ISSN 0975 - 6299 Vol 2 / Issue 4 / Oct - Dec 2011

International Journal of Pharma and Bio Sciences

REVIEW ARTICLE

BIOTECHNOLOGY

THE NOVEL APPROACHES TOWARDS NUCLEIC ACID-BASED THERAPEUTICS & DIAGNOSTICS

S. D. SAWANT*, G. N. PATIL, B. T. TARE AND A. V. PATWA

Post graduate department of pharmaceutical chemistry.
Sinhgad Technical Education Society's Smt. Kashibai Navale College of Pharmacy, Kondhwa (Bk.), Pune- 48.

S. D. SAWANT
Post graduate department of pharmaceutical chemistry.
Sinhgad Technical Education Society's Smt. Kashibai Navale College of Pharmacy, Kondhwa (Bk.), Pune- 48.

***Corresponding author**

ABSTRACT

Even today, new drugs are usually not discovered by rational drug design although this would be the dream of the medically oriented chemist. On an average, it is still necessary to synthesize and test about 10000 new compounds in order to discover a new active substance. In many cases, the active substance is to be directed against proteins such as enzymes, receptors, or ion channels, the structure and mode of action of which are usually very complicated and often incompletely understood. Over past two decades, the use of nucleic acid – based inhibitors of gene expression (antisense agents) has come in and out of fashion. Deep in a patient's genome lies a malfunctioning span of genetic code. If its chemical complement were constructed - a strand of nucleic acid carrying, for instance, the code letter T where the malfunction has an A, and a G where the malfunction has a C and if the strand entered cells and encountered the code, it binds specifically there, by the same interactions - T with A, G with C - responsible for assembling a normal DNA double helix. The resulting hybrid jams the malfunction, preventing it from being expressed as a disease. So, the short spans of "antisense" will be genetic therapy for various diseases such as viral pathogens (Human Immunodeficiency Virus *in vitro* and cytomegalovirus *in vivo*), and variety of hematologic malignancies. Today, it continues to be the basis for seeking a new, genetic kind of magic bullet.

ISSN 0975 - 6299 Vol 2 / Issue 4 / Oct - Dec 2011

KEY WORDS

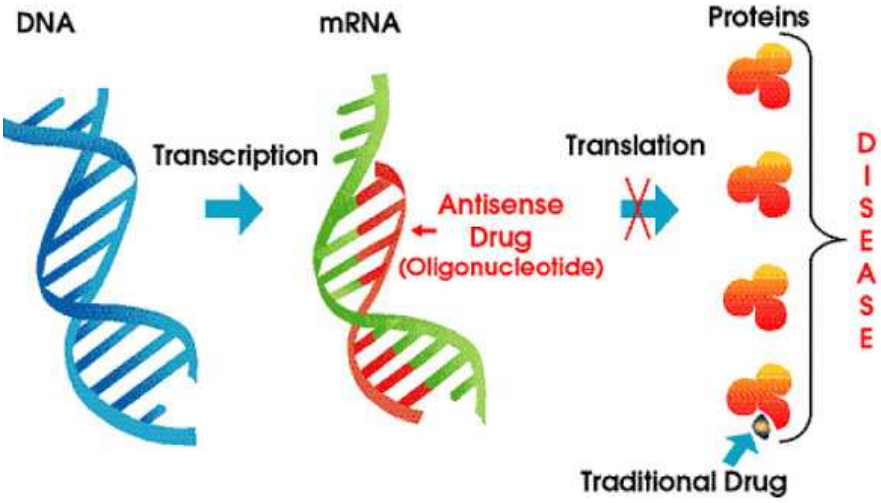
Antisense oligonucleotides, DNAzymes, Peptide Nucleic Acid and Ribozyme.

INTRODUCTION

Basically, gene expression can be interrupted at either of two stages (Figure 1). The first is transcription, the stage at which a gene's code is transferred to a messenger RNA that leaves the cell nucleus. One idea is to engineer antisense capable of interacting with a specific stretch of the DNA double helix, thereby creating a triplex (a triple strand helix) within a gene or in one of its control elements. This in turn may prevent the gene's code from being read. The other interruptable stage is translation, where the messenger acts as an instruction tape enabling the cell, in particular, a ribosome to synthesize protein[1, 2]. The idea is to attack the messenger. If an antisense strand can hybridize with a specific messenger; the resulting duplex may cause a jam in any ribosome reading the message. A related idea is the ribozyme, an RNA that can cleave RNA. Ribozymes were discovered in the late 1970's. The therapeutic application of ribozymes has been studied from viral infections to cancer cells.

