5
Antisense Therapeutics

STANLEY T. CROOKE

Isis Pharmaceuticals, Inc., 2292 Faraday Avenue, Carlsbad, CA 92008, USA

Introduction

The therapeutic use of oligonucleotides represents a new paradigm for drug discovery. The technology focuses on a class of chemicals, oligonucleotides, that have not been studied as potential drugs before, and uses them to intervene in processes that, likewise, have not previously been studied as sites at which drugs might act. Antisense oligonucleotides are designed to modulate information transfer from the gene to protein, in essence, to alter the intermediary metabolism of RNA. Conceptually, interruption of the process at any point from transcription, i.e., transcriptional arrest, to utilization, translational arrest is feasible. However, for a wide variety of reasons transcriptional arrest via DNA strand invasion or triple stranding strategies is chemically, pharmacologically and toxicologically substantially more difficult than intervening at post-transcriptional steps in the intermediary metabolism of RNA. Consequently, virtually all of the pharmacologically significant progress has been achieved with drugs that bind to pre- or mRNAs, that is to say antisense drugs.

The receptor for antisense drugs is a site in a specific mRNA species. Affinity and specificity of binding derive from hybridization interactions and are theoretically much larger than can be achieved with small molecules. In addition to substantial theoretical advantages in specificity, the rational design of antisense drugs is easier than the design of small molecules interacting with proteins. Finally, it is possible to envisage the design of antisense drugs to treat a very broad range of disorders.

Although the field is still in its infancy, it has generated considerable enthusiasm because of the potential specificity of oligonucleotide drugs and the breadth of potential applications. However, enthusiasm about the technology has been tempered by appropriate reservations concerning the ability of the technology to deliver on its promise. In essence, the questions about the technology reduce to: can oligonucleotide analogs be created that have appropriate properties to be drugs; specifically, what are the pharmocokinetic, pharmacologic, and toxicologic properties of these compounds, and what are the scope and potential of the medicinal chemistry of oligonucleotides? Although much remains to be done, the answers to many of the questions are now available.

Biotechnology and Genetic Engineering Reviews – Vol. 15, April 1998
0264-8725/98/15/121-157 $20.00 + $0.00 © Intercept Ltd, P.O. Box 716, Andover, Hampshire SP10 1YG, UK

Molecular mechanisms of antisense drugs

OCCUPANCY ONLY MEDIATED MECHANISMS

Classic competitive antagonists are thought to alter biological activities because they bind to receptors preventing natural agonists from binding and 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.

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 McManaway *et al.*, 1990; Kulka *et al.*, 1989; Zamecnik *et al.*, 1986; Smith *et al.*, 1986. Activities have been reported for anti-c-*myc* and antiviral oligonucleotides with phosphodiester, methylphosphonate and phosphorothioate backbones. Very recently, an oligonucleotide was reported to induce alternative splicing in a cell-free splicing system and, in that system, RNA analyses confirmed the putative mechanism (Dominski and Kole, 1993).

In one study, the factors that determine whether splicing inhibition is effected by an antisense drug were characterized (Hodges and Crooke, 1995). 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 with 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 non-specific effects. Secondly, less effectively spliced introns are better targets than those with strong consensus splicing signals. Thirdly, the 3'-splice site and branchpoint are usually the best sites to which to target to the oligonucleotide to inhibit splicing. Fourthly, RNase H-competent oligonucleotides are usually more potent than even higher affinity oligonucleotides that inhibit by occupancy only.

TRANSLATIONAL ARREST

A mechanism for which many oligonucleotides have been designed is to arrest translation of targeted protein by binding to the translation initiation codon. The positioning of the initiation codon within the area of complementarily of the oligonu-

cleotide and the length of oligonucleotide used have varied considerably. Again, unfortunately, only in relatively few studies have the oligonucleotides been shown to bind to the sites for which they were designed and data that directly support translation arrest as the mechanism has been lacking.

Target RNA species that have been reported to be inhibited by a translational arrest mechanism include HIV, vesicular stomatitis virus (VSV), n-*myc* and a number of normal cellular genes, Agrawal *et al.*, 1988; LeMaitre *et al.*, 1987; Rosolen *et al.*, 1990; Vasanthakumar and Ahmed, 1989; Sburlati *et al.*, 1991; Zheng *et al.*, 1989; Maier *et al.*, 1990. A number of studies have shown that significant number of targets may be inhibited by binding to translation initiation codons. For example, ISIS 1082 hybridizes to the AUG codon for the UL13 gene of herpes virus types 1 and 2. RNase H studies confirmed that it binds selectively in this area. *In vitro* protein synthesis studies confirmed that it inhibited the synthesis of the UL13 protein and studies in HeLa cells showed that it inhibited the growth of herpes type 1 and type 2 with IC_{50} of 200–400 ηM by translation arrest (Mirabelli *et al.*, 1991). Similarly, ISIS 1753, a 30-mer phosphorothioate complementary to the translation initiation codon and surrounding sequences of the E2 gene of bovine papilloma virus, was highly effective and its activity was shown to be due to translation arrest. ISIS 2105, a 20 mer phosphorothioate complementary to the same region in human papilloma virus, was shown to be a very potent inhibitor. Compounds complementary to the translation initiation codon of the E2 gene were the most potent of the more than 50 compounds studied complementary to various other regions in the RNA (Cowsert *et al.*, 1993).

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. However, very little is understood about the precise events that lead to translation arrest.

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.

In one study, oligonucleotides designed to disrupt TAR, showed that several did bind to TAR, disrupt the structure, and inhibit TAR-mediated production of a reporter gene (Vickers *et al.*, 1991). Furthermore, general rules useful in disrupting stem-loop structures were developed as well (Ecker *et al.*, 1992).

Although designed to induce relatively non-specific 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 (Saxena and Ackerman, 1990).

Similarly, oligonucleotides designed to bind to highly conserved sequences in 5.8 S RNA inhibited protein synthesis in rabbit reticulocyte and wheat germ systems (Walker *et al.*, 1990).

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.

5'-CAPPING

A key early step in RNA processing is 5'-capping. This stabilizes pre-mRNA and is important for the stability of mature mRNA. It is also 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 (Westermann *et al.*, 1989). However, again, in no published study has this putative mechanism been rigorously demonstrated. In no published study have the oligonucleotides been shown to bind to the sequences for which they were designed.

Oligonucleotides designed to bind to 5'-cap structures and reagents designed to specifically cleave the unique 5'-cap structure have been reported (Baker, 1993). These studies demonstrate that 5'-cap targeted oligonucleotides were capable of inhibiting the binding of the translation initiation factor eIF-4a (Baker *et al.*, 1992).

INHIBITION OF 3'-POLYADENYLATION

In the 3'-untranslated region of pre-mRNA molecules are sequences that result in the post-transcriptional 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 (Chiang *et al.*, 1991).

OTHER MECHANISMS

In addition to 5'-capping and 3'-adenylation, there are 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 (Zamecnik and Stephenson, 1978). 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 have shown that sequences in the 3'-untranslated region of RNA molecules are often the most sensitive (Zerial *et al.*, 1987; Thuong *et al.*, 1989; Helene and Toulme, 1989). For example, ISIS 1939 is 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, inasmuch a 2'-methoxy analogue 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 (Chiang *et al.*, 1991).

ACTIVATION OF RNASE H

RNase H is an 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 (Crouch and Dirksen, 1985). At least two classes of RNase H have been identified in eukaryotic cells. Multiple enzymes with RNase H activity have been observed in prokaryotes (Crouch and Dirksen, 1985).

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 (Donis-Keller, 1979). 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 (Kawasaki *et al.*, 1993; Sproat *et al.*, 1989). 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 (Morvan *et al.*, 1991; Gagnor *et al.*, 1989). Additionally, backbone modifications influence the ability of oligonucleotides to activate RNase H. Methylphosphonates do not activate RNase H (Maher *et al.*, 1989; Miller, 1989). In contrast, phosphorothioates are excellent substrates (Mirabelli *et al.*, 1991; Stein and Cheng, 1993; Cazenave *et al.*, 1989). In addition, chimeric molecules have been studied as oligonucleotides that bind to RNA and activate RNase H (Quartin *et al.*, 1989; Furdon *et al.*, 1989). 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 (Quartin *et al.*, 1989; Furdon *et al.*, 1989). 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 (Eder and Walder, 1991).

It has also been demonstrated 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 (Monia *et al.*, 1993; Giles and Tidd, 1992). In a recent study, RNase H mediated cleavage of target transcript was more selective when deoxyoligonucleotides comprised of methylphosphonate deoxyoligonucleotide wings and phosphodiester gaps were compared to full phosphodiester oligonucleotides (Giles and Tidd, 1992).

Characteristics of phosphorothioate oligodeoxynucleotides

INTRODUCTION

Of the first generation oligonucleotide analogues, the class that has resulted in the broadest range of activities and about which the most is known, is the phosphorothioate class. Phosphorothioate oligonucleotides were first synthesized in 1969 when a poly rI-rC phosphorothioate was synthesized (De Clercq *et al.*, 1969). This modification clearly achieves the objective of increased nuclease stability. In this class of oligonucleotides, one of the oxygen atoms in the phosphate group is replaced with a sulfur. The resulting compound is negatively charged, is chiral phosphorothioate, and much more resistant to nucleases than the parent phosphodiester (Cohen, 1993).

HYBRIDIZATION

The hybridization of phosphorothioate oligonucleotides to DNA and RNA has been thoroughly characterized (Crooke and Lebleu, 1993; Crooke, 1992; Crooke, 1993). The T_m of a phosphorothioate oligodeoxynucleotide for RNA is approximately 0.5°C less per nucleotide than for a corresponding phosphodiester oligodeoxynucleotide. This reduction in T_m per nucleotide is virtually independent of the number of phosphorothioate units substituted for phosphodiesters. However, sequence context has some influence as the ΔT_m can vary from –0.3–1.0°C depending on sequence. Compared to RNA and RNA duplex formation, a phosphorothioate oligodeoxynucleotide has a T_m approximately –2.2°C lower per unit (Freier, 1993). This means that to be effective *in vitro*, phosphorothioate oligodeoxynucleotides must typically be 17–20 mer in length and that invasion of double-stranded regions in RNA is difficult (Lima *et al.*, 1992; Vickers *et al.*, 1991; Monia *et al.*, 1993; Monia *et al.*, 1992).

Association rates of phosphorothioate oligodeoxynucleotide to unstructured RNA targets are typically 10^6–$10^7 M^{-1}s^{-1}$ independent of oligonucleotide length or sequence (Freier, 1993; Lima *et al.*, 1992). Association rates to structured RNA targets can vary from 10^2–$10^8 M^{-1}s^{-1}$ depending on the structure of the RNA, site of binding in the structure and other factors (Freier, 1993). Said another way, association rates for oligonucleotides that display acceptable affinity constants are sufficient to support biological activity at therapeutically achievable concentrations.

The specificity of hybridization of phosphorothioate oligonucleotides is, in general, slightly greater than phosphodiester analogs. For example, a T-C mismatch results in a 7.7 or 12.8°C reduction in T_m respectively, for a phosphodiester or phosphorothioate oligodeoxynucleotide 18 nucleotides in length with the mismatch centered (Freier, 1993). Thus, from this perspective, the phosphorothioate modification is quite attractive.

INTERACTIONS WITH PROTEINS

Phosphorothioate oligonucleotides bind to proteins. The interactions with proteins can be divided into non-specific, sequence specific and structure-specific binding events, each of which may have different characteristics and effects. Non-specific 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. The affinity of such interactions is low. The Kd for albumin is approximately 200μM, thus, in a similar range with aspirin or penicillin (Crooke *et al.*, 1996; Joos and Hall, 1969). Furthermore, in this study, no competition between phosphorothioate oligonucleotides and several drugs that bind to bovine serum albumin was observed. In this study, binding and competition were determined in an assay in which electrospray mass spectrometry was used. In contrast, in a study in which an equilibrium dissociation constant was derived from an assay using albumin loaded on a CH-sephadex column, the Km ranged from $1 \times 10^{-5} \times 10^{-5}$ M for bovine serum albumin and $2 \times 10^{-3} \times 10^{-4}$ M for human serum albumin. Moreover, warfarin and indomethacin were reported to compete for binding to serum albumin (Srinivasan *et al.*, 1995). Much more work is required before definitive conclusions can be drawn.

Phosphorothioate oligonucleotides can interact with nucleic acid binding proteins such as transcription factors and single-strand nucleic acid binding proteins. However, very little is known about these binding events. Additionally, it has been reported that phosphorothioates bind to an 80 Kd membrane protein that was suggested to be involved in cellular uptake processes (Loke *et al.*, 1989). However, again, little is known about the affinities, sequence or structure specificities of these putative interactions. More recently interactions with 30 Kd and 46 Kd are surface proteins in T15 mouse fibroblasts were reported (Hawley and Gibson, 1996).

Phosphorothioates interact with nucleases and DNA polymerases. These compounds are slowly metabolized by both endo and exonucleases and inhibit these enzymes (Crooke *et al.*, 1995; Crooke, 1992). The inhibition of these enzymes appears to be competitive and this may account for some early data suggesting that phosphorothioates are almost infinitely stable to nucleases. In these studies, the oligonucleotide to enzyme ratio was very high and thus the enzyme was inhibited. Phosphorothioates also bind to RNase H when in an RNA–DNA duplex and the duplex serves as a substrate for RNase H (Gao *et al.*, 1992). At higher concentrations, presumably by binding as a single strand to RNase H, phosphorothioates inhibit the enzyme (Crooke *et al.*, 1995; Crooke, 1992). Again, the oligonucleotides appear to be competitive antagonists for the DNA–RNA substrate.

Phosphorothioates have been shown to be competitive inhibitors of DNA polymerase α and β with respect to the DNA template, and non-competitive inhibitors of DNA polymerases γ and δ (Gao *et al.*, 1992). Despite this inhibition, several studies have suggested that phosphorothioates might serve as primers for polymerases and be extended (Stein and Cheng, 1993; Crooke *et al.*, 1995; Agrawal *et al.*, 1991). In our laboratories, it has been shown extensions 2–3 nucleotides only. At present, a full explanation as to why no longer extensions are observed is not available.

Phosphorothioate oligonucleotides have been reported to be competitive inhibitors for HIV-reverse transcriptase and inhibit RT-associated RNase H activity (Majumdar *et al.*, 1989; Cheng *et al.*, 1991). They have been reported to bind to the cell surface protein, CD4, and to protein kinase C (Stein *et al.*, 1991). Various viral polymerases have also been shown to be inhibited by phosphorothioates (Stein and Cheng, 1993). Additionally, we have shown potent, non-sequence specific inhibition of RNA splicing by phosphorothioates (Hodges and Crooke, 1995).

Like other oligonucleotides, phosphorothioates can adopt a variety of secondary structures. As a general rule, self-complementary oligonucleotides are avoided, if

possible, to avoid duplex formation between oligonucleotides. However, other structures that are less well understood can also form. For example, oligonucleotides containing runs of guanosines can form tetrameric structures called G-quartets, and these appear to interact with a number of proteins with relatively greater affinity than unstructured oligonucleotides (Wyatt *et al.*, 1994).

In conclusion, phosphorothioate oligonucleotides may interact with a wide range of proteins via several types of mechanisms. These interactions may influence the pharmacokinetic, pharmacologic and toxicologic properties of these molecules. They may also complicate studies on the mechanism of action of these drugs, and may obscure an antisense activity. For example, phosphorothioate oligonucleotides were reported to enhance lipopolysaccharide stimulated synthesis or tumour necrosis factor (Hartmann *et al.*, 1996). This would obscure antisense effects on this target.

PHARMACOKINETIC PROPERTIES

To study the pharmacokinetics of phosphorothioate oligonucleotides, a variety of labelling techniques have been used. In some cases, 3'- or 5'^{32}P end-labelled or fluorescently-labelled oligonucleotides have been used in *in vitro* or *in vivo* studies. These are probably less satisfactory than internally labelled compounds because terminal phosphates are rapidly removed by phosphatases and fluorescently labelled oligonucleotides have physico-chemical properties that differ from the unmodified oligonucleotides. Consequently, either uniformly (Cowsert *et al.*, 1993) S-labelled or base-labelled phosphorothioates are preferable for pharmacokinetic studies. In our laboratories, a tritium exchange method that labels a slowly exchanging proton at the C8 position in purines was developed and proved to be quite useful (Graham *et al.*, 1993). Very recently, a method that added radioactive methyl groups via S-adenosyl methionine has also been successfully used (Sands *et al.*, 1994). Finally, advances in extraction, separation and detection methods have resulted in methods that provide excellent pharmacokinetic analyses without radiolabelling (Crooke *et al.*, 1996).

