Oligodeoxynucleotides as Inhibitors of Gene Expression: A Review

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Abstract

The past several years have seen an explosive growth in the application of antisense oligodeoxynucleotides as modulators of gene expression. In this review, we discuss the various classes of compounds currently receiving attention. These include unmodified oligodeoxynucleotides, methylphosphonate-ODN, phosphorothioate-ODN, and o-oligouonucleotides. The relative advantages and disadvantages of each class with regard to parameters such as (a) duplex stability (b) nuclease resistance, and (c) effectiveness of specific gene inhibition are noted. Oligodeoxynucleotides covalently linked to intercalating and other reactive groups are also described, as are possible mechanisms of oligodeoxynucleotide action. Finally, we consider potential future directions for this field and note that it holds the promise for specific gene therapy.

Introduction

During the past 100 years the principle of molecular substitution, i.e., of altering a chemical structure to see what happens, has been developed to new levels of sophistication. However, exquisitely detailed studies of drug mechanisms have all too infrequently led to clinical breakthroughs. New drugs are usually found by screening, but this process depends largely on the tumor model and chance and has had limited success. Most drugs that have been discovered in this way imitate natural cellular substances and operate either by inhibiting key enzymes in biochemical pathways and/or randomly damaging DNA. Due to low therapeutic indices, most antineoplastic drugs are accompanied by serious dose-limiting toxicities. Ideally, drugs are needed which are specifically tumor targeted, thus sparing normal tissue.

One tumor-specific target may be the now well recognized phenomenon of pleiotropic drug resistance. PDR is a matter of great concern, since the resistance to one drug results in resistance to a series of drugs with apparently quite distinct mechanisms of action (1). Consequently PDR can totally frustrate the aims of current chemotherapeutic approaches. Recent work indicates that PDR is associated with biochemical changes intrinsic to the genotype of the chemically transformed cell, which result in the overproduction of certain proteins (2, 3). These proteins are associated with more efficient drug detoxification and confer a much greater degree of survivability upon the resistant cancer cell.

In general, it would be desirable to have a strategy which would allow one to circumvent PDR. Recent advances suggest that such an approach may now be feasible. DNA and proteins, the usual targets of drugs, are among the most stable of biochemical substances, DNA because it is the repository of the genetic information and proteins by virtue of the peptide bond. By contrast, the molecule that acts as the intermediary in genetic expression, mRNA, may be the vulnerable link in the chain and may be a target of attack by a selective agent thereby inhibiting gene expression. It must be noted that inhibition of one copy of mRNA will theoretically be much more efficient in this regard than inhibiting a protein product, since one mRNA gives rise to multiple protein copies.

Parenthetically, but not unimportantly, such an approach might be directly applicable to the inhibition of retroviruses, such as HIV (4), which is the etiological agent of acquired immunodeficiency syndrome and related diseases (5). Viral RNA should have the same vulnerability as mRNA in the cell cytosol. It should be noted that the same compound (sequence) could also inhibit the mRNA of the proviral complementary DNA, hence potentially providing a double target for a putative drug.

Several major developments in molecular biology have made the selective inhibition of gene expression as a chemotherapeutic approach outlined here feasible. They include the development of recombinant DNases, which have allowed DNA and mRNA sequences to be determined. This in turn acted as a stimulus to the development of methods for the automated synthesis of oligodeoxynucleotides (6). Studies of mRNA function led to the understanding of mRNA self-splicing (7) and the realization of the importance of mRNA conformation. This in turn has benefited from the utilization of computer programs to calculate mRNA base-pairing schemes (8), which indicate possible single-stranded accessible regions as likely targets for complementary base binding. Developments in all of these areas combine to provide the basis for this new approach to chemotherapy.

Properties of a Gene-inhibitory Drug

To selectively suppress gene expression the complementary or antisense base sequence to a target genetic message is the basic requirement. One way of attempting to accomplish such inhibition, which has received much attention, is the intracellular expression of an antisense mRNA vector. A discussion of antisense RNA is beyond the scope of this review and may instead be found in Ref. 9. However, this approach runs into serious problems due to the difficulties in controlling expression of such a vector. A much simpler approach is to use an extrinsic oligodeoxynucleotide (Fig. 1) to bring about protein translation arrest. What would be the ideal requirements of such an agent to inhibit gene expression?

Selectivity. The complementary base sequence would be used to provide specificity for binding selectively to the target mRNA molecule; this would require a synthetic oligonucleotide with the requisite sequence. Of course, this presumes knowledge of the mRNA sequence and its accessibility to soluble reagents in the cytosol.

Stability. Since oligonucleotides are readily cleaved by nucleases in vivo (10), an analogue of an oligonucleotide is required that must be sufficiently stable to nuclease to reach the target in effective concentrations. Because of their greater stability...
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Fig. 1. Structures of normal (I) and modified (II-IV) ODNs; B is adenine (A), guanine (G), cytosine (C), or thymine (T). I, normal phosphate linkage; II, ethyl phosphotriester; III, a methylphosphonate; IV, a phosphorothioate. (Note: A phosphonate is, strictly speaking, a phosphorus compound at the P(III) level of oxidation, i.e., lacking an oxygen relative to phosphate; a methyl phosphate is the derivative with a direct P—C bond. In the phosphorothioate, the negative charge is actually shared between the nonesterified O and S atoms.)

Oligodeoxynucleotides, as opposed to oligoribonucleotides, would form the basis of such an analogue.

Solubility. The substance must be soluble in water yet pass through the cell membrane. Although oligonucleotides presumably enter the cell, the fact that they are polyanions could be an impediment to their ready transportability. On the other hand, an oligonucleotide analogue that is too lipophilic may not be useful due to reduced aqueous solubility; clearly a balance is called for between these two extremes.

