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Antisense DNA and RNA agents against picornaviruses

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1. ABSTRACT

Anti-picornaviral antisense agents are part of a broader group of nucleic acid-based molecules developed for sequence-specific inhibition of translation and/or transcription of the target sequence through induced nuclease activity or physical hindrance. Three types of nucleic acid-based gene silencing molecules can be distinguished, including DNA-base antisense oligonucleotides (ASO), nucleic acid enzymes (ribozyme and DNAzyme) and double-stranded small interfering RNA (siRNA or microRNA). These antisense DNA and RNA molecules have been widely studied for gene functional studies and therapeutic purposes. In this review, we focus on drug development using ASO and siRNA strategies to inhibit picornavirus infections. The picornavirus genome organization and life cycle is described, followed by discussion of design considerations, chemical modifications and drug delivery approaches. Recent studies using antisense against picornavirus are reviewed. Finally, we compare the advantages and disadvantages of the antisense agents with those of other therapeutics, taking into consideration their limitations which need to be overcome to achieve the final goal of clinical application.

2. INTRODUCTION

Antiviral antisense drugs, for the purposes of this review, will broadly refer to any nucleic acid oligomer, chemically modified nucleic acid oligomer, or nucleic acid structural analog oligomer that has a base pair sequence complementary to any part of a viral genome or host genome such that expression of the target gene (or an element (s) regulated by the target) is inhibited by the interaction of the target and drug. In particular, the two most prominently used classes of antisense agents, single stranded DNA-based antisense oligonucleotides (ASO) and double stranded small interfering RNA, will be discussed. A third class, nucleic acid enzymes such as DNAzymes and ribozymes, will not be addressed, though in the past some have been tested for antiviral capacity against foot-and-mouth disease virus (FMDV) (1). This review will summarize the use of antiviral antisense drugs designed, studied or applied against viruses from the family *Picornaviridae* in various experimental systems including cultured cells, animal models and humans.

Antisense drugs targeting picornaviruses are a sensible therapeutic strategy to pursue, since the genome of

all picornaviruses consists of a single strand of RNA, the fundamental target of antisense technology, which necessarily must be translated and replicated to propagate the virus. The other major field of antisense drug development is that of anticancer drugs, which typically downregulate oncogenes, growth genes, or inhibitors of apoptosis. However because these genes are typically mutant forms of existing wild type genes, it may be challenging to untangle which antisense candidates do or do not have side effects on normal cells. Viruses, on the other hand, are exogenous agents with foreign genome targets, meaning that the goal of antiviral antisense is to downregulate viral RNA expression to the maximal extent possible.

Most antiviral antisense compounds are being developed with the eventual hope of using them as therapeutic agents in infected individuals to attenuate or clear the infection before it can damage target organs or systems. Although a majority of studies demonstrate the protective effects of antivirals when applied before infection, numerous studies have also shown that antisense antivirals are capable of blocking or attenuating viral replication if applied after viral infection (i.e., after the virus has entered the cell). However, more study is needed to determine how soon an antisense drug must be applied after infection for it to have therapeutic activity. This is likely complicated by the variety of individual responses to both virus and drug, and may be complicated by the extent of the immune response, how rapidly the virus is able to spread in an individual, and how quickly the infection progresses.

Overall, progress in clinical trials of anti-picornaviral antisense has been very limited. Due to the long and stringent process of testing drugs, many tests are still being conducted with first and second generation DNA ASOs (reviewed in (2)). However, a vast majority of preclinical work within the past five years has focused almost exclusively on 3rd generation ASOs or siRNA, which are more consistently effective. To date, the only antisense agent to be approved and distributed as a therapeutic is Vitravene (ISIS2922) for cytomegalovirus-induced retinitis; this oligomer targets the viral IE2 gene (3). In this article, we will review the principles and progress of nucleic acid-based drug development, focusing on ASOs and siRNAs, for the treatment of picornaviral infections.

3. PICORNAVIRUSES

Viruses in the family *Picornaviridae* infect humans and other mammals, and cause a variety of diseases that in some cases can lead to disability or death. Within *Picornaviridae*, nine genera have been established including Rhinovirus, Enterovirus, Cardiovirus, Hepatitis virus, Aphthovirus, Parechovirus, Erbovirus, Kobuvirus and Teschovirus. The first five genera have been widely studied. The most common human viral infections are caused by rhinoviruses which infect the upper respiratory tract and cause the common cold; enteroviruses, which typically are transmitted through the fecal-oral route and enter via the intestinal mucosa, are the second most common of all human viral infections and consist of several highly similar viruses such as coxsackieviruses, poliovirus, echoviruses, and human enteroviruses. Although most picornaviral infections result in mild transient symptoms such as fever, complications can arise making this group a dangerous subset of viruses. Poliovirus is perhaps the most infamous picornavirus, due to its ability to infect the motor neurons and irreversibly paralyze its host. Both coxsackievirus and echovirus commonly cause aseptic meningitis and myocarditis leading to sudden cardiac failure in children. Hepatitits A (HAV) virus mainly infects the liver and causes human hepatitis. Foot-and-mouth disease virus of the genus Aphthovirus can rapidly spread through livestock populations and has resulted in mass cullings in recent years. Cardiovirus contains two species: encephalomyocarditis virus (EMCV) and Theilovirus, which are closely related to aphthoviruses. The remaining four genera have been less studied.

Picornaviruses are non-enveloped, positive sense single-stranded RNA (+ssRNA) viruses. They are icosahedral and approximately 30 nm in size. They share some similarities in gene organization and genome replication mechanisms as other non-enveloped or enveloped +ssRNA viruses such as flaviviruses (e.g. West Nile Virus and hepatitis C), coronaviruses (SARS-CoV) and caliciviruses (e.g., Norwalk Virus). Antisense-based strategies against picornaviruses may be useful against other viruses with a +ssRNA genome, many of which undergo similar translational regulation and posttranslational processing. Antisense drug development, using both DNA and RNA-based therapeutics, is currently very active against hepatitis C virus (4) and SARS-CoV (5, 6). The progress made against these viruses has provided some insight into antisense agents against picornavirus.

3.1. Genome and Proteome

The content and organization of the picornavirus genome and proteome are highly conserved between various genera and share many common elements and mechanisms to promote viral replication and viral infection. The entire genome can be directly translated as a single open reading frame into a large polypeptide precursor. This RNA ranges from 7-8 kilobase pairs in size, approximately 10-12% of which are 5' and 3' non-coding or untranslated regions (UTRs). It is clear that the 5'UTR harbors an internal ribosomal entry site (IRES), which regulates the 7mGpppG cap-independent ribosomal internal initiation of translation of picornaviruses (7, 8) and is necessary for viral replication. The 3'UTR contains kissing-pair structures, which facilitate the transcription and translation of viral RNA through interactions with cellular protein factors (9). Thus, these regions are rational targets for antisense drug design.

The immature polyprotein comprises the viral proteome which contains all the structural and non-structural proteins in a relatively conserved order in picornaviruses. The polyprotein is self-cleaved by a *cis*-acting autoproteolytic mechanism at several regions into precursors P1, P2 and P3. In enterovirus, for example, the

