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Canonical and Non-Canonical Barriers Facing AntimiR Cancer Therapeutics

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Abstract

Once considered genetic “oddities”, microRNAs (miRNAs) are now recognized as key epigenetic regulators of numerous biological processes, including some with a causal link to the pathogenesis, maintenance, and treatment of cancer. The crux of small RNA-based therapeutics lies in the antagonism of potent cellular targets; the main shortcoming of the field in general, lies in ineffective delivery. Inhibition of oncogenic miRNAs is a relatively nascent therapeutic concept, but as with predecessor RNA-based therapies, success hinges on delivery efficacy. This review will describe the canonical (e.g. pharmacokinetics and clearance, cellular uptake, endosome escape, etc.) and non-canonical (e.g. spatial localization and accessibility of miRNA, technical limitations of miRNA inhibition, off-target impacts, etc.) challenges to the delivery of antisense-based anti-miRNA therapeutics (i.e. antimiRs) for the treatment of cancer. Emphasis will be placed on how the current leading antimiR platforms—ranging from naked chemically modified oligonucleotides to nanoscale delivery vehicles—are affected by and overcome these barriers. The perplexity of antimiR delivery presents both engineering and biological hurdles that must be overcome in order to capitalize on the extensive pharmacological benefits of antagonizing tumor-associated miRNAs

Keywords

antimiR; cancer therapy; chemical modification; microRNA inhibition; liposome; polymer nanoparticle; oligonucleotide; oncomiR; therapeutics; tumor targeting

INTRODUCTION

Over the past decade, miRNAs have emerged as promising therapeutic targets for numerous diseases—most notably cancer. MiRNAs are endogenously expressed small (19–25 nucleotides) non-coding RNA molecules that downregulate gene expression using the RNA interference machinery to both repress translation and destabilize target mRNA [1,2]. Ubiquitously expressed in various tissues and cells, miRNAs are involved in the regulation of most—if not all—biological processes. The first link between miRNAs and cancer was identified in 2002 when the Croce group observed that certain miRNAs were deleted or downregulated in chronic lymphocytic leukemia (CLL) [3]. Our group then showed that miRNAs fall into cancer pathways and regulate cancer genes [4]. The Hannon group

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CONFLICTS OF INTEREST

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implicated the miR-17~92 cluster as the first oncogenic miRNAs (i.e. oncomiRs) by showing that overexpression of the cluster accelerated tumorigenesis in B cell lymphoma [5]. Since then, inhibition of miRNAs through binding with complementary oligonucleotides (i.e. antimiRs) has become as an auspicious therapeutic paradigm.

AntimiRs comprise numerous classes of chemically modified oligonucleotides and nucleic acid analogs. This review will focus on some of the most prevalent antimiR technologies: locked nucleic acids (LNAs), 2'-O-methyl (2'-O-Me) oligos, 2'-O-methoxyethyl (2'-O-MOE) oligos, antagomiRs, peptide nucleic acids (PNAs), phosphorodiamidate morpholinos (PMOs), as well as antimiRs associated with nanoscale delivery systems such as liposomes and polymer nanoparticles [6]. LNAs are chemically modified oligonucleotides with an intramolecular methylene bridge connecting the 2'-hydroxyl and 4'-carbon [7]. This entropy-reducing ribose sugar modification locks the stabilized oligo into an RNA-like duplex conformation that binds complementary nucleic acids with an enhanced binding affinity (i.e. 2–8°C melting temperature increase per monomer). An LNA-based antimiR formulation targeted against miR-122 (Miravirsen) is currently in clinical trials for the treatment of Hepatitis C [8]. 2'-O-Me and 2'-O-MOE oligos are chemically modified oligos with a methyl or methoxyethyl group on the 2'-hydroxyl group of the ribose ring [9,10]. Similar to LNAs, these oligos are highly stable and have a higher binding affinity than unmodified nucleic acids—numerous commercially available antimiRs utilize these chemistries. AntagomiRs are 2'-O-Me oligonucleotides with winged (i.e. flanking) phosphorothioated (PS) substitutions in place of phosphodiester linkages and the addition of a 3'-cholesterol moiety. AntagomiRs were among the first antimiRs to show *in vivo* efficacy [11]—3'-cholesterol-modified small interfering RNA (siRNA) molecules have also exhibited success *in vivo* [12]. PNAs and PMOs are charge-neutral nucleic acid analogs that have modified backbones; PNAs have a polyamide backbone and PMOs have sugar groups replaced by morpholinos rings and phosphodiester linkages replaced by phosphorodiamidate linkages [13,14]. Due to their modified backbones, PNAs and PMOs are highly stable and bind complementary nucleic acids with high affinity. Both PNAs and PMOs by themselves typically exhibit poor *in vivo* delivery characteristics, but conjugation to functional molecules such as cell-penetrating peptides (CPPs) is a standard modification that can significantly improve efficacy [15,16]. Liposomes and nanoparticles are synthetic nanocarriers that have been used to deliver numerous small molecule, protein, and nucleic acid therapeutics [17,18]. With a demonstrated capacity to deliver genetic material and manufacturable properties that are suited for tumor-targeted delivery, these nanocarriers show promise as potent antimiR delivery vectors.

