone showed a highly variable staining pattern, and only a few of the cells were completely devoid of CAT protein.

The 5′-nontranslated region (NTR) of hepatitis C virus (HCV) contains important elements that control HCV translation. Antisense oligonucleotides di-

rected against a sequence in the internal ribosomal binding site of the NTR and a portion of the NTR overlapping the core protein translational start site of HCV were prepared (189). Oligonucleotides in the form of asialoglycoprotein-polylysine complexes were administered to Huh7 cells (human hepatoma cell lines), and luciferase activity, generated from a transiently transfected plasmid containing a luciferase gene immediately downstream from an HCV NTR insert, was measured. The results showed inhibition of luciferase activity by 75–99% at 0.01 μM and 0.1 μM doses. Thus, asialoglycoprotein-polylysine complexes of antisense oligonucleotides can be targeted into cells by receptor-mediated endocytosis, and they specifically inhibit protein synthesis.

N. Miscelleneous Conjugates

Puromycin (Fig. 21) is a member of the 3′-amino-3′-deoxyadenosine family of antibiotics and is a potent inhibitor of protein biosynthesis. Inhibition of protein biosynthesis is due to formation of a covalent adduct between the antibiotic and the interrupted premature peptide. A facile method for the synthesis of puromycin-tethered to the 3′ end of oligonucleotides has been described using a solid support derived from puromycin (190). The amino group of puromycin was protected as a fluorenylmethoxycarbonyl FMOC, removed during the oligonucleotide deprotection step. Puromycin-tethered DNA and RNA oligomers were synthesized. Obviously this group will provide increased nuclease resistance to the oligomers. No other biological data are known.

Pamamycin is an antibiotic derived from *Streptomyces aurantiacus* with cell permeation ability. An alkali-mediated hydrolysis product of this antibiotic was conjugated to the 3′ end of antisense oligonucleotides directed against immediate early gene IE 110 of HSV-1 via a phosphodiester or phosphorothioate linkage. This antibiotic has a cationic group in the side chain. A small increase in T_m was observed (191). Cellular uptake and other biological activities are not known.

The ^{76}Br radiobromination of modified oligonucleotides by the use of *N*-succinimidyl 4—(^{76}Br)-bromobenzoate has been described (192). The labeled *N*-succinimidyl bromobenzoate was synthesized from the corresponding trimethyltin compound, *N*-succinimidyl 4-trimethylstannylbenzoate, in 45–60% isolated radiochemical yield with a specific radioactivity of 20–200 GBq/μmol. Both phosphodiester and phosphorothioate 5′-hexylamino-modified oligonucleotides have been conjugated using this intermediate.

Labeling of phosphorothioate antisense oligonucleotides with yttrium 90 has been described as a potential targeted radionuclide therapeutic agent for malignant tumors (193). A 15-mer phosphorothioate antisense oligonucleotide, complementary to the translation start region of the N-myc oncogene mRNA, was conjugated with isothiocyanobenzyl ethylenediamine tetraacetic acid, via a C-5-

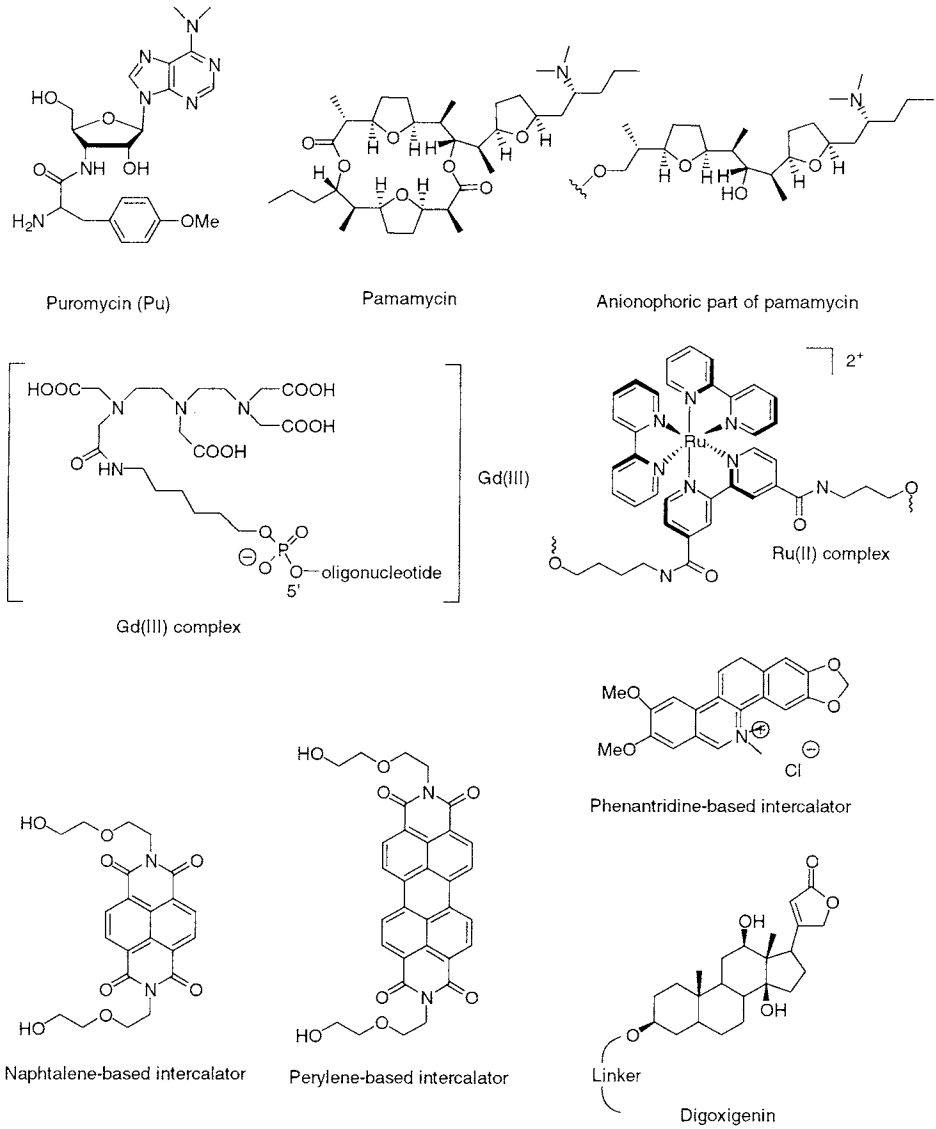


Figure 21 Miscellaneous ligands.

substituted deoxyuridine, and was then labeled with ^{90}Y-acetate. Radiochemical purity was 98.7 ± 0.4% at 72 h after labeling and 90.3 ± 0.9% after 72 h incubation with human normal serum. The ^{90}Y-Bn-EDTA-phosphorothioate antisense oligonucleotide hybridized specifically to a complementary phosphorodiester sense oligonucleotide without loss of binding affinity.

An antisense paramagnetic oligonucleotide analog targeted to a model macromolecular receptor (5S rRNA) was prepared as a contrast agent for magnetic resonance imaging (194). The paramagnetic agent's relaxivity (dependence of the relaxation rate on paramagnetic agent concentration) in the presence and absence of the RNA was measured at different field strengths—1.5 and 6.3 T. The relaxivity of the targeted agent increased specifically in the presence of the RNA receptor (16% at 6.3 T and 15% at 1.5 T). This effect was specific for a paramagnetic oligonucleotide targeted to the receptor and was larger than the relaxivity enhancement due simply to receptor-induced viscosity differences. Maximizing this relaxivity enhancement of tumor-targeted paramagnetic oligonucleotides will aid in contrast agent development for magnetic resonance imaging.

A method of studying intracellular distribution of phosphorothioate oligonucleotides using digoxigenin-label has been described (195) using immunocytochemistry with both confocal and electron microscopy.

Several research groups have reported conjugation of electron transfer reagents such as ruthenium complexes. Benzophenanthridine intercalators (196) have been conjugated by phosphoramidite chemistry. Intercalators based on naphthalene and perylene have been developed using phosphoramidite chemistry (197).

O. Conjugates for Modulating Protein Binding

1. Rationale

As discussed at various points in this review, the interaction of antisense oligonucleotides with serum and cellular proteins determines their pharmacokinetic (transport to and distribution in target tissues) and pharmacodynamic (binding to the mRNA target) properties and hence their eventual pharmacology (1,198). In general, binding of drugs to serum albumin, α_2-macroglobulin, immunoglobulins, and lipoproteins in the bloodstream governs their transport and tissue distributions (199). First-generation antisense compounds, the 2′-deoxyphosphorothioate oligonucleotides (Fig. 2), have relatively avid binding to serum and cellular proteins, which results in their favorable pharmacokinetic properties (1,36,198, 200,201). However, these P=S oligonucleotides also bind to proteins such as thrombin, Factor IX, and Factor H, contributing to the observed dose-limiting side effects such as prolonged clotting time and extent of complement activation (202,203). To make safer and more effective oligonucleotide drugs, it may be

of value to enhance the interaction of these drug molecules with "good" proteins involved in transport and absorption and to minimize the interaction with "bad" proteins responsible for their side effects.

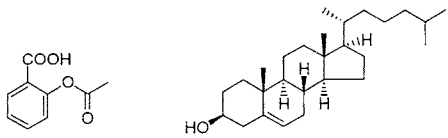
Changing the P=S linkages to the native P=O linkages overcomes some of the side effects and increases the binding affinity to the target RNA (204), but this change also results in the loss of nuclease resistance and consequently more rapid degradation (205) of the drug compound. 2′-*O*-(2-methoxyethyl) (Fig. 2) modifications in the antisense oligonucleotide can offset this undesired sensitivity to nucleolytic degradation. Although the 2′-*O*-MOE provides sufficient nuclease resistance, the replacement of P=S linkages by P=O linkages results in poor phamacokinetic properties, such as limited distribution to organs and faster urinary elimination (206), presumably due to their inability to bind to serum proteins. It would therefore be desirable to improve binding affinity for human serum albumin while reducing phosphorothioate linkage content.

2. Binding to Human Serum Albumin

Human serum albumin, a water-soluble protein with a molecular weight of 66 kD and 585 amino acids as a single chain (207–210), is the most abundant protein in plasma (3.5–5.0 g/100 mL in blood plasma) but also exists in lower concentrations in extravascular fluids. It has a large number of charged amino acids (about 100 negative charges and 100 positive charges) with an isoelectric point of 5.0 and a net negative charge of −15 at plasma pH of 7.4, and attracts both anions and cations. It would be desirable to improve binding affinity of antisense drugs to human serum albumin by using molecules capable of binding to the protein. Aspirin is known to affect the protein binding of phosphorothioate molecules (211). The role of cholesterol in modulating serum protein binding of phosphorothioate oligonucleotides has also been discussed earlier (17,212).

IV. CONCLUSIONS AND PERSPECTIVES

Oligonucleotide conjugation chemistry—attaching proper ligands to modulate the biological activity of oligonucleotides—has proven to be a valuable method-



Aspirin Cholesterol

Figure 22 Examples of ligands capable of modulating protein binding.

ology. In this review, we have shown that conjugating various ligands onto synthetic oligonucleotides can control their pharmacokinetic and pharmacological properties (213,214).

Oligonucleotide conjugates have been evaluated in a wide range of cell culture and in vitro experiments. Efficacy has been evaluated by measuring target RNA reduction and protein reduction and by analyzing effects on nontarget mRNA and protein. Properties related to pharmacokinetics, including cellular uptake and nuclease resistance and binding affinity to proteins related to drug lifetime (such as human serum albumin), have been studied. However, only a limited number of in vivo experiments have been performed. Nevertheless, the value of conjugation chemistry has been clearly demonstrated both in vitro and in vivo. Pendant molecules are capable of altering pharmacokinetics of oligonucleotides and improving their pharmacology.

Conjugated ligands can also cause adverse effects: for example, conjugation of PEG reduced the transfection efficiency of ICAM-1 antisense oligonucleotides and thus decreased antisense activity. The toxicity and side effects of the ligands being conjugated have not been well characterized. For example, some of the intercalators (acidine derivatives) are extremely toxic. Some ligands that enhance protein binding (e.g., aspirin) may cause minor gastrointestinal problems.

In carrying out conjugation chemistry, many types of linkers are used, including chemically and biologically stable linkers (such as amide or carbamate linkers) and bioreversible disulfide linkages. Bioreversible linkages have an advantage in that the ligand is detached from the "oligo cargo" in the cell so that the linker does not have undesirable effects on further antisense oligonucleotide interactions with either target mRNA or protein (for example, RNase H). Chemically and biologically stable linkages are desired, however, if the goal is to improve the protein binding of antisense oligonucleotides for improving pharmacokinetics. Thus, the choice of linkers must be based on the role of the ligand.

Cost and ease of synthesis of some conjugates may be a concern. While synthesis of cholesterol or lipophilic molecules does not pose much synthetic challenge, synthesis of conjugates involving complex molecules such as folic acid, carbohydrate clusters, and peptides creates synthetic challenges in many ways. These ligands have limited solubility in appropriate solvents, require design of orthogonal protecting strategies compatible with oligonucleotide synthesis, and require strategies that limit racemization of the chiral centers in the ligand during oligonucleotide deprotection. Each of these problems has to be addressed and overcome for each new ligand and linker and depends on the chemistry of the antisense oligonucleotide as well.

Many biological interactions relating to ligand conjugation remain to be evaluated. How do these pendant groups (small molecules) or large molecules (peptides) interact with other proteins involved in the antisense action? What is

their role in RNase-H activation? How do they interact with helicases? How do they affect the interaction of oligonucleotides with other RNA regulatory elements? Do they play any role in various steps involved in mRNA editing? How do they affect translation regulation or recoding?

The structures of oligonucleotides carrying pendant groups are not known—it is generally assumed that the structure of oligonucleotide remains unaltered after the conjugation. It is conceivable that a micellar structure is formed when a lipophil-like cholesterol is conjugated to oligonucleotides; similarly when a cationic peptide is attached to highly electron-rich phosphorothioates, the resulting charge neutralization may cause aggregate formation—yet no NMR or crystal structure data are available on this area.

Many medicinal chemistry experiments addressing structure-activity relationships could be initiated based on initial results. For example, additional folate analogs should be evaluated for ability to enhance oral absorption. Unfortunately, all these questions remain unanswered and answers are being sought in only a few laboratories.

In spite of these functional limitations and knowledge-based limitations, antisense oligonucleotide conjugates offer incredible potential for enhancing antisense oligonucleotide technology. Among the chemical analogs evaluated so far, next to the phosphorothioate (both 2′-deoxy and 2′-modified) analogs, *only* conjugates have clearly shown both valuable pharmacological and pharmacokinetic properties. This experimental fact naturally demands enthusiastic preclinical and clinical evaluation of oligonucleotide conjugates for various disease targets.

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Immune Stimulation by Oligonucleotides

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I. INTRODUCTION

Although the immune system possesses exquisitely antigen-specific receptors on T cells and B cells, the generation of such adaptive immune responses relies on the assistance of the innate immune defenses, such as dendritic cells, which must be activated to trigger optimal immune responses. These cells of the innate immune system lack such highly specific antigen receptors, instead relying on a set of "pattern recognition receptors" (PRRs), which have a general ability to detect certain molecular structures that are common to many pathogens, but are not present in self tissues (1,2). Examples of ligands for such PRRs include endotoxins, high-mannose proteins, double-stranded viral RNAs, and, most recently, the unmethylated CpG dinucleotides, in particular base contexts that are prevalent in bacterial and many viral DNAs, but are heavily suppressed and methylated in vertebrate genomes (1–5). Thus, the immune system appears to use the presence of these molecular structures as a "danger signal" that indicates the presence of infection and activates appropriate defense pathways. Aside from the intrinsic scientific interest of these pathways of immune activation, their understanding may give us new tools for therapeutic manipulation of the immune system. Manipulation of PRR-activated pathways may be desirable for the purposes of activating therapeutic responses in cancer or some infectious diseases, but also for preventing undesirable immune activation in other clinical settings. The purpose of this review is to consider just one of these PRR pathways, that mediated by CpG DNA.

II. HISTORY

A. Immune Stimulation by Palindromes and Antisense Oligodeoxynucleotides

Extracts of the attenuated mycobacteria bacillus Calmette-Guerin (BCG) have long been known to induce antitumor immune responses (6,7). To determine the active component of BCG, Tokunaga and colleagues fractionated it into the various components and assayed each of these for their antitumor activity (8). Surprisingly, these studies demonstrated that only the DNA fraction of BCG contained antitumor activity. BCG DNA was further shown to induce NK cell activity and the production of type 1 and type 2 interferons (9). By cloning mycobacterial genes and synthesizing constituent oligodeoxynucleotides (ODN), these investigators concluded that the immune stimulatory effects of BCG DNA could be attributed to the presence of certain self-complementary palindromes in these ODN (10). All of the active palindromes were noted to contain at least one CpG dinucleotide, but methylation of the CpGs was reported to have no influence on the immune stimulatory activities of the DNA (11).

In studies that initially appeared to be completely unrelated, several investigators working with antisense ODN reported unexpected stimulation of lymphocyte proliferation and variable effects on immunoglobulin production. None of these ODN contained the previously described palindromes. In one case, an ODN that was synthesized to be antisense to an immunoglobulin sequence was reported to unexpectedly *increase* the expression of the target gene (12). This ODN also caused a profound induction of B-cell proliferation but inhibited antibody secretion (12). These immune effects did not appear to be due to any antisense mechanism of action because a variety of control ODNs in which various bases were switched had the same immune stimulatory activities. An antisense ODN against the *rev* gene of the human immunodeficiency virus caused a profound degree of B-cell proliferation and massive splenomegaly in vivo in mice (13). In contrast to the inhibitory effects observed in the previous study, the anti-*rev* ODN *induced* production of immunoglobulin. A variety of control ODN lacked any observable immune stimulatory effect. This immune-stimulatory effect was thought to be dependent on the backbone of the ODN because it was only observed if the ODN was synthesized with a nuclease-resistant phosphorothioate (PS) backbone and did not occur if the same sequence was studied with a native phosphodiester DNA backbone (13). At high concentrations, PS ODN can exert sequence-independent immune stimulatory effects, especially on B-cell proliferation (14). However, the lack of effect of the phosphodiester anti-*rev* ODN must be interpreted with caution because of the rapid nuclease degradation of such unmodified ODNs in lymphocytes (15). An antisense ODN against herpes simplex virus (HSV) was also found to induce B-cell proliferation (16). Immune stimulation was observed not only with antisense ODN, but also with a sense ODN against the nuclear factor

κB (NFκB) p65 subunit (17). However, a complementary antisense ODN did not induce the observed B-cell proliferation, immunoglobulin secretion, and in vivo splenomegaly. The sense ODN to NFκB was also noted to trigger the rapid activation of NFκB-binding activity (17). Although these authors were aware of the other reports of immune-stimulatory ODN, no apparent common motif could be discerned that might tie these different observations together (17).

B. Identification of the CpG Motif

I was also attempting to perform antisense experiments when I first observed the immune-stimulatory effects of certain ODN. However, in my own case, the initial results appeared to be consistent with an antisense mechanism of action and the observed biological effects were therefore interpreted in this manner. We had observed that a particular murine endogenous retroviral sequence, mink cell focus-forming (MCF), was highly expressed in mice genetically predisposed to the development of systemic lupus erythematosus, but not in control mice (18). Antisense ODN appeared to be an ideal technology with which to investigate the possibility that some protein product encoded by this retrovirus might possibly have an immune regulatory activity. For my first experiments to test this hypothesis, I synthesized two partly overlapping antisense ODN against the MCF retrovirus and two control ODNs (19). This class of endogenous retrovirus has been reported to contain immunosuppressive domain in the envelope region, and so it seemed possible that inhibition of expression of this gene could release the lymphocytes from these suppressive effects, thereby leading to proliferation. The first experiment with these ODN appeared to be a complete success in that both of the antisense ODNs caused B-cell proliferation and immunoglobulin secretion, but both of the controls were nonstimulatory (19). Further studies confirmed and extended these findings (20,21).

Although our results with these immune-stimulatory oligos were initially interpreted in the context of an antisense mechanism of action, with further studies it became clear that this could not explain all of the observed activities. Several control ODNs synthesized for other experiments showed immune-stimulatory effects with activation of B-cell proliferation and immunoglobulin secretion that was very similar to that induced by the initial "antisense" ODN. Although we initially considered the possibility that these immune-stimulatory control ODN may in fact be antisense to some previously unidentified gene, they showed no homology to any known DNA sequence and did not hybridize to Northern blots of B-cell mRNA. Moreover, certain additional control ODN had similar effects while others did not. It was therefore clear that this was some type of unexpected and sequence-specific but nonantisense activity. The magnitude of the immune stimulatory effect was startling; the strongest ODN were stronger B-cell mitogens than gold standards such as endotoxin. Initially it was unclear whether there was

any common mechanism between the immune-stimulatory effects triggered by these ODN and those reported in the previous studies of ''antisense'' ODN reviewed in the preceding section. Furthermore, very few of the ODN contained any palindromes, so this appeared to be a different type of immune activity than that reported by Tokunaga and colleagues for the activation of NK cells.

To identify the DNA structure responsible for these effects, a series of structure/function analyses were therefore undertaken. Initial studies suggested that some sort of secondary structure such as a stem loop could explain at least some of the immune stimulatory activities caused by the particular ''control'' ODN. This raised the possibility that there may be several different structures or sequences capable of mediating lymphocyte activation. However, after synthesis and testing of several hundred ODN, it eventually became clear that a sufficient sequence element for inducing B-cell activation was a CpG dinucleotide in particular base contexts. With this realization, it quickly became possible to demonstrate that stem loop structures or other secondary structures were not required for immune stimulation. In addition, with the understanding of the role of flanking bases around the CpGs in determining immune stimulation, it became possible to review previous reports of immune stimulatory ''antisense'' ODN and palindromes with a new eye. This comparison revealed that all of the previously reported immune stimulatory sequences also contained CpG dinucleotides, which conformed to the general consensus motif shown in Fig. 1 (22,23). Interestingly, these studies also showed that while in general, increasing the number of stimulatory CpG motifs in an ODN increased the activity of the ODN, the addition of a CpG into an end of an ODN or the addition of a CpG in an unfavorable sequence context could actually reduce the degree of the cell activation (4,24). In all cases, elimination of the CpG dinucleotides from ODN abolished their stimulatory activities (4).

$\mathbf{X_1X_2\underline{CG}X_3X_4}$

"Rules" for optimal stimulatory effects of CpG motifs:
X_1= purine preferred
X_2= not C
X_3= not G, T preferred
X_4= T preferred

Figure 1 Definition of the immune stimulatory CpG motif. A single CpG motif in an ODN is sufficient to trigger strong immune stimulatory effects, especially if the base context of the motif is near optimal. There are some differences in the preferred CpG motifs of phosphorothioate ODN for stimulating rodent and human immune cells. In rodents, an optimal motif is GACGTT, while in humans, GTCGTT is stronger (28).

It is now clear that the immune-stimulatory effects of an ODN depend on the precise bases flanking the CpG dinucleotide ("CpG motif"), on the number and spacing of the CpG motifs, on the presence of poly G sequences in the ODN, and on the ODN backbone (4,25–28). If the ODN has a PO backbone, then CpG dinucleotides are highly immune-stimulatory as long as they are not preceded by a C or followed by a G, in which case the effects are diminished (Table 1). However, there are differences between different CpG motifs, such that optimal motifs can be identified. For activating murine immune responses, the optimal

Table 1 Identification of Optimal Stimulatory CpG Motifs for Murine Cytokine Production and B-Cell Proliferation

ODN #	Sequence 5′-3′	IL-12 (pg/mL)	IL-10 (pg/mL)	B-cell activation (SI)
Media		<20	108	1.0
1916	TCCTGA*CG*TTGAAGT	991	1157	6.8
1929	. . . . . *GC*	<20	65	1.0
1936	. . . . . *ZG*	153	69	1.6
1937	. . Z . . . *CG*	935	377	3.9
1917	. . . . . T*CG*	828	589	4.4
1918	. . . . . G*CG*	776	802	2.8
1919	. . . . . C*CG*	215	684	1.8
1920	. . . . T . *CG*.	748	738	4.5
1921	. . . . A . *CG*	772	644	3.7
1922	. . . . C. *CG*	657	616	3.8
1923	. . . . . *CG*A	571	284	3.5
1924	. . . . . *CG*C	837	497	3.9
1925	. . . . . *CG*G	90	168	1.5
1926	. . . . . *CG* . A	175	269	3.9
1927	. . . . . *CG* . C	332	359	2.4
1928	. . . . . *CG* . G	<20	142	2.3
1930	. . . . . *CG* . . . GG.G	1452	666	1.7
1931	. . . . . *CG* . . CCTTC	2031	923	5.4
1935	. . . . . G*CG*GG	<20	43	1.3
1938	. . . . AG*CG*	485	170	7.8
1939	. . . A . . *CG*	872	414	4.4
1940	. . . . . *CG*GG	<20	23	0.9
1941	. . . . . G*CG*G	<20	34	1.1

Spleen cells from DBA/2 mice were cultured with the indicated ODN for 24 h, and supernatant IL-12 and IL-10 levels were measured by ELISA using antibodies from Pharmingen (San Diego) as described (40). B-cell activation was measured by ^{3}H-uridine incorporation as described (4). The basal cpm in medium-only wells was 524.

CpG motif is GACGTT, and the effects can be further enhanced if the ODN has a TpC dinucleotide on the 5′ end, and is pyrimidine-rich on the 3′ side,especially with thymidines (4,26). If the ODN contains several CpG motifs, they should not be back-to-back, but preferably spaced with at least two bases, preferably Ts. For activating human cells, the same rules pertain except that the optimal motif is GTCGTT (27). A recent study suggests that human T and NK cells may respond to different CpG motifs (29). There is also some evidence that fish may respond to different CpG motifs from mice (30).

C. Immune Recognition of CpG Motifs as a Defense Mechanism

While the identification of the CpG motif explained the way in which certain ODN caused immune stimulatory effects, it raised a new question of what purpose these effects could possibly serve. Was this simply some curiosity of interest only to researchers using antisense ODN, or could immune stimulation by these CpG motifs actually serve some purpose for the immune system? The latter possibility was suggested by the fact that vertebrate and bacterial DNAs differ markedly in their CpG content and methylation (3). While bacterial DNA generally contained the expected frequency of about one CpG dinucleotide per 16 bases, it has long been realized that CpG dinucleotides are markedly suppressed in vertebrate genomes where they occur only about one-fourth as frequently as would be predicted if base utilization was random. Furthermore, the bases flanking CpGs in vertebrate genomes are not random: the most common base preceding a CpG is a C and the most common base following a CpG is a G (31). These types of CpG motifs do not support immune stimulation (Table 1), but actually have an inhibitory effect that neutralizes other immune stimulatory CpG motifs (see the section on CpG-N motifs below). In addition to these differences in CpG content, CpG dinucleotides are not methylated in bacterial DNA, but are routinely methylated at the 5 position of about 70% of the cytosines in vertebrate DNAs (3).

This structural difference between bacterial and vertebrate DNAs suggested the possibility that immune recognition of unmethylated CpG motifs in bacterial DNA could explain the previously reported effects of bacterial DNA on B and NK cells rather than the previously proposed mechanisms involving palindromes reviewed above. To test this hypothesis, we synthesized immune-stimulatory ODN in which the cytosines of the CpG were replaced by 5-methyl cytosine. These ODN showed markedly reduced immune-stimulatory effects, while ODN in which other cytosines outside of the CpG motifs were replaced by 5-methyl cytosine retained full immune-stimulatory capacities (4). Moreover, the immune-stimulatory effects of bacterial DNA are severely attenuated by methylation with CpG methylase (4). Extracts of *Babesia bovis*, like those of many other microbes, are immune-stimulatory. These effects result from the DNA, since they are abol-

ished by treatment of the extracts with nuclease, and can be reproduced with purified *B. bovis* DNA (32). *Drosophila* extracts are also immune-stimulatory, owing to the unmethylated CpG motifs in insect DNA (33). A survey of different genomic DNAs has confirmed that hypomethylation of CpG is required for immune stimulation (34). On the other hand, hypomethylation is not sufficient since hypomethylated vertebrate DNA remains inactive (34). This is consistent with the fact that aside from the fact that CpG motifs are reduced in frequency in vertebrate DNA, they are also skewed such that the CpGs are usually preceded by a C and/or followed by a G, which can have immune-inhibitory activities (24,35).

These data support the concept that immune recognition of CpG motifs triggers protective pathways analogous to those activated by the PRRs that detect endotoxins and other microbial products. In tissues infected by extracellular bacteria or other organisms with unmethylated genomes, a certain amount of cell lysis will likely occur, which could enable the detection of the CpG motifs. However, recognition of CpG motifs does not occur through a cell surface receptor, but rather requires cell uptake and may involve the binding to an intracellular receptor protein (see section on mechanism of action below). This point suggests that immune recognition of CpG motifs may be specialized for defense against intracellular bacteria, parasites, viruses, and retroviruses whose nucleic acids would be more easily detected by an intracellular receptor than would those of extracellular pathogens. If this hypothesis is true, then the immune system should use the recognition of CpG DNA to trigger the Th1-type immune defenses, which are optimal for defense against such intracellular infections (Table 2). Indeed, as will be reviewed in the following section, CpG DNA is an extraordinarily potent inducer of Th1-type immune responses.

III. CELLULAR IMMUNOLOGY OF CpG DNA

A. B Cells

Optimal CpG sequences are extraordinarily strong mitogens for both murine and human B cells (4,27,28,36). CpG ODN can drive more than 95% of B cells into the cell cycle as compared to only about 80% of all B cells using optimal concentrations of LPS (4). CpG DNA and LPS synergize for induction of B-cell proliferation and cytokine production, providing further evidence that these B-cell mitogens work through distinct molecular pathways (A. K. Yi and A. M. Krieg, LPS and CpG DNA synergize for TNF-α production through activation of NF-κB, submitted, J. Immunol.). Moreover, C3H/HeJ mice, which are insensitive to LPS, show normal responses to CpG DNA (4). Some B-cell mitogens, such as 8-substituted guanines, preferentially affect the large, activated subset of B cells (37,38). However, CpG DNA is essentially equally stimulatory to both

Table 2 Characteristics Distinguishing Th1 Versus Th2 Immune Responses

	Cytokine produced by the T cell	Biological activities of the T cell on other cell types	Associated defense mechanisms	Function
Th1	IFN-γ, TNF	Macrophage activation, B-cell activation to produce opsonizing antibodies	NK, CTL	Promote elimination of intracellular infection
Th2	IL-4, IL-5, IL-10	General B-cell activation to produce antibodies	Eosinophils	Defense against parasites

resting and activated B cells, which rapidly enter the G1 phase of the cell cycle (23).

CpG DNA induces B cells to secrete IL-6 and IL-10 within a few hours (39,40). The IL-6 induction is required for the B cells to proceed to secrete IgM (39). The production of IL-10 in response to CpG DNA appears to function as a counterregulatory mechanism that down-regulates the general Th1-like cytokine response (40). Both in vitro and in vivo, this IL-10 reduces the magnitude and duration of IL-12 secretion (41). This counterregulatory mechanism appears to be responsible for the otherwise paradoxical costimulatory effect of cyclosporin-A on CpG-induced IL-12 production. Cyclosporin blocks the CpG-induced B-cell secretion of IL-10, but does not block the CpG-induced macrophage production of IL-12 (40). As a result, cyclosporin prevents the "off" signal from IL-10, resulting in an approximate doubling of the IL-12 response to CpG in vitro and in vivo (40).

The B-cell response to CpG DNA is enhanced by another cytokine, IFN-γ, which is made by CpG-induced NK cells (42). LPS-induced B-cell secretion of IgM is inhibited by addition of IFN-γ (43). In contrast, CpG-induced B-cell secretion of IL-6 and IgM is more than doubled by the addition of exogenous IFN-γ. This costimulatory interaction also occurs in vivo, since mice genetically deficient in IFN-γ produce less than half of the IL-6 and IgM response to CpG DNA as occurs in wild-type mice (44).

In addition to secreting cytokines and Ig, CpG-activated B cells express increased levels of the Fcγ receptor and costimulatory molecules such as class II MHC, CD80, and CD86 (4,45,46). These effects are not unique to rodent B cells, since human B cells up-regulate not only these costimulatory molecules, but also CD40 and CD54 (27). CpG DNA also activates malignant B cells from patients with chronic lymphocytic leukemia, driving them into the cell cycle, inducing cytokine secretion, and up-regulating surface expression of CD40, CD58, CD80, CD86, CD54, and MHC class I (47).

The signals induced by CpG DNA can either oppose or enhance signals induced by activation of the B-cell antigen receptor. In mature peripheral B cells, low concentrations of CpG DNA strongly synergize with signals through the BCR, leading to an approximate 10-fold increase in B-cell proliferation and antigen-specific Ig secretion (4). CpG DNA also synergizes with the BCR for induction of IL-6 secretion, which is required for CpG-induced IgM secretion (39). This synergy between CpG and the BCR is evident as early as the induction of MKK3, MKK4, and MKK6. In murine B cells exposed to both of these stimuli, there is the synergistic induction of JNK, but not ERK (48; A. K. Yi, A. M. Krieg, in preparation).

In contrast to the synergy between CpG and the BCR in mature B cells, CpG DNA can oppose B-cell apoptosis that is triggered by crosslinking of the BCR in certain B-cell lines such as WEHI-231 (49,50). WEHI-231 B cells have

an immature phenotype and are often used as a model for immature B cells that are induced to undergo apoptosis by BCR crosslinking (51) although differences in the signaling pathway used by primary immature B cells and WEHI-231 B cells have been reported (52). In any case, BCR crosslinking in these B cells leads to decreased c-*myc* levels and NF κB activity unless the B cells are rescued by second signals such as LPS or CD40L (53–57). CpG DNA also acts as a second signal to protect WEHI-231 B cells against BCR-induced apoptosis through a mechanism that is associated with the maintenance of NFκB p50/c-Rel heterodimer levels (58,59).

The antiapoptotic effect of CpG DNA on WEHI-231 cells has recently been extended to another murine B cell lymphoma, BKS-2 (60). These investigators demonstrated a similar association of apoptosis protection with NFκB induction. In addition, CpG DNA is shown to induce expression of *egr*-1 mRNA in BKS-2 cells, while an antisense ODN against *egr*-1 blocks the CpG-induced protection against apoptosis (60). Although this experiment was interpreted to suggest a role for *egr*-1 in mediating the protective effect of CpG DNA, there is a potential complication. The antisense sequence used for *egr*-1 contained several poly-G motifs. Since poly-G motifs can oppose the immune stimulatory effects of CpG ODN (25), and since these motifs can also have direct antiproliferative activities on cell lines (61,62), it remains possible that the observed effect of these ODNs may not be due to an antisense mechanism of action.

Mature primary B cells undergo spontaneous apoptosis in tissue culture unless they are rescued by growth factors or other mitogens (63,64). CpG DNA also prevents this spontaneous apoptosis and maintains NFκB expression (26). This protective effect is associated with increased levels of c-*myc*, *egr*-1, c-*Jun*, bcl_{XL}, and *bax* mRNA levels. Inhibition of protein synthesis only partly reduced the apoptosis protection. CpG DNA prevents the normal reduction of the mitochondrial membrane potential, which is associated with spontaneous apoptosis of primary B cells (65). CpG DNA has also been reported to protect B cells against Fas-mediated apoptosis by down-regulating Fas expression on B cells stimulated through CD40 (66).

B. Monocytes, Macrophages, and Dendritic Cells

CpG DNA now appears to be a potent signal for the activation of professional antigen-presenting cells (APCs), which link the innate immune system to the adaptive immune system through their PRRs (5). CpG DNA causes the activation of NFκB in macrophages (67) and initiation of cytokine expression including TNF-α, and in dendritic cells also IL-12 and IL-6 (68,69). CpG DNA also induces dendritic cells to have increased surface expression of MHC class II and the costimulatory molecules CD40, CD80, and CD86 while down-regulating expression of E-cadherin adhesion molecules (68,69). These stimulatory effects of CpG

DNA enhance the ability of the DC to activate allogeneic T cells (68,69). Intracutaneous administration of CpG DNA also leads to in vivo activation of skin Langerhans cells to up-regulate costimulatory molecules and produce IL-12 (68). Interestingly, these direct effects of CpG DNA differ somewhat from LPS in that while LPS induces murine DCs to make large amounts of TNF-α and relatively low amounts of IL-12, CpG DNA activates the opposite pattern of cytokine secretion with lower levels of TNF but higher levels of IL-12 (68). Although CpG DNA can have direct effects on APCs in the absence of other cell types or cytokines, some of the effects of CpG DNA require priming with IFN-γ. For example, macrophage expression of the inducible nitric oxide synthase and production of nitric oxide in response to CpG DNA require IFN-γ priming (70). In addition, CpG DNA can synergize with IFN-γ in driving expression from an HIV-1 long-terminal-repeat reporter construct (70).

The macrophage-like cell line RAW 264 has been used to better understand the mechanism of the IL-12 secretion induced by CpG DNA. Both CpG DNA and LPS activate an IL-12 p40 promoter-reporter construct, and this response is increased two- to fivefold by IFN-γ (71). In this cell line, both LPS and CpG DNA induce similar NFκB activation, although only CpG is a strong activator of IL-12 p40 transcription. Human monocytes respond to both CpG DNA and LPS by secreting TNF and IL-6 and by up-regulating cell surface expression of the adhesion molecule ICAM-1. LPS induces the same activation response, but with quite different kinetics from that induced by CpG DNA. LPS-induced TNF and IL-6 production can be detected by intracellular staining using flow cytometry as early as 4 h (72). In contrast, CpG DNA-stimulated TNF and IL-6 synthesis in human monocytes is not detectable until 18 h. At low concentrations, CpG DNA and LPS show synergy for inducing monocyte cytokine production (72). Even without CpG motifs, the PS ODN backbone can enhance LPS-stimulated TNF production (73).

CpG DNA appears to have generally similar effects on both bone-marrow-derived DCs (69) and skin-derived DCs (68). The fact that the pattern of cytokines produced by DCs in response to CpG DNA is dominated by IL-12 suggests that CpG may be a potent trigger driving DCs to promote Th1-like immune responses. In recent studies, we have demonstrated that CpG DNA is also a potent trigger for the activation of human primary blood DCs. For primary dendritic precursor cells isolated from human blood using magnetic beads, CpG ODN alone were superior to GMCSF in promoting DC survival in tissue culture (74). Thus, CpG appears fully able to substitute for the cytokine requirement for these primary DCs. Moreover, CpG ODN also drove maturation of the primary human DCs as measured by their expression of CD83, class II MHC, and the costimulatory molecules CD40, CD54, and CD86 (74).

Primary human DCs have been divided into the DC1 subset, distinguished by its expression of DC11c, and the DC2 subset, distinguished by its expression

of the IL-3 receptor, CD123. CpG DNA strongly activates the maturation of the DC2 subset, while LPS only activates the DC1 subset, with no detectable effect on the DC2 subset (G. Hartmann, A. Krug, S. Blackwell, J. Vollmer, and A. M. Krieg, submitted, J. Immunol.). Compared with GMCSF, CpG-treated peripheral blood DCs showed enhanced ability to drive T-cell proliferation in an allogeneic mixed-lymphocyte reaction (74).

Another commonly studied type of DC is that which is derived from human peripheral blood monocytes, after culture for approximately 5 days in GMCSF + IL-4. CpG DNA does not appear to activate the expression of costimulatory molecules on these DCs, although it does appear to stimulate the expression of IL-12 and IL-18 mRNA (75). Intriguingly, the addition of CpG DNA to PBMCs from allergic donors results in decreased in vitro production of IgE, suggesting potential clinical utility for CpG DNA in the immunotherapy of allergic diseases (75).

Not all of the effects of CpG DNA on APCs appear to promote antigen processing and presentation. In fact, recent studies indicate that CpG ODN can down-regulate macrophage class II MHC antigen processing and presentation (76,77). In these studies, peritoneal macrophages were cultured for 18 h with CpG ODN and then pulsed with protein antigens. These macrophages had a greatly reduced ability to present specific peptide:MHC-II complexes to T-cell hybridomas and also had decreased levels of cell-surface MHC-II expression but had intact endocytosis (76). Macrophages exposed to CpG DNA for just 2 h had no significant change in their antigen processing and presentation characteristics in these studies. CpG DNA also induces human T cells to produce a heat-labile factor that inhibits macrophage differentiation and adherence (78).

C. Natural Killer Cells

Since the initial observation that mycobacterial DNA activates murine NK cells (79), many additional studies have confirmed and extended this finding, leading to the conclusion that palindromic sequences in mycobacterial DNA were responsible for this NK activation as reviewed above (9–11,80–83). These palindromes were also reported to activate human cells (84). More recent studies have demonstrated that palindromes are not required for this stimulatory activity, but simply CpG motifs in appropriate base contexts, which can include palindromes (25).

In addition to having increased lytic activity, these studies have repeatedly demonstrated IFN-γ production from CpG-stimulated NK cells. In fact, the major source of the initial IFN-γ production in CpG-stimulated murine spleen cells appears to be NK cells (42). The stimulatory effects of CpG DNA on murine NK cells are not direct, but require either the presence of adherent cells or cytokines such as IL-12, TNF-α, or type I interferons, which are derived from adherent APCs (25,42). Thus, CpG DNA appears to act as a costimulatory signal for murine NK cells.

In contrast to this costimulatory effect on *murine* NK cells, CpG motifs have been reported to directly activate highly purified *human* NK cells to produce IFN-γ, to express increased levels of CD69, and to have enhanced cytotoxic activity (29). Nevertheless, this CpG response was enhanced by costimulation of the NK cells with IL-2, IL-12, or anti-CD16 antibodies. The stimulation of human NK cells by these PO CpG ODN appeared to require the presence of palindromes, since stimulation was dramatically reduced if the base sequence of the ODN was changed so as to eliminate the potential to form these secondary structures (29). Although such a finding is consistent with a requirement of the NK cells for a palindrome sequence, it is also possible that the increased effect of palindrome-containing ODNs may be an artifact resulting from the slower degradation of ODNs containing such secondary structures (85).

D. T Cells

Like NK cells, CpG DNA generally has not been reported to have direct stimulatory effects on resting T cells (86,87). An initial report suggesting that CpG DNA directly stimulated cytokine secretion from T cells (88) now appears to have drawn a wrong conclusion because of the use of inadequately purified cell populations. All other investigators who have studied the issue have concluded that CpG DNA does not directly stimulate resting T cells (89). On the other hand, in murine mixed-cell populations, the type I interferons produced by CpG-stimulated adherent cells (presumably dendritic cells and macrophages) stimulate T cells to produce some activation and costimulatory molecules, but also inhibit their proliferative response to TCR ligation (86). However, if T cells are highly purified away from adherent cells, then CpG DNA was initially reported by Wagner and colleagues to synergistically enhance the proliferative response of murine T cells to TCR ligation (90). These costimulatory effects were also observed in T cells from mice lacking CD28, raising the possibility that CpG DNA may substitute for normal T-cell costimulatory signals (90). Interestingly, in contrast to the activation of other cell types by CpG DNA, T-cell activation was reported by these investigators to be resistant to chloroquine inhibition. However, recent studies by the same investigators have led to a reinterpretation of these experimental results. The murine cell costimulatory effect is now reported to be due not to CpG motifs, but rather to G-tetrads present in the ODN studied (91).

The question of the possible costimulatory effects of various ODN on T cells has recently become even more complicated. Iho et al. have recently demonstrated that PO ODN containing certain CpG motifs directly stimulate purified human T cells to proliferate, as long as the T cells are activated through the TCR with a highly crosslinking stimulus such as magnetic beads coated with anti-CD3 (29). This response did not require the presence of IL-2. Surprisingly, some sequences that were not strong stimulators of human NK cells, such as the motifs

CGGCCG, GCGCGC, and TCGCGA, were quite effective at coactivating T cells to secrete IFN-γ (29). This suggests that NK and T cells may detect different subsets of CpG motifs and may express different CpG receptors. Stimulation of these human T cells was abolished if the cytosine in the CpG motif was replaced by 5-methyl cytosine. PO ODN containing poly-G sequences did not show any T-cell costimulatory properties in these studies with human T cells, raising the question of whether the costimulatory effect observed by Wagner and colleagues is limited to murine T cells or to ODN with a phosphorothioate backbone. Further studies will be required to clarify this issue.

IV. MECHANISMS OF ACTION OF CpG DNA

A. Cellular Binding

The identification of potential cell-surface receptors for CpG DNA has been a matter of great interest. Although ODN binding to immune cell membranes can easily be shown, neither we nor other investigators have been able to detect any sequence specificity in the cell-surface binding of ODN; stimulatory CpG ODN and nonstimulatory ODN bind equally well to cell membranes (4,83). Our initial studies in murine cells indicated that CpG ODN immobilized on a solid support could not activate lymphocytes, which indicated that cell uptake was required (4). The only exception to this is that ODN containing G quartets show enhanced binding to lipid bilayers and cells (92,93). In contrast, other investigators have reported that human B cells are stimulated by CpG ODN immobilized on sepharose beads (36). Although these experiments appeared to suggest that CpG ODN may work through a cell-surface receptor, Manzel and MacFarlane have recently found that ODN coupled to sepharose beads can still be taken up by cells in tissue culture, leaving open the possibility that CpG ODN may still work through an intracellular signaling pathway rather than a cell-surface receptor (94). These latter investigators went on to show that CpG ODN that were linked to latex, magnetic, or gold beads could not be taken up and lost their stimulatory activity. Moreover, lipofection of ODN into spleen cells enhances their immune stimulatory effects and makes it possible for very short ODN, which normally are nonstimulatory, to exert a CpG sequence-dependent stimulatory effect (83). Thus, it seems likely that cell uptake is required for the immune stimulatory effects of CpG DNA.

B. Cellular Uptake

As in other cell types, the mechanism of ODN uptake in lymphocytes remains poorly understood. In living lymphocytes, endosomal localization of ODN is usu-

ally most prominent (15,20,95). As with antisense ODN, it is quite possible that the rate-limiting step in triggering immune stimulation is the exit of the CpG ODN from the endosomes. Indeed, measures to enhance the endosomal release of CpG ODN such as delivery with cationic liposomes or conjugation to cholesterol enhances the immune stimulatory effect (20,83). In lymphocytes, ODN uptake is highly inducible by mitogens (96,97). ODN uptake in malignant cells and cell lines is typically higher than that in primary lymphocytes (97).

The rates of ODN uptake differ dramatically between different immune-cell subpopulations. For example, T cells generally have a low rate of ODN uptake compared to B cells or monocytic cells (96–99). However, uptake also appears to be regulated depending on the stage of cell differentiation, at least in B cells (100). Dead or apoptotic cells have extremely high levels of ODN, which are intranuclear and can confound the interpretation of ODN uptake experiments (15).

C. Requirement for Endosomal Acidification/Maturation

One of the unanswered questions in the intracellular trafficking of ODNs is how they are apparently able to exit the endosomes. It is possible that this has something to do with endosomal acidification or other aspects of the intracellular endosomal maturation. To address this possibility, we tested whether monensin, chloroquine, and bafilomycin A, which interfere with endosomal acidification and/or maturation, would affect CpG-induced activation. Surprisingly, these compounds completely blocked the ability of CpG DNA to activate B cells or monocytic cells at low concentrations that did not inhibit stimulation by LPS, anti-CD40, crosslinking of the B-cell-antigen receptor, or by phorbol 12-myristate 13-acetate (101). Other compounds structurally related to chloroquine, such as quinacrine, are even more potent antagonists of CpG-induced immune stimulation (102,103). These compounds appear to act at an extremely early step in the CpG-induced signaling pathway, since their inhibitory effects are already apparent by 5 min, and since they block all of the signaling pathways yet known to be induced by CpG (48,101,104).

D. Activation of the Mitogen-Activated Protein Kinase Pathways

Mitogen-activated protein kinases (MAPKs) are common pathways that have an important role in leukocyte responses to diverse stimuli. Three MAPK pathways can be distinguished: the extracellular receptor kinase pathway (ERK), the p38 MAPK pathway, and the c-Jun NH_2-terminal kinase or JNK pathway. B cells activated by CpG DNA show activation of both the p38 and JNK pathways within 7 min (48). In comparison to B-cell activation by CD40 ligation, CpG DNA

showed a slightly slower onset of MAPK phosphorylation, but a longer duration of activation. Interestingly, the JNK isoforms phosphorylated in response to CpG DNA had apparently identical molecular weights to those phosphorylated in response to CD40 ligation, but were different from those seen in B cells activated through the BCR (48). Human B cells activated by CpG DNA also show activation of the p38 and JNK MAPKs, but not ERK (27). The p38 pathway appears to be required for CpG-induced B-cell cytokine secretion, since this is completely blocked by pretreatment of the cells with a p38 inhibitor (48).

The p38 and JNK pathways also are rapidly activated in macrophages following exposure to CpG DNA (104). Of note, CpG also activates the ERK pathway in primary macrophages and macrophage cell lines, which contributes to CpG-induced TNF production (105). In macrophages, this CpG-induced ERK activity has a negative feedback effect on the IL-12 p40 promoter, resulting in decreased release of IL-12. In CpG-induced DCs, no ERK induction was detectable. To test the hypothesis that ERK activation may have a negative feedback effect on CpG-induced IL-12 synthesis, Hacker et al. chemically activated the ERK pathway in CpG-induced DCs. This resulted in suppressed production of IL-12 (105). These studies suggest that the activation of p38 and JNK MAPKs by CpG DNA contributes to IL-12 production, but that ERK activation can exert a negative feedback effect.

E. Activation of Nuclear Factor κB

Nuclear factor κB (NFκB) is rapidly up-regulated in leukocytes that have been exposed to a broad range of stimuli, and is thought to have a critical role in regulating inflammatory responses (106,107). Given the impressive proinflammatory effects of CpG DNA, it may be expected that it should trigger NFκB induction. Indeed, even prior to the discovery of the CpG motif, McIntyre et al. reported the unexpected observation that in an intended antisense experiment, a control sense ODN to the p65 subunit of NFκB caused a dramatic up-regulation of NFκB activity in murine splenic B cells (17). We later realized that this immune stimulatory ODN contained a CpG motif, and found that this motif was responsible for the observed up-regulation of NFκB activity (A. M. Krieg, R. Narayanan, unpublished data). Further studies have demonstrated that CpG DNA activates NFκB in both macrophages and B cells (59,67). This NFκB activation was associated with the degradation of IκBα and IκBβ (59). In B cells, the dominant form of NFκB induced by CpG DNA appears to be a p50/c-Rel heterodimer, while in macrophages it appears to be a p50/p65 heterodimer (59,108). This NFκB activation results in enhanced transcriptional activity from the human immunodeficiency virus long-terminal repeat (67), and is required for the CpG-induced protection of B cells against apoptosis induced by BCR ligation (59), and for the protection of primary B cells from spontaneous apoptosis in tissue

culture (65). CpG-induced NFκB activation is also seen in human primary B cells (27).

The mechanism through which CpG DNA induces NFκB is unclear. One possibility is related to the production of reactive oxygen species (ROS), which is increased within 5 min of CpG DNA treatment (39). NFκB activation is known to be highly dependent on the redox state of the cell (109,110) suggesting a possible link to the CpG-induced oxidative burst. CpG-induced NFκB activation is blocked by antioxidants, consistent with this hypothesis (59,101).

F. Activation of Transcription and Translation

The MAPK pathways that are activated by CpG DNA have been reported in other systems to lead to the activation of multiple transcription factors, including ATF-1, ATF-2, the cyclic AMP response element binding protein (CREB), Elk-1, Max, and c-Jun (111–113). Some of these factors have already been shown to become phosphorylated and activated in response to CpG DNA treatment of B cells and/or macrophages (48,104). Like NFκB, which is also activated by CpG DNA and B-cell types, these transcription factors are important regulators for the expression of many cellular protooncogenes and proinflammatory cytokines. CpG DNA also induces increased mRNA levels of several other transcription factors, including c-*myc*, Ets-2, C/EBP-β and -δ (101,114). Cooperation between transcription factors of different classes is thought to be an important mechanism in the coordinate regulation of gene expression in response to different stimuli.

So far there have been no detailed analyses of promoter or enhancer function in CpG-stimulated cells to identify the binding sites responsible for these transcriptional responses. However, promoter activity for IL-6 and HIV has been shown to be induced by CpG DNA in macrophages and B cells (39,114). This transcriptional induction appears to be extremely rapid, with increased levels of mRNA within 15 min (26). Among the genes whose RNA expression is increased in B cells are myc, myn, EGR-1, Jun, Bcl-2, Bcl-x_L, IL-6, IL-10, and IL-12 (26,39,40,58,59). Interestingly, although the overall effect of CpG DNA is the strong promotion of Th1-like immune responses, the B-cell production of IL-10 acts to reduce the level of IL-12 secretion that is induced by CpG DNA (40).

CpG DNA also has potent transcription-activating effects on macrophages, leading to the increased transcription of TNF-α, IL-1β, plasminogen activator inhibitor-2, IL-6, IL-12, Type 1 interferons, and several costimulatory and antigen-presenting molecules such as class II MHC, CD80, CD86, and CD40 (41,67,68,86,108,115). As reviewed above, NK cells are induced by CpG DNA to produce IFN-γ. Other cytokines whose expression is induced by CpG DNA, but for whom the cellular sources have not yet been conclusively determined, include IL-1RA, MIP-1β, MCP-1, and IL-18 (116–118).

G. Influence of the ODN Backbone on the Immune Effects of CpG DNA

The strongest effects of CpG DNA on B cells are seen with the use of ODN synthesized with a nuclease-resistant phosphorothioate backbone (PS) (4,119,120). In fact, PS CpG ODN are approximately 200 times more potent for activating murine B-cell proliferation than the corresponding PO CpG ODN (4). The susceptibility of phosphodiester ODN to degradation not only reduces their ability to drive B-cell proliferation, but can even result in an artifact in studies using a ^{3}H thymidine incorporation assay to measure B-cell proliferation. In such assays, phosphodiester DNA can be degraded, releasing free thymidine, which competes for the ^{3}H thymidine and causes a false suppression of incorporation (121). This nonspecific effect is most marked if thymidine nucleotides are present at the 3′ end of an ODN, but can also be observed with high-molecular-weight DNA such as bacterial genomic DNA. Certain exonuclease activities are specific to B lymphocytes, indicating that results obtained using phosphodiester DNA in one cell type cannot necessarily be extrapolated to other cell types (122). Moreover, nuclease activities appear to be higher in human than in mouse cells, with the result that phosphodiester DNA can appear nonstimulatory unless it is added repeatedly (74). This may explain why some investigators have mistaken concluded that human cells do not respond to phosphodiester ODN containing CpG motifs (36).

Interestingly, the backbone dependence of the NK cell effects of CpG DNA is quite different from that for activation of B cells or APCs. The use of the phosphorothioate backbone dramatically increased the stimulatory activity of CpG DNA for activating B cells and APCs. While phosphorothioate backbone CpG DNA can activate NK cells, the magnitude of this activation is often much less than that with phosphodiester backbone DNA. Furthermore, the stimulatory effect of a CpG motif synthesized with phosphodiester DNA can even be abolished when the sequence is synthesized with the phosphorothioate backbone (25,123).

The phosphorothioate ODN backbone changes the biological properties of the ODN compared to normal phosphodiester DNA. The nonspecific binding to a wide variety of proteins is dramatically increased (124,125). Phosphorothioate ODN bind much more avidly to cell membranes, and generally have a much higher degree of cell uptake (15,100,126). The phosphorothioate backbone may have certain sequence-independent immune-stimulatory activities such as the activation of SP1 transcription factor activity (127), inhibition of smooth muscle cell proliferation and migration (62,128), inhibition of basic fibroblast growth factor binding to its receptor (129,130), reduction of the sequence-specific binding of transcription factors to their binding sites (131), inhibition of cellular adhesion to extracellular matrix (132), enhancement of LPS-induced tumor necrosis

factor production (73), and some degree of non-sequence-specific immune stimulation (14).

There are some differences in the immune stimulatory effects of CpG motifs in PS ODN compared to PO ODN. Although the optimal CpG motif for driving murine B-cell proliferation has the formula purine-purine-CG-pyrimidine-pyrimidine, PO ODN bearing suboptimal CpG motifs, such as those in which the CG is preceded by a C or followed by a G, can still drive B-cell proliferation if the ODN concentration is increased (4,26). In fact, even PO ODN in which the CpG motif is methylated retain a limited ability to drive B-cell proliferation that is significantly greater than that present in ODN where the CpG motif has been inverted to a GpC (26). In contrast, apart from the nonspecific effects of the PS backbone, PS ODN bearing suboptimal CpG motifs are less likely to drive high levels of B-cell proliferation and especially Ig secretion, particularly if the CG is followed by a G. PS ODN without CG motifs are also frequently observed to drive the proliferation of murine and human B-cells, although to a more limited degree than that which occurs with CpG ODN (26,36). However, this broad B-cell-stimulatory activity of the PS ODN backbone shows some sequence dependence, as PS ODN with certain types of CpG motifs actually exert a neutralizing activity that diminishes the proliferative response to a stimulatory CpG motif (24). PS ODN comprised solely of CG dinucleotides or those in which the CGs are preceded by a C and/or followed by a G appear to be the most potent neutralizing motifs.

V. THERAPEUTIC APPLICATIONS OF CpG DNA

A. Activation of Innate Immune Defenses Against Infection

The critical role of the innate immune defenses in protecting against infectious agents has been well established for more than a century. Some of the innate immune defenses, such as the complement system, are constitutively expressed and do not require induction. However, many other arms of the innate immune defenses do not appear to be constitutively activated, but are induced upon encounter with infectious agents. For example, activation of front-line innate immune cells leads to the production of IL-6, which in turn triggers the production of acute-phase reactants as well as multiple other protective mechanisms. Previous studies have demonstrated that inactivated bacteria or bacterial products can trigger protective innate immune responses that increase nonspecific resistance to infection (133–135). Presumably, these defensive responses are triggered by host pattern recognition receptors (PRRs), which recognize foreign molecular structures and trigger defensive responses. Unfortunately, most of the nonspecific immune activators previously described were not very well characterized or caused

unacceptable toxicities that would preclude clinical development. Since CpG DNA is a single well-defined molecular entity, and since immune recognition is hypothesized to have evolved as a defense mechanism, it seemed possible that this may be free of some of the undesirable toxic activities associated with previous immune modulators. To investigate this possibility, we tested a model in which BALB/c mice are challenged with a lethal dose of 10^5 colony-forming units (approximately 10 LD_{50}) of *Listeria monocytogenes*, an infection to which these mice are normally highly susceptible. BALB/c mice that were pretreated with as little as 0.3 μg of either bacterial DNA or synthetic CpG ODN were protected against the infectious challenge (136). However, the mice had to be pretreated to be protected against the infectious challenge: mice that were given the CpG DNA at the same time as the challenge or only 24 h before the challenge were not protected, but mice given the CpG at least 48 h prior to the lethal challenge were fully protected. The CpG-induced protection continued for up to 1 month. Protection required the presence of IFN-γ, since mice genetically deficient in IFN-γ were not protected by CpG. The IFN-γ-deficient mice had a normal acute induction of serum IL-12 in response to CpG treatment, but in contrast to the sustained elevated IL-12 levels that were detected in control mice, IL-12 levels in the deficient mice returned to baseline within less than 12 h (136). This suggests that the induction of IFN-γ expression by CpG DNA contributes to the maintenance of IL-12 expression. These results that CpG DNA induces a state of nonspecific resistance to infectious challenge have since been confirmed and extended to another intracellular bacterium, *Francisella tularensis* (137,138). Protection was at least partly dependent on the presence of B cells, and it led to the development of memory responses since animals surviving lethal challenge were protected against later rechallenge. Mice treated with CpG DNA are also protected against challenge with *Leishmania major* through IFN-γ, nitric oxide, and IL-12-dependent mechanisms (139–142). In contrast to the requirement for pretreatment with CpG DNA in the *L. monocytogenes* model, CpG ODN could cure *L. major*–infected BALB/c mice even when administered as late as 15 days after infection.

CpG DNA is a potent inducer of IL-12 production, as reviewed above. IL-12 administration can protect mice against challenge with malaria sporozoites (143). Therefore, it seemed possible that CpG DNA may also provide protection against malaria sporozoite challenge. Indeed, mice treated with CpG DNA were completely resistant to malaria sporozoite challenge within 48 h, although protection was reduced if the challenge occurred a week or more later (144). In contrast to the results with bacterial challenges, mice could be treated with CpG DNA on the day of challenge without loss of protection. Protection was abolished by coadministration of neutralizing antibodies against IL-12 or IFN-γ. CpG DNA treatment also protects mice against challenge with Ebola virus or anthrax (145),

cytomegalovirus, or herpes simplex virus (P. Dunford, personal communication) or the fungus *Coccidioides immitis* (K. Li, personal communication).

In addition to providing nonspecific protection against infectious challenge, CpG ODN have a profound effect on hematopoietic function. Even before the identification of the CpG motif, several investigators using antisense ODN noted the induction of sequence-specific extramedullary hematopoiesis and induction of hematopoietic colony formation (17,146). More recently, these effects have been shown to be CpG specific (147). Mice treated with immune-stimulatory phosphorothioate CpG ODN develop massive splenomegaly and increased spleen granulocyte-macrophage colony-forming units (GM-CFUs) and early erythroid progenitors. CpG had a radioprotective effect in accelerating the recovery of GM-CFUs and enhancing resistance to *Listeria* infection in irradiated mice.

It is noteworthy that, although CpG DNA can protect mice against challenge with numerous intracellular viruses, retroviruses, and parasites, it typically does not protect against challenge with extracellular bacteria. We propose that immune activation by CpG DNA has evolved as a defense mechanism specific to such intracellular pathogens. The activity of CpG DNA in stimulating potent Th1-like immune activation is consistent with the known role of this type of immune response in combating infection by intracellular pathogens. This hypothesis would also explain why the receptor for CpG DNA should be an intracellular molecule instead of a cell-surface molecule. As an intracellular molecule, the CpG receptor would be best positioned to detect the entry of foreign DNA into the cell.

B. Role of CpG DNA as a Vaccine Adjuvant and in DNA Vaccines

Several features of the immune stimulation induced by CpG DNA suggest potential efficacy as a vaccine adjuvant. Although CpG DNA can activate essentially any B cell without regard to its antigen specificity, the synergy observed in B-cell activation through CpG and the BCR suggests that antigen-specific B cells will be preferentially activated. Second, the induction of increased costimulatory molecule expression on B cells and other APCs suggests that these should be more effective at promoting antigen-specific immune responses. The antiapoptotic activity of CpG DNA (58,59) may also help to potentiate sustained B-cell responses. Finally, the generation of a Th1-like cytokine milieu by CpG DNA should promote the generation of these desirable immune responses. The utility of CpG DNA as a vaccine adjuvant has been confirmed in studies using model antigens such as hen egg lysozyme (148) or ovalbumin (87), or heterologous gammaglobulin (149) and β-galactosidase (117). Remarkably, these studies all confirmed that CpG DNA is a stronger Th1-like adjuvant for inducing B-cell and

T-cell responses than the "gold standard," complete Freund's adjuvant. Moreover, CpG DNA accomplished this level of antigen-specific activation without inducing any of the harsh local inflammatory effects seen with Freund's. In addition, coadministration of CpG ODN prevented the induction of IgE responses to allergens, reducing the risk of allergy.

CpG DNA has also been shown to be a very potent adjuvant for inducing responses to infectious-disease antigens including the hepatitis-B surface antigen (45,150), influenza (151), and HIV gp16 (152). CpG DNA is also an effective adjuvant for tumor vaccination and can induce protective immunity against an otherwise lethal tumor challenge (33,153). In therapeutic tumor vaccination models, CpG DNA has shown potent synergistic activity with GMCSF (154), which is consistent with the strong synergy that these two immune activators show in in vitro experiments with murine or human DCs (74). The adjuvant effects of CpG DNA are not limited to mice. CpG ODN adjuvants have enhanced antibody responses against peptide sequences derived from the circumsporozoite protein from *P. falciparum* in a mineral oil emulsion (155) and for a hepatitis B vaccine in orangutans, which are otherwise hyporesponders to the hepatitis-B surface antigen (156). An interesting insight into the evolutionary history of immune recognition of CpG DNA is provided by the finding that fish not only respond to CpG DNA, but may recognize different CpG motifs than do mice (30). This result may be important in the further development of improved vaccination strategies for fish (157).

Polysaccharide antigens represent a special problem in vaccination. Because they cannot be presented in the groove of the MHC molecules, polysaccharides can only activate B cells through crosslinking the surface receptor, which is inefficient at triggering isotype switching. Although high levels of IgM antibodies can be induced by polysaccharide vaccines, these are generally relatively short-lived and are less efficient at fixing complement than some IgG isotypes. Moreover, polysaccharide antigens tend to be very weakly or nonantigenic in the very young and very old populations, which are most at risk of infection. An early report concluded that CpG DNA was an ineffective adjuvant for a polysaccharide vaccine (158). However, these investigators only measured the IgM response, and used an extremely high dose of CpG ODN, 500 μg. This dose of CpG ODN is nearly two orders of magnitude higher than the dose at which the antibody response plateaus, and is well into the dose at which severe toxicity can occur. In our own studies with immunizing mice against a model polysaccharide, TNP-ficoll, we have observed CpG to be a very effective adjuvant at doses of approximately 10–30 μg per mouse (T. J. Waldschmidt and A. M. Krieg, manuscript in preparation). Notably, CpG DNA does not have a great effect on the serum level of IgM antibodies, but causes profound isotype switching with strong induction of IgG3 and other isotypes. Importantly, the adjuvant effect of CpG ODN for

polysaccharide antigens is also prominent in neonatal and very old mice (2 years old) and can be demonstrated in vitro (159).

Because many pathogens enter the body through the mucosal surfaces, there is a great deal of interest in effective adjuvants for mucosal immunization. Although effective mucosal adjuvants such as cholera toxin and the heat-labile enterotoxin have been identified, these are too toxic for human use and require mutagenesis to reduce their severe adverse effects. It is therefore noteworthy that CpG DNA is an extremely effective adjuvant for mucosal immunization, approximately as effective as these other very toxic adjuvants, and yet is extremely well tolerated (151,160,161). Given mucosally, CpG ODN still induces Th1 immune responses at both systemic and mucosal immune surfaces, and also drives strong mucosal IgA responses.

Inducing immunity during the neonatal period is extremely important from the public health standpoint, but extremely difficult to accomplish efficiently. Even with repeated vaccination, immune responses are generally modest in neonatal mice or humans. Moreover, the neonatal immune system tends to be skewed more toward the generation of Th2 responses rather than the more desirable Th1 responses. It is therefore noteworthy that CpG DNA is a highly effective Th1 adjuvant in neonatal mice using the hepatitis-B surface antigen (162,163).

In recent years, there has been a great deal of interest and excitement about the potential for genetic vaccines as an exciting and remarkably simple approach to the generation of antigen-specific immune responses (reviewed in Ref. 164). These so-called ''DNA vaccines'' are simply plasmids that contain a eukaryotic promoter such as the CMV promoter driving expression of a cDNA encoding the antigen of interest. Since these plasmids are grown in bacteria, they will of course be unmethylated. The average plasmid contains several hundred CpG motifs, and recent studies have reported that these motifs are required for the function of the DNA vaccine (165–168). One group has reported that simply cloning an extra two stimulatory CpG motifs into a plasmid is sufficient to dramatically enhance its efficacy (166), but other groups have not reproduced this result (169,170). It would certainly be unexpected for minor changes in the CpG content of a DNA vaccine to alter its efficacy, considering the large number of other stimulatory CpG motifs already present in the plasmid. On the other hand, the amount of CpG DNA in a DNA vaccine seems to be somewhat limiting since addition of *E. coli* DNA or vector DNA to a DNA vaccine dramatically enhances its efficacy in mice (171,172) and primates (173). We have demonstrated that more extensive mutagenesis of plasmids, affecting scores of CpG motifs, can significantly enhance the ability of a DNA vaccine to induce antigen-specific B- and T-cell responses (24). However, addition of too many CpG motifs to a plasmid (50 or more) suppresses the humoral response, possibly because CpG-induced cytokines suppress vector expression (24). Although some investigators

have simply added CpG ODN to DNA vaccines, this approach is generally unsuccessful because of the dose-dependent interference of the phosphorothioate ODN backbone with the uptake and expression of the plasmid (174,175). Changes in the poly-G content of plasmids have not changed their immune stimulatory effects (176). Some investigators have found that addition of just two immune stimulatory CpG motifs to a DNA vaccine can enhance its ability to induce an antigen-specific immune response (166), but most investigators who have examined this issue have found that minor changes in the number of CpG motifs in a plasmid do not effect its function (169,170). In summary, the available data suggest that a DNA vaccine has two essential components: the cDNA insert containing the antigen to which the immune response is to be directed, and the CpG motifs in the plasmid backbone (156,177). In vivo injection of DNA vaccines transfects only a very small fraction of APC to express the encoded antigen, but the CpG motifs in the vaccine activate a large majority of the DCs in the draining lymph nodes (178).

C. Cancer Immunotherapeutic Activities of CpG DNA

1. CpG DNA as an Adjuvant for Cancer Vaccines

Considering the remarkable efficacy of CpG DNA as a Th1-like adjuvant for infectious disease vaccines, it should come as no surprise that CpG DNA is also extremely effective as an adjuvant for cancer vaccines. The first demonstration of the efficacy of CpG DNA as an adjuvant for a tumor antigen was accidental. In studies of *Drosophila* cells that were engineered to function as APCs and present tumor antigens, Sprent and colleagues made the surprising observation that untransfected *Drosophila* cells were highly stimulatory to murine B cells, which up-regulated the expression of costimulatory molecules and could provide bystander costimulation for $CD8^+$T cells (33). They further showed that the *Drosophila* DNA is unmethylated and immune-stimulatory, but loses this property after treatment with CpG methylase. *Drosophila* cells transfected to express a tumor antigen in the context of the appropriate class I MHC could induce MHC-compatible spleen cells to respond to a tumor antigen in vitro, and to mediate tumor rejection in vivo (33). These investigators also confirmed that CpG ODN are more potent adjuvants than CFA for driving Th1-like immune responses (149).

We have evaluated the antitumor adjuvant properties of CpG DNA in the 38C13 murine B-cell lymphoma model that was originally developed by Levy and colleagues (179). In this model, the idiotype (Id) of the 38C13 surface IgM serves as a highly specific tumor-associated antigen, and can be used to immunize syngeneic mice against lethal tumor challenge. For improved antigenicity, the Id is conjugated to keyhole limpet hemocyanin (KLH). In comparing a CpG ODN

to complete Freund's adjuvant (CFA) for immunizing against this Id, we found that both CpG ODN and CFA gave similar high levels of Id-specific antibody (153). The CpG ODN was highly effective when used to immunize via the intradermal or subcutaneous routes, but was slightly less effective through the intraperitoneal route. Although a dose of 25 μg of the ODN was highly effective, a maximal response was seen at doses of 50–100 μg. As might be expected considering the mechanism of action of CpG ODN, optimal antibody responses were seen when the ODN and the Id were injected on the same flank of the mouse, rather than on opposite flanks. Control mice challenged with a lethal dose of tumor cells all died within 1 month of challenge, but mice immunized together with CFA or CpG ODN had prolonged survival including 20 or 40% long-term survival, respectively (153). This slightly higher efficacy of the CpG adjuvant compared to CFA may be related to the fact that CpG induced more than twice as much of the IgG 2A anti-Id isotype while CFA induced higher levels of IgG1. IgG 2A antibodies are generally more effective than IgG1 at activating complement and other antitumor defenses, and usually are preferred for therapeutic applications.

As dramatic as this improvement in survival is, the efficacy of CpG DNA was further improved when the Id was conjugated to GMCSF (154). A GMCSF-Id fusion protein was an extremely effective immunogen for inducing anti-Id antibodies and improved survival. However, almost all of the antibody response was of the IgG1 isotype, and only 30% of mice were long-term survivors of a tumor challenge given 3 days after a single immunization. In contrast, when a CpG ODN was combined with the GMCSF-Id fusion protein, the antibody response was largely IgG2A, the magnitude of the antibody response was increased approximately fivefold, and the long-term survival of the mice was improved from 30% to 70% (154). These results mirror the impressive synergy between GMCSF and CpG DNA in the in vitro studies of DCs referred to above.

2. Activation of NK Cells with CpG DNA for Immunotherapy of NK-sensitive Tumors

Aside from using CpG DNA as an adjuvant, it is also possible that its ability to activate NK cells may lead to antitumor effects. This approach to tumor immunotherapy could be considered to be complementary to tumor vaccination in that the latter strategy requires that the tumor present its specific antigens on MHC, while activated NK cells are most effective against targets that do not express high levels of MHC molecules. To avoid effective CTL antitumor responses, some tumors evolve variants with reduced MHC expression, which potentially would make them susceptible to lysis by activated NK cells. As noted above, phosphodiester CpG DNA is typically a better activator of NK activity than phosphorothioate CpG DNA. However, phosphodiester DNA injected IV is rapidly

degraded and cleared from the circulation with a half-life of about 5 min, limiting its effectiveness (180–182). Plasmids containing CpG motifs can be stabilized by the formation of complexes with cationic lipids, in which case IV administration leads to systemic immune activation with release of Th1 cytokines and activation of NK cells, as long as the CpG motifs in the plasmid are unmethylated (183). These cationic lipid-DNA complexes are efficiently taken up in the pulmonary vascular bed, and cause accumulation of activated NK cells in the lung. These immune-stimulatory effects result in a marked decrease in the number of pulmonary metastases in mice injected IV with experimental fibrosarcoma, melanoma, or colon carcinoma cell lines (183). These antitumor effects were lost in mice depleted of NK cells with anti-asialo G_{m1} antiserum, and in mice genetically deficient in IFN-γ. These studies showed the potential of innate immune activation by CpG DNA for tumor immunotherapy.

We have recently investigated the antitumor effects of a CpG ODN that was optimized for activation of NK cells. Phosphorothioate backbone ODN are much more stable than phosphodiester ODN and are much more potent at activating B cells and DCs, as reviewed above. However, the phosphorothioate backbone is generally less effective at activating NK cells (25). We have found the highest degrees of NK activation with ODN in which the 5′ and 3′ ends are phosphorothioate-modified, and the center portion is phosphodiester. These chimeric ODN have a high degree of nuclease resistance from the phosphorothioate-modified ends, yet also retain potent NK-stimulating effects (25,120). The addition of poly-G motifs to the 5′ and 3′ ends of the ODN substantially improves its ability to activate NK cells (25,92). Using such NK-optimized ODN, we have been able to prevent the development of an otherwise lethal challenge of B16 melanoma in 80% of syngeneic C57 BL/6 mice (Z. K. Ballas, W. Rasmussen, H. Davis, M. Waldschmidt, and A. M. Krieg, submitted). SCID mice were also protected against tumor challenge by CpG DNA, indicating that neither B nor T cells are required. Specific immunity was not generated, since CpG-treated mice that had survived a tumor challenge were not protected against subsequent tumor challenges. Even when treatment was begun 3 days after tumor challenge, 60% of mice could still be cured of disease with CpG treatment.

3. Activation of Antibody-Dependent Cellular Cytotoxicity with CpG DNA for Immunotherapy of NK-Resistant Tumors

Many or even most types of tumors are relatively resistant to NK-mediated lysis. An alternative form of immunotherapy is the use of passive monoclonal antibodies (mAbs) against tumor antigens. Although antitumor mAbs did not live up to early hopes as "magic bullets," steady progress has been made in their human clinical development. Approximately 70 humanized mAbs are currently in human clinical trials, and two mAbs, Rituxin and Herceptin, have been approved by the

U.S. Food and Drug Administration. One of the major mechanisms through which these mAbs are thought to exert their therapeutic effect is antibody-dependent cellular cytotoxicity (ADCC). mAbs specific for tumor-cell surface antigens bind to the tumor cell, which can then be recognized by lymphocytes such as NK cells and neutrophils that express Fc receptors. We hypothesized that the efficacy of antitumor mAbs could be improved by activation of the innate immune system and of Fc-receptor function following treatment with CpG DNA.

We tested this hypothesis in immunocompetent C3H mice in which a syngeneic B-cell lymphoma, 38C13, had been established (184). Several days after tumor implantation, the mice were injected with a dose of CpG ODN sufficient to cause systemic immune activation and enhanced Fc-receptor function. They were then treated with a standard dose of the mAb. Treatment with mAb alone gave only a 10% long-term survival, but mice that had been pretreated with CpG ODN had 70–80% survival (184). Although IL-2 immunotherapy has also been effective at enhancing mAb efficacy, in this model repeated doses of IL-2 were only able to increase survival up to 30–40%. Thus, immunotherapy with IL-2 gave inferior survival compared to the results with CpG DNA. Human clinical trials of this CpG cancer immunotherapy have recently been initiated.

4. Other Cancer Immunotherapeutic Applications of CpG DNA

It is certainly possible to envision other ways in which CpG DNA could be used to induce antitumor responses. Systemic immune activation by CpG DNA can induce high levels of circulating TNF-α and IL-12 (118), which can themselves have antitumor effects. Alternatively, injection of CpG DNA directly into a tumor lesion may activate dendritic cells in or around the tumor, inducing a Th1-like cytokine environment that may overcome the normal immune suppressive effects of the tumor and result in an effective antitumor immune response. In support of this hypothesis, Carpentier et al. have recently demonstrated that daily injection of CpG ODN into neuroblastoma tumor nodules results in tumor regression in about half of the mice (185).

D. Immunotherapy of Allergic Diseases with CpG DNA

Allergic diseases such as asthma result from Th2-type immune responses against otherwise harmless environmental antigens (Table 2). Such responses lead to the generation of Th2 T cells, which produce IL-4 and IL-5 and promote the differentiation of B cells into IgE-secreting cells. This IgE binds to the high-affinity IgE Fc receptor on the surface of mast cells and basophils. Subsequent exposure of these cells to an allergen results in the binding of the allergen by surface IgE, crosslinking of the IgE Fc receptors, and activation and degranulation of the mast cells or basophils. These cells release a variety of preformed

proinflammatory and vasoactive compounds including histamine, prostaglandins, leukotrienes, and cytokines. This results in immediate inflammatory response within 15 min, followed by a secondary late-phase reaction several hours later.

Almost all current therapeutic efforts against allergic disease have been aimed at the control of the symptoms that are triggered by mast cell or basophil degranulation. However, a more fundamental approach to disease therapy would be to prevent the initial generation of the Th2-like immune response against the allergen, or to induce a Th1-like response against the allergen. Since Th1 and Th2 immune responses are typically mutually inhibitory, the induction of a Th1-like immune response to an allergen should suppress the Th2-like response.

The incidence of asthma and allergic diseases (atopy) has been increasing dramatically in industrialized nations over the past few decades (reviewed in Ref. 186). Although the cause of this "atopic epidemic" remains unclear, an explanation that has been gaining increasingly wide acceptance has become known as the "hygiene hypothesis." Briefly, the hygiene hypothesis posits that the proper development and maturation of the immune system requires repeated infections, or exposure to infectious agents during childhood. In children who are overly protected from such infections, the immune system does not develop properly. In the absence of infection, the default pathway of the immature immune system is thought to be toward a Th2-like immune response, which results in atopy (187). The increase in allergy and asthma is thus explained by a reduced frequency of infections during childhood. Evidence supporting the hygiene hypothesis includes the following observations. First, there is an inverse relationship between atopy and immunization or infection with mycobacteria, measles, and hepatitis A. Second, children with multiple older siblings, who are more likely to develop childhood infections early in life, are less likely to develop atopic disease than only children or firstborns. Third, children cared for in day-care settings, who are exposed to large numbers of other children, are also protected against atopy. Fourth, children who are treated with antibiotics in the first 2 years of life have approximately twice the risk of developing allergic disease as those who are not.

How could early childhood infections protect against the development of allergic disease? The answer to this question may lie, at least in part, in the potent Th1-like immune-stimulatory effects of CpG DNA. Childhood bacterial or viral infections that resulted in exposure to CpG DNA may create a Th1-like environment in the airways. Subsequent allergen exposure would lead to the initiation of a Th1-like immune response against the antigen, which would protect against the subsequent development of allergy.

We used a mouse model to test the hypothesis that CpG DNA exposure could prevent the development of allergic disease. Mice were sensitized to a strong Th2-like stimulus, schistosome eggs, by IP injection in the presence or absence of CpG DNA. Upon subsequent inhalation challenge with schistosome

egg antigen (SEA), control mice developed severe eosinophilic airways disease with high levels of Th2-like cytokines in the airways, eosinophilic infiltrates, and evidence of bronchoconstriction (188). In contrast, mice exposed to CpG DNA at the time of the initial sensitization were almost completely protected against the development of eosinophilic airways disease. More importantly, CpG DNA could even block eosinophilic airways disease in mice that had already been exposed to the allergen, suggesting possible utility in the treatment of humans with asthma (188). Mice treated with CpG DNA immunotherapy developed a Th1-like immune response to the SEA instead of the Th2-like immune response, but this was not associated with any apparent airways pathology.

Our initial hypothesis to explain the therapeutic activity of CpG DNA in this model of asthma was that the CpG DNA was working through induction of expression of the Th1-like cytokines IL-12 and IFN-γ. However, more recently we have found that even in mice genetically deficient in either or both of these cytokines, CpG DNA can still prevent allergic disease (189). The only difference from wild-type mice was that the Th1-deficient mice required slightly higher doses of the CpG DNA to prevent disease development. Further studies will be required to determine the mechanism of action of CpG DNA in the absence of these classical Th1-like cytokines. CpG DNA also induces the production of other Th1-like cytokines, such as IL-18 and IFN-α, which may be sufficient to prevent disease development. It is not yet clear whether the ability of CpG DNA to inhibit production of IgE antibodies (190) is dependent upon CpG-induced cytokine production or other mechanisms.

CpG DNA is also effective in preventing this sensitization of mice to ovalbumin (191). The ability of CpG DNA to inhibit tissue and blood eosinophilia in this study was shown to occur within 1 day of CpG administration regardless of whether it was given systemically (IP) or mucosally. Since the conventional treatment for allergic airways disease involves the use of corticosteroids, it is noteworthy that a single dose of CpG DNA inhibited airway eosinophilia at least as effectively as daily injections of steroid for 7 days (191). Moreover, only CpG therapy redirected the immune response toward a Th1-like response. These basic findings have been confirmed by Sur et al., who showed that CpG DNA could also prevent sensitization to ragweed allergen (192). The protective effect of CpG DNA lasted for 6 weeks after its administration, consistent with the previous findings that CpG DNA treatment induces a long-lasting Th1-like state of the immune system. However, in contrast to the finding of Kline et al. that IFN-γ knockout mice were still protected against eosinophilic airways inflammation by CpG DNA, Sur et al. found that administration of CpG ODN to IFN-γ-deficient mice did not protect against airways eosinophilia. The difference between these two studies may lie in the fact that the ODN used by the latter authors contained suboptimal CpG motifs and/or that the dose response to treatment was not determined.

VI. CpG-N MOTIFS COMPRISE ANOTHER CLASS OF IMMUNE-REGULATORY SEQUENCES

Despite the fact that vertebrate DNA has CpG suppression and methylation of most of its CpG dinucleotides, there remain many unmethylated CpG motifs in vertebrate DNA. Assuming that no other factors are involved in determining the immune stimulatory effects of a region of DNA, one might expect that high enough concentrations of vertebrate DNA should therefore be immune stimulatory. However, this is not observed. In fact, vertebrate DNA that has been partially or almost completely unmethylated still has no immune-stimulatory effects (34). These results indicate that vertebrate DNA may contain immune neutralizing sequences that block the immune stimulatory effect of CpG motifs. Indeed, we have observed that addition of equal amounts of vertebrate DNA to bacterial DNA abolishes the induction of cytokine secretion and other immune stimulatory effects (unpublished data).

While comparing the immune effects of DNA from different genomes, we observed that genomic DNA from type 12 adenovirus is roughly as immune stimulatory as bacterial DNA, but that genomic DNA from serotype II adenovirus is nostimulatory (24). In fact, addition of type II adenoviral DNA to *E. coli* DNA virtually abolished the induction of IL-6 or TNF-α secretion. To test the hypothesis that the over- or underrepresentation of particular hexamer sequences may explain the difference in immune activity of these various genomic DNAs, we compared the frequency of all 4096 hexamers in the genomes of humans, *E. coli*, and serotypes 11 and 12 adenovirus. This analysis showed a striking nonrandom distribution of the frequency of different hexamers. The genomes that were immune-stimulatory, type 12 adenovirus and *E. coli*., contained a roughly random distribution of hexamers. However, in the human genome, not all CpG motifs were equally suppressed. The least common CpG motifs in the human genomes were those in which the flanking bases comprised a stimulatory motif, such as GACGTT and AACGTT, which were present at approximately one-fourth of the predicted random frequency. However, other CpG motifs in which the CG was preceded by a C and/or followed by a G were not suppressed in the human genome and were markedly overrepresented (approximately six times the expected frequency) in the type II adenoviral genome (24). This marked overrepresentation of CpG dinucleotides that were either preceded by a C, followed by a G, or arranged back-to-back in the genome of type II adenovirus suggested that these may represent immune neutralizing motifs, or CpG-N motifs. To test this hypothesis, we synthesized ODN containing either CpG-N motifs alone or immune stimulatory (CpG-S) and CpG-N motifs. These studies showed that the presence of a CpG-N motif in an ODN with a CpG-S motif could reduce the level of immune stimulation, and that the addition of ODN containing CpG-N motifs to CpG-S DNA could inhibit the

production of cytokine secretion. Thus, the immune-neutralizing effects of CpG-N DNA can be exerted in *cis* as well as in *trans* (24).

DNA vaccines typically contain several hundred CpG motifs, some of which are in an immunostimulatory context, and others of which are CpG-N motifs. To determine whether the efficacy of a DNA vaccine may be improved by deleting some of these CpG-N motifs, we performed in vitro mutagenesis on a DNA vaccine, eliminating 52 of the 134 CpG-N motifs that were present, and compared this to the parent DNA vaccine, as well as to another vaccine in which 16 or more CpG-S motifs were added after deletion of the 52 CpG-N motifs. These vectors showed progressively increasing immune-stimulatory effects with the deletion of the CpG-N motifs and further addition of 16 CpG-S motifs, but addition of more CpG-S motifs actually resulted in a fall of the antibody response to the DNA vaccine (24). We hypothesize that the addition of too many CpG-S motifs to a DNA vaccine may actually lead to the production of high levels of type I interferons, with resultant inhibition of plasmid expression and loss of immunogenicity. However, further studies will be required to confirm or refute this hypothesis.

Aside from the optimization of DNA vaccines, an understanding of the presence of CpG-S and CpG-N motifs may be important for the generation of improved gene therapy vectors. In the gene therapy field, delivery of plasmid vectors has been associated with highly undesirable immune-stimulatory effects that have been shown to be due to the presence of CpG motifs (193,194). In preliminary studies, we have recently found that the immune stimulatory effects of gene therapy vectors can be significantly reduced by deletion of CpG motifs from the vector, but we have not been able to show any further reduction in immune stimulatory effects by addition of CpG-N motifs to the vectors. These data suggest complex interactions between CpG-S and other DNA sequences in the plasmids, and suggest the need for further studies into the regulation of immune stimulation by CpG DNA and other sequences.

VII. CONCLUSIONS

ODNs are truly remarkable biological molecules. Aside from their ability to hybridize in a sequence-specific fashion with RNA or DNA, ODN can also interact with a wide range of cellular proteins. Some of these interactions are also sequence-specific, while others are nonspecific. In this chapter, we have reviewed the remarkable range of immune stimulatory effects that are triggered by ODN containing unmethylated CpG motifs. Presumably, these effects result from the interaction of CpG ODN with one or more intracellular receptors, but these remain to be identified.

It is now clear that these surprising immune-stimulatory effects of CpG ODN result from their mimicry of pathogen DNA, which in contrast to vertebrate DNA typically contains relatively high levels of CpG dinucleotides that are unmethylated. Aside from revealing the remarkable ability of the immune system to detect subtle differences between the molecular patterns of host cells and those of pathogens, the immune-stimulatory effects of CpG DNA offer new therapeutic possibilities. In animal models, immune-stimulatory CpG ODN have shown remarkable efficacy as vaccine adjuvants and in the immunotherapy of cancer and allergic diseases. Human clinical trials are already underway with encouraging preliminary results. In the coming years, it is hoped that the molecular mechanisms of CpG ODN will become better understood and that their therapeutic promise will be realized.

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18

Pre-mRNA Splicing as a Target for Antisense Oligonucleotides

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I. INTRODUCTION

The results reviewed in this chapter show that antisense oligonucleotides can be used not only in the conventional way, which is for down-regulation of gene expression via RNase H–promoted degradation of targeted mRNA, but also for modification of the splicing patterns of genes. This approach may be useful as a potential therapy for genetic diseases and other disorders involving alternatively spliced genes, as a research tool, and as a positive readout assay for antisense activity.

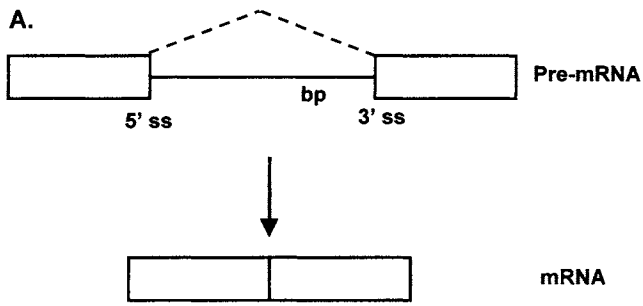
Application of antisense oligonucleotides as down-regulators of gene expression is reviewed elsewhere in this book as well as in recent volumes (Crooke and Bennett, 1996; Stein and Krieg, 1998). Modification of splicing by antisense oligonucleotides and RNAs (Kole, 1997, 1998), including technical protocols (Sierakowska et al., 1999, 2000), have been reviewed previously as well. The first part of this chapter will address the mechanisms of splicing and the suitability of splice sites and other sequence elements as targets for antisense oligonucleotides. The remainder of this chapter will review the uses of antisense oligonucleotides as modifiers of splice site selection.

II. PRE-mRNA SPLICING

Since a detailed discussion of the mechanisms of splicing is beyond the scope of this chapter, the reader is referred to a comprehensive review of eukaryotic

mRNA processing, including constitutive and alternative splicing (Krainer, 1997). The additional references cited below refer to more specific reviews that summarize the vast literature of the subject.

To precisely splice out introns from the pre-mRNA, eukaryotic cells have developed a complex system that involves multiple interactions among many proteins, small nuclear RNAs (snRNAs, which exist as nucleoprotein complexes, snRNPs), and sequences within the pre-mRNA (Staley and Guthrie, 1998; Burge et al., 1999). Sequences within pre-mRNAs that are recognized by the splicing machinery include conserved splice sites at the ends of introns (Fig. 1), branch point sequences within the introns (Fig. 1), and recently identified exon splicing enhancer (ESE) sequences, most frequently found in alternatively spliced exons. The spliceosome, a splicing complex comprised of up to 100 proteins and 5 snRNAs (Burge et al., 1999), brings the 5′ and 3′ splice sites into close proximity of one another and promotes two transesterification reactions involving phosphodiester bonds at the ends of an intron. In the first reaction, the adenosine nucleotide located in the branch point sequence attacks the 5′-most phosphodiester bond at the 5′ splice site. As a result, the upstream exon is displaced by formation of a new covalent phosphodiester bond and the intron is contorted into a lariat struc-



B.

5' Splice Site	$C_{37}A_{58}G_{78}/G_{100}U_{100}A_{57}A_{71}G_{84}U_{47}$
Branch Point	**YNYURAY**
3' Splice Site	$12Y_{70}NC_{74}A_{100}G_{100}/G_{49}$

Figure 1 (A) Splicing of pre-mRNA. Boxes, exons; solid line, intron; dashed line, splicing pathway. The splice sites (5′ss and 3′ss) and the branch point (bp) are indicated. Similar designations are used in subsequent figures. (B) Consensus sequences for splice sites and branch points. The frequencies of occurrence of nucleotides shown at each position are indicated following the pertinent nucleotide. Slashes demarcate the 5′ and 3′ ends of the intron. N, any nucleotide; Y, pyrimidine nucleotide; R, purine nucleotide.

ture. In the second reaction, the nucleoside at the 3′ end of the released exon attacks the phosphodiester bond at the 3′ end of the intron. This releases the intron, which remains in the lariat form, allows formation of a phosphodiester bond with the downstream exon, and results in spliced mRNA. The released lariat is subsequently degraded.

Assembly of the spliceosome begins with base pairing of a nine-nucleotide sequence at the 5′ end of U1 snRNP with the 5′ splice site sequence; U1 snRNP is a complex of 165 nucleotide RNAs and eight or more polypeptides (Krainer, 1997; Yu et al., 1999). This is followed by binding of U2 snRNP, via base pairing of its internal portion, with the branch point sequence located 20–40 nucleotides upstream from the 3′ splice site (Fig. 1). The U2 snRNP binding is stabilized by additional splicing factors such as U2 auxiliary factor, U2AF. Additional essential components of the spliceosome include a tri-snRNP particle U4/U6.U5 and SR proteins, which stabilize the spliceosome and bridge the 3′ and 5′ splice sites. SR proteins have serine-arginine-rich domains that are involved in protein-protein interactions, and RNA binding domains that frequently interact with purine-rich exon-splicing enhancers in some exons (Hoffman and Grabowski, 1992; Zahler et al., 1992, 1993; Fu, 1995; Wang et al., 1995; Manley and Tacke, 1996). Prior to the chemical reactions described above, the spliceosome undergoes several rearrangements, which culminate in dissociation of U1 and U4 snRNPs and positioning of U5 snRNP to base-pair simultaneously with the 5′ and 3′ splice sites.

The fact that splice sites and other pre-mRNA sequences involved in splicing are bound by a large number of splicing factors would suggest that these sequences should be inaccessible to antisense oligonucleotides. However, a large body of work reviewed below indicates that this is not the case. One possible explanation for this seemingly unlikely phenomenon is that the interactions of the splicing factors with pre-mRNA are very dynamic. For each splicing event, the spliceosome is newly assembled on the splice sites and, after splicing, is disassembled into its components that are reused again in another splicing cycle (Moore et al., 1993). Thus, it is likely that the oligonucleotides interfere with the assembly of the spliceosome, preventing its formation rather than displacing the bound splicing factors. This mechanism is supported by early experiments in cell-free splicing extracts; it was shown that the splice sites and branch point became increasingly resistant to oligonucleotide-induced RNase H degradation as the assembly of the spliceosome and the splicing reaction progressed (Ruskin and Green, 1985).

III. ALTERNATIVE SPLICING OF PRE-mRNA

Alternative splicing of multi-intronic pre-mRNA is a complex issue and an in-depth discussion of the process of alternative splicing is beyond the scope of this

chapter. The mechanisms involved in alternative splicing of pre-mRNAs are not completely understood, but a large body of evidence suggests that selection of splice sites is determined by competition between splice sites and/or related splicing sequence elements for splicing factors during spliceosome assembly (see recent reviews by Wang and Manley, 1997; Wang et al., 1997b; Lopez, 1998; Herbert and Rich, 1999). This is supported by observations that both the presence and absence of gene-specific splicing factors (Lopez, 1998) or the levels of constitutive splicing factors (Caceres and Krainer, 1997) may affect the choice of the splicing pathway. Both gene-specific and constitutive splicing factors that play a role in alternative splicing usually belong to the SR family of polypeptides (Zahler et al., 1992; Caceres and Krainer, 1997). These proteins, as well as other splicing factors, are extensively phosphorylated thereby adding an additional regulatory level to alternative splicing (Fu, 1995; Misteli, 1999).

Sequence elements of pre-mRNA that affect splice site selection include exon-splicing enhancers (Berget, 1995), secondary structures (Wang et al., 1997b), and, most importantly, the relative match of the 3′ and 5′ splice sites and branch points to their respective consensus sequences. This is well illustrated by the fact that splicing mutations causing genetic diseases lead to activation of cryptic sites or to skipping of exons bordered by the affected splice sites (Krawczak et al., 1992). On the other hand, experimental mutations that increase the match of a splice site or a branch point to their respective consensus sequences lead to inclusion of otherwise ignored exons (Dominski and Kole, 1992; Hoffman and Grabowski, 1992). These observations indicate that the mutations either decrease or increase the affinity of the affected sequences for the appropriate splicing factors, thereby affecting splice site selection.

There are several possible outcomes of alternative splicing. They include inclusion or exclusion of exons, retention or splicing out of an intron, and selection of alternative 5′ or 3′ splice sites (Fig. 2). Note that an excluded exon becomes a part of the spliced-out intron and that a retained intron becomes part of a coding sequence, i.e., becomes an exon. Thus, it appears that a single mechanism may be responsible for both outcomes. In certain genes, there is competition between alternative splice sites and alternative polyadenylation sites affecting the outcome of both processes (Fig. 2).

IV. MODIFICATION OF PRE-mRNA SPLICING PATTERNS BY ANTISENSE OLIGONUCLEOTIDES

If competition between splice sites for splicing factors plays a major role in alternative splice site selection, then it is probable that blocking a splice site with antisense oligonucleotides will inhibit its use and promote selection of an alter-

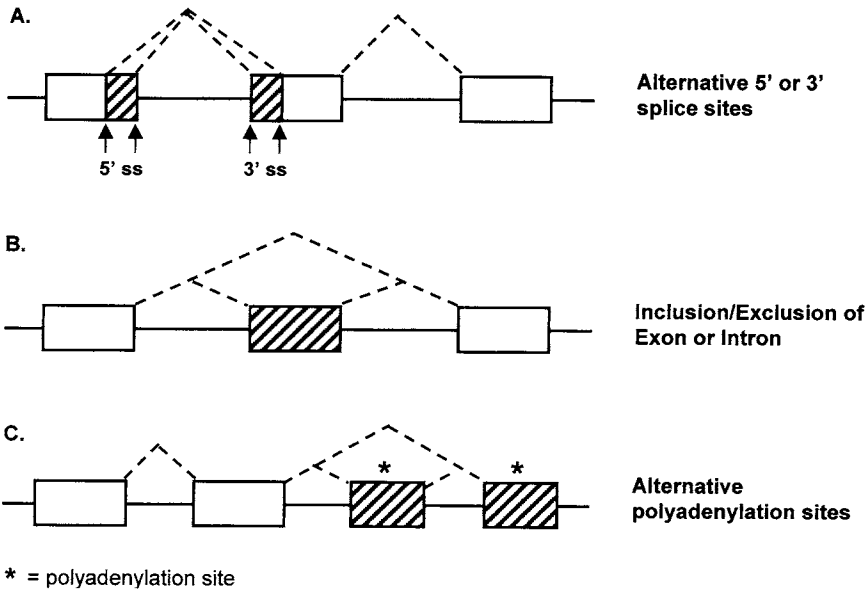


Figure 2 Examples of common alternative pre-mRNA splicing patterns. In A, the arrows indicate alternative 5′ or 3′ splice sites that compete for splicing factors. Hatched boxes, alternatively spliced sequences. In B, the hatched box represents both an alternatively spliced exon and a portion of an intron retained by the use of alternative splice sites within intron sequences. If the exon is excluded, it becomes a part of the spliced out intron. If a portion of an intron is retained, it becomes part of the coding sequence, i.e., an exon. In C, asterisks indicate alternative polyadenylation sites. These sites compete/collaborate with the splicing machinery affecting the final form of the processed mRNA.

native one. By analogy to the alternative splicing pathways discussed above (Fig. 2), several possible outcomes can be expected.

In a multiintron gene whose pre-mRNA is not normally alternatively spliced, the most likely consequence of targeting a 5′ or a 3′ splice site with antisense oligonucleotides is exon skipping. If a cryptic splice site is located nearby, it may be activated instead. Although it has not been shown experimentally, it is also conceivable that oligonucleotide blocking of a splice site may result in intron retention. With some interesting exceptions (see subsequent sections), all of these outcomes should lead to a defective mRNA and therefore to down-regulation of gene expression. Thus, in the above situations, targeting splice sites in pre-mRNA does not offer any particular advantage over the targeting of coding sequences in mRNA. In contrast, pre-mRNAs, which are aber-

rantly spliced owing to mutations that cause genetic disorders or are normally alternatively spliced, provide more interesting targets for antisense oligonucleotides.

In some genetic disorders, point mutations within introns generate new splice sites that redirect the splicing machinery to aberrant splicing pathways even though the correct splice sites still exist and are potentially functional. As a result, a defective mRNA is produced, leading to inappropriate expression or total lack of a gene product. It can be expected, however, that blocking of the aberrant splice sites by antisense oligonucleotides will prevent aberrant splicing and, because of competition from the correct splice sites, will restore correct splicing and generate proper mRNA and functional protein. In this approach, antisense oligonucleotides restore, rather than down-regulate, the expression of a defective gene. In other words, targeting antisense oligonucleotides toward aberrant splice sites represents a potential form of gene therapy for relatively frequent genetic disorders caused by splicing mutations (Krawczak et al., 1992).