Oligodeoxynucleotides and Analogues

This section will focus on the role of oligodeoxynucleotides in regulating gene expression.

Normal Oligodeoxynucleotides. Some of the earliest work in this field was done by Zamecnik and Stephenson (11, 12), who, in the days before automatic synthesizers, made a 13-mer oligodeoxynucleotide complementary to the 5′ and 3′ reiterated terminal sequences of the Rous sarcoma virus 35S RNA. In infected chick embryo fibroblast (CEF) cells (multiplicity of infection, 0.002), there was a 99% decrease in reverse transcriptase activity present in the medium at 192–240 h. At a multiplicity of infection of 0.2, a 90% decrease at 48–96 h was seen. The concentration of the tridecamer (as its blocked isourea) was 10 μg/ml. Some inhibition was seen with the unblocked material, but the effect was not nearly as dramatic. There was no apparent toxicity to the CEF cells, and the inhibitory effect on reverse transcriptase activity was correlated with decreased cell transformation. In a cell-free wheat germ system, the 13-mer (unblocked) was examined for its effects on translation of heated Rous sarcoma virus 70S RNA. A dose-dependent transcription inhibition was observed, most pronounced at 0.05 A_{260} unit of 13-mer (99%). However, there was also a 47% inhibition of globin mRNA synthesis at the same concentration of oligomer, perhaps because there is a partial homology of the 3′-terminal region of the globin mRNA and the 3′-terminal nucleotides in the Rous sarcoma virus 35S mRNA. However, Brome mosaic virus total RNA was inhibited 63%, and endogenous control was inhibited 31% at the same concentration of 13-mer. Random oligomer sequences at about the same concentration showed little effect, but the longest one tested was only a 10-mer. dC_{12-18} did not inhibit translation at the 90% inhibition point of the 13-mer.

In subsequent experiments, Zamecnik et al. (13) synthesized ODNs for use against HIV. The regions of the viral mRNA they considered most susceptible to attack included: (a) the 5′ region adjacent to the lysine tRNA primer binding site; (b) a region which included the primer binding site and the region just 5′ to it; and (c) regions from the splice sites of the pre-mRNA that expresses the 3′ open reading frame (splice acceptor and splice donor sites).

They synthesized several ODNs complementary to a and b: A 12-mer (containing a 3′-terminal deoxythymidine), a 20-mer, and a 26-mer with a 3′-terminal noncomplementary tail of (pA)_{3}. Reverse transcriptase activity and the production of virus-encoded M, 15,000, and M, 24,000 proteins were examined (H9 cells). The greatest inhibition (67% reverse transcriptase, 95% M, 15,000 protein, 88% M, 24,000 protein) was by a 20-mer complementary to the splice acceptor site for the tat-III gene (9 μM). The oligomer containing the 3′-ddT residue (complementary to a region 5′ to the primer) was minimally effective in H9 cells but inhibited reverse transcriptase by 40% in peripheral blood cells (20 μM).

It should be pointed out that most of these experiments with phosphodiester oligodeoxynucleotides were carried out in heat-inactivated medium in order to inactivate any nuclease that were present. The mode of uptake of these charged molecules by cells is still unclear and is the subject of ongoing research.

Methylphosphonate Oligodeoxynucleotides. Concurrent with these experiments on normal ODNs was the elegant work of Miller et al. (14–20), who synthesized and characterized a variety of phosphotriesters and methylphosphonate antisense constructs (see Fig. 1). These compounds, which lack ionizable groups and are uncharged, have increased hydrophobicity, which reportedly confers increased cell membrane permeability upon them. They are presumably passively transported across the cell membrane and are also nuclease resistant, although they are enzymatically depurinated with time. If cell lysates are examined 18 h after treatment, 70% of labeled triitated thymidine was still associated with the intact oligomer (the remainder being in TTP and cellular DNA). However, ethyl phosphotriesters were found to undergo degradative deethylation in cells (21).

Initially, Miller et al. (14–21) synthesized a series of normal 8–12-mer oligodeoxynucleotides and studied the effect on rabbit globin mRNA translation in the reticulocyte lysate and wheat germ systems (0–200 μM ODN, 0.3 mg mRNA). The oligomers constructed were complementary to both α and β chain regions, most included the AUG initiation site and some included sequences near the initiation codon. The oligomers could be used as primers for reverse transcriptase, and the size of the complementary DNA transcripts was usually very close to what was expected. In terms of effect on translation, ODNs complementary to the initiation codon seemed to be most effective. For example, a 12-mer (to α 28–39) at 100 μM inhibited 97% α + 96% β chain production in the reticulocyte and the wheat germ system and 100% α + 100% β in the wheat germ system. At 5 μM oligomer, however, inhibition was sharply reduced. Oligomers complementary to the coding regions or the 3′ noncoding regions of the mRNA had little effect at any concentration used.

Oligodeoxynucleotide methylphosphonates were constructed complementary to the 5′ end, the initiation codon region, the coding region and the polyadenylate tail (dT 6-mer) of the globin message. In general, preannealing the mRNA and the
oligomer increased inhibition 20–30%, as could be expected. What is perhaps more surprising is that at 37°C a 4-mer (to α 37–40, β 54–57, 200 µM) caused inhibition of α and β chain synthesis by 28 and 42%, respectively (Fig. 2). Even more dramatic were the results obtained with a 5-mer, while at the same concentration (200 µM), an 8-mer (to β 49–56) was almost ineffective (11% α and 8% β inhibition, respectively). The results with the shortest oligomers are particularly difficult to understand given the reaction temperature (37°C) and the predicted value of Tm which must be far lower. However, a dT 6-mer showed no inhibitory effect and a random sequence had only a minimal effect (100 µM only). Direct comparison of inhibition of globin mRNA translation by normal oligodeoxynucleotides versus their methylphosphonates showed a slight advantage for the latter, but only in the coding region of the mRNA, where neither are particularly effective.

