Frontiers in Research Review: Cutting-Edge Molecular Approaches to Therapeutics

ANTISENSE OLIGONUCLEOTIDES: FROM DESIGN TO THERAPEUTIC APPLICATION

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SUMMARY

1. An antisense oligonucleotide (ASO) is a short strand of deoxyribonucleotide analogue that hybridizes with the complementary mRNA in a sequence-specific manner via Watson–Crick base pairing. Formation of the ASO–mRNA heteroduplex either triggers RNase H activity, leading to mRNA degradation, induces translational arrest by steric hindrance of ribosomal activity, interferes with mRNA maturation by inhibiting splicing or destabilizes pre-mRNA in the nucleus, resulting in down-regulation of target protein expression.

2. The ASO is not only a useful experimental tool in protein target identification and validation, but also a highly selective therapeutic strategy for diseases with dysregulated protein expression.

3. In the present review, we discuss various theoretical approaches to rational design of ASO, chemical modifications of ASO, ASO delivery systems and ASO-related toxicology. Finally, we survey ASO drugs in various current clinical studies.

Key words: antisense oligonucleotide design, cell-penetrating peptide, dendrimer, gapmer antisense oligonucleotide, liposome, locked nucleic acid, peptide nucleic acid, phosphoroamidate morpholino oligomer, phosphorothioate, RNase H.

INTRODUCTION

An antisense oligonucleotide (ASO) is a single-stranded deoxyribonucleotide (typically 20 bp in length) that is complementary to the target mRNA. Hybridization of ASO to the target mRNA via Watson–Crick base pairing can result in specific inhibition of gene expression by various mechanisms, depending on the chemical make-up of the ASO and location of hybridization, resulting in reduced levels of translation of the target transcript. The ASO is not only a useful tool for studies of loss-of-gene function and target validation, but also highly valuable as a novel therapeutic strategy to treat any disease that is linked to dysregulated gene expression. Antisense oligonucleotide-induced protein knockdown is usually achieved by induction of RNase H endonuclease activity that cleaves the RNA–DNA heteroduplex. This leads to the degradation of target mRNA while leaving the ASO intact. Other ASO mechanisms include translational arrest by steric hindrance of ribosomal activity, interference with mRNA maturation by inhibiting splicing and destabilization of pre-mRNA in the nucleus (Fig. 1).

In the present review, we first discuss a few computational algorithms in ASO design. There are other screening strategies to obtain potent ASO, such as mRNA walking, oligonucleotide array and RNase H mapping, but these approaches are more costly and labour intensive and require expensive automation equipment that many small laboratories may not be able to afford. Rational design of ASO based on computational algorithms that are freely available in the public domain is the most economical approach to ASO design and very often generates potent ASO from a handful of candidates. Because there is no stand-alone program in predicting highly potent ASO, one can increase the ‘hit rate’ using several computer software packages. Unmodified ASOs are susceptible to degradation by nucleases. Therefore, different chemical modifications of ASO have been developed to decrease nuclease cleavage and increase the biostability and potency of the ASO. We then discuss the latest approaches for ASO delivery in vitro and in vivo. Owing to inherent ionic charges of ASO, it is difficult for the ASO to cross the plasma membrane efficiently. As such, the ASO needs to be coupled to a carrier for efficient membrane binding and internalization. Finally, toxicology and clinical studies of ASOs are discussed.

ANTISENSE OLIGONUCLEOTIDE DESIGN

The strength and stability of interactions between the ASO and complementary target mRNA depends on factors such as thermodynamic stability, the secondary structure of the target mRNA transcript and the proximity of the hybridization site to functional motifs on the designated transcript, such as the 5' CAP region or translational start site. We need to consider at least four parameters in ASO design in order to increase the ‘hit rate’: (i) prediction of the secondary structure of the RNA; (ii) identification of preferable RNA secondary local structures; (iii) motifs searching and GC content calculation; and (iv) binding energy (ΔG°) prediction.
Prediction of the secondary structure of RNA

It is generally accepted that effective ASO design depends on accurate prediction of the secondary structure of the RNA. However, a truly reliable algorithm to predict any single mRNA secondary structure and folding pattern is lacking. A widely used mfold program is available in the public domain (http://www.bioinfo.rpi.edu/applications/mfold) that predicts all possible optimal and suboptimal structures of a particular sequence of mRNA. The core algorithm predicts overall minimum free energy, \( \Delta G \), of different possible folding. Conversely, another commonly used computer algorithm is the sfold program (http://sfold.wardsworth.org/index.pl), which predicts only the best secondary structure of the target transcript.