For several reasons, alternatively spliced pre-mRNAs represent a particularly attractive target for antisense oligonucleotides. First, even a small decrease in the concentration of one splice variant induced by an antisense oligonucleotide, by definition, leads to a simultaneous increase in concentration of its counterpart; this results in a major change in the ratio of the alternative splice products. In comparison to oligonucleotide-induced down-regulation of an mRNA, modification of alternative splicing offers amplification of the signal. Second, a large number of genes that are frequently involved in key cellular processes are alternatively spliced. Since the splice variants usually have different functions, the change in their ratio may have significant biological effects in the targeted cell (see Section VIII). Third, as will be discussed in more detail in Section IX, the splice sites and/or other sequence signals involved in alternative splicing are usually "weak," i.e., somewhat distant from the consensus sequences, and therefore more susceptible to antisense oligonucleotides.

V. PROPERTIES OF OLIGONUCLEOTIDES FOR MODIFICATION OF SPLICING

To use oligonucleotides to shift splicing patterns, RNase H, an enzyme that cleaves RNA in RNA-DNA duplexes (Wu et al., 1999, and references cited therein), must not be activated. This is in contrast to the conventional use of antisense oligonucleotides for down-regulation of mRNA, where RNase H activity is often desired. Thus, the antisense oligonucleotides must be modified so that the oligonucleotide-RNA duplex is no longer recognized as a substrate by RNase H.

Several backbone modifications meet this criterion. They include, for example, 2′-*O*-methyl- and other 2′-*O*-substituted oligonucleotides (reviewed in Verma and Eckstein, 1998), methylphosphonate derivatives (Miller, 1998), peptide nucleic acids, PNAs (Nielsen et al., 1993; Hyrup and Nielsen, 1996), and morpholino oligomers (reviewed in Summerton, 1999). Additional RNase H–resistant species may also be found among the close to 200 modifications of oligonucleotides that have been generated (Freier and Altmann, 1997; Verma and Eckstein, 1998). Fortunately, the modifications that inhibit RNase H also render the oligonucleotides resistant to nucleases, thereby increasing their intracellular stability. Furthermore, with the exception of methylphosphonates, the same modifications increase the T_m of the duplexes, increasing the ability of modified oligonucleotides to effectively compete with the splicing factors for the targeted sequence in the pre-mRNAs.

Since the splice site sequences are relatively conserved (Fig. 1; Krainer, 1997), it may be argued that an oligonucleotide targeted to a specific splice site may also bind to splice sites other than the intended one. However, several factors, as well as experimental evidence, suggest that this should not be a critical issue. First, antisense oligonucleotides are usually designed 14–28 nucleotides in length and therefore include sequences antisense not only to the conserved splice site, but also to divergent flanking sequences. Furthermore, only two nucleotides, GU in the 5′ splice site and AG in the 3′ splice site, are conserved in nearly all splice sites (Senapathy et al., 1990) while the remaining nucleotides are represented at much lower frequencies (Fig. 1). Therefore, an oligonucleotide fully complementary to a specific splice site would hybridize to other sites with a number of mismatches, significantly lowering its affinity and effectiveness. Second, if antisense oligonucleotides blocked multiple splice sites and affected proper splicing of a number of mRNAs, including those involved in maintaining cellular homeostasis, cytotoxicity should be expected. However, experiments carried out in this laboratory showed that cellular growth was not inhibited when HeLa cells took up 2′-*O*-methyl-oligoribonucleotide-Lipofectamine (Life Technologies, BRL) complexes targeted to β-globin pre-mRNAs (Sierakowska et al., 1996).

VI. RESTORATION OF CORRECT SPLICING IN MUTANT PRE-mRNAs IN GENETIC DISORDERS

β-Thalassemia is a genetic blood disorder that affects large populations in the Mediterranean, Middle East, and Southeast Asia. Interestingly, the majority of β-thalassemia cases are caused by mutations that affect normal splicing of β-globin pre-mRNA (Weatherall, 1994; Schwartz and Benz, 1995) and, conse-

quently, eliminate or decrease expression of β-globin protein. A mutation of the invariable G of the 5′ splice site of intron 1 (nucleotide 1 of intron 1, IVS1-1) activates three cryptic splice sites, leading to aberrant mRNA and nonfunctional β-globin protein. Even mutations at the less conserved nucleotides 5 and 6 (IVS1-5 and IVS1-6) result in aberrant splicing and activation of the same cryptic 5′ splice sites. Another mutation, IVS1-110 G to A, creates a new 3′ splice site, resulting in mRNA that retains 20 nucleotides of the intron sequence. In intron 2, thalassemic mutations IVS2-654, IVS2-705, and IVS2-745 create aberrant 5′ splice sites and activate a common 3′ cryptic splice site at nucleotide 579 (Schwartz and Benz, 1995). The aberrant 5′ and 3′ splice sites are used predominantly, even though the correct splice sites remain unchanged, resulting in mRNA that retains a portion of the intron of 73, 126, or 165 nucleotides, respectively. This leads to translation of nonfunctional β-globin protein and results in β-thalassemia (Fig. 3; Schwartz and Benz, 1995).

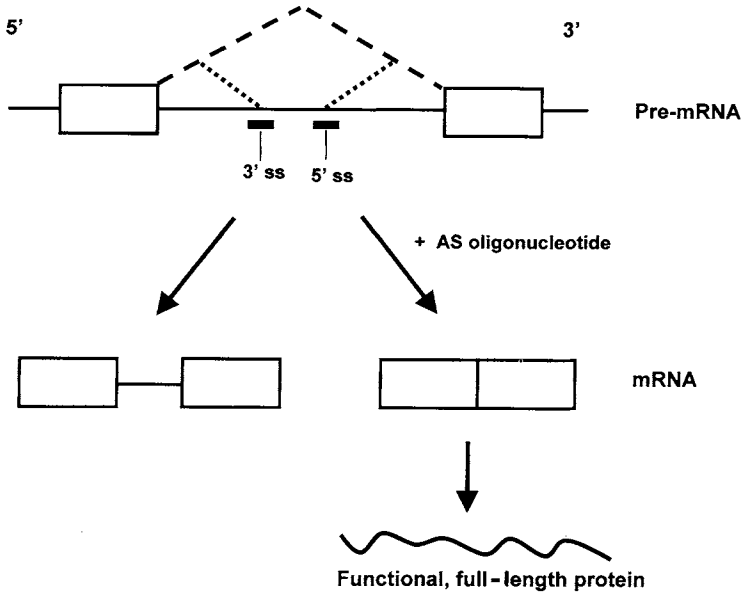


Figure 3 Restoration of splicing of β-globin and CFTR pre-mRNA. Genetic diseases such as β-thalassemia and cystic fibrosis harbor mutations within β-globin and CFTR pre-mRNAs, respectively, that alter the correct splicing pattern of the pre-mRNA, resulting in aberrant mRNA and nonfunctional protein (see text). Blocking the aberrant splice sites with antisense oligonucleotides (heavy bars) inhibits aberrant splicing (dotted line) and promotes correct splicing (dashed line).

Since in the IVS2 mutants the correct splice sites retained the potential to be recognized by the splicing machinery, these pre-mRNAs provided a clinically important system for testing the ability of antisense oligonucleotides to modify the splicing pathways (Fig. 3). Early in vitro experiments, in nuclear splicing extracts from HeLa cells, showed that 18-mer 2′-*O*-methyl-oligoribonucleoside-phosphorothioates antisense to the aberrant 5′ or 3′ splice sites in IVS2-654 or -705 pre-mRNAs nearly completely restored expression of correct β-globin mRNA. The correction was sequence specific and concentration dependent (Dominski and Kole, 1993; reviewed in more detail in Kole, 1998).

Further work showed that transfection of model HeLa, 3T3, and K562 cell lines that stably expressed the IVS2-654 pre-mRNA with oligonucleotide-Lipofectamine complexes targeted against the aberrant 5′ splice site led to efficient correction of β-globin splicing. The effect was concentration dependent and sequence specific; maximum correction of approximately 40% of β-globin mRNA was accomplished (Sierakowska et al., 1996, and unpublished data). Transfection of IVS2-705 and IVS2-745 HeLa cell lines led to even more efficient, virtually 100%, restoration of correct splicing of β-globin mRNA (Sierakowska et al., 1997, 1999). As expected, in all oligonucleotide-treated cells, accumulation of the correctly spliced mRNA resulted in translation of full-length β-globin protein (Sierakowska et al., 1996, 1999; Schmajuk et al., 1999).

In the above experiments, maximal correction was obtained at 0.2–0.4 μM 2′-*O*-methyl-oligoribonucleoside phosphorothioates delivered to the cells with Lipofectamine or other cationic lipid oligonucleotide carriers. Similar delivery of 2′-methoxy-ethoxy-oligoribonucleotides yielded efficient correction of IVS2-654 and IVS2-705 splicing at even lower concentrations, suggesting that the latter backbone promoted higher binding affinity and/or increased resistance to nucleases (Brown-Driver et al., 1999). Neither of these oligonucleotides was effective in correcting aberrant β-globin pre-mRNA splicing if the cells were treated with free oligonucleotides in the culture media (Schmajuk et al., 1999, and unpublished data).

In contrast to the 2′-substituted oligonucleotides with sugar-phosphate backbones, which are negatively charged, morpholino oligonucleotide analogs are neutral and as such cannot be delivered to the cells with cationic lipids. However, when adherent cells were loaded with morpholino oligomers by scrapeloading (Schmajuk et al., 1999; Summerton, 1999), efficient correction of splicing of both IVS2-654 and IVS2-705 was observed. Importantly, direct treatment of HeLa and K562 cells expressing mutant β-globin pre-mRNAs with free morpholino oligomers (5–15 μM) resulted in levels of correction comparable to those achieved by Lipofectamine delivery of 2′-*O*-methyl-substituted oligonucleotides. Although the morpholino derivatives were rather poorly taken up by the cells (their effective concentrations were approximately 40–80 times higher than those required for cationic lipid delivery), they were, nevertheless, the only ones that

had any appreciable effect on splicing under these conditions (Schmajuk et al., 1999).

Recent experiments show that morpholino oligonucleotides are effective not only in cellular models of β-thalassemia based on HeLa or K562 cells but also in the ultimate target cells for this approach, erythroid progenitor cells. The oligomers targeted to IVS2-654 and IVS2-745 aberrant 5′ splice sites were delivered ex vivo to cultured peripheral blood cells from β-thalassemia patients by a procedure analogous to scrapeloading. A concentration-dependent increase in correctly spliced mRNA and correctly translated hemoglobin was detected (Lacerra et al., 2000). Similar results were obtained upon antisense oligonucleotide treatment of cultured bone marrow cells isolated from recently described (Lewis et al., 1998) transgenic mice harboring the human IVS2-654 β-globin gene (H. Sierakowska, G. Lacerra, and R. Kole, unpublished data).

Correction of aberrant splicing was also tested in the context of another genetic disease, cystic fibrosis (Friedman et al., 1999). Over 800 different mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene account for the pathogenesis of the disease. Approximately 14% of the mutations in the CFTR gene affect splicing (Tsui, 1992). Intron mutation, 3849 + 10 kb C to T, appeared particularly suited for repair by antisense oligonucleotides. This mutation creates an aberrant 5′ splice site within intron 19 of the CFTR gene and, similar to β-thalassemic mutations, activates a 3′ cryptic splice site 84 nucleotides upstream from the aberrant 5′ splice site (Fig. 3; Highsmith et al., 1994). The 3849 + 10 kb C-to-T defect has been identified in 1–2% of all cystic fibrosis patients and more frequently in Ashkenazi Jewish populations (Abeliovich et al., 1992).

Several cell lines that modeled the CFTR 3849 + 10 kb mutation were generated and treated with 2′-*O*-methyl-oligribonucleoside-phosphorothiates antisense to the aberrant 5′ and 3′ splice sites (Fig. 3). This treatment yielded correctly spliced CFTR pre-mRNA and correctly translated and processed CFTR protein. Interestingly, combined oligonucleotides targeted to the 3′ and the 5′ aberrant splice sites were more effective than either oligonucleotide alone (Friedman et al., 1999).

An interesting case of aberrant splicing is found in a group of closely related neurodegenerative diseases termed frontotemporal dementia and parkinsonism linked to chromosomes 17 (FTDP-17) (Hutton et al., 1998). Numerous FTDP-17 patient mutations map to the region surrounding the 5′ splice site of exon 10 in a gene of microtubule-binding protein tau. In healthy individuals, the 5′ splice site of exon 10 is sequestered in a stem-loop structure leading to skipping of exon 10 from the spliced mRNA. The mutations disrupt the stem-loop leading to inclusion of exon 10 into tau mRNA and consequently to disease (Grover et al., 1999). Recent work showed that 2′-*O*-methyl-modified oligoribonucleotides complementary to exon 10 sequences potently prevented inclusion of the exon

and restored correct splicing of tau pre-mRNA in cell culture (T. Misteli and B. Kalbfuss, personal communication).

The above work demonstrated that restoration of correct splicing by antisense oligonucleotides is not limited to a single pre-mRNA or a single cell type and may be applicable to several genetic disorders. There are numerous examples of mutations that do not affect correct splice sites and yet lead to aberrant splicing in disorders other than thalassemia or cystic fibrosis (Mitchell et al., 1991; Knebelmann et al., 1995; Satokata et al., 1995; Tassara et al., 1995; Chen et al., 1996; Lee et al., 1996; McConville et al., 1996; Perrin et al., 1996; Roest et al., 1996; Bruggenwirth et al., 1997; Hovnanian et al., 1997; Ikeda et al., 1997; Okuyama et al., 1997; Wang et al., 1997a; O'Neill et al., 1998; Tsuruta et al., 1998; Vervoort et al., 1998; Teraoka et al., 1999; Urban et al., 1999). These mutations may be underrepresented in the databases since genetic mutation screening is frequently limited to coding sequence and adjacent splice sites. More elaborate RNA-based analyses are required for the identification of intron mutations.

Although in the experiments presented above correct splicing is not completely restored, in diseases resulting in a loss of gene function even a modest increase in correct splicing and gene expression may have significant therapeutic value. This is shown, for example, by the fact that transfusion therapy that restores hemoglobin levels to no more than 30% greatly improves the clinical status of the patients (Weatherall, 1994; Schwartz and Benz, 1995).

VII. MODIFICATION OF SPLICING OF *mdx* DYSTROPHIN PRE-mRNA

Human dystrophin pre-mRNA presents an interesting target for antisense modification of splicing pathways. Mutations that disrupt the reading frame of the dystrophin gene and result in prematurely terminated protein lead to a severe muscle disorder, Duchenne muscular dystrophy (DMD). In a milder form of disease, Becker muscular dystrophy (BMD), even large internal deletions are relatively benign, as long as the reading frame is preserved and a truncated but partly functional dystrophin protein is produced (Hoffman et al., 1988; Monaco et al., 1988). It seems likely, therefore, that in-frame skipping of an exon that harbors a stop codon would lead to partial restoration of dystrophin gene expression. Experiments along these lines, oligonucleotide-induced skipping of an offending exon, have been carried out (Dunckley et al., 1998; Wilton et al., 1999; Mann et al., 2001). A *mdx* mouse model of DMD that carries a stop codon in exon 23 of the dystrophin gene (Fig. 4; Sicinski et al., 1989) was used in both investigations discussed below.

Transfection of *mdx* myoblast primary cells with 12-mer (Dunckley et al., 1998) or 18–20-mer (Wilton et al., 1999) 2′-*O*-methyl-oligoribonucleoside phosphorothioates antisense to either the 5′ or the 3′ splice sites of exon 23 yielded

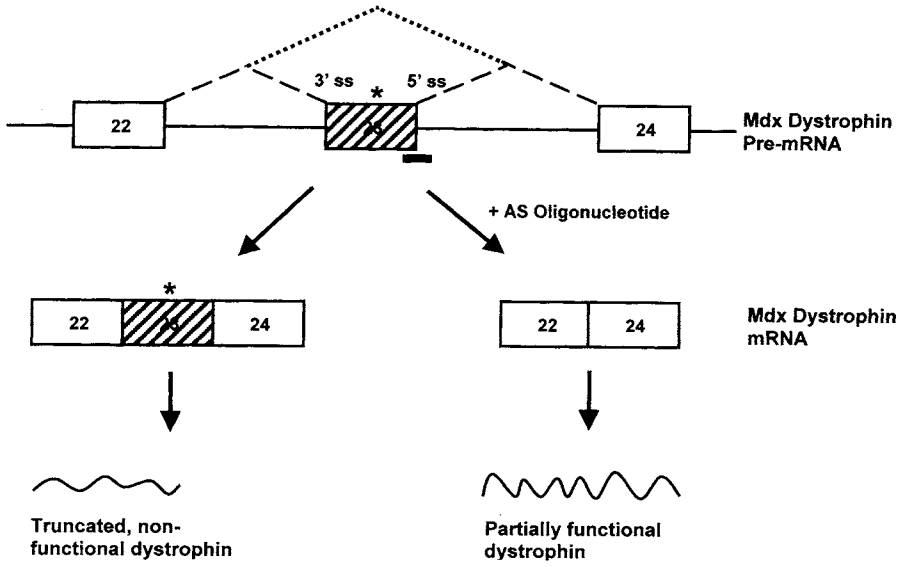


Figure 4 Modification of splicing of mdx dystrophin pre-mRNA. In Duchenne muscular dystrophy (DMD) mutations in the dystrophin gene result in disruption of the reading frame leading to early translation termination and a nonfunctional protein. In a *mdx* mouse model of DMD, a mutation in exon 23 creates a stop codon (asterisk) and prevents further translation. Targeting oligonucleotides (heavy bar) toward the 5′ splice site of exon 23 results in exclusion of the exon during pre-mRNA splicing (dotted line), removing the stop codon and restoring the reading frame.

concentration- and sequence-dependent skipping of exon 23 (Wilton et al., 1999) as well as additional exons (Dunckley et al., 1998). The latter result suggests that the short, 12-mer, oligonucleotides delivered at a relatively high concentration of 1 μM were less sequence specific than the 18–20-mers and bound to similar splice site sequences at the ends of other exons in the dystrophin gene. This interpretation is supported by the fact that in the Wilton et al. (1999) study, skipping of additional exons was also detected at high concentrations of the 18–20-mer oligonucleotides.

The important conclusion from these studies is that antisense oligonucleotides shifted a regular splicing pathway. Thus, they provide evidence that this approach is not limited to aberrant splice sites and therefore, its application may be wider than initially expected. Moreover, shifting splicing by antisense oligonucleotides may be particularly attractive for treatment of DMD since the huge, 2.4-Mb, dystrophin gene will be difficult to manipulate in conventional gene therapy protocols.

VIII. OLIGONUCLEOTIDE-INDUCED MODIFICATION OF ALTERNATIVE SPLICING IN CANCER

Since alternatively spliced genes are very common and frequently play crucial roles in cell growth and homeostasis, the possibility of gene-specific, antisense-induced manipulation of alternative splicing is extremely attractive. Of particular interest are alternatively spliced genes that yield products with opposing functions; one can expect that modification of their splicing will have dramatic biological consequences. Genes involved in cancer that yield either pro- or antiapoptotic products are a good example of these potential targets (reviewed in Jiang and Wu, 1999; Mercatante and Kole, 2000).

Members of the Bcl-2 gene family (Adams and Cory, 1998) play key roles in apoptosis. Overexpression of certain splice variants is associated with a number of cancers. This is the case for the fully spliced form of Bcl-2, which is found in many types of cancers and contributes to resistance to chemotherapy. This variant is strongly antiapoptotic while a truncated splice variant has little or no antiapoptotic activity (Tsujimoto and Croce, 1986). In contrast, a predominant splice variant of Bax results in a protein with proapoptotic properties while the minor one is antiapoptotic (Zhou et al., 1998). Such contrasting functions are also seen in other members of Bcl-2 family such as Bim (O'Connor et al., 1998) and Bcl-x (Boise et al., 1993). Splicing of the latter gene was recently modified by antisense oligonucleotides as discussed below.

Splicing of Bcl-x at two alternative 5′ splice sites in exon 2 (Fig. 5) generates two splice variants, Bcl-xL and Bcl-xS. Use of the downstream alternative 5′ splice site results in a longer product, Bcl-xL, which inhibits programmed cell death, while use of the upstream 5′ splice site results in Bcl-xS, which has strong proapoptotic properties (Boise et al., 1993). Modification of the Bcl-x pre-mRNA splicing pathway was accomplished with antisense 2′-*O*-(2-methoxy)-ethyl oligonucleotides targeted to a region surrounding the Bcl-xL (downstream) splice site (Taylor et al., 1999). The oligonucleotide most effective in shifting pre-mRNA splicing from Bcl-xL to Bcl-xS was complementary to a sequence located 15 nucleotides upstream from the Bcl-xL 5′ splice site. Cellular delivery of the oligonucleotides was with Lipofectin (Life Technologies, BRL).

The physiological effects of the shift in Bcl-xL/-xS splicing were investigated in A549 cells, an epithelial cell line established from human lung carcinoma. Unfortunately, the anti-Bcl-xL oligonucleotide alone did not induce apoptosis, indicating that the expression of Bcl-xS induced by the oligonucleotide was not sufficient to overcome the antiapoptotic stimuli in this cell line. However, the oligonucleotide, at a relatively low concentration (0.1 μM), sensitized the cells to UV and cisplatin treatments (Taylor et al., 1999). Thus, this approach has a potential to provide adjuvant therapy to standard chemotherapeutic protocols. Interestingly, similar experiments performed with 2′-*O*-methyl-oligoribonucleoside phosphorothioates in prostate cancer cell lines yielded an increase in apop-

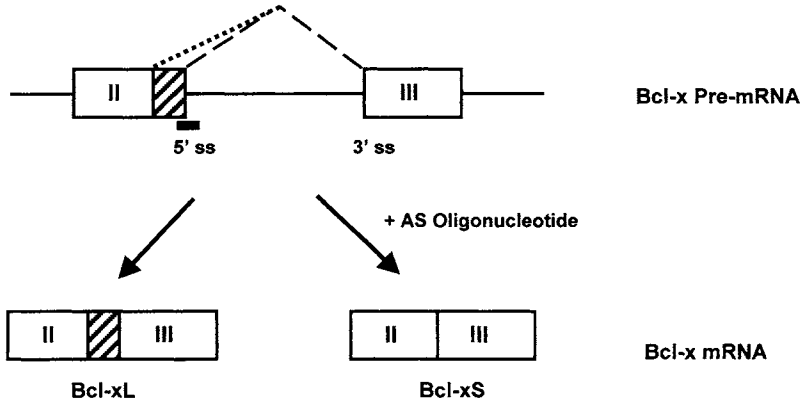


Figure 5 Modification of the splicing patterns of Bcl-x pre-mRNA. Alternative use of two 5′ splice sites generates products with opposing functions. Antiapoptotic Bcl-xL and proapoptotic Bcl-xS are generated when the downstream or upstream 5′ splice sites, respectively, are selected by the splicing machinery. Antisense oligonucleotides (heavy bar) targeted to the downstream 5′ splice site of Bcl-x pre-mRNA induced a shift in splicing from Bcl-xL to Bcl-xS, promoting apoptosis of cancer cells.

totic cells and in cell death without the need for treatment with cytotoxic agents (Mercatante et al., 2001). The mechanisms responsible for the observed differences are under investigation.

The modification of splicing of genes that are normally alternatively spliced is obviously not to be limited to the Bcl-2 family. Other alternatively spliced cancer-related genes such as CD44, cathepsin B, Fas, and WT-1 represent potentially important targets as well (reviewed in Mercatante and Kole, 2000). Modification of apoptotic pathways may also be useful in the treatment of other disorders. This is suggested, for example, by the observation that in lupus, an autoimmune disease, the cells express a secreted rather than membrane-associated splice variant of Fas, which results from alternative splicing (Cheng et al., 1994).

IX. TARGETING OF ANTISENSE OLIGONUCLEOTIDES TO SPLICE SITES

Because of a limited knowledge of the in vivo structure of mRNA, our ability to select appropriate target sites for antisense oligonucleotides is unsatisfactory. The fact that in cells, both pre-mRNAs and mRNAs are always complexed with

proteins (McAfee et al., 1997) that may block sequences and modify the structures that are predicted by energy-based computer algorithms (Zuker, 1994) contributes to this problem. Thus, in spite of recent reports that appear to predict accessible sites in the mRNAs (Milner et al., 1997; Ho et al., 2000), the selection of sites that are susceptible to antisense oligonucleotides still requires the synthesis and screening of a large number of oligonucleotides designed to cover the length of the targeted mRNA molecule (Dean et al., 1998; Monia, 1998). In this regard, pre-mRNA splicing and splice sites offer targets that are limited and better characterized and therefore promise simpler selection of effective oligonucleotides. The results reviewed in this chapter support this assertion and suggest that antisplicing oligonucleotides may be selected in a predictable manner. However, recent work from this laboratory provides instructive illustration of the issues involved and shows that the prediction of activity of oligonucleotides targeted to the splice sites is not yet straightforward.

The studies discussed in Section VI showed that aberrant splicing of β-globin pre-mRNA caused by the generation of the three thalassemic 5′ splice sites, IVS2-654, -705, and -745, is correctable by antisense oligonucleotides. However, a quantitative analysis of the data showed that the accessibility of the 5′ splice sites to antisense oligonucleotides differed dramatically (see Table 1 and Sierakowska et al., 1999). Whereas the effective concentration (EC_{50}) of the oligonucleotide targeted to IVS2-654 splice site was 115 nM, the concentrations of anti-IVS2-705 and ant-IVS2-745 oligonucleotides were 4 and 1 nM, respectively; this was a 25- and 100-fold decrease, respectively. Although the data set

Table 1 Effective Concentration of Antisense Oligonucleotides Targeted to IVS2 Mutant pre-mRNAs

Pre mRNA	5′ splice site	U1[a]	EC_{50} (nM)[b]
IVS2-654	CUGGGUUAAG/GUAAUAGC	7/11	115
IVS2-654con	CUGGGUUAAG/GUAAGUGC	9/11	1420
IVS2-705	UGUAACUGAG/GUAAGAGG	8/11	4
IVS2-705con	UGUAACUGAG/GUAAGUGG	9/11	17
IVS2-745	UACAAUCCAG/GUACCAUU	7/11	1
U1	3′ GUC/CAUUCAUA 5′		

[a] As a measure of splice site "strength" the number of nucleotides within the splice site that base-pair with the 5′ end of U1 snRNA is shown. The relevant fragment of U1 sequence is shown under the splice site sequences.

[b] Concentration of an oligonucleotide that restored 50% of correct splicing. Oligonucleotides were targeted to the aberrant 5′ splice sites of the respective IVS2 mutants; i.e., ON-654 targeted to IVS2-654 pre-mRNA, ON-705 targeted to IVS2-705 pre-mRNA, etc. See Sierakowska et al., 1999, for more details.

Source: Adapted from Sierakowska et al., 1999.

is obviously too small to make any firm interpretations, two trends can be discerned. First, the distance between the 5′ splice site and the upstream 3′ splice site increased in these pre-mRNAs from 73 to 126 to 165 nucleotides. Consistent with the exon definition model (reviewed in Berget, 1995), it seems likely that the interactions of splicing factors bridging the splice sites were weaker for the longer aberrant exons, thereby making the splice sites more accessible to the oligonucleotides. Second, changing the aberrant 654 and 705 5′ splice sites to increase their match to the consensus sequence (654 con and 705 con), and presumably to strengthen their interactions with the splicing factors, has led to a marked increase of EC_{50} values for the oligonucleotides (Sierakowska et al., 1999). One can make an educated guess that these parameters, as well as the strength of the branch points and/or presence or absence of exon-splicing enhancers, ultimately determine accessibility of the splice sites to antisense oligonucleotides.

Since pre-mRNA splicing takes place in the nucleus, it could be argued that targeting antisense oligonucleotides to pre-mRNA will be very inefficient. It would seem that cytoplasmic mRNA may be more accessible to antisense oligonucleotides, and therefore antisense oligonucleotides targeted toward already spliced mRNA located in the cytoplasm might be more efficient. However, several observations, including those previously discussed in this chapter, suggest the nuclear activity of these compounds. For example, the delivery of oligonucleotides by cationic lipids was found to dramatically enhance their antisense effects and simultaneously promote their nuclear accumulation (Bennett et al., 1992; Zelphati and Szoka, 1996). In addition, oligonucleotides microinjected into the cytoplasm (Leonetti et al., 1991; Fisher et al., 1993) or taken up by the cells treated with streptolysin (Spiller and Tidd, 1995) rapidly migrated into the nucleus suggesting that they may be unavailable for interaction with cytoplasmic mRNA. Furthermore, experiments with antisense oligonucleotides targeted to various regions of mRNAs unexpectedly resulted in modification of splicing or nuclear accumulation of the oligonucleotides. An antisense oligonucleotide targeted to the 3′ untranslated region of cytoplasmic E-selectin mRNA inhibited splicing of pre-mRNA in the nucleus instead of down-regulating the mRNA, as was expected to occur (Condon and Bennett, 1996). Another experiment showed that antisense oligonucleotide–mediated down-regulation of PKC-alpha mRNA kinetically coincided with the nuclear accumulation of appropriate antisense oligonucleotide (Marcusson et al., 1998). In yet another experiment, morpholino antisense oligonucleotides targeted to the translation initiation codon and intron 1–exon 2 boundary of c-myc surprisingly resulted in missplicing of the pre-mRNA (Giles et al., 1999). These latter three experimental results strongly suggest that pre-mRNA, which is nuclear localized, is the preferred, if not exclusive, target for antisense oligonucleotides; this implies that antisense oligonucleotides do indeed have nuclear activity.

X. MODIFICATION OF SPLICING PATHWAYS AS A RESEARCH TOOL

Modification of splicing by an antisense oligonucleotide indicates that the latter crossed the cell membrane, entered the nucleus, found the target sequence in the newly transcribed pre-mRNA, and hybridized to it in vivo strongly enough to displace the splicing factors designed to interact with or in the vicinity of the target sequence. Moreover, a new product, alternatively spliced mRNA, is generated, which can be easily detected on a zero or low background. Thus, apart from potential clinical applications, modification of splicing can be used as a rigorous, sequence-specific positive readout assay of antisense activity. This assay was recently perfected by utilizing luciferase activity (Kang et al., 1998) or fluorescence of green fluorescent protein (GFP) (S.H. Kang, P. Sazani, and R. Kole, unpublished), as simplified and easily quantified readouts of antisense activity. This was accomplished by inserting the aberrantly spliced β-globin introns IVS2-705 or IVS2-654 into the coding sequences of luciferase or GFP and generation of the cell lines that expressed the constructs. Because of the aberrant splicing and consequent retention of a fragment of the intron, both the luciferase and the GFP were not functional. However, effective delivery of the oligonucleotides targeted to the aberrant splice sites restored correct splicing and also activity of luciferase (Kang et al., 1998) or GFP (unpublished) proteins. The luciferase activity allowed for the rapid and quantitative analysis of effectiveness of different antisense oligonucleotides and/or delivery agents (Kang et al., 1999) while fluorescent cell sorting or microscopic analysis allowed evaluation of the effects of oligonucleotides in individual cells (P. Sazani and R. Kole, unpublished). Work is in progress to generate transgenic mice based on these constructs. These animals will be extremely useful in investigations of the biovailability, pharmacodynamics, and other important aspects of antisense oligonucleotide activity in vivo.

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Application of Antisense Oligonucleotides to the Study of CNS Protein Function

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I. INTRODUCTION

Antisense oligonucleotides have been widely used to investigate protein function in cell-based systems for more than two decades. Their application to studies in the living brain, however, is much more recent. Since the feasibility of conducting central nervous system (CNS) experiments was first demonstrated, a variety of proteins, including seven-transmembrane receptors, ion channels, transporters, peptide neurotransmitters, G proteins, and growth factors, have been successfully studied. CNS proteins are in many ways suited to exploit the strengths of the antisense approach. Many of these proteins are members of large families with closely related homologs or subtypes. For example, the serotonin and glutamate families of receptors consist of 14 and 8 family members, respectively. Additional protein complexity results from alternative splicing events that generate distinct protein isoforms. The NR1 family of *N*-methyl-D-aspartate ionotropic receptors has eight receptor isoforms. Highly selective agonists and antagonists capable of distinguishing between closely related proteins are unavailable in many of these families.

Although protein subtypes and isoforms are frequently not distinguishable using existing pharmacological ligands, a sufficient degree of diversity exists in the mRNA sequences of related proteins so that exclusive targeting of one specific subtype or isoform can usually be achieved with antisense oligonucleotides. In addition, antisense approaches have the added advantage that oligonucleotides

can be generated even when the mRNA sequences are not full length. This does not, however, imply that all antisense sequences are created equal. On the contrary, like small-molecule drugs, antisense oligonucleotides vary tremendously in their potency, and effort must be expended to identify the most efficacious sequences (1–4).

Antisense experiments are conducted in cell-based systems, in organotypic slice cultures (5), and in animals. Functional effects resulting from antisense inhibition are assessed using cellular, molecular, neurochemical, electrophysiological, and behavioral endpoints. The parameters and issues related to cell-based systems have been addressed by others (6,7). The targeting of proteins in the brain of living animals, however, presents a unique set of issues and challenges, which will be discussed in the following sections.

II. OLIGONUCLEOTIDE CHEMISTRY

Protein concentrations are much lower in cerebral spinal fluid (CSF) than in plasma (8). Phosphodiester oligonucleotides exhibit considerable stability in CSF (9), in contrast to their short half-life in serum. Brain-derived activities of deoxyribonuclease I and endonucleases are also relatively low (10,11), and exogenously administered RNA molecules exhibit some stability in the brain. Vasopressin mRNA injected into the hypothalamus of rats that are genetically deficient in this peptide was stable enough that vasopressin peptide could be translated (12). Phosphodiester oligonucleotides are rarely used in in vivo studies outside of the CNS because of their rapid degradation in the periphery. However, they have been successfully applied to antisense experiments in the brain (13,14) and continue to be widely used for such applications. In spite of their greater stability in the CNS, phosphodiester oligonucleotides are nonetheless degraded (9). Consequently, larger quantities and more frequent administrations of oligonucleotides are required to obtain significant antisense inhibitory effects.

A. Phosphorothioate Oligonucleotides

Phosphorothioate analogs are quite effective at producing antisense inhibitory effects in the brain and many laboratories have employed this chemical analog in their experiments. As discussed in greater detail in the following section, phosphorothioates are significantly more stable than phosphodiesters in tissues of the CNS and can therefore be used at lower concentrations. However, there is increasing evidence that these analogs elicit a variety of CNS-specific toxicities not usually seen with phosphodiester oligonucleotides. Febrile responses (15), induction of inflammatory mediators (16), significant weight loss (17,18), and signs of ill health (chromodacryirrhea, hyperalgesia, decreased motor function, and perineal staining with urine) (17,19) have all been reported. Toxic effects have

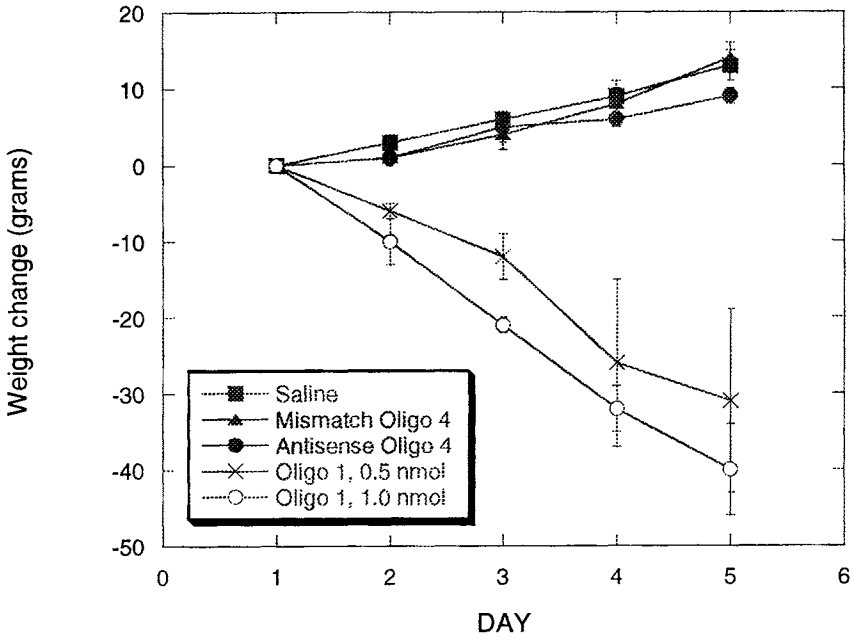


Figure 1 Weight change profile of rats injected with chimeric and phosphorothioate oligonucleotides. A dose of 2.0 nmol of oligonucleotide 4 (antisense and mismatch sequences) was used (17).

also been seen following intrathecal dosing (20). The use of excessive amounts of phosphorothioate or inadequately purified oligonucleotides may be responsible for some of the reported toxicities. In our laboratory, oligonucleotides for in vivo experiments are typically subjected to reverse-phase HPLC or ion-exchange HPLC, cation exchange to sodium, size-exclusion chromatography, and sterile filtration. Oligonucleotide purified in this fashion produced weight loss and symptoms of ill health when administered at doses greater than 1.0 nmol per ventricle per day. Doses of 0.5 nmol of phosphorothioates, which were required to produce a 50% inhibition of the targeted protein, caused weight loss but no other symptoms (17) (Fig. 1). Therefore, the separation between efficacy and toxicity may be quite small with phosphorothioate oligonucleotides.

B. End-Protected Phosphodiesters

Various chemical analogs have been investigated in attempts to circumvent the lack of stability of phosphodiester oligonucleotides and the toxicity of phosphorothioate oligonucleotides. Since digestion by 3′-exonucleases has been shown to

be the major route of oligonucleotide degradation (21), various strategies to protect the 3′ end of phosphodiester oligonucleotides have been examined. Addition of inverted nucleotides at the 3′ end did not appear to significantly protect against nuclease degradation (17). A 5.0-nmol dose of this phosphodiester analog **2**, injected into the lateral ventricle, produced an antisense inhibition of 17% (Table 1). The corresponding phosphorothioate oligonucleotide **1**, at a dose of 0.5 nmol, resulted in a 50% inhibition. Injecting 5.0 nmol of the phosphodiester oligonucleotide twice daily increased the antisense inhibition to 42%. Other laboratories have used one to two phosphorothioate linkages at the 3′ and 5′ ends of phosphodiester oligonucleotides. Such an end-capped oligonucleotide inhibited c-fos immunoreactivity for 2–4 h when injected at a dose of 2.0 nmol into the striatum (22,23). In comparison, an identical dose of phosphodiester oligonucleotide was inactive while the phosphorothioate analog exhibited good activity for up to 10 h (22). These results indicate that protecting the ends of phosphodiester oligonucleotides may confer some resistance against nucleases. Nonetheless, like phosphodiester oligonucleotides, end-protected analogs need to be administered more frequently and at higher doses.

C. 2′-Methoxyribonucleotide-Containing Analogs

Three different chimeric oligonucleotides, each containing six 2′-methoxyribonucleotide phosphodiester residues at either end of the oligonucleotide, were compared (Table 1, **3–5**). The molecules differed from one another in their central region. Oligo **3**, with a central portion consisting of 2′-deoxyribonucleotide phosphodiester bases, was completely inactive (Table 1). A 2.0-nmol dose of oligo **4**, with a 2′-deoxyribonucleotide phosphorothioate central portion, however, produced a 40% inhibitory effect. In distinct contrast to the phosphorothioate oligo **1**, oligo **4** did not cause any weight loss or neurological signs of toxicity in the treated animals (17) (Fig. 1).

D. Antisense Mechanisms in the Brain

Increasing evidence suggests that antisense inhibitory effects associated with 2′-deoxyribonucleotide phosphorothioate–containing oligonucleotides, in cell culture systems, occur through ribonuclease H (RNase H)-type mechanisms (24,25). Neuronal cells in the brain have long been thought to be nondividing, and since RNase H activity is believed to be associated with DNA replication, some neurobiologists have argued that this enzyme may not be expressed in brain tissue. Oligo **5**, containing a central portion of 2′- methoxyribonucleotide phosphorothioate bases, was designed to investigate this issue. This oligonucleotide consists entirely of 2′-methoxyribonucleotide residues, which in several tissue culture studies has been demonstrated to preclude RNase H–mediated antisense mecha-

Table 1 Effect of Chemical Modifications on CRF_2 Receptor Inhibition

Oligo	Chemistry	Dose (nmol)	Frequency	% Reduction
1	TsGsAsCsGsCsAsGsCsGsGsCsAsCsCsAsGsAsCsC	0.5	1×/day	49
2	ToGoAoCoGoCoAoGoCoGoGoCoAoCoCoAoGoAoCoCtca	5.0	1×/day	17
			2×/day	42
3	**ToGoAoCoGoC**oAoGoCoGoGoCoAoCoC**oAoGoAoCoC**	5.0	1 ×/day	2
4	**ToGoAoCoGoC**sAsGsCsGsGsCsAsC**oCoAoGoAoCoC**	2.0	1 ×/day	40
5	**ToGoAoCoGoCsAsGsCsGsGsCsAsCoCoAoGoAoCoC**	2.0	1×/day	8
6	UsGsAsCsGsCsAsGsCsGsGsCsAsCsCsAsGsAsCsC	5.0	1×/day	52
		2.5	1×/day	25

Antisense oligonucleotides of identical sequence, but with different chemical modifications, were injected bilaterally into the lateral ventricle of rats for 5 days. [^{125}I]-sauvagine binding to CRF_2 receptors in the lateral septum was determined by receptor autoradiography. Percent reduction was calculated using binding values from saline-treated animals (17).

2′-Deoxyribonucleotides: capitalized; 2′-methoxyribonucleotides: capitalized and bold; propynyl: capitalized and underlined; 3′-inverted nucleotides with phosphodiester linkages: lower case; phosphodiester: ''o''; phosphorothioate: ''s.''

nisms (26,27). In contrast to oligos **1** and **4**, both of which produced 45–60% reductions in CRF_2 receptor binding and in the CRF_2 receptor mRNA signal (17), a 2.0-nmol dose of oligo **5** had no effect on either measure. These results suggest that the mechanism of antisense inhibition in the brain is consistent with that of an RNase H–type activity. Furthermore, this activity is critical to the antisense inhibition of CRF_2 receptors, as oligonucleotides whose duplexes with RNA are not substrates for RNase H are inactive. The recent finding of ribonuclease H transcripts in the brain (28) lends support to this interpretation.

E. Other Oligonucleotide Chemistries

Oligonucleotides containing C-5 propyne modifications have been reported to be 10-fold more potent than phosphorothioate oligonucleotides with unmodified pyrimidine bases (29). This increased potency was not borne out in in vivo CNS experiments. When a C-5 propyne modified oligonucleotide **6** was administered intracerebroventricularly at a dose of 5.0 nmol per day, a 52% inhibition in CRF_2 receptor binding was obtained. However, the animals lost a significant amount of weight. At 2.5 nmol per day, no weight loss was observed, but antisense inhibition was reduced to 25% (Table 1).

Peptide nucleic acid (PNAs) molecules have been shown to exhibit antisense inhibitory activity in neuronal cells (30). In this report, uptake of PNAs into primary cultures of magnocellular oxytocin neurons was facilitated by a delivery peptide; however, incubation with "naked" PNAs also resulted in cell penetration albeit less efficiently. Interestingly, although unassisted uptake produced considerable punctate staining indicative of sequestration in endosomes, naked PNAs appeared to reduce levels of prepro-oxytocin mRNA to the same levels as peptide-coupled PNAs. PNA antisense molecules conjugated to a delivery peptide and administered intrathecally reduced binding to the galanin type-1 receptor in the dorsal horn of the spinal cord (31). The in vivo data with naked PNAs appears to be somewhat confusing. In one report, naked PNA antisense molecules injected into the brain (1.3 nmol every other day), reduced B_{max} values of the targeted proteins by 40–55% (32). However, a separate study involving naked PNA molecules did not produce any reductions in receptor binding even though the expected behavioral effects were observed (33). PNA molecules do not appear to produce any toxic effects in the CNS (31,33).

Locked nucleic acids (LNAs) are a novel class of nucleic acid analogs that form duplexes with complementary RNA and DNA molecules with high affinity and specificity. These molecules have recently been applied to antisense studies in the CNS (34). Antisense LNAs delivered intracerebroventricularly and intrathecally were efficacious against the δ-opioid receptor in the brain and spinal cord, respectively. These LNA analogs were highly stable in blood, CSF, and bacterial extracts, and did not produce any signs of behavioral or cellular toxicity following in vivo administration.

III. DELIVERY OF OLIGONUCLEOTIDES TO THE CNS

Several parameters limit the efficient transport of molecules across the blood-brain barrier. Polar molecules with high capacity for hydrogen bonding and molecules exceeding a molecular weight threshold of 400–600 Da are generally excluded from the CNS (35). With the exception of PNAs, antisense oligonucleotides are highly charged and their molecular weights typically exceed 6000 Da. Biodistribution and pharmacokinetic studies with phosphorothioate sequences and other oligonucleotide analogs show negligible quantities of oligonucleotide in the brain following various peripheral routes of administration (36–38). Consequently, invasive methods of delivery are required. Intraventricular methods (primarily targeting the lateral and third ventricles) rely on the flow of CSF to distribute the oligonucleotide, principally to periventricular regions. For brain regions remote from the ventricular system, intraparenchymal methods are employed. This approach, however, produces greater tissue injury and hemorrhage. Spinal cord proteins are targeted using intrathecal delivery methods (39).

A. Cellular Stability

While the uptake patterns and extent of distribution of fluorescein-labeled or radiolabeled phosphodiester and phosphorothioate oligonucleotides do not appear to differ greatly, these data need to be considered in the context of the stability of the oligonucleotides. A significant amount of phosphodiester oligonucleotides is degraded in a relatively short period. A ladder of fragments was observed when phosphodiester oligonucleotides were extracted after a 15-min residence time in the brain (40). In addition, when detected using in situ hybridization probes complementary to the injected oligonucleotides, the phosphodiester signal was almost imperceptible at the 24-h time point (41,42). While these data do not necessarily preclude the use of phosphodiester oligonucleotides (these analogs may be quite suitable for studies involving proteins with short half-lives), they highlight the importance of higher quantities and more frequent administration of these analogs. In contrast, extraction experiments (43,44) and in situ hybridization techniques (41,42) indicate that phosphorothioate analogs are stable for at least 24 h in the brain.

B. Intraventricular Administration

Oligonucleotide diffusion and distribution into the brain parenchyma occurs rapidly, and appears to be dependent on dose and volume of injection, but independent of time. Experiments to compare the extent of oligonucleotide diffusion reveal that maximal tissue penetrance is achieved within 1 h after injection (44). While staining of the corpus callosum has been observed (44), white matter tracts including the anterior commisure appear to act as barriers to oligonucleotide dis-

tribution (44,45). A single injection of 20 nmol of phosphorothioate oligonucleotides into the lateral ventricle produces significant penetration of periventricular regions and brain areas in contact with the subarachnoid space (44). However, at doses less likely to produce toxic effects, brain distribution is far more limited. Nonetheless, efficient penetrance to the striatum, septum, and hippocampus, and, to a lesser extent, to hypothalamic regions proximal to the third ventricle can be achieved with 2–5-nmol doses of oligonucleotide (41,44). For more efficient diffusion into hypothalamic tissue, third ventricle injections are often employed. CSF bulk flow is believed to be the major clearance mechanism for intraventricularly administered oligonucleotides. Total CSF volume in a rat is approximately 250 μl (46,47) and the synthesis rate of CSF by the choroid plexus has been estimated to be 180 μl/h (48). Depending on the target site, doses of phosphorothioate oligonucleotides ranging from 0.5 nmol to 5.0 nmol have been reported to produce the desired antisense effects.

C. Intraparenchymal Administration

Distribution studies of oligonucleotide injected into the striatum (43,44), basolateral amygdala (42), and dorsal hippocampus (41) have been reported. Lower quantities of oligonucleotide are required for intraparenchymal administration. Two nanomoles of phosphorothioate oligonucleotide injected into the striatum labeled a roughly spherical region of radius 2–3 mm (44). A 10-fold lower amount of oligonucleotide produced a diffusion sphere with radius of about 1.2 mm. Twenty-four hours after injection, 6% and 4.7% of the total injected dose of oligonucleotide was recovered from the striatum (44) and amygdala (42), respectively. The concentration of oligonucleotide in the amygdala tissue was estimated to be approximately 3.8 mM at the 24-h time point (42). Significant amounts of oligonucleotide have also been observed in perivascular spaces of the brain (40,44), suggesting that uptake by the capillary endothelium is a possible route of oligonucleotide clearance (9).

D. Intrathecal Administration

In contrast to the many studies on oligonucleotide distribution and tissue penetration in rodent brain, few studies have been conducted to address similar issues in the spinal cord. Oligonucleotides are delivered to this region of the CNS through catheters implanted in the subarachnoid space of the spinal cord. The rostral-caudal extent of diffusion of bromophenol blue dye administered intrathecally is dependent on injection volume (39). A frequently used volume of 10 μl results in a spread of 2–4 cm around the catheter tip. In vivo superfusion with a radiolabeled end-capped phosphodiester oligonucleotide showed radioactivity in the dorsal horn at 1 h, with the signal extending to the ventral horn by 6 h (49).

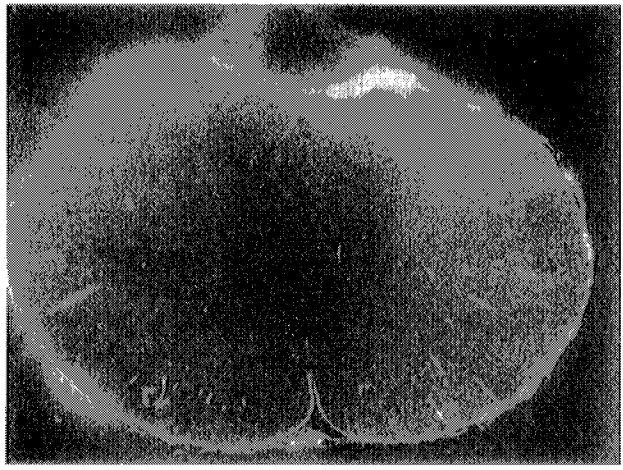
Studies were not conducted to determine the integrity of the oligonucleotide at these time points. In our laboratory, preliminary studies using fluorescein-labeled phosphorothioate oligonucleotides showed uptake into the white and gray matter of the spinal cord, as well as the dorsal root ganglia (Fig. 2). Drugs or dye molecules administered intrathecally do not diffuse into the brain (39), and this is likely to be true for oligonucleotides as well. Doses of 4–6 nmol of phosphodiester oligonucleotide administered twice daily have resulted in significant antisense inhibition in the spinal cord.

E. Bolus Administration Versus Infusion Pump

Oligonucleotides can be administered through bolus injections or by osmotic mini pumps. Pumps infusing 3.0 mM concentrations of phosphorothioate oligonucleotides at a rate of 1 μL/h (72 nmol/day) into the lateral ventricle were lethal to rats (9). Animals appeared to tolerate a 1.5-mM dose although weight loss and other signs of toxicity may have been present. Under steady-state conditions produced at the lower dose, the clearance half-life of phosphorothioate oligonucleotides was determined to be 17 min, and oligonucleotide concentration in the CSF was estimated to be approximately 1.5 μM (9). When oligonucleotides are delivered intracerebroventricularly, large quantities (greater than 25 nmol of phosphorothioate per day) of oligonucleotide are required to achieve significant antisense inhibition (14,50). A direct comparison between the bolus and pump routes of administration was conducted using an antisense sequence designed to target the corticotropin-releasing factor type 2 receptors in the lateral septum. While bolus intraventricular injections of 0.5 nmol/day of the oligonucleotide reduced CRF_2 receptor binding by approximately 55% after 5 days, minipump delivery of 12 nmol of oligonucleotide per day for 4 days produced only a 15% reduction in receptor binding (Ho, unpublished data).

F. Cellular Localization

Double-labeling experiments indicate that oligonucleotides are predominantly taken up by neurons, and to a lesser extent by astrocytes and microglia (43,51). The cellular localization of oligonucleotides is time-dependent. At early time points (1–6 h), oligonucleotide has been detected as diffuse staining in the cytoplasm and in the nucleus (40,43,44). At longer intervals (up to 24 h) following oligonucleotide delivery, the nuclear signal is no longer apparent, while a punctate pattern of localization appears in the cytoplasm. Using electron microscopy, Sommer et al. have characterized these punctate structures as intracellular vesicles (43). Oligonucleotide has been detected in dendritic processes (43,45), and anterograde transport of oligonucleotides to distant terminal fields has also been reported. Twenty-four hours following injection of 1 nmol of phosphorothioate



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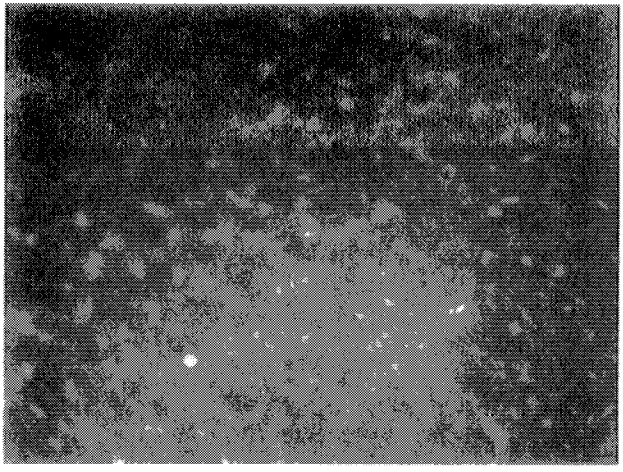
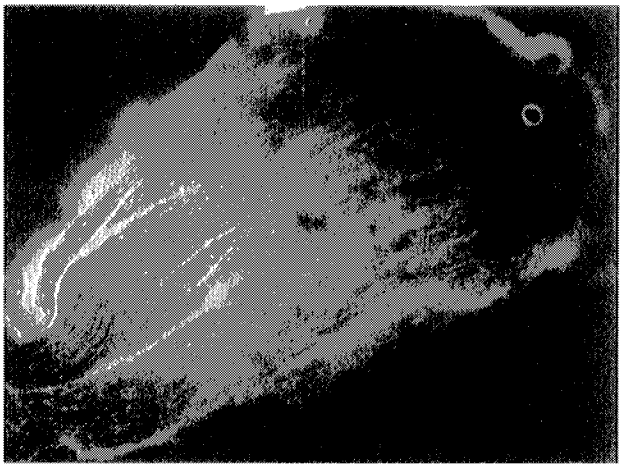


b

Figure 2 Penetrance of a fluorescein-labeled phosphorothioate oligonucleotide 3 h after intrathecal delivery. Ten nanomoles of oligonucleotide (in 10 μl of saline) was injected into the subarachnoid space in the vicinity of the T8 vertebrae. (a) Spinal cord at the level of the T8 vertebrae, 2.5× magnification. (b) Uptake in the gray matter, 20× magnification. (c) The L5 dorsal root ganglion, 2.5× magnification.



c

Figure 2 Continued

oligonucleotide in the striatum, significant oligonucleotide labeling was observed in the ipsilateral substantia nigra (43). Characterization of the intracellular localization of oligonucleotides is prone to artifacts; therefore, care must be exercised when conducting such studies (44,52). Although the mechanisms contributing to the distinct, time-dependent patterns of intracellular distribution are not currently understood, antisense activity appears to correlate with a nuclear and diffuse cytoplasmic localization of oligonucleotide. Using c-fos antisense oligonucleotides, Grzanna et al. showed that robust inhibition of c-fos immunoreactivity occurs at early time points when oligonucleotides are largely localized in the nucleus and cytoplasm, but not in vesicles. At 12 h postinjection and beyond, when high concentrations of oligonucleotides are sequestered in vesicular structures, there is minimal c-fos antisense inhibitory activity (44). Acute time course studies on vasopressin (53) and the angiotensin type 1 receptor (R. Sakai, personal communication) indicate that optimal antisense inhibition in these systems occurs within 3–8 h following oligonucleotide delivery.

G. Crossing the Blood-Brain Barrier

The ability to target discrete brain nuclei can be useful in that it allows the function of proteins in specific brain regions to be studied. In addition, local administration of oligonucleotides can be used to differentiate between the roles of pre- and postsynaptic receptors expressed in the same brain region (54). However,

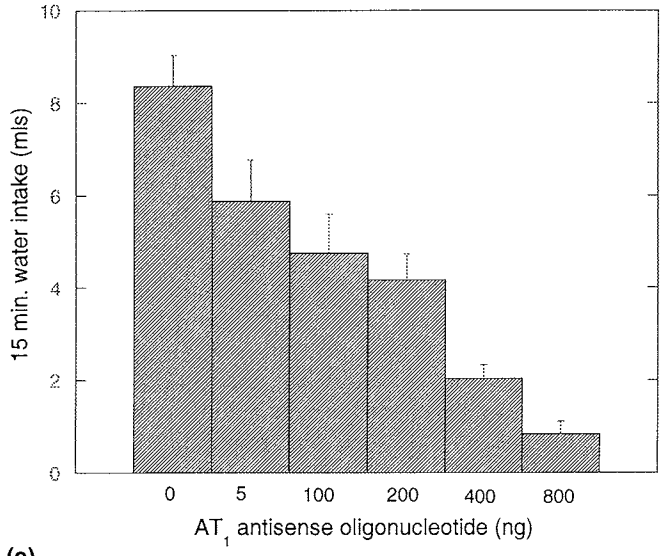
brainwide distribution of antisense oligonucleotides is highly desirable for functional genomics studies. CNS proteins are frequently expressed in multiple brain regions. While inhibition of a protein in one brain region may produce therapeutic benefits, inhibition of the same protein in another brain region may give rise to undesirable side effects. Transport across the blood-brain barrier would provide the most efficient delivery of oligonucleotides to the entire brain. Four hundred miles of capillaries have been estimated to be present in the human brain and the surface area of the network of capillary endothelium is around 12 m^2 (55). Having an antisense molecule capable of penetrating the blood-brain barrier would remove a significant obstacle in the development of antisense therapeutics for treating CNS diseases.

PNAs have previously been reported to have negligible transport across the blood-brain barrier (56). Nonetheless, using an intraperitoneal route of administration, an antisense PNA molecule directed against the neurotensin type 1 receptor mRNA reduced neurotensin receptor binding by 40% in the brain (57). A single 10-mg/kg dose of PNA suppressed the antinociceptive and hypothermal effects of exogenous neurotensin. The presence of PNA molecules in the brain was demonstrated using a gel mobility shift assay (58). If PNAs can be verified by other laboratories to cross the blood-brain barrier, this would represent a significant advance for the antisense CNS field.

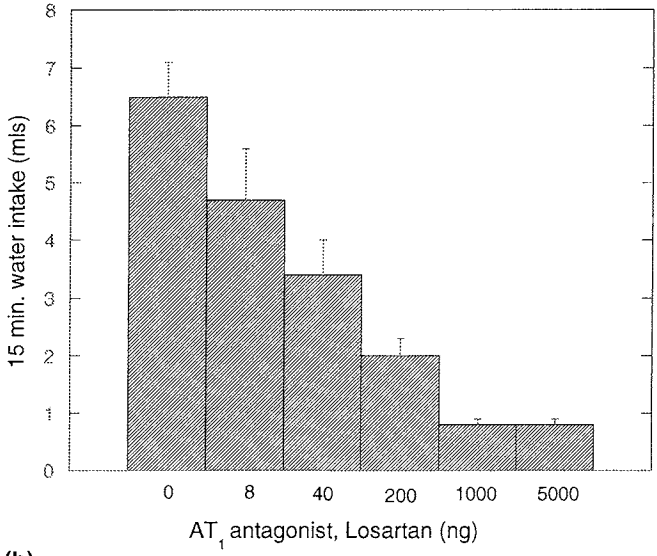
IV. TARGET VALIDATION TOOL

Selective pharmacological ligands are needed to reveal the role of a protein in normal physiology and in the pathophysiology of a disease state. Antisense inhibition of a specific protein should produce pharmacological effects that are equivalent to effects obtained with an inhibitor of that protein. This was verified by studies on the angiotensin type 1 (AT_1) receptor.

Multiple, diverse oligonucleotides that produce sequence-specific antisense inhibition of AT_1 receptors in cells (25) and in vivo (2) have been identified. Antisense oligonucleotides injected into the third ventricle reduced [^{125}I]angiotensin II binding in the hypothalamus, thalamus and septum, and this reduction correlated directly with a decrease in AT_1receptor density (59). Antisense treatment did not change the K_d of the AT_1 receptor for angiotensin II. Functional experiments exploited the fact that maintenance of body fluid homeostasis by central angiotensin II occurs through AT_1 receptors. Intracerebroventricular administration of angiotensin II in rodents elicits an immediate and profound drinking response. When AT_1 antisense oligonucleotides were evaluated in this functional paradigm, the oligonucleotides potently inhibited the drinking response in a dose-dependent manner (Fig. 3a). Losartan, an antihypertensive drug, is highly selective for the AT_1 receptor and displays a 10,000-fold greater affinity for the AT_1 subtype over



(a)



(b)

Figure 3 Antisense oligonucleotides elicit the same physiological responses as receptor-specific small-molecule antagonists. (a) Dose response of an AT_1 antisense oligonucleotide on angiotensin II–induced drinking in the rat. (b) Dose response of Losartan, an AT_1 receptor–specific antagonist, on angiotensin II–induced drinking.

the closely related AT_2 receptor. In the drinking assay, central administration of Losartan (which does not cross the blood-brain barrier) also dose-dependently blocked drinking (Fig. 3b). These results demonstrate that antisense oligonucleotide treatment produces effects that accurately reflect the pharmacological properties of small-molecule drugs. In recent years, antisense oligonucleotide approaches have been used to understand the role and validate the importance of CNS proteins in several disease states. Some of these studies have been chosen for review in the following section.

A. Alzheimer's Disease

This progressive disease is characterized by the abnormal accumulation of plaques of β-amyloid peptide (Aβ) and neurofibrillary tangles in the brain of affected individuals. Amyloid plaque formation is believed to be an important contributor to the neurodegeneration and dementia seen in Alzheimer's patients. The search for potential targets for the therapeutic intervention of this disease has centered on the production, and degradation and clearance, of Aβ, as well as the mechanisms by which Aβ mediates its pathogenic effects.

Formation of Aβ requires the proteolytic cleavage at two different sites of a transmembrane protein called amyloid precursor protein (APP). The two enzymes involved in these cleavages are called β- and γ-secretase (Fig. 4), and until recently, their molecular identity was unknown. Several groups have recently reported the cloning and characterization of an aspartic protease whose properties are consistent with those of the β-secretase enzyme. In cells expressing the Swedish mutant form of APP, a 70–75% antisense inhibition of this enzyme caused a 60–70% reduction of the N-terminal APP cleavage fragment (APPsβ), which results directly from β-secretase activity (60) (Fig. 4). Aβ production, which de-

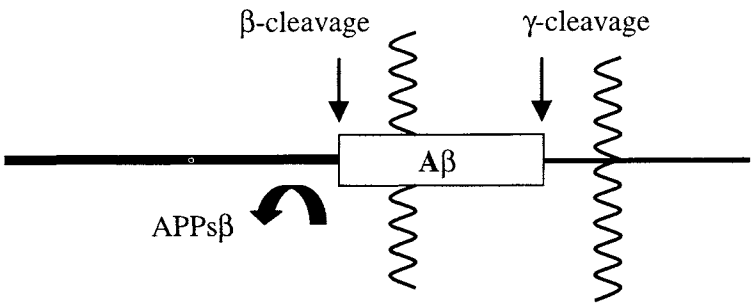


Figure 4 Schematic structure of APP. Cleavage at the β site releases the APPsβ fragment. Cleavage at the β- and γ-sites produces Aβ. Wavy lines represent the cell membrane.

pends on both β- and γ-secretase activity, was reduced by 30–40%. Antisense studies conducted by another laboratory produced similar results in three different cell lines containing wild-type APP or the Swedish mutant form of APP (61). Together with overexpression experiments, these data strongly suggest that activity of the cloned enzyme is predominantly responsible for cleavage of APP at the β site. In addition, inhibition of the enzyme reduces Aβ production, providing important evidence that this protein may be an important drug development target for the treatment of Alzheimer's disease.

Earlier studies have demonstrated that aggregated Aβ induces caspase-dependent apoptosis in neuronal cultures. A broad-spectrum caspase inhibitor blocked Aβ-induced cell death. There are 14 known mammalian caspases, and selective inhibitors and substrates are not available for all of them. Caspase-3 activity was dramatically induced in hippocampal cultures and PC12 cells following Aβ treatment, but a caspase-3 inhibitor did not block the cytotoxic effects of Aβ. This result was confirmed by antisense oligonucleotide experiments (62). However, caspase-2 antisense oligonucleotides prevented the development of Aβ-induced cytotoxicity in primary hippocampal neurons, primary sympathetic neurons, and neuronal PC 12 cells (62). Antisense inhibition of caspase-1 was without effect. The requirement for caspase-2 in Aβ-induced cell death was confirmed using cultured sympathetic neurons taken from caspase-2 null mice.

B. Pain

Current therapies for pain treatment are inadequate for the management of many types of pain and there is a great need for novel analgesics. The multietiological nature of pain implies the existence of many different pain mechanisms and pathways. At least 15 different neurotransmitters/neuromodulators are known to be involved in the spinal cord and ascending and descending pathways associated with pain perception. As a result, a variety of proteins may be suitable targets for the intervention of pain and no single drug is likely to be effective against all types of pain. Some pain targets are expressed in the peripheral nervous system, while others are found in the brain, spinal cord, and dorsal root ganglia (DRG). Among the recently discovered targets for pain are proteins such as the vanniloid type-1 receptor, the $P2X_3$ purinergic receptor, and the PN3/SNS and NaN/SNS2 tetrodotoxin-resistant (TTX-R) sodium channels, all of which have highly restricted patterns of expression. Drugs to these protein targets may offer the potential of producing therapeutic benefits with minimal side effects.

Sodium channel expression in DRG neurons changes dramatically following tissue or nerve injury. Inflammation, in particular, produces an increase in the expression of TTX-R sodium currents. Two TTX-R sodium channels, PN3/SNS and NaN/SNS2, have recently been cloned, and their potential role in the pathophysiology of chronic pain states has been evaluated. In in vitro studies,

incubation of primary cultures of DRG neurons with PN3 antisense oligonucleotides significantly reduced the TTX-R sodium current density as compared to mismatch and sense oligonucleotides (63). In contrast, all three oligonucleotides had no effect on the TTX-sensitive sodium current density and on mean cell body capacitance, which provides a measure of cell body size. Intradermal injections of prostaglandin E_2 into the paws of rats produces mechanical hyperalgesia (excessive pain due to tissue injury). Intrathecal administration of the PN3 antisense oligonucleotide increased the nociceptive threshold and blocked the prostaglandin-E_2 induced hyperalgesia (63).

In a separate study, intrathecal administration of PN3 phosphodiester antisense oligonucleotides (twice a day for 2 days) produced a significant decrease in the immunohistochemical signal of the PN3 channel in the DRG, in contrast to saline or mismatch oligonucleotide-treated animals (64). The immunohistochemical signal for the TTX-sensitive sodium channels, PN1 and PN4, was unaffected. Peripheral nerve injury resulting from spinal nerve ligation produces hyperalgesia and allodynia (pain produced by nonnoxious stimuli). Antisense treatment for 5 days prevented the development of tactile allodynia and thermal hyperalgesia, which developed subsequent to the cessation of antisense treatment. Similar results were obtained in a model for chronic inflammatory pain induced by an injection of complete Freund's adjuvant to the hindpaw. These data strongly indicate a role for PN3 channels in the pathophysiology of neuropathic pain. Antisense treatment did not, however, have any effect on sensory thresholds in sham-operated animals. This suggests that PN3 is unlikely to be involved in normal nociceptive function and that inhibitors of PN3 channels should not interfere with normal pain perception.

Antisense oligonucleotides to the other TTX-R channel, NaN/SNS2, did not block the tactile allodynia and thermal hyperalgesia induced by spinal nerve ligation (64), implying that NaN/SNS2 does not play a prominent role in pain perception following peripheral nerve injury.

The neuropeptide galanin influences a wide range of physiological functions including feeding and pain. It is widely expressed in the CNS and in peripheral tissues, and its effects are mediated through three galanin-binding receptors. In spinal cord, galanin has been proposed to have antinociceptive effects. Peripheral nerve injury induces galanin expression in the DRG and the dorsal horn of the spinal cord where an abundance of galanin type 1 (GAL_1) receptor mRNA has been detected. The galanin type 2 receptor is also expressed in the DRG. In the absence of potent antagonists capable of discriminating between these receptor subtypes, antisense PNA molecules (conjugated to a delivery peptide) were used to target the GAL_1 receptor. When administered intrathecally, the antisense PNA reduced galanin binding in the dorsal horn by about 40% (31). Pain impulses are transmitted through unmyelinated C fibers and direct electrical stimulation of unmyelinated C nociceptors produces a burning or aching pain. Conditioning

stimulation of C fibers produced a brief facilitation of a nociceptive flexor reflex and intrathecal galanin dose-dependently inhibited this facilitation. Antisense inhibition of GAL_1 receptors enhanced this facilitation, showing that the GAL_1 receptor is involved in mediating the inhibitory effects of galanin on C-fiber conditioning stimulation.

C. Affective Disorders

Drugs that are effective in treating depression—monoamine oxidase inhibitors, tricyclic antidepressants and selective serotonin reuptake inhibitors—act on serotonergic and noradrenergic systems of the brain. While these drugs produce immediate effects on their molecular targets, there is usually a lag of several weeks before significant therapeutic benefits are observed. Treatment with a variety of antidepressants produces a significant down-regulation (up to 50%) of the serotonin 2a ($5HT2_a$) receptor in the cortex, and the time course of this down-regulation appears to correlate with the onset of clinical efficacy. To determine whether $5HT2_a$ receptors are involved in the development of antidepressant effects, antisense oligonucleotides were used to down-regulate the $5HT2_a$ receptor. Using a large volume of vehicle (5μL), oligonucleotide was effectively delivered to the subarachnoid spaces and cortex of mice following intracerebroventricular administration. A 40–50% decrease in receptor binding was observed in the frontal, parietal, and piriform cortices of antisense-treated animals (65). Animals were evaluated in the Porsolt forced swim test, an animal model that has been used to identify drugs with antidepressant activity. Mice were placed in a cylinder of water and the amount of time the animals were immobile (as opposed to swimming) was measured. Immobility under these test conditions is thought to reflect a state of despair and a number of antidepressant drugs and treatments have been shown to reduce immobility time in this test. Antisense oligonucleotide–treated animals were significantly less immobile than saline or mismatch oligonucleotide–treated animals and the decrease in immobility time produced by the antisense oligonucleotide was similar in magnitude to the reduction obtained with mianserin, an atypical antidepressant. MDL 100,907, a recently developed $5HT2_a$-selective antagonist, also produced similar results. Evaluation in a locomotor activity test showed that antisense treatment did not increase motor function in antisense-treated animals. These results suggest that down-regulation of $5HT2_a$ receptors alone is sufficient to produce antidepressant-like effects in mice in the forced swim test.

Extensive studies have established the importance of corticotropin-releasing factor (CRF) in regulating the hypothalamic-pituitary-adrenocortical (HPA) system and in mediating the physiological and behavioral responses to stress. Pathophysiology of CRF systems is believed to play an important role in several psychiatric disorders including anxiety and depression. Two high-affinity CRF

receptors have been characterized. CRF_1 receptor function has been studied using transgenic knockouts, antisense oligonucleotides, and small-molecule antagonists. Data from all three approaches indicate that CRF_1 receptors mediate the anxiogenic effects of CRF and CRF_1 receptor antagonists are currently being evaluated clinically. Far less is known about the CRF_2 receptor, which was identified more recently. The abundance of CRF_2 receptor expression in limbic regions (brain areas important in mediating emotional responses including fear, anxiety, and aggression) suggests that this receptor subtype may also play a role in mediating the anxiogenic effects of CRF.

Intracerebroventricular administration of CRF_2 receptor antisense oligonucleotides reduced CRF_2 receptor binding in the lateral septum by 70%. Functional consequences of CRF_2 receptor inhibition were determined in freezing models of anxiety. Rodents adopt a characteristic freezing posture in response to aversive stimuli and the duration of this freezing behavior was used as a measure of the animals' level of anxiety. In the acute anxiety test, where freezing was measured immediately following an aversive stimulus, antisense-treated rats exhibited a significant reduction in freezing duration (3). In the conditioned anxiety test, the aversive stimulus was given prior to the 7-day dosing period. On the day after the last dose, animals returned to the test apparatus in which the aversive stimulus had been previously administered immediately froze. However, the total freezing duration of antisense-treated rats was only half that of vehicle or mismatch oligonucleotide–treated animals. Adrenocorticotropin (ACTH) levels were significantly reduced only in antisense-treated animals. Further studies conducted ruled out decreased pain sensitivity, increased motor function, or impaired learning or memory as possible causes of reduced freezing duration, indicating that antisense-treated animals were most likely exhibiting reduced anxiety. When the dosing period was cut back to 5 days, which reduced the antisense inhibition to 50%, robust anxiolytic effects were no longer seen, suggesting that antisense inhibitory effects greater than 50% are required to reveal a functional role for this receptor.

D. Obesity

The regulation of appetite, satiety, and feeding behavior by the hypothalamus is complex and involves multiple interconnected hypothalamic regions and many neurotransmitters and neuropeptides. Neuropeptide Y (NPY), a 36-amino-acid peptide, is the most abundant neuropeptide in the brain and is believed to be one of the key components in the regulation of food intake. Central administration of NPY produces a potent stimulation of feeding behavior, which can be blocked by antisense oligonucleotides targeted against the neuropeptide. NPY antisense oligonucleotides injected into the arcuate nucleus, a brain area that synthesizes NPY, decreased feeding, fat, and carbohydrate consumption (13). Using a fasting-induced food intake paradigm, antisense oligonucleotides to NPY produced a 40% decrease in basal feeding (66).

Five distinct NPY receptors have been cloned and characterized. The Y1 and Y5 receptors are thought to be the most likely receptors mediating the orexigenic effects of NPY. Early pharmacological data had implicated the Y1 receptor in feeding behavior. However, several antisense studies against this receptor produced conflicting results. The data on the Y5 receptor are less confusing. Two different Y5 antisense sequences produced significant decreases in basal feeding in rats that had been fasted (66). Scrambled, sense, and mismatch controls were without effects. In addition, although Y5 antisense oligonucleotides blocked the feeding induced by exogenously administered NPY, the oligonucleotides were ineffective at blocking feeding induced by galanin administration. This last experiment demonstrates that the suppression in feeding does not arise from a nonspecific toxicity effect. These findings have been substantiated by a second Y5 antisense study (67). In general, antisense studies on obesity targets must be designed and conducted with caution owing to the fact that weight loss and decreases in feeding are known nonspecific consequences of oligonucleotide toxicity.

V. CONCLUSIONS

There is little doubt that antisense oligonucleotides have an important role to play in the elucidation of protein function in the CNS. In the past few years, significant progress has been made toward optimizing the approach for application to the living brain. With the human genome sequencing project nearing completion, there is an even greater need for tools that are capable of uncovering biological function. In the pharmaceutical area, such tools are important for determining which of many proteins should serve as drug discovery targets. The construction of transgenic mice overexpressing or lacking a particular gene product is a well-established approach used for understanding protein function. Antisense oligonucleotides, however, have several advantages over knockout approaches. The generation of transgenic mice requires a significant investment in time and data interpretation is sometimes complicated by compensatory mechanisms that can develop in response to the transgene. The antisense approach is not without its own pitfalls and a clear understanding of the many parameters that impact on an antisense study is important. Figure 5 summarizes these factors and provides relevant references that may be helpful to the novice antisense researcher in planning experiments.

Refinement of antisense oligonucleotides in several areas would further enhance their utility as a CNS tool. More oligonucleotide chemistries with greater stability in biological millieu but with limited toxicity, and which can be easily synthesized and handled, are needed. The ability to achieve brainwide oligonucleotide penetrance, possibly through passage across the blood-brain barrier, would allow the overall effects of specific protein down-regulation to be assessed. Considerable amounts of oligonucleotide in brain tissue are trapped in vesicles. Use

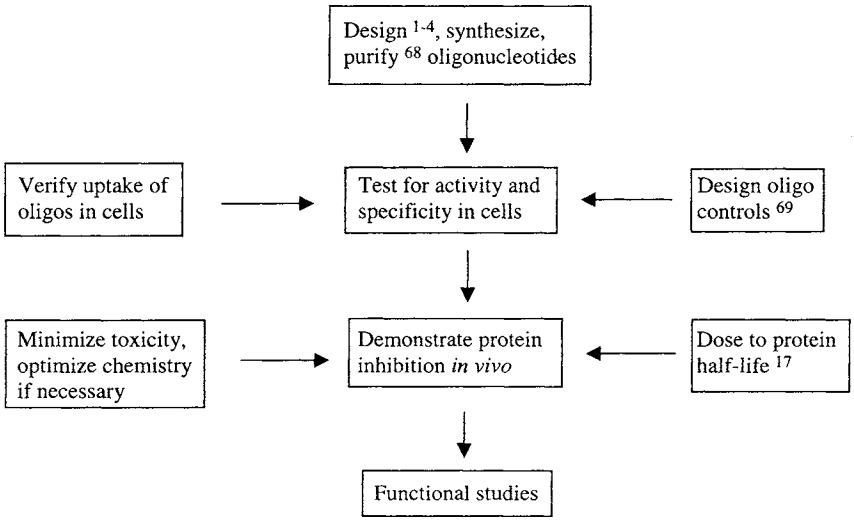


Figure 5 Flow chart of steps in an antisense experiment.

of cationic lipids or related uptake agents that could facilitate oligonucleotide release from such compartments would extend the time course of activity, lower the doses required, and make them more effective agents. The overwhelming majority of antisense experiments involve down-regulation of the targeted protein. Kole, however, has shown that splicing pathways can be modulated with appropriately modified oligonucleotides. Diverting splicing from one pathway to another can result in down-regulation of one protein while up-regulating the expression of a related isoform. Isoforms of CNS proteins could potentially be studied using such an approach. Antisense oligonucleotides are powerful tools for studying protein function. The development of new chemistries and new mechanisms of action, and the increasing number of reports of carefully designed and executed experiments, should serve to firmly establish the general applicability and utility of this technology in biological research.

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20

Antisense Approach to Isoform-Specific Blockade of Acetylcholinesterase

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I. OVERVIEW

Specific inhibition of nervous system enzymes or receptors is often difficult to achieve pharmacologically, especially where the target protein is a particular subtype within a family of closely related gene products. Antisense oligonucleotides attack unique nucleotide sequences rather than three-dimensional protein structures. Thus, they offer a powerful tool to discriminate between closely related proteins derived from homologous genes, polymorphic alleles, or alternative splicing products. The acetylcholine-hydrolyzing enzyme, acetylcholinesterase (AChE), is the molecular target of approved drugs for Alzhiemer's disease (AD) and myasthenia gravis (MG). However, novel findings implicate alternative splicing variants of AChE in the complex etiology of diseases such as AD and MG. Despite the large arsenal of anti-AChE drugs, AChE inhibitors are targeted toward an active site shared by all isoforms. Therefore, isoform-specific inhibitors are not likely to become available in the near future. Thus, the putative roles of different AChE isoforms in health and disease emphasize the potential contribution that antisense technology can make toward improved understanding and strategic approaches to anti-AChE therapeutics.

II. THE NEED FOR SUBTYPE-SPECIFIC PROTEIN TARGETING

The vast complexity of the mammalian central nervous system (CNS) is facilitated by the large variety of neurotransmitters and neurotransmitter receptors in the brain (Barnard, 1988). Molecular heterogeneity in the CNS is generated, in part, by homologous genes, alternative splicing, and combinations of nonidentical subunits that generate heterooligomeric complexes. Thus, closely related receptor isoforms may possess diverse properties with regard to ligand affinity, channel kinetics, or spatiotemporal expression. Moreover, alternative isoforms may be preferentially expressed under various physiological conditions or states of disease (Xie and McCobb, 1998). The same is true for enzyme isoforms catalyzing the various biochemical reactions allowing the brain to maintain homeostasis and respond to external stimuli. The role that specific neurotransmitter or receptor subtypes, or isozymes play in behavior, health, and disease emphasizes the importance of selective drug targeting. Nevertheless, isoform-specific pharmacological inhibitors are often elusive. For that reason, antisense oligonucleotides have become an attractive alternative to classic pharmacology for both basic brain research and drug development.

III. EXPLOITING THE HIGH SPECIFICITY OF ANTISENSE OLIGONUCLEOTIDES

Antisense oligonucleotides have been used to study various families of CNS proteins, including ion channels, neurotransmitter receptors, neuropeptides, and enzymes. Many of these studies have been performed in vivo, using stereotaxic injections or infusions through a surgically implanted cannula. For example, over 40 different potassium channels have been identified in the mammalian CNS. Despite their diverse electrophysiological characteristics, the transmembrane domain of potassium channels has been highly conserved through evolution, making the various subtypes difficult to distinguish pharmacologically. Using multiple intracerebroventricular administrations of isoform-specific antisense oligonucleotides, the ATP-dependent A-type potassium channel Kv1.4 was selectively inactivated in rats, and its role in long-term potentiation and spatial memory distinguished from that of the late-rectifying potassium+ channel Kv1.1 (Meiri et al., 1997, 1998). Similarly, antisense oligonucleotides were used to discriminate between members of the D2 class of dopamine receptors. Since the K_Ds for most agonists and antagonists toward different members of this receptor family are within one order of magnitude, pharmacological discrimination between them is poor. On the other hand, antisense oligonucleotides elicited selective, physiologically significant reductions in either D_2 or D_3 autoreceptors in dopaminergic neu-

rons in the substantia nigra of treated rats (Tepper et al., 1997). An interesting suggestion made in that study was that the limited diffusion of oligonucleotides following intraparenchymal injection can be exploited to specifically target either presynaptic or postsynaptic neurons, an option not afforded by conventional drugs. Indeed, highly specific regional targeting of antisense oligonucleotide effects in the brain has been observed by others (Lamprecht et al., 1997; Lane Ladd et al., 1997). Nevertheless, it should be noted that distant transport of a minor fraction of injected oligonucleotides along projection pathways has been reported (Sommer et al., 1998). In the case of the peptide neurotransmitters neuropeptide Y and galanin, antisense oligonucleotides have been used to down-regulate both the neurotransmitters themselves and specific receptor subtypes (Kalra et al., 2000).

Classification and characterization of the opioid receptors mu, delta, and kappa has been greatly assisted by antisense technology (Pasternak and Pan, 2000). Selective oligonucleotides blocking behavioral or analgesic responses helped map the separate opiate receptors, while oligonucleotides targeting specific exons within a subtype have provided evidence for alternative splicing and additional levels of subtype complexity. For example, oligonucleotides targeting five different exons in the mu opioid receptor were able to discriminate between analgesic effects mediated by endomorphin-1 and morphine (Sanchez-Blazquez et al., 1999). Antisense oligonucleotides targeting specific exons in the neuronal nitrous oxide synthetase gene were similarly employed to differentiate the actions of two isozymes derived by alternative splicing (nNOS-1 and nNOS-2) on morphine analgesia (Kolesnikov et al., 1997). Cyclic nucleotide phosphodiesterases (PDE), some families of which may generate as many as 15 splice variants, have been suggested as another potential area for applied antisense technology (Epstein, 1998). The high specificity of antisense oligonucleotides and the ability to discriminate between closely related gene products made antisense technology a natural tool in approaching the complex biology and therapeutic centrality of acetylcholinesterase.

IV. CURRENT AChE PHARMACOLOGY

Acetylcholinesterase (EC 3.1.1.7) is the enzyme responsible for maintaining tight regulation of neurotransmission at cholinergic synapses by hydrolyzing acetylcholine (ACh). Rapid hydrolysis of ACh acts to reduce the concentration of neurotransmitter at the synapse, preventing overstimulation and tetanic excitation of the postsynaptic nerve or muscle. For this reason, AChE is the target protein of numerous agricultural pesticides and chemical warfare agents (Soreq and Zakut, 1993; Taylor, 1996). For the same reason, however, controlled use of AChE inhibitors has come to play a leading role in therapeutic strategies designed to augment the cholinergic system. The rationale behind clinical use of AChE inhib-

itors is that inactivation of AChE prolongs the half-life of released ACh, thereby enhancing postsynaptic signals. Indeed, anti-AChE therapies have served the medical community relatively well. Nevertheless, inherent limitations restrict the therapeutic utility of pharmacological AChE inhibitors.

V. LIMITATIONS OF AChE PHARMACOLOGY

A. Specificity

One of the principle challenges of classic anti-AChE pharmacology was to overcome the structural and functional homology between AChE and the closely related enzyme butyrylcholinesterase (BChE, EC 3.1.1.8) in designing specific inhibitors (Schwarz et al., 1995; Taylor et al., 1995). AChE and BChE are carboxylesterase type B serine hydrolases sharing 52% identical amino acids (Soreq et al., 1990). Although both enzymes hydrolyze ACh, AChE is very specific in its substrate recognition, while BChE is much more permissive. Owing to its relaxed substrate specificity, BChE interacts to some extent or another with most AChE inhibitors. Thus, the high concentration of plasma BChE has been suggested to serve a scavenging function, protecting AChE from various naturally occurring AChE inhibitors (Loewenstein-Lichtenstein et al., 1995). By the same token, however, BChE will act to scavenge therapeutic anti-AChE drugs, elevating the dose necessary to achieve inhibition at the target organ. Moreover, as BChE is characterized by a large number of allelic polymorphisms with different affinities for anti-AChE compounds, genetic variation was predicted to introduce individual differences in sensitivity to anti-AChE drugs and poisons (Loewenstein-Lichtenstein et al., 1995). The AChE/BChE specificity problem has been partly, but not completely overcome with the development of AChE inhibitors demonstrating up to 1000-fold preference for AChE over BChE (Bryson and Benfield, 1997). Nonetheless, the insignificant sequence homology displayed by AChE and BChE at the level of the gene makes these proteins completely distinguishable, nonoverlapping targets using antisense technology. Furthermore, the question of specificity with regard to AChE inhibitors took an unforseen leap in complexity as data describing the nature and potential consequences of 3′ altenative splicing of AChE mRNA became available (Grisaru et al., 1999).

Cloning of the mammalian ACHE gene revealed three forms of mature mRNA encoding AChE, together accounting for the multitude of known AChE isoforms (Ben Aziz Aloya et al., 1993; Li et al., 1991) (Fig. 1). The intended target of all AChE inhibitors is the ''synaptic'' AChE-S isoform bearing a unique, 40-amino-acid C-terminal peptide encoded by exon 6. A second, erythrocyte-bound form, AChE-E, is encoded by mRNA carrying alternative exon 5. AChE-E presumably participates, together with BChE, in scavenging blood-borne inhibitors, including drugs of abuse (Salmon et al., 1999). Until recently, AChE-S and

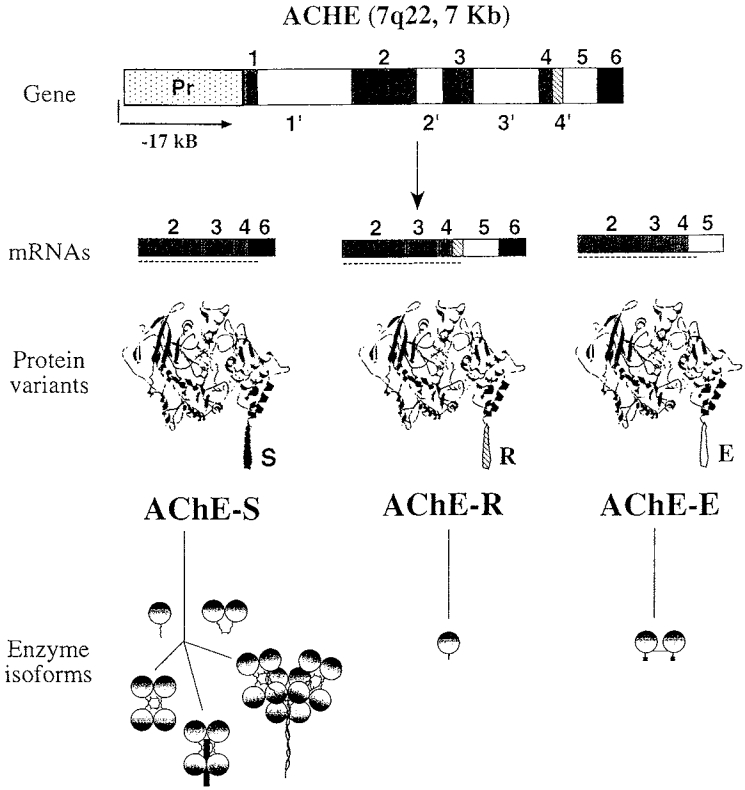

Figure 1 Alternative splicing generates AChE variants. Depicted is the exon/intron structure of the 7-Kb human ACHE gene at chromosomal locus 7q22. Exons are indicated by shaded boxes, introns by white boxes, and the pseudointron 14 (4′) by a hatched box. The extended ACHE promoter (Pr) stretches over 17 Kb upstream of the transcription initiation site and includes, among others, consensus regulatory sites for expression in nervous tissue, muscle, hematopoietic cells, and bone. In addition, the ACHE promoter includes a steroid hormone response element. Alternative splicing yields mRNA transcripts carrying the common coding exons 2-3-4, together with exon 6, intron 4′, or exon 5 encoding the synaptic (S), *readthrough* (R), or erythrocyte (E) AChE variants, respectively. The dotted lines under the RNA schemes indicate the open reading frame of each alternative transcript. The catalytically active core domain of all AChE isoforms is derived from the common exons, resulting in very small differences between the variants in their affinities for both substrates and inhibitors. In contrast, the alternative C-terminal peptides impose diverse biophysical properties upon the various isoforms that determine hydrophobicity, modes of oligomeric assembly, interactions with noncatalytic subunits, and subcellular localization (reviewed by Massoulie et al., 1998).

AChE-E were considered the primary players in the development of anticholinesterase therapies. The big surprise came with the discovery that a rare "*read-through*" mRNA retaining intron 4 in the open reading frame encodes a novel AChE isoform, AChE-R, that is dramatically up-regulated under some physiological conditions, including acute traumatic stress and exposure to AChE inhibitors (Friedman et al., 1996; Kaufer et al., 1998). Using heterologous expression in microinjected *Xenopus* oocytes and embryos, AChE-R was shown to represent a monomeric, hydrophilic, soluble form of catalytically active AChE (Seidman et al., 1995) (Fig. 2). In hippocampal brain slices, elevated AChE-R was associated with suppressed electrophysiological activity 3 h after treatment with the potent AChE inhibitor physostigmine (Kaufer et al., 1998). These studies raised the possibility that AChE-R plays a unique role in the physiological response to stress (Kaufer et al., 1999; Kaufer and Soreq, 1999). In addition, it raised the possibility that the balance between AChE variants carries important implications for health and disease. These studies therefore challenge the pharmaceutical industry to develop isoform-specific AChE inhibitors. Although small differences were found in the affinity of some inhibitors for AChE-S and AChE-R (A. Salmon and H. Soreq, unpublished data), it is yet unclear whether a pharmacological solution to the problem of isoform-specific inhibition is attainable. In contrast, antisense technology offers potential solutions. One approach would be to design exon-specific oligonucleotides to preferentially target exon 6 (AChE-S) or intron 4 (AChE-R). Another option is to exploit the inherent instability of AChE-R mRNA to selectively target this RNA species. The rapid degradation of AChE-R mRNA under treatment of rat phaeochromocytoma (PC12) cells with actinomycin D supported the validity of the latter approach (Grifman and Soreq, 1997).

B. Feedback Overexpression of AChE Alters the Balance and Levels of AChE Isoforms

The discovery that stress and AChE inhibitors elicit pronounced and prolonged feedback expression of AChE-R highlights the complexity of anti-AChE therapeutics on the one hand, and the need to understand the role of AChE-R in long-term responses to stress on the other. To study this issue, we generated transgenic mice overexpressing various AChE isoforms. Mice overexpressing AChE-S in CNS neurons display late-onset cognitive and neuromotor impairments with features reminiscent of human neurological diseases (Andres et al., 1997; Beeri et al., 1995, 1997). More recent studies of transgenic mice overexpressing AChE-R suggest that the balance between AChE-S and AChE-R, and the interplay between them, is itself important in mediating long-term effects (M. Sternfeld et al., 2000).

These studies raise the question of whether it is possible to devise an AChE strategy that does not promote the feedback loop. Inhibitor-mediated enzyme

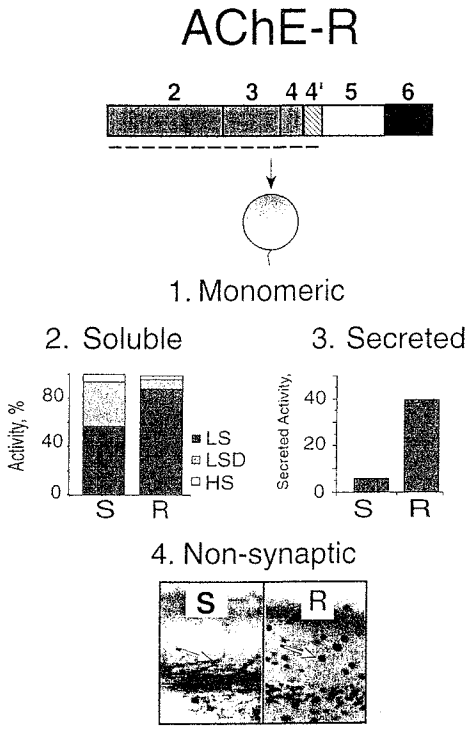

Figure 2 Unique properties of the stress-related *readthrough* AChE. The C-terminal domain of AChE-R, encoded by pseudo-intron 4′, is a 26-amino-acid, hydrophilic peptide lacking a cysteine residue necessary for oligomeric assembly (1). When expressed in microinjected *Xenopus* tadpoles, cDNA encoding AChE-R gave rise to a catalytically active enzyme that appeared almost completely (ca. 90%) in the low-salt (LS) as opposed to detergent-containing (LSD) or high-salt (HS) buffer fraction (2), was secreted in large quantities into the external medium (3), and accumulated in epidermal secretory cells rather than muscle (4) (Seidman et al., 1995). Following both acute stress and exposure to AChE inhibitors, AChE-R is dramatically overexpressed in brain and muscle where its unique properties are presumed to mediate both long- and short-term physiological stress responses.

overproduction appears to be activated by the acute cholinergic stimulation resulting from an abrupt pharmacological blockade of AChE (Kaufer et al., 1998). The inherent instability of AChE-R mRNA suggests that antisense technology could be aimed primarily at AChE-R, leaving AChE-S and cholinergic neurotransmission intact. However, even if the desired target will be AChE-S, it is likely that the pharmacokinetics of antisense inhibition would be less acute and

occur over a longer period than that of anti-AChE drugs. By preventing only de novo synthesis, an antisense oligonucleotide inhibitor would be slower-acting in its depletion of ACh-hydrolyzing potential. In contrast to the abrupt inactivation of catalytic sites taking place with pharmacological inhibitors, the slow action of an antisense inhibitor would conceivably allow the target cell to adapt to gradually increasing concentrations of ACh. Graded inhibition could therefore be expected to minimize or eliminate the feedback response. Furthermore, even if antisense inhibition would elicit a feedback response, the anti-mRNA nature of antisense blockade is such that careful titration of the oligonucleotide dose could be fine-tuned to suppress de novo feedback expression of the ACHE gene.

C. Noncatalytic Activities of AChE

A final issue to contend with when addressing pharmacological inhibition of AChE relates to recently described noncatalytic morphogenic activities of the protein. Data accumulated over the past decade demonstrated that in addition to its long-recognized role in hydrolyzing ACh, AChE possesses profound morphogenic effects on neuronal and synaptic architecture—especially with respect to neurite outgrowth and cell adhesion properties (reviewed by Grisaru et al., 1999; Soreq and Glick, 2000). These activities were proven to be independent of ACh hydrolysis (Sternfeld et al., 1998) and tentatively attributed to sequence homologies between ACHE and a family of cholinesterase-like neuronal cell adhesion molecules that includes *Drosophila* neurotactin and mammalian neuroligins (Darboux et al., 1996; Grifman et al., 1998; Ichtchenko et al., 1996). To validate the concept of using antisense technologies to control morphogenetic processes

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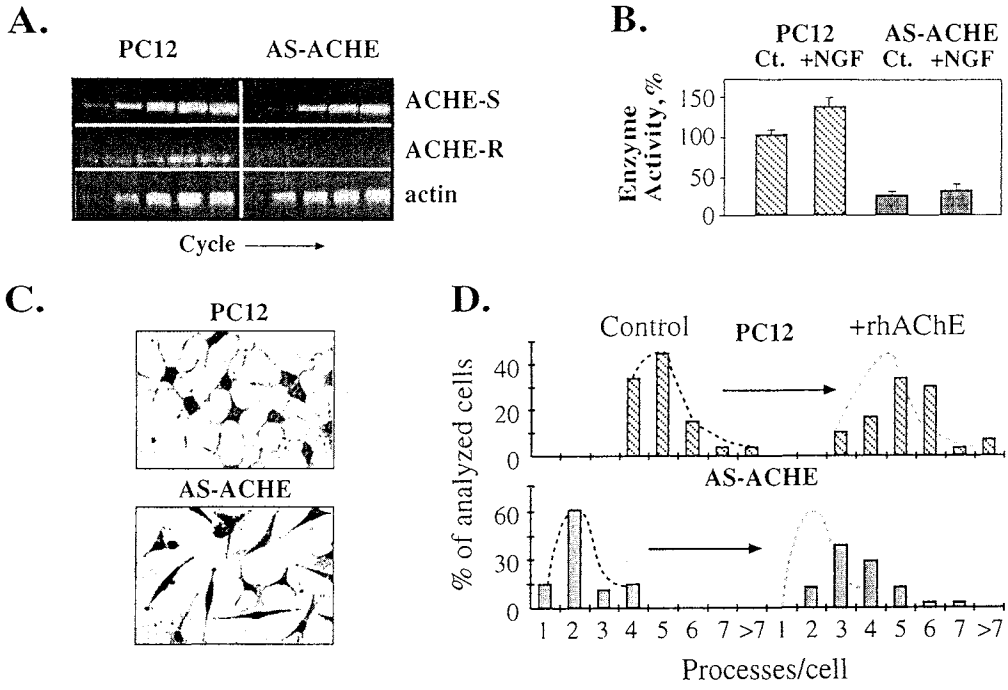
Figure 3 Antisense AChE cRNA arrests neurite extension—proof of concept. PC12 cells stably expressing a 132-bp fragment from the rat ACHE gene in the antisense orientation display dramatically reduced levels of AChE mRNA as determined by RT-PCR (A) and 80% suppression of AChE catalytic activity (B). Note the extreme sensitivity of AChE-R mRNA to antisense-mediated down-regulation compared to AChE-S mRNA. NGF-mediate process extension is prominently blocked by antisense AChE cRNA (C), attributing a morphogenic role to AChE in differentiating PC12. When grown on a collagen matrix infused with purified recombinant human AChE (rhAChE), the neurite-deficient phenotype was partially reversed (D). When retransfected with DNA encoding either catalytically active or inactive AChE-S, or neuroligin, partial recovery was also observed (E). These studies reinforced the notion that AChE shares overlapping, noncatalytic activities with neuroligin-related cell adhesion proteins, and suggest that antisense oligonucleotides may one day be used to intervene in stress or AChE inhibitor-induced changes in neuronal architecture. (After Grifman et al., 1998.)



in the nervous system, we established a model in PC12 cells (Grifman et al., 1998). PC12 cells were stably transfected with a plasmid carrying a 132-base-pair fragment encoding antisense cRNA corresponding to a sequence in exon 6 of the rat ACHE gene. Transfected cells displayed pronounced loss of AChE-R mRNA and significant, but less dramatic, decreases in mRNA encoding AChE-S. Antisense depletion of AChE mRNA was accompanied by a marked reduction in the process of extension normally accompanying nerve growth factor (NGF)-induced differentiation (Fig. 3). Thus, these studies demonstrated pronounced antisense-mediated effects on the cytoarchitecture of these cholinergic neuron-like cells. The abnormal antisense phenotype was partially rescued with exogenous AChE, and by transfection with plasmids directing the expression of either AChE (catalytically active or inactive) or neuroligin, stressing the overlapping functional significance of the cholinesterase-like domain shared by these proteins.

Noncatalytic morphogenic activities of AChE have been tentatively mapped to the peripheral anionic site, and have not yet been shown to be affected by the current anti-AChE therapeutics. Thus, pharmacological inhibitors of AChE block the catalytic activity of the enzyme, but do not necessarily interfere with other biological activities of the protein. On the contrary, since damaging effects of overexpressed AChE may be related to noncatalytic activities, these drugs may actually aggravate certain conditions by elevating the levels of catalytically inactivated AChE via the feedback loop. In that case, antisense technology would be the only approach to offer a potential solution by blocking production of the protein. Figure 4 summarizes the fundamental differences between pharmacological and antisense-based anti-AChE drugs.

