Pharm Res (2011) 28:2966–2982
DOI 10.1007/s11095-011-0549-8

EXPERT REVIEW

Antiviral RNAi: Translating Science Towards Therapeutic Success

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Received: 21 April 2011 / Accepted: 25 July 2011 / Published online: 9 August 2011

ABSTRACT Viruses continuously evolve to contend with an ever-changing environment that involves transmission between hosts and sometimes species, immune responses, and in some cases therapeutic interventions. Given the high mutation rate of viruses relative to the timescales of host evolution and drug development, novel drug classes that are readily screened and translated to the clinic are needed. RNA interference (RNAi)—a natural mechanism for specific degradation of target RNAs that is conserved from plants to invertebrates and vertebrates—can potentially be harnessed to yield therapies with extensive specificity, ease of design, and broad application. In this review, we discuss basic mechanisms of action and therapeutic applications of RNAi, including design considerations and areas for future development in the field.

KEY WORDS antiviral · gene therapy · RNA interference (RNAi) · viral escape

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INTRODUCTION

Viruses are known for their genomic economy and prolific ability to mutate. Related to the former, instead of encoding large numbers of their own factors, viruses rely on a number of host factors to mediate their replication (1–6), a property that limits the number of viral molecules that can be therapeutically targeted, particularly by small molecule therapies. Furthermore, mutation rates as high as 10^{-3} errors per nucleotide per genome replication (7) rapidly endow viruses with impressive sequence diversity, which allows them to evade both host immune responses (8) and sample mutational paths that may yield strains resistant to antiviral therapeutics (9). As a result of the increasing incidence of resistance to the limited number of antiviral drugs (10,11), novel therapies must be explored—particularly ones that are readily designed and tested.

RNA interference (RNAi) is a recently discovered, evolutionarily conserved mechanism for regulating gene expression that has the potential to be harnessed as a therapeutic alternative to antiviral small molecule drugs. This process, in which cells can be primed to identify and degrade RNA in a sequence-specific manner, was first observed in petunias (12). Shortly thereafter, a similar phenomenon was found to have natural antiviral activity in plants (13,14), and the pathway responsible for this activity was identified in *C. elegans* as RNAi (15). The natural antiviral activity of RNAi demonstrated in *C. elegans*, *D. melanogaster*, and *A. aegypti* (16–18)—and importantly conservation of the pathway in vertebrates (19)—makes RNAi a particularly attractive antiviral therapy strategy. In this review, we focus on therapeutic applica-

tions of antiviral RNAi. We will discuss design considerations, including modes of expression and delivery strategies. We will also consider shortcomings in using RNAi as an antiviral therapy and how these challenges may be addressed in therapy design and translation to the clinic.

MECHANISM

The RNAi pathway is a highly conserved cellular mechanism for regulating gene expression. In invertebrate animals (Fig. 1a), long double-stranded RNA (dsRNA) is processed into ~22-nucleotide (nt) short interfering RNA (siRNA) duplexes by a cellular RNase-III enzyme called Dicer (20). One strand of this duplex is loaded into Argonaute as the guide strand to create an active RNA-induced silencing complex (RISC), and the choice of this strand is made based on the thermodynamic properties of the siRNA duplex (21,22). The guide strand is then used to direct RISC to complementary RNAs. Perfectly complementary RNAs are "sliced" by the RNase H-like activity of Argonaute (23), the major component of RISC (24). In vertebrates (Fig. 1b), the presence of long dsRNA precursors induces a non-specific immune response in addition to RNAi (25,26), and RNAi can be specifically triggered by directly introducing siRNA duplexes to cells (27) or through expression of smaller RNAs, such as short hairpin RNAs (shRNAs), that can be processed by Dicer into siRNA duplexes (28).

In a pathway with many similarities to RNAi, endogenously expressed microRNAs (miRNAs) also serve to regulate gene expression in plants and animals (29–31). miRNAs are generally expressed as longer hairpin RNAs called primary miRNAs (pri-miRNAs) and contain several mismatches in the ~33-nt stemloop (32). This pri-miRNA is processed by the cellular complex of Drosha and DGCR8 in animals (33) to generate a shRNA-like molecule called a precursor miRNA (pre-miRNA). The pre-miRNA is then processed by Dicer (34) and loaded into RISC in a manner similar to siRNAs (22). A defining property of miRNAs is that there are typically mismatches between the miRNA guide and the target mRNA, and as a result, miRNAs generally regulate gene expression through repression of translation instead of direct cleavage of the target (35,36), though RNA degradation can occur via alternate mechanisms such as decapping and deadenylation (37).

