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Review

Oligonucleotide Analogues as Potential Chemotherapeutic Agents

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Oligonucleotides specifically bind to complementary sequences of either genomic DNA or genomic RNA through hydrogen bonding of base pairs. In principle, relatively short oligomers (<20 bases) can specifically hybridize with DNA or RNA and thus be used for novel drug design strategies involving targeted interference of genetic expression at the level of transcription or translation. Conceivable chemotherapeutic applications predicated on sequence-specific hybridization ("antisense" inhibition) require oligonucleotide analogues that are resistant to *in vivo* degradation by enzymes such as nucleases. Nuclease-resistant analogues having modified internucleoside linkages (e.g., methylphosphonates or phosphorothioates) or modified nucleosides (e.g., 2′-*O*-methylribose or α-anomers) are now readily available by means of automated synthesis, and there are various classes of pendant groups (e.g., alkylating or intercalating agents) that can be attached to increase the efficacy of these analogues. The present account reviews this area of research by classifying structures and mechanisms of action, with comments on stereochemistry. Biological studies are briefly summarized, and pharmaceutically related topics of interest are noted.

KEY WORDS: antisense oligonucleotides; targeted drugs; translation arrest.

INTRODUCTION

An important goal of drug design is the achievement of highly specific mechanisms of action. DNA and, to a lesser extent, RNA have long been strategic targets for approaches to chemotherapy in view of their central roles in replication, transcription, and translation. Relatively low molecular weight drugs that function by either covalent modification (1) of DNA/RNA or noncovalent binding (2) to double-stranded helical DNA exhibit a degree of sequence specificity less than that found for relatively high molecular weight restriction endonucleases (3), DNA-binding proteins (4), and anti-DNA autoantibodies (5). Analysis of the binding interactions between double-stranded helical DNA and natural products has led to the design of synthetic nonprotein molecules that bind helical DNA of any given sequence and number of base pairs, therefore providing the possibility for new drug development (6). The use of synthetic oligonucleotides, or analogues, therefore represents a comparatively simpler approach to sequence-specific drug binding, which offers the added advantage of being generally applicable to either double- or single-stranded forms of either DNA or RNA.

Oligonucleotides specifically bind to complementary sequences of DNA or RNA through hydrogen bonding of base pairs. Statistically, a sequence of 17 nucleotides should be found only once in the entire human genome (7), and considerably shorter sequences (ca. 12-mers) can in principle be used at the level of mRNA, as only the transcribed DNA has to be considered. The fact that under stringent conditions (thermodynamic control) hybridization of oligonucleotides can be largely abolished by a single base mismatch in DNA and mRNA (8) suggests the possibility of using these relatively short oligomer sequences to achieve a high specificity of binding *in vivo*. Early recognition of this possibility led to investigations published more than 20 years ago in 1967 by Belikova *et al.* (9) regarding synthetic oligonucleotides that had pendant 2-chloroethylamino groups and were designed as sequence-specific DNA/RNA alkylating agents. Also in 1967 there appeared the first of a series of reports by Ts'o and co-workers on various classes of nonionic oligonucleotide analogues, starting with the use of *N*-vinyl derivatives of pyrimidines and purines (10), which have been referred to as "plastic nucleic acid" (11). Halford and Jones (12) during this period published their related work on other types of synthetic nonionic analogues of polynucleotides, which were envisaged as nuclease-resistant compounds that could be taken up by cells more readily than charged oligonucleotides and, therefore, interfere with the function of mRNA in biological systems.

The difficulty of conveniently synthesizing the aforementioned compounds and the newness of DNA/RNA molecular biology at the time were undoubtedly significant impediments to other early investigations of drug design based on sequence-specific hydrogen bonding of nucleobases. This situation has changed dramatically with the advent of new and automated methods for synthesizing oligonucleotides and, importantly, rapid developments in molecular biology that have been made possible by efficient cloning and se-

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quencing techniques. These technological advances in concert with increased understanding of gene expression have led to recent widespread interest in possible chemotherapeutic applications of oligonucleotide analogues. Several groups of investigators have reviewed (11,13–17) their respective work in this area, which has included the design and evaluation of modified oligonucleotides as inhibitors of protein translation by sequence-specific binding to either RNA splice sites or mRNA (11,13,14). These "antisense" or "antimessenger" oligonucleotides are far more simple alternatives to "artificial mRNA-interfering complementary RNA (micRNA)" (18) ("antisense RNA" (19) strategies proposed for the prevention and treatment of diseases (20–23), although both approaches have been the subject of recent scientific meetings (24–26). The possible application of oligonucleotide analogues to cancer chemotherapy has also been recently considered (27).

The present account, which is directed in particular to pharmaceutical researchers, provides a brief summary of the different structural classes of oligonucleotide analogues and indicates the various strategies for mechanisms of action. Attention is placed on the influence of sequence, structure, and stereochemistry of an analogue on its binding to a polynucleotide target. An up-to-date compilation of biological studies is given, and some selected results are discussed. Pharmaceutically related areas of research and development are noted, and some points to consider are given for future synthesis and purification of oligonucleotides on scales that are 10^6 to 10^9 times greater than those currently employed in molecular biology, as will be required for preclinical and clinical testing of these analogues in new drug development programs.