This group of workers also synthesized methylphosphonate oligomers complementary to the initiation codons of VSV N, NS, and G protein mRNAs. They studied the synthesis of five VSV proteins (L, G, NS, N, M) in infected L-cells. When a 9-mer was used (150 µM), a sharp decrease in global viral protein synthesis occurred (visualized autoradiographically). There was no effect on L-cell protein synthesis and no cytotoxicity. That all viral proteins were inhibited was thought to be secondary to a specific viral requirement for the NS protein. The VSV titer was decreased 1 log unit.

ODN methylphosphonates were synthesized to bind to the donor splice junction of the SV40 large T-antigen produced in infected African green monkey kidney cells. A 9-mer inhibited synthesis of the large T-antigen by 19% (elsewhere 19) reported as 45% (25 µM), with a 6-mer showing 30% inhibition. Two 6-mers complementary to the 5’-terminal sequences of U1 RNA were constructed (U1 RNA has been implicated in splicing mammalian and viral pre-RNAs). With these, production of large T-antigen was inhibited by 52 and 29%.

These same authors synthesized an 8-mer ODN methylphosphonate complementary to the acceptor splice junction of the HSV type I immediate-early pre-mRNAs 4 and 5. Vero cells were exposed to various concentrations (0–300 µM) of 8-mer before (1–24 h), during (0 h), or after (1 h) infection with HSV-1 (3 plaque-forming units/cell). With regard to virus titers, a 90% inhibition was seen at 75 µM with no increase at higher concentrations (300 µM; oligomer added before or during infection). Under these same conditions, 25 µM oligomer caused an inhibition of 50%. However, at 1 h postinfection, there was only a 40% decrease in viral titers, even at 150 µM. The effect was sequence specific, and a similar effect was seen in infected fibroblasts. The authors noted that protein synthesis was decreased 10–30% in mock infected cells treated with the oligomer. In infected cells, the rate of protein synthesis remained stable and was not thought to reflect the synthesis of functional or structural viral proteins, both of which were shown to be inhibited (67–95%). There was also a 3.5–4 fold decrease in tritiated thymidine-labeled DNA in infected cells, mostly in the viral fraction, and an increase in the normal cellular fraction. In the untreated cells, no labeled cellular DNA could be observed at all.

Six- and 7-mer ODN methylphosphonates were synthesized complementary to the Shinte-Dalgarone sequence (—AC-CUCCU—) found on the Escherichia coli 16S rRNA at the 3’ end. The sequence influences the binding of the 40S ribosomal subunit to bacterial mRNA and is lacking in the eukaryotic 18S RNA. At 100 µM 6-mer (22°C) polyuridyldirectly polylysinethesine and polyadenylatedirected polyphenylalanine synthesis were suppressed by 39 and 80%, respectively. For the 7-mer at 25 mm, no inhibition was seen. Three- and 4-mers showed little effect. Neither the 6- nor the 7-mers were globin translation inhibitors in the reticulocyte system.

Interestingly, the complexes that inhibited cell-free amino acylation (3- and 4-mer oligodeoxiyadenosine methylphosphonates) to some extent inhibited the growth of the BP6 (transformed Syrian hamster fibroblast) line and the human HTB1080 line (50 µM, 31% for the 3-mer). Two 6-mer antisense U1 RNA oligodeoxynucleotide methylphosphonates, as previously, inhibited the growth of transformed human fibroblasts (53% at 200 µM) and BP6 cells (94% at 75 µM) in culture. In the BP6 cells, RNA synthesis was decreased 66% and protein synthesis 25% (50 µM).

While these results are novel and provocative, the ODN methylphosphonates suffer from several serious drawbacks. (a) They are difficult to synthesize in high yield because they are extremely sensitive to base-catalyzed hydrolysis. The precursors are not yet commercially available, although this may change in the near future. (b) They have relatively low solubility in aqueous media; indeed, M-ODNs without polar terminal phosphate or phosphodiester groups that are longer than 12-mers are virtually insoluble. (c) Phosphorus atoms with four different substituents exhibit diastereoisomerism. The synthesis used for the methylphosphonates is not stereospecific, so that an oligomer of length n has $2^{n-1}$ stereoisomers. Duplexes formed with M-ODNs will therefore be expected to exhibit reduced hybridization compared to normal phosphodiesters (as evidenced by melting temperatures), due partially to steric hindrance by the methyl group that destabilizes the double helix (22). (d) Because of enhanced lipophilicity, they are presumably soluble in intracellular liquid particles and membranes. This, however, may be advantageous in terms of blood-brain barrier penetration.

The above factors may partially explain why oligodeoxynucleotide methylphosphonates require high concentrations (typically 100–300 µM) in order to elicit maximal activity.

Phosphorothioate Oligodeoxynucleotides. S-ODNs, in which one of the nonbridging oxygen atoms in each internucleotide phosphate linkage is replaced by a sulfur atom (Fig. 1), have several properties that make them potentially advantageous antisense analogues: (a) they are relatively stable to cleavage by nuclease [factors up to several thousand have been reported (23)]; (b) they have good aqueous solubility properties; (c) they

Fig. 2. Effect of d-CCAT methylphosphonate tetramer on globin protein synthesis in a rabbit reticulocyte lysate system at 25°C (C) and 37°C (C) (from Ref. 16).
show some depression of $T_m$ and hence can be expected to hybridize well (24); (d) they are stable to base-catalyzed hydrolysis, so that standard blocking groups can be used in synthesis; and (e) the widely used method of automated ODN synthesis using phosphoramidites (6) has been adapted (25) to provide a convenient and cost-effective route to these compounds. The synthesis of the $P=S$ analogue is relatively easy via the phosphoramidite method, since the last step in each synthesis cycle is an oxidation step. Normally this is carried out with iodine in pyridine, but a simple substitution of a sulfur solution leads to direct sulfurization of the phosphate.

