DNA Triple Helix Formation: A Potential Tool for Genetic Repair

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DNA triple helices offer new perspectives towards oligonucleotide-directed gene regulation. Triple helix forming oligonucleotides, which bind to double-stranded DNA, are of special interest since they are targeted to the gene itself rather than to its mRNA product (as in the antisense strategy). However, the poor stability of some of these structures might limit their use under physiological conditions. Specific ligands can intercalate into DNA triple helices and stabilize them. This review summarizes recent advances in this field while also highlighting major obstacles that remain to be overcome, before the application of triplex technology to therapeutic gene repair can be achieved.

Triple helix formation (fig. 1) recently has been the focus of considerable interest because of possible application in developing new molecular biology tools as well as therapeutic agents[1] and because of the possible relevance of H-DNA structures in biological systems[2]. In intermolecular structures, an oligopyrimidine-oligopurine sequence of DNA duplex is bound by a third-strand oligonucleotide in the major groove[3].

Two main types of triple helices have been described, depending on the orientation of the third strand[4]. The first reported triple-helical complexes involved pyrimidic third strand whose binding rests on Hoogsteen hydrogen bonds between a T-A base pair and thymine, and between a C-G base pair and protonated cytosine[5,6]. The (T, C)-containing oligonucleotide binds parallel to the oligopurine strand in the so-called pyrimidine motif. A second category of triple helices contains purines in the third strand, which is oriented antiparallel to the oligopurine strand. C·G×G and T·A×A base triplets are formed after a reverse Hoogsteen hydrogen-bonding scheme[7,8]. Oligonucleotides containing T and G can also form triple helices whose orientation depends on the base sequence[9].

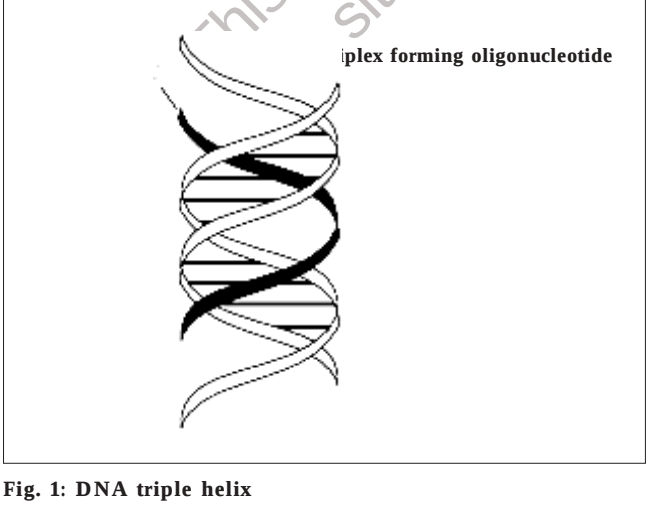

Fig. 1: DNA triple helix

Triple helix formation offers a direct means of selectively manipulating gene expression in cells where DNA triple helices offer new perspectives toward oligonucleotide directed gene regulation (fig. 2). Synthetic triple helix forming oligonucleotides (TFOs) bind with high affinity and specificity to the purine strand in the major groove of homopurine- homopyrimidine sequence in double-stranded DNA[10].

TFOs are good candidates to be used as site-specific DNA-binding agents[11] and they are being investigated for their use as potential therapeutic agents. They have been studied in antisense applications, where they are designed to target mRNAs, antigene applications, where they control gene expression via triple helix formation, and in applications that target proteins, where they are used as aptamers[1,12-15].