STRUCTURAL CLASSES OF OLIGONUCLEOTIDE ANALOGUES

Naturally occurring DNA and RNA have repeating-unit structures that are comprised of base, D-2′-deoxyribose or D-ribose, respectively, and phosphate moieties (Fig. 1, **1**). The naturally found connectivity includes 3′,5′ linkages in the ribose–phosphate "backbone" and attachment of either a purine (adenine, A, or guanine, G) or a pyrimidine (cytosine, C, or thymine, T, for DNA and uracil, U, for RNA) base to the sugar ring via a glycosyl bond in the β configuration. Enzymatic degradation of DNA (28) and RNA (29) by nucleases, which hydrolyze the internucleoside P–O linkages and cause depolymerization, is quite rapid, particularly in serum (28). Consequently, one of the main objectives of using a structurally modified oligonucleotide is to preclude or significantly diminish enzyme-mediated depolymerization *in vivo*. As will be seen from what follows, this can be accomplished in many ways, especially if one considers replacement of the sugar–phosphate backbone with other moieties; however, any protective change in structure must also accommodate the need for adequately strong sequence-specific base pairing between the oligonucleotide analogue and the DNA/RNA targets.

Isostructural. Shown in Fig. 2 are representative internucleoside linkages that contain phosphorus. Synthetic polynucleotides having a thiophosphate linkage (5–7) are resistant (but not impervious) to degradation by nucleases and serum (30–34), which is a property that has been previously utilized to obtain polynucleotide analogues that are enhanced inducers of interferon (30,31,35). Phosphorothioate analogues of DNA with linkage **5** at one or all positions in the chain can be readily synthesized by chemistry (36–38) that has been automated (36,39). The counterpart of **5** containing selenium, **8**, is known to be chemically unstable toward replacement of selenium by oxygen (36,40). Phosphorothioate and phosphoroselenoate groups are isoelectronic with, and close isostructural cognates of, the naturally occurring phosphodiester linkage and, thus, represent subtle perturbations by comparison to methylphosphonate (**9**), phosphoramidate (**10**), and phosphotriester (**11**) groups, which are tetrahedral but are nonionic, are relatively hydrophobic, and have added steric "bulk." Efficient chemistry has been developed for automated syntheses of oligomers having any desired number and location of linkages, **9** (40–42), **10** (37,40), and **11** (40,43–45). Phosphoramidate linkages isomeric with **10** having a P–NH bond to the 5′ CH_2 group are under investigation (J. W. Engels, private communication). The R groups in **10** and **11** are quite variable, whereas only a few counterparts of **9** have been reported,

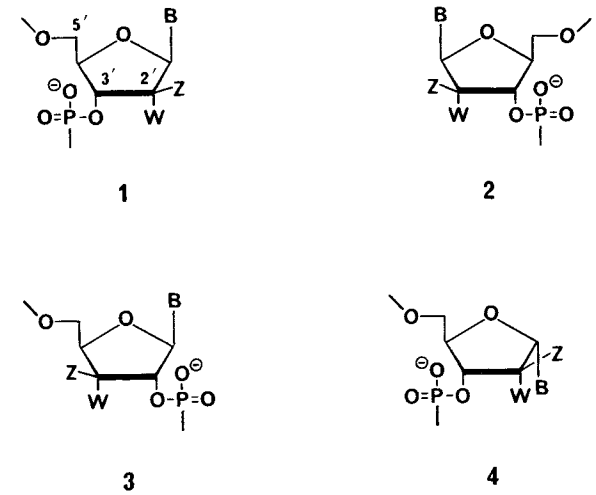

Fig. 1. Representative stereoisomers of naturally occurring (β-anomeric) DNA (**1**; W = Z = H) and RNA (**1**; W = OH, Z = H): L-2′-deoxyribose (**2**; W = H, Z = OH), L-ribose (**2**; W = OH, Z = H) and D-arabinose (**1**; W = H, Z = OH) moieties; 2′,5′-linked analogues of DNA (**3**; W = Z = H) and RNA (**3**; W = OH, Z = H); α configuration in the D-2′-deoxyribose (**4**; W = Z = H) or D-ribose (**4**; W = OH, Z = H) moiety. B is a nucleic acid base.

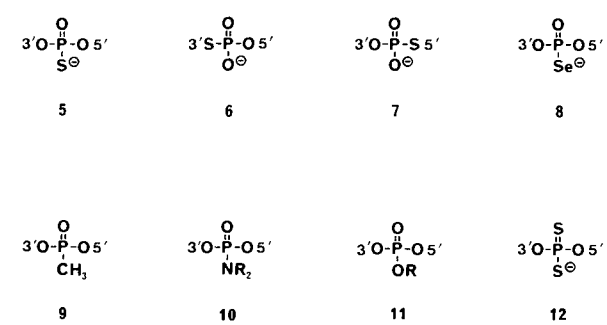

Fig. 2. Representative internucleoside linkages that contain phosphorus.

e.g., triarylmethyl (40) and difluoromethylphosphonate (46) linkages.

In an oligonucleotide analogue, the presence of asymmetric linkages such as 5 and 9 leads to the possibility of 2^n stereoisomers, where n is the number of these linkages. Thus, for example, a 15-mer phosphorothioate analogue containing 14 asymmetric linkages, 5, has 2^{14} or 16,384 stereoisomers. Efficient stereocontrolled chemical syntheses of such oligomers are therefore desirable. Alternative phosphorothioate linkages, 6 and 7, are not asymmetric due to the equivalence of resonance structures $O=P-O^-$ and $^-O-P=O$. By the same token, no linkage isomers exist in the case of a phosphorodithioate, 12.

Relatively few examples of oligoribonucleotide analogues (e.g., with 5 (30,35) have been reported to date, due in part to the inherent difficulties of RNA synthesis. This may soon change in view of the recent availability of highly efficient chemistry suitable for automated RNA synthesis (47). In addition, oligomers of 2′-*O*-methylribonucleotides (1; W = OCH_3, Z = H) are stable to alkaline treatment and RNase digestion, resist degradation by certain nucleotytic enzymes, and have favorable hybridization properties (48). "Chimeric oligonucleotide" analogues can be synthesized by nested incorporation of 2′-deoxy- and 2′-*O*-methylribonucleotide units (49).