The cleaving motif is placed in a strand whose ends are antisense chosen to hybridize with specific messenger code[3].

Figure 1
Antisense Technology



In each instance the goal is straightforward. It must be achieved in a biological system, the living cell, as opposed to a cell-free medium - the goal becomes harder in practice than in theory. First, the antisense therapy must evade breakdown, notably by nucleases, which cleave unprotected nucleic acids into their nucleotide building blocks (also called bases). The enzymes are omnipresent both in the circulation and within cells[4, 5]. To make nucleic acids more resistant to them, a number of strategies have been explored (Figure 2).

Figure 2
Structurally possible DNA modification sites.

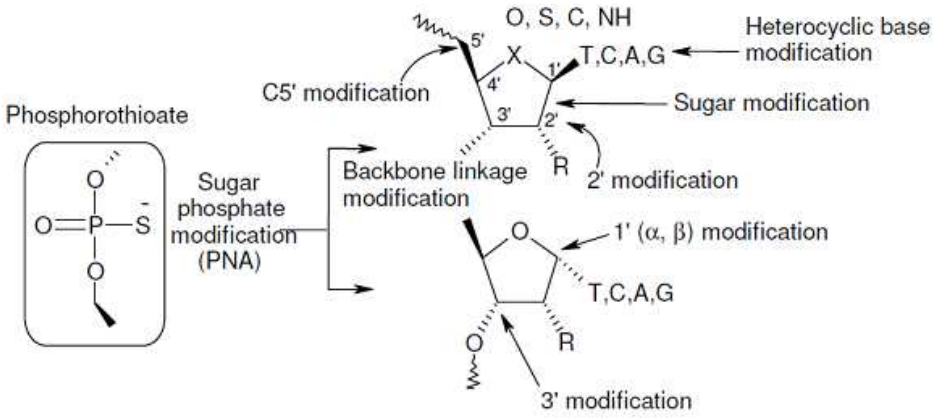
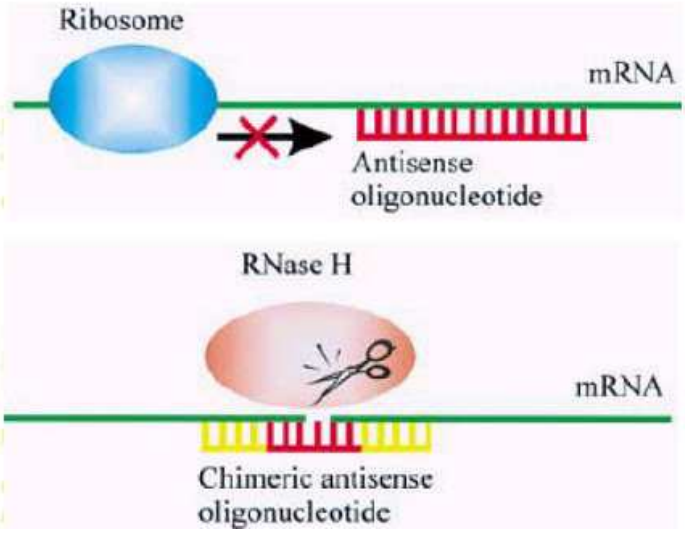


Figure 3 (A)
Translational arrest by blocking the ribosome. (B) RNase H cleavage induced by (chimeric) antisense-oligonucleotides. represents specific gene downregulation or some other action of the administered molecule.