The sequence-specific nature of RNAi provides several advantages in developing antiviral treatments. First, since RNAi acts at the nucleotide level, the only information necessary to begin designing RNAi therapeutics is the target sequence itself, a consideration that can accelerate the rate at which potential therapies enter a drug development pipeline. Second, antiviral small molecules generally act by specific biochemical inhibition of a viral

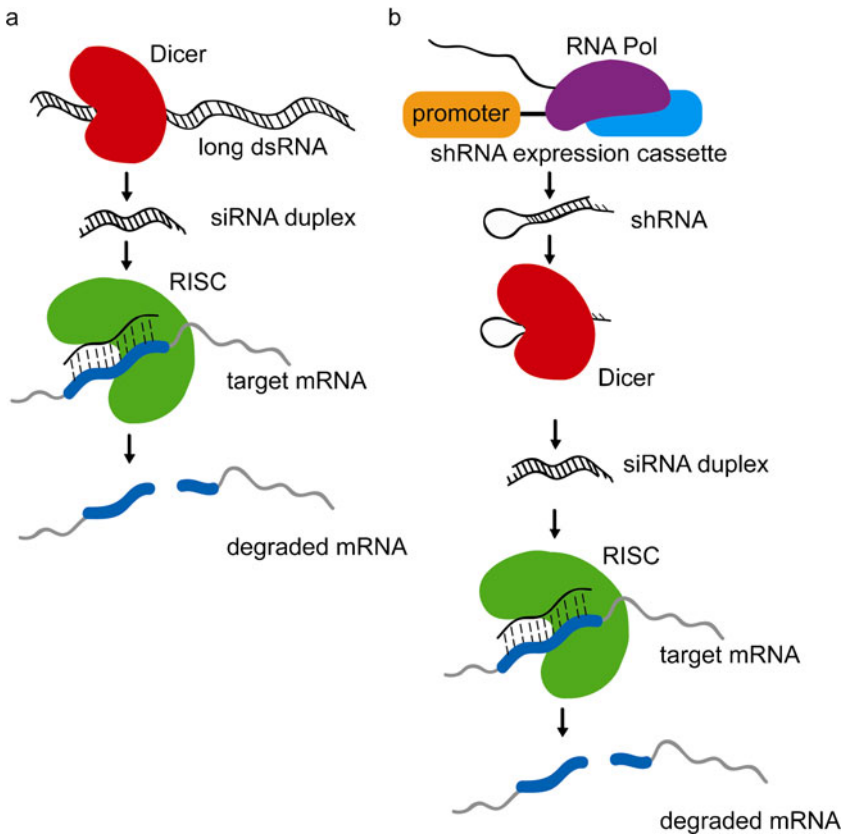

Fig. 1 RNAi mechanism. (**a**) In plants and invertebrates, long dsRNA is processed by Dicer (*red*) into 22-nt siRNA duplexes. The guide strand is chosen based on the thermodynamic properties and loaded into RISC (*green*). RISC then degrades complementary RNAs (*grey* with *blue* target). (**b**) In vertebrates, long dsRNA induces an interferon response. RNAi must be triggered by directly introducing siRNA duplexes or using RNA expression cassettes that produce shRNAs. The shRNAs are processed by Dicer into siRNA duplexes.

protein function, but accompanying non-specific interactions with host factors can result in detrimental side effects that negatively impact long-term patient health and compliance (38). RNAi can certainly have off-target effects through interactions with host mRNAs close in sequence to the viral target (39). However, unlike small molecule-protein interactions, nucleotide sequence and Watson-Crick base pairing provide a straightforward means to differentiate between the host (whose genome sequence is of course now known) and pathogen, and in principle a more specific inhibition of viruses could potentially be achieved and reduce the incidence of side effects.

DESIGN OF THERAPEUTIC RNAi

Viruses infecting a variety of hosts, ranging from mosquitoes to humans, have been therapeutically targeted by RNAi (Table I). The ability of RNAi to target all types of viral genomes (ssDNA, dsDNA, RNA(+), RNA(−) and dsRNA) means this versatile mechanism could be harnessed very broadly as an antiviral therapy. While RNAi targeting of many factors has shown inhibition of viral replication, when translating from proof of principle toward the clinic, it has been helpful to tailor an antiviral RNAi therapy based on the biology of the host-pathogen interaction. Below, we describe design considerations in developing such therapies.

