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DNA and RNA Derivatives to Optimize Distribution and Delivery

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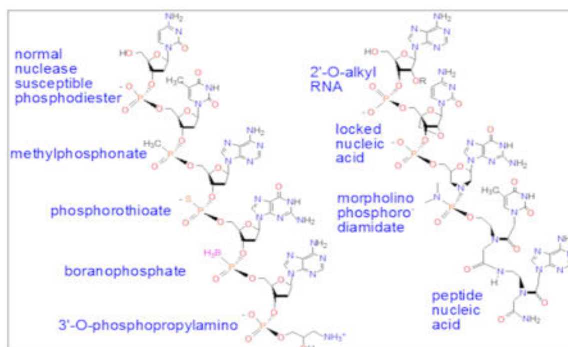
SUMMARY

Synthetic, complementary DNA single strands and short interfering RNA double strands have been found to inhibit the expression of animal, plant, and viral genes in cells, animals, and patients, in a dose dependent and sequence specific manner. DNAs and RNAs, however, are readily digested in biological systems. Hence, chemists are obliged to design and synthesize nuclease-resistant analogs of normal DNA (Fig. 1).

Graphical abstract



I. Antisense DNA and RNA Inhibitors of Gene Expression

The fundamental concept of using synthetic nucleic acids as drugs against specific DNA or RNA sequences was envisioned in a study reporting synthesis and activity of an alkylating derivative of an RNA dinucleotide [1]. Antisense DNA was first successfully utilized to prevent Rous sarcoma virus mRNA translation in chick embryo fibroblast cells [2]. Antisense DNA binding to an RNA target in the nucleus forms a hybrid double strand that is attacked by nuclear ribonuclease H (RNase H), cleaving the RNA in the middle of the DNA-bound sequence (Fig. 2) [3]. Antisense DNAs have been applied since then to interdict the

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expression of a wide variety of viral, bacterial, and animal mRNAs, or miRNAs, in cells, animals, and patients [4–10].

Double stranded RNA (dsRNA) in the form of small interfering RNA (siRNA) or microRNA (miRNA) displays much more potent mRNA silencing than single strand antisense DNA; this mode is called RNA interference (RNAi) [11, 12]. In cells, long dsRNA hairpin loops are cleaved out of transcripts by Drosha in the nucleus, then cleaved by Dicer in the cytoplasm to 20–22 bp duplexes [13]. However, introduction of dsRNA longer than 30 bp into mammalian cells activates a dsRNA-dependent protein kinase, activating the type-1 interferon-response, global shutdown of translation, and ultimately dramatic alteration in cellular metabolism [14]. Chemical synthesis of siRNA duplexes that imitate the Dicer products largely circumvents the non-gene-specific effects [15], yielding gene-specific-silencing in mammalian cells without activating non-specific effects. Processed siRNAs or miRNAs are then trafficked by the dsRNA-binding protein R2D2 to form RNA-induced silencing complexes (RISCs) [11, 13, 16]. RISC is a ribonucleoprotein complex that contains only one of the two strands of the siRNA or miRNA precursor, and proteins of the Argonaut (Ago) family [17]. RISCs then direct mRNA cleavage by siRNA in the middle of the bound mRNA target, or translational inhibition by miRNA (Fig. 3).

Predictions of mRNA secondary structures suggest the existence of loop and bulge sites that might be particularly susceptible to hybridization by antisense DNAs or siRNAs. Secondary structure prediction of antisense DNA correlated with activity against human *MYCC* oncogene mRNA [19–22], human *HRAS* oncogene mRNA [23], and human immunodeficiency virus [24]. With siRNA targeting, sophisticated calculations of mRNA secondary structure, and other sequence considerations, have yielded useful sets of siRNAs [25] that are commercially available. Alternatively, one can walk along the mRNA primary sequence with overlapping DNA or siRNA guide sequences, then screen for the activity of each sequence to determine a lead [9].

II. Nuclease Resistant DNA and RNA Derivatives

In an effort to circumvent rapid nuclease degradation of normal phosphodiester DNA in naturally occurring biological systems [26], a wide variety of backbone modifications of complementary DNA or RNA (Fig. 1) have been synthesized [27]. This spectrum of DNA or RNA analogs all improve the biological stability, solubility, cellular uptake and/or ease of synthesis [28]. The simplest DNA modification involves blocking the 3′ terminus, as with a propylamino adduct (Fig. 1) [29], to prevent attack by 3′ exonucleases, the predominant extracellular degradative mechanism for single strand DNAs [30].

DNA oligomers were first synthesized block by block in solution [31], but are now synthesized by solid phase stepwise synthesis [32] (Fig. 4). The phosphite linkage is then oxidized with iodine in water [32]. Using P(III) phosphoramidite intermediates enables rapid coupling, compared with P(V) phosphotriester intermediates [33].

