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THERAPEUTIC APPLICATIONS OF OLIGONUCLEOTIDES

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INTRODUCTION

First proposed in 1978 by Zamencik & Stephenson (1), oligonucleotide therapeutics represent a new paradigm for drug discovery. The technology focuses on a class of chemicals, oligonucleotides, that has not been studied as potential drugs before and employs them to intervene in biological processes that likewise have not been studied previously as sites at which drugs might act.

The paradigm has resulted in substantial enthusiasm because oligonucleotides may display dramatic increases in affinity and selectivity for their nucleic acid targets compared to traditional drugs. Furthermore, antisense technology may facilitate rational drug design. Table 1 compares affinities and the potential for selectivity of oligonucleotides versus traditional drugs. The comparison is based on average affinities of typical traditional drugs in optimized assays with purified receptors and data derived from a 21-mer phosphorothioate oligonucleotide in binding assays performed in 1M NaCl. Hybridization varies substantially as a function of ionic strength, and the affinities at 100 mm NaCl in the presence of Mg^{2+} for the 21-mer are significantly lower. Furthermore, affinities may be lower in physiological systems with RNA that has secondary structure, so these comparisons present the opportunity in its broadest dimensions.

A number of terms have been coined and often misused to describe various

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Table 1 Affinity and selectivity of traditional and oligonucleotide drugs. Affinity constants were determined as described in text

Traditional drug		Oligonucleotide drug (1M NaCl)	
Affinity for receptor	10^8	Affinity for receptor sequence	10^{29}
Affinity for isotype	10^6 –10^8	Affinity for one base mismatch	10^{26}
Maximum affinity for other proteins	10^6 –10^8	Maximum affinity for nucleic acid binding proteins	10^{12}–10^{13}
ΔKd	1–10^2	Δ kD	10^4–10^{11}

components of the overall approach to using oligonucleotides as therapeutic agents. *Antisense* describes the interaction between oligonucleotides complementary to (sense) pre-mRNA[1] or mRNA molecules; these inhibit the oligonucleotides production of the protein product. The term has been broadened to describe any therapeutic oligonucleotide interaction with nucleic acids. *Triplex* denotes the interaction between oligonucleotides and double-stranded DNA that may result in inhibition of transcription. RNA structures display double-stranded regions, however, and thus the formation of triple-stranded structures in RNA is also possible. *Aptamers* describes the use of oligonucleotides to bind to nucleic acid binding proteins.

SCOPE AND OBJECTIVES OF THE REVIEW

This review focuses strictly on the use of oligonucleotides designed to interact with nucleic acids as therapeutics. The objectives are to place oligonucleotide therapeutics in the context of modern drug discovery and development and to summarize recent progress.

[1]Abbreviations: BFGF, basic fibroblast growth factor; cAMP, cyclic AMP; CH_3-P, methylphosphonate oligonucleotides; EGF, epidermal growth factor; ELAM, endothelial cell adhesion molecule; G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte macrophage colony-stimulating factor; HB, hepatitis B; HIV, human immunodeficiency virus; HSV, herpes simplex virus; HTLV, human T-cell lymphotrophic virus; ICAM, intracellular adhesion molecule; IGF, insulin-like growth factor; IL-1, interleukin 1; introns, intervening sequences; IV, influenza virus; mRNA, messenger ribonucleic acid; P, phosphodiester oligonucleotides; P-acridine, phosphodiester oligonucleotide conjugated with acridine moiety; PCNA, proliferating cell nuclear antigen; PLA_2, phospholipase A_2; P-lipid, phosphodiester oligonucleotide conjugated with lipid moiety; PMA, phorbol mysteric acid; P-S, phosphorothioate oligonucleotides; Rev, regulation of virion protein expression; RSV, Rous sarcoma virus; TAR, Tat response element; TBE, tick-borne encephalitis; Tm, thermal transition point; VSV, vesicular stomatitis.

BASIC CONSIDERATIONS

Conceptually, oligonucleotide drug effects can be rationalized by traditional receptor theory and basic concepts concerning drug action. Within the broad context of pharmacological theory, however, a number of differences influence rational drug design and the potential utility of these agents.

Pharmacodynamics

RNA INTERMEDIARY METABOLISM Oligonucleotides are designed to modulate the information transfer from the gene to protein—in essence, to alter the intermediary metabolism of RNA. Figure 1 summarizes these processes.

RNA intermediary metabolism is initiated with transcription. The transcription initiation complex contains proteins that recognize specific DNA sequences and locally denature double-stranded DNA, thus allowing a member of the RNA polymerase family to transcribe one strand of the DNA (the antisense strand) into a sense pre-mRNA molecule. Usually during transcription, the 5' end of the pre-mRNA is capped by adding a methyl-guanosine and most often by methylation of one or two adjacent sugar residues. This enhances the stability of the pre-mRNA and may play a role in a number of key RNA processing events (2). Between the 5' cap and the site at which translation is initiated is usually a stretch of nucleotides; this area may play a key role in regulating mRNA half-life (3).

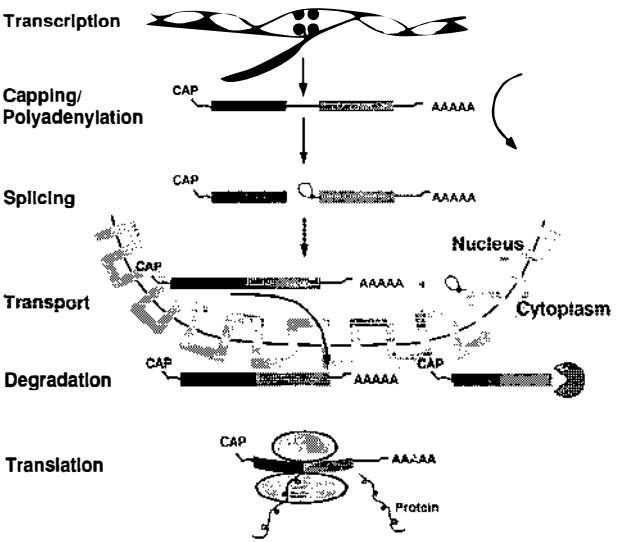


Figure 1 RNA processing.

Similarly, the 3' end of the pre-mRNA usually has a stretch of several hundred nucleotides beyond the translation termination signal. This area often plays an important role in determining mRNA half-life. Moreover, post transcriptionally, most pre-mRNA species are polyadenylated. Polyadenylation stabilizes the RNA, is important in transport of mature mRNA out of the nucleus, and may play important roles in the cytoplasm as well (4, 5).

Because eukaryotic genes usually contain intervening sequences (introns), most pre-mRNA species must have these sequences excised and the mature RNA spliced together. Splicing reactions are complex, highly regulated, and involve specific sequences, small molecular weight RNA species, and numerous proteins. Alternative splicing processes are often used to produce different mature mRNAs and, thus, different proteins. Even though introns are often considered waste, important sequences are conserved, and some introns may play a variety of regulatory roles.

Mature mRNA is exported to the cytoplasm and engages in translation. mRNA half-life varies from a few minutes to many hours and appears to be highly regulated (3).

Each step shown in the pathway is a composite of numerous steps, and each step is theoretically amenable to intervention with oligonucleotides. For virtually no mRNA is the pathway fully defined, however, and available information is insufficient to determine the rate-limiting steps in the intermediary metabolism of any mRNA species (6, 7).

AFFINITY The affinity of oligonucleotides for their receptor sequences results from hybridization interactions. The two major contributors to the free energy of binding are hydrogen bonding (usually Watson-Crick base pairing) and base stacking in the double helix that is formed. As mentioned, affinity is affected by ionic strength. Affinity results from hydrogen bonding between complementary base pairs; the reduction in entropy results from the stacking of the coplanar bases. Consequently, affinity increases as the length of the oligonucleotide receptor complex increases. Affinity also varies as a function of the sequence in the duplex. Nearest neighbor rules allow the prediction of the free energy of binding for DNA-DNA and RNA-RNA hybrids with relatively high precision (8, 9). Less information is available to develop predictions for DNA-RNA duplexes. A common misconception is that DNA-RNA duplexes are more stable than DNA-DNA duplexes. In fact, the relative stability of these duplexes varies as a function of the sequence. RNA-RNA duplexes are typically the most stable (S. M. Freier, unpublished results).

As with other drug-receptor interactions, activity requires a minimum level of affinity. For many targets and types of oligonucleotides, the minimum length of an oligonucleotide may be 12–14 nucleotides.

SPECIFICITY Specificity derives from the selectivity of Watson-Crick or other types of base pairing. The decrease in affinity associated with a mismatched base pair varies as a function of the specific mismatch, the position of the mismatch in a region of complementarity, and the sequence surrounding the mismatch. As an example, Table 2 compares the impact of various mismatches centered in two complementary 18-mers. The $\Delta\Delta G^0_{37}$ or change in Gibbs free energy of binding induced by a single mismatch varies from $^{+}0.2$ to $^{+}4.9$ kcal/mol per-modification at 100 mM NaCl. Thus, a single base mismatch results in a change in affinity of approximately 500-fold (10). Modifications of oligonucleotides may alter specificity. In fact, we have synthesized modified bases with substantially enhanced selectivity and others that display virtually no preferences for different bases.

Table 2 Effects of single-base mismatches on duplex stability. Absorbance vs temperature profiles were measured at 4 μM each strand in 100 mM Na^+, 10 mM phosphate, 0.1 mM, EDTA, pH 7.0.