VI. ANTI-AChE ANTISENSE OLIGONUCLEOTIDES—THE STATE OF THE ART

The lure of highly specific AChE and BChE inhibitors prompted the initiation of an antisense program in our laboratory shortly after the mammalian genes were cloned and sequenced. AChE-targeted antisense oligonucleotides have been shown effective in both in vitro and in vivo paradigms, especially in their effects on the hematopoietic system (Lev Lehman et al., 1994; Soreq et al., 1994). Indeed, antisense oligonucleotides have played an important part in establishing an active role for AChE in hematopoiesis, especially in the genesis of erythroid, lymphocytic, and megakaryocytic lineages. Initially, anti-AChE antisense oligonucleotides were prepared in unmodified phosphodiester or fully phosphorothioated forms (Patinkin et al., 1990) and then in partially phosphorothioated form. By restricting phosphorothioate modification to the three 3′ terminal nucleotides, cytotoxicity was minimized without loss of activity (Ehrlich et al., 1994). Sub-

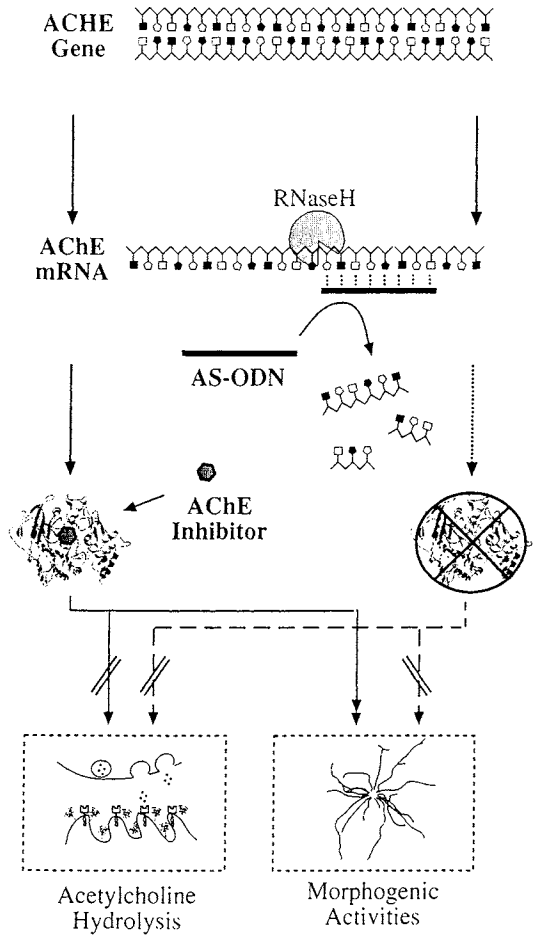


Figure 4 Antisense oligonucleotide approach to anti-AChE drug therapy. AChE displays two independent types of biological activities. Some activities depend on the hydrolysis of ACh; others, on nonenzymatic features of the protein that are presumed to mediate cell adhesion processes. Conventional pharmacology targets the enzyme's catalytic activity either by blocking access of substrate to the active site, or by inactivating the catalytic triad through the formation of nonregenerating enzyme intermediates. Commonly used therapeutic AChE inhibitors do not differentiate between AChE isozymes and may not inhibit the noncatalytic activities of the protein. In contrast, antisense oligonucleotides targeting AChE mRNA block de novo synthesis of AChE protein, thereby blocking both its catalytic and noncatalytic activities. The low cellular abundance of AChE mRNA, the high specificity of antisense agents, and the differential stabilities of the various alternative AChE-encoding mRNAs allow the use of very low doses of oligonucleotides to target specific AChE isoforms.

sequently, 2′-*O*-methyl-modified RNA replaced phosphorothioate modification as the formulation of choice for 3′-capping of AChE targeted oligonucleotides (Grisaru et al., 1999).

In a screen of seven antisense oligonucleotides targeted to various regions in rodent AChE mRNA, PC12 cells were shown to be significantly more vulnerable to antisense effects following NGF-mediated differentiation than naïve PC12 cells (Grifman and Soreq, 1997). Using partly phosphorothioate-protected oligonucleotides and a working concentration of 1 μM oligonucleotide, a maximum inhibition of approximately 30% was achieved. After that study, two oligonucleotides—AS1 and AS3—were selected as the primary antisense agents in our laboratory. Recent experiments using these two oligonucleotides demonstrated even greater inhibition of AChE (up to 50%) at 100–1000-fold lower concentrations of oligonucleotide. Moreover, 2′-*O*-methyl-protected AS1 and AS3 displayed a wide window of effective concentrations (0.02–200 nM) compared to the phosphorothioate-protected oligos (Galyam et al., 2001). In osteosarcoma Saos-2 cells, 2 nM 2′-*O*-methyl-capped antisense oligonucleotides achieved pronounced

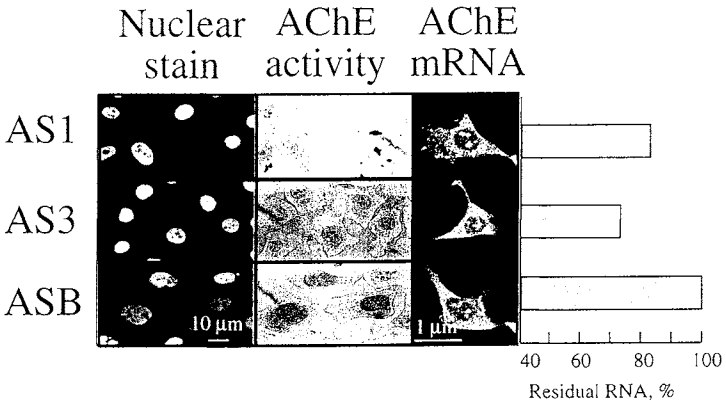


Figure 5 Antisense oligonucleotides suppress AChE activity in osteogenic Saos-2 cells. Human osteosarcoma Saos-2 cells were grown in the presence of anti-AChE oligonucleotides for 12 h and subjected to either histocytochemical staining for catalytically active AChE (middle panel) or in situ hybridization (right panel) with a probe detecting all AChE mRNAs. DAPI was used to mark the nuclei of cells stained for AChE activity (left panel). Confocal microscopy was used to document and quantify the in situ hybridization data as represented in the bar graph to the right. AS1 and AS3 denote 20-mer antisense oligonucleotides with a 3′, 2′-*O*-methyl cap (last three nucleotides) targeting different regions within exon 2 in the ACHE gene (Grifman and Soreg, 1997; Grisaru et al., 1999). ASB is a control, 15-mer antisense oligonucleotide targeting the homologous enzyme BEhE. Proliferation of Saos-2 cells, as determined by BrdU incorporation, was changed under AS1 treatment, supporting a role for AChE in osteogenic development.

blockade of AChE expression that was accompanied by modified cellular proliferation, reinforcing evidence that AChE plays an active role in mammalian osteogenesis (Fig. 5 and Grisaru et al., 1999). The strong antisense effects elicited by extremely low concentrations of oligonucleotide in these various systems greatly reduce the cost of antisense experiments, and can be expected to significantly reduce potential, unwanted side effects. We have therefore extrapolated these in vitro studies to test low-dose administration of anti-AChE oligonucleotides in vivo in animal models of acute psychological stress, chronic low-dose anticholinesterase intoxication, myasthenia gravis, Alzheimer's disease, and closed head injury.

VII. POTENTIAL APPLICATIONS OF AChE ANTISENSE TECHNOLOGIES

A. AChE Poisoning

The association between AChE inhibitors and neuromuscular impairments (Soreq and Glick, 2000) suggests a potential application for AChE-targeted antisense oligonucleotides in treating victims of anti-AChE intoxication. Estimates of illness associated with occupational exposure to organophosphate (OP) anti-AChE pesticides range from 150,000 to 300,000 annually in the United States alone (Feldman, 1999). Accidental ingestion and food contamination are additional sources of OP intoxication with potential long-range health implications. During the Persian Gulf War, several hundred thousand soldiers received prophylactic doses of pyridostigimine to protect them against the threatened use of chemical warfare by Iraq. Although no definitive association has been made between unexplained Gulf War illness and anti-AChE, a recent Rand Corporation report (Document no.MR-1018/2-OSD) prompted the Defense Department to acknowledge that pyridostigmine cannot be ruled out as a possible contributor to some symptoms experienced by Gulf War veterans. Notably, muscle weakness is prominent among the complaints of some Gulf War veterans (Haley et al., 1997). In view of what we know about the AChE feedback loop, the detrimental effects of overexpressed AChE on muscle, and the fact that no coherent approach has been taken to treating muscle weakness among Gulf War veterans, this may represent an appropriate forum in which to examine the utility of antisense therapy for neuromuscular impairments.

B. Neuromuscular Disease

Myasthenia gravis (MG) is a debilitating autoimmune neuromuscular disease characterized by fluctuating muscle weakness and progressive deterioration of neuromotor function (Schonbeck et al., 1990). Symptoms affecting MG patients include drooping eyelids, double vision, difficulty eating or talking, and chronic

fatigue. Episodic, life-threatening, ''myasthenic crisis'' causes respiratory distress that usually requires intensive-care hospitalization. MG is reported to affect approximately 36,000 individuals in the United States (statistics from the Myasthenia Gravis Foundation of America, http://www.myasthenia.org). It has been suggested, however, that MG is underdiagnosed and that the incidence is probably higher. Myasthenia gravis results from autoimmune antibody–mediated depletion of ACh receptors from the neuromusclular junctions. As myasthenia is characterized by understimulation of the muscles, AChE inhibitors have proven an effective palliative treatment for this disease (Evoli et al., 1996). The most commonly used AChE inhibitor for the treatment of MG today is Mestinon. Mestinon is the trade name for the carbamate AChE inhibitor, pyridostigmine. AChE inhibitors address the symptoms of MG, but do not slow its progression. On the contrary, it has been suggested that pyridostigmine may actually contribute to progressive deterioration in muscle function (Swash, 1975). In this light, it is noteworthy that a promising AChE inhibitor for Alzheimer's disease was withdrawn from clinical trials after some patients reported muscle weakness (SCRIP World Pharmaceutical News, no. 2374, p. 19, 1998). In any case, the relief offered by current anticholinesterase drugs is short-lived, and relatively high doses of these medications must be taken up to six times per day for many years.

Recently, we demonstrated that the AChE feedback loop active in brain is similarly active in muscle, and that the AS3 oligonucleotide acts to suppress inhibitor-induced overexpression of AChE in mice (Lev-Lehman et al., 2000). In those experiments, 80 μg/kg AS3 blocked anti-AChE-induced accumulation of catalytically active AChE in muscle by 60%, and largely suppressed the accompanying increase in motor endplates (Fig. 6). This experiment proved that antisense oligonucleotides to AChE can suppress noncatalytic morphogenic activities of the enzyme in vivo. In transgenic mice overproducing AChE in motoneurons, we observed severe deterioration of muscle structure and function (Andres et al., 1997), suggesting that excess AChE may be a factor in the deterioration of muscle function in myasthenic patients. As AChE inhibitors may exacerbate AChE imbalances in the muscle, the potential long-term effects of chronically administered AChE inhibitors should not be ignored. Although continued administration of the inhibitory drug masks the added ACh-degrading activity, it will not necessarily prevent the ill effects of excess noncatalytically active AChE on muscle. Thus, the feedback loop and the role of AChE as a catalytically inert, but biologically active element must be seriously considered in the development of new drugs for MG. In that case, antisense oligonucleotide technology offers a novel approach.

C. Head Trauma

Closed head injury (CHI) is an important cause of death among young adults (Siesjo, 1993; Yakovlev and Faden, 1995) and a prominent risk factor in non-

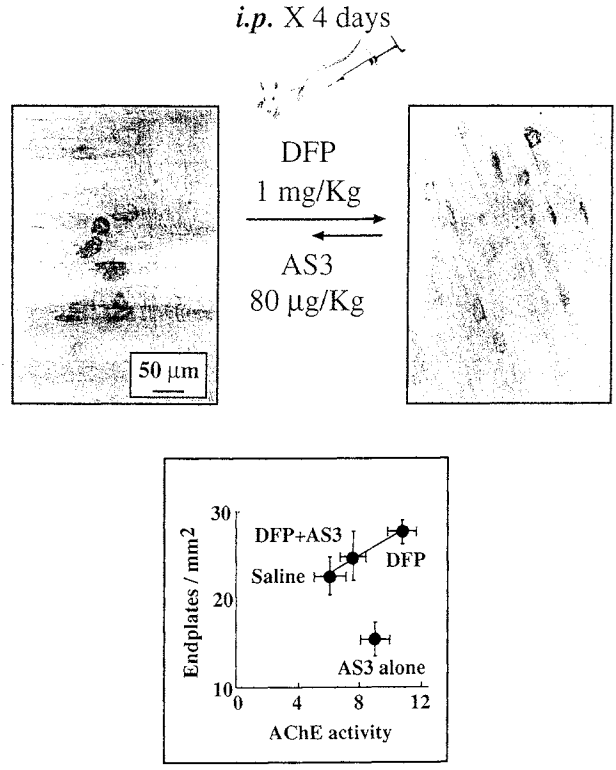


Figure 6 Antisense blockade of anticholinesterase-induced synaptic proliferation. Adult FVB/N mice received four consecutive daily i.p. injections with diisopropylfluorophosphonate (DFP), a potent irreversible AChE inhibitor, with or without coadministration of AS3 (80 µg/kg). Two weeks later, diaphragm muscles were excised and histochemically stained for AChE as a marker of motor endplates. DFP alone promoted feedback elevations in AChE activity as determined in homogenates, neurite outgrowth (not shown), and proliferation of small endplates. Coadministration of partially 2′-*O*-methyl-protected AS3 restrained feedback overexpression of AChE and partly blocked proliferation of motor endplates. These experiments suggest the application of antisense technology to diseases of the neuromuscular junction such as myasthenia gravis and for the prevention of delayed muscle weakness associated with accidental or occupational exposure to AChE inhibitors. (After Lev-Lehman et al., 2000).

familial Alzheimer's disease (Gennarelli and Graham, 1998). Effective emergency strategies must be developed to improve survival, promote recovery, and prevent delayed neurological disorders. Recently, we observed pronounced accumulation of AChE-R mRNA in cortex of mice subjected to controlled head injury (Chen et al., 1996). A single postinjury intracerebroventricular administration of only 0.5 μg AS3 abolished the postinjury accumulation of AChE-R mRNA and the excessive dendritic growth accompanying it (Shohami et al., 2000). In trauma-sensitive AChE transgenic mice, antisense treatment minimized mortality, facilitated neurological recovery, and protected CA3 hippocampal neurons. These findings demonstrate the potential of antisense therapeutics in treating acute injury and suggest antisense blockade of AChE-R for limiting the detrimental consequences of various traumatic insults to the nervous system.

D. Neurodegenerative Disease

Alzheimer's disease (AD) is a debilitating neurodegenerative disease characterized by progressive deterioration of cognitive faculties including learning, short-term memory, problem solving, and abstract thinking. The average lifetime cost approaches $175,000 per patient and total AD patient care amounts to approximately $100 billion per year. AD currently affects 4 million Americans and is currently considered the fourth leading cause of death in the United States (Statistics from the Alzheimer's Association, http://alz.org). The cholinergic theory of AD suggests that the selective destruction of cholinergic neurons in AD results in a relative deficit of ACh in brain regions that mediate learning and memory functions (Coyle et al., 1983). The primary approach to treating AD has therefore aimed to augment the cholinergic system with AChE inhibitors. Indeed, the only currently appeared drugs for AD are potent AChE inhibitors (Giacobini, 1998). Nevertheless, the value of cholinesterase therapy is limited to about 1 year, and to mildly affected patients.

The discovery of a role for AChE in neurite growth is particularly significant in view of the fact that abnormal neurite projections are characteristic features of the Alzheimer's brain, as are abnormal deposits of AChE at sites of senile plaque formation—the principal histopathological hallmark of AD (Wright et al., 1993). In vitro, AChE was even shown to mediate the aggregation of β-amyloid protein, the major component of AD plaques (Campos et al., 1998; Inestrosa et al., 1996). Thus, emerging evidence suggests that AChE may play a role in the etiology of AD that goes beyond the scope of the cholinergic theory. Taking the feedback loop and noncatalytic activities of AChE into consideration, AChE as an active player in the progress of AD may explain the overall disappointing performance of AChE inhibitors in providing effective long-term relief. Using surgically implanted cannulae to deliver nanomolar quantities of AS3 to the cere-

brospinal fluid of cognitively impaired transgenic mice (Beeri et al. 1995), we are testing the effects of antisense therapy on performance in behavioral models such as social exploration and conditioned taste aversion. Indeed, the potential applications of antisense technology to treating diseases of the central nervous system are enticing (McCarthy, 1998; Seidman et al., 1999). Nevertheless, the challenge of bringing oligonucleotide therapeutics to AD in particular, and to neurodegenerative disease in general, faces yet-unresolved technical limitations. Among the most difficult issues to resolve is the poor transport of oligonucleotides across the blood-brain-barrier (Soreq et al., 2000).

VIII. SUMMARY

Developments in our knowledge of the molecular and cellular mechanisms of action of AChE have allowed us to identify the inherent limitations of conventional pharmacological blockade of AChE catalytic activity. These limitations are based on the discovery of noncatalytic activities of AChE isoforms, and on the existence of a feedback loop leading to overexpression of AChE following acute inhibition of enzymatic activity. At the same time, cloning of the ACHE gene opened the door to novel strategies to contain undesired AChE activities based on antisense technology. These hand-in-hand advances in AChE basic research afford us the opportunity to think ahead to the application of antisense oligonucleotides to future anti-AChE therapeutics. An antisense approach to the control of AChE expression promises to address the previously unappreciated role of AChE variants in the etiology of disease, something that pharmacological approaches have not yet begun to explore. Given the unique challenges of AChE-based therapeutics and the highly relevant answers that antisense technology offers to these challenges, AChE represents a promising target with which to promote the development of therapeutic antisense for the central and peripheral nervous systems.

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21

Serine/Threonine Protein Phosphatases

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I. INTRODUCTION

Reversible phosphorylation is a mechanism widely utilized by eukaryotic cells to control the biological activity of numerous regulatory and structural proteins. In mammalian cells, complex signaling networks have evolved in which the site-specific phosphorylation of key cell-cycle-regulatory proteins facilitate or impede the propagation of growth-promoting and growth-arresting signals. Accordingly, understanding the cellular functions of the individual protein kinases and protein phosphatases that control reversible phosphorylation is critical to understanding how the growth-promoting signals are propagated or attenuated. Although studies with semiselective inhibitors suggest that the serine/threonine protein phosphatases are highly regulated and responsive enzymes, determining the role of individual phosphatases in the regulation of cell growth has been difficult owing to the lack of truly specific inhibitors. With the advent of modern antisense technology, however, isotype-selective inhibitors are finally becoming available. Thus, we now have the tools necessary to study the cellular functions of individual phosphatases in growth control pathways. In turn, this may lead to the development of agents useful in altering the cell-cycle-regulatory machinery of cells undergoing aberrant proliferation.

Employing antisense oligonucleotides that specifically inhibit the expression of serine/threonine protein phosphatase type 5 (PP5) and type 1 gamma 1 (PP1γ1), it is now clear that both participate in the regulation of cell cycle progression. While we are just beginning to understand how PP1γ1 regulates a step in late cytokinesis, a key role for PP5 in the suppression of cellular growth is emerg-

ing. Studies employing antisense oligonucleotides to suppress the expression of PP5 (ISIS 15534) have revealed that PP5 plays a major role in the regulation of both p53- and glucocorticoid receptor (GR)-induced signaling cascades that promote growth suppression. Following treatment with ISIS 15534, both p53- and dexamethasone-induced growth suppression is augmented, with growth arrest associated with the transcriptional induction of $p21^{WAF1/Cip1}$. Gel-shift analysis revealed a marked increase in the association of both p53 and glucocorticoid receptors (GR) with DNA encoding their respective consensus recognition sequences. Transient transfection studies conducted with GR-luciferase reporter plasmids revealed that suppression of PP5 expression induces GR-transcriptional activity, without the addition of hormone. Furthermore, dexamethasone-mediated induction of GR-reporter activity increases to a level that is ~10 times greater than the maximal response obtainable in the presence of PP5. Dexamethasone-mediated growth arrest also correlates with an increase in p53 phosphorylation, and comparative studies in p53-wild type, p53-defective, and p53-deficient cell lines indicate that either (1) p53 participates in GR-mediated induction of $p21^{WAF1/Cip1}$, with the hyperphosphorylation of basal p53 induced by glucocorticoids sufficient for the propagation of an antiproliferative response when PP5 expression is inhibited, or (2) PP5 acts where p53-mediated and GRinduced signaling networks converge to regulate the transcriptional induction of $p21^{WAF1/Cip1}$. Either way, the data obtained with the antisense oligonucleotides have revealed that PP5 promotes cellular proliferation by inhibiting both glucocorticoid and p53-mediated signaling pathways leading to $p21^{WAF1/Cip1}$-mediated growth arrest.

In eukaryotic cells, the biological activity of numerous regulatory and structural proteins is influenced by reversible phosphorylation. Phosphorylation occurs principally on serine, threonine, and tyrosine residues, and the reaction is catalyzed by a large number of relatively substrate specific protein kinases. Dephosphorylation is catalyzed by a more limited number of serine/threonine, tyrosine, or dual-specificity protein phosphatases, with the known kinases greatly outnumbering the known phosphatases. Together, these enzymes are recognized as principal regulators of diverse cellular processes including metabolism, ion-channel activity, membrane transport, the transcription of genes, cell cycle progression, and even processes as complex as learning and memory.

Since reversible phosphorylation affects virtually all aspects of normal physiology, it is not surprising that many protein kinases have been identified as potential molecular targets for the development of therapeutics useful in the treatment of disorders that occur in humans. However, the protein phosphatases, particularly the serine/threonine protein phosphatases (PPases), have received far less attention. This lack of attention can be attributed, in part, to the initial misconception that serine/threonine PPases act as nonspecific housekeeping enzymes that function simply to ''mop up'' after their ''substrate specific'' kinase counterparts. This misconception has been rectified by numerous studies clearly

demonstrating that most, if not all, protein phosphatases are highly regulated and responsive enzymes. Furthermore, with respect to the serine/threonine protein kinases, the serine/threonine PPases are more limited in number and appear to have less redundancy of function. Therefore, serine/threonine PPases may be more suitable targets for the development of drugs to regulate cellular processes controlled by reversible phosphorylation.

II. THE SERINE/THREONINE PPase FAMILY

The traditional classification of serine/threonine PPases is based on the biochemical characteristics of the catalytic subunit and relies on the use of specific inhibitors and a limited amount of substrate specificity that can be demonstrated in vitro (1–3). Based on these criteria, two major classes, type 1 (PP1) and type 2 (PP2), have been established. The catalytic subunit of PP1 preferentially dephosphorylates the beta subunit of phosphorylase kinase and is sensitive to inhibition by nanomolar concentrations of two acid- and heat- stable cytosolic proteins, inhibitor 1 and inhibitor 2. In contrast, the PP2 family is classified based on the ability to preferentially dephosphorylate the alpha subunit of phosphorylase kinase and the lack of inhibition by inhibitors 1 and 2.

PP2 comprises three subtypes [PP2A, PP2B (also referred to as calcineurin), and PP2C] that are distinguished from each other by their requirements for divalent cations and their sensitivities to a number of natural toxins, such as the complex polyether okadaic acid (for review see Ref. 4). The type PP2A family of enzymes is highly sensitive to inhibition by okadaic acid (K_i = 0.2 nM), while PP2B is relatively insensitive (K_i = 10 μM), and PP2C is completely resistant to the inhibitory effects of okadaic acid. In addition, the activity of PP2B and PP2C is dependent on the presence of divalent cations. PP2B has an absolute requirement for Ca^{2+}/calmodulin, while PP2C activity is dependent on Mg^{2+}. In contrast, both PP1 and PP2A are active in the presence of mM concentrations of EGTA/EDTA, indicating that the catalytic activities of these PPases is not regulated directly by either calcium or magnesium.

Several lines of evidence indicate that the current nomenclature system needs updating. First, genetic and cloning studies have revealed that the primary amino acid sequences of PP1, PP2A, and PP2B are related, while PP2C belongs to a distinct gene family (Fig. 1) (3,5). Second, four isoforms of PP1, two isoforms of PP2A, three isoforms of PP2B, and at least six isoforms of PP2C have been identified. In addition, four novel phosphatases that are structurally related to the PP1/PP2A/PP2B gene family have been identified. Unfortunately, attempts to revise the nomenclature system to accommodate these findings have not been widely accepted. As a result, the naming system that has evolved is somewhat arbitrary and controversial.

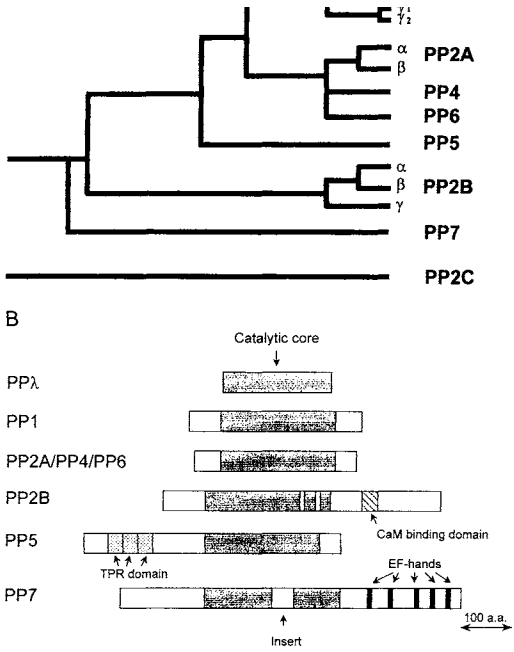

Figure 1 Schematic representation of the structural relationship of known human serine/threonine protein phosphatases based on the similarity of their primary amino acid sequences. (A) Phylogenic tree. To date two human gene families encoding serine/threonine protein phosphatases have been identified. One family, the PP1/PP7 family, contains at least 13 members that are encoded by 12 genes. This gene family is divided into seven groups of related enzymes designated as PP1, PP2A, PP2B (calcineurin), PP4, PP5, PP6, and PP7. In mammals, four isoforms of PP1, which demonstrate >90% identity, two isoforms of PP2A, with >97% identity, and three isoforms of PP2B, with >80% identity, have been identified. Molecular studies have also identified four additional PPases, designated PP4, PP5, PP6, and PP7. PP4 and PP6 are structurally related to PP2A, sharing 65% and 57% identity at the level of their primary amino acid sequence, respectively. The second family of enzymes, the PP2C gene family, contains at least six distinct genes encoding enzymes that are dependent on Mg^{2+} for activity and not sensitive to okadaic acid or microcystin-LR. (B) Homology shared by the PP1/PP7 serine/threonine protein phosphatases. A schematic representation showing similarity within the catalytic subunits of the known human serine/threonine protein phosphatases (PPases). PPases belonging to the PP1–PP7 family of enzymes contain a common catalytic core domain that is conserved with λ-PPase (97,98). PP1, PP2A, PP4, and PP6 are highly homologous enzymes, differing primarily in their C- and N-terminal regions. PP2B differs in that it contains a Ca^{2+}-calmodulin-binding domain in its C-terminal region and two small divergent regions (open boxes) in the catalytic domain near the okadaic acid/microcystin–binding domain. PP5 possesses three tetratricopeptide (TPR) domains in the amino-terminal region. PP7 differs from all of the other PPase families in that it contains EF-hand motifs in the C-terminal region (filled squares) and a 44-amino-acid insert in the catalytic core domain (open box). (C) Sequence similarity between the catalytic core domain of the PP1/PP7 family of human PPases. Residues that are identical in all of the PPases compared are

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PP2Aα PVTVCGDVHG QFHDLMELFR IGGKSPDTN. YLFMGDYVDR GYYSVETVTL
PP4   PVTVCGDIHG QFYDLKELFR VGGDVPERN. YLFMGDFVDR GFYSVETFLL
PP6   PVTVCGDIHG QFYDLCELFR TGGQVPDTN. YIFMGDFVDR GYYSLETFTY
PP5   KITVCGDTHG QFYDLLNIFE LNGLPSETNP YIFNGDFVDR GSFSVEVILT
PP2Bα PVTVCGDIHG QFFDLMKLFE VGGSPANTR. YLFLGDYVDR GYFSIECVLY
PP7   EVTICGDLHG KLDDLFLIFY KNGLPSERNP YVFNGDFVDR GKNSIEILMI
          CGD HG    DL   F    G         Y F GD VDR G  S E

      51                                                  100
PP1α  LLAYKIKYPE NFFLLRGNHE CASINRIYGF YDECKRRY.. .NIKLWKTFT
PP2Aα LVALKVRYRE RITILRGNHE SRQITQVYGF YDECLRKY.. GNANVWKYFT
PP4   LLALKVRYPD RITLIRGNHE SRQITQVYGF YDECLRKY.. GSVTVWRYCT
PP6   LLALKAKWPD RITLLRGNHE SRQITQVYGF YDECQTKY.. GNANAWRYCT
PP5   LFGFKLLYPD HFHLLRGNHE TDNMNQIYGF EGEVKAKY.. .TAQMYELFS
PP2Bα LWALKILYPK TLFLLRGNHE CRHLTEYFTF KQECKIKY.. .SERVYDACM
PP7   LCVSFLVYPN DLHLNRGNHE DFMMNLRYGF TKEILHKYKL HGKRILQILE
      L                RGNHE          F   E    Y

      101                                                 150
PP1α  DCFNCLPIAA IVDEKIFCCH GGL.SPDLQS MEQIRRMRPT ..........
PP2Aα DLFDYLPLTA LVDGQIFCLH GGL.SPSIDT LDHIRADRLQ ..........
PP4   EIFDYLSLSA IIDGKIFCVH GGL.SPSIQT LDQIRTDRKQ ..........
PP6   KVFDMLTVAA LIDEQILCVH GGL.SPDIKT LDQIRTERNQ ..........
PP5   EVFEWLPLAQ CINGKVLIMH GGLFSEDGVT LDDIRKERNR ..........
PP2Bα DAFDCLPLAA LMNQQFLCVH GGL.SPEINT LDDIRKDRFK ..........
PP7   EFYAWLPIGT IVDNEILVIH GGI.SETTDL NLLHRVERNK MKSVLIPPTE
          L              H GG  S         R  R

      151                                                 200
PP1α  .......... .......... .......... ...IDVPDQG LLCDLLWSDP
PP2Aα .......... .......... .......... ...LEVPHEG PMCDLLWSDP
PP4   .......... .......... .......... ...IEVPHDG PMCDLLWSDP
PP6   .......... .......... .......... ...IEIPHKG AFCDLVWSDP
PP5   .......... .......... .......... ...IQPPDSG PMCDLLWSDP
PP2Bα .......... .......... .......... ...LEPPAYG PMCDILWSDP
PP7   TNRDHDTDSK HNKVGVTFNA HGRIKTNGSP TEHLTEHEWE QIIDILWSDP
                                                    DI WSDP

      201                                                 250
PP1α  DKDVQGWGEN D.......RG VSFTFGAEVV AKFLHKHDLD LICRAHQVVE
PP2Aα DDR.GGWGIS P.......RG AGYTFGQDIS ETFNHANGLT LVSRAHQLVM
PP4   ED.TTGWGVS P.......RG AGYLFGSDVV AQFNAANDID MICRAHQLVM
PP6   EDVDT.WAIS P.......RG AGWLFGAKVT NEFVHINNLK LICRAHQLVH
PP5   QPQ.NGRSIS K.......RG VSCQFGPDVT KAFLEENNLD YIIRSHEVKA
PP2Bα LEDFGNEKTQ EHFTHNTVRG CSYFYSYPAV CEFLQHNNLL SILRAHEAQD
PP7   RGKNGCFP.. .....NTCRG GGCYFGPDVT SKILNKYQLK MLIRSHECKP
                        RG                           R H

      251                                 284
PP1α  DGYEFFAKRQ. .....LVTLF SAPNYCGEFD NAGA
PP2Aα EGYNWCHDRN. .....VVTIF SAPNYCYRCG NQAA
PP4   EGYKWHFNET. .....VLTVW SAPNYCYRCG NVAA
PP6   EGYKFMFDEK. .....LVTVW SAPNYCYRCG NIAS
PP5   EGYEVAHGGR. .....CVTVF SAPNYCDQMG NKAS
PP2Bα AGYRMYRKSQT TGFPSLITIF SAPNYLDVYN NKAA
PP7   EGYEICHDGK. .....VVTIF SASNYYEEGS NRGA
       GY               T   SA NY       N
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indicated in boldface type. PP2A is human protein phosphatase type 2A; PP1 is human protein phosphatase type 1α; PP2B is human protein phosphatase type 2B; PP4 is human protein phosphatase type 4; PP5 is human protein phosphatase type 5; PP6 is human protein phosphatase type 6; PP7 is human protein phosphatase type 7. (Reprinted, in part, from Ref. 25. Copyright 1998 by the American Society for Biochemistry and Molecular Biology Inc.)

Much of the confusion in nomenclature involves the isoforms of PP1. Originally, two isoforms of PP1 were cloned from rabbit cDNA and were designated PP1α and PP1β (6). In rat, cDNA cloning experiments identified a homologue to PP1α and three additional isoforms that were termed PPγ1, PPγ2, and PP1δ (8). The PPγ1 and PPγ2 isoforms are encoded by one gene and are produced by alternative splicing of the same primary transcript. Subsequent studies revealed that PP1β was a cloning artifact (7), and a cDNA encoding a rabbit homologue of the PP1δ isoform identified in rat was cloned and termed PP1β (9). Thus, PP1β and PP1δ designate the same isoform of PP1. In humans all four isoforms of PP1 have been identified, and the isoforms are designated PP1α, PP1δ (also referenced as PP1β), PP1γ1, and PP1γ2. The human isoforms demonstrate >90% identity, and all four subtypes are extremely conserved in nature, sharing ~80% identity with PP1 homologues identified in yeast and even ~60% identity with homologous phosphatases expressed in plants (10).

Less controversy is associated with the designations assigned to the other PPases. To date, two isoforms of mammalian PP2A (PP2Aα, PP2Aβ), which share >97% identity, have been identified (11–14). Like PP1, both isoforms of PP2A are highly conserved in nature, with PP2A in humans and yeast sharing ~80% identity at the level of their primary amino acid sequences. Molecular and genetic studies have also identified two additional PPases, designated PP4 (PPX) and PP6, that are structurally related to PP2A (15,16). Comparison of the predicted primary amino acid sequences of PP4 and PP6 indicate that they share 65% and 57% identity with PP2A, respectively. In addition, the human gene encoding PP4 shares structural similarity with the genes encoding PP2A (17). Thus, one might consider PP4 an isoform of PP2A. Nonetheless, since recent studies indicate that the regulatory proteins that interact freely with either isoform of PP2A are distinct from those that interact with PP4 (18), PP4 and PP6 are currently classified as distinct families.

Like PP1 and PP2A, three isoforms of PP2B (also called calcineurin) with >80% identity have been identified. (19–23). In addition, two other PPases, designated PP5 (16,24) and PP7 (25), have been placed in the PP1/PP2A/PP2B family of enzymes. Both PP5 and PP7 contain a catalytic domain common to the PP1/PP2A/PP2B family of PPases (Fig. 1B). The extended N-terminal domain of PP5 has an autoinhibitory function and contains tetratricopeptide repeat motifs (TPR), which may serve to influence the interaction of PP5 with other proteins. PP7 is homologous to the *Drosophila* retinal degeneration C gene product (rdgC), and it contains unique N- and C-terminal regions. The C-terminal region contains multiple putative Ca^{2+}-binding sites (EF-hand motifs). Although calcium binding to these EF-hand domains has not been demonstrated directly, PP7 activity is dependent on Mg^{2+} and activated by calcium (25). Unlike the other known PPases, which are expressed ubiquitously, the expression of PP7 is restricted to retina and developing fetal brain. Similar to PP2B and PP2C, PP7 is resistant to

inhibition by okadaic acid and microcystin-LR. Both PP4 and PP5 are sensitive to okadaic acid and microcystin-LR. A PPase originally designated as PP3 based on its biochemical properties and immunological studies (26) is likely the C-terminal region of bovine PP5 produced by cleavage of the N-terminal region during purification by contaminating protease.

III. SEMISELECTIVE PPase INHIBITORS

Studies with semiselective PPase inhibitors suggest that type-specific antisense oligonucleotides may be useful as antitumor agents. To date, several compounds that inhibit the activity of the PP1-PP5 family of PPases have been identified. Most of these compounds are natural products produced by cyanobacteria (i.e., >30 structural variants of microcystin and nodularin), dinoflagelates (i.e, okadaic acid), *Streptomyces* (i.e., tautomycin and fostriecin), or insects (i.e., cantharidin). The nonselective inhibitors (notably, calyculin A, microcystin-LR, and nodularin) are all highly toxic. Therefore, their use as potential pharmacological agents is not promising. In contrast, some of the less toxic inhibitors are more selective, and two (cantharidin and fostriecin) have demonstrated antitumor activity both in vitro and in vivo.

A. Cantharidin

In China, Mylabris, the dried body of the Chinese blister beetle, is sold in a concoction that has been used for more than 2000 years as a traditional medicine for the treatment of cancer (27). The active constituent of mylabris, cantharidin (exo, exo-2,3-dimethyl-7-oxabicyclo[2.2.1]heptane-2,3-dicarboxylic acid anhydride), is produced by as many as 1500 different species of blister beetles, with the Spanish fly, *Cantharis vesicatoria*, probably being the best known (27,28). As in China, cantharidin also has a history of medical usage in Europe during the 1800s. However, by the early 1900s, cantharidin was considered obsolete and too toxic for use as an internal medicine (28).

Although cantharidin has been developed into an effective treatment for warts (29–33), further exploration of its potential for medical use remained dormant until the early 1990s. Renewed interest in cantharidin then emerged when it was shown to bind with high affinity to a specific and saturable binding site in a cytosolic fraction produced from homogenized mouse liver (34,35). This fraction was subsequently purified, and the high-affinity cantharidin-binding protein was identified as protein phosphatase type 2A (36). Further characterization of the inhibitory activity of cantharidin on a number of PPases revealed that cantharidin is a strong inhibitor of PP1 and PP2A and a weak inhibitor of PP2B (37). Studies of the molecular actions of cantharidin-mediated antitumor activity

revealed that cantharidin treatment of CHO cells induces the formation of aberrant mitotic spindles, which is associated with G2/M-phase growth arrest and the induction of apoptosis (38). Clinically, as an antitumor agent, cantharidin was found effective in primary hepatoma but limited by severe toxicity in mucous membranes (39). Current studies designed to determine whether a new generation of cantharidin analogs with less toxicity are effective as anticancer agents should prove interesting (39).

B. Fostriecin

Fostriecin, 3{2H-pyran-2-one,5,6-dihydro-6-[3,6,13-trihydroxy-3-methyl-4-(phosphonooxy)-1,7,9,11-tridecatetraenyl]}, also referred to as Cl-920, NSC 339638, or PD 110,161, was discovered in the course of a screening program designed to identify new antitumor agents contained in the fermentation broth of an actinomycete, *Streptomyces pulveraceus* (subspecies *fostreus*). Fostriecin was originally identified as a water-soluble component of an extract that inhibited the growth of murine L1210 lymphoid and P388 lymphocytic leukemia cells in tissue culture and had marked antileukemic activity in mice (40–42). Studies of the structure/activity relationship of fostriecin revealed that removal of the phosphate ester or disturbances to the lactone ring resulted in a considerable loss of cytotoxic activity (40), while removal of the primary alcohol moiety (PD113, 270) had little effect on cytotoxicity against L1210 and HCT-8 cells in vitro. Together, these studies suggest that both the phosphate ester and the lactone ring contribute to the biological activity of fostriecin.

Initial biochemical studies revealed that fostriecin produced a marked inhibition of macromolecular synthesis (40,43). However, no effects on DNA polymerase, RNA polymerase, or enzymes within the purine or pyrimidine biosynthetic pathways were noted, and the mechanism of nucleic acid synthesis inhibition remained elusive (40,43,44). Subsequent studies revealed that fostriecin treatment at a cytostatic concentration (1.5μM for 24 h) results in a marked accumulation of cells in the G2-M phase of the cell cycle (45). In CHO cells, fostriecin-induced G2/M-growth arrest is associated with the appearance of cells containing multiple aberrant mitotic spindles (38), and higher concentrations (25–500 μM) of fostriecin triggered endonucleolytic DNA degradation, resulting in apoptotic cell death (46–48). The ability of fostriecin to induce apoptotic cell death is addressed in more detail in a recent review of the preclinical data obtained with fostriecin (49).

Although several early reports revealed that fostriecin-mediated growth arrest is associated with inhibition of topoisomerase II (for a review see Ref. 49), more recent studies suggest that the antitumor activity of fostriecin may arise from the inhibition of certain PPases (38,50–53). Detailed studies conducted with both the purified catalytic subunits of mammalian PPase and endogenous PPases contained in dilute, whole-cell extracts reveal that fostriecin is a potent inhibitor

of PP2A (IC_{50} = 1.5–3.2 nM) and weak inhibitor of PP1 (IC_{50} = 131 μM) (38,50,53). Fostriecin also has potent inhibitory activity against the purified catalytic subunit of PP4 ($IC_{50} \cong 3.0$ nM) (50), and preliminary data suggest that fostriecin inhibits the activity of PP5 (unpublished observation). Fostriecin also inhibits endogenous, divalent cation-insensitive PPase activity of dilute, whole-cell extracts of RINm5F insulinoma (53) and CHO (38) cells, rat and pig myocyte homogenates (54,55), and homogenates of rabbit ventricular biopsies (55). The divalent, cation-independent PPase activity contained in these homogenates is derived primarily from PP1, PP2A, PP4, PP5, and possibly PP6 (55), and the inhibition kinetics obtained were similar to predicted values for a homogenate composed of these enzymes (38,53,55). The kinetics of fostriecin-induced inhibition of PP2A, PP4, and PP5 correlate well with the ability of fostriecin to inhibit tumor cell proliferation both in vivo and in vitro, suggesting that additional studies into the relationship of these PPases to cell cycle progression is warranted. In addition, several of the aberrant cellular effects observed following treatment with fostriecin [e.g., the formation of aberrant mitotic spindles (38) and the induction of apoptosis (46,47,56–58)] can be mimicked by treatment with other PPase inhibitors (i.e., okadaic acid and cantharidin), when applied at concentrations that inhibit PPase activity to a comparable extent (38,47,53,59,60,61,62). Still, based on in vitro investigations with fostriecin and the limited data available from the phase I clinical trial, it is not yet clear whether blood concentrations of fostriecin predicted to have antitumor activity via PPase inhibition and achievable at doses of 30–40 mg/m^2 in mice can be reached in humans (63).

IV. AGENTS THAT DECREASE EXPRESSION OF PPases

Antisense oligonucleotides that inhibit the expression of human serine/threonine PPases have been identified and characterized. The studies, conducted with both fostriecin and cantharidin, imply that the development of compounds with inhibitory activity against certain PPases may have antitumor activity in humans. However, owing to toxic side effects, semiselective inhibitors may not prove clinically useful. Through the development of agents that decrease the expression of a particular PPase, it is hoped that compounds with antitumor activity and less systemic toxicity can be developed. Yet, one important question remains unanswered: which type or isoform should be targeted?

A. Identification of Antisense Oligonucleotides that Inhibit the Expression of PP5

Inhibition studies conducted with fostriecin indicate that PP5 activity is inhibited at concentrations demonstrated to have antitumor activity in mice. Furthermore, Northern blot analysis of A549 cells reveals that the expression of PP5 is elevated

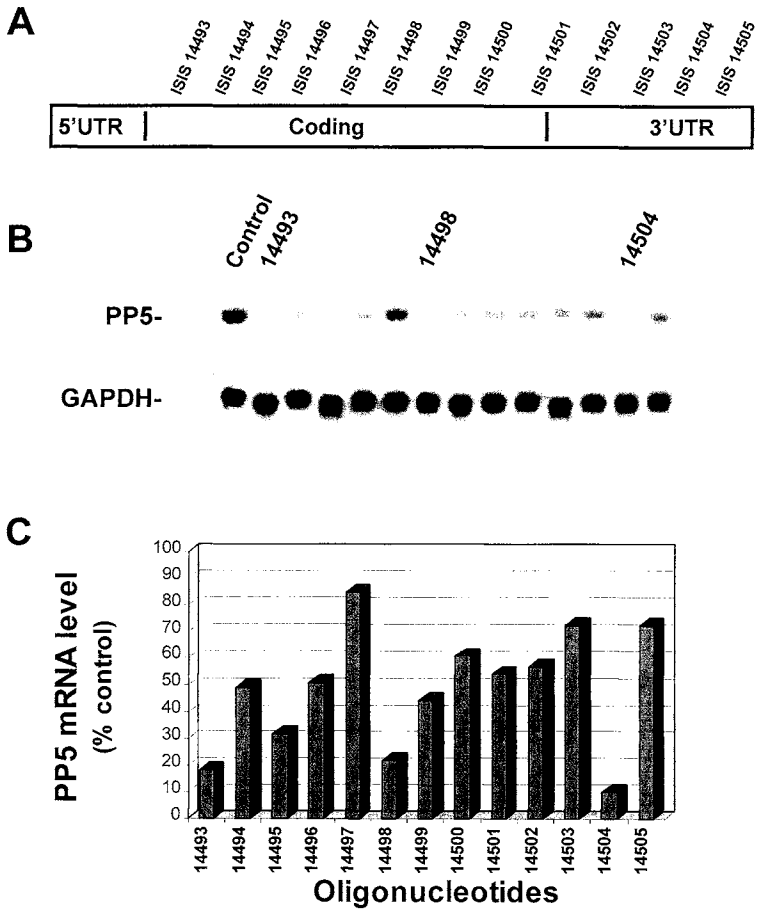
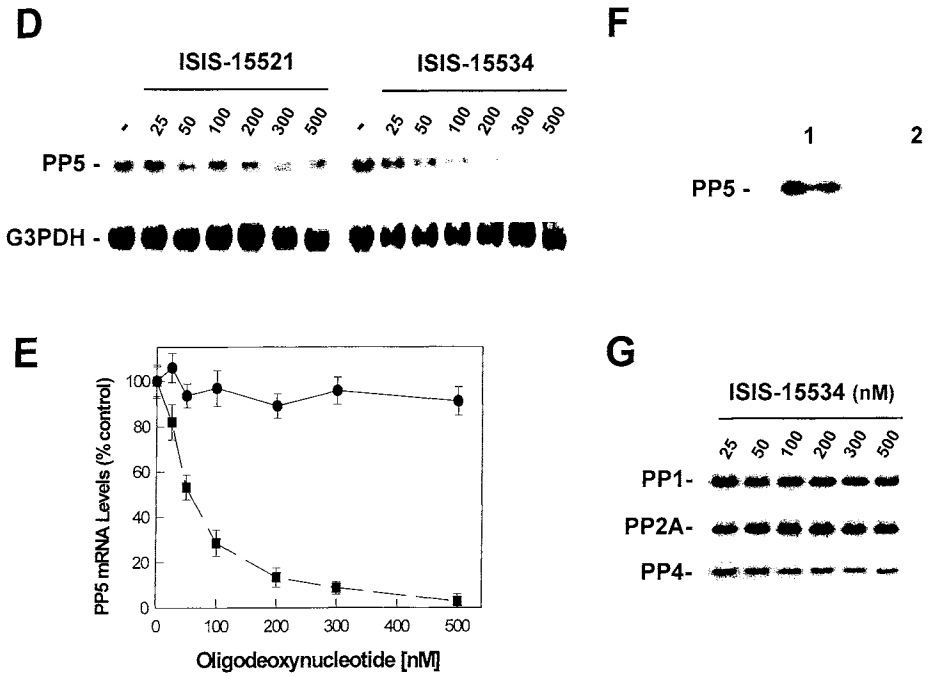


Figure 2 Inhibition of PP5 mRNA and protein expression by treatment with phosphorothioate antisense oligodeoxynucleotides. (A) Relative positioning of the predicted hybridization sites within the human PP5 mRNA of 13 antisense oligodeoxynucleotides that were evaluated for their ability to inhibit PP5 mRNA expression in cultured A549 tumor cells. (B) Identification of antisense oligodeoxynucleotides that inhibit the expression of PP5 mRNA. A549 cells were treated with the indicated antisense oligodeoxynucleotides at a concentration of 300 nM. RNA was prepared 24 h later and analyzed for PP5 and GAPDH mRNA levels by Northern blot analysis. Control cells were treated with random oligodeoxynucleotides. (C) PP5 mRNA levels from Northern blot analysis expressed as a percentage of the levels of PP5 mRNA in control cells following normalization to G3PDH. (D) Inhibition of PP5 mRNA levels by ISIS 15534. A549 cells were treated with increasing concentrations (25–500 nM) of ISIS 15534 (GGGCCCTATTGCTTGAGTGG) or a mismatched control analog, ISIS 15521 (GTGCGATCGTTGCGGTTAGC), that con-



tains 13 base changes (mismatches) within the 15534 sequence. Total mRNA was prepared 24 h later and analyzed for PP5 and G3PDH mRNA levels by Northern blot analysis: (–) indicates untreated cells. (E) Quantification of PP5 mRNA levels after normalization to G3PDH in A549 cells following treatment with increasing concentrations of ISIS 15534 (■) or a mismatched control analog of ISIS 15534, ISIS 15521 (●). (F) Western blot analysis of PP5 protein levels in A549 cells. Cells were treated with the mismatch control oligodeoxynucleotides (1) or ISIS 15534 at a concentration of 300 nM (2), and protein extracts were prepared 24 h later. Each lane contained 40 μg of protein. (G) Target-specific inhibition of PP5 mRNA. A549 cells were treated with increasing concentrations (25–500 nM) of ISIS 15534, and total mRNA was prepared 24 h later and analyzed for PP1, PP2A, and PP4 mRNA levels by Northern blot analysis. (From Ref. 67. Copyright 1998 by the American Society for Biochemistry and Molecular Biology Inc.)

in proliferating cells and suppressed under conditions that arrest cell growth (i.e., upon density-induced growth suppression). Thus, PP5 was chosen as an initial target.

To identify antisense oligonucleotides that potently inhibit the expression of PP5, "chimeric" oligonucleotides 20 bases in length, containing eight central phosphorothioate oligodeoxy residues ("gap") flanked by six 2′-methoxyethyl

residues on the 3′ and 5′ ends (''wings''), were synthesized. The oligonucleotides tested were designed to hybridize to unique regions of human PP5 mRNA and targeted the protein coding region, the 5′-untranslated region, or the 3′-untranslated region (Fig. 2). The ''chimeric'' modifications were chosen because, as discussed in more detail in other chapters of this volume, such modifications have been shown to enhance the potency of antisense oligonucleotides targeting mRNAs encoding other proteins (64,65). Because phosphorothioate oligonucleotides commonly act through an RNase H–dependent mRNA cleavage mechanism in cells, the ability of each oligonucleotide to specifically inhibit the expression of PP5 was initially determined by Northern blot analysis probing for levels of PP5 mRNA in A549 cells. For these studies 50–70% confluent cell cultures were treated with oligonucleotides (300 nM) in a solution containing 15 μg/ml DOTMA/DOPE (Lipofectin; GIBCO-BRL). The cationic lipids (DOTMA/DOPE; Lipofectin) were used to facilitate the uptake of the oligonucleotides in the cultured cells (66). PP5 mRNA levels were then assessed by Northern blot analysis using a [^{32}P]probe for human PP5 generated from the full-length coding region of PP5 and recognizing a single ~2.3-kb transcript. Probe binding was visualized by autoradiography, and each membrane was then stripped and reprobed with a [^{32}P]-labeled glyceraldehyde-3-phosphate dehydrogenase (G3PDH) cDNA probe to confirm equal loading.

A comparison of PP5 mRNA levels in A549 human lung carcinoma cells treated with 300 nM PP5-specific antisense oligonucleotides in the presence of cationic lipids is shown in Fig. 2B. In the initial screen of ~20 oligonucleotides, the reduction in PP5 mRNA levels obtained in response to treatment was varied. Treatment with some oligonucleotides had little or no effect on the inhibition of PP5 mRNA levels, while other oligonucleotides had a moderate effect and a few had pronounced effects (Fig. 2B and 2C). The antisense oligonucleotide with the most potent activity against PP5 mRNA identified in this series was ISIS 14504, which targets a region contained in the 3′-untranslated region of PP5. To increase the potency of 14504, oligonucleotides containing additional modifications were synthesized and tested. These studies revealed that when the ''gap'' region was increased from eight to 10 contiguous phosphorothioate nucleotides (ISIS 15534), the potency of inhibition was improved. Thus, ISIS 15534 was chosen for future studies. Time course studies indicated that the mRNA was effectively decreased by 8–10 h, while the Western blot analysis indicates that the treatment of cells with ISIS 15534 also effectively decreased PP5 protein levels after 24–36 h (Fig. 2F).

1. Specificity of PP5 Antisense Inhibition

To test the specificity of ISIS 15534 treatment on the expression of PP5 mRNA, mismatch control analogs were synthesized. The mismatch controls (i.e., ISIS

15521) have the same base composition as ISIS 15534 but with sequences non-complementary to PP5. As illustrated in Fig. 2D and 2E, the dose-dependent reduction of PP5 mRNA levels observed after treatment with ISIS 15534 was not observed following treatment with the mismatch controls, suggesting that the observed effect was not due to the chemical composition of the oligonucleotides. Similarly, since the sequence targeted by ISIS 15534 is not contained in the structurally related PPases (PP1, PP2A, PP4), ISIS 15534 would not be expected to inhibit the expression of these proteins if it is inhibiting the expression of PP5 mRNA via an antisense mechanism. To test this, mRNA levels of PP1, PP2A, and PP4 were evaluated following treatment with 0–500 nM ISIS 15534. As depicted in Fig. 2D and 2G, even at a concentration of 500 nM, ISIS 15534 has no effect on the expression of PP1, PP2A, PP4, or G3PDH mRNA levels.

2. Cellular Effect of ISIS 15534

Satisfied that ISIS 15534 acts as a specific and potent inhibitior of PP5 mRNA expression, we explored the cellular effect produced by a decrease in PP5 expression. First, the effect of ISIS 15534 on cell growth was tested in A549 cells treated one time with ISIS 15534 or its mismatched control at a concentration of 300 nM. The number of cells was then assessed daily over a 5-day period. This single treatment with ISIS 15534 resulted in a marked suppression of A549 cell proliferation (Fig. 3), with growth suppression observed for 3 days. After day 4, the cells started to proliferate, and the renewed growth was associated with the recovery of PP5 mRNA expression. The reason for the recovery of PP5 expression is not known. However, it may be due to the extrusion of the antisense oligonucleotides from the cells, nuclease-mediated degradation of ISIS 15534 after a single application of the oligonucleotides, or the proliferation of a small percentage of cells that failed to absorb the oligonucleotides during the initial treatment. In contrast, treatment with the mismatched control oligonucleotides did not affect cellular proliferation.

3. ISIS 15534–Mediated Inhibition of Cell Growth Correlates with the Induction of $p21^{WAF/Cip1}$

Unlike the effect produced following treatment with fostriecin or cantharidin (38), flow cytometry analysis of ISIS 15534–treated cells did not reveal G2/M-phase growth arrest. In contrast, growth suppression was delayed at the G1/S-phase transition. Further exploration of possible mechanisms by which the inhibition of PP5 expression resulted in growth suppression revealed a marked inverse relationship between the expression of PP5 and the expression of a cyclin-dependent kinase (Cdk) inhibitor protein, $p21^{WAF/Cip1}$ (67). As depicted in Fig. 4, following treatment with ISIS 15534, the amount of $p21^{WAF/Cip1}$ mRNA contained in logarithmically growing A549 cells increases in a dose-dependent manner to a

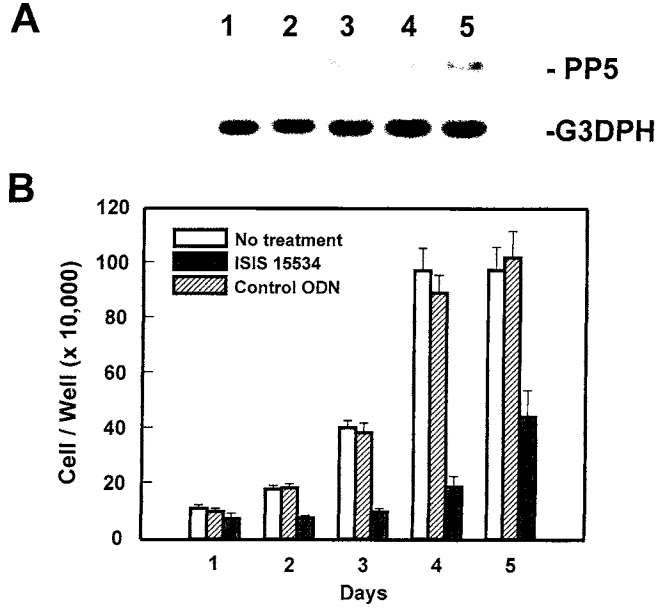


Figure 3 Antiproliferative effects of PP5 antisense in A549 cells. (A) Recovery of PP5 mRNA with time after treatment of A549 cells with ISIS 15534. A549 cells were treated one time with ISIS 15534 at a concentration of 300 nM at time 0. At days 1, 2, 3, 4, and 5 total RNA was prepared and analyzed for PP5 and G3PDH mRNA levels by Northern blot analysis. (B) Time course for the antiproliferative effects of ISIS 15534 in A549 cells. Cells in log-phase growth were treated with a single dose (300 nM) at time 0 with ISIS 15534 or the mismatched control oligodeoxynucleotide analog of ISIS 15534 (described in legend to Fig. 2). Cell number was determined after days 1, 2, 3, 4, and 5 following treatment. Each point represents the mean of triplicate cultures with error bars representing the SD. (Reprinted from Ref. 67. Copyright 1998 by the American Society for Biochemistry and Molecular Biology Inc.)

level >10-fold higher than that observed in cells treated with the mismatched control oligonucleotides. This increase in $p21^{WAF/Cip1}$ mRNA levels correlates well with the does-dependent inhibition of PP5 mRNA expression (Fig. 4), suggesting that there is an inverse relationship between the expression of PP5 and the expression of $p21^{WAF/Cip1}$.

The $p21^{WAF1/Cip1}$ gene encodes an inhibitor of most cyclin-dependent kinases (68–72) and has been implicated in both G1/S- and G2/M-phase growth arrest of many cell types. One pathway leading to the induction of $p21^{WAF1/Cip1}$ expression is mediated by the p53 tumor suppressor protein, which functions as a transcription

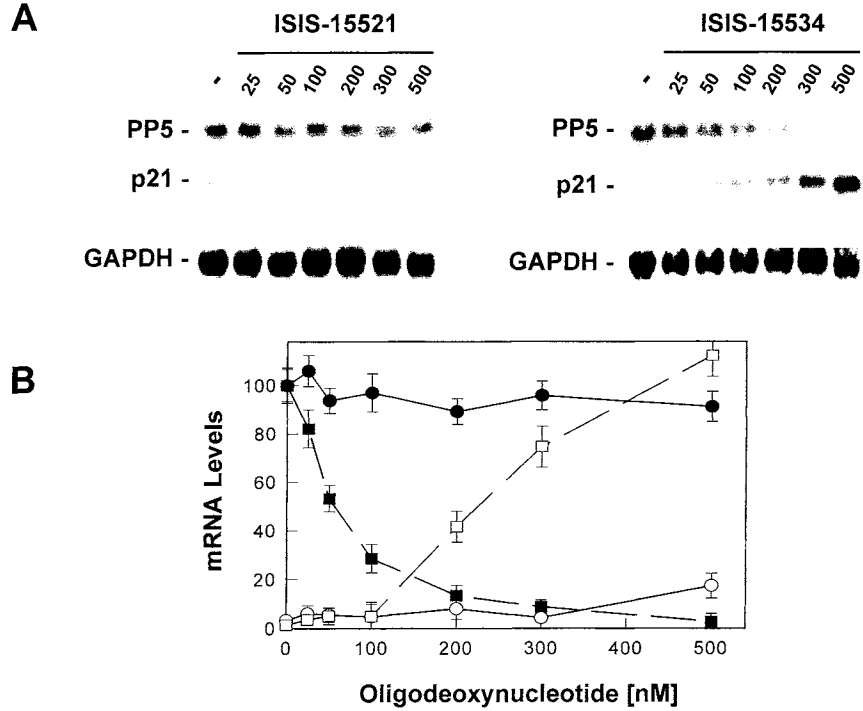


Figure 4 Inhibition of PP5 expression induces the expression of $p21^{WAF/Cip1}$. (A) A549 cells in log-phase growth were treated with increasing concentrations (25–500 nM) of ISIS 15534 or a mismatched control (ISIS 15521) analog of ISIS 15534. Total RNA was prepared 24 h later and analyzed for PP5, $p21^{WAF/Cip1}$, and GAPDH mRNA levels by Northern blot analysis. (B) Quantification of PP5 mRNA and $p21^{WAF/Cip1}$ mRNA levels after normalization to GAPDH mRNA levels in A549 cells following treatment with increasing concentrations of ISIS 15534 (■, □), or a mismatched control analog of ISIS 15534 (●, ○). Filled symbols indicate PP5 mRNA and open symbols indicate $p21^{WAF/Cip1}$ mRNA. (From Ref. 67. Copyright 1998 by the American Society for Biochemistry and Molecular Biology Inc.)

factor to control the expression of p53-responsive genes. As part of a cellular response to DNA damage (73,74), the ensuing accumulation of nuclear p53 induces the synthesis of p53-responsive genes, one of which is $p21^{WAF/Cip1}$. In turn, $p21^{WAF/Cip1}$ inhibits the activity of G1/Cdk activity (cyclin D/Cdk4,6 and/or CyclinE/Cdk2), inducing cell growth arrest late in the G1 stage of the cell cycle (75). Recent studies in fibroblasts (76), osteoscarcoma (77), and rat hepatoma cells (78) have demonstrated that dexamethasone-mediated growth suppression

is also associated with increased expression of $p21^{WAF1/Cip1}$, suggesting that $p21^{WAF1/Cip1}$ participates in a glucocorticoid receptor–induced signaling network(s) that regulates G1-growth suppression.

Further characterization of the mechanism by which the inhibition of PP5 gene expression results in increased $p21^{WAF/Cip1}$ expression revealed that the increase in $p21^{WAF/Cip1}$ mRNA levels observed after treatment with ISIS 15534 does not require a concomitant increase in p53 expression (67). However, in A549 cells, increased $p21^{WAF/Cip1}$ expression is associated with both enhanced phosphorylation of p53 and enhanced binding of p53 to DNA-containing p53 recognition sequences (67). This is consistent with reports in other cell types where enhanced p53 phosphorylation increases both the DNA binding activity and the transcriptional activity of p53 (79–81). Studies conducted in p53-deficient T-24 cells (79,82), p53-null human fibroblasts (TR9), and a stable cell line, derived from MDAH041 p53-null human fibroblasts that contain tetracyline-regulated transactivator and operator plasmids to control the expression of wild-type p53 (TR9-7 cells) (83), reveal that, in the absence of p53, ISIS 15534 treatment does not induce G1-growth arrest or the induction of $p21^{WAF/Cip1}$ (67). However, when wild-type p53 is reintroduced into TR9-7 cells at a low level [one that is insufficient to induce $p21^{WAF/Cip1}$ or cell cycle arrest on its own (83,84)], then treatment with ISIS 15534 induces both the expression of $p21^{WAF/Cip1}$ mRNA and growth suppression. In contrast, when the expression of wild-type p53 is totally suppressed, ISIS 15534–mediated inhibition of PP5 expression does not induce $p21^{WAF/Cip1}$ or G1-growth arrest. Together, these data suggest that when the expression of PP5 is suppressed the ensuing hyperphosphorylated form of p53 is capable of inducing $p21^{WAF/Cip1}$ expression and growth arrest.

4. PP5 and Glucocorticoid Receptor Signaling Cascades Leading to the Induction of $p21^{WAF1/Cip1}$

In addition to p53, ligand-induced glucocorticoid receptor (GR) activation has also been linked to the inhibition of cell proliferation via the transcriptional induction of $p21^{WAF1/Cip1}$. Since PP5 coimmunoprecipitates with the GR–heat shock protein 90 (hsp 90) complex (85,86) and both PP5 and hsp-90 are retained by microcystin-Sepharose affinity chromatography (Honkanen et al., unpublished observation), the relationship of GR, PP5, p53, and $p21^{WAF1/Cip1}$-mediated growth arrest was explored. To test the potential role of PP5 in glucocorticoid-mediated growth arrest, the effect of dexamethasone and ISIS 15534 on the growth rates of A549, T-24, and TR9-7 cells was assessed. As observed following treatment with ISIS 15534, dexamethasone treatment inhibited the proliferation of A549 cells, while having no apparent effect on the growth rate of T-24 or TR9 cells (Fig. 5). In addition, Northern blot analysis revealed that dexamethasone treatment produces an increase in the expression of $p21^{WAF1/Cip1}$ in A549 cells, while

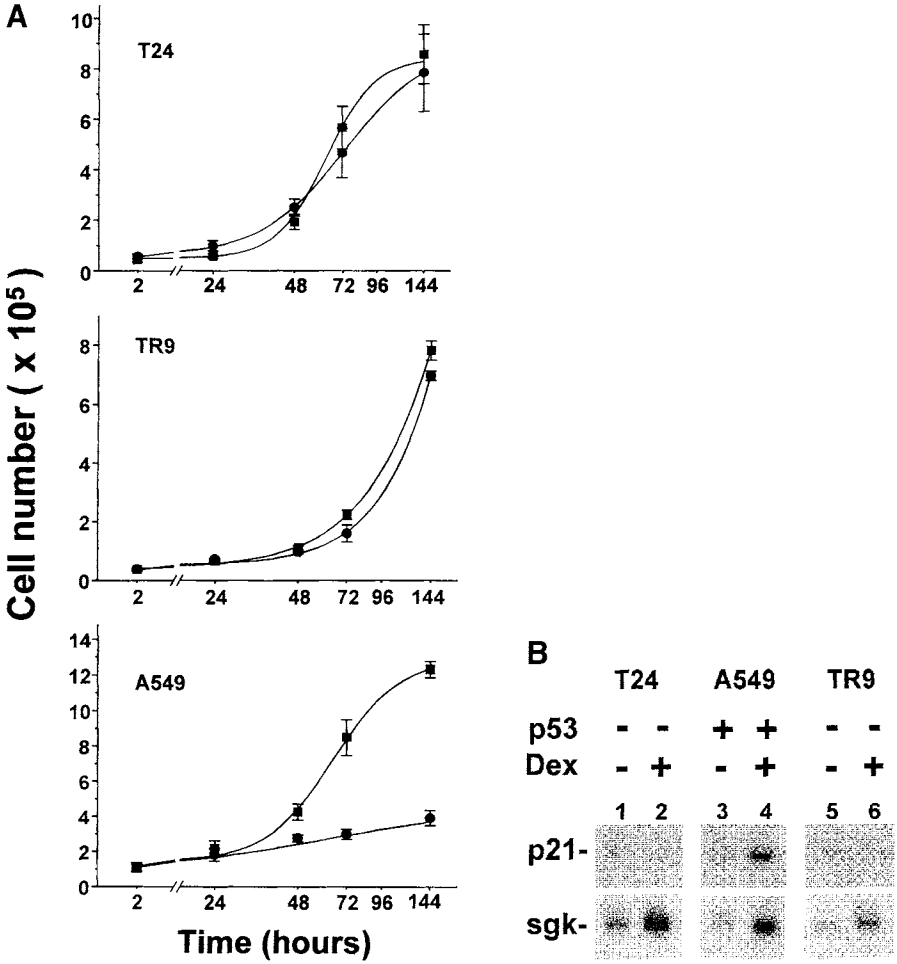
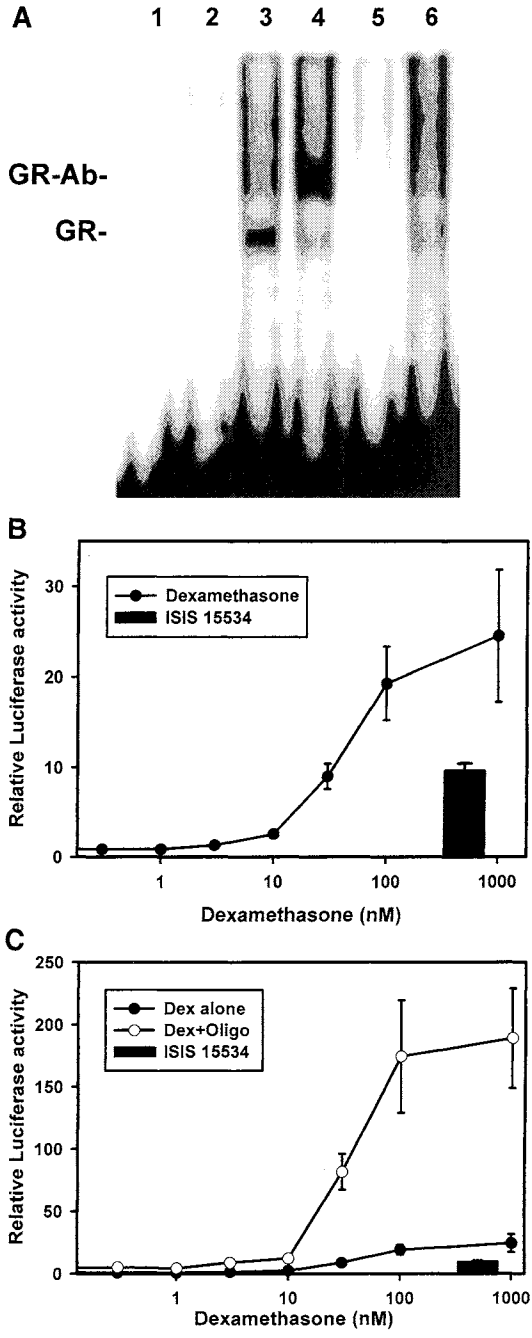


Figure 5 The antiproliferative effect of dexamethasone correlates with the presence of wild-type p53 and the expression of $p21^{WAF1/Cip1}$ mRNA. (A) The effect of dexamethasone on cell proliferation in p53-defective T-24 human bladder carcinoma, p53-null TR9 human fibroblasts, and p53 wild-type A549 human lung carcinoma cells was determined by treating cells in log-phase growth with a single dose of 100 nM dexamethasone and counting the number of cells in treated (●) and untreated (■) cultures for 6 consecutive days following treatment. Each point represents the mean of triplicate dishes with error bars representing SE. (B) Comparison of $p21^{WAF1/Cip1}$ (p21) and serum glucocorticoid inducible protein kinase (sgk) mRNA levels in p53-competent (Lanes 3, 4) and p53-deficient (Lanes 1, 2, 5, 6) cell cultures after dexamethasone treatment. Cell cultures in log-phase growth were treated with 100 nM dexamethasone (+) or vehicle alone as a control (−). After 24 h total mRNA was prepared and analyzed for $p21^{WAF1/Cip1}$ and sgk mRNA levels by Northern analysis. (From Ref. 87. Copyright 1999 by American Chemical Society.)



no increase was observed in the two p53-defective cell lines tested (87). Binding studies with [^{3}H]dexamethasone indicated that both T-24 and TR9 cells express GRs, and dexamethasone treatment induced the expression of serum/glucocorticoid-inducible protein kinase (sgk) in all three cell lines. Therefore, all three cell types appear to express functional GR, and the inability of dexamethasone to induce $p21^{WAF1/Cip1}$ expression/growth arrest in T-24 and TR9 cells is not due to the lack of GRs.

5. Effect of PP5 on the Binding of Dexamethasone to the GR

To explore the relationship of PP5 and GR activation in the induction of $p21^{WAF1/Cip1}$ further, binding studies were conducted with [^{3}H]dexamethasone in A549 cells pretreated with ISIS 15534 or mismatched controls (ISIS 15521). These studies revealed no differences in the specific binding of [^{3}H]dexamethasone in control, ISIS 15534–treated, and ISIS 15521–treated cells (87), suggesting that

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Figure 6 (A) Inhibition of PP5 expression enhances glucocorticoid-receptor binding to DNA. Nuclear extracts were prepared from A549 cell cultures treated with mismatched control oligodeoxynucleotides (ISIS 15521), ISIS 15534, or 1 μM dexamethasone. After 16 h, the ability of GR to bind DNA was analyzed by gel-mobility shift assay. Lane 1, no protein control: migration of ^{32}P-GRE probe in the absence of nuclear extracts; Lane 2, control: nuclear extracts prepared from mismatch control oligodeoxynucleotides treated cells (ISIS 15521; 500 nM); Lane 3, ISIS 15534–induced gel shift: nuclear extracts from cells treated with 300 nM ISIS 15534; Lane 4, GR-antibody-induced supershift: nuclear extracts used in Lane 3 after further incubation with an antibody generated against GR; Lane 5, excess cold probe control: samples treated in an identical manner as in Lane 3 after incubation in the presence of excess nonradioactive GRE probe; Lane 6, dexamethasone-induced supershift; nuclear extracts from cells treated with 1 μM dexamethasone following incubation with the GR antibody used in Lane 4. (B) Stimulation of GRE promoter activity with dexamethasone or ISIS 15534. A549 cells were transiently transfected with GR luciferase reporter plasmid, MMTV-luc. Twenty-four hours later, the cells were treated with a single dose of the indicated amount of dexamethasone (●) for 2 h at 37°C, harvested, washed twice with PBS, and lysed. Aliquots (150 μl) of cell extracts were then assayed for luciferase activity. The relative light units of the cell extract were calculated as an average of three independent experiments, performed in triplicate, and presented as relative luciferase activity with error bars indicating SD. Luciferase activity in cells treated with 500 nM ISIS 15534 in the absence of dexamethasone is indicated by the solid bar. (C) Inhibition of PP5 expression enhances glucocorticoid stimulation of GR-reporter plasmids. The transient transfection studies described in B were repeated with an additional experimental group (○) in which PP5 mRNA expression was inhibited by treatment with 500 nM ISIS 15534 18 h prior to the addition of dexamethasone. Note the difference in scale on the Y-axis. (From Ref. 87. Copyright 1999 by American Chemical Society.)

the suppression of PP5 expression has no apparent effect on hormone binding. Similarly, dexamethasone did not affect the expression of PP5. However, when the expression of PP5 was suppressed by treatment with ISIS 15534, mobility gel-shift studies revealed a marked increase in the association of nuclear GR and DNA-containing consensus glucocorticoid recognition elements (GRE). The enhanced association of nuclear GR and GRE occurs even in the absence of dexamethasone and indicates that: (1) the inhibition of PP5 expression alone is sufficient to enhance the association of GR with GRE, and/or (2) a decrease in PP5 expression facilitates the nuclear accumulation of GR.

To determine whether the observed increase in the association between nuclear GR and DNA-containing GRE results in increased transcriptional activity, transient transfection studies were conducted in A549 cells using glucocorticoid-responsive reporter plasmids (MMTV-luc). As shown in Fig. 6, dexamethasone treatment produced a dose-dependent stimulation of transcriptional activity, with the addition of 100 nM dexamethasone producing an ~20-fold increase in luciferase activity. Treatment with ISIS 15534 alone also enhanced GR-reporter plasmid activity (Fig. 6B). However, GR-reporter plasmid activation was more pronounced after treatment with dexamethasone than after treatment with ISIS 15534 (Fig. 6B). Transient transfection studies, conducted with GR-reporter plasmids in A549 cells 24 h after the expression of PP5 was inhibited with ISIS 15534, revealed that dexamethasone treatment induced a dose-dependent increase in GR-reporter plasmid activation. The maximal increase in luciferase activity was ~200-fold above the control and ~10 times greater than the maximum response achieved with dexamethasone alone (Fig. 6C). Gel-shift analysis of nuclear extracts following the combined treatment did not reveal an increase in GR/GRE interactions beyond that produced by treatment with ISIS 15534 alone. Therefore, inhibition of PP5 expression appears to enhance GR-induced gene expression. However, it is not yet clear whether both dexamethasone and ISIS 15534 induce the expression of $p21^{WAF1/Cip1}$ by exerting an effect on parallel pathways that have a common endpoint or, alternatively, whether PP5 participates directly in a GR-mediated signaling cascade leading to the induction of $p21^{WAF1/Cip1}$ and growth arrest (Fig. 7).

In summary, studies with antisense oligonucleotides have provided valuable insight into the cellular functions of PP5 that were impossible to discern with available semiselective small-molecule inhibitors. The studies conducted with ISIS 15534 have revealed that PP5 is a key regulator of cell growth, affecting both glucocorticoid-induced and p53-mediated cellular responses controlling the expression of $p21^{WAF1/Cip1}$. Although the suppression of PP5 expression inhibits cellular proliferation, it failed to induce G2/M-phase growth arrest, as achieved with fostriecin and okadaic acid prior to the onset of apoptosis. Therefore, if the formation of aberrant mitotic spindles and G2/M-phase growth arrest is indeed associated with the antitumor activity of fostriecin, it appears to result from the

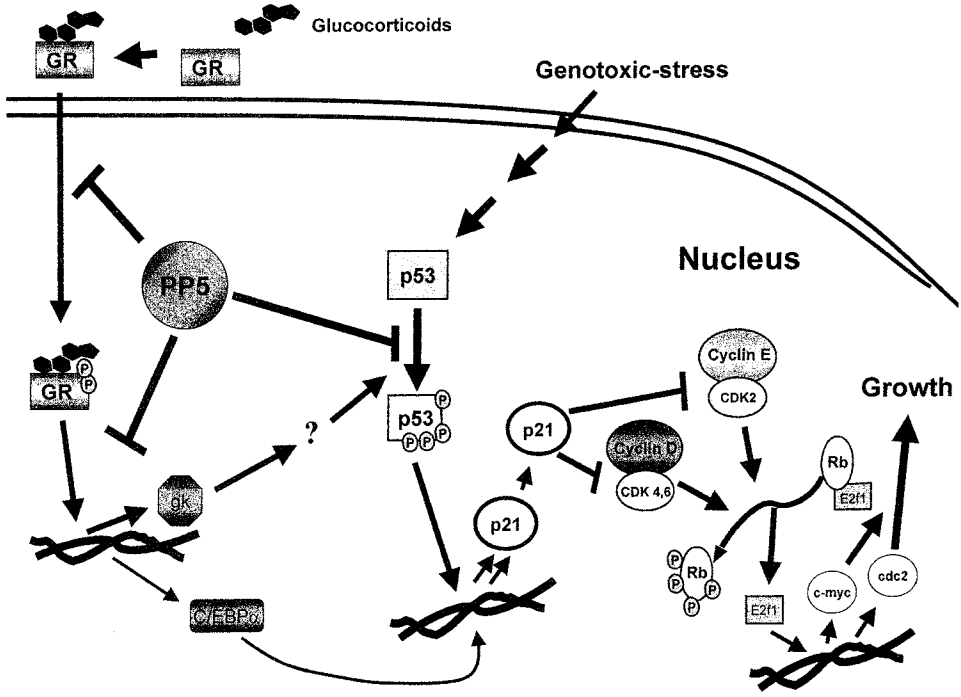

Figure 7 Proposed role of PP5 in the regulation of G1-growth arrest initiated by glucocorticoids or genotoxic stress. The increase in PP5 expression that occurs during log-phase cell growth functions to inhibit glucocorticoid-mediated growth arrest by blocking the association of the ligand-activated GR complex with specific recognition sequences in the promoter of glucocorticoid responsive genes and/or the formation of an active GR-transcriptional complex. This inhibits the expression of a GR-inducible protein kinase (gk) or a signal transduction cascade that results in activation of a kinase that catalyzes the phosphorylation of p53. In the absence of PP5, the hyperphosphorylated form of p53 produced has increased transcriptional activity, which allows the basal amount of p53 to induce the expression of the cyclin/CDK inhibitor protein, $p21^{WAF1/Cip1}$ (p21). $p21^{WAF1/Cip1}$ inhibits the activity of G1-cyclin/CDK complexes (cyclin D/CKD4, 6; cyclin E/CDK2). In turn, this prevents the hyperphosphorylation of the Rb-tumor suppressor protein and facilitates G1 cell cycle arrest. In response to genotoxic stress, the amount of p53 increases owing to a decrease in degradation rate and, in some instances, an increase in p53 expression (51–58). In the presence of PP5, the increased amount of p53 may be necessary to "override" the growth-promoting effects of PP5. (From Ref. 87. Copyright 1999 by American Chemical Society.)

inhibition of a PPase other than PP5. However, a role for PP5 in G2/M-phase cell growth has not been ruled out. Because the inhibition of PP5 expression arrests cell growth prior to the onset of S phase, it is still possible that PP5 also participates in events later in the cell cycle that are not apparent owing to suppression of growth prior to the onset of S phase.

B. Identification of Antisense Oligonucleotides that Inhibit the Expression of PP1 γ1

In lower eukaryotes (*Schizosaccharomyces pombe, Saccharomyces cerivisiae, Aspergillus nidulans*, and *Drosophila*), mutations in the genes encoding protein phosphatases homologous to mammalian PP1 produce aberrations during mitosis and anaphase (88–94). Similarly, studies of microtubule dynamics during mitosis in *Xenopus* egg extracts, and studies with PP1-neutralizing antibodies in rat embryo fibroblasts, suggest that PP1 activity is necessary for the completion of mitosis (93–95). In A549 cells, as observed with PP5, the expression of PP1γ1, but not PP1 δ or PP1 α, is elevated during log-phase growth. In addition, although the concentration of fostriecin associated with antitumor activity in animals produces only a partial inhibitory effect on the purified catalytic subunit of PP1, "purified PP1" is likely composed of a mixture that contains the α, δ, and γ isoforms. Thus, the partial inhibition produced by fostriecin could reflect the inhibition of a fostriecin-sensitive isoform contained in the mixture.

To identify antisense oligonucleotides that inhibit the expression of PP1γ1, 12 oligonucleotides, 20 bases in length and predicted to hybridize to different regions of human PP1γ1 mRNA, were synthesized. Like the oligonucleotides targeting PP5, the oligonucleotides tested were designed to target specific regions in the protein-coding region, the 5′-untranslated, or the 3′-untranslated region of human PP1γ1 mRNAs, and were "chimeric" 2′-*O*-(2-methoxy)ethylphosphothioate oligonucleotides, containing eight central phosphorothioate oligodeoxy residues ("oligodeoxy gap"), flanked by six 2′-*O*-(2-methoxy) residues on the 3′ and 5′ ends. Similarly the ability of each oligonucleotide to specifically inhibit the expression of PP1γ1 was determined by Northern blot analysis, probing for levels of PP1γ1 mRNA using a PP1γ1-specific cDNA probe that forms a hybrid with a single ~2.4-kb transcript.

As observed with antisense oligonucleotides targeting PP5, the response to treatment was varied, yet two, ISIS 14435 and ISIS 14439, potently (IC_{50} ~ 50 nM) inhibited the expression of PP1γ1 (96). The specificity of both ISIS 14435 and ISIS 14439 for PP1γ1 was demonstrated by both Northern blot analysis and ribonuclease protection assays (96), and the cellular effects of ISIS 14435 treatment were then assessed. Similar to the effect observed following the inhibition of PP5 expression, treatment with ISIS 14435 resulted in the inhibition of A549

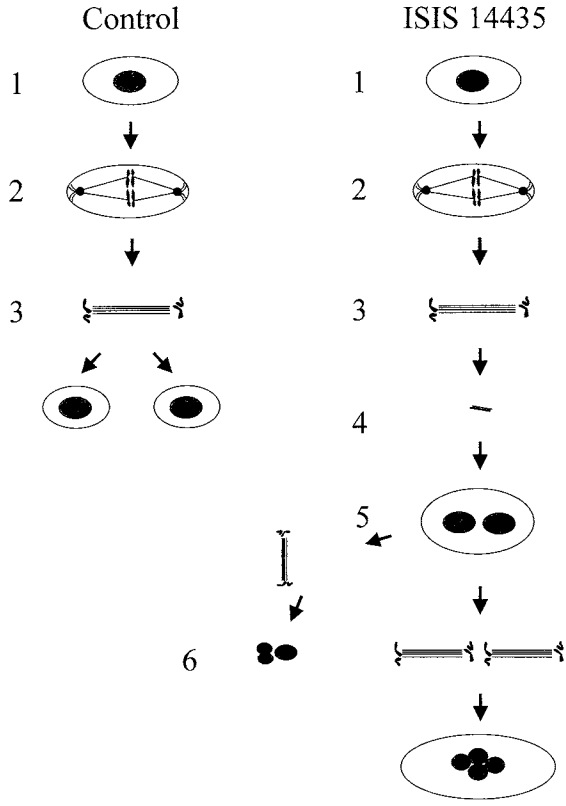

Figure 8 Diagram of cell division illustrating the effect of treatment with ISIS 14435 on cell division. Time-lapse video microscopy revealed that a reduction of PP1γ1 expression had no apparent affect on cell cycle progression into mitosis (1), the formation of mitotic spindles (2), or the onset of telophase (3). However, unlike cells treated with the mismatch control oligonucleotides (control), cells treated with the antisense oligonucleotides targeting PP1γ1 arrest in cytokinesis after the formation of a deep cleavage furrow (4). After ~140 min, the contractile ring appears to relax and two apparently normal nuclei reform (5). Most of the dikaryons die without further replication. However, a few (<1%) undergo a ''second attempt at cellular replication'' that fails and results in production of multinucleated cells (6). (From Ref. 96. Copyright 2000 by the American Society for Biochemistry and Molecular Biology Inc.)

cell proliferation. However, the effect produced by the inhibition of PP1γ1 expression was markedly different than that produced by ISIS 15534. Following a single treatment with 300–500 nM ISIS 14435, growth arrest occurred after ~48 h. Fluorescence-activated cell sorter analysis (FACS analysis) revealed no evidence of G1/S-phase growth arrest. In contrast, when the expression of PP1γ1 was suppressed, an increase in the accumulation of cells with 4 N DNA was observed (96). Immunostaining with antitubulin antibodies revealed that the suppression of PP1γ1 expression had no apparent effect on the formation of mitotic spindles or the appearance of prophase microtubules (96). Thus, again we have failed to produce a fostriecin-like effect. However, following treatment with ISIS 14435, there was a marked increase in the number of cells blocked in the late stages of cytokinesis (96). Time-lapse video microscopy of ISIS 14435–treated cells revealed that, following the delay in late cytokinesis, the deep cleavage furrow relaxes. Then, two distinct nuclei reform. Most of the dikaryons that are formed do not undergo a second round of nuclear replication. Yet, occasionally, multinucleated cells are produced by a second aberrant attempt at cell division (Fig. 8). Thus, although the inhibition of PP1γ1 does not appear to affect mitotic spindle formation, the studies with ISIS 14435 imply that PP1γ1 plays a critical role during the late stages of cytokinesis. Future studies designed to determine the substrates utilized by PP1γ1 and how the regulation of PP1γ1 activity is coordinated with that of other cellular proteins that regulate the onset and progression of cytokinesis should prove interesting.

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22

Pharmacological Activities of Antisense Drugs: Inflammatory Diseases

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I. INTRODUCTION

A. Inflammatory Processes

Inflammation is fundamentally a protective process, designed to facilitate the body's ability to respond to perceived physical or microbial threats. The classic hallmarks of inflammation—redness, swelling, heat, and pain—occur because of increases in blood flow and capillary permeability, the leakage of plasma proteins, and migration of white blood cells into the site of injury. The outcome of a particular inflammatory reaction depends on a complex interplay of many factors (summarized in Fig. 1). These can include the types of leukocytes migrating into the tissue; their interactions with each other, parenchymal cells, and the extracellular matrix; the particular "soup" of cytokines, chemokines, reactive oxygen species, and plasma proteins elicited; host genetics; the nature of the antigen or foreign body that elicits the inflammatory response; and its ability to be cleared. When inflammatory processes become clinically apparent, it usually means the body has been besieged by either a large amount of a foreign substance, a foreign substance in an unusual location, or one that is difficult to eliminate. When inflammation is dysregulated, disease or even death can result.

Inflammatory processes can be either acute or chronic. Both have the potential to become dysregulated. Acute inflammation typically involves the innate immune system, while chronic inflammation mobilizes cells of the adaptive (antigen-specific) immune response. Acute inflammatory foci are usually dominated by neutrophils, but other phagocytic cells such as eosinophils, monocytes, and macrophages may also migrate to the site of injury. They are attracted there

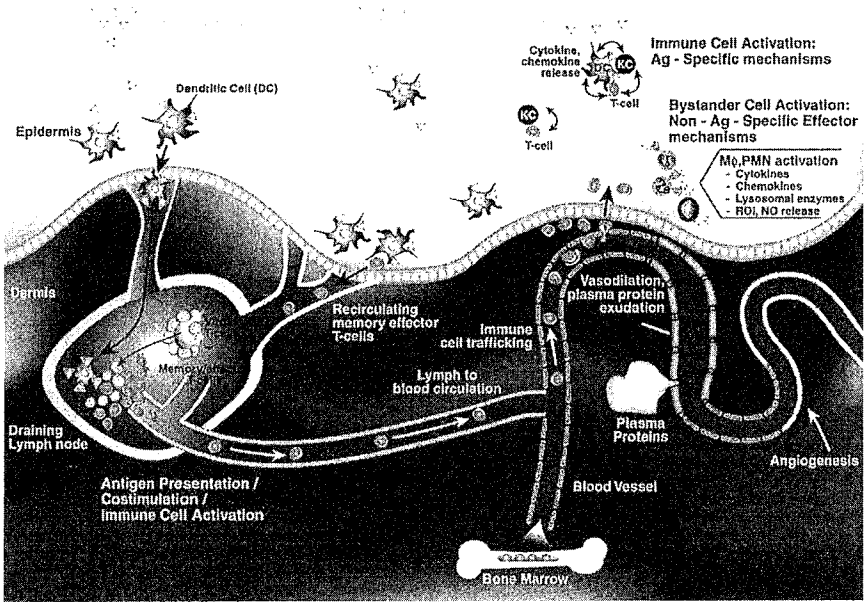

Figure 1 The complex interplay of factors contributing to an inflammatory reaction are illustrated for a hypothetical immune response occuring in the skin. Both antigen-specific and antigen-nonspecific cells are mobilized by the invading antigen. The antigen-specific response is generated when epidermal dendritic cells (Langerhans' cells) pick up antigen and transport it to draining lymph nodes, where it is presented to virgin T cells that have entered the lymph node via high endothelial venules. The lymph node provides the proper microenvironment for the virgin T cells to undergo clonal expansion and mature into memory and effector cells. These cells leave the lymph node, enter the bloodstream, and circulate until they are attracted back into the skin by cytokines, chemokines, and other inflammatory proteins elicited in the skin by cells of the innate (antigen nonspecific) immune system and resident skin cells such as epidermal keratinocytes. Neutrophils and macrophages at the site of antigen invasion secrete cytokines, chemokines, lysosomal enzymes, and reactive oxygen and nitrogen species in an effort to clear the antigen. This contributes to the activation of keratinocytes, which become capable of presenting antigen to memory T cells. Extravasation into an inflammatory site is not antigen-restricted: any T (or B) cell expressing appropriate homing receptors and adhesion molecules can be recruited, and antigen-specific T cells may actually represent a small percentage of the overall lymphocytic infiltrate. Blood vessels are also affected by the soup of inflammatory molecules generated in the skin. Some inflammatory cytokines can elicit the growth of new blood vessels (angiogenesis), while endothelial cells lining blood vessels also become activated and express adhesion molecules that serve to attract circulating leukocytes into the site. Blood flow and capillary permeability are also increased, and plasma proteins that have chemoattractant and inflammatory properties are exuded into the skin. DC, dendritic cell; KC, keratinocyte; mϕ, macrophage; PMN, polymorphonuclear leukocyte; ROI, reactive oxygen intermediates; NO, nitric oxide.

by chemotactic factors, which can include complement C5a, platelet-activating factor, leukotrienes, bacterially derived peptides (e.g., fmet-leu-phe), and cytokines like IL-8. Phagocytic cells can recognize invaders by cell surface receptors that allow them to attach nonspecifically to a variety of microorganisms. The phagocytic process is greatly facilitated, however, when the microbe is opsonized by antibody or the C3b component of complement. Once internalized, bacteria and parasites are usually killed by a combination of reactive oxygen intermediates, enzymes, and other proteins contained in cell granules or lysosomes.

Chronic inflammation occurs when an antigenic stimulus persists. The antigen can be either a microbial pathogen resistant to elimination or, in the case of autoimmune diseases, a self-antigen. Although chronic inflammation can involve a wide variety of white blood cells, macrophages and lymphocytes usually occur in greater numbers than they do in the neutrophil-dominated acute inflammatory lesion. The adaptive immune response plays a more central role in chronic inflammation than it does in most acute scenarios. T and B lymphocytes bearing unique cell surface receptors for antigen interact not only with the antigen and with one another, but also with macrophages and other antigen-presenting cells in an effort to clear the antigen. These cells impart a unique set of surface receptors, cytokines, chemokines, and effector mechanisms to the inflammatory milieu.

Neither the innate nor adaptive immune systems act in isolation during acute or chronic inflammation. Antibodies produced by B cells, for instance, help phagocytes recognize and bind to their targets. Activated antigen-specific T cells can produce cytokines that attract other leukocytes of the innate immune system to the inflammatory site and activate macrophages. Macrophages, in turn, can facilitate antigen recognition by T cells by migrating to lymphoid organs, and processing and presenting the antigen in a form and an environment that facilitates an immune response (see Fig. 2).

The cells of the innate and adaptive immune systems, the products they produce, as well as their interactions with various plasma enzyme systems involved in inflammation (e.g., the complement, clotting, fibrinolytic, and kinin cascades) all represent potential points for intervention in inflammatory diseases. A particularly attractive strategy for modulating these various inflammatory targets involves the use of antisense oligonucleotides. The antisense approach is useful not only as a route to anti-inflammatory drug development, but also as a tool for dissecting the role of particular molecules in inflammation, since antisense oligonucleotides have the potential for highly specific down-regulation of defined molecular targets.

Over the last 5 years, the antisense field has matured considerably, and the number of studies using antisense oligonucleotides to modulate various inflammatory targets and diseases has burgeoned. This chapter will review some of the recent antisense strategies used to down-regulate molecular targets that could play a role in inflammation. (For earlier reviews in this arena, see Refs. 1–5).

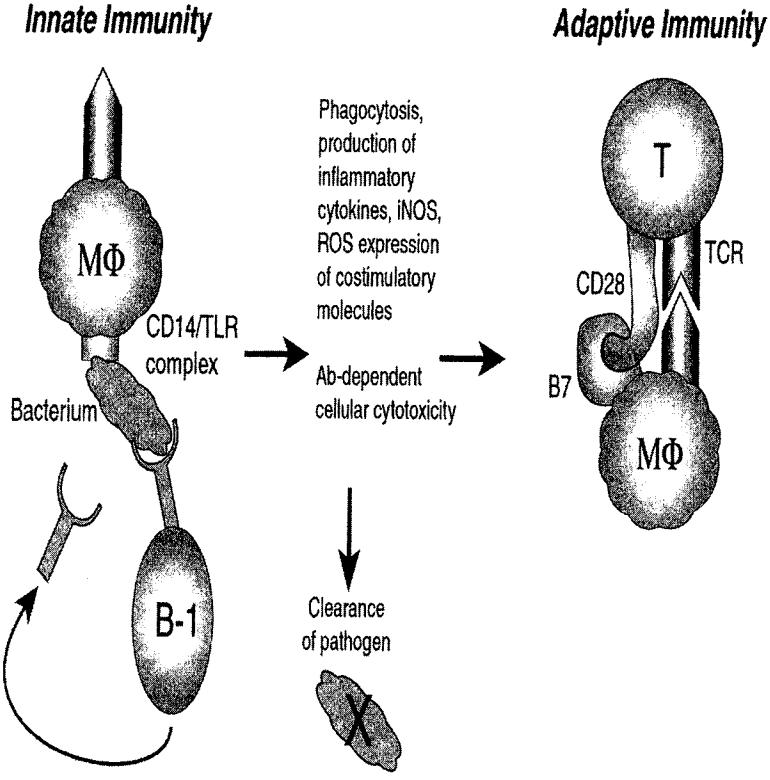

Figure 2 Communication between the innate and adaptive systems of immunity. Until recently, specific molecules that mediate cognate interaction between cells of the innate and acquired (or adaptive) arms of immunity were unknown. The identification of human toll-like receptors (TLRs) and the delineation of their ability to signal CD14-mediated recognition of pathogens such as bacteria by cells of the innate immune system have provided an example of such an interaction (reviewed in Ref. 238). This signal leads to the induction of cell surface molecules that act as danger signals to alert T lymphocytes to respond, evoking the adaptive immune response (239). CD28-B7 interactions act as costimulatory signals for antigen-activated T cells and promote T-cell clonal proliferation, cytokine secretion, and expression of surface molecules that provide signals to B lymphocytes to foster their effector functions. Other aspects of the innate immune response also exist, such as the presence of B-1 type B lymphocytes, which secrete low-avidity antibodies exhibiting specificity for common bacterial components and which also aid in the clearance of these pathogens. MHC, major histocompatibility complex; Mϕ, macrophage; iNOS, inducible nitric oxide synthetase; ROS, reactive oxygen species; T, T lymphocyte; TCR, T-cell receptor.

Both in vitro and in vivo studies will be covered in this review, and where appropriate, the limitations of the experimental approaches will be cited and the strength of the data commented upon. To provide a quick reference for the reader, Table 1 summarizes the major in vivo antisense experiments reviewed below.

II. MOLECULAR TARGETS

A. Antigen Presentation and Recognition

1. Major Histocompatibility Complex

Mammalian immune responses are initiated by recognition of antigens presented to T lymphocytes by accessory cells termed antigen-presenting cells (APCs). These cells are dendritic cells, tissue macrophages, and monocyte-derived cells principally, but other types of cells including B lymphocytes, endothelial, and epithelial cells may also act as APCs under certain conditions. The major histocompatibility complex (MHC) antigens, named for their role in determining the compatibility of tissue grafts in transplantation, are critical molecules in antigen presentation that function to assure specificity of a host immune response by displaying peptide antigen fragments for the lymphocyte effector cells in the context of self. T cells receiving the antigen signal in the context of appropriate MHC antigens undergo clonal expansion, secrete cytokines, and up-regulate their receptors for soluble growth factors, cytokines, and costimulatory molecules that drive T-cell differentiation and effector function. Thus, recognition of antigen by T cells is termed signal 1 and the costimulatory signals from APCs are termed signal 2. Signal 1 in the absence of signal 2 leads to limited clonal expansion followed by anergy or apoptosis.

The MHC in mammals consists of class I and class II groups of cell surface antigens, as well as the class III genes that encode complement proteins. Class I antigens are expressed on most cells whereas class II are primarily restricted in expression to APCs. Class I MHC molecules interact with $CD8^+$ cytotoxic T cells, which combat viral infections and provide cell-mediated immune responses, while the class II MHC antigens recognize T helper cells involved in providing help for the generation of humoral (or antibody-mediated) immunity as well as cell-mediated responses. In the human, there are three class II MHC gene products (HLA-DR, -DQ, and -DP); in the mouse, class II antigens are termed Ia molecules.

Historically, various antisense approaches have been utilized to study the role of the MHC in the generation of immune responses. Hambor et al. generated a T-cell clone stably transfected with an episomal expression vector encoding antisense DNA to CD8 and observed that stimulation of this clone with alloantigen resulted in loss of cytotoxic and proliferative responses along with decreased

Table 1 In Vivo Studies Using Antisense Oligonucleotides: Inflammatory Targets

Therapeutic area	Molecular target	Animal model	Ref. (section)
Colitis	NF-κB p65	Mouse TNBS colitis; IL 10−/− mouse colitis	Neurath et al., 1996 (II C v. c(3))
	NF-κB p65	*E. histolytica* infection in human intestinal xenografts in SCID mice	Seydel et al., 1998 (II C v. c(3))
	ICAM-1	Mouse DSS colitis	Bennett et al., 1997 (II F i. a(5))
Lung inflammation	Adenosine A1 receptor	Rabbit dust mite–induced asthma	Nyce et al., 1997 (II C iv. b)
	IL-4, IL-5	Rat ovalbumin-induced asthma	Molet et al., 1999 (II C ii. b)
	IL-5	Mouse ovalbumin-induced asthma	Karras et al., 2000 (II C ii. b)
	ICAM-1	Mouse LPS-induced lung inflammation	Kumasaka et al., 1996 (II F i. a(4))
	TNF-α	Mouse LPS-induced lung inflammation	Qin et al., 2000 (II C i. b)
Central nervous system inflammation	MCP-1	Rat traumatic brain injury	Ghirnikar et al., 1998 (II F ii.)
	iNOS	Mouse experimental autoimmune encephalomyelitis	Ding et al., 1998 (II C vi. a)
	c-fos	Rat carrageenan-induced inflammation	Gillardon et al., 1997 (II C v. c(2))
	IP-10	Rat experimental autoimmune encephalomyelitis	Wojcik et al., 1996 (II F ii.)

