Antisense antivirals: future oligonucleotides-based therapeutics for viral infectious diseases

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Despite the availability of antiviral chemotherapies, human pathogenic viral infections remain a global health problem, causing formidable morbidity and mortality worldwide. Of particular concern are those virus types developing (multi)drug-resistance, eg. herpesviruses, hepatitis C virus (HCV), and human immunodeficiency virus (HIV), that fail almost all the available antiviral drugs in clinical practice. Novel antiviral strategy of using single-stranded antisense oligo(deoxy)nucleotides (ASOs) as gene silencers has been a prospective area of anti-infective study, which has shown great advantages in providing fast-respond postexposure therapeutics for emerging viruses. This chapter briefly introduces the important technological advances in the development of antisense antivirals (including antisense mechanism, chemical modification of ASOs), highlights the identified potential targets for different virus types and the therapeutic efficacy of ASOs that have reached clinical trials with antiviral protocols, and discusses the problems and challenges in future anti-infective application.

Keywords antisense oligo(deoxy)nucleotides; pathogenic virus; steric blocking; phosphorothioate (PS); phosphorodiamidate morpholino oligomers (PMOs); cell penetrating peptide (CPP); host-directed antiviral strategy.

1. Introduction

Pathogenic viruses are significant etiology of different types of infectious diseases, causing tremendous burdens on human health care globally. In spite of the extensive efforts on developing effective antiviral therapeutics (including small-molecule antiviral chemicals and vaccines), a number of known viruses that claim millions of lives every year, e.g. the human immunodeficiency virus (HIV), dengue virus, hepatitis B and C virus [1], and West Nile virus, still lack efficient or accredited drug treatments. Also, the emergence of virus types/strains with (multi)drug resistance fails the available antiviral treatment for infected patients and aggravates drug paucity. Further, effective vaccines or postexposure therapeutics are still inadequate, especially for lethal outbreaks of new strains that cause medical care disasters, e.g. the severe acute respiratory syndrome coronavirus (SARS-CoV) and the avian influenza viruses.

Antisense antiviral strategy is considered a very attractive means of fulfilling the aims of offering timely therapeutic countermeasures to prevent virus propagation [2]. Antisense oligonucleotides (ASOs) -based antiviral therapeutices are normally 15-30 nucleotides in length and specifically bind to complementary DNA/RNA by Watson–Crick hybridization to arrest translational processes either by inducing cleavage mechanisms or by sterically blocking [3], thereafter inhibit viral growth. Viral genomic regions involved in transcription or genomic-replication processes are usually targeted by ASOs, which act as gene-silencers interfering with the virological processes to inhibit production of infectious virus particles in infected cells, potentially reducing cytopathic effects.

The use of ASOs as potential antisense antiviral therapeutics was first documented in 1978, when Zamecnik and Stephenson observed the viral growth inhibition of a phosphodiester oligodeoxynucleotide composed of 13 nucleotides (a 13-mer) designed to block Rous sarcoma virus replication [4,5]. The availability of a wider range of oligonucleotide analogs has stimulated target validation and development of ASO-based antiviral agents, the efficacy of which have been recoganized in different virus types *in vitro* as well as *in vivo* [6]. However, ever since the approval to use Vitravene (fomivirsen, ISIS 2922), an ASO composed of 21 phosphorothioate oligonucleotides (PS-ONs) against CMV IE2, by FDA in 1998 for local treatment of cytomegalovirus (CMV)-induced retinitis, progress in antisense antivirals has been relatively slow. Despite that major challenges involved in target identification and efficient delivery system development have received considerable studies, numerous new challenges in clinical trials rose [7], including off-target effects, resistance and pharmaco-issues (e.g., systematic toxicity, bio-distribution, and pharmacokinetics).

In this chapter, we provide an overview of the many important aspects of antisense antivirals, highlighting specific studies of interest (e.g. antisense mechanism in viruses, development of ASOs constructs, effective targets identification, refinement of efficient delivery systems, and problems in clinical trials, etc.) over the past three decades. Specifically, with antisense targeting host genes involved in viral infection as a promising field, we provide certain insight into the development of potential adjuvant ASO-based therapeutics for antiviral drugs.

2. Antiviral ASOs: a brief overview of structural chemistry and mechnisms

Practical application of ASOs has required modifications of DNAs or RNAs, with the aim of retaining the hybridization capacity while increasing stability. Therefore, major modifications concerning the backbone, phosphodiester bond, and sugar ring have been introduced and investigated, giving births to three generations of nucleic acid analogs for synthesis of ASO oligomers (Figure 1). ASO-based antiviral agents are usually designed to arrest translational processes [3] (i) either by inducing cleavage mechanisms involving endogenous cellular nucleases such as ribonuclease H (RNAse H) or RNase P (only if external guide sequences are coupled to the oligonucleotide), or (ii) by sterically blocking enzymes involved in target gene translation. The specific gene-silencing mechanism of ASOs-based therapeutics depends on the structural chemistry and special designs.