X strand: 5'-d(CTC GTA CCx TTC CGG TCC)-3' fully phosphorothioate X = dA, dC, dG, or T

Y strand: 5'-(GGA CCG GAA yGG TAC GAG)-3' fully RNA y = A, C, G, or U

X	Y	T_m[1](°C)	ΔT_m(°C)	ΔG^0_{37} (kcal/mol)	$\Delta\Delta G^0_{37}$ (kcal/mol)
dT	rA	53.6	—	−11.6	
dT	rC	40.8	−12.8	−8.9	+2.6
dT	rG	50.0	−3.6	−10.5	+1.1
dT	rU	41.9	−11.7	−9.1	+2.5
dG	rC	56.9	—	−13.1	—
dG	rA	42.3	−14.6	−8.9	+4.2
dG	rG	45.0	−11.8	−9.3	+3.8
dG	rU	45.7	−11.1	−9.8	+3.3
dC	rG	59.0	—	−12.5	—
dC	rA	43.3	−15.7	−9.3	+3.2
dC	rC	39.5	−19.4	−8.7	+3.9
dC	rU	40.0	−19.0	−8.7	+3.8
dA	rU	52.7	—	−11.4	—
dA	rA	42.7	−10.0	−9.3	+2.2
dA	rC	42.7	−10.0	−9.1	+2.3
dA	rG	44.5	−8.1	−9.3	+2.1

[1] T_ms and free energies of duplex formation were obtained from fits of the absorbance vs. temperature data to a two-state model with linear sloping baselines. Reported parameters are averages of at least three experiments.

Based on the differences in affinity of oligonucleotides for their complementary target sequence, calculations suggest that unmodified oligodeoxynucleotides between 11–15 in length should be able to bind selectively to a single RNA species in the cell (11). Studies in our laboratories have demonstrated that affinities predicted by nearest neighbor analyses are highly useful in rational drug design (10). For example, by using strategies based on nearest neighbor predictions, oligonucleotides can be designed that selectively inhibit the production of mutant RAS containing a single base change in the mRNA vs. normal RAS in cells in tissue culture (B. P. Monia et al, manuscript submitted).

NUCLEIC ACID SELECTIVITY The 2'-hydroxyl in RNA results in the sugar assuming a different conformation from that in DNA. RNA-RNA duplexes assume an A-form double helix whereas DNA-DNA duplexes assume a B-form double helix. Consequently, oligonucleotides can be modified to bind more tightly to RNA or DNA sequences. For example, Table 2 shows the effect of 2'-modifications at every position of a 15-mer on T_m and ΔG^0_{37} for DNA and RNA targets and demonstrates that 2'-O-methyl substitutions increase T_m for RNA by 1.4° per modification compared to DNA (12).

RNA STRUCTURE RNA can assume a variety of secondary structures deriving from intramolecular base pairing. The simplest structures are stem-loops in which double-stranded regions are interspersed with loops and random coils. More complex structures described as *pseudoknots* also form (13). These structures are profoundly important in determining RNA function and influencing the ability of oligonucleotides to bind to their RNA targets. The types of effects of bound oligonucleotides on RNA function are affected by RNA structures as well.

Pharmacokinetics

As with any other class of drugs, oligonucleotide drugs must attain a sufficient concentration at their receptor for a sufficient period to display activity. Inasmuch as most of the targets for oligonucleotides are intracellular, oligonucleotides must be relatively stable in and outside the cell and must be able to traverse the cellular membrane.

NUCLEASE STABILITY Oligonucleotides may be degraded by nucleases. Nucleases that degrade DNA or RNA from either the 5' or 3' terminus are known as *exonucleases;* those that cleave internally are *endonucleases.* Numerous nucleases exist and have been shown to degrade oligonucleotides. Although in serum the dominant nuclease activity is 3' exonuclease, in cells and other bodily fluids 3' and 5' exonucleases and endonucleases are present.

In serum, phosphodiester oligodeoxynucleotides are rapidly degraded. The rate of degradation varies as function of the sequence and length of the oligonucleotide and the type of serum (14–16). Typically, half-lives of phosphodiester oligodeoxynucleotides range from 15 to 60 min in most sera. Heat inactivation of serum reduces the rate of degradation of oligonucleotides. Oligoribonucleotides are significantly less stable than oligodeoxynucleotides.

Work from many laboratories has demonstrated that a wide range of modifications may be used to enhance the stability of oligonucleotides. Phosphate modifications have been shown to result in marked increases in stability (see Table 3). Phosphorothioate oligonucleotides have been shown to be extremely stable in media, cells and cell extracts, serum, various tissues, urine and stable to most nucleases (16–20). The half-life of phosphorothioate oligonucleotides is greater than 24 hr in nearly all environments tested. Furthermore, phosphorothioates have been shown to be stable to various restriction endonucleases when in duplexes. In general, one of the diastereomers is cleaved slowly and the other is entirely resistant (21–24).

The non-ionic methylphosphonate analogs have also been shown to be extremely stable to nucleases (25–31). Again, these oligonucleotides are diasteresomeric at each modified phosphate, and the R isomer is slightly more sensitive than the S isomer to degradation by nucleases (16, 32).

Table 3 Effects of 2' modifications on hybridization and stability. Duplex hybridization was evaluated from absorbance vs. temperature profiles at 260 nm in 100 mM Na^+, 10 mM phosphate, 0.1 mM EDTA, pH 7.0 at 8 μM strand concentration.

		Hybridization		Serum stability
Modification[1]	Positions	T_m[2] vs DNA (°C)	T_m vs RNA (°C)	$T_{1/2}$
Phosphodiester	All	50.5	39.0	1 h
Phosphorothioate	All	43.2		>24 h
2'-O-nonyl dA	12 13 14		41.3	60 h
2'-O-allyl	12 13 14	50.3	40.8	10 h
2'-O-benzyl	12 13 14	45.5	37.8	18 h
2'-O-aminopropyl	12 13 14	53.7	42.0	1 h
2'-fluoro P + S	2'F in 12 13 14 P = S in *all*	47.2	36.5	>>24 h

[1] 15 mer: CGA CTA TGC AAA AAC

[2] T_m is the temperature at which half the strands are in the duplex state and half are in the coil state. T_m was obtained from a nonlinear least squares fit of the experimental data to a modified two-state model with linear sloping baselines (224)

Other classes of modifications that have been reported to result in substantial nuclease stability include the phosphoramidates (19, 33) and isopropyl phosphate triesters (34, 35). Interestingly, ethylphosphate triesters were shown to be cleaved after being deethylated in cells (27, 36). Oligonucleotides containing α-anomers in the sugar moiety are substantially more stable in serum and cells than natural phosphodiesters (14–15, 37–41).

Modifications at the 2'-position of the sugar have also been shown to enhance nuclease stability (42). 2'-O-methyl-oligonucleotides were shown to be significantly more resistant than unmodified oligonucleotides, and 2'-O-allyl modified oligonucleotides were even more stable (43). In studies in our laboratory, a large number of 2'-modifications have been characterized. 2'-O-methyl analogs were highly resistant to nucleases in serum and cells. Modifications as bulky as nonyl groups were shown to have only a minor negative effect on hybridization and to impart high levels of nuclease stability In contrast, 2'-fluoro derivatives were nearly as sensitive to nucleases as unmodified oligoribonucleotides (12, 44). Table 3 provides a comparison of affinities to RNA and stabilities in serum for several 2' modified oligonucleotides (45). Although numerous other modifications have been studied, either insufficient data concerning hybridization properties or nuclease stabilities have been reported to support conclusions or their hybridization properties were unattractive. For example, open ring sugar analogs of adenosine were reported to be nuclease stable (46). Acyclic pentofuranosyl modified oligonucleotides were reported to be nuclease resistant, but the T_m for these oligonucleotides was reduced 9–15 degrees per modification (47). Other acyclic sugars have been reported but, again, the hybridization properties were poor (48). Carbocyclic modified oligothymidylates were reported to be nuclease resistant and to hybridize to oligodeoxyadenosine with higher affinity than natural oligodeoxy-thymidylate (49, 50), but studies on mixed sequences have not been reported.

A wide variety of phosphate replacements have also been studied. In earlier work, the phosphodiester was replaced with esters, amides, and various polymeric materials, but these modifications were not designed to be used as antisense oligonucleotides and, therefore, are largely unattractive (12, 16). More recently, formacetal replacement of the phosphate has been reported by two groups to result in oligonucleotides with acceptable hybridization properties and nuclease resistance (51–53).

Other modifications for which little information is available include sulfonamide replacement of phosphate (54), diphosphonate dinucleotides (55), acetamide linkages (56, 57), and phosphonyl methyl linkages (58). These and other modifications are discussed in detail in two recent reviews (12, 16). In our laboratories, a number of other novel backbone modifications have been synthesized. Given the number of novel synthetic approaches and molecules

and the number of laboratories now involved, a substantial increase in the repertoire of backbone-modified oligonucleotides with desirable properties is likely in the near future.

In addition to uniform modifications, a number of pendant groups at the 5' and/or 3' termini and more recently in internal positions of oligonucleotides have been reported to enhance nuclease stability. Modifications include intercalating agents (59–62) and poly-L-lysine (63, 64) at the 5' or 3' terminus and a number of modifications such as amino-alkoxy (65), anthraquinone (66), and alkyl groups (45). Moreover, heterocycle modifications, including pendant groups from the N2 site of guanine (67, 68), pendant groups from 3-deazaguanine (69), and 5- and 6-position modifications of deoxycytidine and thymidine (70), have shown increased stability to nucleases of varying levels.

In conclusion, numerous medicinal chemical strategies can be employed to create oligonucleotides with varying degrees of nuclease stability. The choice of the modification(s) employed is dictated by the level of stability desired and other desired properties of the oligonucleotides. It is now possible to design oligonucleotides that display excellent hybridization characteristics and half-lives, that range from minutes to several days when oligonucleotides are incubated with nucleases, serum, cells, or cell extracts.

INTRACELLULAR STABILITY Although considerable confusion and controversy exist with regard to the stability of oligonucleotides in cells in tissue culture and the ability to predict intracellular stability of oligonucleotides based on stability in sera, a consensus opinion is emerging. The nuclease activity of sera derived from different species varies. Fetal calf serum is more active than mouse serum, and human serum appears to have the least nuclease activity (G. D. Hoke, unpublished observations). All sera display substantial nuclease activity, however, and there are significant lot-to-lot variations. In all sera tested, 3' exonucleases constitute the primary nuclease activity (12, 16, 71). In a number of publications, fetal calf serum used in tissue culture experiments has been heated to inactivate nucleases. Again, however, conditions were not standardized, and in some lots of sera, heating to 65°C for 30 min does not inactivate all nucleases (16).