NUCLEASE STABILITY

The principal metabolic pathway for oligonucleotides is cleavage via endo and exonucleases. Phosphorothioate oligonucleotides, while quite stable to various nucleases are competitive inhibitors of nucleases (Crooke *et al.*, 1995; Gao *et al.*, 1992; Hoke *et al.*, 1991; Wickstrom, 1986; Campbell *et al.*, 1990). Consequently, the stability of phosphorothioate oligonucleotides to nucleases is probably a bit less than initially thought, as high concentrations (that inhibited nucleases) of oligonucleotides were employed in the early studies. Similarly, phosphorothioate oligonucleotides are degraded slowly by cells in tissue culture with a half-life of 12–24 hours and are slowly metabolized in animals (Crooke *et al.*, 1995; Cossum *et al.*, 1993; Hoke *et al.*, 1991). The pattern of metabolites suggests primarily exonuclease activity with perhaps modest contributions by endonucleases. However, a number of lines of evidence suggest that, in many cells and tissues, endonucleases play an important role in the metabolism of oligonucleotides. For example, 3'- and 5'-modified oligonucleotides with phosphodiester backbones have been shown to be relatively rapidly degraded in cells and after administration to animals (Sands *et al.*, 1995; Miyao *et al.*, 1995). Thus,

strategies in which oligonucleotides are modified at only the 3'- and 5'-terminus as a means of enhancing stability have not proven to be successful.

IN VITRO CELLULAR UPTAKE

Phosphorothioate oligonucleotides are taken up by a wide range of cells *in vitro* (Crooke, 1991; Crooke *et al.*, 1995; Gao *et al.*, 1992; Crooke, 1993; Neckers, 1993). Uptake of phosphorothioate oligonucleotides into a prokaryote, *vibrio parahaemolticus*, has been reported as has uptake into *Schistosoma Mansoni* (Chrisey *et al.*, 1993; Tao *et al.*, 1995). Uptake is time and temperature dependent. It is also influenced by cell type, cell-culture conditions, media and sequence, and length of the oligonucleotide (Crooke *et al.*, 1995). No obvious correlation between the lineage of cells, whether the cells are transformed or whether the cells are virally infected, and uptake has been identified (Crooke *et al.*, 1995). Nor are the factors that result in differences in uptake of different sequences of oligonucleotide understood. Although several studies have suggested that receptor-mediated endocytosis may be a significant mechanism of cellular uptake, the data are not yet compelling enough to conclude that receptor-mediated endocytosis accounts for a significant portion of the uptake in most cells (Loke *et al.*, 1989).

Numerous studies have shown that phosphorothioate oligonucleotides distribute broadly in most cells once taken up (Crooke *et al.*, 1995; Crooke, 1993). Again, however, significant differences in subcellular distribution between various types of cells have been noted.

Cationic lipids and other approaches have been used to enhance uptake of phosphorothioate oligonucleotides in cells that take up little oligonucleotide *in vitro* (Bennett *et al.*, 1993; Bennett *et al.*, 1992; Quattrone *et al.*, 1994). Again, however, there are substantial variations from cell type to cell type. Other approaches to enhanced intracellular uptake *in vitro* have included streptolysin D treatment of cells and the use of dextran sulphate and other liposome formulations as well as physical means such as microinjections (Crooke, 1995; Giles *et al.*, 1995; Wang *et al.*, 1995).

IN VIVO PHARMACOKINETICS

Phosphorothioate oligonucleotides bind to serum albumin and α-2 macroglobulin. The apparent affinity for albumin is quite low (200–400 μM) and comparable to the low affinity binding observed for a number of drugs, e.g. aspirin, penicillin (Crooke *et al.*, 1996; Joos and Hall, 1969; Srinivasan *et al.*, 1995). Serum protein binding therefore, provides a repository for these drugs and prevents rapid renal excretion. As serum protein binding is satiable, at higher doses, intact oligomer may be found in urine (Agrawal *et al.*, 1991; Iversen, 1991). $T_m T_m$ Studies in our laboratory suggest that in rats, oligonucleotides administered intravenously at doses of 15–20 mg/kg saturate the serum protein binding capacity (Leeds, unpublished data).

Phosphorothioate oligonucleotides are rapidly and extensively absorbed after parenteral administration. For example, in rats, after an intradermal dose 3.6 mg/kg of ^{14}C-ISIS 2105, a 20 mer phosphorothioate, approximately 70% of the dose was absorbed within 4 hours and total systemic bioavailability was in excess of 90% (Cossum *et al.*, 1994). After intradermal injection in man, absorption of ISIS 2105 was

similar to that observed in rats (Crooke *et al.*, 1994). Subcutaneous administration to rats and monkeys results in somewhat lower bioavailability and greater distribution to lymph as would be expected (Leeds, unpublished observations).

Distribution of phosphorothioate oligonucleotides from blood after absorption or intravenous administration is extremely rapid. Distribution half-lives of less than one hour, have been reported by several laboratories (Cossum *et al.*, 1993; Cossum *et al.*, 1994; Agrawal *et al.*, 1991; Iversen, 1991). Blood and plasma clearance is multi-exponential with a terminal elimination half life from 40–60 hours in all species except man. In man, the terminal elimination half-life may be somewhat longer (Crooke *et al.*, 1994).

Phosphorothioates distribute broadly to all peripheral tissues. Liver, kidney, bone marrow, skeletal muscle and skin accumulate the highest percentage of a dose, but other tissues accumulate small quantities of drug (Cossum *et al.*, 1993; Cossum *et al.*, 1994). No evidence of significant penetration of the blood brain barrier has been reported. The rates of incorporation and clearance from tissues vary as a function of the organ studied, with liver accumulating drug most rapidly (20% of a dose within 1–2 hours) and other tissues accumulating drug more slowly.

Similarly, elimination of drug is more rapid from liver than any other tissue, e.g. terminal half life from liver: 62 hours; from renal medulla: 156 hours. The distribution into the kidney has been studied more extensively and drug shown to be present in Bowman's capsule, the proximal convoluted tubule, the bush border membrane and within renal tubular epithelial cells (Rappaport *et al.*, 1995). The data suggested that the oligonucleotides are filtered by the glomerulus, then reabsorbed by the proximal convoluted tubule epithelial cells. Moreover, the authors suggested that reabsorption might be mediated by interactions with specific proteins in the bush border membranes.

At relatively low doses, clearance of phosphorothioate oligonucleotides is due primarily to metabolism (Cossum *et al.*, 1993; Cossum *et al.*, 1994; Iversen, 1991). Metabolism is mediated by exo- and endonucleases that result in shorter oligonucleotides and, ultimately, nucleosides that are degraded by normal metabolic pathways. Although no direct evidence of base excision or modification has been reported, these are theoretical possibilities that may occur. In one study, a larger molecular weight radioactive material was observed in urine, but not fully characterized (Agrawal *et al.*, 1991). The potential for conjugation reactions and extension of oligonucleotides via these drugs serving as primers for polymerases must be explored in more detail. In a very thorough study, 20 nucleotide phosphodiester and phosphorothioate oligonucleotides were administered intravenously at a dose of 6 mg/kg to mice. The oligonucleotides were internally labelled with ^{3}H-CH_3 by methylation of an internal deoxycytidine residue using Hha1 methylase and S-(^{3}H) adenosyl methionine (Sands *et al.*, 1994). The observations for the phosphorothioate oligonucleotide were entirely consistent with those made in our studies. Additionally, in this paper, autoradiographic analyses showed drug in renal cortical cells (Sands *et al.*, 1994).

One study of prolonged infusions of a phosphorothioate oligonucleotide to human beings has been reported (Bayever *et al.*, 1993). In this study, five patients with leukemia were given ten-day intravenous infusions at a dose of 0.05 mg/kg/hour. Elimination half lives reportedly varied from 5.9 to 14.7 days. Urinary recovery of

radioactivity was reported to be 30–60% of the total dose, with 30% of the radioactivity being intact drug. Metabolites in urine included both higher and lower molecular weight compounds. In contrast, when GEM-91 (a 25-mer phosphorothioate oligodeoxynucleotide) was administered to humans as a two hour i.v. infusion at a dose of 0.1 mg/kg, a peak plasma concentration of 295.8 mg/ml was observed at the cessation of the infusion. Plasma clearance of total radioactivity was biexponential with initial and terminal eliminations half-lives of 0.18 and 26.71 hours, respectively. However, degradation was extensive and intact drug pharmacokinetic models were not presented. Nearly 50% of the administered radioactivity was recovered in urine, but most of the radioactivity represented degradates. No intact drug was found in the urine at any time (Zhang *et al.*, 1995).

In a more recent study in which the level of intact drug was carefully evaluated using capillary gel electrophoresis, the pharmacokinetics of ISIS 2302, a 20-mer phosphorothioate oligodeoxynucleotide, after a 2 hour infusion, were determined. Doses from 0.06 mg/kg to 2.0 mg/kg were studied and the peak plasma concentrations were shown to increase linearly with dose with the 2 mg/kg dose resulting in peak plasma concentrations of intact drug of approximately 9.5 μg/ml. Clearance from plasma, however, was dose dependent with the 2 mg/kg dose having a clearance of 1.28 ml $min^{-1}kg^{-1}$, while that of 0.5 mg/kg was 2.07 ml $min^{-1}kg^{-1}$. Essentially, no intact drug was found in urine.

The two most recent studies differ from the initial report in several facets. Although a number of factors may explain the discrepancies, the most likely explanation is related to the evolution of assay methodology, not to difference between compounds. Overall, the behavior of phosphorothioates in the plasma of humans appears to be similar to that in other species.

In addition to the pharmacological effects that have been observed with phosphorothioate oligonucleotides, there are a number of lines of evidence supporting the notion that these drugs enter cells in various organs. As an example, *Figure 1* shows autoradiographic, fluorescent and immunohistochemical data demonstrating the intracellular location of phosphorothioate oligonucleotides in renal proximal convoluted tubular cells. Similar results have been observed in liver, skin and bone marrow in similar studies. Using radiolabelled drugs and isolated perfused rat liver cells, uptake into parenchymal and non-parenchymal cells of the liver has also been reported (Takakura *et al.*, 1996).

PHARMACOLOGICAL ACTIVITIES

Selection of sites at which optimal antisense activity may be induced in a RNA molecule is complex, dependent on terminating mechanism and influenced by the chemical class of the oligonucleotide. Each RNA appears to display unique patterns of sites of sensitivity. Within the phosphorothioate oligodeoxynucleotide chemical class, studies in our laboratory have shown antisense activity can vary from undetectable to 100% by shifting an oligonucleotide by just a few bases in the RNA target (Chiang *et al.*, 1991; Crooke, 1992; Bennett and Crooke, 1996). Although significant progress has been made in developing general rules that help define potentially optimal sites in RNA species, to a large extent, this remains an empirical process that must be performed for each RNA target and every new chemical class of oligonucleotides.

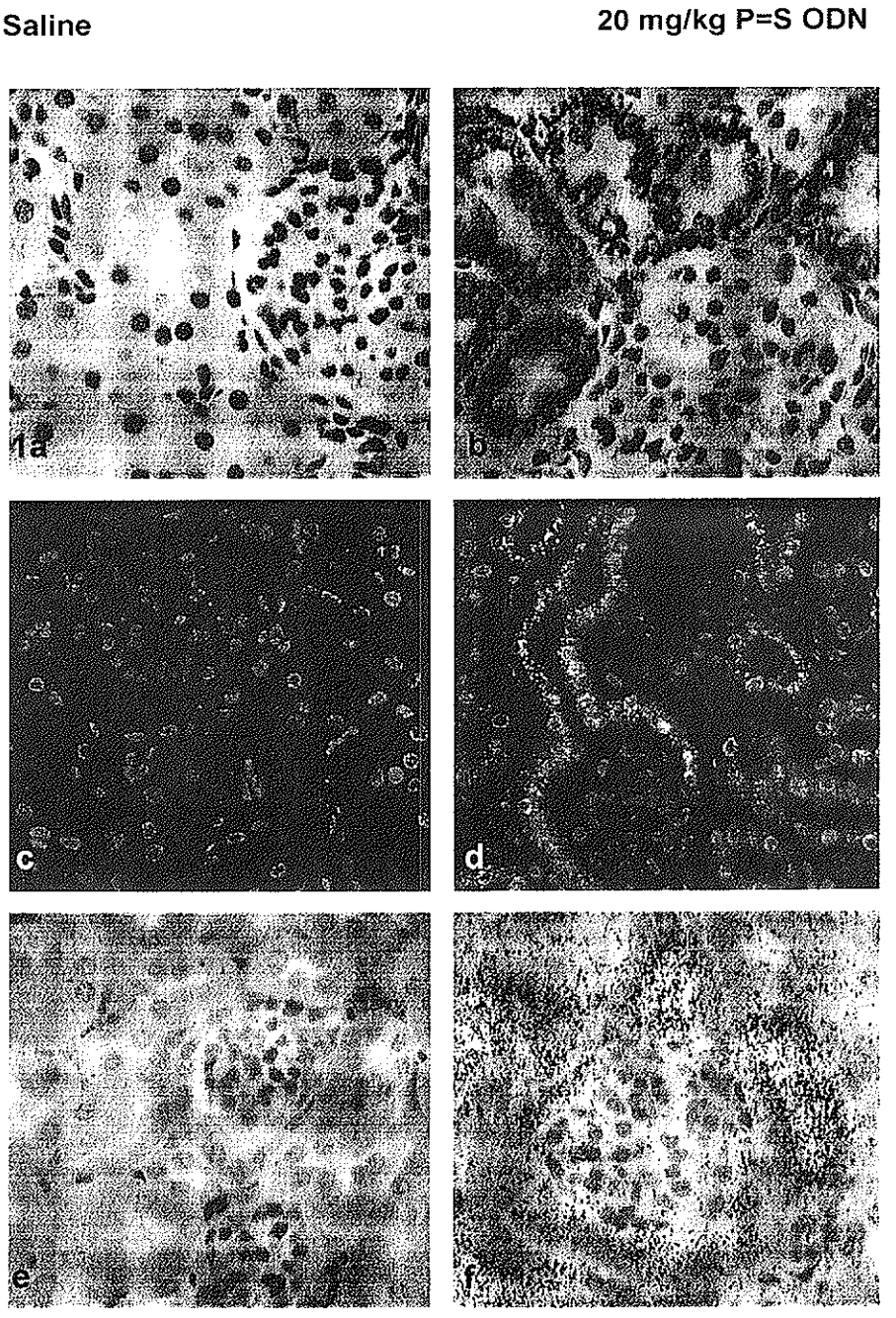

Figure 1. Phosphorothioate oligodeoxynucleotide distribution in kidney.

Phosphorothioates have also been shown to have effects inconsistent with the antisense mechanism for which they were designed. Some of these effects are due to sequence or are structure specific. Others are due to non-specific interactions with proteins. These effects are particularly prominent in *in vitro* tests for antiviral activity as often high concentrations of cells, viruses and oligonucleotides are coincubated (Azad *et al.*, 1993; Wagner *et al.*, 1993). Human immune deficiency virus (HIV) is particularly problematic as many oligonucleotides bind to the gp120 protein (Wyatt *et al.*, 1994). However, the potential for confusion arising from the misinterpretation of an activity as being due to an antisense mechanism when it is due to non-antisense effects is certainly not limited to antiviral or just *in vitro* tests (Barton and Lemoine, 1995; Burgess *et al.*, 1995; Hertl *et al.*, 1995). Again, these data simply urge caution and argue for careful dose response curves, direct analyses of target protein or RNA and inclusion of appropriate controls before drawing conclusions concerning the mechanisms of action of oligonucleotide-based drugs. In addition to protein interactions, other factors, such as overrepresented sequences of RNA and unusual structures that may be adopted by oligonucleotides, can contribute to unexpected results (Wyatt *et al.*, 1994).

Given the variability in cellular uptake of oligonucleotides, the variability in potency as a function of binding site in an RNA target and potential non-antisense activities of oligonucleotides, careful evaluation of dose–response curves and clear demonstration of the antisense mechanism are required before drawing conclusions from *in vitro* experiments. Nevertheless, numerous well-controlled studies have been reported in which antisense activity was conclusively demonstrated. As many of these studies have been reviewed previously, suffice it to say that antisense effects of phosphorothioate oligodeoxynucleotides against a variety of targets are well documented (Crooke and Lebleu, 1993; Stein and Cheng, 1993; Crooke, 1992; Crooke, 1993; Crooke, 1995; Nagel *et al.*, 1993).