Target Choice

It is ideal to choose an RNAi target that is essential for viral replication, which could be a viral or host factor. When targeting viral factors, it is important to consider at what point in the viral life cycle the target will be accessible for RNAi-mediated degradation. For example, in addition to using RNAi to target newly transcribed viral mRNAs, viruses with positive sense RNA genomes, such as hepatitis C virus (HCV) and human immunodeficiency virus (HIV), may be susceptible to degradation even earlier, during their initial infection and entry into a cell (40). In addition, alternative splicing of the viral genome may mean that some viral targets are present in all splice variants, while some are only present in early or late gene expression (41). Furthermore, recent computational modeling suggests that targeting regions that are transcribed early or included in many transcripts can help to maximize the impact of therapeutic RNA by degrading viral RNA early and often (42,43). Although viral factors represent clear targets that are likely to be essential to replication and are readily distinguished from host factors, the rapid evolution of viruses means that these targets can be mutated and selected for resistance to RNAi. While we will discuss viral resistance to RNAi in more detail below, two options to avoid resistance are to target a highly conserved viral factor or a less mutable host factor.

The primary advantage of a host target is that its sequence is constant relative to the rapidly evolving viral genome, though one clear complication is that it may be required for host function and health. Some host factors may not be essential, as is the case with inhibiting CCR5 expression in HIV infection (44,45). In other cases, host factors essential for maintenance of cell health can serve as effective targets if the level of knockdown required to inhibit viral replication does not affect the endogenous function of the factor, as was the case *in vitro* with knocking down the transcription elongation factors Cdk9 and CyclinT1 to suppress HIV infection (46). Nonetheless, when inhibiting host factors, careful screening for undesirable side effects should be conducted in cell culture and animal models prior to implementation in humans. Finally, as mentioned above, modeling efforts suggest that targeting early points of the viral life cycle is essential for therapeutic efficacy (42,43), and host factors used for viral entry or genome replication represent additional options that act early in the viral life cycle.

With the maturation of high throughput screening methods, a number of large-scale RNAi screens have recently been conducted to identify host factors involved in viral infections for HIV, Influenza A, HCV, and West Nile virus (WNV) (1–6), and hits emerging from these screens could feed into target validation efforts. In addition, expanding the types of viruses and the host cell lines used for such screens will increase the number of potential host targets for antiviral RNAi therapy, improve confidence in the screens when common hits emerge in multiple screens (47), and provide insights into whether particular factors are or are not essential to the healthy function of host cells.

Upon choosing a host or viral target for knockdown, a specific 19 to 22-nt sequence must be identified. Secondary structure of the target RNA, as well as the thermodynamic stability of both the siRNA duplex and guide-target duplex, can greatly affect the efficacy of RNAi. Generally, the RNAi target should be as unstructured as possible, particularly at the termini of the ~22-nt target region, in order to improve target accessibility to RISC (48,49). Additional thermodynamic properties associated with active siRNAs include low GC content, asymmetric instability of the siRNA duplex (meaning an effective duplex has lower internal stability at the 5′ end compared to the 3′ end of the antisense strand (50)), and asymmetric interactions between the guide strand and the target RNA within RISC (such that the 5′ end of the guide stand is responsible for surpassing a thermodynamic threshold for stability with the target RNA for effective degradation (48)). An optimal target will also have minimal off-target effects, just as an ideal small molecule pharmaceutical will have minimal interactions with targets beyond those therapeutically

Table I Plant and Animal Viruses Targeted with RNAi. Virus Name, Target Gene(s), Mode of RNAi Induction and Biological System are Listed