Matsukura et al. (26) synthesized a series of phosphorothioate-ODNs complementary to sequences of the art/trs genes of HIV; these genes are apparently essential for viral replication. They also examined normal as well as one methylphosphonate counterpart to these sequences. The factors of base composition and chain length were evaluated with homopolymers $dA$, $dC$, and $dT$ in three lengths (5-, 14-, and 28-mers). An oligo-$N$-methylthymidine phosphorothioate analogue was synthesized in order to assess the role of hybridization in the antiviral effect, this sequence gives no measurable $T_m$ when annealed to its complement. ATH8 cells were used as targets for the cytopathic effect of HIV. At 25$\mu M$, a phosphorothioate 14-mer complementary to the art/trs region (trivial name ODN-1) inhibited the cytopathic effect of the virus by 95% as compared to 4 and 10% for the normal ODN and M-ODN at the same concentration, respectively. Similar results were seen with a 14-mer S-ODN downstream to a sequence 12 nucleotides downstream of the complement of ODN-1. However, there was some cytotoxicity associated with the active S-ODNs (10–20%). The S-ODN which contained $N$-methylthymidine showed no anti-HIV activity, suggesting that hydrogen bonding with the complementary strand was critical for inhibition. In subsequent experiments, a 14-mer with the same base composition as ODN-1 but with <70% homology with any sequence in the human T-cell leukemia virus III BH10 clone either as sense or antisense was equally efficacious in viral infection (25$\mu M$), as was the sense counterpart of ODN-1. The former observation is somewhat difficult to rationalize but it may be related to the observed proportional increase of viral inhibition with increasing GC content of the analogue. Interestingly, $dC_{28}$-phosphorothioate was one of the most potent of all compounds tested, with no measurable cytotoxicity and 100% inhibition of viral cytopathic effect at a concentration as low as 3$\mu M$ (Fig. 3).

Inhibition was also observed for $S-dA_{14}$ and $S-dA_{28}$ but was not as pronounced, while $S-dA_5$ and $S-dC_5$ were ineffective. In general, for both homopolymers and mixed-base constructs, longer sequences were found to be more effective than shorter sequences at the same molar concentration (28 > 14 > 5; Fig. 3). S-$dC_5$ was shown by Southern blotting to decrease the $T_m$ of viral DNA at concentrations as low as 1$\mu M$. Furthermore, S-$dC_{14}$ (5$\mu M$) seemed to synergize with 2',3'-dideoxyadenosine (2$\mu M$) in providing complete protection against the viral cytopathic effect; each alone was only marginally effective.

The precise mechanism(s) of the phosphorothioate-ODN antiviral effect at the present time remain uncertain. It appears that more than one mechanism may be operative, including direct inhibition of primer binding to reverse transcriptase as well as translation arrest. It should be noted that a sequence-independent inhibitory mechanism is not strictly an antisense mechanism but might instead be termed an "anti-retroviral arrest." In that respect the non-sequence-specific mechanism involving polymerase inhibition might be considered similar to the “antitemplate” inhibition described for polymerases using polynucleotides, including partially thiolated (on the pyrimidine) polyctydilic acid (27).

Marcus-Sekura et al. (28) used a plasmid containing the gene for expression of the bacterial enzyme CAT coupled to either an SV40 or a HIV enhancer. They constructed phosphorothioate and methylphosphonate oligomers complementary to a 21-base sequence which included the initiation site. (The methylphosphonate oligo had a 5′-terminal phosphodiester linkage.) The most effective methylphosphonate oligomer was a 15-mer, which produced 65% inhibition of CAT activity (30$\mu M$). A similar concentration of 15-mer phosphorothioate produced 84% inhibition and was far more effective than a normal ODN.

Marcus-Sekura et al. (28) also noted that an $N$-methylpyrimidine phosphorothioate derivative produced significant levels of inhibition of CAT activity, in contrast to the results with HIV described above (26). This result and the possibility that the S-ODNs are directly inhibiting reverse transcriptase III (see above) suggest the general point that these oligomers might well interact with cellular enzymes (23), as well as with nucleic acids.