Using a combination of both mfold and sfold, one can determine the most frequently occurring secondary structure of the target mRNA with minimal overall free energy as a potential ASO target site.

Identification of preferable mRNA local secondary structures

An effective ASO should be designed at the regions where mRNA is accessible for hybridization. Local structures accessible to ASOs are those usually located at the terminal end, internal loops, joint sequences, hairpins and bulges of 10 or more consecutive nucleotides.
nucleotides. In conjunction with mfold, a new software (TargetFinder; http://www.bioit.org.cn/ao/targetfinder.com) has recently been developed to facilitate ASO target site selection based on the method of mRNA accessible site tagging (MAST). Yang et al. have recently demonstrated that potent ASO target sites can be found in highly conserved local motifs, whereas ASO targeting at variable local motifs may lead to non-sequence specific effects. Therefore, in order to increase the ‘hit rate’ of potent ASO design, one needs to look for locally conserved structures among various optimal and suboptimal mRNA predicted secondary structures.

**Motifs determination and GC content calculation**

After confirmation of the accessible conserved local secondary structures and the corresponding sequences of ASOs (approximately 20 bp), one can settle on some well-defined activity enhancing motifs and discard those activity decreasing motifs in the ASOs. Matveeva et al. analysed data collected from > 1000 experiments using phosphorothioate (PS)-modified ASOs and found a positive correlation between ASO-mediated mRNA knockdown and the presence of CCAC, TCCC, ACTC, GCCA and CTCT motifs in the ASOs. Conversely, the presence of GGGG (G-quartets formation), ACTG, AAA and TAA motifs in ASOs weakened ASO activity. Although it is believed that the formation of the ASO–mRNA heteroduplex stimulates RNase H activity, leading to target mRNA degradation, found that RNase H activity is sequence independent. Instead, GC content is strongly correlated with the thermodynamic stability of the ASO–mRNA duplexes and RNase H activity. observed strong ASO effects with a minimum of 11 G or C residues in 20 bp ASOs, whereas poor inhibition was observed by ASOs having nine or fewer G or C residues.

**Binding energy (ΔG°37) prediction**

Successful ASO design also needs to take into consideration thermodynamic energy. Software for calculating thermodynamic properties between the ASO and mRNA target sequence is available. The program OligoWalk from the package RNAstructure 3.5 (http://www.cgb.ki.se/AOpredict) has been developed to calculate binding energy of ASO/ASO and ASO/mRNA. To design a potent ASO, the binding energy between the ASO and mRNA should be \( \Delta G_{37} \geq -8 \text{ kcal/mol} \), whereas the energy for binding between ASOs should be \( \Delta G_{37} \geq -1.1 \text{ kcal/mol} \). By using two large databases from ISIS Pharmaceuticals (Carlsbad, CA, USA) and the published literature, Matveeve et al. showed that the hit rate of developing a potent and active ASO is six- and threefold higher, respectively, if the above criteria are met. In addition, by using this algorithm alone, Fei and Zhang were able to design ASO for the downregulation of vascular endothelial growth factor protein expression with a success rate higher than 85%.

To circumvent a tedious ASO design approach using multiple computer algorithms, a fast and handy ASO prediction based on a neural network has been developed using on a broad range of parameters, including base composition, RNA–ASO binding energy, RNA–ASO terminal properties, ASO–ASO binding properties and 10 verified sequence motifs correlated with efficacy and RNase H accessibilities. The prediction server interface is available at http://www.cgb.ki.se/AOpredict. Although this model can predict effective ASOs with > 50% gene expression inhibition with a success rate of 92%, some effective sequences may be missed because the selective criteria of this program are too stringent. Furthermore, there is a lack of thermodynamic consideration in this network in correlating dimer energy with efficacy.

In conclusion, there is no reliable stand-alone algorithm to accurately predict ASO. In practice, ASOs have to be tried and screened, so that some companies, such as ISIS Pharmaceuticals, have performed gene-walking and have screened hundreds of ASOs against one gene. Using this linear ‘shot-gun’ approach, only 2–5% of the oligonucleotides are generally found to be potent ASOs. However, by combining the above theoretical criteria using multiple computational algorithms, one can markedly increase the hit rate of highly potent ASOs.