Virus[a]	Target	Mode	Model	Reference
ALCV	L Polymerase, Z mRNA	si-, shRNA	HEK 293T, Vero, A549	(150)
ALV	tvb, env(B)	shRNA	DF-1 cells	(151)
BDV	VP1	shRNA	Vero cells	(152)
Coxsackie	CRE (2C)	shRNA	BALB/c	(153)
Coxsackie	3C pro	siRNA	Rhabdomyosarcoma cells	(154)
Dengue	pre-membrance CDS	IR RNA	Aedes aegypti	(64)
Dengue	Env	siRNA	Human DCs, NOD/SCID humanized mice	(155)
EAV	ORF1, ORF2b, ORF7	si-, shRNA	APH-R, BHK-21 cells	(156)
EBV	Zta	shRNA	NA NPCs, 293A	(157)
ENT-70	3Dpol	siRNA	Rhabdomyosarcoma cells	(158)
ENT-71	3′UTR, 2C, 3C, 3D	siRNA	Rhabdomyosarcoma cells	(159)
ENT-71	3Dpol	si-, sh-, lhRNA	Suckling mice	(160)
ENT-71	VP1, VP2	siRNA	Rhabdomyosarcoma cells	(161)
FMDV	1D, Pol3D	shRNA	Guinea pig, Pigs	(81)
FMDV	VP1	shRNA	BHK-21 cells, suckling mice	(162)
FMDV	3P, 3D	siRNA	BHK-21 cells	(163)
GB Virus B	5′ UTR	siRNA	Marmoset primate model	(72)
HBV	HBsAg/POL	shRNA	HepG2.2.15 cells	(164)
HBV	pre S2/S	shRNA	Huh-7 cells	(165)
HBV	Core, Pol, X	shRNA	C57BL/6J, NOD SCID mouse model	(166)
HBV	X, Core	shRNA	Huh-7 cells	(167)
HBV	Pol, X	shRNA	Huh-7,	(168)
HBV	HBsAg, pre-genomic RNA	shRNA	HepG2.2.15 cells, BALB/c	(169)
HCMV	UL54, IE2	siRNA	primary fibroblasts, U373 cells	(170)
HCV	5′ NTR, IRES	siRNA	En5-3, 2-3c cells	(171)
HCV	IRES, NS5b, CD81	shRNA	Huh-7 cells	(111)
HCV	NS3, NS5B	siRNA	Huh-7 cells	(172)
HCV	NS5B	siRNA	Huh-7 HCV Replicon cells	(173)
HCV	C	shRNA	HepG2	(83)
HCV	La, PTB, hVAP-33	shRNA	Huh-7 cells	(174)
HCV	NS5b	siRNA	Huh-7	(96)
HCV	IRES, NS5b, CD81	shRNA	Huh-6, Huh-7, NOD/SCID mice	(107)
HCV	E2, NS3	tsiRNA	Huh-7	(175)
HCV	IRES, NS5b	siRNA	Huh-7, HepG2, HeLa	(176)
HEV	helicase, replicase, 3′CAE	shRNA	HepG2	(177)
HIV-1	Tat	shRNA	H9 cells	(94)
HIV-1	Gag	siRNA	PBMCs	(178)
HIV-1	TRBP	siRNA	HeLa cells	(99)
HIV-1	Nef	shRNA	SupT1 cells	(179)
HIV-1	Tat	shRNA	human	(66)
HIV-1	ALIX, ATG16, TRBP	shRNA	HEK293T, SupT1 cells	(100)
HIV-1	RT	siRNA	HeLa	(126)
HIV-1	Vif, TAR, Nef	siRNA	Magi cells, PBMCs	(40)
HIV-1	CCR5	siRNA	BLT mice	(84)
HIV-1	TAR	shRNA	SupT1s	(89)
HIV-1	Tat	shRNA	CD34+ HSCs	(180)
HIV-1	LTR, Gag, Pol, Vif, Tat, Env, Vpu	shRNA	HEK 293A	(112)
HIV-1	LTR, Gag, Pol, Vif, Env, Nef	siRNA	HeLa	(109)
HIV-1	Pol, U3	sh-, lhRNA	MT-4, PBMCs	(181)

Table 1 (continued)

