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COMMENTARY

Antisense Oligonucleotides: Is the Glass Half Full or Half Empty?

C. *Frank Bennett**
ISIS PHARMACEUTICALS, CARLSBAD, CA 92008.

ABSTRACT. Antisense oligonucleotides are widely used as tools to explore the pharmacological effects of inhibiting expression of a selected gene product. In addition, they are being investigated as therapeutic agents for the treatment of viral infections, cancers, and inflammatory disorders. Proof that the pharmacological effects produced by the oligonucleotides are attributable to an antisense mechanism of action requires careful experimentation. Central to this problem is the finding that oligonucleotides are capable of interacting with and modulating function of specific proteins in both a sequence-independent and -dependent manner. Despite these undesired interactions, it has been possible to demonstrate that oligonucleotides are capable of binding to a specific RNA in cultured cells, or within tissues, resulting in selective reduction of the targeted gene product and pharmacological activity. In general, these oligonucleotides were identified after a selection process in which multiple oligonucleotides targeting different regions on the RNA were evaluated for direct inhibition of targeted gene product, resulting in the identification of a potent and selective oligonucleotide. Similar to other drug-receptor interactions, selection of the most potent inhibitor results in an increase in the signal-to-noise ratio, yielding increased confidence that activity observed is the result of a desired effect of the inhibitor. With careful selection, proper controls, and careful dose-response curves it is possible to utilize antisense oligonucleotides as effective research tools and potentially as therapeutic agents. BIOCHEM PHARMACOL 55;1:9–19, 1998.

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Solving the genetic code and identification of methods for manipulating genetic information in cells have provided the framework for the current revolution in biomedical sciences. One of the most direct applications of this information is the design of short oligonucleotides (12–25 bases in length) that hybridize to a specific mRNA by Watson-Crick base pairing rules. Upon binding to the mRNA in the cell, the antisense oligonucleotide prevents expression of a protein product encoded by the targeted RNA. Antisense oligonucleotides have the potential to be a truly rational approach for drug design in that the rules for binding have been well characterized. Conceptually, such an approach is very attractive, in that all that is needed to develop an inhibitor is the sequence of the RNA of interest and the ability to synthesize the oligonucleotides. In fact, numerous studies have been published claiming inhibition of a wide variety of gene products with antisense oligonucleotides both in cell culture based experiments and *in vivo* [1–3]. However, it has become increasingly clear that like any other scientific endeavor, practicing antisense technology requires careful experimental design and interpretation of the results, in that some of these published studies probably identified pharmacological activity of the oligonucleotide which cannot be attributed to an antisense effect. Because of these findings, there has been some criticism of the technology in the scientific literature [4–6]. Although some of these criticisms have validity, it is important to put them in perspective. The ultimate questions are (1) whether the mechanism of action for antisense drugs can be well enough defined to allow their use as research tools, and (2) will antisense oligonucleotides be useful therapeutic agents. I will attempt to answer these two questions in this commentary and, in so doing, discuss what some of the early perceived issues were for antisense technologies, which of these have proven to be valid issues, where some of the pitfalls have occurred in using antisense oligonucleotides, and finally what the prospects are for the future of the technology. I will not attempt to perform a comprehensive review of the topics, but rather use this as a forum to stimulate additional discussion and focus attention on what I feel are important issues the technology faces in 1997.

* Correspondence: Dr. C. Frank Bennett, ISIS Pharmaceuticals, 2292 Faraday Ave., Carlsbad, CA 92008. Tel. (760) 603-2336; FAX (760) 931-0209.

ANTISENSE OLIGONUCLEOTIDES: PERCEIVED AND REAL ISSUES

Pharmacokinetics

CAN STABLE OLIGONUCLEOTIDE ANALOGS BE IDENTIFIED? Protection of the oligonucleotide from nuclease degradation has been one of the major accomplishments, resulting

from the application of medicinal chemistry to antisense technology. Chemical modifications have been identified that decrease degradation of the oligonucleotides, some even before the application of oligonucleotides for regulation of gene expression was fully appreciated. Some of the early modifications include phosphorothioate linkages [7, 8], phosphotriesters [9], methylphosphonate linkages [10, 11], and α-oligonucleotides [12]. More recently additional modifications have been identified that exhibit nuclease resistance, such as phosphorodithioate [13], additional 2′-modifications [14–17], 2′-5′ linkages [18], MMI† [19], formacetal [20], N3′ → P5′ phosphoramidate [21], amide 3 [22], and PNA [23].

Of these modifications, phosphorothioate oligodeoxynucleotides are the most widely used and currently account for all but one of the antisense oligonucleotides currently in the clinic. The stability of phosphorothioate oligodeoxynucleotides varies depending on the cell line or tissue investigated. In cell-based assays, phosphorothioate oligodeoxynucleotides have been reported to reduce expression of targeted mRNA for 24–48 hr [17, 24, 25]. If longer suppression of target RNA is desired, then cells either should be retreated with the oligonucleotide or additionally modified oligonucleotides can be used such as 2′-alkoxy [15, 25] or modifications in which the phosphorus has been replaced. *In vivo*, phosphorothioate oligodeoxynucleotides are metabolized in both serum and tissues [26–28]. The tissue half-life for intact drug is somewhat variable depending upon the sequence of the oligonucleotide and the tissue being studied [26–28]. More nuclease-resistant modifications would be expected to exhibit tissue half-lives significantly greater than these values [26, 29, 30]. With these modifications, dosing frequency could be reduced for better patient convenience. Thus, with a large number of modified oligonucleotides to select from, the degree of nuclease resistance desired in an oligonucleotide can be tailored to meet specific needs.

DO OLIGONUCLEOTIDES GET INTO CELLS? Alternatively, will oligonucleotides reach their intracellular target in sufficient amounts to inhibit expression of the targeted RNA? More importantly, are the pharmacokinetics of the oligonucleotide favorable enough to allow use for *in vivo* applications? Like most other drugs, the mechanisms by which oligonucleotides and their analogs interact with plasma components, distribute to tissues, and accumulate within cells are poorly understood. The pharmacokinetics of phosphorothioate oligodeoxynucleotides have been well described in terms of general plasma kinetics and tissue distribution [28, 31–34]. Phosphorothioate oligodeoxynucleotides are rapidly distributed out of plasma to most peripheral tissues, with liver and kidney accumulating the highest concentrations of oligonucleotide. Significant concentrations are also obtained in other tissues such as intestine, spleen, skin, and bone marrow. Thus, phosphorothioate oligodeoxynucleotides readily accumulate in a broad range of tissues.

Information on where within tissues the compounds accumulate is beginning to appear [35–37]. These studies suggest that at early times, up to 2 hr, the oligonucleotide is both associated with extracellular components and localized intracellularly [37]. At later times the bulk of the oligonucleotide is localized inside cells within the tissues. As expected, the oligonucleotides do not distribute uniformly within a tissue but accumulate within certain cell populations. Best characterized is the kidney, in which the oligonucleotide accumulates within epithelial cells of the proximal convoluted tubule [33, 37, 38]. Within other tissues, the oligonucleotide clearly localizes within specific cell populations; however, identification of the cell populations is more difficult.

Does enough oligonucleotide accumulate within cells to inhibit expression of targeted gene products? A very rough estimate for mRNA concentration in a cell that expresses 10,000 copies of a target mRNA (a relatively abundant mRNA) would be 20 nM. At a pharmacologically relevant dose of 3–6 mg/kg, the bulk concentration of oligonucleotide in kidney and liver is approximately 5–15 and 1–5 μM, respectively. Concentrations in other tissues range from less than 0.05 μM (brain) to 1 μM. One should next recognize that the oligonucleotide is accumulating within certain cell types within tissues, which could increase the actual cellular concentration at least 10-fold for some cell types. These concentrations, in theory, would be far in excess of target mRNA concentrations and therefore should inhibit targeted gene expression within tissues if they have access to the mRNA. This latter point is the one issue that has generated the most controversy, i.e. do oligonucleotides that are internalized in cells have access to the target RNA? Unfortunately, the answer to this question is complicated, but appears to be affirmative for some cell types within tissues.