IN VIVO PHARMACOLOGICAL ACTIVITIES

A relatively large number of reports of *in vivo* activities of phosphorothioate oligonucleotides have now appeared documenting activities both after local and systemic administration (*Table 1*) (Crooke, 1995). However, for only a few of these reports have sufficient studies been performed to draw relatively firm conclusions concerning the mechanism of action. Consequently, the few reports that provide sufficient data to support a relatively firm conclusion with regard to mechanism of action will now be reviewed. Local effects have been reported for phosphorothioate and methylphosphonate oligonucleotides. A phosphorothioate oligonucleotide designed to inhibit c-myb production and applied locally was shown to inhibit intimal accumulation in the rat carotid artery (Simons *et al.*, 1992). In this study, a Northern blot analysis showed a significant reduction in c-*myb* RNA in animals treated with the antisense compound, but no effect by a control oligonucleotide. In a recent study, the effects of the oligonucleotide were suggested to be due to a non-antisense mechanism (Burgess *et al.*, 1995). However, only one dose level was studied, so much remains to be done before definitive conclusions are possible. Similar effects were reported for phosphorothioate oligodeoxynucleotides designed to inhibit cyclin-dependent kinases (CDC-2 and CDK-2). Again, the antisense oligonucleotide inhibited intimal

Table 1. Reported activity of antisense oligonucleotides in animal models

Target	Route	Specie	Ref.
Cardiovascular models			
c-*myb*	Topically	rat	(Simons *et al.*, 1992)
cdc2 kinase	Topically	rat	(Morishita *et al.*, 1993)
PCNA	Topically	rat	(Morishita *et al.*, 1993)
cdc2 kinase	Topically	rat	(Abe *et al.*, 1994)
CDK2	Topically	rat	(Abe *et al.*, 1994)
Cyclin B_1	Topically	rat	(Morishita *et al.*, 1994)
PCNA	Topically	rat	(Simons *et al.*, 1994)
Angiotensin 1 receptor	Intracerebral	rat	(Gyurko *et al.*, 1993)
Angiotensinogen	Intracerebral	rat	(Phillips *et al.*, 1994)
c-*fos*	Intracerebral	rat	(Suzuki *et al.*, 1994)
Inflammatory models			
Type I IL-1 receptor	Intradermal	mouse	(Burch and Mahan, 1991)
ICAM-1	Intravenous	mouse	(Stepkowski *et al.*, 1994)
ICAM-1	Intravenous	mouse	(Kumasaka *et al.*, 1996)
ICAM-1	Intravenous	mouse	(Katz *et al.*, 1995)
ICAM-1	Intravenous	mouse	(Stepkowski *et al.*, 1995)
ICAM-1	Intravenous	mouse	(Bennett *et al.*, 1997)
Adenosine A_1 receptor	Aerosol	rabbit	(Nyce and Metzger, 1997)
Cancer models			
N-*myc*	Subcutaneous	mouse	(Whitesell *et al.*, 1991)
NF-kB p65	Intraperitoneal	mouse	(Kitajima *et al.*, 1992)
c-*myb*	Subcutaneous	mouse	(Ratajczak *et al.*, 1992)
p120 nucleolar antigen	Intraperitoneal	mouse	(Perlaky *et al.*, 1993)
NK-kB p65	Subcutaneous	mouse	(Higgins *et al.*, 1993)
protein kinase C-α	Intraperitoneal	mouse	(Dean and McKay, 1994)
c-*myb*	Subcutaneous	mouse	(Hijiya *et al.*, 1994)
Ha-*ras*	Intratumor	mouse	(Schwab *et al.*, 1994)
BCR-ABL	Intravenous	mouse	(Skorski *et al.*, 1994)
PTHrP	Intraventricular	rat	(Akino *et al.*, 1996)
c-raf kinase	Intravenous	mouse	(Monia *et al.*, 1995)
protein kinase C-α	Intravenous	mouse	(Dean *et al.*, 1996)
protein kinase C-α	Intravenous	mouse	(Yazaki *et al.*, 1996)
Neurological models			
c-*fos*	Intracerebral	rat	(Chiasson *et al.*, 1992)
SNAP-25	Intracerebral	chicken	(Osen-Sand *et al.*, 1993)
Kinesin heavy chain	Intravitreal	rabbit	(Amaratunga *et al.*, 1993)
Arginine vasopressin	Intracerebral	rat	(Flanagan *et al.*, 1993)
c-*fos*	Intracerebral	rat	(Heilig *et al.*, 1993)
Progesterone receptor	Intracerebral	rat	(Pollio *et al.*, 1993)
Dopamine D_2 receptor	Intracerebral	rat	(Zhang and Creese, 1993)
Y-Y1 receptor	Intracerebral	rat	(Wahlestedt *et al.*, 1993)
Neuropeptide Y	Intracerebral	rat	(Akabayashi *et al.*, 1994)
k-opioid receptor	Intracerebral	rat	(Adams *et al.*, 1994)
IGF-1	Intracerebral	rat	(Castro-Alamancos and Torres-Aleman, 1994)
c-*fos*	Intraspinal	rat	(Gillardon *et al.*, 1994)
c-*fos*	Intracerebral	rat	(Hooper *et al.*, 1994)
c-*fos*	Intraspinal	rat	(Woodburn *et al.*, 1994)
NMDA receptor	Intracerebral	rat	(Kindy, 1994)
CREB	Intracerebral	rat	(Konradi *et al.*, 1994)
Delta-opioid receptor	Intracerebral	mice	(Lai *et al.*, 1994)
Progesterone receptor	Intracerebral	rat	(Mani *et al.*, 1994)
GAD65	Intracerebral	rat	(McCarthy *et al.*, 1994)
GAD67	Intracerebral	rat	(McCarthy *et al.*, 1994)

AT1-angiotensin receptor	Intracerebral	rat	(Sakai *et al.*, 1995)
Tryptophan hydroxylase	Intracerebral	mouse	(McCarthy *et al.*, 1995)
AT1-angiotensin receptor	Intracerebral	rat	(Ambuhl *et al.*, 1995)
CRH_1-corticotropin-releasing hormone receptor	Intracerebral	rat	(Liebsch *et al.*, 1995)
opiod receptor	Intracerebral	rat	(Cha *et al.*, 1995)
opiod receptor	Intracerebral	mouse	(Mizoguchi *et al.*, 1995)
Oxytocin	Intracerebral	rat	(Morris *et al.*, 1995)
Oxytocin	Intracerebral	rat	(Neumann *et al.*, 1994)
Substance P receptor	Intracerebral	rat	(Ogo *et al.*, 1994)
tyrosine hydroxylase	Intracerebral	rat	(Skutella *et al.*, 1994)
c-*jun*	Intracerebral	rat	(Tischmeyer *et al.*, 1994)
D_1 dopamine receptor	Intracerebral	mouse	(Zhang *et al.*, 1994)
D_2 dopamine receptor	Intracerebral	mouse	(Zhou *et al.*, 1994)
D_2 dopamine receptor	Intracerebral	mouse	(Weiss *et al.*, 1993)
D_2 dopamine receptor	Intracerebral	mouse	(Qin *et al.*, 1995)
Viral models			
HSV-1		mouse	(Kulka *et al.*, 1989)
Tick-born encephalitis		mouse	(Vlassov, 1989)
Duck hepatitis virus	Intravenous	duck	(Offensperger *et al.*, 1993)

thickening and cyclin-dependent kinase activity, while a control oligonucleotide had no effect (Abe *et al.*, 1994). Additionally, local administration of a phosphorothioate oligonucleotide designed to inhibit N-*myc* resulted in reduction in N-*myc* expression and slower growth of a subcutaneously transplanted human tumor in nude mice (Whitesell *et al.*, 1991).

Antisense oligonucleotides administered intraventricularly have been reported to induce a variety of effects in the central nervous system. Intraventricular injection of antisense oligonucleotides to neuropeptide-y-y1 receptors reduced the density of the receptors and resulted in behavioural signs of anxiety (Wahlestedt *et al.*, 1993). Similarly, an antisense oligonucleotide designed to bind to NMDA-R1 receptor channel RNA inhibited the synthesis of these channels and reduced the volume of focal ischemia produced by occlusion of the middle cerebral artery in rats (Wahlestedt *et al.*, 1993).

In a series of well-controlled studies, antisense oligonucleotides administered intraventricularly selectively inhibited dopamine type-2 receptor expression, dopamine type-2 receptor RNA levels and behavioral effects in animals with chemical lesions. Controls included randomized oligonucleotides and the observation that no effects were observed on dopamine type-1 receptor or RNA levels (Weiss *et al.*, 1993; Zhou *et al.*, 1994; Qin *et al.*, 1995). This laboratory also reported the selective reduction of dopamine type 1 receptor and RNA levels with the appropriate oligonucleotide (Zhang *et al.*, 1994).

Similar observations were reported in studies on AT-1 angiotensin receptors and tryptophan hydroxylase. In studies in rats, direct observations of AT-1 and AT-2 receptor densities in various sites in the brain after administration of different doses of phosphorothioate antisense, sense and scrambled oligonucleotides were reported (Ambuhl *et al.*, 1995). Again, in rats, intraventricular administration of phosphorothioate antisense oligonucleotide resulted in a decrease in tryptophan hydroxylase levels in the brain, while a scrambled control did not (McCarthy *et al.*, 1995).

Injection of antisense oligonucleotides to synaptosomal-associated protein-25 into

the vitreous body of rat embryos reduced the expression of the protein and inhibited neurite elongation by rat cortical neurons (Osen-Sand *et al.*, 1993).

Aerosol administration to rabbits of an antisense phosphorothioate oligodeoxynucleotide designed to inhibit the production of adenosine A_1 receptor has been reported to reduce receptor numbers in the airway smooth muscle and to inhibit adenosine, house dust mite allergen and histamine induced bronchoconstriction (Nyce and Metzger, 1997). Neither control nor oligonucleotide complementary to bradykinin B_2 receptors reduced adenosine A_1 receptors' density although the oligonucleotides complementary to bradykinin in B_2 receptor mRNA reduced the density of these receptors.

In addition to local and regional effects of antisense oligonucleotides, a growing number of well-controlled studies have demonstrated systemic effects of phosphorothioate oligodeoxynucleotides. Expression of interleukin-1 in mice was inhibited by systemic administration of antisense oligonucleotides (Burch and Mahan, 1991). Oligonucleotides to the NF-kB p65 subunit administered intraperitoneally at 40 mg/kg every three days slowed tumour growth in mice transgenic for the human T-cell leukemia viruses (Kitajima *et al.*, 1992). Similar results with other antisense oligonucleotides were shown in another *in vivo* tumour model after either prolonged subcutaneous infusion or intermittent subcutaneous injection (Higgins *et al.*, 1993).

Several recent reports further extend the studies of phosphorothioate oligonucleotides as anti-tumor agents in mice. In one study, a phosphorothioate oligonucleotide directed to inhibition of the bcr-abl oncogene was administered at a dose of 1 mg/day for nine days intravenously to immunodeficient mice injected with human leukemic cells. The drug was shown to inhibit the development of leukemic colonies in the mice and to selectively reduce bcr-abl RNA levels in peripheral blood lymphocytes, spleen, bone marrow, liver, lungs and brain (Skorski *et al.*, 1994). However, it is possible that the effects on the RNA levels were secondary to effects on the growth of various cell types. In the second study, a phosphorothioate oligonucleotide antisense to the protooncogene myb, inhibited the growth of human melanoma in mice. Again, myb mRNA levels appeared to be selectively reduced (Hijiya *et al.*, 1994).

A number of studies that directly examined target RNA levels, target protein levels and pharmacological effects using a wide range of control oligonucleotides and examination of the effects on closely-related isotypes have been completed. Single and chronic daily administration of a phosphorothioate oligonucleotide designed to inhibit mouse protein kinase C-α, (PKC-α), selectively inhibited expression of PKC-α RNA in mouse liver without effects on any other isotype. The effects lasted at least 24 hours after a dose and a clear dose response curve was observed with a dose of 10–15 mg/kg intraperitoneally reducing PKC-α RNA levels in liver by 50% 24 hours after a dose (Dean and McKay, 1994).

A phosphorothioate oligonucleotide designed to inhibit human PKC-α expression selectively inhibited expression of PKC-α RNA and PKC-α protein in human tumour cell lines implanted subcutaneously in nude mice after intravenous administration (Dean *et al.*, 1996). In these studies, effects on RNA and protein levels were highly specific and observed at doses lower than 6 mg/kg/day and antitumor effects were detected at doses as low as 0.6 mg/kg/day. A large number of control oligonucleotides failed to show activity.

In a similar series of studies, Monia *et al.* demonstrated highly specific loss of human c-*raf* kinase RNA in human tumour xenografts and antitumour activity that correlated with the loss of RNA. Moreover, a series of control oligonucleotides with 1–7 mismatches showed decreasing potency *in vitro* and precisely the same rank order potencies *in vivo* (Monia *et al.*, 1995; Monia *et al.*, 1996).

Finally, a single injection of a phosphorothioate oligonucleotide designed to inhibit c-AMP-dependent protein kinase type 1 was reported to selectively reduce RNA and protein levels in human tumour xenografts and to reduce tumour growth (Nesterova and Cho-Chung, 1995).

Thus, there is a growing body of evidence that phosphorothioate oligonucleotides can induce potent systemic and local effects *in vivo*. More importantly, there are now a number of studies with sufficient controls and direct observation of target RNA and protein levels to suggest highly specific effects that are difficult to explain via any mechanisms other than antisense. As would be expected, the potency of these effects varies depending on the target, the organ and the endpoint measured as well as the route of administration and the time after a dose when the effect is measured.

TOXICOLOGICAL PROPERTIES

The acute LD_{50} in mice of all phosphorothioate oligonucleotides tested to date is in excess of 500 mg/kg (Kornbrust, unpublished observations). In rodents, evaluation of the acute and chronic toxicities of multiple phosphorothioate oligonucleotides administered by multiple routes has taken place (Henry *et al.*, 1997; in press). The consistent dose limiting toxicity was immune stimulation manifested by lymphoid hyperplasia, spelnomegaly and a multi-organ monocellular infiltrate. These effects occurred only with chronic dosing at doses >20 mg/kg and were dose dependent. The liver and kidney were the organs most prominently affected by monocellular infiltrates. All of these effects appeared to be reversible and chronic intradermal administration appeared to be the most toxic route, probably because of high local concentrations of the drugs resulting in local cytokine release and initiation of a cytokine cascade. There were no obvious effects of sequence. At doses of 100 mg/kg and greater, minor increases in liver enzyme levels and mild thrombocytopenia were also observed.

In monkeys, however, the toxicological profile of phosphorothioate oligonucleotides is quite different. The most prominent dose limiting side effect is sporadic reductions in blood pressure associated with bradycardia. When these events are observed, they are often associated with activation of C-5 complement and they are dose related and peak plasma concentration related. This appears to be related to the activation of the alternative pathway (Henry *et al.*, in press). All phosphorothioate oligonucleotides tested to date appear to induce these effects through there may be slight variations in potency as a function of sequence and/or length (Cornish *et al.*, 1993; Galbraith *et al.*, 1994; Henry *et al.*, in press).

A second prominent toxicologic effect in the monkey is the prolongation of activated partial thromboplastin time. At higher doses, evidence of clotting abnormalities is observed. Again, these effects are dose and peak plasma concentration dependent (Galbraith *et al.*, 1994; Henry *et al.*, in press). Although no evidence of sequence dependence has been observed, there appears to be a linear correlation

between number of phosphorothioate linkages and potency between 18 and 25 nucleotides (Nicklin, unpublished observations). The mechanisms responsible for these effects are likely very complex, but preliminary data suggest that direct interactions with thrombin may be at least partially responsible for the effects observed (Henry *et al.*, submitted).

In humans, again the toxicological profile differs a bit. When ISIS 2922 is administered intravitreally to patients with cytomegalovirus retinitis, the most common adverse event is anterior chamber inflammation easily managed with steroids. A relatively rare and dose-related adverse event is morphological changes in the retina associated with loss in peripheral vision (Hutcherson *et al.*, 1995).

ISIS 2105, a 20-mer phosphorothioate designed to inhibit the replication of human papilloma viruses that cause genital warts, is administered intradermally at doses as high as 3 mg/wart weekly for three weeks, essentially no toxicities have been observed, including remarkably, a complete absence of local inflammation (Grillone, unpublished results).

Every other day administration of 2-hour intravenous infusions of ISIS 2302 at doses as high as 2 mg/kg resulted in no significant toxicities including no evidence of immune stimulation and no hypotension. A slight subclinical increase in APTT was observed at the 2 mg/kg dose (Glover *et al.*, 1996).

