DRUG DISCOVERY TODAY TARGETS

Chemical modification of gene silencing oligonucleotides for drug discovery and development

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Gene silencing, the specific inhibition of unwanted gene expression by blocking mRNA activity, has long appeared to be an ideal strategy to leverage new genomic knowledge for drug discovery and development. But effective delivery has continuously been a limiting factor. In the past two decades, valuable progress has been made through the development of various chemically modified single-stranded antisense oligonucleotides, with improved properties such as enhanced stability, higher affinity and lower toxicity. Although short interfering RNA (siRNA) can provide better specificity and stronger efficacy by means of RNA interference (RNAi), *in vivo* delivery of siRNA often relies on plasmids or vectors, both of which present therapeutic safety risks. This review presents a brief history of gene silencing from PS-ODN through siRNA, introduces DNP-RNA – a more potent and easily delivered gene silencing platform – and compares its performance with that of siRNA and other AS-oligonucleotides.

Because their sequence is complementary to that of mRNA, antisense oligonucleotides (ONs) could be the ideal inhibitors for suppressing the expression of specific genes as well as the growth of targeted malignant cells [1]. However, their successful therapeutic application has been delayed owing to problems of delivery. Natural ONs are not membrane-permeable, and even after delivery into cells with the help of amphipathic cations or liposomes, antisense ONs (AS-ON) can still be hydrolyzed by cellular nucleases before reaching their intended target mRNAs. To improve their bioavailability, various types of chemically modified AS-ONs such as phosphorothioate oligodeoxynucleotide (PS-ODN), mixed backbone oligonucleotide (MBO) and morpholino (MF) have been produced with significant bioavailability improvements [2–6]. However, chemical modification has also led to undesirable complications such as decreased sequence-specificity and general toxicity.

The discovery of RNA interference (RNAi) started a new era in antisense technology [7–9]. Double-stranded short interfering RNAs (siRNA) were found to silence their target mRNA almost 100 times more effectively than single-stranded antisense PS-ODN [10], even though only the antisense strand of siRNA is incorporated in the RNA-induced silencing complex (RISC) [11]. Delivery, however, is still a problem as successful administration of RNAi often requires the use of plasmids or viral vectors; these delivery methods carry the risk of random integration into chromosomal DNA, a serious threat to therapeutic safety [12].

The challenge, therefore, remains to develop a chemically modified gene silencing platform that can be effectively delivered and which can approach or exceed the gene expression inhibition potency and specificity of siRNA.

Earlier generations of chemically modified antisense ONs

Many types of chemically modified AS-ONs have been produced and tested to date. Modifications range from changes in the overall electronic charge

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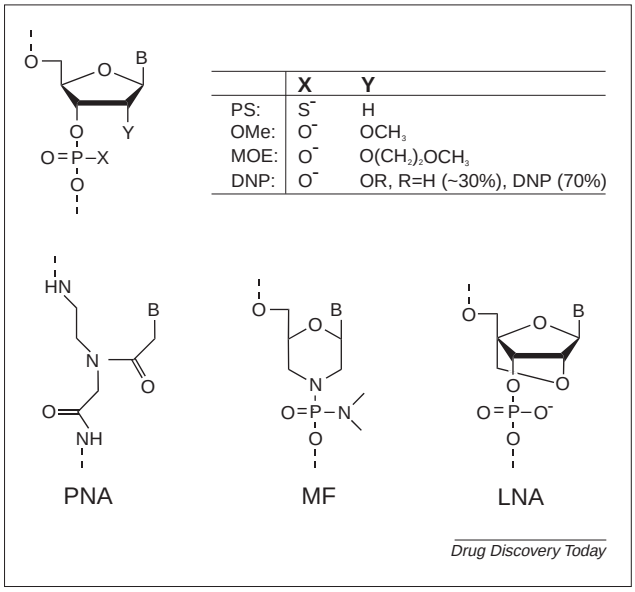

FIGURE 1

Nucleic acid analogs discussed in this review. B denotes base. Abbreviations: PS, phosphorothioate DNA; OMe, 2′-O-methyl RNA; MOE, 2′-O-methoxy-ethyl RNA; DNP, 2′-O-(2,4-dinitrophenyl)-RNA; PNA, peptide nucleic acid; MF, morpholino phosphoroamidate; LNA, locked nucleic acid

of the oligonucleotide to the incorporation of nonphosphate oligonucleotide backbones. The structures of the often cited chemical modifications are illustrated in Figure 1. They are commonly grouped into three 'generations' based upon the type of modification: AS-ON analogues with altered phosphate backbones, modified sugars (especially at the 2′ position of the ribose), or AS-ONs containing unnatural bases. The major technological goals for gene silencing platforms in all cases are: resistance to nuclease digestion, good cellular uptake, satisfactory hybridization affinity to the target nucleic acids, binding specificity and low toxicity [13].