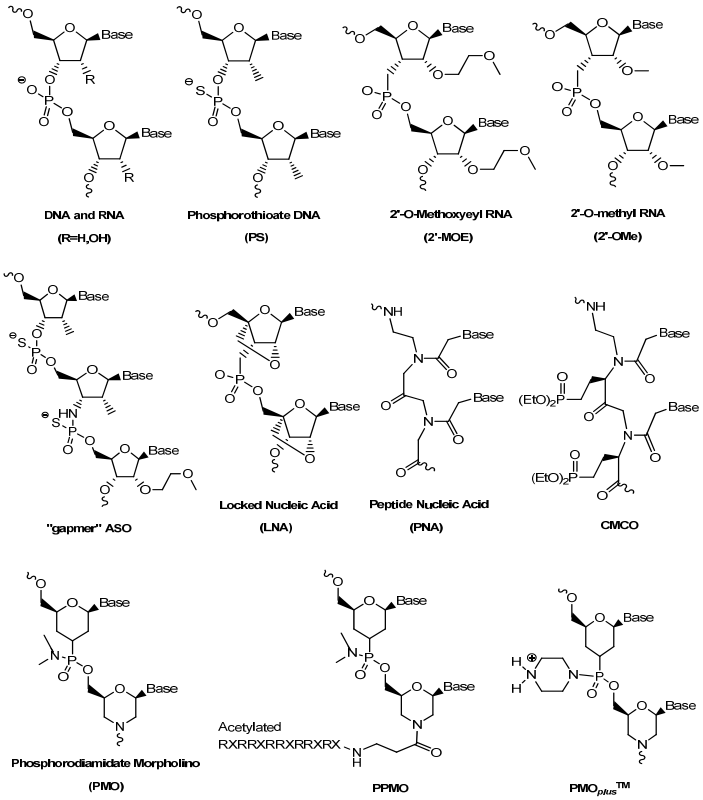

Fig. 1 Oligonucleotide chemistries. Phosphorothioate (PS) backbones, as well as 2′-O-methoxyethyl (2′-MOE) and 2′-O-methyl (2′-OMe) substituents are negatively charged oligonucleotides that increase resistance to degradation and promote protein binding to target RNA (see top panel). Locked nucleic acid (LNA) modification markedly increases the binding of the oligonucleotide to the targeted mRNA(see middle panel). Peptide nucleic acid (PNA) modification uses non-charged achiral peptide (polyamide) backbone, resulting in a good structural mimics of DNA resistant to degradation (see bottom panel). Introduction of phosphonic ester residues into a PNA backbone (CMCO) improves the solubility of the PNAs and the penetration of the compounds throug the cell membrane (see bottom panel). In phosphorodiamidate morpholino oligomers (PMOs), ribose (RNA) or deoxyribose (DNA) is replaced with morpholine rings, and the phosphorothioate or phosphodiester (RNA) groups are replaced with uncharged phosphorodiamidate groups, resulting in a compound that is neutral and very resistant to degradation (see bottom panel). Positively charged piperazine residues in positively charged PMOs (PMO*plus*), or positively charged arginine-rich peptides in peptide-conjugated PMOs (PPMOs), dramatically improve the intracellular uptake of the oligomers (see bottom panel).

2.1. Phosphorothioates (PS): the first-generation cleavage inducer

The PS DNA constructs, where a non-bridging oxygen atom in the DNA backbone is replaced by sulfur atom (Figure 1), otherwise result in strongly enhanced stability against enzymatic degradation. In spite of the loss in hybridization affinity (0.3-1 ◦C drop in Tm per modification), the sensitivity to RNase H cleavage of the hybrid between a PS and its RNA complement has been largely preservd. Most of the eraly literature on researaches in antisense antivirals use PS-modified oligonucleotides, regarding this innovative modification that offers tailored ASOs with a balance of characteristics of hybridization affinity, hydrophobicity and the capacity to recruit RNase H mediated hydrolysis of the target RNA. Meanwhile, the first generation of ASO-based drugs that have progressed to clinical trials have been PS compounds [8,9].

PS constructs have received thorough evaluations on biomedical properties. However, serious non-specific effects related to its structure forced them to step out of stage. The negative-charged character of PS shows decreased affinity for their target mRNA and lowered efficiency in cellular uptake. Most importantly, it causes nonspecific interaction with serum and intracellular proteins, leading to strong immunostimulatory response or unexpected spurious off-target effects. Additionally, with variation in different sequences, this backbone imparts a significant, hybridization-independent toxicity profile, including increased coagulation time, pro-inflammatory effects, and activation of the complement pathway. Several PS-ASO drug candidates progressed into clinical phases during 1990 to 2010, but only one drug fomivirsen made itself to market (although the drug was discontinued in 2004 as the market for the drug diminished). Nonetheless, the development of PS has been continued in some companies with known disadvantages, mainly due to its low cost at large-scale synthesis.

2.2. Phosphorodiamidate morpholino oligomers (PMOs): the third-generation steric-blocker

Most of the second (e.g., 2′-O-methoxyethyl and 2′-O-methyl) and third (e.g., locked nucleic acid, peptide nucleic acid and PMO) generation ASO constructs are steric-blocking ONs that are unable to recruit RNaseH and therefore are limited to inhibition of translation. The creation of gapmers (Figure 1), which are now applicable to many ASO constrcuts, endows the oligomers with conjugated 5'-phosphorylated-2',5'-linked oligoadenylate to recruit of 2-5A dependent RNase L [10,11]. Numerous studies have suggested that a steric-blocking antisense strategy may be effective against viral pathogens, evidenced by the success of third-generation ASOs as effective antisense antivirals, especially the morpholino ASOs (i.e. PMOs, peptide-PMOs, and positively-charged PMO_{plus}, as illustrated in Figure 1).

PMOs are DNA-like oligomers with six-membered morpholine rings substituting for the deoxyribose, and with neutral phosphorodiamidate intersubunit linkages. These structural modifications confer high stability, solubility, and affinity to target mRNA sequences, which are desirable qualities for clinical use. The morpholino ASOs are capable of forming a stable duplex with complementry sequences and thereby sterically blocking access of other biomolecules to specific regions of viral DNA/RNA, or of disrupting important RNA structures. The first report of antiviral activity by a PMO was in 2001[12], and the *in vivo* activity of PMO against a variety of viral pathogens has been demonstrated therafter. However, none has yet been licensed for treatment of viral infection or other indications.

3. ASOs-based antiviral therapeutics

3.1. Targets identification and validation

Unlike traditional antiviral drugs that target only a limited number of proteins, the ASOs-based antiviral therapeutics can be designed to be highly specific ligands targeting to any of the viral genes in order to inhibit viral growth. Although there is not a fully rational approach to RNA target sequence selection, multiple techniques impact on success, including computer models of RNA secondary structure, nucleotide hybridization efficiency and frequency comparation. Antisense activity correlates well with hybridization strength, while RNA secondary structure does not necessarily predict optimal antisense activity. Additionally, modifications of ASOs have resulted in a variety of non-antisense antiviral activities, therefore, it is essential to define an antisense activity with sequence-specificity of action and selective inhibition of targeted gene expression.