THERAPEUTIC INDEX

In *Figure 2*, an attempt to put the toxicities and their dose response relationships in a therapeutic context is shown. This is particularly important as considerable confusion has arisen concerning the potential utility of phosphorothioate oligonucleotides for selected therapeutic purposes deriving from unsophisticated interpretation of toxicological data. As can be readily seen, the immune stimulation induced by these compounds appears to be particularly prominent in rodents and unlikely to be dose limiting in humans. Nor, to date, have hypotensive events been observed. Thus, this toxicity appears to occur at lower doses in monkeys than man and certainly is not dose limiting in man.

Based on experience to date, the dose-limiting toxicity in man will likely be clotting abnormalities and this will be associated with peak plasma concentrations well in excess of 10 μg/ml. In animals, pharmacological activities have been observed with i.v. bolus doses from 0.006 mg/kg to 10–15 mg/kg depending on the target, the endpoint, the organ studied and the time after a dose when the effect is measured. Thus, it would appear that phosphorothioate oligonucleotides have a therapeutic index that supports their evaluation for a number of therapeutic indications.

Conclusions

Phosphorothioate oligonucleotides have perhaps outperformed many expectations. They display attractive parenteral pharmacokinetic properties. They have produced potent systemic effects in a number of animal models and, in many experiments, the antisense mechanism has been directly demonstrated as the hoped-for selectivity. Further, these compounds appear to display satisfactory therapeutic indices for many indications.

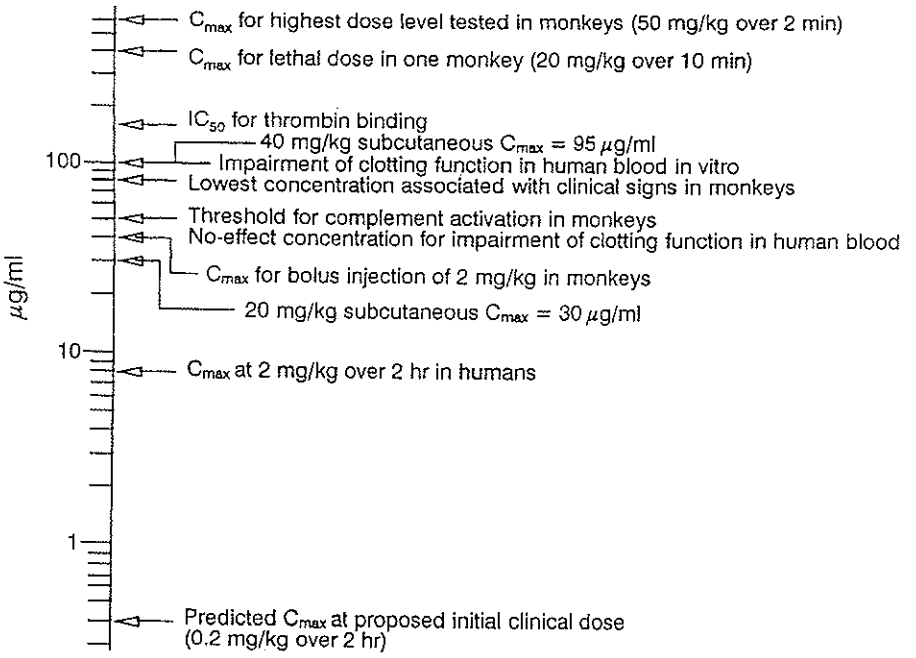

Figure 2. Plasma concentrations of ISIS 2302 at which various activities are observed. These concentrations are determined by extracting plasma and analysing by capillary gel electrophoresis and represent intact ISIS 2302.

Nevertheless, phosphorothioates clearly have significant limits (*Table 2*). Pharmacodynamically, they have relatively low affinity per nucleotide unit. This means that longer oligonucleotides are required for biological activity and that invasion of many RNA structures may not be possible. At higher concentrations, these compounds inhibit RNase H as well. Thus, the higher end of the pharmacologic dose response curve is lost. Pharmacokinetically, phosphorothioates do not cross the blood brain barrier, are not significantly orally bioavailable and may display dose-dependent

Table 2. Phosphorothioate oligonucleotides

Limits
• Pharmacodynamic
– Low affinity per nucleotide unit
– Inhibition of RNase H at high concentrations
• Pharmacokinetic
– Limited bioavailability
– Limited blood brain barrier penetration
– Dose-dependent pharmacokinetics
– Possible drug-drug interactions
• Toxicologic
– Release of cytokines
– Complement associated effects on blood pressure?
– Clotting effects

pharmacokinetics. Toxicologically, clearly the release of cytokines, activation of complement and interference with clotting will pose dose limits if they are encountered in the clinic.

As several clinical trials are in progress with phosphorothioates and others will be initiated shortly, we shall soon have more definitive information about the activities, toxicities and value of this class of antisense drugs in human beings.

The medicinal chemistry of oligonucleotides

INTRODUCTION

The core of any rational drug discovery program is medicinal chemistry. Although the synthesis of modified nucleic acids has been a subject of interest for some time, the intense focus on the medicinal chemistry of oligonucleotides dates perhaps to no more than five years prior to this chapter. Consequently, the scope of medicinal chemistry has recently expanded enormously, but the biological data to support conclusions about synthetic strategies are only beginning to emerge.

Modifications in the base, sugar and phosphate moieties of oligonucleotides have been reported. The subjects of medicinal chemical programs include approaches to create enhanced affinity and more selective affinity for RNA or duplex structures, the ability to cleave nucleic acid targets, enhanced nuclease stability, cellular uptake and distribution, and *in vivo* tissue distribution, metabolism and clearance.

HETEROCYCLE MODIFICATIONS

Pyrimidine modifications

A relatively large number of modified pyrimidines have been synthesized and are now incorporated into oligonucleotides and evaluated. The principle sites of modification are C-2, C-4, C-5 and C-6. These and other nucleoside analogs have recently been thoroughly reviewed (Sanghvi, 1993). Consequently, a very brief summary of the analogues that displayed interesting properties is incorporated here.

Inasmuch as the C-2 position is involved in Watson–Crick hybridization, C-2 modified pyrimidine containing oligonucleotides have shown unattractive hybridization properties. An oligonucleotide containing 2-thiothymidine was found to hybridize well to DNA and, in fact, even better to RNA (T_m 1.5°C modification (Swayze *et al.*, unpublished results).

In contrast, several modifications in the 4 position that have interesting properties have been reported. 4-thiopyrimidines have been incorporated into oligonucleotides with no significant negative effect on hybridization (Nikiforov and Connolly, 1991). A bicyclic and an N4-methoxy analog of cytosine were shown to hybridize with both purine bases in DNA with T_m's approximately equal to natural base pairs (Lin and Brown, 1989). Additionally, a fluorescent base has been incorporated into oligonucleotides and shown to enhance DNA–DNA duplex stability (Inoue *et al.*, 1985).

A large number of modifications at the C-5 position have also been reported including halogenated nucleosides. Although the stability of duplexes may be enhanced by incorporating 5-halogenated nucleosides, the occasional mispairing with G

and the potential that the oligonucleotide might degrade and release toxic nucleosides analogs cause concern (Sanghvi, 1993).

Furthermore, oligonucleotides containing 5-propynylpyrimidine modifications have been shown to enhance the duplex stability (ΔT_m 1.6°C/modification, Wagner *et al.*, 1993) and support the RNase H activity. The 5-heteroarylpyrimidines were also shown to influence the stability of duplexes (Wagner *et al.*, 1993; Gutierrez *et al.*, 1994). A more dramatic influence was reported for the tricyclic 2'-deoxycytidine analogs, exhibiting an enhancement of 2–5°C/modification depending on the positioning of the modified bases (Lin *et al.*, 1995). It is believed that the enhanced binding properties of these analogues is due to extended stacking and increased hydrophobic interactions.

In general, as expected, modifications in the C-6 position of pyrimidines are highly duplex destabilizing (Sanghvi *et al.*, 1993). Oligonucleotides containing 6-aza pyrimidines have been shown to reduce T_m by 1–2°C per modification, but to enhance the nuclease stability of oligonucleotides and to support RNase H induced degradation of RNA targets (Sanghvi, 1993).

Purine modifications

Although numerous purine analogues have been synthesized, when incorporated into oligonucleotides, they usually have resulted in destabilization of duplexes. However, there are a few exceptions, where a purine modification had a stabilizing effect. A brief summary of some of these analogues is discussed below.

Generally, N1 modifications of purine moiety has resulted in destabilization of the duplex (Manoharan, 1993). Similarly, C2 modifications have usually resulted in destabilization. However, 2-6-diaminopurine has been reported to enhance hybridization by approximately 1°C per modification when paired with T (Sproat *et al.*, 1991). Of the 3-position substituted bases reported to date, only the 3-deaza adenosine analog has been shown to have no negative effective on hybridization.

Modifications at the C-6 and C-7 positions have likewise resulted in only a few interesting bases from the point of view of hybridization. Inosine has been shown to have little effect on duplex stability, but because it can pair and stack with all four normal DNA bases, it behaves as a universal base and creates an ambiguous position in an oligonucleotide (Martin *et al.*, 1985). Incorporation of 7-deaza inosine into oligonucleotides was destabilizing, and this was considered to be due to its relatively hydrophobic nature (SantaLucia *et al.*, 1991). 7-Deaza guanine was similarly destabilizing, but when 8-aza-7-deaza guanine was incorporated into oligonucleotides, it enhanced hybridizations (Seela *et al.*, 1989). Thus, on occasion, introduction of more than one modification in a nucleobase may compensate for destabilizing effects of some modifications. Interestingly, 7-iodo 7-deazaguanine residue was recently incorporated into oligonucleotides and shown to enhance the binding affinity dramatically (ΔT_m 10.0°C/modification compared to 7-deazaguanine) (Seela *et al.*, 1995). The increase in T_m value was attributed to (i) the hydrophobic nature of the modification, (ii) increased stacking interaction and (iii) favourable pKa of the base.

In contrast, some C8 substituted bases have yielded improved nuclease resistance when incorporated in oligonucleotides, but seem to be somewhat destabilizing (Sanghvi, 1993).

OLIGONUCLEOTIDE CONJUGATES

Although conjugation of various functionalities to oligonucleotides has been reported to achieve a number of important objectives, the data supporting some of the claims are limited and generalizations are not possible based on the data presently available.

Nuclease stability

Numerous 3'-modifications have been reported to enhance the stability of oligonucleotides in serum (Manoharan, 1993). Both neutral and charged substituents have been reported to stabilize oligonucleotides in serum and, as a general rule, the stability of a conjugated oligonucleotide tends to be greater as bulkier substituents are added. Inasmuch as the principal nuclease in serum is a 3'-exonuclease, it is not surprising that 5'-modifications have resulted in significantly less stabilization. Internal modifications of base, sugar and backbone have also been reported to enhance nuclease stability at or near the modified nucleoside (Manoharan, 1993). In a recent study, thiono triester (adamantyl, cholesteryl and others) modified oligonucleotides have shown improved nuclease stability, cellular association and binding affinity (Zhang *et al.*, 1995).

The demonstration that modifications may induce nuclease stability sufficient to enhance activity in cells in tissue culture and in animals has proven to be much more complicated because of the presence of 5'-exonucleases and endonucleases. In the author's laboratory, 3'-modifications and internal point modifications have not provided sufficient nuclease stability to demonstrate pharmacological activity in cells (Hoke *et al.*, 1991). Even a 5-nucleotide long phosphodiester gap in the middle of a phosphorothioate oligonucleotide resulted in sufficient loss of nuclease resistance to cause complete loss of pharmacological activity (Monia *et al.*, 1992).

In mice, neither a 5'-cholesterol nor 5'-C18 amine conjugate altered the metabolic rate of a phosphorothioate oligodeoxynucleotide in liver, kidney or plasma (Crooke *et al.*, 1996). Furthermore, blocking the 3'- and 5'-termini of a phosphodiester oligonucleotide did not markedly enhance the nuclease stability of the parent compound in mice (Sands *et al.*, 1995). However, 3'-modification of a phosphorothioate oligonucleotide was reported to enhance its stability in mice relative to the parent phosphorothioate (Temsamani *et al.*, 1993). Moreover, a phosphorothioate oligonucleotide with a 3'-hairpin loop was reported to be more stable in rats than its parent (Zhang *et al.*, 1995). Thus, 3'-modifications may enhance the stability of the relatively stable phosphorothioates sufficiently to be of value.

Enhanced cellular uptake

Although oligonucleotides have been shown to be taken up by a number of cell lines in tissue culture, with perhaps the most compelling data relating to phosphorothioate oligonucleotides, a clear objective has been to improve cellular uptake of oligonucleotides (Crooke, 1991; Crooke *et al.*, 1994). Inasmuch as the mechanisms of cellular uptake of oligonucleotides are still very poorly understood, the medicinal chemistry approaches have been largely empirical and based on many unproven assumptions.

Because phosphodiester and phosphorothioate oligonucleotides are water soluble, the conjugation of lipophilic substituents to enhance membrane permeability has been a subject of considerable interest. Unfortunately, studies in this area have not been systematic and, at present, there is precious little information about the changes in physicochemical properties of oligonucleotides actually effected by specific lipid conjugates. Phospholipids, cholesterol and cholesterol derivatives, cholic acid and simple alkyl chains have been conjugated to oligonucleotides at various sites in the oligonucleotide. The effects of these modifications on cellular uptake have been assessed using fluorescent, or radiolabeled, oligonucleotides or by measuring pharmacological activities. From the perspective of medicinal chemistry, very few systematic studies have been performed. The activities of short alkyl chains, adamantine, daunomycin, fluorescein, cholesterol and porphyrin conjugated oligonucleotides were compared in one study (Boutorine *et al.*, 1991). A cholesterol modification was reported to be more effective at enhancing uptake than the other substituents. It also seems likely that the effects of various conjugates on cellular uptake may be affected by the cell type and target studied. For example, we have studied cholic acid conjugates of phosphorothioate deoxyoligonucleotides or phosphorothioate 2'-methoxy oligonucleotides and observed enhanced activity against HIV and no effect on the activity of ICAM directed oligonucleotides.

Additionally, polycationic substitutions and various groups designed to bind to cellular carrier systems have been synthesized. Although many compounds have been synthesized, the data reported to date are insufficient to draw firm conclusions about the value of such approaches or structure activity relationships (Manoharan, 1993).

RNA cleaving groups

Oligonucleotide conjugates were recently reported to act as artificial ribonucleases, albeit in low efficiencies (De Mesmaeker *et al.*, 1995). Conjugation of chemically reactive groups such as alkylating agents, photoinduced azides, prophine and psoralene have been utilized extensively to effect a cross-linking of oligonucleotide and the target RNA. In principle, this treatment may lead to translation arrest. In addition, lanthanides and complexes thereof have been reported to cleave RNA via a hydrolytic pathway. Recently, a novel europium complex was covalently linked to an oligonucleotide and shown to cleave 88% of the complementary RNA at physiological pH (Hall *et al.*, 1994).

In vivo *effects*

To date, relatively few studies have been reported *in vivo*. The properties of a 5'-cholesterol and 5'-C-18 amine conjugates of a 20-mer phosphorothioate oligodeoxynucleotide have been determined in mice. Both compounds increased the fraction of an i.v. bolus dose found in the liver. The cholesterol conjugate resulted in more than 80% the dose accumulating in the liver. Neither conjugate enhanced stability in plasma, liver or kidney (Crooke *et al.*, 1996). The only significant change in the toxicity profile was a slight increase in effects on serum transamineses and histopathological changes indicative of slight liver toxicity associated with the cholesterol conjugate (Henry *et al.*, 1997). A 5'-cholesterol phosphorothioate conju-

gate was also recently reported to have a longer elimination half-life, to be more potent and to induce greater liver toxicity in rats (Desjardins *et al.*, 1995).

SUGAR MODIFICATIONS

The focus of second generation oligonucleotide modifications has centred on the sugar moiety. In oligonucleotides, pentofuranose sugar ring occupies a central connecting manifold that also positions the nucleobases for effective stacking. Recently, a symposium series has been published on the carbohydrate modifications in antisense research which covers this topic in great detail (Sanghvi and Cook, 1994). Therefore, the content of the following discussion is restricted to a summary of the main events in this area.

A growing number of oligonucleotides in which the pentofuranose ring is modified or replaced have been reported (Breslauer *et al.*, 1986). Uniform modifications at the 2'-position have been shown to enhance hybridization to RNA, and in some cases, to enhance nuclease resistance (Breslauer *et al.*, 1986). Chimeric oligonucleotides containing 2'-deoxyoligonucleotide gaps with 2'-modified wings have been shown to be more potent than parent molecules (Monia *et al.*, 1993).