First generation antisense molecules

These were designed to make the internucleotide linkages, the backbone to which the nucleotide bases are covalently attached, more resistant to nuclease attack. They were modified by replacing one of the non-bridging oxygen atoms in the phosphate group with either a sulphur or a methyl group (Figure 1). Those DNA analogues with a sulfur group are known as phosphorothioate oligodeoxynucleotides (PS-ODNs), and are the most widely used AS-ON to date.

The main advantages of PS-ODNs are resistance against nucleases, ability to recruit RNase H to cleave target mRNA, ease of synthesis and attractive pharmacokinetic properties. Weaknesses of PS-ODNs include lower binding affinity as measured by melting temperature (T_m) than its corresponding phosphodiester oligonucleotide, and a tendency to exhibit generally undesirable sequence-independent properties *in vivo*, such as immune stimulation, complement activation and cellular toxicity [14–16].

PS-ODNs have been studied in a number of disease models, both *in vivo* and *in vitro*, with promising results [14]. The first FDA-approved antisense drug, Vitravene from Isis (Carlsbad, CA, USA.) and the majority of antisense compounds in clinical trials to date are based on this chemistry.

Second generation antisense molecules

These contain nucleotides with alkyl modification at the 2′ position of the ribose. 2′-O-methyl and 2′-O-methoxyethyl RNAs are the most important representatives. The significant improvements are a reduction in general toxicity, increased hybrid stability, and increased nuclease resistance. However, these desirable properties are counterbalanced by the fact that 2′-O-methyl RNA cannot induce RNase H cleavage of the target mRNA. For most early antisense approaches, target RNA cleavage by RNase H was deemed necessary to achieve sufficient antisense potency. Use of RNAs with only the 2′-O-alkyl modification has been largely discontinued.

In order to induce RNase H cleavage, mixed backbone ONs (MBOs) were developed by surrounding a phosphorothioate-modified deoxyribose core that retains RNase H activity with nuclease-resistant arms, such as 2′-*O*-methyl (OMe) ribonucleosides [17,18]. The choice of oligonucleotide modification and its placement is critical to their properties. The antisense activity, pharmacokinetics, *in vivo* degradation and safety profile of an MBO can be modulated by combining appropriate oligonucleotide segments and backbone modifications at defined sites; this can lead to improved therapeutic effectiveness. GEM231, which targets RI_a/PKA, is built on an MBO platform and is being developed by Hybridon (Cambridge, MA, USA) as a cancer therapeutic [19]. It is an 18-mer oligonucleotide with four OMe ribonucleosides at both the 3′- and 5′- ends surrounding the remaining deoxynucleosides, all with phosphorothioate internucleotide linkages. Several Phase I and II clinical trials using GEM231 are being conducted to treat solid cancers, either as a monotherapy or in combination with other marketed chemotherapeutics.

Third generation antisense agents

These are DNA and RNA analogues with modified phosphate linkages or riboses as well as nucleotides with a completely different chemical moiety replacing the furanose ring. Peptide nucleic acids (PNA), morpholino phosphoroamidates (MF) and locked nucleic acids (LNA) are three interesting RNA/DNA analogues in this class that attract much attention. These compounds are essentially nuclease resistant while maintaining good hybridization affinity with their complementary mRNA.

PNAs and MFs represent a more radical approach to the nuclease-resistance problem, as the phosphodiester linkage is completely replaced with a polyamide (peptide) or phosphoroamidate backbone (Figure 1) [6,20]. They both form

tight bonds with their RNA targets and probably exert their effects by blocking translation, as neither molecule effectively activates RNase H. Whether it is necessary to preserve the ability of these molecules to activate RNase H is controversial, but many workers in the field still believe that molecules with this capability are likely to be more effective, at least in clinical settings. Noncharged backbones prevent PNAs and MFs from binding to proteins that normally recognize polyanions, which are a major source of nonspecific interactions. However, due to the electrostatically neutral property, solubility and cellular uptake are serious problems. Improved intracellular delivery could be obtained by coupling these ONs with certain ligands (e.g. a four-lysine tail), or by mechanical disturbance of the cell membrane (e.g. electroporation).