Multiple opportunities during RNA processing may be vulnerable to ASO binding. Generally, gene targets for ASOs include those involved in viral replication, early gene expression and viral coat proteins. It has been validated that RNA splice donor or receptor sites, 5' capping, 3' adenylation, translation start and termination sites, and ribozome entry are the most targeted sites due to higher sensitivity for antisense inhibition. This part summarizes the notable findings of genetic locations that provided the most sensitive target sites for ASO intervention against various important pathogenic viruses.

3.1.1. Targets for DNA viruses

A DNA virus is a virus that has DNA as its genetic material and replicates using a DNA-dependent DNA polymerase. The nucleic acid is usually double-stranded DNA (dsDNA). Genome organization within this group varies considerably. Some have circular genomes (*Baculoviridae*, *Papovaviridae* and *Polydnaviridae*) while others have linear genomes (*Adenoviridae*, *Herpesviridae* and some phages). Some important pathogenic DNA viruses, including hepatitis B virus (HBV), herpes simplex virus (HSV), Epstein–Barr virus (EBV), human cytomegalovirus (HCMV), Marek's disease virus (MDV), and human papillomavirus (HPV), have been studied for the possibility of antisense inhibition. ASOs have been evaluated against different sites of the viral genome, and demonstrated differing success (Table 1).

Table 1 Summary of effective ASO target sites in double-stranded DNA viruses.

Productive ASO target region(s)[a]	**ASOs**[b]	**Delivery Method**[c]	**Ref.**
1. Hepatitis B virus, HBV (Family *Hepadnaviridae*)			
cap site/SPII and initiator/gene S	AS-ODN, PS-ODN	—	Goodarzi G et al, 1990
5' region of pre-S gene (795-812nt)	PS-ODN	—/liposome	Offensperger WB et al, 1993/Soni PN et al, 1998
preS1 open reading frame, S gene, C gene	PS-ODN	—	Korba BE et al, 1995
S gene	Antisense RNAs	lentiviral vectors	Nash KL et al, 2005
S and C genes	LNA	liposome	Deng YB et al, 2009
Pre-S region (951-968 nt)	PS-ODN	—	He L et al, 1998
upstream sequence of the encapsidation site (1849-1864 nt)	AS-ODN, 2'-allyl ODN	ASGP-R delivery system	Madon J et al, 1996

U_5-like region (1980-1905 nt) in pre C and C region	ligand PS-ASO	liver-targeting ligand Gal-PLL	Zhoug S et al, 1998 Zhong S et al, 2002
pol gene (2468-2487 nt)	poly-DNP-RNA	—	Xin W et al, 1998
pregenomic RNA	Antisense RNAs	—	zu PJ et al, 1998
core promoter (1734-1754 nt)	TFO	—	Yang L et al, 1999
initiation sites of pre C initiation sites of pregenomic RNA	PS-ODN		
initiation site, DR2, and EN II in X gene	PS-ODN	—	Liu S et al, 2001
initiation site of HBV X gene	PS-ODN	—	Matsukura M et al, 1995
X and P regions	Antisense RNAs	pLXSN	Zhao W et al, 2005
bulge (2567-2581nt) and upper stem (2573-2587 nt) of encapsidation signal ε	PNA	CPP	Robaczewska M et al, 2005
	PS-ODN	—	

2. Herpes simplex virus, HSV (Family *Herpesviridae*)

IE 4 and 5 pre-mRNA	intron:exon junction of the splice acceptor	octamer MODN	—	Smith CC et al, 1986
		dodecamers/psoralen derivatized ON		Kulka M et al, 1989
	splice donor site and translation initiation site	MODN		Kulka M et al, 1993
		2'-OMe with methylphosphonate internucleoside connection		Kean JM et al, 1995
		PO-ODN with minihairpins at the 3'-end		Poddevin B et al, 1994
TIS of IE 1 mRNA		PO-ODN with 2 residues of PS-ODN at 5' and 3' end	—	Peyman A et al, 1995
		end-capping and pyrimidine protection		Peyman A et al, 1997
IE 3 mRNA		PO-ODN	—	Cantin EM et al, 1992
IE 1 and 3 mRNA		MODN	—	Feng CP et al, 1996
splicing acceptor site of IE 5 mRNA		PO-ODN, MODN,PS-ODN	—	Shoji Y et al, 1996
		PS-ODN	geraniol	Shoji Y et al, 1998
KSHV IE transcription activator Rta mRNA		2'-OMe modified EGS	—	Zhu J et al, 2004
internal AUG codon of UL13 mRNA		PS-ODN, 5' or/and 3' modified PS-ODN	—	Hoke GD et al, 1991
5' noncoding region of HSV-1 UL29 mRNA		PS-ODN	—	Flores-Aguilar M et al, 1997
TIS of HSV-1 ICP0 or ICP27mRNA		PMO	Arg-rich peptide P7	Moerdyk-Schauwecker M et al, 2009
TIS of HSV-1 UL30 and UL39				Eide K et al, 2010
TIS of Vmw65 mRNA		ODN/PS-ODN	—	Draper KG et al, 1990/Kmetz ME et al, 1991
TIS of large subunit of RR1		AS-ON	—	Aurelian L et al, 2000
vIL6 mRNA	ORF-K2 TIS (−14 to 7 nt)	PMO	Arg-rich peptide P7 or P4	Zhang YJ et al, 2008
	5' UTR (−106 to −84, −42 to −20nt)			
DNA polymerase		PS homo-ODN	—	Gao WY et al, 1989; Gao WY et al, 1990
virus adsorption		GT-ODN	—	Fennewald SM et al, 1995

3. Epstein-Barr virus, EBV (Family *Herpesviridae*)

coding region(3' of AUG in mRNA)	PO-ODN, PS-ODN	—	Pagano JS et al, 1992
EBNA-1 RNA	UM AS-ODN	—	Roth G et al, 1994
translation initiation codons and their flanking sequences of BZLF1 transcript	PO-ODN, PS-ODN	—	Diabata M et al, 1996

TIS of LMP1 mRNA	UM /AS-ON	—/ lipid-based and receptor-mediated delivery systems	Mattia E et al, 1997/ Galletti R et al, 2007