Nothing is apparently known about oligomers that contain the isostructural cognates of a phosphodiester linkage which are represented by the general formula 3′0-M(X)(Y)-05′, where M = C, Si, or S, except for one reported (50) example of a dimer in the case with silicon.

Stereochemical. Altering the stereochemistry of an oligomer relative to naturally occurring DNA/RNA (Fig. 1) is a second general approach by which adequate stability against nuclease may sometimes be obtained. The 2′,5′-linked oligonucleotides (3) that are of interest in studies (51) related to interferon are subject to rapid enzymatic degradation unless additionally modified, as in the case of phosphorothioate analogues (31,33). Compared to 3, very little is known for analogues containing the unnatural L sugar moieties (2). Oligomers of L-2′-dU have been shown to be much more resistant to snake venom phosphodiesterase than were oligomers of D-2′-dU, although no evidence was found for binding of the former compounds to poly(dA) (52). Oligoarabinonucleotides (1; W = H, Z = OH) are likewise not well known but are now accessible by an automated synthetic method (53). These oligomers are stable to alkaline treatment but undergo degradation by spleen and snake venom phosphodiesterases (53,54).

Oligomers of α-2′-deoxynucleotides or "α-DNA" (4), which Sequin (55) predicted in 1973 could form a duplex with natural "β-DNA" (but with parallel, head-to-head orientation), were found shortly thereafter to exhibit nuclease resistance (56). These compounds have attracted much recent attention (57–68) and can now be automatically assembled by the use of commercially available DNA synthesizers (64) and chemistry that allows sulfurization (36) for incorporation of phosphorothioate linkages (5) should additional stability against nucleases be desirable.

Miscellaneous. An essentially unlimited variety of polymers with nucleobase-containing side chains is conceivable. Replacement of the phosphodiester linkage with a carboxymethyl moiety [Fig. 3, 13; W–Z = $OCH_2C(0)$] was investigated more than two decades ago by Jones and coworkers (12,69,70). Renewed interest in these structures has led to the synthesis of carbamate-linked homooligomers [13; W–Z = OC(0)NH] of dT (71) and dC (72). A hexamer in the dT case was not readily soluble in water and did not appear to bind to complementary DNA or RNA, while a hexameric dC case was water soluble (presumably via protonation of the exocyclic amino group) and apparently bound to complementary DNA and RNA. These carbamate analogues are envisaged as offering an advantage due to there being a single stereoisomer (71,72), although possible complications from slowly interconverting conformational isomers need to be considered. Such complications are less likely for internucleoside thioether linkages: 3′C–CH_2CH_2S–C5′ (73).

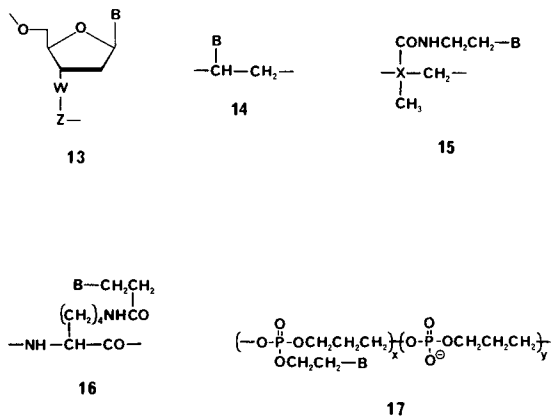

Fig. 3. Miscellaneous general structures having a nucleic acid base (B) attached by a side chain to the polymer backbone.

More radical departures from the naturally occurring backbone in DNA/RNA have been investigated by Jones and co-workers (74), who prepared an acryloyluridine–acrylamide copolymer with pendant nucleobases. Inaki and Takemota (75) have expanded the scope of the latter approach by synthesizing poly-*N*-vinyl (14), polymethacrylate (15; X = C), polyethyleneimine (15; X = N, no CH_3), poly(amino acid) [e.g., poly(L-lysine); 16], poly(vinyl alcohol), and other derivatives. Poly(alkylene phosphates) represented by 17 have been recently reported (76,77) to have a high solubility in water. Investigations of 14–17 have apparently been limited to physical studies using homopolymers, which are easily synthesized compared to mixed-base sequences, which are of interest as antisense oligomers.

That terminally modified structures may have adequate *in vivo* resistance to enzymatic degradation by nuclease has been investigated by Zamecnic and co-workers (78), who prepared phosphodiester-linked oligodeoxynucleotides with 5′ and 3′ isourea "blocking groups." More recently it was reported (42) that a ca. 100-fold increase in stability toward exonuclease was obtained by the incorporation of only two methylphosphonate linkages at both the 5′ and the 3′ ends of an otherwise unmodified oligodeoxynucleotide in the 20-mer range. It remains to be demonstrated whether this or other terminal modifications (e.g., 5) afford similar protection *in vivo*, although some encouraging indications have been ob-

tained with 3′-blocked (alkylphosphate) and 5′-blocked (methylthiophosphate) oligonucleotides injected into *Xenopus* oocytes (67).

FACTORS THAT INFLUENCE BINDING OF OLIGONUCLEOTIDE ANALOGUES TO DNA/RNA TARGETS

The structure and stability of oligonucleotide duplexes are frequently studied by nuclear magnetic resonance (NMR) techniques (79–81), although for many purposes it is adequate simply to use UV spectroscopy to measure the increase in absorbance that occurs upon dissociation or "melting" of a duplex (82). A plot of this change in absorbance vs temperature generally gives an "S"-shaped melting curve that has an inflection point indicating the temperature, T_m, at which the duplex is half-dissociated, at the specified concentration of oligomers and added salt. Melting curves obtained over a range of salt concentrations can then be used to plot T_m vs log[metal ion activity] in order to obtain thermodynamic constants for duplex formation.