According to the *in vivo* studies performed to date, PNAs appear to be nontoxic and can target a broad range of mRNA sequences effectively, but the clearance *in vivo* is rapid compared with that of PS-ODN or MBO, thus limiting *in vivo* effectiveness. The seemingly negative property of binding to certain proteins protects PS-ODN or MBO from filtration, thus increasing their serum half-life. Whereas PNAs do not appear to have gene silencing advantages over other chemically modified ONs, their ability to recognize duplex DNA makes PNAs promising candidates for modulating gene expression or inducing mutations by strand invasion of chromosomal duplex DNA [21].

Effective gene knock-down by antisense MFs *in vivo* has been shown, and therapeutic compounds are being developed. One such MF targets the *c-myc* oncogene [2] and is currently being tested in phase I and II clinical trials by AVI BioPharma (Portland, OR, USA).

LNAs, also known as bridged nucleic acids (BNA) are another type of novel high-affinity molecule that provide major improvements in a number of key properties. LNAs contain a methylene bridge connecting the 2′-oxygen of the ribose with the 4′-carbon (Figure 1). This design leads to improved binding to complementary DNA and RNA sequences [22]. Like other 2′ modifications, the 2′–4′ linkage reduces or eliminates activation of mRNA cleavage by RNase H. However, because LNA bases are added by standard DNA/RNA synthesis protocols, it is straightforward to design chimeric 'gapmers', in which a central DNA portion is flanked by LNA in order to enhance the stability of binding. Such chimeric LNA-containing ONs allow the high affinity of LNA binding to be combined with the ability of DNA to recruit RNase H.

In the first *in vivo* study reported for an LNA, an efficient knock-down of the rat delta opioid receptor was achieved in the absence of any detectable toxic reactions in rat brain [23]. Subsequently, full LNA ONs have been successfully used *in vivo* to block the translation of an RNA polymerase [24]. These ONs inhibited tumor growth in a xenograft model with an effective concentration that was five times lower than was found previously for the corresponding PS-ODN.

RNAi and traditional antisense technology

RNAi is the process by which double-stranded RNAs target mRNAs for destruction in a sequence-dependent manner [7-9]. The mechanism of RNAi involves processing of long (~ 500–1000 nucleotides) dsRNA into fairly short 21–25 bp 'trigger' fragments by a nuclease called DICER. These small RNAs (referred to as short interfering RNAs or siRNAs) subsequently assemble with a large, multicomponent nuclease complex named RISC. An ATP-generated unwinding of the siRNA activates the RISC, which in turn binds to the complementary transcript by base-pairing interactions between the siRNA antisense strand and the mRNA. The bound mRNA is cleaved, and sequence specific degradation of mRNA results in gene silencing [25]. In mammalian systems, siRNA has been suggested for use to avoid broad nonspecific reactions [7].

siRNAs were found to silence their target mRNAs almost 100 times more effectively than the single-stranded antisense PS-ODNs, even though only the antisense strand of siRNA is incorporated in the RISC. This large difference in effectiveness appears to have made many single-stranded antisense ONs look obsolete. However, this is difficult to understand unless we assume that they inhibit by different molecular mechanisms. AS-ONs typically inhibit gene expression either by steric blockade of the ribosome, or by cleaving the mRNA by RNase H-type of mechanism. siRNA cannot be hydrolyzed by RNase H, but it cleaves its mRNA much more efficiently via an ATP-driven RNAi mechanism [26–27]. The RNase H mechanism requires an antisense oligomer with at least four or five deoxy residues, whereas the RNAi mechanism requires either an antisense oligomer with single-stranded RNA (ssRNA) backbone or homologous double-stranded RNA (dsRNA) as the platform for its base pairing. For the same sequence targeting the same mRNA in the same type of mammalian cells, the RNAi mechanism is much more effective than the RNase H mechanism.

Native ssRNA can be rapidly hydrolyzed by RNases, whereas siRNA remains more stable due to intramolecular hybridization until it is guided into the RISC and has the sense strand removed. On the other hand, if the antisense strand can be stabilized by chemical modification without interfering with its basic biochemical properties, the resulting antisense ssRNA could be just as potent or even a better inhibitor of gene expression than the native siRNA.