AntimiRs are a relatively nascent class of therapeutics, so their development has the advantage of dovetailing off of the successes and failures of similar technologies; such as the more established nucleic acid therapeutic-based platforms of antisense oligonucleotides and siRNAs [19–21]. Current antimiR technologies are extensions of these related systems; they face many of the same physiological and cellular barriers. However, this likeness can also be an encumbrance to innovation, since the process of therapeutic miRNA inhibition has its own exclusive challenges that require consideration. This review will detail these canonical and non-canonical delivery barriers facing antimiR-based therapeutics, with specific attention placed on the inhibition of oncogenic miRNAs in tumors.

CANONICAL BARRIERS

Systemic Stability

Systemic administration is the most attractive option for delivery of anticancer therapeutics, including antimiRs. In the bloodstream, unmodified phosphodiester oligonucleotides have a half-life of just a few minutes; however, oligonucleotides modified with a PS substitution

have a markedly improved circulation time with an initial (distributional phase) half-life of 3–30 min and then a terminal (elimination phase) clearance half-life of several to 24 hours [22,23]. In general, these pharmacokinetic parameters are similar between rodents, primates, and humans [24]. PNAs and PMOs have shorter half-lives than PS-modified oligos, but this can be improved by conjugation to functional ligands such as CPPs or stretches of cationic amino acids (typically lysines or arginines) [25–28]. Nanocarriers can also improve circulation time of encapsulated oligos. Liposomes and nanoparticles generally have circulation half-lives approaching 20 hours [29,30]. Although through engineering enhancements such as coating the surface with polyethylene glycol (PEG), some nanovehicles have exhibited a terminal half-life of 55 hours in humans [31]. Circulating antimiRs face numerous modes of clearance that decrease half-life and obstruct productive tumor delivery. One obstruction is the presence of plasma nucleases that rapidly degrade unmodified nucleic acids [32]. PS-modifications, most 2'-hydroxyl modifications, and the charge-neutral backbones of PNAs and PMOs all prevent nuclease degradation. Nuclease protection is also an intrinsic benefit of incorporating oligonucleotides with nanovehicles; even phosphodiester-based oligonucleotides exhibit nuclease-resistance when encapsulated within or adsorbed to the surface of nanocarriers [33]. Overall, stability in circulation is generally not a limiting factor for current developed antimiR technologies.

Renal Excretion

Most systemically delivered antimiRs are deposited in the kidneys and liver. Size is one of the main parameters that affect renal clearance of antimiRs. Due to protein binding (typically to albumin), PS-modified oligonucleotides generally show less renal clearance and excretion than non-PS oligos, PNAs, and PMOs (Fig. (1)) [23,32]. Renal filterability is a function of hydrodynamic diameter with a cut-off of 5–6 nanometers for globular proteins; the renal system clears ~75% of intravenous myoglobin (~3.8-nanometer hydrodynamic diameter) while clearing less than 0.3% of human serum albumin (~7.3-nanometer hydrodynamic diameter) [34]. Binding of PS-modified oligonucleotides to albumin is saturable and can be modulated by titrating the number of PS substitutions [23,35]. Note that in addition to PS, other chemical modifications have been shown to improve protein binding capacity and aid in avoiding renal clearance [32]. Also, due to their larger size (typically between 50–150 nanometers), most nanovehicles are not readily cleared by the kidneys; Choi *et al.* identified a renal filtration threshold for nanoparticles with a hydrodynamic diameter less than 5.5 nanometers [34]. Similar to stability in plasma, current antimiRs can successfully be engineered to avoid renal excretion.

Reticuloendothelial System

Clearance by the liver is perhaps the main physiological obstacle for existing antimiR technologies. This is mainly due to the reticuloendothelial system (RES), also known as the mononuclear phagocyte system (MPS), which functions to clear foreign and cellular debris from the body via circulating monocytes and tissue macrophages [22,32]. After systemic administration, naked oligonucleotides that are not cleared by renal filtration are often enriched in the highly fenestrated liver, with the majority of the oligos localized within resident macrophages (i.e. Kupffer cells) [32]. Although there is some debate, these macrophages have been shown to bind oligonucleotides via scavenger receptors [36,37]. Certain chemical modifications can aid in RES avoidance; for example, Bijsterboesch *et al.* showed that cholesterol conjugation to PS-modified oligonucleotides resulted in a redistribution of the majority of the oligos to hepatocytes and endothelial cells within the liver [38]. Interestingly, this altered distribution may be a result of cholesterol-mediated binding to lipoproteins that target to lipoprotein receptors within the liver [10,12].