Phosphodiester modifications to protect the internucleoside linkage include methylphosphonates [35], phosphorothioates [36], or boranophosphates [37] (Fig. 1). Although these modifications increase the *in vivo* half-life of oligonucleotides, they also

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weaken hybridization to the RNA target sites due to the creation of chiral phosphorus diastereomers [38].

Nucleases can also be inhibited by replacing deoxyriboses with modified riboses (Fig. 1). Notable examples include 2′-O-methyl ribose [39], 2′-O-methoxyethyl ribose [40], 2′-fluoro ribose [41], 2′-fluoro arabinose [42], and 2′-4′-cyclo-methoxy ribose (locked nucleic acid, LNA) [43]. All those modifications strengthen hybridization, as well as providing nuclease resistance [44]. On the other hand, RNase H susceptibility is lost, limiting the antisense effect to steric blocking.

Nuclease resistance also results from replacing the deoxyribose phosphodiester backbone with morpholino phosphorodiamidates [45]. Morpholino phosphorodiamidates display slightly reduced hybridization properties and reasonable base specificity [46]. Their weaker hybridization properties require long (20–25 nt) sequences for efficacy, but have shown efficacy against a broad spectrum of mRNAs, such as zebrafish embryo mRNAs [47] and Ebola virus mRNAs in mice and guinea pigs [48].

Instead of modifying the backbone, the attachment of the base may be reversed from above the deoxyribose ring to below, changing the natural β-anomer to the α-anomer, which achieves nuclease resistance without loss of base pairing effectiveness [49, 50]. The unusual α-oligodeoxynucleotides have been found capable of antisense inhibition of β-globin mRNA translation, independent of RNase H activity [51].

The most radical modifications are found in peptide nucleic acids (PNA), where both the phosphodiester linkages and sugars are replaced with a peptide-like backbone of (N-2-aminoethyl) glycine units, with the bases directly attached by methylene-carbonyl linkers (Fig. 1) [52]. Compared with other DNA or RNA derivatives, PNAs display the highest T_ms for duplexes formed with single-stranded DNA or RNA [53]. Hence, sequences as short at 12 bases allow strong, specific hybridization [53]. Alternating hydroyprolyl/phosphono PNAs provide a more soluble, polyanionic version effective in zebrafish embryos [54].

Each of these structural changes affects not only nuclease susceptibility, but also cellular uptake, cellular trafficking, and RNase H activation [28]. Among the derivatives described, only phosphodiester, phosphorothioate, and boranophosphate DNAs direct RNase H degradation of hybridized RNA.

III. Methylphosphonates

Uncharged methylphosphonate DNAs (Fig. 1) are powerfully resistant to nucleases, enter animal cells, and specifically inhibited translation of rabbit globin mRNA [55], *HRAS* mRNA [56], and human immunodeficiency virus mRNA [57], along with several other mRNAs [35]. Methylphosphonate DNA 15mers targeted against a predicted loop at the initiation codon of murine c-*myc* mRNA displayed sequence-specific knockdown of c-Myc protein in the circulating lymphocytes of Eμ-*myc* transgenic mice [21].

However, relatively high methylphosphonate DNA concentrations are required for significant inhibition. One would have expected that the greater longevity, more efficient

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cellular uptake, and lack of charge on methylphosphonate DNAs would make them much more effective inhibitors of mRNA translation than normal DNAs. In cell-free extracts, nevertheless, racemic methylphosphonate DNAs are much less effective than normal DNAs [58], where nuclease sensitivity and cellular uptake are irrelevant. The key is that normal DNAs, hybridized to mRNAs, enjoy the advantage of RNase H attack on the mRNA partner in the duplex [59]. Methylphosphonate DNAs, however, are limited to steric blocking of mRNAs to ribosomal translation [60].

Furthermore, standard coupling of methylphosphonate DNA monomers yields racemic mixtures of Rp and Sp diastereomers at each phosphorus atom [61]. This problem occurs with every asymmetric backbone derivative.

Molecular dynamics [62], nuclear magnetic resonance spectra [63], and crystallography [64] of separated diastereomers revealed that the Sp methyl hinders base stacking by pushing against the deoxyribose ring and base (Fig. 5). The Rp methyl, on the other hand, extends away from the deoxyribose ring and base.