As in the case of unmodified oligonucleotide probes and primers commonly used in molecular biology, the design of structurally modified oligonucleotide analogues as potential inhibitors of genetic expression should avoid selection of sequences that have a substantial amount of either external or internal self-complementarity, which can lead to either bimolecular self-association or intramolecular "hairpin" formation (80,81), respectively. The bimolecular process is favored by high concentrations of oligomer, whereas the intramolecular process is concentration independent and relatively fast, which makes it especially problematic. Both of these processes can obviously influence the transport and uptake by cells, as well as decrease the efficiency of binding to the target. The evaluation of self-complementarity should take into account Watson–Crick A · T and G · C base pairing and formation of stable "mismatched" non-Watson–Crick base pairs, especially G · T but also A · C and G · A. These alternative base pairs can potentially lead to increased levels of nonspecific analogue–target binding and should be dealt with, if possible, in computer-assisted homology searches. Runs of five or more purine bases in an oligonucleotide are also potentially problematic, since stacking interactions can lead to secondary structures. Another possible complication to be aware of is the formation of imperfect, but stable, "gapped" duplexes in which a base residue in one strand lacks a complement in the opposite strand (83).

It is generally believed that the order of increasing stability of oligonucleotide·polynucleotide complexes is deoxyribo · deoxyribo < deoxyribo · ribo < ribo · ribo, although at this time the magnitudes of these differences are not known with certainty. From empirical data (7,84) regarding T_m and base-pair content, an unmodified A,T-containing oligodeoxyribonucleotide bound to DNA is expected to have a $T_m \geqq 37°C$, at physiological salt and 1.5×10^{-4} *M* strand concentrations, when oligomer chain lengths are in the range of ca. 12–15, although a significant sequence dependence has been found (84). These chain lengths are roughly halved for the G,C case (7). As will be seen below, antisense sequences of mixed-base composition in these size ranges, and longer (ca. 20- to 30-mers), have been found to elicit biological effects, although there are insufficient data to form conclusions about size optima. Relatively long sequences (e.g., ≧50-mers) may prove to be problematic inasmuch as short segments could bind to complementary nontarget sequences if kinetic rather than thermodynamic control is operative in a particular biological application. Transformation of genomic DNA may also be a size-related factor, as shown in recent studies with yeast using 10- to 73-mers (85).

The influence on T_m of converting a natural phosphodiester linkage to one with the general formula 3′O-P(O)(X)-O5′ should depend on the electronic nature of X, its steric "bulk," and the absolute stereochemistry at phosphorus. An electronically neutral (e.g., X = OCH_2CH_3 or CH_3) or positively charged substituent (e.g., X = $NHCH_2CH_2NH_3$) (86) should decrease the dependence of T_m on the concentration of salt, in the medium, in a predictable manner based on polyelectrolyte theory. If the absolute stereochemistry at phosphorus (*R* or *S* configuration) is such that substituent X is oriented "inward," toward the helix, then inspection of model duplexes, such as B-form DNA, indicates the possibility of destabilization (lowered T_m) due to steric repulsion. In contrast, orientation of substituent X "outward," away from the helix, appears to be a sterically favorable situation. Experimental evidence for these differential steric effects with X = CH_3 had been reported (87). That the absolute stereochemistry at phosphorous in the case with X = CH_3 has an effect on T_m was originally demonstrated by Miller and co-workers (88), who synthesized a pair of stereochemically homogeneous (stereoregular) 10-mers of dT having alternating phosphodiester and methylphosphonate linkages, either all *R* or all *S*. The complex formed by poly(rA) or poly(dA) and one of these isomers, presumably with inward P–CH_3 groups, had T_m values ca. 20–30°C lower than those complexes formed with the other isomer. Although these data have been commonly used to argue that stereoregular methylphosphonate and, by extension, other analogues will exhibit increased biological potency and less nonspecific binding, relative to the stereorandom analogue mixture, direct experimental evidence for these effects has apparently not been reported. It should also be noted that the aforementioned data of Miller *et al.* (88) can be reasonably attributed to special structural effects operative in sequences of nonalternating A · T(U) base pairs, which are not present in mixed-base sequences of A, G, C, and T(U) (89). The relatively small effect of stereorandom *R* and *S* methylphosphonate linkages is evident from the ΔT_m values given in Table I for oligomers **19b** and **22c** having alternating phosphodiester and stereorandom methylphosphonate linkages, which showed only 7–9°C lowering of duplex stability relative to the unmodified duplex. Even compounds with stereorandom methylphosphonate linkages at all positions, except one terminus, along either a single chain (**22d**) or both chains (**21c**) gave ΔT_m values of only 10–12°C. The ΔT_m values for **21a** and **21b** are essentially identical, which demonstrates the absence of a measurable stability difference for a methylphosphonate linkage flanked by A,T vs T,T (see, however, Ref. 89). The approximately equal ΔT_m values (8–9°C) for phosphorothioate analogues **18**, **19a**, and **20** suggest that the lowering of duplex stability is insensitive to both the percentage of G · C base pairs and the chain length. Interestingly, these phosphorothioate-induced ΔT_m values are comparable in magnitude to the ΔT_m for alternating

Table I. Duplex Stability (T_m) of Oligonucleotide Analogues[a]

Compound	*n*-mer	Sequence, 5′ → 3′	G,C (%)	T_m (°C)[b]	ΔT_m (°C)[c]
18	11	TsAsTsTsCsAsGsTsCsAsT	27	28.3	8.7
19a	11	TsAsTsTsCsCsGsTsCsAsT	36	36.3	8.3
19b		TpAmTpTmCpCmGpTmCpAmT		37.5	7.1
20	14	TsCsGsTsCsGsCsTsGsTsCsTsCsC	64	56.0	8.5
21a[d]	14	TpApAmTpTpApAmTpApAmTpTpA	0	25.6	4.0
21b		TpApApTmTpApApTmTpApApTmTpA		25.8	3.8
21c		TmAmAmTmTmAmAmTmTmAmAmTmTpA		ca 20	ca 10
22a	14	GpAeTpTeTpTeTpTeTpCeTpCeCpAeT	27	43.6	5.6
22b		GpAiTpTiTpTiTpTiTpCiTpCiCpAiT		42.9	6.3
22c		GpAmTpTmTpTmTpTmTpCmTpCmCpAmT		40.5	8.7
22d		GpAmTmTmTmTmTmTmCmTmCmCmAmT		37.6	11.6

[a] s, phosphorothioate; p, phosphodiester; m, methylphosphonate; e, ethyl phosphotriester; i, isopropyl phosphotriester. Each modified linkage has *R* and *S* configurations.