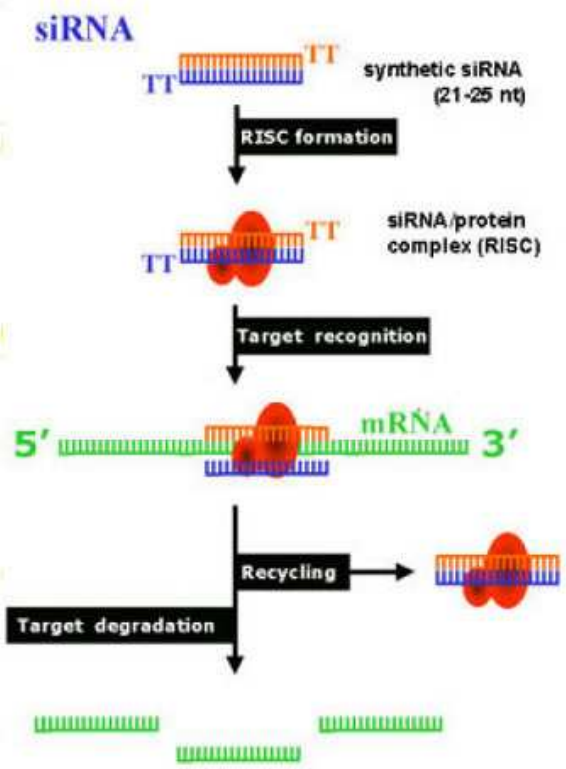


Figure 4
Mechanism of RNA interference

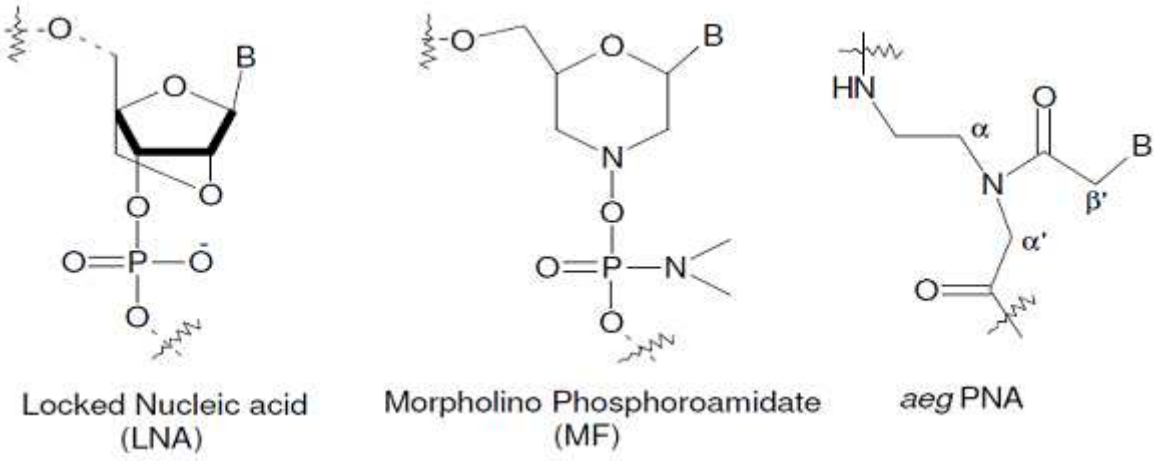


Figure 5
Third generation antisense oligonucleotides

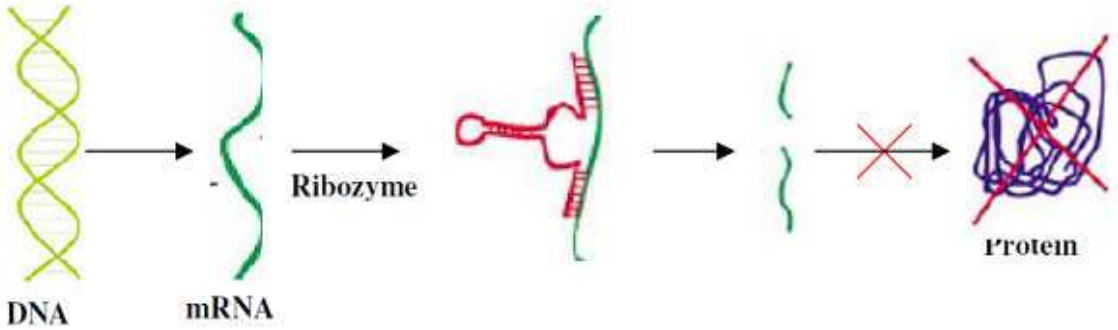
For example, each of the bridges connecting successive bases can be given a sulfur atom instead of the normal oxygen (creating phosphorothioate bridges). The change may give a nucleic acid a lifetime of several days instead of mere hours. An important hurdle that has to be overcome for successful antisense applications is the cellular uptake of the molecules. Naked nucleic acids have a strong electric charge, a negative charge arising from phosphate groups in their chemical backbone. This property tends to make a molecule soluble in water but insoluble in lipid, and therefore "unwilling" to pass through cell membrane, which is a lipid bilayer. Cellular uptake of nucleic acids appears nevertheless to be a natural phenomenon. At the cell surface, a receptor-like mechanism may be involved. Still, such processes are inefficient. Initially, it tends to be trapped in endosomes, from which it may get sorted to lysosomes for intracellular degradation. Only a small fraction may evade this fate, *via* endosomal rupture[6, 7, 8, 9]. Free in the cytoplasm, it may then enter the nucleus, apparently by diffusing through a nuclear pore. In cultured cells, internalization of naked DNA is usually inefficient due to the charged oligonucleotides having to cross a hydrophobic cell membrane. A number of methods have therefore been developed for in vitro and in vivo delivery of oligonucleotides. By far, the most commonly and successfully used delivery systems are liposomes and charged lipids, which can either encapsulate nucleic acids within their aqueous center or form lipid–nucleic acid complexes as a result of opposing charges. These complexes are usually internalized by endocytosis. For efficient release of the oligonucleotides from the endosomal compartment, many transfection reagents contain helper lipids that disrupt the endosomal membrane and help to set the oligonucleotides free[10, 11]. A number of macromolar delivery systems have been developed recently that mediate a highly efficient cellular uptake and protect the bound oligonucleotides against degradation in biological fluids. Examples of these new agents are dendrimers with highly branched three dimensional structures, biodegradable polymers and oligonucleotide-binding nanoparticles. In addition, pluoronic gel as a depot reservoir can be used to deliver oligonucleotides over a prolonged period Further, polymers for the delivery of antisense-oligonucleotides consist of amino acids or sugars[12]. Evidence has been provided, however, that the structural properties of a peptide conjugated to an