Thus, all-Rp methylphosphonate DNAs should exhibit stronger hybridization and greater water-solubility than racemic oligomers. Stereospecific coupling by a variety of pentavalent [65–67] and trivalent [68, 69] routes have been reported. dT_8 with all-Rp methylphosphonate linkages, except for a central racemic T, displayed a melting temperature of 38°C when hybridized to normal dA_{15}, under physiological conditions where normal dT_8:dA_{15} showed a melting temperature of 13°C, comparable to the racemic methylphosphonate dT_8, and the Sp-enriched dT_8 revealed a melting temperature of less than 2°C [70]. Similarly, dCCAAACA with all-Rp methylphosphonate linkages hybridized to normal dpTGTTTGGC in a physiological buffer yielded a melting temperature of 30.5°C, compared with 21°C for normal dCCAAACA, or 12.5°C for all-Sp dCCAAACA [71]. Hybridizing to normal RNAs, both all-Rp dCTCTCTCTCTCTCTA and all-Rp dAGAGAGAGAGAGAGT gave melting temperatures 10°C higher than their racemic equivalents, while the all-Sp versions displayed melting temperatures 10°C lower [72]. These results illustrate the power of stereochemistry in the hybridization of DNA derivatives with chiral linkages.

Nevertheless, stereospecific scaleup is daunting. A racemic methylphosphonate DNA linkage at the 3′ end of a 2′-O-methyl RNA-DNA-2′-O-methyl RNA chimera provided 3′-nuclease resistance to an RNase H-active sequence targeted to the 3′-side of HIV Rev response element (RRE) stem-loop IIB RNA [73]. Internal introduction of alternating 5′-O-methylphosphonate linkages in dCAGCTGCTTTTGGGATTCCGTTG hybridized to miR-191 enhanced the melting temperature while maintaining RNase H activity [74]. The development of RNase H-active DNA sequences including methylphosphonate residues that provide nuclease resistance invites translation to animal models.

IV. Phosphorothioates

Phosphorothioate DNAs [36] (Fig. 1) represent a modification with polyanionic character similar to normal phosphodiester DNAs [75], but lower nuclease sensitivity [76]. To create a 3′-5′ phosphorothioate link in DNA during solid phase synthesis, one oxidizes the P(III)

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phosphite intermediate with a sulfur donor such as tetraethylthiuram disulfide (TETD) [77], instead of iodine and water [32].

Phosphorothioate DNAs also lose hybridization strength due to racemic linkages, but retain RNase H activity [60]. All-Rp dAGATGTTTGAGCTCT hybridized to its RNA complement or a 475 nt RNA including the complement showed higher melting temperatures and greater RNase H cleavage of the RNA than the duplexes with racemic dAGATGTTTGAGCTCT or all-Sp dAGATGTTTGAGCTCT [78]. Due to the R/S naming convention, the S atom in an Rp phosphorothioate linkage is pseudoaxial, while the Sp S atom is pseudoequatorial, the reverse of the methylphosphonate or boranophosphonate situation.

To compensate for the lower activity of racemic phosphorothioate DNAs, sequences of 20–22 nucleotides are often chosen. Phosphorothioate DNAs were first reported to inhibit human immunodeficiency virus mRNA [79–81], influenza virus mRNA [82], protein kinase C mRNA [83], and transferrin receptor mRNA [84] in human cells, but with noticeable non-sequence-specific effects. Phosphorothioate DNA 15mers targeted against the murine c-*myc* mRNA initiation codon did, however, show sequence-specific knockdown of c-Myc protein and prevention of lymphoma onset in Eμ-*myc* transgenic mice [85].

Despite their efficacy, however, phosphorothioate DNAs exert off-target effects due to nonspecific protein binding [86], complement binding, inflammation, and inhibition of clotting [87]. The known modes of phosphorothioate toxicity invite further modifications to reduce sulfur content in therapeutic DNAs.

In clinical trials, phosphorothioate DNAs have been administered to humans to knock down mRNAs encoding Bcl-2, PKCα, c-RAF, PKA, and survivin [88], as well as apolipoprotein B-100 [89], CMV IE2 [90], HIV *gag* [89], ICAM1 [91], IGF1R [92], c-Myb [93], c-Myc [94], p53 [95], H-Ras [96], and VEGF [97]. The US FDA has approved two for patients: the CMV IE2 21mer fomiversen for retinitis [90], and apolipoprotein B-100 20mer mipomersen [89].

V. Boranophosphonates

Boranophosphonate DNAs [98] (Fig. 1) are isosteric with methylphosphonate DNAs, but the BH_3 group exhibits a negative charge, isoelectronic with the oxygen of the phosphodiester group [37]. Aside from normal phosphodiester DNA and phosphorothioate DNA, only the boranophosphonate modification also exhibits RNase H activity [99] Further, it increases lipophilicity while maintaining binding to the targeted mRNA and exhibits a relatively low toxicity [37]. A solid phase H-phosphonate synthesis is possible [100], but enzymatic coupling has proved more facile [37]. Synthetic limitations have precluded cellular tests of boranophosphonate DNA activity.