Another factor that has contributed to confusion is that a variety of labeling methods and analytical techniques have been employed. Studies have employed 3'^{32}P and 5'^{32}P labeled oligonucleotides, 5'^{35}S labeled oligonucleotides, and oligonucleotides labeled with fluorescent pendant groups at the 5' terminus (14–16, 72). Relatively few studies have used uniformly labeled oligonucleotides. Furthermore, relatively few studies have rigorously separated intact oligonucleotides from degradation products, and even fewer have performed careful kinetic studies.

Studies in our laboratory have employed either phosphodiester oligonucleotides uniformly labeled with ^{32}P or phosphorothioate oligonucleotides uniformly labeled with ^{35}S. The kinetics of degradation have been studied with several cell lines in vitro and cytoplasmic and nuclear extracts derived from HeLa cells. In contrast to a number of studies, in all cells studied to date, phosphodiester oligonucleotides were degraded within 15–30 min of incubation (71, 73). In contrast, phosphorothioate oligonucleotides of 15, 21, and 30 nucleotides in length and various sequences were stable for at least 24 hr when incubated with various cells. In studies in HeLa cells in which ISIS 1082, a 21-mer phosphorothioate, was incubated with the cells, then extracted from cells at various time points and analyzed on polyacrylamide gels, the compound was intact for four days (73).

Methylphosphonate oligonucleotides have also been shown to be stable in a variety of cells lines and extracts (14). No other class of oligonucleotides, however, has been sufficiently studied to allow definitive conclusions.

CELLULAR UPTAKE AND DISTRIBUTION Antisense oligonucleotides typically are 15–30 nucleotides long and thus have molecular weights that range from 4500–9000 daltons. The charge carried by phosphodiesters is, of course, negative and they are highly water soluble. The charge and hydrophilicity of modified oligonucleotides vary depending on the modifications. Consequently, membrane transport and cellular distribution are likely to vary widely as a function of the modifications introduced into oligonucleotides. For the two classes of modified oligonucleotides for which significant data have been reported—methylphosphonates and phosphorothioates—this is clearly the case. For both classes of oligonucleotides, the evidence is compelling that they do enter many cells at pharmacologically relevant concentrations.

Methylphosphonates are uncharged and lipophilic. Although thought to be taken up by most cells in tissue culture via passive diffusion, detailed studies of the kinetics of cellular uptake, distribution, and metabolism of uniformly labeled methylphosphonates have not been reported. Studies in Syrian hamster fibroblasts on oligonucleotides 3–9 nucleotides in length showed linear cell association for 1 hr, then reduced uptake. At equilibrium, the intracellular concentration of oligonucleotide was reported to be equivalent to the extracellular concentration (27, 74). In another study, a 21-mer methylphosphonate labeled with ^{32}P at the 5' terminus was reported to be taken up by CV-1 cells. Cell association was linear for 2 hr. Unfortunately, however, studies proving that the cell-associated radioactivity represented intact oligonucleotide were not presented. Nor were detailed studies on characteristics of uptake or intracellular distribution presented (75).

Phosphorothioates are negatively charged, but because of the sulfur atoms

they may be slightly more lipophilic than phosphodiesters and tend to bind nonspecifically to serum proteins. Studies in our laboratories have shown that phosphorothioate oligonucleotides bind to serum albumin and that in the presence of serum albumin, cell-association is reduced (73; G. D. Hoke et al, unpublished observations).

Studies employing a 28-mer phosphorothioate deoxycytidine that was uniformly labeled with ^{35}S demonstrated that when HeLa cells were incubated with 1 μM of the drug, significant intracellular concentrations were achieved. Cellular uptake was linear, reaching a plateau of 60 p mole/10^6 cells in 6 hr. Adsorption to the cell membrane was minimal. Uptake was also concentration-dependent, reaching a plateau at approximately 1 μM. The drug associated with HeLa cells was intact for 24 hr and was located in both nuclei and cytoplasm. Infection with herpes simplex virus type 2, but not type 1, increased cellular uptake (76).

Studies in our laboratories have confirmed and extended the observations on phosphorothioate oligonucleotides. The cellular uptake, distribution, and metabolism of ISIS 1082, a uniformly ^{35}S labeled 21-mer phosphorothioate with a mixed antisense sequence, have been characterized in HeLa cells and HeLa S_3 cells, a variant line conditioned to growth in suspension. Incubation of HeLa cells with 5 μM of the drug resulted in approximately 8% of input radioactivity being associated with the cells. Cell association was linear for approximately 8 hr, and approximately 20% of the cell-associated radioactivity appeared to be adsorbed to the membrane. Uptake was temperature-dependent, required viable cells, and was inhibited by metabolic poisons. Uptake was concentration-dependent, and was linear to 10 μM. Uptake was influenced slightly by calcium and magnesium and was saturable. Natural oligonucleotides and methylphosphonates did not compete for uptake while other phosphorothioates competed; however, different length and sequence phosphorotioates competed differently (73, 77).

We have also studied other phosphorothioates of various lengths and other cell lines. HL 60 cells appear to take up less phosphorothiate oligonucleotides than HeLa cells and HeLa S_3 cells take up very little drug (73). Although not directly compared, human umbilical vein endothelial cells also appear to take up less drug than HeLa cells. Thus, there is considerable variation in the extent of uptake as a function of cell type.

In all cells studied, and with all uniformly labeled phosphorothioate oligonucleotides of varying size and sequences, we have shown that these drugs are stable in cells and cytoplasmic and nuclear extracts. In HeLa cells, no degradation of intracellular ISIS 1082 was observed for four days (73). Preliminary studies confirmed that these oligonucleotides distributed to both cytoplasm and nuclei and showed that there is an active temperature-dependent efflux process as well (77, 78).

When incubated with cells in the absence of serum or heat-inactivated serum, several laboratories have reported the apparent uptake of phosphodiester deoxyoligonucleotides. Moreover, a number of laboratories have reported activities for phosphodiester oligonucleotides that apparently were due to cellular uptake and intracellular activities. The studies on cellular uptake are not fully convincing, however Loke et al (79) studied deoxythymidine oligonucleotides ranging from 3–20 nucleotides in length and labeled with acridine at the 3' terminus. They incubated HL60 and three other hematopoietic cell lines with 12.5 μM of the acridine labeled drug and used flow-cytometric analyses of acridine fluorescence to quantitate cellular uptake. Uptake was reported to decrease as the length of the oligonucleotide increased and to vary as a function of the cell type. Uptake achieved a plateau in HL60 cells in 50 hr and was inhibited by polynucleotides of any length. The authors concluded that the oligonucleotides were taken up by an endocytotic mechanism. Unfortunately, the stability of the oligonucleotide-acridine conjugate was not rigorously documented. Nor were possible effects of acridine in the uptake of the oligonucleotide rigorously explored. Additionally, possible quenching or enhancement of the fluorescence of acridine by cellular interactions was not explored. Finally, extrapolations from homopolymers to mixed sequences have not been proven to be valid.

Another study employing phosphodiester oligonucleotides reached similar conclusions (80). Again, for most of the experiments, oligodeoxythymidines of 8 to 16 nucleotides in length were incubated with L929 mouse fibroblasts in the absence of serum. Maximal uptake occurred within 2 hr and upon incubation with fresh medium, cell-associated ^{32}P was released. Substantial degradation of the 5' labeled oligonucleotide was observed within 2 hr, and the authors concluded that approximately 20% of the radioactivity was in nuclei. Again, the authors concluded that the most likely mechanism of uptake was endocytosis (80).

Other pendant modifications of phosphodiester oligonucleotides have also been studied. A 9-mer labeled with acridine at the 3' terminus was reported to be taken up by *Trypanosome brucei* (61). More recently, the same group has reported that a 9-mer coupled at the 3' terminus to acridine via a dodecanal linker was more active in cells expressing mutated RAS than a 9-mer with a 3' acridine only (81). 3' poly-L-lysine-oligonucleotides have been reported to be stable to serum nucleases and to have enhanced activity as compared to phosphodiesters. Uptake was not studied, however, (63, 82, 83). In a later publication, the uptake of a poly-L-lysine oligonucleotide conjugate was enhanced compared to the unmodified oligonucleotide (84). When used to treat cells other than L929 cells, however, poly-L-lysine conjugates were inactive (64).

A number of lipid conjugates have also been studied. 5' linked triethylam-

monium 1,2 di-O-hexadicyl-rac-glycerol-3-H-phosphonate oligonucleotides were taken up 8–10-fold more than unmodified oligonucleotides by L929 cells and were more active against varicella zoster viral infections, albeit at high concentrations (85). An oligonucleotide linked at the 5' terminus to an undecyl residue was reported to be active, but no uptake or stability studies were reported (86).

The intracellular fate of oligonucleotides injected into oocytes and the uptake of oligonucleotides into oocytes have also been studied. When injected into *Xenopus* oocytes, unmodified oligonucleotides were degraded within 1 min primarily by 3' exonuclease digestion, but other nucleolytic activities were also present (87, 88). Interestingly, in this system, even phosphorothioate oligonucleotides were reported to be degraded, albeit much more slowly than phosphodiesters (89). These observations were extended in studies on oligodeoxynucleotides injected into CV-1 endothelial cells. A 28-mer oligonucleotide of either phosphodiester, phosphorothioate, or methylphosphonate type was injected into the cytoplasm of these cells. All three types of oligonucleotides localized to the nucleus in a temperature- but not energy-dependent fashion. The methylphosphonate oligonucleotide concentrated in regions of genomic DNA, in contrast to the two other oligonucleotides that co-localized with small nuclear ribonucleoproteins (90). Uptake of unmodified oligonucleotides by pre-implantation embryos was reported to be virtually nil (91).