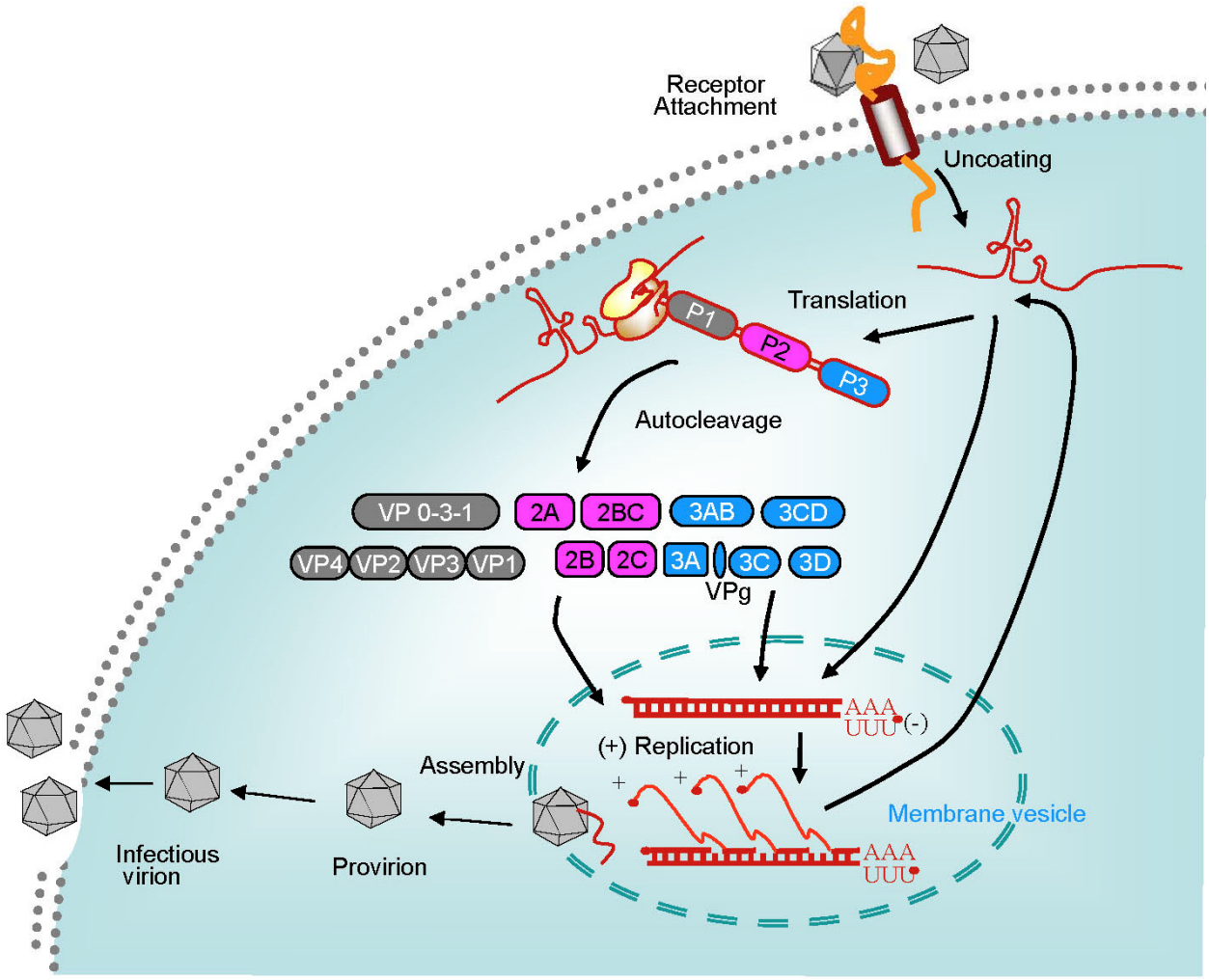

Figure 1. Summary of enterovirus life cycle. Infection begins with virus attachment to its receptor (s), followed by uncoating and release of its RNA genome in the cytoplasm where it is translated by host machinery through a cap-independent mechanism. The resulting polyprotein is processed through cleavage in *cis*, releasing discrete structural (gray) and nonstructural (pink, blue) proteins. The genome is replicated in membrane vesicles by RNA-dependent RNA polymerase 3D, creating a negative strand intermediate template, which is then used for positive strand replication through the same mechanism. The positive RNA genome is packaged into the assembled capsid to form provirion and released upon maturation by cell lysis.

three precursors are further cleaved by 2A and 3C proteases to produce individual proteins: P1 is processed into structural proteins VP4, VP2, VP3 and VP1 that make up the viral capsid (coat) (for certain picornaviruses, a polypeptide called L protein is located immediately preceding the P1 region); P2 and P3 are processed into nonstructural proteins including protease 2A, viroporin 2B, NTPase 2C, 3A, RNA replication primer 3B (uridylated VPg peptide (10)), protease 3C, and RNA polymerase 3D (11, 12) In addition, the polyprotein is processed into intermediates with unique roles. For example, the 3CD peptide is a precursor containing the 3C and 3D amino acid sequences, and is believed to cleave other viral peptide precursors in their final discrete forms. Another intermediate precursor is 3AB, which binds to 3D RNA polymerase and stimulates its activity (13).Viral proteases are also responsible for cleaving a multitude of host cellular proteins, causing the shutdown of host cell cap-dependent translation and eventually, cell death and viral particle release. Thus, viral proteases play an important role in viral life cycle (see discussion below) and pathogenesis and are important targets for drug action.

3.2. Viral Life Cycle

Picornaviruses share many common replication mechanisms and have highly similar life cycles (summarized in Figure 1 and Table 1). The first step of an infection is the binding of the receptor. The binding of an enterovirus to its receptor causes a conformational change in the capsid, whereby the VP4 component is released and the VP1 component is uncovered, further facilitating membrane interaction. Many viruses such as FMDV (14) and rhinovirus type 2 (15) use clathrin-mediated endocytosis while others such as some Coxsackie B viruses

Table 1. Summary of common picornaviruses and their associated targets and mechanisms of entry

Genus	Species	Receptor	Entry	Target	Drugs
Enterovirus	Poliovirus	Poliovirus receptor (PVR) CD155 (114)	Actin & tyrosine kinase dependent endocytosis (17)	Gastrointestinal, route to primary motor neurons; lymph nodes	Vaccine
	Coxsackievirus	Coxsackie and adenovirus receptor (CAR) (115); decay accelerating factor (DAF) (116); ICAM-1	Caveolin-mediated endocytosis (16)	Gastrointestinal route to heart, pancreas, meninges	None
Rhinovirus	Rhinovirus A, B	ICAM-1 (CD54) (117), LDLR	Clathrin-dependent endocytosis	Upper respiratory epithelium	Pleconaril (118)
Hepatovirus	Hepatitis A virus (HAV)	HAVcr-1/T-cell immunoglobin mucin 1 (119)	Uncharacterized	Gastrointestinal route to Liver	Vaccine
Cardiovirus	Encephalo- myocarditis virus (EMCV)	Vascular cell adhesion molecule (120), CD106	Uncharacterized	Brain, heart, pancreas	None
Aphthovirus	Foot-and-mouth-disease virus (FMDV)	Integrins (α,β family) (121)	Clathrin-mediated endocytosis (14)	Hoof, mouth & tongue; lymph nodes	Vaccine (122)

require caveolin and not clathrin (16), and poliovirus does not require either but is dependent on actin and tyrosine kinase activity (17). Once inside, the virus is trafficked through the early endosome pathway and uncoats. The sites of replication for picornaviruses are membranous vesicles in the cytoplasm. Because they are positive, single-stranded RNA viruses, the host cell translation machinery can immediately bind and translate the full proteome.

Picornaviruses can shut down translation of host cellular proteins in the very early stages of infection, favoring their own gene translation by host machinery. The common strategy employed by picornaviruses is the cleavage of eukaryotic cell translation initiation factor 4GI (eIF4GI) (a cap-binding protein) by viral proteases to prevent the cap-dependent translation initiation complex from forming. Since picornaviral translation depends on the IRES present in the 5'UTR of the genome but not on the cap structure, cleavage of eIF4GI does not hinder viral RNA translation. Picornaviruses can also inhibit host cell gene expression through cleavage of a number of cellular transcription initiation actors and activators (18) (19)

In addition to polypeptide translation, picornaviruses must also copy their RNA genome to fully replicate. Replication occurs inside replication complexes, which consist of viral nonstructural proteins inside membranous vesicles formed by viral protein intermediate 2BC (20). The mature 3D polymerase first uridylates the VPg leader protein using the *oril cis*-acting RNA element (in Polio, a cloverleaf in the coding region of 2C), which in turn serves as a primer for 3D synthesis of the picornavirus negative RNA strand, based on the positive RNA genome as a template. The genome temporarily exists as a double-stranded intermediate, which may trigger the host cell dsRNA defense response. Negative strands are then primed with urdiylated VPg and used as templates in the synthesis of positive strand genomes by 3D, which are then packaged into assembled virions consisting of structural proteins.