Although they avoid the kidneys, generally, circulating nanovehicles can be taken up by the RES within minutes of administration [39]. This rapid clearance is mostly due to the formation of a corona of non-specifically adsorbed proteins (which includes immunoglobulins, complement components, and serum proteins) that is recognized by the RES. This opsonization process typically leads to phagocytosis of nanovehicles by macrophages within the liver [39]. One of the most well-known methods for avoiding RES clearance of nanocarriers is through the use of a PEG surface coating which provides a hydrophilic shield that sterically and electrostatically occludes the opsonization process and enhances circulation time [40]. As a result, PEGylation has become a standard practice during liposome and polymer nanoparticle fabrication (Fig. (1)).

Because of this proclivity for liver accumulation, some of the most promising antimiR-based therapies involve targets within the liver. For example, in non-human primates, Elmen *et al.* delivered naked PS-modified LNA-based antimiRs to inhibit miR-122 enriched in the liver, which resulted in depletion of miR-122 in hepatocytes and the expected phenotype of lowered plasma cholesterol levels [41]. This progress bodes well for the utility of anti-miR therapies; however, for the treatment of non-liver cancer, more efficient delivery systems must be developed.

Tumor Microenvironment

Tumors are enigmatic therapeutic targets. The tumor microenvironment comprises dynamic and heterogeneous cell populations (e.g. normal and cancer-associated stromal cells, immune cells, in addition to neoplastic cells); tortuous and poorly assembled vasculature beds; poor lymphatics; as well as regions of necrosis, hypoxia, acidity, and high interstitial fluid pressure. Paradoxically, some of these factors contribute to the ability of systemically administered nanoscale vehicles to preferentially distribute to tumors. Tumor blood vessels are more disorganized than normal blood vessels; some tumors have endothelial fenestrations (i.e. pores) ranging from 100–700 nanometers [42]. The enhanced permeability and retention (EPR) effect capitalizes on this vasculature leakiness (Fig. (1)). The EPR effect posits that macromolecules and nanovehicles of a given permissive size can escape out of blood vessels within a tumor, and then stay there because of ineffective lymphatic drainage [43]. In general, free oligonucleotides do not show pronounced tumor enrichment due to the EPR effect [44]. However, at sufficient doses some studies have demonstrated the ability of naked antimiRs to inhibit miRNAs in tumors. For example, Zhang *et al.* systemically delivered LNA antimiRs against miR-155 which limited the growth of subcutaneous xenograft lymphoma tumors [45]. Capitalizing on the EPR effect, numerous antimiR therapies directed against miRNAs within tumors have utilized nanocarriers. Anand *et al.* systemically administered lipid nanoparticles loaded with 2'-O-Me antimiRs against miR-132 to delay tumor growth and suppress angiogenesis in an orthotopic xenograft breast cancer model [46]. Plummer *et al.* systemically delivered PEGylated liposomes loaded with antimiRs against miR-10b and miR-196b to attenuate angiogenesis-mediated tumor growth in a breast cancer tumor model [47]. Also, we developed PEGylated, CPP-coated biodegradable polymer nanoparticles that delivered PNA antimiRs against miR-155 to attenuate the growth of xenograft pre-B cell lymphoma tumors in mice [48].

Size, charge, and shape can greatly influence the delivery efficacy of nanocarriers to tumors. Generally, smaller nanoparticles exhibit better tumor extravasation and transport than larger nanoparticles [49]. Charge-neutral nanoparticles typically distribute farther and more homogeneously than charged nanoparticles due to a lack of non-specific electrostatic interactions with the tumor extracellular matrix [50]. Also, some studies have shown that malleable nanostructures may diffuse throughout tumors better than rigid nanoparticles [50]. Delivery of nanovehicles to tumors is also constrained by the tissue type. For example, the

brain exhibits an exponential decrease in delivery efficiency with increasing nanovehicle size; nanoparticles delivered to the lungs often get trapped in capillary beds; and lymph nodes exhibit substantially different nanovehicle trafficking depending on the administration route [6]. Nanoparticles also have difficulty in accessing disseminated metastases, which generally exhibit a diminished EPR effect [6]. In line with PEGylation-based avoidance of RES clearance, nanovehicles can be engineered to more effectively treat these confounding tissues [51].