Other sugar modifications include α-oligonucleotides, carbocyclic oligonucleotides and hexapyranosyl oligonucleotides (Breslauer *et al.*, 1986). Of these, α-oligonucleotides have been most extensively studied. They hybridize in parallel fashion to single stranded DNA and RNA and are nuclease resistant. However, they have been reported to be oligonucleotides designed to inhibit Ha-*ras* expression. All these oligonucleotides support RNase H and, as can be seen, a direct correlation between affinity and potency exists.

A growing number of oligonucleotides in which the C-2'-position of the sugar ring is modified have been reported (Manoharan, 1993; De Mesmaeker *et al.*, 1995). These modifications include lipophilic alkyl groups, intercalators, amphipathic amino-alkyl tethers, positively charged polyamines, highly electronegative fluoro or fluoro alkyl moities, and sterically bulky methylthio derivatives. The beneficial effects of a C-2'-substitution on the antisense oligonucleotide cellular uptake, nuclease resistance and binding affinity have been well documented in the literature. In addition, excellent review articles have appeared in the last few years on the synthesis and properties of C-2'-modified oligonucleotides (De Mesmaeker *et al.*, 1995; Lamond and Sproat, 1993; Sproat and Lamond, 1993; Parmentier *et al.*, 1994).

Other modifications of the sugar moiety have also been studied including other sites as well as more substantial modifications. However, much less is known about the antisense effects of these modifications (Crooke, 1995).

2'-Methoxy-substituted phosphorothioate oligonucleotides have recently been reported to be more stable in mice than their parent compounds and to display enhanced oral bioavailability (Zhang *et al.*, 1995; Agrawal *et al.*, 1995). The analogues displayed tissue distribution similar to that of the parent phosphorothioate.

Similarly, the pharmacokinetics of 2'-propoxy modified phosphodiester and phosphorothioate deoxynucleotides have been compared (Crooke *et al.*, 1996). As expected, the 2'-propoxy modification increased lipophilicity and nuclease resistance. In fact, in mice the 2'-propoxy phosphorothioate was too stable in liver or kidney to measure an elimination half-life.

The 2'-propoxy phosphodiester was much less stable than the parent phosphorothioate in all organs except the kidney in which the 2'-propoxy phosphodiester was remarkably stable. The 2'-propoxy phosphodiester did not bind to albumin significantly, while the affinity of the phosphorothioate for albumin was enhanced. The only difference in toxicity between the analogues was a slight increase in renal toxicity associated with the 2'-propoxy phosphodiester analogue. (Henry *et al.*, in press).

Incorporation of the 2'-methoxyethyoxy group into oligonucleotides increased the T_m by 1.1°C/modification when hybridized to the complement RNA. In a similar manner, several other 2'-O-alkoxy modifications have been reported to enhance the affinity (Martin, 1995). The increase in affinity with these modifications was attributed to (i) the favourable gauche effect of side chain and (ii) additional solvation of the alkoxy substituent in water.

More substantial carbohydrate modifications have also been studied. Hexose-containing oligonucleotides were created and found to have very low affinity for RNA (Pitsch *et al.*, 1995). Also, the 4'-oxygen has been replaced with sulfur. Although a single substitution of a 4'-thio modified nucleoside resulted in destabilization of a duplex, incorporation of two 4'-thio modified nucleosides increased the affinity of the duplex (Bellon *et al.*, 1994). Finally, bicyclic sugars have been synthesized with the hope that preorganization into more rigid structures would enhance hybridization. Several of these modifications have been reported to enhance hybridization (Sanghvi and Cook, 1994).

BACKBONE MODIFICATIONS

Substantial progress in creating new backbones for oligonucleotides that replace the phosphate or the sugar-phosphate unit has been made. The objectives of these programs are to improve hybridization by removing the negative charge, enhance stability and potentially improve pharmacokinetics.

For a review of the backbone modifications reported to date, please see (Crooke, 1995; Sanghvi and Cook, 1994). Suffice it to say that numerous modifications have been made that replace phosphate, retain hybridization, alter charge and enhance stability. Since these modifications are now being evaluated *in vitro* and *in vivo*, a preliminary assessment should be possible shortly.

Replacement of the entire sugar-phosphate unit has also been accomplished and the oligonucleotides produced have displayed very interesting characteristics. PNA oligonucleotides have been shown to bind to single-stranded DNA and RNA with extraordinary affinity and high sequence specificity. They have been shown to be able to invade some double-stranded nucleic acid structures. PNA oligonucleotides can form triple-stranded structures with DNA or RNA.

PNA oligonucleotides were shown to be able to act as antisense and transcriptional inhibitors when microinjected in cells (Hanvey *et al.*, 1992). PNA oligonucleotides appear to be quite stable to nucleases and peptidases as well.

In summary, then, in the past five years, enormous advances in the medicinal chemistry of oligonucleotides has been reported. Modifications at nearly every position in oligonucleotides have been attempted and numerous potentially interesting analogues have been identified. Although it is far too early to determine which of the

modifications may be most useful for particular purposes, it is clear that a wealth of new chemicals is available for systematic evaluation and that these studies should provide important insights into the SAR of oligonucleotide analogues.

Conclusions

Although many more questions about antisense remain to be answered than are answered, progress has continued to be gratifying. 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.

References

ABE, J., ZHOU, W., TAGUCHI, J., TAKUWA, N., MIKI, K., OKAZAKI, H., KUROKAWA, K., KUMADA, M. AND TAKUWA, Y. (1994). 'Suppression of neointimal smooth muscle cell accumulation *in vivo* by antisense CDC2 and CDK2 oligonucleotides in rat carotid artery.' *Biochemical and Biophysical Research Communications* **198**, 16–24.

ADAMS, J.U., CHEN, X.H., DERIEL, J.K., ADLER, M.W. AND LIU-CHEN, L.Y. (1994). '*In vivo* treatment with antisense oligodeoxynucleotide to kappa-opioid receptors inhibited kappa-agonist-induced analgesia in rats.' *Regulatory Peptides* **54**, 1–2.

AGRAWAL, S., GOODCHILD, J., CIVEIRA, M.P., THORNTON, A.H., SARIN, P.S. AND ZAMECNIK. P.C. (1988). 'Oligodeoxynucleoside phosphoramidates and phosphorothioates as inhibitors of human immunodeficiency virus.' *Proceedings of the National Academy of Sciences of the USA* **85**, 7079–7083.

AGRAWAL, S., TEMSAMANI, J. AND TANG, J.Y. (1991). 'Pharmacokinetics, biodistribution, and stability of oligodeoxynucleotide phosphorothioates in mice.' *Proceedings of the National Academy of Sciences USA* **88**, 7595–7599.

AGRAWAL, S., ZHANG, X., LU, Z., ZHAO, H., TAMBURIN, J.M., YAN, J., CAI, H., DIASIO, R.B., HABUS, I., JIANG, Z., IYER, R.P., YU, D. AND ZHANG, R. (1995). 'Absorption, tissue distribution and *in vivo* stability in rats of a hybrid antisense oligonucleotide following oral administration.' *Biochemical Pharmacology* **50**(4), 571–576.

AKABAYASHI, A., WAHLESTEDT, C., ALEXANDER, J.T. AND LEIBOWITZ, S.F. (1994). 'Specific inhibition of endogenous neuropeptide Y synthesis in arcuate nucleus by antisense oligonucleotides suppresses feeding behavior and insulin secretion.' *Molecular Brain Research* **21**, 55–61.

AKINO, K., OHTSURU, A., YANO, H., OZEKI, S., NAMBA, H., NAKASHIMA, M., ITO, M., MATSUMOTO, T. AND YAMASHITA, S. (1996). 'Antisense inhibition of parathyroid hormone-related peptide gene expression reduces malignant pituitary tumor progression and metastases in the rat.' *Cancer Research* **56**(1), 77–86.

AMARATUNGA, A., MORIN, P.J., KOSIK, K.S. AND FINE, R.E. (1993). 'Inhibition of kinesin synthesis and rapid anterograde axonal transport *in vivo* by an antisense oligonucleotide.' *Journal of Biological Chemistry* **268(23)**, 17427–17430.

AMBUHL, P., GYURKO, R. AND PHILLIPS, M.I. (1995). 'A decrease in angiotensin receptor binding in rat brain nuclei by antisense oligonucleotides to the angiotensin AT1 receptor.' *Regulatory Peptides* **59**(2), 171–182.

AZAD, R.F., DRIVER, V.B., TANAKA, K., CROOKE, R.M. AND ANDERSON, K.P. (1993). 'Antiviral activity of a phosphorothioate oligonucleotide complementary to RNA of the human cytomegalovirus major immediate-early region.' *Antimicrobial Agents and Chemotherapy* **37**(9), 1945–1954.

BAKER, B.F. (1993). ' "Decapitation" of a 5'-Capped Oligoribonucleotide by o-Penanthroline:Cu(II).' *Journal of Biological Chemistry* **115**, 3378–3379.

BAKER, B.F., MIRAGLIA, L. AND HAGEDORN, C.H. (1992). 'Modulation of eucaryotic initiation factor-4E binding to 5'-capped oligoribonucleotides by modified anti-sense oligonucleotides.' *Journal of Biological Chemistry* **267**, 11495–11499.

BARTON, C.M. AND LEMOINE, N.R. (1995). 'Antisense oligonucleotides directed against p53 have antiproliferative effects unrelated to effects on p53 expression.' *British Journal of Cancer* **71**, 429–437.

BAYEVER, E., IVERSEN, P.L., BISHOP, M.R., SHARP, J.G., TEWARY, H.K., ARNESON, M.A., PIRRUCCELLO, S.J., RUDDON, R.W., KESSINGER, A. AND ZON, G. (1993). 'Systemic administration of a phosphorothioate oligonucleotide with a sequence complementary to p53 for acute myelogenous leukemia and myelodysplastic syndrome: initial results of a phase I trial.' *Antisense Research Development* **3**(4), 383–390.

BELLON, L., LEYDIER, C. AND BARASCUT, J.L. (1994). 4-Thio RNA: a novel class of sugar-modified B-RNA. In *Carbohydrate Modifications in Antisense Research*, ed. Y.S. Sanghvi and P.D. Cook, pp. 68–79. Washington, DC: American Chemical Society.

BENNETT, C.F., CHIANG, M-Y., CHAN, H. AND GRIMM, S. (1993). 'Use of cationic lipids to enhance the biological activity of antisense oligonucleotides.' *Journal of Liposome Research* **3**, 85–102.

BENNETT, C.F., CHIANG, M-Y., CHAN, H., SHOEMAKER, J.E.E. AND MIRABELLI, C.K. (1992). 'Cationic lipids enhance cellular uptake and activity of phosphorothioate antisense oligonucleotides.' *Molecular Pharmacology* **41**, 1023–1033.

BENNETT, C.F. AND CROOKE, S.T. (1996). 'Oligonucleotide-based inhibitors of cytokine expression and function.' In *Therapeutic Modulation of Cytokines*, ed. B. Henderson and M.W. Bodmer, pp. 171–193. Boca Raton: CRC Press.

BENNETT, C.F., KORNBRUST, D., HENRY, S., STECKER, K., HOWARD, R., COOPER, S., DUTSON, S., HALL, W. AND JACOBY, H.I. (1997). 'An ICAM-1 antisense oligonucleotide prevents and reverses dextran sulfate sodium-induced colitis in mice.' *Journal of Pharmacology and Experimental Therapy* **280**(2), 988–1000.

BOUTORINE, A., HUET, C. AND SAISON, T. (1991). *Cell penetration studies of oligonucleotide derivatized with cholesterol and porphyrins*. In Nucleic Acid Therapeutics.

BRESLAUER, K.J., FRANK, R., BLOCKER, H. AND MARKY, L.A. (1986). 'Predicting DNA duplex stability from base sequence.' *Proceedings of the National Academy of Sciences, USA* **83**, 3746–3750.

BURCH, R.M. AND MAHAN, L.C. (1991). 'Oligonucleotides antisense to the interleukin 1 receptor mRNA block the effects of interleukin 1 in cultured murine and human fibroblasts and in mice.' *Journal of Clinical Investigations* **88**, 1190–1196.

BURGESS, T.L., FISHER, E.F., ROSS, S.L., BREADY, J.V., QIAN, Y.X., BAYEWITCH, L.A., COHEN, A.M., HERRERA, C.J., HU, S.S.F., KRAMER, T.B., LOTT, F.D., MARTIN, F.H., PIERCE, G.F., SIMONET, L. AND FARRELL, C.L. (1995). 'The antiproliferative activity of c-myb and c-myc antisense oligonucleotides in smooth muscle cells is caused by a nonantisense mechanism.' *Proceedings of the National Academy of Sciences, USA* **92**, 4051–4055.

CAMPBELL, J.M., BACON, T.A. AND WICKSTROM, E. (1990). 'Oligodeoxynucleoside phosphorothioate stability in subcellular extracts, culture media, sera and cerebrospinal fluid.' *Journal of Biochemical and Biophysical Methods* **20**, 259–267.

CASTRO-ALAMANCOS, M.A. AND TORRES-ALEMAN, I. (1994). 'Learning of the conditioned eye-blink response is impaired by an antisense insulin-like growth factor I oligonucleotide.' *Proceedings of the National Academy of Sciences, USA* **91**, 10203–10207.

CAZENAVE, C., STEIN, C.A., LOREAU, N., THUONG, N.T., NECKERS, L.M., SUBASINGHE, C., H,LŠNE, C., COHEN, J.S. AND TOULM, J.J. (1989). 'Comparative inhibition of rabbit globin mRNA translation by modified antisense oligodeoxynucleotides.' *Nucleic Acids Research* **17**, 4255–4273.

CHA, X.Y., XU, H., NI, Q., PARTILLA, J.S., RICE, K.C., MATECKA, D., CALDERON, S.N., PORRECA, F., LAI, J. AND ROTHMAN, R.B. (1995). 'Opioid peptide receptor studies. 4. Antisense oligodeoxynucleotide to the delta opioid receptor delineates opioid receptor subtypes.' *Regulatory Peptides* **59**(2), 247–253.

CHENG, Y., GAO, W. AND HAN, F. (1991). 'Phosphorothioate oligonucleotides as potential

antiviral compounds against human immunodeficiency virus and herpes viruses.' *Nucleosides Nucleotides* **10**, 155–166.

CHIANG, M.Y., CHAN, H., ZOUNES, M.A., FREIER, S.M., LIMA, W.F. AND BENNETT, C.F. (1991). 'Antisense oligonucleotides inhibit intercellular adhesion molecule 1 expression by two distinct mechanisms.' *Journal of Biological Chemistry* **266**, 18162–18171.

CHIASSON, B.J., HOOPER, M.L., MURPHY, P.R. AND ROBERTSON, H.A. (1992). 'Antisense oligonucleotide eliminates *in vivo* expression of c-fos in mammalian brain.' *European Journal of Pharmacology* **227**, 451–453.

CHRISEY, L.A., WALZ, S.E., PAZIRANDEH, M. AND CAMPBELL, J.R. (1993). 'Internalization of oligodeoxyribonucleotides by *Vibrio parahemolyticus*.' *Antisense Research and Development* **3**, 367–381.

COHEN, J.S. (1993). 'Phosphorothioate oligodeoxynucleotides.' In *Antisense Research and Applications*, ed. S.T. Crooke and B. Lebleu, pp. 205–222. Boca Raton: CRC Press.

CORNISH, K.G., IVERSEN, P., SMITH, L., ARNESON, M. AND BAYEVER, E. (1993). 'Cardiovascular effects of a phosphorothioate oligonucleotide to p53 in the conscious rhesus monkey.' *Pharmacology Communications* **3**, 239–247.

COSSUM, P.A., SASMOR, H., DELLINGER, D., TRUONG, L., CUMMINS, L., OWENS, S.R., MARKHAM, P.M., SHEA, J.P. AND CROOKE, S. (1993). 'Disposition of the ^{14}C-labeled phosphorothioate oligonucleotide ISIS 2105 after intravenous administration to rats.' *Journal of Pharmacology and Experimental Therapeutics* **267**, 1181–1190.

COSSUM, P.A., TRUONG, L., OWENS, S.R., MARKHAM, P.M., SHEA, J.P. AND CROOKE, S.T. (1994). 'Pharmacokinetics of a ^{14}C-Labeled phosphorothioate oligonucleotide, ISIS 2105, after intradermal administration to rats.' *Journal of Pharmacology and Experimental Therapy* **269**, 89–94.