Virus[a]	Target	Mode	Model	Reference
HIV-1	Gag, Pol, Vif, Rev, Env, Gag	shRNA	HeLa, HEK293FT	(58)
HIV-1	Tat, Nef, LTR, Pol	lhRNA	HEK 293T	(121)
HIV-1	5′ UTR, Gag, Pol, Tat/Rev	shRNA	SupT1, PBMCs	(110)
HIV-1	5′ UTR, Gag, Pol, Vif, Tat/Rev Nef/LTR	shRNA	SupT1	(182)
HIV-1	CycT1, CDK-9	siRNA	HeLa	(46)
HIV-1	CXCR4, FasL	siRNA	SX22-1, HEK293-005	(101)
HIV-1	PARP-1	siRNA	HeLa, J111	(102)
HIV-1	Arp2/3	shRNA	HEK293, H9 cells	(104)
HIV-1	Sam68	shRNA	HEK293T, HeLa SSKH	(105)
HIV-1	hRIP	siRNA	HeLa, HL2/3, Jurkat, primary macrophages	(183)
HIV-1	CCR5, Vif, Tat	siRNA	ND/SCID/IL2rgamma−/− Hu-PBL mice	(67)
HIV-1	CD4, CCR5, CXCR4	shRNA	Magi, PBMCs	(44)
HIV-1	Int, mut-Int	shRNA	SupT1	(123)
HIV-1	Pol, Tat, Rev, Nef	esh-, lhRNA	SupT1	(184)
HIV-1	5′LTR, Gag, Pol, Tat/Rev	shRNA	SupT1	(185)
HIV-1	Gag, Pro, Int, Tat/Rev	shRNA	SupT1	(186)
HIV-1	Env/Rev	shRNA	SupT1	(187)
HIV-1	Nef, mut-Nef	shRNA	SupT1	(124)
HPV	E6, E7	siRNA	Human cervical carcinoma cells	(188)
HPV	E6	si-, shRNA	HeLa	(189)
HRV-16	5′UTR, VP1-4, 2A, 2C, 3A, 3C, 3D, 4B, 5B	siRNA	HeLa	(190)
HSV-1	glycoprotein E	siRNA	human keratinocytes, *in vitro*	(191)
HTLV-1	Gag, Env	siRNA	HEK293	(192)
HTLV-1	TORC3	siRNA	HEK293T	(103)
HV-6B	U38 DNA Polymerase	siRNA	SupT1	(193)
Influenza A	NP, PA, PB-1,	siRNA	C57BL/6	(194)
Influenza A	M2, NP	shRNA	MDCK	(195)
Influenza A	NP, PA	siRNA	BALB/cAnNR	(196)
Influenza A	PB1, PB2, PA, NP, MP	siRNA	A549 cells	(197)
Influenza A	NP, M2	siRNA	MDCK cells, BALB/c	(198)
Influenza A	Caveolin-1	shRNA	MDCK cells	(106)
JEV	Env	si-, shRNA	BHK-21, Neuro2A, Vero cells, BALB/c	(67)
JEV	Env	siRNA-peptide	Neuro2A cells	(73)
Marburg	NP, VP35, VP30	siRNA	HeLa CCL-2, Vero cells	(199)
MDV	gB, UL29	shRNA	chicken embryo fibroblasts, chickens	(119)
Monkeypox	A6R, E8L	siRNA	LLC-MK2 cells, *in vitro*	(200)
NDV	Matrix	shRNA	Chicken embryo fibroblasts	(201)
ONNV	P3	dsRNA	Anopheles gambiae	(147)
Parainfluenza	P	siRNA	A549 cells, BALB/c	(62)
Parainfluenza	F, HN	siRNA	A549 cells	(202)
PCV-1	Rep	shRNA	PK15 cells	(203)
PCV-2	ORF1, ORF2	shRNA	PK15 cells, BALB/c	(204)
PCV-2	Rep	shRNA	PK15 cells	(203)
PEMV	2C, 2B, 3C and 3D	siRNA	BHK-21 cells	(205)
Polio	Capsid, P3	siRNA	HeLa S3, P19 mouse carcinoma cells	(206)
Polio	Capsid, P3	siRNA	HeLa S3, MEFs	(95)
PPV	P1, HC-Pro	IR RNA	Nicotiana benthamiana	(63)
PRRSV	ORF 7	shRNA	MARC-145	(207)
Rabies	Nucleocapsid	shRNA	Neuro2A cells	(208)

Table I (continued)

Virus[a]	Target	Mode	Model	Reference
Rotavirus	VP4, VP7	siRNA	MA104	(209)
Rotavirus	VP4	siRNA	MA104	(210)
RSV	P	siRNA	A549 cells, BALB/c	(62)
RSV	Nucleocapsid	siRNA	human	(143)
RSV	P,F	siRNA	A549 cells	(211)
SARS-CoV	Replicase 1A	siRNA	FRhk-4 cells	(212)
SARS-CoV	S, E, M and N	shRNA	FRhk-4 cells	(213)
SARS-CoV	RdRP	shRNA	293, HeLa, Vero-E6 cells	(214)
SARS-CoV	Leader, TRS, 3′UTR, Spike	siRNA	Vero E6	(98)
SARS-CoV	ORF1b, ORF2	siRNA	FRhk-4 cells	(215)
VSV	M, RdRP	siRNA	HEp-2	(202)
WNV	3′ UTR	siRNA	Vero cells, *in vitro*	(216)
WNV	Nucleocapsid	si-, shRNA	BHK-21, Neuro2A, Vero cells, BALB/c	(217)
YHV	Protease, Polymerase, Helicase	dsRNA	Shrimp Primary Cells	(218)