Numerous investigators have characterized oligonucleotide internalization in mammalian cells. These studies, not unexpectedly, have yielded conflicting results in that different methods were used for evaluation of oligonucleotide internalization, different cell lines were used, and different oligonucleotides were used. The one consistent conclusion that can be drawn from these studies is that all mammalian cells investigated are capable of internalizing phosphorothioate oligodeoxynucleotides by an active process. The intracellular fate of the oligonucleotide is controversial. Some studies suggest that oligonucleotides are internalized by a "receptor" mediated or adsorptive endocytosis pathway in which the oligonucleotide is retained within membrane-bound intracellular vesicles [39–42], while other studies suggest that the oligonucleotide either uses alternative methods for gaining entry into cells or escapes from the cytoplasmic vesicles [43–45]. Our studies would suggest that there are multiple competing mechanisms by which

† *Abbreviations:* CMV, cytomegalovirus; ICAM-1, intercellular adhesion molecule-1; MMI, methylene (methylimino); NK cells, natural killer cells; and PNA, peptide nucleic acid.

oligonucleotides are internalized in the cells [46]. For the majority of cultured cell lines that we have investigated, we observed localization of fluorescently labeled oligonucleotides in cytoplasmic structures and failed to observe specific antisense effects in the absence of a facilitator such as cationic lipids. However, we and others have demonstrated that some specific cell types accumulate phosphorothioate oligonucleotides in the cell nucleus without use of cationic lipids or other transfection methods [44, 45]. We have also observed that *in vivo* administered oligonucleotide exhibits both patterns of distribution within specific cell types in tissues [37]. These data are supported by pharmacological studies in which it is possible to demonstrate reductions in target RNA or protein expression in tissues following systemic administration of the antisense oligonucleotide [47–56]. It is unlikely that there will be a single mechanism by which oligonucleotides become internalized in cells. Furthermore, data would suggest that behavior of oligonucleotides in cell culture does not predict their behavior *in vivo*.

Pharmacology

MECHANISM OF ACTION. What is the mechanism by which antisense oligonucleotides inhibit expression of the targeted gene product? Is occupancy of the RNA by the oligonucleotide sufficient or does the oligonucleotide need to exploit a catalytic activity such as RNase H? There are multiple theoretical mechanisms by which oligonucleotides can be used to regulate expression of target genes [57–59]. Perhaps the most widely used mechanism is cleavage of the targeted RNA by RNase H. Although it has not been unequivocally demonstrated that reduction or cleavage of the targeted RNA in cells is mediated by RNase H, there is a great deal of evidence to support such a conclusion, including direct demonstration of a reduction in target mRNA, demonstration of appropriate cleavage products [60–62], and use of modified oligonucleotides that do not support RNase H activity [24, 63, 64]. In addition to RNase H, mammalian cells express a variety of other RNases, which could be exploited to selectively inhibit expression of a targeted gene. Examples include RNase L [65] and double-stranded RNases.

There are also steric mechanisms by which oligonucleotides can prevent expression. Translation arrest, in which the oligonucleotide binds to the target mRNA and blocks movement of the ribosome and subsequently translation of the mRNA, is one of the most cited mechanisms of action. Several studies have been able to document that oligonucleotides are capable of inhibiting translation in an *in vitro* translation assay, including experiments by Ochoa and colleagues in 1961 [66]. However, it is more difficult to prove this as a mechanism in cell-based assays. Reduction in targeted protein by an oligonucleotide but no reduction in mRNA has been used as evidence for a translation arrest mechanism. Alternatively, demonstration of a selective reduction in target protein by modified oligonucleotides that do not support RNase H has also been used as evidence for a translation arrest mechanism. However, in both cases other mechanisms of action could account for these observations. Therefore, evidence directly demonstrating a translation arrest mechanism in cell culture or *in vivo* is circumstantial.

A somewhat related mechanism is prevention of ribosome assemble on the mRNA. Baker *et al.* [67] utilized uniformly 2′-modified oligonucleotides, which do not support RNase H activity, to target the 5′-terminus of ICAM-1 RNA [67]. These oligonucleotides very effectively inhibited ICAM-1 protein expression by markedly changing the polysome profile of ICAM-1 mRNA, shifting it from a higher molecular weight polysome pool to a lower molecular weight pool.

Regulating pre-mRNA maturation is also a potential mechanism by which oligonucleotides may inhibit or alter gene expression by sterically blocking recognition of the RNA. Kole and colleagues [68] have performed a series of studies demonstrating that uniformly modified 2′-O-methyl phosphorothioate oligonucleotides, which do not support RNase H, can alter the splicing of a thalassemic β-globin mRNA in mammalian cells. The oligonucleotide was used to mask a splice site so that an alternative site was utilized. Hodges and Crooke [69] have also reported similar findings in which they demonstrated that 2′-O-methyl oligonucleotides are capable of selectively blocking splicing of an adenovirus transcript.

These results demonstrate that there are multiple antisense mechanisms by which oligonucleotides can inhibit expression of genes. It is possible to utilize endogenous enzymes in cells to provide catalytic activity as a mechanism, or sterically blocking a critical regulatory element can be a very effective mechanism for inhibiting expression. The mechanism of action is dependent in part on where on the RNA the oligonucleotide hybridizes as well as the type of oligonucleotide chemistry used.

DO OLIGONUCLEOTIDES PRODUCE PHARMACOLOGICAL EFFECTS BY AN ANTISENSE MECHANISM OF ACTION, IN VITRO AND IN VIVO? There are multiple published experiments that demonstrate direct reduction in targeted gene expression by oligonucleotides, in which it is difficult to conclude that they are working by any other mechanism [as examples, see Refs. 24, 47, 63, 64, and 70–79]. In general, these studies had in common several of the following experiments to provide evidence that the oligonucleotide was, in fact, working by an antisense mechanism of action. (1) In these experiments, the oligonucleotides were selected for potency following a screen in which the expression of the target gene was directly analyzed, rather than indirect assays. By selecting for more potent oligonucleotides the signal-to-noise ratio is increased, allowing for characterization of pharmacological effects due to inhibition of the targeted gene product. This selection process at this time is rather empirical and should be conducted regardless of the type of chemistry. (2) These studies demonstrate reduction in

either the mRNA which codes for the protein of interest or the protein itself. (3) Control oligonucleotides were examined for activity, in some cases multiple controls. (4) In some experiments rank order potency comparisons were performed between *in vitro* and *in vivo* studies. (5) The studies demonstrate that the effects of the antisense oligonucleotide are specific for the targeted gene, in that related gene products were not inhibited. (6) Dose–response curves were performed with the oligonucleotide rather than single points. (7) The observed pharmacological activity was consistent with what was known about the biology of the target. (8) In most of the cell-based assays, either cationic lipids or microinjection was used to facilitate delivery of the oligonucleotide to the cytoplasm of the cell.

Demonstrating that the oligonucleotide is producing a pharmacological effect by an antisense mechanism of action *in vivo* is more difficult than in cell-based assays. Nevertheless, studies are being published, demonstrating pharmacological activity of oligonucleotides in *in vivo* models strongly supporting an antisense mechanism of action in which many of these same criteria were applied [3, 47–56]. If the studies are examined in aggregate, it is hard not to conclude that oligonucleotides are capable of inhibiting gene expression *in vitro* and *in vivo* by an antisense mechanism of action.