Just as with the methylphosphonate and phosphorothioate modifications, introduction of the BH_3 group creates a racemic mixture of chiral centers at the phosphorus, weakening hybridization to RNA. A successful effort to prepare an all-Sp boranophosphonate DNA, with the pseudoaxial borano group pointing towards the helix, as in the Sp methylphosphonate (Fig. 5), yielded an oligomer that retained RNase H activity [101].

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Structures of DNA:RNA duplexes that include a single Rp or Sp boranophosphonate were determined by two-dimensional NMR spectroscopy, yielding helical properties midway between A-form or B-form [102]. Specific NOE evidence of base contacts placed the Sp BH_3 group in the major groove. In contrast, the pseudoequatorial Rp BH_3 group appeared to point away from the DNA, predicting steric clashes with critical RNase H sidechains, suggesting no RNase H activity with an all-Rp boranophosphonate DNA [102].

VI. Other Backbone Modifications

DNA oligomers with triazole internucleotide linkages show nuclease resistance, melting temperatures almost as high as unmodified DNA, and recognition by polymerases [103]. Instead of a triazole, introduction of nucleosyl-3′ amido methyl amino linkages yields a zwitterionic backbone that can hybridize with normal DNA, sensitive to mismatches [104]. For siRNA applications, RNA oligomers with neutral phosphodiester-thioester linkages displayed serum stability, albumin binding, cellular uptake, intracellular hydrolysis to normal RNA phosphodiesters, and RNAi activity in mouse livers [105].

VII. 2′-O-Alkyls

Aside from backbone modifications, the deoxyribose may also be modified to a 2′-O-alkyl ribose (Fig. 1), strengthening hybridization and resisting nuclease attack [44, 106]. The 2′-O-alkyl modifications maintain chirality of the ribose 2′ carbon. The 2′-O-alkyl RNAs differ sufficiently from DNA to preclude RNase H activity, and from RNA to preclude RISC activity. Thus, 2′-O-alkyl RNAs serve as steric inhibitors of RNA translation [44], RNA reverse transcription [107], or RNA splicing [108]. In particular, 2′-O-methyl RNAs successfully induced excision of an exon bearing a nonsense mutation from dystrophin mRNA of the *mdx* mouse model for Duchenne muscular dystrophy [109], demonstrating a route to therapy for muscular dystrophy in patients.

2′-O-alkyl RNA/phosphorothioate DNA/2′-O-alkyl RNA chimeras demonstrated that a combination of nuclease resistant components and parts that elicit RNase H activity, sometimes called gapmers, improve the potency of antisense DNAs [110, 111]. This chimeric approach has been applied successfully in animal trials targeting apolipoprotein B-100 mRNA in hypercholesterolemia [112], *DM1* mRNA in myotonic dystrophy [113], and against huntingtin mRNA in Huntington's disease [114], and in human trials against apolipoprotein C-III mRNA in severe hypertriglyceridemia and familial chylomicronemia [115], and against transthyretin mRNA in transthyretin-associated polyneuropathy [116].

VIII. Locked Nucleic Acids

Bridging the methyl carbon of a 2′-O-methyl ribose to the 4′ carbon yields a locked nucleic acid (LNA) (Fig. 1) [117]. The methylene bridge below the ribose ring constrains pseudorotation, favoring exclusively the C3′-endo, Northern, A-form conformer [43]. The locked-in A-form elevates 3′ stacking, thermodynamic stability, and thus melting temperatures of LNA:RNA and LNA:DNA duplexes [118].

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The slightly unnatural sugar structure imparts resistance to 3′ exonucleases [119], the prominent degradative agent in blood. As with other 2′-O-alkyl RNA forms, LNA lacks RNase H or RISC activity, but LNA/DNA/RNA gapmers have displayed antisense efficacy against mRNAs [120, 121] and microRNAs [122, 123]. Exonuclease protection on the 3′ end of siRNA sense, or passenger, strands increased lifetime in blood, and thus potency [124].

On the other hand, LNA-protected siRNA also elevated off-target transcriptome effects, relative to unmodified siRNA, in mice bearing pancreatic cancer xenografts transformed to express enhanced green fluorescent protein (EGFP) mRNA as a target [125]. This is a natural result of strong LNA binding to RNA. For clinical application, one must shorten the LNA segments, and spike as many DNA residues into the guide and passenger strands as efficacy will allow.

IX. Morpholino Phosphorodiamidates

Greater resistance to nucleases and other degradative enzymes in blood, liver cells, and target cells was achieved by the design and synthesis of morpholino phosphorodiamidate oligomers (Fig. 1) [45]. Replacing the ribose with morpholine, and the phosphodiester with phosphorodiamidate, precluded nuclease recognition, or activity with RNase H or RISC, but enabled high solubility in water despite their lack of charge, due to their strong polarity [45].