[a] *ALCV* Arenavirus Lymphocytic Choriomeningitis Virus, *ALV* Avian Leukosis Virus, *BDV* Bursal Disease Virus, *ENT-70* Enterovirus 70, *ENT-71* Enterovirus 71, *EBV* Epstein Barr Virus, *EAV* Equine Arteritis Virus, *FMDV* Foot and Mouth Disease Virus, *HBV* Hepatitis B Virus, *HCMV* Human Cytomegalovirus, *HCV* Hepatitis C Virus, *HEV* Hepatitis E Virus, *HV-6B* Herpesvirus 6B, *HSV-1* Herpes Simplex Virus 1, *HIV-1* Human Immunodeficiency Virus 1, *HPV* Human Papilloma Virus, *HTLV-1* Human T Lymphotropic Virus 1, *HRV-16* Human Rhinovirus 16, *JEV* Japanese Encephalitis Virus, *MDV* Marek's Disease Virus, *NDV* Newcastle Disease Virus, *ONNV* O'nyong nyong virus, *PCV-1* Porcine Circovirus 1, *PCV-2* Porcine Circovirus 2, *PEMV* Porcine Encephalomyocarditis Virus, *PPV* Plum Pox Virus, *PRRSV* Porcine Reproductive and Respiratory Syndrome Virus, *RSV* Respiratory Syncytial Virus, *SARS-CoV* Severe Acute Respiratory Syndrome Corona Virus, *VSV* Vesicular Stomatitis Virus, *WNV* West Nile Virus, *YHV* Yellow Head Virus. This Table is Not Comprehensive, But is Meant to Provide Both Breadth and Depth of Viruses Targeted by RNAi in the Last 10 Years

desired (39). Checking for sequence homology between the target and the host genome can minimize these effects. Furthermore, by leveraging the degree to which a specific position and the nature of mismatch between the guide strand and target RNA can affect siRNA activity and specificity, one can potentially distinguish the target from off-target regions that exhibit high degrees of sequence similarity (51).

Computational efforts can also aid in improving target prediction. There are a number of general-use websites for siRNA design developed by industry and academia that consider thermodynamic requirements of the siRNA and potential off-target effects, though these resources are not designed to address the need to target highly conserved regions in viruses to mitigate viral escape. Two design services have been developed to fill the niche for antiviral RNAi therapy design. siVirus is a web-based application that streamlines antiviral RNAi design by implementing several algorithms for creating functional siRNAs, targeting highly conserved regions of the virus, and considering potential off-target effects (52). Currently, the software has sequence data to identify highly conserved regions of HIV, SARS, HCV, and influenza, and as second and even third generation sequencing technologies lower the cost of large-scale sequencing (53), the known genetic diversity of many viruses and the capabilities of siVirus will presumably grow. Another freely available piece of software specifically for viral siRNA design is CAPSID (54), which searches for active siRNAs with minimized potential off-target effects in highly conserved regions of the viral genome, with guidance from user-provided sample virus sequences. This flexibility means CAPSID is more widely applicable to different viruses; however, limited user-provided sequence data could bias the results. It should be noted that the impact of computational modeling on antiviral siRNA design is not limited to target selection, and additional insights that other classes of computational work have provided in addition to siRNA sequence selection will be discussed below.

Library approaches can also be used to identify effective targets that do not necessarily conform to the general thermodynamic and structural rules outlined above by probing every possible 19 to 22-nt target individually. The ever-decreasing cost of oligonucleotide synthesis and multiplexing technology brings the potential for complete siRNA coverage of shorter viral genomes into reach (55,56). Alternatively, methods for generating such libraries using enzymatic approaches have been developed such that near complete siRNA coverage can be achieved by processing genomic DNA or cDNA into shRNAs using a combination of specialized restriction endonucleases and loop adaptors (57–61).

Method of Induction

The method of RNAi induction, or the mechanism by which the RNAi pathway is triggered for therapeutic purposes, can also have considerable impacts on efficacy, depending on the type of infection being treated. As mentioned earlier, RNAi can be triggered using a variety of effector agents, including synthetic siRNAs and gene-encoded shRNAs, and the choice among such options can have important implications for therapeutic applications. For example, acute, existing infections can be treated by administration of synthetic siRNAs, though delays in the initiation of treatment following the initial infection can limit the therapeutic benefits (62). On the other hand, treatment of chronic infections and prophylactic use of RNAi to prevent the inception of a viral infection may require sustained induction through repeated delivery of synthetic siRNAs or possibly gene-encoded effector agents such as shRNAs (though the risk/benefit ratio of the latter must be considered). Such sustained induction could be accomplished in invertebrates by genetically engineering inverted repeat RNA (IR RNA) or siRNA expression cassettes into the host cell genome (63–65). For vertebrates, sustained induction will likely be accomplished by gene-encoded siRNA expression in genetically engineered cells (66) or periodic administration of synthetic siRNAs (67,68). Depending on the nature of the host (invertebrate *versus* vertebrate), the nature of the infection (acute or chronic), the treatment objective (preventative or pre-existing), and the necessity and difficulty of maintaining sustained expression (single or repeated administration), one method of RNAi induction may be more desirable than another.