[b] Refers to (analogue)–(unmodified complement) duplex, except for self-complementary compound **23**, which forms an (analogue)–(analogue) duplex. The oligomer concentration was 1.5×10^{-4} *M*. Measured in 10 m*M* piperazine-*N*,*N*-bis-(2-ethanesulfonic acid) (Pipes) buffer at pH 7.0 that contained 1 m*M* EDTA and 0.2 *M* NaCl, unless specified otherwise.

[c] Relative to the unmodified duplex (data not shown); a positive value implies that the analogue gave a lower T_m.

[d] T_m and ΔT_m refer to buffer containing 0.1 *M* NaCl.

phosphodiester and stereorandom methylphosphonate linkages. The magnitude and possible steric origin of ΔT_m for a single *R* and *S* phosphorothioate linkage has been reported (90). Interestingly, there is no significant difference in ΔT_m for **22a–22c**, which have alternating phosphodiester and stereorandom ethyl phosphotriester, isopropyl phosphotriester, and methylphosphonate linkages, respectively. The magnitude and possible steric origins of the ΔT_m for single *R* or *S* ethyl phosphotriester (91,92) and isopropyl phosphotriester (93) linkages have been reported.

DESIGN STRATEGIES FOR MECHANISMS OF ACTION

Compared to naturally occurring polyanionic oligonucleotides, nonionic compounds of the type originally reported by Jones *et al.* (12) and Ts'o and Miller and co-workers (11,13,14) are expected to associate more strongly with target DNA/RNA in water since the (analogue) · (target) duplex has one-half the negative charge density. On the other hand, the repulsive forces between negatively charged phosphodiester groups are rapidly diminished by increasing concentrations of counterions such as Na^+, and at physiological salt concentrations the potential advantage of better binding by a nonionic analogue may actually be small or negligible. Oligonucleotides with pendant alkylating groups represent an early (9) but still pursued (94–96) strategy to obtain essentially irreversible binding to target. The use of chemically reactive alkylative groups faces potential problems due to undesired alkylation reactions that occur prior to hybridization. Summerton and Bartlett (97–100) proposed attachment of α-haloketal moieties to C residues in an oligomer "carrier" for conversion to α-haloketones in a posthybridization "activation" step to be followed by alkylation of G in a final cross-linking step. The major drawback of this approach is that activation is not the consequence of hybridization. Webb and Matteucci (101,102) have more recently investigated a clever strategy for what they call "hybridization-triggered" alkylation, wherein a modified oligomer is designed to be essentially unreactive until it is constrained in a double helix and then forms a stable covalent bond with its complementary sequence in the target DNA/RNA. The N^4,N^4-ethanocytosine residue they used to test the strategy has an aziridinyl moiety that is apparently made more electrophilic upon hydrogen bonding to G. The aziridinyl group was found to alkylate a G residue in the hybridized target strand; however, the 30-hr half-life for cross-linking at room temperature was regarded by these investigators to be too slow for inhibition of mRNA, and they suggested that a different group needs to be designed.

An interesting, albeit exotic, variant of hybridization-triggered destruction of target DNA/RNA is to use an oligomer having an attached "abzyme" (103) that would be designed to recognize and cleave the unique sequences of the duplexes afforded by hybridization of the analogue to either DNA or RNA. A similar idea is seen in the recently described (104,105) attachment of staphylococcal nuclease to an oligomer in order to deliver selectively the nucleotidic enzyme to defined target sites on RNA and single-stranded DNA. In lieu of an attached abzyme or nuclease, it might suffice to have a pendant synthetic peptide that mimics the enzymatic reactivity after hybridization has occurred.

A strategy for noncovalently "locking" an oligomer onto the target strand following hybridization involves the use of an attached intercalator or helical-groove binder, which can bind to the duplex by "internal" insertion between adjacent base pairs or bind to "external" nucleobase and phosphate contact elements, respectively. The intercalator, 2-methoxy-6-chloro-9-aminoacridine, which has been attached to the 3′ end of oligomers, has been studied in detail over the past few years (106–108). Attachment of phenanthridinium intercalators to either a phosphoramidate linkage, which is the subject of a patent (109), or the 3′ end (110) of an oligomer has also been investigated, as well as coattachment of a phenazine intercalator and an alkylating group at both termini of an oligomer (95).

Intercalation by a photoactivatable psoralen attached to an oligomer can afford light (360 nm)-induced cross-linking with either target DNA (111,112) or target RNA (113), as

described by Miller and Ts'o (113) for a methylphosphonate-modified oligomer targeted against viral mRNA in a cell culture. In an interesting variation of this general approach, natural β and synthetic α-octathymidylates were functionalized with a photocross-linker, *p*-azidophenacyl, and an intercalator for added stabilization (68). It was shown that, with this triple function molecule, it is possible to recognize an oligopurine · oligopyrimidine sequence in a DNA double helix, via local triple-helix formation, and to target photochemical reactions to specific sequences in both double- and single-stranded nucleic acids (68). Despite the obvious limitations of using photochemical strategies, there may nevertheless be some applications in treatment of diseases of the skin, eye, and other tissues that are accessible by direct irradiation or with the aid of optical fibers. A chemical version of this approach involves functionalization of an oligomer with one component of a free radical-generating system, the latter of which can be completed after hybridization to the target, by addition of the other reaction component(s). Reported examples include the use of EDTA-Fe bonded to either a methylphosphonate analogue (113) or an otherwise unmodified oligonucleotide (114–116) and either phenanthroline-Cu (117) or phorphyrin-Fe (118) attached to an otherwise unmodified oligonucleotide.