Skin inflammation	ICAM-1	Mouse contact hypersensitivity	Klimuk et al., 2000 (II F i. a(3))
	IL-1 receptor	Mouse IL-1-induced skin inflammation	Burch et al., 1991 (II C iii. c)
	ICAM-1	Mouse cytotoxic dermatitis	Christofidou-Solomidou et al., 1997 (II F i. a(3))
Shock	iNOS	Rat LPS-induced hypotensive shock	Hoque et al., 1998 (II C vi. a)
Ocular disease	VEGF	Mouse model of retinopathy of prematurity	Robinson et al., 1996 (II D)
Lupus	NF-kB p50	BXSB mouse model of autoantibody production	Khaled et al., 1997 (II C v. c(3))
Transplantation rejection	ICAM-1	Mouse heart allograft	Stepkowski et al., 1994 (II F i. a(1))
	ICAM-1	Mouse islet cell allograft	Stepkowski et al., 1998 (II F i. a(1))
	ICAM-1	Rat heart, kidney allograft	Stepkowski et al., 1997 (II F i. a(1))
	ICAM-1	Rat heart allograft	Mann et al., 1999 (II F i. a(2))
	Cdc2 kinase	Mouse heart allograft (arteriopathy)	Suzuki et al., 1999 (II F i. e)
	Cdk2 kinase	Mouse heart allograft (neointimal formation)	Suzuki et al., 1997 (II F i. e)
	Granzyme C	Mouse interstitial nephritis	Bailey and Kelley, 1997 (II E)
Reperfusion injury	ICAM-1	Rat kidney isograft reperfusion injury	Dragun et al., 1998 (II F i. a(2))
	ICAM-1	Rat heart allograft reperfusion injury	Feeley et al., 1999 (II F i.a(2))

IL-2 and IL-2 receptor expression (6). Direct targeting of the MHC accomplished in cells using antisense expression vectors resulted in altered expression of DR dimers and DR/DQ heterodimers, respectively (7), while targeting Ia^b using a similar approach or by generating mice expressing an antisense Ia^b transgene delayed B-lymphocyte development by an unknown mechanism (8,9).

Transporter Associated with Antigen Processing (TAP)-2. Several laboratories have investigated factors regulating the expression of MHC molecules in cells using an antisense approach. These approaches have involved either targeting TAP genes encoding proteins that are involved in the intracellular transport of antigenic peptides to the MHC molecules or the soluble factors that regulate this process. Antisense against TAP-2 was found to inhibit MHC class I expression on the cell surface (10). Although no reduction of mRNA for TAP-2 was shown in antisense-treated cells, protein levels of MHC class I antigens were decreased, as shown by flow cytometry, to levels similar to those found in TAP-deficient cell lines. TAP-2 antisense-treated primary adherent splenocytes pulsed with peptide to stabilize surface MHC class I expression were highly effective in eliciting cytotoxic T lymphocytes (CTLs) and inducing protective antitumor immunity in syngeneic mice, presumably because the majority of MHC class I molecules were then associated with the peptide. These results, however, should be interpreted with caution because the antisense 25 mer used in these studies did contain a canonical CpG immunostimulatory sequence (11), while the control oligonucleotide contained three noncanonical CpG motifs. Because of the documented induction of cytokine secretion and lymphocyte activation by CpG sequences in murine systems, contribution of this sequence motif to the antisense-induced effects is possible.

Interleukin (IL)-10. In contrast, targeting IL-10 with an antisense expression vector produced increased TAP-dependent translocation of peptides and expression of MHC class I molecules (12). In a similar study, Hauber and coworkers utilized antisense technology to investigate the regulatory function of IL-10 in adherent and nonadherent mononuclear blood cells derived from two patients that were MHC class II–deficient (13). Isolated cells from the patients showed a six- to sevenfold higher expression of IL-10 in comparison to healthy control cells. Given the role of IL-10 in the down-regulation of the Th1 response, the authors speculated that IL-10 was playing a role in the impaired expression of the MHC genes. Treatment of the patients' cells with an anti-IL-10 phosphorothioate oligonucleotide that targeted the AUG region reduced spontaneous IL-10 production by approximately 95% in comparison to cells treated with the sense control. As a consequence of antisense treatment and IL-10 reduction, expression of several MHC class II genes was up-regulated at the mRNA level in the nonadherent cells; however, these changes in expression of mRNA for MHC class II genes did not result in increased expression of protein, apparently due to lack of

complete mRNA processing. These results indicated that additional factors were involved in regulation of the MHC II genes in these patients.

Regulatory Factor of X Box Motif (RFX)-1. In a particularly thorough study, interferon-γ-induced up-regulation of MHC II antigens was blocked in human THP-1 monocytes by antisense targeting of the RFX-1 regulatory factor that binds the essential X box motif of the MHC class II promoters (14). Eighteen antisense oligonucleotides or their sense complements 14–20 base pairs in length targeting the translation initiation site or sequences within the coding region were compared by dose-response analysis, and one antisense 14 mer was found to inhibit HLA-DR expression by 74%, as measured by flow cytometry. Intracellular oligonucleotide uptake was documented by radiolabel analysis in cell extracts. The antisense was found not to affect expression of MHC class I molecules or to inhibit constitutive expression of DR. In addition, a 72-h time lag between antisense treatment and IFN-γ induction was required for the antisense effect, suggesting inhibition of de novo synthesis of RFX-1 following IFN-γ treatment, and the presence of a long-lived intracellular depot of DR protein.

Altogether, the preceding experimental evidence suggests that modulation both of the level of MHC expression and of its function in immune responses can be achieved by antisense technology. As corollaries, antisense intervention in MHC-induced signaling responses or in the targeting of MHC alleles associated with inflammatory disease has not generally been undertaken and represents an attractive potential approach.

2. T-Cell Antigen Receptor (TCR) and TCR-Mediated Signal Transduction

In the last several decades, an intense focus has been placed on understanding the roles of the T- and B-cell antigen receptors (BCR) in regulating immunity. As a result, the components that comprise the TCR and BCR are relatively well understood today and many signaling events emanating from antigen receptors have been delineated (discussed below). With regard to therapeutics, T-cell targeting has been the favored route and most of these strategies have concentrated on interfering with signals generated from the TCR.

The TCR is a multisubunit protein complex consisting of a polymorphic heterodimer of αβ chains in noncovalent association with the invariant ζ chain and the γ, δ, and ε chains of the CD3 complex (15). The intracellular tails of the ζ and CD3 component chains contain immunoglobulin receptor family tyrosine-based activation motifs (ITAMS) crucial for TCR coupling to tyrosine kinases that are essential for all types of TCR signaling (16,17). The ITAMS mediate TCR-stimulated activation of the *src* tyrosine kinases p59fyn and p56lck and ZAP-70, which together initiate signaling down multiple pathways. Downstream signaling from the TCR includes inositol lipid metabolism, leading to calcium

signaling and protein kinase C (PKC) activation, as well as activation of phosphatidylinositol 3′-hydroxyl kinase (PI 3-K) pathway, activation of guanine nucleotide–binding proteins such as *ras*, initiation of the *ras*- and *raf* kinase-mediated mitogen activated protein kinase (MAPK) pathways, and activation of Vav and Cbl protooncogenes (18). Signals from many of these pathways converge in the nucleus at the level of transcription factor activation and gene regulation. TCR ligation induces expression of the T-cell growth factor interleukin-2 (IL-2) and its receptor (IL-2R). The IL-2 gene is regulated by multiple transcription factors, including AP-1, NF-κB, Oct-1, and nuclear factor of activated T cells (NF-AT), such that its expression cannot be induced by any one signaling pathway. Thus, the TCR conveys information from antigen recognition events to the nucleus by utilizing many signal transduction pathways that interpret both qualitative and quantitative input. In turn, these signals are integrated with those emanating from costimulatory molecule interactions and cytokine and growth factor receptors to ultimately control the cellular response.

Protein Tyrosine Kinases (PTKs). Because of their proximal position in the TCR signaling cascade, independent targeting of p59*fyn* and p56*lck* with antisense has been studied. Stable expression of antisense against p59*fyn* in a T-cell clone was found to impair antigen- and TCR monoclonal antibody–induced tyrosine phosphorylation of intracellular substrates (19). In this study, reduced *lck* kinase activity was also associated with *fyn* antisense treatment even though equivalent amounts of *lck* protein were present in all experimental groups. This result suggests the importance of protein interactions in signaling outputs and indicates that the specificity of an antisense approach can be lost at the functional level and result in a more broad inhibitory action. A similar study performed in the Jurkat human T-cell line found that transfection of an antisense p59*fyn* construct blocked calcium influx following TCR crosslinking (20). However, in this study, a full-length p59*fyn* antisense construct was utilized, which may differ from targeting approaches using a shorter sequence or a short oligonucleotide. p56*lck* antisense oligonucleotides have been shown to block TCR-induced apoptosis (21), and produce arrest at the G2/M checkpoint of the cell cycle in nontransformed T lymphocytes (22). Clearly, inhibition of TCR triggered PTKs results in dramatic immunosuppression and this strategy will need to be further examined for its suitability for specific diseases as well as its applicability in animal models.

Protein tyrosine phosphatases (PTPs) such as CD45 and hematopoietic tyrosine phosphatase (HePTP) also regulate PTK-mediated signaling events after TCR ligation. Targeting these molecules with antisense oligonucleotides represents a separate strategy for altering TCR signaling. Down-regulation of IL-2 production in Jurkat T cells has been shown following antisense depletion of all CD45 isoforms (23). This study utilized CD45 antisense expression vectors and

showed dose-dependent effects on IL-2 production. Reconstitution with a CD45 expression plasmid restored wild-type IL-2 levels. Because of the negative regulatory role of CD45 on TCR signaling in general and the unique activities of the CD45 isoforms that arise by alternative splicing and are differentially expressed on T-cell subsets, this is an intriguing area of further investigation for potential immunotherapy of immune deficiency diseases with compromised T-cell function or for boosting anticancer responses.

Protein Kinase C. PKC is a family of serine/threonine kinases that is made up of at least 12 different isoenzymes that are expressed in tissue-specific fashion and respond to different physiological stimuli (24,25). Several of the PKC enzymes have been studied in T cells using an antisense approach and PKC-β, ε, and ζ have been shown to play important roles in T-cell responses to IL-2 (26). Moreover, PKC-α inhibition with a stably expressed antisense vector has been explored in conjunction with pharmacological activation of TCR signaling pathways using phorbol ester and calcium ionophore in Jurkat T cells. Expression of the antisense construct was verified in stable transfectants and was shown to result in reduced mRNA expression of PKC-α but not other PKC isoforms. In this system, PKC-α was shown to control IL-2 receptor-α expression, TNF-α production, and the induction of an IL-2 promoter-driven reporter gene (27).

Ras-Raf-MAPK Pathway. There has been a large effort in recent years spent on defining the intracellular signaling pathways that utilize the *ras* family of G proteins, the cytoplasmic *raf* kinases, and downstream MAP kinases to effect changes in cell function. However, little work has been performed using antisense inhibition of the *ras-raf*-MAPK components involved in TCR signaling. One study that evaluated treatment of Th1 clones with an anti-*c-raf* phosphorothioate-modified oligonucleotide found that *c-raf* knockdown inhibited production of IFN-γ upon activation with anti-CD3 antibody, whereas the random sequence control did not (28). IL-2 secretion, however, was unaffected by oligonucleotide treatment. This pathway has been shown to be crucial for T-cell activation and development through control of IL-2 gene transcription triggered by the TCR (29). Further analysis under more robust conditions, such as an APC coculture assay to evaluate costimulatory signals, seems appropriate given the effects of *c-raf* knockdown on IFN-γ production activated through anti-CD3 stimulation alone.

Transcription Factors. A number of transcription factors cooperate to regulate both the expression and the function of the TCR. Antisense targeting of individual transcription factors has provided novel information relevant for modulating T-cell responsiveness. Reduction of *c-myb* levels using an antisense oligonucleotide resulted in impaired activity of the TCR delta chain E3 enhancer

that is required for expression of the δ gene (30). Antisense-mediated inhibition of *c-myb* levels was demonstrated in Jurkat T cells by Western blot (75% reduction) and no effects on other enhancer constructs were observed, indicating antisense specificity. A relatively high concentration of antisense oligonucleotide was required (50 μM) owing to the use of suspension cells; however, no effect on proliferation was noted at this concentration. In another approach, an antisense expression plasmid driven by its own promoter targeting *nur*77, an immediate early gene, blocked TCR-induced apoptosis in a T-cell hybridoma (31). The sense construct showed no activitiy in protecting cells and the antisense was shown to down-regulate *nur*77 protein expression by greater than 70%. *c-myc* has also been implicated in the control of both T- and B-cell apoptosis induced via antigen receptor crosslinking, as shown by antisense approaches (32,33). Thus, specific inhibition at the level of factors that directly regulate TCR-mediated transcription is also a promising strategy for antisense.

3. TCR Alleles Associated with Diseases

There has been debate over whether specific TCR Vβ genes play a causal role in the pathogenesis of rheumatoid arthritis and multiple sclerosis (34,35). Evidence linking Vβ gene usage with inflammatory disease may be stronger in certain types of psoriasis and in systemic lupus erythematosus (36,37). Although there has been no attempt to date to selectively deplete a T-cell subset or clone using an antisense strategy based on a TCR polymorphism, a study has shown that antisense targeting of the transcriptional start sites of TCR Vα or β chains utilized by various T-cell clones produced effects in a cell-specific manner (38). In these experiments, the expression of TCR/CD3 complex on the cell surface was also blocked by treatment with antisense oligonucleotides directed against the ζ or γ chains, indicating that more thorough investigation into this approach is warranted.

B. T-Cell Costimulation

Lymphocytes receive costimulatory signals through cell-cell contact with other immune and nonimmune cells. While several costimulatory signals play distinct roles in the immune response, a large focus of attention has recently been placed on the contribution of signals delivered by APCs through the T-cell CD28 molecule. This type of costimulatory signaling is required to prevent the induction of unresponsiveness or tolerance (termed anergy) following antigen receptor ligation in T cells (39), and is required to achieve T-cell clonal expansion, lymphokine secretion, and effector function.

The B7 family of molecules, B7-1 (CD80) and B7-2 (CD86), expressed largely on APCs, interact with CD28 to provide the major costimulatory signal

for augmenting the T-cell response (40). However, B7 molecules share a dual specificity for CTLA-4, an additional T-cell counterreceptor that delivers signals that oppose those delivered through CD28, and the higher affinity of B7 for CTLA-4 in comparison to CD28 suggests that a complex interplay between the relative affinities and expression profiles of these molecules coordinates the B7-mediated costimulatory response. Recent data underscore the potential utility of modulating this pathway in disease in that monoclonal antibody and soluble protein antagonists of this pathway enable long-term graft survival and suppress autoimmunity and inflammatory responses in vivo (41–43).

1. CD28

Several strategies have been attempted to interfere with productive CD28 signaling in vitro. Oligonucleotides that contain two guanosine tetrads separated by four bases can function as molecular decoys in cells to bind the transcription factors Sp-1 and Egr-1 and block CD28 gene transcription by a nonantisense mechanism (44). In contrast, antisense strategies have been directed at blocking CD28-induced responses by targeting downstream signaling components. Antisense targeting of adenosine 3′,5′-monophosphate (cAMP)-specific phosphodiesterase-7 (PDE7), which is triggered by CD3 and CD28 coligation, resulted in reduced T-cell proliferation and IL-2 secretion, most likely due to increased levels of cAMP since blocking PKA-dependent cAMP signaling pathways reversed this effect (45). The authors showed the antiproliferative antisense effect in both the HUT78 T-cell line and primary T cells. However, no dose-response effect was shown in the study, which mainly utilized a 24-mer antisense sequence that contained a noncanonical CpG sequence in the 5′ end. Antisense oligonucleotide-mediated inhibition of ZAP-70 in Jurkat T cells has also shown that CD28 costimulation increases tyrosine phosphorylation and activation of Vav and p38 MAPK through activation of ZAP-70, indicating that ZAP-70 is a critical integrator of the TCR and CD28 signaling pathways (46); 18-mer antisense and scrambled control sequences were utilized in this study and the control sequence but not the antisense sequence contained a noncanonical CpG motif. Relatively high concentrations of the oligonucleotides were used in these studies (20 μg/mL), owing to the lack of transfection aids. Finally, antisense inhibition of the NF-κB subunits p50 and p105 was found to significantly reduce IL-2-dependent T-cell proliferation and IL-2 receptor α-chain expression in primary human T cells stimulated simultaneously through CD2 and CD28 (47). The antisense oligonucleotides used in the study were non-phosphorothioate-modified 21 mers directed at the translation initiation sites. While statistical differences were observed, nonspecific inhibition of the proliferative response was apparent. To date, there have been no published studies directly investigating antisense inhibition of CD28 or the B7 molecules.

C. Activation and Effector Phases of the Inflammatory Response

1. Cytokines

Interferon Gamma (IFN-γ). IFN-γ is expressed by certain subpopulations of T cells and natural killer cells upon activation and costimulation by antigen-presenting cells. Soluble factors that regulate production of IFN-γ include IL-2 and IL-12, which up-regulate its expression, and IL-4, IL-10, and PGE_2, which down-regulate it. IFN-γ is a key component and marker of the Th1 immune response, playing an important role in the differentiation and activation of T cells, B cells, NK cells, neutrophils, and monocytes. Nonleukocytic cells such as epithelial and endothelial cells also respond to IFN-γ by up-regulating a variety of genes including MHC II, TNF-α, CD40, cyclooxygenase 2, iNOS, and certain adhesion molecules. The cytokine has a modest antiproliferative effect on most cell types, with the exception of certain T cells.

Although this cytokine has a primary role in the Th1 response, few studies have used antisense oligonucleotides to down-regulate IFN-γ. In a recent report, Kuo and co-workers utilized an anti-IFN-γ oligonucleotide to demonstrate the autocrine role of the cytokine in expression of iNOS in IL-1β-treated rat hepatocytes (48). They first demonstrated that anti-IFN-γ oligonucleotide treatment effectively reduced IL-1β-induced expression of the target cytokine to basal levels, whereas the sense control oligonucleotide had no effect on expression. Further analysis showed that antisense knockdown of IFN-γ expression also inhibited expression of the inducible isoform of nitric oxide synthase (iNOS) and subsequent production of nitrite in a sequence-specific manner. In vivo effects of an antisense oligonucleotide to IFN-γ have yet to reported.

Tumor Necrosis Factor Alpha (TNF-α). TNF-α is a pleiotropic cytokine whose overproduction has been implicated in the development and progression of many inflammatory, infectious, and autoimmune diseases (49,50). TNF-α is expressed by a number of immune cells (e.g., macrophages, monocytes, T cells, and mast cells), as well as certain nonimmune cells under pathophysiological conditions. A humanized monoclonal antibody to TNF-α (51,52) and a soluble TNF receptor decoy (53,54) have recently shown clinical utility in patients suffering from Crohn's disease and rheumatoid arthritis, respectively). In both these instances, improvement in disease was demonstrated. The long-term treatment profile of these new anti-TNF drugs is not known, however. Formation of antibodies to either protein construct may limit their usefulness with repeated dosing. In this respect, antisense oligonucleotides may prove to be a preferred platform for suppression of this cytokine in chronic inflammatory and autoimmune diseases.

Unlike many other genes of interest, antisense efforts against TNF-α have been greatly facilitated by the early availability of the complete sequence of the

human, rat, and mouse genes. Sequence information is also available to varying degrees for many other species, providing many opportunities for the in vivo evaluation of anti-TNF oligonucleotide pharmacology once an active compound has been identified. To date, a variety of antisense oligonucleotides have been reported that reduce human, mouse, or rat TNF-α expression in cell culture. The range of activity observed has been broad, however, ranging from 36% to 90% reduction in expression levels. This variation appears to be based upon the use of different cell types and transfection methods, the number of sites screened for activity, and the type and degree of oligonucleotide modifications (56–61).

Recently it has been reported that administration of an anti-TNF-α morpholino oligomer into mice, by intranasal insufflation, inhibited TNF-α protein expression resulting from exposure to aerosolized endotoxin by approximately 30%, as measured from the bronchoaveolar lavage fluid (62). Reduction of TNF-α protein levels occurred in a dose-responsive manner and was sequence-specific, as shown by comparison to a scramble morpholino control. Unlike oligodeoxynucleotides, morpholino oligomers are not substrates for RNase H or other endogenous enzymes, and reduce target protein expression via an occupancy-only mechanism (63). Incomplete inhibition was attributed to heterogeneous delivery of the oligomers to the cells throughout the lungs by the intranasal insufflation procedure. The effect of anti-TNF-α oligomer treatment on LPS-induced pulmonary inflammation, as measured by lung lavage neutrophil counts, was dependent on the time between treatment with the oligomer and exposure to LPS. The sequence specificity of the effect of morpholino treatment on pulmonary inflammation was not determined.

In an in vitro study, bacterial lipopolysaccharide (LPS)-induced TNF-α production from lung alveolar epithelial cells was dose-dependently inhibited in a sequence-specific manner by an 18-mer TNF-α antisense oligonucleotide (first described by Rojanasakul et al., as noted above) targeted to the translation initiation site (64). Lipofectin-mediated transfection was also found to block macrophage inflammatory protein (MIP)-2 production by these cells, similar to coincubation with TNF-α polyclonal or monoclonal antibodies. However, the magnitude of inhibition of either TNF-α or MIP-2 protein was no better than 50% in this study. These observations suggest that MIP-2 expression is controlled in part in an autoregulatory fashion by TNF-α produced from the epithelial cells following LPS stimulation, as might occur during an acute inflammatory reaction.

TNF-α antisense has also been shown to protect against LPS-induced, Kupffer cell–mediated hepatocyte injury in vitro (65). In this hepatocyte–Kupffer cell coculture model, TNF-α released from Kupffer cells after endotoxin exposure was implicated in compromised hepatocyte mitochondrial energy synthesis, as measured by decreased oxidative phosphorylation. The mitochondrial dysfunction was followed by positive propidium iodide staining, indicating loss of membrane barrier function and lethality. In these experiments, 18-mer antisense and

sense oligonucleotides were utilized that were free of CpG motifs and incubated in cultures in the absence of transfection enhancers. However, the authors did not show a dose-response effect. Antisense treatment (at 20 μM) of Kupffer cells but not hepatocytes resulted in reduction of TNF-α protein levels, as would be expected since the Kupffer cells are the major source of TNF-α in this system. TNF-α antisense treatment of Kupffer cells was also found to attenuate the decrease in oxidative phosphorylation, pointing to a role for TNF-α in endotoxin-induced liver injury.

2. Interleukins

Interleukin 1 Alpha (IL-1α). IL-1α activates multiple signal transduction pathways and cellular processes upon binding to the type I IL-1 receptor. Recently, it has been found that skin fibroblasts isolated from patients with systemic sclerosis, a connective tissue disease that results in excessive formation of extracellular matrix, constitutively express IL-1α. IL-1α is known to activate expression of several cytokines and growth factors in human dermal fibroblasts, including those that cause production of extracellular matrix. Kawaguchi and colleagues hypothesized that IL-1α expression in these fibroblasts acted in an autocrine manner to promote production of collagen and thus sclerosis of the skin. To test this hypothesis, they treated these fibroblast cells with a phosphorothioate-modified antisense oligonucleotide complementary to the AUG region of the IL-1α transcript, and then examined the effects of treatment on expression of two factors that promote synthesis of collagen, IL-6 and PDGF-α (66). IL-1α antisense oligonucleotide treatment reduced target IL-1α mRNA and protein levels in a dose- and sequence-dependent manner. Further analysis showed that antisense reduction of IL-1α levels reduced expression of IL-6 at both the RNA and protein level, and PDGF-α at the RNA level.

Interleukin 4 (IL-4) and 5 (IL-5). Recent evidence suggests that CD4 T cells secreting cytokines typical of a T helper 2 type of immune response (i.e., IL-4, IL-5, IL-13) may be critical in asthma, a chronic inflammatory disease of the airways characterized by bronchial hyperreactivity and inflammatory cell infiltration. IL-4 is known to promote T helper 2 cell development and the immediate bronchorestrictor response through IgE- and mast cell–mediated pathways. IL-5, on the other hand, governs release of eosinophils from the bone marrow, as well as their maturation and degranulation at sites of inflammation, and IL-5 expression has been correlated with lung tissue damage and late-phase airway hyperreactivity responses in humans. Several attempts have been made to use antisense oligonucleotides to modulate the Th2 response in asthma models by targeting either IL-4 or IL-5.

In a rat model of asthma, investigators treated CD4 cells ex vivo with antisense oligonucleotides specific to either IL-4 or IL-5, and then injected the cells

back into rats prior to challenge with an ovalbumin aerosol. They found a significant reduction in airway resistance, airway eosinophilia, and both IL-4 and IL-5 expression when the rats received CD4 cells treated with IL-4 but not IL-5 antisense, or a control oligonucleotide (67). IL-5 expression in lung lavage fluid was only slightly decreased by the transfer of IL-5 antisense–treated T cells, suggesting that the early production of IL-4, but not IL-5, may be critical in the late-phase response. Results may differ, however, when animals are treated systemically with antisense oligonucleotide. Our own group has found that a systemically administered methoxy-ethyl–modified phosphorothiate oligonucleotide to IL-5 was effective in reducing both lung eosinophilia and the late-phase response in a mouse model of asthma (68). These results may differ from those of Molet et al. because systemically administered IL-5 antisense is likely to have a different site of action than antisense oligonucleotide administered ex vivo to isolated T cells. Likely target cells of systemically administered IL-5 antisense could include eosinophils and/or mast cells, both of which can secrete IL-5, or precursor cells in bone marrow.

Interleukin 6 (IL-6). IL-6 is a pleiotropic cytokine that plays a role in immune responses, acute-phase reactions, and hematopoiesis. IL-6 expression is observed in a wide variety of cell types, including lymphocytes, monocytes, osteoblasts, keratinocytes, fibroblasts, and hepatocytes in response to mitogenic and immunogenic stimuli. It is also expressed in transformed cells such as myelomas, glioblastomas, and melanomas where it may act in an autocrine fashion.

Two reports describe the use of antisense oligonucleotides to reduce expression and function of human IL-6 in cell culture. In the most recent report, the investigators used a phosphorothioate-modified 15-base oligonucleotide, complementary to a sequence in the coding region of the transcript, to reduce IL-6 production in B cells stimulated with CD40 antibodies and IL-4 (69). Although treatment of the B45 B-cell hybridoma with the oligonucleotide resulted in reduction of IL-6 production, this activity may have been due to the nonantisense effects of a string of guanine residues nested in this particular oligonucleotide sequence (70,71). The control oligonucleotide did not produce this kind of activity. Treatment of activated tonsillar B cells with the anti-IL-6 oligonucleotide was shown to result in a reduction in IgE production.

Reduction of proteoglycan synthesis by chondrocytes is believed to play a role in the loss of cartilage during rheumatoid arthritis. Exposure of chrondrocytes to IL-1 effectively inhibits proteoglycan synthesis, and also activates expression of many inflammatory genes, including IL-6. With this in mind, Nietfeld and co-workersdd evaluated the effects of an IL-6 antisense oligonucleotide on synthesis of proteoglycans in cartilage explant cultures induced with IL-1 (72). Pretreatment of the explants with the IL-6 phosphorothioate-modified antisense oligonucleotide inhibited IL-1-induced production of IL-6 protein in a dose- and

sequence-specific manner. Further analysis showed that antisense reduction of IL-6 expression interfered with the inhibition of proteoglycan synthesis that occurred upon IL-1 induction. Antisense efficacy was notably good in these experiments considering the highly electronegative extracellular matrix that surrounds the chrondrocytes in cartilage, given the overall negative charge of oligonucleotides. The accessibility of the cells to the oligonucleotide may be an attribute of the explants, however, since chondrocyte uptake is not observed in vivo, as determined by IHC staining of fixed tissue (M. Butler, ISIS Pharmaceuticals, unpublished results).

Interleukin 10 (IL-10). IL-10 is an immunomodulatory cytokine produced by Th2 cells and activated monocytes that negatively regulates production of cytokines and genes that are predominantly expressed in a Th1 response. Overexpression of IL-10 is thought to be an underlying determinant of poor immune responses to *Staphylococcus aureus* (73), to certain tumors (74,75), and to certain viral infections in which the virus expresses an IL-10 homolog, (76,77). In contrast, IL-10 or IL-10 receptor deficiencies are thought to contribute to certain inflammatory diseases that are dominated by a Th1 response (78).

Several research groups have utilized antisense oligonucleotides to reduce expression of IL-10 in cell culture. In addition to work by the Hauber group and Salazar-Onfray and colleagues, discussed earlier, that showed pharmacological effects by IL-10 antisense on MHC expression, Tsuchiya and co-workers identified two antisense oligonucleotides that efficiently reduced IL-10 mRNA and protein levels in phorbol ester–activated U937 cells, a transformed monocytic cell line. These oligonucleotides were found to reduce IL-10 levels in a dose- and sequence-specific manner (79). The compounds, which targeted sites in the coding region and the 3′ untranslated region, were both more active than those targeted to the AUG region.

3. Cytokine Receptors

Tumor Necrosis Factor Receptor 1 (TNF-R1, or p55). TNF-R1 is one of two TNF receptors that is expressed in most cell types and interacts with tumor necrosis factor to mediate a broad range of cellular responses. The two receptors, TNF-R1 and -R2, have distinct cytoplasmic domains, and as such, interact with different sets of signal transduction components. Most cellular responses, including apoptosis, activation of second-messenger systems, and up-regulated expression of inflammation-related genes by activation of the NF-κB and AP-1 transcription factor pathways (80), have been attributed to the interaction of TNF with TNF-R1. In two recent reports, Ojwang and co-workers evaluated the effects of anti-TNF-R1 oligonucleotides in a transformed human lung embryonic fibroblast cell line, and in human newborn foreskin fibroblasts (81,82). In these studies, the investigators evaluated a number of differentially modified analogs of an

oligodeoxynucleotide that targeted the poly(A) signal site of the TNF-R1 mRNA. These modifications included C5-propynyl or -hexynyl derivatives of 2′-deoxyuridine, the C5-propynyl derivative of 2′-deoxycytidine, and the 7-deaza-7-propynyl derivative of 2′-deoxy guanosine. Although antisense knockdown of target mRNA and protein was 50% or less, reduction of TNF-α-induced IL-6 and IL-8 expression by treatment with these anti-TNF-R1 oligonucleotides ranged from 40 to 80%. Antisense treatment was selective for TNF-R1, as shown by selective decrease of TNF-R1 protein in comparison to TNF-R2 and lack of effect on IL-1-induced IL-6 production. The modest activity of these oligonucleotides against TNF-R1 may be a reflection of the metabolism or turnover rate of the protein. In this respect, only a minor subpopulation of TNF-R1 has been shown to be expressed in the plasma membrane of endothelial cells and monocytic U937 cells. The majority of the protein is associated with the Golgi apparatus (83). The significance of this distribution pattern is unknown, but it may have an impact on antisense efficacy.

Tumor Necrosis Factor Receptor 2 (TNF-R2 or p75). Less is known about the functional role of TNF-R2 in comparison to TNF-R1. Antisense knockout of TNF-R2 in human neuronal-like cells increased the degree of cell death induced by its ligand, TNF-α, and cell injury induced by hypoxic conditions or exposure to the β-amyloid peptide (84). Treatment was selective for TNF-R2 in comparison to TNF-R1, as shown by radioimmunoprecipitation and Western blot, respectively. These results indicate that TNF-R2 may protect human neurons from cell death.

Interleukin Receptor I, Type I (IL-1RI). There are two IL-1 receptor proteins, type I and type II. Type I transduces signals across the cell membrane, whereas type II acts as a decoy or ligand sink. One of the earliest in vivo demonstrations of antisense efficacy involved the type I interleukin-1 receptor (85). These experiments involved subcutaneous injection of an 18-base phosphorothioate oligonucleotide that targeted the AUG region of the type 1 IL-1R. IL-1-induced neutrophil migration into skin was reduced by 37%. Although the authors did not investigate whether antisense treatment affected IL-1R expression in skin, a human-specific IL-1R oligo that contained five mismatched bases to the murine sequence failed to modulate neutrophil influx, suggesting an antisense mechanism in this system.

A more recent report (86) has described the evaluation of 36 phosphorothioate-modified oligonucleotides for activity toward IL-1RI in A549 cells and normal human dermal fibroblasts, where target sites were located in the 5′ UTR, coding region, splice junctions, and 3′ UTR of the transcript. In these experiments, antisense activity, as measured by reduction of RNA levels, was observed only at sites in the 3′ UTR. The oligonucleotide targeting the AUG region, as previously described, was inactive. Activity of the lead compound, ISIS 8807, was dose- and

sequence-dependent, and treatment of A549 cells with this compound reduced IL-1R1 protein and mRNA by 60 and 90%, respectively. In addition, this oligonucleotide reduced IL-1α-induced expression of ICAM-1 mRNA in A549 cells by 66%, in comparison to saline or control oligonucleotide-treated cells.

Interleukin 4 Receptor, Alpha Chain (IL-4Rα). Interleukin 4 (IL-4) plays a critical role in differentiation of T cells to the Th2 phenotype and induces isotype switching in B cells, resulting in IgE production. IL-4 binds with high affinity and specificity to the IL-4 receptor alpha chain (IL-4Rα), which then activates distinct signal transduction pathways through formation of a heteromeric receptor complex with either the common gamma chain (utilized by IL-2, IL-7, IL-9, and IL-15) or IL-13 receptor alpha chain (IL-13Rα). To date, one research group has utilized antisense oligonucleotides to down-regulate IL-4Rα expression in B cells (87). In this study, three phosphorothioate-modified antisense oligonucleotides were tested for activity in Daudi cells, which constitutively express the receptor. The oligonucleotide that targeted the AUG region was the most effective at reduction of receptor surface expression. This oligonucleotide was evaluated for its ability to inhibit production of IgE by normal B cells costimulated with IL-4 and CD40 monoclonal antibody. A dose-dependent decrease in IgE protein production was observed in the antisense-treated group, with no effect on IgG production. Reduction of total Cε mRNA and mature Cε mRNA levels was observed, but germ-line Cε was unaffected. These investigators, however, did not determine whether inhibition of IgE production was sequence-dependent.

4. Other Receptors

CD14. CD14 is a glycosol phosphatidyl inositol (GPI)-anchored membrane protein that functions in the innate immune response. It binds LPS, a component of the outer membrane of Gram-negative bacteria. Recent evidence indicates that CD14 is also involved in recognizing membrane components of Gram-positive bacteria such as peptidoglycan (88–90). CD14 lacks an intracellular signaling domain and interacts with other proteins, such as the Toll-like receptors 2 and 4 (91; also see Fig. 2), that can cooperatively activate LPS-induced intracellular signaling.

Periodontal disease is an infectious disease caused by Gram-negative bacteria that is characterized by the occurrence of local bone resorption. LPS can trigger bone resorption through stimulation of the production of proinflammatory cytokines like TNF and IL-1. Recently, Hanazawa and colleagues found that treatment of mouse calvarial cells with a phosphorothioate-modified antisense oligonucleotide complementary to the AUG region of CD14 reduced LPS-induced bone resorption by 60%, with concomitant reduction of LPS-induced

expression of IL-1β mRNA (92). The effects of antisense treatment were sequence-specific, as determined by comparison to a sense control oligonucleotide.

Adenosine A_1 Receptor. Adenosine is involved in normal physiological processes, but elevated concentrations of this endogeneous purine are found in the bronchoalveolar lavage fluid of asthmatics, and asthmatic individuals respond to adenosine challenge with marked airway obstruction. Antisense oligonucleotides targeting the adenosine A_1 receptor have been shown to be effective inhibitors of asthma in a rabbit model of the disease. Aerosolized administration of a 21-mer phosphorothioate oligonucleotide specific to the adenosine A_1 receptor desensitized rabbits to subsequent challenge with either adenosine or dust mite allergen. The effect was sequence-specific and investigators were able to show a decrease in adenosine A_1 receptor–binding sites in airway smooth muscle tissue with antisense treatment (93).

5. Intracellular Signal Transduction Components

The cell response to external stimuli, such as cytokines, growth factors or cell-cell contact, is often a result of receptor-mediated activation of one or more intracellular signal pathways. Inactivation of a pivotal component of a given signal transduction pathway is one strategy for modulating a cell's response to such stimuli. One advantage of this strategy is that certain transduction components (e.g. receptor-associated factors, adaptor proteins, protein scaffolds, kinases, and transcription factors) may be shared by several signal pathways that are activated by different stimuli. These signals may occur at different phases, or in different cell types, of an immune response. Thus targeting a shared internal component may have a more profound effect than targeting a single ligand or a single receptor. A potential disadvantage of this targeting strategy is that receptor-mediated signaling often involves activation of many different transduction pathways that lead to activation of a variety of processes that define the total response. This occurs either directly through multiple protein interactions with the receptor or indirectly as a result of signal branch points downstream of the receptor complex. In the last few years a fair number of reports have emerged that have utilized antisense oligonucleotides for dissecting the role of different signal transduction components in an immune response. These investigations have been directed towards a wide variety of cell types under a number of different conditions, or stimuli.

GTP-Binding Proteins.

Ha-Ras. Ha-ras is one of three members of a GTP-binding protein family that is involved in regulation of cell proliferation and differentiation in response to a variety of extracellular stimuli, including cytokines such as TNF-α (94). Ras

proteins have been shown to activate the raf/MEK/ERK pathway and MEK kinase/JNK kinase/JNK pathway, as well as the NF-κB pathway (95–97). Antisense oligonucleotide–mediated knockdown of Ha-ras mRNA and protein has shown that this GTP-binding protein contributes to TNF-induced expression of E-selectin, ICAM-1, and VCAM-1 in human dermal microvascular cells, as shown by reduction of mRNA and protein (98). Of the three adhesion molecules, E-selectin mRNA levels showed the greatest reduction (approximately 85%), followed by VCAM (75%) and ICAM-1 (30%). Antisense activity was dose- and sequence-dependent.

Rho A. The Rho family of small GTPase proteins includes Rho A, B, C, D, G, Rac1, Rac 2, Rac 3, and Cdc42. These G proteins are recognized primarily for their role in reorganization of the actin cytoskeleton and assembly of integrin-containing focal adhesions (99,100). The function of individual Rho family members in cells has yet to be unequivocally defined. 2′ chimeric antisense oligonucleotides have been identified for Rho A and Rac 1 (101). Use of these compounds has shown that Rho A, and not Rac 1, plays a partial role (30–38%) in the activation of JNK activity in human A549 cells exposed to reactive oxygen species (H_2O_2) or to ultraviolet light, but not to IL-1β.

Kinases.

Txk. Txk is a member of the Tec family of nonreceptor tyrosine kinases (102–104). This family of kinases contain the Src homology 3 (SH3), SH2, and tyrosine kinase catalytic domains that characterize the Src superfamily of kinases. Expression of this family of kinases has been shown to be restricted to hematopoietic cells. In this respect, Txk is expressed preferentially by mast cells and by Th1/Th0 cells with IFN-γ-producing potential. Kashiwakura and colleagues recently showed sequence-specific reduction of Txk protein expression in normal human peripheral blood T cells treated with a phosphorothioate-modified oligonucleotide specific for Txk (105). Pretreating normal human peripheral blood T cells, antigen-specific Th1 clones, or Th0 clones with this oligonucleotide resulted in inhibition of IFN-γ production upon activation with PHA or antigen in the presence of irradiated autologous PBMCs. Oligonucleotide treatment did not affect IL-2 and IL-4 production by these cells.

IL-1 Receptor Associated Kinase 2 (IRAK-2). IRAK-2 is one of three members of a serine/threonine kinase family that has been identified as a signal transduction component of the Toll and IL-1 receptor family members involved in activation of the NF-κB pathway (106,107). IRAK-2 and IRAK-1 are ubiquitously expressed, whereas the most recently identified member, IRAK-M, is expressed only in cells of myeloid origin (108). All three members have been shown to interact with Myd88, a recruiter or adapter protein of the TLR and IL-1R complexes (109,110). IRAK-1 knockout mice maintain partial responsiveness to IL-1 and IL-18, indicating the existence of redundant pathways in signal transduc-

tion. Antisense technologies provide an efficient means to further delineate the functional roles of the three IRAKs. In this respect, a recent report described the effects of an IRAK-2 antisense oligonucleotide on IL-1-induced NF-κB nuclear translocation and ICAM-1 protein expression in human umbilical vein endothelial cells (111). RT-PCR analysis showed a modest sequence-specific reduction (less than 50%) of the IRAK-2 transcript as a consequence of treatment of the cells with a phosphorothioate-modified antisense oligonucleotide complementary to the AUG region of the target transcript. Regardless, the investigators found that the IL-1-induced increase in nuclear NF-κB and ICAM-1 protein levels was significantly reduced by treatment with the anti-IRAK-2 oligonucleotide in a dose-responsive manner.

Hematopoietic Cell Kinase (Hck). Hck is one of several members of the Src family of tyrosine kinases involved in activation and function of cells of myeloid origin, such as neutrophils and macrophages (112). Treatment of the macrophage cell line RAW264.7 with a phosphorothioate-modified antisense oligonucleotide that targeted the AUG region of the Hck transcript effectively inhibited phosphorylation of the *vav* protooncogene upon induction with LPS or IFN-γ (113). Although not shown, the authors indicated that LPS-induced TNF expression was also inhibited by anti-Hck oligonucleotide treatment. These results appear contrary to what has been observed in mice with triple null mutations in the hck, fgr, and lyn genes. LPS activation of ERK1/2, JNK, and NF-κB activity in isolated macrophages from these mice was unaffected (114).

Tyrosine Kinase 2 (Tyk2) and Janus Kinase 2 (Jak2). Tyk2 and Jak2 are two members of the Janus kinase (Jak) family of nonreceptor tyrosine kinases that act as signal transduction components for a variety of cytokine receptors that lack intrinsic tyrosine kinase activity (115). These include the Type I cytokine receptors for IL-2, -3, -4, -5, -6, -7, -9, -11, -12, -13, GM-CSF, G-CSF, and LIF; and the Type II cytokine receptors for IL-10, IFN-α, IFN-β, and IFN-γ. Both Type I and II receptors are usually composed of two or more chains, with the ligand-binding alpha chain associating with a second chain that is often a shared component between receptors, such as gp130 for the IL-6 family of receptors, the common gamma chain of the IL-2 receptor for the IL-2, IL-4, IL-7, IL-9, and IL-15 receptors, or the common beta chain for the IL-3, IL-5, and GM-CSF receptors, respectively. Different combinations of these receptor chains and their associated transduction components, i.e., the Jak/STATS, are thought to be the basis of both the redundant and differential cell-type-dependent responses to each cytokine. A significant amount of research has been directed toward identifying the individual members of the Jak and STAT families that are activated in response to a given cytokine. Tyk2 and Jak2 have been shown to be activated in peripheral blood monocytes upon induction with IL-13, as shown by immunoprecipitation and antiphosphotyrosine blotting techniques. Antisense oligonucleotides were utilized to determine the functional role of Tyk2 and Jak2 in the IL-

13-induced up-regulation of 15-lipooxygenase expression and activity (116). Treatment of the isolated monocytes with phosphorothioate-modified oligonucleotides specific for either Tyk2 or Jak2 mRNA resulted in 75–85% reduction of each respective target protein in a sequence-specific manner. Antisense-dependent reduction of either tyrosine kinase completely inhibited IL-13-induced expression of 15-lipoxygenase protein. This result supports a mechanism wherein the two kinases are activated by phosphorylation of each other (117). The effect of the Jak antisense oligonucleotides under multicytokine conditions or in in vivo models of inflammation, in which the individual kinases are likely involved in more than one signal transduction pathway, has yet to be reported.

Protein Kinase C. The PKC family has been implicated in LPS-induced expression of inducible nitric oxide synthase and the subsequent rise in nitric oxide levels seen in both macrophages and astrocytes. Macrophages express the α, βI, δ, η, and ζ PKC isoforms, whereas astrocytes express the α, θ, δ, η, and ζ PKC isoforms. To determine which PKC isoforms were functionally activated by LPS in each cell type, Chen and colleagues targeted the PKC isozyme(s) that had previously been indicated as candidates by nonspecific PKC down-regulation with phorbol ester. In murine RAW264.7 macrophages, TPA treatment suggested the potential involvement of PKC-α, βI, and δ, but not η (118). Treatment of the cells with phosphorothioate-modified oligonucleotides specific for each of the four isozymes showed that iNOS expression was equally dependent on all three isozymes, and confirmed the lack of involvement of PKC-η. A greater, but not complete reduction of iNOS expression and NO production (75%), was achieved by treating the cells with a combination of the PKC-α-, βI, and δ-specific antisense oligonucleotides. Knockout of each of the three isozymes involved in iNOS expression also dramatically reduced formation of NK-κB DNA-protein complexes, whereas knockout of PKC-η or the control oligonucleotides had no effect. In primary rat astrocytes, TPA treatment suggested the potential involvement only of PKC-η (119). Treatment of the cells with an anti-PKC-η oligonucleotide confirmed the isozyme's role in LPS-induced iNOS expression. However, the effect of PKC-η-targeted antisense treatment on iNOS protein expression and NO production was modest (40% reduction). This result may have been due to incomplete reduction of the target protein, as revealed by Western blot.

The MAP kinase phosphatase-1 (MKP-1) appears to negatively regulate LPS stimulation of bone marrow–derived macrophage MAP kinase cascades in a PKC-dependent manner (120). Bone marrow macrophages were found to express only PKC-βI, -ε, and -ζ. Treatment of the cells with antisense oligonucleotides specific for each of the three isozymes showed that LPS-induced MKP-1 expression was dependent upon PKC-ε, and not βI and ζ. PKC-ε antisense treatment also resulted in increased activity of the MAP kinases ERK-1 and 2, which are known substrates for MKP-1 (121).

PKC activity has also been implicated in ligand-induced signal transduction pathways that lead to generation of superoxide anion radicals in neutrophils. Activated neutrophils generate oxygen radicals through action of the respiratory burst enzyme, NADPH oxidase ($p47^{phox}$). Phosphorylation and translocation of the enzyme to b cytochrome-containing membranes are essential for its activation and the generation of superoxide anion radicals. The anion is metabolized by superoxide dismutase (SOD). Excess levels of this reactive oxygen species may result from either the excessive activity of $p47^{phox}$ or the diminished activity of SOD. PMNs obtained from asthmatic subjects demonstrate a heightened respiratory burst with increased superoxide generation compared to those from nonasthmatics. Recently, Korchak and colleagues showed that selective antisense-mediated depletion of PKC-β in differentiated HL60 cells reduced or delayed generation of superoxide anion radicals upon activation with the chemotactic tripeptide (fMLP) or upon treatment with phorbol ester (PMA), respectively (122). Ligand-induced phosphorylation and nuclear translocation of $p47^{phox}$ was also reduced. Depleting this member of the PKC family had no effect on ligand-induced cell adherence to fibronectin or fMLP-induced degranulation.

c-Raf. c-Raf, or Raf-1, is a member of the Raf serine/threonine kinase family (a-Raf, b-Raf, and c-Raf; 123) that has been identified as a component of growth factor (c-Kit, IL-3, GM-CSF, IL-5), cytokine (TNF-α), and TCR-activated signal transduction pathways. A variety of kinases can potentiate Raf activity, including those of the *ras*, *src*, and protein kinase C gene families. Antisense knockdown of c-Raf has been found to suppress proliferation and/or activation of a variety of immune and nonimmune cell types, including bone marrow cells (124), eosinophils (125), T cells (28), and endothelial cells (98).

Two research groups have evaluated c-Raf in terms of its role in the cellular response to IL-3/GM-CSF and IL-5 in bone marrow cells and eosinophils, respectively. Binding of these cytokines to their respective receptors has been shown to rapidly induce activation of multiple intracellular signaling pathways, including the Ras-Raf-ERK, JAK/STAT, phosphatidylinositol 3-kinase/PKB, JNK/SAPK, and p38 signaling pathways. Identifying critical components of these signals and their contribution to the related cellular responses is thus of great interest.

Normal murine bone marrow cells treated with a phosphorothioate-modified oligonucleotide that targets the AUG region of the c-Raf transcript inhibited IL-3-induced cell proliferation in a dose- and sequence-specific manner (124). Further evaluation of the function of c-Raf in progenitor cells derived from human bone marrow was performed, utilizing either Lin-/Kit+ or CD34+ cells. Treatment of Lin-/Kit+ cells with the c-Raf antisense oligonucleotide significantly inhibited colony formation (88–97%) upon stimulation with IL-3, IL-3 + SLF (steel factor), or GM-CSF. In contrast, only partial inhibition of colony formation was observed in c-Raf antisense-treated CD34+ cells upon stimulation

with a combination of either IL-3 or GM-CSF with SLF. This result is intriguing because c-Kit, the receptor for SLF, is known to activate multiple signal pathways, including the Raf/MAP kinases, upon ligand binding. Nonoverlapping signals emanating from the different receptors are likely the basis of this result. As a technical note, the investigators improved antisense activity by treatment of the cells twice over the 14-day assay period. The difference in activity between treatment protocols was attributed to the compensation for nuclease degradation of the oligonucleotide over time.

In a more recent study, Pazdrak and colleagues found that treating primary eosinophils with the same c-Raf antisense oligonucleotide inhibited IL-5-induced cell survival (34% viability for antisense- vs 74% for control-treated cells; 125). This treatment also reduced IL-5-induced CD11b adhesion molecule expression and the phorbol ester–dependent degranulation process of IL-5-primed cells, as measured by eosinophil cationic protein release.

A recent study by Monia and colleagues showed that expression of cell adhesion molecules in TNF-α-induced human dermal microvascular endothelial cells is dependent upon c-Raf activity (98). Treating cells with a 2′chimeric c-Raf antisense oligonucleotide significantly reduced E-selectin and VCAM expression at both the RNA and protein levels. In contrast, ICAM-1 RNA and protein expression levels were only partly reduced by this treatment. Antisense reduction of c-Raf was shown to inhibit TNF-α-induced activity of ERK and JNK, but not p38 MAPK. Additional experiments with the small-molecule MEK1/2 inhibitor, PD98059, and JNK-1 and -2 antisense oligonucleotides indicated that TNF-α induction of E-selectin expression was also dependent on activation of JNK2. The specific interactions involved in the cross-talk between c-Raf activity and TNF signaling and gene expression remain to be fully defined.

Transcription Factors.

Signal Transducer and Activator of Transcription 6 (STAT6). STAT6 is one of a set of transcription factors that are substrates for the Janus kinase (JAK) family of tyrosine kinases (126). Targeted disruption of the STAT6 gene in mice has been shown to impair both IL-4 and IL-13 signaling, and the Th2 immune response. STAT6-deficient mice thus do not develop bronchial hyperresponsiveness after antigen sensitization and challenge. Recently, Hill and colleagues identified a 2′MOE gapmer antisense oligonucleotide that reduces both human and murine STAT6 RNA and protein levels in vitro in a dose- and sequence-specific manner (127). The identified oligonucleotide is complementary to a sequence 20 nucleotides in length that is located in the coding region of both transcripts. This antisense oligonucleotide effectively reduced STAT6 mRNA and protein in a human Burkitt lymphoma B-cell line and caused a down-regulation in the IL-4-induced expression of germline Cε mRNA, a precursor to IgE production. This

effect was clearly due to reduction of STAT6 protein since levels of the other members of the STAT family (STAT-1α/β, -2, -3, and -5a/b) were unchanged, and the mismatch control oligonucleotide had no effect on STAT6 RNA or protein levels or expression of the germline Cε mRNA.

c-Fos. c-Fos is a basic-region leucine-zipper protein (bZIP) known to promote transcription of a variety of genes involved in inflammation, such as IL-2, IL-5, GM-CSF, IFN-γ, TCR, and a number of matrix metalloproteinases. It is known to dimerize with c-Jun to form the activator protein-1 (AP-1) transcription factor complex (128,129). c-Fos is encoded by an immediate early gene whose expression is induced rapidly and transiently in normal quiescent cells upon stimulation with mitogens and cytokines. Expression of c-Fos has been observed in chronically inflamed rheumatoid synovium (130) as well as sites distal to acute inflammation, such as spinal cord neurons (131,132).

When *c-fos* antisense oligonucleotides were continuously infused around the spinal cord, expression of c-Fos protein was reduced by approximately 50% in spinal dorsal horn neurons (133). The effect was not seen with a scrambled control oligonucleotide. In these experiments, the oligonucleotide backbone was phosphorothioate-modified only on the two terminal bases at the 5′ and 3′ ends. Oligonucleotide was either superfused into silicone-backed chambers surrounding the lumbar spinal cord or systemically administered by minipumps draining into a paraspinal pocket. Investigators used a carrageenan injection to produce peripheral inflammation, and they speculate that the local increase in blood–spinal cord barrier permeability produced by the carrageenan may have allowed the oligonucleotides to cross the blood–spinal cord barrier.

In cell culture, treatment of synovial fibroblasts from rheumatoid arthritis patients with a phosphorothioate-modified oligonucleotide complementary to the AUG region of *c-fos* mRNA was shown to inhibit IL-1β-induced cell proliferation, as measured by thymidine incorporation, in a dose- and sequence-specific manner (134). Loss of AP-1-binding activity was observed in the cells treated with the *c-fos* antisense oligonucleotide, whereas no loss of AP-1 activity was observed in cells treated with the sense control.

Nuclear Factor Kappa B (NFκB). NF-κB is a ubiquitously expressed transcription factor that regulates the expression of a large number of gene products found in many inflammatory settings. Genes regulated by NF-κB include the cytokines IL-1, IL-2, IL-6, IL-8, GM-CSF, and TNF; the adhesion molecules ICAM-1, VCAM-1, and E-selectin; and a number of acute-phase proteins (reviewed in Ref. 135). Members of the NF-κB/Rel transcription factor superfamily are functionally active as homo- or heterodimers. NF-κB is the heterodimer formed by the p50 and p65 molecules. In resting cells, NF-κB exists as a trimeric complex in the cytoplasm through its interaction with IκB. IκB becomes phos-

phorylated upon cell activation, however, which causes its degradation and the subsequent translocation of NF-κB into the cell nucleus, where it activates transcription of genes with NF-κB recognition elements in their promoters.

The p65 subunit of NF-κB was the target of some of the earliest in vivo antisense experiments. While these studies focused on implicating NF-κB in tumorigenesis, in vitro work by other investigators showed that murine embryonic stem cells treated with antisense oligonucleotides to p65 showed marked changes in their adhesive capacity, with complete detachment of cells from extracellular matrix materials (136). Another study investigated the adherence and proliferative capacities of vascular smooth muscle cells (VSMC), and found that p65 antisense, but not sense oligonucleotide or p50 antisense, inhibited VSMC adherence and proliferation in a concentration-dependent manner (137).

It was subsequently demonstrated, however, that the p65 oligonucleotides used in these early studies may have been causing pharmacological activity at least in part through nonantisense mechanisms. Both of the aforementioned studies used p65 antisense oligonucleotides containing four consecutive guanine residues. The so-called G string is now somewhat notorious for creating nonantisense effects (70), and this was shown specifically to be the case with the p65 antisense oligonucleotide (71,138).

Later studies targeting p65 have avoided the G-quartet motif, however. One of the most impressive studies used p65 antisense to block several different models of colitis in mice (139). In this work, a single intravenous injection or intracolonic application of p65 antisense reversed clinical symptoms in mice with trinitrobenzene sulfonic acid (TNBS)-induced colitis. The effect was greater than that produced by single or daily administration of glucocorticoids. Macrophages isolated from the intestine of antisense oligonucleotide-treated mice produced significantly lower amounts of IL-1, IL-6, and TNF-α. The same oligonucleotide could also reverse clinical and histological scores in IL-10-deficient mice that develop a chronic intestinal inflammation. In both models, the mismatched control oligonucleotides were without effect.

In another animal model study, severe gut inflammation induced by *Entamoeba histolytica* infection was established in human intestinal xenografts on SCID mice. Human intestinal epithelial cell inflammation was inhibited by the intraluminal administration of an antisense oligonucleotide to p65 (sans G quartet). This blocked the production of human IL-1β and IL-8, inhibited neutrophil influx into the xenografts, and lowered intestinal permeability to dextran. Although p65 mRNA, protein levels, and/or nuclear translocation were not evaluated in this study, a control oligonucleotide was without effect on changing the levels of cytokine production, neutrophil influx, and intestinal permeability (140).

The first demonstration of in vivo activity for an antisense oligonucleotide that acts via an RNase H–independent mechanism (63) has recently been reported

for a 16-mer N3′-P5′ phosphoramidate that targets the AUG region of the p65 subunit of NF-κB (141). In this study the investigators evaluated the effects of single-dose antisense treatment on LPS-induced expression of IL-6 in mice. Treatment of mice with 900 μg of oligonucleotide 24 h prior to LPS administration yielded the greatest reduction in IL-6 expression. Eight hours post-LPS, serum IL-6 levels were reduced nearly to basal levels in the phosphoramidate NF-κB antisense-treated mice, whereas the scrambled phosphoramidate control had only a slight effect on IL-6 levels as compared to the untreated mice. A chimeric phosphoramidate-phosphodiester antisense analog, which may act as a substrate for RNase H, was as effective as the fully modified phosphoramidate oligonucleotide when mice were treated with oligonucleotide 2 h prior to LPS administration but not as effective with treatment 24 h prior to LPS exposure. Variation in activity with respect to treatment and induction profile may be due to differences in resistance to nucleases.

An interesting approach to NF-κB blockade was attempted by a group who used synthetic double-stranded oligodeoxynucleotides as "decoy" cis elements to block NF-κB binding to promoter regions of genes critical to reperfusion injury that occurs after myocardial infarction (142). The double-stranded phosphorothioate NF-κB decoy (20 bases long, consensus binding site) was incorporated into liposomes containing hemagglutinating virus of Japan (HVJ) to facilitate oligonucleotide delivery to infused rat hearts. Transfection with NF-κB decoy, but not a scrambled decoy oligonucleotide, was found to significantly reduce the area of infarction that ensued following coronary artery occlusion. Myocardial infarction could not be inhibited with an antisense oligonucleotide (single-stranded) directed against rat-inducible nitric oxide synthase (iNOS). The NF-κB decoy also resulted in a marked decrease in IL-6 and VCAM mRNA to levels similar to those seen in uninfarcted heart tissue, and it reduced neutrophil migration into the infarcted area.

Three members of the NF-κB/Rel transcription factor family have been targeted by antisense oligonucleotides in vitro: the p65 subunit, the p50 subunit, and c-Rel. Two research groups have looked at the effects of antisense treatment on expression of genes involved in prostaglandin synthesis in IL-1β-stimulated rheumatoid synovial fibroblasts. The most comprehensive study, with respect to number of NF-κB targets, was performed by Roshak and colleagues, who evaluated the effects of p65, p50, and c-Rel antisense oligonucleotides, as well as a double-stranded DNA decoy (22 mer) of the classic NF-κB consensus sequence motif (143). As endpoints, they analyzed genes whose promoters contained either the classic NF-κB motif (Cox-2 and $cPLA_2$) or the alternative NF-κB motif (IL-8). Treatment of these cells with the p65 antisense oligonucleotide inhibited production of both prostaglandin E2 and IL-8 in a dose-responsive manner. Further experimentation showed that this oligonucleotide significantly suppressed IL-1β induction of Cox-2 and $cPLA_2$ mRNA expression, with complementary reduction

of Cox-2 protein but not $cPLA_2$ protein. In contrast, treating the cells with the c-Rel antisense oligonucleotide only reduced IL-8 production. Neither the p50 antisense nor the control oligonucleotides had a significant impact on these endpoints. These results supported previous studies that showed a higher affinity for the consensus site by the p65/p50 heterodimer, and for the alternative site by the p65/c-Rel heterodimer. As such, this revealed the inherent advantage of targeting the common subunit of each heterodimer, p65, and also showed the limitations of the double-stranded DNA decoy.

The effects of the same p65 antisense oligonucleotide on Cox-2 expression in IL-1β-induced rheumatoid arthritis synovial fibroblasts were evaluated by a second group in a more limited study (144). In this case, reduction of Cox-2 protein levels in the p65 antisense-treated cells was moderate in comparison to the sense control oligonucleotide-treated cells 4 h after IL-1 induction. In contrast to the study by Roshak et al., these investigators treated the cells with the oligonucleotides in the absence of cationic lipids, so any differences in oligonucleotide uptake efficiency and intracellular distribution could have contributed to the differing results. Differences in the period of time between oligonucleotide treatment and IL-1β induction could also have resulted in variable antisense efficacy given that there are preexisting amounts of the p65 subunit in the resting cell complexed to IκB.

Antisense reduction of the NF-κB p50 subunit has also been evaluated in primary murine splenic B cells and the transformed B-cell line WEHI 231. Cells were treated in vitro with a 21-mer phosphorothioate targeting p50. The group was able to achieve about a 50% reduction in p50 protein levels, but only when the cells were first activated with LPS (145). p50 antisense treatment was also efficacious in B cells isolated from the mouse BXSB strain, an animal that develops B-cell hyperproliferation and autoantibody production reminiscent of that seen in human systemic lupus erythematosus. In B cells from this strain, NF-κB levels were specifically reduced approximately 50% with antisense treatment, IgM and IgG immunoglobulin levels decreased to a similar extent, and double-stranded DNA autoantibodies were reduced 90%. LPS treatment was not necessary to maximize oligonucleotide uptake and facilitate target protein reduction in this strain of mice, but it was required when WEHI cells or B cells from nonautoimmune mice were used. This implies that cells either activated by exogenous mitogens or stimulated by an inflammatory or autoimmune process in vivo could be better targets for antisense treatment.

6. Other Inflammatory Mediators

Free Radicals.

Inducible Nitric Oxide Synthase (iNOS). Nitric oxide is a short-lived free radical that is generated by a family of enzymes referred to as nitric oxide syn-

thases (NOS). NO has emerged as an important mediator involved in vascular, platelet, bone, neural, and immune function. As such, deregulation of its production has been found in a variety of pathophysiological states. There are three known NOS isoforms, endothelial-derived NOS (eNOS), neuronal-derived NOS (nNOS), and inducible NOS (iNOS). eNOS and nNOS are constitutively expressed isoforms that are calcium/calmodulin dependent, whereas iNOS is a Ca/calmodulin-independent isoform whose expression is induced by inflammatory stimuli, such as cytokines and endotoxin. Isoform-specific inhibition of iNOS expression and activity by antisense oligonucleotides has been demonstrated in vivo and in vitro.

iNOS has been implicated in the pathogenesis of multiple sclerosis (MS), and in an animal model of MS, experimental autoimmune encephalomyelitis (EAE), administration of iNOS inhibitors or nitric oxide scavengers has ameliorated disease (146). In a mouse model of EAE induced by the adoptive transfer of T cells specific for myelin basic protein, intraventricular injection was used to deliver oligonucleotides targeted to iNOS (147). Antisense treatment in this mouse model caused a significant reduction in clinical scores, and blocked iNOS mRNA and protein synthesis, as well as iNOS enzyme activity within the central nervous system. Neither sense nor scrambled oligonucleotide sequences affected disease scores or iNOS expression, and the protein and enzyme activity level of constitutive neuronal nitric oxide synthase was not affected by the iNOS oligonucleotide, strongly implicating an antisense mechanism in this model. Mice in these experiments were only monitored for 10 days, however, so it is unknown whether the iNOS antisense treatment will permanently modulate clinical signs of EAE or prevent clinical relapses.

Another group used antisense oligonucleotides targeting iNOS to explore their therapeutic efficacy in a rat model of LPS-induced hypotensive shock (148). LPS administration can cause impaired vascular contractility. Vascular hyporeactivity to catecholamines and other vasoconstrictive agents is a characteristic feature of human septic shock that contributes to the high mortality rate associated with this condition. Rats pretreated with iNOS antisense oligonucleotide were protected from LPS-induced vascular hyporeactivity to norepiniphrine. iNOS antisense also prevented the hemorrhaging that was caused by LPS, and the oligonucleotide inhibited iNOS activity and iNOS protein expression. A mismatch control oligonucleotide was without effect on these parameters. Some care should be taken in interpreting these results, however, since the iNOS antisense sequence contained a G quartet near its 5′ end.

The effects of iNOS expression on cell viability and nitrite production have also been evaluated in green monkey renal tubular epithelial cells exposed to LPS or hydrogen peroxide (149). In this application, the antisense oligonucleotide was a phoshorothioate-modified 24-mer that complemented the AUG region of the human iNOS transcript (owing to lack of availability of the monkey iNOS

sequence). iNOS protein expression levels were assessed by immunocytochemical staining of cells pretreated with iNOS antisense oligonucleotides versus scramble controls. iNOS expression was significantly reduced in a sequence-specific manner. Investigators also evaluated the effects of oligonucleotide treatment on LPS- or H_2O_2-induced production of nitrite, and expression of NADPH-diaphorase and cell viability during oxidative stress. Under these conditions, nitrite levels were maintained at basal levels by pretreatment of the cells with the antisense oligonucleotide. Antisense pretreatment also maintained cell viability under conditions of oxidative stress. Although not stated, some effect was apparently seen on unstimulateld cells by treatment with the iNOS antisense oligonucleotide because constitutive nitrite expression decreased, and cell viability increased in a sequence-specific manner.

Prostaglandins and Leukotrienes. Prostaglandins (PG) and leukotrienes (LT) are two classes of lipids that act as potent mediators of both normal and pathological immune responses (150). Prostaglandins are expressed by both immune and nonimmune cells, whereas the leukotrienes are primarily expressed by immune cells. Both classes of lipid mediators are generated from arachidonic acid (AA) by cyclooxygenases (151) and lipoxygenases (LOX), respectively. AA levels are tightly regulated through de novo synthesis, dietary intake, or enzymatic metabolism of phospholipids (primarily by members of the phospholipase A family). Several of the enzymes that are involved in generating these lipid mediators during an immune response have been targeted by antisense oligonucleotides in cell culture experiments.

Phospholipase A2 (PLA_2). An expanding number of PLA_2 enzymes have been identified that generate arachidonic acid from the phospholipid pool (152). Identifying the specific member(s) of the family that play a role in a given inflammatory response or immune-based activation pathway versus those involved in homeostatic processes has therefore received a fair amount of attention. Several investigators have discovered, with the aid of isotype-specific antisense oligonucleotides and small-molecule enzyme inhibitors, that the enzymatic source of AA that leads to synthesis of each class of lipid mediator may be isotype-specific and depends on cell type, species, and mechanism of activation. For example, Marshall and co-workers showed that primary human monocytes stimulated with either the calcium ionophore A23187 or zymosan produced both leukotrienes and prostaglandins. Reduction of the 85-kDa cytosolic Group IV PLA_2 enzyme ($cPLA_2$) by treatment with an antisense oligonucleotide that targeted the AUG site of the transcript inhibited production only of the prostaglandins but not the leukotrienes, as measured by PGE_2 and LTC_4 levels, respectively (153). This result was supported by similar effects of the small-molecule enzyme inhibitor arachidonyl trifluoromethyl ketone, which is specific for $cPLA_2$. Interestingly, treatment of the cells with the

secretory Type II PLA_2 inhibitor SB 203347 resulted in loss of leukotriene production, but not prostaglandin production. This effect of anti-$cPLA_2$ oligonucleotide treatment on eicosanoid production has also been observed in LPS-plus-PMA-treated U937 cells, a transformed monocytic line (154).

These results contrast to observations made using peritoneal macrophages isolated from Group IV $cPLA_2$-deficient mice (155). As with human PBMCs, $cPLA_2$ (+/+) peritoneal macrophages produce both prostaglandins (PGE_2) and leukotrienes (Cys-LTs) upon stimulation with A23187. The $cPLA_2$ (−/−) macrophages, however, showed a marked decrease in A23187-induced production of both lipid mediators. The basis for this difference is unclear. It may be due to differences in species or the origination of the hematopoietic cells. Alternatively, the $cPLA_2$ inhibitors may not have sufficiently reduced enzyme activity in the PBMCs. In this respect, treatment with the anti-PLA_2 oligonucleotide did not completely eliminate the target protein as shown by Western analysis (153,154). In both cases, further optimization of the anti-$cPLA_2$ oligonucleotide activity as well as a comparison to oligonucleotides that target other members of the PLA_2 family, e.g., Type II, may clarify the given observations and results.

In another system, Dennis and co-workers have also investigated the function of different PLA_2 isotypes through the use of antisense oligonucleotides. Their reports focused on the expression and function of the Group IV, V, and VI PLA_2 isozymes in mouse $P388D_1$ cells, an activated macrophage cell line that does not express the secretory Group II isozymes. This research has shown that selective reduction of the secretory Group V isotype by antisense oligonucleotide treatment results in reduction of PGE_2 synthesis. In contrast, antisense-directed reduction of the cytosolic Group VI PLA_2 in $P388D_1$ cells showed that this isotype does not play a significant role in the PAF activation pathway, but instead is involved in the homeostatic phospholipid fatty acid remodeling procesos (156). Group V PLA_2 activity was also shown to mediate production of prostaglandin PGD_2 in activated murine mast cells. Herschman and co-workers found sequence-specific reduction of PGD_2 expression with the antisense oligonucleotide utilized in the previously described macrophage experiments (157).

Further investigation of the Group V isozyme in a selected $P388D_1$ subclone showed that both its expression and its subsequent activity leading to immediate and delayed synthesis of prostaglandins is dependent upon activation of cytosolic Group IV $cPLA_2$ (158). The pivotal role of the cytosolic Group IV $cPLA_2$, in inducing a second PLA_2 isotype has also been found in cytokine-activated rat 3Y1 fibroblasts cells by Kuwata and co-workers (159). In this study, antisense-directed inhibition of secretory Group IIA PLA_2 showed this isotype to be the source of AA for delayed production of prostaglandin PGE_2, by Cox-2. These studies collectively implicate the Group IV $cPLA_2$ isotype as a pivotal upstream component of prostaglandin synthesis in several cell types, under a variety of stimuli.

D. Angiogenesis

Angiogenesis, or the growth of new blood vessels, is a process that occurs during normal physiological processes such as fetal development, exercise-induced muscle hypertrophy, and menstrual-cycle endometrial proliferation, but may also play a key role in various inflammatory scenarios. Cells can secrete a variety of angiogenic factors that induce the endothelial cell migration and proliferation common to new blood vessel growth. These angiogenic factors include both acidic- and basic-fibroblast growth factor (bFGF), transforming growth factor-α, TNF-α, epidermal growth factor, vascular endothelial growth factor (VEGF), and various extracellular matrix proteins.

1. VEGF

Dysregulated angiogenesis is critical to the development of several different ocular diseases, including macular degeneration, diabetic retinopathy, and corneal neovascularization. The role of vascular endothelial growth factor (VEGF) was investigated in a mouse model of retinopathy of prematurity. Antisense oligonucleotides targeting VEGF were injected intravitreally into the eyes of neonatal mice prior to the onset of proliferative retinopathy. The VEGF antisense treatment caused a statistically significant reduction in retinal VEGF levels and in the level of retinal neovascularization, which was not seen when control oligonucleotides were injected (160). The inhibition of neovascularization was, however, incomplete, implying that other angiogenic factors could be involved in this model.

2. Sp1/NF-κB

Short-term exposure of human microvascular endothelial cells to low doses of TNF-α results in formation of tube-like structures in vitro. Increased expression of the angiogenic factors IL-8, VEGF, and bFGF is observed under these conditions at the mRNA and protein levels. Treatment of the microvascular endothelial cells with antisense oligonucleotides that target the transcription factors involved in TNF-induced production of these angiogenic factors results in down-regulation of their expression, in particular that of IL-8 and VEGF, as well as inhibition of tubular morphogenesis (161). Sequence-specific inhibition of TNF-induced microvascular cell tube formation was greatest (60% inhibition) when cells were pretreated with the antisense oligonucleotides to either Sp1 or the p65 subunit of NF-κB. Less inhibition (30%) was observed in cells pretreated with a c-Jun antisense oligonucleotide.

E. Apoptosis and Its Role in Inflammatory Disease

The process of programmed cell death (apoptosis) is critical for the development and maintenance of the immune system. Disorders of the normal death pathway

can lead to multiple disease states. In addition to defects in this pathway that can lead to uncontrolled lymphoid cell growth covered in other chapters, imbalances in apoptosis have been observed in inflammatory conditions, although apoptosis itself is not generally believed to be immunogenic (162). In this regard, heightened apoptosis occurs as a result of the inflammatory pathology of viral hepatitis and endotoxin-induced liver failure as well as during allograft rejection (163–168). Conversely, the absence of normal apoptotic signaling can result in the induction of autoimmunity and uncontrolled inflammation (169–172). The development of disease during both heightened and reduced levels of apoptosis in these cases depends on whether the programmed cell death involves the immune or nonimmune tissue (e.g., the hepatocytes or allograft).

1. Granzyme C

Antisense techniques have reproducibly been shown to alter apoptotic processes in lymphocytes. For example, nephritogenic cytotoxic T lymphocytes can induce the granzyme C–mediated apoptosis of cultured renal proximal tubular epithelial cells. An antisense oligonucleotide targeted against granzyme C was found to inhibit the cytotoxic behavior of a nephritogenic T cell clone in vitro and also in vivo, following subcapsular transfer of the in vitro antisense-treated T-cell clone to recipient kidneys (173). A sense oligonucleotide was without effect and neither oligonucleotide caused cytotoxicity in vitro. Interestingly, antisense-mediated inhibition of cytoxicity was more potent in vivo than in vitro. The authors speculate that this may be the result of the inefficient killing of the tubular epithelial cell barrier, preventing entry of the nephritogenic T cells into the kidney interstitium, or that granzymes may be more important cytotoxic mediators in vivo than they are in vitro.

2. *c-myc*

In an approach using primary B lymphocytes, antisense to *c-myc* specifically inhibited both spontaneous and anti-IgM-induced programmed cell death and also prevented the anti-IgM-triggered increase in Myc protein levels known to correlate with the induction of apoptosis in these cells (174). Inhibition of NK cell proliferation in the presence of IL-2 was shown to occur by apoptotic signaling through the FcγRIIIA receptor (175). This process may control NK cell expansion during an antibody-dependent immune response, and was demonstrated to involve IL-2-induced *c-myc*, since *c-myc* antisense treatment during IL-2 stimulation inhibited FcγRIIIA-induced cell death. Again, CpG motifs were present in both the 26-mer antisense and 6-base mismatch sequences, although reduction of *c-myc* protein levels was shown in mixed lymphocyte cultures (since levels in IL-2-treated NK cells are low).

3. Bcl-2 family

Bcl-2. Antisense oligonucleotide targeting of bcl-2 family members has been attempted by several groups investigating the role of these proteins in apoptosis. Natural killer (NK) cells play a role in human diseases such as cancer, asthma, and natural killer cell lymphocytosis (176–178). Defects in apoptosis of NK cells or their expression of apoptosis-inducing ligands may contribute to disease pathology. Studies evaluating the role of *c-kit* ligand in human NK cell survival have shown that this cytokine suppresses apoptosis through up-regulation of *bcl-2* (179) and *bcl-2*-directed antisense treatment blocked this protective effect of *c-kit* ligand. However, the *bcl-2* antisense 20 mer contained two near-canonical CpG motifs while the sense version contained one CpG motif.

Bcl-x. Targeting genes that regulate apoptosis pathways with antisense may also impact inflammatory processes with granulocytic components. The anti-apoptotic effects of granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-5 on primary human eosinophils can be inhibited by treatment with *bcl-xL* antisense oligonucleotide (180). The 20-mer antisense oligonucleotide alone had no effect on cell viability under the culture conditions used and the antisense-specific occurrence of apoptosis was confirmed by analysis of cellular morphology. Importantly, no difference in the effect was observed between eosinophils from normal or allergic patients, although antisense inhibition of cytokine-mediated cell survival was maximally only 34%.

Bax. Inhibition of expression of the bcl-2 family member Bax with antisense oligonucleotides has been shown to delay neutrophil apoptosis (181). In this study, two 20-mer antisense or scrambled control sequences were incubated together with isolated human neutrophils in the absence of uptake enhancers. Both of the antisense and one of the control sequences contained noncanonical CpG motifs. However, these investigators also found that Bax expression is down-regulated under inflammatory disease conditions in bronchoalveolar neutrophils obtained from cystic fibrosis patients compared to neutrophils from donors without disease, and that Bax is reexpressed following cytokine withdrawal, a known inducer of neutrophil apoptosis, suggesting that the reported Bax antisense effect is real.

4. Lyn Kinase

Wei et al. have shown that in primary human neutrophils, antisense inhibition of Lyn kinase resulted in insensitivity to the antiapoptotic effect of GM-CSF (182), whereas antisense targeting of Hck or Fgr kinases had no effect. Although the Lyn antisense compound contained two noncanonical CpG motifs, Lyn protein levels were reduced following culture with the 20 mer. On the other hand, Lyn antisense treatment did not affect expression of Hck, suggesting oligonucleo-

tide specificity. Cotreatment of neutrophils with GM-CSF and Lyn antisense blocked GM-CSF–mediated survival, while scrambled control had no effect. This was shown by DNA fragmentation and by morphologic analyses. Together with the explosion of knowledge of the molecular events governing apoptosis in various biological systems over the past several years, these investigations underscore the potential utility of antisense modulation of cell death pathways in inflammation.

F. Immune Cell Trafficking

1. Cell Adhesion Molecules

During inflammation, leukocytes extravasate from the circulation and enter tissue through a sequential process involving the interactions of adhesion molecules and their ligands expressed both on the endothelium of blood vessels and on the surface of the leukocytes. The emigration of white blood cells through postcapillary venules involves three distinct steps: a reversible adhesion or rolling of leukocytes along the surface of endothelial cells, activation of the leukocytes leading to their firm adhesion to the endothelium, and subsequent diapedesis of white blood cells through the venules and into tissue. The initial rolling steps are primarily mediated by the selectin family of adhesion molecules (E-, P-, and L-selectin) expressed on either endothelial cells or leukocytes. The selectins interact with carbohydrate ligands expressed on the cognate cell type. Firm adhesion to the vasculature and diapedesis are mediated by members of the integrin and immunoglobulin superfamilies of adhesion molecules. Ig superfamily members include ICAM-1 and VCAM-1, frequently expressed on inflamed vascular endothelium. Prominent integrin members involved in inflammatory process include VLA-4, $\alpha 4\beta 7$, and the $\beta 2$ integrin family members LFA-1 and MAC-1. For more extensive reviews on this process, see Refs. 183–186.

Some of the earliest work using antisense oligonucleotides to block adhesion molecule expression was directed against adhesion molecules expressed on vascular endothelial cells. Two different groups identified phosphorothioate antisense oligonucleotides specific for ICAM-1, VCAM-1, and E-selectin, and demonstrated target reduction in human umbilical vein endothelial cells (HUVEC) (187,188). These oligonucleotides were capable of reducing the adhesion of monocytes, U937 cells, or HL-60 cells to cytokine-activated HUVECs.

ICAM-1. ICAM-1 is an adhesion molecule expressed at low levels on resting endothelial cells that is markedly up-regulated in response to inflammatory mediators like TNF-α, IL-1, and IFN-γ. It can also become up-regulated on skin keratinocytes during inflammation, a process that may be important in mediating the retention of T cells in the epidermis during autoimmune diseases like psoriasis.

ICAM-1 antisense can block the interactions of immune cells with nonimmune cells. This was seen in an in vitro model of T-cell binding to intestinal fibroblasts (189). The fibroblasts expressed ICAM-1 and VCAM-1 after being exposed to immune cell–derived supernatants. T-cell binding to the fibroblasts was found to be nearly exclusively dependent on ICAM-1, and an ICAM-1 antisense oligonucleotide specifically and dose-dependently inhibited ICAM-1 expression and T-cell binding. The experiments suggested a potentially important regulatory role of fibroblasts in mucosal immunity and their potential as targets for therapeutic intervention during intestinal inflammatory diseases.

Allograft Survival. Perhaps the largest and most convincing body of in vivo data with antisense oligonucleotides exists for ICAM-1. Antisense inhibition using phosphorothioate oligonucleotides targeted to the 3′-untranslated region of ICAM-1 has shown efficacy in a number of rodent allograft models. Many of these studies employed minipumps for oligonucleotide delivery. Endothelial cells in allografts exhibit markedly increased ICAM-1 expression, and this facilitates interactions with circulating host leukocytes expressing the β2-integrins (LFA-1, MAC-1, and CD11c) and subsequent migration of these cells through the graft vasculature and into the tissue parenchyma.

Antisense to mouse ICAM-1 has proved effective in models of heart, kidney, and pancreatic allograft survival. In a mouse model of heterotopic heart allograft survival, treatment of mice for 14 days by continuous intravenous infusion of antisense oligonucleotide to ICAM-1 prolonged allograft survival from 7.7 ± 1.4 days to a maximum of 23.0 ± 7.5 days at a dose of 5 mg/kg/day (190). Two control oligonucleotides failed to prolong allograft survival, and the effects of the ICAM-1 ASO were synergistic when combined with treatment with monoclonal antibody to LFA-1, in which case allografts survived indefinitely (more than 200 days).

The same antisense oligonucleotide (ISIS 3082) also proved effective in prolonging the survival of mouse islet cell allografts. Whereas untreated streptozotocin-induced diabetic C3H mice rejected pancreatic islets from C57BL/10 mice at 10.7 ± 2.3 days, treatment with 5 mg/kg ISIS 3082 daily for 7 days prolonged islet survival to 28.9 ± 12.0 days. The combination of ICAM-1 antisense with LFA-1 mAb once again induced tolerance to islet allografts, prolonging their survival beyond 100 days (5).

A phosphorothioate antisense oligonucleotide (ISIS 9125) targeting the same region in rat ICAM-1 mRNA as the mouse oligonucleotide cited aboved was effective in prolonging the survival of rat heart and kidney allografts (191). When hearts from Lewis rats were transplanted into ACI recipients, survival was prolonged from 8.8 ± 0.8 days in untreated controls to 16.8 ± 3.5 days in recipients dosed daily for 7 days by minipump infusion of 10 mg/kg/day. Treatment with a scrambled control oligonucleotide had no effect on prolonging graft survival. A 14-day intravenous infusion of ISIS 9125 produced better results in a

rat kidney allograft model, with prolongation of Lewis rat allografts in ACI recipients from 8.7 ± 0.8 days in untreated animals to 50.4 + 21.6 days. In both cases, scrambled control oligonucleotides were without effect. Interestingly, when treatment was restricted to preoperative perfusion of the transplanted kidney (20 mg/2 mL), recipient allograft survival was prolonged to 35 days.

Ischemia/Reperfusion Injury. Ischemic reperfusion injury is a common by-product of organ transplantation. It can cause graft injury at the outset of transplantation, and there is some evidence to suggest it may hasten the process of allograft rejection (192). The pathogenesis of ischemia/reperfusion injury involves the up-regulation of cytokines and adhesion molecules, and evidence from animal models of kidney reperfusion injury suggests that ICAM-1 may be a key player.

Phosphorothioate antisense to rat ICAM-1 (also targeting the 3′ UTR but with a different sequence than ISIS 9125 described above) was successfully used to prevent reperfusion injury in renal isografts from Lewis rats (193). Kidney donor animals in this experiment were treated with either ICAM-1 antisense oligonucleotide, reverse sense oligonucleotide, or saline 6 h before nephrectomy. The kidneys were subjected to an hour of warm ischemia and 30 min of cold ischemia, a procedure that causes delayed graft function. Six weeks after the kidneys were transplanted into syngeneic recipients ($N = 16$/group), half of the animals receiving control oligonucleotide and saline vehicle were dead, while all but two rats in the antisense-treated group survived to 20 weeks. The ICAM-1 antisense treatment also improved kidney function (measured by serum creatinine concentrations and protein excretion in the urine) at every time point measured when compared to saline or control oligonucleotide-treated survivors.

Another group also used ICAM-1 antisense treatment to reduce reperfusion injury in rat heart allografts, and compared this therapy to treatment with mAb to LFA-1 administered systemically (194). This group employed a novel method of hyperbaric pressure to infuse oligonucleotide into the graft prior to transplantation. The antisense treatment caused a decrease in ICAM-1 protein expression in grafts removed 72 h after transplantation. Signs of reperfusion injury, including myeloperoxidase activity and contraction band necrosis, were also reduced, and the antisense treatment appeared somewhat more effective in this regard than treatment with LFA-1 mAb alone.

In a second study involving the hyperbaric pressure method for transfecting antisense oligonucleotides ex vivo, transfection efficiency of a fluorescently labeled oligonucleotide was measured in human saphenous vein and rat myocardium, and found to be 90% and 50%, respectively. Nuclear localization of the oligonucleotide was also documented. When antisense to rat ICAM-1 was delivered to rat hearts by hyperbaric pressure, a widely distributed, sequence-specific inhibition of ICAM-1 mRNA and protein levels was achieved after allotransplantation. Although the myocardial ICAM-1 expression was not completely down-

regulated, the 50% decrease obtained correlated nicely with the measured efficiency of antisense uptake (195).

Skin Models of Inflammation. ICAM-1 is expressed constitutively at low levels in epidermal keratinocytes, but expression levels increase markedly in these cells during inflammation. IFN-γ-inducible ICAM-1 protein expression could be reduced up to 50% by ICAM-1 antisense in cultured keratinocytes, a process only partly dependent on the commonly used antisense transfection reagent lipofectin (196). These results suggest that antisense compounds may interact with keratinocytes differently than they do with other cell types. Antisense targeting of ICAM-1 has also proven effective in modulating models of skin inflammation in vivo in both mouse and human systems. In a murine model of a hapten-induced contact hypersensitivity reaction, a mouse-specific ICAM-1 antisense oligonucleotide encapsulated in liposomes (ISIS 3082) was evaluated for its ability to prevent the ear-swelling reaction. Ear swelling and cellular infiltration in mice treated with empty liposomes or liposome-encapsulated control oligonucleotide were not reduced when compared to untreated controls. The mice that received ISIS 3082, however, exhibited near-baseline levels of ear thickness and leukocyte infiltration, similar to those observed in mice treated with topically applied corticosteroid. The ICAM-1 antisense produced this effect only when it was encapsulated in liposomes (197). This may have been due to the enhanced circulation half-life and targeting to the inflammation site that were reported.

Antisense-mediated inhibition of human ICAM-1 was also effective in a model of cytotoxic dermatitis induced by the microinjection of heterologous human lymphocytes into human skin xenografted onto immunodeficient SCID mice. In this model, injection sites develop a progressive T-cell accumulation culminating in a cytotoxic dermatitis that bears some resemblance to human lichen planus. Migration of T cells into the epidermis in this model is linked to the induction of ICAM-1 by keratinocytes. The intraepidermal T-cell migration and subsequent lesion formation in this model were partially abrogated by systemic administration of antisense to ICAM-1 (198).

Lung Inflammation. ICAM-1 may become up-regulated in lung tissue as a sequela to bacterial infection. This process was modeled in mice treated with endotoxin, and the efficacy of ICAM-1 blockade was compared in mice treated with antisense to ICAM-1 (ISIS 3082), ICAM-1-specific monoclonal antibodies, or ICAM-1 mutant mice. The antisense oligonucleotides inhibited up-regulation of ICAM-1 mRNA in a dose-dependent manner. Neutrophil emigration into alveolar spaces at 24 h was inhibited by 59%, similar to the level of inhibition achieved using ICAM-1 mAb (199). No inhibition of neutrophil emigration was observed in the knockout mice, suggesting that the mutant mice may have developed ICAM-1-independent pathways to compensate for the long-term loss of ICAM-1. The incomplete blockade of ICAM-1 by antibody or antisense further

suggests that alternative adhesion pathways that do not rely on ICAM-1 are important for leukocyte migration into lungs.

Colitis. Increased ICAM-1 expression has been detected in both ulcerative colitis and Crohn's disease. A murine-specific ICAM-1 antisense oligonucleotide, ISIS 3082, was evaluated in a mouse model of colitis induced by the administration of 5% dextran sulfate in the animals' drinking water for 5–7 days. This produces colitis characterized by focal areas of inflammation and crypt abscesses, and an increased expression of ICAM-1. Prophylactic treatment of DSS-treated mice with ICAM-1 antisense produced a dose-dependent reduction in the clinical signs of colitis, with maximal effects occurring at a dose of only 1 mg/kg/day. Reductions in ICAM-1 immunostaining and infiltrating leukocytes were observed, while scrambled control oligonucleotides failed to modify the course of the disease (200).

The pharmacological activity of ICAM-1 antisense in such a wide range of in vivo models as those outlined above has prompted clinical trials evaluating ICAM-1 blockade for a number of indications, including Crohn's disease and ulcerative colitis. The status of trials involving ISIS 2302, a 20-mer phosphorothioate oligonucleotide targeted to the 3′ UTR of human ICAM-1, is reviewed in another chapter in this volume.

E-Selectin. Antisense inhibitors of human E-selectin have been identified that interfere with the cytokine-activated binding of leukocytes to HUVECs (187,188). A group studying the adhesion of colon cancer cells to E-selectin used a novel antisense approach to block expression of the E-selectin ligand sialosyl Le(a) on tumor cells (201). They transfected a colon carcinoma cell line with high expression of sialosyl Le(a) with an expression vector containing a fragment of cDNA for alpha 1,3/4-fucosyltransferase in the antisense orientation. After transfection, the cell line failed to express sialosyl Le(a) antigen, and lacked alpha 1,3/4-fucosyltransferase activity. This completely abolished adhesion of the cancer cells to E-selectin.

PECAM-1. PECAM-1 is a member of the immunoglobulin superfamily of adhesion molecules and is constitutively expressed on endothelial cells. Antisense knockdown of PECAM-1 in murine bEND.3 cells had a fairly dramatic effect on the morphology of these cells, as well as their ability to proliferate, migrate, and cause hemangiomas (202). The antisense-mediated changes in PECAM-1 expression were accompanied by an up-regulation of thrombospondin-1 and its antiangiogenic receptor CD36 in the cells. PECAM-1 is also expressed on monocytes and macrophages, and treatment of the promonocytic cell line U-937 with PECAM-1 antisense was found to reduce the TGF-β-induced homotypic aggregations of these cells (203).

β2 *Integrins.* Less work has been directed toward adhesion targets expressed on leukocytes. However, one group developed an antisense molecule specific for the CD11a chain of LFA-1 (204). They used this oligonucleotide to

show that when CD11a expression was decreased on a human B-cell line, the homotypic adhesion of these cells caused by CD40 ligation was reduced, and the protective response induced by CD40 ligation prior to anti-IgM antibody treatment was abrogated. This implied that LFA-1/ICAM-1–dependent cell adhesion induced by signaling through CD40 played an important role in the inhibition of anti-IgM–induced apoptosis of these cells.

Adhesion Molecule Regulation. Antisense technology has also been used to dissect the regulation of adhesion molecule expression. In a study in A549 cells, an antisense oligonucleotide specific for human PKC-α was used to examine the role of PKC-α in mediating phorbol ester–induced changes of ICAM-1 mRNA levels (205). Phorbol ester treatment caused a 10–20-fold increase in ICAM-1 mRNA in A549 cells, but when PKC-α levels were depleted by antisense treatment, this increase in ICAM-1 expression was abrogated, demonstrating the critical role of the PKC-α isoform in the process.

The hyperproliferation of vascular smooth muscle cells is a critical process underlying the development of atherosclerosis and the narrowing of arteries that frequently occurs after balloon angioplasty and heart transplantation. Adhesion molecules are often up-regulated in the diseased arteries, implicating an inflammatory process. A number of groups have been active in using antisense oligonucleotides to prevent restenosis injury, coronary graft arteriopathy, and atherosclerosis. A group from Japan focused its efforts on some of the cell-cycle-regulatory genes. In one set of experiments, they found that antisense specific for cell division cycle (cdc) 2 kinase could be used to inhibit the expression of ICAM-1 and VCAM-1 adhesion molecules in transplanted heart allografts (206). They used the hemagglutinating virus of Japan (HVJ) liposome method to deliver antisense oligonucleotides to infused hearts prior to transplantation.

In another series of experiments, cyclin-dependent kinase 2 (cdk2) was targeted (207). Oligonucleotides were again infused into mouse hearts prior to allotransplantation, and delivered via the HVJ liposome method. Thirty days after transplantation, cdk2 antisense caused a dramatic decrease in neointimal formation in the allografts, accompanied by reduced VCAM-1 expression. These effects were not seen in control oligonucleotide-treated allografts. Targeting cell cycle genes by antisense therapy thus seems to be an effective strategy in reducing both vascular smooth muscle cell proliferation and adhesion molecule up-regulation seen in cardiac graft arteriopathy. (For a more extensive review of antisense applications in vascular system diseases, see Ref. 208.)

2. Chemokines and Chemokine Receptors

Chemokines are small-molecular-weight chemoattractant substances that direct leukocyte trafficking and function to activate lymphocytes, inhibit human immunodeficiency virus-1 (HIV-1) infection, and promote the proinflammatory phenotype of macrophages, vascular smooth muscle cells, and endothelial cells

(209–211). In addition, these molecules also influence angiogenesis, collagen production, and hematopoietic cell precursor proliferation.

The chemokines fall into four families based on the presence of a cysteine amino acid motif near their amino terminus: the α-chemokines, with a configuration C-X-C, the β-chemokines, with a C-C configuration, and more recently, members with only a single cysteine or a C-X-3C configuration (211). Chemokine receptors are of the seven-transmembrane spanning type and are coupled to trimeric guanine-nucleotide-binding (G) proteins (212–214). Unique chemokine receptor expression profiles have been documented on different types of leukocytes and individual receptor types have been shown to bind to more than one chemokine of the same or even different families, indicating that redundancy is a common feature of the system. With regard to HIV-1 resistance, CXC receptor-4 (CXCR4) and CC receptor-5 (CCR5) and others have been shown to act as coreceptors for HIV-1 entry into T cells and macrophages, respectively (215–217).

Monocyte Chemotactic Protein (MCP)-1. The application of antisense technology to chemokine-mediated processes has focused chiefly on monocyte chemotactic protein-1 (MCP-1) and interleukin-8 (IL-8), two of the earliest described members of the family. MCP-1, a CC chemokine, has been shown to regulate chemotaxis of monocytes, T lymphocytes, and basophils (218). In an in vivo model of traumatic brain injury in rats, MCP-1 expression has been linked to macrophage infiltration and inflammation of the CNS. Administration of MCP-1 antisense but not sense oligodeoxynucleotides for 3 days via an implanted Alzet miniosmotic pump prior to traumatic brain injury inhibited the induction of gliosis, as measured by glial fibrillary acidic protein immunostaining, and reduced the number of macrophages that were able to cross the damaged blood-brain barrier (219). In addition, a role for MCP-1 in recruitment of proinflammatory monocytes to atherosclerotic plaques was suggested by studies showing that MCP-1 antisense oligonucleotides or neutralizing antibodies inhibited monocyte chemotactic activity produced by cultured rat aortic vascular smooth muscle cells treated with platelet-derived growth factor (PDGF), using a Boyden chamber assay (220). These investigators showed that two separate 18-mer antisense sequences devoid of CpG motifs produced similar effects and that antisense treatment resulted in decreased levels of PDGF-induced MCP-1 mRNA. Published data also claim that MCP-1 can directly modulate lymphocyte activation, as MCP-1 antisense oligonucleotide treatment of either lung fibroblasts or splenic macrophages both selectively inhibits MCP-1 synthesis by these cells and enhances $CD4^+$ T-cell proliferation (221). However, in this study, the antisense 29-mer sequence contains a near-canonical CpG motif whereas the sense oligonucleotide does not.

Macrophage Inflammatory Protein (MIP)-1α, β. The related CC chemokines, MIP-1α and β, are spontaneously expressed by adult $CD4^+$ T-cell leukemia cells (ATL). Antisense oligonucleotides directed at MIP-1α and β have been

shown to reduce ATL adhesion to endothelial cells and inhibit expression of the spontaneously activated form of LFA-1, events that are hypothesized to control pathologic ATL extravasation into tissues (222). Inhibition of either MIP-1α or MIP-1β with these antisense 15 mers was found to result in decreased ICAM-1-dependent adhesion to endothelial cells. The investigators directly measured transendothelial migration using microchemotaxis chambers precoated with activated endothelial cells and found that either MIP-1α or MIP-1β antisense pretreatment of ATL cells blocked their spontaneous transmigration across the endothelial layer. Thus, these activities appear to be mediated by endogenous production of these chemokines by the leukemic cells. However, in this study, it is not clear whether the observed effects are due to an antisense mechanism, since target mRNA or protein reduction following antisense treatment was not shown and the antisense but not the sense sequences did contain noncanonical CpG motifs.

IL-8. Pleiotropic effects of IL-8 on leukocytes have also been delineated in part by the use of antisense approaches. An IL-8 antisense oligonucleotide was found to specifically block the production of monocyte-derived angiogenic activity (223). Hypoxia-induced IL-8 production by epithelial cells was blocked by treatment with IL-8 antisense and this resulted in decreased adherence of polymorphonuclear cells (PMNs), suggesting that IL-8 acts as a chemotactic signal for PMNs to aggregate at the basal surface of epithelia (224). Furthermore, an antisense but not a scrambled IL-8 antisense oligonucleotide was found to inhibit endometrial stromal cell proliferation in vitro and this effect was reversed by addition of recombinant IL-8 (225).

IFN-γ-Inducible Protein (IP)-10/crg-2. Antisense oligonucleotides targeting the chemokine crg-2 (more recently named interferon-γ-inducible protein, or IP-10) were evaluated in a rodent model of experimental autoimmune encephalomyelitis (EAE) (226). Diester backbone and phosphorothioate backbone oligonucleotides targeting the same region of IP-10 mRNA were compared, and the oligonucleotides were injected into the CNS by osmotic minipump infusion into the lumbar subarachnoid space.

The phosphorothioate, but not the diester backbone, oligonucleotides proved to be nonspecifically toxic to CNS tissue. Although rats treated with phosphorothioate oligonucleotides developed paralytic symptoms typical of EAE, the paralysis occurred in rats given both IP-10 antisense oligonucleotides and control oligonucleotides. Moreover, when rats were not immunized to develop EAE but were given the IP-10 phosphorothioate antisense oligonucleotide, they developed paralysis as well. A histological examination of rat spinal cords showed that the phosphorothioate oligonucleotides were causing an atypical necrosis of CNS parenchymal tissue instead of the expected inflammatory infiltration of mononuclear cells, with the necrosis presumably causing the paralytic symptoms. Inter-

estingly, the IP-10 phosphorothioate antisense did cause a specific reduction in target mRNA in spinal cord when compared to a scrambled control sequence.

The diester backbone oligonucleotides used by Wojcik et al. did not cause CNS tissue necrosis, and the antisense sequence produced a statistically significant reduction in the disease activity (i.e., a reduction in paralytic symptoms) compared to the control oligonucleotide-dosed group. Although the diester IP-10 antisense failed to reduce IP-10 mRNA levels when sampling was conducted at the height of clinical disease, a significant decrease in target mRNA was achieved when IP-10 levels were measured earlier in the disease course. Although it is somewhat surprising that a diester backbone oligonucleotide would have sufficient potency to produce the effects that it did in these experiments, little is known about nuclease activity in cerebrospinal fluid.

This was one of the first studies using antisense oligonucleotides to treat EAE, and the results obtained suggest that some complex interactions may be occurring between the locally administered phosphorothioate oligonucleotides and the inflamed CNS tissue. With respect to the toxicity observed with the intrathecally administered phosphorothioates in this model, it is worth noting that other groups have directly injected phosphorothioate oligonucleotides into the CNS without observing any local tissue necrosis (e.g., Ref. 147). Our group, moreover, has had extensive experience using systemically administered phosphorothioate oligonucleotides in mouse models of EAE without any such toxicity. The toxicity seen by Wojcik et al. may therefore be related to interactions between specific factors induced during the EAE model and the intrathecal injection of phosphorothioates. Alternatively, but less likely, is the possibility that heightened IP-10 target reduction with the phosphorothioate oligonucleotides could result in CNS toxicity and tissue destruction.

Although somewhat limited in scope, together these studies support the multifunctional nature of the chemokine molecules in experimental model systems, and indicate the potential for therapeutic benefit through pharmacological intervention in this pathway. A challenge for the future development of antisense or other drugs targeted to chemokines or chemokine receptors will be to further understand the functional redundancy of the chemokine system in inflammation.

III. FUTURE DIRECTIONS

A. Application of Antisense Oligonucleotides to Inflammatory Diseases—Novel Strategies for the Use of Antisense Oligonucleotides

Antisense oligonucleotides are recognized as valuable tools for delineation of the roles of individual genes in normal and abnormal cellular processes. However, improvements in the understanding of antisense pharmacokinetics, distribution,

and medicinal chemistry have opened the door to greater clinical application in a wide spectrum of diseases and disorders. The increased stability and potency of second- and third-generation antisense molecules compared to first-generation phosphorothioates, coupled with enhanced knowledge of transfection conditions and in vivo disposition of oligonucleotides, have enabled the more robust use of antisense technology for both gene validation and human therapeutics.