$\alpha$-Oligodeoxynucleotides. Another class of oligodeoxynucleotides are the $\alpha$-analogues (Fig. 4) (29–33), in which the $\beta$ linkage formed by the deoxyribose moiety with the purine or pyrimidine base in natively occurring DNA is transposed into the unnatural $\alpha$ linkage. Interestingly, $\alpha$-oligodeoxynucleotides form parallel rather than antiparallel duplexes with $\beta$-DNA. These $\alpha$-oligodeoxynucleotides are also highly nuclease resistant. Under similar reaction conditions, a $\beta$ 6-mer ODN was 89% degraded at 10 min by calf spleen phosphodiesterase, while the $\alpha$-anomer was essentially unchanged; with S$_2$O$_8$ (10 min), the $\beta$-DNA was entirely degraded whereas the $\alpha$ form was >90% intact. With snake venom phosphodiesterase, the $\beta$ isomer was degraded 30 times faster than the $\alpha$. Unfortunately, one serious problem with $\alpha$-DNA is its cost; one supply house
allocation of the acridine to the UUAA triple tandem repeat sequences which are contained in the regulatory site of the gene 32 mRNA of phage T4 (r1 = UUAAA, r2 = UUAAAUUAAA, r3 = UUAAAUUAAA). A derivatized acridine (Acr) was covalently linked to the 3'-end of the molecule. In brief, r3Acr was by far the most effective in inhibiting protein synthesis in a cell-free Escherichia coli system. The synthesis of trichloroacetic acid-precipitable material was decreased by 78% in the presence of 20 μM r3Acr compared to only 35% in the presence of r3. The order of inhibition was r1Acr < r2Acr < r3Acr, and r1 had only a very slight effect. This parallels the order of stabilities, as Tm for r3 = 39°C (but there is only a 4°C increment for r3Acr. For r2 versus r2Acr, the increment is 11.5°C). Oligomers with unrelated sequences had little effect, but 90 μM acridine derivative did block translation. However, nonspecific effects on transcription were present in this system, possibly due to direct interaction of the acridine-containing moiety with RNA polymerase. Specific effects on translation were observed using a M, 26,000 protein as a standard. As determined by autoradiography, this protein was decreased 20% after exposure to r2Acr while the gene 32 polypeptide was decreased 60%. Plasmid pKH13-encoded β-lactamase was not affected by r2Acr. In another plasmid (pTB10), there was no specific target for the oligomers, and β-lactamase and the T4 rIIB-encoded polypeptides were affected by about 20%. The oligomers were susceptible to endo- and 5'-nucleases and were hydrolyzed during incubation.

A similar set of experiments were attempted with the bla (or amp R) gene from transposon Tn5. Twelve base pairs (+2 to −10, where No. 1 is the transcription initiation site) are disrupted in the open complex. Helene et al. (36) constructed two ODNs with 3′ covalently linked derivatized acridines. One was a 6-mer (−4 to +2) and the other a nonamer (−7 to +2). Both inhibited initiation of transcription (for the 9-mer, 60%, 80 μM), but the nonamer, unlike the hexamer, also had a small effect on a control lac fragment. Paradoxically, a 6-mer shifted one nucleotide (−3 to +3) had no effect on bla transcription initiation.

Cazenave et al. (37) expanded this work with experiments using acridine-linked oligodeoxynucleotides directed against various regions of the β-globin message. For example, a 7-mer (complementary to the β-globin base sequence 45–51) and an 11-mer (sequence 44–54) each linked at the 3' ends to a derivatized acridine were used. Note that both these sequences are immediately 5' to the initiation codon region.

Injection of the Acr-11-mer plus rabbit globin mRNA into frog oocytes produced a 50% inhibition of β-globin synthesis at 0.5 μM (concentration of ODN inside the oocyte). An 11-mer without the acridine had no effect, as did an Acr-7-mer at 100 μM or an Acr-11-mer not complementary to the globin sequence. Injection of acridine alone (50 μM) also produced no effect. These last observations demonstrated the specific nature of α-thymidine at almost 30 times the cost of normal thymidine.

Covalently Linked ODN Analogues. A further pharmacological concept in relation to the use of ODN analogues is to covalently attach a chemotherapeutic agent. Several examples of the covalent linking of active groups to ODNs have been accomplished.

Asseline et al. (34) constructed ODNs of the type (Tp)n(CH2)mAcr where Acr is a derivatized acridine and is covalently linked to the oligomer at the 3' terminus (Fig. 5). They also prepared (Tp)nCH2CH3 as a comparison. The Tm values for the duplex with poly(rA) are given in Table 1. Note that the Tm of the 4-mer with a linked intercalator is similar to that of a 12-mer without. Furthermore, the optimal number of methylene groups separating the oligomer from intercalator seems to be 5 (no difference with m = 6 was claimed, but the data were not shown), and the binding stabilization secondary to the intercalation rapidly fell off with increasing ODN length (the Tm of the 12-mer is only slightly different than that of the 8-mer).

In a subsequent paper, Toulme et al. (35) synthesized ODNs complementary to the UUAAA triple tandem repeat sequences which are contained in the regulatory site of the gene 32 mRNA of phage T4 (r1 = UUAAA, r2 = UUAAAUUAAA, r3 = UUAAAUUAAA). A derivatized acridine (Acr) was covalently linked to the 3' end of the molecule. In brief, r3Acr was by far the most effective in inhibiting protein synthesis in a cell-free Escherichia coli system. The synthesis of trichloroacetic acid-precipitable material was decreased by 78% in the presence of 20 μM r3Acr compared to only 35% in the presence of r3. The order of inhibition was r1Acr < r2Acr < r3Acr, and r1 had only a very slight effect. This parallels the order of stabilities, as Tm for r3 = 39°C (but there is only a 4°C increment for r3Acr. For r2 versus r2Acr, the increment is 11.5°C). Oligomers with unrelated sequences had little effect, but 90 μM acridine derivative did block translation. However, nonspecific effects on transcription were present in this system, possibly due to direct interaction of the acridine-containing moiety with RNA polymerase. Specific effects on translation were observed using a M, 26,000 protein as a standard. As determined by autoradiography, this protein was decreased 20% after exposure to r2Acr while the gene 32 polypeptide was decreased 60%. Plasmid pKH13-encoded β-lactamase was not affected by r2Acr. In another plasmid (pTB10), there was no specific target for the oligomers, and β-lactamase and the T4 rIIB-encoded polypeptides were affected by about 20%. The oligomers were susceptible to endo- and 5'-nucleases and were hydrolyzed during incubation.

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In a subsequent paper, Toulme et al. (35) synthesized ODNs...
of the effect. The relative lack of inhibition for the underivatized 11-mer was ascribed by the authors as perhaps due to the increased $T_m$ for the Acr-11-mer compound (36°C versus 31°C). Injection of ODNs linked to derivatized acridine at >15 μM did, however, result in a nonspecific decrease in the synthesis of high molecular weight (>50,000) proteins.