Liposomes and related formulations have been shown to enhance cellular uptake of oligonucleotides in vitro. Loke et al (92) compared the uptake of phosphodiester and phosphorothioate deoxythymydine heptamers into HL-60 cells by using oligonucleotides coupled to 2-methyloxy-6-chloro 9-(5-hydroxypentyl) amino acridine and monitoring with flow cytometry. They did not determine the integrity of the oligonucleotides, but reached the conclusion that phosphodiester dT_7 was taken up by HL-60 cells much more effectively than phosphorothioate d-T_7, and that uptake plateaued at 50 hr. They reported increased anti-c-*myc* activity of phosphorothioate oligonucleotides after loading them in phosphatidyl serine liposomes. The uptake of a tetramer 2'-5' deoxyadenylate into L1210 cells was reported to be increased by loading the oligo-adenylate into *Staphyloccus aureus* protein A-crosslinked phospholipid vesicles (93). In our laboratories, we have shown that lipofectin, a cationic lipid mixture, can significantly increase the uptake and activity of phosphorothioate oligonucleotides in several cell lines. It also alters the intracellular distribution of these nucleotides (78).

With the exception of methylphosphonates, the conclusion from studies that have addressed the mechanisms of uptake of oligonucleotides is that the most likely mechanism is receptor-mediated endocytosis. In fact, in one study an 80-kd protein that appeared to bind oligonucleotides was partially purified

and postulated to be a "receptor" (79). The evidence supporting this mechanism is limited, however, and data are insufficient to conclude that receptor-mediated endocytosis is the most common or only mechanism of uptake of charged oligonucleotides in most cells.

In conclusion, although many questions remain to be answered, it appears that many cells in tissue culture may take up oligonucleotides at pharmacologically relevant concentrations. Clearly, oligonucleotides of different types behave differently and there are substantial variations as a function of cell type. Moreover, length and specific sequences may alter uptake, and pendant modifications may profoundly influence cellular uptake.

Once in the cell, it would seem that oligonucleotides distribute to the cytoplasm and the nuclei. In most if not all cells, phosphodiester oligonucleotides are rapidly degraded whereas methylphosphonates and phosphorothioates are much more stable. Again, pendant modifications may alter the rate of intracellular degradation and distribution.

Mechanisms of uptake and distribution are poorly understood. Clearly, however, multiple mechanisms may play a role, and different types of oligonucleotides may behave very differently.

Novel formulations may enhance cellular uptake. Liposomes and cationic lipids significantly enhance uptake and may alter the mechanisms of uptake and intracellular fate of oligonucleotides.

IN VIVO PHARMACOKINETICS Preliminary in vivo pharmacokinetic data are now available on methylphosphonate and phosphorothioate oligonucleotides. A 12-mer ^{3}H-labeled methylphosphonate injected in the tail vein of mice was rapidly cleared as intact oligonucleotide and distributed broadly to all tissues except the brain (94).

More extensive studies have been performed on ^{35}S-labeled phosphorothioates in rats. A true distribution phase of 15–25 min was observed after a single IV dose of a 27-mer followed by a prolonged elimination phase of 20–40 hr (94). The prolonged elimination phase may result from the binding of phosphorothioates to serum proteins. Phosphorothioates distrubuted broadly to all tissues except the brain and were eliminated in the urine intact. Phosphorothioates were rapidly and extensively absorbed after IM and IP administration (94).

Repeated daily doses of 50 mg/kg of a 27-mer phosphorothioate to mice resulted in similar distribution and elimination kinetics but slight differences in tissue concentrations from single dose studies. Liver, kidney, spleen, and lung were the organs with highest concentrations. Again, the drug was excreted intact in the urine (94).

Continuous osmotic pump administration of the same compound subcutaneously for 4 wk at doses of 50–150 mg resulted in similar pharmacokinetics (94).

Studies with ISIS 1082, a 21-mer phosphorothioate, in mice showed that when applied to the cornea in a sodium acetate buffer, significant adsorption to the cornea and absorption into the aqueous and vitreous humors occurred. Moreover, significant systemic bioavailability was observed (78). In rabbit, as much as 25% of an applied ocular dose was systemically bioavailable (unpublished observations). Post absorption pharmacokinetics were equivalent to IV pharmacokinetics.

Recently, a 20-mer phosphodiester was administered intravenously to rabbits. Clearance from blood was rapid and, after 90 min, 16% of the dose was found in the urine and was intact. In blood, at least 17% of the drug was estimated to be completely degraded within 5 min (95).

Toxicology

IN VITRO

Phosphodiesters Very little information has been published on the in vitro toxicities of unmodified oligonucleotides. In most systems, the oligonucleotides are thought to be rapidly degraded. When a 15-mer complementary to a c-myc sequence was incubated with human lymphocytes at 30 μM for 4 hr, no toxicity was observed. Longer incubation (24 hr) in 10% serum resulted in reduced ^{3}H-thymidine incorporation, but the authors concluded that this was probably due to dilution of the thymidine pool by thymidine liberated after rapid degradation of the oligonucleotide (96).

The incubation of a transformed leukemic cell line with 50 μM of a 20-mer complementary to a sequence in the BCL-1 proto-oncogene was reported to result in no decrease in viability as judged by trypan blue exclusion (97).

Methylphosphonates Incubation of Vero cells with 30 μM and lower concentrations of an 8-mer methylphosphonate for 24 hr resulted in no decrease in growth rate or cell count; however, 48 hr incubation resulted in 40% inhibition of growth rate (98). Similarly, neither of three 9-mers had any effect on L929 cell plating efficiency or protein synthesis after 16 or 40 hr incubations with 150 μM of drug (99). Incubation of T15 cells with 80 μM of a 9-mer directed against N-ras for 48 hr produced no effect on protein synthesis or viability (100). Similar results were reported for HT29 cells.

Inasmuch as methylphosphonate oligonucleotides have, when they have displayed activity, effective concentrations of 50–100 μM, the therapeutic index in vitro may be rather modest. Much more detailed studies are required before reaching final conclusions, however.

Phosphorothioates Phosphorothioate oligonucleotides bind to a variety of proteins, including serum albumin. In cell free protein translation experiments, they have been shown to induce nonspecific inhibition of protein synthesis (11, 101, 102). In wheat germ and rabbit reticulocyte lysate assays,

concentrations as high as 100 nM of a 17-mer phosphorothioate targeted to the protein mRNA inhibited globin synthesis relatively specifically. At 10 μM, nonspecific effects were observed (103). The nonspecific effects of phosphorothioates in these assays are length-dependent, as a 5-mer was much less potent than the 14-mers and dC28 appeared to be the most potent phosphorothioate oligonucleotide tested. In studies in our laboratories, we have made similar observations with a number of phosphorothioate oligonucleotides (G. D. Hoke et al, unpublished observations).

Phosphorothioate oligonucleotides have also been shown to inhibit DNA polymerases, reverse transcriptases, and nucleases when incubated in cell free systems (76, 77, 104).

Despite the potential nonspecific interactions of phosphorothioate oligonucleotides with cellular proteins, a wide variety of compounds have been shown to have excellent therapeutic indices. Microinjection of nanomolar concentrations of a 17-mer into *Xenopus* oocytes inhibited β-globin synthesis. When 16 μM of the compound were injected, however, protein synthesis was aborted and the oocytes underwent extensive cytolysis (89).

Incubation of cells in vitro with phosphorothioate oligonucleotides has likewise resulted in toxicities only at concentrations much higher than those at which therapeutic activities were observed. Human mononuclear cells were unaffected after 20 hr of incubation with 25 μM of several 15-mers (105). T697 cells were unaffected by a three-day exposure to 25 μM of a 20-mer (97, 106).

In our laboratories, we have determined the effects of ISIS 1082, a 21-mer phosphorothioate that inhibits herpes simplex virus types 1 and 2 infections in HeLa cells at 200–400 nM, on HeLa cell viability, DNA synthesis, RNA synthesis, protein synthesis, and energy metabolism. At no concentration below 500 μM were statistically significant effects observed after incubation for 96 hr. Exposure of HeLa cells to 500 μM ISIS for 48 hr resulted in 20% inhibition of protein synthesis (77). Similar results were observed in other cell lines.

Table 4 presents results from studies on 20 phosphodiester or phosphorothioate oligonucleotides targeted to various regions in the 5-lipoxygenase gene. Again, most of the phosphorothioates displayed toxicities only at 50 μM and greater. The exceptions to this rule were three 30-mers that inhibited cell growth at 10–35 μM. Clearly, one can conclude from this study that toxicity was time- and concentration-dependent and that, with longer exposures in particular, phosphorothioates were more toxic than their phosphodiester analogs (73). T4

We have identified other factors that influence the toxicity of phosphorothioates. Cell type may alter toxicity significantly. A comparison of the toxic effects of a 15-mer phosphorothioate on HL60 cells, U937 cells, and RBL-1

Table 4 In vitro toxicities of 5-

Compound	Oligonucleotide Class[b]	Length	Sequence	AT:GC	24 hr	48 hr	72 hr	96 hr
1787	PD	15	5'GTGTGCCACCAGGAG-3'	1:2	21.5	18.5	16.0	14.4
1788	PS	15			>100	35.0	25.0	19.0
1789	PD	30	AATGGTGAATCTCAC		>100	>100	>100	>100
1790	PS	30	GTGTGCCACCAGCAG	1:1.1	>100	>100	15.0	11.8
1795	PD	15	TGCCAGTGATTCATG	1:0.88	63.0	39.5	34.0	26.0
1796	PS	15			>100	>100	50.0	35.0
1797	PD	30	GGTCTTCCTGCCAGT		>100	>100	50.0	74.0
1789	PS	30	GATTCATGACCCGCT	1:1.31	>100	20.0	10.0	10.0
1799	PD	15	GTCCTGATGGCTTCC	1:1.5	28.0	25.0	22.0	22.0
1800	PS	15			>50.0	50.0	50.0	34.0
1801	PD	30	GTCCTGATGGCTTCC		>50.0	>50.0	>50.0	>50.0
1802	PS	30	CACACCAGGAGCCCG	1:2.0	35.0	27.0	21.0	3.9
1812	PD	15	GTTGCTGCTTGGTGT	1:1.14	29.0	17.0	16.0	18.0
1813	PS	15			>50.0	32.0	25.0	43.0
1814	PD	30	ATTTGCTGTTGCTGC		>50.0	32.0	25.0	43.0
1815	PS	30	TTGGTGTGGAAATGC	1:0.88	10.0	9.0	10.0	11.0
1816	PD	15	AGGTGTCCGCATCTA	1:1.14	>50.0	>50.0	>50.0	>50.0
1817	PS	15			32.0	>50.0	>50.0	>50.0
1818	PD	30	TCGGCGCGGCGGTCC	1:2.33	>50.0	>50.0	>50.0	>50.0
1819	PS	30	AGGTGTCCGCATCTA		15.0	19.0	19.0	20.0

[a] HL 60 cells were incubated in 96 well plates with increasing concentrations of oligonucleotides (1–50 or 100 μM) in the presence of 10% fetal bovine serum. Viability of the cells was determined at each time point by trypan blue exclusion. IC_{50} values were obtained by plotting percentage control cell number vs. drug concentration.