In addition to the recent implementation of antisense strategies for target validation (227) and gene functionalization, the ability of antisense technology to specifically target distinct isoforms of gene families critical for immune signaling has also begun to be explored. Whereas it is often challenging or indeed impossible to design small-molecule drugs that can discriminate between different related proteins from a gene family, mRNA sequences of related genes are readily divergent enough to allow specific antisense molecules to be engineered. Further, antisense strategies in addition to inhibition of gene expression have been recently developed that have therapeutic promise for inflammatory diseases, such as the use of antisense oligonucleotides to modulate pre-mRNA splicing. In an example of this approach with direct relevance for inflammatory disease, antisense targeted to a region near a splice site unique to the membrane-bound isoform of the murine IL-5 receptor α-chain was found to redirect splicing events, resulting in selective inhibition of the membrane but not soluble forms of the IL-5 receptor (228). This strategy represents a novel approach for blocking IL-5-mediated inflammatory responses.

The advent of molecular biological techniques has resulted in the delineation of multiple cell signaling pathways in immune cells that influence inflammation. With this information has come the appreciation of common components of signaling pathways that propagate cross-talk following cell activation through specific receptors. One of the unique benefits of current antisense technology is its application in dissecting the relative importance of signaling components at the molecular level in a controlled fashion within a particular pathway. Coupling antisense gene inhibition with multifaceted gene expression profiling, as can be performed using arrays, allows the rapid procurement of both pharmacological and toxicological information relevant for both drug discovery and development. Furthermore, an antisense drug development approach provides an additional powerful advantage—the antisense molecule serves not only as a research discovery tool but also as a development compound, obviating the need for the time-consuming process of traditional drug screening commonly used for small-molecule drugs. The implementation of these strategies holds great promise for accelerating the development of novel therapeutic compounds to treat inflammatory diseases.

B. Antisense Oligonucleotide Delivery and Distribution

Although phosphorothioate oligonucleotides are widely distributed to tissues following systemic injection, they do not go everywhere, nor do they enter all tissues

in equal amounts (229,230). Phosphorothioate oligonucleotides bind rapidly to serum proteins, but spend little time in the serum ($t_{1/2}$ 30–60 min) before being taken up by tissues. Following systemic injection, the majority winds up in kidney and liver, but substantial amounts of oligonucleotide can also be found in bone marrow, skin, spleen, lymph nodes, intestine, and skeletal muscle. They do not get into the brain. There is also variable uptake of oligonucleotides by individual cells within a tissue (231). The successful application of antisense therapy to inflammatory diseases requires a serious consideration of the ability of the oligonucleotide to reach the cells and tissues being targeted. For certain conditions, tissue-specific formulations or drug delivery systems may be required to achieve pharmacological effects.

Because oligonucleotides fail to cross the intact blood-brain barrier, many investigators have used direct injection techniques to get antisense oligonucleotides into central nervous system tissues. This has included intrathecal (226), intraventricular (147), intrathalamic (232), and intracerebral (233) administration. While these methods are useful experimentally, they are likely to prove less than palatable in the clinical setting. There is some reason to believe that these draconian delivery techniques may not be necessary for certain inflammatory diseases of the central nervous system. In multiple sclerosis, for instance, the blood-brain barrier (which normally precludes oligonucleotide entry into the CNS) is severely disrupted. In our own laboratory, using a mouse model of multiple sclerosis (EAE), we have been able to detect oligonucleotide within CNS parenchyma following systemic delivery. The antisense oligonucleotide was seen in endothelial cells in spinal cord white matter, and in microglial cells (M. Butler, ISIS Pharmaceuticals, unpublished observations). However, for a disease like multiple sclerosis, oligonucleotide localization within the CNS may not even be necessary. It is quite possible that systemic delivery of oligonucleotide to certain targets expressed in peripheral lymphoid tissues (e.g., lymph nodes or spleen) could provide a pharmacologically beneficial effect. This assumes, of course, that cells from these tissues traffic back into the CNS to exacerbate the disease underway there.

Liposomes have been used to increase the circulation half-life of oligonucleotides and improve their delivery into tissues such as skin (197). Coating the liposomes with viral fusion proteins is a variation on this approach (234). Formulations may also be used to change oligonucleotide uptake in organs like the skin. Pressurized systems have been used in ex vivo settings to introduce unformulated oligonucleotides into cardiovascular tissues (195). Aerosolized oligonucleotide delivery is an approach that may prove useful in the treatment of diseases such as asthma. Some of these alternative delivery systems will be discussed in greater detail in another chapter.

Finally, the application of antisense technology to the treatment of inflammatory diseases demands particular care in the selection and screening of nucleotide sequences that will be incorporated into the antisense drugs. It is now known

that unmethylated CpG dinucleotidees can produce an immunostimulatory response characterized by lymphoid hyperplasia, splenomegaly, mononuclear cell infiltration into several tissues, and a propensity toward a Th1 type of cytokine response (11). Oligonucleotide-induced immune stimulation is much more pronounced in mouse systems than it is in humans (235). Failure to control for CpG effects could hamper the identification of oligonucleotides that have a true antisense effect on different inflammatory targets. However, this phenomenon can be successfully controlled by chemical modification of oligonucleotides, such as methylation of cytosine residues (236,237).

Antisense as a drug development platform for inflammatory diseases is a novel approach. One anti-inflammatory antisense drug (ISIS 2302, specific for ICAM-1) is currently being evaluated in several clinical trials, including Crohn's disease, inflammatory bowel disease, renal allograft rejection, and psoriasis. Other anti-inflammatory antisense drugs are likely to enter the drug pipeline in the near future. Advances in the field will be greatly facilitated by the genomic revolution.

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Respirable Antisense Oligonucleotides (RASONs)

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I. INTRODUCTION

Respirable antisense oligonucleotides (RASONs) represent a novel class of therapeutics for the treatment of respiratory diseases (1,2). They represent a subset of the wider class of antisense oligonucleotides undergoing clinical, and now commercial, development (3–7). RASONs are effective at delivered doses that are generally reduced as compared to other routes of administration, e.g., the intravenous route, and represent a relatively noninvasive delivery method. The low-dose effectiveness of RASONs may relate to their interaction with pulmonary surfactant, a lipid-containing sol with detergent-like properties that lines the pulmonary alveoli. The physicochemical properties of RASONs permit aerosolization by a number of delivery technologies, from nebulizers to dry-powder inhalers. Preliminary inhalation toxicology data in primates, obtained using an aqueous formulation and a Pari Star nebulizer, indicate that RASONs can be delivered effectively and safely to the deep lung.

II. THE POTENTIAL ROLE OF PULMONARY SURFACTANT IN RASON UPTAKE AND DELIVERY

RASONs delivered to the respiratory tract as aerosols may mitigate the problem of intracellular distribution by an interesting mechanism: interaction with pulmonary surfactant. The unique distribution of surfactant, and the potential for surfac-

tant to enhance the uptake and delivery of RASONs throughout the alveoli, may identify the lung as an excellent target for antisense therapeutics.

The synthesis of surfactant takes place in the alveolar epithelial type II cells, which represent approximately 10% of the population of cells in the lung parenchyma. Surfactant is 90% composed of zwitterionic lipids such as dipalmitoyl phosphatidylcholine, which, at lung pH, have cationic properties. Cationic lipids are well known to enhance the uptake and delivery of oligonucleotides into cells (8,9). Furthermore, surfactant has an extremely rapid rate of recycling between the lung surface/air interface and the type II pneumocytes. In the adult lung, approximately 50% of the surfactant phosphatidylcholine is degraded by macrophages and type II cells via lysosomal pathways (10). The other 50% is recycled back into lamellar bodies for resecretion (11). RASONs delivered via inhalation may therefore have their uptake and distribution enhanced by interaction with surfactant. While these arguments are offered as a potential explanation for the dramatically lower doses of RASON shown to be efficacious in vivo (as compared to i.v. administration), actual proof of this mechanism remains elusive. Traditional experimental designs in which one would assess the effects of surfactant removal and its reconstitution upon RASON uptake alter the in vivo system too drastically to reliably validate or invalidate the hypothesis.

III. TARGET RESPIRATORY DISEASES

Respiratory disease accounts for a significant percentage of domestic health care spending in developed countries. In the United States, for example, asthma, one of the more prominent respiratory diseases, affects up to 20 million Americans and accounts for greater than 1% of all U.S. health care costs. Inflammatory conditions of the lung represent particularly interesting targets for RASON therapeutics, and EpiGenesis has devoted the bulk of its efforts in this area. Table 1 shows a partial listing of respiratory diseases representing unmet medical needs that could be favorably impacted by the development of new drugs, and for which EpiGenesis is developing RASON-based therapeutics.

IV. DELIVERY DEVICES

EpiGenesis has had considerable experience administering RASONs via nebulizer, and has employed a variety of different nebulizer configurations in its nonclinical studies, including devices manufactured by MedicAid (HaloLite), Pari (Pari LC Star, PARI LC+), and DeVilbliss (Model 646). Ready solubility in aqueous solutions over a wide range of concentrations has made the jet nebulizer a convenient device for nonclinical studies employing RASONs. We have em-

Table 1 Respiratory Diseases Against Which EpiGenesis Is Developing RASON-Based Therapeutics

Adult respiratory distress syndrome
Infant respiratory distress syndrome
Asthma
Influenza virus infection
Respiratory syncytial virus infection
Chronic bronchitis
Bronchiolitis
Hypersensitivity pneumonitis
Chronic obstructive pulmonary disease
Pulmonary fibrosis
Pulmonary hypertension
Pneumonia
Lung cancer

ployed concentrations of phosphorothioate RASONs up to 50 mg/mL before experiencing delivery difficulties with nebulizer devices.

Using typical air-driven nebulizers (e.g., the Pari Star), RASON aerosols have been shown by autoradiographic analysis to distribute quite evenly throughout the lung, reaching primary, secondary, and tertiary bronchi in a more or less uniform fashion. Commercial applications of RASON technology may utilize nebulizer, dry-powder inhaler, or metered-dose-inhaler configurations, depending on a variety of factors, including quantity of drug required for efficacy, duration of effect of the applied RASON, patient compliance, and other considerations.

V. EPI 2010

EPI 2010 (Durason) is a 20-mer phosphorothioate RASON that targets the adenosine A1 receptor and for which clinical trials have begun as an asthma preventive. The adenosine A1 receptor is an underrecognized but fundamental mediator of asthma, and offers a number of features that make it an attractive target for RASON therapeutic intervention. For example, the adenosine A1 receptor is not expressed in normal lung, but is expressed in asthmatic lung. It apparently becomes discordantly expressed early in the pathogenesis of asthma, and is involved in all three of the major sequelae of asthma: bronchoconstriction, inflammation, and surfactant depletion. Inasmuch as adenosine, the activating ligand for this receptor, is also significantly up-regulated in the lungs of asthmatics, the A1 receptor may undergo a more or less constitutive activation in the asthmatic state.

In addition to these pathophysiological features that make the adenosine A1 receptor an attractive target for RASON intervention in asthma, other molecular pharmacology considerations may combine to increase its attractiveness for such intervention. For example, the half-life of the adenosine A1 receptor protein is approximately 6 h, such that one might expect to be able to effect diminution of receptor function fairly rapidly following cessation of messenger RNA function for this protein. Furthermore, once the up-regulated levels of this receptor are returned to normal via EPI 2010 intervention, there is a relatively long duration of effect averaging 7 days. This may permit once-per-week dosing, which would represent a significant advantage over currently available asthma drugs. Low-to-moderate levels of expressed message and active, membrane-embedded protein may also contribute to the effectiveness of the adenosine A1 receptor as a target for RASON intervention.

VI. BIODISTRIBUTION

Using EPI 2010 as an example, RASONs provide an interesting pattern of distribution, and one that may present particular advantages for their therapeutic application. We have shown that [^{35}S]-labeled EPI-2010 administered by nebulizer (for example, an air-driven Aerogen AeroNeb) distributes widely throughout the lung of experimental animals. Furthermore, little bioactive EPI 2010 escapes the lung when doses are kept moderately low (e.g., less than 10 mg/kg), although there may be some threshold lung dose above which RASON is distributed to the systemic circulation with higher efficiency. The half-life of [^{35}S] label in the lung following nebulization of [^{35}]-labeled EPI-2010 in rabbits is about 30 h, and radioactivity is rapidly cleared via the urine thereafter, with very little accumulation ($\leq$1%) in the liver and kidneys. After inhalation of much higher doses (40–400 mg/kg) of nonlabeled EPI 2010 to nonhuman primates, blood levels of intact RASON are detectable in most, but not all, animals. The lung thus acts as a reservoir from which only limited amounts of intact EPI 2010 appear to escape. Extraction of RASON from tissues, followed by postlabeling and gel electrophoresis, indicate that extensive nuclease degradation occurs within the lung, potentially accounting for the lack of systemic effects observed (see below).

VII. TOXICOLOGY

One expected toxicology of phosphorothioates involves the induction of hypotension, particularly in primates, when higher doses are employed. This induction of hypotension is thought to be caused by activation of complement. With i.v. administration of this class of compounds as a rapid push, threshold levels neces-

sary for the activation of complement may be exceeded prior to reaching an efficacious dose unless the infusion rate is prolonged (e.g., 1–2 h). Inhalation offers a quite different pharmacodynamic pattern, with blood levels of RASONs at their peak 2–4 h following inhalation. EpiGenesis' data on the inhalation toxicology of RASONs, to be described in detail elsewhere, indicates that inhaled doses up to the maximum feasible were not associated with the cardiovascular effects generally associated with this class of compounds. RASONs deposited in the lung following aerosolization appear to leach from this environment at a relatively slow rate, apparently avoiding the activation of complement, as judged by the lack of hypotension induced. Furthermore, RASONs do not appear to alter cardiovascular parameters, which might be expected from systemic attenuation of the adenosine A1 receptor, as judged by Cardiomax-2 monitoring of heart rate, mean arterial pressure, cardiac output, stroke volume, total peripheral resistance, or contractility (data to be reported elsewhere). Similarly, baroreceptor reflex function, as judged by i.v. administration of nitroprusside or phenylephrine, remains unaffected by inhalation of efficacious doses of the RASON EPI-2010.

A. *des*Adenosine RASONs

A cardinal feature of hyperreactive airways (e.g., occurring in asthma and certain other respiratory diseases) is their hypersensitivity to adenosine. Recently, adenosine has been approved for the treatment of supraventricular tachycardia, and as a pharmacological stressor used in assessment of cardiovascular function. Coinciding with this therapeutic use of adenosine, numerous incidents of life-threatening bronchoconstriction have occurred in asthma patients who were administered adenosine for therapeutic purposes. Consequently, adenosine is now considered to be contraindicated in patients with hyperresponsive airways.

Most RASONs undergo degradation at a rate consistent with the nuclease resistance of their chemistries. Phosphorothioate RASONs appear to be virtually totally degraded within the lung parenchyma, at least at moderate inhaled dosages, while other chemistries may provide sufficient nuclease resistance to permit undegraded RASON to be systemically distributed. Applications of RASONs in which the target exists systemically and not within the lung may utilize chemistries providing maximized resistance to nuclease degradation, while RASONs targeting receptor mRNAs that exist within the lung may fruitfully employ phosphorothioate or other similar, less resistant chemistries.

When RASONs target lung receptor mRNAs and less resistant chemistries are utilized to prevent bioactive amounts of RASON from leaving the lung, the adenosine content of such molecules may become a critical issue. Release of adenosine from phosphorothioate and similarly less nuclease-resistant RASONs during degradation can stimulate bronchoconstriction in hyperreactive airways,

and could be problematic for the development of RASON therapeutics against diseases such as asthma.

To counteract this problem, EpiGenesis has developed the *des*Adenosine RASON approach for use in the treatment of diseases characterized by hyperreactive airways. This technology utilizes RASONs designed to disinclude adenosine from their structures. The lack of adenosine in *des*Adenosine RASONs prevents adenosine-mediated bronchoconstriction from occurring as a consequence of RASON degradation.

VIII. CONCLUSIONS

RASONs represent a new and potentially very useful tool in the treatment of a wide array of medically important respiratory diseases. The respirable route may overcome some of the difficulties encountered in the therapeutic application of antisense oligonucleotides. In this regard, the lung may represent a unique target for antisense administration because it is lined with surfactant that may, by virtue of its cationic lipid properties (and/or its specific protein constituents), enhance oligonucleotide uptake and delivery. EPI-2010 is a RASON targeting the adenosine A1 receptor and indicated for the prophylaxis of asthma. Its durable effect, averaging 7 days, gives it the potential to be a safe, effective, once-per-week treatment for this disease. RASONs offer a novel therapeutic modality with the potential to improve the treatment of a wide array of respiratory diseases.

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Combined Antisense Therapy and Chemotherapy in Animal Models

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I. INTRODUCTION

There are over a dozen antisense preparations in human clinical trials for the potential treatment of cancer or cancer-causing viruses (1,2) and one FDA-approved antisense preparation for the intraocular treatment of cytomegalic virus (CMV) retinitis on the market, Vitrovene (Fomivirsen), of the Isis/Ciba Vision collaboration (1). These examples suggest that rational and specific "targeting" may supplement empirical anticancer drug searches. Several additional advantages of the antisense approach are apparent. It may be possible to select targets that complement the inhibitory effects of other currently accepted anticancer drugs such as the antimetabolites. Such targeting may completely eliminate an essential function of a cancer cell that cannot be achieved by either treatment alone thereby achieving a supra-additive or synergistic cell-killing result. In addition, the combined use of antisense and an accepted anticancer drug may allow these anticancer drugs to be used at lower concentrations, thereby reducing their unwanted effects on normal proliferating populations such as hair follicles, intestinal epithelial cells, and hematopoetic cells. This in turn may lead to greater compliance, prolonged treatment regimens, and generally more flexible and efficacious clinical protocols. Here we briefly review the combined use of antisense and chemotherapy agents and summarize experience in vivo in the use of antisense complementary to N-terminal Jun kinase in combination with cisplatin (*cis*-diamine-dichloro platinum, CDDP). A review of combination antisense oligonucleotide and low-dose chemotherapy of hematological conditions has appeared

recently (4). An important review and guide to the NIH database with information on the mechanism of action of over 70,000 compounds, including cluster analyses of the responses of 60 NIH test cell lines, has been provided (5,6).

II. ANTISENSE c-myc

The first extensive studies of the combined use of antisense oligodexoynucleotides (ODNs) and chemotherapeutic agents examined phosphorothioate antisense c-myc ODNs in combination with cisplatin (*cis*-diaminedichloroplatinum, CDDP) (7–9). T24 bladder carcinoma cells exhibit high steady-state levels of c-myc in vitro and are resistant to chemotherapy (8). Treatment with antisense oligonucleotides (8 h at 10 μM oligonucleotide) but not sense oligonucleotides nearly eliminated c-myc mRNA expression (8), providing one criterion that an antisense mechanism was functioning. Treatment of T24 cells with antisense c-myc in combination with mitomycin C, Adriamycin, or 5-flourouracil (for a guide to chemotherapeutic agents, see Ref. 10) did not overcome the resistance of these cells to these chemotherapeutic agents. However, the combination of antisense c-myc and cisplatin did lead to marked inhibition of growth as judged by an MTT assay, which is a tetrazolium dye reduction assay proportional, predominantly, to mitochondrial lactate dehydrogenase (10,11). Thus, MTT results are proportional to viable cell mass resulting from the sum of cytostatic and cytocidal effects on proliferation.

The supra-additive nature of the results was judged by the isobologram criterion (12,13,15). Isobolograms, or "identical effect graphs," are constructed by plotting the concentration of one component of a mixture necessary to achieve a fractional effect such as an ED_{50} against the concentration of the second component of the mixture. Thus, the intercepts are just the ED_{50} concentrations of each component alone and the "diagonal" line connecting these points indicates the results expected for "simple addition" of two components that inhibit independent mechanisms (e.g., mutually exclusive components) (15). Observations falling significantly "below" the line indicate less of one or both components of an applied mixture achieve the ED_{50} result, which may come about, for example, by blocking interacting mechanisms (mutually nonexclusive components). These results are termed "supra-additive," "potentiated," or "synergistic." Conversely, observations above the diagonal line indicate "antagonism." A succinct review of methods and applications has been proved by Gessner (15).

Thus, the combination of antisense c-myc ODNs with cisplatin proved to be synergistic when applied to cisplatin-resistant T24 cells as well as when applied to freshly isolated cells from the urinary bladder tumors of two patients (8). Antisense c-myc treatment led to twice the intracellular accumulation of cisplatin compared to the sense control oligonucleotide treatment, suggesting the basis of

antisense c-myc–stimulated synergism may be related to intracellular accumulation specific for cisplatin (nonmutually exclusive components). By present standards, these studies used high concentrations of oligonucleotides associated with additional affects in vitro (16) and would be impractical in vivo (17).

Increased generality for these observations comes from the studies of primary melanoma tumor cells and the corresponding primary cultures of cells isolated from cisplatin-resistant lymph node metastases derived from the same patient (18). Xenografts of the metastatic cells were insensitive to treatment with cisplatin whereas the xenografts of the pimary tumor cells were reduced in weight by 70% upon cisplatin treatment. However, in the case of the metastatic cells, combined antisense c-myc ODN and cisplatin treatment (3.3 mg/kg/day cisplatin i.p., days 8–10; 0.5–1.0 mg ODN i.v. days 9–16) led to increased apoptosis and 65% inhibition of tumor weight compared to controls. It was concluded that the studies indicate a potential role of c-myc in promoting resistance to cisplatin in melanoma and demonstrate a potential treatment method (18).

III. ANTISENSE c-myb

The c-myb transcription factor is elevated in a variety of hematopoetic malignancies as well as certain colon carcinomas (19). Phosphorothioate antisense c-myb ODNs (10 μM, 3 days), but not the sense control oligonucleotides, suppress steady-state message in LoVo Dx colon carcinoma cells in vitro (14). Both antisense c-myb and cisplatin treatments impede the growth of LoVo Dx cells in culture and combined treatment produces additive but not synergistic inhibition of growth by the isobologram criterion. In vivo administration of antisense c-myb, but not sense ODNs, to tumor-bearing mice (10 mg/kg daily i.v.) led to 39% decreased tumor weight after 14 days, which is very similar to the 41% inhibition observed for treatment with cisplatin alone (3.3 mg/kg t.i.d. for 3 days). However, as for the in vitro results, combined in vivo therapy resulted in a 62% inhibition and synergism was not observed. Nieborowska-Skorska et al. (20) reported highly effective cell killing of BV173 Philadelphia chromosome positive cells in vitro by combined antisense c-myb and antisense complementary to the breakpoint of the bcr/abl transcript (see also CML, below).

IV. ANTISENSE PROTEIN KINASE A TYPE I

Protein kinase A type I (PKA-I) appears to cooperate in growth promotion of several tumors (21). Antisense complementary to the regulatory subunit of PKA-I, RIα, blocks tumor cell growth in vitro and in vivo. Tortora et al. (21) investigated cooperativity by employing low (0.1 μM) and noninhibitory doses of anti-

sense RIα ODNs in combination with a variety of chemotherapeutic agents. Synergistic in vitro growth inhibition, defined as inhibition greater than the sum of individual effects, and apoptosis were seen when assessed 12 days following treatment with antisense RIα ODNs (0.1 μM, 12 h) but not following treatment with mismatched control ODNs in combination with cisplatin, other platinum-based compounds, topoisomerase II–selective inhibitory drugs, or taxanes. In contrast, synergism was not observed with 5-fluorouracil, methotrexate, vincristine, or camptothecin, indicating a degree of specificity. Based on these results, an in vivo trial using GEO colon carcinoma cell xenografts in athymic mice was carried out. Established subcutaneous tumors of approximately 0.4 cm^3 were treated with paclitaxel (20 mg/kg, i.p. once, the maximum tolerated dose in mice) alone or followed by antisense or control oligonucleotides (10 mg/kg, i.p., days 2–6) and the results were followed for 60 days. Tumor growth inhibition was apparent in all the expected cases. Tumor growth was characterized by approximately linear growth curves with a single slope over the 67-day observation period even though treatment ended on day 6.

The lower slope of the growth curve for the combined antisense RIα ODN-paclitaxel treatment group was less than the slopes observed following either treatment alone or following the control oligonucleotide-paclitaxel treatment. Growth was significantly inhibited for the combined antisense RIα ODN-paclitaxel treatment case compared to all other cases. A greater-than-additive effect, however, was not quantitatively evaluated nor was the effect of treatment on gene product levels. While synergism was not demonstrated, additive effects of these low-dose and short-interval treatment regimens on tumor growth were apparent. The apparent prolonged effect of regimen on tumor growth rate following cessation of treatment is of interest.

Paclitaxel and cisplatin had similar effects on cell growth in vitro (21). The growth inhibitory effects of these two compounds also have been examined in combination with a nonantisense inhibitor of PKA-I, 8-chloro-cyclic-3′,5′-adenosine monophosphate (8-Cl-cAMP) (22,23). 8-Cl-cAMP is considered relatively selective for inhibition of PKA-I and blocks the growth of a wide variety of cancer cell types in vitro and in vivo (21–23). Indeed, both paclitaxel and cisplatin when combined with 8-Cl-cAMP exhibited synergistic inhibition of cell growth in vitro (15). Using the GEO colon carcinoma xenograft model, sequential treatment with 8-Cl-cAMP (1 mg/dose biweekly for 2 weeks) and paclitaxel (0.4 mg/dose/cycle) for three cycles was compared to treatment with either regimen alone. The separate regimens led to approximately 25% tumor growth reduction compared to vehicle each after 54 days whereas the combined treatment led to 59% tumor growth reduction, which was judged to be significant compared to either drug alone. Whether the result represented an effect significantly greater than the sum of the separate treatments was not addressed. The results, therefore,

are similar to those of previous studies (19) showing additivity of the treatment of established xenografts with antisense RIα ODN of PKA-I and paclitaxel.

A three-agent study of 8-Cl-cAMP, antisense RNA complementary to the epidermal growth factor receptor ligand, CRIPTO, and a monoclonal antibody that blocks activation of the EGF receptor, C225, has been applied to GEO human colon carcinoma cells (24). The antibody was originally developed by Mendelshon and co-workers and effectively inhibits activation of the EGF receptor (25). C225 alone is in extensive phase II clinical trials sponsored by ImClone Systems Incorporated, including combination of C225 with irradiation for the treatment of head and neck cancers and breast carcinoma. The antisense compound was of the "mixed backbone" formulation of Hybridon, Inc., composed of 25-nucleotide-long ODNs where the first and last thirds of the molecule contain phosphorothioate backbone linkages and bear methoxy groups at the 2′ position (26, see also S. Agrawal, this volume). Treatment of GEO cells with each agent alone (0.2 μM antisense; 0.5 μg/ml C225; 2.5 μM 8-Cl-cAMP; 3 days) or with any combination of two agents was not able to induce apoptosis. In contrast, treatment with all three compounds was associated with a three- to fivefold decrease in growth and an approximately threefold increase in DNA fragmentation. It was concluded that the use of agents directed against different but related pathways efficiently inhibited growth and caused apoptosis (24).

V. CHRONIC LYMPHATIC LEUKEMIA

The chronic phase of human chronic lymphatic leukemia (CML) is a slowly progressive B-cell leukemia that commonly exhibits translocation of chromosomes 9 and 12 leading to a product visible in karyotype preparations, the Philadelphia (Pa) chromosome. The fusion occurs within an area originally designated the breakpoint cluster region (bcr) thereby leading to expression of a 210-kDa fusion product containing the ABL gene product (bcr/abl), a tyrosine kinase of the phosphoinositol-3-kinase family. CML cells are nearly euploid in contrast to the markedly aneuploid distribution found in many solid tumors suggesting less diverse oncogenic mechanisms. Calabretta and associates (27,28) have shown that treatment of CML cells in vitro with a suboptimal dose of 4-hydroxycyclophosphamide (mafosfamide, an in vitro active form of cyclophosphamide) and antisense bcr/abl phosphorothioate ODNs leads to suppression of p210 and to cell killing greater than the sum of that for each treatment alone. The analysis was extended to treatment of SCID mice inoculated with primary human CML cells derived from blast crisis phase (28). Three weeks after inoculation, the mice were treated with a suboptimal dose of cyclophosphamide and/or followed by 12 daily treatments with antisense bcr/abl ODN (0.1 mg). The survival of tumor-

bearing SCID mice was greater than that for the sum of individual treatments consistent with the in vitro results. A mechanistic basis for the synergistic effect was suggested by the observation that antisense mediated suppression of p210 levels in vitro. In addition, it was observed that the amount of antisense oligonucleotide recovered from the bone marrow was increased in the presence of mafosfamide, suggesting that mafosfamide enhanced cellular uptake (28). Another potential mechanistic basis was suggested by the observations that antisense-mediated suppression of the bcr/abl product is associated with decreased Bcl-2, and that the use of mafosfamide is associated with increased p53 and BAX thereby favoring increased apoptosis (28). In addition, these studies utilize high concentrations of antisense for prolonged periods, leaving open the possibility of additional effects. Further complexity is revealed by the recent observation that activation of the N-terminal Jun kinase (JNK) pathway, an alternate MAP kinase pathway that is a common proapoptic pathway in a variety of normal cells, has been observed to be required for transformation by the bcr/abl oncogene (29). Thus, JNK appears to be a rational target in this and other (30–33) solid tumors. The sum of results identifies several potential targets in bcr/abl transformed cells.

Iversen and coworkers examined the effect of antisense bcr/abl ODNs on K562 CML cells in combination with various chemotherapeutic agents (4). Antisense bcr/abl ODN treatment (ca. 20 μM, 34 h) caused complete elimination of target protein. The combination antisense bcr/abl ODNs with 4-hydroxyperoxyphophamide, a DNA alkylating agent that promotes apoptosis, led to supra-additive inhibition of proliferation in vitro. On the other hand, antisense bcr/abl ODNs in combination with idarubicin, a topoisomerase inhibitor, led to strictly additive inhibition of growth. Thus, these contrasting results may indicate that 4-hydroxyperoxyphophamide promotes apoptosis by a mechanism that is partly blocked by the p210 product whereas idarubicin may act through a pathway that is independent of p210 (4). Another potential complication capable of producing either cooperative or antagonistic effects in combined therapy is direct interaction of the chemotherapeutic agent with antisense oligonucleotides. Preliminary studies suggest that mitoxantrone, idarubicin, and doxorubicin can directly interact with an oligonucleotide complementary to p53 mRNA with strong, mild, and very weak affinities, respectively (4). Thus, when HL-60 cells were incubated in the presence of the antisense p53 ODN, there was a protective effect against various concentrations of mitoxantrone. This result is thought to be due to a strong interaction of mitoxantrone with the antisense ODN (4). Similarly, Rao et al. (quoted in Ref. 4) observed an increased uptake of antisense c-myc into K562 cells only in the presence of 4-hydroxyperoxyphosphamide indicating that a possible interaction between the chemotherapeutic agent and the antisense oligonucleotides may have produced a complex that is more readily taken up by K562 cells. Other ODN-drug complexes with unique activities are well known

(34). Whether these interactions may be related to the use of ODNs in the micromolar range was not determined (16).

VI. Bcl-2

The sum of observations indicates that the combination of antisense ODNs with chemotherapeutic agents often may lead to a number of potential interactions other than the desired synergistic cell killing. Stahel and co-workers (35) carried out a systematic study of the effects of antisense Bcl2 ODN in combination with three different chemotherapeutic agents as applied to three different small cell lung cancer (SCLC) lines. Western analyses confirmed that antisense Bcl-2 alone, an unmodified 20-mer phosphorothioate at low concentrations (0.075–0.150 μM for 48 h), specifically reduced target protein. Dose-response curves for the effects of each drug alone and in combination with antisense Bcl-2 on viability of all three cells lines were determined. The presence or absence of synergistic effects was judged by analysis of "combination indices." For this analysis, the data were linearized by replotting as the log form of a generalized progress or overall reaction equation consistent with a receptor-mediated mechanism ($\log(f/1 - f)$ versus log dose, where f is the fractional viability). At the ordinate value of median effect [i.e., where $\log(f/1 - f) = 0$], the ratio of the log of the dose of chemotherapeutic agent in combination with antisense to that of the chemotherapeutic agent alone is taken as the combination index (CI, CI $\sim \log(IC_{50})_{combo}/\log IC_{50})_{chemo}$). Thus, CI < 1 indicates synergism, CI $= 0$ indicates additive mechanisms, and CI > 1 indicates antagonism. CI provides a quantitative comparison of rank-order data. Since the linearized results are amenable to regression analysis, the probabilities of the CI may be determined. Finally, the slopes of the log plots provide information about whether two drugs have different modes of action or act independently (mutually exclusive or mutually nonexclusive).

Based on combination indices that were significantly less than 1, synergistic decreases in viability were observed for antisense Bcl-2 ODNs in combination with etoposide, doxorubicin, or cisplatin for SCLC cell lines SW2 and NCI-H82. Most drugs were used in the nanomolar range and control ODNs consisting of a sense, scrambled, and four-base mismatch ODNs were without affect. The control results rule out, for example, the possibility that the synergistic increase in cell killing could be due to enhanced uptake of the chemotherapeutic agents in the presence of lipid transfection reagents (36). Interestingly, for the cell line with the lowest basal level of Bcl-2, NCI-H69, all three possible combinations of antisense Bcl-2 with the chemotherapeutic agents led to somewhat antagonistic interactions. Thus, these studies utilize basic pharmacodynamic principles (37) to analyze a two-component system. The results provide quantitative criteria for the existence of synergistic cell killing in concentration ranges consistent with in

vivo use and well below the levels associated with nontarget-specific interactions (16). Notably, the results also reveal that important tumor-specific differences occur as exemplified by the antagonistic effects observed only with NCI-H69 cells.

The analysis has not been extended to in vivo studies. However, Gleave et al. (38) applied a 20-mer phosphorothioate ODN complementary to the 20 residues of the translation initiation site of Bcl-2 prepared by Isis Pharmaceuticals, in combination with androgen ablation or taxane in an androgen-dependent tumor model—the Shionogi model. In this model, male syngeneic and immunocompetent mice are inoculated subcutaneously with mouse *mammary* carcinoma cells, which are allowed to develop tumors. Castration causes marked tumor regression, which spontaneously regrows from residual tumor about 35 days after castration. Bcl-2 is up-regulated five-to sevenfold 4–7 days after castration and maintained at a twofold elevated level throughout the androgen-independent growth phase. In vitro, Lipofectin-mediated antisense mouse Bcl-2 treatment of the tumor cells in the nanomolar range leads to reduced protein and up to 94% reduction of steady-state Bcl-2 mRNA in a dose-dependent manner, whereas a two-base mismatch control was without effect. Taxol treatment promotes the phosphorylation and presume inactivation of Bcl-2. The combined treatment at 0.5 μM antisense Bcl-2 significantly decreased viability. In vivo antisense Bcl-2 treatment at 12.5 mg/kg i.p. daily from the time of castration hastens regression and blocks regrowth by over 90%. Thus, the effect of androgen ablation is greatly extended. The combined treatment led to one-twelfth of the regrowth of the antisense-alone treatment group and to one-eighth of the regrowth of the Taxol + mismatch ODN–treated group. Bcl-2 protein and mRNA reduction and Bcl-2 protein phosphorylation within treated tumors was confirmed by Western and Northern analyses. The findings were taken to suggest that antisense Bcl-2 ODNs sensitizes the tumors to taxanes over and above the effects of taxane on the phosphorylation of Bcl-2.

Several clinical trials are underway that are designed to assess the toxicity and efficacy of antisense Bcl-2 in combination with chemotherapeutic agents. Genta Incorporated is carrying out a variety of trials with an ODN phosphorothioate also complementary to the first six codons of the Bcl-2 ORF (G3139) (39,40). These phase I and II trials utilize G3139 in combination with dacarbazine (melanoma), Taxol (prostate carcinoma), Taxotere (prostate, breast, and SCLC), fludarabine (AML), and Rituxan (non-Hodgkin's lymphoma, NHL), an anti-CD20 monoclonal antibody that reduces CD20+ pre-B cells and mature B cells and has proven efficacious against chemotherapy-refractory NHL. Preliminary phase I results for the treatment of NHL with antisense Bcl-2 alone indicate that when the antisense agent is administered to eight cohorts for 14 days by subcutaneous infusion (4.6–195.8 mg/M^2/day), the Bcl-2 protein is reduced in seven of 16 assessable patients (40). The reduction occurred in tumor cells of lymph nodes

of two patients as well as in peripheral blood or bone marrow mononuclear cells in five patients (40). These affects were observed at doses (~37 mg/M^2/day) below those associated with significant side effects (17). Genta notes that it is the first company to have documented reduction of target protein in tumor and other tissues attributable to systemic antisense treatment of patients thereby achieving an important "proof-of-principle." Indeed there are in general few confirmatory measurements of control of target mRNa or protein in in vivo systems. One complete response, two minor responses, nine cases of stable disease, and nine cases of progressive disease were noted (40). These studies are being extended to antisense Bcl-2 in combination with Rituxan. The results for the combined therapies are not known yet. Finally, the efficacies of cisplatin and G3139 have been compared in a SCID mouse model of Merkel cell carcinoma (41). The results for cisplatin treatment alone were similar to those for sense and two-base mismatch control ODN treatments whereas G3139 treatment (10 mg/kg/day continuous ALZET pump infusion for 4 weeks) led to substantially decreased tumor growth or complete remission. Combined cisplatin-G3139 treatment was not described (41).

VII. MDM2

MDM2 is a gene product with an ability to bind the p53 tumor suppressor protein and inhibit its ability to act as a transcription factor thereby limiting the expression of p21CIP^1/WAF^1 and other cell-cycle-regulating gene products. Since DNA damage causes the activation of wild-type p53, antisense-mediated elimination of MDM2 might be expected to promote synergistic results when combined with suitable DNA damage-enhancing agents. Chen et al. (42) developed a 20-mer phosphorothioate oligonucleotide complementary to the coding region of MDM2, which was chosen following a screening ("gene walk") of a number of candidate ODNs. This optimal compound exhibits an IC_{50} of <0.2 μM for the reduction of MDM2 mRNA and protein in JAR cells, which is associated with a significant increase in p21 CIP^1/WAF^1. The combined use of antisense MDM2 ODNs and a topoisomerase I inhibitor that enhances DNA strand breaks led to greater-than-additive activation of p53 and decreased survival of JAR cells. Thus, combined therapy may enhance the effects of DNA damaging agents.

In support of this approach, it has been shown that the stress-activated N-terminal JNK pathway is required for repair of chemotherapy-induced DNA damage (30,43) and that specific inhibition of the phosphorylation of c-Jun, a major JNK substrate, greatly enhances the cell-killing properties of a adenovirus expression vector for wild-type p53 (43). Thus, the antisense MDM2 approach (42), which is limited to tumor retaining wild-type p53 allele, potentially may be extended to tumors deficient in wild-type p53.

VIII. MULTIDRUG RESISTANCE-1 PROTEIN

HL-60/Vinc human leukemia cells are resistant to vincristine and overexpress P-170, which is thought to effectively transport vincristine from the cells (44). Treatment (~18 μM day 0, ~9 μM o.d. days 1–7) with an 18-mer phosphorothioate antisense multidrug resistance-1 protein (MDR1) ODN inhibited P-170 expression and restored sensitivity of the cells to vincristine in vitro whereas both sense and scrambled control ODNs were without significant effect. In vivo reversal of resistance was obtained in SCID mice that had been inoculated with the HL-60/Vinc cells and systemically treated with antisense MDR1 ODNs plus vincristine. Survival was significantly prolonged for the mice that received antisense MDR1 plus vincristine whereas treatment with antisense MDR1 alone or scrambled control ODN with or without vincristine had no effect on survival. These results suggest that the use of antisense MDR1 in combination with standard antineoplastic drugs might be useful in reversing MDR in vitro and in vivo.

IX. PROTEIN KINASE C

Antisense ODNs that suppress expression of most isozymes of protein kinase C-alpha (PKCα) have been prepared and the well-characterized compound of Isis Pharmaceuticals complementary to PKCα (ISIS3521) is currently in phase II trials for application to a variety of solid tumor types (1,38,38a). Early studies utilizing MCF-7 cells resistant to doxorubicin revealed increased sensitivity to the drug upon expression of vector-derived antisense PKCα RNA (39). Vector-derived antisense PKCα RNA that was stably expressed in Moser human colon carcinoma cells led to down-regulation of the target protein and increased sensitivity to several anticancer drugs including mitomycin-C, 5-fluorouracil, and vincristine (45). Similarly, the PKCα inhibitor safingol sensitizes gastric carcinoma cells to mitomycin-C leading to enhanced apoptosis (46). These examples suggest that PKCα is an appropriate target for further analysis of combined treatment antisense PKCα ODN and a variety of chemotherapeutic agents.

X. c-RAF KINASE

As an upstream member of the MAP kinase signal transduction pathway that is commonly activated by growth factors, c-Raf kinase has been investigated as a potential target for treatment of cancer by antisense ODNs. For example, ISIS5132 is a 20-mer phosphorothioate ODN complementary to the 3′ untranslated region of the human c-Raf kinase gene and is currently in phase II clinical trials for treatment of a variety of solid human tumors (47). Moreover, animal

model studies indicate that the Isis Pharmaceuticals sequence may enhance radiotherapy (47–49). Antisense c-Raf Kinase ODN alone greatly retards the growth of established human laryngeal squamous cell (SQ-20) xenografts in athymic mice when administered i.v. as a liposomal formulation (6–10 mg/kg i.v. alternate days $\geq$ 12 days). A somewhat more pronounced effect was obtained by irradiation alone (3.8 Gy daily for 18 days). However the combined treatment promoted tumor *regression* to an average size of less than half the volume of the pretreatment tumors. Western analysis confirmed that c-Raf kinase protein of the tumors also was reduced to less than half (27.7–35.5%) of control doses. Significant side effects of the systemic treatment with or without irradiation were not apparent. The effect of the treatment regimen on the endogenous murine c-Raf kinase protein level was not described. These studies indicate that combined treatment of antisense c-Raf kinase ODN and irradiation produced an effect that was not observed by either treatment alone. Additional antisense c-Raf kinase ODN studies in combination with chemotherapy are ongoing (S. Frier and N. Dean, personal communication).

XI. c-JUN N-TERMINAL KINASE

N-terminal phosphorylation of the transcription factor and protooncogene c-Jun at serines 63 and 73 by c-Jun N-terminal kinase (JNK) (51–53) greatly enhances the transactivation potential of c-Jun (54,55). Roles of JNK in stress responses and apoptosis as well as survival, growth, and transformation have been postulated (56,57). The activation of c-Jun by N-terminal phosphorylation is an essential event for the transformation of primary fibroblasts and other model cell systems by activated oncogenes such as src, Ha-ras, c-raf, and v-sis (52,58–60). There is considerable evidence that JNK may be an important mediator of transformation not only by activated Ras (59,60) but also by activated forms of Cdc42 (61–63), Rac (59,61–63), and C3G (64–66) as well as the oncogenic GTPases Vav and Dbl (67–69), the oncogenic proteins v-Fps/Fes (70), v-Crk (64–66), and the Met oncogene (71).

Although less information is available, there are indications that JNK may play an essential role in certain human cancers (60,29–32,72–76). The bcr/abl leukemia oncogene activates JNK and requires c-Jun for transformation implicating the JNK pathway in transformation by a human leukemia oncogene (29). JNK is constitutively active in a variety of solid human tumor cells (30,31). Moreover, EGF appears to activate JNK in a phosphoinositol 3-kinase-dependent manner (73,74). Similarly, expression of the EGF receptor variant type III, a constitutively active naturally occurring mutation that is found in many types of human tumors, leads to transformation of NIH-3T3 cells, constitutive activation of JNK, and down-regulation of the MAPK/ERK pathway (75). Inhibitors of phosphoi-

nositol-3-kinase reverse these effects (75). In the case of A549 NSCLC cells, EGF causes preferential activation of JNK and a twofold enhancement of four characteristics of transformation: proliferation (31,32), tumor-take rate (32), growth of xenograft (32), and colony formation in soft agar (32). The in vitro effects are completely inhibited by expression of a nonphosphorylatable derivative of c-Jun, c-Jun (S63A, S73A), or by application of specific antisense JNK-2 oligonucleotides but not by antisense JNK-1 oligonucleotides, indicating an essential role of JNK-2 in transformation (31,32). Although commonly implicated in apoptosis, especially in cells of neural and hematopoietic origin (56,57), in solid tumors the mechanisms associated with apoptosis are commonly ineffective (77,78) leaving open the possibility that cellular variants that utilize JNK as a growth-promoting pathway may come to predominate in the tumor cell population.

We carried out an in vivo trial using phosphorothioate antisense ODNs complementary to JNK-1 or JNK-2 selected on the basis of a "gene walk" procedure and characterized in various cells lines including nine prostate carcinomas (79), gliolbastoma (33), and others (31,32). Groups of 10 (control groups, antisense JNK-1, or JNK-2 alone) or 15 animals (all other groups) were inoculated with PC3 human prostate carcinoma cells that were then allowed to develop established tumors of 200 mm^3 on average (Fig. 1). The groups were treated (arrow, Fig. 1) with each antisense JNK ODN alone (total ODN = 25.0 mg/kg, i.p., daily, all antisense treated cases) or with antisense JNK-1 + JNK-2 ODNs or cisplatin alone or combined antisense JNK-1 + JNK-2 ODNs + cisplatin. All treatments led to significantly (ANOVA, all data of a given curve) reduced tumor growth compared to vehicle or scrambled control ODN (Fig. 1). As for the in vitro studies, treatment with antisense JNK-2 ODN was significantly better than antisense JNK-1 alone or cisplatin alone (Fig. 1). Treatment with antisense JNK-2 alone led to 81% inhibition (based on integrated area of growth curves) whereas treatment with antisense JNK-1 ODN alone or with cisplatin alone only caused about 50% inhibition. Combined antisense JNK-1 + JNK-2 ODNs led to nearly 80% inhibition of tumor volume growth over the 22-day treatment course. The marked inhibition of growth following antisense JNK2 or combined antisense treatment provides little room to discriminate a further effect upon addition of cisplatin. However, the complete combination of antisense JNK-1 + JNK-2 ODNs + cisplatin led to 89% inhibition or a further reduction of about half the remaining growth potential of the tumors. The increased inhibition is not significant compared to antisense JNK-1 + JNK-2 ODN treatment and corresponds to the result expected for an additive relationship. Western analysis (data not shown) and direct enzyme activity assays (Fig. 2) of tumor tissue from antisense and scrambled control ODN–treated tumor confirmed a fivefold decrease in enzyme activity in the antisense ODN–treated tumors compared to the scrambled ODN control case. Interestingly, cisplatin, a known activator of JNK (20), is associated

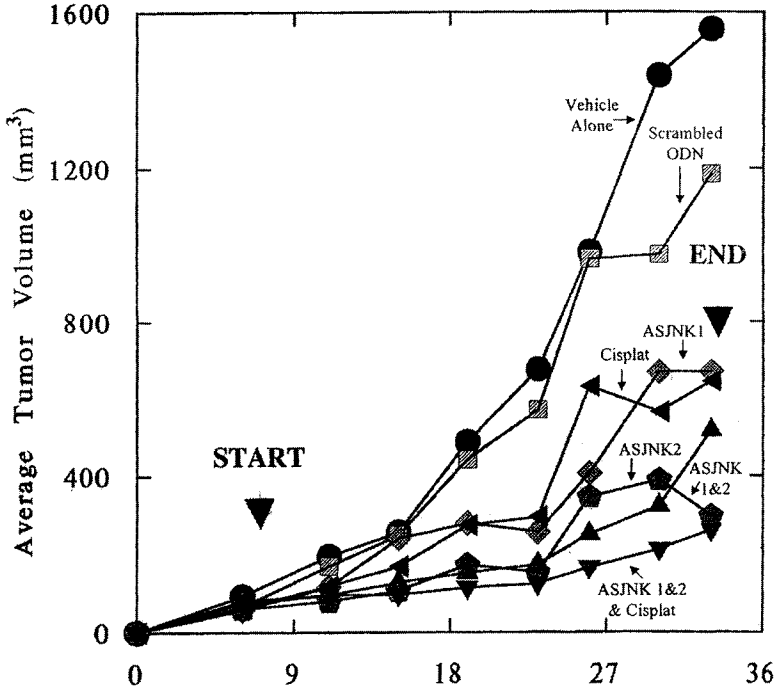

Figure 1 Inhibition of established tumor growth by antisense JNK ODNs. Groups of 10 (control groups, JNK-1 ODN group) or 15 animals were inoculated with 10^6 human pC3 prostate carcinoma cells and allowed to grow to an average size of 100 mm^3 when systemic treatment with antisense JNK ODNs by i.p. injection at 25 mg/Kg was started and continued 5–7 days. On day 7 the indicated groups also received cisplatin i.p. (Platinol) at 0.4 mg/kg. The average tumor sizes of each group are plotted and all data were applied to an ANOVA analysis. Probabilities were corrected for multiple comparisons (Bonferroni adjusted). The percent inhibition of growth was calculated by integration and comparison of the integrated area to that for the PBS control. All comparisons revealed probabilities less than the critical value except for antisense JNK-1 ODN group versus cisplatin, antisense JNK-2 ODN versus combined antisense, and combined antisense JNK-1 + JNK-2 + cisplatin group versus combined antisense or antisense JNK-2 alone.

with *increased* JNK activity, perhaps owing to strong activation of residual JNK protein. If so, this effect may explain the additive but not synergistic result of the combined antisense JNK ODN-cisplatin treatment group. It is concluded that, as for in vitro studies, JNK is an important growth pathway for human prostate carcinoma leading to prominent cytostatic effects following in vivo treatment with antisense JNK ODNs. Also similar to in vitro studies of a series of nine

JNK Kinase Activity of Antisense-Treated Human Prostate Carcinoma Xenografts

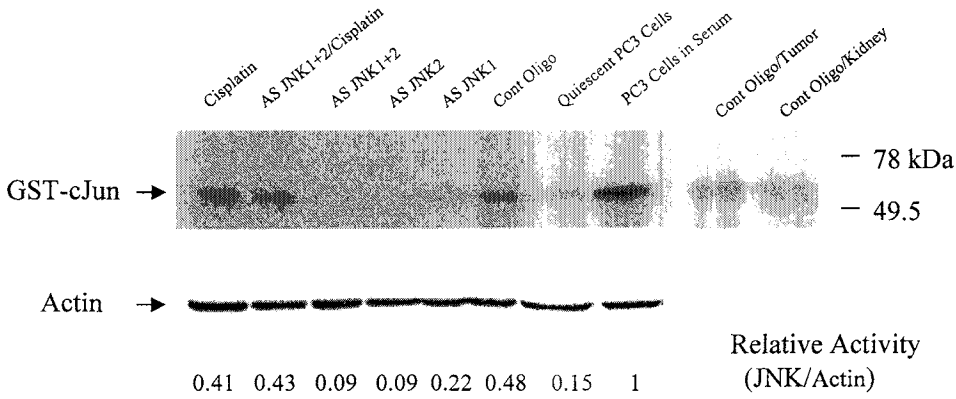

Figure 2 JNK kinase activity assay of human prostate tumor (xenograft) tissue from mice treated systemically with antisense JNK ODNs. JNK kinase activity was determined by immunoprecipitation of 32P-labeled GST-c-Jun protein from tumor extracts that had been incubated for 30 min in the presence of purified GST-c-Jun and 32P-ATP and separated by electrophoresis and used to form the autoradiograph shown here. β-Actin was determined as a measure of the amount of cellular material applied to the electrophoresis gel. Cultures PC3 cells exhibit high constitutive activity, which was given a relative kinase activity value of 1. Tumors of scrambled control ODN-treated animals exhibit about half this value likely due to dilution by stromal elements. All antisense JNK ODN-treated tumors exhibit a further fivefold reduction in activity indicating an antisense-mediated reduction in JNK. Addition of cisplatin is associated with no significant decrease in activity perhaps due to the known JNK-activating property (30) of cisplatin (see text). "AS" indicates antisense ODN complementary to JNK-1 or JNK-2 or the combination of these.

human prostate carcinoma cell lines (79), JNK-2 is the major JNK isozyme family mediating growth of tumors. Finally, antisense JNK combines additively with cisplatin to promote near-complete cytostasis. This system and the role of JNK in human prostate carcinoma appear worthy of considerable continued characterization.

XII. SUMMARY

It is over 12 years since the first application of antisense RNA in vivo to block tumor growth via plasmid-derived RNA complementary to the c-fos protooncogene (80). While plasmid-derived antisense and mRNA is an area of intense research for gene therapy, including cancer gene therapy, ODNs have proven versatile and amenable to conventional drug development. Among the potential advantages is the combined use of antisense ODNs and chemotherapeutic agents.

This brief and incomplete review provides examples of promising results as well as a variety of complications that have emerged including direct interaction between the ODNs and chemotherapeutic agents. These complications are usually associated with the use of antisense and/or chemotherapeutic agents at relatively high concentrations, i.e., $\geq$micromolar, where even relatively weak and nontarget specific interactions with association constants on the order of only 10^5–10^6 may account for an appreciable fraction of the original amount of ODN in the system. A variety of effects associated with studies at this level have been noted (16). In clinical applications, concentrations on this scale only occur transiently in the plasma immediately following bolus i.v. infusion (cf. Ref. 17; Fig. 1). Suggested criteria for the development of high-affinity antisense ODNs emphasize the use of a ''gene walk'' procedure (17,81), the use of dose-response and rank-order potency comparisons, and the confirmation that target RNA is specifically eliminated (17). Dose-response analyses provide a basis for detecting the emergence of unwanted concentration-dependent effects. These criteria are even more important for the analysis of multicomponent systems.

Several convenient methods of judging dose-response results including statistical criteria are provided by the isobologram (12,15) and median-effect methods (13,35). In several cases, substantial ''synergistic'' results and/or results supported by one or more of the quantitative criteria have been observed (35,48,49). These results clearly show that synergism, including the inhibition of xenograft growth, can be obtained. Although there are too few studies for a confident analysis, it appears that the rational choice of an antisense sequence for combination therapy may be to target a pathway with some shared function to that of the pathway inhibited by the chemotherapeutic agent and not to target a mutually exclusive mechanism. The goal is to block partly redundant routes thereby creating the complete inhibition of an essential function.

On the other hand, *antagonism* between two components of an otherwise successful combination has been observed in specific cell types (35). The notion that tumors even of histopathologically closely related types may have unique responses and gene expression patterns following exposure to well-studied and widely used chemotherapeutic agents has been recognized recently and supported by cluster analysis array data (5,6). This potential for individual tumor responses

may limit the range of application of even a very successful combination. More importantly, the potential for individual variation in response to a given treatment emphasizes the need for systematic pharmacodynamic studies and, perhaps, an abbreviated screen when considering application to individual patients. This may be a small price to pay for the decreased dose, decreased side effects, and greatly increased therapeutic response that a successful application may lead to.

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Antisense Oligodesoxynucleotide Strategies in Renal and Cardiovascular Disease

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I. INTRODUCTION

In cardiovascular medicine the targeting of specific molecules in the vessel wall to influence pathophysiological mechanisms of disease is an attractive and promising therapeutic approach. The endothelium is a direct interface between the blood and the vessel wall and may be directly targeted by small RNA molecules. Antisense oligodesoxynucleotides (ODN) provide a novel strategy to inhibit RNA transcription and thereby the synthesis of the gene product. Because antisense ODN hybridize with the mRNA strand, they are highly specific. Their backbone structure has been modified to phosphorothioates or phosphoamides so that they can better withstand degradation after delivery. We have shown that antisense ODN are a useful research tool to elucidate intracellular processes. The example we provide involves the inhibition of PKC signaling. Furthermore, we have shown the potential clinical utility of antisense treatment. We successfully inhibited the expression of the surface adhesion molecule ICAM-1 with antisense ODN in a model of reperfusion injury. This model is highly applicable to the problem of delayed graft function in humans. However, ''getting there'' is a major problem. Cationic substances such as lipofectin have worked sufficiently well in the experimental setting. Viral gene transfer offers a possibility; however, viruses produce

an additional series of problems. Liposomes may not provide sufficient transfer efficiency. Coating liposomes with viral fusion proteins may offer an ideal way to deliver the goods into the cytoplasm of the target cell.

The endothelium outlines all blood vessels and is the interface between the circulating blood cells and the vascular wall. These properties render the endothelial cells a target tissue for gene transfer. The selective inhibition of a specific protein provides the possibility to analyze its physiological and pathophysiological function and offers a defined therapeutic target. The use of antisense oligodeoxynucleotides (ODN) for the blockade of gene expression was introduced in 1978 by Zamecnik and Stephenson (1). Owing to the specificity of Watson-Crick base-pair hybridization, antisense ODN have been used extensively in attempts to inhibit expression of distinct genes both in vitro and in vivo. Although their precise mechanism of action has not been clarified, antisense ODN offer considerable promise as novel molecular therapeutic agents against diseases including AIDS, cancer, and inflammatory disorders. Furthermore, antisense ODN have been used in renal and cardiovascular therapeutic agents (2). In this chapter, we will discuss several aspects of our experience using antisense ODN in the understanding of renal and vascular pathophysiology as well as in experimental therapeutic protocols. We will first address recent improvements in the design of RNA molecules with modified properties. Second, we will demonstrate how the specificity of antisense ODN can be used to dissect molecular mechanisms of disease and how specificity offers new possibilities of drug treatment. Third, we will discuss the endothelium as a potential target tissue for antisense therapy. Finally, we will introduce new, nonviral, gene transfer techniques that enhance ODN uptake under experimental conditions and may be useful in future therapeutic trials.

II. CHEMICAL MODIFICATION OF ODN

The naturally occurring phosphodiester-linked ODN that were used intially are degraded rapidly by cellular nucleases and therefore cannot be used as in vivo therapeutic agents. Thus, chemically modified antisense ODN were developed that are more resistant to endogenous degradation. An example of such stable analogs are oligonucleotides with a phosphorothioate-modified backbone. These compounds are relatively stable and are the first generation of antisense compounds used in clinical trials (2). However, phosphorothioates are not ideal and possess several properties that are not suitable for therapeutic purposes. The main disadvantages of phosphorothioates are low binding affinity for stranded RNA and double-stranded DNA targets compared to natural phosphodiesters (2) and nucleotide independent binding to a variety of cellular proteins (3). Subsequently, DNA analogs with nonphosphodiester backbones have been developed. A larger

number of derivatives are now available in which the phosphodiester linkage has been replaced but the deoxyribose structure is retained. These derivatives include compounds ranging from phosphate backbone (phosphodithioates, chimeric methylphosphonate-phosphodiester, peptide nucleic acids) and 5-propynol-pyrimidine containing oligomers to sugar modifications (2′-substituted ribonucleosides, a-configuration) (4). However, only a few of these structures, such as those having a thioformacetal or a carboxamide linkage, appear to be good structural DNA mimics.

Recently, a new type of deoxyoligonucleotide analog was synthesized with a modification of the phosphate backbone, where the O3′-P bonds are replaced by N3′-P linkages (5,6). These so-called phosphoramidate analogs show several promising features. They have an achiral phosphorus-containing, negatively charged backbone and therefore exhibit good water solubility. In addition, they feature improved resistance to nuclease degradation. Thus they form very stable sequence-specific duplexes with single-stranded DNA, RNA, and with themselves. The phosphoramidate analogs are also able to form stable triplexes with double-stranded DNA and RNA under nearly physiological conditions (7). Phosphoramidates are also more digestion resistant and display less protein binding than phosphorothioates (8,9). A major problem is the large-scale synthesis of these compounds. We have focused our efforts on the synthesis of phosphoramidate ODN with a method that employs a phosphoramidite amine-exchange reaction (10). This method utilizes the corresponding monomethoxytrityl-protected 3′-amino-2′,3′-dideoxynucleoside-5′-phosphoramidites as building blocks.

III. SPECIFICITY

Antisense ODN are directed against distinct molecular entities. Thus, the effective therapeutic use of antisense ODN offers the possibility to directly interfere with the molecular mechanisms of the pathophysiological process (11). Such an approach allows very specific hypothesis testing. The specificity of the approach enabled us to directly target molecules with similar properties and structure such as kinase isoforms from the same kinase family, small GTP-binding proteins, or subtypes of receptors. We have used the unique property of antisense treatment to dissect the function of the various protein kinase C (PKC) isoforms in endothelial cells. PKC is a group of calcium- and phospholipid-dependent protein kinases (isoforms) wth a broad substrate specificity. PKC isoforms are involved in signal transduction response. The enzyme family was first described by Nishizuka (12). PKC is ubiquitously distributed and plays an important role in the control or regulation of many different biological processes (12). In endothelial cells, PKC has been implicated in the expression and regulation of adhesion molecules (13), in the expression of endothelin-1 (14), and in the proliferative response to hor-

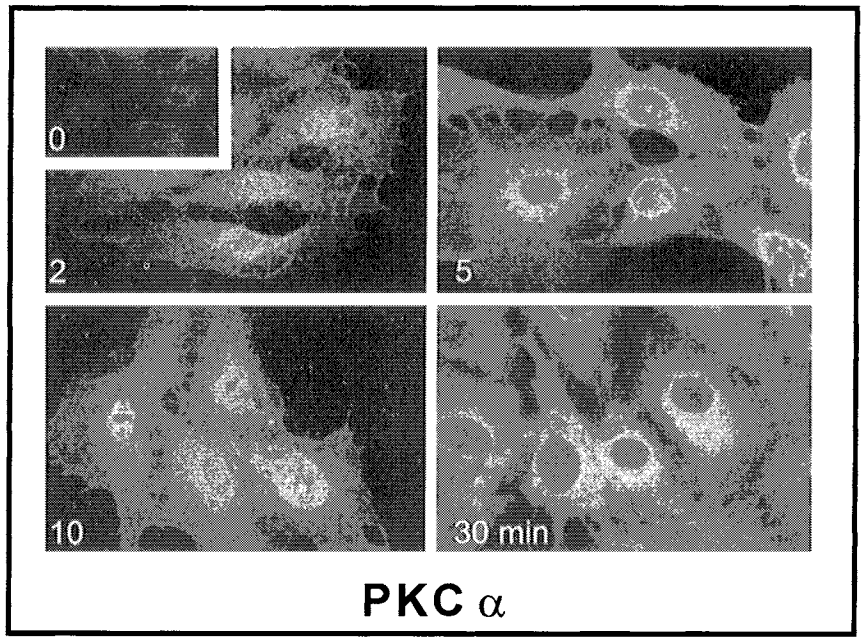
mones and growth factors (15). Furthermore, endothelial PKC appears to mediate the intracellular effects of shear stress (8) and may also be important to angiogenesis (16). Investigating PKC is difficult because it is not a single entity, but consists of several distinct isoforms with different regulatory and biochemical properties (13). The PC isoforms are expressed on separate genes and may play different roles in cell signaling and cell function (12).

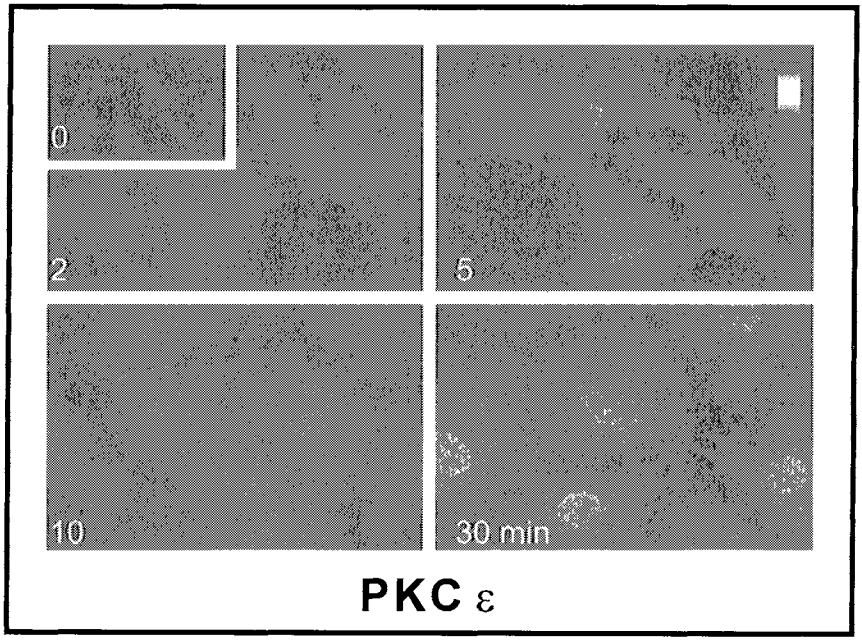
At present, the mammalian PKC family consists of 12 different polypeptides, α, βI, φII, γ, δ, ε, ζ, η, θ, τ, λ, and μ. An analysis of isoform expression and distribution is necessary to investigate PKC's role in signaling. Since PKC plays an important role in the intracellular signal transduction pathways of the endothelium and is involved in various functions of the edothelium such as expression of adhesion molecules and regulation of the permeability barrier, defining which PKC isoform mediates a specific signal is of considerable interest. However, thus far no specific inhibitors for the different PKC isoforms are available.

This problem would appear ideal for an antisense ODN-based approach. We observed that hyperglycemia increased endothelial cell-layer permeability via a PKC-mediated mechanism. To determine which PKC isoform was responsible, we relied on an antisense strategy. We concentrated on the PKC isoforms α and ε, because our confocal immunofluorescent data implicated those isoforms. These data are reviewed in Fig. 1. We included antisense to PKC ζ as an additional control. We selected an antisense ODN (ISIS 3521) against the human 3′-untranslated region derived form the human PKC α sequence (European Molecular Biology Laboratories database, Heidelberg, FRG). The antisense sequence used for PKC α was (5′ GTT.CTC.GCT.GGT.GAG.TTT CA 3′). The sense ODN sequence (5 ′ TG.AAA.CTC. ACC.AGC.GAG.AAC 3′), a reverse ODN sequence (5′ AC.TTT. GAG.TGG.TCG.CTC.TTG 3′), and a scrambled version (5′ GAG.TTG. CTT.GCT. TAT. CGG. TC 3′) were used as control. The antisense sequence used for PKC ε against the human AUG start codon was (5′ GCC.ATT.-GAA.CAC.TAC CAT 3′).

Figure 2 shows a western blot analysis of PKC α, PKC ε, and PKC ζ after transfection with antisense ODN. Antisense ODN led to a down-regulation of the respective PKC isoforms to 40–30% as compared to control. In contrast,

Figure 1 Effect of high glucose (20 mM) on the intracellular distribution of PKC isoform α, ε, and ζ under control conditions (left panel, control), and after 5, 10, and 30 min. High glucose induced changes in intracellular distribution of PKC isoform α and ε. In contrast, PKC ζ was not influenced by high glucose. The graded color bar indicates different PKC concentrations whereby blue, green, yellow, and red represent increasing PKC concentrations, respectively. (Original figure was in color.)





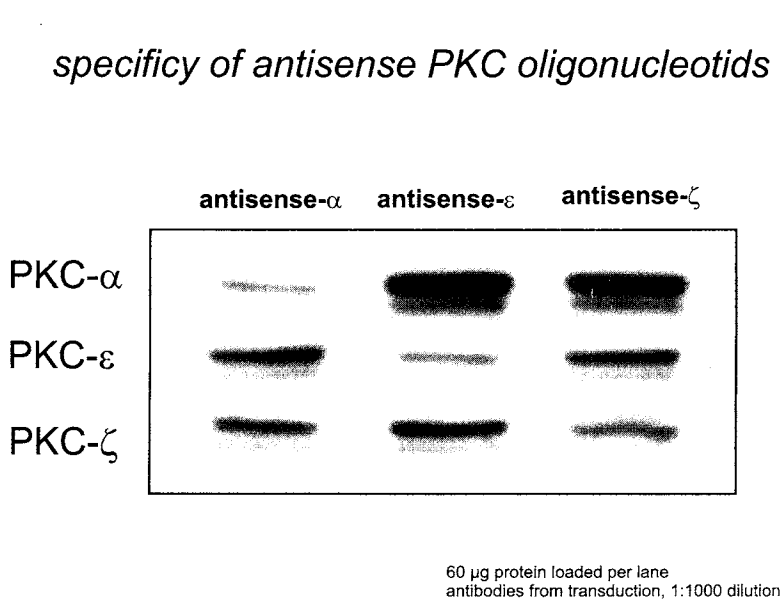


Figure 2 Western blot analysis of antisense ODN against PKC α, ε, and ζ in endothelial cells. Western blots were stained with PKC-specific antibodies as indicated. Antisense ODN against PKC α led to a significant down-regulation of PKC α, while PKC ε and ζ protein levels were not influenced. Antisense ODN against PKC e and PKC ζ, respectively, also led to a specific inhibition of PKC isoform expression without influencing the protein levels of the other PKC isoforms.

protein levels of PKC ε were not affected by exposure of the endothelial cells to antisense ODN against PKC α.

We then used these antisense ODN to influence the glucose-induced increase in endothelial cell permeability, as shown in Fig. 3. Antisense ODN for PKC α almost completely inhibited the increase in glucose-induced endothelial cell permeability. Sense and scrambled ODN for PKC α had no effect on the glucose-induced permeability. In contrast to the effects of antisense against PKC α, the antisense ODN against PKC ε did not reduce the glucose-induced permeability significantly. This experience demonstrates that an antisense ODN approach can be used for delineation of the specific PKC effects in signal transduction and cell physiology. Early experiments have shown that this approach can also be used in vivo. Intraperitoneal injection of ODN in mice caused a dose-dependent, ODN sequence-dependent reduction in PKC α mRNA (17). Thus, this approach may possibly be applied to prevent glucose-induced vascular changes in vivo. We are now using this approach for the treatment of diabetes-induced changes in the rat.

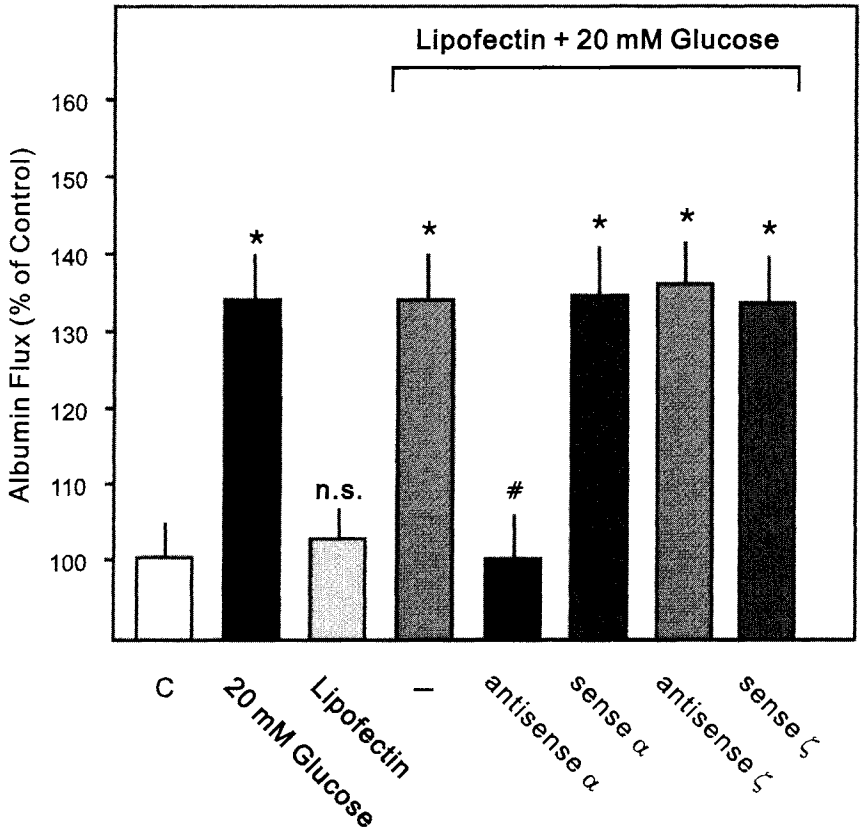


Figure 3 Effect of antisense ODN against PKC α, sense, scrambled ODN, or antisense against PKC ε and ζ on glucose (20 mM)-induced endothelial cell permeability. Endothelial cells were exposed to ODN with lipofectin (10 mg/mL) 24 h before exposure to 20 mM glucose. Antisense against PKC α significantly reduced the glucose-induced permeability ($p < 0.01$), while the control sense and scrambled ODN, the ODN against PKC ε and PKC ζ, or lipofectin alone had no significant effect.

The utility of antisense to block intracellular signaling processes is, of course, not limited to PKC. Other examples include antisense directed at inhibiting the production of GTP-binding proteins, other kinases, and transcription factors. Several groups have shown that antisense against cdc2 kinases exert specific effects in vascular tissue (18,19). Nakajima et al., have used antisense ODN for the inhibition of NFκB (20). In addition, antisense can also be used to investigate subtypes of membrane-bound receptors such as the FGF receptor family

(21,22) or the rapidly expanding family of VEGF receptors (23). Still other examples include targeting of proteases or components of the cell cycle (24–26).

IV. TARGETING

Identifying potential targets for antisense strategies is easy; however, getting the antisense to the targets can be insurmountably difficult. Fortunately, since antisense ODN are small molecules they cross the cell membrane more easily than other genetic material. When injected into the venous circulation, antisense ODN demonstrate a rather high first-pass effect and are rapidly taken up by the liver. However, about 1–3% of the antisense ODN reaches the coronary circulation, and, between 0.5 and 1% arrives in the kidney after intravenous injection. Our own experiments show that addition of cationic lipids increases the percentage of renal uptake considerably. The uptake into the renal vasculature takes place within minutes. Most of the circulating ODN are taken up by the endothelium. In our experience, even the intimal layers of vascular cells are not reached by circulating antisentse ODN. However, other groups have observed an antisense effect in media vascular smooth muscle cells (27). Conceivably, injured vascular tissue or damaged endothelial cells may exhibit altered uptake characteristics. In addition, cells that are adjacent to the endothelium and are not shielded by the basal lamina, such as mesangial cells in the kidney, may also take up circulating ODN (28). The rapid uptake by the endothelium makes this tissue a suitable target for antisense. The endothelium may be an interesting target for gene therapy because endothelial cells play a major role in the development of all chronic vascular and renal diseases.

Adhesion of leukocytes to the endothelium plays an important role in such diverse processes as inflammation, transplantation, and atherosclerosis. Reperfusion injury involves activated leukocytes with enhanced adhesiveness to endothelium (29). Adhesion-molecule-mediated, neutrophil endothelial binding is inherent to this process (30). The leukocyte β2 integrin complex (CD11/CD18) interacts with the endothelial ligand intercellular adhesion molecule-1 (ICAM-1). The initial rolling of neutrophils is mediated by the selectins, while CD11/CD18-ICAM-1 interactions are responsible for leukocyte adhesion and diapedesis (31). Studies in liver, brain, and myocardium showed that ICAM-1 is upregulated during ischemia-reperfusion (30). Antibodies against either CD11/CD18 or ICAM-1 prevented tissue damage and protected organ function in other studies (32–34). We used antisense ODN for ICAM-1 to influence the expression of adhesion molecule and to prevent reperfusion injury in the ischemic kidney of the rat. Phosphorothioate ODN were used and an antisense ODN (ISIS 1939) against the human 3′ untranslated region derived from the rat ICAM sequence RSICAM and the human ICAM-1 sequence HSICAM01 (European Molecular

Biology Laboraotries database, Heidelberg, FRG) was selected (35,36). For the rat experiments, we compared rat and human sequence data and used the rat homologue to ISIS 1939 (5′ ACC GGA TAT CAC ACC TTC CT 3′). The reverse ODN sequence was used as control. As in the previous experiments we used a cationic lipid solution to enhance ODN uptake.

From preliminary in vitro experiments, a lipofectin concentration of 0.8 mg/mg DNA and a ODN concentration of 2 mg/kg body weight was chosen for the in vitro studies. Figure 4 shows the effect of verse and antisense oligonucleotides on expression of ICAM-1 in renal cortical vessels 24 h after 30 min of ischemia. The saline-injected control showed ICAM-1 staining along with vascular intima. This staining was decreased in antisense ODN–treated animals. The reverse ODN–treated animals, on the other hand, showed prominent ICAM-1 staining. In antisense-treated animals, renal function was preserved and perivascular leukocyte infiltration was inhibited.

We believe that the antisense approach to acute renal failure and reperfusion injury could have great clinical utility. We do not envision prophylactic preoperative antisense ODN treamtent in patients. Instead, we believe such an approach would be both more valuable and practical in transplantation medicine. For instance, cadaveric donor kidney are routinely stored in Collins or similar solutions for 12–72 h before transplantation. Delayed graft function from ischemia and reperfusion injury is a major posttransplant problem and has a direct negative impact on long-term graft survival (36). Transplanted hearts and livers also are subject to reperfusion injury and ICAM-1 seems to play a role in acute and chronic rejection (38–40). The antisense ODN treatment is not subject to the same immunological problems that accompany the use of antibodies directed against adhesion molecules. We would envision a multiple antisense ODN treatment of transplant grafts directed against a variety of adhesion molecules associated with reperfusion injury.

V. TRANSFER

Our experiments demonstrate that antisense ODN can be successfully used for the treatment of endothelial cell disorders. However, a limiting step in these investigations is still the low uptake of ODN in the endothelium. Relatively high concentrations of ODN have to be injected to achieve a significant down-regulation of the targeted protein. More effective gene transfer techniques may reduce the costs of antisense ODN therapeutic approaches and may help to target cells within the vascular wall or other organs. For gene transfer two main approaches, viral gene transfer and nonviral techniques, have been used. Viral gene transfer techniques show high efficiency, but potentially cause viral infection, activation of oncogenes, and autoimmune response. Newman et al. showed that adenovirus-

mediated gene transfer into rabbit arteries results in prolonged vascular cell activation, inflammation, and neointimal hyperplasia (41). Liposome vectors contain no viral sequences and possess the desired safety profile (43). However, the liposomal approach routinely results in efficiencies below 1% (43,44). Several techniques have been put forward to enhance gene uptake by liposomes. Improvement of the liposomal method was demonstrated by Kaneda et al. (45) preparing DNA-loaded liposomes together with gangliosides and inactivated Sendia virus particles.

Several reports have recently demonstrated that Sendai virus–coated liposomes can mediate transfer of DNA, antisense ODN, and double-stranded DNA as a decoy. An HVJ liposome can encapsulate DNA up to 100 kbp (46). Using HVJ liposomes associated with gangliosides and the nuclear protein HMG-1 (nonhistone chromosomal protein, high-mobility group 1), Kaneda et al. successfully introduced the entire human insulin gene into adult rat liver. The transcript amount of the insulin gene cointroduced with HMG-1 was more than 10 times greater that of the gene cointroduced with bovine serum albumin alone. In vivo insulin gene expression was also possible (46). Tomita et al. used the same approach in introducing a reporter gene into rat kidney. In 1993, Isaka et al. demonstrated that TGF-b or PDGFb cDNA can be transported into rat kidney with HVJ liposomes to induce glomerulosclerosis.

The HVJ-liposome method of gene transfer has also been used successfully in the cardiovascular system. For instance, the cDNAs of the angiotensin-converting enzyme (ACE) and renin genes were transfected into cultured vascular smooth muscle cells in vitro as well as into rat carotid artery in organ culture (47). Morishita et al. measured increased ACT activity after transfection of ACE into intact rat carotid arteries. They also demonstrated that high levels of atrial natriuretic peptide (ANP) were secreted by cultured endothelial cells (47). HVJ liposomes loaded with eNOScDNA restored eNOS expression in the vessel wall and inhibited neointimal vascular lesion after balloon injury (48).

The HVJ-liposome-mediated transfer is also applicable for antisense ODN delivery. For instance, this delivery system was also used for antisense ODN directed at cdk 2 kinase oligonucleotides. The cyclin-dependent kinase is activated in the red carotid artery after balloon angioplasty injury and is probably responsible for smooth muscle proliferation. Morishita et al. showed that intimal hyperplasia after vascular injury is inhibited by specific antisense oligonucleotides (49). Furthermore, a gene therapy strategy using a transcription factor decoy of the E2F binding site inhibits smooth muscle proliferation in vivo (50).

A major disadvantage of this promising gene transfer approach is the tedious and time-consuming preparation of the viral particles. Thus far, no preparation that would pass GMP criteria for human use has been demonstrated. We have therefore started to develop a gene delivery system containing neutra lipo-

somes and *recombinant* viral surface fusion proteins. Yeagle reported that a fusion peptide, isolated from the remainder of F1, destabilizes membranes (51). Therefore, he hypothesized that contact between the hydrophobic sequence of the fusion peptide of F and the target membrane is capable of substituting for bilayer-bilayer contact. The fusion process differs from the recepor-mediated endocytosis with fusion of endosomes and lysosomes at acidic pH used in influenza virus (52).

Expression of recombinant viral fusion proteins is not an easy task. Ponimaskin et al. used a baculovirus system and found incomplete processing and membrane transport of F protein, due to the different glycosylation and protein transport pathways in invertebrate cells (52). Construction of recombinant vaccinia virus expressing Sendai F protein resulted in a biologically inactive protein (53), probably because the severe cytopathic effects of vaccinia virus disturb expression of the activity. Using PCR mutagenesis, we added a factor Xa cleavage site into the F cDNA to obtain the correctly processed and active F protein. For future purification of the protein we fused a histidine sequence coding at the end of the F and HN cDNA. The vector pcDNA3 carries the corresponding cDNAs under the control of the cytomegalovirus promoter. Furthermore, we cloned our constructs into the vector pZeoSV2. Genes cloned into pZeoSV2 are expressed from the Simian virus 40 early promoter for high-level transient and stable expression in mammalian cells. Then, it should be possible to use the purified proteins to generate fusion protein–coupled liposomes. Our method differs from commonly used HVJ liposomes because we do not use the whole inactivated Sendai virus. Instead of this, we integrate the two recombinant proteins from Sendai virus into the liposomes. In vivo experiments will show whether these constructs will diminish cell toxicity, immune response, and inflammation.

VI. PERSPECTIVES

Antisense strategies have been termed the "poor man's road to gene therapy." The ODN have been made more robust by means of a phosphoramidate backbone. The tremendous specificity of antisense ODN was demonstrated in the PKC experiments we conducted, as well as in a host of studies by other investigators. Since the inhibition of mRNA transcription by antisense ODN is transient, the therapeutic potential may be limited. However, our approach to use antisense to inhibit the development of reperfusion injury and thereby delayed graft function in transplanted kidneys may be an ideal clinical setting to use antisense ODN. As in all "gene therapy" strategies, delivery is a serious problem. We believe that eventually, liposomes coated with viral fusion proteins will offer an acceptable, highly efficient delivery system.

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26

The Development of Antisense Oligonucleotides as Antivirals

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I. INTRODUCTION

Traditionally, selection of antiviral agents has been the product of massive random screening rather than rational drug design. However, there remains a significant unmet need and great potential to design and develop more specific antiviral agents that will be advantageous in terms of both reduction in toxicity and improvement in target selectivity and specificity. As we move into the next generation of drug design the ability to inhibit genes that are implicated in various viral infections is becoming a reality. Viruses make excellent targets for antisense oligonucleotides since virally encoded proteins are unique and without similar counterparts at the cellular level thus limiting the possibility of unwanted interactions with normal cell functions. Although Vitravene is the first antiviral antisense oligonucleotide to be marketed, a number of other compounds targeting a variety of viral diseases are in various stages of preclinical and clinical development. Even with an ever-increasing number of drugs in development, many viral diseases are still in need of new therapeutics. In some cases no appropriate therapy exists. Many antisense oligonucleotides have been investigated in vitro and in vivo targeting a number of different indications. The infectious agents being investigated as potential antisense clinical targets include: human papillomavirus (HPV), cytomegalovirus (CMV), human immunodeficiency virus (HIV-2), hepatitis B (HBV), hepatitis C (HCV), influenza A, and herpes simplex virus (HSV). Hopefully, this is only the beginning of a new focus on novel therapeutics that target inhibition of viral replication utilizing antisense oligonucleotides.

Fomivirsen (Vitravene), the first antisense oligonucleotide to have successfully advanced through clinical trials, received marketing approval in the United States (August 26, 1998), Europe (July 1999), Brazil, Switzerland, and Australia. Vitravene is indicated for the treatment of CMV retinitis in patients with acquired immune deficiency syndrome (AIDS). The value in the development and approval of the first antisense drug extends well beyond the target indication and market. The approval of Vitravene demonstrates that there is broader value created from the development of these unique chemical entities. The strategy used in obtaining approval of Vitravene, and described in this chapter, is an example of how the development of a new class of compounds targeted to a unique receptor forms the foundation of a broad opportunity for drug development. As well, a review of other antiviral antisense compounds in clinical or preclinical development summarizes the potential for use of these in the treatment of a variety of viral diseases. Some of these have been discussed in a number of earlier reviews. (1–4).

II. THE TARGET

For the vast majority of currently approved drugs, the primary target is a protein receptor. In the new paradigm known as antisense technology the target is a ribonucleotide strand. The new "receptor," messenger RNA (mRNA), is not so much a new target as a previously unexploited target. While traditional drugs are competitive antagonists that bind to disease-causing proteins subsequently blocking the action of natural agonists, they may also bind to other proteins with unwanted toxicological effects or reactions. In contrast, antisense oligonucleotides are designed to be highly selective and specific (5). Further, when designing an oligonucleotide, it is important to choose a target that is unique, i.e., different from the other targeted proteins for currently used drugs. For example, in choosing a target for CMV it was useful to consider that DNA polymerase was already targeted by three different currently marketed compounds, ganciclovir, foscarnet, and cidofovir. As a consequence, a resistant mutation to one of these drugs would likely result in cross-resistance to the others. With this in mind, a unique mRNA sequence coding for viral proteins essential for replication of cytomegalovirus was chosen as the target for designing potential anti-CMV drugs. Several candidate antisense oligonucleotides targeted to the mRNA sequence specific for these proteins were designed utilizing first-generation-chemistry phosphorothioate oligonucleotides, which were optimized for enhanced pharmacological activity and stability to nuclease degradation. Numerous phosphorothioates were tested in a variety of in vitro assays prior to selection of ISIS 2922 (fomivirsen sodium) as a development compound (6,7).

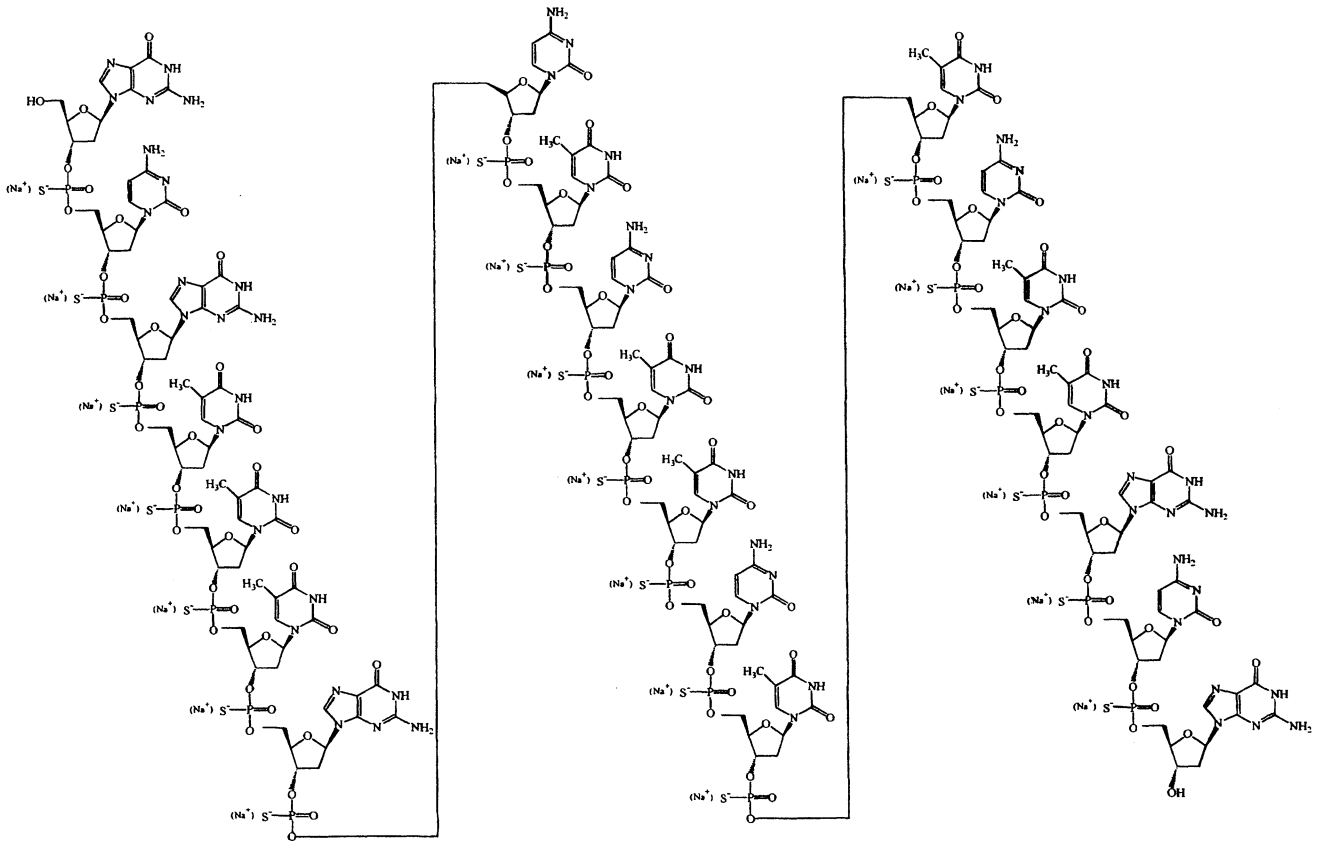

Figure 1 Structural formula of fomivirsen sodium. Vitravene (fomivirsen sodium intravitreal injectable) is a sterile, aqueous, preservative-free, bicarbonate-buffered solution for intravitreal injection. Fomivirsen sodium is a phosphorothioate oligonucleotide, 21 nucleotides in length, with the following sequence: 5′-GCG TTT GCT CTT CTT CTT GCG-3′.

Fomivirsen is a phosphorothioate oligonucleotide, 21 nucleotides in length, with the following sequence: 5′-GCG TTT GCT CTT CTT CTT GCG-3′ (Fig. 1). Fomivirsen is designed to be complementary to the mRNA sequence encoding the message for the proteins of the major immediate early (IE2) region of CMV. This sequence, unique to CMV (there is no known complementarity to any other mRNA), codes for proteins that are essential to replication of the CMV virus. It is these proteins that play an immunoregulatory role during viral replication. Thus, by selectively inhibiting the production of the IE proteins, human CMV replication is inhibited. In the case of fomivirsen, a unique mechanism of action as well as a unique target allowed for the development of a compound that is effective in the treatment of CMV particularly in patients who may not be responsive to other therapies.

During the development of traditional drugs, once a compound or group of compounds is selected based on in vitro data, an assessment of in vivo efficacy in an appropriate animal model is the next step. Human CMV is a virus that replicates only in human tissue types. Thus, in the case of CMV retinitis in an immunocompromised patient no relevant animal model exists that could be used to demonstrate in vivo pharmacology. Without the benefit of antiviral activity in an animal model, demonstration of viral inhibition by fomivirsen in in vitro studies (6) provided the foundation to proceed with pharmacokinetic and toxicology studies in animals and, ultimately, clinical studies.

III. ANTISENSE OLIGONUCLEOTIDES AS ANTIVIRAL AGENTS

Much work with antisense oligonucleotides as antiviral agents has been done in vivo and some compounds have been tested in clinical trials. The antiviral activity of phosphorothioate oligonucleotides has been evaluated in a number of systems prior to initiation of clinical trials. The viruses studied include: HPV (8), influenza A (9), HBV (10), HCV (11), and CMV (6,12). To date, clinical trials with antisense oligonucelotides as antiviral agents have evaluated HIV (Gem 91), HPV (ISIS 2105, aforvirsen), CMV (ISIS 2922 and GEM 132), and HCV (ISIS 14803).

The first antiviral antisense oligonucleotide to be evaluated in clinical trials was ISIS 2105 (afovirsen sodium) targeted to HPV 6 and 11, the causative viral strains in genital warts. As the target was a local infection, afovirsen was given as an intradermal injection directly to the wart site. The development of this compound represented the first in local delivery to the target for a local viral infection. Treatment with this compound in more than 400 patients resulted in few side effects other than induration and mild edema.

The moribidity and mortality that results from acute respiratory disease caused by the influenza A virus suggests that antisense oligonucleotide may pro-

vide a much needed therapy. Mizuta et al. (9) were able to demonstrate antiviral efficacy of a 20-mer antisense oligonucleotide complementary to sequences surrounding the translation initiation codons of the viral PB2 gene. Following intravenous injection of the 20-mer in mice infected with influenza A, these authors demonstrated efficacy as measured by a decrease in target mRNA levels as well as a reduction in viral titer and a reduction in pulmonary damage.

Infection with hepatitis B frequently manifests as chronic hepatitis and liver cirrhosis. HBV is the causative agent that contributes to hepatocellular carcinoma, and, as such, the development of an effective therapy for HBV is a major goal in the treatment of this disease. Moriya et al. (10) utilized a transgenic mouse system as a hepatocellular carcinoma model to evaluate the potential for antisense oligonucleotides to suppress HBV gene expression. Injections with a specific antisense oligonucleotide not only inhibited the target gene expression in the liver of these animals, but as well, prevented preneoplastic lesions from developing. Others have used primary duck hepatocytes and infected ducklings to demonstrate the efficacy of antisense oligonucleotides against HBV (13,14). Treatment with an antisense oligonucleotide targeted to the 5′ region of the preS gene of duck HBV both in vitro and in vivo resulted in dose-dependent inhibition of viral replication and subsantial decrease in viral DNA. These results suggest that such targets may be useful in the clinic to address the prevention of hepatocellular carcinoma.

Another potential target is the HCV, the major cause of non-A, non-B hepatitis. The virus that causes hepatitis C, a member of the Flaviviridae family, affects more than four million people in the United States. In other geographic locations the prevalence is considered to be higher (15). This virus is also thought to be associated with the development of chronic liver diseases, cirrhosis, and hepatocellular carcinoma (16–18). The treatment of HCV is limited by the side effects of the currently approved drugs, interferon-α alone or in combination with ribavirin. Consequently, there is a need to develop new and better-tolerated therapies for this chronic infection.

Vidalin and co-workers (19) used two different cell-free translation systems and several different chemically modified oligonucleotides to evaluate the ability of antisense compounds to inhibit HCV translation. These studies were done in both a rabbit reticulocyte lysate and a wheat germ extract. The ability of these compounds to inhibit HCV RNA translation provided further evidence that specific antiviral agents have the potential to be effective in vivo systems.

Efficacy of selected compounds was demonstrated in a mouse model (11). These authors provided evidence for inhibition of HCV gene expression by antisense oligonucleotides using two antisense oligonucleotides, ISIS 14803 and ISIS 6547, in mice infected with vaccinia virus vector containing a portion of the HCV genome. Both compounds were 20 nucleotides in length with the same sequence and were complementary to the HCV translation initiation codon. One of the

compounds, ISIS 14803, was modified so it contains 5-methyl-cytosine in place of cytosine. It has been suggested that this type of modification reduces the possibility of immunostimulatory activity (20,21). Both the modified (ISIS 14803) and the unmodified (ISIS 6547) oligonucleotides showed similar inhibition of HCV gene expression in infected mice, reducing HCV expression in a dose-dependent manner. Thus, the potential to inhibit HCV gene expression in animals suggested that one of these compounds would make a good candidate for further development. Because of the desirability to reduce immunostimulatory effects in humans and because of the increased potency demonstrated with ISIS 14803 (11), this compound was chosen as the candidate with which to initiate clinical trials. ISIS 14803 is the first antiviral antisense oligonucleotide given by the intravenous route of administration for the treatment of HCV to begin testing in humans. This study is in a very early stage and no results are available.

Antisense oligonucleotides may also be useful in the treatment of HIV-1. Several different target sites have been studied utilizing in vitro cell systems. These targets include gag, pol, rev, tat, and tar within the HIV genes (22,23). Kim et al. (22) demonstrated that antiviral activity in HIV-infected cells was dependent both on the target sequence chosen (e.g., antisense oligonucleotides targeted to rev or tat) and on the length of the oligomer. Junker et al. (23) evaluated antiviral efficacy in retrovirally transduced human T cells. Their results demonstrated that both rev and gag targets provide a reasonable approach for the development of anti-HIV therapies. Sequence-specific inhibition of HIV-1 viral replication was investigated using a gene-expression modulator identified as GEM91 (Hybridon, Inc.). This 25-mer phosphorothioate oligonucleotide is targeted to the translation initiation site of the gag mRNA. Lisziewicz et al. (24) demonstrated sequence-specific inhibition of HIV-1 replication using GEM91 in (1) an immortalized T-cell line, (2) primary lymphocytes, and (3) primary macrophages. Together with data from safety and pharmacokinetic studies these results formed the basis for initiation of clinical trials with this compound for the treatment of AIDS. GEM91 was administered to over 250 subjects prior to the discontinuation of its development in July 1997.

IV. HUMAN PHARMACOKINETICS OF FOMIVIRSEN

In the absence of an assay for nonradiolabeled oligonucleotides, previous pharmacokinetic studies in animals and humans utilized radiolabeled compounds. The first study in humans employed ^{14}C-labeled afovirsen (ISIS 2105), a compound that was in clinical trials for the treatment of genital warts (25). In this study, patients were treated with a single intradermal injection of ^{14}C-labeled afovirsen directly to the wart. Tissue samples were removed to determine presence of intact drug by high-pressure liquid chromatography analysis of extracts. Results showed

that intact afovirsen was found in wart tissue for at least 72 h after single injection. These data helped to support the study design utilized in the clinical trials, once- to thrice-weekly dosing. However, the use of radiolabeled compounds limited the ability to interpret results further particularly with regard to metabolism. The development of an assay to evaluate absorption, distribution, metabolism, and excretion of nonradiolabeled compounds provided many advantages over the use of radiolabeled oligonucleotides (26,27). These authors demonstrated that it is possible to obtain accurate and precise quantitation of another compound, fomivirsen, and metabolites as small as n-9 mers, using a capillary gel electrophoresis–ultraviolet method in human vitreous humor and human plasma (28). Development of this method allowed for more precise measurement of fomivirsen and its metabolites.

Animal studies showed that following a single intravitreal injection of fomivirsen the vitreous is cleared of oligonucleotide with first-order kinetics and drug is primarily distributed to the retina in both rabbits and monkeys (29). As a result of metabolism, fomivirsen is cleared from the retina (29), suggesting that retinal accumulation is not likely if fomivirsen is dosed once every 2 weeks or less frequently. Importantly, the animal pharmacokinetic data predict that following intravitreal injections of fomivirsen in humans, therapeutic levels of 0.1 μM will be surpassed througout the treatment period with predicted concentrations ranging from 5.0 to 0.6 mM (29,30).

Understanding that the targeted virus replicates at the level of the retina provided the basis for the intravitreal route of administration of this drug. With this in mind, the human pharmacokinetic study was designed as a single-dose intravitreal injection. In this human pharmacokinetic study several questions were addressed: (1) Following a single intravitreal injection what was the rate of clearance from the vitreous? (2) What was the degree, if any, of systemic bioavailability? (3) Would infrequent dosing allow for therapeutics levels for fomivirsen to remain in the target tissue?

The human pharmacokinetic study was conducted in 31 patients who were scheduled to receive a primary Vitrasert (ChironVision). These patients received a single dose of fomivirsen at 336 h, 168 h, 48 h, or 1 h prior to surgery for the implant. The dose of fomivirsen was based on the degree of retinal involvement as determined by the treating physician. Patients (n = 15) with less than 25% retinal involvement received 165 μg fomivirsen while patients (n = 16) with 25% or greater retinal involvement received 330 μg fomivirsen. The authors (31) reported that the concentration of fomivirsen in the vitreous 1 h after a 165 μg dose ranged from 1.23 μM to 11.82 μM and from 0.30 to 32.7 μM 1 h after a single 330 μg injection. The mean concentration of fomivirsen measured in the vitreous 7 days after a single injection of 165 μg fomivirsen was 0.07 μM and by 17 days following this dose fomivirsen was below the level of quantitation, i.e., less than 25 nM. Similar results were reported following a single injection

of 300 μg fomivirsen with a mean concentration, 7 days after the dose, of 0.27 μM. By 14 days after the dose the concentration was below the limit of quantitation in one sample and at 0.06 μM in another. This time-dependent decrease in vitreal concentration of fomivirsen is thought to be primarily due to (1) uptake by the retina, (2) outflow through the anterior chamber, and (3) metabolism within the vitreous.

No plasma samples taken from any patient at any time point showed evidence of fomivirsen nor were there detectable levels of oligonucleotide metabolites in the plasma samples at any time point. These results indicate that the risk of systemic adverse effects following intravitreal dosing with fomivirsen is unlikely. Importantly, these results suggest that in a patient population treated with a large variety of therapeutic agents there is minimal risk of systemic drug-drug interactions.

Thus, following a single intravitreal injection of fomivirsen to the site of infection the drug is rapidly cleared from the vitreous via metabolism and uptake into the retina, and there is little, if any, systemic bioavailability.