The efficacy of antimiR delivery strategies will not solely hinge on overcoming the aforementioned physiological barriers. As has been shown with various naked antimiRs, tissues (e.g. tumors) with a relatively poor enrichment of antimiRs can still be susceptible to pharmacological effects. Therefore, understanding antimiR delivery at a cellular and biochemical level can greatly inform the development of successful technologies.

Cell Membrane

Regardless of their chemistry or transport vector, antimiRs generally face the same canonical cellular delivery barriers as typical polar macromolecules. Traversing the cell membrane often precedes the interaction between traditional anticancer drugs and their targets. Lipophilic drugs such as doxorubicin can readily enter cells by passive diffusion [52,53], while other chemotherapeutics such as cisplatin, camptothecin, and methotrexate enter cells by hijacking active transporters normally used for physiological substrates [54–56]. AntimiRs cannot capitalize on these modes of intracellular transport as effectively as chemotherapeutics. Size and polarity preclude their ability to diffuse across lipid bilayers [57]. Doxorubicin is smaller than 600 Daltons, while full-length antimiRs can range in size between 6000–8000 Daltons. As a lipophilic drug, doxorubicin has an octanol-water partition coefficient (log P) of 0.92, while the log P for just a single adenosine nucleotide of 2'-O-Me is -4.15 and -4.39 for LNA. Despite their methyl-based modifications these antimiR oligonucleotides are considerably hydrophilic. With charge-neutral backbones, PNAs and PMOs are more lipophilic (PNA adenosine monomer log P is -3.43 and -2.72 for PMO) than nucleotide-based antimiRs; however, they also do not efficiently diffuse across lipid bilayers.

Since the 1990's, both charged and uncharged oligonucleotides have been shown to enter cells via active endocytosis processes [58–62]. The predominant forms of oligonucleotide endocytic entry are through non-specific adsorptive endocytosis and pinocytosis (i.e. fluid-phase endocytosis) [60,63]. Notably, charged oligonucleotides that adsorb to the cell surface are typically endocytosed more efficiently than uncharged oligos, and this uptake can be attenuated by competition with other polyanions, including other oligonucleotides [59,62]. However, Koller *et al.* recently surmised that non-specific electrostatics might not be the only determinant for cellular uptake. They found that entry of PS-modified 2'-O-MOE oligonucleotides into hepatocytes occurred through both productive and non-productive pathways, and competition with polyanions seemingly only perturbed the non-productive pathway. Additionally, they demonstrated that productive endocytic uptake relied on the adaptor protein, AP2M1 [64]. Adsorptive endocytosis of oligonucleotides is saturable, which may be due to occupancy of cell surface binding moieties such as heparin-binding proteins (which have a nanomolar binding affinity for PS oligos) or oligonucleotide-specific receptors [60,65]. At saturating oligo concentrations, pinocytosis becomes the predominant endocytic uptake pathway [63]. For naked oligonucleotides, these uptake modes are generally not efficient, particularly in cell culture settings.

Although there have been several *in vivo* studies in which PS-modified LNAs and antagomiRs have effectively inhibited intracellular miRNAs [11,41,66–68], the cellular uptake pathways for these antimiRs have not been fully elucidated. These LNAs likely enter

cells via endocytosis facilitated by their PS-modifications and there is some evidence that the cholesterol moiety of antagomiRs associates with lipoproteins to facilitate cell entry via lipoprotein receptor-mediated endocytosis [12]. Certain techniques can improve cellular delivery of antimiR oligonucleotides such as experimental manipulations (e.g. gymnosis [69]) or the addition of cell uptake-enhancing agents (e.g. Endo-Porter [70]); however, methods such as these are not readily translatable to *in vivo* antimiR delivery.