oligonucleotide do not significantly alter its ability to cross mammalian plasma membranes. Therefore, aspects, other than improved translocation across the membrane, are likely to be responsible for enhanced biological activity of peptide–oligonucleotide derivatives[13]. There it must encounter its target. If the entire gene or messenger were unshielded and linear, success would be far easier. The target's code would be fully exposed. In fact, both DNA and RNA are elaborately folded and also are covered with regulatory proteins. Hence, a targeted sequence may be concealed. In RNA, the problem is especially severe. In that current capacities to predict how it folds in a living cell are still quite primitive. For selecting a target sequence, the usual strategy has therefore been trial and error. A series of tests of antisense spans commencing at or near a known start site for gene transcription or translation, in the hope that such areas may be relatively open, since the cell's own machinery gains access there[14, 15, 16].

For interaction between the therapy and its target to yield a stable hybrid, further conditions arise. Stability increases, for instance, with the proportion of G-C interactions, each involving three hydrogen bonds instead of the two of an A-T interaction. The minimum length of a therapeutic

antisense strand is also dictated by that of the human genome. In a genome, totaling three billion nucleotides, a strand shorter than 12 to 15 bases would likely make multiple, perhaps harmful matches in irrelevant genes or messengers. For reasons of both recognition and stability, the usual length has been 13 to 30 bases[17].

Once a hybrid forms, it must disable its target. Antisense designed to target messenger RNA may succeed by supporting the binding of RNase H, a nuclear enzyme that cleaves the messenger. In fact, some studies suggest that RNase H is essential. If the antisense is a single-stranded DNA, it will emerge intact and may go on to participate in the destruction of further messengers[18, 19, 20]. (Likewise, ribozymes as shown in figure 3 may destroy one messenger after another, emerging unscathed themselves, in the fashion of an enzyme.).

Figure 6
Catalytic ribozymes target RNA



Mechanism of antisense activity

Certainly, some sort of message degradation is desirable. Otherwise, unraveling of a hybrid might sooner or later enable genetic instructions to be translated into disease related protein. As it happens, ribosomes have intrinsic "unwindase" activity, presumably to facilitate the reading of tangled messages. To forestall unwinding, investigators have sought to engineer antisense for tight binding. Since DNA/RNA duplexes are weaker than RNA/RNA duplexes, attempts have been made to engineer DNA that more closely resembles RNA[21, 22].