DNP-RNAs

Poly-2′-O-(2,4-dinitrophenyl)-oligoribonucleotide (DNP-RNA) represents a promising new gene silencing platform (Figure 1). It can be easily synthesized by *in vitro* transcription, using a DNA template containing a T7 promoter, followed by a controlled reaction with 2,4-dinitro-1-fluorobenzene. It has been found that DNP-RNAs with a DNP:nucleotide molar ratio of about 0.7 can spontaneously cross viral envelopes and mammalian cell membranes.

DNP-RNAs are resistant to degradation by ribonucleases including RNases A, B, H, S, T_1 and T_2 and Phosphodiesterases I and II [28] and hybridize with their complementary RNA with greater affinity than DNA, MBO or underivatized RNA, as shown in the thermodenaturation curves in Figure 2 [29]. Several antisense DNP-RNAs, including those targeting the RI_α subunit of cAMP-dependent protein kinase (RI_α/PKA), Bcl-2, IGFR, erbB-2 and c-myc were synthesized and found to be effective anti-cancer or antiviral agents, acting in a sequence-specific and concentration-dependent way with no general toxicity in the effective concentration range [29–35].

Molecular modeling of a helical segment of DNP-RNA suggests that the hydrophobic dipolar DNP-groups are located at the cylindrical surface of the helix pointing outwards; this could be the reason for the observed improvement of the membrane permeability of the oligonucleotide compared to non-modified ONs. Ashun and colleagues observed that a 300-mer, DNP-[^{14}C] poly[A], diffuses spontaneously into human lymphocytes in the absence of transfection agents [36]. By contrast, a hydrophilic [^{14}C] poly [A] chain of equal length did not diffuse into lymphocytes under the same conditions. Ru and colleagues showed that a 20-mer DNP-RNA can permeate into the cytoplasm of MDA-MB-231 breast cancer cells in the absence of transfection agents [30].

Inhibition of cancer cell growth *in vitro* and induced apoptosis in targeted cancer cells

When a particular gene is overexpressed in cancer cells, blocking its expression with antisense oligonucleotide or siRNA is a way of inhibiting cancer growth. RI_α/PKA is often overexpressed in many cancer cell lines and is often associated with cancer cell proliferation [3, 37].

Based on early work of PS-ODN [2] and MBO [4, 5], one DNP derivatized antisense compound, DNP001, was synthesized [31]. Its base sequence is complementary to that of ribonucleotides 110–130 in the mRNA of RI_α/PKA. This membrane-permeable and RNase-resistant compound was found to inhibit cell growth with an IC_{50} value of 0.05 nM in MCF-7 cells and 3 nM in A549 cells. The control DNP-RNAs with sense or mismatched base sequences were inactive (see Figure 3). Furthermore, RT–PCR and western blot data showed that treatment of MCF-7 and A549 cells abolished the steady-state level of RI_α/PKA mRNA and RI_α protein, while control DNP-RNAs had no effect [31,29].

Similar to the observed apoptosis triggered by antisense MBO [38], by using TUNEL assay, Ru and colleagues showed that antisense DNP-RNA targeting to RI_α/PKA induced apoptosis in human breast cancer SK-Br-3 cells without affecting the non-tumorigenic MCF-10A cells [32].

It is not known exactly how antisense DNP-RNA induces apoptosis, but western blot analysis of DNP001 showed activation of caspase 8, the cleavage of Bid and decreased concentration of Bcl-2 [31]. All these proteins play important roles in the pathways of apoptosis. These observations suggest that these compounds could be developed as anticancer agents.

Inhibition of cancer growth *in vivo*, prevention of metastasis and reduced mortality

Inhibition of cancer growth *in vivo* by RI_α/PKA-targeting DNP-RNA was studied in SCID mice bearing human breast cancer (MDA-MB-231) xenografts by Ru and colleagues [30]. The results demonstrate sequence specific and concentration-dependent inhibition of the xenograft growth and reduced mortality as compared to untreated or random sequence-treated mice.

Histological examination showed that all six of the untreated mice had bone marrow metastasis of breast cancer cells, whereas no metastatic tumor was found from any of the six antisense-compound-treated animals, These improved mortality and 100% metatasis prevention results suggest that antisense DNP-RNAs deserve continued study as anti-cancer agents.