One of the main goals of incorporating antimiRs with nanoscale delivery vectors is to facilitate intracellular transport. Liposomes promote cellular uptake of encapsulated agents via multiple possible routes dictated by the lipid composition: cell fusion, adsorption, and endocytosis [17,71]. Cell fusion involves direct mixing of the liposome bilayer with the plasma membrane to release contents of the aqueous core into the cytosol. Liposomes adsorbed on cell surfaces can be destabilized to locally release their payload, which can then be consumed by cells via pinocytosis. However, for both lipid- and polymer-based nanovehicles endocytosis is the predominant mode of cell entry [17,72–76]. Nanovehicles can be engineered to be efficiently endocytosed by cells. The size of the nanostructure has a direct impact on cell uptake pathways. In studies with non-phagocytic B16 melanoma cells, Rejman *et al.* demonstrated that nanoscale beads between 50–500 nm in diameter were endocytosed in a size-dependent manner. Nanoparticles that were smaller than 200 nm utilized clathrin-mediated endocytosis, while larger particles up to 500 nm in diameter used caveolae-dependent processes [75]. Nanovehicle shape can also impact cellular uptake. Gratton *et al.* have shown that rod-like structures may have an uptake advantage over spheres [77]. However, manipulating nanostructure geometry is technically challenging since most liposomes and nanoparticles adopt their spherical shape as a result of the hydrophobic effect and minimization of water-exposed surface area. Lastly, surface charge can have a profound impact on cellular uptake. Most polymer and lipid nanovehicles used in nucleic acid delivery are cationic; this net positive surface charge has been shown to promote cell surface adsorption—as well as condense the nucleic acid payload [75,78,79]. Notably, Jin *et al.* and Su *et al.* have delivered antimiRs *in vitro* using nanoparticles that are derivatized with cationic lysine residues [80,81]. In addition to these physical properties, almost all nanodelivery platforms can be further enhanced with modulating agents that directly facilitate cellular uptake. For the past few decades, liposomes have been coated with functional ligands that aid in cell uptake such as folate, which triggers receptor-mediated endocytosis via the folate receptor [82,83]. The previously mentioned study by Plummer *et al.* utilized liposomes that were coated with RGD, which actively binds to integrins in the tumor neovasculature [47]. Also, our antimiR-delivering nanoparticles were coated with a CPP that specifically enhanced uptake into hard-to-transfect lymphocytes [48].

Current technologies can effectively deliver antimiRs into cells; however, since endocytosis is the principle route of entry for most systems, once in a cell, the next challenge to overcome is escape from endosomes and endolysosomal degradation.

Endosomal Trafficking

Despite the multitude of platforms for delivering antimiRs a complete understanding of how these antimiRs exit endosomes is unclear. In the simplest of summations, there are three possible fates for endocytosed antimiRs: degradation, export out of the cell, and escape from the endosome (Fig. (2)). Both chemical modifications and incorporation into nanovehicles inherently protect antimiRs from nuclease attack. The 2'-hydroxyl group on the ribose ring of RNA is involved in both hydrolysis and ribonuclease cleavage. Protection of the 2'-hydroxyl group in chemically-stabilized RNA oligonucleotides such as 2'-O-Me, 2'-O-MOE, and LNA prevents nuclease degradation. PS modifications also impart nuclease resistance [84]. As nucleic acid analogs, PMOs and PNAs are not natural substrates for

nucleases. Additionally, nanovehicles present a steric barrier between associated antimiRs and nucleases. Although current antimiR delivery platforms generally preclude nuclease degradation, recycling of antimiRs to lysosomes remains an unwanted endocytic fate. Extracellular recycling of endocytosed antimiRs is not well-studied; however, oligonucleotide efflux processes have previously been demonstrated [85], and it has been suggested that oligonucleotides that are associated with certain membrane proteins may be exported outside of cells along with the recycled carrier protein [58]. Although there is some evidence that antimiRs may silence target miRNAs within (or proximal to) endosomes after fusion of antimiR-containing endosomes with miRNA-containing vesicles [27], escape from endosomes is generally the desired fate of antimiRs that intend to interact with cytosolic miRNAs.

Although endocytic trafficking is a highly regulated process, endosome membrane leakiness during vesicle fusion events may present opportunities for leakage and escape of antimiRs [86]. It is not clear if chemically-modified antimiRs such as LNA, 2'-O-Me, and 2'-O-MOE possess active endosome escape capabilities; on the contrary, nanovehicles can be specifically engineered to escape vesicular sequestration. The most well-known—yet highly debated—mechanism whereby nanoparticles can escape endosomes is the proton sponge effect [87]. The sponge hypothesis postulates that polycations with an intrinsically high buffering capacity, such as polyethyleneimine, produce an influx of protons and counter ions during endosome acidification, which in turn leads to osmotic swelling and endosome disruption. Although rupturing endosomes can theoretically facilitate cytosolic delivery, this may also contribute to the crippling toxicities associated with polycation-dense delivery systems [88]. Nanovehicles can also be endowed with pH-sensitive peptides (e.g. HA2 and JTS-1 [89–91]) and polymers (e.g. poly(propylacrylic acid) [92]) that specifically induce endosome membrane fusion or destabilization. As with the proton sponge effect, these agents are triggered by endosomal acidification, but unlike the sponge effect, control over ligand density and the polymer blend ratio can limit cytotoxicity. A strategy for endosome escape that is unique to liposomes involves endomembrane fusion. Hafez *et al.* proposed a fusion mechanism that involves lipid exchange between cationic liposomes and anionic phospholipids comprising the inner leaflet of endosomal membranes [78]. This fusion event is triggered by endosome maturation because of the observation that anionic lipids primarily reside on the cytoplasmic leaflet of the plasma membrane and early endosomes; however, late endosomes are more enriched for anionic lipids on their inner leaflet [93]. Furthermore, liposomes may avoid endolysosomal processing altogether via the process of plasma membrane fusion. This unconventional uptake route is supported by studies Lu *et al.* and Ming *et al.* in which productive intracellular payload delivery occurred via non-endocytic pathways [94,95].