Mature viruses are released upon the death of the cell, which typically occurs after extensive stress from viral proteases which activate cell death cascades during the replication process. This begins as early as six hours postinfection. The viral protein 2B (21) also degrades the membrane, destabilizing ion gradients and facilitating mature virion release from dead cells whereby new cells may be infected.

4. DNA ANTISENSE OLIGONUCLEOTIDES

DNA ASOs silence gene translation by binding to their target complementary single stranded mRNA sequence and preventing its translation in one of two ways. First, a DNA ASO can hybridize to its target and recruit endogenous cellular ribonuclease H which specifically degrades the RNA strand of RNA-DNA heteroduplexes. The DNA ASO is then freed to bind another copy of that RNA target, allowing the effect to be cumulative over time. While a majority of RNAse H is found in the nucleus where it plays a role in DNA replication, the enzyme has also been found in the cytoplasm, presumably from nuclear leakage or newly synthesized RNAse H translated by cytoplasmic machinery (22, 23). Secondly, DNA-analogue ASOs or DNA ASOs with modified chemistries (see below) have DNA-RNA interaction geometries that are too atypical for RNAse H to recognize. However, they can still effectively compete for translation machinery by binding to start codons or positive regulatory elements like the viral IRES. This blockage has a 1:1 stoichiometry which is not cumulative over time. Though ASOs predominantly silence gene expression at the translation level, gene transcription is also affected in certain conditions. Most notably, non-essential exons bearing premature stop mutations can be skipped by interfering with mRNA splicing at exon-intron junctions using ASOs directed to splicing sites. This approach has perhaps been most successful in the study of muscular dystrophy, where ASOs targeting splice sites flanking exon 23 are among the most promising therapeutics for the disease and are currently being tested in humans (24).

Since the viral genome is a single-stranded RNA molecule, it is directly affected by antisense-mediated downregulation by the same mechanism as normal cellular RNAs. In cultured cells transfected with RNAse H-recruiting DNA ASOs, maximal degradation of target RNA occurred at 8 hours posttransfection, with RNA levels returning to baseline during the 48-96 hour period (25). The most effective targets of DNA ASOs and DNA-

analogue ASOs are the untranslated regulatory regions of the genome such as the 5'UTR, or the viral initiation codon AUG region (26). Since certain chemically modified ASOs and DNA-analogue ASOs cannot recruit RNAse H they are only effective as translation initiation blockers at the IRES or start codon.

Choosing a target for ASOs is typically based on knowledge about functional sites (for example, the AUG start codon is the functional location necessary for correct translation). These sites, and areas around these sites, can be predicted by various software modeling programs such as AOBase (27) (http://www.bioit.org.cn/ao/aobase) and sfold (28) http://sfold.wadsworth.org , although the efficacy of ASO candidates is best evaluated empirically. As expected, the presence of highly ordered secondary structure in many viral RNA genomes makes it difficult for complimentary ASO sequences to bind and affect gene translation (25); however it is also true that a high affinity ASO can still overcome secondary structure and achieve some knockdown effect (29). Chemical modifications (see below) that affect affinity will also affect the ability of the ASO to overcome secondary structure. DNA ASOs that mediate RNAse H-dependent degradation are very effective against the IRES or start codon in FMDV and CVB3 (30, 31).

5. SMALL INTERFERING RNA

Small interfering RNAs (siRNAs) are short double-stranded RNA molecules that participate in a natural cellular posttranscriptional silencing mechanism called RNA interference (RNAi). This phenomenon was first seen in plants and later in invertebrates, and is believed to be an innate reaction to the foreign double-stranded RNA (dsRNA) structures that result from viral infection. dsRNA is formed transiently during viral genome replication and also exists in conserved RNA secondary structures such as hairpin loops. Viral RNA can be cleaved by the Dicer family of RNase III-like enzymes into viRNAs of 21-28 nts (reviewed in (32)). A multimeric complex of argonaute family nucleases called RNA-induced silencing complex (RISC) incorporates the antisense strand (strand complementary to an RNA target) of the siRNA and degrades mRNA sequences to which it is complementary (33).

In mammals, dsRNA longer than 30 nts triggers broad antiviral defenses including the release of interferon and activation of the RNase L and protein kinase PKR pathways, which lead to a global inhibition of mRNA translation and apoptosis (34). No viRNAs have been identified in mammalian systems, but the RISC RNAi machinery can still be recruited through the application of exogenous pre-synthesized dsRNA 21mers siRNA containing an antisense strand complimentary to the viral genome.

Mammalian cells also recruit the RNAi pathway using another group of small RNA molecules called microRNA (miRNA). miRNAs are processed from endogenous non-coding RNA sequences (primRNA), some of which are complimentary to viral RNA sequences. Their maturation starts in the nucleus, with cleavage by the RNase III endonuclease Drosha, into 60-70 nt stem loop intermediates with 5' phosphate and 2 nt 3' overhangs. After transportation into the cytoplasm, this pre-miRNA containing a short hairpin structure is further processed by Dicer and incorporated into the RISC to inhibit mRNA expression through mechanisms similar to siRNA (35), but while siRNAs act via target degradation, miRNAs act mainly through translation repression of target mRNA due to partial complementarity to the message (36, 37). To date, more than 500 human miRNAs have been characterized, though bioinformatic analysis predicts that there could be over 1000 (38, 39). It is estimated that as many as 30% of all protein-coding genes are regulated by miRNAs, either in a positive or a negative way (40). Thus miRNAs play an important role in human disease development. A number studies have indicated that miRNAs can be used not only as effective agents for therapy but also as targets for antisense action. Currently, a majority of studies are focused on cancer-related miRNA characterization (37, 41) and some other areas. For viral infections, investigations mainly focus on HIV (42), HCV (43) and some large DNA viruses such as herpes virus, (44). We have not seen such reports on picornaviruses thus far.

While interest in the traditional DNA-based antiviral antisense field has slowed in recent years, siRNAs have been developing rapidly in parallel. In many cases, RNAi has replaced DNA-based antisense as the gene silencing tool of choice in a majority of biological systems. In 2002, siRNAs were first tested as antiviral agents against the coding region of poliovirus (45); this study opened the door for successful siRNA silencing of many other picornavirus genes. Both structural and nonstructural proteins appear to be effective antiviral targets. The theoretical design of siRNAs has been thoroughly explored (46). Briefly, up to eight parameters influence efficacy, such as GC-content, lack of inverted repeats, and sense-strand integrity at specific bases called the seed region. These can now be computed by predictive software; various such programs are available online – for example, a recently developed SiVirus (http://siVirus.RNAi.jp), which specifically designs antiviral siRNA (47).

In cultured cells transfected with siRNA, maximal knockdown effect is typically achieved 20-30 hours slower than DNA ASOs, usually between 24-48 hours posttransfection (25). The duration of the siRNA effect is believed to depend significantly on the dilution of the siRNA through cell division – for example, in dividing cancer cell lines, silencing lasts on the order of days, whereas in nondividing fibroblasts a target may be silenced for weeks (48). In animal systems, siRNA confers antiviral effect as long as it remains detectable in the animal system (49); this length can depend on the method and efficiency of delivery, and again is more stable in nondividing differentiated cells than in actively dividing tumor cells (48). Like ASOs, siRNAs are also sensitive to secondary structure in their mRNA targets, and there is a linear inverse relationship between siRNA efficacy and free energy contained in secondary structure bonds.