TFOs can also be used in gene therapy where they

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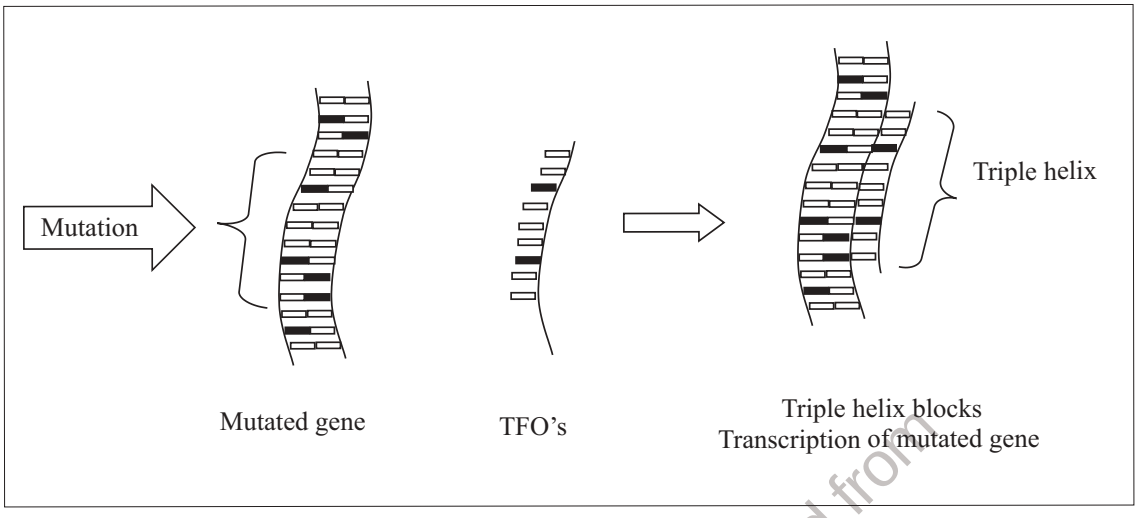

Fig. 2: TFO's preventing transcription of mutated gene

target DNA sequence of mutated gene to prevent its transcription. Purine-rich tracts are frequently found in gene promoter regions and TFOs directed to these regulatory sites have been shown to selectively reduce transcription of the targeted genes, likely by blocking binding of transcriptional activators and/or formation of initiation complexes. Triplex-mediated modulation of transcription has potential application in therapy since it can be used; for example, to reduce levels of proteins thought to be important in disease processes. TFOs can also be used as molecular tools for studying gene expression and they have been proved to be effective in various gene-targeting strategies in living cells. TFOs can bind to polypurine/polypyrimidine regions in DNA in a sequence-specific manner. The specificity of this binding raises the possibility of using triplex formation for directed genome modification, with the ultimate goal of repairing genetic defects in human cells. Several studies have demonstrated that treatment of mammalian cells with TFOs can provoke DNA repair and recombination, in a manner that can be exploited to introduce, desired sequence changes. A number of studies have been reported in which oligonucleotides were utilized as antigene compounds within the cells[2].

Formation of triple helix DNA:

Triple helix formation is a result of oligoinucleotides binding with a high specificity of recognition to the major groove of double helical DNA by forming Hoogsteen-type bonds with purine bases of the Watson-Crick base pairs, the compound rationally designed for artificial regulation of gene expression[16]. Triplex formation requires Mg^{2+}ions whereas it is inhibited by K^{+}ions.

In the triple helix or antigene strategy, the oligonucleotide binds in the major groove of double-stranded DNA via Hoogsteen hydrogen bonding to form a triple helix[5,6]. TFOs bind homopurine-homopyrimidine sequences in double-stranded DNA. There are four structural motifs for triplex formation that have been described based on the third-strand composition and its orientation relative to the purine-rich strand of the duplex. Purine motif TFOs (those that are comprised of G and A) form G*G:C and A*A:T triplets and bind in antiparallel orientation with regard to the purine strand of the duplex. On the other hand, pyrimidine motif TFOs (C/T) form triplexes in parallel orientation and generally only at low pH due to the necessity of the cytosine bases being protonated in order to form Hoogsteen bonds; they form C^{+}*G:C and T*A:T triplets. Finally, mixed purine and pyrimidine TFOs bind in either parallel or antiparallel orientation and form G*G:C and T*A:T triplets. The orientation in which the mixed motif TFOs bind, is dependent upon the number of GpA and ApG steps in the homopurine tract[17]. The antiparallel orientation is favored by a greater number of steps, while a low number of steps favor the parallel orientation[18].