**CHEMICAL MODIFICATIONS OF ASO**

An unmodified ASO is rapidly attacked by all types of nucleases in biological fluid and its overall charged property prevents it from penetrating through the cell membrane. Various chemical modifications have been developed to enhance nuclease resistance, prolong tissue half-life, increase affinity and potency and reduce non-sequence-specific toxicity (Fig. 2).

**First-generation ASOs**

First generation ASOs 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. Phosphorothioate modification confers higher resistance to the ASO against nuclease degradation, leading to higher bioavailability of the oligonucleotide. Phosphorothioate-modified ASOs promote RNase H-mediated cleavage of target mRNA. However, this modification may slightly reduce the affinity of the ASO for its mRNA target because the melting temperature of the ASO–mRNA heteroduplex decreases by approximately 0.5°C per nucleotide. Phosphorothioate-modified ASOs have also been reported to produce non-specific effects by interactions with cell surface and intracellular proteins. Despite these disadvantages, PS modification is the most widely performed chemical modification of ASOs for loss-of-function studies in vitro and in vivo for gene target identification and validation. Indeed, intravitreous fomiviren, a 21 bp first generation PS-modified ASO, is currently the only ASO drug approved for clinical use.

**Second-generation ASOs**

To further enhance nuclease resistance and increase binding affinity for target mRNA, second-generation ASOs with 2′-alkyl modifications of the ribose were developed. 2′-O-Methyl (2′-OME) and 2′-O-Methoxethyl (2′-MOE) modifications of PS-modified ASOs are the two most widely studied second-generation ASOs. Unexpectedly, 2′-OME and 2′-MOE substitutions do not support RNase H-mediated cleavage of target mRNA, which dampens the efficacy of the ASO.

To circumvent this shortcoming, a chimeric ASO 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’ ASO allows RNase H to sit in the central gap to execute target-specific mRNA degradation; meanwhile, the ‘wings’ resist nuclease cleavage of ASO by 2′-alkyl modifications at both
ends. Extensive studies have been performed in vivo to assess the stability and toxicity of these modified ASOs.1,26

### Third-generation ASOs

To further enhance target affinity, nuclease resistance, biostability and pharmacokinetics, a third generation of ASO 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 ASOs.2,27

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.28,29 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. 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 antigen effects by hybridizing with double-stranded DNA in four possible configurations, including triplex, triplex invasion, duplex invasion and double duplex invasion,29,30 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,30–32 but its efficacy in vivo remains to be determined.

Locked nucleic acid is a conformationally restricted nucleotide containing a 2’-O,4’-C-methylene bridge in the β-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.33 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. It has been shown that the chimeric LNA/DNA/LNA gapmer with seven 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 ASO potency, target accessibility and nuclease resistance.34 Among the nine members of the LNA molecular family, α-1-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.35–37

Phosphoroamidate morpholino oligomer represents a non-charged ASO agent in which the ribose sugar is replaced by a six-membered morpholino ring and the phosphodiester bond is replaced by a phosphoroamidate linkage.38 Phosphoroamidate morpholino oligomer does not support RNase H activity, such that its ASO effect is primarily mediated by steric interference of ribosomal assembly resulting in translational arrest. 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.39 Phosphoroamidate morpholino oligomer has demonstrated antisense efficacy in animal models in vivo and in human clinical trials.40,41

### DELIVERY OF ASO

Unmodified naked ASO has a net negative charge and can barely penetrate the plasma membrane. Cellular uptake of ASO is primarily