Physiological clearance, cellular uptake, and subsequent endosome escape thwart the effective delivery of naked oligomers and nanovehicles. In addition to these principle barriers, antimiRs face numerous challenges that are unique to microRNA-directed therapeutics.

NON-CANONICAL BARRIERS

Spatial Localization of Target miRNAs

A hindrance to the success of antimiR technologies that is unique to miRNAs is the spatial availability of the miRNA target. It is overly simplistic to envision miRNAs as molecules freely floating around in the intracellular milieu; in actuality, single-stranded miRNAs associate with numerous cellular components such as P-bodies, GW-bodies, and even exosomes (Fig. (2)). P-bodies (cytoplasmic) and GW-bodies (residing near endosomes and multivesicular bodies) are foci comprising mRNAs, miRNAs, and specific proteins that

contribute to the silencing and degradation of mRNAs. Much of the machinery (including Argonaute proteins) required for miRNA function is found within these aggregates [96–98], and miRNAs are thought to congregate and localize in P-bodies and GW-bodies following binding to mRNA [27]. Also, Gibbings *et al.* showed that GW-bodies are enriched for the protein, GW182, which may function as a “temporal lock” that occludes association of miRNA-loaded AGO with other miRNAs [99]. So miRNAs associated with P-bodies and GW-bodies might not be sterically accessible to antimiR binding. Several studies have mapped the possible association of various oligonucleotides with P-bodies. Both Krutzfeldt *et al.* and Koller *et al.* showed that antagomiRs and 2'-O-MOE oligos do not co-localize with P-bodies [64]. On the contrary, Stein *et al.* observed LNA-based antisense oligonucleotides within P-bodies [100]; however, it is unclear if this localization was specific to the cell line, oligonucleotide composition, or cell culture delivery method (i.e. gymnosins). Consistent with the notion that miRNAs within P-bodies and GW-bodies are mRNA-bound, it seems more reasonable that antimiRs bind to miRNAs in upstream or downstream events.

Recent studies have shown that miRNAs can be sequestered within exosomes (Fig. (2)) [101–103]. Exosomes are secreted vesicles of cellular origin that have been proposed to perform a variety of biological functions namely involving the transfer of genetic information between cells [102]. Various cancer-associated miRNAs have been found within exosomes. Ohshima *et al.* identified enrichment of the let-7 miRNA family within exosomes of a gastric cancer cell line; they posited that these tumor suppressive miRNAs are jettisoned into the extracellular environment via exosomes in order to maintain an oncogenic state [104]. Oncogenic miRNAs have also been found within exosomes; Umezu *et al.* showed that K562 leukemia cells secrete exosomes enriched with miR-92a, which is an established effector of oncogenesis [105]. Exosome-associated miRNAs are considered promising cancer biomarkers [106]; in fact, malignant cells can excrete exosomes enriched with different miRNA compositions than non-malignant cells [103]. As their exact functions are elucidated, exosome-associated miRNAs may also prove to be useful therapeutic targets. Unfortunately, these miRNAs may be inaccessible to antimiR delivered using current technologies. In addition to providing a vesicular barrier, exosomes that contain miRNAs may reside in the extracellular environment and even in the bloodstream [102,107].

Furthermore, miRNAs have also been shown to localize in the nucleus and mitochondria [108,109]. All of these factors challenge the paradigm of targeting anticancer antimiRs strictly to the cytosol of tumor cells by presenting a window of spatial and temporal miRNA accessibility.

Caveats of miRNA Inhibition

As previously mentioned, inhibition of disease-associated miRNAs with antimiRs is a promising therapeutic strategy that has garnered much excitement over the past few years. However, there are some inherent limitations of antimiR-based therapy that must be considered. Some antimiRs have been shown to elicit degradation of targeted miRNA [110], but most antimiRs simply bind complementary miRNAs with high affinity and form stable duplexes that occlude downstream mRNA silencing. Unless there is a melting or degradation process, most antimiRs function in a one-to-one fashion [111]. Given that cancers can express thousands of copies of a single oncomiR per cell, achieving pharmacological effects from miRNA inhibition may require high antimiR doses. Gapmer antimiRs have been designed in an effort to overcome this stoichiometric limitation. Oligonucleotides with fully modified 2'-hydroxyl groups typically cannot elicit RNase H degradation of hybridized RNA; however, so-called gapmers are chemically modified oligos

that contain stretches of deoxyribonucleotides (with or without PS modifications) that can induce RNase H activity [112]. Gapmers can trigger degradation of mRNA [113], but have seen limited success in the inhibition of miRNAs, which may be due to steric inaccessibility of RISC-bound miRNA to RNase H [9]. Due to this, gapmers may be more successful in targeting pri- or pre-miRNA than mature miRNA molecules.