Just like methylphosphonate DNA, phosphorothioate DNA, and boranophosphonate DNA, the phosphorodiamidate linkages are synthesized as a racemic mixture. Thus, hybridization with RNA is weaker than with normal DNA, resulting in the requirement for long (20–25 nt) antisense sequences for knockdown efficacy [46].

Lack of a negative charge on the neutral phosphorodiamidate linkages resulted in poor cellular uptake [46], as with methylphosphonate DNA and peptide nucleic acids. Pressure injection of antisense morpholino phosphorodiamidate oligomers targeting mRNAs of interest into the yolk or zygote of embryos of zebrafish (*Danio rerio*) [45], African clawed frogs (*Xenopus* sp.), tunicates (*Ciona* sp.), sea urchins (*Strongylocentrotus* sp.) and mice has enabled genome-wide, sequence-based, reverse genetic screens in these organisms.

Morpholino phosphorodiamidates are frequently targeted to the start codon or 5′-UTR to block translation [45], to the snRNP or splice-regulatory binding sites of pre-mRNA to redirect splicing [126], or to the guide-strand precursors of miRNA [127] to block their maturation and activity. Numerous knockdown studies have successfully phenocopied a number of mutants [128], but not all morpholino phosphorodiamidate sequences block mRNA translation efficiently, and some nonspecific mistargeting effects have been observed.

MYCC mRNA expression in tumors has been explored as a therapeutic target of morpholino phosphorodiamidate oligomers, using a 20mer version of the phosphodiester [19], methylphosphonate [21], and phosphorothioate [22, 85] 15mers used earlier. The anti-*MYCC* morpholino phosphorodiamidate ablated c-Myc protein in human PC-3 prostate cancer xenografts, reducing tumor burden by 75–80% [129]. The followup phase I safety

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trial in healthy volunteers revealed no toxicity or serious adverse events upon intravenous infusion [129]. A second phase I clinical study for pharmacokinetics and tumor bioavailability showed significant concentrations of intact anti-*MYCC* morpholino phosphorodiamidate oligomer in resected prostate and breast tumor tissues [130]. As before, no serious adverse events were reported [130].

Antisense morpholino phosphorodiamidate oligomers have also shown efficacy against several Ebola virus mRNAs in mice and guinea pigs [48]. Simultaneous knockdown of Ebola VP24, V35, and RNA polymerase L mRNAs protected mice and macaques from lethal infection [131]. 28 doses of the VP24 agent [48] are available. Pairs of morpholino phosphorodiamidate oligomers against Ebola and Marburg viruses were safe and well tolerated at up to 4.5 mg/kg in phase I trials with healthy male and female volunteers [132].

An antisense morpholino phosphorodiamidate oligomer, called eteplirsen, was designed to skip mutant exon 51 in *DMD* mRNA, which encodes dystrophin. Lack of intact dystrophin causes Duchenne muscular dystrophy, primarily in boys. A placebo-controlled phase IIb trial of eteplirsen at 50 mg/kg/week achieved 52% dystrophin-positive leg muscle fibers, and 67 meters improvement in the 6-minute walking test, in n=4 boys carrying mutant *DMD* after 24 weeks of treatment, and was well tolerated [133]. Participants (n=160) are currently being recruited for a phase III trial [134].

X. Peptide Nucleic Acids

The most radical modifications are found in peptide nucleic acids (PNA) (Fig. 1) where both the phosphodiester linkages and sugars are replaced with a peptide-like backbone of (N-2-aminoethyl) glycine units, with the bases directly attached by methylene-carbonyl linkers [52]. Compared with other oligonucleotide derivatives, PNAs display the highest T_ms for duplexes formed with single-stranded DNA or RNA [53]. The strength and precision of hybridization by PNA 12mers enables single mismatch specificity [135–140].

PNA structure differs so much from DNA, RNA, or peptides, that proteases and nucleases fail to recognize or hydrolyze PNAs [141]. PNA-Tat peptides tested for toxicity in immunocompentent mice were non-immunogenic [142], non-mutagenic, non-clastogenic, and non-teratogenic [143]. These characteristics all favor application of PNAs in diagnosis and therapy.

While unmodified PNAs demonstrate antisense activity *in vitro* [144], activity in cells, however, requires microinjection of PNAs into the cytosol or nucleus. This stems from poor cellular uptake [145], which was ten times less efficient than uptake of phosphorothioate DNA in a variety of mammalian cells [146]. To alleviate this situation, cellular uptake can be improved by addition of a variety of ligands [147, 148]. Receptor-specific uptake into cells has been demonstrated for PNA-peptide chimeras [136, 138, 148, 149].