POTENCY. How much can the affinity of an oligonucleotide be improved over natural DNA or RNA and will this improvement translate to improved potency in pharmacological assays? This is another area in which application of medicinal chemistry has been quite successful [80]. There are a number of modifications that exhibit greater affinity than oligodeoxynucleotides for RNA, including various sugar modifications [14, 15, 81–83], heterocyclic modifications [76, 84–86], phosphoramidates [21], phosphate replacements [87–89], and sugar phosphate replacements [23]. In that potency in cellular based assays and *in vivo* is dependent on factors in addition to target affinity, the increase in affinity does not always translate to increase in potency in pharmacological assays. In many cases, the oligonucleotides need to be further modified with phosphorothioate linkages or other backbone modifications to protect from nuclease degradation. One concern with higher affinity oligonucleotides was that they would lose specificity [90]; however, this has not been borne out. In cases where it has been examined, the modified oligonucleotide has exhibited equal or greater selectivity for its Watson–Crick hybridization partner [91].

Toxicology

WILL OLIGONUCLEOTIDES HYBRIDIZE TO NON-TARGET RNA MOLECULES? In theory an oligonucleotide 11–15 bases in length should uniquely hybridize to a given mRNA, while an oligonucleotide 15–19 bases in length should hybridize to a unique DNA sequence depending on the A + T and G + C content [92]. These conclusions were based upon the assumption that the oligonucleotide would have equal access to all sites on the target RNA or DNA and whatever terminating mechanisms are utilized, they do not exhibit any sequence dependence. Both of these assumptions have proven to be incorrect. Multiple experiments have indicated that not all sites on a target RNA are equally accessible to the oligonucleotide either *in vitro* or *in vivo* [24, 63, 70, 72, 79, 93–96]. In addition, terminating mechanisms appear to be highly dependent upon the context of the sequence within the RNA, especially for oligonucleotides that do not utilize RNase H [24, 63, 67]. Therefore, it is difficult to predict what the chances are that a given oligonucleotide will hybridize to a non-target RNA and affect expression. In our experience this has not been a major issue. It is probably more important to optimize length of the oligonucleotide based upon potency, rather than selectivity for target RNA. This will vary depending on the chemistry used. For phosphorothioate oligodeoxynucleotides, optimal length may range from 15 to 21 bases in length, while higher affinity analogs would allow use of shorter oligomers [79].

INTERACTION WITH OTHER NON-TARGET MOLECULES. It should be appreciated that oligonucleotides, like any other molecules, are capable of interacting with non-target molecules [5, 6, 97–102]. In some cases, these interactions can be quite sequence specific, such as aptamers [103–105], while in other instances the oligonucleotides interact with molecules in a sequence-independent manner. Interaction with non-nucleic acid targets can contribute to their overall pharmacological activity, at times making it difficult to interpret which activity is due to the antisense effect and which is due to a non-antisense effect. Incorporating multiple controls into the experiment is important in drawing any conclusions.

There are a variety of proteins with which oligonucleotides interact in an apparently sequence-independent manner, including DNA polymerases, laminin, and CD4 [106, 107]. In many cases phosphorothioate oligodeoxynucleotides were more potent than phosphodiester oligodeoxynucleotides, suggesting that the sulfur substitution enhances these interactions. From a toxicological perspective, these interactions have not at this time been proven to result in untoward effects. The primary acute toxicities that are a concern are interaction with proteins in the clotting cascade, resulting in an anti-coagulant effect, and interaction with a protein or proteins resulting in complement activation [3]. In both cases it is unknown which proteins the oligonucleotides are interacting with to produce these effects, although candidate proteins have been identified.

Recently, several papers have been published demonstrating that pharmacological activities originally assumed to be due to an antisense effect of the oligonucleotide were, in fact, due to interaction with proteins [100–102, 107, 108]. In many cases, this activity can be attributed to the presence of a consecutive series of guanine residues in the oligonucleotide, which are capable of forming a four-

stranded structure termed a G quartet [109], although it has not been demonstrated conclusively that these oligonucleotides do form such a structure. Oligonucleotides that contain three or more guanine residues in a row can also hybridize to an RNA target, complicating interpretation of data generated from such an oligonucleotide. A second sequence motif that has been demonstrated to produce pharmacological activity by a non-antisense mechanism of action is a CpG motif in which the CG residues are flanked by two purines on the 5′-end and two pyrimidines on the 3′-end [102]. Oligonucleotides containing such motifs have been shown to be very potent B lymphocyte mitogens and activators of NK cells, although the latter activity appears to be more restricted [110]. In our experience, all phosphorothioate oligodeoxynucleotides examined will produce some degree of immune stimulation. With the proper sequence context, the oligonucleotide can produce very profound immune stimulation [111]. Rodents appear to be more sensitive to this effect than primates [112].

ECONOMICS. Will oligonucleotide synthesis be commercially viable for human therapeutics? For treatment of disease with a high morbidity and a large unmet medical need the markets would support an antisense oligonucleotide; however, for markets with low morbidity or a met medical need it would be questionable whether the economics would support an antisense oligonucleotide, or many other types of drugs for that matter. At this time it is difficult to predict what the full burden of costs will be for the manufacture of a phosphorothioate oligodeoxynucleotide at the time of commercialization of the first systemically administered oligonucleotide. In 1990, it was estimated that the actual cost of synthesizing a 28-mer phosphorothioate oligodeoxynucleotide was $42,700 per gram [113]. Improvements in process research and cost reductions associated with scale have dramatically reduced the costs of manufacture approximately 40-fold compared with what they were in 1990. It is anticipated that costs can be reduced by another 8- to 10-fold upon implementation of processes required to manufacture at the ton per year scale. Although this cost may be high compared with that of traditional small molecules, it is similar to or even cheaper than other biotechnology products and is commercially feasible for many clinical indications.

ANTISENSE OLIGONUCLEOTIDES: CURRENT ISSUES

Hopefully it is apparent from the above discussion that most of the early concerns about antisense oligonucleotides have been addressed and that there have not been any issues that have arisen preventing application of the technology either as a research tool (if experiments are properly controlled) or, more importantly, as a therapeutic agent. The largest body of information is known about first-generation phosphorothioate oligodeoxynucleotides. Large-scale synthesis of phosphorothioate oligodeoxynucleotides is feasible, they appear to be well tolerated at therapeutically relevant doses, they produce predicted pharmacological effects, and they are broadly distributed to tissues. In fact, it is possible that a first generation phosphorothioate oligodeoxynucleotide will be on the market before the year 2000. Although phosphorothioate oligodeoxynucleotides appear to be acceptable as drugs or research tools, there are several properties that can be improved for broader applicability. As discussed above, second and third generation oligonucleotide chemistries are already demonstrating improved properties, as are some advanced formulations. Following is a brief discussion of some of the limitations of phosphorothioate oligodeoxynucleotides or current modifications. Whenever possible, solutions that are in hand or appear to be well on their way to solving the issue are highlighted.

Pharmacokinetics

DELIVERY ROUTES. Current applications of oligonucleotides utilize parenteral dosage forms. While this route of administration will be acceptable for the treatment of many diseases, alternate dosage forms that are more convenient for the patient will expand the application of antisense oligonucleotides. Agrawal and colleagues [114] published some very intriguing work in which they demonstrated that phosphorothioate 2′-O-methyl oligonucleotides exhibit enhanced stability to nucleases in the gastrointestinal tract and enhanced oral bioavailibility. Extension of these observations by either additional chemical modifications or formulations may further improve oral bioavailibility, so that it is not unrealistic to envisage oral dosage forms for oligonucleotides. In addition, investigating other non-parenteral routes of administration may provide additional opportunities.

TISSUE DISTRIBUTION. Although phosphorothioate oligodeoxynucleotides are broadly distributed to many peripheral tissues, it may be desirable either to selectively target a tissue with an antisense oligonucleotide or to target tissues that do not achieve high concentrations, such as brain or even lung. There are multiple ways this might be achieved, including chemical modifications and formulations. In addition, it may be desirable to direct the oligonucleotide to specific cell types in the tissue or make more of the oligonucleotide within a tissue available for hybridization to target mRNA. Numerous studies have been published demonstrating selective targeting of tissues or cell types within tissues for other types of agents using antibodies, peptides, carbohydrates, or vitamins demonstrating technical feasibility. It still remains to be determined whether these types of approaches will be commercially viable in terms of enhancing delivery of antisense oligonucleotides.