4. Human cytomegalovirus, HCMV (Family *Herpesviridae*)

mRNA of DNA polymerase gene	PS-ODN	—	Azad RF et al, 1993
RNA transcripts of IE1			
IE2 (170120-170140 nt)	(modified)PS-ODN		Detrick B et al, 2001
the intron-exon boundary of HCMV genes UL36 and UL37 (UL36ANTI)	PS-ODN	—	Pari GS et al, 1995; Smith JA et al, 1995
	modified PS-ON	cholesteryl moiety	Zhang Y et al, 1997
ORF UL83	AS RNA (stably expressed)	—	Dal MP et al, 1996
UL36 mRNA	AS-ODN	—	Yao JX et al, 2004

5. Human papillomavirus, HPV (Family *Papillomaviridae*)

TIS of the E2 mRNA for HPV 6 and 11	PS-ODN	—	Cowsert LM et al, 1993
PV E1 helicase transcript	PS-ODN	—	Lewis EJ et al, 2000
E1 TIS	PS-ODN	—	Roberts S et al, 1997
	2'-OCH_3 hybrid of PS-ODN		
HPV16 E6 RNA	AS RNA	lipofectamine	Cho CW et al, 2002
HPV11 E6/E7 RNA	AS-ODN	lipofectamine	Clawson GA et al, 2004

[a] C gene, HBV core antigen (HBcAg) gene;S gene, HBV surface antigen (HBsAg) gene; cap site/SPII, the cap site of mRNA transcribed from the SPII promoter; initiator/gene S, the translation initiation site of the S gene; nt, nucleotides; pol gene, polymerase gene; IE, immediate-early; vIL6, viral Interleukin 6; ORF, open reading frame; TIS, translation initiation site; UTR, untranslated region; RR1, ribonucleotide reductase 1; EBNA-1, Epstein-Barr virus nuclear antigen 1; BZLF1, ; LMP1, latent membrane protein 1; UL, unique long gene; Vmw65, virion tegument protein.

[b] ASO, antisense oligonucleotide; AS-ODN, antisense oligodeoxynucleotide; PS-ODN, phosphotioates oligodeoxynucleotide; poly-DNP-RNA, poly-2'-O-(2,4-dinitrophenyl)-oligoribonucleotide; PNA, peptide nucleic acid; LNA, locked nucleic acid; MODN, methyl phosphonate oligodeoxynucleotide; PO-ODN, phosphodiester oligodeoxynucleotide; PO-ODN with minihairpins at the 3'-end, PO-ODNs constituting of a 5-dodecameric sequence, flanked at the 3'-end by octameric sequences adopting hairpin-like structures; GT-ODN, ODNs composed entirely of deoxyguanosine and thymidine, but not specifically designed to act as antisense agents,with either natural PO or PS modified internucleoside linkages; PMO, phosphorodiamidate morpholino oligomers; EGS, external guided sequence that activate RNase P cleavage of targeted mRNA; UM, unmodified.

[c] CPP, cell penetrating peptide; ASGP-R, asialoglycoprotein receptor; NAcGlcNH2.BSA, N-acetylglucosamine derivative of bovine serum albumin; Gal-PLL, alactosylated poly-L-lysine; Arg-rich peptides include P7, CH_3CONH-$(RXR)_4$-XB, P4, $R_5F_2R_4$, NH_2-RRRRRRFFRRRRC-$CONH_2$ (R stands for arginine, X stands for 6-aminohexanoic acid, F stands for phenylalanine, C stands for cysteine, and B stands for beta-alanine); pLXSN, Retrovirus vector; "—", no delivery method used.

3.1.2. Targets for RNA viruses

While Human immunodeficiency virus (HIV) remains the RNA virus with the largest impact on global public health, nonretroviral RNA viruses including dengue, measles, respiratory syncytial, hepatitis C [13], and influenza A viruses are currently major pathogens causing of infectious disease with high mortality. Despite the availability of vaccines and small molecule drugs for limited viruses, the development of safe and effective therapeutic treatment against non-retroviral RNA viruses has been comparatively scanty [14]. The historical context of ASO targeting the RNA sequence of HIV (Table 2) and pathogenic non-retroviral RNA viruses (Table 3) is summarized below.

Table 2 Summary of effective ASO target sites in human immunodeficiency virus (HIV).

Productive ASO target region(s)[a]		**ASOs[b]**	**Delivery Method[c]**	**Ref.**
splice acceptor sites (upstream of $tRNA^{Lys}$ for PBS)		UM-ODN	—	Zamecnik PC et al, 1986
Human $tRNA^{Lys3}$ including the 3' terminus, TΨC stem-loop and variable loop (41-76 nt)				Wei X et al, 2000
Pre-PBS region for $tRNA^{Lys3}$ primer (164-183 nt)		2'-OMe		Freund F et al, 2001
exon I of *art*/*trs* genes (6018-6032 nt)		M-ODN,PS-ODN	—	Matsukura M et al, 1987
		Phosphoro-selenoate		Mori K et al, 1989
ends of retrovirus RNA (R region)	Poly(A) signal (9183-9202nt)	AS-ODN	—	Agrawal S et al, 1988
	Cap (1-20nt)			