To disable a gene itself, as contrasted with its messengers, special problems arise. As noted, the idea is to devise a single strand that will bind to a gene's double strand, creating a triplex. Since the double strand has all of its crossties intact, standard base pairing cannot occur. Instead, there must be charge interactions between the therapy and the gene[23]. These occur by a process called Hoogsteen binding, which in turn requires that the gene have a long run of the bases A and G. For this reason, the strategy can be directed only at certain code sequences. In cell-free systems, triplexing at such sites is readily demonstrated. In the living cell, however, DNA is highly coiled and packaged in protein. One wonders whether a minute, specific part of it is sure to be accessible[24, 25, 26].

A series of inefficient steps lies between drug administration and successful down regulation of gene expression. If the individual problems are challenging, the need to address all of them simultaneously is even more so. Consider the first problem, that of

avoiding breakdown. As mentioned, phosphorothioate modification of the bridges in DNA will make them resistant to nucleases. It also preserves a capacity to recruit RNase H. Unfortunately; it keeps DNA lipophobic, impeding cellular uptake[27]. Methylphosphonate bridges are likewise resistant to nucleases. By removing negative charges, they make DNA lipophilic. Unfortunately, they block RNase H. Possible modifications are, however, nearly limitless. Indeed, the bridges can be completely replaced by a chain resembling a string of the amino acid glycine, in essence converting any nucleic acid into a "protein" that carries genetic code. Since the bases project correctly, they can hybridize. Unfortunately, the construct does not cross cell membrane and fails to activate RNase H. Even when antisense appears to exhibit a biologic effect inhibited cell growth or even cell death, there remains the question of whether the effect represents specific gene downregulation or some other action of the administered molecule[28, 29, 30].

In the light of above discussion, the question of roles existing for these technologies, such as antisense oligonucleotides, ribozymes, DNAzymes versus RNAi is an important one. The various unique potential advantages and disadvantages for each of the technologies are summarized in Table 1.

Table-1

Relative strengths and weaknesses of antisense technologies

Approaches	Advantages	Disadvantages
Antisense ODNs	Can be modified to improve selectivity and efficacy. Can be targeted to introns. Easy to make.	Can induce interferon. Can bind proteins. Only exogenous delivery possible. Off-target effects.
Ribozymes	Can discriminate single base polymorphisms Can be used to correct defects Sequence can be appended to change target specificity Simple catalytic domain. Can target introns/subcellular compartments	Require GUC triplet – limits choice of target. Binds protein.
DNAzymes	Inexpensive to make Good catalytic properties. Can be modified for Systemic delivery.	Only exogenous activity. Off target effects?
RNAi	Effective at low concentrations. Bypasses interferon pathway. Can be delivered by multiple pathways. Tissue specific expression possible. Non-toxic? Lasts longer?	Can not target nuclear RNAs or introns. No option for improving if target refractory. Some reports of off target effects