Once the best motif for binding a particular target sequence is established, problems with natural phosphodiester oligonucleotides limit the success of the antigene approach and the therapeutic applications of oligonucleotides in general. Oligonucleotides with the natural phosphodiester backbone are susceptible to endo- and exonucleases. The predominant activity that degrades oligonucleotides is 3'-exonuclease activity, but endonuclease activity has also been observed in some settings[19,20]. Thus, for application as therapeutics *in vivo*

TFOs must be able to resist both exonuclease and endonuclease activity in order to reach their target. A backbone modification that confers nuclease resistance but allows binding to double-stranded DNA with high affinity is required for the *in vivo* applications of TFOs.

Phosphorodiamidate morpholino oligomers are modified backbone oligonucleotides that have previously been investigated as antisense agents[21,22]. Morpholino oligonucleotides have an uncharged backbone in which the deoxyribose sugar of DNA is replaced by a six-membered ring and the phosphodiester linkage is replaced by a phosphorodiamidate linkage[23] (fig. 3). Morpholino oligonucleotides are resistant to enzymatic degradation[24] and appear to function as antisense agents by arresting translation or interfering with pre-mRNA splicing rather than by activating RNase H[25,26]. They have been successfully delivered to tissue culture cells by methods that physically disrupt the cell membrane, and one study comparing several of these methods found that scrape-loading was the most efficient method of delivery; however, because the morpholino backbone is uncharged, cationic lipids are not effective mediators of morpholino oligonucleotide uptake in cells[27]. A recent report demonstrated triplex formation by a morpholino oligonucleotide and, because of the non-ionic backbone, these studies showed that the morpholino oligonucleotide was capable of triplex formation in the absence of magnesium[28].

Cations have been shown to play an important role in triple helix formation. When phosphodiester oligonucleotides are used as TFOs, magnesium is generally required for triplex formation with purine and mixed motif TFOs; it also speeds the reaction and stabilizes the triplex formed with pyrimidine motif TFOs[29-31]. Other divalent cations have been shown to function in the same capacity as magnesium with regard to triplex formation[32]. Magnesium occurs at a concentration of ~0.8 mM in the cell and ~1.5 mM in the blood[33], but most *in vitro* triplex reactions are performed in 5-10 mM $MgCl_2$. Potassium occurs in the cell at a concentration of ~140 mM, and at 4 mM in the blood. High concentrations of potassium can inhibit triplex formation with guanine-rich oligonucleotides designed as TFOs by favoring other secondary DNA structures, such as dimers and quadruplexes[18,34-38]. It will be necessary to overcome the limited ability of phosphodiester TFOs to form a triplex in low magnesium and high potassium for them to be effective under physiological conditions. In a recent study of morpholino TFOs, triplex formation was demonstrated in the absence of magnesium and in the presence of potassium. These properties make morpholino TFOs good candidates for further study as antigene therapeutics.

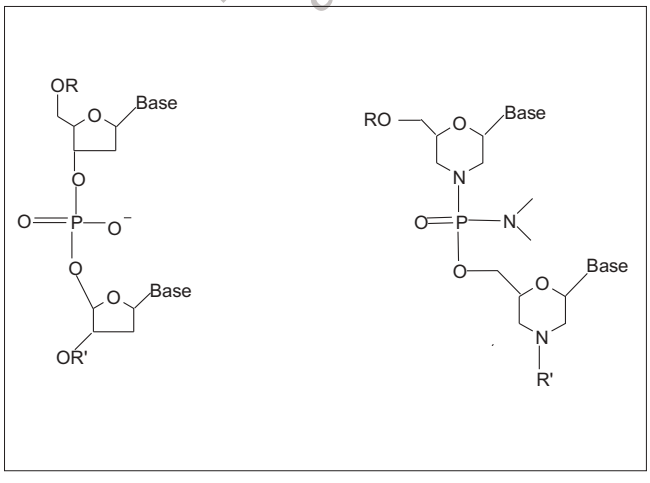

Fig. 3: Structural presentation of phosphodiester DNA and morpholino

Molecular modeling:

DNA triplex structure can be constructed by molecular modeling techniques by using coordinates that correctly take into account the sugar conformation of (T,C)-motif triple helices[39]. This structure is closer to a B-form DNA as reported by NMR studies[40,41] as compared to the structure proposed[42], based on fiber X-ray diffraction. The JUMNA program allows constructing DNA structures according to their helical parameters[43]. An intercalation site can be easily created in the triplex by doubling the rise parameter for two adjacent T·A×T base triplets (rise = 6.8 Å), and subsequently decreasing the twist parameter between these two triplets from 34° to 16° to reduce bond distance constraints.

Using molecular modeling, one can demonstrate the possibility of forming a parallel triple helix in which the single strand interacts with the intact duplex in the minor groove, via novel base interactions[44].

JUMNA uses a mixture of helical and internal coordinates (valence and dihedral angles) to describe nucleic acid flexibility. The helical parameters position each 3'-monophosphate nucleotide with respect to a fixed-axis system. Junctions between successive nucleotides are maintained with quadratic restraints on the O5'-C5' distances. In addition to a reduced number of variables with respect to Cartesian coordinate programs, the choice of physically meaningful variables allows large, concerted conformational moves during minimization, together with an efficient control of the structure and easy introduction of

constraints or restraints. Available tools include both adiabatic mapping and combinatorial searches with respect to chosen structural parameters. Particularities of the Flex force field include the presence of a specific term to account for the angular dependence of hydrogen bonding and the possibility of electrostatic energy screening with a sigmoidal dielectric function[45,46], $\varepsilon(R) = D-(D-D_0)/2[(RS)^2+2RS+2] \exp(-RS)$, where R is the distance between two charges. The slope S, the plateau value at long distance D, and the initial value D_0 of the function are adjustable, with default values of 0.16, 80, and 1, respectively, using two assumptions already employed for constructing the minor-groove triple helix[47]. First, the base triplets are restrained to be coplanar to avoid any possible interbase triplet interactions. Such interactions easily form during the construction of stretched helices but cannot play a role in recognition or strand exchange, since these processes are independent of the overall sequence. It has been checked out that the optimized structure of the minor-groove triplex is independent of these restraints. The second assumption, in line with the stoichiometry of RecA/DNA complexes, which shows three nucleotides per RecA monomer, is the use of trinucleotide helical symmetry. For this reason preliminary studies have been limited to sequences with trinucleotide repeat.

Specific restraints or constraints are needed for triplex construction and manipulation. These include the "plateau" restraints and the trinucleotide symmetry constraints, described previously[48]. The "plateau" restraint maintains the co-planarity of the bases forming a triplet, while allowing the rotations and displacements required for base pair switching. The trinucleotide symmetry constraint implies the equivalence of the variables describing each successive group of three nucleotides. Stretching dsDNA, so that the twist decreases and the minor groove opens have been previously achieved by restraining the distance between the terminal O3' atoms of the trinucleotide symmetry unit. This restraint has been slightly modified because the O3'-O3' distance can be altered by a lateral displacement of the backbones during strand exchange. In a recent work, only the component of the O3'-O3' vector parallel to the helix axis was restrained. The restraints on the groove width, calibrated with the help of numerical Poisson-Boltzmann electrostatic calculations, were used to avoid groove narrowing due to the lack of explicit solvent molecules[47].

Base pair switching is studied by base rotation, using the approach defined by Bernet *et al*[48]. This involves a restraint applied to the angle θ between the glycosidic bond (purine: C1'-N9 or pyrimidine: C1'-N1) and the vector joining the two C1' atoms of a base pair, projected on the plane perpendicular to a local helical axis. θ has a value of 55° in canonical B-DNA. Modeling base pair switching for a chosen base involves an adiabatic variation of θ from 65° to 10° by steps of 2° while maintaining both "plateau" and stretching restraints.