Table 2. Summary of common nucleic acid chemical modifications in antisense agents

Modification/analog	Conformation	Stability	Affinity	Efficacy	Test Progress
Phosphorothioate	Chiral B-DNA (123, 124)	Nuclease resistant	↓ Affinity	RNAse H recruiting	Picornavirus *in vitro*, *in vivo (26)*; HCV in clinical trials (125)
Ribose 2' OMe or alkyl; an electronegative group at the 2' position is critical for function	C3'-endo polymorphism (126)	Exo, endo-nuclease repellant (steric) ↑thermo stability (↑ hydration in minor groove) (126)	↑RNA affinity (↑ hydration in minor groove)	Steric block only, no RNAse H recruiting	Picornavirus *in vitro*; cancer, TNF-α in clinical trials
Locked nucleic acid	Rigid C3'-endo (127)	Highly stable	High affinity	High; chimeras can activate RNAse H (128)	HCV *in vivo (*58)
Peptide nucleic acid	Rigid P-form helix (129)	Not recognized by nucleases or peptidases (129)	Extremely high; PNA>DNA>RNA; will displace a DNA duplex and/or form triplex (129)	High; steric hindrance and disruption of secondary structure	HIV *in vitro (*61)
Phosphorodiamidate Morpholino	Unknown	Not recognized by nucleases	↑RNA affinity	No RNAse H recruiting; steric block of translation	Picornavirus *in vitro, in vivo*

siRNA antiviral strategies have had considerable success in animal models, effectively inhibiting coxsackievirus B3 (CVB3) (49), FMDV (50), SARS coronavirus (6), RSV (51), herpes virus (52) and others. To our knowledge, there are no ongoing clinical trials of siRNA agents against picornaviruses.

6. CHEMICAL MODIFICATIONS OF ANTISENSE

Both DNA ASOs and siRNA are highly susceptible to degradation before they are able to affect their target sequence. To protect ASOs, chemical modifications have been made to various parts of the nucleotide. A summary of the commonly used modifications are listed in Table 2 and illustrated in Figure 2, and the biochemical impact of these and other modifications are extensively reviewed elsewhere (53). Each modification is intended to increase one or more of the compound's nuclease resistance, thermal stability, target specificity, sequence affinity and cellular uptake, although a modification designed to improve one of these factors may harm another.

The first generation of ASOs has a modified backbone that confounds the recognition of the standard phosphodiester linkage by nucleases, but maintains the overall activity of an unmodified ASO. The phosphorothioate linkage, replacing a non-bridging oxygen with a sulfur atom, achieved this effect. The second generation of ASOs has an additional alkyl moiety to the 2' carbon of the ribose sugar ring to increase conformational stability and target affinity, and block the backbone from nuclease attack. The moiety (2'R) can vary in length and contain other bonds or charges; the chemical properties of more than 20 possible configurations of 2'R have been examined. A third generation of ASOs has significantly altered nucleotide compositions. The most extensively tested 3rd generation ASOs against picornaviruses are those of the morpholino group, which have a morpholine ring in place of the deoxyribose sugar. Due to many industry-academia collaborations established by AVI-Biopharma, a proprietary antisense morpholino with phosphorodiamidate backbone linkages (PMO) has been tested against CVB3 (54) and FMDV (55), as well as other +ssRNA viruses such as SARS-CoV (5), West Nile and Dengue viruses (56).

The inter-study consistency of the PMO ASO chemistry as well as the antiviral testing methodology makes comparative evaluations of these compounds relatively straightforward compared to individual studies using custom-developed ASOs that contain a variable mixture of other ASOs modifications. The morpholino structure cannot be recognized by nucleases, is extremely stable and can maintain antiviral activity in cultured cells days after entering the cell, when the cells themselves die from overconfluence (54). Morpholinos have an increased affinity for RNA compared to natural nucleic acid oligos but cannot induce cellular RNase H activity. Many custom designed antiviral oligos have successfully inhibited viral replication in cells and/or in animals, but none have progressed to clinical trials, likely due to the cytotoxic side effects of the PMO molecule or its associated drug carrier molecule.

Another configuration of third-generation ASOs is the locked nucleic acid (LNA) designed to be conformationally frozen with extremely high affinity for target sequences. Various studies have shown that LNA oligos have high stability, affinity and specificity *in vitro* and *in vivo*, with particular research focus on hepatitis C virus, however in animal systems they are also hepatotoxic (57, 58); reviewed in (59). To our knowledge, ASOs containing the LNA chemistry have not been used against picornaviruses. Peptide nucleic acids (PNA) have abandoned the ring structure altogether in favor of a non-cyclical structure with a peptide backbone. PNA have a neutral charge, which has both advantages and drawbacks; the neutral molecules are not very soluble in water and will therefore tend to form hydrophobic aggregates. Neutrality also prevents them from easily passing through cell membranes, limiting their application as therapeutics in the absence of a carrier molecule. Diffusions studies have shown that both PNA and unmodified DNA ASOs are poorly taken up across the membrane (60). At the same time, the neutrality greatly increases PNA affinity for other nucleic acids because the negatively charged strand is not repulsed by another negatively charged strand. PNA are currently being refined with various delivery methods in order to overcome their limitations and have been used as antiviral agents against HIV (61).

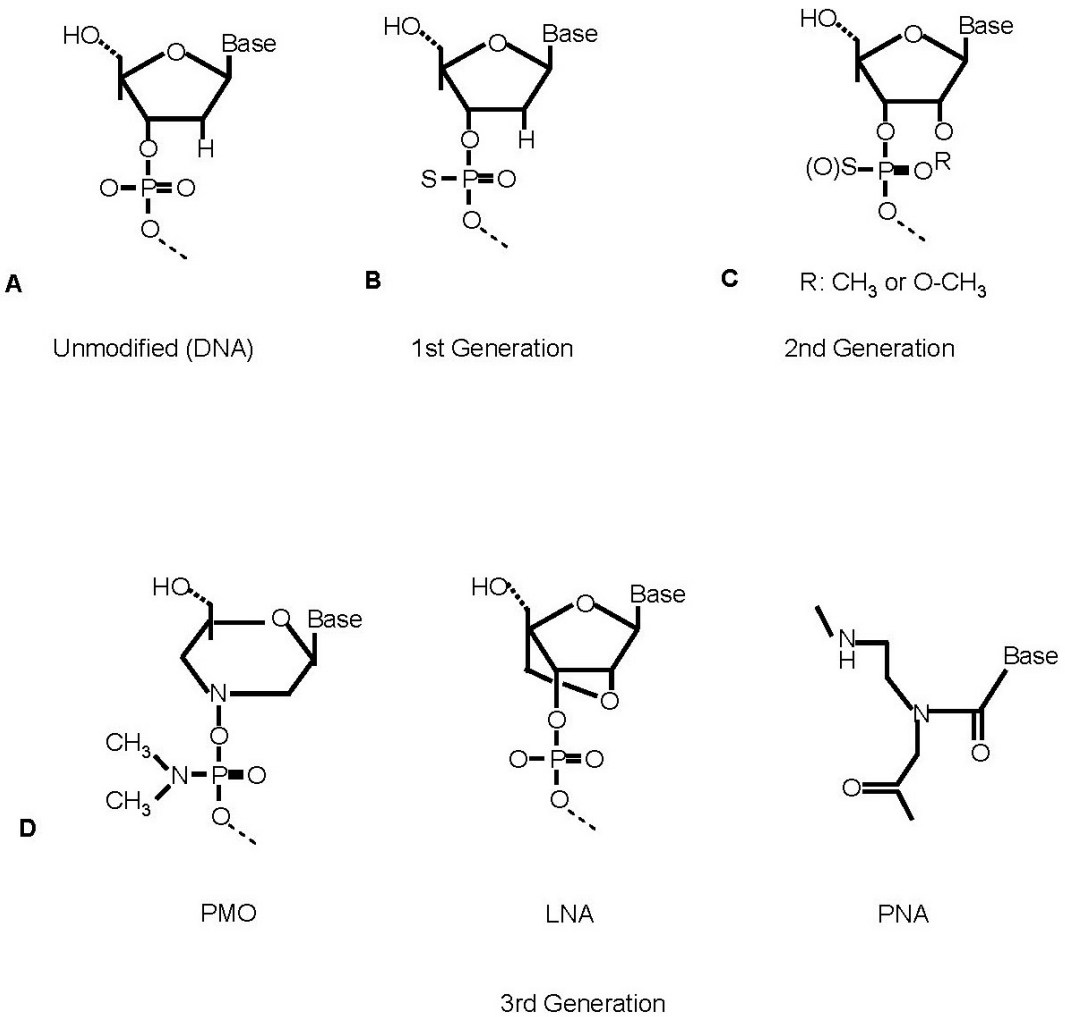

Figure 2. Commonly used nucleotide modifications and nucleotide analogues in antisense oligo synthesis. A, unmodified DNA. B, phosphorothioate modification uses a sulfur atom to evade nuclease recognition. C, 2'-O-Methyl modification sterically hinders nuclease access. (equivalent ribonucleotide modifications are used for siRNA).D, phosphorodiamidate modification with morpholine ring is a neutral nucleotide analogue completely resistant to nuclease degradation. E, locked nucleic acids are locked into the C3 endo conformation and typically replace flanking nucleotides around a core of unmodified DNA in chimeric mixmer oligos. F, peptide nucleic acids are DNA analogues with extremely high affinity for both DNA and RNA and can invade existing genoming strands.