Method of Delivery

Once the mode of induction is chosen, a delivery strategy must also be determined. Synthetic siRNAs can simply be administered as naked RNA, though poor RNA stability can limit the efficacy of this approach. siRNA stability can be improved by chemical modification (69–71), and delivery can be enhanced by encapsulation of the siRNA in synthetic vehicles such as cationic liposomes (72), though directing the siRNAs to specific cells of interest remains a challenge. To address this problem, siRNAs have been conjugated to targeting antibodies, peptides, or aptamers that target particular cell types or infected cells (67,68,73).

For gene-encoded RNAi effectors, naked plasmid DNA administration is an option, though delivery efficiency is typically very limited. For higher efficiency, expression cassettes can be delivered via viral vectors (66). The use of viral vectors can further improve one's ability to target cells of interest. For example, lentiviral vectors can be used for systemic delivery to both dividing and non-dividing cells, while retroviral vectors limit infection to dividing cell populations such as stem cells (74). Systemic delivery generally results in targeting the liver and spleen (75), and recently discovered variants of vectors such as adeno-associated virus (AAV) can also transduce muscle and even the central nervous system upon systemic injection (76). Alternatively, direct injection into the desired tissue could aid in targeting particular cells of therapeutic interest (77). In the future, systemic or direct administration could also aid in transitioning from the *ex vivo* delivery approach recently used to generate hematopoietic stem cells protected from HIV replication (66) into a more readily administered *in vivo* delivery method. Despite their delivery advantages, lenti- and retroviral vectors can pose a risk of genotoxicity due to vector integration (78). This risk can be decreased through the use of other viral vectors, such as AAV, that provide stable gene expression from episomal viral genomes without an explicit need for integration (79), engineered retroviral vectors that exhibit zinc finger-mediated site-specific integration (80), or adenoviral vectors for transient gene expression (81). Cellular expression of siRNAs also affords further degrees of control. For example, tissue-specific and viral infection-specific promoters have been shown to drive siRNA production only in therapeutically relevant cells (82,83), thus reducing the risk of off-target effects. Delivery can be further improved by functionalization of the delivery vehicle via chemical or protein conjugation (84,85), vector pseudotyping (73,86), or engineering the viral capsid (87,88). Targeted delivery can reduce the total amount of siRNA or expression cassette required for treatment, thus reducing the potential risk of off-target effects, as well as potentially the cost of therapy.

Additional Computational Insight

Computational models of antiviral RNAi have also provided significant insights into therapy design. Two studies have considered long-term antiviral RNAi therapy and identified critical constraints on therapy parameters that could significantly affect therapy outcome. In the first investigation, a stochastic model was developed to understand how specific therapy parameters impact efficacy of and viral escape from an anti-HIV siRNA (42). This model was the first of its kind to simulate the molecular level detail of virus replication and response to therapeutic RNAi. Depending on RNAi inhibition efficiency, the model suggests that two to four targets are required to maintain long-term inhibition without escape. It also predicts that a threshold exists for the size of the reservoir of unprotected cells (cells not harboring siRNAs) tolerable for therapy success, and this prediction was subsequently validated *in vitro* (89). A more recent study simulates HIV infection in lymphoid tissue (90). This larger *in vivo*-like model evaluates efficacy in a biologically relevant engraftment efficiency range of 1% to

20% and finds that larger reservoirs of unprotected cells may not be detrimental to therapy efficacy, provided that the RNAi therapy is able to degrade incoming viral genomes prior to integration. This latter capability is debated (40,91), however, and RNAi therapy may need to be combined with another therapy capable of targeting the incoming viral genome for full efficacy. Further testing of predictions and expansion of these models to simulate other viruses and alternative modes of RNAi induction will continuously improve their predictive power and applicability.