In the wheat germ system, Acr-11-mer at 1 μM decreased β-globin synthesis 90% (30 min, 25°C), while α-globin synthesis was unaffected. However, the acridine-modified compound was no more effective than the underivatized 11-mer in this system. The authors state that “... the differences in the wheat germ system and oocytes indicated that the relative stability of the mRNA oligonucleotide could not account for the hybrid arrested translation in the two expression systems.” In subsequent experiments, incubation of β rabbit globin mRNA in the wheat germ system with ODNs complementary to the β-globin message led to loss of intact mRNA and to shorter mRNA fragments as seen by Northern blotting. Using a 17-mer complementary to the 3–19 base sequence, an mRNA shortened by some 60 nucleotides compared to intact β-globin mRNA was seen. A 500-nucleotide mRNA fragment was detected by a probe complementary to the 3’ end of the β-globin message following incubation with a 17-mer complementary to the base sequence 113–129. In oocytes, injection of a mixture of rabbit globin mRNA and this 17-mer resulted in a gradual decrease of intact β mRNA with time. No decrease was seen in the absence of injected ODNs. No mRNA fragments could be seen, which the authors suggested was perhaps due to their rapid degradation in oocytes. In the wheat germ system, Acr-ODNs gave results similar to those for the underivatized ODN. There was little difference between the two types of compounds in the oocyte system as well. The authors concluded that RNase H activity, acting to cleave the mRNA strand of the duplex, accounted for these observations. This presupposes that the presence of the acridine moiety had little or no effect on RNase H activity, an assumption that does not seem unreasonable. RNase H activity is an attractive potential mechanism to explain what has been labeled “translation arrest,” and may help to resolve the question of variability of results seen in the various test systems. However, other factors, such as cellular permeability (in the case of Acr-linked versus underivatized ODNs), are also probably of significance.

α-Oligodeoxynucleotides have also been linked to an intercalating group (38). The authors synthesized α(Tp)ₙ(CH₂)₅Acr (n = 7, 8). They noted that α-deoxyxogilocmers formed more stable duplexes (as measured by $T_m$s) with poly(rA) than do β-deoxynucleotides but slightly less stable complexes with poly-L-lysine. These workers synthesized ODNs with poly-L-lysine (M, 41). These oligomers (14-, 12-, 10-, 8-, and 6-mers) were complementary to positions 261–74 of a 303-nucleotide strand which was part of the tick-borne encephalitis virus. The intercalating group N(2-chloroethylamine) was attached to the 5’ end. Through an amine linker, an intercalating phenazine derivative could be attached to the 3’ end. In addition, a series of oligodeoxynucleotides (14-, 12-, 10-, 8-, and 6-mers) were constructed only containing the alkylator (either at the 5’ or 3’ end). With the 5’-linked oligomers in 20–30-fold excess over target strand, it could be shown that the alkylation group was delivered to the first nucleotide adjacent to the complementary sequences. Neighboring nucleotides were affected to a low extent. Compounds with 3’-alkylators delivered them to a position three nucleotides upstream on the complementary strand, but also alkylated at a guanosine residue 80 nucleotides from the target sequence. This residue may abut the target sequence in three dimensions. The authors noted, however, that at 37°C, 6- and 8-mers bearing 3’-alkylators do not react with the complementary strand, presumably because the reaction conditions exceeded the $T_m$. However, attachment of intercalating groups enhanced the binding. Thus, with a derivatized phenazine in the 5’ position an alkylator in the 3’ position, both the 6- and 8-mers now alkylated the DNA fragment, and the reaction was efficient even at 40°C; yields may have approached 80%. Small amounts of products modified at other positions both up and downstream were seen at 37°C (8-mer, 3’-alkylator plus 5’-intercalator). At 50°C, only a single product was seen, but the alkylation efficiency appeared to diminish.

Webb and Matteucci (42) have described a modified pyrimidine base that results in interstrand cross-linking upon duplex formation. They synthesized the novel 5-methyl-1',N²,N⁴-ethanoxytocine (C) base prepared from a cytosine triazole intermediate and incorporated it into an oligomer. They were able to show that the otherwise stable C alkylates the complementary strand upon hybridization. This is an example of a new class of modified base alkylators.

A somewhat different approach was taken by Lemaitre et al. (43). These workers synthesized ODNs with poly-l-lysine (M, 14,000, 66 amino acid residues) covalently attached to the 3’ terminus through a morpholine ring. One ODN (a 15-mer) was complementary to the 5’ end of a mRNA coding for the VSV N-protein. The other, a 13-mer, was complementary to a sequence located in the middle of the message. The average ODN/lysine molar ratio was 0.5 ODNs were incubated with L929 cells 2 h prior to infection; the authors state that the conjugates were efficient 2 h after infection but the data were not shown.

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Virus titers were determined 24 h later. A dose-dependent reduction of VSV yield was observed, with >95% inhibition achieved at an oligomer concentration of 400 nM. No effect was seen with the ODN-polylysine conjugate directed at the internal sequence of the gene. Cellular protein synthesis in treated, infected cells was, as in Ref. 19, actually increased, again, presumably resulting from reversal of a VSV-induced shutoff of host protein synthesis. However, in those experiments, ODN methylphosphonates had to be added at 100-fold higher concentration to reduce VSV multiplication in mouse L-cells.