Another limitation of miRNA inhibition is the transient nature of inhibiting epigenetic effector molecules that are continually expressed by cells. Coupled with stoichiometric limitations, a cell depleted of a specific miRNA can continue to produce more and eventually saturate the delivered antimiRs. Related therapeutic strategies (e.g. Imatinib) that attenuate expressed products like tyrosine kinases have seen success due to proper dosing and the crippling nature of inhibiting the intended target. Similarly, selection of lynchpin miRNAs will be critical for successful therapy. Cancer cells can become addicted to key miRNAs such that inhibition of the miRNA will result in cell death or reversion of the disease phenotype [114,115]. MicroRNAs also can work in concert with other therapeutics [116,117]. Due to high loading capacity, liposomes and polymer nanoparticles are potential delivery systems for combining antimiRs with small molecule or macromolecule drugs. Additionally, these nanosystems can aid in overcoming the transient nature of miRNA inhibition through controlled release of encapsulated agents gradually over time [118]. In particular, biodegradable polymer nanoparticles have been shown to exhibit sustained release of antimiR PNAs and PMOs—essentially acting as an antimiR reservoir [119]. These transient issues can also be alleviated using expression vectors for miRNA inhibitors such as miRNA sponges, miRZips, and tough decoy RNAs that can stably integrate into the genome [120–122]. However, these virus-based systems must overcome numerous safety hurdles that are avoided by most synthetic antimiR platforms [123]. Ultimately, dosing and target selection will be paramount factors that determine the efficacy of antimiR therapeutics.

Downstream Impacts of miRNA Inhibition

When inhibiting miRNAs, one must consider the downstream impacts of this attenuation and the resultant loss-of-function phenotypes. A single miRNA can downregulate numerous target genes with various biological functions. The function of miRNAs can be context-dependent; in fact, some miRNAs can function as both an oncogene and tumor suppressor. In aggressive CLL, miR-29 has been identified as a tumor suppressor that downregulates the expression of the oncogene, TCL1 [124]. On the contrary, in various cancers such as indolent CLL, acute myeloid leukemia, and breast cancer, miR-29 is overexpressed and has been implicated as an oncogene [124]. Various miRNAs are also involved in feedback loops. The oncogenic miR-17~92 cluster is a component of a multi-level negative feedback circuit in which miR-17~92 downregulates Myc and E2F, while Myc and E2F induce expression of miR-17~92 [125]. Therefore, inhibition of miR-17~92 may upregulate Myc and E2F, which ultimately can yield higher expression levels of the miR-17~92 oncomiR cluster. In antimiR therapeutics, it is standard practice to identify and evaluate the direct gene targets affected by the miRNA. However, broader analysis is also prudent. The process of blocking miRNAs with high affinity antimiRs may occlude the endogenous RNAi machinery (e.g. miRNA-bound to RISC) and produce global cellular impacts on un-targeted RNAi processes [126]. Also, some nanoscale delivery vectors have been identified to induce unintentional genetic changes by themselves [127,128], which can certainly be detrimental when trying to affect specific processes regulated by a target miRNA. As antimiR systems are further developed in the future, toxicogenomics and global expression analysis should be noted forms of characterization and safety profiling.

Caveats of AntimiR Technologies

As with any therapeutic, the antimiR delivery strategies detailed here all have limitations intrinsic to their chemistry or material composition. In general, cationic liposomes and nanoparticles can have unfavorable biodistributions *in vivo* and can be toxic to cells [88,129,130]. These problems can be alleviated by shielding or limiting the excess positive charge (e.g. PEGylated liposomes [17]). Charge-neutral PNA and PMO are typically rapidly cleared by renal excretion and do not enter cells as effectively as anionic oligonucleotides [32]. Conjugating PNAs and PMOs to functional ligands (e.g. cell penetrating moieties) can improve delivery [15,131,132]. And, although PS-modified LNA, 2'-O-Me, and 2'-O-MOE are currently the most developed and utilized antimiR technologies, they exhibit a reliance on serum and cellular proteins that prompts detailed mechanistic investigation [32]. It is possible to over-engineer these systems: coating nanovehicles with too much PEG can prevent cell uptake and drug release; conjugating ligands to charge-neutral oligomers can decrease binding affinity and specificity; and adding PS modifications to anionic oligonucleotides can also decrease binding efficacy (e.g. each PS-modification imparts a slight decrease in the relative binding affinity). Regardless, through meticulous engineering and optimization, all of these systems have demonstrated success in inhibiting miRNAs.