	5’ UTR (54-73nt)			
splice acceptor site	upstream of the *env* initiator (7947-7966nt)			
	tat initiator (5349-5368nt)	PS-ODN	cholesteryl group	Letsinger RL et al, 1989
first splice acceptor site of the *tat-3* gene		M-ODN	—	Zaia JA et al, 1988
capped 5' end and splice acceptor site of *tat*		M-ODN	—	Sarin PS et al, 1988
splicing acceptor site	5350-5380 nt	2’-OMe/PS	—	Shibahara S et al, 1989
	5346-5380 nt	patch derivative		
env (5585-9153nt) covering the complete exon II of the tat gene near the 3’ terminus		AS-ON	CD3 MAb-targeted liposome	Renneisen K et al, 1990
U3 Enhancer Element (-93 to -83 nt)		M-ODN	—	Laurence J et al, 1991
tat initiator (5402-5410nt)				
splice acceptor site of exon II of gene *tat*		AS-ODN	—	Daum T et al, 1992
translation initiation site of *tat*		ON	poly(L-lysine) conjugated	Degols G et al, 1992
p18/p24 junction in gag, active site of HIV protease in *pol*, first exon of *re*v gene		PS-ODN	—	Kinchington D et al, 1992
conserved sequence of *env*, negative sense viral RNA,5'-terminus of gene *rev*, poly(A) sequences		ON	lipophilic groups conjugated	Svinarchuk FP et al, 1993
RRE	stem I, initial portion of stem loop II	PS-ODN	—	Li G et al, 1993
	stem loop II			
TIR of *gag* (324-362nt)		Self-stabilized ODN	—	Tang JY et al, 1993
TIR of *gag* (776-802nt)		PS-ODN	—	Lisziewicz J et al, 1993
rev1 (5970-5997nt)				Weichold FF et al, 1995
rev and *tat* gene		AS-ODN, PS-ODN	immunoliposome	Zelphati O et al, 1993
initiation codon region of *rev* gene		α and β phosphodiester ON		Zelphati O et al, 1994
tat gene		modifiedAS-ODN	Influenza derived fusogenic peptide	Bongartz JP et al, 1994
gag (p24 leader, 1185-1214nt), *pol* (p24 leader, 1185-1214nt), *rev* (splice acceptor, 4486-4505nt), *tat* (splice acceptor, 5348-5367nt; translation start, 5550-5569nt),*tar* (1-20nt, 15-34nt, 40-59nt)		PS-ODN	—	Boiziau C et al, 1995
initiation site of *rev*		PS-ODN	—	Matsukura M et al, 1995
1-20 region of HIV RNA		DNA	MAb linked	Morelli D et al, 1995
5’-LTR (262-281nt)		PS-ODN	—	Anazodo MI et al, 1995
U5 region (149-174 nt) PrePBS region (224-238 nt)		ODN	poly(L-lysine) conjugated	Bordier B et al, 1992; Bordier B et al, 1995
non-regulatory region (1189-1208nt)		PS-ODN	—	Anazodo MI et al, 1995
			cationic lipid	Anazodo MI et al, 1995; Anazodo MI et al, 1995
DIS loop	272-280 nt	RNA, DNA/RNA	—	Skripkin E et al, 1996
	272-290	LNA, LNA/DNA		Elmen J et al, 2004
5’-end of HIV Rev mRNA		PS-ODN	CD4 MAb-targeted liposome/ DLS-liposome	Selvam MP et al, 1996/ Lavigne C et al, 1997
stem-loop structure of TAR element at the 5' end of RNAs (11-35 nt, 20-35 nt)		ODN	—	Boulme F et al, 1997
PBS and U5 region		PNA, PNA-DNA chimera	—	Lee R et al, 1998
RRE		AS-ODN	CD4 coupled pH-sensitive liposome	Duzgunes N et al, 1999
near the 3' end of *gag-pol* transframe region (2262-2276 nt, highly conserved)		PNA	Peptide conjugated	Sei S et al, 2000
			erythrocytes carriers	Fraternale A et al, 2009
Srev + SDIS (245-270 nt) + SPac (295-324 nt)		AS-ODN,PS-ODN	DLS carrier system	Lavigne C et al, 2001

apical stem-loop of 39-mer TAR RNA	20-45 nt	UM	—	Vickers T et al, 1991
	21-36 nt	PS, OMe, NP, PNA		Boulme F et al, 1998
		OMe/LNA (α-L-LNA and S-LNA)		Arzumanov A et al, 2003
	25-36 nt	OMe, PNA,OMe /pC,OMe /LNA		Arzumanov A et al, 2001
	22-36 nt	mr-AOMP		Hamma T et al, 2003
minimal functional TAR sequence comprising the apical stem-loop and bulge regions (18-34 nt)		PNA	—	Mayhood T et al, 2000
			transportan conjugated	Chaubey B et al, 2005; Kaushik N et al, 2002a; Kaushik N et al, 2002b
			penetratin	Tripathi S et al, 2005
TAR and SL3 loop of stem-loops in 5'-UTR		2'-OMe/LNA	—	Brown D et al, 2005
PPT		ODN A	—	Heinrich J et al, 2009; Matskevich AA et al, 2006
downstream from PBSD (203-222 nt), major SD (278-297 nt), *gag* (AUG, 326-345 nt)		LNA antisense gap-mer	— /lipofectamine2000	Jakobsen MR et al, 2007
5'-UTR	TAR (16-45 nt)	ODN	pNL4-3plasmid complexed with Fugene HD	José A. Reyes-Darias et al,2012
	Poly(A) (95-66 nt)			
	PBS (210-239 nt, 107-136 nt, 142-171nt, 178-207 nt)			
	DIS (243-252 nt)			
	SD (274-303 nt)			
	Psi (304-333 nt)			
	AUG (325-348 nt)			

[a] PBS, primer binding site; nt, nucleotides; *pol* gene, polymerase gene; RRE, Rev-responsive element; LTR, long terminal repeat; TAR, the *trans*-activating responsive; TIR, translational initiation region; Srev, a 28-mer ODN complementary to 5'-end of HIV Rev mRNA; SDIS, a 26-mer ODN complementary to a highly conserved sequence localized between the primer binding site and the major splice donor site; SPac, a 30-mer ODN complementary to a sequence localized between the major splice donor site and the first ATG gag initiation codon and corresponds to the packaging signal psi (ψ); DIS, dimerization initiation site; PPT, polyp-urine tract; TAR, Tat protein binding site; SL3 loop, primary packaging element binding the Gag polyprotein; PBSD, the primer binding site; DIS, dimerization initiation site; SD, splice donor site; Psi, major packaging signal.
[b] for repeated abbreviation please refer to note for Table 2; mr-AOMP, oligo-2'-O-methylribonucleosides that have alternating methylphosphonate/phosphodiester linkages; Self-stabilized ODN, oligodeoxynucleotid analog that has hairpin loop structures at 3' ends; ODN A, oligodeoxynucleotide A consists of a 25-mer antisense and a 25-mer passenger strand, connected by four thymidines (T4), phosphorothioated at each end (three bases) and in the T4 linker; pC, propynylC substituted.
[c] MAb, monoclonal antibody; "—", no delivery method used.