Synergy of adjacent target-bound oligomers has been reported by Maher and Dolnick (119), who studied oligonucleotides of length 11–20 that are complementary to various sites near the 5′ end of human dihydrofolate reductase (DHFR). Length and concentration were found to be important variables in hybrid arrest of DHFR mRNA, while the exact target site near the 5′ end of the mRNA was unimportant. Combinations of oligomers whose binding sites were separated by ≥16 nucleotide residues selectively inhibited translation but showed no synergy, whereas combinations with binding sites that were contiguous or separated by only one or two nucleotide residues exhibited approximately fourfold more inhibition relative to the individual oligomers.

A different approach to synergism involves a degradable antisense oligonucleotide for generating, for example, an antiviral nucleotide substrate analogue. This idea is seen in nuclease-mediated release of either 2′,3′-dideoxythymidine attached to the 3′ end of an otherwise unmodified oligodeoxyribonucleotide (120) or 5-fluoro-2′-deoxyuridine incorporated throughout an oligodeoxyribonucleotide (121,122).

RNase H, a nuclease capable of cleaving the RNA strand of a DNA · RNA hybrid (123), has been implicated in the primary mechanism of "hybrid arrest of translation" in the wheat germ cell-free systems (124,125). Additionally, using *Xenopus* oocytes, 15- to 30-mer oligodeoxyribonucleotides complementary to endogenous mRNA were found to stimulate degradation of the mRNA by means of RNase H (125). This depolymerization was rapid and complete (at appropriate oligomer concentrations), and any portion of the messages could be targeted. Such oligomer-induced activity has obvious relevance in antiviral applications. Retrovirus-encoded reverse transcriptase, carried into cells with the virion particle, exhibits DNA polymerase and an associated RNase H activity (126). The DNA polymerase activity can copy the RNA sequence, while the RNase H activity degrades the RNA of the resultant DNA · RNA hybrid to allow DNA-directed formation of a double-stranded DNA. The interesting implication that modified oligodeoxyribonucleotides might be used to prevent such integration, via induction of RNase H-mediated cutting of viral RNA, must contend with the problem of designing an analogue that functions as an RNase H inducer and binds adequately to RNA but is resistant to degradation by nuclease. α-DNA oligomers are resistant to nucleases and form hybrids with RNA but the hybrids are not susceptible to cleavage by RNase H (67). In contrast, DNase-stable phosphorothioate-modified oligodeoxyribonucleotides form duplexes with RNA that are very susceptible to RNase H digestion, which suggests that these analogues may prove to have antiviral properties by virtue of an RNase H mechanism of action (127). Another likely candidate is suggested by the recent finding (49) that RNA, when specifically bound by a complementary "chimeric oligonucleotide" analogue containing four 2′-deoxyribo- and six flanking 2′-*O*-methylribonucleotides (m), viz., m(CAG)d(GTAA)mGU, underwent endonucleotidic sequence-specific cleavage by RNase H. In contrast, no cleavage of RNA by RNase H was detected when the analogue was composed entirely of 2′-*O*-methylribonucleotide residues.

BIOLOGICAL EFFECTS OF OLIGONUCLEOTIDES AND ANALOGUES THEREOF

Virtually all of the early investigations of the biological activity of oligonucleotide analogues were conducted by Ts'o and Miller and their colleagues, who studied compounds modified with either ethyl phosphotriester (**11**; R = CH_2CH_3) or methylphosphonate (**9**) linkages, the latter of which were named "Matagen," an acronym for masking tape for gene expression. This pioneering work, which has been reviewed several times (11,13,14,113), focused on **9** (due in part to deethyation of **10**) and showed, in a cell-free translation assay (rabbit reticulocyte lysate), that 8- to 12-mer oligonucleoside methylphosphonates complementary to either the 5′ end or the initiation codon (but not coding) regions could specifically inhibit synthesis of rabbit globin at concentrations of roughly 100 μ*M* and that this inhibition of translation was also possible in cells (128,129). More recently, anti-initiation-codon targeting of oligonucleoside methylphosphonates has been extended to vesicular stomatitis virus (VSV) mRNAs both in a cell-free system and in virus-infected mouse L cells, wherein the inhibition of protein translation required ca. 100–150 μ*M* oligomer (130). The feasibility of inhibiting mRNA biosynthesis by targeting these nonionic oligomers at the splice junctions of pre-mRNA has been demonstrated with an 8-mer using herpes simplex virus (HSV) type 1 and African green monkey kidney cells, wherein virus titers were decreased by 50, 90, and 99% upon treatment with 25, 75, and 300 μ*M* oligomer, respectively (131). Anti-mRNA Matagen against HSV-1 has also apparently prevented expression of herpetic lesions when applied in the form of cream to the HSV-infected ear of a mouse, with reduction of the yield of virus in both the skin region and the ganglion region of the ear (113).