siRNAs have also been engineered with chemical modifications similar to those used in DNA-based ASOs. Since siRNA is rapidly degraded in the bloodstream, both phosphorothioate and 2'O-methyl modifications are used as protection against nuclease attack (62, 63). Because the most important strand in siRNA-RISC activity is the antisense strand (complimentary to the target sequence), the sense strand can be most easily modified without significantly reducing overall efficacy. The maximum amount of chemical modification resulting in the best stability-efficacy tradeoff is best determined empirically and has previously been studied by analyzing siRNA degradation products in human plasma (64).

The mixing or spacing of normal nucleotides with modified nucleotides in ASOs or siRNA is a common strategy to protect the drug at key sites but leave the original biological activity intact. Called mixmer, chimeric, or gapmer ASOs, they are typically stretches of phosphorothioate or regular phosphodiester ssDNA, flanked on both sides by modified bases such as 2nd generation 2'OMe phosphorothioate nucleotides or locked nucleic acids. This confers much of the affinity and protection of the methylated ribose with the RNAse H recruiting activity of PS ASOs, and chimeric siRNA are able to activate the RNAi pathway in cells.

7. ADVANTAGES AND DISADVANTAGES OF ANTISENSE

A number of different strategies have been attempted to develop new drugs to inhibit picornaviral replication. Currently, the only promising candidate for clinical application in humans is pleconaril, a small-

molecule competitor of the antireceptor harbored in the viral VP1 capsid protein for the HRV receptor. Thus this compound can block HRV attachment to cell membrane. Another strategy is the development of viral protease inhibitors since viral proteases play an important role in viral replication and pathogenesis. However, the development of such inhibitors highly depends on the elucidation of picornavirus protease crystal structures, of which only a few are available (65, 66). Another possible approach is to use soluble receptor peptides to directly compete with viral access to the true receptor on the cell surface; however this approach needs the application of the drug prior to infection to successfully block CVB3 entry and replication in mice and prevent viral myocarditis and pancreatitis (67). Still other drugs have focused primarily on amplifying the host immune responses to infection through interferon or similar treatments; these will not be discussed here.

Theoretically, the principal appeal of antisense compounds as drugs is the ability to customize their sequence to a uniquely specific target sequence. Thus, it may achieve a specific downregulation of a given gene. Antisense is expected to form Watson-Crick base pairing only with complementary nucleotide sequences that are an exact match, with diminishing affinity as the base-pairing specificity is lost. Unfortunately, a high degree of specificity is also a downfall: picornaviruses and other RNA viruses often have highly error-prone polymerases which facilitate rapid generation of escape mutants to the sequence-specific antisense treatments (68-70). Several studies outlined in section 9 attempt to address this issue by targeting multiple conserved regions of the viral genome, or targeting the viral receptor of the host.

siRNAs frequently have off-target effects, silencing genes other than the target and potentially interfering with normal host cell processes (reviewed in (64, 71)). Thus when designing novel antisense compounds, it may be most practical to target several regions of the viral gene or gene-regulatory element to be downregulated, and subsequently screen a battery of candidates for the efficacy of the desired knockdown effect compared to the incidence of nonspecific toxic effects. Many companies that offer commercially available siRNA offer a certain subset that has been experimentally validated by the company for off-target effects (Ambion, Dharmacon). DNA ASOs also have nonspecific effects *in vivo*. In particular, early generation phosphorothioate ASOs bind to intracellular proteins, making them unavailable for antisense silencing of the target and interfering with normal cellular processes, with a majority being bound by human replication protein A (RPA). Under certain conditions, phosphorothioate-modified ASOs have up to 300 fold greater binding affinity for proteins with DNA-binding properties, compared to native ssDNA (72). The alkylation of the ribose (the 2'OMe second generation modification) also increases the affinity of DNA oligos for DNA-binding proteins, but significantly attenuates the protein binding of phosphorothioate alone (73). Similarly, 2'OMe-PS-ASO hybrid ASOs have a significantly lower affinity for RPA, and a significantly higher affinity for their target, compared to PS-ASO without ribose methylation (74). When administered systemically, PS-ASOs are fairly stable against degradation in the serum, but can elicit an immune response at therapeutic-level doses. This has led to the termination of several clinical studies requiring systemic application of DNA ASOs.

The toxicity of antisense has been reviewed previously (75, 76). Detailed information about the true toxicity of these compounds is limited, since such toxicity represents negative data with respect to progress in antisense, and are usually underreported compared to positive data. The harmful effects of antisense are dose dependent (77). However, the exact toxic dose is difficult to generalize, because many ASOs are chimeras custom designed to consist of a combination of first, second and third generation chemistries that may contribute different adverse reactions *in vivo* or in clinical trials. More importantly, toxicity is also dependent on the method of delivery (see below). Nearly all recent *in vivo* studies use some forms of delivery or targeting systems and in some cases, the delivery system is more toxic and/or immunogenic than the antisense oligo itself. However, the lack of a specific delivery vehicle may increase antisense uptake in collateral organs, leading to increased nonspecific side effects.

8. DELIVERY SYSTEMS

The dilemma of any antiviral or antimicrobial drug is that greater dosages increase both drug efficacy as well as toxic side effects. Since many ASOs have had undesirable immunogenic or other side effects in clinical trials, other methods of delivering therapeutics need to be explored to lower the effective dose. There are two primary considerations that would improve the efficiency of ASO or siRNA drugs: increased cell uptake and greater target organ specificity. An increase in either is expected to reduce the overall levels of antisense required and therefore reduce side-effects.

Antisense oligos are not efficiently internalized by cells. Even modified oligos applied systemically that avoid degradation and arrive at their target intact are typically unable to pass through cellular membranes without additional assistance of a carrier molecule or other delivery vehicle. Negatively charged DNA or modified DNA ASOs are highly polar, causing a high repulsive force with the hydrophobic region of the lipid bilayer. Recent 3rd generation ASOs such as PMO and PNA have a neutral charge, but they still contain significant polar components, and empirically cross the membrane at approximately the same rate as regular ASOs (60). Similarly, siRNA is inefficiently taken up in some cell populations, but is readily internalized in others. In order to demonstrate proof-in-principle that an ASO or siRNA agent can inhibit viral replication, many studies use reagents such as lipid-based cell transfection reagents or conjugated penetrating peptides to facilitate drug entry into infected cell populations. However, an effective consistent *in vivo* and clinical delivery system combining both cell targeting and cell entry mechanisms is still under development. The