V. CLINICAL EFFICACY OF FOMIVIRSEN

CMV retinitis is a local manifestation of a systemic CMV infection and is the primary evidence of this infection (32,33). Although the exact mechanism by which retinal cells become infected is not fully understood, studies on autopsy samples suggest that a likely pathway is via retinal vascular endothelial cells (34). Nevertheless, the primary site of ocular infection is the retina. The ocular manifestation of CMV results in the destruction of retinal cells, hence the term "CMV retinitis."

In immunocompromised patients, for example, patients with AIDS, CMV is usually pathogenic. Infection with this member of the Herpesviridae family often results in various clinical features. The diagnosis of CMV retinitis is based on clinical identification typical characteristics of opaque, yellow-white lesions in the retina, sometimes associated with vessel sheathing and hemorrhage. This is a local infection that usually occurs when the patient's CD4 cell count is low. Vitravene was developed to be administered locally by intravitreal injections to treat the infection at the site where extensive replication of the virus is likely to cause blindness.

Clinical trials with fomivirsen were initiated prior to the era of potent antiretroviral therapy in which the combination of protease inhibitor, nucleoside, and nonnucleoside reverse transcriptase inhibitors was used to elicit immune restoration in patients with AIDS. Thus, the treatment regimen became known as highly active antiretroviral therapy (HAART).

Following Phase II studies in patients with AIDS and CMV retinitis unresponsive to other therapies, Phase III studies with fomivirsen were initiated in 1995. The development strategy for Phase III included randomized, controlled studies in two different patient subsets. The design of the primary pivotal trial was identical to trials with ganciclovir, foscarnet, and cidofovir for the treatment of CMV retinitis. Patients with newly diagnosed, peripheral CMV retinitis were randomly assigned to either an immediate-treatment group or a deferred-treatment group. Utilization of this study design provided a basis for comparison of the effect of treating CMV retinitis at the time the diagnosis is first made to the course of the untreated infection during a time when the retinitis is not immediately sight-threatening. In another randomized controlled clinical trial, patients with active CMV retinitis that had been previously treated were assigned to one of two dosing regimens in a dose schedule comparison.

As with the assessment of the other drugs for treatment of CMV retinitis, double-masked studies were not possible. Similarly, fundus photographs were evaluated by a single ophthalmologist at the Fundus Photograph Reading Unit of the Jules Stein Eye Institute Clinical Research Center, UCLA School of Medicine. These photos were read in a masked fashion and according to standard criteria of retinitis progression and were used to provide an independent assessment of the treatment outcome. Additional criteria, assessed by the treating ophthalmologist, were used to determine efficacy. These criteria were (1) retinal detachment in an area of active CMV retinitis and (2) CMV optic neuritis along with a decrease in visual acuity to 20/200 or worse.

Fomivirsen intravitral injection was effective in the treatment of newly diagnosed, previously untreated CMV retinitis (35, 36). In a study design that followed a 2:1 randomization scheme, 18 patients received one injection of 165 μg of fomivirsen weekly for three doses followed by every-other-week maintenance dosing at the same dose. In the control arm (delayed-treatment group) 10 patients were monitored until the disease progressed, at which time they were given the option to receive the same dose of fomivirsen and according to the same schedule as those in the immediate-treatment group. A protocol-defined interim analysis demonstrated that the difference in treatment effect between the two groups was statistically significant ($p = 0.0001$). The estimated median time to CMV retinitis progression (Kaplan-Meier analysis) was 71 days for patients who received immediate treatment and 13 days for those whose treatment was delayed (Table 1).

The study design of the dose schedule comparison evaluated a higher dose of fomivirsen, 330 μg, given either as an induction once weeky for three doses followed by a maintenance dose every other week, or as an induction every other week for two doses followed by monthly maintenance. The results from the protocol-defined interim efficacy analysis showed that there was no difference in time to CMV retinitis progression for patients treated according to one or the other

Table 1 Fomivirsen (165 μg) Efficacy in Patients with Peripheral, Newly Diagnosed CMV Retinits, Previously Untreated[a]

Group	Dose	Schedule	Number of patients	Median time to progression (days)
Immediate treatment	165 μg	Weekly for 3 weeks, then every other week for maintenance	18	71
Delayed treatment		No treatment, observation until progression, option to treat as above after progression	10	13

[a] Estimated median time to CMV retinitis progression by Kaplan-Meier analysis.

dosing schedule (37). For patients treated on either schedule, the interpolated estimated median time to CMV retinits progression was 90 days (Table 2).

The results demonstrate that fomivirsen (Vitravene) is an effective treatment for CMV retinitis in patients with AIDS. Treatment dose and schedule are dependent on whether the infection is newly diagnosed and previously untreated or advanced, active, and previously treated.

The safety profile of fomivirsen was also established from these clinical trials. Patients who enrolled in a total of six clinical trials were given complete ophthalmic examinations conducted at regularly scheduled intervals. Ocular signs were also evaluated during the examination. Systemic adverse events were evalu-

Table 2 Fomivirsen (330 μg) Efficacy in Patients with Advanced, Active CMV Retinits, Previously Treated[a]

Group	Dose	Schedule	Number of patients	Median time to progression (days)
Regimen A	300 μg	Weekly for 3 weeks then every other week for maintenance	34	90
Regimen B	330 μg	Every other week for 2 doses then monthly for maintenance	20	90

[a] Estimated median time to CMV retinitis progression by Kaplan-Meier analysis.

Table 3 Safety Table (Most Frequent and Clinically Important)

Ocular adverse effect	165 μg	330 μg
Number of patients, total percent incidence	92(%)	238(%)
Anterior chamber inflammation	12	22
Increased intraocular pressure	12	22
Retinal detachment	7	13
Vitritis	5	12
Uveitis	2	6

ated through the assessment of clinical laboratory values. Consistent with plasma pharmacokinetic data in humans, there were no reports of systemic adverse events considered to be related to fomivirsen in patients treated with either 165-μg or 330-μg doses (37–39).

A total of 92 patients were treated with 165 μg fomivirsen and 238 patients were treated with 330 μg fomivirsen. Evaluation of the safety profile for patients in these trials demonostrated that the local ocular adverse events reported were predominantly mild to moderate, and either resolved spontaneously or were manageable with topical medications (37). The most frequently reported, clinically important, ocular adverse events (Table 3) included: anterior chamber inflammation (12% at 165 μg and 22% at 330 μg), increased intraocular pressure (12% at 165 μg and 22% at 330 μg), vitritis (5% at 165 μg and 12% at 330 μg), and retinal detachment (7% at 165 μg and 13% at 330 μg). Ocular adverse events that were less frequently observed included: cataract (9% at 165 μg and 11% at 330 μg), cystoid macular edema (2% at both 165 μg and 330 μg), uveitis (2% at 165 μg and 6% at 330 μg), and visual field defects (2% at 165 μg and 3% at 330 μg). In this patient population the relationship of these ocular adverse events to study drug or underlying infection was difficult to determine (37). Nevertheless, the safety profile of fomivirsen is one that consists of events that are clinically manageable.

VI. CONCLUSION

The approval of the first antiviral antisense ODN designed to inhibit viral replication by selectively and specifically blocking proteins that are essential for viral replication is an important achievement. The marketing of Vitravene worldwide provides an alternative to nonselective and often toxic nucleoside antivirals that were the only treatment for CMV retinitis previously available. Even more sig-

nificant is the start of a new era of rational drug design utilizing a chemistry that allows for the optimization of treatment and the opportunity to treat a wide variety of other viral infections. We have now initiated the development of what is hopefully the next antisense oligonucleotide to be used in the treatment of a viral infection, ISIS 14803 for hepatitis C. Perhaps this will open the window to a wide range of systemically administered antiviral antisense oligonucleotides.

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Clinical Activities in Patients with Solid Tumors or Lymphoma

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I. INTRODUCTION

The last decade has seen the introduction of a number of new chemotherapeutic and biological agents to treat patients with advanced cancer. While this has provided patients and their physicians with more treatment options, and true improvements in outcome, cure for most advanced tumors remains a distant goal, and the toxicity of new agents is still often considerable. For some tumors, progress, though significant, has been grudging indeed. For example, 10 years of medical research and drug development have improved the outlook for patients with advanced non-small-cell lung cancer (NSCLC) from (approximately) a 15% response rate, median survival of 6 months, and 20% 1-year survivorship at the start of the decade to (approximately) a 25% response rate, median survival of 8 months, and 33% 1-year survivorship at decade's end (1). Clearly, much work remains to be done, and new drugs to treat cancer are still needed.

Advances in tumor biology have identified many attractive molecular targets for new drug discovery and development. Antisense oligonucleotides, unlike conventional drugs that may bind to more than one molecular target, are particularly attractive as exquisitely selective drugs. An antisense oligonucleotide binds specifically to a region of its target mRNA by Watson-Crick hybridization. This reduces the production of the corresponding protein, most commonly by RNase H–mediated degradation of the target mRNA. Because of the specificity of hybridization, the expression of a single member of a gene family (e.g., one of several enzyme isotypes) can be inhibited without affecting the others. This selectivity, in turn, makes it possible that the efficacy observed with an antisense

oligonucleotide will be obtained with less toxicity than with a conventional cytotoxic chemotherapy agent.

A number of "first-generation" phosphorothioate oligonucleotides are currently undergoing clinical testing for the treatment of patients with advanced cancers. The chemistry, preclinical data, and excellent clinical safety profiles of these agents are reviewed elsewhere in this volume. This chapter will review the current evidence that these drugs are active against human solid tumors or lymphoma.

II. AGENTS IN CLINICAL DEVELOPMENT

Antisense drugs that are currently in clinical trials for patients with solid tumors or lymphoma are listed, with their main characteristics briefly summarized, in Table 1. These drugs are being developed as single agents (Table 2), and in combination with standard cancer chemotherapy regimens (Table 3). Single-agent antitumor activity has been observed in several trials, and promising data have been obtained in early trials of combination regimens.

A. ISIS 3521 (Isis Pharmaceuticals)

ISIS 3521 is a 20-mer phosphorothioate oligodeoxyribonucleotide (5′-GTTCTCGCTGGTGAGTTTCA-3′) that binds to a sequence in the 3′-untranslated region of the human mRNA for protein kinase C-α (PKC-α). PKC-α is one of a family of cytoplasmic serine-threonine kinases that are responsible for signal transduction in response to a variety of cellular stimuli, leading to a range of responses including cellular proliferation, differentiation, membrane transport, and gene expression. The PKC-α isozyme, which is expressed in a wide range of tissues, is found in many transformed cell lines (2,3). Increased PKC-α expression has been reported in human breast cancer specimens (4), and PKC-α expression has been linked to the proliferation of tumor cell lines in vitro (5–7) and to enhanced tumorigenicity of malignant cells in vivo. The PKC family has been implicated in malignant transformation and tumor growth by studies that did not differentiate among the various isozymes (8–10), and a number of PKC inhibitors (e.g., staurosporine analogs) have been tested in clinical trials. However, these compounds are not specific for PKC isoforms, nor, potentially, for the PKC family overall.

Two Phase 1 trials of single-agent ISIS 3521 were conducted, using two different schedules: 2-h intravenous (IV) infusion, repeated Monday-Wednesday-Friday, 3 weeks out of 4, and 21-day continuous IV infusion, followed by 1 week off. Antitumor activity was observed with both schedules in Phase 1. The continuous-infusion schedule was chosen for Phase 2 because of its potential for

Table 1 Characteristics of Anticancer Oligonucleotides in Clinical Development

Drug	ISIS 3521	ISIS 5132	ISIS 2503	G3139	GEM 231	GTI-2040	MG98	ANGIOZYME
Company/ sponsor	Isis	Isis	Isis	Genta	Hybridon	Lorus Therapeutics	MethylGene	Ribozyme Pharmaceuticals
Backbone chemistry[a]	P=S	P=S	P=S	P=S	P=S; 2′-*O*-methyl ribose at 5′ and 3′ ends	P=S	P=S; 2′-*O*-methyl ribose at 5′ and 3′ ends	
Length	20-mer	20-mer	20-mer	18-mer	18-mer			
Molecular target	Protein kinase C-alpha	c-*raf*	H-*ras*	*bcl*-2	Protein kinase A	Ribonucleotide reductase	DNA methyltransferase	Vascular endothelial growth factor
Mechanism of target mRNA reduction	RNase H	RNase H	RNase H	RNase H	RNase H	RNase H	RNase H	
Phase of clinical development	2 → 3	2	2	2–3	2	1–2	1	1
Indications	Non-small-cell lung cancer Low-grade or follicular non-Hodgkin's lymphoma Other solid tumors	Solid tumors	Pancreatic cancer Non-small-cell lung cancer Breast cancer Colon cancer	Non-Hodgkin's lymphoma	Advanced cancers	Advanced cancers	Advanced cancers	Advanced cancers

[a] P=S: phosphorothioate.

Table 2 Single-Agent Antitumor Activity in Clinical Trials of Antisense Oligonucleotides

Oligo/target	Schedule	Tumor	Efficacy results	Ref.
ISIS 3521/PKC-α	2 h IV infusion tiw, 3 weeks out of 4	Lymphoma	Complete response in 2 of 2 patients with low-grade disease: one continuing 30+ months after start of treatment, one with skin recurrence 15 months after start of treatment	11
		Non-small-cell lung	Stable disease for 6 months	
	2 day CIV, 3 weeks out of 4	Ovarian	Partial response: time to progression (TTP): 11 months, marker only disease with 75% ↓ in CA-125. TTP: 7 months, marker only disease with 40% ↓ in CA-125. TTP: 7 months, 80% ↓ in CA-125 with stable measurable disease	12
ISIS 5132/C-*Raf*	2 h IV infusion tiw, 3 weeks out of 4	Colon	Stable disease for 7 months, 31% decrease of CEA, concurrent ↓ C-*raf* expression in peripheral blood mononuclear cells	41, 42
		Renal	Stable disease for 9 months, concurrent ↓ C-*raf* expression in peripheral blood mononuclear cells	
	21 day CIV, 3 weeks out of 4	Ovarian	97% decrease in CA-125 with stable evaluable disease, TTP: 10 months	40
		Pancreatic	Stable disease for 10 months	
		Renal	Stable disease for 9 months	
ISIS 2503/H-*Ras*	14 day CIV, 2 weeks out of 3	Sarcoma	Stable disease for 10 cycles	31
		Pancreatic	Stable disease for 9 cycles	
		Colon	Stable disease for 8 cycles	
		Mesothelioma	Stable disease for 6 cycles	
	24 h CIV, repeated weekly	Melanoma	Melanoma: stable disease for 7 cycles	32
G3139/*bcl*-2	14-day SC infusion	Lymphoma	Complete response: 1/17 patients, 11 stable disease (including 2 minor responses), 6/17 patients with improved lymphoma symptoms, 7/16 patients with ↓ Bcl-2 protein in PBMC, bone marrow, or lymph node	48

maintaining continuous tissue exposure to oligonucleotide, at plasma concentrations well below those associated with toxicity in primate studies.

Complete responses (complete disappearance of all evidence of tumor) were observed in two patients with relapsed/refractory low-grade non-Hodgkin's lymphoma (NHL) in Phase 1 trials of ISIS-3521 (11). (These two patients, treated with the 2-h infusion schedule, were the only two with low-grade NHL included in the Phase 1 trials, which were open to patients with a variety of advanced cancers.) Lymphoma had persisted in both, despite extensive prior chemotherapy, including high-dose therapy with peripheral blood stem cell transfer in one case. These patients received 17 and nine cycles of treatment, respectively. The former patient remained relapse-free 12 months after completing treatment (time to progression, 30+ months from the start of treatment). The other had a relapse in the form of a skin lesion 4 months after completing treatment. This lesion was surgically resected, and the patient was free of further relapse 4 months later.

Based on these results, a Phase 2 trial of ISIS 3521 in low-grade or follicular NHL have been initiated, using the continuous-infusion schedule. In the early stages of one trial, an additional patient had a partial response ($\geq$50% decrease in the cross-sectional areas of tumors on computed tomography), which developed gradually over 10 cycles of therapy (unpublished data, Isis Pharmaceuticals Inc.). These trials will determine further the activity of ISIS 3521 in low-grade lymphoma, including the potential for durable complete responses as observed in the Phase 1 trial.

Objective antitumor activity has also been observed in patients with ovarian cancer treated in Phase 1–2 trials of ISIS 3521 by the continuous-infusion schedule. A partial response occurred in a patient with ovarian cancer whose tumor size had doubled in the month prior to starting treatment with ISIS-3521 (12). Her disease was stable for 4 months after the start of treatment, then developed an extensive central necrosis, and then decreased in size until she had a 60% reduction in the size of her tumor after eight cycles of treatment. Her serum level of the tumor marker CA-125, which was initially 107 units/mL, increased to 620 U/mL after three cycles of therapy but subsequently declined to 209 after nine cycles. The patient received a total of 11 cycles of therapy, without treatment-related toxicity, before being removed from the study with disease progression. Three other patients with ovarian cancer had declines in CA-125 of 80%, 76%, and 40%, respectively (Ref. 12 and unpublished data, Isis Pharmaceuticals Inc.). Elevated CA-125 was the only evidence of disease in two of these patients. The third patient had stable measurable tumor throughout the treatment period.

Typically, patients enrolled in Phase 1 trials in cancer have disease that has failed to respond to several treatment regimens. Consequently, the responses that were observed in the Phase 1 trials of ISIS 3521 are promising. Accordingly, Phase 2 single-agent trials of ISIS 3521 in a number of tumor types have been initiated. However, anticancer agents are frequently used not only singly, but also

Table 3 Clinical Trials of Oligonucleotides in Combination with Other Anticancer Agents

Oligo	Oligo dose/schedule	Additional agents	Additional agents' dose/schedule	Phase of trial	Tumor(s)	Results/status
ISIS 3521	1–2 mg/kg/d CIV days 1–14, 21-day cycle	Carboplatin Paclitaxel	Carboplatin AUC 5–6, Paclitaxel 175 mg/m^2 IV q 21 days	I–II	NSCLC, other solid tumors	11/19 PRs (58%), plus 10 patients on therapy. ISIS 3521 does not add to the toxicity of carbo/taxol. See text.
ISIS 3521	2 mg/kg/d CIV days 1–14, 21-day cycle	Carboplatin Paclitaxel	Carboplatin AUC 6, Paclitaxel 175 mg/m^2 IV q 21 days	III	NSCLC	Planned
ISIS 3521	2 mg/kg/d CIV days 1–14, 21-day cycle	Gemcitabine Cisplatin	Gemcitabine, 1000–1250 mg/m^2 Cisplatin, 80–100 mg/m^2 IV q 21 days	I–II	NSCLC, other solid tumors	In progress
ISIS 3521	2 mg/kg/d CIV days 1–14, 21-day cycle	Docetaxel	75 mg/m^2 IV q 21 days	II	NSCLC	Planned
ISIS 3521	1–2 mg/kg/d CIV days 1–21, 28-day cycle	5-FU Leucovorin	5-FU 425 mg/m^2 IV, Leucovorin 20 mg/m^2 IV days 1–5	I	Solid tumors	Combination well tolerated; 2 PR in adenocarcinoma of colon/unknown primary; ref. 17
ISIS 2503	4–6 mg/kg/d CIV days 1–14, 21-day cycle	Gemcitabine	1000 mg/m^2 IV days 1, 8	I	Solid tumors	Minor response in breast cancer; prolonged stable disease in esophageal, lung, unknown primary, sarcoma tumors. Ref. 33
ISIS 2503	6 mg/kg/d CIV days 1–14, 21-day cycle	Gemcitabine	1000 mg/m^2 IV days 1, 8	II	Pancreatic	Planned

ISIS 2503	6 mg/kg/d CIV days 1–14, 21-day cycle	Docetaxel	75–100 mg/m^2 IV q 21 days	I	Solid tumors	Planned
ISIS 5132	2–3 mg/kg/d CIV days 1–14, 21-day cycle	Carboplatin Paclitaxel	Carboplatin AUC 6, Paclitaxel 175–200 mg/m^2 IV q 21 days	I	Solid tumors	Acceptable toxicity; 2 mg/kg/d ISIS 5132 and 175 mg/m^2 recommended for further study
ISIS 5132	1–3 mg/kg/d CIV days 1–21, 28-day cycle	5-FU Leucovorin	5-FU 425 mg/m^2 IV, Leucovorin 20 mg/m^2 IV days 1–5	I	Solid tumors	Acceptable toxicity; 4 patients with stable disease (pancreatic, colon, 2 renal cell)
G3139	1–3 mg/kg/d, 21-day CIV, 28-day cycle	Docetaxel	35 mg/m^2 IV day 8, 15, and 22 of each cycle	I	Breast/other solid tumors	In progress; see text and reference 50.
G3139	Dose escalation, CIV days 1–14 or 1–21	Paclitaxel	Weekly IV infusion	I–II	Prostate, other solid tumors	In progress; see text and reference 51.
G3139	CIV, or bid SC	Dacarbazine	IV day 5 of therapy	I–II	Melanoma	ICR, 2 PR in 1st 14 patients. ↓ Bcl-2 protein in serial tumor biopsies reported. Reference 49.
G3139	Dose escalation, CIV days 1–10 (28-day cycle)	Fludarabane, Cytarabine	Dose escalation, fludarabine IV followed by cytarabine, days 6–10	I	Acute myeloid or lymphoid leukemia	In progress
G3139	Dose escalation, CIV days 1–7 (21-day cycle)	Irinotecan	Dose escalation, 90-minute IV day 6 of cycle	I–II	Colorectal	In progress
G3139	CIV days 1–7 (21-day cycle)	Paclitaxel	3-hour IV day 6 of cycle	I–II	Small cell lung	In progress
GEM231	2-hour IV days 1, 4, 8, 11, 15, 18	Paclitaxel	1-hour IV day 1, q 3 weeks	I	Solid tumors	In progress
GEM231	2-h IV days 1, 4, 8, 11, 15, 18	Docetaxel	1-h IV day 1, q 3 weeks	I	Solid tumors	In progress

in two- or three-drug regimens, in combination with other agents. Because of this, a Phase 1 trial of ISIS 3521 in combination with the chemotherapy drugs carboplatin and paclitaxel (Taxol) was initiated early in development. Phosphorothioate oligos are excellent candidates for development in combination with chemotherapeutic agents because of their safety and nonoverlapping mechanism of action. Carboplatin and paclitaxel are standard therapy for advanced ovarian cancer and NSCLC. The addition of ISIS 3521 was considered of interest based on the activity observed in these diseases in the Phase 1 single-agent trials. (In addition to the activity in ovarian cancer, one patient with NSCLC in the Phase 1 single-agent trials had stable disease for eight cycles of therapy before progressing.) ISIS 3521 at the Phase 2 dose, 2.0 mg/kg/day, administered by 14-day continuous infusion every 21 days, was well tolerated in combination with standard doses of carboplatin [dosed to a calculated area under the curve (AUC) of 6.0, administered IV every 21 days] and paclitaxel (175 mg/m^2 IV every 21 days) (13).

Seven of 10 patients with previously untreated NSCLC enrolled in this Phase 1 trial had partial responses (13), and the trial was expanded to obtain additional efficacy data in patients with NSCLC. Preliminary efficacy data from this ongoing trial are available (Ref. 14 and unpublished data, Isis Pharmaceuticals Inc.). At this writing, 11 partial responses (per protocol definition of 50% reduction in bidimensionally measurable tumor) have been observed among a total of 19 patients with NSCLC who are evaluable for response in this trial and who have completed therapy (58% response rate). One patient (one of whom remains on treatment in cycle 3) have had a minor response, and stable disease has been the best response for four patients. Treatment is ongoing for 10 additional patients, who may yet develop a complete or partial response with continued therapy. One of these has had a minor response, two have stable disease to date, and seven are still in the early stages of treatment, prior to their first scheduled response evaluation. Patient accrual to this trial is continuing at this writing.

Of particular note, and importance, in this trial is the survival of patients with NSCLC who have been treated. The U.S. Food and Drug Administration has generally required that new drugs to treat NSCLC demonstrate a survival advantage to establish efficacy, particularly when the new drug is combined with approved therapy of known efficacy. In the present trial of ISIS 3521 plus carboplatin and paclitaxel, the median survivorship (measured from the time of entry into the trial) of the first 29 NSCLC patients is approximately 19 months (Fig. 1). This includes nine patients who have survived for greater than 1 year (range 12.2+ to 25.8+ months for these nine patients). The median time to tumor progression of the group is 7.1 months. These data appear to compare favorably with historical data from a recent, randomized trial of carboplatin and paclitaxel in NSCLC, or with prior data from small, single-arm Phase 2 studies of carboplatin and paclitaxel (15,16) (Table 4). However, such comparisons must be

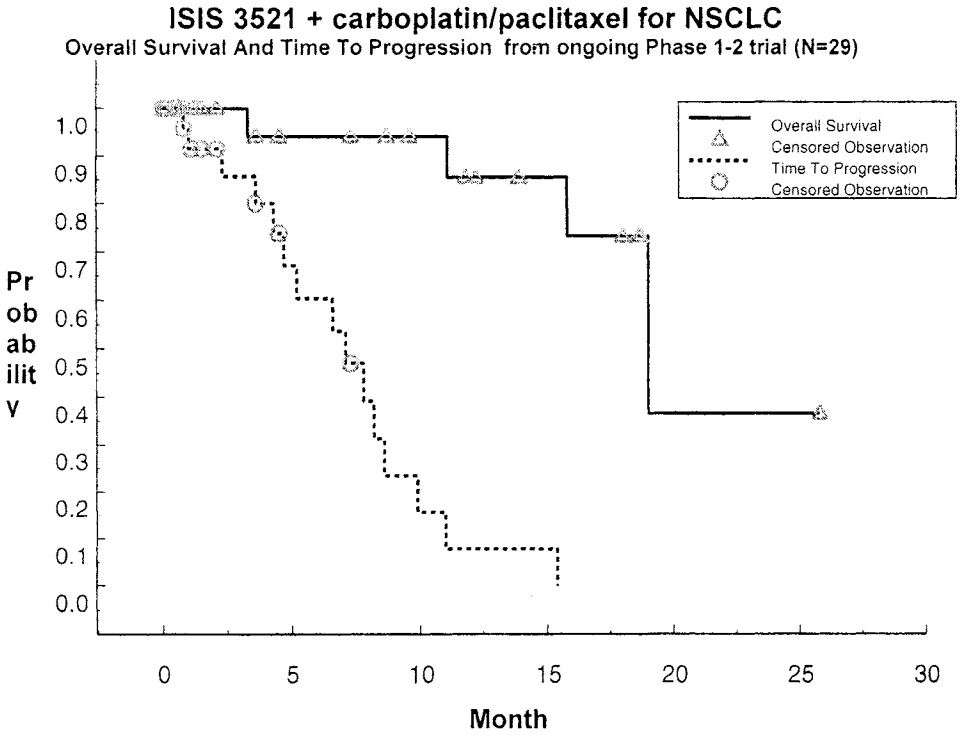

Figure 1 Kaplan-Meier plot of time to progression and overall survival of patients with non-small-cell lung cancer (NSCLC) from ongoing Phase 1–2 trial of ISIS 3521 in combination with carboplatin and paclitaxel. Data are for 29 patients as of May 12, 2000.

made with caution, and a sufficiently powered prospective, randomized trial of carboplatin and paclitaxel, with or without ISIS 3521, will be necessary to establish any added contribution of ISIS 3521 to the efficacy of the chemotherapy. Such a trial is currently in the planning stages. Additional trials of ISIS 3521 in combination with other chemotherapy regimens for advanced NSCLC will also be conducted to determine further the potential role of ISIS 3521 in the treatment of this disease.

ISIS-3521 has also been studied in combination with 5-fluorouracil (5-FU) and leucovorin (LV), which are commonly used to treat colorectal cancer and other gastrointestinal malignancies, in a Phase 1 trial (17). In this study, ISIS 3521 was well tolerated at the recommended Phase 2 dose of 2 mg/kg/day by 21-day continuous infusion, in combination with a standard regimen of 5-FU

Table 4 Descriptive Comparison of Historical Efficacy Results with Carboplatin/Paclitaxel for Advanced NSCLC

Trial(s)	Response rate (%)	Median time to progression (mo)	Median survival (mo)	% of patients alive ≥ 1 year	Ref.
Phase 3 trial, carbo/taxol vs. vinorelbine/platinum (n = 184 receiving carbo/taxol)	27%	Not stated	8	36	15
Single-arm Phase 2 trials of carbo/taxol (8 trials, appx. n = 30–60 per trial)	46%	Not stated	9.5	40	Reviewed in Ref. 16
ISIS 3521 + carbo/taxol (n = 29, ongoing)	58% of 1st 19 patients; 10 others on study	7.1	19	To be determined: currently 9 of 1st 19 patients alive > 1 year	14, and unpublished data, Isis Pharmaceuticals Inc.

and LV. Two patients—one with colon cancer and one with adenocarcinoma of unknown primary site—had partial responses in this trial.

B. ISIS 2503 (Isis Pharmaceuticals)

ISIS 2503 is a 20-mer phosphorothioate oligodeoxyribonucleotide (5′-TCCGTCATCGCTCCTCAGGG-3′) that binds to a sequence in the translation initiation region of the human mRNA for H-*ras*. The *ras* gene family (principally H-*ras*, K-*ras*, and N-*ras*) encode a set of closely related plasma-membrane-associated, guanine-nucleotide-binding proteins that normally cycle between an active GTP bound state and an inactive GDP bound state (18–20). The *ras* genes may be responsible for malignant transformation by a number of mechanisms. Best-characterized among these are point mutations affecting amino acid positions 12, 13, or 61, which lock Ras into the active state and lead to sustained mitogenic signaling through the Raf/MAP kinase pathway (19). However, it has been reported that signaling through phosphoinositol-3 kinase (PI-3 kinase) also mediates Ras activity, and may be important in tumor formation and maintenance (21–25). A recent report suggests that c-Raf is relatively more important in signaling downstream of K-Ras, while PI-3 kinase is more important to the downstream signaling of H-Ras (26).

Human cancers demonstrate multiple abnormalities that involve *ras* gene products directly or indirectly, including activating mutation, notably of K-*ras*, in up to 90% of pancreatic cancers, 35% of colorectal cancers, 25–30% of NSCLC, and other tumors (27,28); overexpression of Ras protein (including H-Ras) (29); and overexpression of growth factors and their receptors, whose downstream signaling is mediated by *ras* pathways (30). In human tumor xenograft models, ISIS 2503 demonstrated activity against both K-Ras mutant (Mia-PaCa-2 pancreatic, Panc-1 pancreatic, Calu-1 lung) and nonmutant tumors (HT-29 colon (ras normal), H-69 lung (ras normal), MDA-MB-231 breast (ras status unknown), suggesting that ISIS 2503 is an attractive potential treatment for a broad range of human cancers (data on file, Isis Pharmaceuticals Inc.).

In Phase 1 single-agent trials, ISIS 2503 was administered to patients with a variety of advanced cancers by 14-day continuous IV infusion, in a 21-day cycle, or by weekly 24-h IV infusion. Treatment by both schedules was well tolerated. Four patients treated with ISIS 2503 by the 14-day continuous-infusion schedule—with pancreatic cancer, sarcoma, colon cancer, and mesothelioma—had stable disease on therapy for 9, 10, 8, and 6 3-week cycles, respectively (31). Notably, the patient with pancreatic cancer had persistent disease after prior therapy with gemcitabine, which is currently the standard treatment for advanced pancreatic cancer. One patient, with melanoma, treated by the weekly 24-h infusion schedule, had stable disease for seven cycles (21 weeks) before developing progressive disease (32).

A Phase 1 trial of ISIS 2503, administered by the 14-day continuous-infusion schedule, in combination with gemcitabine has been conducted (33). The combination was well tolerated, with minimal toxicity, by the 18 patients with advanced cancer who were treated on this study. On this study, a patient with persistent breast cancer despite prior high-dose chemotherapy has demonstrated improvement in her tumor. This improvement—80% reduction in subcutaneous metastases and 40% reduction in a liver metastasis—does not yet meet the criteria for a partial response, but the patient remains on study, in her twelfth 3-week cycle of chemotherapy, at this writing. Four other patients—with esophageal cancer, NSCLC, tumor of unknown primary site, and sarcoma—remain on study with stable disease after 11, 6, 7, and 3 cycles of treatment, respectively.

Phase 2 single-agent trials of ISIS 2503—again by the 14-day continuous-infusion schedule—are in progress in patients with pancreatic, NSCLC, colon, and breast cancers, and a Phase 2 trial of ISIS 2503 in combination with gemcitabine is planned for patients with advanced pancreatic cancer. These trials will estimate the response rate of these tumors to treatment with ISIS 2503. The Phase 1 results suggest that stabilization of disease, which may lead to improved survival or a more indolent disease course, could be an important effect of ISIS 2503, and the current trials will also determine the number of patients with prolonged stable disease. In addition, clinical trials combining ISIS 2503 with taxane chemotherapeutic agents [paclitaxel (Taxol) or docetaxel (Taxotere)] will be conducted. The trials employing taxanes will attempt to extend observations made in an H-*ras* transgenic mouse model, in which female mice bearing the H-*ras* transgene spontaneously develop mammary carcinomas (34). In that model, ISIS 2503 demonstrated antitumor activity, and significantly increased the antitumor activity of paclitaxel when the two were given in combination. The increased antitumor activity was reflected in a significant increase in the percentage of tumor cells undergoing apoptosis. Tumor expression of H-Ras protein was decreased after administration of ISIS 2503 in these studies, consistent with an antisense mechanism of action.

C. ISIS 5132 (Isis Pharmaceuticals)

ISIS 5132 (5′-TCCCGCCTGTGACATGCATT-3′) is a 20-mer phosphorothioate oligonucleotide that hybridizes to a portion of the 3′-untranslated region of human C-*raf* mRNA. The *raf* kinase genes (A-, B-, and C-*raf*) code for serine-threonine protein kinases that mediate signaling from Ras, upstream, through a cascade of downstream reactions collectively referred to as the MAP kinase signaling pathway (35). Because *ras* mutations are present in a high proportion of human cancers, the Raf proteins, as direct downstream efectors, are potentially important targets for antitumor therapy (36,37). Furthermore, *raf* mutations are trans-

forming in vitro and are associated with human tumors (38), and *raf* has been reported in small-cell lung cancer and breast cancer (39).

In Phase 1 clinical trials of ISIS 5132 administered by 21-day continuous infusion, or by 2-h infusion three times per week, prolonged stable disease was observed in patients with ovarian, pancreatic, renal, and colon cancers. A patient with refractory ovarian cancer who received ISIS 5132 by 21-day continuous infusion demonstrated a decline of 97% (from 1490 to 47 U/mL) in serum CA-125 with therapy. She received 10 months of therapy with ISIS 5132 before her disease progressed. This patient also had nonmeasurable peritoneal studding with tumor, which remained stable on therapy, with improvement in a small amount of ascites. She had previously received multiple treatment regimens with approved chemotherapeutic agents, which had afforded more modest declines (up to approximately 50–60%) in her CA-125 level. A second patient, with pancreatic cancer, remained on study with stable disease for 10 months before progressing, and a third, with renal cell cancer, had stable disease for 9 months before progressing (40).

In the 2-h infusion study of ISIS 5132 (41,42), two patients with progressive cancer, despite receiving several prior treatment regimens, had prolonged stable disease while receiving ISIS 5132 and remained on treatment for eight and 10 cycles, respectively, without cumulative toxicity. One, a man with colorectal cancer, had a decline in his blood level of the tumor marker carcinoembryonic antigen (CEA) for 7 months, along with reduction of measurable disease not meeting criteria for a partial response. A woman with renal cell carcinoma had stable disease before developing a new bone metastasis after 10 months of therapy. Both patients demonstrated a decline in the expression of c-*raf* mRNA, as determined by reverse transcriptase polymerase chain reaction (RT-PCR), in peripheral blood mononuclear cells (PBMC) during therapy with ISIS 5132. These declines—to $<10\%$ of baseline in the patient with colon cancer, and to 12% of baseline in the patient with renal cell cancer—followed a time course that paralleled the patients' clinical benefit. Of a total of 30 patients treated in this study, c-*raf* mRNA expression was studied in the 14 patients treated at the highest doses (2.5–6.0 mg/kg IV on Monday-Wednesday-Friday, 3 weeks out of 4). Significant declines in c-*raf* mRNA (median, 42% of baseline after one dose of ISIS 5132 and 26% of baseline after two doses) were observed in PBMCs from 13 of these 14 patients. A dose-response effect was not apparent in these patients at the dose levels analyzed. While it is not possible to extrapolate reliably from results in PBMC to results in tumor tissue, the data from this trial are consistent with an antisense mechanism of action in the cells tested.

ISIS 5132 is being studied in Phase 2 single-agent trials against a variety of tumor types, and in Phase 1 trials in combination with carboplatin/paclitaxel or 5-FU/LV (Table 3) (43 and data on file, Isis Pharmaceuticals Inc.).

D. G3139 (Genta)

The *bcl*-2 gene is a member of a family of genes encoding protein products that function as key regulators of programmed cell death (apoptosis). The Bcl-2 protein has been identified as an ''antiapoptotic'' protein that is overexpressed in a number of tumor types, most notably non-Hodgkin's lymphoma, and is believed to contribute to decreased sensitivity to chemotherapeutic agents (44). Moreover, studies in transgenic mice suggest that Bcl-2 overexpression plays an etiological role in lymphoma (45). Therefore, a drug targeting *bcl*-2 is of interest as monotherapy, or as a chemosensitizer, in combination with currently available antitumor agents.

G3139 is an 18-mer phosphorothioate oligonucleotide that targets the first six codons of the open reading frame of the human *bcl*-2 mRNA. Based on animal studies in which G3139 eradicated lymphoma from SCID mice (46,47) and subsequent studies demonstrating increased chemosensitivity of human melanoma xenografts to decarbazine (with decreased Bcl-2 protein expression in tumor tissue), G3139 is being developed for use as a single agent in patients with non-Hodgkin's lymphoma, and in combination with chemotherapy for a variety of solid tumors and lymphoma.

Initial results of a Phase 1 trial of G3139 in non-Hodgkin's lymphoma (NHL) were previously available (47), and final results, including additional patients since the initial report, have recently been published (48). This study included 21 patients with NHL, including 17 with low-grade or follicular disease, three with intermediate-grade, diffuse large-cell NHL, and one with mantle cell lymphoma. The patients were treated with between 4.6 and 195.8 mg/m^2/day of G3139 by 14-day subcutaneous (SC) infusion (approximately equivalent to 0.15–5.6 mg/kg/day). One of the 17 patients with low-grade disease had a complete response. This patient was treated at a dose of 73.6 mg/m^2/day (approximately 2 mg/kg/day). He had resolution of nodal and bone marrow disease, and was reported to have improvement in lymphoma-related symptoms (alcohol intolerance). His response was durable, persisting for 3 years without further treatment for lymphoma. Eleven other patients had stable disease, two of whom were reported to have minor responses (tumor improvement that was insufficient to meet criteria for a partial response). These two patients were treated at 36.8 and 110.4 mg/m^2/day; the latter dose was the recommended Phase 2 dose. Minor response or stable disease was observed over a total treatment period of 6 weeks, and the time to lymphoma progression was not specified for these patients. A total of six patients (including the complete responder but not including the two minor responders) were reported to have had improvement in lymphoma-related symptoms. Overall, there was no apparent relationship of tumor improvement, symptom improvement, or stable disease to dose.

An attempt was made in this trial to determine the effects of G3139 on the expression of the Bcl-2 protein in patients' cells. Circulating lymphoma cells, bone marrow aspirates, or fine-needle aspirate specimens from lymph nodes were obtained from 16 patients. Bcl-2 expression in B cells obtained from these sources was assessed by fluorescence-activated cell sorting (FACS) analysis before and after treatment. Reduced Bcl-2 expression of at least 10% (range 12–47%) was reported in one or two tissues obtained from seven of the 16 patients. Overall, reduced Bcl-2 expression was observed in nine of 29 tissue specimens: 5/10 PBMC specimens, 2/8 bone marrow aspirates, and 2/11 lymph node aspirates. In one of the minor responders, Bcl-2 expression was reduced 47% in the lymph node aspirate, but not in the PBMCs or bone marrow. The other minor responder showed no effect on Bcl-2 expression in lymph node (the only tissue studied in that patient). Five of the other six patients with a reported effect on Bcl-2 expression had stable disease; the sixth had progressive disease. (Bcl-2 expression could not be studied in the complete responder.) Effects on Bcl-2 expression in this study did not appear to be related to the dose of G3139.

G3139 is also being studied in combination with chemotherapeutic agents, and results from three Phase 1 trials have been presented in scientific forums. One biopsy-confirmed complete response and two partial responses were reported among 14 patients with melanoma (overall response rate 21%) treated in a dose-finding study of G3139 in combination with decarbazine (DTIC) (49). Response duration greater than 1 year was reported, along with a median survival of 7+ months in the 14 patients. Three additional patients were reported to have had minor responses. In this trial, G3139 was administered by continuous intravenous infusion or twice-daily subcutaneous injection, with DTIC administered at a standard dose after 5 days of G3139. Some of the patients treated had serial biopsies of melanoma lesions before therapy and after 5 days of G3139, and, at doses $\geq$ 1.7 mg/kg/day, lower levels of Bcl-2 expression, by Western blot, were reported in some of the day 5 biopsies when compared with the pretreatment biopsies. In preliminary results from a Phase 1 trial of G3139 plus docetaxel, two partial responses among five patients with breast cancer have been observed, and reduced Bcl-2 expression in peripheral blood lymphocytes has been reported at G3139 doses $\geq$ 2 mg/kg/day by 21-day continuous infusion (50). In a Phase 1 trial of G3139 by 14–21-day continuous infusion in combination with weekly paclitaxel, one "clinical and radiographic" response (degree or tumor type not reported) was observed among 35 solid tumor patients, and reduced Bcl-2 expression was reported after 1 week of treatment with G3139 at a dose of 4.1 mg/kg/day (51).

The chemotherapeutic agent used in each of these Phase 1 combination trials has independent antitumor activity, and further data are needed first to estimate tumor response rates when G3139 is added to chemotherapy, then to deter-

mine more formally the efficacy of such combinations. In addition, the potential for treating low-grade NHL with single-agent G3139 awaits further data. A Phase 2 trial in NHL is in progress, as are several Phase 1–2 trials of G3139 in combination with chemotherapy (Table 3).

E. GEM231 (Hybridon)

GEM231 is an 18-mer phosphorothioate oligonucleotide (5′-GCGUGCCTCCT-CACUGGG-3′) directed against the mRNA for the regulatory subunit α of type I protein kinase A (PKA-I). PKA-I has been reported to play an important role in cell proliferation and neoplastic transformation (52). It is overexpressed in a variety of human cancers and is correlated with unfavorable prognosis (Refs. 17–19 from CCR paper). Referred to in the literature as a "mixed backbone oligonucleotide" (MBO), GEM 231 is a DNA/RNA hybrid oligo that is further "mixed" with regard to the ribose rings of the 18 nucleotides. Four on each end (underlined in the sequence above) are modified with a 2′-*O*-methyl group.

GEM231 is in early clinical development for the treatment of advanced cancers, and results of a Phase 1 trial have recently been published (53). Thirteen patients (three with NSCLC, three with renal cell cancer, three with sarcoma, and four with other tumor types) received GEM231 at doses of 20–360 mg/m^2 by 2-h IV infusion twice weekly for between 3.5 and 10.5 weeks (average, 5.7 weeks). There were no objective antitumor responses. One patient with colon cancer demonstrated stabilization of a previously rising level of the serum tumor marker carcinoembryonic antigen (CEA) after 8 weeks of treatment. Additional Phase 1 trials of GEM231 as a single agent, or in combination with chemotherapy, are in progress or planned.

F. GTI 2040 (Lorus Therapeutics)

GTI 2040 is a phosphorothioate oligonucleotide that targets the R2 component of ribonucleotide reductase. R2 is required for ribonucleotide reduction and also appears to play a role in the MAP kinase signaling pathway. GTI-2040 demonstrated activity in a number of mouse tumor models (54) and is currently in a Phase 1–2 clinical trial in patients with advanced cancers.

G. MG 98 (MethylGene)

Like GEM231, MG 98 (initially developed by Hybridon and subsequently licensed to MethylGene, a Canadian spinout company of Hybridon) is a "mixed backbone oligonucleotide" with 2′-*O*-methyl modifications on several of the 5′- and 3′-nucleotides. MG 98 (previously designated HYB 101584) targets cytosine

DNA methyltransferase, a regulatory protein that has been implicated in cell growth and differentiation and that is overexpressed in some tumors. MG 98 has been reported to inhibit DNA methyltransferase activity and tumor growth in vitro and in vivo (55) and is currently being tested in a Phase 1 trial in patients with advanced cancer. One partial response was reported in a patient with renal cell cancer.

H. Angiozyme (Ribozyme Pharmaceuticals, Inc.)

Angiozyme is a hammerhead ribozyme that targets the mRNA for the Flt-1 receptor for vascular endothelial growth factor (VEGF). Ribozymes differ from "classic" antisense drugs in that, while both types of agents bind to the target sequence by Watson-Crick hybridization, ribozymes are small catalytic RNA molecules capable of cleaving the target mRNA after binding, without requiring cellular RNases. A Phase 1 trial of Angiozyme, administered at doses of 100 or 300 mg/m^2 as a single dose IV or SC to patients with advanced cancer, has recently been reported (56). Treatment was well tolerated, but no anticancer activity was reported. A multidose Phase 1 trial by SC administration is in progress in patients with advanced solid tumors.

III. OUTLOOK AND FUTURE DIRECTIONS

Several phosphorothioate oligonucleotides have been safely administered to patients with advanced cancer, and the clinical activity observed to date provides cause to hope that one or more of these agents will find a place, as single-agent therapy or in combination with other drugs, in the medical oncologist's armamentarium. Data from several clinical trials are consistent with an antisense mechanism of action for these drugs. Ultimately, however, the utility of these agents will be determined by clinical outcomes, particularly survival, in pivotal clinical trials. Trials that are currently in progress or planned will provide important efficacy data for these "first-generation" antisense agents.

The clinical experience to date should be considered part of the beginning of the story of antisense treatment for cancer. In addition to new trials with the drugs currently in the clinic, clinical trials of new antisense drugs against other molecular targets can be expected in the near future. Chemical modifications of these agents that increase their affinity for mRNA and prolong their tissue half-life are likely to make less frequent administration possible (57–59). Development of an oral formulation with acceptable bioavailability (currently in progress) will also make antisense drugs more convenient to administer. The fuller story, yet to be written, promises to be rich.

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Nucleic Acid Therapeutics for the Treatment of Human Leukemia

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I. INTRODUCTION

The development of simple, reliable tools for modifying gene expression "on demand" would represent a major technical advance for cell biologists. Since much progress has been made in understanding the molecular pathogenesis of many diseases, we may easily hypothesize that these same tools could be of tremendous importance to clinicians as well. For example, many genes responsible for cellular transformation have been identified (1). If the function of these genes were shown to be either completely, or relatively, tumor specific, they would become legitimate targets for therapeutic manipulation of their expression. More-effective, less-toxic cancer treatments could reasonably result.

Of the genes we have targeted using the antisense oligodeoxynucleotide (ODN) strategy (2–5), c-myb is of particular interest (6). The c-*myb* protooncogene is the normal cellular homologue of v-myb, the transforming oncogene of the avian myeloblastosis virus (AMV) and avian leukemia virus E26. Myb is a member of a family of transcription factors composed of at least two other highly homologous genes designated A-myb and B-myb (7). Located on chromosome 6q in humans, c-myb's predominant transcript encodes a ~75-kDa nuclear binding protein (Myb) that recognizes the core consensus sequence 5′-PyAAC(G/Py)G-3′ (8).

Myb protein consists of three primary functional domains (9) (Fig. 1). The NH_2 terminus contains a DNA-binding domain, an acidic transactivation domain is found in the midportion, and a negative regulatory domain is localized to the carboxy terminus. Interestingly, the carboxy terminus is deleted in the v-myb-

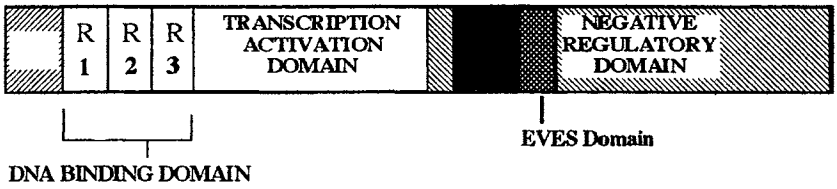

Figure 1.

encoded protein thereby contributing to the transforming ability of the gene. A clinical example supporting this hypothesis has recently been described (10).

Investigations with other deletion and point mutants have suggested that loss of Myb's ability to bind DNA, and to interact with proteins in its environment, are important transforming stimuli (11–13). With regard to the latter point, Dash et al. (14) have reported that the Myb amino and carboxy termini interact with each other through a highly conserved phosphorylation site in the carboxy terminus called the EVES motif. Adapter proteins with a competing EVES site, such as the p100 coactivator, can block the folding mediated by this site and allow Myb to adapt an open, "on"-type configuration. At the same time, the coactivator protein can facilitate interaction with other proteins that modulate Myb's transactivating abilities. It is also of interest that different v-myb mutants will cause different leukemic phenotypes depending on the cell type in which they are expressed. In aggregate, these data suggest the possibility that interactions between Myb and other, as yet unidentified, adapter proteins may play important regulatory roles in Myb's transactivation or repressor functions. Based on these data, it is reasonable to hypothesize that some of these adapter proteins may be unique to the leukemic cell environment. Again, with the concern that Myb is expressed in both normal and malignant cells, our desire to identify leukemic-cell-specific partner proteins is compelling for obvious reasons.

Myb's ability to transactivate genes important for hematopoietic cell proliferation (15–18), and differentiation (19–24), including c-kit (25), may also play a role in its ability to transform cells. Recent work reporting that AMV's v-myb transactivates the homeobox gene GBX2 and that GBX2 in turn mediates autocrine growth and monocytic differentation by transactivation of myelomonocytic growth factor gene (cMGF) illustrates this point (26). Based on these aggregate data, and the considerations discussed above, we have also hypothesized that Myb may transactivate different, or additional, gene targets in leukemic cells. This prompts our search for Myb transactivation targets in leukemic cells since genes of this type may be more tumor-cell-specific, and regardless would be

legitimate targets for ODN-mediated, therapeutically motivated disruption strategies.

II. PRECLINICAL STUDIES WITH ANTISENSE Myb ODN

My laboratory has helped popularize the use of ODN as a research tool, and potential therapeutic. The translational relevance of our findings has been ascertained with in vitro and in vivo models and data extrapolated from these studies have been of great help in the design of Phase I/II clinical trials testing the utility of a c-myb–targeted ODN. The evolution of this work is documented succinctly below.

A. In Vivo Treatment Modeling

ODN were tested for potential clinical efficacy in human leukemia–SCID mouse chimeras (27). Survival of control mice was 6 ± 3 days (mean ± SD) after development of overt disease and was unaltered by treatment with control sequence ODN. Animals treated with c-myb antisense ODN survived ≥3.5 times longer and had altered disease phenotype (Fig. 2). In animals receiving c-myb

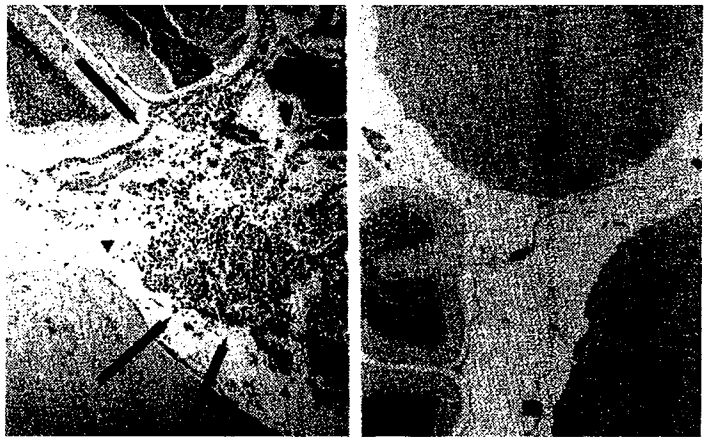

Figure 2. Composite photomicrographs (×400) of mouse brain obtained from chimeric SCID-hu K562 leukemia-bearing mice treated with sense (left) or antisense (right) phosphorothioate c-myb ODN. Note extensive meningeal and subarachnoid infiltration with leukemic blasts in left panel (black arrows) and lack of involvement in right panel.

antisense ODN the central nervous system and ovaries had markedly less leukemic cell infiltration. Clinical utility for the c-myb–targeted ODN was suggested, and helped justify proceeding to Phase I clinical trial of the ODN.

B. Pharmacokinetic/Dynamic Studies

Our ability to detect ODN in tissues and changes in a target gene's expression in tumor tissue has been documented previously (28, Fig. 3). To correlate ODN uptake versus effects on c-myb mRNA expression versus tumor growth, tumor-bearing animals were infused with c-myb AS-ODN (500 μg/day) for 7 days. On days 7, 9, and 11 postinfusion an animal was sacrificed and its tumor excised to determine tissue c-myb mRNA levels.

As shown in Fig. 3, c-myb mRNA levels were measurably decreased (normalized to β-actin) in comparison to expression in controls. When the infusion stopped, myb mRNA level remained low for ~2 days and then began to recover. mRNA recovery was likely related to tissue ODN concentration falling below a critical level. Target down-regulation was clearly specific, but transient, as expected. An important goal of these studies will be to determine the length of time that gene suppression is required for a useful clinical effect.

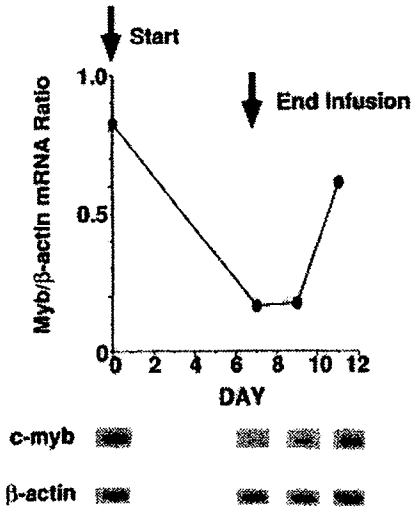

Figure 3.

C. Uptake and Intracellular Trafficking Studies

We have previously studied ODN uptake and intracellular trafficking in the past and will do so as we have reported (29).

The kinetics and localization of ODN/mRNA interactions has been investigated using a novel experimental system recently reported by our laboratory (30). Briefly, we synthesized "molecular beacon" (MB) reporter ODN with matched fluorescent donor and acceptor chromophores on their 5′ and 3′ ends (Fig. 4). In the absence of a complementary nucleic acid strand, the MB remains in a stem-loop conformation where fluorescence resonance energy transfer (FRET) prevents signal emission. Upon hybridization with a complementary sequence, the stem loop opens increasing the physical distance between the donor and acceptor moieties, thereby reducing FRET and allowing a detectable signal to be emitted

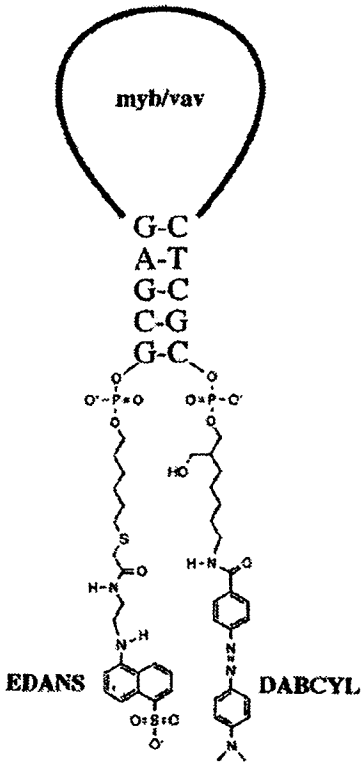

Figure 4.

when the beacon is excited by light of the appropriate wavelength. Solution hybridization studies revealed that in the presence of a complementary strand, targeted MB could yield up to a 60-fold increase in fluorescence intensity in comparison to control MB. Real-time detection of MB/target mRNA hybridizaton in living cells was attempted by microinjecting MB targeted to the vav protooncogene, or control MB, into K562 human leukemia cells. Within 15 min, confocal microscopy revealed fluorescence in cells injected with targeted, but not control, MB. These studies suggested that real-time visualization and localization of OLG/mRNA interactions were possible.

III. INITIAL CLINICAL EXPERIENCES WITH PHOSPHOROTHIOATE-MODIFIED ANTISENSE ODN

A. Bone Marrow Purging with Antisense ODN—Illustrative Examples

After extensive preclinical development (see above), we initiated clinical trials to evaluate the effectiveness of ODN drugs for the treatment of human leukemia. A phosphorothioate modification of the anti-myb ODN is being used as a marrow purging agent in chronic-phase (CP) or accelerated-phase (AP) chronic myelogenous leukemia (CML) patients (pts). As of this writing, we have carried out a total of 18 bone marrow autografts. The marrow employed has been purged for either 24 (13 pts) or 72 h (5 pts) with the ODN. We have also carried out 20 intravenous infusions. The clinical outcomes of these studies will be presented. In brief, major cytogenetic responses were observed in five out of eight evaluable pts who underwent 24-h marrow purges. Of these seven out of eight are still alive up to 4 years postprocedure. The 72-h purging regimen was very effective in vitro but was associated with a prolonged period of marrow hypoplasia. The results of these studies are being prepared for publication.

The course of the first patient we treated is summarized below for illustrative purposes: A 51-year-old interferon-intolerant African-American man with Ph+ CML was autografted with c-myb antisense ODN purged marrow. Prior to transplant, his marrow cells were 100% Ph+; bcr/abl mRNA was detected by PCR. After purging, his marrow cells were Ph−; bcr/abl mRNA was undetectable by PCR (Fig. 5). The patient was prepared for autotransplant with busulfan and cyclophosphamide. He engrafted 12 days after receiving the ODN purged marrow. Approximately 5 years later the patient has evidence of partial cytogenetic relapse (~50% of metaphases are Ph+) but he remains in a complete, unmaintained, hematological remission.

Figure 5. Ethidium bromide–stained agarose gel containing RT-PCR products derived from K562 cells (lane 1, positive bcr/abl control, and patient marrow cells post-ODN purge (lane 2). Note absence of amplifiable bcr/abl band, with positive □-actin from same material. Lane 3 is a negative control conducted with water instead of RNA. Mol wt markers are in extreme left lane.

B. Study of Infused Myb Antisense ODN in Refractory Leukemia Patients

In a Phase I study, a c-myb–targeted phosphorothioate ODN has been administered to 20 patients with CML blast crisis (BC), or other refractory leukemias, by constant intravenous infusion. Dose levels ranged between 0.3 mg/kg/day × 7 days and 2.0 mg/kg/day × 7 days. Hypotension was not observed during the infusions. No consistent metabolic abnormality or coagulopathy was noted. Two patients had incidents questionably related to the study ODN (one transient renal failure, one pericarditis) but these were not observed again when the patients were re-treated at the same or higher doses. One CML blast crisis patient, referred from the Deaconess Hospital in Boston, received anti-myb ODN at a dose of 0.3 mg/kg/d × 7 days q 3 weeks, as well as *intermittent* low-dose hydroxyurea (~1–2 g/day). The patient received a total of nine infusion cycles before control of her disease was lost. She survived ~12 months with excellent quality of life. The life expectancy of blast crisis is typically measured in weeks, regardless of therapy employed.

C. Pharmcokinetic Data

Hybridization is a simple and accurate method of detecting ODN in fluids and tissues. Serum or cells are isolated from the patient using standard methods. DNA

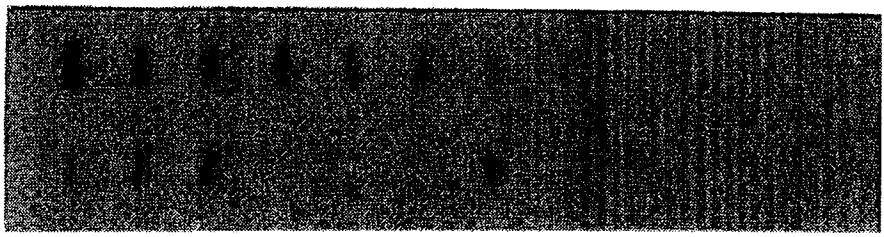

Figure 6. Detection of c-myb AS ODN in serum and peripheral blood of patient receiving ODN infusion, 0.3 mg/kg/day × 7 days. Row A: Lanes 1–8, DNA standards (μg)-[12.5, 3.13, 1.56, 0.78, 0.39, 0.195, 0.09, 0.062 respectively]. Row B: Lanes 1–3 (replicate samples of 250 μl of serum-densitometric comparison with known standards suggests a serum level of 34 μg/mL). Lane 7: Material present in 10^7 peripheral blood cells' densitometric analysis suggests ~0.5 pg/cell. The method gives acceptable accuracy and reproducibility when compared with HPLC.

extracted from cells or serum is slot blotted and hybridized using standard methods (Fig. 6).

D. Toxicity Studies

In agreement with other reports in the literature (31,32), the phosphorothioate ODN was well tolerated from doses of 0.3–2 mg/kg/day × 7 days when administered by continuous infusion.

Table 1 Clinical Outcome of Refractory Leukemia Patients Receiving Anti-myb ODN Infusions as Described in Text

Diagnosis	# patients	Cycles delivered
Refractory AML	7	2 ± 1
Blast-phase CML	7	2.7 ± 2.9
Accelerated-phase CML	6	4.0 ± 1
	Total = 20	Mean: 2.9 ± 2

Progressive disease (<2 cycles): 8/20 pts (40%).
"Stabilization" of disease (>2 cycles): 12/20 pts (60%).
"Stabilization" with hematological improvement: 5/20 pts (25%).

E. Clinical Outcome of Refractory Leukemia Patients Treated with ODN Infusions

Though only 20 patients have been studied, we have followed outcomes of the patients who were infused with the OLG. The number of patients, diagnosis, and number of infusions the patients were able to receive are tabulated in Table 1. Note that one cycle = 1 week of drug infusion followed by 3 weeks of rest. Patients received no other therapy. Outcomes were classified as progressive, disease stabilization, or stabilization with hematological improvement. Patients with accelerated-phase CML would have received additional cycles of therapy but the study was stopped prematurely because the drug supply was discontinued.

IV. CONCLUSIONS

Many challenges remain to be addressed before nucleic acid–based therapeutics become a reality. A significant problem in this field is the limited ability to deliver ODN into cells and have them reach their target. Biological inefficiency is the predictable result of these problems. Nevertheless, ODN can escape from the vesicles intact, enter the cytoplasm, and then diffuse into the nucleus where they presumably acquire their mRNA, or in the case of decoys, protein target. The processes that regulate such trafficking and ultimately govern whether and where an oligonucleotide can interact with its target reamin poorly understood and need elucidation.

Efficient delivery of antisense molecules will not solve all the problems, however. For an ODN to hybridize with its mRNA target, it must find an accessible sequence. Sequence accessibility is at least in part a function of mRNA physical structure, which is dictated in turn by internal base composition and associated proteins in the living cell. Attempts to describe the in vivo structure of RNA, in contrast to DNA, have been fraught with difficulty. Accordingly, mRNA targeting is largely a random process, accounting for many experiments where the addition of an ODN yields no effect on expression. Strategies to address this fundamental problem are at present under development.

Those of us who care for patients with cancer are constantly reminded of the importance of work focused on the mission of making anticancer therapeutics less morbid and less deadly to those unfortunate individuals who must endure it. Investigators are following many paths toward this goal, including strategies designed to interrupt the most fundamental of processes within a cell, gene expression. When this goal is finally accomplished, as it must be, it will truly represent the culmination of many small steps toward a truly giant leap for humankind.

ACKNOWLEDGMENTS

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ISIS 2302, an Antisense Inhibitor of Intercellular Adhesion Molecule 1 (ICAM-1)

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I. BACKGROUND

A. Intercellular Adhesion Molecule 1 (ICAM-1)

ICAM-1, a member of the immunoglobulin superfamily, is an inducible transmembrane glycoprotein constitutively expressed at low levels on vascular endothelial cells and on a subset of leukocytes (1–3). In response to proinflammatory mediators, many cell types up-regulate expression of ICAM-1 on their surface. The primary counterligands for ICAM-1 are the beta-2 integrins, LFA-1 and Mac-1, expressed on leukocytes (4–6).

ICAM-1 serves multiple functions in the propagation of inflammatory processes, the best characterized being facilitation of leukocyte emigration from the intravascular space in response to inflammatory stimuli (7–9). ICAM-1 also appears to provide an important secondary signal during antigen presentation (10–12), and to play an important facilitatory role in cytotoxic T-cell (13), NK-cell (14), and neutrophil-mediated (15) damage to target cells. Any or all of the above functions make ICAM-1 a theoretically attractive therapeutic target for a broad spectrum of inflammatory and autoimmune diseases.

Numerous studies have demonstrated an increase in ICAM-1 expression within involved tissue from patients suffering from a wide range of diseases with an inflammatory component, including inflammatory dermatoses, inflammatory bowel disease, rheumatoid arthritis, glomerulonephritis, systemic lupus, vasculi-

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tis, atherosclerosis, organ allograft rejection, multiple sclerosis, and reperfusion injury (16–32).

ICAM-1 monoclonal antibodies have been used to demonstrate beneficial effects in a variety of animal models of disease, including pulmonary inflammation and asthma (33,34), prevention of allograft rejection (35,36), nephritis (37,38), ischemic injury (39,40), arthritis (41), and contact dermatitis (42), further suggesting that inhibitors of ICAM-1 function or expression could have broad therapeutic utility. A murine ICAM-1 antibody (BIRR-1) has also demonstrated evidence of efficacy in Phase 1/2 studies in rheumatoid arthritis (43) and renal transplantation (44), but owing to immunogenicity was not adequately tolerated upon repeated administration to permit further development in rheumatoid arthritis (45). In a large renal transplant study, five daily infusions against a background of standard immunosuppression was not effective in reducing the incidence of acute rejection (46), perhaps because the course of therapy was too short, the antibody activated endothelial cells by crosslinking surface ICAM-1, or the development of antidrug antibodies limited the duration of action.

B. ISIS 2302 and Animal Analogs

1. Pharmacology

ISIS 2302 is 20-base phosphorothioate oligodeoxynucleotide (sequence 5′ GCCCAAGCTGGCATCCCTCA 3′) that inhibits (ICAM-1) expression through an antisense mechanism of action (47,48). ISIS 2302 is designed to specifically hybridize to a sequence in the 3′ untranslated region of the human ICAM-1 message.

ISIS 2302 selectively inhibits cytokine-induced ICAM-1 expression on a wide variety of human cells in vitro (49–51). Functional effects of ICAM-1 down-regulation can also be demonstrated in vitro: inhibition of leukocyte adherence to human endothelial cells (47,52) and intestinal fibroblasts (53), and decreased T-cell proliferation to IL-4/GM-CSF–treated monocytes (dendritic cells, Isis Pharmaceuticals, unpublished data). A murine analog, ISIS 3082, has been shown to be active in multiple models of inflammation including prolongation of cardiac allograft survival (54), carrageenan-induced neutrophil infiltration (Isis Pharmaceuticals, unpublished data), dextran sulfate-induced colitis (55), endotoxin-induced neutrophil migration (56), and collagen-induced arthritis (Isis Pharmaceuticals, unpublished observations). In each study, control oligonucleotides failed to demonstrate pharmacological activity, suggesting that the anti-inflammatory activity of ISIS 3082 was due to inhibition of ICAM-1 expression. In the endotoxin pneumonitis and the dextran sulfate–induced colitis models, down-regulation of either ICAM-1 message or protein in involved tissue was sought and demonstrated. A rat analog, ISIS 9125, was also effective in the prevention

of rat cardiac and renal allograft rejection (57). Rat analogs have also been effective in preventing renal reperfusion injury in this species (58–60).

2. Pharmacodynamics

As an antisense drug, ISIS 2302 inhibits de novo synthesis of ICAM-1, and pharmacological activity must therefore await turnover of already expressed protein. As an inducible protein with very low constitutive expression, an in vitro assessment of the pharmacokinetics of down-regulation of ICAM-1 message and protein is further complicated by the need for induction. For constitutively expressed proteins, peak effects on message content in vitro are generally seen after 4–6 h of drug exposure with other phosphorothioate oligodeoxynucleotides (ODNs). In cell culture with human endothelial cells, inhibition of ICAM-1 message and protein induction by TNF-α is seen after a 4-h exposure to ISIS 2302, with an IC_{50} of ~25 nM (47). In animal models of disease, therapeutic activity was generally seen with doses ranging from 0.03 to 10 mg/kg, depending upon the model, administered 3 times weekly to daily over 1–2 weeks. Down-regulation of ICAM-1 message, protein, or function was also demonstrated in target tissue from the models in which it was investigated.

3. Pharmacokinetics, Metabolism, and Toxicology

ISIS 2302 behaves similarly to all other phosphorothioate ODNs, and similarly across species (61,62). In rodents and monkeys, ISIS 2302 and other phosphorothioate ODNs are poorly absorbed (<5%) after oral administration, but well absorbed after intraperitoneal, intradermal, or subcutaneous injection. In blood, ISIS 2302 is highly protein bound (>99%), with low-affinity binding primarily to albumin. The compound does not cross an intact blood-brain barrier, but is otherwise broadly distributed, with principal uptake in the liver, kidney, and spleen. The plasma distribution half-life ranges from ~30 to 45 min in animals, but the tissue half-life is much longer, varying from tissue to tissue with a range of ~1–5 days. ISIS 2302 is metabolized as a native nucleic acid, by nuclease, primarily 3′ exonuclease, digestion with subsequent nucleotide metabolism. Urinary and biliary excretion of intact drug and its primary metabolites, chain-shortened oligonucleotides, is minimal. A ^{14}C label at the 2′ position of the thymine residues of another first-generation phosphorothioate ODN was primarily eliminated as exhaled CO_2 in the rat (63). Collectively, these data suggested that an every-other-day to 3-times-weekly dose regimen would be appropriate for humans, with minimal tissue accumulation.

In planning the Phase I study, it was important to include the effective dose range in animals and to take into account the toxicities observed in monkeys. In cynomolgus monkeys, doses of ISIS 2302 up to 50 mg/kg every other day for

1 month (64) and 20 mg/kg for 6 months (Isis Pharmaceuticals, unpublished data) have been investigated. The cynomolgus monkey ICAM-1 message differs for only one of 20 bases in the targeted region of the message, and ISIS 2302 therefore has substantial pharmacological activity (IC_{50} ~ twice that of the simian analog) in vitro on simian endothelial cells (ISIS Pharmaceuticals, unpublished data). ISIS 2302 was very well tolerated, with the principal toxicities consistent with other phosphorothioate ODNs and due to nonspecific protein binding in blood: transient (2–4 h) and modest prolongation of the activated partial thromboplastin time (aPTT) due to inhibition of intrinsic tenase complex activity (65), and alternative pathway complement activation, probably due to inhibition of Factor H and/or I activity, inhibitory proteins of the alternative pathway (66). Both laboratory effects were clearly related to peak plasma drug levels, with a threshold of ~50 μg/mL for complement activation, and ~30 μg/mL for prolongation of aPTT. Humans in vivo and/or in vitro appear to be more sensitive to the anticoagulant effect and less sensitive to the complement-activating effect of phosphorothioate antisense drugs.

II. CLINICAL STUDIES WITH ISIS 2302

A. Phase 1

In planning initial exposure in humans, it was felt that a peak plasma level of 10–15 μg/mL, or approximately 20–30% of the threshold for complement activation in monkeys, would provide an adequate margin of safety for initial Phase 1 studies. Extrapolating from monkey data, it was projected that a 2-mg/kg, 2-h infusion would produce such peak drug levels in humans. Furthermore, 2 mg/kg was within the therapeutic range in animal models. A 2 mg/kg by 2-h i.v. infusion was therefore selected as an appropriate target regimen for the initial Phase 1 trial.

In a Phase 1 i.v. study, single and multiple (four every-other-day doses) doses of ISIS 2302 from 0.06 to 2 mg/kg were administered by 2-h intravenous infusion (67). All doses were well tolerated, and dose-limiting toxicity was not observed. As expected, dose-related, modest (~50% above control at the 2 mg/kg dose), and transient (2–4 h) increases in aPTT were observed with infusion, and threshold effects on complement activation (C3a but not C5a generation) were observed. Pharmacokinetic behavior was predictable, and very similar to that in monkeys. ISIS 2302 was well behaved: peak plasma levels were related to dose (10 μg/mL at 2 mg/kg), and the plasma distribution half-life was 60 min.

In parallel with the Phase 2 i.v. trials, a Phase 1 subcutaneous trial was conducted with the saline formulation. ISIS 2302 was well tolerated subcutaneously in monkeys. Plasma bioavailability as compared to i.v. administration was

~50%, with a time to maximal plasma concentration of 1–3 h after injection. In the Phase 1 study, pharmacokinetic behavior was similar to that observed in monkeys, but dose-limiting toxicity was observed at 2 mg/kg every other day (68). All concentrations of ISIS 2302 from 50 to 200 mg/mL caused local erythema and edema. The maximum well-tolerated dose was 0.5 mg/kg daily: higher doses were associated with regional lymphadenopathy, and the 2 mg/kg dose also caused moderate C3a (but not C5a) generation and was associated with constitutional symptoms. It is presumed that regional lymphadenopathy and complement activation were due at least in part to direct complement activation: mg/mL or mg/g concentrations of drug were likely achieved in regional lymph and lymph nodes, well above the 50 μg/mL threshold for complement activation defined in monkeys.

B. Phase 2a Trials

Phase 2a trials were conducted in four indications: Crohn's disease, rheumatoid arthritis, prophylaxis of acute renal allograft rejection, and psoriasis.

To increase the opportunity to demonstrate a therapeutic effect, drug exposure in these trials was maximized within the constraints of available 1-month toxicology data and our experience in normal volunteers. The animal data limited us to 1 month of exposure in North America. Threshold effects on complement activation (C3a generation) and 50% increases in aPTT with a 2-h infusion of 2 mg/kg in normal volunteers argued that initial exposure in patients should be limited to this dose and regimen. Such an increase in aPTT was a concern in inflammatory bowel disease and the perioperative state, and it was unknown whether patients with autoimmune and inflammatory conditions might be more or less sensitive to alternative pathway complement activation. Further, 2 mg/kg is within the therapeutic range in mice, and with similar pharmacokinetics in humans and mice, including drug exposure at a given mg/kg dose, it was reasonable to expect that this dose level would be therapeutic in humans. The Phase 2a trials were therefore designed as fixed-dose-within-patient, dose-escalation studies, generally beginning at 0.5 mg/kg and escalating to 1 and 2 mg/kg every other day i.v. in successive cohorts.

These studies enrolled refractory patients, with efficacy being assessed by standard, well-accepted outcome measures. All but the psoriasis trial was double-blinded, placebo-controlled, and randomized (3:1; study drug:placebo), and all but the renal transplant study involved a 4-week treatment period: treatment in the renal transplant study was for 2 weeks owing to logistical considerations—many patients came from a long distance. In addition, the renal transplant study differed from the other studies by specifying two additional lower-dose groups of 0.05 and 0.1 mg/kg, and by providing for a Phase 1 and a Phase 2 segment.

In each study, 17–52 patients were enrolled at one or two centers. Patients were followed for up to 6 months after the treatment period or until disease relapsed or failed to respond to study drug.

1. Crohn's Disease

This study was a 20-patient, double-blinded, placebo-controlled, randomized (3:1; study drug:placebo), single-center study that enrolled steroid-dependent patients with moderately active Crohn's disease despite background corticosteroids (≤40 mg prednisone or equivalent/day) (69). Four patients each were assigned to the 0.5 and 1 mg/kg dose group, and the remaining 12 patients to the 2 mg/kg dose group, and all patients received 13 i.v. infusions of ISIS 2302 or placebo over 26 days. Patients were then followed for a total of 6 months. Moderately active was defined as a Crohn's Disease Activity Index (CDAI) ≥ 200 and ≤ 350. Background 5-ASA drugs in stable dosage were also permitted. Corticosteroids were to remain stable during the 26-day treatment period. The primary efficacy measures were the CDAI and corticosteroid dosage. Endoscopic Index of Severity (EIS) and the Inflammatory Bowel Disease Questionnaire, a quality of life measure, were also followed.

The CDAI is a validated clinical instrument (70,71) that is widely accepted as the "gold standard" in evaluating clinical disease activity and response. It is a composite score based upon patient diaries and more objective measures, and can range from 0 to approximately 600. A CDAI of <150 is widely accepted as defining disease remission, and a decrease of 70–100 points as a response.

Over the investigated dose range of 0.5–2 mg/kg there was no consistent evidence of a dose response for clinical measures, and data are therefore presented for the combined ISIS 2302 group as compared to placebo. At the end of the treatment period, seven of 15 (47%) ISIS 2302–treated and one of five placebo-treated patients, a patient already in remission at baseline though with active disease at screening, were in remission. At the end of month 6, five of the seven ISIS 2302-treated remitters were still in remission, and a sixth patient had a CDAI of 156. At the end of the treatment period, exercising blinded clinical judgment, the investigator chose to increase the mean corticosteroid dosage in the placebo group and decrease steroid dosage in the ISIS 2302 group. These differences persisted throughout the remaining 5 months of the study. At the end of the study (month 6), five (33%) ISIS 2302–treated and none of the four remaining placebo-treated patients were completely weaned from corticosteroids.

Supportive trends favoring ISIS 2302 over placebo were also observed in other clinical (EIS and IBDQ) and pharmacological measures. Mucosal biopsies from the same area of the intestine were obtained at baseline and day 26, immunohistochemically stained for ICAM-1, and qualitatively assessed by a blinded pathologist as to whether ICAM-1 expression had increased, remained constant, or

decreased. Statistically significant reductions in ICAM-1 expression were observed in the ISIS 2302 as compared to the placebo group ($p = 0.033$); ICAM-1 was judged to be reduced in seven of nine patients receiving the 2 mg/kg dose as compared to one of the five placebo patients.

Two patients with upper gastrointestinal obstructive symptoms due to gastroduodenal Crohn's disease experienced resolution of these symptoms, and the two patients with an open enterocutaneous fistula at baseline experienced closure: all four patients received ISIS 2302.

By amendment to the protocol, upon disease relapse after completion of the original trial, patients were offered open-label treatment with ISIS 2302 at their originally assigned dose and schedule (72). Seven patients availed themselves of retreatment 7–13 months after their original therapy: four of these patients (responders) had experienced sustained remission (three) or response (one), two patients (nonresponders) had experienced only short-lived responses to original treatment with ISIS 2302, and the one placebo-treated patient had experienced a late and short-lived remission. During the treatment protocol, all four previous responders again experienced a sustained remission or response that lasted at least through the 6-month retreatment trial, as did the previously placebo-treated patient. As expected, the two previous nonresponders experienced only short-lived improvements in their disease activity. ISIS 2302 continued to be well tolerated upon retreatment. These data, though open-label and limited, suggest that retreatment with ISIS 2302 is both safe and effective in previous responders.

Based upon the encouraging safety and efficacy profile of ISIS 2302 in the pilot study, a 300-patient pivotal trial exploring the steroid-sparing and remission-inducing qualities of ISIS 2302 was initiated, as well as two smaller studies investigating short-duration (5 day) i.v. as well as low-dose (0.5 mg/kg) s.c. administration.

2. Rheumatoid Arthritis

The rheumatoid arthritis trial was conducted at two centers in patients with active disease ($\geq$10 swollen joints plus at least two of the following: tender joints $\geq$12, morning stiffness $\geq$1 h, ESR $\geq$25 for men and 35 for women) despite NSAIDs $\pm$ low-dose corticosteroids $\pm$ approved second-line agents. This was a placebo-controlled, double-blinded trial that recruited 43 patients (73). The study was initially designed for 20 patients; four each at the 0.5 and 1 mg/kg level and the remainder at the 2 mg/kg level. The study was subsequently expanded to 40 patients after the 2 mg/kg dose level had been reached. An interim analysis of the first 20 patients suggested that the 0.5 mg/kg dose might be the most effective, so all additional patients were then assigned to this dose group. Due to commitments to patients, enrollment was extended to 43. These modifications resulted in 11 placebo-treated patients, and 32 ISIS 2302-treated patients (10 received 0.5

mg/kg, 3 received 1 mg/kg, and 19 received 2 mg/kg). The treatment regimen, duration of treatment, and follow-up period were the same as for the Crohn's disease study. Efficacy was primarily assessed by composite scoring (Paulus) (74).

Patients enrolled in this study were typical of patients enrolled in studies of this nature, with long-standing, refractory disease. Patients averaged approximately 22 swollen joints and were an average of approximately 58 years old at baseline. By individual efficacy measures and Paulus 20 criteria, similar improvements were seen in all treatment groups during the treatment period and month 2. By month 4, the ISIS 2302–treated groups were faring better than the placebo-treated patients, with the best results observed in the 0.5 mg/kg group. From months 4 to 6, Paulus 20 response rates were fairly constant, averaging ~40% for the 0.5 mg/kg group, ~20% for the 2mg/kg group, and ~10% for the placebo group. As in the Crohn's study, ISIS 2302 was well tolerated.

Although the rates of response seen late in this trial are comparable to that observed for many traditional second-line agents, they are modest compared to that recently reported for the TNF-alpha–directed therapies etanercept and infliximab. The delayed differentiation from placebo observed in this trial asks the question of what the results of more prolonged therapy might be. Further trials in rheumatoid arthritis with ICAM-1 antisense await the development of an orally bioavailable formulation or chemistry.

3. Psoriasis

Owing to anticipated difficulty with patient acceptance of a placebo control in this disease, the psoriasis study was the only Phase 2a study that was open-labeled and uncontrolled. Seventeen patients with moderately active (5–40% body surface area involvement) plaque-type psoriasis vulgaris, resistant to medium-potency topical corticosteroids, were enrolled (Isis Pharmaceuticals, unpublished data). Patients were washed out from all antipsoriatic medication prior to baseline. All patients received 13 infusions of ISIS 2302 over 26 days: four patients received 0.5 mg/kg, three patients 1 mg/kg, and ten patients 2 mg/kg. There was no evidence of a dose response. At the end of the treatment period, an approximate 30% reduction in the Psoriasis Area and Severity Index (PASI) (75) was observed that was not long-lived. This may represent a modest therapeutic effect or a placebo effect.

It was known at the outset of the i.v. trial that phosphorothioate ODNs do not distribute well to dermis, particularly epidermis with parenteral administration, but owing to molecular size and charge, it was thought that delivery via a topical formulation could not be readily achieved. However, a topical formulation has now been developed that achieves drug concentrations in the dermis and epidermis 2–3 logs higher than that achieved with i.v. dosing; with the topical

formulation, unlike with the i.v. route, substantial inhibition of ICAM-1 up-regulation can be demonstrated in mouse skin and in human skin xenografts on SCID mice (103). Animal topical tolerability studies have completed, and a topical paired plaque comparison clinical trial in patients with mild psoriasis has begun.

Sixty patients with mild psoriasis (5–15% of body surface area) with two symmetrical plaques on the trunk or upper extremities (proximal to the elbows and knees) are being enrolled. Five different concentrations of ISIS 2302 ranging from 0.03% to 4% drug in a cream formulation are applied once daily for 90 days or until the plaque clears. Placebo constitutes the sixth treatment. Each of two symmetrical plaques in a given patient are randomized to receive a different concentration of drug or placebo. The study design alllows for both intra- and interpatient comparisons. Since there are 120 plaques in this study (two from each patient), 20 plaques in the study will receive each concentration of study drug or placebo. Patients will then be followed until the end of month 6. In this way, both the kinetics of onset of action and duration of action can be assessed. If this study is positive, Phase 3 studies could commence utilizing one or two concentrations of drug compared to placebo.

4. Renal Transplantation

The prophylaxis of acute renal cadaveric allograft rejection protocol is being conducted at a single center as a double-blinded, placebo-controlled, randomized (3:1) study. Other than indication, it differs from the other Phase 2a studies in four respects: (1) since most patients come from a distance, therapy is limited to 2 weeks for logistical reasons; (2) the study is divided into a Phase 1 and a Phase 2 segment; (3) two lower-dose groups (0.05 and 0.1 mg/kg) are included; and (4) owing to the unknown potential for complement activation and bleeding in the immediate perioperative period, the first two infusions are administered over 6 h, the second two infusions over 4 h, and the remaining three infusions over 2 h to reduce maximum plasma concentrations of drug during the immediate perioperative and postoperative periods.

In the Phase 1 segment that is now completed, patients with stable cadaveric renal allografts at least 6 months postoperatively were administered a single 6-h infusion of ISIS 2302 followed 1 week later by a 2-week-every-other-day regimen against a stable background of prednisone and cyclosporin A (CsA). Four patients each were assigned to the 0.05, 0.5, 1, and 2 mg/kg dose groups. All doses were well tolerated. There was no evidence of a pharmacological or pharmacokinetic interaction between ISIS 2302 and CsA: baseline CsA blood levels were not changed by concomitant administration of ISIS 2302, and at a given dosage, ISIS 2302 blood levels were similar to blood levels observed in normal

volunteers. Furthermore, no patient experienced a rejection episode during or after treatment with ISIS 2302. As predicted, dose-related, transient increases in aPTT similar in magnitude to increases experienced by normal volunteers at equivalent doses were observed in the renal transplant patients.

In the Phase 2 segment, four patients were assigned to the 0.05 and 0.1 mg/kg group, eight patients each to the 0.5 and 1 mg/kg dose groups, and 12 patients to the 2 mg/kg dose group. De novo renal transplant patients, recipients of cadaveric allografts, were administered seven every-other-day infusions of ISIS 2302, beginning during the transplant procedure, in addition to their usual regimen of prednisone and CsA. Patients were followed for 6 months posttransplantation to assess the incidence of acute rejection in this time period.

The incidence of acute rejection has been validated as an early surrogate for graft survival (76–78), and served as the basis for the recent FDA approvals of mycophenolate mofetil, two different IL-2 receptor antibodies for the prophylaxis of acute renal allograft rejection, and sirolimus (79–86). With standard therapy, consisting of prednisone and cyclosporin A ± azathioprine, the incidence of acute rejection within the first 3–6 months posttransplantation is ~40–50%.

Interpretation of this study is complicated by a low rate of acute rejection in the placebo arm (two of nine patients, or 22.2%), a rate of rejection approximately half of what would have been expected from historical controls. The rate of rejection for all ISIS 2302 patients was nine of 27, or 33.3%, representing a reduction from historical controls, but a somewhat higher rate of rejection than that observed in the placebo patients. For reasons that have not been delineated but may have to do with immune response genes, effectiveness of immunosuppressive regimens, and/or histocompatibility between donor and recipient, graft survival, although improving in all races, remains considerably shorter in African-Americans than in recipients with other genetic backgrounds (87). Higher rates of rejection have been reported with mycophenolate mofetil in African-Americans than in Caucasians (88). Interestingly, in the present study, acute rejection was observed in only one of 11 Caucasian recipients (9.1%) treated with ISIS 2302 and two of five placebo-treated Caucasians (40%). Further study is being contemplated, with stratification of patients by race and employment of higher dosages of ISIS 2302.

5. Ulcerative Colitis

A placebo-controlled, double-blinded enema study in distal ulcerative colitis is under way. ISIS 2302 by enema is taken up in mg/g concentrations by intestinal epithelium in experimental animals, and is well tolerated. At this time it is not known whether a topical approach with ISIS 2302 will be effective in this disease, but an antisense to NF-κB administered by edema was reported to be effective

in treating established colitis in two mouse models: IL-10–deficient mice and TNBS-induced colitis (89).

In the distal ulcerative colitis study, 40 patients are being randomized to 28 nightly enemas, and will then be followed through the end of month 6. This is a dose-escalation study with cohorts of 10 patients being sequentially randomized 4:1 to ISIS 2302 or placebo in a 75-mL enema to deliver 60 mL. Concentrations of ISIS 2302 are 0.5, 1, 2, and 4 mg/mL. Roughly translating into doses of 0.5–4 mg/kg. Patients will be assessed periodically by clinical scoring and sigmoidoscopy. Mucosal biopsies will also be assessed for ICAM-1 expression, and if feasible, drug concentrations.

6. Asthma

ISIS 2302 and other ODNs readily lend themselves to aerosolization. Preliminary data in animals demonstrate good tolerability, local drug uptake, and pharmacology via the inhaled route. Antisense compounds to ICAM-1 and other inflammatory/immune molecules are being investigated preclinically to select the most promising molecular target(s) for clinical development. If ISIS 2302 is chosen for development, then Phase 1/2 asthma studies could begin within a year.

C. Phase 2b Crohn's Disease

A 299-patient, randomized, double-blinded, placebo controlled 6-month study in steroid-dependent (10–40 mg/day of prednisone or equivalent for at least 3 months), active Crohn's disease (CDAI 200–350) was just completed (CS9). The study design was a modification of the North American Methotrexate Study (Feagan et al.). Beginning on day 8, patients in the high-dose corticosteroid stratum (20–40 mg/day) were assigned to 20 mg/day of prednisone and were tapered by 2.5 mg/day/week thereafter as tolerated, so that patients had the opportunity to be at a dosage of zero at the beginning of week 10. Patients in the low-dose stratum (10–19 mg/day) remained on their entry level of prednisone, and began tapering dosage by 2.5 mg/day/week on the same protocol day that a patient entering in the high-dose stratum would have tapered to a lower dosage. In this way all patients had the opportunity to be completely weaned from corticosteroids on the same protocol day. A 4- and a 2-week regimen of ISIS 2302, 2 mg/kg i.v. three times weekly during months 1 and 3 were compared to placebo. The primary endpoint in this trial was a combined endpoint, complete clinical remission at the end of week 14, defined as a CDAI < 150 *and* a steroid dose of zero.

A limited interim analysis of the first 150 patients was preagreed with the FDA so that a decision could be taken as to whether to begin the approximate 1-year preparations required for potential commercial-scale manufacturing if this

pivotal-quality trial proved successful. At interim analysis, a similar proportion of patients in the 2- and 4-week arms achieved steroid-free remission at the end of week 14. Combining the data from the two arms, the rate of steroid-free remission was 29% in ISIS 2302–treated patients and 14% in placebo-treated patients ($p = 0.047$), projecting a highly significant p-value at the end of the study if the pattern persisted. Unfortunately, in the second half of the study the response pattern reversed (12.2% vs. 25.5%, ISIS 2302 vs. placebo, $p = 0.063$) resulting in overall rates of 20.7% and 19.8%, respectively. A similar pattern was observed for premature discontinuations for lack of efficacy or disease progression: for ISIS 2302 versus placebo, 16.0% versus 34.0% for the first half of the study ($p = 0.0203$), 20.4% versus 5.9% for the second half of the study ($p = 0.0295$), and 18.2% versus 19.8% in the final analysis. Differences between the two halves of the study in the rate of steroid-free remission for ISIS 2302 patients (29.0% vs. 12.2%, $p = 0.047$) and in the rate of premature discontinuations in placebo-treated patients (34.0% vs. 5.9%, $p = 0.0004$) were highly statistically different, strongly suggesting that the observed differences were not due to chance. Errors in dosing, randomization, and programming were ruled out, and integrity of the drug supply was confirmed. Logistic regression analysis and serological profiling revealed rates of steroid-free remission that was significantly greater in ISIS 2302–treated women than men (25.4% vs. 12.5%, $p = 0.04$), and in ISIS 2302–treated ASCA+ p-ANCA− patients than in patients with all other serological patterns (28.3% vs. 14.8%, $p = 0.04$). Rates of steroid-free remission in ASCA+ p-ANCA-patients in the 2-week arm were 44% for women and 30% for men, although the numbers of patients in these subsets was relatively small (16 and 13, respectively).

Estrogen, but not progesterone, has been demonstrated to increase TNF-alpha–induced ICAM-1 expression and leukocyte adhesion to human umbilical vein endothelial cells in vitro (90), and may explain why women might be more effectively treated with a therapy that inhibits ICAM-1 expression. Interestingly, a markedly negative effect of birth control pills on maintenance of Crohn's disease has been reported (91). The fact that differences were only seen within treatment, and not across treatment (i.e., compared to placebo), obviously weakens an inference of a gender effect in CS9.

ASCA (anti-*Saccharomyces cerevesiae* antibodies) and p-ANCA (peripheral antineutrophil cytoplasmic antibodies) are two antibodies that have recently been reported to be relatively sensitive (~55–65%) and highly specific (~95%) for Crohn's disease and ulcerative colitis, respectively (92–102). In our study, only approximately 46% of the patients were ASCA+, approximately 9% of these patients having an antibody to p-ANCA as well. Approximately 40% of the patients had neither marker, and about 15% were positive for p-ANCA and negative for ASCA. The clinical value of serological testing for ASCA and p-ANCA is being debated, but if ASCA positivity defines a serological subset that

is responsive to ISIS 2302 then the merits for disease categorization would not be relevant to identifying patients who might respond to ISIS 2302.

Although subject to the usual criticism for a post hoc analysis, investigation of the relationship between drug exposure and outcome has led to a possible explanation for the failure of this study. Area under the curve (AUC) per dose was consistent within patients but increased with obesity index and female gender, resulting in a 5-fold range in per dose exposure in this study (from 13.49 to 96.09 μg·hr/mL). Although few patients appear to have received adequate exposure, achievement of the primary endpoint was strongly related to exposure per dose ($P = 0.007$), with rates of steroid-free remission at the end of week 14 ranging from 15.6% (15/96) in patients with an AUC per dose ≤ 40, to 38% (5/13) of those with an AUC > 60, to 55% (5/9) to those with an AUC > 65, to 75% (3/4) of those with an AUC > 75 μg·hr/mL. Compared to the overall placebo rate of 19.8%, the response rate for the AUC > 65 μg·hr/mL subgroup was significantly greater ($P = 0.023$). Similar rates were observed in the 2- and the 4-week groups in the AUC > 65 μg·hr/mL subgroup: 3 of 5 (60%) of the patients in the 2-week arm and 2 of 4 patients (50%) in the 4-week arm achieved the primary endpoint of steroid-free remission. Increasing AUC per dose was also significantly related to improvements at the end of week 14 in CDAI and IBDQ. Mean duration of remission increased with exposure, but the results were not significant.

Two smaller trials examined the effectiveness of shorter, more concentrated i.v. dosing and low-dose subcutaneous regimens, respectively. Both trials were identical in design to the 299-patient trial, except in the dose regimens employed and in the number of patients per treatment arm (30). In the short-induction i.v. trial (CS10), daily doses of 2 mg/kg for 5 days, 3 days, and 1 day were compared to placebo. In the subcutaneous trial (CS11), ISIS 2302 at a dosage of 0.5 mg/kg was administered daily 5 days per week for 4 weeks, 2 weeks, and 1 week, and for 2 days, and was compared to placebo. Both studies were discontinued early (85 of 120 planned patients in CS10, 75 of 150 for CS11) to focus efforts on completing the pivotal trial (CS9) after interim analyses demonstrated substantially less efficacy for the primary endpoint (steroid-free remission at the end of week 14) than was observed at interim in the pivotal trial. Analyses of these trials is ongoing, and interestingly, although the overall results were disappointing, there is evidence of efficacy in CS11, the subcutaneous dosing trial.

In CS11, trends favoring ISIS 2302 over placebo were seen in the more stringent efficacy variables. At week 14, two of 60 patients treated with ISIS 2302 (3.3%) but none of 15 placebo patients were in steroid-free remission, and at week 26, the end of the study, eight ISIS 2302 patients (13.3%) and only one placebo patient met criteria for this endpoint. Rates of low-dose steroid-dependent remission (prednisone ≤ 10 mg/day) also favored ISIS 2302 over placebo at weeks 14 and 26, 8.3 versus 0% and 21.7 versus 13.3%, respectively. By these

criteria and average CDAI over time, the 1- and 2-week regimens had the best response. Although the average total steroid consumption by weeks 14 and 26 was similar among treatment groups, median steroid consumption did favor ISIS 2302 over placebo (1752 vs. 2533 mg and 2969 vs. 4553 mg, respectively, at these time points). Disease progression as an adverse event or reason for termination was almost twice as frequent in placebo as in ISIS 2302–treated patients (40 vs. 22%, respectively). Twenty-three percent of ISIS 2302–treated and no placebo-treated patient experienced injection site reactions, usually mild or moderate, but one patient discontinued from the study owing to severe injection site reactions. Higher doses may therefore not be tolerated (2 mg/kg s.c. was not tolerated in the Phase 1 trial), but the data suggest that higher doses or a longer duration of treatment might be more effective.

III. SAFETY AND TOLERABILITY

ISIS 2302 has now been administered to ~400 patients, 310 of whom have Crohn's disease, and to approximately 60 healthy volunteers. ISIS 2302 has been well tolerated. The only consistent drug effect is a transient, approximate 10–15-s increase in aPTT at the 2 mg/kg dose that has been without clinical sequelae. This is not surprising since the prolongations in aPTT are at the lower end of target prolongation for heparin anticoagulation therapy, and last only a few hours. There has been no clinical evidence of complement activation by the i.v. route, although there is evidence of modest complement activation (C3a but not C5a generation) at the 1 and 2 mg/kg dosages observed only in the Phase 2 rheumatoid arthritis study. Although carcinogenesis and serious infection remain theoretical possibilities owing to impairment of leukocyte trafficking and possibly T-cell responses, overall rates of infection are comparable with placebo, and there has been no clinical evidence of carcinogenesis.

Apparent hypersensitivity reactions, presumptively IgE mediated, have been observed in six patients, consisting primarily of urticaria but which included transient hypotension in one patient. There has been no clinical or laboratory evidence of the development of IgG or IgM antidrug antibodies in the clinical studies to date. In the Phase 1 i.v. study, no antidrug antibodies were detected by IgG and IgM ELISA on serum taken 14 days after the last of four every-other-day doses, and have been detected in only one of approximately 100 patients assayed to date in the 299-patient Crohn's disease trial. The only other apparent drug effect is minor cutaneous vasodilatation. Some 10–12% of ISIS 2302–treated patients versus ~4% of placebo patients have been reported to experience occasional and mild facial flushing or other minor vasodilatory events during study drug infusion, which have not resulted in discontinuation or slowing of the study drug infusions. These are usually reported as single episodes, but

may occur repeatedly. As noted earlier, injection site reactions were common in the subcutaneous trial (23% of patients), and one patient discontinued owing to severe local reactions.

IV. CONCLUSION

ISIS 2302, an antisense inhibitor of ICAM-1, is the first systemic antisense drug to demonstrate evidence of efficacy in controlled clinical trials. ISIS 2302 may be a promising new drug for the treatment of Crohn's disease, with a very favorable safety profile. Taken together, the phase 2 data, and especially the data from the phase 2b trial (CS9), suggest that ISIS 2302 may be effective in higher dose in Crohn's disease, particularly if exposure exceeds 65 μg·hr/mL. As a dosage of 2 mg/kg was well tolerated, additional studies are underway to investigate the safety and efficacy of higher doses of ISIS 2302 in Crohn's disease.

Phase 2a i.v. studies and preclinical data also suggest efficacy of ISIS 2302 in other diseases and by other routes of administration. Although ISIS 2302 may be effective in the treatment of rheumatoid arthritis, activity was insufficient to justify further development for now with the intravenous route, but is supportive of further study in rheumatoid arthritis if an oral formulation is achieved. A Phase 2a trial in the prophylaxis of acute renal transplant rejection suggests that ISIS 2302 may be effective in this setting, especially in caucasians, but a low rate of rejection in the placebo group and limited numbers of patients preclude any firm conclusions. Additional studies at higher doses are being contemplated, perhaps stratified by race as graft survival in Afro-Americans is considerably shorter than in other races. An ongoing trial by the topical route will assess the importance of ICAM-1 in the maintenance of psoriasis, and an ongoing trial by enema will assess efficacy in distal ulcerative colitis. Delivery by the aerosolized route appears to be feasible, well tolerated, and effective in animals. Future clinical trials in asthma with an antisense compound targeting ICAM-1 or another molecular target are anticipated.

Antisense technology offers the potential for rapid and highly efficient drug discovery, absolute target specificity, and a wide therapeutic index in the treatment of a broad spectrum of human diseases. Clinical trials with second-generation antisense drugs to TNF-alpha and the alpha-4 integrin are being planned over the next year. Although topical delivery to skin, lung, and colon is feasible and about to be tested clinically, antisense technology continues to be limited by the need for parenteral delivery to achieve systemic activity. Preclinical testing with solution-based technologies suggests that 25% oral bioavailability may be an attainable goal. If this can be achieved with a solid dosage form, the horizons

for antisense therapy will be considerably expanded in inflammatory as well as other diseases.