Early studies by Zamecnik and co-workers (78,132) using unmodified (or terminally blocked) oligodeoxyribonucleotides also provided evidence for inhibition of translation

in a cell-free system. Moreover, they showed that a 10-mer, complementary to the 3′- and 5′-reiterated terminal sequences of Rous sarcoma virus RNA, inhibited virus production in chick embryo fibroblast tissue cultures in the low micromolecular range of concentrations. These observations have been followed by a number of model studies of unmodified oligodeoxynucleotides as inhibitors of mRNA translation in cell-free systems: influenza A RNA polymerase with globin mRNA (133) and either VSV (134) or human dihydrofolate reductase (119) or Sendai virus nucleocapsid protein and phosphoprotein (135) mRNAs in rabbit reticulocyte lysate. The involvement of RNase H-mediated degradation of mRNA in wheat germ (125) and reticulocyte translation systems as well as *Xenopus* oocytes has been studied with β- and α-DNA oligomers. Unmodified DNA oligomers as anti-mRNAs have also been investigated with the c-*myc* gene in human leukemic cells (HL-60) (136,137) and human T lymphocytes (138). Unmodified anti-c-myc oligodeoxynucleotides were previously patented as antitumor agents, based on model experiments with mice (139), as have unmodified anti-HSV-1 oligodeoxynucleotides in a method for inhibiting propagation of virus and as antiviral agents, also based on experiments with mice (140). Interestingly, these reported *in vivo* activities were obtained with injections of only low microgram amounts of oligomer.

Similarly impressive activity has been found in cell cultures for 0.1 μ*M* concentrations of an unmodified oligodeoxyribonucleotide conjugate with poly(L-lysine) directed against the initiation region of a VSV mRNA (141). Attachment of an intercalator to an otherwise unmodified, anti-mRNA oligomer is known to inhibit specifically mRNA translation *in vitro* (106,107), and this has recently been extended to investigations of specific inhibition of the cytopathic effect of type A influenza viruses in cell culture, wherein activity of a 7-mer was obtained at ≥50μ*M* concentrations (108). Anti-mRNA oligodeoxyribonucleotides with pendant alkylating groups reportedly specifically suppress the expression of immunoglobulins upon treatment of mouse myeloma cells (142) and have an effect of viral modification of chicken fibroblasts infected with an influenza virus (143).

The possibility of using unmodified oligodeoxyribonucleotides complementary to viral RNA or proviral DNA to inhibit the replication of human immunodeficiency virus (HIV) in cultured human cells has been studied by Zamecnik and co-workers (120). Of the sequences that were investigated, a 20-mer complementary to the splice acceptor site was found to give the greatest inhibition in H9 cells, at 9 μ*M*. These findings were soon followed by the discovery by Matsukura *et al.* (34) that a 28-mer phosphorothioate (5)-linked homopolymer of deoxycytidine, at only 1 μ*M* in cell cultures, exhibited potent anti-HIV activity and completely inhibited detectable *de novo* viral DNA synthesis as shown by Southern blot analysis. The possibility that this activity, which is apparently not sequence specific, may involve degradation of RNA by RNase H has been addressed by model studies (127). On the other hand, recent experiments with infected cells have indicated that inhibition of HIV p24 *gag* protein expression by antisense *trs/art* (144) phosphorothioate analogues of oligodeoxyribonucleotides is sequence specific, which suggests that at least two mechanisms of action may be operative (145).

In a comparative study an unmodified oligoribonucleotide and its counterparts modified with either phosphorothioate (5), methylphosphonate (9), or alkyl phosphotriester (11) linkages, the anti-chloramphenicol activity encoded in a plasmid was assayed after transfection of CV-1 cells (146). At a 30 μ*M* concentration, the order of decreasing activity was 5 > 11 > 9 > unmodified phosphodiester.

AREAS FOR PHARMACEUTICAL RESEARCH AND DEVELOPMENT

Published studies of the uptake, distribution, and metabolism of antisense oligomers have apparently been limited to only a few examples employing either cell cultures or simple organisms (11,13,34,120,146). Apparent uptake, which is measured in minutes, is faster than what may have been intuitively expected, especially in cases with polyanionic oligomers. The mechanisms for uptake are not established at this time and most likely differ for ionic (energy-dependent?) (78) and nonionic oligomers (passive diffusion) (11). Much more work needs to be done in these areas as well as investigation of possible untoward antigenicity resulting from metabolic degradation with subsequent formation of high molecular weight conjugates.

The polyanionic antisense compounds, such as terminally protected or phosphorothioate-modified or α-anomer oligonucleotides, are in a gross sense structurally similar to double-stranded polyribonucleotides, which have been investigated as interferon inducers (147–149). Their negatively charged backbone is likewise mimicked by carboxylic acid polymers, which modulate a variety of biological responses related to host defense mechanisms (150). Although phosphorothioate analogues are known (34) not to induce interferons, the aforementioned structural similarities suggest that there may at least be some pharmacological and toxicological commonalities amongst all of these polyanions. Poly(A) · poly(U) does not elicit toxic symptoms in mice injected at doses of 250 mg/kg, and in ^{51}Cr labeling studies with rabbits it has been found to have a long lifetime (measured in days), with localization mainly in spleen cells, where there was equal partitioning between nuclei and cytoplasm (149). The molecular weights of these biologically active polyribonucleotides are in the range of several million daltons, which is roughly 500 times the average size of typical 10- to 20-mer antisense oligomers (3500–7000 daltons); consequently the pharmacokinetics and bioactivity of the antisense compounds might be better anticipated from the behavior of comparably sized (1000- to 10,000-dalton) carboxylic acid polymers that have been studied (150). Interestingly, the calcium salt of divinylether maleic anhydride copolymer was approximately twofold less toxic than the sodium salt. In general, carboxylic acid polymers are biologically active when administered i.v. or i.p. but are totally ineffective if given orally (150). The pharmacokinetics of radiolabeled carboxylic acid polymers by i.v. or i.p. injection indicate a difference in the time for organs to achieve peak levels, together with reticuloendothelial distribution and concentration primarily in the liver and spleen; uptake by macrophages was inhibited by either lower temperatures or 2,4-dinitrophenol and has a rate consistent with an absorptive pinocytosis mechanism (150). A similar inhibitory effect

by 2,4-dinitrophenol has been reported for uptake of oligodeoxyribonucleotides (78). Obtaining radiolabeled oligonucleotide analogues for pharmacokinetic and distribution measurements could take advantage of a simple synthetic method (36) for incorporation of one (or more) ^{35}S-labeled phosphorothioate linkages as well as ^{3}H incorporation into oligomer nucleobases by a simple exchange method using labeled water (151).