This approach has been expanded by attaching iron EDTA or analogues to the oligomer. These can give rise to reactive oxygen-containing radicals in the vicinity of the DNA. Boutorin et al. (44) synthesized oligo(dT)₈ covalently linked to EDTA. The authors claim 50% “degradation” of polydeoxyadenylate by the iron EDTA complex (5 h), and 14% of polydeoxyribonucleotide, indicating some nonspecificity. The reagent itself also underwent autodestruction with time. Dreyer and Dervan (45) attached an EDTA moiety to C-5 of thymidine through an N-(2-aminoethyl)propionamide linker. The synthesis is not trivial and the cleavage reaction requires 10 mM Fe(II), and 4 mM dithiothreitol. These authors constructed a 19-mer complementary to the EcoR1/RsaI restriction fragment of pBR322. Cleavage occurred to some extent over the entire length of the hybridized template strand, with two maxima about four bases 5’ and 3’ of the modified thymidine, which is the central point (500-fold excess of iron EDTA oligomer complex). At a 50-fold excess, the cleavage efficiency markedly decreased.

Chi-hong and Sigman (46) used 5-glycylamido-1,10-o-phenanthroline covalently linked to the 3’ terminus of a 21-mer targeted to positions 1–21 of the single-stranded M13mp8 phage DNA. The predominant products were formed at positions 21, 22, 23, and 24 but the cleavage reaction requires 1.0 mM cuprous ion and 2.5 mM 3-mercaptopropionic acid. The results indicated, however, that the cleavage species being produced was not freely diffusible. Chu and Orgel (47) covalently attached EDTA and diethylentriamine-pentaacetic acid to the 3’ terminus of the oligomer. This reaction, as previously, also requires 1 mM Fe(II) and 5 mM dithiothreitol. Cleavage efficiencies were uniform over the range of molar excess of oligomer from 7 to 70, but at lower ratios the yield of cleavage products decreased. At a 20–30-fold molar excess, there was no change when [Fe(II)] was lowered from 0.06 mM to 0.01 mM, but the reaction efficiency decreased markedly at [Fe(II)] = 0.001 mM. The authors note that achieving complete specificity with EDTA complexes could be problematic: the Fe(II) of the EDTA complex may not be tethered to a single nucleotide site; OH radicals may diffuse in many directions; and the highly efficient cleavage reagents required may “commit suicide” by destroying their own backbones.

Towards a New Chemotherapy?

From the above discussions it appears implicit that ODNs enter cells, specifically interact with their complementary mRNA molecules, and render them useless for translation, although such a mechanism may be somewhat harder to prove in practice. Synthetic ODNs, as discussed, may eventually find clinical use in the treatment of HIV and related retroviral disorders. Indeed, it is conceivable that many types of mRNA molecules of clinical interest may fall prey to specific inhibition.

A basic issue requiring further investigation is the question of optimal location of the inhibitory oligomer, namely, the complementary strand. Wickstrom et al. (48) calculated the secondary structure for the 5’ half of the VSV mRNA. Nucleotides 17–31 formed a single strand bulge, and nucleotides 37–46, which contained the initiation codon, formed a small bulge. They constructed an ODN complementary to the former and observed 50% inhibition of overall VSV mRNA translation at about 14 µM. However, the same oligomer nonspecifically inhibited Brome mosaic virus mRNA translation in the reticulocyte system. The issue is clearly not resolved, but on balance, oligomers complementary to regions containing the initiation codon and/or neighboring sequences seem well worth investigating.

An attempt to understand some of the mechanistic aspects of ODN inhibition was made by Haeuptle et al. (49). Initially, in an in vitro system, chicken lysozyme mRNA was transcribed from plasmid pDSS4 with RNA polymerase. The transcribed message was translated in the wheat germ system in the presence or absence of a 1–50-fold excess of a 5-, 10-, 15-, or 20-mer ODN complementary to the mRNA sequence just 3’ of the unique SstI restriction site. The 5-mer was unable to arrest translation; the 10- and 15-mers at 50-fold excess and the 20-mer at 10-fold excess gave rise to what were shown to be truncated polypeptides. Addition of an unrelated oligomer yielded a full-length lysozyme. Because, for each oligomer, the authors observed only one species of truncated polypeptide, they favored the idea that the ribosomes coming upon the blocked site may dissociate, allowing the trailing ribosomes to continue translating until the blocked site is reached. This may allow for repeated cycles of initiation and elongation.

Unfortunately, in the reticulocyte lysate system, 50% of the translated products were full-length polypeptides. Addition of an unrelated oligomer yielded a full-length lysozyme. Because, for each oligomer, the authors observed only one species of truncated polypeptide, they favored the idea that the ribosomes coming upon the blocked site may dissociate, allowing the trailing ribosomes to continue translating until the blocked site is reached. This may allow for repeated cycles of initiation and elongation.

In a similar set of experiments, these authors (49) also constructed an li-CAT fusion protein which consisted of two fragments of human invariant chain (209 amino acids each) fused out of frame to the CAT protein so that protein synthesis is terminated after 62 amino acids. An ODN was constructed complementary to the 3’ end of the li fragments. The “fully arrested” peptide is one li unit (li-1). The first read-through product is an in-tandem li-li dimer (li-2). The full-fusion protein requires read through of these two blocks. In fact, experimentally, li-1 was heavily represented, li-2 was seen, but the double read through was not seen at all (wheat germ system). Unfortunately, in the reticulocyte lysate system, 50% of the translated products were full-length polypeptides.

Kawasaki (50) constructed multiple oligoribo- or oligodeoxyribonucleotides (18–23-mers) which were all very effective (in µM concentrations) in blocking the appearance of interleukin 2 activity in Xenopus oocytes. Wang et al. (51) found a highly imaginative use for ODNs; they synthesized several ODNs on the basis of the amino acid sequence of tumor necrosis factor (64 total, each a 14-mer, 4 pools of 16, to complement all possible coding sequences for 5 amino acids). They hybridized each pool of probes to tumor necrosis factor mRNA, and then injected the mixture into Xenopus oocytes. They then assayed the oocyte extracts to determine the pool that inhibited tumor necrosis factor and identified 1 pool of 14-mers that inhibited
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The synthesis 5-fold. A specific 14-mer was eventually isolated and used to clone the gene.