Obstacles and limitations encountered in triple helix formation:

Biological applications of TFOs are compromised by fundamental biophysical considerations, as well as limitations imposed by physiological conditions. Triplex formation involves the approach and binding of a negatively charged third strand to a double-negatively charged duplex. Neutralization of charge repulsion is typically provided experimentally by levels of Mg^{++} (5-10 mM) that are much higher than what is thought to be available in cells[49]. Furthermore, triplex formation involves conformational changes on the part of the third strand, and some distortion of the underlying duplex[40,49-52]. Pyrimidine motif triplexes are unstable at physiological pH because of the requirement for cytosine protonation that occurs at relatively acidic pH (pKa = 4.5). This is necessary for the second Hoogsteen hydrogen bond, although the resultant positive charge apparently makes the more important contribution to triplex stability[53]. Pyrimidine motif triplexes containing adjacent cytosines are often less stable than those with isolated cytosines. Traditionally this has been ascribed to charge-charge repulsion effects[54], although a recent study suggests incomplete protonation of adjacent cytosines may be the critical factor[55]. In addition, purine motif third strands (which are G rich) may form G tetrads in physiological levels of K^+, which inhibit triplex formation[56]. All these factors impose kinetic barriers on triplex formation and reduce the stability of triplexes once formed (most triplexes, even under optimal conditions *in vitro*, are less stable than the underlying duplex[57].

Strategies to counteract the limitations:

The first and foremost problem encountered in triple helical antigene strategy is the instability of the triplex formed by the TFOs under physiological conditions which consequently limits the utilization of this very fascinating strategy meant for gene correction to variable extent. Hence various approaches and strategies have been proposed to confer stability to the triple helical structure formed.

Oligonucleotide directed triple helices could be stabilized by using nucleic acid ligands that selectively stabilize triple helices. For example, ethidium bromide has been shown to bind and stabilize a triple helix made of poly (dT)·poly (dA)× poly (dT), which contains only T·A×T triplets[58]. However, this compound poorly stabilizes (or even destabilizes) the triple helices containing both T·A×T and C·G×C+ base triplets, probably as a result of electrostatic repulsion[59]. Benzopyridoindole derivatives were the first molecules reported to strongly stabilize this latter type of triple helices even though they have a preference for T·A×T stretches[60]. Several other intercalators[61] as well as various DNA minor groove ligands[62-64] have also been shown to bind to DNA triple helices. For example triple helices can be stabilized by chemical modification of oligonucleotides such as, psoralene attached to oligonucleotides has been shown to enhance their biological activity following UV irradiation[65,66]. Intercalators usually stabilize to a greater extent triple helices containing T·A×T triplets, whereas minor groove binders usually destabilize triplexes, except in a particular case where the triple helix involved an RNA strand[67]. Because no structural data are available on triple helix-ligand complexes, not much is known about the interactions that direct specific intercalation into triple helices. BPI derivatives have been shown to intercalate between T·A×T base triplets by excitation fluorescence energy transfer from base triplets to ligands[60,68] and by linear and circular dichroism[69]. Pyrimidine-parallel morpholino oligonucleotides were found to be able to form a triplex with duplex target. As expected, this motif required a low pH for triplex formation, as required by the pyrimidine-parallel motif phosphodiester TFO. It may be possible to overcome this pH dependence with such substitutions as 5-methylcytosine for the cytosines in the TFO[70].

An alternative approach by which triple helices can be stabilized is via chemical modifications of oligonucleotides, such as covalent attachment of an acridine molecule[71]. It has been shown that acridine substitution strongly increases the inhibition of restrictin enzyme cleavage and also it does not impair sequence specificity for triplex formation[71].