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New Routes and Novel Formulations for Delivery of Antisense Oligonucleotides

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I. INTRODUCTION

Initial efforts in the creation of antisense therapeutics have focused on fundamental questions such as safety, efficacy, and technology costs, as would be expected for any emerging technology with a significant potential to create a new therapeutic paradigm. Clinical proof of efficacy has been established with the approval of the first antisense oligonucleotide therapeutic, Vitravene (fomivirsen), indicated for the local treatment of cytomegalovirus (CMV) retinitis in patients with AIDs (1). With proof of principle now established and a dramatic and continuing fall in the cost of goods, it is appropriate that we now address the area of new delivery routes and novel oligonucleotide formulations. Such advances will impact oligonucleotide biopharmaceutics and broadly expand the utility of antisense technology. Early research in drug delivery systems focused on a perceived weakness of the technology: cellular uptake. In vitro observations of phosphorothioate oligodeoxynucleotide led many working in the area to conclude that because of metabolic instability, size, and charge, oligonucleotides would not be adequately taken up into the targeted cells or tissues necessary to exert pharmacological activity. An ever-expanding body of in vivo animal and clinical data, however, has shown that even simple saline solutions delivered parenterally can lead to effective oligonucleotide uptake.

While such simple formulations are efficacious for some parenteral applications, the development of novel formulations and delivery routes is opening a

wider variety of target tissues and cells to treatment with oligonucleotides through increased efficiency and stability. In this chapter we will address a variety of challenges and opportunities in the targeting and delivery of antisense oligonucleotides for a wide range of diseases.

II. PHYSICAL-CHEMICAL PROPERTIES

In the development of any drug product it is essential that the fundamental physical and chemical properties of the drug molecule and of prototype drug formulations be determined. Such data often dictate subsequent development events and underscore the choices considered for dosage form design. This requirement is especially evident for oligonucleotides, which represent a new class of compounds yet to be fully characterized. With respect to the inherent properties of the unformulated oligonucleotide, there is sufficient evidence that we may assign common properties for all oligonucleotides for a given class of oligonucleotide chemistry. That is, data consistently demonstrate that all "first generation" or phosphorothioate oligodeoxynucleotides exhibit similar physiochemical properties. Likewise, newer chemistries appear to have conformity within their defined chemistry, such as for sugar-modified chemistries (e.g., 2′-*O*-methoxyethyl) or for backbone-modified chemistries (methylphosphonates).

Phosphorothioate oligodeoxynucleotides are synthesized as complex mixtures of diastereomers. In the solid state they are amorphous, electrostatic, hygroscopic solids with low-bulk densities, possessing very high surface areas, and poorly defined melting points. Their good chemical stability allows them to be stored as lyophilized or spray-dried powders or as concentrated, sterile solutions; more than 2 years of storage is possible at refrigerated temperatures.

Owing to their polyanionic nature, phosphorothioate oligodeoxynucleotides are extremely water-soluble under neutral and basic conditions. Consequently, drug-product concentrations are often only limited by an increase in solution viscosity at very high concentrations, e.g., >300 mg/mL. Extremes of pH and ionic strength influence the apparent solubility. In acidic environments such as the stomach, internucleotide linkages are partly neutralized by protonation. This results in a marked decrease in oligonucleotide solubility, an event that can be easily avoided by elevating the pH.

Phosphorothioate oligonucleotide degradation has been primarily attributed to two mechanisms: desulfurization and acid-catalyzed hydrolysis. Desulfurization of the backbone, observed at elevated temperatures and under intense ultraviolet light, leads to (pharmacologically active) oligonucleotides primarily containing a single phosphate diester linkage. Under acidic conditions, depurination may occur, resulting in oligonucleotides containing one or more abasic sites. Such sites are then susceptible to base-mediated cleavage of the adjacent phos-

phorothioate linkages. Both the desulfurization and hydrolysis pathways can be monitored by a variety of analytical techniques including, for example, capillary gel electrophoresis, anion exchange, and ion-pair liquid chromatography and liquid chromatography–mass spectroscopy.

All of the physical-chemical characteristics discussed here are important to designing viable, efficacious formulations with acceptable storage and in vivo stability. Each delivery system presents its own formulation challenges, as will be seen in the remainder of this chapter.

III. LOCAL ADMINISTRATION

A. Brain

In general, the blood-brain barrier (BBB) is only permeable to lipophilic molecules of molecular weight <600 Da, while even small water-soluble molecules generally cannot be transported (2). This would seem to preclude delivery of oligonucleotides to the brain from the systemic circulation without the assistance of specialized drug delivery systems.

Unmodified phosphodiester oligodeoxynucleotides have been directly injected intracerebrally to down-regulate the expression of neurotransmitters in a sequence-specific manner (3). While phosphodiester oligodeoxynucleotides are rapidly degraded in the brain, phosphorothioate oligodeoxynucleotides are more metabolically stable, but are nevertheless rapidly cleared via the cerebrospinal fluid bulk flow. Whitesell et al. demonstrated extensive brain penetration and marked cellular uptake after continuous intracerebral infusions with a miniosmotic pump (4). Direct intracerebral administration has been utilized by Sommer et al. (5) to study phosphorothioate oligodeoxynucleotides directed to c-fos and by Mustafa and Dar (6) to down-regulate the adenosine A1 receptor. One report in the literature details the accumulation of systemically administered phosphorothioate oligodeoxynucleotides in implanted subcutaneous and intracranial glioblastoma tumors in mice (7). A similar phosphorothioate oligonucleotide was shown not to cross the BBB in normal animals (8), so it was hypothesized that the presence of a glioma may sufficiently disrupt the BBB to allow pharmacological concentrations of oligonucleotide to accumulate within the tumor. Accumulation was sufficient in this model to demonstrate antitumor activity.

Boado et al. (9) devised delivery systems based on conjugates of streptavidin and the OX26 monoclonal antibody directed to the transferrin receptor as a carrier for the transport of oligonucleotides. These delivery systems were found to transport peptide nucleic acid antisense molecules, but not phosphorothioate oligonucleotides, across the BBB. The authors attributed this difference to preferential binding of phosphorothioate oligonucleotide to plasma protein instead of the antibody complex, which reduced transport.

B. Ocular

The first approved antisense oligonucleotide therapeutic, Vitravene (fomivirsen) is indicated for the local treatment of CMV retinitis in patients with AIDs (1). Local treatment is accomplished by intravitreal injection of only 50 μL of a sterile, aqueous solution with a 30-gauge needle. Fomivirsen is cleared from the vitreous in rabbits over the course of 7–10 days, by a combination of tissue distribution and metabolism. In the eye, fomivirsen concentrations were greatest in the retina and iris. Fomivirsen was detectable in retina within hours after injection, and concentrations increased over 3–5 days. Metabolism is the primary route of elimination from the eye. Metabolites of fomivirsen are detected in the retina and vitreous in animals. Systemic exposure to fomivirsen following single or repeated intravitreal injections in monkeys was below limits of quantitation. In monkeys treated every other week for up to 3 months with fomivirsen there were isolated instances when fomivirsen's metabolites were observed in liver, kidney, and plasma at a concentration near the level of detection (10).

C. Pulmonary Delivery

The ability to efficiently deliver oligonucleotides to the lungs would open a series of important diseases to antisense treatment. Delivey to lung tissue can be by way of intravenous administration or by the pulmonary route. Parenteral delivery of oligonucleotide does not lead to substantial lung uptake, and is therefore not an ideal route for treatment of disease in lung tissue. After a 0.8 mg/kg i.v. bolus injection, the concentration in lung tissue has been shown to be less than 5 μg/g (11), a concentration approaching pharmacologically active levels. Direct administration to the pulmonary airways through inhalation would potentially increase local concentrations, and also serve as a less invasive route of administration.

The pulmonary route is an increasingly attractive option for both local and systemic delivery of a wide variety of macromolecules such as oligonucleotides. Delivery options include nebulizers, metered-dose inhalers, and dry-powder inhalers. Any of these pulmonary devices are far less invasive than parenteral administration and, in addition to potentially increasing efficacy, are likely to increase patient compliance.

1. Formulation Considerations

Wu-Pong and Byron have contributed a review of the issues associated with pulmonary delivery of oligonucleotides (12). Since oligonucleotides are freely soluble and highly hygroscopic, it would be reasonable to assume first dosage forms will rely upon aerosolization of simple aqueous solutions. Our data suggest that commercially available nebulization devices will generate suitable aerosolizations of oligonucleotide solutions at concentrations of up to 180 mg/mL (Table

Table 1 Nebulization of ISIS 2503 Solutions by Healthline Nebulizer Model NEB-3A at 10 L/min

Concentration (mg/mL)	MMAD (μm)	GSD (μm)	% particles <5 μm	Solution delivery rate (mg/min)
100	1.02	1.96	98.6	258
150	0.77	2.27	98.0	213
180	0.70	2.29	97.8	198

MMAD = mass mean aerodynamic diameter; GSD = geometric standard deviation.
Source: Ref. 13.

1) (13). Ultrasonic and jet nebulizations were found to have essentially no effect on phosphorothioate stability of the oligonucleotide over 40 min (Fig. 1), which is longer than typical treatment times.

Depending on particle size, delivery may primarily be to the upper airways or to the alveoli, where systemic delivery may also be possible (12,14). Particles with diameters of approximately 5–10 μm will generally deposit in the upper airways, while those in the range of approximately 0.5–5 μm will reach the alveoli. Smaller particles are often exhaled; larger particles are generally swallowed. Owing to the relatively small doses of drug that may conveniently be delivered

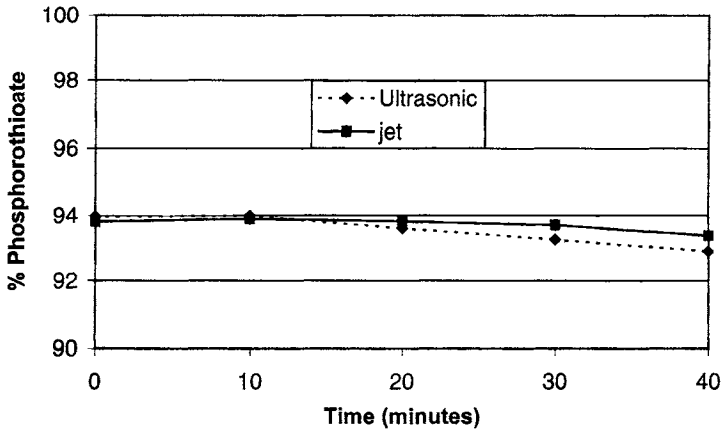

Figure 1 Effect of different nebulization methods on phosphorothioate oligonucleotide stability (13).

by aerosolization, this route may be more practical for treatment of the local tissue rather than systemic dosing for oligonucleotides.

2. Deposition and Uptake

The bioavailability of simple solutions of a phosphorothioate oligonucleotide, ISIS-3521 (CGP 46128A), in rats by intratracheal administration was compared to subcutaneous, oral gavage, and intraperitoneal administration (15). Intratracheal delivery, a model for pulmonary delivery, demonstrated the highest bioavailability of all nonparenteral routes evaluated (Table 2). Since no morphological or pathological changes were observed in the trachea or respiratory tract even at the highest dose studied, the dose-dependent bioavailability was attributed to saturation of the local tissue uptake pathways. A similar study (16) using Brown-Norway rats determined that the cellular uptake of the phosphorothioate oligodeoxynucleotide ISIS-2105 was specific: predominantly into macrophages, and to a lesser extent eosinophils. Nonspecific binding to mucus and the intercellular tissue matrix was observed only at 15 min after dosing. Cellular uptake was also observed in both tracheal and bronchial epithelium. These studies raise several important biopharmaceutic questions: (1) Can oligonucleotides be efficiently delivered by a means other than direct infusion into lung? (2) What is the tolerability of daily multiple dosing? (3) Which cell types and/or pulmonary tissues accumulate oligonucleotide? (4) Is there potential for systemic bioavailability?

Table 2 Effect of ISIS 3521 Dose and Route of Administration on Plasma and Tissue Bioavailabilities

Administration route	Dose (mg/kg)	% bioavailability compared to i.v. over 360 min	% bioavailability based on tissue accumulation over 360 min
i.v.	0.6	100.0	95.9
Subcutaneous	0.6	30.9	24.9
Intraperitoneal	0.6	28.1	59.4
Oral gavage	6.0	0.3	0.1
Intraduodenal	6.0	0.7	0.9
Intraileal	6.0	0.1	2.4
Intratracheal	0.06	3.2[a]	2.7
Intratracheal	0.6	16.5[a]	21.1
Intratracheal	6.0	39.8[a]	23.0

[a] Absorption was not complete at 360 min; therefore, these values are considered an underestimate.
Source: Data taken from Ref. 15.

At Isis Pharmaceuticals Inc., antisense compounds were successfully delivered to cells of the rat lung using simple aqueous formulations aerosolized by commercial nebulization equipment (17). Single or multiple nose-only administration led to significant oligonucleotide concentrations in lung tissues (Table 3) and bronchiolar lavage fluid with low or mild toxicity, depending on dose. Using immunohistochemistry, oligonucleotides could be visualized in pneumocytes, vascular endothelium, and alveolar macrophages. Single and multiple exposures differed only in the amount of oligonucleotide present and not the distribution pattern. Experiments with doses as low as 1–3 mg/kg also demonstrated that significant concentrations of phosphorothioate oligodeoxynucleotide can be deposited (over 50 μg oligonucleotide/g of tissue) and maintained (20 h or greater half-life) in lung, with little if any systemic exposure. Oligonucleotide was localized to bronchiolar epithelium and alveolar epithelium and endothelium. At doses of 12 mg/kg, systemic distribution was found, with plasma levels as high as 4.2 μg/mL and liver concentrations as high as 30 μg/g. Toxicity was mild at the 12 mg/kg level and minimal to absent at doses of 3 mg/kg or below. Taken together, these studies indicate that pulmonary delivery of oligonucleotides directly to the lungs for local activity can be accomplished safely with doses lower than those required for intravenous administration. It would appear that pulmonary dosing of antisense oligonucleotides for local therapeutic benefit is within reach of currently available technologies. Furthermore, the use of newer oligonucleotide chemis-

Table 3 Oligonucleotide Concentrations in Lung Tissue Following a Single, 30-min, Nose-Only Inhalation Exposure to ISIS 2105, a 20-Base Phosphorothioate

Dose level	Time point	Conc. of total oligonucleotide	% intact
1.2 mg/kg	EOE	55.7	79
	2 h	52.4	63
	8 h	34.3	48
	24 h	21.8	25
12 mg/kg	EOE	153.6	86
	2 h	86.0	72
	8 h	68.7	49
	24 h	78.3	33

Dose level expressed as the pulmonary dose, i.e., amount estimated to be deposited in lung. Necropsy conducted at the end of exposure (EOE) or at selected time intervals after exposure. Oligonucleotide concentration data expressed as mean μg oligonucleotide/g lung tissue. n = 3–5 animals. Percent intact is the relative amount of parent, 20 mer of the total oligonucleotide (ISIS 2105 plus metabolites). *Source:* Ref. 17.

tries, such as the sugar-modified (2′-*O*-methoxyethyl) derivatives that significantly enhance tissue accumulation will allow higher steady-state concentrations or less frequent dosing to be achieved, owing to their longer tissue elimination half-lives.

3. Demonstration of Pharmacology by the Pulmonary Route

In addition to these studies that characterize deposition and uptake, several reports in the literature demonstrate antisense pharmacology using the pulmonary route. Administration of an aerosolized phosphorothioate oligonucleotide (antisense to adenosine A_1 receptor) desensitized rabbits to subsequent challenge with either adenosine or dust mite allergen. Rabbits treated with the sequence-specific oligonucleotide demonstrated a dose-dependent desensitization, while animals treated with a mismatched sequence control showed no desensitization (18). Specifically, the authors showed that aerosols created with ordinary nebulizers (which create 80% of droplets < 5 μm in diameter) effectively reach the bronchial smooth muscle in sufficient quantities to specifically attenuate the target up to 75% (19). Similar results were found using bradykinin B_2 receptor–specific oligonucleotide and a mismatched control (18). In another approach to oligonucleotide delivery to the lungs, pharmacology was demonstrated by inserting an antisense mRNA complementary to the ras protooncogene into adenovirus vector particles. These vectors were delivered intratracheally to nude mice that had been previously inoculated with a human lung carcinoma. While 90% of the mice treated with a virus expressing a control sequence grew tumors, 87% of those treated with the virus expressing the antisense sequence were tumor free (20).

The literature reviewed here show that both formulated and unformulated (simple aqueous solution) oligonucleotides can be delivered to the lungs where they subsequently suppress local gene expression, opening a wide variety of diseases to antisense therapy.

IV. ADVANCED PARENTERAL DELIVERY

In animal studies, there is overwhelming evidence of biological activity of oligodeoxynucleotides upon intravenous injection of simple solutions. Given the excellent solution stability and solubility possessed by phosphorothioate oligodeoxynucleotides, it has been relatively straightforward to formulate these first-generation drug products in support of early clinical trials. Simple, buffered solutions have been successfully used in clinical studies by intravenous, intravitreal, and subcutaneous injections. Simple aqueous formulations of oligodeoxynucleotide are generally administered by slow infusion, rather than intravenous bolus, to control peak plasma levels below those associated with acute toxicity.

A. Cellular-Based Delivery for Targeting, Uptake, and Release

As pointed out earlier, targeting oligonucleotide molecules among cell populations is often unnecessary since specificity for the target is inherent and precise. However, other considerations, such as dose conservation and/or masking of nonspecific effects and toxicity, may provide justification for specific formulations of oligonucleotides for certain applications. Based upon the belief that formulations would be required to optimize therapeutic benefits of oligonucleotides (i.e., organ accumulation, sustained action, increased cellular uptake, etc.), a significant body of literature has been generated regarding the use of liposomal structures to alter the biopharmaceutics of oligonucleotides.

1. Cationic Complexes

Until recently, there existed an academic debate as to whether oligonucleotides would reach their sites of action in intact cells. This question has stimulated a great deal of research interest in the areas of targeting, cellular uptake, and release. It is now an accepted technique to use cationic lipids for cell transfection in in vitro screening assays (21). Similarly, the use of a cationic polymer, polyethylenimine, for increasing cellular transfection in culture and in newborn mice has been reported (22).

Cationic lipoplexes bind ionically to oligonucleotides owing to the electrostatic interaction between positively charged head groups on lipids and negatively charged phosphates or "phosphorothioate" groups on oligonucleotides. Complexation of the oligonucleotide can be quantitatively achieved without extensive purification. Entrapment of oligonucleotide into cationic liposomes enhances uptake into several cell types in vitro (23–25), and has been shown to deliver the oligonucleotide to the nucleus (26,27). Other studies point out the importance of the charge ratio of the complex on the extent of nuclear accumulation (27). In vivo, the utility of cationic lipids to delivery oligonucleotides is limited owing to sequestration of material in lung and immediate uptake of released material by the intensely phagocytic Kupffer cells lining the sinusoids of the liver (28). Additionally, interaction of the complex with blood components leads to serum sensitivity and cytotoxicity (29,30).

2. Anionic or Charge-Neutral Liposomes

There are fewer examples of oligonucleotide delivery by anionic or charge-neutral liposomes. In one example, oligonucleotides encapsulated into cardiolipin-containing anionic liposomes were shown to have a 7–18-fold increase in uptake versus unencapsulated liposome by human leukemia MOLT-3 cells in vitro. The intracellular release of oligonucleotides in vitro was also facilitated by liposomes

composed of cardiolipin, phosphatidylcholine, and cholesterol. Significant delivery to the nucleus and down-regulation of the *mdr1* gene, which encodes a plasma membrane glycoprotein (Pgp), was achieved, as evidenced by inhibition of Pgp synthesis and doxorubicin resistance in human multidrug-resistant ovarian cancer SKVLB cells (31,32).

Another limitation of these liposomes is their low efficiency in encapsulation of the phosphorothioate oligodeoxynucleotides, typically on the order of 10–20%. However, hydrophobic *p*-ethoxyoligonucleotides (Fig. 2) have been incorporated with ~90% efficiency into dioleoylphosphatidylcholine liposomes and injected intravenously into rats (33). Governed by the behavior of the liposomes, the oligonucleotide clearance from plasma, not unexpectedly, closely fits a two-

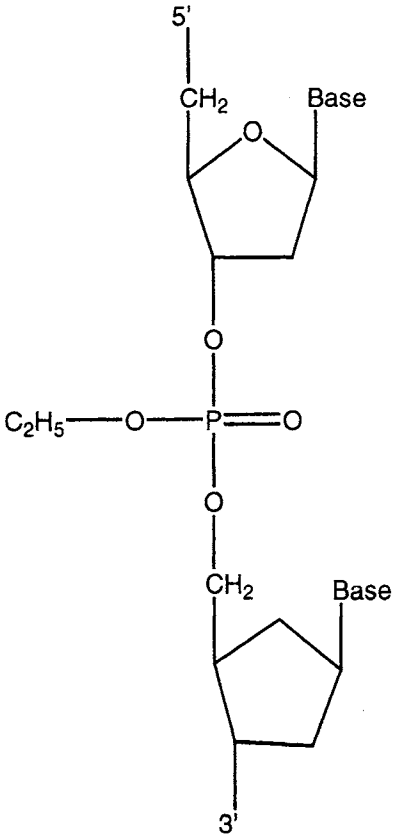

Figure 2 Structure of *p*-ethoxyoligodeoxynucleotide (based on Ref. 33).

compartment model, with a terminal phase half-life of around 7 h and the highest tissue concentrations found in the spleen and liver.

To take advantage of the propensity of liposomes to accumulate in the liver, Soni et al. investigated the liver target hepatitis B (34). Ducks were injected with either free or liposome-entrapped 18-mer phosphorothioate oligodeoxynucleotide, which was known to inhibit duck hepatitis B virus (DHBV). Liposome entrapment increased delivery of oligodeoxynucleotide to the liver by a factor of 6–10, depending on the time point. Retention in the air sacs increased by a similar factor, while other organs examined showed little difference. However, both the liposomal antisense and non-liposomal-scrambled control showed inhibition of DHBV replication in the study, raising the possibility of nonsequence effects in this model.

Ponnappa et al. described liposomes (mean diameter 1000–1470 nm), consisting of dipalmitoylphosphatidylcholine, cholesterol, and dimyristoylphosphatidylglycerol, targeted toward Kupffer cells (35). In this study, over 65% of the liver-associated oligonucleotide, representing 40% of the total oligonucleotide dose, was found in Kupffer cells; relative accumulation of phosphorothioate oligonucleotide in these cells was 200-fold that of the remaining combined body tissues.

3. Long-Circulating Liposomes

Passive targeting of oligonucleotide has been achieved using liposome-encapsulated therapeutics. Increased circulation times of these materials and leaky vasculatures associated with infection, inflammation and tumor physiology are considered to be factors impacting accumulation at these sites (36–38). In our laboratories we have synthesized a variety of long-circulating liposomal formulations containing phosphorothioate oligonucleotides. All liposomal formulations protected the circulating reservoir of oligonucleotide from degradation and allowed for greater tumor accumulation (Fig. 3). The percent of parent or intact oligonucleotide was found to be proportional to the liposomes' mean circulation time. For a selected formulation, further study of tumor growth suppression demonstrated the feasibility of lowering the dosing regimen (dose and/or frequency) as compared to unformulated drug (Fig. 4).

One caution regarding these observations is worth noting. Because the mononuclear phagocytic system is largely responsible for clearing these materials from circulation, misleading data regarding circulation time may be obtained in species with less-evolved systems. It is therefore prudent to confirm formulation performance in higher-order species. Liposome-encapsulated and unencapsulated ISIS 2503, a 20-mer phosphorothioate oligodeoxynucleotide directed to Ha-*ras*, was administered to rhesus monkeys by intravenous infusion (39). The blood, plasma, and tissue concentrations of oligonucleotide demonstrate that Stealth li-

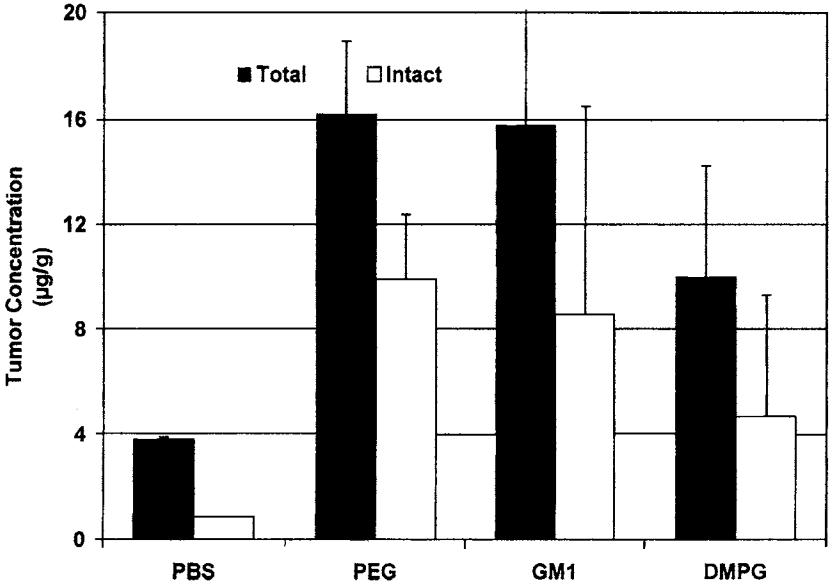

Figure 3 MiaPaCa tumor concentration of ISIS 2105 oligonucleotide 24 h after single 19.5 mg/kg i.v. dose of liposomal formulations or phosphate-buffered saline solutions in mice. PBS, phosphate buffered saline; PEG, disteroylphosphatidylcholine-polyethyleneglycol; GM1, monosialoganglioside-GM_1; DMPG, dimethylphosphatidylglycerol.

posomes protect the oligonucleotide from nucleases in blood and tissues, slow tissue uptake, and slow the rate of clearance from the systemic circulation.

4. pH-Sensitive Liposomes

In general, liposomes are primarily taken up by endocytosis into endosomes and are eventually degraded in the lysosomes (40). In an attempt to avoid this destruction, several investigators have created reactive liposomal structures to take advantage of changes in the endosome microenvironment. pH-sensitive liposomes have membranes that have been designed to destabilize when the pH drops, putatively then emptying their contents into the endosome and cytosol. These pH-sensitive liposomes have been used to deliver antisense oligonucleotides. In one in vitro example, pH-sensitive liposomes composed of oleic acid, dioleoylphosphatidylcholine, and cholesterol were used to encapsulate oligonucleotide targeted against Friend retrovirus. Liposome-encapsulated oligonucleotide inhibited viral spreading, whereas free oligonucleotide and non-pH-sensitive liposomes

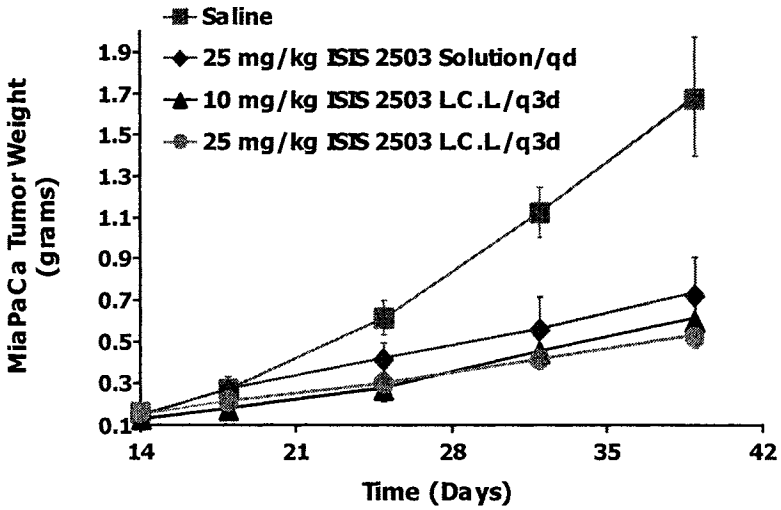

Figure 4 Pharmacological effects of long-circulating liposomes (L.C.L) (101).

were ineffective (41). Similarly, pH-sensitive liposomes encapsulating the anti-env (retroviral envelope protein) oligonucleotide were found to inhibit viral spreading at low concentration in infected Dunni cells (42).

The major limitation of pH-sensitive liposomes in vivo is their instability in plasma (43,44). This problem has been overcome by adding poly(ethylene glycol)distearoylphophatidylethanolamine (PEG-PE) into the formulation (45). PEG-PE is thought to coat the surface of liposomes thereby preventing the interaction of liposomes with blood components. This reduced interaction leads to increased stability and plasma half-life of liposomes. When injected intravenously into rats, the pH-sensitive liposomes composed of cholesteryl hemisuccinate, dioleoylphosphatidylcholine, and PEG-PE had similar pharmacokinetics parameters as non-pH-sensitive sterically stabilized liposomes. The regular pH-sensitive liposomes without PEG-PE were cleared rapidly from the circulation.

Looking more closely at the fate of liposomes taken up by endosomes, Lee et al. recently reported a novel approach to releasing endosomal contents into the cytoplasm after macrophage uptake (46). A 58-kDa protein, LLO, isolated from *Listeria monocytogenes* was incorporated into pH-sensitive liposomes containing a fluorescent dye. It could be determined that as the endosome began to acidify, the pH-sensitive liposomes released their contents into the endosome; however, this delivery was ineffective in producing a pharmacological change. However, when the LLO protein was included in the liposomes' payload, the

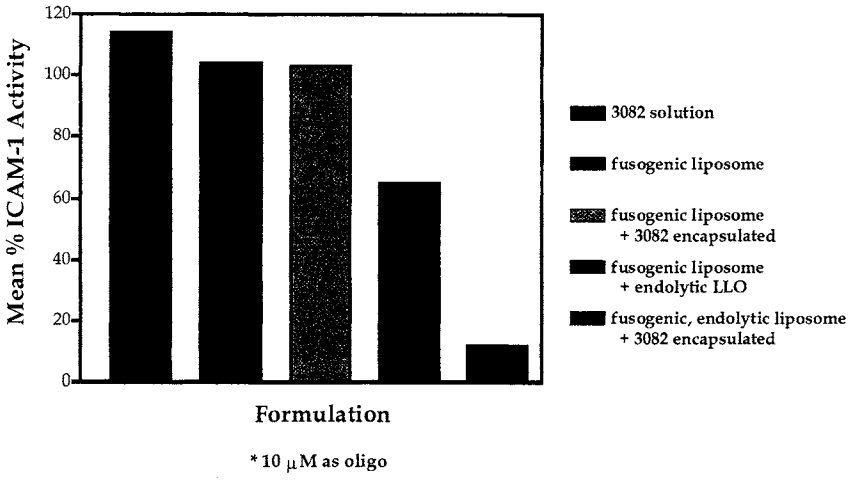

Figure 5 ICAM-1 inhibition in murine macrophages mediated by liposome formulations.

liposome/endosome contents were more effectively released into the cytosol as evidenced by ICAM down-regulation (Fig. 5).

5. Cell-Surface-Targeted Liposomes

Immunoliposomes are liposomes conjugated to antibodies. These liposomes have been used for delivery of oligonucleotides to specific targets (47–51). Problems with this approach include inhibition of cellular uptake by the high-molecular-weight antibody, cost, and poor encapsulation efficiency. An alternative approach utilizes tagging the liposome with a ligand of interest. Cellular uptake of oligonucleotides encapsulatled in dipalmitoylphosphatidylcholine/cholesterol liposomes containing folate-PEG-PE conjugate was ninefold higher than oligonucleotide encapsulated in nonfolated liposomes and 16 times higher than unencapsulated oligonucleotide (52). Using this system and oligonucleotide directed against epidermal growth factor, these authors also established the correlation between cellular uptake and growth inhibition in vitro.

More recently, an oligonucleotide with specificity to the hepatitis B virus was targeted to liver hepatocytes using triglyceride-rich emulsions containing recombinant apoE. This formulated complex mimics endogenous chylomicrons with respect to selective uptake as mediated by apoE receptors (53). Intravenous delivery in C57Bl/6 mice resulted in a sixfold reduction of the extrahepatic deposition and a nearly sixfold increase in uptake by hepatocytes.

6. Protein-Coated Microbubbles

Oligonucleotides have been noncovalently conjugated to protein-encapsulated, gas-filled microbubbles for targeted delivery (54,55). Rats were injected with phenobarbital alone or with phenobarbital together with microbubbles conjugated with antisense oligonucleotides targeting the cytochrome P-450 gene sequence, to alter the metabolism of hexobarbital. Phenobarbital stimulates CYP IIB1 MRNA, and as a result, hexobarbital, which is hydroxylated by CYP IIB1, is more quickly metabolized and its sedative effect reduced. Rats pretreated with phenobarbital and the oligo against CYP IIB1, which was not microbubble conjugated, showed little change in hexobarbital-induced sleep time because CYP IIB1 was induced. In contrast, rats given 1/20th dose of microbubble-conjugated oligo had greatly increased sleep time relative to rats given unconjugated oligo (55). This suggests that the conjugated oligonucleotide was a more potent inhibitor of CYP IIB1 induction.

7. Fusogenic Liposomes

Nakamura et al. designed fusogenic liposomes by conjugating inactivated hemagglutinating virus of Japan (HVJ) conjugated to liposomes containing fluorescence-labeled phosphorothioate oligodeoxynucleotide (56). In this way they hoped to promote fusion of the liposomes with target cells and increase delivery of oligonucleotide. Direct injection of liposomes was found to increase the uptake of oligonucleotide to ligament scars in rabbits. In related work, these authors showed increased uptake of oligonucleotides in the kidney and cardiac muscle after intra-arterial injection of these systems in the rat (57,58).

As with the other delivery systems mentioned above, the eventual usefulness (by incremental improvements) of a particular approach will be determined as we further define the mechanisms and governing restrictions for the inter- and intracellular trafficking of oligonucleotides.

B. Sustained-Release Subcutaneous Formulations

Subcutaneous administration is preferable to the current constant infusion or i.v. bolus injection techniques due to its greater convenience and lower costs. While greater potency is required than that demonstrated by the first-generation compounds to have a reasonable depo volume, interesting formulation progress has been reported in the literature. A subcutaneous injection of the phosphorothioate c-*myc* antisense oligonucleotide, complexed with zinc and microencapsulated with the biodegradable polymer poly(D,L-lactide-co-glycolic acid), was found to be significantly more efficacious than an intravenous administration of unencapsulated drug in human melanoma and leukemia xenografts in immunocompromised mice (59). The microencapsulated oligonucleotide provided a sustained-

release formulation that was effective at lower doses and less frequent dosing than the unformulated (solution) oligonucleotide.

V. TOPICAL DELIVERY

The barrier properties of human skin have long been an area of multidisciplinary research. Skin is one of the most difficult biological membranes to penetrate, primarily owing to the presence of stratum corneum (SC). The SC is composed of corneocytes laid in a brick-and-mortar arrangement with layers of lipid. The corneocytes are partially dehydrated, anuclear, metabolically active cells completely filled with bundles of keratin with a thick and insoluble envelope replacing the cell membrane (60). The primary lipids in the stratum corneum are ceramides, free sterols, free fatty acids, and triglycerides (61), which form lamellar lipid sheets between the corneocytes. Unaltered, these unique structural features of SC provide an excellent barrier to the penetration of most molecules, particularly large, hydrophilic molecules such as oligonucleotides.

Physical alteration in the SC, of which its integrity is the primary barrier to transport of molecules to the skin, can therefore result in improved skin penetration. For example, tape stripping and abrasion by repeated brushing reduced the SC barrier sufficiently to allow penetration of naked plasmid DNA. Uptake of this plasmid produced gene expression changes in skin at a level comparable to that after intradermal injection of naked plasmid DNA (62). Nonplasmid DNA-like oligonucleotides can also be made to penetrate, using physical removal of the SC barrier (63,64).

A. Altering the Thermodynamic Properties of Oligonucleotide Molecules

Improving skin penetration by increasing lipid partitioning has been evaluated using two techniques that alter the thermodynamic properties of oligonucleotide molecules. A complex of phosphorothioate oligonucleotide with hydrophobic cations, such as benzalkonium chloride, resulted in increased penetration through isolated hairless mouse skin. This was explained on the basis of greater partitioning into the lipid phase (63). Chemical modification of oligonucleotides to eliminate the negative charges, when used with chemical penetration enhancers such as ethanol and dimethyl sulfoxide, also resulted in a size-dependent increase in the penetration of oligonucleotide into the skin (64).

B. Electrical Field for Alteration of Skin Permeability

Iontophoresis, which involves application of an electric field across the skin to induce electrochemical transport of charged molecules, has been studied exten-

sively for transdermal delivery of phosphorothioate oligonucleotides (65). Transdermal delivery of oligonucleotides was shown to be size dependent with steady-state flux values ranging from 2 to 26 pmol/cm^2 in isolated hairless mouse skin. The steady-state flux may also depend on the specific sequence, as well as the base composition, of the oligonucleotide. Direct experimental verification is necessary to determine whether there is a contribution of sequence specificity and possibly also secondary structure to the iontophoretic profile (66,67).

The transport behavior of a series of oligonucleotides through human epidermal membrane and a synthetic model membrane system (Nuclepore membranes) was investigated (68). Electromobilities of the oligonucleotides were found to be smaller than expected. Results suggested the existence of pores with significantly larger effective pore radii than had been estimated in earlier studies with small-molecular-weight model permeants.

Electroporation, a technique using much higher voltage than iontophoresis, causes the formation of transient aqueous pathways in skin lipids, providing therapeutic levels (>1 μM) of oligonucleotides in the viable tissues of the skin (69). Although locally high levels can be obtained by electroporation, the practical issues associated with its use in vivo, such as dose delivered, area treated, and tolerability, may limit its practical utility.

C. Formulations for the Alteration of Skin Permeability

Chemical penetration enhancers have recently been studied for increasing transdermal delivery of oligonucleotides or other polar macromolecules (70). Chemically induced transdermal penetration results from a transient reduction in the barrier properties of the SC. The reduction may be attributed to a variety of factors such as opening of intercellular junctions due to hydration (71), solubilization of SC lipids (72,73), or increased lipid bilayer fluidization (74,75). Combining various surfactants and cosolvents can be used to achieve skin penetration, resulting in therapeutically relevant concentrations of phosphorothioate oligonucleotides in the viable epidermis and dermis. Table 4 shows the results of epidermal and dermal penetration of ISIS 2302 (a 20-mer phosphorothioate oligonucleotide targeted against human ICAM-1 mRNA) as a function of chemical enhancers. Pretreatment of skin with enhancer resulted in an increase in epidermal and dermal levels of drug with higher levels of drug in epidermis than dermis (Table 4, Formulations 1–6). Terpenes as a class appear to be better penetration enhancers than other groups of compounds including azone (results not shown). Formulation of Enhancer 4 incorporates isopropyl myristate into a cream with the aid of nonionic surfactants; application of this cream for the duration of the study resulted in a large increase in the dermal levels of drug as compared to pretreatment (Formulations 7–10). Formulating the oligonucleotide with enhancer was more effective than enhancer pretreatment alone (Formulations 11, 12). The effect of formulation structure is demonstrated in Fig. 6. Topical application of these for-

Table 4 Effect of Selected Enhancers and Formulations on Penetration of ISIS 2302 in Isolated Hairless Mouse Skin[a]

	Formulation (with 1 mg ISIS 2302)	Epidermis (μg/g[b])	Dermis (μg/g[b])
1	Buffer	79.4	6.0
2	Enhancer 1 pretreatment	272.0	111.5
3	Enhancer 2 pretreatment	460.2	28.5
4	Enhancer 3 pretreatment	378.9	38.8
5	Enhancer 4 pretreatment	361.9	25.6
6	Enhancer 5 pretreatment	378.9	19.0
7	5% enhancer 4[c] cream	199.7	267.3
8	10% enhancer 4 cream	371.1	382.1
9	30% enhancer 4 cream	261.0	246.9
10	48% enhancer 4 cream	243.7	267.3
11	10% enhancer 4[d] emulsion	188.7	124.2
12	35% enhancer 4 emulsion	204.4	152.5

[a] Permeation for 24 h.
[b] Mean, $n = 3–6$.
[c] Also contains nonionic surfactants to form the cream.
[d] No surfactants added.

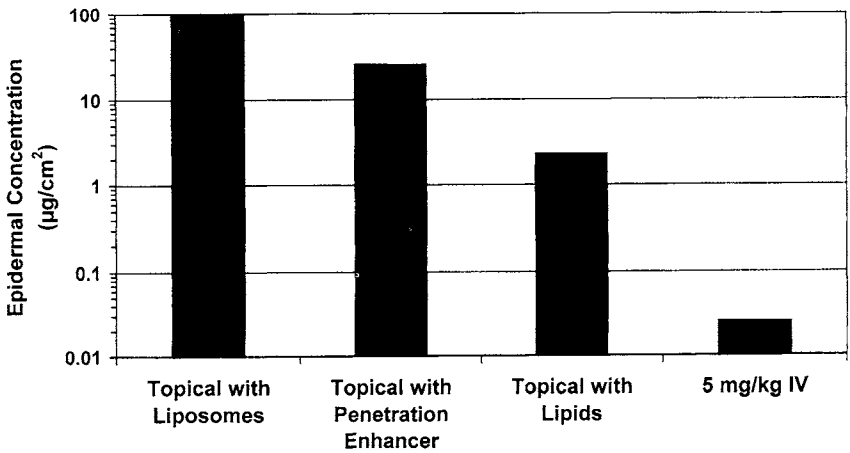

Figure 6 Epidermal concentrations of ISIS 2302 in mouse skin.

mulations resulted in significantly higher epidermal and dermal levels of ISIS 2302 than those achieved by an intravenous injection even at the highest dose studied, suggesting that topical administration of phosphorothioate oligonucleotides is more efficient in reaching all layers of the skin than systemic administration.

Liposomes have also been studied to transport oligonucleotides into the skin (76). They can increase the fluidity of skin lipid layers (similar to chemical enhancers) to facilitate transdermal permeation and can also carry encapsulated molecules through the appendageal pathway (77,78). Table 5 summarizes the results of studies conducted to evaluate the effect of phospholipids on penetration of ISIS 2302 in skin. A mixture of ISIS 2302 with a lipid suspension of anionic or neutral lipids resulted in a slight increase in accumulation in epidermis and dermis. The neutral lipid mixture appears to be slightly better than the anionic mixture (Formulations 1, 2). Application of liposomes formulated with anionic and neutral lipids resulted in a 10-fold-higher accumulation in the epidermis (Formulations 3, 4). This may be attributed to the ability of liposomes to mix with intercellular lipid layers and increase their fluidity, thereby allowing greater penetration of oligonucleotide. A mixture of empty fusogenic liposomes made from dioleylphosphatidylethanolamine (DOPE) with ISIS 2302 resulted in a 23-fold-higher amount of drug accumulation in the dermis and 11-fold-higher accumulation in the epidermis as compared to application of drug without liposomes (Formulation 5).

At Isis Pharmaceuticals Inc. (79) a cream formulation of the anti-ICAM-1 oligodeoxynucleotide ISIS 2302 effectively inhibited TNF-α-induced expression of ICAM-1 when applied topically to human skin transplanted on SCID mice. The reductions in ICAM-1 mRNA in skin were dose-dependent and se-

Table 5 Effect of Phospholipids on Penetration of ISIS 2302 in Isolatled Hairless Mouse Skin[a]

	Formulations (mixed with 1 mg ISIS 2302)	Epidermis ratio[b]	Dermis ratio[b]
1	Anionic lipid mixture DPPC:DMPC:Chol (3:1:1)	1	<1
2	Neutral lipid mixture DPPC:DMPG:Chol (3:1:1)	3	4
3	Anionic liposomes[c] DPPC:DMPC:Chol (3:1:1)	10	4
4	Neutral liposomes[c] DPPC:DMPG:Chol (3:1:1)	10	4
5	Fusogenic liposomes egg-PC:DOPE:Chol (3:1:1)	11	23

[a] Permeation for 24 h.

[b] Ratio of oligonucleotide penetrated with phospholipids to its aqueous control.

[c] Contains a mixture of encapsulated and free oligonucleotide.

Source: Ref. 79.

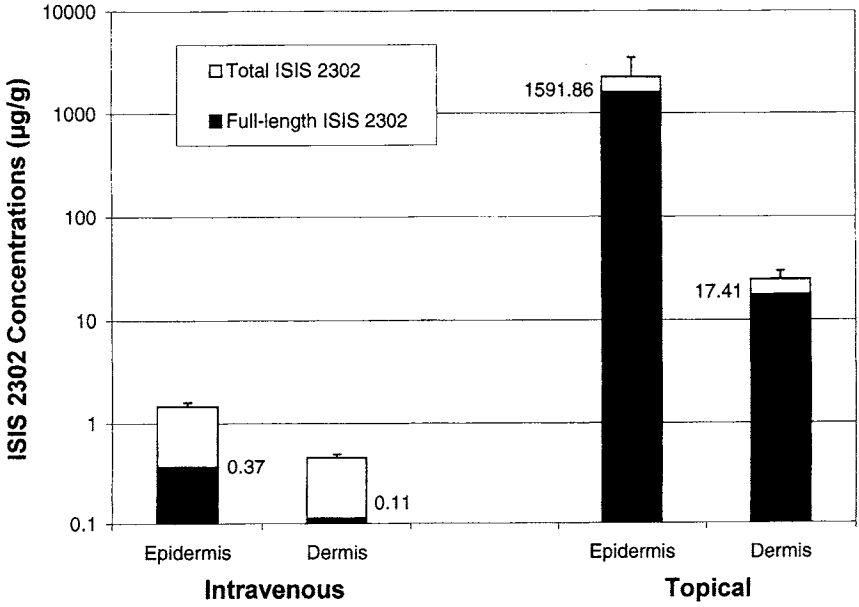

Figure 7 Skin concentrations (log scale) of oligonucleotide after i.v. or topical delivery of a 2% cream of ISIS 2302 (79). Concentration of total and full-length oligonucleotide (ISIS 2302) in epidermis and dermis after intravenous and topical administration to hairless mice. The number next to the solid bar shows the concentration of full-length oligonucleotide in µg/g. Topical dosing delivered 4300-fold greater intact ISIS 2302 to epidermis and 156-fold greater intact ISIS 2302 to dermis than i.v. dosing at 2 mg/kg.

quence-specific. Intravenous administration of the drug did not reduce ICAM-1 mRNA, putatively owing to insufficient drug concentrations in skin. These results are explained by the rapid and significantly higher accumulation of oligodeoxynucleotide in the epidermis and dermis produced by topical delivery. Quantitatively, the topical application of a 2% oligodeoxynucleotide cream resulted in >1000-fold-greater concentration of total oligodeoxynucleotide in epidermis than an intravenous dose at 2 mg/kg. This difference is even greater if only the intact material is considered, as drug administered by the topical route shows less metabolism. Even when we consider the deeper dermis, the topical route was able to deliver >100 fold the intact drug (Fig. 7).

D. Pharmacology by the Topical Route

Using a human skin transplant, TNF-α stimulation model, Mehta et al. (79) demonstrated a dose-dependent suppression of ICAM up-regulation (Fig. 8). In a

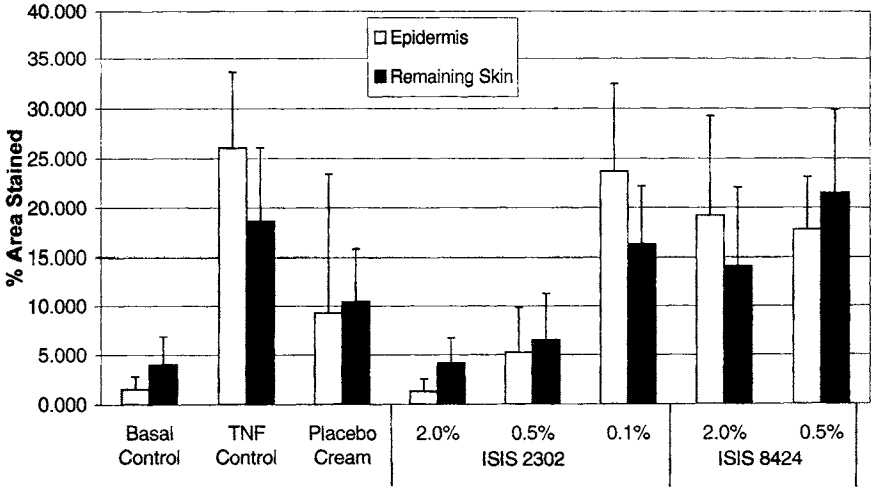

Figure 8 Percentage of area stained by anti-ICAM-1 antibody in sections of skin treated with various formulation prior to induction of ICAM-1 by TNF-α (79). Only the groups treated with topical ISIS 2302 at 2% ($p < 0.00001$ for epidermis and remaining skin) and 0.5% ($p < 0.00001$ for epidermis and $p = 0.0002$ remaining skin) dose strengths showed a significant lowering of ICAM-1 close to the basal levels. A very low concentration of ISIS 2302 (0.1%) cream and cream with ISIS 8424 (a scrambled control sequence of ISIS 2302) showed little reduction in ICAM-1. The differences between epidermis and dermis were not significant in any group.

hairless mouse model, the mouse-specific sequence for ICAM applied with a topical enhancing formulation was used to suppress up-regulation of mRNA after TNF-α stimulation (Fig. 9). The pharmacological effects were dose dependent and vehicle or scrambled control oligonucleotides have no effect when applied topically in the same formulation.

In summary, it is now feasible to deliver therapeutically relevant amounts of antisense oligonucleotides to the skin using a number of different delivery techniques and formulations. In addition, our results with ISIS 2302 show a dose-dependent pharmacological effect consistent with the antisenes mechanism of action. Clinical trials are underway to assess pharmacology and tissue kinetics of ISIS 2302 in human indications. When considering dose utilization, topical delivery is superior to systemic administration for targeting oligonucleotides to the epidermis and dermis. Taken together, these results establish that delivery systems providing significant concentrations of oligonucleotides in target tissues result in specific pharmacological effects. These tools, antisense and specific topical formulations, provide a new approach for the elucidation of the molecular basis of skin disease and the creation of a new class of dermal therapeutics.

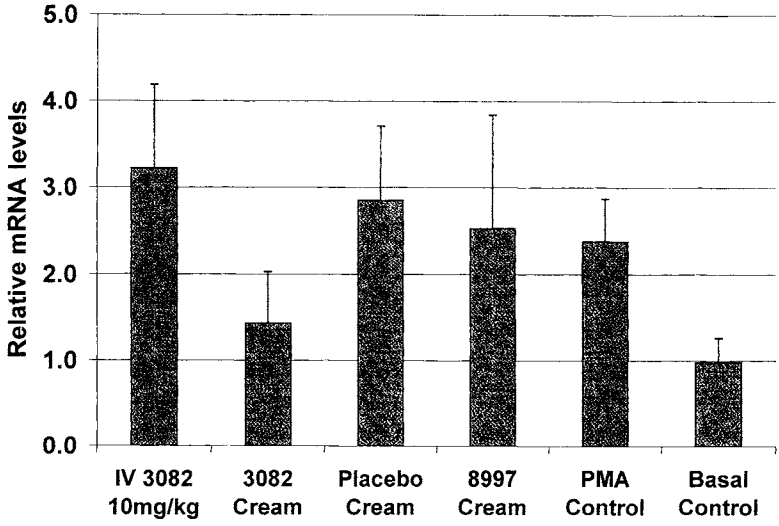

Figure 9 ICAM-1 mRNA levels in mice skin treated with anti-ICAM-1 oligodeoxynucleotide. Only the group treated with topical ISIS 3082 cream showed a lowering of ICAM-1 mRNA ($p = 0.01$ against i.v. and $p = 0.04$ against placebo cream) to a level that was not different from basal level. When compared to PMA control, intravenous dosing of ISIS 3082, placebo cream, and cream with the ISIS 8997 (scrambled control) showed no reduction in ICAM-1 mRNA (79).

VI. ORAL AND GASTROINTESTINAL DELIVERY

Oral and rectal dosage forms are ideal for patient convenience and compliance. For traditional small molecules, these routes have been used for both systemic delivery and delivery to local gastrointestinal (GI) tissues. For large, polyanionic, hydrophilic oligonucleotide molecules, a variety of issues must be considered when designing such dosage forms, particularly to enable systemic delivery by the oral route. Among these are chemical instability and precipitation at gastric pH, metabolic instability within the intestinal environment, low intestinal permeability, high protein binding, and first-pass hepatic clearance. These challenges have led many to conclude that effective oral systemic administration of oligonucleotides is not feasible (80). Indeed, the early reports of oral bioavailability approaching 35% from gavage solutions containing radioactive phosphorothioate oligodeoxynucleotides in rats were consequently determined to be the uptake of degraded forms (81). The above-mentioned barriers to oral bioavailability of oligonucleotides were delineated by Nicklin et al. (82). Of these, our experience demonstrates that two stand out as critical: instability in the GI tract and low

permeability across the intestinal mucosa. However, progress is being made to address and/or understand each of these barriers by way of changes to oligonucleotide chemistry and use of appropriate formulations.

A. Presystemic Metabolism

Natural DNA and RNA are rapidly digested by the ubiquitous nucleases found within the gut. As a consequence, oligonucleotides require stabilization to achieve a reasonable GI residence time to allow absorption to occur. To impart enzyme resistance, modifications can be made to the phosphate backbone, the nucleotide sugar, or both. It has been demonstrated that such modifications need to be incorporated at only a subset of the sites available on the oligonucleotide molecule. Such hybrid structures have demonstrated significantly improved nuclease stability. This was shown by Zhang et al. for both backbone modifications (methylphosphonates) and sugar-modified (2′-*O*-methyl) oligonucleotides (83,84). However, care must be taken to ensure that mRNA binding affinity is not impaired by such modifications—particularly modifications involving an increase in the size of the 2′-*O*-substituent. Nonetheless, a surprising discovery was that 2′-*O*-alkoxyalkyl substituents (i.e., groups related to ethylene glycol) retain or improve upon the RNA-binding affinity and have greatly improved nuclease resistance. The 2′-*O*-methoxyethyl (MOE) derivative was proposed as a promising candidate from this class of derivatives (85) and has since been evaluated for presystemic enzyme stability within the digestive system of the rat. This stability is proportional to the degree of sugar substitution (Fig. 10).

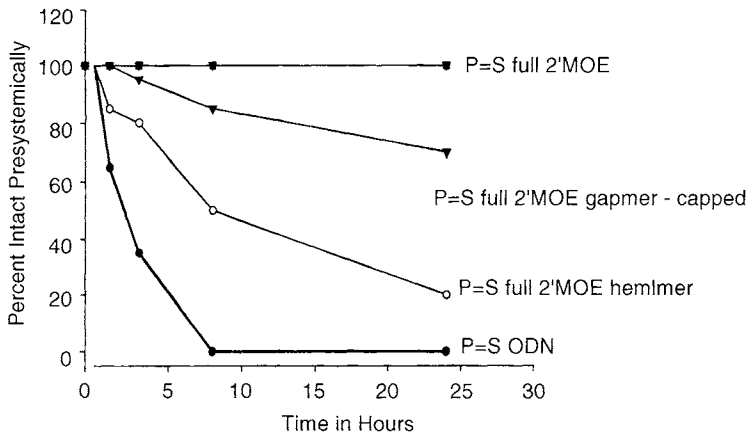

Figure 10 Stability of various oligonucleotide chemistries in the rat intestine prior to absorption.

Relative to the unmodified phosphorothioate oligodeoxynucleotides, these data demonstrate the increasing benefit of MOE substituents present as either a hemi-mer, with MOEs at the 3′ termini only; or a gap-mer, with MOEs present at both the 3′ and 5′ termini; and last, a full MOE substitution at every sugar location (86). These derivatives possess promising properties but are still awaiting clinical demonstration of their utility as therapeutic agents. Selection of a specific chemistry for development depends upon a number of criteria, of which nuclease stability is but one.

An alternative approach to improving enzyme stability of oligonucleotides is by way of formulations that impose a physical barrier, preventing nuclease from reaching the oligonucleotide cleavage site (see, for example, Refs. 87–89). Examples of such formulations include: (1) conventional modified-release dosage forms that incorporate the oligonucleotide within the solid matrix of the dosage unit, and (2) complexed oligonucleotides with cationic species formulated through a process referred to as complex coacervation. In addition to enzyme stability, such formulations have been purported to impart additional characteristics favorable to oligonucleotide absorption. These include: modified delivery (e.g., site-specific or increased retention by bioadhesion), a change in oligonucleotide physicochemical properties (e.g., charge neutralization or lipophilicity), and a change or impact on the absorbing membrane itself (e.g., opening of tight junctions or fluidization of mucosal membrane). These concepts are discussed more fully below.

B. Permeability

The physicochemical properties of phosphorothioate oligonucleotides present a significant barrier to their GI absorption into the systemic circulation or the lymphatics. These properties include their large size and molecular weight (i.e., approximately 7 kDa for 20 mers), hydrophilic nature (log $D_{o/w}$ approximating -3.5), and multiple ionization pk_as (e.g., unpublished titration data, using a Sirius $GlpK_a$ instrument on a 20-mer sequence, noted over 17 pk_as for the unmodified oligodeoxynucleotide and over 32 pk_as for the MOE hemi-mer form). While the baseline permeability values for 20-mer oligodeoxynucleotides approximate 2×10^{-6} cm/s in the rat small intestine (mucosal-to-serosal), this can be improved by chemistry modifications (Fig. 11); these values are still far below those of compounds exhibiting high permeability, such as naproxen (90).

The use of formulations can further improve upon this permeability. When formulating oligonucleotide drugs to improve oral bioavailability, the mechanism of oligonucleotide absorption, either paracellular via the epithelial tight junctions or transcellular by direct passage through the lipid membrane bilayer, must be considered. By using paracellular and transcellular models appropriate for water-soluble, hydrophilic macromolecules, it was suggested that oligonucleotides pre-

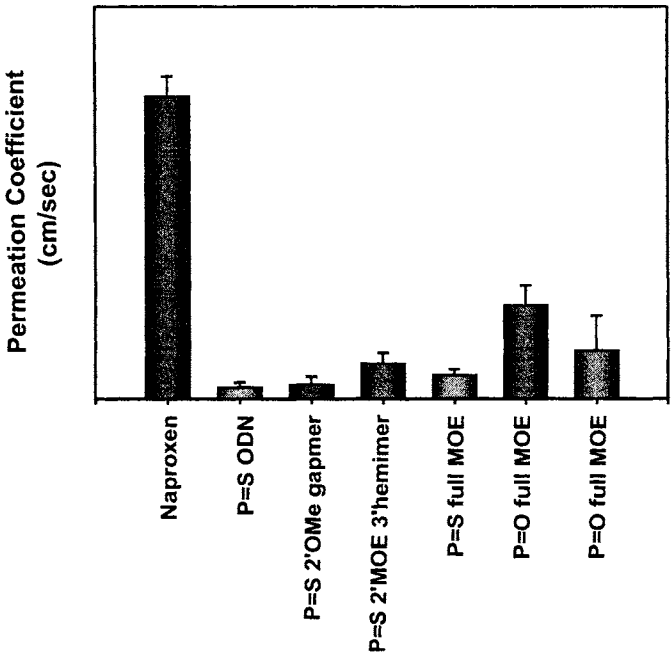

Figure 11 Effect of oligonucleotide chemistry on permeability in the rat intestine in situ (90).

dominantly traverse GI epithelium via the paracellular route (91–94). In this regard, formulation design considerations involve the selection of those permeation enhancers (PEs) that facilitate paracellular transport and meet other formulation criteria including: suitable biopharmaceutics, safety considerations, manufacturability, physical and chemical stability, and practicality of the dosage-form configuration (i.e., regarding production costs, dosing regimen, patient compliance, etc.).

Initial attempts at selecting PEs have identified certain surfactants, such as bile salts and fatty acids, that appear to facilitate oligonucleotide absorption. The advantages of these components are many in that they are endogenous to foods and body constituents plus the literature is rich with information about the use and exposure of these two classes of compounds (95). The precise mechanism of action for these PEs is unknown but is believed to involve a disruption of the mucus-layer barrier, an increase in the fluidity of the mucosal membrane, and potentially opening the paracellular, tight junction opening. The mucolytic effect coupled with the increased membrane fluidity imparted by these excipients ap-

pears to allow increased concentrations of oligonucleotide to enter the villi crypt wells. Experimental data suggest that tight junctions at the villi crypts are larger and therefore more permeable than their counterparts at the villi tips (96). When PE-formulated drug is administered orally to mice, the oligonucleotide can be visualized by immunohistochemistry throughout the brush border areas of the murine ileum (94). This enhanced intimacy of the oligonucleotide to the absorbing surface would increase its GI residence time and therefore its potential absorption via entry into the paracellular tight junctions.

C. Systemic Bioavailability

While significant oral bioavailability has not yet been demonstrated in humans, numerous model systems are being employed to obtain basic information on enhancers and other substituents that will enable the oral administration route. A useful animal model is the intrajejunally (IJ) ported canine. While not strictly oral administration, the IJ-dosed dog is considered important in that the dog model is a large species and therefore more representative than the rat model of what may be expected in humans. In this model significant oligonucleotide absorption has been observed after IJ administration of a solution containing both PEs and the 20-mer ISIS 2302 oligodeoxynucleotide. Plasma concentrations of the intact oligodeoxynucleotide and of a hemi-MOE derivative with the same sequence (ISIS 15839) are shown in Fig. 12, where it also appears that the MOE oligonucleotide plasma half-life is longer than that of the unmodified oligonucleotide.

When this formulation was assembled as a conventional tablet taking over 1 h to dissolve, the resultant plasma concentrations after oral dosing were negligible (data not shown). Upon reformulation to promote immediate dissolution (in less than 15 min) the absolute bioavailability approached 3%, as indicated by the plasma concentration plots in Fig. 12. These results, obtained with a rudimentary solid dosage form, are ~40% that obtained with the IJ dosing. From these results, it may be concluded that the following factors are important for oligonucleotide absorption in the above PE system: coincident presentation of oligonucleotide with the PE components, high concentrations at the absorbing site, and enteric protection to prevent component dilution and oligonucleotide exposure to low pH, which has been associated with rapid degradation. Plasma concentrations represent the absorption and distribution of oligonucleotide, but not the true tissue or body elimination. A more relevant metric for pharmacokinetics and bioavailability would be the total tissue levels. This is particularly true for oligonucleotide chemistries that are rapidly cleared from the bloodstream but are accumulated in target tissues or organs.

In line with this hypothesis, a study was performed to evaluate both plasma pharmacokinetics and tissue distribution of ISIS 2302 and ISIS 15839, a phosphorothioate oligodeoxynucleotide and a MOE hemi-mer with the same sequence.

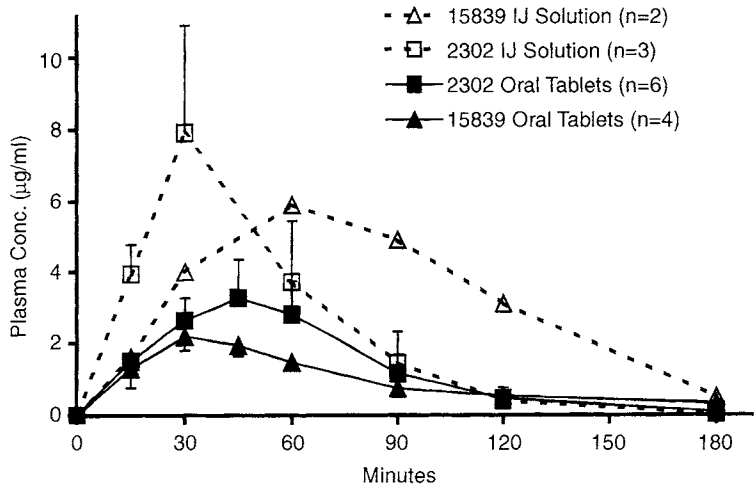

Figure 12 Intact oligonucleotide in dogs after either intrajejunal (IJ) administration of a solution formulation (open symbols with broken lines, 10 mg/kg) or oral administration of a tablet formulation (filled symbols with solid lines, ~15 mg/kg) of two oligonucleotide chemistries. ISIS 2302 (squares) is a phosphorothioate oligodeoxynucleotide; ISIS-15839 (triangles) is a hemimer MOE derivative with the same sequence. Error bars are standard error.

Pharmacokinetic parameters for each chemistry were assessed in beagle dogs during and following 14-day oral dosing of tablets containing oligonucleotide and PEs in enteric-coated tablets (97). The in vitro performance of these tablets was such that they remained intact in acid medium, yet rapidly dissolved (~15 min) at pH 6.5. In plasma, the ISIS 15839 gave approximately 150% the bioavailability found for ISIS 2302 (Table 6). However, the end-of-study oligonucleotide tissue concentrations for both liver and kidney cortex were approximately 10-fold higher for ISIS 15839 than for ISIS 2302 (Table 7).

Table 6 Summary of Plasma Oligonucleotide Pharmacokinetics After the First Single 200-mg Oral Dose in a Multiple-Dose Study

	ISIS 15839	ISIS 2302
AUC (µg*min/mL)	91	62
Cmax (µg/ml)	1.2	1.1
% Plasma BAV	1.4%	0.1–3%

* Represents a multiplication symbol.

Table 7 Tissue Oligonucleotide Concentrations and Approximate Bioavailabilities Based on Data Obtained at End of 2-Week Daily Dosing Study in Dogs

Oligonucleotide chemistry (20-base phosphorothioates)	Liver conc (μg/g)	Kidney cortex conc (μg/g)	%BAV[a]
ISIS 2302—antisense to ICAM-1 (deoxy)	4.9	12	1.3
ISIS 15839—antisense to ICAM-1 (hemimer MOE of ISIS 2302)	33	109	5.5

[a] Average bioavailabilities as ratio of final tissue concentrations (oral to i.v.), dose adjusted.

In this multiple-dosing study, the distribution of oligonucleotides out of the plasma circulation was very rapid with a distribution half-life of 30–60 min. Therefore, as mentioned earlier, the bioavailability assessments based upon plasma data may be inaccurate and/or misleading for this class of compounds and this relative range of absolute bioavailabilities. The bioavailability determined from sentinel tissues, such as the kidney and liver, may be a more credible representation of the true systemic exposure since these tissues reflect true elimination kinetics as well as accumulation to steady-state concentrations during chronic dosing. On this basis, our data indicate that bioavailabilities based upon organ concentrations (after oral and i.v. dosing) would be 50% higher for the kidney and 100% higher for the liver than those calculated by plasma AUCs.

In consideration of the practical difficulties involved in experimental determinations of organ bioavailability, we have performed simulations of organ accumulation after oral dosing of first-generation phosphorothioate oligodeoxynucleotide (Fig. 13) versus a MOE gapmer (Fig. 14). Each simulation is generated

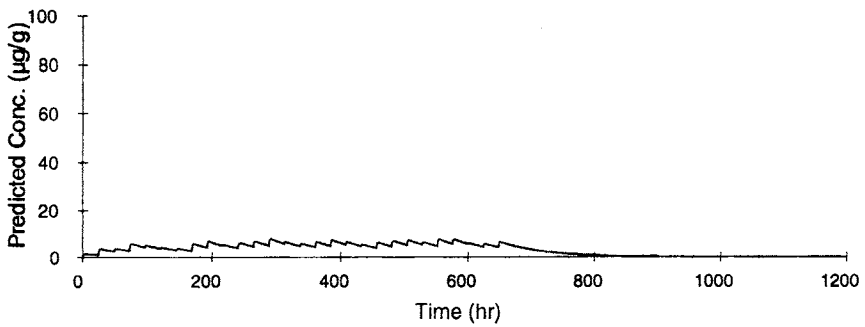

Figure 13 Simulation of tissue oligonucleotide concentrations for a daily oral dosing regimen of 1 month duration for a oligodeoxynucleotide at 2 mg/kg using a variable bioavailability input metric randomly generated from 2 to 15% (geometric mean 7%).

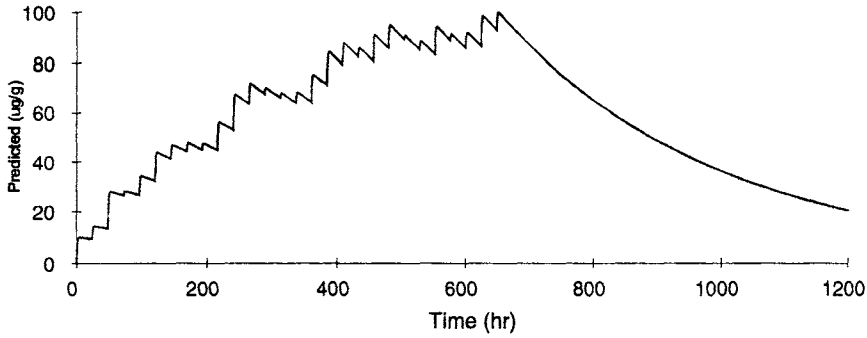

Figure 14 Simulation of tissue oligonucleotide concentrations for a daily oral dosing regimen of 1 month duration for a MOE gapmer chemistry at 2 mg/kg using a variable bioavailability input metric randomly generated from 2 to 15% (geometric mean 7%).

assuming a random-input metric of 2–15% oral bioavailability (geometric mean 7%) with all other pharmacokinetic parameters consistent for these two chemistries remaining fixed. The data indicate that even with variable absorption, the long half-life, which provides improved accumulation in tissue, makes it feasible to give the newer chemistry MOE gapmer compounds by the oral route.

To place this model in context we have recently dosed humans with first-generation phosphorothioate oligodeoxynucleotide solutions containing enhancers. This clinical study involved the administration of test solutions directly to the human jejunum by way of intubations. Preliminary analysis of the resultant oligonucleotide plasma concentrations after dosing via the intrajejunal route versus the intravenous route indicates bioavailabilities that are higher than those obtained by the comparable dog IJ model (unpublished data). Furthermore, it has been demonstrated that the presence of an intestinal tube will affect GI motility (98). Read et al. determined that, in the presence of an intestinal tube, peristalsis is induced in the small intestine enhancing the transit of food to the colon. The impact of such enhanced mid-gut motility on the absorption of formulated oligonucleotide solutions is unknown but is believed to diminish absorption due to the concomitant dilutional affect that such motility would have on the formulation's enhancers. For these reasons it is possible that these human data, as well as the dog model, are both underpredictors of what may be possible for orally dosed formulation bioavailabilities in humans. It appears certain that oral dosing using new chemistries of oligonucleotides will ultimately be available for a variety of indications and that oral dosing using first-generation oligodeoxynucleotides may be possible for selected indications, such as for certain sentinel organs or for local GI indications.

D. Local GI Uptake

Direct delivery of oligonucleotides to GI tissues, by way of oral or rectal dosage forms, may lead to significantly higher local oligonucleotide concentrations than doses by the parenteral route (94). These dosage forms may therefore be effective approaches to treat a variety of local GI disease indications. Such pathologies include certain malabsorption syndromes, inflammatory conditions, and carcinomas. A variety of sophisticated oral dosage forms may be pursued to more conveniently target oligonucleotide for a variety of local GI indications.

The local treatment of GI disorders, delivered through retention enema, is a commonly practiced therapeutic approach for conditions such as ulcerative colitis. Enema administration allows sustained retention of drug in the distal colon. Neurath et al. (99) locally administered a single, 150-μg dose of the antisense NF-κB phosphorothioate oligodeoxynucleotide targeted to mice with trinitrobenzene sulfonate (TNBS)-induced colitis. The oligonucleotide abrogated the clinical and histological signs of colitis more effectively than daily glucocorticoids, suggesting a treatment for Crohn's disease. Zhang and Agrawal have introduced oligonucleotides into the colon of rats as solutions and suppositories to test stability and uptake (81,100). Their work demonstrated the necessity of stabilizing the oligonucleotides to intestinal degradation.

A retention enema formulation to treat inflammation locally within the colon is currently under investigation at Isis Pharmaceuticals Inc. Absorption into local tissues was achieved in animal enema models using oligonucleotides in an aqueous buffered, viscous formulation. Two hours after a 1-h retention, levels of intact oligonucleotide in healthy tissue were about 100-fold higher than those achieved with an intravenous injection of an equivalent or higher dose (Table 8)

Table 8 Dog Colon Tissue Biopsy Concentrations of Intact ISIS 2302 Oligonucleotide After Single-Dose Intravenous (2 and 10 mg/kg) or Retention Enema (10 mg/kg) Administration

Formulation (enemas at 5 mg/mL oligonucleotide)	CGE (μg/g)	
	3 h	24 h
1.5% hydroxypropylmethylcellulose	660	7
1.0% carrageenan	558	3
Emulsion with Captex, Labrasol, and Crill	224	1
0.5% Tween 80, 0.75% HPMC	621	6
5% sorbitol, 0.75% HPMC	417	1
IV dosing at 2 mg/kg	2	NA
IV dosing at 10 mg/kg	11	0.6

(94). Furthermore, the presence of oligonucleotide was still evident the following day in this single-dose study.

In further support of this nonsystemic therapeutic approach are observations in mice (dextran sodium sulfate colitis model) and rats (TNBS colitis model) that locally administered solutions of oligonucleotides accumulate to a greater extent in inflamed GI tissue than in normal tissue (94). In the rat colitis model, ISIS 2302 in water was administered by intravenous or intracolonic injection. Oligonucleotide concentration was determined in liver and colon (Figs. 15 and 16). While there was no significant difference in tissue or plasma concentrations between normal and colitic rats when given oligonucleotide intravenously, large differences were found between normal and colitic rats for intracolonic administration. Concentrations in the colon, in particular, were considerably higher in the diseased tissue. The data also suggest that diseased tissue may be targeted with simple oral formulations, without the need for permeation enhancers. On

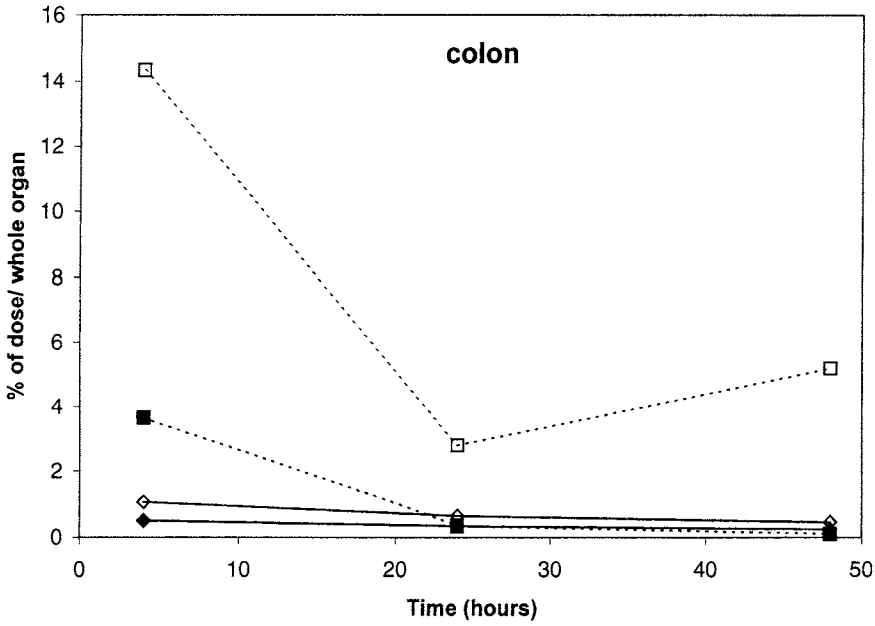

Figure 15 Rat colon tissue oligonucleotide concentrations as a percent of administered radioactive dose for normal rats (filled symbols) or treated with TNBS (open symbols) as a model for colitis. Solid lines represent intravenous route and broken lines designate rectal dosing—both at 100 mg/kg of ISIS 2302 with nonexchangeable ^{3}H label.

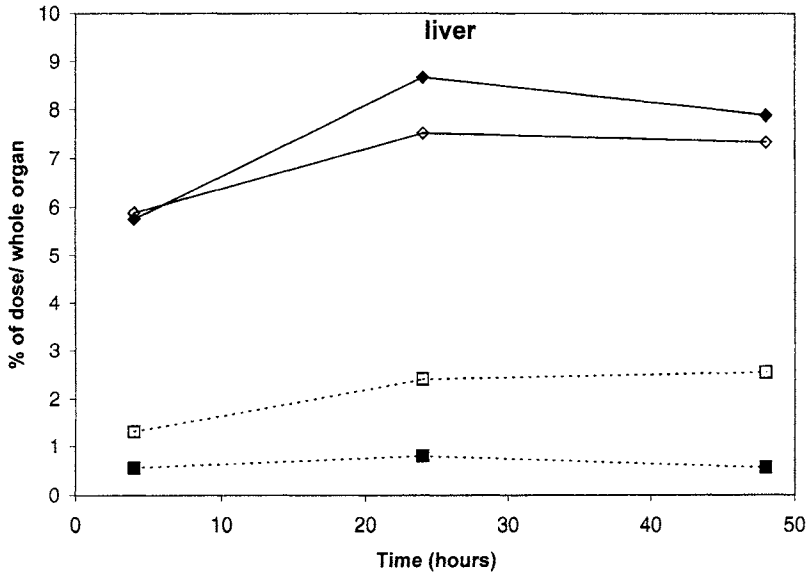

Figure 16 Rat liver tissue oligonucleotide concentrations as a percent of administered radioactive dose for normal rats (filled symbols) or treated with TNBS (open symbols) as a model for colitis. Solid lines represent intravenous route and broken lines designate rectal dosing—both at 100 mg/kg of ISIS 2302 with nonexchangeable ^{3}H label.

the basis of these observations we have recently begun clinical trials of ISIS 2302 in a retention enema formulation for ulcerative colitis.

VII. CONCLUSIONS

To date, clinical administration of antisense oligonucleotides has focused on local or parenteral routes using simple saline solutions. Intravenous dosing in animals has been shown to lead to accumulation of oligonucleotide in specific cells, such as endothelial and phagocytic cells, and organs such as the kidney and liver. These observations accommodate obvious targets for early clinical applications, for example liver diseases such as hepatitis C and the intraocular disease CMV retinitis.

Animal models have shown that formulations and delivery routes can be used to alter the tissue distribution of oligonucleotides. By making use of the knowledge gained through these studies, antisense oligonucleotides may be selectively delivered to a variety of target indications that would otherwise not be

accessible, such as the lung, skin, and colon. Isis Pharmaceuticals has recently begun early clinical trials with new topical and enema formulations to take advantage of these new opportunities.

Novel formulations designed using fundamental biopharmaceutical principles and guidance from relevant animal models make it possible to target those tissues that would fail to accumulate oligonucleotides from parenteral delivery of simple aqueous solutions. They also enable new routes to be practically considered that have the potential for increased patient convenience and compliance, as well as reduced treatment costs, with delivery methods such as topical, pulmonary, and oral.

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31
DNA-Binding Molecules

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I. INTRODUCTION

Cellular responses are mainly triggered through specific molecular interactions that result in targeted biochemical changes of signaling proteins, primarily through chemical modifications or binding events that alter their properties. Signals are passed along a complex network of pathways that ultimately leads to an altered level of gene expression by influencing the accessibility of DNA, changing the composition of present transcription factors, and/or changing their functional properties through modification.

Over the past years the scientific technology platform has expanded at a rapid pace. Sequence information, differential expression patterns, proteomics, and other methodologies allow us to increasingly understand biological phenomena at the molecular level. In principle a living organism is driven by the finely tuned expression level of its genome as defined by three parameters for each individual gene: *level*, *timing*, and *localization* of expression. The understanding of these parameters for the complete human genome, in particular the differences between diseased and healthy individuals, will deliver an abundance of new targets for drug discovery. So far, roughly 500 targets have been addressed in drug discovery, representing only an estimated 5–10% of all potential molecular targets that will be discovered mainly as a consequence of the Human Genome Project (1).

The cell-surface glycoprotein HER-2/neu is an excellent example of a disease-related molecular target. This receptor protein was reported to be overexpressed in roughly 30% of human breast cancers (2–4). Targeting the HER-2/neu receptor in malignant cells by a monoclonal antibody was shown to inhibit

cell growth and the ability to form colonies. This discovery was successfully translated into a therapeutic application by the design of a humanized monoclonal antibody targeting HER-2/neu (Herceptin) that was approved by the FDA in 1998 for the treatment of metastatic breast cancer.

Traditionally, mainly proteins served as molecular targets for therapeutics. Within a closely related family such as tyrosine kinases, the acquired knowledge in drug discovery for a defined protein will be beneficial for new, structurally related targets. However, a rapidly increasing number of proteins fail to produce hits or leads in drug discovery by high-throughput screening approaches. Such proteins often lack a defined binding pocket for low-molecular-weight compounds and mainly function through protein-protein interaction of a large, shallow surface area. This lack of feasibility for a number of attractive targets and the desire for a truly general approach in drug discovery make nucleic acids, DNA and RNA, highly attractive as molecular receptors. Whereas RNA as target is extensively captured in this book, this chapter aims at providing a brief overview on DNA-binding molecules, mainly focusing on newer approaches that target DNA with the aim to specifically control gene expression.

Even though proteins have been the main focus of drug discovery, DNA as molecular receptor for drugs is not a new concept and from all known mechanisms of therapeutics, roughly 2% are based on interactions with DNA (1). Aside from DNA-binding proteins, Nature provided a structural variety of molecules that bind DNA (Fig. 1) and, as a consequence, have a profound biological activity. A number of excellent review articles have been written on DNA-binding molecules or drugs (5–11) to which the reader is referred for more detailed information on specific classes of compounds.

Within a cell, DNA is largely bound to proteins that restrict its general accessibility. The core structure or nucleosome was initially proposed (12) and later structurally resolved at high resolution (13–15). It consists of roughly 200 base pairs of DNA wrapped twice around an octameric complex of histone proteins H2A, H2B, H3, and H4. These disk-like nucleosomes are central structural elements of chromatin whose accessibility is governed by a variety of mechanisms like histone acetylation and deacetylation, chromatin remodeling, or the formation of heterochromatin (for an excellent review see Ref. 16).

The transcriptional control of a specific gene is determined by the ordered assembly of DNA-binding proteins on the regulatory or promoter regions that are located upstream of the coding region (for reviews see Refs. 17–22). Activator or repressor proteins bind to their DNA target sites within the regulatory domain and orchestrate the level of transcription by controlling the recruitment of the basal transcription complex. The fact that transcription cannot be extrapolated from in vitro experiments to cell-based or in vivo studies indicates the importance of chromatin structure in the overall process of gene expression.

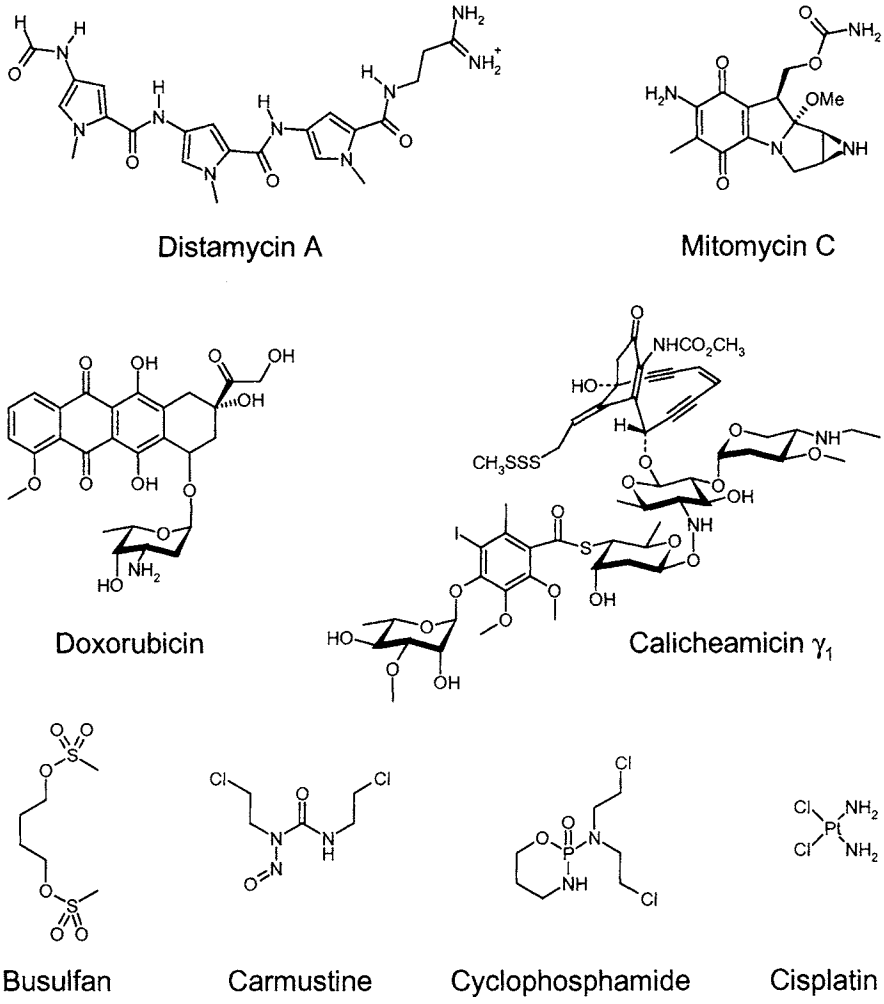

Figure 1 Selected representation of biologically active compounds that interact with double-stranded DNA.

The transcriptional level of a large number of genes is controlled by small molecules, primarily through highly specific interactions with membrane-bound or soluble receptors that trigger a cascade of signaling events, ultimately stimulating or inhibiting the expression of a particular set of genes. The disruption or modulation of DNA-protein interactions should be a generally applicable strategy to interfere with the mentioned processes crucial for transciptional regulation.

The attractiveness of aiming at a highly conserved and structurally defined target, namely DNA, is obvious but also raises some key issues. Especially the predominant question on specificity needs to be addressed. How discriminative will a particular molecule bind to a match target site as opposed to a structurally similar mismatch site? What is the required length of a target site to obtain sufficient functional specificity? In particular this latter question is interesting conceptionally as various hypotheses will inevitably lead to different conclusions. In theory, a binding site size of 17 base pairs should occur only once in the total human genome (23) and could be considered as upper limit for a DNA-targeting approach. In an ideal hypothetical case one individual molecule would interfere with the expressional level of only one gene. Taking into account that there are roughly 30,000 individual genes of which only approximately 1/3 are transcriptionally active, a realistic target number for an optimal binding site is more likely between eight and 12 base pairs. Even this range of interaction on double-helical DNA is demanding as a molecular entity needs to cover between 40 and 60 Å of groove space, requiring a compound with at least 600 Dalton molecular weight. On the other hand, DNA-binding molecules like mitomycin C or doxorubicin are clinically used to treat cancer and therefore have been shown to possess therapeutic use despite lacking a high level of sequence specificity (albeit with some drastic side effects). The functional specificity in a complex organism like human beings is influenced not only by molecular interactions on cellular DNA or its molecular receptor but is governed as well by a variety of other properties like, for example, metabolic stability, biodistribution, and pharmacokinetic behavior of the drug molecule.

Different approaches have been discovered over the past decades that allow the direct design of a variety of DNA-binding molecules. The following sections will briefly cover some of the most promising approaches (some of them are extensively covered in this book as individual chapters) with the main focus on minor groove-binding polyamides as the most promising approach reported to date (24).

II. DNA-BINDING PROTEINS

Nature chose a variety of structurally distinct proteins to recognize double helical DNA with high affinity and specificity (Fig. 2). The wider major groove allows more base-pair-specific interactions for sequence readout and, not surprisingly, is the prime site for protein interactions, even though additional minor groove contacts are found in a subset of these proteins (for selected examples see Fig. 2,b–d). Particularly interesting are zinc finger proteins as visualized with the structure of the complex between Zif268, a three-finger protein, and its target DNA (Fig. 2a) (25–27). The modular occurrence of these structurally defined

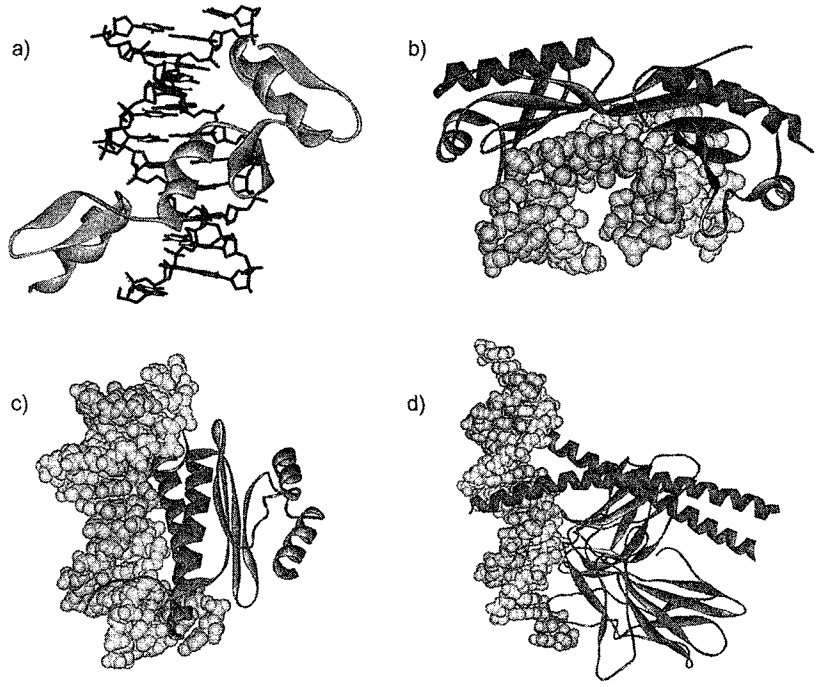

Figure 2 Different DNA-binding proteins based on the retrieved coordinates from the Protein Data Bank (PDB). (a) Zinc finger protein Zif268 bound to its target DNA site (PDB code 1A1L) (25,26). (b) Structure of a human TATA-box-binding protein/TATA element complex (28) (PDB code 1CDW). (c) Structure of the serum response factor core bound to DNA (29) (PDB code 1SRS). (d) Ternary protein complex between NFAT, jun, and fos bound to DNA (30) (PDB code 1A02).

fingers makes them ideal candidates to explore the opportunity of creating proteins with similar structure but altered binding preferences. A single zinc finger domain of approximately 30 amino acids consists of an α-helix and a short two-stranded antiparallel β-sheet that are held together by hydrophobic interactions and a Cys_2His_2 coordinated zinc atom. The α-helix of this domain is facing into the major groove enabling the side chains to make specific contacts mainly with the edges of three base pairs (5′-GNN-3′). The use of combinatorial technologies and/or structure-based rational design (31–33) allowed the creation of new zinc finger proteins that recognize target sites of up to 18 base pairs with high affinity and sequence selectivity.

Transcription factors act frequently through the combination of DNA-binding domains for the exact localization on genomic DNA and linked effector do-

mains that either activate or repress transcription at or near that site. In analogy to this strategy used by nature, new proteins have been created consisting of designed zinc finger proteins that bind to 18 base pairs of DNA and either the activation domain of herpes simplex virus VP16 or the repression domain Krüppel-associated box (KRAB). These fusion proteins were shown to be effective activators or repressors in mammalian cells after transfection of the corresponding constructs, both in gene reporter assays [erbB-2 (34)] and on the endogenous genes [erbB-2 and erbB-3 (35)]. Such gene switches will have a great potential as tools in biology (e.g., functional genomics or diagnostics) but most likely will be limited to gene therapeutic approaches for the treatment of human diseases.

III. TRIPLE-HELIX-FORMING OLIGONUCLEOTIDES

Shortly after Watson and Crick published the structure of double-stranded DNA, homopurine-homopyrimidine nucleic acids were reported to form stable triple helices under appropriate conditions (36). The third strand is accommodated in the wide major groove allowing the third base to specifically form hydrogen bonds with the purine base of the duplex (Fig. 3). This potential for molecular

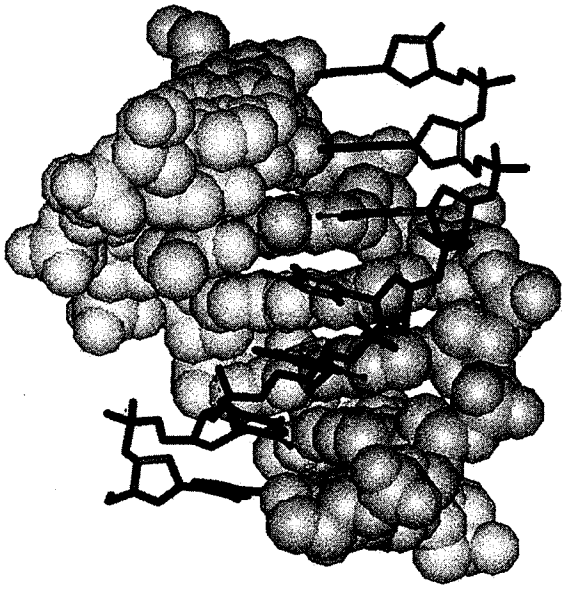

Figure 3 Solution structure of a Py-Pu-Py triplex (37) (PDB code 177D); for clarity the loops were omitted in this illustration.

recognition was used to design oligonucleotides that were able to specifically bind to complementary stretches of double-stranded DNA (38,39). The ability to bind to DNA at preselected sites and interfere with protein binding is highly attractive for the regulation of gene expression. However, the sequence requirement to target homopurine stretches, marginal binding affinity under physiological conditions, lack of oligonucleotide stability against nuclease degradation, and extremely slow binding kinetics reduced the initial excitement of triplex-forming oligonucleotides as tools to control gene expression under physiological conditions. Selected chemical modifications such as intercalator conjugates (40), phosphoramidates (41), or 2′-aminoethyl ribonucleosides (42) improved some of these properties and a limited number of experiments have been published that indicate the potential of this approach to interfere with gene expression (for reviews see the chapter in this volume by Glazer and Ref. 43). The major issues that will limit a general use of triplex-forming oligonucleotides are the difficulty of cellular penetration for this class of compounds and the current restriction to target exclusively homopurine sequences on double-stranded DNA.

IV. PEPTIDE NUCLEIC ACIDS

During the intense search for oligonucleotide modifications with improved properties for antisense or triplex applications, a remarkable new analog was discovered lacking both the phosphodiester backbone and the carbohydrate moiety (44). The structural simplicity and properties of these achiral peptide nucleic acids (PNA) were stunning; they not only bound to single-stranded DNA or RNA with high affinity but could also displace double-stranded A-tracts and form a local triplex (for reviews see the chapter in this book by Nielsen and Refs. 45–47). The expansion of this initial strand displacement approach was achieved in an elegant way by using a double-duplex invasion of two complementary PNA strands (48). To optimize the overall binding energy, adenine and thymine were replaced in the PNA strands by diaminopurine and thiouracil to increase the binding affinity for DNA and simultaneously destabilize the duplex between the two PNA strands. Even though these results look encouraging at first glance, a general application to control gene expression is unlikely to work based on the requirement of low ionic strength for this double-duplex invasion that cannot be achieved under physiological conditions.

V. SMALL DNA-BINDING MOLECULES

A variety of compounds have been discovered that bind to double-stranded DNA. In principle, they can be divided into three classes based on their molecular recog-

nition of DNA: molecules governed by electrostatic interactions, intercalators, and minor groove binders. This classification is not strict as many compounds use more than one of these interactive modes to bind to DNA. From a functional perspective, biologically and clinically relevant compounds fall in three groups of compounds that either alkylate or modify DNA, induce single- or double-strand breaks, or simply bind to DNA by either intercalation or minor groove interaction. It is noteworthy that small molecules usually do not bind to the roughly 11-Å-wide major groove of B-DNA as an optimal fit would require a much larger molecular entity such as structural features of proteins.

DNA-binding molecules were mainly discovered by serendipity as a result of screening efforts for antibacterial or antiproliferative activity of new compounds isolated from natural sources. The capability of these molecules to target double-stranded DNA was mostly discovered and investigated after biological activity was established in animal models. The following paragraphs will briefly list the main classes of small molecules interacting with DNA and should serve only as a brief overview of this topic that has been covered more extensively in a number of excellent reviews (5,7–9,11,49–53).

The oldest class of compounds with a therapeutic use and known to interact with DNA are alkylating or DNA-adduct-forming agents like cyclophosphamide, cisplatin, busulfan, or carmustine. Sulfur mustard was first synthesized in 1854 and the vesicant properties were discovered more than 30 years later. Between World Wars I and II extensive studies of the biological and chemical actions of the nitrogen mustards were conducted and the marked cytotoxic effect on lymphoid tissue initiated experiments on transplanted lymphosarcoma in mice. Clinical trials were started in 1942 and over the following decades thousands of different nitrogen mustards were synthesized and a few of these agents proved to be useful for clinical applications to treat particular neoplasms. Other classes of compounds like ethyleneimines, alkyl sulfonates, nitrosoureas, and triazenes were added to these DNA-adduct-forming agents. Common features of all these molecules are relatively low molecular weight, no high affinity for DNA combined with the ability to form covalent adducts and crosslinks, a limited sequence specificity, and, as a consequence, a small therapeutic index or a relatively high level of general cytotoxicity.

Another class of compounds that interact with DNA in a relatively nonspecific way are intercalators, represented by the well-studied acridines, actinomycin D, and the natural product doxorubicin (54–58). The latter belongs to the family of anthracycline antibiotics or antitumor agents that were isolated from various *Streptomyces* strains. Anthracyclines have indicator-like properties and consist of a core tetracyclic anthraquinone choromophore carrying one or multiple carbohydrate substituents. They interfere with DNA replication and transcription by binding to double-stranded DNA via intercalation, preferably at alternating py-

rimidine-purine tracts, placing the carbohydrate moiety in the minor groove. In addition, they interfere with topoisomerase II activity (59). Owing to an intense interest in their biological behavior and shortcomings in the clinical application (cardiotoxic side effects and development of resistance) efforts were initiated to improve the therapeutic profile, albeit with little success. It is worthwhile to note that these molecules most likely do not exercise their biological function exclusively through DNA interactions but do bind to plasma and intercellular membranes as well (58). Activation of the chromophore by either topoisomerase or the generation of free radicals generates single- or double-strand breaks. The question arises of the importance of DNA damage in relation to the observed biological activity as this might be a critical mechanistic property of these therapeutically useful molecules.

Additional classes of DNA-modifying compounds have been discovered over the years that bind with existing but limited selectivity to DNA. Among those are bleomycins, mitomycin C, or the ene-diyne antibiotics like calicheamicin γ_1 or esperamicin A_1. The latter family of antibiotics is not only interesting based on the structurally unique ene-diyne warhead that induces single- or double-strand breaks in DNA but also owing to the extended carbohydrate moieties that bind in the minor groove and provide a limited sequence selectivity. Despite the incredible potency to inhibit tumor growth in animal models, these compounds have not yet found a successful application in the clinic.

A. Polyamides

1. Introduction

An interesting new class of antitumor antibiotics was discovered with the *N*-methyl pyrrole amides distamycin A and related compounds. Originally isolated roughly 40 years ago by Arcamone, these structurally simple and achiral compounds revealed an interesting variety of biological properties (60,61). Distamycin A binds with micromolar affinities to A/T-rich DNA sequences (62–65), is readily taken up by mammalian cells and transported to the nucleus, inhibits DNA- and RNA-dependent DNA synthesis in vitro, and can affect nucleosome (66) and chromatin structure (67,68). Activity was reported against DNA viruses, some retroviruses, and Gram-positive, bacteria and the compound was clinically evaluated as a topical agent, providing its efficacy in the treatment of herpes simplex, herpes zoster, and chickenpox viral infections. Despite these activities distamycin A shows little cytotoxic effects and only marginal antitumor activity. Additional biological activities like the influence on the expressional level of human estrogen receptor (69), the inhibition of human DNA ligase (70), or the displacement of essential transcription factors (71) were discovered. Based on

all these investigations it is obvious that distamycin A represents a fascinating class of molecules but the question arises why this natural product never took the hurdle to a successful application in the clinic.

A combination of factors might have contributed to the final clinical failure: lack of stability based on hydrolytic formamide cleavage followed by oxidation of the terminal aminopyrrole, lack of sufficient sequence specificity, and/or moderate binding affinity to DNA in the low micromolar range. These are selected properties and reflect a critical analysis of the observed experimental behavior of distamycin A. Increased stability paired with improved sequence selectivity are achievable goals for this class of molecules and might lead to a new class of powerful therapeutics as outlined below. A striking attractiveness of distamycin analogs or polyamides lies in the ease of synthesis setting these molecules apart from more complex structures such as calicheamicin γ_1 (72). The combination of readily available building blocks with a reliable assembly of the final molecules on solid support allows a rapid production of new analogs. Consequently, iterative cycles to optimize selected compounds for multiple parameters—e.g., the DNA-binding property or metabolic stability—are feasible and enable the optimization of polyamides within a short time frame.

Despite all these attractive features of distamycin and its analogs, mainly properties like binding affinity and specificity had to be greatly improved to compete with nature's transcription factors. As these proteins bind with low-nanomolar or high-picomolar affinities to their target sites on double-stranded DNA, the affinity of polyamides needed to be improved by about three to four orders of magnitude. In addition, regarding functional specificity, i.e., the inhibition of a specific gene expression within the human genome, their specificity for a broader variety of predetermined DNA sequences and their binding-site size had to be optimized. Several research groups started to address these issues and have played a key role in the analysis of the binding mode of polyamides as well as in the optimization of their DNA-binding affinity and specificity.

Dickerson and co-workers provided the first structural evidence on how this class of molecules interacts with DNA (73,74). They demonstrated by X-ray crystallography that netropsin (Fig. 1), the natural product containing two *N*-methyl pyrrole rings, forms a 1 : 1 complex in the narrow minor groove of an A/T-rich DNA fragment.