Treatment of murine leukemia and duck hepatitis B

Two antisense DNP-RNAs have been synthesized and used successfully to treat the retroviral disease murine leukemia (*MLV*) [33]. *MLV*-infected mice that were left untreated all died within 6 months, whereas those mice that were treated with the DNP-ssRNA survived in good health. Autopsy

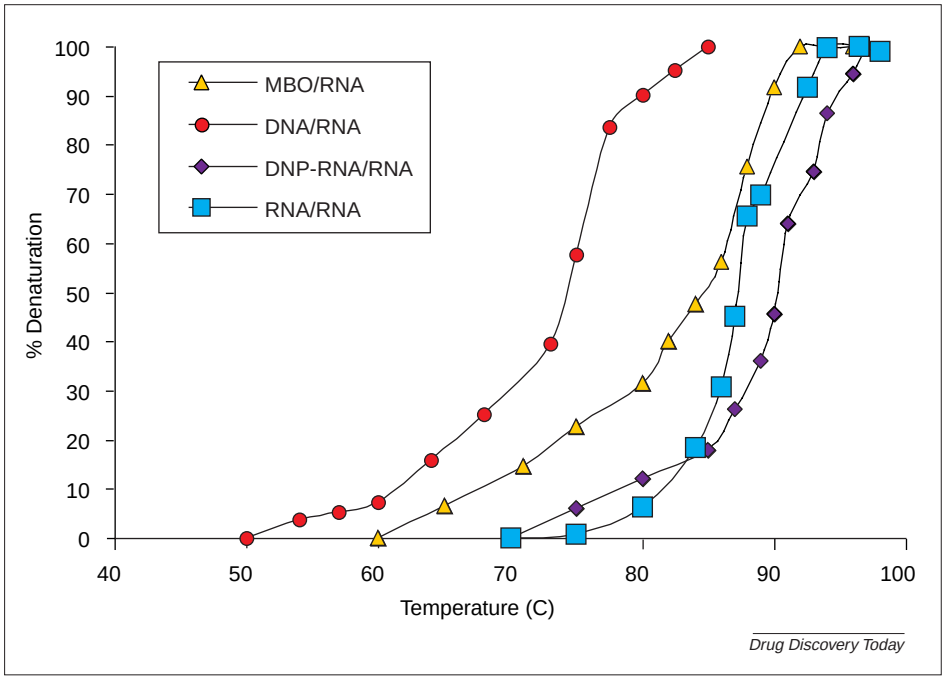

FIGURE 2

Thermodenaturation profile of RNA duplexes DNA–RNA, MBO–RNA, RNA–RNA, DNP-RNA–RNA.