Pharmacology

POTENCY. There are at least three mechanisms by which the potency of antisense oligonucleotides can be improved *in vivo*: increase affinity for its receptor, decrease non-receptor interactions, or increase efficiency for delivery to the subcellular compartment in which the receptor is localized. As discussed above, there are numerous modifications that enhance affinity for the target RNA in the cell, demonstrating improved activity in cell-based assays. Some of these modifications also demonstrate decreased interaction with various proteins, simultaneously accomplishing two goals. Enhancing delivery to the intracellular target is still an area in which little progress has been made. Preliminary studies suggest that cholesterol conjugation to oligonucleotides improves delivery to target RNA [115, 116]. In cell culture based experiments, cationic lipid formulations have been shown to facilitate intracellular delivery of charged oligonucleotides to the target RNA. However, the utility of these formulations for enhancing intracellular delivery of oligonucleotides *in vivo* has yet to be demonstrated, although they will significantly alter the tissue distribution [117]. Ultimately, I feel that additional improvements will be forthcoming.

MORE EFFICIENT IDENTIFICATION OF ACTIVE OLIGONUCLEOTIDE. As discussed previously, numerous studies have demonstrated that not all target sites on a mRNA are equally accessible. This is an issue not only for phosphorothioate oligodeoxynucleotides, but also for all oligonucleotides. Currently, high affinity target sites on RNA are identified empirically through screening multiple oligonucleotides. In that it is not economical to screen for all possible oligonucleotides, it is never known if the highest affinity oligonucleotide possible has been identified. It would be highly desired to improve on this method, ideally through predictive computer algorithms. However, because current understanding of RNA structure and accessibility inside a cell is very limited, it is unlikely that this will be forthcoming in the next 5 years. Other methods for identifying target sites on RNA, such as combinatorial approaches or arrays [95, 118], may provide solutions in the near term.

Toxicology

CLEARANCE FROM NON-TARGET TISSUES. Although phosphorothioate oligodeoxynucleotides have the desirable property of being stable towards serum and cellular nucleases, in some cases this stability may ultimately produce a detrimental effect. Although there are no published studies demonstrating that this is a problem, it could be rationalized that if the rates of tissue clearance from a non-target tissue are slower than the rates of clearance from a target tissue, accumulation of oligonucleotide in non-target tissue would occur when dosed optimally for the target tissue. In particular, accumulation in kidney is a concern in that clearance rates from kidney may be slower than from other tissues [112, 119]. Both medicinal chemistry modifications and formulations have shown potential promise to change the tissue distribution away from the kidney.

CHRONIC IMMUNE STIMULATION. Although immune stimulation is much more pronounced in rodents than in primates, there is some concern about chronic, low-level stimulation of the immune system. Alternatively, for some immune-mediated disorders, the oligonucleotide, at clinically relevant doses, may prime the immune system to respond to other stimuli. In that modified oligonucleotides have been demonstrated to abrogate this immune stimulation [102, 120, 121], this should be less of an issue with newer oligonucleotides.

INTERACTION WITH PLASMA PROTEINS. Currently there are two types of plasma protein interactions that are a concern for phosphorothioate oligodeoxynucleotides: interaction with protein components of the clotting cascade, resulting in anti-coagulant effects, and interaction with proteins resulting in complement activation. In both cases, the critical protein component with which the oligonucleotide interacts has not been identified. There are a number of suspected candidates, but more information is needed to confirm that interaction with the protein produces the undesirable effect. Several sugar and phosphate backbone modified oligonucleotides have been demonstrated to exhibit fewer anti-coagulant effects and less potential for inducing complement. At the same time, these same modifications also increase potency.

It should be pointed out that interaction with plasma proteins may not be an entirely undesirable property of the oligonucleotide. Interaction with plasma protein likely prevents filtration of unbound oligonucleotide in the kidney and excretion into the urine. Furthermore, interaction with plasma proteins may facilitate movement out of the vasculature through transcytosis and may enhance cellular uptake. If the binding to plasma proteins is low affinity [26, 122], then the oligonucleotide can partition off the plasma proteins and ultimately bind the target RNA.

METABOLISM OF MODIFIED NUCLEOSIDES. There is a rich literature in the use of modified nucleosides and nucleotides for anti-viral and anti-cancer agents, demonstrating that modified nucleosides can be cytotoxic. Therefore, there should be some concern that some of the oligonucleotides incorporating modified nucleosides or internucleosidic linkage may exert unwanted toxicities through release of these modified residues upon degradation. The oligonucleotide would, in essence, serve as a prodrug for the release of cytotoxic nucleotides [123].

FUTURE OF ANTISENSE OLIGONUCLEOTIDES

Is the glass half full or half empty? I feel that the technology is at a transition. There have been many lessons learned in how to utilize the technology and what the limitations are.

With careful selection of antisense oligonucleotides and proper controls it is possible to demonstrate a reduction in target gene expression by an antisense mechanism of action both *in vitro* and *in vivo*, validating the concept. With the explosion of information coming from genome sequencing efforts, a method for rapidly identifying the biological function and validating interesting targets for larger drug discovery efforts will be needed. Antisense oligonucleotides are the most logical tool for answering these questions. With properly conducted experiments, antisense oligonucleotides should provide valuable information in this regard. Newer oligonucleotide chemistries provide greater signal-to-noise ratios in the *in vitro* assays. It is clear that no single oligonucleotide modification will solve all the issues the technology faces; therefore, it is important to have a number of chemical modifications available for application to specific projects. These modifications can be used in a matrix approach in which the oligonucleotide can be tailored for specific needs.

In addition, the lead oligonucleotides also have the potential for progressing on to the clinic. One advantage that antisense oligonucleotides have in this regard is that chemical synthesis, preclinical pharmacokinetics, and to a large extent toxicology are modular from molecular target to molecular target. Once an investment has been made for a specific compound, the costs incurred for additional compounds against additional molecular targets are considerably less. How broadly useful antisense oligonucleotides will be for the treatment of human diseases is still an unanswered question. An oligonucleotide is already benefiting patients with CMV retinitis.* Preliminary results from other studies are encouraging as well. As with any other drug that enters the clinic, it is likely there will be many more failures than successes. It is unrealistic to expect otherwise, as there are many factors, in addition to technological issues, which control the success of a drug. However, it is likely that antisense oligonucleotides will be a part of the pharmacopoeia in the future, providing benefit to patients. Therefore, it is my perspective that the glass is actually more than half full and there is a promising future for antisense technologies.