[b] PD = Phosphodiester; PS = Phosporothioate.

cells showed considerable variation in sensitivity; HL60 cells were the most sensitive. As phosphorothioates bind to serum albumin, in the presence of 10% fetal calf serum, a 15-mer produced no cytotoxicity after 24 hr of incubation at 100 μM. In the presence of 2.5% fetal calf serum, the IC_{50} was 19 μM. Finally, the purity of the oligonucleotide has a significant effect. Purification of oligonucleotides in triethyl ammonium buffers with trityl-on HPLC followed by removal of the trityl groups in triethyl ammonium may result in substantial contamination with triethyl ammonium ions, which are toxic to cells (73). Others have alluded to batch-to-batch variations and the potential that contaminants might contribute to toxicities, but they have not identified potential toxins (60, 97, 106–108).

Pendant group modified oligonucleotides Limited information is available concerning the effects of pendant groups on the toxicities of oligonucleotides. An acridine conjugated 7-mer phosphodiester was reported to produce no toxicities at 100 μM even though the free acridine had an lC_{50} for cell viability of 2 μM (109). Two 11-mer phosphorodiesters that were covalently attached to an undecyl group at the 5' terminus had no apparent toxic effect on MDCK cells at 100 μM (86). 5'terminal phospholipid conjugates of both phosphodiester and phosphorothioate oligonucleotides produced little toxicity in L292 cells when incubated at 50 to 100 μM (85). In contrast, a phosphodiester 15 mer linked to poly-L-lysine was toxic to L929 cells at 1 μM (84).

Table 5 summarizes published data concerning the in vitro toxicology.

IN VIVO Although only preliminary toxicologic data are available, considerably more information should soon be available, as several compounds are currently in preclinical development.

Single-dose toxicity studies in mice were reported for phosphodiester (19), methylphosphonate (110), phosphomorpholidate, and phosphorothioate oligonucleotides. Unmodified oligonucleotides resulted in deaths in two of four treated mice at 160 mg/kg and all four mice treated with 640 mg/kg IV. Within three days after injection, a phosphorothioate oligonucleotide resulted in equivalent toxicities to the phosphodiester. The other analogs produced similar toxicologic effects with slight differences in doses.

Single doses of as much as 3.5 mg of a 27-mer complementary to the REV gene of HIV given IV or IP produce no toxicities in rats. Daily injections of 50 mg/kg IV of the same compound for 12 days in mice resulted in no observable toxicities. This 27-mer was also administered via a subcutaneous osmotic pump designed to administer up to 150 mg at a constant rate for 4 wk to rats. Again, no toxicities in any organ were observed (94).

ISIS 1082, 1 21-mer phosphorothioate targeted to inhibit herpes virus types 1 and 2, has been administered topically to mouse and rabbit eyes for as much

as 21 days and resulted in no ocular toxicities. In rabbits, other organs were examined, and no effects were observed. Given the extensive bioavilability of ISIS 1082 in rabbits after ocular administration, this constitutes a significant observation.

Single doses of ISIS 2105, a 20-mer phosphorothioate active against human papilloma viruses, were administered intradermally and resulted in no local or systemic toxicities.

Consequently, a growing body of data supports the contention that at least single doses of phosphorothioate oligonucleotides may be given to mice, rats, and rabbits without significant acute or subacute toxicities.

MUTAGENICITY Virtually no data have been published on the potential mutagenicity of oligonucleotides. A 27-mer phosphorothioate was reported to be negative in an Ames assay in the presence or absence of a liver metabolic activation system at doses as high as 5 mg/plate (101).

P. Iverson (personal communication; 101) compared a number of oligonucleotide types and related chemicals in hamster lung fibroblasts. Unfortunately, although this study has been cited, the primary data have never been published, and thus it is difficult to draw any conclusion.

Mechanisms of Action of Oligonucleotides Interacting with Nucleic Acid Targets

The mechanisms by which interactions of oligonucleotides with nucleic acids may induce biological effects are complex and potentially numerous. Furthermore, very little is currently understood about the roles of various mechanisms or the factors that may determine which mechanisms are involved after oligonucleotides bind to their receptor sequences. Consequently, a discussion of mechanisms remains largely theoretical. Although a number of potential schemes to classify mechanisms of action might be employed, I prefer a scheme based on drug-receptor concepts.

OCCUPANCY-ONLY MEDIATED MECHANISMS Classic competitive antagonists are thought to alter biological activities because they bind to receptors, thereby preventing natural agonists from binding and inducing normal biological processes. Binding of oligonucleotides to specific sequences may inhibit the interaction of the RNA or DNA 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.

Transcriptional arrest Oligonucleotides may bind to DNA and prevent either initiation or elongation of transcription by preventing effective binding of factors required for transcription, thus producing transcriptional arrest.

Table 5 In Vitro toxicology of antisense oligonucleotides

Oligonucleotide class	Length	Concentration	Target	Cell type	Time	Toxicity assessment	References
Phosphodiester	15	30 μM	c-*myc*	Human T cells	4 hours	Nontoxic	99
Phosphodiester	20	150 μM	βCL2	697 cells	3 days	Nontoxic	100
Phosphorothioate	20	25 μM	βCL2	697 cells	3 days	Nontoxic	
Phosphodiester	23	1–30 ng	Vg 1	*Xenopus* oocytes	2 days	Nontoxic @ low concentrations, i.e. <5 ng	92
Phosphorothioate	23	1–30 ng	Vg1	*Xenopus* oocytes	2 days	Toxic @ 15–30 ng	
Phosphodiester-acridine conjugate	7	50–100 μM	Type A influenza	MDCK	3 days	Nontoxic	112
Acridine alone		2 μM		MDCK	3 days	Toxic	
Methylphosphonate	9	150 μM	VSV	L929	16.40 hours	Nontoxic	102
Methylphosphonate	9	80 μM	N-*ras*	T15	48 hours	Nontoxic	103
Methylphosphonate	9	80 μM	N-*ras*	HT29	48 hours	Nontoxic	
Phosphodiester	14	1–25 μM	HIV	ATH8	7 days	Minor toxicity @ all conc. (<35%)	20
Methylphosphonate	14	1–25 μM			7 days	Minor toxicity @ all conc. (<27%)	

Phosphorothioate (heteropolymer)	14	1–25 μM			7 days	Nontoxic	
Phosphorothioate (homopolymer)	14–28	1–25 μM			7 days	Nontoxic	76
Phosphorothioate (homooligomer)	28	3–50 μM	HSV-2	HeLa S_3	72 hours	Nontoxic	
Phosphorothioate (antisense)	20	4–100 μg/ml	HIV	H9, MOLT3	96 hours	Nontoxic	19
Phosphorothioate (nonsense)	20	4-100 μg/ml		H9, MOLT3	96 hours	Nontoxic (4 μg/ml) Toxic (20 μg–5% 100 μg-67%)	
Phosphodiester homooligomer (dT)	15	4–100 μg/ml		H9, MOLT3	96 hours	Toxic	
Phosphodiester homooligomer (dA)	15	4–100 μg/ml		H9, MOLT3	96 hours	Minor toxicity	
Phosphormorpholidate homooligomer (dT)	15	4–100 μg/ml		H9, MOLT3	96 hours	Toxic	
Phosphormorpholidate homooligomer (dG)	15	4–100 μg/ml		H9, MOLT3	96 hours	Toxic	
Phosphorothioate	17	16 μM	β-Globin	*Xenopus* oocytes	>6 hours	Toxic	106
Phosphorothioate	15	0.1–25 μM	ILIβ	Human blood monocytes	20 hours	Nontoxic	108

It is possible that oligonucleotides could bind to segments of DNA that are partially denatured by the transcription complex, although this is highly unlikely. The initiation and elongation of transcription require a complex set of proteins and other factors, and it is difficult to conceive of a mechanism by which oligonucleotides might compete effectively against the transcriptional machinery for these single-stranded regions. Nevertheless, despite the improbability of such an event, reports of activities have been made that can be explained most simply by this mechanism (112, 113). Additionally, Helene and colleagues (114) reported that hexanucleotides to nonanucleotides with acridine derivatives at the 3' terminus inhibited transcription of the β-lactamase gene. When the RNA polymerase was preincubated with the oligonucleotide-acridine adducts, however, they observed nonspecific inhibition (115).

The alternative to seeking transient single-stranded regions or to attempting to denature a double-stranded region of DNA is to inhibit transcription by interacting with double-stranded DNA, i.e. forming triple-stranded structures. To form triple-stranded structures, hydrogen bonds other than Watson-Crick must be formed. In most current triple–strand motifs, the oligonucleotide becomes the third strand by recognizing hydrogen bonding donor/receptor sites on a purine reference strand and lying in the major groove (116--124). Alternative motifs have also been proposed. For example, Hogan and colleagues (125) proposed that a purine-rich oligonucleotide can form a triplex structure based upon the purines in the oligonucleotide base pairing in parallel fashion with the purines in the duplex DNA. Studies by Dervan's group (126), however, suggested that the purine-rich oligonucleotide bound to the duplex DNA with an antiparallel orientation.

The formation of triple-stranded structures by using natural nucleosides requires runs of purines Watson-Crick-hydrogen-bonded to their complementary pyrimidines. When cytidine is used to form a triple strand with a G-C base pair, it must be protonated; this occurs at nonphysiological acidic conditions (121). Furthermore, all motifs employ one or more "weak" hydrogen bonds. Thus, to achieve sufficient stability, relatively long triple-strand structures are required.