An unmodified antisense oligonucleotide is rapidly attacked by all types of nucleases in biological fluid and its overall charged property prevents it from penetrating through the cell membrane[31]. Various chemical modifications have been developed to enhance nuclease resistance, prolong tissue half-life, increase affinity and potency and reduce non-sequence specific toxicity. First generation antisense oligonucleotides (AS-ODN) are those containing a PS-modified backbone, in which one of the non-bridging oxygen atoms in the phosphodiester bond is replaced by a sulphur atom[32, 33]. Phosphorothioate (PS) modification confers higher resistance to the AS-ODN against nuclease degradation, leading to higher bioavailability of the oligonucleotide. Phosphorothioate-modified antisense oligonucleotide promote RNase H-mediated cleavage of target mRNA. However, this modification may slightly reduce the affinity of the antisense oligonucleotide for its mRNA target because the melting temperature of the AS-ODN–mRNA heteroduplex decreases by approximately 0.5^0 C per nucleotide. Phosphorothioate-modified antisense oligonucleotides have also been reported to produce non-specific effects by interactions with cell surface and intracellular proteins[34, 35]. Despite these disadvantages, PS modification is the most widely performed chemical modification of antisense oligonucleotide for loss-of-function studies *in vitro* and *in vivo* for gene target identification and validation. Indeed, intravitreous fomivirsen, a 21 bp[36]. First generation PS-modified antisense oligonucleotide, is currently the only AS-ODN drug approved for clinical use. To further enhance nuclease resistance and increase binding affinity for target mRNA, second-generation AS-ODNs with 2'-alkyl modifications of the ribose were developed. 2'-*O*-Methyl (2'-OMe) and 2'-O-Methoxyethyl (2'-MOE) modifications of PS-modified AS-ODNs are the two most widely studied second-generation AS-ODNs. Unexpectedly, 2'-OMe and 2'-MOE substitutions do not support RNase H-mediated cleavage of target mRNA, which dampens the efficacy of the AS-ODN. To circumvent this shortcoming, a chimeric AS-ODN was developed in which a central 'gap' region consisting of approximately 10 PS-modified 2'-deoxynucleotides is flanked on both sides (5' and 3' directions) by approximately five nucleotide 'wings'. The wings are composed of 2'-OMe or 2'-MOE PS-modified nucleotides. This chimeric 'gapmer' AS-ODN allows RNase H to sit in the central gap to execute target-specific mRNA degradation; meanwhile, the 'wings' resist nuclease cleavage of AS-ODN by 2'-alkyl modifications at both ends[37, 38]. Extensive studies have been performed *in vivo* to assess the stability and toxicity of these modified AS-ODNs. To further enhance target affinity, nuclease resistance, biostability and pharmacokinetics, a third generation of AS-ODN was developed mainly by chemical modifications of the furanose ring of the nucleotide. Peptide nucleic acid (PNA), locked nucleic acid (LNA) and phosphoroamidate morpholino oligomer (PMO) are the three most studied third-generation AS-ODNs. Peptide nucleic acid is a synthetic DNA mimic in which the phosphodiester backbone is replaced with a flexible pseudopeptide polymer (*N*-(2-aminoethyl) glycine) and nucleobases are attached to the backbone via methylene carbonyl linkage. Peptide nucleic acid is a non-charged nucleotide analogue that can hybridize complementary DNA or RNA with higher affinity and specificity than unmodified DNA–DNA and DNA–RNA duplexes. In addition, PNA demonstrates high biostability in biological fluid because it is not degraded by nucleases or peptidases[39, 40]. Peptide nucleic acid exerts its antisense effect by forming a sequence-specific duplex with mRNA, which mainly causes steric hindrance

of translational machinery leading to protein knockdown because it is not a substrate for RNase H. Furthermore, PNA can elicit antigene effects by hybridizing with double-stranded DNA in four possible configurations, including triplex, triplex invasion, duplex invasion and double duplex invasion, resulting in transcriptional arrest. Substantial data have revealed the effectiveness of PNA in gene silencing in various *ex vivo* models and in genetic and cytogenetic analyses, but its efficacy *in vivo* remains to be determined[41]. Locked nucleic acid is a conformationally restricted nucleotide containing a 2'-O,4'-*C*-methylene bridge in the β-D-ribofuranosyl configuration. This modification greatly enhances its hybridization affinity towards target mRNA and DNA, with a substantial increase in the thermal stability of the duplexes. In addition, LNA is resistant to nuclease degradation. Like any 2'-O ribose modification, LNA is not a substrate for RNase H. Notwithstanding; LNA monomer can be freely incorporated into RNA and DNA to form chimeric oligonucleotides resulting in restoration of RNase H-mediated cleavage of mRNA[42, 43, 44]. It has been shown that the chimeric LNA/DNA/LNA gapmer with 7 to 10 PS-modified DNA central gaps flanked by three to four LNA oligomers on both ends provides highly efficient mRNA cleavage, in addition to high AS-ODN potency, target accessibility and nuclease resistance. Among the nine members of the LNA molecular family, α-L-LNA is the stereoisomer of β-D-LNA and has been shown to demonstrate the highest efficacy in mRNA knockdown in both *in vitro* and *in vivo* studies, making it one of the most promising LNA antisense agents[45, 46]. Phosphoroamidate morpholino oligomer represents a non-charged AS-ODN agent in which the ribose sugar is replaced by a six-membered morpholino ring and the phosphodiester bond is replaced by a phosphoroamidate linkage. Phosphoroamidate morpholino oligomer does not support RNaes H activity, such that its AS-ODN effect is primarily mediated by steric interference of ribosomal assembly resulting in translational arrest[47]. This chemical modification also confers excellent resistance to nucleases and proteases in biological fluid. Phosphoroamidate morpholino oligomer does not readily enter mammalian cells in culture, but a recent study using an arginine-rich peptide (ARP) conjugation to PMO markedly enhanced its cellular uptake and antisense potency by increasing the thermal stability of the ARP–PMO–mRNA heteroduplex. Phosphoroamidate morpholino oligomer has demonstrated antisense efficacy in animal models *in vivo* and in human clinical trials[48, 49].