Based on this 1 : 1 binding model Dickerson and Lown proposed that the replacement of a pyrrole heterocycle (Py) in netropsin by *N*-methylimidazole (Im) should allow the recognition of a G·C base pair (74,75). However, the DNA-binding specificity of imidazole-containing netropsin/distamycin analogs could not be rationalized using the 1 : 1 model. For instance, Dervan and co-workers synthesized the molecule containing an imidazole followed by two pyrrole units (ImPyPy) and examined its binding affinity and specificity against a library of DNA-binding sites by quantitative footprint titration and affinity cleavage experi-

ments (76,77). Unexpectedly, ImPyPy bound to (A/T)G(A/T)C(A/T) instead of the predicted site. The affinity cleavage experiment further revealed that ImPyPy bound with twofold symmetry rather than a single orientation as expected for the 1:1 model. This initially confusing result was clarified by the Wemmer group, who discovered by NMR that the natural product distamycin A bound to the minor groove of DNA in a 2:1 as well as in a 1:1 stoichiometry, depending on the concentration (78). These findings indicated that ImPyPy also bound as an antiparallel 2:1 dimer to the sequence 5′-TGTCA-3′ allowing an excellent explanation of the observed experimental results. The discovery of this 2:1 binding stoichiometry of polyamides to double-stranded DNA marked a breakthrough in the polyamide field that subsequently led to significant discoveries.

Pioneering efforts were mainly reported from the groups of Lown and Dervan. Whereas Lown approached this class of molecules (lexitropsins) from a medicinal chemistry point of view, the Dervan group was dedicated for the last 25 years to explore various approaches that would allow the design and synthesis of molecules with high binding affinity and specificity for selected target sites on double-stranded DNA. The following sections present a brief overview on key achievements of polyamides that can be considered as scientific foundation for a new class of compounds with the potential of therapeutic benefits.

2. Pairing Rules

Encouraged by the initial results with the simple distamycin analog ImPyPy, Dervan and associates prepared several imidazole-containing polyamides and established a first set of pairing rules for polyamides binding to DNA (79). An imidazole opposite a pyrrole targets a G·C base pair whereas a pyrrole opposite an imidazole recognizes a C·G base pair. A Py/Py pair is partly degenerated and recognizes both A·T and T·A pairs in preference to G·C and C·G. The antiparallel 2:1 motif with the polyamide-oriented N-C with respect to the 5′-3′ direction of the DNA helix in the minor groove was confirmed by NMR for ImPyPy (76). More recently a high-resolution X-ray crystal structure revealed the hydrogen bond between the N(3) of imidazole and the exocyclic amino group of guanine and a total of three hydrogen bonds between an Im/Py pair and the edge of the G·C base pair (80).

The A·T/T·A degeneracy of the Py/Py pair constituted an unsolved problem for a long time. The minor groove has even been thought to lack sufficient information for a complete recognition code (81). Despite these speculations, the A·T/T·A degeneracy was successfully resolved and the polyamide/DNA pairing code completed (82). In a rational design approach the new aromatic amino acid *N*-methyl-3-hydroxypyrrole (Hp) was introduced to the repertoire of monomeric building blocks. As anticipated, an Hp/Py pair recognizes a T·A pair while a Py/Hp base pair targets an A·T bp (Fig. 4). Both pairs disfavor binding to either

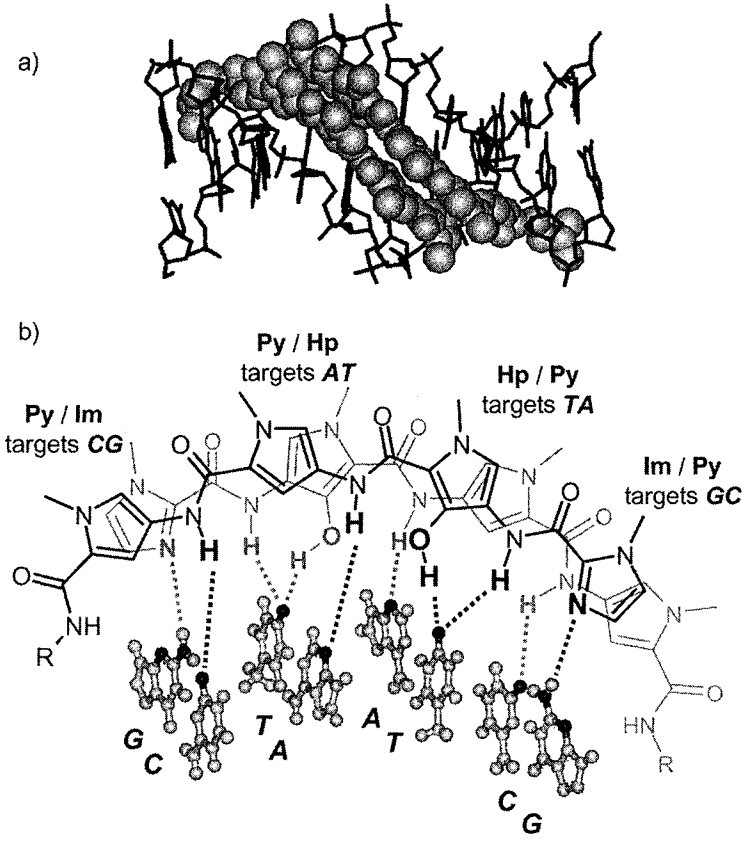

Figure 4 (a) High-resolution X-ray crystal structure of the polyamide ImHpPyPy-β-Dp recognizing the DNA sequence 5′-AGTACT-3′ as a dimer; all four Watson-Crick base pairs are discriminated in the minor groove (83). (b) Schematic representation of the interaction of the two ligand molecules and the minor groove of DNA. The newly introduced Hp/Py pair forms three specific hydrogen bonds to the T·A base pair.

G·C or C·G. As confirmed by a high-resolution X-ray crystal structure, the hydroxyl group of Hp fits tightly into the asymmetrical cleft between thymine and adenine and the Hp unit forms two hydrogen bonds to O(2) of thymine (82,83). In fact, there are three specific hydrogen bonds between the Hp/Py pair and the T·A base pair. Figure 4 illustrates the shape-selective recognition of the DNA match sequence 5′-CATG-3′ by two ImHpPyPy molecules, indicating the base-pair-specific hydrogen bonds that force the correct side-by-side alignment in the

minor groove. The ability to recognize all four Watson-Crick base pairs therefore made it possible in theory to target any sequence on genomic DNA.

3. Affinity and Specificity

Transcription factors typically bind at nanomolar concentrations to their target sites within the regulatory elements of the human genome. An isolated factor often contacts about six to eight base pairs with only moderate specificity with respect to a single base-pair mismatch site. The specificity for a predetermined sequence within the genome arises from a highly cooperative binding as a multi-protein complex to larger DNA sequences (84).

In contrast, the affinity of unlinked three-ring dimers such as distamycin A or ImPyPy to their respective match sites is modest (low micromolar). By connecting the carboxyl and amino termini of two such molecules with a γ-amino butyric acid linker, the affinity could be increased by two to three orders of magnitude (85). These covalently linked molecules bind in a hairpin-like conformation to DNA conserving the antiparallel side-by-side alignment of the aromatic amino acid units as confirmed by NMR (86). As with extended polyamides, the hairpin compounds are preferably oriented N-C with respect to the 5′-3′ direction. In special cases, a reversed binding orientation has been observed as well (87).

Such hairpin polyamides exhibit an extraordinary affinity for their target site that increases with the number of heteroaromatic units. In a key study, Dervan et al. demonstrated that the eight-ring hairpin polyamides shown in Fig. 5 bind their predetermined match sites at subnanomolar concentrations while target sites containing a single base-pair mismatch are discriminated by seven- and 90-fold, respectively (88). It was subsequently found that the γ-turn element (loop) and the carboxy-terminal "β-linker" (tail) prefer to bind to A·T or T·A base pairs and consequently add to the overall binding specificity of hairpin polyamides (89).

4. Binding-Site Size

To achieve an optimal functional specificity, i.e., the transcriptional modulation of one specific gene within the human genome, the optimal binding-site size is a key issue. Purely based on statistics a single match site within the whole genome should consist of about 17 contiguous base pairs. However, in the context of chromatin only a fraction of the genome is most likely accessible for DNA-binding molecules. In addition, first results indicate that only molecules binding to important sites within the *cis*-regulatory elements interfere with gene trancription (vide infra). Thus, the part of the genome that is relevant as a target for therapeutic agents may be significantly smaller than the whole genome and consequently targeting eight to 12 base pairs might be optimal (vide supra for a more extensive discussion).

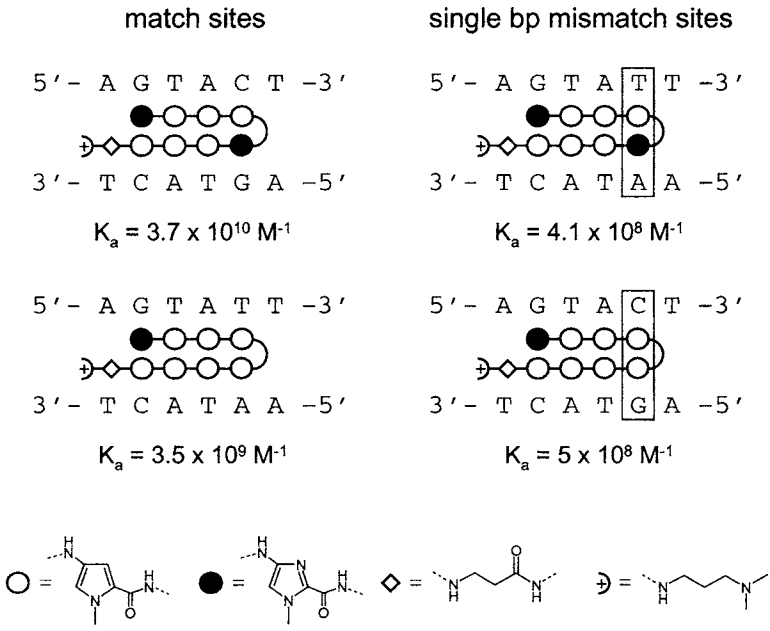

Figure 5 Example of two eight-ring hairpin polyamides recognizing their match sites with affinities 10–100-fold higher with respect to the corresponding single-base-pair mismatch site.

In a first effort to increase the binding site size of polyamides, Dervan and co-workers have found that the binding affinity maximizes at a contiguous ring number of five and decreases as the lengths of the crescent-shaped molecules and the corresponding DNA binding-site size increase (90). A high-resolution X-ray crystal structure of the four-ring polyamide ImImPyPy-β-Dp bound as a dimer to a six-base DNA site revealed that the curvature of the polyamide is slightly overwound with respect to the DNA (80). This finding is consistent with unfavorable interactions for molecules containing more than five consecutive rings. This problem has been overcome by the introduction of the conformationally flexible β-alanine (β) unit to the polyamide building blocks (91–93); replacing an internal Py/Py pair by the more flexible β/β combination "relaxes" the ligand curvature in a way that the polyamide can optimally adjust to the curvature of the DNA helix. An NMR study has recently confirmed the extended, antiparallel 2:1 binding motif of ImPyPy-β-PyPyPy-β-Dp with the aromatic rings fully overlapped (86). The β/β pair has been shown to favor A·T and T·A with

respect to G·C/C·G recognition by at least 20-fold. In some cases, a side-by-side pairing of the aliphatic β-alanine with Py or Im has also been found to compensate for sequence composition effects. For instance, the ligand Im-β-ImPy-γ-Im-β-ImPy-β-Dp binds with a 100-fold-increased equilibrium association constant to its G,C-rich match site 5′-TGCGCA-3′ as compared to ImPyImPy-γ-ImPyImPy-β-Dp ($3.7 \times 10^9\ M^{-1}$ vs. $3.7 \times 10^7\ M^{-1}$) (94). In summary, the flexible β-alanine allows the generation of polyamides that bind to longer target sequences with subnanomolar affinities. An example of various binding motives is represented by the slipped dimer motif (Fig. 6c) (93) in which two identical polyamides bind cooperatively to larger DNA sequences without significantly increasing the molecular weight as compared to an eight-ring hairpin polyamide (92).

An alternative approach to increase the binding-site size was pursued by the covalent tail-to-turn linkage of two hairpin polyamides. For example, two six-ring hairpins were connected by a five-amino valeric acid linker to yield a

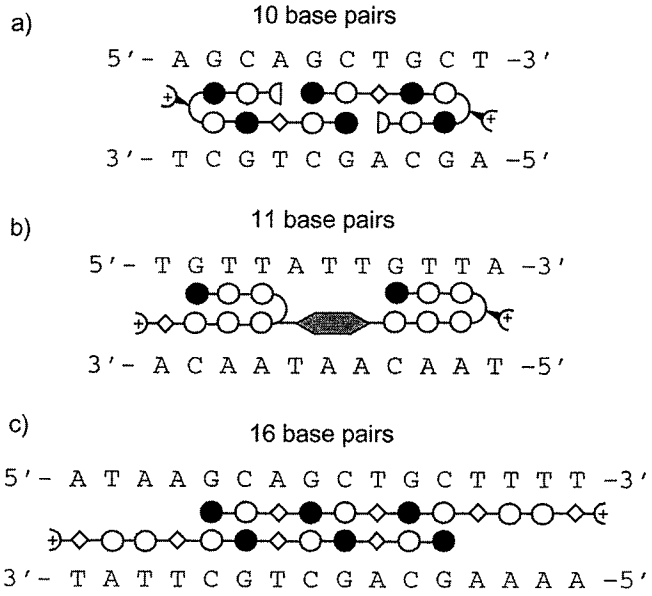

Figure 6 Selected polyamides designed to recognize longer stretches of double-stranded DNA. (a) A cooperatively binding hairpin dimer recognizing a 10-bp DNA sequence. (b) Two covalently linked six-ring hairpin polyamides target 11 bp. (c) A partially overlapping extended homodimer recognizing 16 bp.

tandem-hairpin polyamide that bound its 11-bp target site with an equilibrium constant of 1×10^{12} M^{-1} and >4500-fold specificity versus a double-base-pair mismatch sequence (Fig. 6b) (95).

5. Inhibition of Protein/DNA Interaction

DNA-binding proteins can roughly be divided into three categories according to their DNA-binding mode; proteins binding exclusively to the major groove, to the minor groove, and those contacting both grooves. For example, the TATA-box-binding protein (TBP, Fig. 2b), which plays a crucial role in the transcription of most genes, binds exclusively to the minor groove forcing the DNA to bend significantly. A considerable number of proteins contact both grooves such as the nine-zinc-finger transcription factor TFIIIA or the ets protein family. Basic leucine zipper proteins (bZIP) represent a class of proteins that contact exclusively the major groove of DNA (e.g., jun and fos, Fig. 2d).

Analogs of distamycin that bear polyamine chains at the central Py unit of the tripyrrole oligoamide and bind A,T-rich DNA sequences have been found to inhibit DNA binding of proteins (96,97). In addition, it has been demonstrated that Im-containing polyamides are able to competitively inhibit protein/DNA recognition in cases where minor groove contacts are essential for protein binding (98). This constituted an advance in the field as Im-Py polyamides have the potential to target a broad variety of predetermined DNA sequences with high specificity. This was experimentally demonstrated with the TBP, the lymphoid-enhancer binding factor 1 (LEF-1), and the stimulatory factor Ets-1. All these proteins that bind within the HIV-1 enhancer/promoter element can efficiently be inhibited from DNA binding by the correspondingly designed Im-Py polyamides (99). TBP and LEF-1 both bind to the minor groove of DNA while Ets-1, a member of the ets protein family, binds primarily the major groove with additional contacts in the minor groove (100). The DNA recognition site of these proteins is not likely to be an optimal target sequence for polyamides as they are conserved in promoters of many genes. Rather, the sequences flanking and partly overlapping with the protein-core-binding site are gene-specific and therefore provide better target sites (101,102). In addition, as observed, for example, with the TATA-box-binding protein or the serum response factor (Fig. 2, b and c), binding of many transcription factors induces a distortion of DNA that potentially could be prevented in an allosteric manner by appropriately bound polyamides (103).

Similarly to Ets-1, the nine-zinc-finger transcription factor TFIIIA binds to both grooves; fingers 4 and 6 bind in or across the minor groove and are required for high-affinity binding whereas the other zinc fingers exclusively contact the major groove. An eight-ring hairpin polyamide targeting the binding region of the minor groove contacting finger 4 has been found to inhibit TFIIIA binding greater than 90% at a concentration of 5 nM (98,104). In contrast, an

eight-ring hairpin polyamide and a recombinant protein containing only the three-amino-terminal, major-groove-binding zinc fingers of TFIIIA can simultaneously occupy the TFIIIA-binding site.

As polyamides fit tightly into the minor groove it is not surprising that they can simultaneously bind to DNA with a protein that exclusively binds to the major groove. Earlier studies also demonstrated that the three-ring homodimer ImPyPy co-occupies the DNA helix with the basic-region leucine zipper protein GCN4 (105). Therefore, the question arose how polyamides could competitively interfere with the large family major-groove-binding proteins. This problem was solved in an elegant way by equipping polyamides with an Arg-Pro-Arg tripeptidic substituent that can interfere efficiently with GCN4 binding in the adjacent major groove (106). It was rationalized that the correctly positioned Arg-Pro-Arg unit delivers a positive patch to the DNA backbone and severely disturbs the protein backbone interaction. Thus, a subtle alteration of the DNA microenvironment at a precise location within a specific DNA sequence can result in both gene-specific and protein-specific targeting.

6. Inhibition of Gene Expression

Polyamides target a large variety of predetermined DNA sequences with sufficient affinity and specificity to interfere with protein/DNA complexes. If designed to bind immediately flanking to the core-binding site of an important transcription factor or activator, they may compete selectively with this protein and affect the assembly of the transcriptional complex that is responsible for the recruitment of RNA polymerase II. Thus, they potentially offer a general approach to specifically regulate the transcription of an individual gene.

In a first case study the TFIIIA-activated transcription of 5S RNA genes by RNA polymerase III was investigated. An eight-ring hairpin polyamide that is known to interfere with the TFIIIA/DNA complex was shown to inhibit TFIIIA-mediated transcription in vitro by greater than 80% at 60 nM (98). Under these conditions only a small degree of nonspecific inhibition of tRNA transcription was observed. As expected, mismatch polyamides did not interfere with 5S RNA transcription at concentrations up to 60 nM. In cultured *Xenopus* kidney cells the match polyamide had a pronounced and selective effect on 5S gene transcription at concentrations as low as 100 nM. At higher concentration a general decrease in the transcriptional activity was observed. At concentrations up to 1 μM, the polyamide displayed no cellular toxicity, as measured by cell density. These results provided the first evidence that polyamides in general and eight-ring hairpin molecules in particular are able to penetrate eukaryotic cells and selectively interfere with gene expression.

Encouraged by these results, Dervan and Gottesfeld examined whether polyamides could also regulate specific genes transcribed by RNA polymerase

II in human cells. The two eight-ring hairpin polyamides that inhibit Ets-1, LEF-1 and TBP upon binding adjacent to the binding sites within the HIV-1 enhancer/promoter element were shown to inhibit HIV-1 transcription in a cell-free assay (107). When used in combination at 1 μM each, the two polyamides inhibited viral replication by more than 99% in isolated human peripheral blood lymphocytes as determined by ELISA quantification of the viral capsid protein p24. Again, no obvious toxicity was observed even at prolonged exposure of cells to various polyamides.

Notably, a control polyamide that does not interfere with LEF-1, TBP, and Ets-1 binding, but binds within the HIV-1 RNA-coding region, does not inhibit gene transcription. In addition, the active polyamide does not inhibit a set of seven control genes in which the DNA sequences flanking to the previously mentioned transcription factors are different. These results indicate that the process of transcription inhibition by designed polyamides is specific for the promoter region as well as for the polyamide.

VI. CONCLUSIONS AND OUTLOOK

An organism and its functions can be described by its complete genome according to the time, level, and space of expression for each individual gene. An imbalance of these fine-tuned and interconnected expressional patterns is often associated with diseases. Thus, the ability to interfere specifically with the expressional level of an individual gene or a gene family will undoubtedly become extremely powerful for disease management. In particular the increasing knowledge of the human genome and its disease-related alterations, combined with powerful diagnostic techniques, will form the basis to successfully address the regulation of gene expression. To date, however, the opportunities to target DNA as a molecular receptor for drug discovery are still limited; promising approaches such as engineered zinc finger proteins, triplex-forming oligonucleotides, strand-invading peptide nucleic acids, and intercalating and/or minor-groove-binding small molecules have been reported. Only the last class of agents provided a few compounds of therapeutic value that are used clinically in oncology, despite severe side effects.

Polyamides represent a new class of DNA-interacting molecules that combine a number of promising features. They can be designed to bind a broad range of predetermined DNA sequences with high binding affinity and specificity at a relatively low molecular weight. They are easy to synthesize by solid-phase chemistry and have been shown to readily penetrate cellular membranes without the requirement of formulation. First experiments performed under cell-free conditions and in tissue cultures indicate the potential of this class of compounds as a new generation of therapeutics acting on DNA by specific interference with

gene expression. The relatively facile preparation of polyamides will allow further optimization of this class of molecules for various applications. Data on metabolic stability, pharmacokinetic behavior, efficacy, safety pharmacology, and toxicology will eventually unravel the real potential of polyamides as future pharmaceuticals.

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Targeted Genome Modification via Triple Helix Formation

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I. INTRODUCTION

Most oligonucleotide-based gene therapy strategies target RNA. In this chapter, we describe the use of triplex-forming oligonucleotides (TFOs) to target specific sequences in duplex DNA. TFOs can direct damage to specific sites in DNA and induce mutations in a gene as well as promote homologous recombination at selected sites.

II. TRIPLEX-FORMING OLIGONUCLEOTIDES

A. Specificity

Triplexes were first observed in the 1950s by Felsenfeld and colleagues (1). They showed that two poly(U) strands of RNA could associate with one poly(A) strand in a poly[U:A:U] triple-helical structure. Subsequent work by several groups established that sequences other than polynucleotides could form triple helices and helped to determine the third strand-binding code (2–6). Triple-helix-forming oligonucleotides (TFOs) bind in the major groove of DNA to polypurine/polypyrimidine sequence motifs by forming specific non-Watson-Crick hydrogen bonds, called Hoogsteen base pairs, with the polypurine strand. Polypurine third strands bind in an antiparallel orientation (forming reverse Hoogsteen hydrogen bonds) relative to the polypurine strand of the double helix, while polypyrimidine third strands bind in a parallel orientation (forming Hoogsteen hydrogen bonds). In the purine motif, G forms hydrogen bonds with G in a G:C pase pair, and A

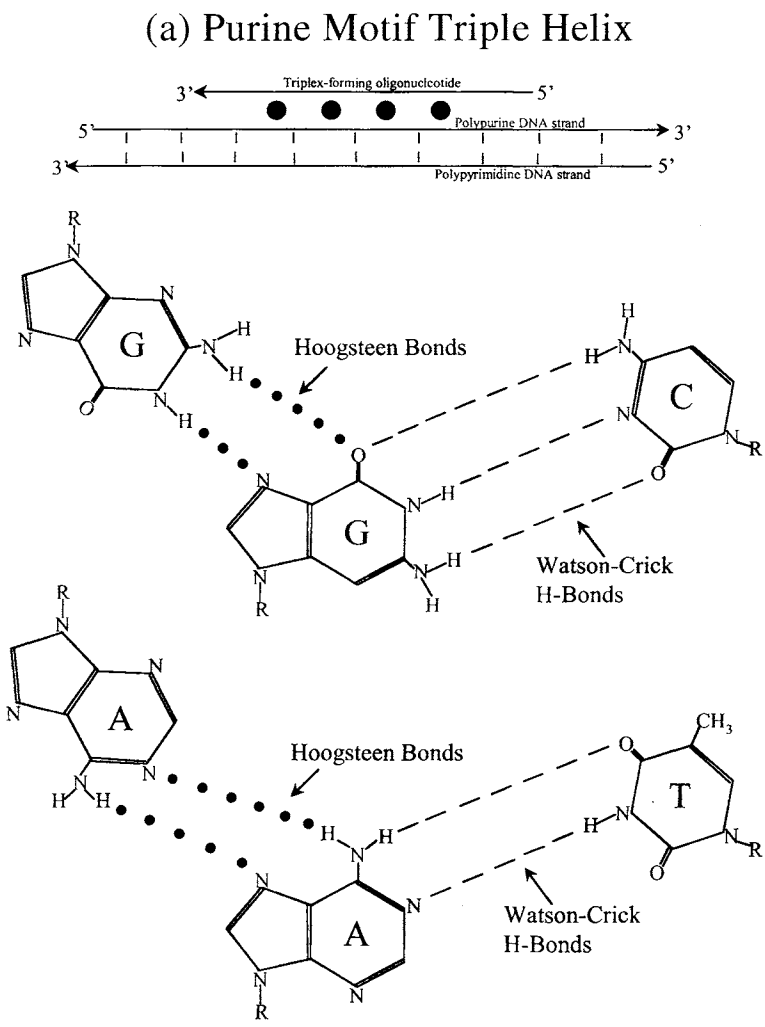
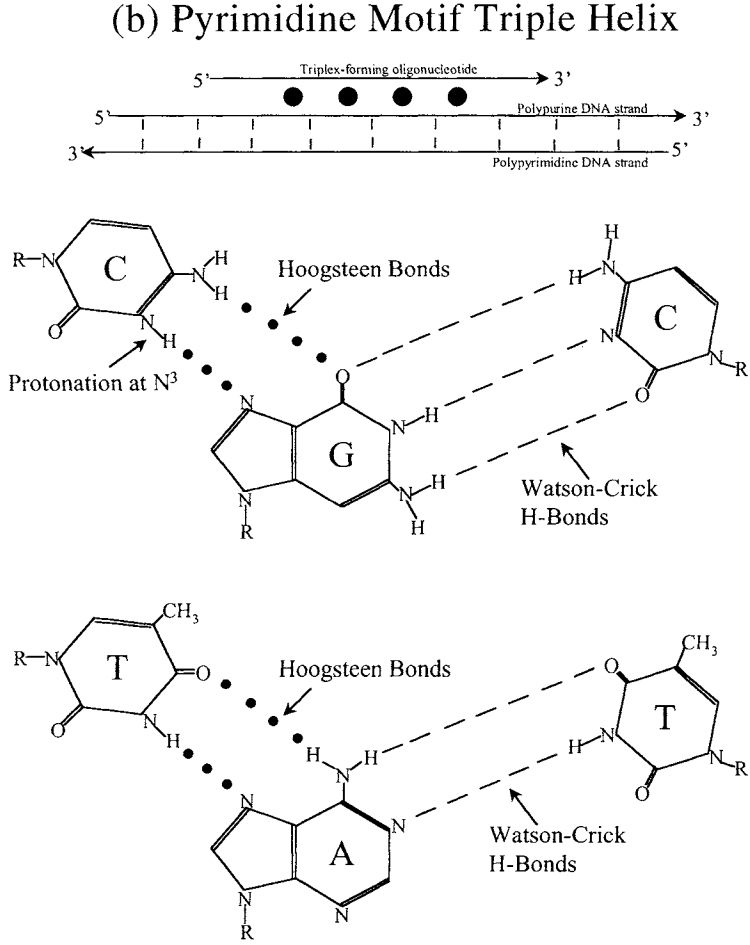


Figure 1 Schematic illustration of triple-helix formation in the purine and pyrimidine motifs.

and T form hydrogen bonds with A in an A:T base pair. In the pyrimidine motif, C forms hydrogen bonds with G in a G:C pair, and T forms hydrogen bonds with A in an A:T base pair (Fig. 1). TFOs are very specific for their target sites in duplex DNA and have been shown to mediate site-specific cleavage of yeast or human chromosomes (7,8).



B. Stability

Triplexes form several orders of magnitude more slowly than duplexes, but once formed, are very stable and can inhibit DNA-binding proteins (9–13). Factors that affect binding affinity include the sequence of the polypurine site, the length of the oligonucleotide, and the magnesium concentration (2,13). Divalent cations, such as magnesium, and polyamines, like spermine and spermidine, stabilize triplex formation by overcoming the repulsion between the negatively charged strands of the Watson-Crick duplex and the negatively charged backbone of the oligonucleotide (14).

Optimal binding of pyrimidine TFOs occurs at a pH below 6 because cytosine must be protonated at the N3 position to form Hoogsteen hydrogen bonds with guanine in the duplex (15). Base modifications have been made that help to alleviate this pH dependence so that stable triplexes can be formed at physiological pH. For example, substitution of 5-methylcytosine for cytosine allows stable triplex formation at physiological pH by facilitating interactions between the methyl groups of the modified cytosine and adjacent thymidines (16).

While purine motif triplexes can form readily at physiological pH, triplex formation by G-rich oligonucleotides is inhibited by physiological concentrations of potassium (17,18). Potassium appears to favor aggregation of G-rich oligonucleotides by stabilizing the formation of G-quartet structures (19,20). The modified base 7-deazaxanthine, instead of adenine in the purine motif, can help overcome potassium inhibition (21).

Backbone modifications that help to stabilize triplex formation in vivo have also been made. One such modification is replacement of the phosphodiester linkage with a phosphoramidite linkage in which the 3′ sugar oxygen is replaced with a nitrogen. Dagle and Weeks showed that TFOs with a positively charged backbone, composed of *N,N*-diethylethylenediamine (DEED) phosphoramidite linkages, form more stable triplexes in vitro than unmodified oligonucleotides and are not inhibited by physiological levels of potassium (22). Phosphorothioate linkages, in which one of the phosphate oxygens is replaced by a sulfur group, confer nuclease resistance and, if they are stereoregular, can in some cases increase triplex stability as well (23,24). Other work suggests that G-rich third strands with phosphorothioate backbones bind less well than their phosphodiester counterparts (25).

III. GENE "KNOCKOUT" BY TFO

A. The Transcription Inhibition Approach

The highly specific and stable binding of TFOs to DNA has made them very attractive antigene agents. In the 1980s, Michael Hogan and colleagues demonstrated that a purine-rich oligonucleotide could bind specifically to a sequence within the c-*myc* gene and inhibit transcription in vitro (5). Since then, TFOs have been shown to form triplexes in various gene promoters and inhibit regulatory protein binding (26–30). There is also some evidence that triplexes can block polymerases and inhibit replication or transcription elongation (31–37). A number of experiments have demonstrated the ability of TFOs to inhibit gene expression on plasmid targets in vitro and in vivo (12,38–40). Michael Hogan and associates showed that a specific TFO targeted to the IL-2 receptor alpha gene promoter could inhibit transcription of the endogenous gene in lymphocytes (41), and transcriptional inhibition by TFOs has since been observed at additional en-

dogenous loci (42–45). Transcriptional inhibition by triplex formation offers an advantage over the antisense approach owing to the small number of targets per cell.

B. Site-Specific Mutagenesis by TFOs

One limiting factor in the use of TFOs for long-term transcription inhibition is the requirement for constant administration owing to their short intracellular half-life. An alternative antigene approach is to use TFOs to induce site-specific mutations in a gene. The advantage of this technique is that a low level of triplex formation could have dramatic effects and the change is permanent and heritable.

1. Mutagenesis of an Extrachromosomal SupF Gene Using TFOs Linked to Psoralen

Third-strand binding can confer sequence specificity on a generally nonspecific mutagen. Our laboratory and others have used TFOs to deliver a tethered mutagen, psoralen, to a gene and site-specifically introduce DNA damage (46–49). Psoralen intercalates into DNA and can covalently crosslink thymines on both strands at 5′ ApT 3′ sites. Triplex-directed gene targeting using psoralen-linked TFOs was first demonstrated by Helene and colleagues (50,51).

To study the ability of psoralen-linked TFOs to induce site-specific mutations in a gene, we have targeted a polypurine/polypyrimidine run in the *Escherichia coli supF* reporter gene. The *supF* gene encodes an amber suppressor tyrosine tRNA. In bacteria with an amber mutuation in the *lacZ* gene, ClacZ125(am), the *supF* gene produces blue colonies on plates containing X-gal and IPTG, while mutant versions produce white colonies. Rates of mutagenesis are measured by the number of white colonies among blue colonies.

We initially used a 10-nucleotide, psoralen-linked, purine TFO, psoralen-AG10, to target *supF* within the genome of a lambda phage vector (46). The psoralen was photoactivated to generate a DNA adduct, and in vitro packaging allowed growth of the phage in ClacZ125(am) bacteria to fix the adduct into a mutation. The observed rate of mutagenesis with psoralen-AG10 was about 0.23%, which was at least 100-fold higher than in an untargeted gene. Ninety-six percent of the mutations were in the targeted region and 56% were found to be T-A to A-T transversions at the expected site of psoralen crosslinking.

TFOs were also shown to stimulate site-specific mutagenesis in mammalian cells. We used an SV40 shuttle vector, pSP189, which contained the *supF* gene and could be replicated in mammalian cells, due to the SV40 origin and T-antigen gene, as well as in bacterial cells, due to the pBR327 origin (47). Psoralen-AG10 was incubated with pSP189 in vitro, and the psoralen was photoactivated to form crosslinks at the target site. When monkey COS cells were transfected with the

plasmid-TFO complex, mutagenesis of the *supF* gene was observed at frequencies as high as 7%. The highest mutation frequencies were obtained when the ratio of oligonucleotide to vector was highest, suggesting that the mutagenesis effect is dependent on TFO concentration. A nonspecific purine oligonucleotide, psoralen-mix30, did not induce mutagenesis of the *supF* gene above background. All of the mutations were in the targeted region and 55% were T-A to A-T transversions at the expected site of psoralen crosslinking.

Despite these promising results, the 10-nucleotide TFO proved to be ineffective at stimulating mutagenesis of the *supF* gene in an assay for intracellular triplex formation. This was presumed to be due to its low-affinity binding in vitro with a Kd of about 8×10^{-7}M. A modified *supF* gene with a 30-nucleotide polypurine/polypyrimidine run, containing only two mismatches, was created (52). A 30-nucleotide purine TFO, psoralen-AG30, bound to this target (*supFG1*) in vitro with a dissociation constant in the nanomolar range, which is comparable to the Kds of many DNA-binding proteins.

Psoralen-AG30 was added to the growth medium of COS cells already transfected with an SV40 shuttle vector containing the modified *supF* gene (pSupFG1) (Fig. 2), and the cells were UVA irradiated after 2 h (52). The plasmid was rescued 48 h later and used to transform ClacZ125(am) bacteria. Psoralen-AG30 was capable of intracellular triplex formation as measured by its ability to induce mutations in the *supF* gene at frequencies around 1–2% (about 70-

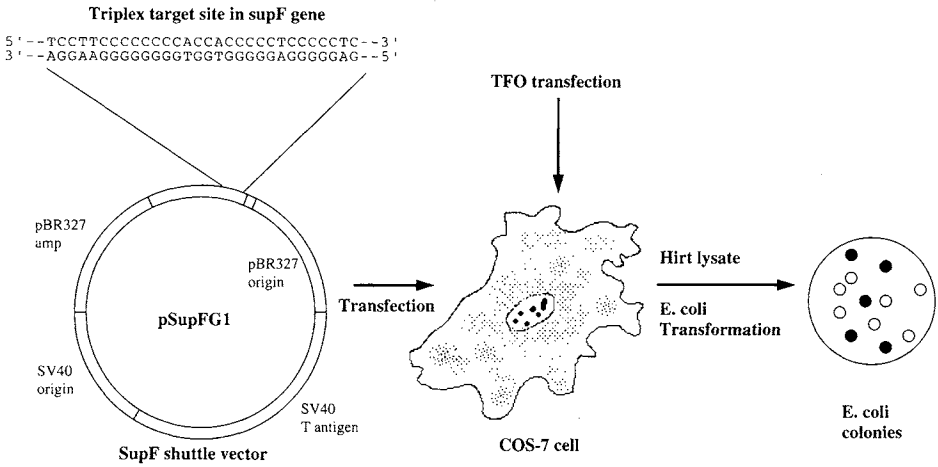


Figure 2 Shuttle vector protocol for assaying triplex-targeted mutagenesis in an SV40-based shuttle vector carrying the *supF* mutation reporter gene.

fold above background). Nearly 70% of the total mutations were T-A to A-T transversions at the expected site of psoralen crosslinking.

In addition to demonstrating the feasibility of triplex-mediated gene targeting in cells, this work showed that the in vitro binding affinities of TFOs are highly correlated with their abilities to induce mutagenesis in vivo. Since the frequencies of mutagenesis were increased when the plasmid was preincubated with the TFO prior to transfection of COS cells, the in vivo activity of TFOs may be limited by the efficiency of oligonucleotide delivery as well as chromatinization of the target.

2. Repair of TFO-Induced DNA Damage: Influence of the Third Strand on Repair

The ability of plasmids treated with psoralen-TFOs to be replicated in mammalian cells indicates that triplex-mediated psoralen damage is repaired. Sandor and Bredberg showed that a plasmid covalently crosslinked in vitro by a 22-nucleotide, psoralen-linked TFO was replicated and repaired in human cells, although the recovery of replicated vectors was significantly reduced when psoralen-linked TFOs were designed to introduce damage at both ends of the *supF* gene (49). Degols et al. showed that psoralen adducts targeted by a 15-nucleotide TFO were removed within a few hours from a plasmid transfected into human cells, but the role of the third strand in this process was unclear (53).

In mammalian cells, psoralen adducts and many types of DNA damage are repaired by the nucleotide excision repair (NER) pathway (54). The excision repair patch generated by NER is generally between 28 and 30 bases (55). We wondered if the length of the TFO would influence the repair of our psoralen adducts.

We compared the spectra of mutations obtained by psoralen-AG10, which is shorter than the size of the usual NER repair patch, and by psoralen-AG30, which is longer than the size of the normal NER repair patch (56). Both molecules direct psoralen adducts to the same target site in the *supF* gene. In addition, we used an oligonucleotide that could subsequently be detached from the psoralen adduct by disulfide bond reduction, allowing us to compare the effect of the psoralen adduct alone at the same target site on mutagenesis. The SV40 shuttle vector, pSupFG1, was preincubated with the corresponding TFO and UVA irradiated to photoactivate the psoralen. Monkey COS cells were transfected with the plasmid-TFO complex by electroporation, and the vector was recovered after 48 h. Sequencing of the mutants showed that the pattern of mutagenesis by psoralen-AG10 was similar to that produced by a psoralen adduct alone at the same site but differed from mutagenesis by psoralen-AG30. While the mutations produced by psoralen-AG10 plus UVA were mainly A-T to T-A transversions, those pro-

duced by psoralen-AG30 were more diverse, with mutations occurring over several base pairs around the psoralen intercalation site. This suggested that repair of a triplex-targeted psoralen adduct can be influenced by the associated third strand. The pattern of mutations also differed when monoadducts were formed versus crosslinks targeted to the same site. Others recently showed that the orientation of psoralen crosslinks relative to the transcribed and coding strands also influences mutagenesis (57).

We studied the repair of the shuttle vector-psoralen-TFO substrate in human cell extracts in vitro to determine whether some of the observed differences in mutation spectra could be due to the inability of the psoralen-AG30 lesion to be excised (58). The TFOs were 3′ radiolabeled and incubated in HeLa cell extracts. Analysis by denaturing gel electrophoresis revealed that psoralen-AG30 could inhibit excision of the damaged DNA. This suggested that the incision steps of NER can be blocked by the presence of a high-affinity third strand of sufficient length.

Mutagenesis by psoralen-TFOs may be dependent on an error-prone repair pathway. Barre et al. showed that interstrand crosslinks formed by TFOs in the *ura3* gene on a plasmid in yeast cells were poorly repaired and resulted in high levels of mutagenesis (59). These mutations were shown to be generated by the error-prone repair (EPR) pathway, alone or in combination with NER. In yeast strains with an inactive *rad18* gene, which encodes a protein that controls damage-avoidance repair and induced mutagenesis with RAD6, the mutant plasmids disappeared, indicating that EPR played an important role in the mutagenesis.

Using TFOs to target a *supF*-containing, SV40 shuttle vector in XP Variant (XPV) cells, our laboratory has shown that EPR may also be important for triplex-induced mutagenesis in mammalian cells (60). XPV cells have recently been found to contain mutations in the gene encoding an error-free polymerase (61). Cells lacking this polymerase appear to be more sensitive to ultraviolet (UV) damage, possibly owing to a defect in lesion bypass during replication (62). We saw an increased frequency of mutagenesis of our episomal *supF* gene by psoralen-AG30 in XPV cells (30%) relative to normal cells (11.5%), suggesting that the error-free polymerase plays an important role in preventing mutagenesis (60). The role of the triple helix itself in this process is unclear because a psoralen adduct alone targeted to the same site produces an increase in mutagenesis frequency between normal and XPV cells. From all of this work, we can conclude that repair of triplex-mediated DNA damage is a complex process involving multiple competing pathways.

3. Triplex Formation Itself Stimulates Repair and Mutagenesis

Triplex formation itself, without a tethered mutagen, appears to be mutagenic, and the NER and transcription-coupled repair (TCR) pathways are critical for

the induced mutagenesis (63). COS cells were transfected with pSupFG1 by electroporation. After 24 h, AG10, AG20, or AG30 was added to the growth medium and the vector DNA was rescued 48 h later. Observed rates of *supF* mutagenesis were 0.07% (about threefold above background), 0.11% (about fivefold above background), and 0.27% (about 14-fold above background) with AG10, AG20, and AG30, respectively. This work showed that, as with the psoralen-linked TFOs, the level of induced mutagenesis correlates with TFO affinity.

To determine whether the mutagenic effect was a consequence of the stimulation of DNA repair by triple-helix formation, we utilized cell lines deficient in NER [xeroderma pigmentosum group A (XPA) cells] or in TCR [Cockayne's syndrome group B (CSB) cells] (63). No mutagenesis of the SV40-vector *supF* gene above the background rate was detected in the XPA or CSB cells treated with AG10, AG20, or AG30. This indicated that the NER and TCR pathways are critical for triplex-induced mutagenesis. Equally interesting was the finding that triplex formation itself could stimulate DNA repair synthesis in human cell extracts (63). Preformed triplexes were added to HeLa extracts supplemented with [alpha-32P] dCTP. AG30 stimulated labeling of pSupFG1, but not a control plasmid, pUC19. The vector was then used as a substrate for in vitro transcription assays. The induced repair synthesis correlated with the inhibition of transcription in these extracts. The ability of triple-helix formation, without any other associated DNA damage, to stimulate mutagenesis by provoking repair is evidence that high-affinity DNA-binding ligands may be useful tools for genome modification.

4. TFO-Induced Mutagenesis at Chromosomal Sites

TFOs have been shown to induce mutagenesis at chromosomal sites in vivo (64,65). Our laboratory used TFOs to target a chromosomal *supF* gene. A mouse fibroblast line (3340) was constructed containing about 15 copies of lambda *supFG1* (a lambda vector containing the *supFG1* mutation reporter gene) (66). The TFOs were introduced into these cells by passive uptake and the cells were UVA irradiated to photoactivate the psoralen (64). After 48 h, the replicated and repaired phage DNA was isolated from genomic DNA and packaged into phage particles using lambda packaging extracts. The phage particles were propagated in ClacZ125(am) bacteria. Mutagenesis was measured by the number of white plaques out of the total plaques on X-Gal and IPTG plates (Fig. 3). An induction of mutagenesis about 10-fold above background was achieved with pso-AG30 (about 5×10^{-4}) in both the presence and absence of UVA, indicating that the TFO alone was mutagenic. No induction of mutagenesis was observed with a scrambled-sequence purine TFO, psoralen-SCR30, and the nontargeted phage gene, *cII*, was unaffected. Sequencing revealed that the majority of mutations were single- or double-base-pair insertions or deletions in the triplex-binding site.

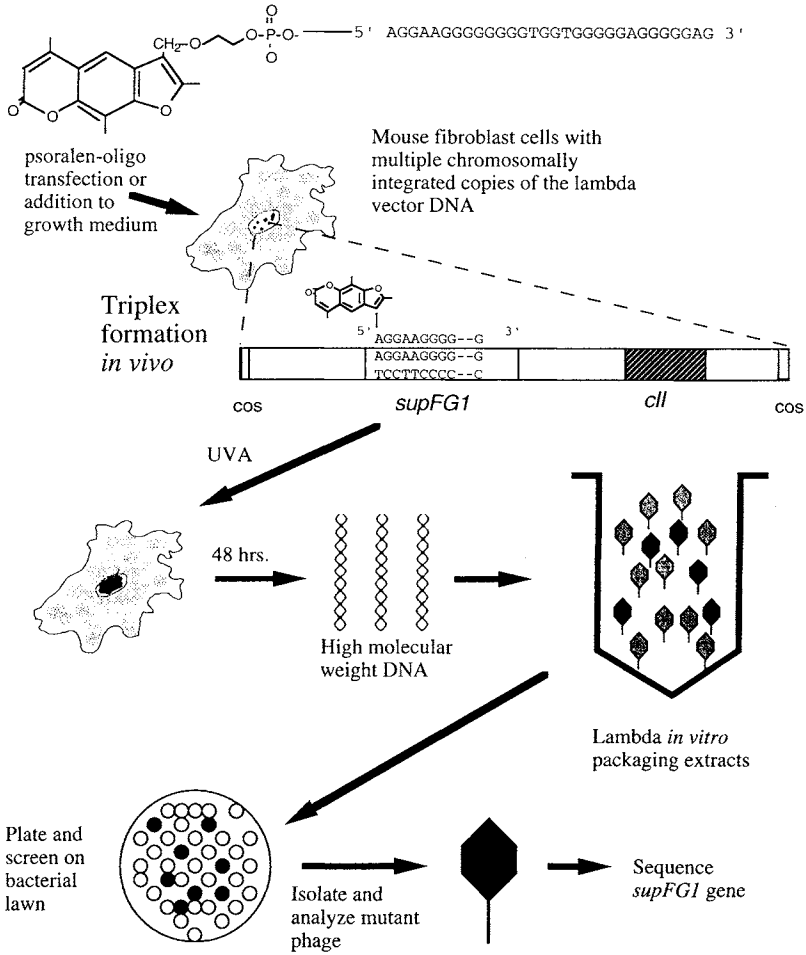


Figure 3 Chromosomal *supF* TFO-mediated targeting and mutagenesis assay using a lambda shuttle vector.

Majumdar et al. demonstrated TFO-mediated chromosomal gene targeting at an endogenous locus (65). They designed a psoralen-conjugated pyrimidine TFO to bind in an intron immediately adjacent to the smallest exon of the HPRT gene in CHO cells. Mutations at the psoralen target site would activate the AG splice acceptor site. Mutagenesis was measured by resistance to 6-thioguanine. An induction of mutagenesis about 10-fold above background was achieved with

specific TFOs (about 1×10^{-4}). Sequencing analysis showed that 85% of the mutant colonies had mutations in the triplex target region.

The results of these experiments support the concept that at least some chromosomal sites are accessible to gene targeting by TFOs in vivo. Further work to enhance the efficiency of triplex formation at chromosomal sites will be necessary to improve the frequency of TFO-mediated gene modification.

5. Site-Specific Mutagenesis by Peptide Nucleic Acids

Peptide nucleic acids (PNAs) are an alternative to modified DNA or RNA oligonucleotides for triplex formation. PNAs are nonionic nucleic acid analogs in which the sugar-phosphate backbone is replaced by an *N*-aminoethylglycine-based polyamide structure (67). PNAs bind with higher affinity to polypurine DNA sequences than DNA or RNA oligonucleotides and can bind in both the parallel and antiparallel orientation (68). Homopyrimidine PNA oligomers form very stable $(PNA)_2$DNA triple helices with complementary DNA by the Watson-Crick base pairing of one PNA strand and the Hoogsteen base pairing of the other PNA strand to the polypurine strand of the DNA duplex (68). This binding occurs by displacement of the pyrimidine strand of the DNA duplex and formation of a D-loop type structure, called the P-loop (69). PNAs are also shown to have increased resistance to cellular proteases and nucleases (70).

Because of their high-affinity binding to nucleic acids and resistance to cellular proteases and nucleases, PNAs are attractive candidates for use as antigene agents. PNAs have been shown, in vitro, to inhibit transcription initiation and elongation and to block DNA polymerase (71–73). They can inhibit binding of sequence-specific binding proteins, like restriction endonucleases (74). PNAs have also been shown to inhibit transcription and translation in vivo (71,75), but studies reporting their intracellular effects have been limited, mainly because of the difficulty of cellular PNA delivery.

Dimeric PNAs, in which two PNA strands are connected by a flexible linker, can form PNA/DNA/PNA triplexes, called PNA clamps, at polypurine DNA sequences with very high affinity (76). Our laboratory designed eight- and 10-mer dimeric PNAs to form clamps on the purine-rich strand of the *E. coli supFG1* gene (77). We used mouse fibroblasts (3340) with 15 chromosomally integrated copies of the lambda phage shuttle vector containing the *supFG1* gene. The cells were permeabilized with streptolysin O and cellular uptake of PNAs was confirmed by fluorescein labelling and fluorescent microscopy. After 48 h, the phage vector was rescued. *SupF* mutagenesis was determined by counting white plaques on Xgal and IPTG plates after infection of ClacZ125(am) bacteria. PNA-8 and PNA-10 induced mutations in the *supF* gene at a chromosomal site in mammalian cells with a frequency of 0.1% (10-fold above background). No

mutagenesis was detected at the nontargeted phage gene, *cII*. Sequence analysis showed that the majority of mutations were located within the PNA-binding site.

6. Induction of Mutagenesis by TFOs in Mice

We recently found that TFOs are capable of inducing specific mutations in the somatic cells of an animal. We used transgenic mice (3340) with multiple copies of a chromosomally integrated lambda *supFG1* vector, containing the 30 base-pair triplex target site within the *supFG1* gene (78). The mice were given daily intraperitoneal (i.p.) injections with 1 mg of either AG30 or the nonspecific control oligonucleotide, SCR30, for 5 consecutive days. Ten days after the last injection, the mice were sacrificed and tissues were collected. Lambda packaging extracts were used to generate phage particles, which were then propagated in ClacZ125(am) bacteria. Mutations in the *supFG1* gene were identified by the appearance of white plaques on X-gal and IPTG plates. The combined mutation frequencies in a variety of tissues were about fivefold higher when the mice were treated with AG30 (2.7×10^{-4}) than when they were treated with SCR30 (5×10^{-5}). The level of mutagenesis observed in tissues of mice treated with SCR30 did not differ significantly from the level of mutagenesis in tissues of mice treated with phosphate-buffered saline (PBS) (3×10^{-5}). Therefore, nonspecific oligonucleotides do not appear to be mutagenic. No mutagenesis above background was observed in the brain tissue of mice treated with AG30. This was consistent with previous results showing that oligonucleotides do not efficiently cross the blood-brain barrier after i.p. injection (79). Nearly 85% of the mutations in the *supF* gene were clustered within the triplex target site (78). The effects of AG30 are specific to the target gene because no induction of mutagenesis above background was observed in another mutation reporter gene, *cII*, when the mice were treated with AG30. Given these results, it appears that gene modification via triple-helix formation is a feasible approach to gene knockout in animals. The development of modified oligonucleotides with enhanced binding affinity and improved cellular uptake will be of critical value for improved efficiency.

IV. GENE CORRECTION OR ACTIVATION BY TFOS

In addition to gene knockout, a goal of many gene therapy strategies is the correction or activation of mutated or unexpressed genes. Recent work has shown that TFOs may be useful for these approaches.

A. Transcriptional Activation by Triplex Formation

Some recent experiments have demonstrated the feasibility of gene activation using triplex-forming molecules. In one study, a 20-nucleotide TFO, targeted to

a polypurine stretch of the vpx gene of HIV-2, was able to activate expression of a luciferase reporter gene when covalently linked to three repeats of a minimal transcriptional activation domain from herpes simplex virus protein 16 (VP16) (80). The TFO-peptide conjugate was incubated with the plasmid pVPX-fos(-40)Luc to allow triplex formation. This plasmid contained the polypurine stretch of the HIV-2 vpx gene followed by a minimal c-fos promoter and the luciferase gene. NIH-3T3 cells were then electroporated with the preformed triplex, and luciferase activity was measured. They achieved about a threefold induction of expression in vivo with the TFO-peptide.

The D-loop structures formed by PNA binding and strand displacement have also been found to be able to initiate gene transcription in vitro (81), and more recently, in vivo (82). Wang et al. used PNAs designed to bind to the 5′-flanking region of the gamma-globin gene to induce expression of a GFP reporter gene construct (82). The plasmid, pUSAG3, containing the 5′-flanking region of the gamma-globlin gene upstream of the GFP gene, was incubated with PNA-1 (eight-mer) or PNA-2 (10-mer) and microinjected into the nucleus of CV1 monkey kidney fibroblast cells. GFP was expressed in 30% of these cells as compared with 0% when the plasmid was injected alone. These PNAs were also shown to induce expression of the endogenous gamma-globin gene in K562 human erythroleukemia cells. The PNAs were introduced into the cells by electroporation and the increase in expression after 2 days was two- to eightfold above background.

B. Triplex-Induced Recombination

Triplex formation appears to also stimulate recombination (83–85). This is of particular interest because a major limitation of gene therapy strategies is the low frequency of homologous recombination in mammalian cells (86). The frequency of homologous recombination can be enhanced by DNA damage to the target site from UV irradiation, alkylating agents, and photoreactive molecules like psoralen (87–89) as well as by site-specific endonucleases (90–94). We hypothesized that the mechanism by which triplexes stimulated DNA repair and mutagenesis could also sensitize a site to homologous recombination. This would be useful because many genes are likely to have a polypurine:polypyrimidine run for targeting by TFOs.

1. Intramolecular Recombination Between Two Mutant Genes Carried as Direct Repeats

Our laboratory created a construct to test the ability of TFOs to stimulate intramolecular homologous recombination in mammalian cells (85). We used an SV40 shuttle vector with two mutant copies of the *supF* gene carried as direct repeats. The upstream mutant *supF* gene, *supF1*, contained a C-to-G point mutation at

nucleotide position 163, and the downstream mutant gene, *supF2*, contained a G-to-A point mutation at position 115 (Fig. 4A). At the 3′ end of *supF1* was the 30-base-pair polypurine sequence (positions 167–196) for high-affinity triplex binding. Monkey COS-7 cells were transfected with this plasmid by electroporation. The psoralen-linked oligonucleotides were then added to the growth medium of the cells in suspension to allow intracellular triplex formation. After 2 h to

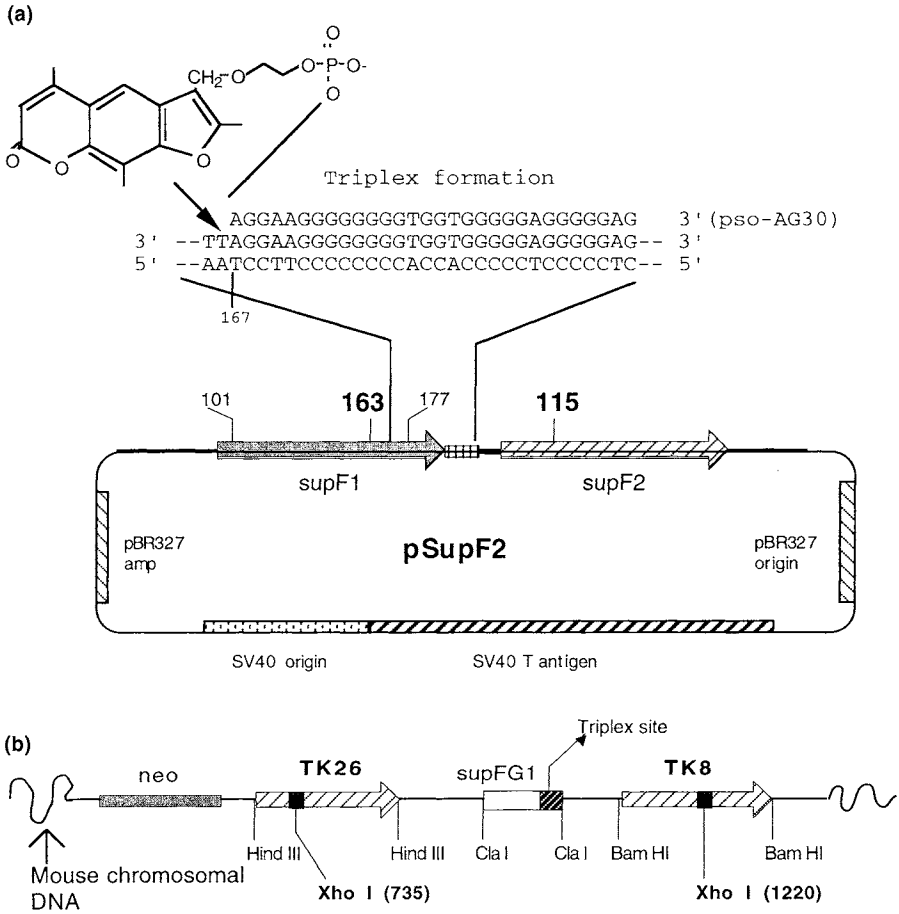


Figure 4 Substrates for detecting triplex-induced recombination in mammalian cells. (A) SV40-based shuttle vector containing two mutant copies of the *supF* gene. (B) Chromosomal locus in mouse cells engineered to contain two mutant copies of the herpes simplex virus thymidine kinase gene (TK).

allow the TFOs to enter the cells and bind to the triplex site, the cells were irradiated with UVA to photoactivate the psoralen and form covalent crosslinks in the DNA. The plasmid DNA was isolated after 48 h and used to transform ClacZ125(am) bacteria. Since the point mutation in *supF1* did not completely inactivate the gene, faint blue colonies could be seen on X-gal and IPTG plates with the parental vector alone. Therefore, recombination events yielding a wild-type *supF* gene (blue colonies) and events yielding a completely inactivated *supF* gene (pure white colonies) could both be identified. Mutations as well as recombination events were detected. The high-affinity TFO, pso-AG30, stimulated recombination to generate a wild-type *supF* gene nearly 25-fold. All of the blue colonies tested had given rise to functional *supF* genes by a gene conversion event in which no vector sequences are lost. Similarly, pso-AG30 simulated the appearance of pure white colonies about 30-fold above background. Ten percent of the nonparental white colonies contained only a single *supF* gene, suggesting that at least some nonconservative recombination events were occurring. However, most of the white colonies had retained both copies of the *supF* gene, and sequence analysis revealed that most of these colonies had undergone mutation rather than recombination events. Overall, recombinants (blue and white) were induced at a frequency of 0.58% and mutations were induced at a frequency of 0.77%.

Reversion frequencies of the *supF1* gene, carried as a single *supF* gene in a shuttle vector otherwise identical to pSupF2, were measured (85). After intracellular incubation with pso-AG30, only about 0.06% of the colonies were blue. Therefore, the blue colonies observed in pSupF2 must have arisen from a nonmutational event, such as gene conversion. Interestingly, exposure to visible light (which results in mainly monoadducts and few crosslinks) after addition of TFOs did not yield blue recombinants above background.

The third strand appears to be important for the induction of recombination (85). A psoralen-conjugated TFO was designed to bind at the 3′ end of the *supF1* gene in pSupF2 and synthesized to contain a disulfide linker between the psoralen and the oligonucleotide. No blue recombinants above the background frequency were observed when the TFO-bound vector was treated with DTT to detach the oligonucleotide from the psoralen adduct. The differences observed between monoadducts and crosslinks, and between the presence of the triplex oligonucleotide and the targeted psoralen alone, may have to do with the strand bias in repair.

The psoralen does not seem to be required for the stimulation of recombination however, and similar results were obtained when a non-psoralen-conjugated TFO was used in the experiment (95). We used the plasmid pSupFAR, which is similar to the pSupF2 vector, except that it is lacking polymorphisms at positions 102 and 177. Cells pretransfected with pSupFAR were then transfected with the specific TFO, AG30. After 48 h, the shuttle vector DNA was isolated from the cells and analyzed for supF function by the assay previously described. Blue

recombinants were generated at a frequency of 0.37% (fivefold above background) when AG30 was used. No induction of recombination above background was observed with a nonspecific purine oligonucleotide.

This effect was dependent on nucleotide excision repair (95). In XPA-deficient human cell lines, no induction of recombination above background was observed with AG30. However, when the XPA deficiency was corrected by expression of wild-type XPA cDNA, AG30 was able to stimulate recombination. Recombination induced by psoralen triplexes is only partly dependent on NER, however. In XPA-deficient cell lines, psoralen triplexes still stimulate recombination to a substantial degree.

These results suggested that high-affinity TFOs might stimulate recombination at a chromosomal site. Our laboratory constructed a mouse LTK-cell line with two mutant copies of the herpes simplex virus thymidine kinase (TK) gene as direct repeats in a single locus (Fig. 4B). A triplex-binding site was situated between the two genes. Specific TFOs, with or without psoralen, were found to stimulate recombination between the tandem TK genes at frequencies around 1% (2500–3000-fold above background) when microinjected into the cells (96).

2. Intermolecular Recombination Using a TFO Tethered to a Donor Fragment

Since most genes do not exist as direct repeats, we were also interested in examining the ability of TFOs to stimulate intermolecular homologous recombination. Sandor and Bredberg showed that psoralen damage mediated by triplex formation at a site in a mutant *supF* gene carried on an SV40 shuttle vector could induce a low frequency (threefold above background) of intermolecular homologous recombination when the plasmid was cotransfected into human cells with another plasmid containing the wild-type *supF* gene (97).

Several research groups have used short fragments of DNA or DNA/RNA hybrids as recombination substrates for gene correction (98,99). We designed an oligonucleotide that covalently tethers a TFO to a DNA donor fragment (100). We hypothesized that in addition to positioning the donor fragment, the TFO could enhance recombination by inducing repair at the target site. An SV40 shuttle vector containing a mutant *supF* gene with a G-to-C point mutation at position 144 was used for this experiment. The plasmid was preincubated with the corresponding oligonucleotide, and COS cells were transfected with the complex using the cationic lipid reagent, Lipofectamine. After incubation for 48 h, the cells were lysed and plasmid DNA was rescued and used to transform ClacZ125(am) bacteria. Reversion frequencies were calculated as the number of blue colonies among white ones on X-gal and IPTG plates. The frequency of spontaneous reversion in this plasmid was about 0.003%. A single-stranded donor tethered to a specific TFO, A-AG30, and a double-stranded donor tethered to a specific TFO,

A/B-AG30, stimulated reversion to frequencies of 0.17% and 0.68% (57-fold and 227-fold above background), respectively. The reversion frequencies with the single-stranded or double-stranded donors alone were significantly lower: 0.051%, 0.062%, and 0.17% respectively. The reversion frequencies with the double-stranded donor domain tethered to a nonspecific TFO, Mix30, which should not form a triplex in the *supF* gene, were similar to those attained with the donor alone (0.15%). There was no significant reversion above background when the vector was incubated with the TFO domain, AG30, alone, and little activity above background was observed when AG30 was tethered to a random donor fragment or when the TFO and donor fragment were not tethered. Therefore, the two domains of the tethered donor appear to work synergistically to stimulate recombination. Much of the stimulation provided by the TFO domain is dependent on nucleotide excision repair (NER). The A/B-AG30 TFO did not induce reversion at a frequency higher than A/B-Mix30 in XPA-mutant cells.

Surprisingly, while the double-stranded donor appeared to be more effective when the triplexes were preformed, the single-stranded donor fragment was better at inducing recombination when the triplexes were allowed to form within the cell (100). The frequencies of reversion were significantly lower when the triplexes were not preformed, suggesting that oligonucleotide transfection efficiency and accessibility of chromatinized targets may be limiting factors.

Correction of the gene sequence is dependent on the donor fragment sequence because a specific point mutation at position 144 of the donor could be transferred to the wild-type *supF* gene in the shuttle vector, pSupFG1, at a frequency of about 0.64% (100). We found that a tethered donor TFO could also generate a novel SalI restriction site in the *supF* gene, suggesting that the tethered donor TFO has the potential to correct complex mutations involving more than a single point mutation.

V. PHYSICAL DETECTION OF TRIPLEX FORMATION AT CHROMOSOMAL SITES IN CELLS

Since DNA in cells is typically bound by histones and tightly packed into chromatin, many sites may be inaccessible to triplex formation. Several in vitro studies have suggested that triplex formation and nucleosome formation are competing processes (101–103). Westin and associates looked at the effects of triplex DNA on the structure of in vitro reconstituted nucleosomes. Their results showed that nucleosomes could not assemble within the MMTV promoter when a 16-base-pair pyrimidine-rich oligonucleotide was bound (101). However, when nucleosomes were formed prior to TFO addition, triplex formation was inhibited. Consistent with this result, Espinas and co-workers showed that formation of triplexes

on sequences already organized into nucleosomes was effective only at relatively high salt concentration, when nucleosomes are partially destabilized (102). A more recent study by Brown et al. indicated that triplexes can only be formed at sites located toward the ends of nucleosomal DNA fragments (103).

Despite the ability of nucleosomes to inhibit triplex formation in vitro, recent experiments have directly demonstrated the ability of intermolecular triplexes to form on chromatinized plasmids, or intact chromatin in vivo (104–107). Guieysse et al. demonstrated direct binding and photo-induced crosslinking of a psoralen-linked TFO to a site in the IL-2 receptor alpha subunit promoter in human cells (104). They used a primer extension assay, in which taq polymerase is stopped at psoralen adducts, to quantitate the plasmid DNA that had reacted with the psoralen of the TFO. Cells were electroporated with the plasmid and then incubated with the TFO. When the cells were irradiated for 30 min with 390 nm UV light, 20% of the plasmid was detected as crosslinked to the oligonucleotide.

Giovannangeli et al. showed in vivo triplex formation at a 15-base-pair HIV-1 target site in the chromosome (105). They used an assay in which the triplex-mediated psoralen crosslink prevented cleavage of the target sequence by the DraI restriction endonuclease, and the level of cleavage inhibition was quantitated by Southern blot analysis. The cells were permeabilized with digitonin to facilitate TFO uptake. Up to 30% of the triplex site could be protected from DraI cleavage at the highest concentrations of TFO.

Belousov et al. showed that triplexes could be formed in the gene for the chemokine receptor CCR5 in permeabilized mammalian cells (106). They detected triplex formation by a ligation-mediated PCR assay that allows detection of the exact site of TFO reaction. They used a phenylacetate mustard–conjugated TFO to alkylate a specific guanine adjacent to the triplex site. Cells were permeabilized with streptolysin to facilitate sufficient entry of the TFO. When a 20-μM concentration of TFO was used, 25% of the targets were modified at the expected site.

In a recent paper, Oh and Hanawalt demonstrated direct binding of a seemingly low-affinity psoralen-TFO to a chromosomal site in intact human cells (107). To detect triplex formation, they used a PCR assay, called single-strand ligation PCR (ssligPCR), that relies on the inability of taq polymerase to replicate past psoralen adducts in the DNA. Cells were transfected with psoralen-conjugated TFOs using cationic lipids and UVA irradiated. They observed 15% adduct formation in the human interstitial collagenase gene with their best TFO.

From this work, it appears that chromatin structure is dynamic enough to allow triplex formation at chromosomal sites in mammalian cells. However, some sites may be more accessible to TFOs than others. A better understanding of the factors that influence triplex formation at chromosomal sites will be critical for the improvement of TFO-mediated gene-targeting strategies.

VI. LIMITATIONS AND FUTURE CHALLENGES

A. Identification of Disease-Related Targets: Moving Beyond Model Systems

A potential problem with TFO-based gene targeting is that binding is limited to homopurine-homopyrimidine stretches of duplex DNA. Synthetic base analogs have been made that bind to inverted base pairs and minimize triplex destabilization by mismatches in the polypurine sequence (108). 2-Deoxynebularine is able to specifically bind at a C:G inversion (109), and imidazole can stabilize a T:A inversion in a poly(G) stretch (110). Intercalators have also been shown to overcome mismatches in the target sequence (111). In addition, mixed-sequence duplex DNA, consisting of tracts of purines followed by tracts of pyrimidines, can be recognized by alternate-strand triple-helix formation (112,113). Oligonucleotides can be synthesized with linkers that allow binding to adjacent purine tracts on opposite strands.

Even given the limitations of the binding code, however, most human genes are sufficiently large that they are likely to contain a polypurine/polypyrimidine run. Novel backbone modifications that increase triplex stability may allow stable triplex formation at shorter polypurine tracts. For instance, the high-affinity binding of PNAs allowed triplex-induced mutagenesis in vivo at relatively short (eight to 10 base pairs) target sites in the duplex (77). In addition, many of the oligonucleotide modifications designed to enhance antigene activity (described elsewhere in this book) may prove to be useful for triplex formation.

B. Oligonucleotide Delivery

The introduction of TFOs into cells is a significant challenge. Low-level entry of DNA oligonucleotides into mammalian cells in culture can occur by passive uptake from growth medium (64,114). However, modifications that improve the nuclease resistance or in vivo binding affinity of TFOs may also hinder cellular uptake (36,77). Improvements in transfection efficiency have been observed using electroporation (115) or various lipid carrier agents (116). Permeabilization of the cell using streptolysin O has been shown to be effective for delivery of oligodeoxynucleotides and PNAs (77,117).

Peptide-oligonucleotide conjugates have been made that facilitate nuclear entry (118,119). Zanta et al. recently showed that transfection could be enhanced 10–1000-fold using a gene containing a nuclear localization signal peptide (119). Branden et al. created a PNA oligonucleotide with the SV40 nuclear localization signal (NLS) (118). Covalent attachment of the NLS to the PNA increased the nuclear uptake of the oligonucleotide or PNA-bound plasmid up to eightfold. Tethering of an oligonucleotide to a peptide that facilitates penetration of the plasma membrane may also be effective. Schwarze et al. showed that the B-

galactosidase protein could be delivered to all tissues in mice when fused to the protein transduction domain from the HIV TAT protein (120).

We found that microinjection of TFOs directly into the nuclei of mouse fibroblast cells could stimulate homologous recombination between two mutant TK genes carried as direct repeats in a chromosomal locus at a frequency of 1% (2500-fold above background) when the TFOs were targeted to a site between the two repeats (96). In contrast, delivery of the TFOs to the cells using Geneporter, a cationic lipid transfection reagent, resulted in only a sevenfold induction of recombination above background. This indicates that inefficient cellular uptake of TFOs may significantly limit their in vivo activity. New methods of oligonucleotide delivery will be crucial for the improvement of triplex-mediated gene-targeting strategies.

VII. CONCLUSIONS

The work reviewed in this chapter demonstrates the ability of triplex-forming oligonucleotides to site-specifically modify genes in mammalian cells. Further work to identify accessible targets, facilitate targeting of mixed sequences, and improve cellular delivery of TFOs will be critical for realization of the therapeutic potential of this technology.

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Intracellular Ribozyme Applications

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I. INTRODUCTION

Over the last decade ribozymes have been widely employed in a variety of systems. Their use as tools to control gene expression is both enticing and simple in concept. Indeed, ribozymes can be designed to specifically base-pair with and cleave any given RNA target, with the result of producing either an irreversible inactivation of the targeted RNA, or in some cases, trans-splicing of a corrective segment of RNA onto a defective messenger RNA. Delivery of preformed ribozymes or ribozyme-encoding genes can be accomplished via several routes, including lipofection, transfection, and viral vector–mediated transduction. Thus, the combination of specificity, the possibilities of endogeneous synthesis or exogenous delivery, their capacity for multiple turnover of targeted sequences, and their broad targeting capabilities make ribozymes extremely attractive genetic tools as well as potential therapeutic agents.

II. RNA CATALYTIC MOTIFS

Five catalytic motifs have been utilized as trans-acting ribozymes. These are the group I intron, RNase P, the self-cleaving domains of plant viroids (comprised of the hammerhead and hairpin ribozyme), the self-cleaving domain of the hepatitis delta virus reviewed in (Refs. 1–3) and the *Neurospora* VS RNA (4,5).

A. Group I Introns

The group I introns are defined by complex, but phylogenetically conserved, sequence homologies and secondary structures, but many, if not all, require protein factors to splice efficiently in vivo (6). The best-characterized group I intron, and perhaps the most thoroughly studied ribozyme, is the intervening sequence of *Tetrahymena thermophila*. This was the first ribozyme for which the cis-cleaving reaction was converted to a trans-reaction (6,7). A structure at 5 Å resolution of the *Tetrahymena* intron has recently been solved by X-ray crystallography, and has revealed a striking similarity between its active site and the active sites found in protein enzymes (8). While this RNA enzyme has been engineered to cleave a variety of substrates, including single-stranded DNA (9), its application in biological systems has been limited by its complexity and a lack of specificity in substrate pairing. These problems have not yet been solved, but recent applications of the group I intron for trans-splicing of corrective sequences onto defective messenger RNAs have created enhanced interest in this new biological application of this ribozyme (Fig. 1) (12–12).

B. RNase P

RNase P is a ubiquitous enzyme whose primary cellular function is processing the 5′ leader sequence from pre-tRNA transcripts (13). The enzyme itself does not need to be modified to carry out reactions in trans, since this is the only ribozyme known to bind and cleave free substrate molecules in nature. In contrast to the other ribozymes, the substrate needs to be modified to create a structure that mimics the pre-tRNA substrates normally cleaved by this enzyme. RNAs that are used for this purpose have been designated external guide sequences (14,15). RNase P has been used for site-specific cleavage of intracellular RNAs that formed substrates with external guide sequences (16–18) that are added in trans and bind the target RNA, directing cleavage by endogenous RNase P. Another use of RNase P takes advantage of the known catalytic activity of the M1 RNA component of the *Escherichia coli* enzyme. This RNA has been modified by in vitro evolution and selection to create ribozymes with cis-appended (on the same strand of RNA as the catalytic center) guide sequences, creating a new type of holoribozyme (19). Liu and Altman have demonstrated that mammalian cells expressing the modified *E. coli* M1 RNA effectively target and destroy targeted transcripts (16). With these modifications, the M1 RNA itself can function as a sequence-specific ribozyme in human cells, thereby adding another member to the family of genetically engineered ribozymes with functional utility.

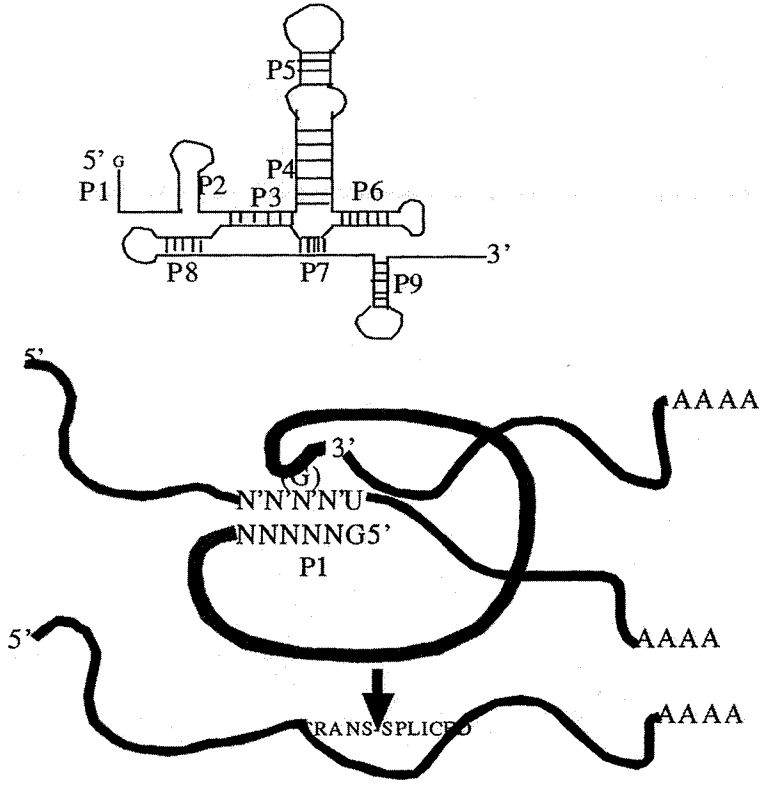


Figure 1 Generalized group I intron structure. The regions of helical pairing are designated P1–P9. Base-paired regions are indicated by vertical or horizontal lines. The group I intron has been modified to pair with and cleave RNAs in trans by modifying the intron portion of P1 to be complementary to the target sequence of interest. When an exon is attached to the modified intron, it can pair with the target RNA, cleave the target, and trans-splice the appended 3′ exon as depicted. See text for discussion and references.

C. Hammerhead and Hairpin Ribozymes

For intracellular applications, the hammerhead and the hairpin ribozyme have thus far proven to be the most popular [see recent reviews (20–25)]. These ribozymes are depicted in Fig. 2. The structure of the hammerhead ribozyme has been well understood for quite some time (26,27). This ribozyme is the simplest, and has therefore been the most popular for intracellular applications. The catalytic core of the hairpin ribozyme is slightly more complex than that of the ham-

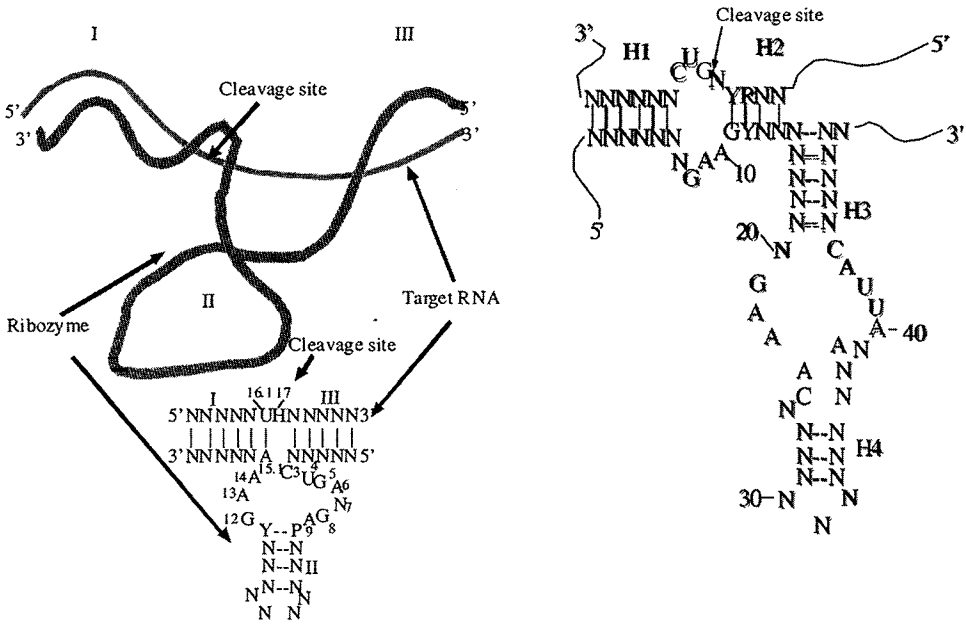


Figure 2 Generalized structures for trans-cleaving hammerhead (left) and hairpin (right) ribozymes. The ribozyme structures are paired with target RNAs and the sites of ribozyme-directed cleavage are indicated. A schematic diagram of the three-dimensional structure of the hammerhead ribozyme is depicted above the secondary structure. The corresponding stems I, II, and III are indicated. N = A, C, G, or U. Pairing of the ribozymes with the targets is via complementary Watson-Crick base pairing. At the site of cleavage for the hammerhead ribozyme H = A, C, or U. Y = pyrimidine and R = purine on both ribozymes. The numbering of specific nucleotides on both the hammerhead and hairpin ribozymes is also indicated.

merhead (28–31), but nonetheless lends itself well to trans-cleavage reactions. The hairpin also catalyzes a more efficient ligation reaction than the hammerhead, and thus may be more amenable to in vitro evolution and selection schemes (32,33) that can be used to increase ribozyme efficiency.

The hammerhead ribozyme has a catalytic core consisting of 11 conserved nucleotides flanked by three duplexes (Fig. 2). Extensive in vitro biochemical analyses of the hammerhead ribozyme reaction mechanism along with exhaustive mutational analyses have demonstrated that the highly conserved bases in the catalytic domain are essential for optimal catalytic activity. These studies have provided insight into how this rather simple RNA can function as a site-specific RNA-cleaving agent. In vitro biochemical analyses of the hammerhead ribozyme

reaction mechanism have been complemented by the acquisition of X-ray crystallographic structures (29,34–37). These revealed an approximately Y-shaped structure with stems I and III forming the arms of the Y and stem II forming the base (Fig. 2). Several unique base-pairing and base-stacking interactions in the catalytic core confirm the importance of the strong conservation of bases within this region. An interesting feature of the structure is a uridine or "U" turn characteristic of the anticodon stem of many transfer RNA molecules. In the hammerhead ribozyme, this turn is stabilized by a hydrogen bond from the uridine base to an oxygen atom of the phosphate following A6, and by a second hydrogen bond from the 2′ hydroxyl of the U4 to the N7 position of A6. Alteration of any of the nucleotides involved in the U turn effectively abolishes catalytic activity, suggesting a functional role for this structure in catalysis. The cleavage rate (about one per minute) is relatively independent of the primary sequence of the binding arms of the ribozymes, provided the ribozyme forms stable base pairs surrounding the site of cleavage. Biochemical, structural, and kinetic studies support the role of the hammerhead catalytic domain as a magnesium coordination center. The magnesium molecules bound by and adjacent to the catalytic core may be involved in the chemistry of the cleavage reaction. The proposed cleavage mechanism invokes a proton abstraction from the scissile 2′ OH at the site of cleavage, which allows a nucleophilic attack on the adjacent phosphorous atom at the 3′ position, resulting in a 2′3′ cyclic phosphate and a 5′ OH. Interestingly, the crystallographic data cannot provide the details for the cleavage mechanism but they do reveal a rather simple RNA structure that undergoes conformational changes from a ground state to an active enzyme upon binding of magnesium.

III. USING RIBOZYMES IN TRANS-CLEAVAGE REACTIONS

A. Sequence Specificity

The interactions of all the trans-acting ribozymes with target RNAs are mediated through the simple rules of Watson-Crick base pairing. The ribozyme reaction is comprised of a series of sequential steps: binding of the ribozyme to the target, cleavage, and release of the cleavage products (38). Simple principles govern the rate of each step. The rates of target binding and the release of each cleavage product follow the general rules of nucleic acid hybridization. For optimal ribozyme activity of the hammerhead and hairpin ribozymes, the lengths of the substrate binding arms should allow sufficiently stable ribozyme-substrate base pairing to facilitate cleavage, yet allow rapid dissociation of the cleaved products. Extensive mutational analyses of the hammerhead and hairpin ribozymes and their sites of cleavage have been carried out (39–41). For the hammerhead, cleavage can occur after any NUH triplet, where N = any nucleotide and H = A, C, or U. However, the kinetics of the reaction can vary significantly (up to one or

more orders of magnitude) with different triplet-flanking sequence combinations. The hairpin ribozyme has a strong requirement for a G immediately 3′ of the scissile bond, and cleaves most efficiently 5′ of GUC (42). Mismatches in the helices adjacent to the site of cleavage can strongly inhibit the ribozyme reactions. A point mutation at the site of cleavage will abolish the cleavage reaction for either ribozyme. Investigators have taken advantage of this property of the hammerhead ribozyme to discriminate between cleavage of mutant versus wild-type RNAs.

Mismatches in the ribozyme-binding arms have been used to discriminate between normal and single nucleotide polymorphisms (SNPs). An example of this is provided by studies of ribozyme-mediated inhibition of a mutant form of rhodopsin in a rat model. A point mutation in this protein is responsible for a single amino acid change that eventually leads to blindness. In the rat model, the amino acid change did not create a ribozyme cleavage site, but a neutral polymorphism in the rhodopsin mutant allele did create a hammerhead cleavage site (NUH). By taking advantage of this difference between the wild-type and mutant rhodopsin alleles the investigators were able to selectively down-regulate the mutant rhodopsin, thereby significantly retarding the progression to blindness characteristic of this disease (43). To date, hammerhead and hairpin ribozymes have been reported to effectively down-regulate a variety of RNA targets both in cell culture and in vivo and modified group I introns have been utilized to repair mutant RNAs in cell culture (44,45). Because of their proven efficacy in preclinical studies, both the hammerhead and hairpin ribozymes are currently being tested in human clinical trials.

B. Getting Ribozymes to Work in Cells

The major limitations to effective intracellular ribozyme use are target site accessibility (46,47) and colocalization of ribozymes with target RNAs (48). These specific problems as well as strategies for expression and delivery of ribozyme genes are discussed below.

C. Determining Accessible Sites for Ribozyme Pairing in Target RNAs

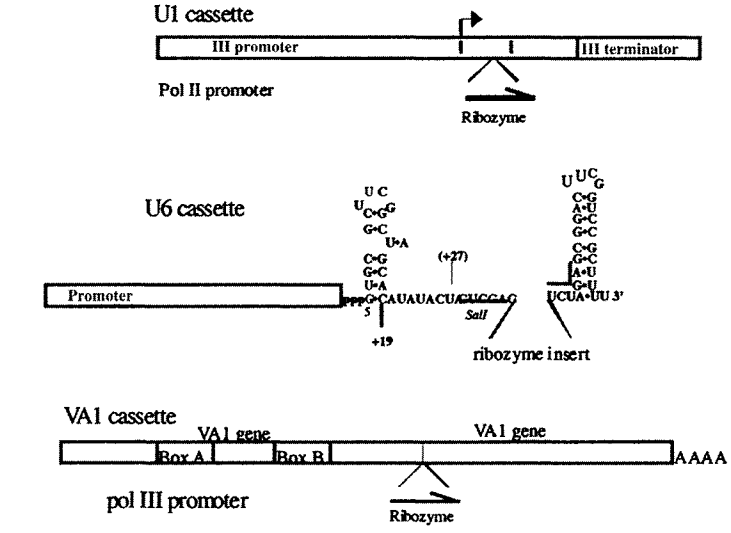
For most ribozyme experiments, the rate-limiting step is the choice of a good cleavage site. For ribozymes to be effective in down-regulating gene expression, the targeted region(s) of the RNA need to be accessible to the binding and subsequent cleavage of the ribozyme. RNA within the cell is complexed with heterogeneous nuclear proteins (hnRNPs) and small nuclear ribonuclear proteins (snRNPs) within the nucleus, and with hnRNPs and the translational apparatus in the cytoplasm. Portions of the RNA transcripts may also be sequestered in

secondary and tertiary structures, thus making them inaccessible to ribozyme interaction. Predicting sites that are accessible to ribozyme binding and cleavage is problematic. The use of computer RNA-folding programs such as MFOLD can be useful in determining regions of strong secondary structure, but these programs thus far have not facilitated prediction of the most accessible sites in the target RNA. The use of random ribozyme libraries to identify sites on target RNAs accessible to pairing has to date been the most direct approach for ribozyme target site determination (46). Alternatively, chemical probing of the target structures has also been used to identify sites on RNAs accessible to base pairing (49). The binding of a pool of random DNA oligos to in vitro–prepared RNA followed by incubation with the enzyme RNase H, which cleaves the RNA only within DNA-RNA hybrids, has also been used as a probe for sites accessible to base pairing on a target mRNA (50).

Ideally, any approach for identifying a ribozyme cleavage site should take advantage of the RNA in its native, protein-associated state. We therefore have devised a strategy for identifying accessible sites in a message utilizing native mRNAs in cellular extracts (51). The principle underlying this approach is that RNAs associated with proteins in their native state provide the best substrates for identifying sequences accessible to antisense RNA or DNA base pairing. Binding of DNA oligos and subsequent degradation by RNase H present in the extracts can be used to identify regions along any target RNA of interest that are most accessible to base pairing by an antisense agent. The DNA oligos are inexpensive to make, and a large number of potential sites can be rapidly screened by this procedure. The most accessible target sites are expected to generate cleavage products most rapidly during a time course. Once effective deoxyribonucleotide oligo pairing sites have been identified, ribozyme genes corresponding to these sites can be made in an appropriate plasmid harboring either the bacteriophage T7 or T3 promoters. The ribozymes are then transcribed and tested for cleavage activity in the same extracts against the native mRNAs (52). Thus far, we have observed perfect correlation between the results obtained using the DNA oligonucleotides in extracts with the most active ribozymes in the same extracts and consequently in cell culture (92).

D. Ribozyme Design and Expression

Once verification of the ribozyme activity to the chosen target sites in cell extracts is obtained, the next step is cloning of the ribozyme gene into an expression cassette. Selecting an appropriate cassette for intracellular gene expression is an important step for obtaining intracellular efficacy. The expression of catalytic RNAs has been successfully directed by Pol II and Pol III promoters. Figure 3 depicts several of the promoters that we and others have used successfully for functional ribozyme expression. Inducible, repressible, or tissue-specific pro-



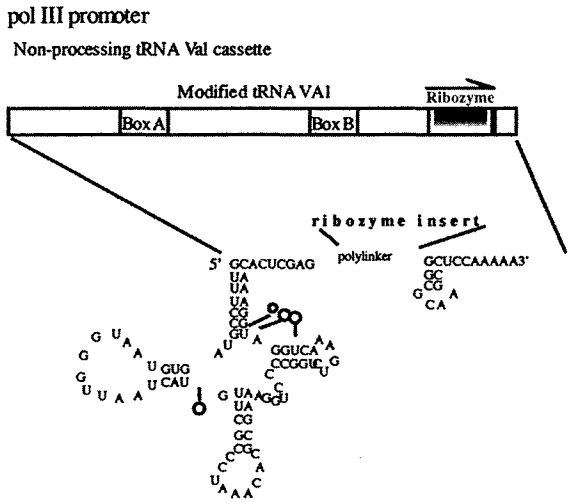


Figure 3 Ribozyme expression cassettes. Diagrammatic presentations of the Pol II U1 snRNA promoter, along with the Pol III U6 snRNA, adenoviral VA1 gene, and tRNA^{Val} promoters, are presented. Positions where ribozyme-coding sequences can be inserted are indicated as are the directions of transcription. The lollipop structures in the tRNA^{Val} cassette represent bases that have been modified to prevent 3′ end processing of the tRNA^{Val}-ribozyme chimeric transcripts (see text for reference and details).

moters can be used to confer temporal, cell-specific expression. Each of these systems has some unique feature that distinguishes it from the others. For Pol II promoters, the addition of a 5′ cap and 3′ poly A tail may be important for stability and intracellular cytoplasmic localization. However, a disadvantage of Pol II expression cassettes is that the sequence elements required to achieve efficient transcription often extend for hundreds of nucleotides. Appended 5′ UTR and 3′ UTR sequences could affect proper folding of the ribozyme. Some investigators have circumvented this problem by incorporating cis-cleaving ribozymes into the primary transcripts (53,54). A possible compromise for using a Pol II promoter without the trimming ribozymes can be achieved with mammalian snRNA Pol II promoters such as the U1 promoter. This is a relatively strong promoter that is ubiquitously expressed in a variety of cell types of both human and murine origin. Even though the U1 promoter normally drives transcription of a small nuclear RNA, it can be used for transcribing capped ribozyme transcripts either by replacing the U1 sequence with the ribozyme sequence, or by inserting the ribozyme within the stable U1 coding region (55,56). The intracellular distribution of U1-promoted ribozymes embedded in the U1 sequence is both cytoplasmic and nuclear. If the appropriate signals in U1 snRNA are included, the ribozyme transcript will shuttle to the cytoplasm and back into the nucleus.

Expressing ribozymes from Pol III cassettes can lead to high intracellular levels of ribozymes. Depending upon the type of Pol III expression unit chosen, transcripts can be localized to either the nucleus or the cytoplasm. As examples, ribozymes expressed from the human U6 snRNA promoter can be engineered to have either nuclear or cytoplasmic localization (55,57). The U6 promoter is a somewhat unusual Pol III promoter since all of the regulatory elements are contained within sequences 5′ to the transcribed U6 sequence. This allows engineering of a variety of ribozyme-containing transcripts to be expressed from this strong promoter. The U6 snRNA transcript is normally capped with a gamma-methyl group on the initiating triphosphate (58). By including the first 27 nucleotides of U6 snRNA in the ribozyme transcripts, these can be effectively localized to the nucleus, whereas eliminating just nucleotides 20–27 results in more random nuclear-cytoplasmic localization (55,57).

Most other Pol III promoters harbor the transcriptional regulatory elements within the transcribed region. This is true for tRNAs and the adenoviral VA1 RNA, both of which have been utilized for ribozyme expression. The adenoviral VA1 promoter has been engineered to allow incorporation of ribozymes or antisense RNAs within a highly structured stem loop (59,60). Substitution of a ribozyme for the loop and part of the stem eliminates the cellular kinase inhibitory domain, thus making this a useful vector for ribozyme expression. An important feature of the VA system utilized in this way is that the transcripts are predominantly cytoplasmic (59,60).

Transfer RNAs also maintain the Pol III regulatory elements within the mature coding sequence of the tRNA. For use in ribozyme expression, the ribozyme is most often inserted either within the anticodon loop or in the aminoacyl acceptor stem (55,61–63). A useful Pol III expression system is a modified version of mammalian $tRNA^{Val}$ (28). This Pol III cassette allows cloning of the ribozyme downstream of the structural portion of the tRNA. The tRNA itself is not end-processed since there are several base changes in the tRNA portion that perturb its tertiary structure and prevent end processing, while still allowing expression. Figure 3 depicts the essential features of each of these promoters.

IV. RIBOZYME TRAFFICKING AND INTRACELLULAR LOCALIZATION

Successful ribozyme-mediated inhibition is also dependent on the colocalization of the ribozyme transcripts with the targeted RNAs (for a review see Ref. 64). The use of retroviral packaging and dimerization signals for colocalizing ribozymes to targets has been shown to be a powerful approach to enhance ribozyme effectiveness and is a paradigm for the importance of colocalizing ribozyme and substrate (48,65,66). For nonviral targets, there are strategies for directing intracellular localization and subcompartmentalization that can be achieved by adding specific signals to the RNA transcripts. For instance, nuclear localization or colocalization of ribozymes with pre-mRNAs can be achieved by incorporating the ribozyme within the body of a snRNA such as U1 such that the ribozyme is directed to a site adjacent to the 5′ splice site of a targeted message (56,67). Localization signals, which are often found in the 3′ UTR of certain messenger RNAs (68), can be attached to the 3′ end of the ribozyme transcript to direct its intracellular trafficking (69). By utilizing the same 3′ signal on the ribozyme that is present on the localized target, enhanced ribozyme efficiency in a cell can be achieved. Using this approach, up to a fourfold enhancement in ribozyme efficacy utilizing matched 3′ UTRs was observed. This strategy is, of course, applicable only to messages with known subcellular localization properties. For other targets, more general localization of ribozyme and transcripts should be attempted. Empirical testing of expression patterns that provide cytoplasmic, nuclear, or mixed nuclear-cytoplasmic localization of the ribozyme is advisable for ribozymes directed at targets that do not have discrete subcellular compartmentalization.

In summary, in designing a cassette for endogenous expression of ribozymes the following should be considered:

1. The choice of promoter-expression cassette: Pol II versus Pol III. This choice should be based upon desired levels of expression, desired intra-

cellular localization, and whether or not the ribozyme is to be part of a chimeric transcript.

2. The choice of the promoter should take into account the delivery vehicle. Size constraints in retroviral or other viral vectors may necessitate use of Pol III or specialized Pol II promoters like the U1 promoter.
3. The choice of sequences appended to the ribozyme transcript will dictate the trafficking and target colocalization of the ribozyme.

V. DELIVERY OF RIBOZYME GENES

There are two basic approaches for delivery of ribozyme genes into cells: (1) transfection of the DNA encoding the ribozyme gene(s) or; (2) viral vector–mediated transduction of the ribozyme genes into the desired cells.

A. Viral Vectors

Several classes of viral vectors are being exploited for delivery of genes in vivo, including DNA (adenoviruses, herpes virus, adeno-associated virus) and RNA retroviral and lentiviral vectors. It is beyond the scope of this chapter to review the advantages and disadvantages of each system. For an overview, the reader is referred to review articles by Morgan and Anderson (70), Bahner (71), and Chatterjee and Wong (72). General concerns are associated with the use of viral vectors such as residual infectivity, toxicity, and rescue of infectivity by recombination. Each vector system has its own set of advantages and disadvantages that ultimately dictate its use in a specific application. To date the most extensively utilized viral vectors have been murine-derived amphotropic retroviruses. These viral vectors can infect a wide variety of cell types; they integrate into the host chromosome and, thus, are potentially capable of long-term expression. The integration process requires cell replication, restricting the use of these vectors to actively dividing cells. Lentiviral vectors that are useful for gene therapy have recently been shown to readily transduce nondividing cells (73). Retroviral vectors are currently the preferred method for gene transfer into cultured cells and primary cells in culture because of the efficiency of transduction and integration in the host genome. The retroviral genome includes only a few genes (gag, pol, and env), the promoter and enhancer sequences contained in the long terminal repeats (LTR), splicing acceptor and donor sites, and the packaging signal. Thus, the design of a retroviral vector is relatively simple. The expression cassette containing the selected ribozyme(s) can be cloned into the U3 region of the 3′ LTR to generate a double-copy insert or in between the two LTR sequences (55,74,75). Although potentially capable of long-term expression, the LTR viral promoters are subject to progressive silencing after chromosomal integration. Specific modi-

fications have been made to a Moloney murine–derived retroviral vector in an attempt to alleviate this problem (76,77). These modifications resulted in enhanced expression of an inserted gene in murine embryonic carcinoma cells (EC), embryonic stem cells, and fibroblast cells (76,78).

Following the construction of the retroviral viral vector, it must be packaged into virion that can be transduced into the appropriate target cells. Packaging can be carried out either by transient transfection (79) or by establishing permanent packaging cell lines (80). While transient transfection is very time efficient and also allows the use of human cell lines as the producer of viral particles, variations in transfection efficiencies may provide an undesirable variable when comparing several constructs. The use of packaging cell lines and cocultivation of the packaging cells and the transductants is more time consuming, but provides more reproducible transduction efficiencies.

B. Nonviral Delivery

For nonviral vector delivery of ribozyme genes into tissue culture cells, standard calcium phosphate or electroporation transfection systems are generally employed. These techniques are well described in a variety of laboratory manuals and will not be discussed here.

VI. DETERMINING IN VIVO RIBOZYME FUNCTION

The intracellular effectiveness of ribozyme function can be determined both by a reduction in the level of a target RNA and by measurement of the reduction of a functional activity encoded by that RNA. In carrying out intracellular experiments, a mutant, noncleaving form of the ribozyme should always be used as a control for antisense activity, and when possible, the reduction in the RNA level should be confirmed by a reduction in the level of the encoded protein. Noncleaving, crippled ribozymes are created by mutating one or more nucleotides in the catalytic domains of the hammerhead or hairpin ribozymes (39, 41). Standard techniques such as Northern gel, RNase protection, primer extension, and RT-PCR can be performed to analyze in vivo ribozyme function. The reduction in the level of target RNA can and should be confirmed by the corresponding reduction of the encoded protein. RNA analysis is not by itself conclusive because it is possible that the cleavage occurs during the RNA extraction. Every effort should be made to protect against this, such as low pH and added EDTA. The use of stable, ribozyme-expressing clones allows more accurate determinations than those obtained from transiently transfected cells. In both

cases, a nontargeted RNA (and protein if possible) should be used as internal standards.

VII. SYNTHETIC RIBOZYMES

Many exciting developments in the chemical synthesis of RNA and modified forms of RNA have taken place over the past several years. Molecules with long-term stabilities in serum or intracellular environments have been synthesized. Several of these backbone-modified ribozymes still maintain the site specificity and catalytic turnover features of unmodified RNAs, and some have enhanced catalytic properties (81–85). Modifications of the backbones have resulted in ribozymes that can be delivered in vivo in the absence of carrier agents. One such nuclease-stabilized ribozyme has been developed against the VEGF receptor subtype Flt-1 mRNA. Pharmacokinetic studies in mice have demonstrated that this modified ribozyme can be administered systemically via intravenous or intraperitoneal injections with uptake in several tissues and a serum half-life of several hours (86). This ribozyme and another targeted against hepatitis C virus are currently in early-phase clinical trials. Progress in scaled-up synthesis of synthetic ribozymes coupled with many developments in new catalytic motifs and increased targeting possibilities make these molecules promising candidates as highly target-specific drugs that can be developed for the treatment of a variety of diseases.

VIII. CONCLUDING REMARKS

In contrast to the rather extensive knowledge of the rules governing effective ribozyme function in vitro, the rules for predicting ribozyme efficacy in an intracellular or in vivo environment are just beginning to emerge. Even though it is recognized that a number of parameters are able to influence the intracellular functioning of ribozymes, obtaining optimal in vivo efficacy still requires empirical testing of targeting, expression, and localization strategies. The results from carefully controlled cell culture as well as a limited number of in vivo studies suggest effective intracellular ribozyme applications require strategies for high-level expression and/or novel targeting approaches. Even in the absence of a well-defined set of rules, elegant experiments demonstrating ribozyme efficacy in vivo have been conducted in model organisms including *Drosophila* (87), zebrafish (88), and mice (89–91). As we learn more about methods for optimizing ribozyme-target interactions in cells, more successful applications of ribozymes as genetic tools and as therapeutic agents will be forthcoming.

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