The principal theoretical advantage of triple helical inhibition schemes is that transcription represents the first step in the intermediary metabolism of RNA and may, therefore, provide substantial leverage for drug therapy. The other advantages that have been suggested are much more speculative. For example, it has been suggested that the smaller number of genes (one or two) compared to the number of mRNA molecules (usually less than 1000) per cell is an advantage for approaches that inhibit transcription. This suggestion ignores the kinetics of the targets, however. Genes have an infinite half-life relative to cell life. RNA molecules are synthesized and degraded with

varying kinetics. Furthermore, a variety of mechanisms exist to assure that even covalent modifications of DNA are repaired. Another concept has been that triple helixes in DNA might produce permanent biological effects. That even alkylating and DNA-cleaving anticancer drugs do not produce permanent effects points to the speciousness of this notion.

A number of theoretical disadvantages of triple helical inhibition of transcription have also been enumerated. Sequence specific binding is not yet possible, as runs of homopyrimidines are required. These sequences may play important regulatory roles in DNA, as they are much more abundant than statistically predicted (16). Longer term, a more substantial problem may simply be gaining sequence-specific access to DNA in chromatin. Additionally, deliberate interactions with the genome raise concerns about mutagenicity, carcinogenicity, and teratogenicity, which, in most therapeutic settings, are of considerable importance.

Several strategies have been developed to circumvent the requirement for purine-pyrimidine runs and other limitations. For example, purine oligonucleotides form triplex structures at higher pH values than pyrimidine-rich oligonucleotides (125, 126). Similarly, pyrimidine-rich oligonucleotides, in which 2'-O-methyl pseudoisocytidine was substituted for 2' deoxycytidine, formed triplex structures as neutral pH (127). Oligonucleotides with linkers that allow crossover of the oligopyrimidine from one strand of the duplex to the other have been reported and this motif suggested to be a solution to a broader sequence repertoire (128). To enhance the stability of triple helices, intercalators and photoactivatable crosslinkers and alkylators have been conjugated to oligo pyrimidines (129–131). To increase potency and enable identification of sites of binding, a number of cleavage moieties have been conjugated to oligopyrimidines (132–137). Finally, to enhance nuclease stability, methylphosphonates (138) and α-oligonucleotides (136) have been shown or suggested to form triple helices.

In addition to cleavage of DNA in vitro by triplex-forming oligonucleotides coupled to cleavage reagents and alkylation induced by oligonucleotide-coupled alkylators, several other methods have been used to show triplex formation. These include agarose affinity column purification (139), NMR (140), protection from uv dimerization (141), solution hybridization (142), inhibition of binding of DNA-binding proteins (143), inhibition of restriction endonucleases (144), and repression of c-myc transcription in vitro (125). Recently, a 28-mer phosphodiester stabilized at the 3' end by alanine and directed to enhancer elements for the IL-2 receptor gene was shown to inhibit the transcription of the gene when incubated with human lymphocytes. The authors reported evidence for selectivity to oligonucleotides as well (145).

Obviously, triple-helix-based inhibition of transcription is of potential therapeutic importance, particularly for targets that for a variety of reasons

may be difficult to inhibit at the post-transcriptional level. Substantial medicinal chemistry must be completed, however, to create oligonucleotides that can interact with duplex structures in a sequence-specific fashion without requiring special motifs. Once this is accomplished, of course, additional studies must show that the other theoretical limitations discussed above can be overcome.

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 spliceosmes. 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 presented 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 (146–149). Activities have been reported for anti-c-*myc* and antiviral oligonucleotides with phosphodiesters, methylphosphonates, and phosphorothioates.

Translational arrest Without question, the mechanism for which the majority of oligonucleotides have been designed is translational arrest. Oligonucleotides have been designed to bind to the translational initiation codon. The positioning of the initiation codon within the area of complementarity of the oligonucleotide and the length of the 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 other data that support translation arrest as the mechanism reported.

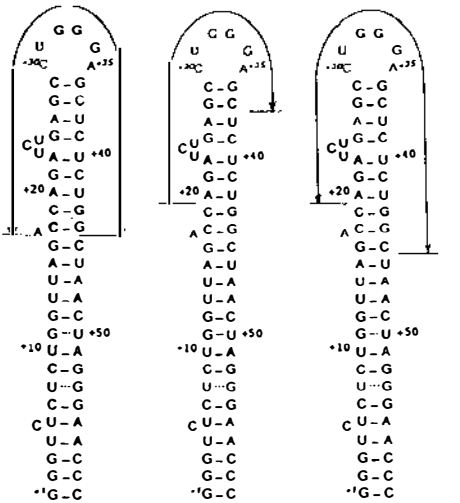
Target RNA species that have been reported to be inhibited include HIV (19), vesicular stomatitus virus (VSV) (82), n-*myc* (150), and a number of normal cellular genes (151–154).

In our laboratories, we have shown that a 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. Studies with RNaseH confirmed that ISIS 1082 binds selectively in this area. In vitro protein synthesis studies confirmed that ISIS 1082 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 an IC_{50} of 200–400 nm

by translation arrest (155). 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 were the most potent of the more than 50 compounds studied complementary to various other regions in the RNA (156).

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. Recent studies on several compounds have provided data that unambiguously demonstrate that this mechanism can result in potent antisense drugs.

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 (Figure 2). These structures play crucial roles in a variety of functions. They are used to provide additional stability for



length	28	18	26
P=S	1972	1308	1307
P=O	1971	1116	1118

Figure 2 Antisense oligonucleotides directed against the HIV TAR element. The oligonucleotide sequences are complementary to the tar sequences where indicated.

RNA and as recognition motifs for a number of proteins, nucleic acids, and ribonucleoproteins that participate in the intermediary metabolism and activities of RNA species. Thus, given the potential general activity of the mechanism, it is surprising that occupancy-based disruption RNA has not been more extensively exploited.

As an example, we designed a series of oligonucleotides that bind to the important stem-loop in all RNA species in HIV, and TAR element. We synthesized a number of oligonucleotides designed to disrupt TAR, and showed that several indeed did bind to TAR, disrupt the structure, and inhibit TAR-mediated production of a reporter gene (157). Futhermore, general rules useful in disrupting stem-loop structures were developed as well.

Although designed to induce relatively nonspecific cytotoxic effects, two other examples are noteworthy. Oligonucleotides designed to bind to a 17 nucleotide loop in *Xenopus* 28S RNA, required for ribosome stability and protein synthesis, inhibited protein synthesis when injected into *Xenopus* oocytes (158). Similarly, oligonucleotides designed to bind to highly conserved sequences in 5.8S RNA inhibited protein synthesis in rabbit reticulocyte and wheat germ systems (159).

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. As RNA intermediary metabolism is better understood, many other regulatory features and mechanisms will probably be identified.

5' Capping A key early step in RNA processing is 5' capping (Figure 1). This stabilizes pre-mRNA and is important for the stability of mature mRNA. It also is important in binding to the nuclear matrix and nuclear transport of mRNA. 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, presumbly by inhibiting the binding of proteins required to cap the RNA. Again, however, this putative mechanism has not been rigorously demonstrated in any published study. In fact, none of the oligonucleotides have been shown in any published study to bind to the sequences for which they were designed. 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 (160).

In studies in our laboratory, we have designed oligonucleotides to bind to 5' cap structures and reagents to specifically cleave the unique 5' cap structure (161).

Inhibition of 3' polyadenylation In the 3' untranslated region of pre-mRNA molecules, there 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.

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

Zamecnik & Stephenson (1) reported that a 13-mer targeted to untranslated 3' and 5' terminal sequences in Rous sarcoma viruses was active. Oligonucleotides that were conjugated to an acridine derivative and targeted to a 3'-terminal sequence in type A influenza viruses were reported to be active (109, 162, 163). Against several RNA targets, studies in our laboratories have shown that sequences in the 3' untranslated region of RNA molecules are often the most sensitive. For example, ISIS 1939, a 20-mer phosphorothioate that binds to and appears to disrupt a predicted stem-loop structure in the 3' untranslated region of the mRNA for ICAM, is a potent antisense inhibitor. However, inasmuch as a 2'-O-methyl analog of ISIS 1939 was much less active, it is likely that in addition to destabilization to cellular nucleolytic activity, activation of RNase H (see below) is also involved in the activity of ISIS 1939 (164).

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 (for review see 165). At least two classes of RNase H have been identified in eukaryotic cells. Those in yeast and multiple enzymes with RNase H activity have been observed in prokaryotes (165). Furthermore, data suggest that there are multiple isozymes in eukaryotic cells.

Although RNase H is involved in DNA replication, it may play other roles in the cell and is found in the cytoplasm as well as the nucleus (166). The concentration of the enzyme in the nucleus is thought to be greater, however, and some of the enzyme found in cytoplasmic preparations may be due to nuclear leakage.

RNase H activity is quite variable. It is absent or minimal in rabbit reticulocytes (167) but present in wheat germ extracts (165) in a wide range of

cells (16). The level of RNase H varies as a function of development, differentiation, and rate of cell division (165). In HL60 cells, for example, the level of activity in undifferentiated cells is greatest; it is relatively high in DMSO and vitamin D--differentiated cells, and much lower in PMA-differentiated cells (G. D. Hoke et al, unpublished observations).

The precise recognition elements for RNase H are unknown; however, it has been shown that oligonucleotides with DNA-like properties as short as tetramers can activate RNase H (168). Changes in the sugar influence RNase H activation, as sugar modifications that result in RNA-like oligonucleotides, e.g. 2'-fluoro or 2'-O-methyl, do not appear to serve as a substrate for RNase H (44, 169). 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 (41, 170). Additionally, backbone modifications influence the ability of oligonucleotides to activate RNase H. Methylphosphonates are not substrates for RNase H (74, 143). In contrast, phosphorothioates are excellent substrates (106, 155, 171; G. D. Hoke unpublished observations). More recently, chimeric molecules have been studied as substrates for RNase H (172, 173). A single ribonucleotide in a sequence of deoxyribonucleotides was recently shown to be sufficient to serve as a substrate for RNase H when bound to its complementary deoxyoligonucleotide (174).