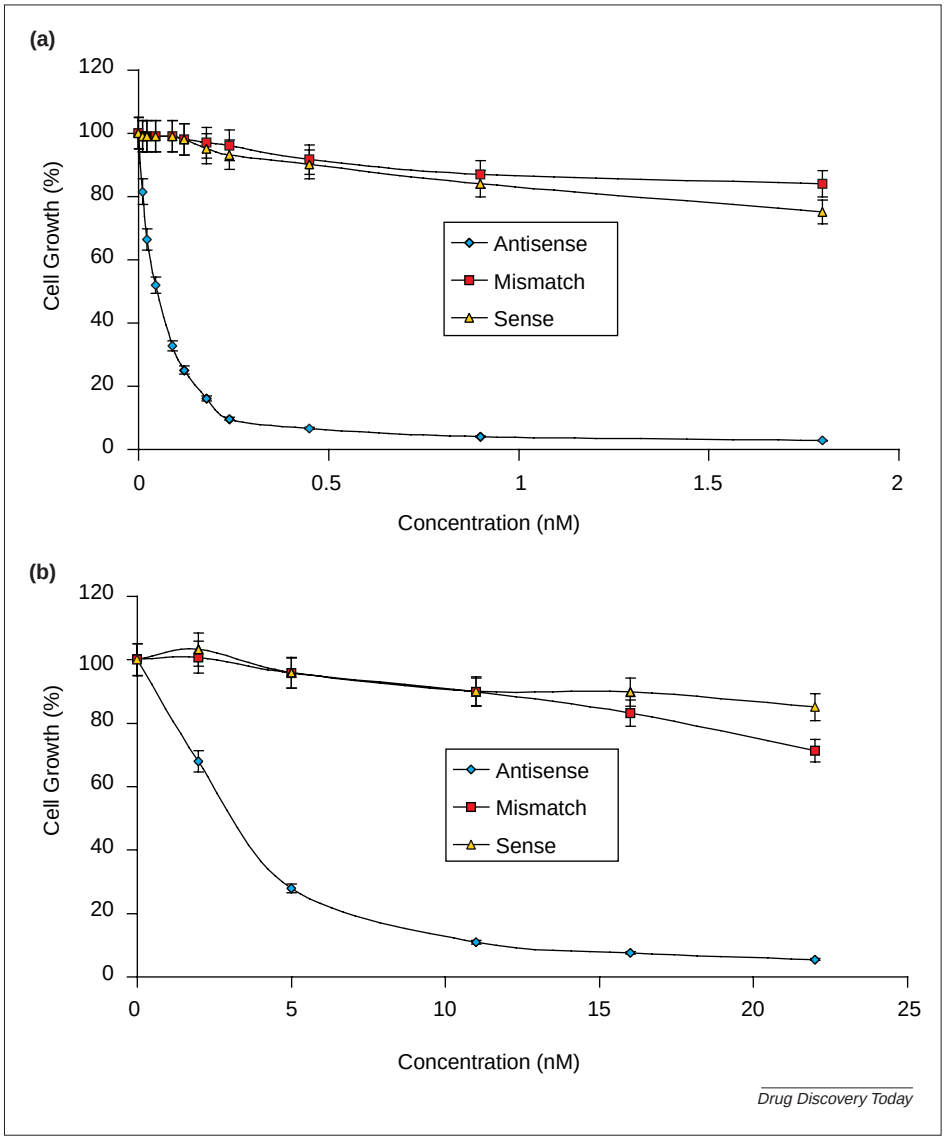

FIGURE 3

Sequence and concentration dependence of the inhibition of cell growth by DNP001. (a) MCF-7 breast cancer cells. **(b)** A549 lung adenocarcinoma cells. The cells were treated with different concentrations of poly(DNP-RNA)s in the presence of OLIGOGECTAMINE. After seven more days of incubation, the cells were collected and counted with a Coulter counter. Data are expressed as the percentage of growth inhibition in reference to the growth of untreated control cells. Data are presented as means ± SD of four independent determinations.

and RT–PCR assays showed that either i.p. or oral administration of the DNP-ssRNA eliminated not only viremia but also the integrated viral genome in bone marrow.

Successful results were also obtained in the duck hepatitis model. Based on the previous work of antisense PS-ODN to inhibit *DHBV* replication in ducks [39], a DNP-RNA was designed and tested [34]. After three weeks daily i.v. injections followed by 10 months recession, *DHBV* was still absent from ducks of the antisense group, and their damaged livers had returned to normal.

DNP-RNA vs competing gene silencing platforms

To determine the impact of the platform on the ability of different ONs to inhibit the growth of cancer cells, a series of ON homologues with identical base sequence but different gene silencing platforms (MBO, DNP-ssRNA, siRNA and DNP-siRNA) were tested under the same experimental conditions [29]. GEM231 (Hybridon, Inc., Cambridge, MA, www.hybridon.com), an antisense therapeutic currently in Phase I and II clinical trials, which targets the same region as DNP001, was chosen for comparison purposes. According to the IC_{50} values in Table 1, the inhibition efficacy of homologous ONs targeting the same gene in the same cell line and assayed by the same methods lines up in the following decreasing order: DNP-siRNA ≈ DNP-ssRNA > siRNA > ssRNA.

DNP001 is much more potent than AS-MBO, the MBO homologue. As DNP001 and AS-MBO are identical in sequence, their large difference in IC_{50} can only be due to different platforms. DNP001 has an RNA platform with ~ 70% of its 2′-OH groups covalently linked to dinitrophenyl (DNP) groups for increased stability and membrane-permeability. However, the other ~30% of its 2′-OH groups remain free. RNAi requires free 2′-OH groups to trigger the ATP-driven RNAi action [40]. In contrast, AS-MBO has no free 2′-OH groups to generate RNAi action and can therefore only inhibit gene expression less effectively by competitive binding or RNase H mechanisms.

DNP-derivatization improves binding affinity to the target mRNA thus increasing gene silencing efficiency. The thermal denaturation profile in Figure 2 shows that the DNP-RNA/RNA duplex exhibits higher binding affinity than MBO/RNA, DNA/RNA and RNA/RNA duplexes which would be formed by MBOs, PS/ODNs and unmodified siRNAs respectively.