Table 2 is showing the drugs which are in the pipeline of clinical trials. However, inefficient delivery and stability, causing limited efficacy, as well as undesired side effects, have stood in the way of broader uptake of antisense gene silencing for drug discovery and development[50]. All three generations of antisense oligonucleotides have gone through preclinical toxicological studies and, in fact, some of these antisense oligonucleotides have entered clinical trials[51]. In general, antisense oligonucleotide drugs produce dose-dependent, transient and mild-to-moderate toxicities manifested in rodents, primates and humans. The most common acute toxicities associated with antisense oligonucleotides administration *in vivo* are activation of the transient complement cascade and inhibition of the clotting cascade. Both these toxic effects are dependent on antisense oligonucleotide backbone chemistry, but are antisense oligonucleotide sequence independent[52, 53]. The toxicities are largely due to the non-specific binding properties of phoshorothioate antisense oligonucleotides to proteins at high plasma concentrations. The phoshorothioate antisense oligonucleotides may interact with

factor H, a circulating negative regulatory factor, thus facilitating activation of the complement cascade via an alternative pathway, resulting in increased complement split products, such as C3a and C5a, and subsequent cardiovascular events, such as hypotension. Conversely, phoshorothioate antisense oligonucleotides, with its polyanionic characteristics, binds to multiple coagulation factors, such as VIIIa, IXa X and II, leading to a transient self-limited prolongation of activated partial thromboplastin times[54, 55]. Another frequently occurring subchronic toxicity is immune stimulation, manifested as splenomegaly, lymphoid hyperplasia and diffused multi-organ mixed mononuclear cell infiltrates. This is due to an unmethylated cytosine–phosphorous–guanine (CpG) motif in the antisense oligonucleotide sequence that can be recognized by Toll-like receptor-9 in immune cells, resulting in the release of cytokines (interleukin (IL)- 6, IL-12 and interferon-g), B cell proliferation, antibody production and activation of T lymphocyte and natural killer (NK) cells[56, 57]. The immunostimulatory effects of CpG are further amplified when the ummethylated CpG is flanked by two 5' purines and two 3' pyrimidines (e.g. AACGTT).50 Newer generations of antisense oligonucleotide have been designed to circumvent this side effect by exclusion of the CpG motif or by methylation of cytosine to reduce the immune stimulatory effects. In addition, introduction of LNA into the phoshorothioate antisense oligonucleotides has been shown to reduce, and even eliminate, CpG dinucleotide-mediated immune stimulation. Other mild and self-limiting toxicities usually observed at high plasma antisense oligonucleotide concentrations are thrombocytopenia, enhanced liver enzyme (e.g. aspartate aminotransferase and alanine aminotransferase) levels and hyperglycaemia[58].

Table- 2

Complete profile of antisense drugs

Product	Company	Target Disease	Disease	Chemistry	Status
Vitravene (Fomivirsen)	ISIS Pharmaceuticals	CMV- IE2	CMV Retinitis	PS DNA	Approved
Afinitac(ISIS 3521)	ISIS	PKC-a	Cancer	PS DNA	Phase III
Genasense	Genta Bcl2	Bcl2	Cancer	PS DNA	Phase III
Alicaforsen (ISIS 2302)	ISIS	ICAM-1	Psoriasis, crohn's disease, ulcerative colities	PS DNA	Phase II/III
ISIS 14803	ISIS	Antiviral	Hepatitis C	PS DNA	Phase II
ISIS 2503	ISIS	H-ras	Cancer	PS DNA	Phase II
MG98 DNA	Methylgene	DNA methyl Trasferase	Solid tumors	PS DNA	Phase II
EPI-2010	Epigenesis Pharmaceuticals	Adenosine A1 receptor	Asthma	PS DNA	Phase II
GTI 2040	Lorus Therapeutics	Ribonucleotide Reductase (R2)	Cancer	PS DNA	Phase II
ISIS 104838	ISIS	TNF α	Rheumatoid Arthritis, Psoriasis	2nd generation	Phase II
Avi4126	AVI Biopharma	c-myc	Restenosis,	3rd generation	Phase I/II