COWSERT, L.M., FOX, M.C., ZON, G. AND MIRABELLI, C.K. (1993). '*In vitro* evaluation of phosphorothioate oligonucleotides targeted to the E2 mRNA of papillomavirus: potential treatment of genital warts.' *Antimicrobial Agents Chemotherapy* **37**, 171–177.

CROOKE, R.M. (1991). '*In vitro* toxicology and pharmacokinetics of antisense oligonucleotides.' *Anti-Cancer Drug Design* **6**, 609–646.

CROOKE, R.M. (1993). Cellular uptake, distribution and metabolism of phosphorothioate, phosphodiester, and methylphosphonate oligonucleotides. In *Antisense Research and Applications*, ed. S.T. Crooke and B. Lebleu, pp. 427–449. Boca Raton: CRC Press.

CROOKE, R.M. (1993). *In vitro* and *in vivo* toxicology of first generation analogs. In *Antisense Research and Applications*, ed. S.T. Crooke and B. Lebleu, pp. 471–492. Boca Raton: CRC Press.

CROOKE, R.M., GRAHAM, M.J., COOKE, M.E. AND CROOKE, S.T. (1995). '*In vitro* pharmacokinetics of phosphorothioate antisense oligonucleotides.' *Journal of Pharmacology and Experimental Therapy* **275**(1), 462–473.

CROOKE, S.T. (1992). 'Therapeutic applications of oligonucleotides.' *Annual Review of Pharmacology and Toxicology* **32**, 329–376.

CROOKE, S.T. (1993). 'Progress toward oligonucleotide therapeutics: pharmacodynamic properties.' *The FASEB Journal* **7**, 533–539.

CROOKE, S.T. (1995). Oligonucleotide Therapeutics. In *Burger's Medicinal Chemistry and Drug Discovery, Vol. 1*, ed. M.E. Wolff, pp. 863–900. New York: Wiley and Sons, Inc.

CROOKE, S.T. (1995). *Therapeutic Applications of Oligonucleotides.* Austin, R.G. Landes Company.

CROOKE, S.T., GRAHAM, M.J., ZUCKERMAN, J.E., BROOKS, D., CONKLIN, B.S., CUMMINS, L.L., GREIG, M.J., GUINOSSO, C.J., KORNBRUST, D., MANOHARAN, M., SASMOR, H.M., SCHLEICH, T., TIVEL, K.L. AND GRIFFEY, R.H. (1996). 'Pharmacokinetic properties of several novel oligonucleotide analogs in mice.' *Journal of Pharmacology and Experimental Therapeutics* **277**(2), 923–937.

CROOKE, S.T., GRILLONE, L.R., TENDOLKAR, A., GARRETT, A., FRATKIN, M.J., LEEDS, J. AND BARR, W.H. (1994). 'A pharmacokinetic evaluation of 14C-labeled afovirsen sodium in patients with genital warts.' *Clinical and Pharmaceutical Therapeutics* **56**, 641–646.

CROOKE, S.T. AND LEBLEU, B. (1993). *Antisense Research and Applications.* Boca Raton: CRC Press.

CROOKE, S.T., LEMONIDIS, K.M., NIELSON, L., GRIFFEY, R. AND MONIA, B.P. (1995). 'Kinetic characteristics of E. coli RNase H1: cleavage of various antisense oligonucleotides–RNA duplexes.' *Biochemical Journal* **312**(2), 599–608.

CROUCH, R.J. AND DIRKSEN, M.L. (1985). Ribonucleases H. In *Nucleases*, ed. S.M. Linn and R.J. Roberts, p. 211. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

DE CLERCQ, E., ECKSTEIN, F. AND MERIGAN, T.C. (1969). 'Interferon induction increased through chemical modification of synthetic polyribonucleotide.' *Science* **165**, 1137–1140.

DE MESMAEKER, A., HAENER, R., MARTIN, P. AND MOSER, H.E. (1995). 'Antisense Oligonucleotides.' *Accounts of Chemical Research* **28**(9), 366–74.

DEAN, N.M. AND MCKAY, R. (1994). 'Inhibition of protein kinase C-alpha expression in mice after systemic administration of phosphorothioate antisense oligodeoxynucleotides.' *Proceedings of the National Academy of Sciences, USA* **91**, 11762–11766.

DEAN, N.M., MCKAY, R., MIRAGLIA, L., HOWARD, R., COOPER, S., GIDDINGS, J., NICKLIN, P., MEISTER, L., ZEIL, R., GEIGER, T., MULLER, M. AND FABBRO, D. (1996). 'Inhibition of growth of human tumor cell lines in nude mice by an antisense oligonucleotide inhibitor of PKC-alpha expression.' *Cancer Research* **56**(15), 3499–3507.

DESJARDINS, J., MATA, J., BROWN, T., GRAHAM, D., ZON, G. AND IVERSEN, P. (1995). 'Cholesteryl-conjugated phosphorothioate oligodeoxynucleotides modulate CYP2B1 expression *in vivo*.' *Journal of Drug Targeting* **2**, 477–485.

DOMINSKI, Z. AND KOLE, R. (1993). 'Restoration of correct splicing in thalassemic pre-mRNA by antisense oligonucleotides.' *Proceedings of the National Academy of Sciences, USA* **90**, 8673–8677.

DONIS-KELLER, H. (1979). 'Site specific enzymatic cleavage of RNA.' *Nucleic Acids Research* **7**(1), 179–192.

ECKER, D.J. (1993). Strategies for invasion of RNA secondary structure. In *Antisense Research and Applications*, ed. S.T. Crooke and R. Lebleu, pp. 387–400. Boca Raton: CRC Press.

ECKER, D.J., VICKERS, T.A., BRUICE, T.W., FREIER, S.M., JENISON, R.D., MANOHARAN, M. AND ZOUNES, M. (1992). 'Pseudo-half-knot formation with RNA.' *Science* **257**, 958–961.

EDER, P.S. AND WALDER, J.A. (1991). 'Ribonuclease H from K562 human erythroleukemia cells.' *Journal of Biological Chemistry* **266**, 6472–6479.

FLANAGAN, L.M., MCCARTHY, M.M., BROOKS, P.J., PFAFF, D.W. AND MCEWEN, B.S. (1993). 'Arginine vasopressin levels after daily infusions of antisense oligonucleotides into the supraoptic nucleus.' *Annals of NY Academy of Sciences* **689**, 520–521.

FREIER, S.M. (1993). Hybridization considerations affecting antisense drugs. In *Antisense Research and Applications*, ed. S.T. Crooke and B. Lebleu, pp. 67–82. Boca Raton: CRC Press.

FURDON, P.J., DOMINSKI, Z. AND KOLE, R. (1989). 'RNase H cleavage of RNA hybridized to oligonucleotides containing methylphosphonate, phosphorothioate and phosphodiester bonds.' *Nucleic Acids Research* **17**, 9193–9204.

GAGNOR, C., RAYNER, B., LEONETTI, J.P., IMBACH, J.L. AND LEBLEU, B. (1989). 'Alpha-DNA.IX: Parallel annealing of alpha-anomeric oligodeoxyribonucleotides to natural mRNA is required for interference in RNase H mediated hydrolysis and reverse transcription.' *Nucleic Acids Research* **17**(13), 5107–14.

GALBRAITH, W.M., HOBSON, W.C., GICLAS, P.C., SCHECHTER, P.J. AND AGRAWAL, S. (1994). 'Complement activation and hemodynamic changes following intravenous administration of phosphorothioate oligonucleotides in the monkey.' *Antisense Research Development* **4**(3), 201–206.

GAO, W.Y., HAN, F.S., STORM, C., EGAN, W. AND CHENG, Y.C. (1992). 'Phosphorothioate oligonucleotides are inhibitors of human DNA polymerases and RNase H: Implications for antisense technology.' *Molecular Pharmacology* **41**, 223–229.

GILES, R.V., SPILLER, D.G. AND TIDD, D.M. (1995). 'Detection of ribonuclease H-generated mRNA fragments in human leukemia cells following reversible membrane permeabilization in the presence of antisense oligodeoxynucleotides.' *Antisense Research and Development* **5**, 23–31.

GILES, R.V. AND TIDD, D.M. (1992). 'Increased specificity for antisense oligodeoxynucleotide targeting of RNA cleavage by RNase H using chimeric methylphosphonodiester/phosph odiester structures.' *Nucleic Acids Research* **20**, 763–770.

GILLARDON, F., BECK, H., UHLMANN, E., HERDEGEN, T., SANDKOHLER, J., PEYMAN, A. AND ZIMMERMANN, M. (1994). 'Inhibition of c-fos protein expression in rat spinal cord by antisense oligodeoxynucleotide superfusion.' *European Journal of Neurosciences* **6**, 880–884.

GLOVER, J.M., LEEDS, J.M., MANT, T.G.K., KISNER, D.L., ZUCKERMAN, J., LEVIN, A.A. AND SHANAHAN, W.R. (1996). 'Phase I safety and pharmacokinetic profile of an ICAM-1 antisense oligodeoxynucleotide (ISIS 2302).' *Journal of Pharmacology and Experimental Therapy (in press).*

GRAHAM, M.J., FREIER, S.M., CROOKE, R.M., ECKER, D.J., MASLOVA, R.N. AND LESNIK, E.A. (1993). 'Tritium labeling of antisense oligonucleotides by exchange with tritiated water.' *Nucleic Acids Research* **21**, 3737–3743.

GUTIERREZ, A.J., TERHORST, T.J., MATTEUCCI, M.D. AND FROEHLER, B.C. (1994). '5-heteroaryl-2'-deoxyuridine analogs. Synthesis and incorporation into high-affinity oligonucleotides.' *Journal of the American Chemical Society* **116**, 5540–5544.

GYURKO, R., WIELBO, D. AND PHILLIPS, M.I. (1993). 'Antisense inhibition of AT1 receptor mRNA and angiotensinogen mRNA in the brain of spontaneously hypertensive rats reduces hypertension of neurogenic origin.' *Regulatory Peptides* **49**, 167–174.

HALL, J., HUSKEN, D., PIELES, U., MOSER, H.E. AND HANER, R. (1994). 'Efficient sequence-specific cleavage of RNA using novel europium complexes conjugated to oligonucleotides.' *Chemical Biology* **1**(3), 185–90.

HANVEY, J.C., PEFFER, N.C., BISI, J.E., THOMSON, S.A., CADILLA, R., JOSEY, J.A., RICCA, D.J., HASSMAN, C.F., BONHAM, M.A., AU, K.G., CARTER, S.G., BRUCKENSTEIN, D.A., BOYD, A.L., NOBLE, S.A. AND BABISS, L.E. (1992). 'Antisense and antigene properties of peptide nucleic acids.' *Science* **258**, 1481–1485.

HARTMANN, G., KRUG, A., WALLER-FONTAINE, K. AND ENDRES, S. (1996). 'Oligodeoxynucleotides enhance lipopolysaccharide-stimulated synthesis of tumor necrosis factor: dependence on phosphorothioate modification and reversal by Heparin.' *Molecular Medicine* **2**(4), 429–438.

HAWLEY, P. AND GIBSON, I. (1996). 'Interaction of oligodeoxynucleotides with mammilian cells.' *Antisense and Nucleic Drug Development* **6**, 185–195.

HEILIG, M., ENGEL, J.A. AND SODERPALM, B. (1993). 'C-fos antisense in the nucleus accumbens blocks the locomotor stimulant action of cocaine.' *European Journal of Pharmacology* **236**, 339–340.

HELENE, C. AND TOULME, J.J. (1989). Control of gene expression by oligonucleotides covalently linked to intercalating agents and nucleic acid-cleaving reagents. In *Oligonucleotides: Antisense Inhibitors of Gene Expression*, ed. J.S. Cohen, pp. 137–172. Boca Raton: CRC Press.

HENRY, S., GICLAS, P.C., LEEDS, J., PANGBURN, M., AULETTA, C., LEVIN, A.A. AND KOMBRUST, D.J. (1997). 'Activation of the alternative pathway of complement by a phosphorothioate oligonucleotide: potential mechanism of action.' *Journal of Pharmacology and Experimental Therapy* **281**, 810–816.

HENRY, S., NOVOTNY, W., LEEDS, J., AULETTA, C. AND KOMBRUST, D.J. (Submitted). 'Inhibition of clotting parameters by a phosphorothioate oligonucleotide.' *Antisense Nucleic Acid Drug Developments.*

HENRY, S.P., GRILLONE, L.R., ORR, J.L., BRUNNER, R.H. AND KOMBRUST, D.J. (1997). 'Comparison of the toxicity profiles of ISIS 1082 and ISIS 2105, phosphorothioate oligonucleotides, following subacute intradermal administration in Sprague–Dawley rats.' *Toxicology* **116**(1–3), 77–88.

HENRY, S.P., TAYLOR, J., MIDGLEY, L., LEVIN, A.A. AND KOMBRUST, D.J. (In Press). 'Evaluation of the toxicity profile of ISIS 2302, a phosphorothioate oligonucleotide in a 4-week study in CD-1 mice.' *Antisense Nucleic Acid Drug Developments.*

HENRY, S.P., ZUCKERMAN, J.E., ROJKO, J., HALL, W.C., HARMAN, R.J., KITCHEN, D. AND CROOKE, S.T. (1997). 'Toxicologic properties of several novel oligonucleotide analogs in mice.' *Anti-Cancer Drug Design* **12**(1), 1–14.

HERTL, M., NECKERS, L.M. AND KATZ, S.I. (1995). 'Inhibition of interferon-gamma-induced intercellular adhesion molecule-1 expression on human keratinocytes by phosphorothioate antisense oligodeoxynucleotides is the consequence of antisense-spe-

cific and antisense-non-specific effects.' *Journal of Investigative Dermatology* **104**, 813–818.

HIGGINS, K.A., PEREZ, J.R., COLEMAN, T.A., DORSHKIND, K., MCCOMAS, W.A., SARMIENTO, U.M., ROSEN, C.A. AND NARAYANAN, R. (1993). 'Antisense inhibition of the p65 subunit of NF-kappaB blocks tumorigenicity and causes tumor regression.' *Proceedings of the National Academy of Sciences, USA* **90**, 9901–9905.

HIJIYA, N., ZHANG, J., RATAJCZAK, M.Z., KANT, J.A., DERIEL, K., HERLYN, M., ZON, G. AND GEWIRTZ, A.M. (1994). 'Biologic and therapeutic significance of MYB expression in human melanoma.' *Proceedings of the National Academy of Sciences, USA* **91**, 4499–4503.

HODGES, D. AND CROOKE, S.T. (1995). 'Inhibition of splicing of wild-type and mutated luciferase-adenovirus pre-mRNA by antisense oligonucleotides.' *Molecular Pharmacology* **48**, 905–918.

HOKE, G.D., DRAPER, K., FREIER, S.M., GONZALEZ, C., DRIVER, V.B., ZOUNES, M.C. AND ECKER, D.J. (1991). 'Effects of phosphorothioate capping on antisense oligonucleotide stability, hybridization and antiviral efficacy versus herpes simplex virus infection.' *Nucleic Acids Research* **19**, 5743–5748.

HOOPER, M.L., CHIASSON, B.J., ROBERTSON, H.A. (1994). 'Infusion into the brain of an antisense oligonucleotide to the immediate-early gene c-fos suppresses production of Fos and produces a behavioral effect.' *Neuroscience* **63**, 917–924.

HUTCHERSON, S.L., PALESTINE, A.G., CANTRILL, H.L., LIEBERMAN, R.M., HOLLAND, G.N. AND ANDERSON, K.P. (1995). 'Antisense oligonucleotide safety and efficacy for CMV retinitis in AIDS patients.' *35th ICAAC*, 204.

INOUE, H., IMURA, A. AND OHTSUKA, E. (1985). 'Synthesis and hybridization of dodecadeoxyribonucleotides containing a fluorescent pyridopyrimidine deoxynucleoside.' *Nucleic Acids Research* **13**(19), 7119–7128.

IVERSEN, P. (1991). '*In vivo* studies with phosphorothioate oligonucleotides: pharmacokinetics prologue.' *Anticancer Drug Design* **6**(6), 531–8.

JOOS, R.W. AND HALL, W.H. (1969). 'Determination of binding constants of serum albumin for penicillin.' *Journal of Pharmacology and Experimental Therapeutics* **166**, 113.

KATZ, S.M., BROWNE, B., PHAM, T., WANG, M.E., BENNETT, C.F., STEPKOWSKI, S.M. AND KAHAN, B.D. (1995). 'Efficacy of ICAM-1 antisense oligonucleotide in pancreatic islet transplanation.' *Transplant Proceedings* **27**(6), 3214.