Table 3 Summary of effective ASO target sites in major pathogenic non-retroviral RNA viruses.

Productive ASO target region(s)[a]	**ASOs[b]**	**Delivery Method[c]**	**Ref.**
NON-RETROVIRAL POSITIVE-SENSE SINGLE STRANDED RNA VIRUS			
1. Hepatitis C virus (HCV, Family *Flaviviridae*)			
5' NCR (38-65, 134-175, 312-339 nt), core open reading frame (341-377 nt)	ODN	—	Wakita T et al, 1994
core region containing initiator codon AUG region (342-361 nt), upstream of AUG (321-340 nt), downstream of initiator AUG (346-363 nt)	PS-ODN	—	Mizutani T et al, 1995
5' NCR (from HCV 355 to 364-374 nt)	DNA, 2'-F, PS	—	Lima WF et al, 1997
5' NCR: core coding region (371-388 nt)	PS-ODN	—, lipofection method	Alt M et al, 1997
5' NCR: 3' end of the NCR (326-348 nt)	PS-ODN		Alt M et al, 1995
5' NCR: 3' end of the NCR (326-348 nt)	B-, M-,S-ODN		Alt M et al, 1999; Caselmann WH et al, 1997; Caselmann WH et al, 2000
5' NCR: (326-342 nt)	modified AS-ODN	Cholesterol/ bile acids conjugated	Lehmann TJ et al, 2001/ Lehmann TJ et al, 2001
NS3 protease RNA (1916-1950 nt)	AS-ODN	—	Heintges T et al, 2001
stem loop of 5' NCR (260-279 nt)	PS-ODN	—	Hanecak R et al, 1996
initiator codon region of 5' NCR (330-349 nt)	PS-ODN		

initiator codon region of 5' NCR (340-359 nt)	2'-Methoxye-thoxy ON		
initiator codon region of 5' NCR (330-349 nt)	PS-ODN, 5-methylcytidine modified PS-ON	—	Zhang H et al, 1999 Amin MA et al, 2005
domain IV of the IRES region in 5' NCR (containing the translation initiation codon, 345-365 nt)	MPO	—	McCaffrey AP et al, 2003
	5-methylcytidine-modified PS-ON		Soler M et al, 2004 McHutchison JG et al, 2006; Witherell GW, 2001
stem loop III of IRES region in 5' NCR (260-273 nt)	2'-OMe	—	Tallet-Lopez B et al, 2003
IRES region in 5' NCR (197-207 nt, 314-330 nt, 333-349 nt)	PNA, chimeric LNA	lipid-mediated transfection	Nulf CJ et al, 2004
stem loop IIId (264-282 nt) and initiator codon region (326-348 nt) of IRES region in 5' NCR	PS-ODN	—	el-Awady MK et al, 2006
IV loop region of IRES (340-349 nt)	PS-ODN, PNA*	—	Alotte C et al, 2008
		lipid-mediated transfection	
stem loop III region at 5'-end of X-RNA (1-17 nt)	PNA	CPP	Ahn DG et al, 2011b
25-40 nt of IRES (1-350 nt) containing the distal and proximal miR-122 binding sites	LNA	—	Laxton C et al, 2011
2. West Nile virus (WNV, Family *Flaviviridae*,)			
5'-terminal (first 20 nt at 5'End) or the 3'-terminal element involved in a potential genome cyclizing interaction (3'CSI)	PMO	Arg-rich peptide $R_5F_2R_4$	Deas TS et al, 2005; Deas TS et al, 2007
G-rich region of RCS2/CS2, CS1 near 3'-terminus	PS-ON with partial deoxyguano-sines	G-quartets formation	Torrence PF et al, 2006
3. Dengue virus (DENV, Family *Flaviviridae*,)			
5'-terminal (first 20 nt at 5'End) 3'-terminal element involved in a potential genome cyclizing interaction (3'CSI)	PMO	Arg-rich peptide $R_5F_2R_4$	Deas TS ey al, 2005; Kinney RM et al, 2005
top of the terminal 3'stem-loop (3'SLT)	PMO	Arg-rich peptide R_9F_2C	Holden KL et al, 2006
5'SL- and 3'CS-region (22-24 mer)	PMO	Arg-rich peptide $R_5F_2R_4$	Stein DA et al, 2008
4. Coxsackie virus groups A and B (CVA and CVB, Family *Picornaviruses*)			
pyrimidine-rich tract (S(CB1)), structural protein VP(1) (S(CB4)) , initiation of translation (S(CB2))	ODN	—	Sun H et al, 2000
proximal terminus of the 5' UTR (1-20 nt) IRES region (557-576 nt) translation initiation codon AUG region including 9 nt of the 5' UTR (733-752 nt)	PS-ODN	lipofectin transfection	Wang A et al, 2001
3' proximal terminus of the 3' UTR (7380-7399 nt)		—	Yuan J et al, 2004
3' portion of IRES region (570-590 mer)	PMO	Arg-rich peptide P7	Yuan J et al, 2006
4. foot-and-mouth disease virus (FMDV, Family *Picornaviruses*)			
Second AUG of polyprotein gene	DNA and RNA	cytoplasmic microinjection	Gutierrez A et al, 1993
domain 5 of the IRES (1015-1035 nt), first and second AUG of polyprotein gene (1036-1056 nt, 1121-1142 nt)	PMO	Arg-rich peptide R_9F_2 C	Vagnozzi A et al, 2007
5. SARS-associated coronavirus (SARS Co-V, Family *Coronaviridae*)			
TRS region in 5' UTR (53-72 nt, 56-76 nt)	PMO	Arg-rich peptide R_9F_2Cor R_5F_2 R_4	Neuman BW et al, 2005; Neuman BW et al, 2006
-1 PRF (encompassing loop3, 13458-13472 nt)	PNA	$Tat_{57–49}$	Ahn DG et al, 2011a