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<th>Second generation</th>
<th>Third generation</th>
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Fig. 2 Chemical modifications of antisense oligonucleotides.
an adsorptive endocytosis process. Phosphorothioate modification of ASOs not only enhances nuclease resistance, but also promotes adsorption of ASOs to cell surface proteins, resulting in higher internalization of the ASOs. Peptide nucleic acid and PMO are non-charged oligonucleotides that do not interact well with cell surface proteins, making them even more difficult for adsorptive endocytosis. The amount of ASO that enters cells is so low that a variety of delivery strategies has been devised to enhance cellular uptake of ASOs and the ensuing mRNA knockdown (for a review, see Lysik and Wu-Pong). Mechanical techniques, like electroporation and microinjection, are very useful in delivering ASOs into cell cultures in vitro, but are impractical for in vivo studies. In contrast, chemical-mediated ASO delivery has been tested extensively in both in vitro and in vivo studies. Cationic lipid carriers like N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTMA) and N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium methyl sulphate (DOTAP) are the most widely used vectors for ASO internalization. Upon entry into the intracellular milieu in the form of an endosome, the ASO needs to escape from the endosomal vesicles to avoid lysosomal degradation so as to interact directly with target mRNA in the cytosol and in the nucleus. Diolylphosphatidylethanolamine (DOPE) has been added into the lipid carrier formulation to promote endosomal membrane destabilization, leading to release of the ASO into the cytosol. Indeed, a pH-sensitive fusogenic liposome preparation consisting of DOPE and an amphiphatic lipid, such as cholesteryl hemisuccinate, supports fusion of the liposomal and endosomal membranes at a pH below 5.5, resulting in ASO escape and enhanced mRNA knockdown efficacy. The use of microparticles, such as biodegradable copolymer poly(d,l-lactide-coglycolide) in sustained-release ASO delivery has also been investigated. The ASOs, encapsulated in microspheres ranging from 10 to 60 μm, are released gradually with enhanced serum stability, increased cytosolic and nuclear delivery and prolonged duration of ASO action in both in vitro and in vivo models.

Covalent conjugation of ASO to a macromolecule like dendrimer and to cell-penetrating peptides (CPP) has been shown to promote cellular uptake of the ASO. Dendrimers are spherical and highly branched polymers with cationic polyamidoamine moieties capable of forming a covalent complex with the ASO. In contrast with the liposome formulation, the dendrimer–ASO complex is stable and active in the presence of serum. It enhances ASO cellular delivery into the cytosol and nucleus and increases the retention time of ASO in the cells. Conversely, CPP is a short peptide sequence (< 30 amino acids) with net positive charge that allows rapid translocation of a large molecule like the ASO through the cell membrane via an energy dependent pathway. Commonly used CPP include penetratin (RQIKIWFQNRRMKWKK), HIV TAT peptide 48–60 (GRKKRRQRRRPQ) and transportan (GWTLSAGYLLGKN-LKALAAALAKKIL-amide). The ASOs can be directly conjugated to any of these CPP via formation of disulphide bridge. By far, PNA is the most frequently used ASO in evaluating CPP-mediated mRNA knockdown in both in vitro and in vivo studies.

Despite the advances in liposome technology, the most critical challenge for the ASO to be an effective therapeutic is for it to be delivered to the site of action and to produce expected efficacy in vivo. There is a new trend of using topical application of ASOs as the most popular mode of administration. In fact, the first clinically approved ASO, formivirsen, is administered intravitreally. More recent studies have revealed that alicaforsen, a PS-modified intercellular adhesion molecule-1 ASO, produced promising acute and long-term benefits in ulcerative colitis patients when given locally in an enema preparation. AP 12009, a PS-modified transforming growth factor-β2 ASO, induced prolonged and complete brain tumour remission when given directly into the brain and TPI-ASMB, an ASO targeted to cytokine receptors, is being evaluated as an inhalational therapy for asthma.

TOXICOLOGY OF ASO

All three generations of ASOs have gone through preclinical toxicological studies and, in fact, some of these ASOs have entered clinical trials. In general, ASO drugs produce dose-dependent, transient and mild-to-moderate toxicities manifested in rodents, primates and humans. The most common acute toxicities associated with ASO administration in vivo are activation of the transient complement cascade and inhibition of the clotting cascade. Both these toxic effects are dependent on ASO backbone chemistry, but are ASO sequence independent. The toxicities are largely due to the non-specific binding properties of PS-ASOs to proteins at high plasma concentrations. The PS-ASO 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, PS-ASO, with its polyamionic 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. 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 ASO 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-γ), B cell proliferation, antibody production and activation of T lymphocyte and natural killer (NK) cells. The immunostimulatory effects of CpG are further amplified when the unmethylated CpG is flanked by two 5′ purines and two 3′ pyrimidines (e.g. AAGCTT). Newer generations of ASOs 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 PS-ASO has been shown to reduce, and even eliminate, CpG dinucleotide-mediated immune stimulation. Other mild and self-limiting toxicities usually observed at high plasma ASO concentrations are thrombocytopenia, enhanced liver enzyme (e.g. aspartate aminotransferase and alanine aminotransferase) levels and hyperglycaemia.