KAWASAKI, A.M., CASPER, M.D., FREIER, S.M., LESNIK, E.A., ZOUNES, M.C., CUMMINS, L.L., GONZALEZ, C. AND COOK, P.D. (1993). 'Uniformly modified 2'-deoxy-2'-fluoro phosphorothioate oligonucleotides as nuclease-resistant antisense compounds with high affinity and specificity for RNA targets.' *Journal of Medical Chemistry* **36**, 831–841.

KINDY, M.S. (1994). 'NMDA receptor inhibition using antisense oligonucleotides prevents delayed neuronal death in gerbil hippocampus following cerebral ischemia.' *Neuroscience Research Communications* **14**, 175–183.

KITAJIMA, I., SHINOHARA, T., BILAKOVICS, J., BROWN, D.A., XIAO, X. AND NERENBERG, M. (1992). 'Ablation of transplanted HTLV-1 tax-transformed tumors in mice by antisense inhibition of NF-KB.' *Science* **258**, 1792–1795.

KONRADI, C., COLE, R.L., HECKERS, S. AND HYMAN, S.E. (1994). 'Amphetamine regulates gene expression in rat striatum via transcription factor CREB.' *Journal of Neuroscience* **14**, 5623–5634.

KULKA, M., SMITH, C.C., AURELIAN, L., FISHELEVICH, R., MEADE, K., MILLER, P. AND TS'O, P.O.P. (1989). 'Site specificity of the inhibitory effects of oligo(nucleoside methylphosphonate)s complementary to the acceptor splice junction of herpes simplex virus type 1 immediate early mRNA 4.' *Proceedings of the National Academy of Sciences, USA* **86**, 6868–6872.

KUMASAKA, T., QUINLAN, W.M., DOYLE, N.A., CONDON, T.P., SLIGH, J., TAKEI, F., BEAUD, BENNETT, C.F. AND DOERSCHUK, C.M. (1996). '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.' *Journal of Clinical Investigations* **97**(10), 2362–2369.

LAI, J., BILSKY, E.J., ROTHMAN, R.B. AND PORRECA, F. (1994). 'Treatment with antisense

oligodeoxynucleotide to the opioid δ receptor selectively inhibits δ_2-agonist antinociception.' *Neuroreport* **5**, 1049–1052.

LAMOND, A.I. AND SPROAT, B.S. (1993). 'Antisense oligonucleotides made of 2'-O-alkylRNA: their properties and applications in RNA biochemistry.' *FEBS Letters* **325**, 123–127.

LEMAITRE, M., BAYARD, B. AND LEBLEU, B. (1987). 'Specific antiviral activity of a poly(L-lysine)-conjugated oligodeoxyribonucleotide sequence complementary to vesicular stomatitis virus N protein mRNA initiation site.' *Proceedings of the National Academy of Sciences, USA* **84**, 648–652.

LIEBSCH, G., LANDGRAF, R., GERSTBERGER, R., PROBST, J.C., WOTJAK, C.T., ENGELMANN, M., HOLSBOER, F. AND MONTKOWSKI, A. (1995). 'Chronic infusion of a CRH1 receptor antisense oligodeoxynucleotide into the central nucleus of the amygdala anxiety-related behavior in socially defeated rats.' *Regulatory Peptides* **59**(2), 229–239.

LIMA, W.F., MONIA, B.P., ECKER, D.J. AND FREIER, S.M. (1992). 'Implication of RNA structure on antisense oligonucleotide hybridization kinetics.' *Biochemistry* **31**, 12055–12061.

LIN, K.Y., JONES, R.J. AND MATTEUCCI, M. (1995). 'Tricyclic-2'-deoxycytidine analogs: Synthesis and incorporation into oligodeoxynucleotides which have endhanced binding to complementary RNA.' *Journal of the American Chemical Society* **117**, 3873–3874.

LIN, P.K.T. AND BROWN, D.M. (1989). 'Synthesis and duplex stability of oligonucleotides containing cytosine-thymine analogues.' *Nucleic Acids Research* **17**, 10373–10383.

LOKE, S.L., STEIN, C.A., ZHANG, X.H., MORI, K., NAKANISHI, M., SUBASINGHE, C., COHEN, J.S. AND NECKERS, L.M. (1989). 'Characterization of oligonucleotide transport into living cells.' *Proceedings of the National Academy of Sciences, USA* **86**, 3474–3478.

MCCARTHY, M.M., MASTERS, D.B., RIMVALL, K., SCHWARTZ-GIBLIN, S. AND PFAFF, D.W. (1994). 'Intracerebral administration of antisense oligodeoxynucleotides to GAD_{65} and GAD_{67} mRNAs modulate reproductive behavior in the female rat.' *Brain Research* **636**, 209–220.

MCCARTHY, M.M., NIELSEN, D.A. AND GOLDMAN, D. (1995). 'Antisense oligonucleotide inhibition of tryptophan hydroxylase activity in mouse brain.' *Regulatory Peptides* **59**(2), 163–170.

MCMANAWAY, M.E., NECKERS, L.M., LOKE, S.L., AL-NASSER, A.A., REDNER, R.L., SHIRAMIZU, B.T., GOLDSCHMIDTS, W.L., HUBER, B.E., BHATIA, K. AND MAGRATH, I.T. (1990). 'Tumour-specific inhibition of lymphoma growth by an antisense oligodeoxynucleotide.' *Lancet* **335**, 808–811.

MAHER, L.J., III, WOLD, B. DERVAN, P.B. (1989). 'Inhibition of DNA binding proteins by oligonucleotide-directed triple helix formation.' *Science* **245**, 725–730.

MAIER, J.A.M., VOULALAS, P., ROEDER, D. AND MACIAG, T. (1990). 'Extension of the life-span of human endothelial cells by an interleukin-1α antisense oligomer.' *Science* **249**, 1570–1574.

MAJUMDAR, C., STEIN, C.A., COHEN, J.S., BRODER, S. AND WILSON, S.H. (1989). 'Stepwise mechanism of HIV Reverse transcriptase: primer function of phosphorothioate oligodeoxynucleotide.' *Biochemistry* **28**, 1340–1346.

MANI, S.K., BLAUSTEIN, J.D., ALLEN, J.M.C., LAW, S.W., O'MALLEY, B.W. AND CLARK, J.H. (1994). 'Inhibition of rat sexual behavior by antisense oligonucleotides to the progesterone receptor.' *Endocrinology* **135**, 1409–1414.

MANOHARAN, M. (1993). Designer antisense oligonucleotides: conjugation chemistry and functionality placement. In *Antisense Research and Applications*, ed. S.T. Crooke and B. Lebleu, pp. 303–349. Boca Raton: CRC Press.

MARTIN, F.H., CASTRO, M.M., ABOUL-ELA, F. AND TINOCO, I.J. (1985). 'Base pairing involving deoxyinosine: implications for probe.' *Nucleic Acids Research* **13**, 8927–8938.

MARTIN, P. (1995). 'Ein neuer zugang zu 2'-O-alkylribonucleosiden und eigenschaften deren oligonucleotide.' *Helvetica Chimica Acta* **78**, 486–489.

MILLER, P.S. (1989). Non-ionic antisense oligonucleotides. In *Oligodeoxynucleotides: Antisense Inhibitors of Gene Expression*, ed. J.S. Cohen, p. 79. CRC Press.

MIRABELLI, C.K., BENNETT, C.F., ANDERSON, K. AND CROOKE, S.T. (1991). '*In vitro* and *in vivo* pharmacologic activities of antisense oligonucleotides.' *Anti-Cancer Drug Design* **6**, 647–661.

MIYAO, T., TAKAKURA, Y., AKIYAMA, T., YONEDA, F., SEZAKI, H. AND HASHIDA, M. (1995).

'Stability and pharmacokinetic characteristics of oligonucleotides modified at terminal linkages in mice.' *Antisense Research Developments* **5**(2), 115–21.

MIZOGUCHI, H., NARITA, M., NAGASE, H. AND TSENG, L.F. (1995). 'Antisense oligodeoxynucleotide to a delta-opioid receptor blocks the antinociception induced by cold water swimming.' *Regulatory Peptides* **59**(2), 255–259.

MONIA, B., JOHNSTON, J.F., SASMOR, H. AND CUMMINS, L.L. (1996). 'Nuclease resistance and antisense activity of modified oligonucleotides targeted to Ha-ras.' *Journal of Biological Chemistry* **24**(14), 14533–14540.

MONIA, B.P., JOHNSTON, J.F., ECKER, D.J., ZOUNES, M., LIMA, W.F. AND FREIER, S.M. (1992). 'Selective inhibition of mutant Ha-ras mRNA expression by antisense oligonucleotides.' *Journal of Biological Chemistry* **267**, 19954–19962.

MONIA, B.P., JOHNSTON, J.F., GEIGER, T., MULLER, M. AND FABBRO, D. (1995). 'Antitumor activity of a phosphorothioate oligodeoxynucleotide targeted against C-raf kinase.' *Nature Medicine* **2**(6), 668–675.

MONIA, B.P., LESNIK, E.A., GONZALEZ, C., LIMA, W.F., MCGEE, D., GUINOSSO, C.J., KAWASAKI, A.M., COOK, P.D. AND FREIER, S.M. (1993). 'Evaluation of 2(modified oligonucleotides containing deoxy gaps as antisense inhibitors of gene expression.' *Journal of Biological Chemistry* **268**, 14514–14522.

MORISHITA, R., GIBBONS, G.H., ELLISON, K.E., NAKAJIMA, M., ZHANG, L., KANEDA, Y., OGIHARA, T. AND DZAU, V.J. (1993). 'Single intraluminal delivery of antisense cdc2 kinase and proliferating-cell nuclear antigen oligonucleotides results in chronic inhibition of neointimal hyperplasia.' *Proceedings of the National Academy of Sciences, USA* **90**, 8474–8478.

MORISHITA, R., GIBBONS, G.H., KANEDA, Y., OGIHARA, T. AND DZAU, V.J. (1994). 'Pharmacokinetics of antisense oligodeoxyribonucleotides (cyclin B1 and CDC 2 kinase) in the vessel wall *in vivo*: enhanced therapeutic utility for restenosis by HVJ-liposome delivery.' *Gene* **149**, 13–19.

MORRIS, M., LI, P., BARRETT, C. AND CALLAHAN, M.F. (1995). 'Oxytocin antisense reduces salt intake in the baroreceptor-denervated rat.' *Regulatory Peptides* **59**(2), 261–266.

MORVAN, F., RAYNER, B. AND IMBACH, J.L. (1991). 'Alpha-oligonucleotides: a unique class of modified chimeric nucleic acids.' *Anticancer Drug Design* **6**(6), 521–9.

NAGEL, K.M., HOLSTAD, S.G. AND ISENBERG, K.E. (1993). 'Oligonucleotide pharmacotherapy: an antigene strategy.' *Pharmacotherapy* **13**(3), 177–188.

NECKERS, L.M. (1993). Cellular internalization of oligodeoxynucleotides. In *Antisense Research and Applications*, ed. S.T. Crooke and B. Lebleu, pp. 451–460. Boca Raton: CRC Press.

NESTEROVA, M. AND CHO-CHUNG, Y.S. (1995). 'A single-injection protein kinase A-directed antisense treatment to inhibit tumor growth.' *Nature Medicine* **1**, 528–533.

NEUMANN, I., PORTER, D.W.F., LANDGRAF, R. AND PITTMAN, Q.J. (1994). 'Rapid effect on suckling of an oxytocin antisense oligonucleotide administered into rat supraoptic nucleus.' *American Journal of Physiology* **267**, R852–R858.

NIKIFOROV, T.T. AND CONNOLLY, B.A. (1991). 'The synthesis of oligodeoxynucleotides containing 4-thiothymidine residues.' *Tetrahedron Letters* **32**(31), 3851–4.

NYCE, J.W. AND METZGER, W.J. (1997). 'DNA antisense therapy for asthma in an animal model.' *Nature* **385**(6618), 721–725.

OFFENSPERGER, W.B., OFFENSPERGER, S., WALTER, E., TEUBNER, K., IGLOI, G., BLUM, H.E. AND GEROK, W. (1993). '*In vivo* inhibition of duck hepatitis B virus replication and gene expression by phosphorothioate modified antisense oligodeoxynucleotides.' *EMBO Journal* **12**, 1257–1262.

OGO, H., HIRAI, Y., MIKI, S., NISHIO, H., AKIYAMA, M. AND NAKATA, Y. (1994). 'Modulation of substance P/neurokinin-1 receptor in human astrocytoma cells by antisense oligodeoxynucleotides.' *General Pharmacology* **25**, 1131–1135.

OSEN-SAND, A., CATSICAS, M., STAPLE, J.K., JONES, K.A., AYALA, G., KNOWLES, J., GRENNINGLOH, G. AND CATSICAS, S. (1993). 'Inhibition of axonal growth by SNAP-25 antisense oligonucleotides *in vitro* and *in vivo*.' *Nature* **364**, 445–448.

OSEN-SAND, A., CATSICAST, M., STAPLE, J.K., JONES, K.A., AYALA, G., KNOWLES, J.,

GRENNINGLOH, G. AND CATSICAS, S. (1993). 'Inhibition of axonla growth by SNAP-25 antisense oligonucleotides *in vitro* and *in vivo*.' *Nature* **364**, 445–448.

PARMENTIER, G., SCHMITT, G., DOLLE, F. AND LUU, B. (1994). 'A convergent synthesis of 2'-O-methyl uridine.' *Tetrahedron* **50**(18), 5361–8.

PERLAKY, L., SAIJO, Y., BUSCH, R.K., BENNETT, C.F., MIRABELLI, C.K., CROOKE, S.T. AND BUSCH, H.(1993). 'Growth inhibition of human tumor cell lines by antisense oligonucleotides designed to inhibit p120 expression.' *Anti-Cancer Drug Design* **8**, 3–14.

PHILLIPS, M.I., WIELBO, D. AND GYURKO, R. (1994). 'Antisense inhibition of hypertension: a new strategy for renin-angiotensin candidate genes.' *Kidney International* **46**, 1554–1556.

PITSCH, S., KRISHNAMURTHY, R., BOLLI, M., WENDEBORN, S., HOLZNER, A., MINTON, M., LESUEUR, C., SCHLOENVOGT, I., JAUN, B. AND ESCHENMOSER, A. (1995). 'Pyranosyl-RNA ('p-RNA'): base-pairing selectivity and potential to replicate.' *Helvetica Chimica Acta* **78**(7), 1621–35.

POLLIO, G., XUE, P., ZANISI, M., NICOLIN, A. AND MAGGI, A. (1993). 'Antisense oligonucleotide blocks progesterone-induced lordosis behavior in ovariectomized rats.' *Molecular Brain Research* **19**, 135–139.

QIN, Z.H., ZHOU, L.W., ZHANG, S.P., WANG, Y. AND WEISS, B. (1995). 'D2 dopamine receptor antisense oligodeoxynucleotide inhibits the synthesis of a functional pool of D2 dopamine receptors.' *Molecular Pharmacology* **48**(4), 730–737.

QUARTIN, R.S., BRAKEL, C.L. AND WETMUR, J.G. (1989). 'Number and distribution of methylphosphonate linkages in oligodeoxynucleotides affect exo- and endonuclease sensitivity and ability to form RNase H substrates.' *Nucleic Acids Research* **17**, 7253–7262.

QUATTRONE, A., PAPUCCI, L., SCHIAVONE, N., MINI, E. AND CAPACCIOLI, S. (1994). 'Intracellular enhancement of intact antisense oligonucleotide steady-state levels by cationic lipids.' *Anti-Cancer Drug Design* **9**, 549–553.

RAPPAPORT, J., HANSS, B., KOPP, J.B., COPELAND, T.D., BRUGGEMAN, L.A., COFFMAN, T.M. AND KLOTMAN, P.E. (1995). 'Transport of phosphorothioate oligonucleotides in kidney: Implications for molecular therapy.' *Kidney International* **47**, 1462–1469.

RATAJCZAK, M.Z., KANT, J.A., LUGER, S.M., HUIYA, N., ZHANG, J., ZON, G. AND GEWIRTZ, A.M. (1992). '*In vivo* treatment of human leukemia in a scid mouse model with c-myb antisense oligodeoxynucleotides.' *Proceedings of the National Academy of Sciences, USA* **89**, 11823–11827.

ROSOLEN, A., WHITESELL, L., IKEGAKI, N., KENNETT, R.H. AND NECKERS, L.M. (1990). 'Antisense inhibition of single copy N-myc expression results in decreased cell growth without reduction of c-myc protein in a neuroepithelioma cell line.' *Cancer Research* **50**(19), 6316–22.