APPLICATIONS OF TRIPLE HELIX DNA

The formation of intermolecular DNA triple helices offers the possibility of designing compounds with extensive sequence recognition properties, which may be useful as antigene agents or tools in molecular biology[72]. During the past decade, a new approach using DNA analogues, as therapeutic agents, is emerging in medicinal chemistry. This is based on regulating expression of genes of disease-related proteins/enzymes by blocking their transcription (antigene) or translation (antisense) (fig. 4). It is affected through sequence-specific binding of complementary oligonucleotides to either DNA duplex via triplex formation to inhibit production of mRNA or interfere in the translation of the latter to proteins. Since oligonucleotides do not enter cells easily and are amenable to destruction by cellular nucleases, a variety of chemically modified analogues of oligonucleotides are being designed, synthesized and evaluated for development as therapeutic agents.

The specific recognition of homopurine-homo pyrimidine regions in duplex DNA by triplex-forming oligonucleotides (TFOs) provides an attractive strategy for genetic manipulation, with the ultimate goal of repairing genetic defects in human cells.[57] The ability to target mutations may prove useful, as a tool for studying DNA repair, and as a technique for gene therapy and genetic engineering[73].

Efficient tools based on triple helices were developed for various biochemical applications such as the development of highly specific artificial nucleases. The antigene strategy remains one of the most fascinating fields of triplex application to selectively control gene expression (Table 1). Targeting of genomic sequences is now proved to be a valuable concept on a still limited number of studies; local mutagenesis is in this respect an interesting application of triplex-forming oligonucleotides, on cell cultures[74].

Antigene strategies focus primarily on gene targeting by

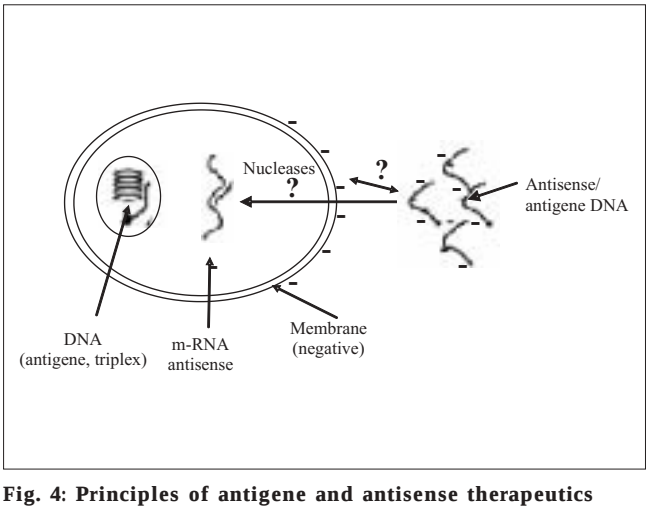

Fig. 4: Principles of antigene and antisense therapeutics

TABLE 1: ANTIGENE NUCLEIC ACID STUDIES WITHIN EUKARYOTIC CELLS[80]

Target gene	Cell line	Oligomer size, and modifications
Transfected genes	HSB2 cells (T-cell)	15-mer acridine
CAT gene/ IL -2R	HeLa cells	orpsoralen linked
promoter	cv-1 cells	21-mer
CAT gene/(6-16) IRE		38-mer,
CAT gene/tk promoter		cholesterol
PRE upstream		
Endogenous genes		
IL-2R	Human lymphocytes	28-mer
c-myc	HeLa cells	27-mer
SV 40 T Ag	Tsa 8 cells	15,20-mer PNAs
Antivirals		
SV 40	CV-1 MT4	8-mer, acridine
HIV-1		31,38-mers

homologous recombination[75] or by triple helix-forming oligodeoxynucleotides[76,77]. For many technical reasons, including very limited gene accessibility within the highly condensed, protein-wrapped chromosomal structure, the clinical application of these methods has not progressed rapidly. Kielkopf *et al* have recently described an alternative approach, using polyamides that can diffuse into the nucleus and recognize specific DNA sequences[16]. Although very exciting, this methodology is still in its infancy and its ultimate clinical utility remains unknown[78].