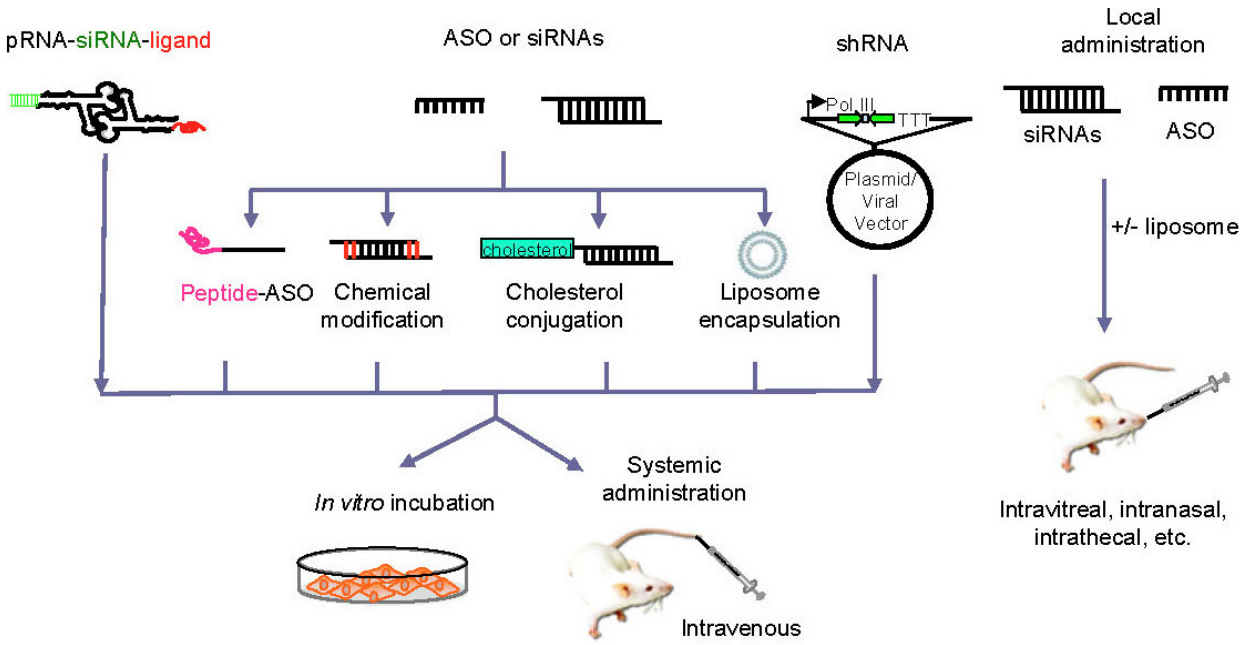

Figure 3. Antisense delivery strategies. Chemically modified or non-modified ASOs or siRNAs are delivered *in vitro* and *in vivo* after liposome encapsulation and/or conjugation with either penetrating peptides or cholesterol. SiRNAs can also be delivered by systemic and local administration of viral vector expressing the shRNA or the pRNA multimers linked with both siRNAs and receptor ligands.

following will briefly discuss several antisense drug delivery systems (Figure 3):

8.1. Liposomes

Based on transfection reagents, lipid-based delivery methods have also been used to transport DNA ASOs and siRNA. Common lipid molecules such as DOTAP N- (1- (2,3-dioleoyloxy)propyl,N,N,N-trimethylammonium chloride) - DOTMA N- (1- (2,3-dioleyloxy)propyl) N,N,N-trimethylammonium chloride and SAINT-2 N-methyl-4 (dioleyl)methylpyridiniumchloride, vary slightly by head-group charge and tail-group organization. The amphipathic lipids, similar to those of a biological membrane, are mixed with antisense solution and form liposomes composed of a bilayer encapsulating the negatively charged DNA or RNA (reviewed in (78)). The interaction between nucleic acid and cationic lipid forms stable lipoplexes resistant to nuclease degradation. Lipoplexes are able to bind with target cells, possibly through negatively charged surface proteins such as proteoglycans; entry is poorly understood, but some liposomes use a clatherin-dependent and caveolin-mediated endocytosis before releasing their antisense contents into the cytoplasm for gene silencing activity.

Lipid-based nanoplexes, containing catioinic polyethylene glycolated-polyethyleneimine (PEI-P) have been used to deliver siRNA. Custom nanoplexes can be designed with peptide ligands such as Arg-Gly-Asp conjugated to the PEG to deliver the nanoplexes to their target (79). Furthermore, PEI is able to form non-covalent interpolylectrolyte complexes with DNA or RNA (80). For this reason PEI with various molecular weights and other modifications has been used as transfection reagents *in vitro* and *in vivo* to establish its efficacy for nucleic acid delivery.

Proprietary liposome technology is being developed in the biotechnology sector as a method for delivering siRNA. Protiva Biotherapeutics (Burnaby, British Columbia) is marketing pre-clinical and clinical grade stable nucleic-acid-lipid particles (SNALPs). SNALPs are spherical lipid bilayer structures approximately 140 nm in size, composed of both cationic and neutral lipids, with an outer shell of PEG-based hydrophobic molecules. These lipoplexes have been used by Sirna therapeutics (Boulder, Colorado) to deliver 2'-O-Me modified siRNAs against Heptatis B Virus (HBV) in mice (81). The Atufect lipid (Atugen, Berlin) has a highly charged head group that can efficiently deliver siRNA in lipoplexes when mixed with uncharged helper lipids. Transfection with Atufect increases cellular uptake of Cy3-labeled siRNAs by approximately 1000-fold and furthermore directs siRNAs to late endosomes, rather than lysosomes where naked siRNAs typically accumulate. Atuplexes also improved biodistribution of labeled siRNA and immunogenic responses (interferon, cytokines) were not detected *in vivo* (82).

8.2. Viral vectors

Viruses themselves have gained significant attention as tools that can be manipulated to deliver a variety of gene therapies – either as a supplement for a deficient or mutant gene in its host, or as short hairpin RNA (shRNA) which will be processed into siRNA and

subsequently silence its target (reviewed in (83)). Perhaps one of the most widely used viral vectors is adenovirus. Adenovirus is capable of transiently delivering a DNA sequence to the nucleus of a variety of dividing and nondividing cell types and its immunogenicity has been greatly improved in newer-generation vectors with deleted early genes (E1-E4). The DNA is transcribed by RNA polymerase II, driven by the CMV promoter, and may code for a single long mRNA which is self complimentary and can fold into shRNA. Furthermore, the coxsackie and adenovirus receptor (CAR) is a common receptor to both coxsackie B viruses and adenoviruses 2 and 5, making adeno-based vectors (Ad5) an appealing choice to specifically target cell populations infected with CVB3. Fechner and colleagues took advantage of this fact by delivering an AdV anti-CAR shRNA to cardiac myocytes, demonstrating that subsequent challenge with CVB3 was blocked (84). The viral genome itself has also been targeted with Ad5-shRNA; in cultured cells and guinea pigs, Ad5-shRNA transduction against FMDV polymerase or structural protein 1D has a significant protective effect (85).

Adeno-associated virus (AAV), a parvovirus that does not cause human disease, is a promising alternative for gene therapies and is likely the safest viral vector since it also has low toxicity and low immunogenicity. Although the AAV vector can only carry up to a 5kb transgene, this is more than sufficient to deliver multiple shRNA sequences. AAV can also be pseudotyped, by packaging the genome of the extensively characterized AAV-2 into the capsid of another AAV serotype. In this way the tropism of the viral vector can be partially customized, since AAVs of different serotypes infect a large range of tissues, with tropism for serotypes 1-8 already characterized in the eye, lung, muscle, liver, pancreas, and CNS (86).

A third major class of shRNA delivery vectors is the Lentivirus, a retrovirus usually derived from HIV-1 or -2. Lentivirus can transduce dividing and non-dividing cells. They possess an intrinsic ability to cross the nuclear membrane and integrate the sequence it is carrying into the genome of its delivery target, resulting in its constitutive expression under the control of the PolIII transcription promoter. This continual expression could remove the need to reapply transient treatments as with Adeno-mediated delivery. However, there is an added risk of insertional mutagenesis leading to cancer. Currently, lentivirus delivery of shRNA typically is not able to exceed 80-85% knockdown (87) if directed against endogenous genes. To date, the main drawback of all viral delivery vectors are their lack of target cell specificity, as they transduce a variety of cell types in the body. Furthermore, the large-scale production and packaging of recombinant viral particles remains a challenge for practical distribution since these viruses are engineered to be non-replicating.