NON-RETROVIRAL NEGATIVE-SENSE SINGLE STRANDED RNA VIRUS			
1. Ebola virus, EBOV (Family *Filoviridae*)			
near or overlapping the AUG translational start site of VP24, VP35, and RNA polymerase L	PMO	—	Warfield KL et al, 2006
translational start site region of VP35 positive-sense RNA (3136-3115 nt)	PMO	—/Arg-rich peptide R_9F_2C	Enterlein S et al, 2006
5' to, overlapping, or immediately downstream of the translational start codon of VP24	PMO /PMO*plus*	Arg-rich peptide P7/ —	Swenson DL et al, 2009
eVP24 (10331-10349 nt) and eVP35 (3133-3152 nt) transcripts	PMO*plus*	—	Warren TK et al, 2010
2. Marburg virus, MARV (Family *Filoviridae*)			
NP24 (73-95 nt)/ VP24 (10204-10224 nt) transcripts	PMO*plus*	—	Warren TK et al, 2010
3. Respiratory Syncytial Virus, RSV (Family Paramyxoviridae)			
start site for NS2 and P genes of RSV genomic RNA	PS-ODN	—	Jairath S et al, 1997
GS present at the 5' terminus of L gene mRNA to 13 nt into the coding sequence	PMO	Arg-rich peptide P7	Lai SH et al, 2008
4. Influenza A virus, FLUAV (Family Orthomyxoviridae)			
loop-forming site of the polymerase 3 mRNA	ON	undecyl residue conjugated	Kabanov AV et al, 1990
the juxtaposed sequences in the 5'-terminus of the molecule around and upstream of the initiation codon	ON	—	Vlasov VV et al, 1991
AUG initiation codons of PB2 and PA	ODN,PS-ODN	—	Hatta T et al, 1995
AUG initiation codon sites of NP protein, RNA polymerase (PB1, PB2, and PA) genes	PS-ODN	liposome	Hatta T et al, 1997
PB1, PB2, PA and NP genes	PS-ODN	liposome	Abe T et al, 1998
surrounding the translation initiation codons of the viral PB2 or PA genes	PS-ODN	Tfx-10	Mizuta T et al, 1999
AUG initiation codons of PB1, PB2, PA and NP	PS-ODN	liposome	Abe T et al, 1999
3'/5' terminal sequences	PS-ODN	—	Chen Z et al, 2000
AUG initiation codons of PB2	PS-ODN	—/DMRIE-C	Mizuta T et al, 2000
AUG initiation codons of PB2 and PA	PS-ODN	Tfx-10	Abe T et al, 2001
AUG translation start site region of PB1 mRNA, 3'-terminal region of NP viral genome RNA	PMO	Arg-rich peptide P7 or R_5F_2 R_4	Ge Q et al, 2006
viral mRNA encoding heamagglutinin protein	AS-ON	liposome	Wong JP et al, 2007
the translation start site region of PB1 or NP mRNA or the 3'-terminal region of NP viral RNA	PMO	Arg-rich peptide P7	Gabriel G et al, 2008
5'-terminal conserved sequence	PS-ODN	—	Duan M et al, 2008
the AUG translation start site region of the polymerase subunit PB1 mRNA	PMO	Arg-rich peptide P7	Lupfer C et al, 2008
the 22 terminal nt at the 3' end of the NP virion RNA			
packaging signals in the 5' end of viral PB2 RNA (2279-2293 nt)	PS-ODN	liposome	Giannecchini S et al, 2009
5' ends of segments 2 or 3 (complementary to the 3'-coding regions of PB1 and PA, respectively)	PS-ODN	liposome	Giannecchini S et al, 2011

[a] NCR, non coding region; IRES, internal ribosome entry site; RCS2/CS2, complementary sequence at the 5'- and 3'- terminus respectively for cyclization of viral RNA by base pairing; CS1, an intervening 22 nucleotide sequence following CS2 for cyclization mediation; UTR, untranslated region; eVP, ZEBOV viral protein; NP, nucleoprotein; ORF, open reading frame; GP, glycoprotein; GS, gene-start sequence.

[b] for repeated abbreviation please refer to note for Table 2; S-ODN, B-ODN, M-ODN, polar phosphorothioates (S), non-polar methyl- (M) or benzylphosphonates (B) modifications partially made in the 23-mer ODN that contained only six modified nucleotides which are located at the ODN termini or are scattered along the molecule; PMO*plus*, positively charged PMOs containing piperazine linkages within the molecular backbone.

[c] for repeated abbreviation please refer to note for Table 2; CPP, cell penetrating peptide; G-quartets formation, four consecutive guanosine residues (GGGG) at the terminus of AS-O(D)N that enhance the termini stability and cellular uptake; HIV Tat peptide, Tat48–60 (GRKKRRQRRRPPQ), Tat57–49 (RRRQRRKKR); Tfx-10 and DMRIE-C, cationic liposome ; "—", no delivery method used.

3.2. Problems in development

Many reports have documented issues currently preventing the further development of antiviral ASOs. Thus, this part is to briefly summarize these notable findings with a focus on the specific challenges listed below.

3.2.1. CPP-mediated delivery strategy

The poor intracellular uptake of ASOs is a major impediment to their use as antiviral therapeutics. As shown in Table 2 and 3, many different efficient and tissue-specific delivery systems have been evaluated in viral infection cultures, including antibody-coupled pH-sensitive liposome encapsulation, lipid-mediated carrier system, and tissue-targeting ligand conjugated system. Despite their conceptual advantages, these complex delivery systems lack *in vivo* evaluation data, and stand less superiorities in practical manipulation, when factors of systematic application in humans, manufacturing, quality control, and costs are considered. Delivery systems with simpler structures, higher efficiency, and excellent *in vivo* tolerance are more welcomed, in which the CPP-mediated delivery system and PMO_{plus}^{TM} are representative candidates.