ANTSENSE OLIGONUCLEOTIDE DRUGS IN CLINICAL TRIALS

At present, formivirsen (Vitravene; Isis Pharmaceuticals) is the first and only US Food and Drug Administration (FDA)-approved ASO drug indicated for the treatment of peripheral cytomegalovirus retinitis in AIDS patients by local intravitreal administration. With the approval of this novel therapeutic strategy, numerous clinical trials (mainly on first-generation ASO drugs) have been conducted for the treatment of diseases such as cancer, viral infections, autoimmune disorders and allergic asthma (Table 1). Although most of the
first-generation ASOs failed to survive the clinical trials, partly due to suboptimal pharmacokinetic, pharmacodynamic and toxicological profiles, there are a few first-generation ASOs that have demonstrated promising therapeutic potential. One of these is an 18 bp PS-ASO oblimerson (G3139; Genasense; Genta Incorporated, Berkeley Heights, NJ, USA) targeting the first six codons of the bcl-2 mRNA open reading frame, which has shown promising therapeutic effects in various types of cancer. Bcl-2 is overexpressed in cancer cells, controls the anti-apoptotic pathway and contributes tumour resistance to chemotherapy. By knocking down bcl-2 protein, oblimerson increases the efficacy of cytotoxic agents in the treatment of cancers such as multiple myeloma and chronic lymphocytic leukaemia.54,55

Another PS-ASO, namely GTI-2040, is a 20 bp PS-ASO targeting the coding region of human ribonucleotide reductase (RNR) R2 subunit component mRNA. The RNR is composed of R1 and R2 components and is critical for the production of 2′-deoxyribonucleoside 5′-triphosphates required for DNA synthesis.56 The R2 subunit appears to determine the malignant potential of tumour cells via positive cooperation with activated oncogenes such as c-myc and H-ras. Overexpression of the R2 subunit is also associated with an increased drug resistance property of cancer cells. It has been shown that GTI-2040 inhibits the growth of various tumours in xenograft models.57 Phase I/II trials using GTI-204 in combination with anticancer drugs in patients with various types of cancer are currently in progress.58

Another first-generation ASO currently in clinical trial is LErrAON (NeoPharm, Waukegan, IL, USA), a cationic liposome-encapsulated raf-1 proto-oncogene ASO. Dysregulated raf-1 activity has been implicated in oncogenic transformation, increased resistance to cytotoxic agents and angiogenesis in human tumours. The ASO targeted at raf-1 inhibits tumour growth and increases sensitivity to both chemotherapy and radiotherapy. A phase I study59 of LErrAON revealed a hypersensitivity reaction and dose-dependent thrombocytopenia. Future clinical trials of this ASO depend on an improvement of the liposomal formulation.58

In addition, newer generations of ASO have entered into clinical trials. OGX-011 is a second-generation 2′-MOE gapmer ASO targeting the translation initiation site of human clusterin mRNA. Clusterin plays a critical role in tumorigenesis and disease progression. It is upregulated in various types of cancer, such as prostate cancer and breast cancer, and in response to stress, such as radiation therapy and chemotherapy.59,60 Clusterin overexpression confers resistance to anticancer treatments, whereas ASO-mediated clusterin knockdown enhanced the efficacy of anticancer agents. A phase I study of OGX-011 in patients with localized prostate cancer showed that it was well-tolerated and reduced clusterin expression in primary prostate tumours.59 AVI-4126 is a non-charged 20 bp third-generation PMO targeting c-myc mRNA. The c-myc protein is upregulated in human solid tumours, such as prostate cancer and breast cancer, leukaemias and lymphomas, and plays a critical role in controlling cell proliferation, differentiation and apoptosis. AVI-4126 has demonstrated beneficial effects in tumour models of lung cancer and prostate cancer.41,61 A phase I clinical study of AVI-4126 revealed significant tumour accumulation of PMO in breast and prostate tissues and a favourable safety profile devoid of common side-effects,61 such as activation of the complement cascade, inhibition of the clotting cascade, thrombocytopenia and hypotension associated with PS-ASO.41,61

With a better understanding of ASO design, chemical modifications of ASO, ASO delivery systems and human pharmacokinetic and pharmacodynamic profiles of ASO and numerous ASO candidates being tested in both preclinical and clinical stages for a variety of human diseases,1,3,27 the prospect for ASOs to become a major therapeutic modality is very bright.

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REFERENCES