8.3. Conjugation of cell-penetrating peptides or cholesterol

Certain peptides consisting of positively charged amino acids have the ability to pass through the cell membrane, carrying with them any antisense drugs to which they are conjugated. The HIV-TAT peptide is known to have this property, and several laboratories have taken advantage of the TAT chemistry as a cell-delivery signal. Other positively charged peptide sequences, such as arginine-rich peptides (ARPs), have a similar effect and are able to transport antisense past the cell membrane with a high efficiency. Work from our laboratory has demonstrated the cell penetrance of ARP-conjugated, FITC-tagged PMOs by confocal imaging in HeLa and HL-1 cardiomyocytes, as well as tissue distribution in mouse hearts, also by confocal imaging of slides (54). AVI Biopharma is pursuing arginine rich peptides as conjugates to the 3rd generation phosphorodiamidate morpholino antisense oligos, which are taken up by cells with high efficiency. Both TAT and ARPs are not very stable in human serum, but arginine can be interspersed with 6-aminohexanoic acid (X) or β-alanine to increase stability while maintaining activity (88).

A similar strategy for enhancing the efficiency of antisense delivery is the conjugation of siRNA with derivatives of cholesterol, lithocholic or lauric acid (89). The cholesterol attachment seems to stabilize siRNA by binding to human serum albumin, resulting increased uptake by the liver. Soutschek and coworkers conjugated cholesterol to the 3' end of the sense strand of siRNA. Intravenous injections of this conjugate in mice resulted in uptake into multiple organs and efficiently reduced apolipoprotein B levels in the liver and jejunum (90). This approach has not been used for picornaviruses.

For drug administration *in vivo*, a number of different approaches have been developed. In the majority of studies performed in mice, rapid infusion by hydrodynamic injection of siRNA or siRNA coding vectors achieves the best delivery efficiency (91). In this method, a large volume of nucleic acid, usually about 8-12% of the body weight, is rapidly injected via the tail vein. Several groups have used this technique to successfully introduce siRNAs or siRNA-expressing plasmid into mice and demonstrated an effective silencing of target genes (92, 93). However, delivery is restricted to highly perfused tissues, such as liver, spleen or kidneys and the technique is not clinically transferable to humans.

8.4. Modular nano-scale vectors – pRNA

In order to integrate the two concepts of cell membrane permeability and target organ specificity, modular multimeric delivery vectors capable of packaging several functional groups (therapeutic sequence and receptor ligand) have been developed recently. One such vector is the packaging RNA (pRNA) of the bacteriphage Φ29, which is a partially double-stranded RNA molecule of approximately 100 bps (94). As a ribonucleic acid-based vector, pRNA may be naturally extended to contain siRNA sequences at its 5' and 3' terminal regions that will be cleaved by Dicer into siRNAs, much in the same way as shRNA. pRNA naturally forms dimers, trimers and hexamers through complementary intermolecular base-pairing between their stem-loop regions (called right and left handed loops). The sequence of the pRNA can be

engineered to carry a siRNA or a receptor ligand, which can form dimers or trimers consisting of siRNA:ligand, where siRNA is directed against a viral sequence and the ligand binds to a target cell receptor, mediating drug specific internalization by endocyotsis. This technique has been successful in the silencing of oncogenes *in vitro* and *ex vivo* using folate receptor-mediated endocytosis (94). We have used pRNA containing siRNA sequences tartgeting viral RNA in our laboratory and found that coxsackievirus replication in cells is strongly inhibited (data not published).

9. ANTISENSE AGAINST SPECIFIC PICORNAVIRUSES

Individual picornaviruses have received varying amounts of antiviral research attention in recent years, often due to the availability of alternative treatments or vaccines, as well as the overall commonality of specific species. The most frequently tested antisense-based drug candidates of the past several years for both picornaviruses and other prevalent viruses are 3rd generation PMO ASOs and siRNAs. Individual experiments for evaluation of antipicornavirals are detailed below.

9.1. Hepatitis A

Since the advent of an effective, universally available vaccine to Hepatitis A in 1994 and a combination HAV-HAB vaccination in 1996, interest in developing a therapeutic has waned. The success of the vaccine is in part because only a single serotype of hepatitis A exists. However, studies have shown that siRNA targeting the IRES of HAV inhibits translation of the HAV genome *in vitro (*95). siRNA targeting the 2C-1 stem loop, a highly exposed region of the HAV genome, also inhibits viral replication in cultured cells by up to 4-log, with the effect diminishing over a course of 12 days (68).

9.2. Poliovirus

Poliovirus has been eradicated in western industrialized nations, although outbreaks do still exist in underdeveloped countries. PV has a well-known vaccine based on Jonas Salk's "Inactivated Polio" vaccine propagated in cells and then inactivated with formalin treatment. This treatment does not prevent infection and transmission through the gastrointestinal (GI), fecal-oral route; however, it does prevent infection of the motor neurons and viremia, preventing the paralytic effects of PV. An oral vaccine based on a temperature-attenuated PV has also been used successfully, and also acts through the GI system. The WHO has been spearheading a worldwide campaign to completely eradicate polio and remove the need for future vaccination; however the effort is still underway. Overall the need and interest to pursue a PV antisense therapeutic remains low given the proven efficacy of vaccination. Since PV has been studied most extensively in molecular biology and pathogenesis than other picornavirus, researchers usually use PV as a model system to perform a variety of studies.

A seminal paper was published in Nature in 2002, demonstrating the use of siRNA against poliovirus capsid protein or RNA-dependent RNA polymerase to transiently inhibit viral replication in cultured HeLa cells for 24 hours (>48 hours if combined) (45). However, PV efficiently mutates to escape the application of siRNA, which is typically very sensitive to mismatches, particularly in the central region of the siRNA oligo. Recent study with siRNA has demonstrated that pools of multiple siRNAs against highly conserved regions can overcome escape mutations, and indeed completely cure cultured cells in a persistent model of PV infection even after several months of PV incubation and the presence of mutated virus (96).

9.3. Rhinovirus

Although over 100 different known serotypes of human rhinovirus (HRV) exist, and the symptoms of HRV infection are relatively mild and brief (upper respiratory tract infection with little cytolytic effect), the virus is so common that interest in developing a therapeutic has been high. However, due to the variability in serotypes, the favored approach is to inhibit the virus entry or viral 3C protease with small-molecule pharmacological inhibitors, rather than attempt a sequence-specific treatment that may be easily avoided by mutation. Accordingly, clinical trials to date have focused on such inhibitors, in particular the WIN family of capsid-binding drugs and the intranasal spray based on 3C protease inhibitor ruprintivir. In recent years, a small molecule inhibitor of the viral structural protein VP1, named Pleconaril, has entered phase II clinical trials. This compound can block HRV interaction with host cell surface receptor and then block viral entry (97).

Currently, no clinical trials have used an antisense approach against HRV. *In vitro*, there are no reported uses of DNA ASOs inhibiting rhinovirus; however, siRNA has been tested in cultured cells against serotype 16 of HRV. Targeting various coding regions in the HRV genome significantly inhibited viral titre released in supernatants of HRV-infected HeLa cells, with particular efficacy against sequences in the VP1, VP4, 2C and 3D regions (98)

9.4. Coxsackievirus

Coxsackieviruses represent one of the groups that could have significant potential for antiviral antisense therapeutic applications, since there is no available vaccine, there are demonstrated conserved targets in the genome, and there are demonstrated positive effects of antisense treatments *in vitro* and *in vivo*. Furthermore, coxsackievirus infection can have several serious debilitating or lethal outcomes; type A coxsackieviruses can infect skeletal myocytes, causing flaccid paralysis. Type B coxsackievirus typically causes pancreatitis, aseptic meningitis, and myocarditis. Acute myocarditis from coxsackie B infection is a surprisingly common cause of sudden death and is particularly dangerous to children and young adults; in addition, viral myocarditis often enters its late phase, dilated cardiomyopathy, resulting in heart failure requiring transplant. Thus, the need for a treatment is pressing.