SAKAI, R.R., MA, L.Y., HE, P.F. AND FLUHARTY, S.J. (1995). 'Intracerebroventricular administration of angiotensin type 1 (AT1) receptor antisense oligonucleotides attenuate thirst in the rat.' *Regulatory Peptides* **59**(2), 183–192.

SANDS, H., GOREY-FERET, L.J., COCUZZA, A.J., HOBBS, F.W., CHIDESTER, D. AND TRAINOR, G.L. (1994). 'Biodistribution and metabolism of internally ^{3}H-labeled oligonucleotides. I. Comparison of a phosphodiester and a phosphorothioate.' *Molecular Pharmacology* **45**, 932–943.

SANDS, H., GOREY-FERET, L.J., HO, S.P., BAO, Y., COCUZZA, A.J., CHIDESTER, D. AND HOBBS, F.W. (1995). 'Biodistribution and metabolism of internally ^{3}H-labeled oligonucleotides. II. 3',5-blocked oligonucleotides.' *Molecular Pharmacology* **47**, 636–646.

SANGHVI, Y.S. (1993). Heterocyclic base modifications in nucleic acids and their applications in antisense oligonucleotides. In *Antisense Research and Applications*, ed. S.T. Crooke and B. Lebleu, pp. 273–288. Boca Raton: CRC Press.

SANGHVI, Y.S. AND COOK, P.D. (eds.) (1994). *Carbohydrate Modifications in Antisense Research, ACS Symposium Series No. 580*, pp. 232. Washington, DC: American Chemical Society.

SANGHVI, Y.S., HOKE, G.D., FREIER, S.M., ZOUNES, M.C., GONZALEZ, C., CUMMINS, L., SASMOR, H. AND COOK, P.D. (1993). 'Antisense oligodeoxynucleotides: Synthesis, biophysical and biological evaluation of oligodeoxynucleotides containing modified pyrimidines.' *Nucleic Acids Research* **21**, 3197–3203.

SANTALUCIA, J., JR., KIERZEK, R. AND TURNER, D.H. (1991). 'Functional group substitutions as probes of hydrogen bonding between GA mismatches in RNA internal loops.' *Journal of the American Chemical Society* **113**, 4313–4322.

SAXENA, S.K. AND ACKERMAN, E.J. (1990). 'Microinjected oligonucleotides complementary to the α-sarcin loop of 28 S RNA abolish protein synthesis in *Xenopus* oocytes.' *Journal of Biological Chemistry* **265**, 3263–3269.

SBURLATI, A.R., MANROW, R.E. AND BERGER, S.L. (1991). 'Prothymosin alpha antisense oligomers inhibit myeloma cell division.' *Proceedings of the National Academy of Sciences, USA* **88**, 253–257.

SCHWAB, G., CHAVANY, C., DUROUX, I., GOUBIN, G., LEBEAU, J., HELENE, C. AND SAISON-BEHMOARAS, T. (1994). 'Antisense oligonucleotides adsorbed to polyalkylcyanoacrylate nanoparticles specifically inhibit mutated Ha-*ras*-mediated cell proliferation and tumorigenicity in nude mice.' *Proceedings of the National Academy of Sciences, USA* **91**(22), 10460–10464.

SEELA, F., KAISER, K. AND BINDIG, U. (1989). '2'-Deoxy-.beta.-D-ribofuranosides of N6-methylated 7-deazaadenine and 8-aza-7-deazaadenine: solid-phase synthesis of oligodeoxyribonucleotides and properties of self-complementary duplexes.' *Helvetica Chimica Acta* **72**(5), 868–81.

SEELA, F., RAMZAEVA, N. AND CHEN, Y. (1995). 'Oligonucleotide duplex stability controlled by the 7-substituents of 7-deazaguanine bases.' *Bioorganic Medical Chemical Letters* **5**(24), 3049–52.

SIMONS, M., EDELMAN, E.R., DEKEYSER, J.L., LANGER, R., ROSENBERG, R.D. (1992). 'Antisense c-myb oligonucleotides inhibit arterial smooth muscle cell accumulation *in vivo*.' *Nature* **359**, 67–70.

SIMONS, M., EDELMAN, E.R. AND ROSENBERG, R.D. (1994). 'Antisense proliferating cell nuclear antigen oligonucleotides inhibit intimal hyperplasia in a rat carotid artery injury model.' *Journal of Clinical Investigations* **93**, 2351–2356.

SKORSKI, T., NIEBOROWSKA-SKORSKA, M., NICOLAIDES, N.C., SZCZYLIK, C., IVERSEN, P., IOZZO, R.V., ZON, G. AND CALABRETTA, B. (1994). 'Suppression of Philadelphia leukemia cell growth in mice by *BCR-ABL* antisense oligodeoxynucleotide.' *Proceedings of the National Academy of Sciences, USA* **91**, 4504–4508.

SKUTELLA, T., PROBST, J.C., JIRIKOWSKI, G.F., HOLSBOER, F. AND SPANAGEL, R. (1994). 'Ventral tegmental area (VTA) injections of tyrosine hydroxylase phosphorothioate antisense oligonucleotide suppress operant behavior in rats.' *Neuroscience Letters* **167**, 55–58.

SMITH, C.C., AURELIAN, L., REDDY, M.P., MILLER, P.S. AND TS'O, P.O.P. (1986). 'Antiviral effect of an oligo(nucleoside methylphosphonate) complementary to the splice junction of herpes simplex virus type 1 immediate early pre-mRNAs 4 and 5.' *Proceedings of the National Academy of Sciences, USA* **83**, 2787–2791.

SPROAT, B.S., IRIBARREN, A.M., GARCIA, R.G. AND BEIJER, B. (1991). 'New synthetic routes to synthons suitable for 2'-0-allyloligoribonucleotide assembly.' *Nucleic Acids Research* **19**(4), 733–738.

SPROAT, B.S., LAMOND, A.I. (1993). 2'-O-alkyloligoribonucleotides. In *Antisense Research and Applications*, ed. S.T. Crooke and B. Lebleu, pp. 351–362. Boca Raton: CRC Press.

SPROAT, B.S., LAMOND, A.I., BEIJER, B., NEUNER, P. AND RYDER, U. (1989). 'Highly efficient chemical synthesis of 2'-O-methyloligoribonucleotides and tetrabiotinylated derivatives, novel probes that are resistant to degradation by RNA or DNA specific nucleases.' *Nucleic Acids Research* **17**, 3373–3386.

SRINIVASAN, S.K., TEWARY, H.K. AND IVERSEN, P.L. (1995). 'Characterization of binding sites, extent of binding, and drug interactions of oligonucleotides with albumin.' *Antisense Research Developments* **5**(2), 131–9.

STEIN, C.A. AND CHENG, Y.C. (1993). 'Antisense oligonucleotides as therapeutic agents-Is the bullet really magical?' *Science* **261**, 1004–1012.

STEIN, C.A., NECKERS, M., NAIR, B.C., MUMBAUER, S., HOKE, G. AND PAL, R. (1991). 'Phosphorothioate Oligodeoxycytidine Interferes with Binding of HIV-1 gp120 to CD4.' *Journal of Acquired Immune Deficiency Syndromes* **4**, 686–693.

STEPKOWSKI, S.M., TU, Y., CONDON, T.P. AND BENNETT, C.F. (1994). 'Blocking of heart

allograft rejection by intercellular adhesion molecule-1 antisense oligonucleotides alone or in combination with other immunosuppressive modalities.' *Journal of Immunology* **153**, 5336–5346.

STEPKOWSKI, S.M., TU, Y., CONDON, T.P. AND BENNETT, C.F. (1995). 'Induction of transplantation tolerance by treatment with ICAM-1 antisense oligonucleotides and anti-LFA-1 monoclonal antibodies.' *Transplant Proceedings* **27**, 113.

SUZUKI, S., PILOWSKY, P., MINSON, J., ARNOLDA, L., LLEWELLYN-SMITH, I.J. AND CHALMERS, J. (1994). 'c-fos antisense in rostral ventral medulla reduces arterial blood pressure.' *American Journal of Physiology* **266**(4 Pt 2), R1418–R1422.

TAKAKURA, Y., MAHATO, R.I., YOSHIDA, M., KANAMARU, T. AND HASHIDA, M. (1996). 'Uptake characteristics of oligonucleotides in the isolated rat liver perfusion system.' *Antisense Nucleic Acid Drug Developments* **6**, 177–183.

TAO, L.F., MARX, K.A., WONGWIT, W., JIANG, Z., AGRAWAL, S. AND COLEMAN, R.M. (1995). 'Uptake, intracellular distribution, and stability of oligodeoxynucleotide phosphorothioate by *Schistosoma mansoni*.' *Antisense Research Developments* **5**(2), 123–9.

TEMSAMANI, J., TANG, J., PADMAPRIYA, A., KUBERT, M. AND AGRAWAL, S. (1993). 'Pharmacokinetics, biodistribution, and stability of capped oligodeoxynucleotide phosphorothioates in mice.' *Antisense Research and Development* **3**, 277–284.

THUONG, N.T., ASSELINE, U. AND MONTENEY-GARESTIER, T. (1989). Oligodeoxynucleotides covalently linked to intercalating and reactive substances: synthesis, characterization and physicochemical studies. In *Oligodeoxynucleotides: Antisense Inhibitors of Gene Expression*, p. 25. CRC Press.

TISCHMEYER, W., GRIMM, R., SCHICKNICK, H., BRYSCH, W. AND SCHLINGENSIEPEN, K.H. (1994). 'Sequence-specific impairment of learning by c-jun antisense oligonucleotides.' *Neuroreport* **5**, 1501–1504.

VASANTHAKUMAR, G. AND AHMED, N.K. (1989). 'Modulation of drug resistance in a daunorubicin resistant subline with oligonucleoside methylphosphonates [published erratum appears in *Cancer Communications*, 1990,2(8):295].' *Cancer Communications* **1**(4), 225–32.

VICKERS, T., BAKER, B.F., COOK, P.D., ZOUNES, M., BUCKHEIT, R.W., JR., GERMANY, J., ECKER AND D.J. (1991). 'Inhibition of HIV-LTR gene expression by oligonucleotides targeted to the TAR element.' *Nucleic Acids Research* **19**, 3359–3368.

VLASSOV, V.V. (1989). Inhibition of tick-borne viral encephalitis expression using covalently linked oligonucleotide analogs. Oligodeoxynucleotides as antisense inhibitor of gene expression. Therapeutic implications (unpublished conference proceedings).

WAGNER, R.W., MATTEUCCI, M.D., LEWIS, J.G., GUTIERREZ, A.J., MOULDS, C. AND FROEHLER, B.C. (1993). 'Antisense gene inhibition by oligonucleotides containing C-5 propyne pyrimidines.' *Science* **260**, 1510–1513.

WAHLESTEDT, C., PICH, E.M., KOOB, G.F., YEE, F. AND HEILIG, M. (1993). 'Modulation of anxiety and neuropeptide Y-Y1 receptors by antisense oligodeoxynucleotides.' *Science* **259**, 528–531.

WALKER, K., ELELA, S.A. AND NAZAR, R.N. (1990). 'Inhibition of Protein Synthesis by Anti-5.8 S rRNA Oligodeoxyribonucleotides.' *Journal of Biological Chemistry* **265**, 2428–2430.

WANG, S., LEE, R.J., CAUCHON, G., GORENSTEIN, D.G. AND LOW, P.S. (1995). 'Delivery of antisense oligodeoxyribonucleotides against the human epidermal growth factor receptor into cultured KB cells with liposomes conjugated to folate via polyethylene glycol.' *Proceedings of the National Academy of Sciences, USA* **92**, 3318–3322.

WEISS, B., ZHOU, L.W., ZHANG, S.P. AND QIN, Z.H. (1993). 'Antisense oligodeoxynucleotide inhibits D2 dopamine receptor-mediated behavior and D2 messenger RNA.' *Neuroscience* **55**, 607–612.

WESTERMANN, P., GROSS, B. AND HOINKIS, G. (1989). 'Inhibition of expression of SV40 virus large T-antigen by antisense oligodeoxyribonucleotides.' *Biomedica et Biochimica Acta* **48**(1), 85–93.

WHITESELL, L., ROSOLEN, A., NECKERS, L.M. (1991). '*In vivo* modulation of N-myc expression by continous perfusion with an antisense oligonucleotide.' *Antisense Research and Development* **1**, 343–350.

WICKSTROM, E. (1986). 'Oligodeoxynucleotide stability in subcellular extracts and culture media.' *Journal of Biochemical and Biophysical Methods* **13**, 97–102.

WOODBURN, V.L., HUNTER, J.C., DURIEUX, C., POAT, J.A. AND HUGHES, J. (1994). 'The effect of C-FOS antisense in the formalin-paw test.' *Regulatory Peptides* **54**(1 Pt. 2), 327–328.

WYATT, J.R., VICKERS, T.A., ROBERSON, J.L., BUCKHEIT, R.W., JR., KLIMKAIT, T., DEBAETS, E., DAVIS, P.W., RAYNER, B., IMBACH, J.L. AND ECKER, D.J. (1994). 'Combinatorially selected guanosine-quartet structure is a potent inhibitor of human immunodeficiency virus envelope-mediated cell fusion.' *Proceedings of the National Academy of Sciences, USA* 91: 1356–1360.

YAZAKI, T., AHMAD, S., CHAHLAVI, A., ZYLBER-KATZ, E., DEAN, N.M., RABKIN, S.D., MARTUZA, R.L. AND GLAZER, R.I. (1996). 'Treatment of glioblastoma U-87 by systemic administration of an antisense protein kinase C-α phosphorothioate oligodeoxynucleotide.' *Molecular Pharmacology* **50**(2), 236–242.

ZAMECNIK, P.C., GOODCHILD, J., TAGUCHI, Y. AND SARIN, P.S. (1986). 'Inhibition of replication and expression of human T-cell lymphotropic virus type III in cultured cells by exogenous synthetic oligonucleotides complementary to viral RNA.' *Proceedings of the National Academy of Sciences, USA* **83**, 4143–4146.

ZAMECNIK, P.C. AND STEPHENSON, M.L. (1978). 'Inhibition of Rous sarcoma virus replication and cell transformation by a specific oligodeoxynbucleotide.' *Proceedings of the National Academy of Sciences, USA* **75**, 289–294.

ZERIAL, A., THUONG, N.T. AND HELENE, C. (1987). 'Selective inhibition of the cytopathic effect of type A influenza viruses by oligodeoxynucleotides covalently linked to an intercalating agent.' *Nucleic Acids Research* (23), 9909–19.

ZHANG, M. AND CREESE, I. (1993). 'Antisense oligodeoxynucleotide reduces brain dopamine D2 receptors: Behavioral correlates.' *Neuroscience Letters* **161**, 223–226.

ZHANG, R., LU, Z., ZHANG, X., ZHAO, H., DIASIO, R.B., LIU, T., JIANG, Z. AND AGRAWAL, S. (1995). '*In vivo* stability and disposition of a self-stabilized oligodeoxynucleotide phosphorothioate in rats.' *Clinical Chemistry* **41**(6, Pt. 1), 836–843.

ZHANG, R., YAN, J., SHAHINIAN, H., AMIN, G., LU, Z., LIU, T., SAAG, M.S., JIANG, Z., TEMSAMANI, J., MARTIN, R.R., SCHECHTER, P.J., AGRAWAL, S. AND DIASIO, R.B. (1995). 'Pharmacokinetics of an anti-human immunodeficiency virus antisense oligodeoxynucleotide phosphorothioate (GEM 91) in HIV-infected subjects.' *Clinical Pharmacology Therapy* **58**(1), 44–53.

ZHANG, S.P., ZHOU, L.W. AND WEISS, B. (1994). 'Oligodeoxynucleotide antisense to the D1 dopamine receptor mRNA inhibits D_1 dopamine receptor-mediated behaviors in normal mice and in mice lesioned with 6-hydroxydopamine.' *Journal of Pharmacology and Experimental Therapeutic* **271**, 1462–1470.

ZHENG, H., SAHAI, B.M., KILGANNON, P., FOTEDAR, A. AND GREEN, D.R. (1989). 'Specific inhibition of cell-surface T-cell receptor expression by antisense oligodeoxynucleotides and its effect on the production of an antigen-specific regulatory T-cell factor.' *Proceedings of the National Academy of Sciences, USA* **86**, 3758–3762.

ZHOU, L.W., ZHANG, S.P., QIN, Z.H. AND WEISS, B. (1994). '*In vivo* administration of an oligodeoxynucleotide antisense to the D_2 dopamine receptor messenger RNA inhibits D_2 dopamine receptor-mediated behavior and the expression of D_2 dopamine receptors in mouse striatum.' *Journal of Pharmacology and Experimental Therapeutics* **268**, 1015–1023.