For cellular internalization without a receptor ligand, negatively charged alternating phosphonate PNA-*trans*-4-hydroxy-L-proline PNA analogs (HypNA-pPNA) were synthesized and characterized [150]. The negatively charged HypNA-pPNAs display

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excellent hybridization properties toward DNA and RNA while preserving the high single mismatch discrimination and nuclease/protease resistance of PNAs [150–153].

Strong, sequence-specific knockdown of *chordin, notail*, *uroD*, and *bozozok* developmental mRNAs in zebrafish embryos was achieved by microinjection of alternating phosphonate PNA monomers and *trans*-4-hydroxy-L-proline PNA monomers (HypNA-pPNAs) [153]. Even a single mismatch in a PNA abrogated activity, both for the latter four developmental mRNAs, and for the zebrafish orthologs of oncogenes *CCND1* [154] and *TP53* [155].

XI. Conjugates with targeting agents

Cellular uptake of modified DNAs can be increased by conjugation with a wide variety of ligands. For example, the activity of alkylating DNAs was enhanced by conjugation of hydrophobic, neutral cholesterol moieties at the 3′ end [156]. On the other hand, addition of a hydrophilic, positively charged poly(L-lysine) tail to the 3′ end of a single-stranded DNA elevated its activity against vesicular stomatitis virus in cell culture [157]. The same general improvement in cellular uptake of modified DNAs can be mediated by shorter positively charged cell-penetrating peptides (CPP), such as Tat 48–60 [158], penetratin [159], transportan [160], ApoE 141–150 [161], or just tetralysine [40, 162].

Receptor-mediated endocytosis of modified DNAs can be directed by conjugation of the natural ligands biotin [163] or folate [164]. Furthermore, conjugated peptides that are fragments of known protein ligands have also served as effective ligands for receptor-mediated endocytosis, such as octreotide, an octapeptide mimic of somatostatin [165, 166], JB3, a tridecapeptide mimic of insulin-like growth factor 1 [167], JB9, a tetrapeptide mimic of insulin-like growth factor 1 [135, 148, 168, 169], or DAMGO, an enkephalin analog [149].

Bifunctional conjugation of receptor ligands and imaging moieties to modified DNAs, particularly PNAs, has enabled imaging of particular mRNAs in specific cells that overexpress the target receptor. Coupling a chelator to the N-terminus of PNA 12mers against particular oncogene mRNAs, and a JB9 mimic of insulin-like growth factor 1, allowed ^{99m}Tc SPECT imaging of *CCND1* and *MYCC* mRNAs in breast cancer xenografts [136, 137], and *KRAS2* mutant mRNA in pancreatic xenografts [170]. The same strategy provided ^{64}Cu PET images of *CCND1* mRNA in breast cancer xenografts [139] and *KRAS2* mutant mRNA in pancreatic xenografts [138]. Following a similar plan, chelator-PNA-octreotate yielded ^{111}In SPECT images of *BCL2* mRNA in lymphoma xenografts [165]. Applying the same bifunctional principle to fluorescence imaging, a DAMGO-PNA-fluorophore construct yielded fluorescent images of *MAOA* mRNA in neuronal cells that express μ-opoid receptor [149].

XII. Conclusions

Antisense DNA derivatives show great promise for gene-specific knockdown therapy. In the beginning, great skepticism existed about the possibility of achieving antisense DNA inhibition of mRNA translation in cell-free extracts. When this barrier was overcome, the question of cellular uptake arose. It should have been impossible for charged oligomers to

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enter cells, yet it was found that uptake mechanisms operate in all cells. Degradation of single-stranded DNAs was expected to be extremely rapid within cells, but this did not turn out to be true. Furthermore, single-stranded DNAs taken up by cells penetrated not only the cytoplasm, but also the nucleus, opening up the possibility of interdicting both mRNA processing and even transcription.

On the other hand, the observed rapid degradation of DNA by serum nucleases, and the inefficiency of cellular uptake, underscored the need for some modes of derivatization to permit application of DNA oligomers as practical therapeutics. A wide variety of bulky modifications of the 5′ and 3′ termini have been found to inhibit exonucleases, and to accelerate cellular uptake. Similarly, alteration of even a few of the internucleotide phosphodiester linkages to less susceptible derivatives, particularly at the 3′ end, markedly increases the lifetime of single-stranded DNAs in the presence of serum.

Some examples of backbone and end group modifications have been found effective in animal models, and have proceeded to clinical trials. Two sequences have been approved for clinical use by FDA; several more are in late phase III trials.