Several variations of antisense oligomers have been studied in our laboratory showing very promising

efficacy, although none have reached clinical trials to date. First generation ASOs applied *in vitro* targeting the 5' or 3' end of the genome, the IRES and the AUG start codon region reduced viral RNA replication detected by RT-PCR, viral titre detected by plaque assay, and viral protein production detected by western blot (31).When applied intravenously, the same PS ASOs in mice protectively inhibited viral RNA levels, myocarditic lesions, and viral titre in heart tissue (26).Peptide-linked morpholino oligomers (PPMOs) have had similar success, and can also easily enter cells via their membrane-penetrating arginine-rich peptides without any additional delivery vehicle. In collaboration with AVI BioPharma, we have demonstrated that PPMOs targeting the CVB3 IRES inhibit viral replication and viral titre by approximately 2-log in cells in a dose-dependent, sequence specific manner and can maintain activity up to 4-5 days after application. Similarly, viral titres in infected mouse hearts were 2-log lower in groups receiving antiviral PPMOs, compared to groups receiving scrambled PPMOs or PBS (54). PNAs, another class of 3rd generation ASOs, have also been tested against CVB3 in HeLa cells using lysine-flanked 12-mers to increase cell uptake (99). Because of its extremely high affinity for natural RNA, PNA is able to invade and displace normal secondary structure that is a strict requirement for the highly ordered 5'UTR structure containing the CVB3 IRES. PNAs applied before infection protected cells from the cytopathic effects of CVB3 challenge in a dose-dependent manner, reducing cell death by approximately 50% at 12.5 μM and 80% at 50 μM.

siRNA against 2A protease significantly inhibited viral replication and viral titre when transiently transfected in cultured HeLa cells (>92% and 2-log factor, respectively). As with PV, the antiviral effect was disrupted by mutations in the central strand region, and mismatch was tolerated near the 3' end and not the 5' end (100); furthermore, the siRNA effect is mediated by the antisense strand to the viral genome, rather than the sense strand complimentary to the viral negative strand intermediate. This finding was further conformed by anther report (101). When applied systemically to mice, siRNA targeting 2A had a significant protective effect if applied six and fourteen hours after infection, including reduced viral replication and tissue injury, as well as increased survival (49). shRNA processed into siRNA was also effective against CVB3 3D RNA polymerase and structural protein VP1, both in cells and mice, where viral pancreatitis was significantly reduced (102). Schubert and colleagues used the SiDEx double expression vector to simultaneously transfect two siRNA sequences targeting the CVB3 3D polymerase sequence in a GFP reporter construct. Double expression of both siRNAs successfully suppressed reporter expression despite the intentional introduction of an artificial point mutation (simulating an escape mutation) that caused a mismatch with one of the two siRNAs (103).

9.5. Foot-and-Mouth Disease Virus

Also called hoof-and-mouth disease virus, FMDV infects cloven-hooved ungulates and primarily affects the agricultural industry by infecting livestock. Transmission to humans is extremely rare, and the species barrier in FMDV infection has remained intact over hundreds of years of close human-animal exposure, with the nature of this barrier remaining largely unknown. Although vaccination programs are used to curtail outbreaks in livestock, the variability of the virus strains and problems inactivating 100% of the vaccine has led to some infections escaping these preventative measures. Therefore, FMDV is a reasonable candidate for antiviral antisense therapies in animals.

Several antisense candidates have been tested for this purpose. Early unmodified RNA-based antisense against FMDV transiently suppressed viral replication in BHK cells in a sequence-specific and dose-dependent manner. Effective targets were either strand of the 3'UTR, or the sense strand of the 5'UTR (104), with inhibition approaching 90%. A similar inhibitory effect was achieved by another report through combination of 5' transcript with either sense or antisense RNA from the 3' region of the genome (105). Other early experiments using unmodified DNA ASOs surprisingly achieved a range of only 30-50% inhibition of FMDV when directed against the AUG start codon region (30). Recent work has focused on morpholino ASOs linked to cell-penetrating peptides (PPMOs). PPMOs targeting certain conserved regions across six FMDV serotypes were selected and screened for efficacy. PPMOs against the AUG start codon were particularly effective, reducing viral titres by a factor of 4-log at concentrations of 1μM in the A serotype. The most versatile PPMO targeted the 5D stem loop just upstream of the start codon, reducing viral replication significantly in all seven tested serotypes, suggesting that the requirement for the 5D region is the most conserved or most PMO-accessible region (55).

siRNA targeting FMDV VP1 inhibited viral replication by 80-90% in cells and also reduced susceptibility of suckling mice to FMDV infection (50). Moreover, delivery of shRNA against FMDV 1D structural protein or 3D polymerase via adenoviral delivery vector is protective against subsequent LD50 viral challenge in swine and guinea pigs (85). The nonstructural protein 2B is also an effective target to inhibit FMDV replication. Transfection of a plasmid containing shRNA against 2B in cultured cells inhibits subsequent infectious virion production by 97%, and this inhibition does not depend on interferon-activated genes such as PKR, which had no change in levels after transfection (106).

In order to design siRNA that could inhibit all serotypes of FMDV, Kahana and colleagues performed a bioinformatic search for regions of 22 bp with 100% homology across serotypes, and found three such regions – one within the 3B coding region and two within the 3D coding region. As measured by real time RT-qPCR, the most effective of these siRNA reduced FMDV mRNA levels by 80-92% in cultured cells infected with the O1 serotype. A mixture of all three siRNAs reached 98% inhibition of viral RNA compared to control (107). In a

similar experiment, Liu et al. selected conserved sequences in the VP4, VPg, POL, and 3′UTR regions using conventional NCBI BLASTN to find stretches that were 85-98% homologous between four serotypes: O, A, C and Asia 1. The custom siRNA were applied individually and the 50% tissue culture infectious dose was measured, however, no single sequence proved highly effective against all serotypes tested, suggesting a mixture may be required (108).

9.6. Other Picornaviruses

Other enteroviruses are often highly related to PV and CV – for example, coxsackievirus A23 was reclassified as echovirus 9, and echovirus 28 was reclassified as rhinovirus 1A. Many of these related picornaviruses have also been subject to similar antisense treatments, particularly siRNA which can be easily custom-made to order from several biotech companies. For example, human enterovirus B replication was significantly inhibited in cultured cells by targeting the *cis*-acting replication element (CRE) for the viral 2C protein (109).

Significant RNA interference work has been done against enterovirus 71 (EV71), the major causative agent of hand foot and mouth disease in infants. Transfection of a DNA plasmid coding for shRNA targeting EV71 VP1 or 3D proteins inhibited viral protein production in cultured cells (110). Direct transfection of various 19mer siRNA against nonstructural genes also inhibits EV71 RNA replication and viral titre in a dose-dependent manner (111) in cultured cells, whereas 27mer siRNA proved even more effective at 10-fold lower concentrations (112). In suckling mice, both 19-mer siRNA and shRNA targeting the 3D RNA polymerase of enterovirus 71 significantly inhibits infection and development of hand, foot and mouth disease (113), without evoking an immunogenic response to the therapy.

10. CONCLUSIONS

Both DNA and siRNA antisense oligomers against picornaviruses are viable and effective agents to inhibit viral replication. At present, no such drugs against picornavirus have been approved for distribution, primarily due to a number of issues that need to be resolved. These include drug stability, toxicity, specific delivery and off-targeting. Despite the challenges that remain, the use of Vitravene for treatment of CMV infection indicates that there is a theoretical and practical basis for effective anti-picornavirals, but also that new work will be required to refine efficacy and reduce side effects. Developments in these areas have led to a number of antisense oligo-based therapeutics in phase I, II and III clinical trials for cancers, cardiovascular diseases, diabetes and infectious diseases. For viral infection, HCV and RSV treatments have entered phase I clinical trials, both of which have promising futures (http://assuragen.com/therapeutics/thera_trials.html). With the progress in improved drug design, chemical modification and specific delivery, effective anti-picornavirals will be eventually developed and this timeline will not be very long.

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Abbreviations: ASO – Antisense oligonucleotide, siRNA – small interfering RNA, PV – poliovirus, HRV – Human rhinovirus, HAV – Hepatitis A Virus, SARS CoV – severe acute respiratory syndrome coronavirus, CV – coxsackievirus, FMDV – foot and mouth disease virus, IRES – internal ribosomal entry site, +ssRNA – positive, single-stranded RNA, ARP – arginine rich peptide, TAT – Human immunodeficiency transactivator protein, PPMO – peptide conjugated phosphorodiamidate morpholino oligomer, LNA – locked nucleic acid, PNA – peptide nucleic acid, RSV – respiratory syncytial virus, CMV – cytomegalovirus

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