CONCLUSION

As a class of anticancer drugs, antimiRs must overcome the numerous roadblocks to miRNA-based tumor therapeutics. This arduous undertaking involves canonical physiological pharmacokinetic and cellular uptake barriers, as well as non-canonical barriers such as intracellular miRNA localization and trafficking, off-target toxicities, and other intrinsic limitations. Furthermore, additional hurdles are likely to emerge as antimiR technologies develop. The field appears up to the task. Given the heterogeneity of cancer, there may be no so-called magic bullet; however, as our understanding of miRNA and cancer biology continues to evolve, it is not too much of a stretch to proclaim miRNAs as magic targets. In fact, delivery of synthetic miRNAs and miRNA precursors (that function as tumor suppressors) is another promising anticancer approach that is hindered by many of the same barriers facing antimiRs. Ultimate clinical success in the field of miRNA therapeutics will truly rely on the convergence of basic and translational research.

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ABBREVIATIONS

2'-O-Me	RNA with methylated 2'-hydroxyl group
2'-O-MOE	RNA with methoxyethyl-modified 2'-hydroxyl group
CLL	chronic lymphocytic leukemia
CPP	cell-penetrating peptide
EPR	enhanced permeability and retention
miRNA	microRNA
mRNA	messenger RNA
oncomiR	oncogenic microRNA
LNA	locked nucleic acid

Log P	octanol-water partition coefficient
PEG	polyethylene glycol
PMO	phosphorodiamidate morpholino oligomer
PNA	peptide nucleic acid
PS	phosphorothioate
siRNA	small interfering RNA

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Fig. (1).

Physiological fates of systemically administered antimiRs. Chemically-modified antimiRs are generally removed from circulation by renal excretion and RES clearance; interaction with certain serum proteins leads to avoidance of these barriers, e.g. binding to albumin can prevent renal excretion. Nanovehicles that deliver antimiRs are typically removed from circulation by opsonization and subsequent RES clearance; this can be prevented by certain functional modifications, e.g. PEGylation obstructs recognition by the RES system. Although albumin binding and PEGylation improve circulation, it is unclear if these modifications directly facilitate tumor targeting. However, tumors are characterized by a leaky vasculature and poor lymphatic drainage, which facilitates passive accumulation of macromolecules and nanovehicles.

Fig. (2). Cellular uptake and miRNA availability. AntimiR oligos and nanovehicles typically enter cells via endocytosis, which principally results in endolysosomal degradation or recycling. Therefore, escape from endocytic vesicles is necessary to inhibit cytosolic miRNAs. However, target accessibility may be obstructed by miRNA association with P-bodies and GW-bodies, as well as sequestration of miRNAs within cell-derived vesicles, such as exosomes.

Table 1Barriers to antimiR therapeutics for inhibiting oncomiRs *in vivo*

AntimiR Delivery Barriers		Measures Taken to Overcome Delivery Barriers	
		AntimiR Oligos	Nanovehicles
Canonical	Instability in serum (i.e. nuclease degradation)	Stability-enhancing chemical modifications ^{a,b}	Association with nanovehicles obstructs nuclease interactions ^c
	Renal filtration	Binding to serum proteins ^d	Size-based exclusion ^e
	RES clearance (typically in the liver)	Binding to serum proteins ^d	PEGylation to prevent RES recognition ^f
	Extravastion and accumulation in tumors	Unclear	Size-based utilization of the EPR effect ^g
	Cellular uptake	Modification with cell uptake-enhancing moieties ^{h,i}	
	Endosome trafficking and escape	Escape from leaky vesicles ^j	Escape via vesicle destabilization ^{k,l}
Non-Canonical	Inaccessibility of free single-stranded miRNA (e.g. associated with P-bodies or GW-bodies)	Administration when miRNA is sterically accessible	
	Localization of miRNA in vesicles or organelles (e.g. exosomes, nucleus, and mitochondria)		
	One-to-one antimiR binding stoichiometry	Administration of sufficiently high antimiR dose	
	Transient nature of miRNA inhibition	Successive antimiR administrations	Gradual and sustained release of antimiRs
	Off-target effects of miRNA inhibition and antimiRs	Proper miRNA target selection and safety profiling	

^a[9];^b[10];^c[33];^d[32];^e[34];^f[40];^g[43];^h[12];ⁱ[48];^j[86];^k[87];^l[89]