TABLE 1

Comparative efficacy of DNP-RNAs with other gene silencing platforms

Oligonucleotide	IC_{50}(MCF-7)	IC_{50} (A549)
AS-DNP-ssRNA (DNP-001)	0.05 nM	3 nM
DNP-siRNA	0.15 nM	2.5 nM
siRNA	0.3 nM	15 nM
AS-MBO	30 nM	80 nM
GEM 231	45 nM	100 nM

The primary structures of the oligos are: DNP-001: 5′-poly-DNP-GGCUGCGUGCCUCCUCACUGG; AS-MBO: 5′-GGCUGCGTGGCCGCCTCCTCACUGG; GEM 231: 5′-GCGUGCCTCCTCACUGGC, where the underlined nucleosides are O-methylribonucleotides, and the remaining are deoxyribonucleosides. All internuclear linkages in AS-MBO and GEM 231 are phosphorothioate.

TABLE 2

Comparison of DNP-RNA with siRNA	
Potency	DNP-ssRNA ≈ DNP-siRNA > siRNA
Stability	DNP-RNA > siRNA DNP-ssRNAs are more resistant than siRNA to hydrolysis by ribonucleases.
Hybridization affinity	DNP-RNA > siRNA DNP-RNA/RNA duplexes show higher hybridization affinity than native RNA/RNA duplexes formed by siRNA
Target specificity	DNP-ssRNA ≈ siRNA DNP-ssRNA and siRNA show similar position-dependent disruption of gene inhibition resulting from single-base mismatches.
Ease of delivery	DNP-ssRNA > siRNA Delivery of DNP-ssRNA does not require the use of transfection agents and has been demonstrated *in vivo* intraperitoneally, intravenously and orally. (see examples below)
In vivo DNP-ssRNA results:	
Anti-cancer	Human breast cancer / SCID mouse xenograft: tumour growth inhibition; reduced mortality; prevention of metastasis
Anti-viral	Murine leukemia and duck hepatitis B: elimination of viremia; removal of integrated viral DNA from bone marrow/liver/spleen; restored organ histology

DNP-derivatization also improves the performance of double-stranded RNA, as the efficacy of the homologous DNP derivatized siRNA (DNP-siRNA) is higher than that of the corresponding unmodified siRNA platform.

Similar sequence specificity between DNP-RNAs and siRNAs

The high sequence specificity of siRNA has been demonstrated by many investigators using chemically modified or single-base mutated ONs [40–44]. To study the sequence specificity of AS DNP-ssRNA, a series of 21nt ssRNA and DNP-ssRNA were synthesized, each with the same sequence as DNP001 but with a single mismatching base. The impact of the mismatch on the inhibition of cell growth and protein synthesis is highly position-dependent. A single-mismatch mutation at either position 19 or position 20 renders the ssRNA or DNP-ssRNA completely inactive. This finding supports the hypothesis that the double-stranded and single-stranded RNA system might act through a common pathway [45].

Concluding remarks

Selective knockdown of gene expression has long been regarded as a potent tool for genomic research and target validation. PS-ODNs and their single-stranded successors were viewed to be a fast and versatile approach to specific knockdown for research use as well as therapeutic candidates. 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. Due to much higher gene silencing efficacy, improved specificity and reduced side-effects, RNAi has taken much drug discovery and development attention away from single-stranded antisense platforms. However, recent reports indicate that gene silencing by double-stranded and single-stranded RNAs might act through a common pathway [11,45]. Single-stranded antisense DNP-RNA (DNP-ssRNA) has been successfully used for the silencing of a variety of genes [29,31,32] and has the added benefit of delivery and *in vivo* effectiveness without use of plasmids or vectors [30,33,34,35].

Both forms of DNP-RNA, DNP-ssRNA (antisense) and DNP-siRNA, outperform previous generations of antisense and unmodified siRNA in gene silencing potency, stability, hybridization affinity and ease of delivery, and they show equivalent target sequence specificity to siRNA (see Table 2). In addition, successful animal models suggest their usefulness for *in vivo* research and human applications. However, given that roughly equivalent performance is achievable by antisense DNP-ssRNA with only half of the molecular mass of DNP-siRNA, the single-stranded form would appear to be a more economical and therefore preferable choice.

Chemically modified gene silencing ONs, DNP-RNAs in particular, therefore show good potential to substantively improve the drug discovery and development feasibility and value of gene silencing with applicability to *in vivo* target validation, diagnostics and therapeutics.

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