References

1. Crooke ST, Therapeutic applications of oligonucleotides. *Annu Rev Pharmacol Toxicol* **32:** 329–376, 1992.
2. Stein CA and Cheng Y-C, Antisense oligonucleotides as therapeutic agents-Is the bullet really magical? *Science* **261:** 1004–1012, 1993.
3. Crooke ST and Bennett CF, Progress in antisense oligonucleotide therapeutics. *Annu Rev Pharmacol Toxicol* **36:** 107–129, 1996.
4. Sharma HW and Narayanan R, The therapeutic potential of antisense oligonucleotides. *Bioessays* **17:** 1055–1063, 1995.
5. Stein CA and Krieg AM, Problems in interpretation of data derived from *in vitro* and *in vivo* use of antisense oligodeoxynucleotides. *Antisense Res Dev* **4:** 67–69, 1994.
6. Stein CA, Does antisense exist? *Nature Med* **1:** 1119–1121, 1995.
7. De Clercq E, Eckstein F and Merigan TC, Interferon induction increased through chemical modification of synthetic polyribonucleotide. *Science* **165:** 1137–1140, 1969.
8. Eckstein F, Schulz HH, Ruterjans H, Haar W and Maurer W, Stereochemistry of the transesterification step of ribonuclease T_1. *Biochemistry* **11:** 3507–3512, 1972.
9. Letsinger RL and Miller PS, Protecting groups for nucleosides used in synthesizing oligonucleotides. *J Am Chem Soc* **91:** 3356–3359, 1969.
10. Miller PS, Yano J, Yano E, Carroll C, Jayaraman K and Ts'o POP, Nonionic nucleic acid analogues. Synthesis and characterization of dideoxyribonucleoside methylphosphonates. *Biochemistry* **18:** 5134–5143, 1979.
11. Jayaraman K, McParland K, Miller P and Ts'o POP, Selective inhibition of *Escherichia coli* protein synthesis and growth by nonionic oligonucleotides complementary to the 3′ end of 16S rRNA. *Proc Natl Acad Sci USA* **78:** 1537–1541, 1981.
12. Bertrand J-R, Imbach J-L, Paoletti C and Malvy C, Comparative activity of α- and β-anomeric oligonucleotides on rabbit β globin synthesis: Inhibitory effect of cap targeted α-oligonucleotides. *Biochem Biophys Res Commun* **164:** 311–318, 1989.
13. Marshall WS and Caruthers MH, Phosphorodithioate DNA as a potential therapeutic drug. *Science* **259:** 1564–1570, 1993.
14. Iribarren AM, Sproat BS, Neuner P, Sulston I, Ryder U and Lamond AI, 2′-O-Alkyl oligoribonucleotides as antisense probes. *Proc Natl Acad Sci USA* **87:** 7747–7751, 1990.
15. Altmann K-H, Dean NM, Fabbro D, Freier SM, Geiger T, Haner R, Husken D, Martin P, Monia BP, Muller M, Natt F, Nicklin P, Phillips J, Pieles U, Sasmor H and Moser HE, Second generation of antisense oligonucleotides: From nuclease resistance to biological efficacy in animals. *Chimia* **50:** 168–176, 1996.
16. Cummins LL, Owens SR, Risen LM, Lesnik EA, Freier SM, McGee D, Guinosso CJ and Cook PD, Characterization of fully 2′-modified oligoribonucleotide hetero- and homoduplex hybridization and nuclease sensitivity. *Nucleic Acids Res* **23:** 2019–2024, 1995.
17. McKay RA, Cummins LL, Graham MJ, Lesnik EA, Owens SR, Winniman M and Dean NM, Enhanced activity of an antisense oligonucleotide targeting murine protein kinase C-α by the incorporation of 2′-O-propyl modifications. *Nucleic Acid Res* **24:** 411–417, 1996.
18. Alul R and Hoke GD, (2′-5′)-Oligo-3′-deoxynucleotides: Selective binding to single-stranded RNA but not DNA. *Antisense Res Dev* **5:** 3–11, 1995.
19. Debart F, Vasseur J-J, Sanghvi YS and Cook PD, Synthesis and incorporation of methyleneoxyl (methylimino) linked thymidine dimer into antisense oligonucleotides. *Bioorg Med Chem Lett* **2:** 1479–1482, 1992.
20. Matteucci M, Lin K-Y, Butcher S and Moulds C, Deoxyoligonucleotides bearing neutral analogues of phosphodiester linkages recognize duplex DNA via triple-helix formation. *J Am Chem Soc* **113:** 7767–7768, 1991.
21. Gryaznov SM, Lloyd DH, Chen J-K, Schultz RG, DeDionisio LA, Ratmeyer L and Wilson WD, Oligonucleotide N3′ → P5′ phosphoramidates. *Proc Natl Acad Sci USA* **92:** 5798–5802, 1995.
22. De Mesmaeker A, Waldner A, Lebreton J, Fritsch V and Wolf RM, Novel backbone replacements for oligonucleotides. In: *Carbohydrate Modifications in Antisense Research*, ACS *Symposium Series No. 580* (Eds. Sanghvi YS and Cook

* Hutcherson SL, Palestine AG, Cantrill HL, Lieberman RM, Holland GN and Anderson KP, Antisense oligonucleotide safety and efficacy for CMV retinitis in AIDS patients. *35th ICAAC*, p. 204, 1995.