Therapeutic applications of antigene technology:

Triple helix DNA has attracted attention because of potential application of TFOs as therapeutic agents, for application such as intracellular gene targeting, as rational chemical solutions to sequence specific recognition of a DNA duplex and identification of genes that are responsible for cell growth and malignant transformation[80-82]. With this knowledge has come a natural desire to translate this information into new, target-specific therapeutic strategies for the treatment of cancer, cardiovascular disease, and other common maladies of humankind (Table 2). The recent development of a relatively specific biochemical inhibitor of the bcr/abl protein tyrosine kinase in patients with chronic myelogenous leukemia is a stunning example of this quest[83]. For therapies aimed directly at replacement, repair, or disabling of disease-causing genes, progress has been much slower and a success equivalent to the biochemical bcr/abl inhibitor has yet to be achieved. The reasons for this are complex and vary with the type of gene-directed therapy being used[78].

Stephenson and Zamecnik[84] showed that a short (13nt) DNA oligonucleotide reverse complementary in sequence

TABLE 2: SOME DISEASES AMENABLE FOR TREATMENT BY DNA THERAPEUTICS

Disease	Cause
Cancer	Uncontrolled cell growth from mutational activation and activation of oncogenes
Viral infection	Replication of virus in host cells e.g., HIV, HSC, influenza
Endocrinological	Abnormal levels of-renin, angiotensinase or vasopressin precursor (high blood pressure)-transforming growth factor (kidney failure)-growth hormone (acromegaly)-gastrins (ulcers)
Bacterial	Antibiotic resistant tuberculosis, mycoplasmas-blocking of 3'terminus of 16s RNA
Neurological	Lesins in β-amyloid gene (Alzhiemer's disease)'
Autoimmune	Inadvertant production of antibodies against normal tissues (degradation of host tissue - arthritis, myasthenia gravis), blocking β-cell, Ig cell or T-cell receptor genes by antisense
Parasite	Haem polymerase production (malaria-blocking expressions of haem polymerase), sleeping sickness (trypansoma)

(antisense) to the Rous sarcoma virus could inhibit viral replication in culture. One of the most important properties of antisense and antigene oligonucleotides in their use as therapeutics is their nuclease resistance. Phosphorothioate oligonucleotides are the most common type of oligonucleotides having relatively high nuclease resistance and have been introduced to the market as drugs against cytomegalovirus-induced retinitis[85]. Oligonucleotides with an 2'-O,4'-C- ethylene nucleic acid (ENA) residue at the second position from the 3' end show much higher nuclease resistance than those with an locked nucleic acid (LNA) residue at the same position[86]. Although Kurreck *et al* reported that LNA oligonucleotides were stable in the human serum[87], partially modified ENA oligonucleotides were much more nuclease-resistant than LNA oligonucleotides in rat plasma. Moreover, oligonucleotides contiguously modified with ENA residues at the 3' and 5' end show more stability than those partially modified. Thus, ENA oligonucleotides have high potential as antisense and antigene agents that can be used *in vivo*[88]. Sequence-specific triplex formation can be applied for gene targeting, gene silencing and mutagenesis[73].

Future prospects:

Oligonucleotides can bind site-specifically to a target gene of interest by triple helix formation. Triplex-forming oligonucleotides currently are designed to bind to the HER-2 (human epidermal growth factor receptor 2)/ neu gene, a gene that is over-expressed in a wide

variety of human tumors, including non small cell lung cancer, breast cancer, ovarian cancer, and GI tumors. This strategy will be widely applicable to prevent the expression of many oncogenes or other cancer-related genes. These "antigene" oligonucleotides now are being used to deliver DNA-alkylating agents to specific bases in the HER-2/neu promoter and coding sequence, to prevent transcription initiation and elongation. In particular, triplex-forming oligonucleotides have been used to deliver nitrogen mustard, such as chlorambucil, to a specific guanine base in the HER-2/neu gene to prevent gene expression. Target-specific anti-cancer strategy involving antigene oligonucleotides coupled to DNA active drugs will prove to be a milestone in the near future.

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