Poly(L-lysine), a well-known polycationic drug carrier (152), has been used at a molecular weight of ca. 30,000 daltons to form a ternary complex with poly(I) · poly(C) and carboxymethylcellulose that was 10 times more resistant to RNase degradation and more biologically effective compared to poly(I) · poly(C) alone (147). Conjugation of poly(L-lysine) with an oligodeoxyribonucleotide directed against a viral mRNA likewise had a remarkable potentiating effect, which presumably is dependent on the average size of the poly(L-lysine) that was used (ca. 14,000 daltons), although little is actually known about the pathway for internalization or metabolism of the conjugate (141).

Current research on bioerodible polymers (153) for controlled release of large molecules (>1000 daltons), such as polypeptide hormones, may have some bearing on better delivery of antisense oligomers. More important, perhaps, are targeted delivery and achievement of a relatively high intracellular concentration of antisense oligomers, as these factors are widely believed to be the most critical for successful development of antisense oligomers as drugs. Liposomes have several important functional characteristics that define their potential as drug carriers (154): localized slow release upon direct application (e.g., skin or eye) (155), site avoidance, which lowers toxicity to sensitive tissues (e.g., heart, kidneys, and gut) (156,157), clearance by the reticuloendothelial system for targeting of the liver and spleen, and site-directed targeting via attached ligands. An indication of the feasibility of employing liposomes for site-directed targeting of antisense oligonucleotide analogues can be seen in the recent use of a pH-sensitive "immunoliposome" (antibody-coated liposome) to deliver specifically DNA (a plasmid-encoded reporter gene) to lymphoma cells in mice (158). Other packaging strategies include utilization of liposomes with polyamine and acridinyl–polyamine headgroups that electrostatically bind DNA to the liposome's exterior surface (159) and attachment of receptor ligands for receptor-mediated endocytosis to achieve specific internalization (160). The pharmacokinetics of the latter case has been studied in isolated, perfused rat liver experiments using liposomes containing digalactosyldiacylglycerol for efficient targeting via the hepatic asialoglycoprotein receptor, with minimal vesicle leakage, as shown with carboxylfluoroscein (160). A nonliposome variant of this approach is represented by the use of an asialoglycoprotein–poly(L-lysine) conjugate to bind noncovalently DNA (a plasmid-encoded reporter gene) for delivery to human hepatoma cells, which have the asialoglycoprotein receptor, but not to either control hepatoma cells or fibroblasts, which lack the receptor (161). The advent of "stealth liposomes" (154), which have a biochemical coating to mimic red blood cells and thereby circulate freely for prolonged periods, may additionally prove to be useful for targeting of antisense oligomers. Magnetic drug targeting (162) via appropriately derivatized liposomes and external fields provides other options to consider.

Hydrogels (163) are a possible alternative to liposomes for packaging antisense oligomers and can be functionally elaborated by attachment of antibodies and receptor ligands for site-directed targeting and uptake by cells. These unusual gels can be slightly warmed (e.g., 40°C) in order to exceed the lower critical solution temperature, which causes release of structured, bound water from the polymer backbone, with concomitant expulsion of dissolved drug.

PRACTICAL CONSIDERATIONS

It is likely that, in the very near-future, antisense oligonucleotide analogues will be found to have sufficient activity in cell culture experiments to warrant extensive *in vivo* pharmacological, biological, and toxicological testing. This necessitates practical considerations of the synthesis, purification, characterization, and quality assurance of 10- to 100-g quantities of test material, at the outset, and further scale-up to presumably hundreds of kilograms or more if such a drug is developed and commercialized for widespread clinical usage as, for example, an antiviral or anticancer agent. To put such material requirements into perspective, it should be noted that oligonucleotides are generally prepared on only the microgram to milligram scale for most current applications in basic and applied research. Upscaling and modification of commercially available equipment for automated or semiautomated synthesis of DNA and/or peptides can accommodate the short-term material needs, although significant cost-saving improvements need to be implemented at the level of monomer production and consumption. The much more challenging technical problem is postsynthesis isolation and purification of the oligonucleotide analogue by large-scale, cost-effective processes. Polyacrylamide gel electrophoresis and ion-exchange chromatography of oligonucleotide analogues with, for example, methylphosphonate and phosphorothioate linkages face formidable problems in scale-up and subsequent sample processing to remove denaturants, salts, etc. By comparison, reversed-phase high-performance liquid chromatography with a volatile buffer is preferable, although here again, there needs to be development of postchromatographic processing techniques that are cost effective. To some extent the solutions to these technical problems in isolation and purification will be facilitated by analogous process technology which is now being developed for purifying synthetic peptides and genetically engineered proteins intended for use in humans. On the other hand, the fundamental chemical differences between oligonucleotide analogues and peptides/proteins will require uniquely different approaches to product characterization, proof of the correct sequence, and determination of the level of purity of the final products. Guidelines and points to consider for these issues will continue to come from the Food and Drug Administration (FDA), although at the present time a final decision has apparently not been made as to whether antisense oligonucleotide analogues will be considered by the FDA to be drugs or whether they will be considered biological products (164). There are some reasons to expect that they will be classified

as biological products (164), although this judgment may not occur until the first IND is filed with the FDA.

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