PD), pp. 24–39. American Chemical Society, Washington, DC, 1994.
23. Nielsen PE, Egholm M, Berg RH and Buchardt O, Sequence-selective recognition of DNA by strand displacement with a thymine-substituted polyamide. *Science* **254:** 1497–1500, 1991.
24. Dean NM, McKay R, Condon TP and Bennett CF, Inhibition of protein kinase C-α expression in human A549 cells by antisense oligonucleotides inhibits induction of intercellular adhesion molecule 1 (ICAM-1) mRNA by phorbol esters. *J Biol Chem* **269:** 16416–16424, 1994.
25. Monia BP, Johnston JF, Sasmor H and Cummins LL, Nuclease resistance and antisense activity of modified oligonucleotides targeted to Ha-*ras*. *J Biol Chem* **271:** 14533–14540, 1996.
26. Crooke ST, Graham MJ, Zuckerman JE, Brooks D, Conklin BS, Cummins LL, Greig MJ, Guinosso CJ, Kornbrust D, Manoharan M, Sasmor HM, Schleich T, Tivel KL and Griffey RH, Pharmacokinetic properties of several novel oligonucleotide analogs in mice. *J Pharmacol Exp Ther* **277:** 923–937, 1996.
27. Zhang R, Diasio RB, Lu Z, Liu T, Jiang Z, Galbraith WM and Agrawal S, Pharmacokinetics and tissue distribution in rats of an oligodeoxynucleotide phosphorothioate (GEM 91) developed as a therapeutic agent for human immunodeficiency virus type-1. *Biochem Pharmacol* **49:** 929–939, 1995.
28. Cossum PA, Sasmor H, Dellinger D, Truong L, Cummins L, Owens SR, Markham PM, Shea JP and Crooke S, Disposition of the ^{14}C-labeled phosphorothioate oligonucleotide ISIS 2105 after intravenous administration to rats. *J Pharmacol Exp Ther* **267:** 1181–1190, 1993.
29. Zhang R, Lu Z, Zhao H, Zhang X, Diasio RB, Habus I, Jiang Z, Iyer RP, Yu D and Agrawal S, *In vivo* stability, disposition and metabolism of a "hybrid" oligonucleotide phosphorothioate in rats. *Biochem Pharmacol* **50:** 545–556, 1995.
30. Zhang R, Iyer RP, Yu D, Tan W, Zhang X, Lu Z, Zhao H and Agrawal S, Pharmacokinetics and tissue distribution of a chimeric oligodeoxynucleoside phosphorothioate in rats after intravenous administration. *J Pharmacol Exp Ther* **278:** 971–979, 1996.
31. Agrawal S, Temsamani J and Tang JY, Pharmacokinetics, biodistribution, and stability of oligodeoxynucleotide phosphorothioates in mice. *Proc Natl Acad Sci USA* **88:** 7595–7599, 1991.
32. Cossum PA, Truong L, Owens SR, Markham PM, Shea JP and Crooke ST, Pharmacokinetics of a ^{14}C-labeled phosphorothioate oligonucleotide, ISIS 2105, after intradermal administration to rats. *J Pharmacol Exp Ther* **269:** 89–94, 1994.
33. Sands H, Gorey-Feret LJ, Cocuzza AJ, Hobbs FW, Chidester D and Trainor GL, Biodistribution and metabolism of internally ^{3}H-labeled oligonucleotides. I. Comparison of a phosphodiester and a phosphorothioate. *Mol Pharmacol* **45:** 932–943, 1994.
34. Srinivasan SK and Iversen P, Review of *in vivo* pharmacokinetics and toxicology of phosphorothioate oligonucleotides. *J Clin Lab Anal* **9:** 129–137, 1995.
35. Plenat F, Klein-Monhoven N, Marie B, Vignaud J-M and Duprez A, Cell and tissue distribution of synthetic oligonucleotides in healthy and tumor-bearing nude mice. *Am J Pathol* **147:** 124–135, 1995.
36. Rifai A, Brysch W, Fadden K, Clark J and Schlingensiepen K-H, Clearance kinetics, biodistribution, and organ saturability of phosphorothioate oligodeoxynucleotides in mice. *Am J Pathol* **149:** 717–725, 1996.
37. Butler M, Stecker K and Bennett CF, Histological localization of phosphorothioate oligodeoxynucleotides in normal rodent tissue. *Nucleosides Nucleotides*, in press.
38. Oberbauer R, Schreiner GF and Meyer TW, Renal uptake of an 18-mer phosphorothioate oligonucleotide. *Kidney Int* **48:** 1226–1232, 1995.
39. Shoji Y, Akhtar S, Periasamy A, Herman B and Juliano RL, Mechanism of cellular uptake of modified oligodeoxynucleotides containing methylphosphonate linkages. *Nucleic Acids Res* **19:** 5543–5550, 1991.
40. Loke SL, Stein CA, Zhang XH, Mori K, Nakanishi M, Subasinghe C, Cohen JS and Neckers LM, Characterization of oligonucleotide transport into living cells. *Proc Natl Acad Sci USA* **86:** 3474–3478, 1989.
41. Stein CA, Tonkinson JL, Zhang L-M, Yakubov L, Gervasoni J, Taub R and Rotenberg SA, Dynamics of the internalization of phosphodiester oligodeoxynucleotides in HL60 cells. *Biochemistry* **32:** 4855–4861, 1993.
42. Bennett CF, Chiang M-Y, Chan H, Shoemaker JEE and Mirabelli CK, Cationic lipids enhance cellular uptake and activity of phosphorothioate antisense oligonucleotides. *Mol Pharmacol* **41:** 1023–1033, 1992.
43. Wu-Pong S, Weiss TL and Hunt CA, Calcium dependent cellular uptake of a c-*myc* antisense oligonucleotide. *Cell Mol Biol* **40:** 843–850, 1994.
44. Nestle FO, Mitra RS, Bennett CF, Chan H and Nickoloff BJ, Cationic lipid is not required for uptake and selective inhibitory activity of ICAM-1 phosphorothioate antisense oligonucleotides in keratinocytes. *J Invest Dermatol* **103:** 569–575, 1994.
45. Noonberg SB, Garovoy MR and Hunt CA, Characteristics of oligonucleotide uptake in human keratinocyte cultures. *J Invest Dermatol* **101:** 727–731, 1993.
46. Crooke RM, Graham MJ, Cooke ME and Crooke ST, *In vitro* pharmacokinetics of phosphorothioate antisense oligonucleotides. *J Pharmacol Exp Ther* **275:** 462–473, 1995.
47. Dean NM and McKay R, Inhibition of protein kinase C-α expression in mice after systemic administration of phosphorothioate antisense oligodeoxynucleotides. *Proc Natl Acad Sci USA* **91:** 11762–11766, 1994.
48. Yazaki T, Ahmad S, Chahlavi A, Zylber-Katz E, Dean NM, Rabkin SD, Martuza RL and Glazer RI, Treatment of glioblastoma U-87 by systemic administration of an antisense protein kinase C-α phosphorothioate oligodeoxynucleotide. *Mol Pharmacol* **50:** 236–242, 1996.
49. Dean N, McKay R, Miraglia L, Howard R, Cooper S, Giddings J, Nicklin P, Meister L, Ziel R, Geiger T, Muller M and Fabbro D, Inhibition of growth of human tumor cell lines in nude mice by an antisense oligonucleotide inhibitor of protein kinase C-α expression. *Cancer Res* **56:** 3499–3507, 1996.
50. Monia BP, Johnston JF, Geiger T, Muller M and Fabbro D, Antitumor activity of a phosphorothioate oligodeoxynucleotide targeted against C-*raf* kinase. *Nature Med* **2:** 668–675, 1995.
51. Kumasaka T, Quinlan WM, Doyle NA, Condon TP, Sligh J, Takei F, Beaudet AL, Bennett CF and Doerschuk CM, The role of the intercellular adhesion molecule-1 (ICAM-1) in endotoxin-induced pneumonia evaluated using ICAM-1 antisense oligonucleotides, anti-ICAM-1 monoclonal antibodies, and ICAM-1 mutant mice. *J Clin Invest* **97:** 2362–2369, 1996.
52. Nesterova M and Cho-Chung YS, A single-injection protein kinase A-directed antisense treatment to inhibit tumor growth. *Nature Med* **1:** 528–533, 1995.
53. Neurath MF, Pettersson S, Meyerzum Büschenfelde K-H and Strober W, Local administration of antisense phosphorothioate oligonucleotides to the p65 subunit of NF-κB abrogates established experimental colitis in mice. *Nature Med* **2:** 998–1004, 1996.
54. Noiri E, Peresleni T, Miller F and Goligorsky MS, *In vivo*