Maher and Dolnick (52) synthesized ODNs complementary to various positions near the 5' end of the dihydrofolate reductase mRNA, some of which included the initiation codon. These ranged in length from 11 to 20 mers. Maximum protein inhibition in the reticulocyte lysate system, corresponding to a 5-fold decrease in DHFR synthesis, was best achieved with the 20-mer at 200 μM. The position of the ODN within the first 100 nucleotides of the DHFR mRNA was relatively unimportant but the oligomer length was most significant. Combinations of ODNs with binding sites that were separated by 16 or more nucleotides were no better than each alone. Those with binding sites that are contiguous or separated by 1–2 nucleotides were much more effective than each individually, and best results were obtained when the oligomers were contiguous. Strikingly, two contiguous 14-mers, each at 25 μM, inhibited DHFR mRNA translation as well as a 20-mer to the same region at 200 μM.

In an experiment that presages novel therapeutic uses for ODNs, Cornelissen et al. (53) constructed ODNs complementary to the 5'-terminal sequence of 35 nucleotides of Trypanosoma brucei mRNA. (12 22, 34 mers; it should be noted that probably all T. brucei mRNA molecules contain the same 5'-terminal sequences). The 22- and 34-mers inhibited translation 95–100% (wheat germ system, 15–30 μM). There was no effect on Brome mosaic virus mRNA translation.

As part of an effort to suppress the effects of expression of oncogenes, anti-myc ODNs have been constructed. Wickstrom et al. (54) reported that a 15-mer ODN complementary to the c-myc initiation codon and downstream region specifically inhibited HL-60 proliferation. Heikila et al. (55), using the same 15-mer, noted that it inhibited mitogen-induced c-myc mRNA expression in T-lymphocytes and prevented S-phase entry. There was no associated inhibition of transcriptional activation of the interleukin 2 receptor or transferrin receptor genes. The substitution of phosphorothioate ODNs in place of normal ODNs for the suppression of oncogene expression can be anticipated to result in more efficient inhibition from the viewpoints of both stability and dose. This assumes no other structural effects of the substitution of S for O in the phosphate moiety, which is the most conservative substitution one can conceive. The potential of this approach at this point seems great.

Covalently attaching a reactive group such as iron EDTA (see above) converts the ODN into an artificial endonuclease (Fig. 6). In a theoretical sense, the ODN can be used in effect as a delivery system. For example, it can be linked, by methods now under development, to a known drug such as Adriamycin. At this juncture it is not known if this will be a more efficient delivery system than simply providing the drug alone, but it is certainly worthy of testing.

A more subtle approach would be to attach a chemical group to the oligomer that can result in localized catalytic hydrolysis of RNA, more specific than the use of a radical-producing group such as iron EDTA. An example of such a group is imidazole, which is known to be involved in phosphodiester hydrolysis in the active site of RNase (56, 57). In such a case the selective-sequence ODN analogue could be used to deliver an irreversible inhibitor to the mRNA intermediate in viral or gene expression. This presupposes the absence of crucial normal genes utilizing the same sequence, or at best that similar sequences are not at crucial positions in the genome. However, the approach is by definition infinitely more selective than the usual drug therapy approach. It would also be less restrictive in terms of amount of substance required at the target site than the simple M- or S-ODNs, since reversible inhibition requires one copy per mRNA, while for irreversible inhibition the same molecule can theoretically recycle and destroy potentially many mRNA copies. It is unknown what precise molecular geometry will be most effective in producing mRNA cleavage. The length of methylene chain in the linker for an imidazoyl moiety may be estimated from the results of Helene et al. (34, 58), who found that a 5-methylene linker was most efficient for intercalation. Clearly in order to cleave the phosphodiester bond a longer linker may be needed. A DNA-RNA hybrid is generally expected to be in the A-form, and the interstrand phosphate-phosphate distance across the major groove is about 18 Å. This would require about 10–12 methylene groups between the P atom and the putative imidazole moiety. The result will also depend on whether or not the methylene chain binds to the surface of the DNA or is free in solution. By contrast, in RNase, the distance between the P-O atom of the bound RNA and the imidazole N is about 2.6 Å (57), which is a major reason for the efficiency of the enzyme. However, the intention in this case is to convert an ODN into a specific RNase, i.e., confer enzymatic activity upon a DNA derivative.

Many variations are possible in such a putative drug (Table 2). These variations suggest a whole series of molecules, most of which have never previously been synthesized, which might have potentially important therapeutic properties. Such molecules might have unusual mechanisms in vivo, including hybridization as well as polymerase inhibition or RNase H activation. Such mechanisms might well overcome the current hiatus in cancer chemotherapy due to the inherent development of multiple drug resistance to drugs of natural origins. The combination of a carefully designed catalytic group together with the use of nuclease-resistant phosphodiester analogue linkages such as phosphorothioates, as well as new synthetic methods (59), would appear to provide the basis for a new approach to chemotherapy. These compounds suggest a "magic bullet" in which the specificity is not derived from a protein (enzyme, receptor, or antibody site), but depends on the base sequence and genetic information and mechanisms of the cell.

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Table 2 Possible variations in the ODN-LR analogue

| No. of R groups—could be just one or a mixture of groups. |
| Location of R group—at 5' end, middle, or at 3' end of ODN analogue, attached to P, and/or on sugar, and/or base. |
willingshine their synthetic expertise. We also thank Claude Helene for permission to reproduce Figs. 5 and 6.

References


Oligodeoxynucleotides as Inhibitors of Gene Expression: A Review

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