Despite the information about RNase H and the demonstrations that many oligonucleotides may activate RNase H in lysate and purified assays (168, 175–177), relatively little is known about the role of structural features in RNA targets in activating RNase H. There is little direct proof that RNase H activation is, in fact, the mechanism of action of oligonucleotides in cells. Recent studies in our laboratories provide additional, albeit indirect, insights into these questions. ISIS 1939 is a 20-mer phosphorothioate complementary to a sequence in the 3' untranslated region of ICAM-1 RNA. It inhibits ICAM production in human umbilical vein endothelial cells, and northern blots demonstrate that ICAM-1 mRNA is rapidly degraded. A 2'-O-methyl analog of ISIS 1939 displays higher affinity for the RNA than the phosphorothioate, is stable in cells, but inhibits ICAM-1 protein production much less potently than ISIS 1939. It is likely that ISIS 1939 destabilizes the RNA and activates RNase H. In contrast, ISIS 1570, an 18-mer phosphorothioate that is complementary to the translation initiation codon of the ICAM-1 message, inhibited production of the protein but caused no degradation of the RNA. Thus, two oligonucleotides that are capable of activating RNase H had different effects, depending on the site in the mRNA to which they bound (164).

COVALENT MODIFICATION OF THE TARGET NUCLEIC ACID BY THE OLIGONUCLEOTIDE A large number of oligonucleotides conjugated to alkylating

and photoactive alkylating species have been synthesized and tested for effects on purified nucleic acids and intracellular nucleic acid targets (162, 178). The potential advantage of such modifications is, of course, enhanced potency. The potential disadvantages are equally obvious: nonspecific alkylation in vivo and resulting toxicities.

A variety of alkylating agents have been used to modify single-stranded DNA covalently and have been shown to induce alkylation at sequences predicted by the complementary oligonucleotide to which they were attached (178–182). Similar alkylators have been employed to modify double-stranded DNA covalently after triplex formation (125, 137, 183, 184).

Photoactivatable crosslinkers and platinates have been coupled to oligonucleotides and shown to crosslink sequence-specifically as well. Photoactivatable crosslinkers coupled to phosphodiesters, methyl-phosphonates, and phosphorothioates have been shown to produce sequence-specific crosslinking (59, 130, 185–190). Photoreactive crosslinking has also been demonstrated for double-stranded DNA after triplex formation (136, 191).

Preliminary data suggesting that covalent modifications of nucleic acids in cells is feasible and may enhance the potency of oligonucleotides have also been reported. Psoralen-linked methylphosphonate oligonucleotides were reported to be significantly more potent than methylphosphonate oligonucleotides in inhibiting rabbit globin mRNA in rabbit reticulocyte lysate assay (33). Psoralen-linked methylphosphonates were also reported to be more potent in inhibiting herpes simplex virus infection in HeLa cells in tissue culture (147). Additionally, although it did not produce covalent modification, a 9-mer phosphodiester conjugated with an intercalator inhibited mutant Ha-ras synthesis in T-24 bladder carcinoma cells (81).

OLIGONUCLEOTIDE-INDUCED CLEAVAGE OF NUCLEIC ACID TARGETS

Another attractive mechanism by which the potency of oligonucleotides might be increased is to synthesize derivatives that cleave their nucleic acid targets directly. Several potential chemical mechanisms are being studied, and positive results have been reported.

The mechanism that has been most broadly studied is to conjugate oligonucleotides to chelators of redox-active metals and generate activated oxygen species that can cleave nucleic acids. Dervan and colleagues have developed EDTA-conjugated oligonucleotides that cleave double-stranded DNA sequence specifically after triplex formation (124, 137). Dervan and others have employed EDTA-oligonucleotide conjugates to cleave single-stranded DNA (192, 193). It is thought that EDTA chelates iron, which generates hydroxyl radicals that cleave the DNA; however, the cleavage occurs at several nucleotides near the nucleotide at which EDTA is attached.

In the presence of copper, oligonucleotides that are conjugated to 2,10-phenantholine also cleave DNA with some sequence specificity (129,

133–135, 194, 195), as do porphyrin-linked oligonucleotides when exposed to light (196–198). Phorphyrin-linked oligonucleotides, however, oxidize bases and induce crosslinks as well as cleave the phosphodiester backbone.

To date, no reports have demonstrated selective cleavage of an RNA or enhanced potency of oligonucleotides in cells using oligonucleotides and cleaving moieties that employ these mechanisms. Studies in progress in a number of laboratories will probably soon explore this question.

Another mechanism that may be intrinsically more attractive for therapeutic applications, particularly for cleavage of RNA targets, is a mechanism analogous to that used by many ribonucleases, nucleotodiyltransferases, phosphotransferases, and ribozymes.

Ribozymes are oligoribonucleotides or RNA species capable of cleaving themselves or other RNA molecules (199). Furthermore, the Tetrahymena ribozyme has been shown to cleave DNA, but at a slower rate than RNA (200). Although several classes of ribozymes have been identified that differ with regard to substrate specificity, the use of internal or external guanosine, and other characteristics, they all employ similar enzymatic mechanisms. Cleavage and ligation involve a Mg^{2+}-dependent transesterification with nucleophilic attack by the 3'-hydroxyl of guanosine (200).

The notion that we might design a relatively small ribozyme that could interact with desired sequences as a therapeutic was given impetus by studies that showed activity for ribozymes as short as a 19-mer (201) and by the demonstration that ribozyme activity can be retained after substitutions such as phosphorothioates are introduced (200).

Other approaches to creating oligonucleotides that cleave RNA targets are to synthesize oligonucleotides with appropriate adducts positioned to catalyze degradation via acid-base mechanisms. Substantial progress is being made in this area as well (P. D. Cook et al, unpublished observations).

A few studies have attempted to compare activities of oligonucleotides targeted to different receptor sequences in the same RNA. In our laboratories, we have shown that the most sensitive site in ICAM mRNA appears to be the 3' untranslated region (164). Against PLA_2, the most active molecules are also directed to sequences in the 3' untranslated region. In contrast, the most active molecules against ELAM are in the 5' untranslated region (C. F. Bennett et al, unpublished observations). However, oligonucleotides directed to the 5' cap site, translation initiation codon, and coding regions have also shown activity (for review see 202, 203).

In conclusion, an array of potential post-binding mechanisms have already been identified for oligonucleotides. For specific oligonucleotides, however, insufficient data are available to draw firm conclusions about mechanisms. More than one mechanism may very well play a role in the activity of a given oligonucleotide. Many additional mechanisms are likely to be identified as

progress continues. It is important to consider the structure and function of receptor sequences in designing oligonucleotides and to continue to study potential mechanisms in detail. Clearly, RNase H may play a role in the mechanisms of many oligonucleotides, but, equally clearly, it is not critical for the activity of others. In the future, the mechanisms for which oligonucleotides are designed will probably be optimized for each target and class of oligonucleotide.

MEDICINAL CHEMISTRY

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 three years prior to this writing. 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. As several excellent reviews have been published recently, I focus here strictly on design features and progress in evaluating various approaches to enhance the properties of oligonucleotides as drugs (12, 16, 202, 203).

Modifications in the base, sugar, and phosphate moieties of oligonucleotides have been reported. The subject 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.

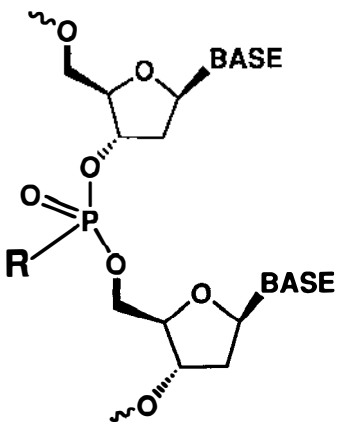
Modifications of the Phosphate

Table 6 shows the structures of various phosphate analogs. The properties of phosphodiester, phosphorothioate, and methylphosphonate analogs have been discussed extensively. More recently, phosphorodithioates have been synthesized and reported to be nuclease-resistant and to form stable duplexes with complementary DNA (204). Of course, a potential advantage of the phosphorodithioates is the lack of a chiral center. Another interesting recent modification is the replacement of one of the nonbonding oxygen atoms with a borane group (205). A dimer was reported to be nuclease-resistant, but little additional information is available.

Modifications of the Pentofuranose Linker

Modifications of oligonucleotides that replace phosphorous may be attractive because they support the design of oligonucleotides that may have a range of

Table 6 Phosphate modifications and analogs



Structure	R	Name
1	O^-	Phosphodiester
2	S^-	Phosphorothioate
3	Me	Methyl phosphonate
4	N (alkyl)	Phosphoramidate
5	S and O → S	Phosphorodithioate
6	H_3B	Boranophosphate

charges from nonionic to negatively or positively charged. In theory, reducing the anionic character of oligonucleotides may enhance hybridization and pharmacokinetic properties.

As discussed above, earlier modifications were not specifically directed to the needs of oligonucleotide drugs. Recent modifications have attempted to maintain the geometry and spacing required to support hybridization. Replacement of the phosphorous dioxygen moiety with a methylene group has been reported by two groups (51–53). This "formacetal" linkage forms a stable duplex with DNA and to be nuclease-resistant, but it is not amenable to additional modifications without creating chiral centers and would result in a water-insoluable molecule if fully substituted throughout an oligonucleotide. Other one-for-one substitutions have been reported, but the substitutions are either less interesting or have not been evaluated sufficiently to determine their potential (for review see 12).

More complex substitutions have also been reported recently, including two atom substitutions in which the phosphorous and 5' oxygen atoms were replaced by a sulfonamide linkage (54) or methyl sulfoxyl linkage (206). A thymine tetramer in which the phosphate backbone structure was replaced with dimethylene sulfonate was recently found to hybridize with natural oligonucleotides (207). Additionally, an acetamide group has been substituted for the backbone phosphate structure in a dinucleoside (207). Finally, a great many other substitutions in the backbone have been made and will probably be published in the next year, so the repertoire of compounds should continue to increase.