			cancer, Polycystic kidney disease		
Gem92	Hybridon	HIV gag	AIDS	2nd generation	Phase I
GTI 2051	Lorus Therapeutics	Ribonucleotide Reductase (R1)	Cancer	PS DNA	Phase I
Avi4557	AVI BioPharma	CYP 3A4	Metabolic redirection of approved drugs	3rd generation	Phase I

PS DNA = Phosphorothioate DNA, 2nd generation = 2'-O-methyl RNA or 2'-O-ethoxy-ethyl RNA and 3rd generation = LNA/MF/PNA

Due to nucleic acid's relative simplicity, fair rapidity, solid reliability, universal workability and remarkable sensitivity, DNA diagnostics hold the distinct position in the area of molecular *in vitro* diagnostics. Today, they are the most rapidly developing branch in this field, DNA probes are expected to account for 12–15% of a multibillion *in vitro* diagnostics market worldwide. Dozens of biotech companies are now involved in the DNA diagnostic business and their number has also increased remarkably during the past few years. Such a promising trend is well represented in the *Expert Review Molecular Diagnostics* journal, which regularly reviews various aspects of DNA diagnostics ranging from PCR-based techniques, DNA microarrays and micro fluidics to fluorescent *in situ* hybridization (FISH), peptide nucleic acid (PNA) technology , SNP detection, DNA nanotechnology, molecular beacons, invasive cleavage assays, rolling circle amplification (RCA) and many others[59, 60, 61]. All these approaches are milestones in the development of DNA diagnostics and, according to our own opinion; they will play an important role in the medical practice, public health, pharmaceutical industry, and forensics and bio defense in the 21st century. As an intrinsic part of DNA technology, DNA diagnostics are rooted in the April 1953 discovery of the intertwined double-stranded structure of DNA biopolymers, five decades of which we are now celebrating. This breakthrough was well prepared by earlier key developments in molecular biology, biochemistry, genetics and related fields[62, 63]. Yet, it was the genius of Watson and Crick, who ultimately recognized all the previous data as a whole and, based on this understanding, finally realized that DNA is a double helix and who also comprehended the deep biological impact of the new insight. Namely this elegant structure and the complementarily rules directing its formation ushered in the advent of DNA diagnostics[64, 65]. One can see that DNA technology in general, and DNA diagnostics in particular, embody a set of notable technological and methodological advances. Starting from the DNA double helix discovery 50 years ago, the developments revolutionized the entire field of bio-analysis and bio-diagnostics. Indeed, DNA testing is now possible on a single molecule and thousands of diagnostic reactions can be performed at once, thus allowing a range of characteristics to be rapidly and simultaneously determined[66]. Moreover, current DNA tests are conducted not only in the laboratory or in the clinic: some can also be run at home using portable disposable devices, such as the DNA paternity 'do-it-yourself' kits.

CONCLUSION

After a long period of ups and downs, antisense technologies have gained increasing attention in recent years. Advances in recombinant DNA technology

and synthetic chemistry have led to novel nucleic acid drugs that inhibit gene expression and protein function as evidenced by various studies. Major improvements have been achieved by the development of modified nucleotides that provide high target affinity, enhanced bio-stability and low toxicity. By developing a suitable drug delivery system, these drugs can emerge as novel therapeutics. Today, the antisense oligonucleotide technology has emerged as a valid approach to selectively modulate gene expression. By adhering to a strict set of specific rules, ongoing *in vitro* studies using antisense oligonucleotides permit the characterization of new targets and new potential therapeutic compounds. The number of *in vitro* experiments has increased continuously, and this has led to numerous therapeutic trials, a few of which now appear preliminarily to be positive. However, the optimal use of antisense oligonucleotides in the treatment of disease requires the resolution of problems relating to effective design, enhanced biological activity, and efficient target delivery. We are sure that this hope continues to shed light on ways to increase therapeutic efficacy and specificity over the diseases like cancer, AIDS and many more.

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