targeting of inducible NO synthase with oligodeoxynucleotides protects rat kidney against ischemia. *J Clin Invest* **97:** 2377–2383, 1996.
55. Christofidou-Solomidou M, Albelda SM, Bennett FC and Murphy GF, Experimental production and modulation of human cytotoxic dermatitis in human-murine chimeras. *Am J Pathol* **150:** 631–639, 1997.
56. Bennett CF, Kornbrust D, Henry S, Stecker K, Howard R, Cooper S, Dutson S, Hall W and Jacoby HI, An ICAM-1 antisense oligonucleotide prevents and reverses dextran sulfate sodium-induced colitis in mice. *J Pharmacol Exp Ther* **280:** 988–1000, 1997.
57. Helene C and Toulme J-J, Specific regulation of gene expression by antisense, sense and antigene nucleic acids. *Biochim Biophys Acta* **1049:** 99–125, 1990.
58. Bennett CF and Crooke ST, Regulation of endothelial cell adhesion molecule expression with antisense oligonucleotides. *Adv Pharmacol* **28:** 1–43, 1994.
59. Crooke ST, *Therapeutic Applications of Oligonucleotides*. R.G. Landes Co., Austin, 1995.
60. Stewart AJ, Canitrot Y, Baracchini E, Dean NM, Deeley RG and Cole SPC, Reduction of expression of the multidrug resistance protein, MRP, in human tumor cells by antisense phosphorothioate oligonucleotides. *Biochem Pharmacol* **51:** 461–469, 1996.
61. Condon TP and Bennett CF, Altered mRNA splicing and inhibition of human E-selectin expression by an antisense oligonucleotide in human umbilical vein endothelial cells. *J Biol Chem* **271:** 30398–30403, 1996.
62. Giles RV, Spiller DG and Tidd DM, Detection of ribonuclease H-generated mRNA fragments in human leukemia cells following reversible membrane permeabilization in the presence of antisense oligodeoxynucleotides. *Antisense Res Dev* **5:** 23–31, 1995.
63. Chiang M-Y, Chan H, Zounes MA, Freier SM, Lima WF and Bennett CF, Antisense oligonucleotides inhibit intercellular adhesion molecule 1 expression by two distinct mechanisms. *J Biol Chem* **266:** 18162–18171, 1991.
64. Monia BP, Lesnik EA, Gonzalez C, Lima WF, McGee D, Guinosso CJ, Kawasaki AM, Cook PD and Freier SM, Evaluation of 2′-modified oligonucleotides containing 2′-deoxy gaps as antisense inhibitors of gene expression. *J Biol Chem* **268:** 14514–14522, 1993.
65. Torrence PF, Maitra RK, Lesiak K, Khamnei S, Zhou A and Silverman RH, Targeting RNA for degradation with a (2′-5′) oligoadenylate-antisense chimera. *Proc Natl Acad Sci USA* **90:** 1300–1304, 1993.
66. Lengyel P, Speyer JF and Ochoa S, Synthetic polynucleotides and the amino acid code. *Proc Natl Acad Sci USA* **47:** 1936–1942, 1961.
67. Baker BF, Lot SF, Condon TP, Cheng-Flournoy S, Lesnik E, Sasmor HM and Bennett CF, 2′-O-(2-Methoxy)ethyl modified anti-ICAM-1 oligonucleotides selectively increase the ICAM-1 mRNA level and inhibit formation of the ICAM-1 translation initiation complex in HUVECs. *J Biol Chem,* **272:** 11994–12000, 1997.
68. Sierakowska H, Sambade MJ, Agrawal S and Kole R, Repair of thalassemic human β-globin mRNA in mammalian cells by antisense oligonucleotides. *Proc Natl Acad Sci USA* **93:** 12840–12844, 1996.
69. Hodges D and Crooke ST, Inhibition of splicing of wild-type and mutated luciferase-adenovirus pre-mRNA by antisense oligonucleotides. *Mol Pharmacol* **48:** 905–918, 1995.
70. Bennett CF, Condon T, Grimm S, Chan H and Chiang M-Y, Inhibition of endothelial cell-leukocyte adhesion molecule expression with antisense oligonucleotides. *J Immunol* **152:** 3530–3540, 1994.
71. Monia BP, Johnston JF, Ecker DJ, Zounes MA, Lima WF and Freier SM, Selective inhibition of mutant Ha-*ras* mRNA expression by antisense oligonucleotides. *J Biol Chem* **267:** 19954–19962, 1992.
72. Monia BP, Johnston JF, Geiger T, Muller M and Fabbro D, Antitumor activity of a phosphorothioate antisense oligodeoxynucleotide targeted against C-*raf* kinase. *Nature Med* **2:** 668–675, 1996.
73. Monia BP, Sasmor H, Johnston JF, Freier SM, Lesnik EA, Muller M, Geiger T, Altmann K-H, Moser H and Fabbro D, Sequence-specific antitumor activity of a phosphorothioate oligodeoxyribonucleotide targeted to human C-*raf* kinase supports an antisense mechanism of action *in vivo*. *Proc Natl Acad Sci USA* **93:** 15481–15484, 1996.
74. Barbour SE and Dennis EA, Antisense inhibition of group II phospholipase A_2 expression blocks the production of prostaglandin E_2 by P388D_1 cells. *J Biol Chem* **268:** 21875–21882, 1993.
75. Colige A, Sokolov BP, Nugent P, Baserga R and Prockop DJ, Use of an antisense oligonucleotide to inhibit expression of a mutated human procollagen gene (COL1A1) in transfected mouse 3T3 cells. *Biochemistry* **32:** 7–11, 1993.
76. Wagner RW, Matteucci MD, Lewis JG, Gutierrez AJ, Moulds C and Froehler BC, Antisense gene inhibition by oligonucleotides containing C-5 propyne pyrimidines. *Science* **260:** 1510–1513, 1993.
77. Fenster SD, Wagner RW, Froehler BC and Chin DJ, Inhibition of human immunodeficiency virus type-1 *env* expression by C-5 propyne oligonucleotides specific for rev-response element stem-loop V. *Biochemistry* **33:** 8391–8398, 1994.
78. Duff JL, Monia BP and Berk BC, Mitogen-activated protein (MAP) kinase is regulated by the MAP kinase phosphatase (MKP-1) in vascular smooth muscle cells. Effect of actinomycin D and antisense oligonucleotides. *J Biol Chem* **270:** 7161–7166, 1995.
79. Wagner RW, Matteucci MD, Grant D, Huang T and Froehler BC, Potent and selective inhibition of gene expression by an antisense heptanucleotide. *Nature Biotech* **14:** 840–844, 1996.
80. Matteucci M, Structural modifications toward improved antisense oligonucleotides. *Perspect Drug Dis Des* **4:** 1–16, 1996.
81. Kawasaki AM, Casper MD, Freier SM, Lesnik EA, Zounes MC, Cummins LL, Gonzalez C and Cook PD, Uniformly modified 2′-deoxy-2′-fluoro phosphorothioate oligonucleotides as nuclease-resistant antisense compounds with high affinity and specificity for RNA targets. *J Med Chem* **36:** 831–841, 1993.
82. Blencowe BJ, Sproat BS, Ryder U, Barabino S and Lamond AI, Antisense probing of the human U4/U6 snRNP with biotinylated 2′-OMe RNA oligonucleotides. *Cell* **59:** 531–539, 1989.
83. Lesnik EA, Guinosso CJ, Kawasaki AM, Sasmor H, Zounes M, Cummins LL, Ecker DJ, Cook PD and Freier SM, Oligodeoxynucleotides containing 2′-O-modified adenosine: Synthesis and effects on stability of DNA:RNA duplexes. *Biochemistry* **32:** 7832–7838, 1993.
84. Sanghvi YS, Heterocyclic base modifications in nucleic acids and their applications in antisense oligonucleotides. In: *Antisense Research and Applications* (Eds. Crooke ST and Lebleu B), pp. 273–288. CRC Press, Boca Raton, 1993.
85. Ramasamy KS, Zounes M, Gonzalez C, Freier SM, Lesnik EA, Cummins LL, Griffey RH, Monia BP and Cook PD, Remarkable enhancement of binding affinity of heterocycle-modified DNA to DNA and RNA. Synthesis, characterization and biophysical evaluation of *N2*-imidazolylpropylguanine and *N2*-imidazolylpropyl-2-aminoadenine modified oligonucleotides. *Tetrahedron Lett* **35:** 215–218, 1994.