Pentofuranose (Sugar) Modifications

A significant number of modifications have recently been reported. In essence, these derive from two strategies with different objectives. Oligonucleotides in which the sugars are modified uniformly throughout are designed to enhance affinity to RNA targets by facilitating the formation of a more stable helix. They also may enhance nuclease stability and membrane permeability, but these outcomes are usually secondary to the hybridization goals. In contrast, pendant modifications have also been synthesized primarily to enhance pharmacokinetics or to introduce alkylating or cleaving moieties. In any event, the sugar at the 2' position is clearly an attractive site for medicinal chemistry. Some of the properties of a few of the 2' modified oligonucleotides are shown in Table 3. This remains a fertile area for medicinal chemistry, and additional advances are likely.

More substantive alterations in sugar and even replacement of sugar are also possible. Of course, α-oligonucleotides represent one type of modification (41), but numerous other approaches are feasible. Carbocyclic (49, 50) and acyclic (47, 48) structures have been reported.

Heterocyclic Modifications

Numerous heterocyclic modifications have also been described. Many of these have been designed to enhance affinity and/or alter specificity (224). Other modifications have been developed to attach pendant modifications that may alkylate, intercalate, or cleave, as well as others that may enhance pharmacokinetic properties.

Conclusions

In conclusion, it is clear that an enormous scope for medicinal chemistry exists and that the major programs are already beginning to pay dividends.

ACTIVITIES OF OLIGONUCLEOTIDES

In the past several years, scores of articles have been published demonstrating the activity of a large number of oligonucleotides in a variety of systems. A number of excellent reviews have summarized the activities of these compounds (16, 202, 203, 208). The activities of oligonucleotides in assays of cell-free protein synthesis and after injection into cells of several types have also been summarized. Consequently, I provide a brief summary of the activities of oligonucleotides in cell-based assays and a comment on the limited in vivo data reported to date.

Activities in Cells in Tissue Culture

To date, oligonucleotides have been found to inhibit the growth of a large number of viruses in tissue culture, the expression of numerous oncogenes, a variety of normal cellular genes, and a number of transfected reporter genes controlled by several regulatory elements. The oligonucleotides used, the cells employed, and the receptor sequences, concentrations, and conditions have differed widely. Only a few of the studies have reported detailed dose response curves and conditions. Studies for which sufficient information was presented are summarized in Table 7.

The data presented in Table 7 support only a few generalizations, as follows:

1. Even though phosphodiesters are relatively rapidly degraded, a number of laboratories have reported activities for unmodified phosphodiester oligonucleotides in cells incubated in the absence of serum. The concentrations required to display activity were typically greater than 10 μM.
2. A variety of modified oligonucleotides have been reported to be active. Methylphosphonates appear to be less potent than phosphorothioates, but considerable variation has been noted, depending on the system. Conjugation of alkylators and intercalators to phosphodiesters and methylphosphates increases potency. Lipophilic and poly-lysine conjugates have also displayed enhanced activities.
3. Oligonucleotides have demonstrated a broad array of activities against viral targets, oncogenes, normal host gene products, and various transfected genes. Thus, clear evidence supports the broad, potential applicability of these drugs.
4. Although the data from studies incorporated in Table 7 are limited, when combined with the in vitro toxicologic data, the therapeutic indexes of phosphorothioates appear to be quite high in vitro. Methylphosphonates appear to have lower therapeutic indexes. Too few data are available to draw conclusions about other classes of oligonucleotides.
5. Very few data support putative mechanisms of action, and generalizations concerning desired mechanisms of action are not possible. Nevertheless, a variety of mechanisms of action may be employed by oligonucleotides to result in significant biological activities.

In Vivo Activities

Two earlier investigations have suggested in vivo activities of antisense drugs against viral infections. Although no data were reported, a methylphosphonate oligonucleotide was indicated to have been active in a mouse model of herpes simplex virus I infection (147). Another study claimed in vivo activity against tick-borne encephalitis virus (213).

Table 7 Summary of antisense oligonucleotide Activities (in vitro)[a]

Target	Cell type	Serum	Oligo types	Length	Concentration	Reference
Viruses						
HTLV-III	H9 cells	–	P	12–26	5–50 mg/ml	148
HIV	H-T cells	+	PS	14–28	0.5 1 μM	20
HIV (gag/pol)	H-T cells	+	PS	18–24	1–10 μM	211
HIV	H9 cells	+	PS, others	20	4–20 μg/ml	19
HIV	CZM cells	+	PS	18–28	10 μM	157
Herpes simplex	Vero cells	+	CH_3P	7	50–100 μM	98
Herpes simplex	HeLa cells	+	PS	28	1–10 μM (non-antisense)	76
Herpes simplex	Vero cells	+	CH_3P	12	20–50 μM	15
Herpes simplex	Vero cells	+	CH_3P psoralen	12	5 μM	15
Herpes simplex	HeLa cells	+	PS	21	0.2–4 μM	155
Vesicular stomatitis	L929 cells	+	CH_3 P	9	25–50 μM	99
Vesicular stomatitis	L929 cells	+	P-lipid	11	50–150 μM	85
Vesicular stomatitis	L929 cells	+	P-poly *l*-lysine	10–15	0.1 μM	212
Influenza	MDCK cells	+	P-acridine	11	50 μM	109
Tick-borne encephalitis	—	+	Various	Var.	0.1–1 μM	213
SV40	MDCK cells	+	CH_3P	6–9	25 μM	214
Rous	Chicken fibroblasts	+	Various	Var.	10 μM	■
Hepatitis B	Alexander	+	P	15	8.5 μM	215
Bovine papilloma virus	C-127 cells	+	PS	4–30	0.01–1 μM	209
Oncogenes						
c-*myc*	T-lymphocytes	+	P	15	30 μM	96
c-*myc*	HL-60 cells	+	P, PS	15	10 μM	216
c-*myc*	Burkitt cells	■	P	21	100 μM	146

c-*myb*	PMBC	+	P	18	40 mg/ml	217
BCL-2	L697 cells	■	P, PS	20	25–150 μM	97
N-*myc*	Neuroblastoma cells	+	P	15	1–5 μM	155
N-*ras*	T15 cells	+	CH_3P	9	Inactive	100
Host genes						
Multiple drug Resistance	MCF-1 cells	+	PS	15		218
PCNA (cyclin)	3T3	+	P	18	30 μM	219
Prothymosin	Human myeloma cells	−	P	22		153
T cell receptor	T cells	+	P	22		153
Gm CSF	Endothelial cells	−	P	15, 18	10^{-5}M	153
CSF-1	*FL-ras/myc* cells	+	P	?	?	220
EGF receptor		+	P	13	30 μM	221
BFGF	Human astrocytes	−	P	15	10–75 μM	229
β Globin	Rabbit reticulocytes	+	CH_3P	9	100 μM	222
TAU	Neurons	−	P	20–25	3–50 μM	228
cAMP-Protein kinase II β	HL-60 cells	+	P	21	15 μM	210
Myeloblastin	HL-60 cells	+	P	18	■	227
Phospholipase A_2 activating protein	$BC3H_1$	+	P	25	25 μM	233
ICAM-1	A549 HVEC tymphocytes	−	PS	18–20	0.01–1 μM	154
IL-2	T-lymphocytes	−	P	15	5 μM	72
IL-1α	HUVEC	+	P	18	10 μM	154
IL-1β	Monocytes	+	PS	15	0.1–2.5 μM	105

Table 7 (*Continued*)

Target	Cell type	Serum	Oligo types	Length	Concentration	Reference
IGF-1	Myoblasts		P	15	10 μM	225
Perforin	T-lymphocytes		P	18	5–35 μM	226
Other						
Chloramphenicol acetyl transferase	CV-1 cells	+	P, PS, CH_3P	21	5–30 μM	75
Placental alkaline phosphatase driven by HIV TAR	SK-mel-2 cells	+	PS	18–28	0.25–5 μM	157
Chloramphenicol Acetyl transferase Driven by human papilloma virus E2 responsive element	C-127 and CV-1 cells	+	PS	14–20	1–10 μM	209

[a] Abbreviations: cAMP, cyclic AMP; CH_3-P, methylphosphonate oligonucleotides; EGF, epidermal growth factor; G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte macrophage colony-stimulating factor; HB, hepatitis B; HIV, human immunodeficiency virus; HSV, herpes simplex virus; HTLV, human T-cell lymphotrphic virus; IV, influenza virus; P, phosphodiester oligonucleotides; P-acridine, phosphodiester oligonucleotide conjugated with acridine moiety; P-lipid, phosphodiester oligonucleotide conjugated with lipid moiety; P-S, phosphorothioate oligonucleotides; PCNA, proliferating cell nuclear antigen; PMA, phorbol mysteric acid; RSV, Rous sarcoma virus; TAR, Tat response element; TBE, tick-borne encephalitis; VSV, vesicular stomatitis.

Topical application of ISIS 1082 in an aqueous buffer to the cornea of mice infected with herpes virus I inhibited viral growth in a concentration-dependent fashion and cured the infection at concentrations greater than 1% (230). The activity of ISIS 1082 was equivalent to trifluorothymidine and was associated with no local or systemic toxicities.

CONCLUSIONS

Oligonucleotides designed to interact with nucleic acid receptors represent a potentially revolutionary advance in pharmacotherapy. Advances in the recent past and the intense, current focus assure that the paradigm will be fully explored.

Oligonucleotides have already been shown to work in vitro and have proven to be invaluable pharmacologic tools. The progress in resolving the basic pharmacological questions relating to oligonucleotide therapeutics and in resolving issues that will influence the commercialization of new drugs of this class has been impressive. Moreover, advances in medicinal chemistry are exciting and argue that exciting new classes of these drugs are forthcoming.

Much remains to be learned; a great deal remains to be accomplished before the paradigm is fully proven and the opportunity it represents realized. In the coming years, the key tasks will be (*a*) to place oligonucleotide therapeutics on a solid pharmacological footing by performing careful dose response curves in well-designed experiments, and (*b*) to advance the development of oligonucleotide pharmaceutic products to the point that the paradigm can be tested in man.

There is now cause for considerable optimism that the promise of oligonucleotide therapeutics may be realized.

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