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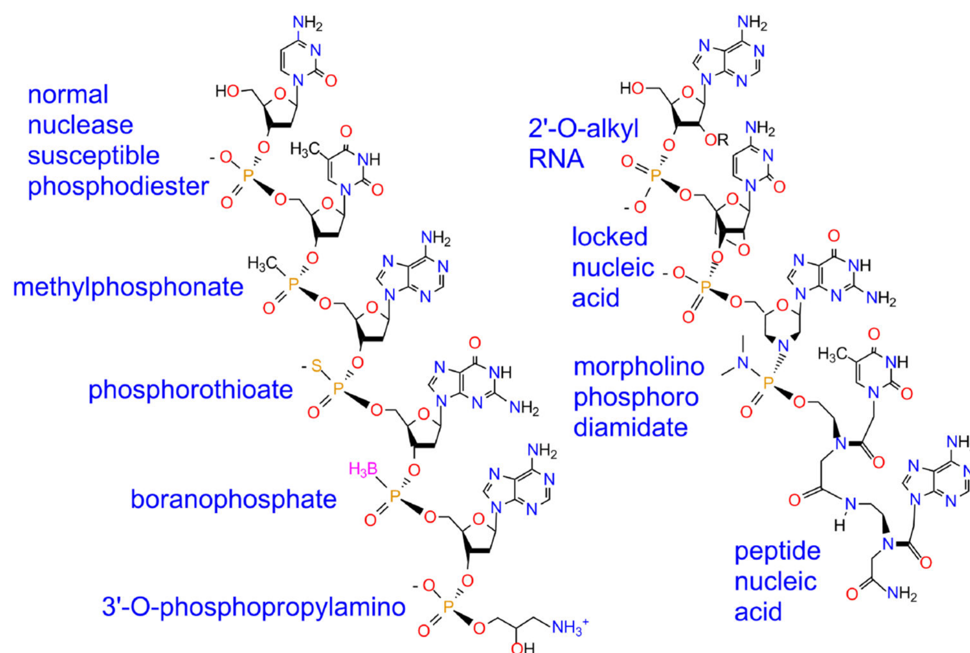

Fig. 1.
Examples of DNA and RNA backbone derivatives.

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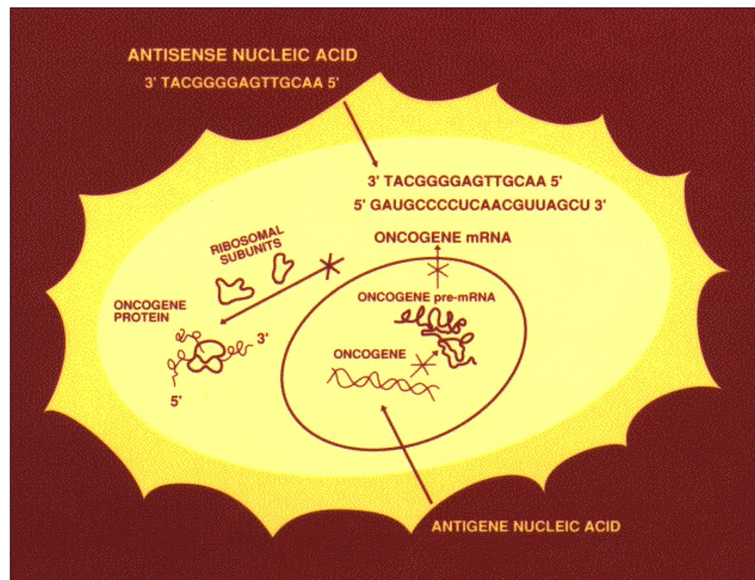

Fig. 2.
Antisense DNA interdiction of mRNA translation in cells, from [4] (cover).

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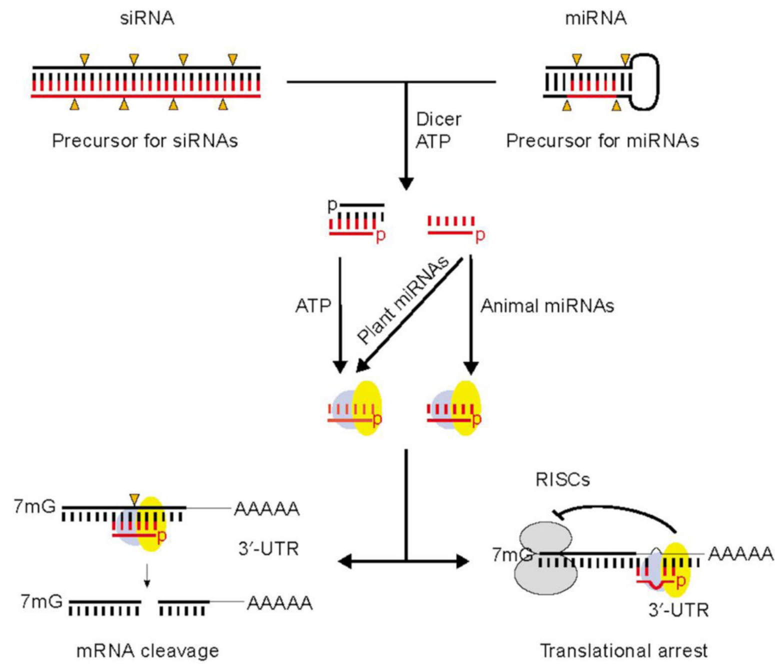

Fig. 3.