86. Kutyavin IV, Rhinehart RL, Lukhtanov EA, Gorn VV, Myer RB and Gamper HB, Oligonucleotides containing 2-aminoadenine and 2-thiothymine act as selectively binding complementary agents. *Biochemistry* **35:** 11170–11176, 1996.
87. Jones RJ, Lin K-Y, Milligan JF, Wadwani S and Matteucci MD, Synthesis and binding properties of pyrimidine oligodeoxynucleoside analogs containing neutral phosphodiester replacements. The formacetal and 3′-thioformacetal internucleoside linkages. *J Org Chem* **58:** 2983–2991, 1993.
88. Vasseur J-J, Debart F, Sanghvi YS and Cook PD, Oligonucleosides: Synthesis of a novel methylhydroxylamine-linked nucleoside dimer and its incorporation into antisense sequences. *J Am Chem Soc* **114:** 4006–4007, 1992.
89. De Mesmaeker A, Waldner A, Lebreton J, Hoffmann P, Fritsch V, Wolf RM and Freier SM, Amides as a new type of backbone modification in oligonucleotides. *Angew Chem Int Ed* **33:** 226–229, 1994.
90. Meier C and Engels JW, Peptide nucleic acids (PNAs)–Unusual properties of nonionic oligonucleotide analogues. *Angew Chem Int Edn* **31:** 1008–1010, 1992.
91. Egholm M, Buchardt O, Christensen L, Behrens C, Freier SM, Driver DA, Berg RH, Kim SK, Norden B and Nielsen PE, PNA hybridizes to complementary oligonucleotides obeying the Watson-Crick hydrogen-bonding rules. *Nature* **365:** 566–568, 1993.
92. Helene C and Toulme J-J, Control of gene expression by oligonucleotides covalently linked to intercalating agents and nucleic acid-cleaving reagents. In: *Oligonucleotides: Antisense Inhibitors of Gene Expression* (Ed. Cohen JS), pp. 137–172. CRC Press, Boca Raton, 1989.
93. Goodchild J, Carroll E III and Greenberg JR, Inhibition of rabbit β-globin synthesis by complementary oligonucleotides: Identification of mRNA sites sensitive to inhibition. *Arch Biochem Biophys* **263:** 401–409, 1988.
94. Bacon TA and Wickstrom E, Walking along human c-*myc* mRNA with antisense oligodeoxynucleotides: Maximum efficacy at the 5′ cap region. *Oncogene Res* **6:** 13–19, 1991.
95. Lima WF, Brown-Driver V, Fox M, Hanecak R and Bruice TW, Combinatorial screening and rational optimization for hybridization to folded hepatitis C virus RNA of oligonucleotides with biological antisense activity. *J Biol Chem* **272:** 626–638, 1997.
96. Lima WF, Monia BP, Ecker DJ and Freier SM, Implication of RNA structure on antisense oligonucleotide hybridization kinetics. *Biochemistry* **31:** 12055–12061, 1992.
97. Gao W-Y, Han F-S, Storm C, Egan W and Cheng Y-C, Phosphorothioate oligonucleotides are inhibitors of human DNA polymerases and RNase H: Implications for antisense technology. *Mol Pharmacol* **41:** 223–229, 1992.
98. Perez JR, Li Y, Stein CA, Majumder S, Van Oorschot A and Narayanan R, Sequence-independent induction of Sp1 transcription factor activity by phosphorothioate oligodeoxynucleotides. *Proc Natl Acad Sci USA* **91:** 5957–5961, 1994.
99. Guvakova MA, Yakubov LA, Vlodavsky I, Tonkinson JL and Stein CA, Phosphorothioate oligodeoxynucleotides bind to basic fibroblast growth factor, inhibit its binding to cell surface receptors, and remove it from low affinity binding sites on extracellular matrix. *J Biol Chem* **270:** 2620–2627, 1995.
100. Bennett CF, Chiang M-Y, Wilson-Lingardo L and Wyatt JR, Sequence specific inhibition of human type II phospholipase A_2 enzyme activity by phosphorothioate oligonucleotides. *Nucleic Acids Res* **22:** 3202–3209, 1994.
101. Burgess TL, Fisher EF, Ross SL, Bready JV, Qian Y, Bayewitch LA, Cohen AM, Herrera CJ, Hu SS-F, Kramer TB, Lott FD, Martin FH, Pierce GF, Simonet L and Farrell CL, The antiproliferative activity of c-myb and c-myc antisense oligonucleotides in smooth muscle cells is caused by a nonantisense mechanism. *Proc Natl Acad Sci USA* **92:** 4051–4055, 1995.
102. Krieg AM, Yi A-K, Matson S, Waldschmidt TJ, Bishop GA, Teasdale R, Koretzky GA and Klinman DM, CpG motifs in bacterial DNA trigger direct B-cell activation. *Nature* **374:** 546–549, 1995.
103. Bock LC, Griffin LC, Latham JA, Vermaas EH and Toole JJ, Selection of single-stranded DNA molecules that bind and inhibit human thrombin. *Nature* **355:** 564–566, 1992.
104. Ellington AD and Szostak JW, Selection *in vitro* of single-stranded DNA molecules that fold into specific ligand-binding structures. *Nature* **355:** 850–852, 1992.
105. Ellington AD, Aptamers achieve the desired recognition. *Curr Biol* **4:** 427–429, 1994.
106. Benimetskaya L, Tonkinson JL, Koziolkiewicz M, Karwowski B, Guga P, Zeltser R, Stec W and Stein CA, Binding of phosphorothioate oligodeoxynucleotides to basic fibroblast growth factor, recombinant soluble CD4, laminin and fibronectin is P-chirality independent. *Nucleic Acids Res* **23:** 4239–4245, 1995.
107. Stein CA, Phosphorothioate antisense oligodeoxynucleotides: Questions of specificity. *Trends Biotechnol* **14:** 147–149, 1996.
108. Maltese J-Y, Sharma HW, Vassilev L and Narayanan R, Sequence context of antisense RelA/NF-κB phosphorothioates determines specificity. *Nucleic Acids Res* **23:** 1146–1151, 1995.
109. Williamson JR, Guanine quartets. *Curr Opin Struct Biol* **3:** 357–362, 1993.
110. Ballas ZK, Rasmussen WL and Krieg AM, Induction of NK activity in murine and human cells by CpG motifs in oligodeoxynucleotides and bacterial DNA. *J Immunol* **157:** 1840–1845, 1996.
111. Monteith DK, Henry SP, Howard RB, Flournoy S, Levin AA, Bennett CF and Crooke ST, Immune stimulation–A class effect of phosphorothioate oligonucleotides in rodents. *Anticancer Drug Des*, **12:** 421–432, 1997.
112. Levin AA, Monteith DK, Leeds JM, Nicklin PL, Geary RS, Butler M, Templin MV and Henry SP, Toxicity of oligodeoxynucleotide therapeutic agents. In: *Antisense Research and Applications* (Ed. Crooke ST). Springer, Heidelberg, in press.
113. Geiser T, Large-scale economic synthesis of antisense phosphorothioate analogues of DNA for preclinical investigations. *Ann NY Acad Sci* **616:** 173–183, 1990.
114. Agrawal S, Zhang X, Lu Z, Zhao H, Tamburin JM, Yan J, Cai H, Diasio RB, Habus I, Jiang Z, Iyer RP, Yu D and Zhang R, Absorption, tissue distribution and *in vivo* stability in rats of a hybrid antisense oligonucleotide following oral administration. *Biochem Pharmacol* **50:** 571–576, 1995.
115. Letsinger RL, Zhang GR, Sun DK, Ikeuchi T and Sarin PS, Cholesteryl-conjugated oligonucleotides: Synthesis, properties, and activity as inhibitors of replication of human immunodeficiency virus in cell culture. *Proc Natl Acad Sci USA* **86:** 6553–6556, 1989.
116. Alahari SK, Dean NM, Fisher MH, Delong R, Manoharan M, Tivel KL and Juliano RL, Inhibition of expression of the multidrug resistance-associated P-glycoprotein by phosphorothioate and 5′-cholesterol-conjugated phosphorothioate antisense oligonucleotides. *Mol Pharmacol* **50:** 808–819, 1996.
117. Bennett CF, Zuckerman JE, Kornbrust D, Sasmor H, Leeds JM and Crooke ST, Pharmacokinetics in mice of a [^{3}H]-labeled phosphorothioate oligonucleotide formulated in the presence and absence of a cationic lipid. *J Control Rel* **41:** 121–130, 1996.

118. Southern EM, DNA fingerprinting by hybridization to oligonucleotide arrays. *Electrophoresis* **16:** 1539–1542, 1995.
119. Geary R, Leeds J, Henry SP, Monteith DK and Levin AA, Antisense oligonucleotide inhibitors for the treatment of cancer: 1) Pharmacokinetic properties of phosphorothioate oligodeoxynucleotides. *Anticancer Drug Des*, **12:** 383–393, 1997.
120. Zhao Q, Temsamani J, Iadarola PL, Jiang Z and Agrawal S, Effect of different chemically modified oligodeoxynucleotides on immune stimulation. *Biochem Pharmacol* **51:** 173–182, 1996.
121. Boggs RT, McGraw K, Condon T, Flournoy S, Villiet P, Bennett CF and Monia BP, Characterization and modulation of immune stimulation by modified oligonucleotides. *Antisense Nucleic Acid Drug Dev*, in press.
122. Kumar S, Tewary HK and Iversen PL, Characterization of binding sites, extent of binding, and drug interactions of oligonucleotides with albumin. *Antisense Res Dev* **5:** 131–139, 1995.
123. Gmeiner WH, Sahasrabudhe P, Pon RT, Sonntag J, Srinivasan S and Iversen PL, Preparation of oligomeric 2′-deoxy-5-fluorouridylate of defined length and backbone composition: A novel pro-drug form of the potent anti-cancer drug 2′-deoxy-5-fluorouridylate. *Nucleosides Nucleotides* **14:** 243–253, 1995.