With efficient delivery of ASOs into viral cells realized by the advent of peptide-ASO conjugate (PASO), the use of PASO as antisense antivirals has intensified. However, analyses have shown CPP is susceptible to degradation in cells, serum or tissue homogenate, which impacts its effectiveness by preventing the efficient escape of conjugates from endosomal or lysosomal vesicles [15,16]. Besides, PASOs are more poorly tolerated *in vivo*, as illustrated by the dose-dependent reductions in weight, behavioral alterations, and mild liver histopathology following repeated high dose administration in mice [17]. Thus, various attempts have been taken to introduce non-natural amino acids or other chemical changes in CPP to offset toxicity and intracellular distribution, though with varying degrees of success [16,18]. Notably, PMO*plus*^{TM}, which contain positively-charged piperazine groups along the molecular backbone (Figure 1 and [19]), has recently been shown to be well tolerated and provide improved efficacy in numerous in vivo viral infection models [20,21]. Presumably, broad implications for therapeutic development of PMO*plus*^{TM} against viruses and other highly pathogenic microbes can be highly expected.

3.2.2. Virus resistance to ASOs

Viral resistance could conceivably occur through the propagation of minority variants containing nucleotide polymorphisms that prevent the efficient binding of ASOs to their target sequences. In 1998, Mulamba GB and et al firstly reported that a HCMV mutant isolated for resistance (10-fold) to the ASO fomivirsen (ISIS 2922) exhibited cross-resistance to a PS modified derivative of fomivirsen with an identical base sequence [22]. Several reports document the rapid selection of RNA viruses containing compensatory mutations that enabled these viruses to overcome PMO antiviral activity following serial passage in cultured cells, including SARS CoV [23,24], WNV [17], poliovirus type 1 (PV1) [25], and FMDV [26]. Although the high affinity of modified ASOs may compensate for single mutation caused decrease in biding affiniy, rigorous deep-sequence-based investigations are in need to characterize the occurrence and prevalence of potentially resistant minority quasispecies in virus challenge stocks and in viruses isolated from infected humans.

3.2.3. Other pharmaco-issues

As an antiviral pharmaceutical agent described for humans, ASOs must have favorable pharmacokinetic and safety profiles. Important pharmaco-issues, including *in vivo* toxicity, biodistribution, and pharmacokinetic behavior, are needed to be addressed. Among the many ASO types, the pharmaceutical properties and pharmacological behavior of antiviral PMOs have been the most extensively assessed and reported [27]. It has been reported that various PMO produced no serious drug-related adverse events in over ten phase I and II human clinical studies [27]. However, the number of reports of significant antiviral efficacy by PMO is low, making it imperative to evaluate the pharmacologic behavior of PPMO. Antiviral regimens of PPMO were shown to cause few ill effects when delivered i.v., i.p. or i.n. to non-infected mice. Generally, PPMOs are well tolerated by animal recipients, although toxicity can be observed as the dose increases [28]. The nature of the treatment regimen may affect PPMO toxicity, e.g., no treatment-associated toxicity was observed when PPMOs were administered to healthy mice, whereas, significant toxicity was observed when PPMO treatment followed viral challenge [29]. Limited studies have reported the tissue distribution pattern of PPMO (e.g. high level in liver, kidney, and spleen, and lower level in lung and heart), which may be attributed to differences between the routes of administration and the rate of blood perfusion to an organ. The pharmacokinetic profile of PPMO was characterized by rapid distribution from the vascular space to tissues, with an initial distribution (plasma) half-life that averaged about 1.5 h and an elimination (tissue) half-life averaging about 6 h [28,30]. Also, the level of antiviral activity by ASOs administered at various times in relation to the time of virus inoculation has been addressed. Studies of PPMO in cell cultures and *in vivo* [27] indicate that the antiviral activity of PPMO diminishes in concert with the period of time that elapses after infection and before treatment. However, several *in vivo* studies showed that PPMO treatment beginning after infection did cause a significant reduction in viral replication or severity of disease [27].

4. Concluding remarks

The austere genomes of RNA viruses, as well as the highly conserved sequences of defined viral elements and/or structures involved in functional regulation of translation, RNA synthesis, mRNA capping, and virion assembly [27], are fundamentally advantagous prerequisites for antisense inhibition. Thus in all, inhibition of viral translation could therefore be expected to have severe consequences for viral replication, and those critical motifs constitute appealing targets for sequence-specific intervention of steric-blocking ASOs. However, improvement of the efficacy, specificity and delivery of antiviral ASOs witnessed in the past three decades are still lagged behind in finding their way through the FDA approval process and into the clinic. Specifically, a detailed screen for unwanted side effects are requiring larger investment of time and money.

Viruses and their host cells have evolved for millions of years, and host factors that participate in viral infection are an underappreciated target pool for antiviral therapy. Amount of evidence have shown that plenty types of host factors (i.e, genes, proteins, miRNAs, cytokines, etc.) are highly involved in viral entry, viral gene expression, virion assembly, and release. Meanwhile, viruses manipulate host factors to modulate host gene expression and defenses, thereby they have developed unique mechanisms to escape from ASOs. Thus, in addition to viral targets, inhibitory antisense reagents can be directed against host factors. Recent researches utilizing ASOs-based host-directed antiviral strategy has been applied successfully to suppress replication of several viruses *in vitro* and *in vivo*, e.g., antisense targeting host factors of heat sock protein 60 (Hsp60) [31], asialoglycoprotein receptor 1 (ASGPR1) [32], abhydrolase domain containing 2 (ABHD2) and epiregulin (EREG) [33], miR-210 and miR-199a-3p [34] for inhibiting HBV, antisense targeting of gamma interferon [35], interleukin-6 [35], interleukin-1[35], Interleukin-4, tissue necrosis factor-alpha (TNF-α) [35], and miR-28, miR-125b, miR-150, miR-223 and miR-382 [36] for inhibiting HIV, antisense targeting of TNF-α [37] [38] for inhibiting HSV-1. Notably, miR-122 inhibitors Miravirsen [39], is the first miRNA-targeted LNA drug to receive investigational new drug approval by the FDA, which will enable phase II studies for the treatment of HCV. Given the immense interest in ASO as an antiviral therapeutic modality, the coming years are likely to see an increasing range of clinical applications of ASO as an advanced and simplified system with integrated functionality.

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