Actions of small silencing RNAs in cells. (Left) mRNA cleavage specified by a siRNA. Orange arrowhead indicates site of cleavage. (Right) Translational arrest specified by miRNAs or siRNA, from [18]. 7 mG: 7-methyl guanine; AAAAA: poly-adenosine tail; p: 5′ phosphate.

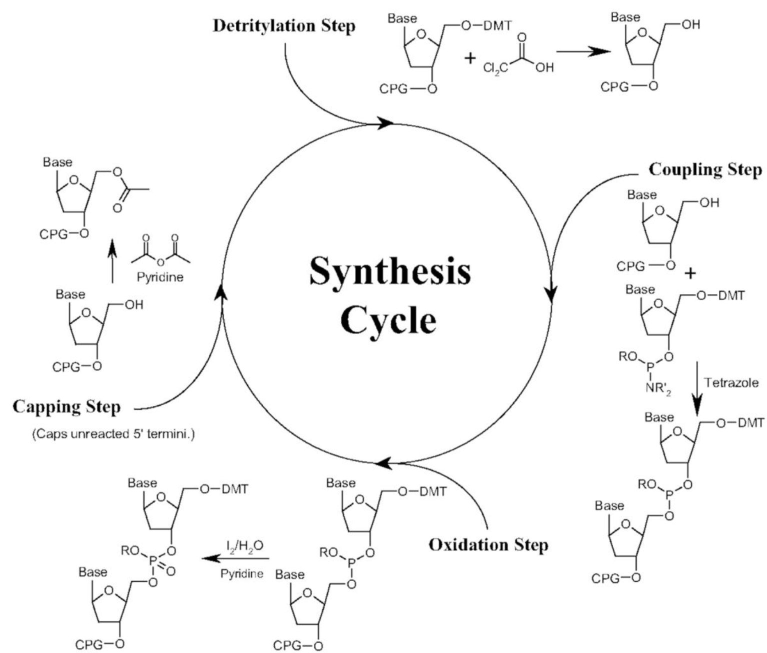

Fig. 4.

Stepwise solid phase synthesis of short DNAs by phosphoramidite method, from [34].

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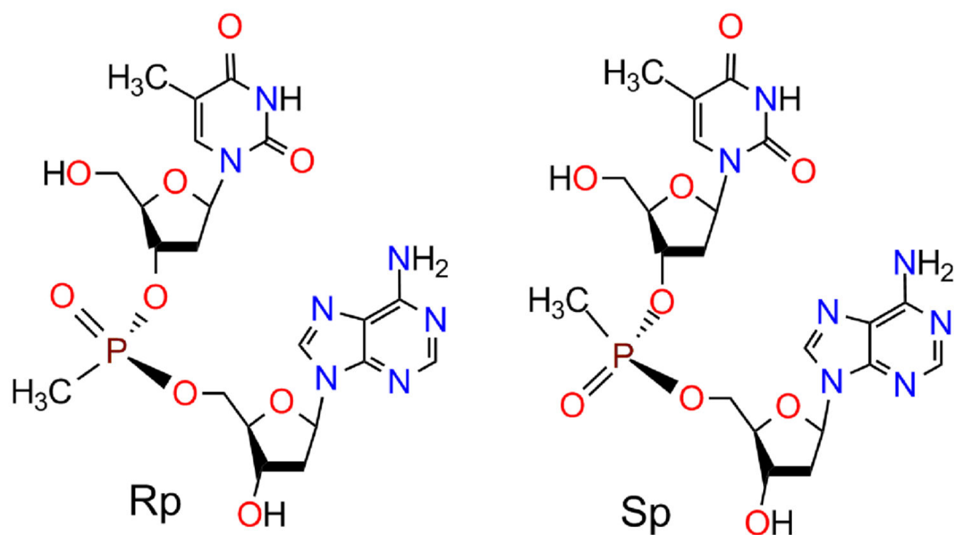

Fig. 5.

Stereo view of pseudoequatorial Rp (left) and pseudoaxial Sp (right) diastereomers of dTmpA.