COMPLICATIONS

Viral Escape

The ease of RNAi design can increase the number of therapies in a development pipeline by providing a large number of potential siRNA targets within a single viral gene that can readily be tested in cell culture and preclinical animal models; however, these therapies face the same challenges that conventional antiviral therapies encounter in the clinic: viral escape. A single nucleotide substitution in an RNAi target site within the viral genome can result in complete loss of interference, depending on the location and the nature of the resulting mismatch (92). In addition to nucleotide substitution in the target (Fig. 2), viral resistance has emerged by deletion of the target, indirect structural rearrangement of the targeted region (93), and even mutation of promoter/enhancer elements far from the target site to increase viral gene transcription and thereby overwhelm the RNAi machinery with elevated numbers of viral transcripts (89). Such viral escape from RNAi suppression has been documented in HIV, HCV, and poliovirus (94–96). In addition, both RNA and DNA viruses pose the additional problem of pre-existing quasispecies diversity (97,98), such that RNAi-resistant clones may already be present within the host prior to therapeutic RNA administration (9).

A number of strategies have been suggested to circumvent or prevent the emergence of resistance to RNAi. As mentioned above, viruses often co-opt many host factors to facilitate viral replication, and targeting host factors involved in viral replication instead of viral factors is likely to reduce the risk of viral escape, as a virus cannot mutate a host factor. Such a strategy has been demonstrated to inhibit replication of HIV, human T lymphotropic virus (HTLV), Influenza A, and HCV (44,84,99–107).

An effective host target is not always available or sufficient to circumvent viral escape, and another approach is to target highly conserved regions of the viral genome. Regions constrained by overlapping reading frames, important functional codons (start codons, primer binding sites), and structured nucleic acid features (IRES, packaging signals, splicing sites, export signals) may be less able to

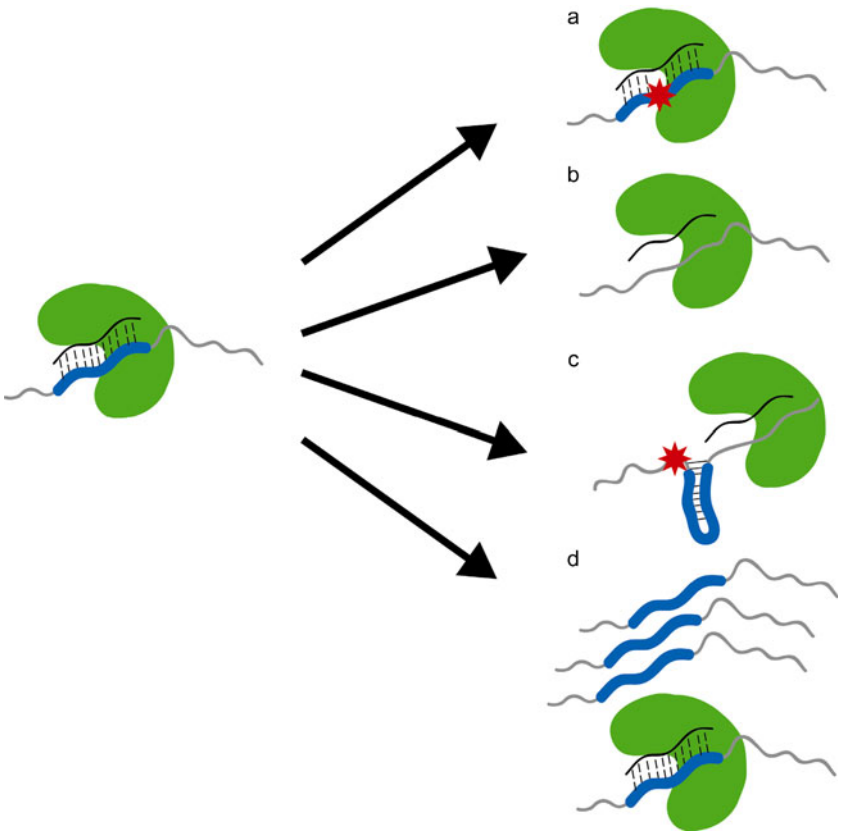

Fig. 2 Mechanisms of viral escape from RNAi. The viral RNA is shown in grey, and the region targeted by RNAi is highlighted in blue. (**a**) A point mutation (*red star*) within the target can partially interrupt base-pairing between the guide and target RNAs. (**b**) Deletion of a non-essential target can eliminate base-pairing between the guide and target. (**c**) A point mutation outside the target can result in a structural rearrangement of the target making it inaccessible to the guide strand for base-pairing. (**d**) Mutation of the viral promoter can increase the total number of viral transcripts produced by the virus and overwhelm the RNAi pathway.

tolerate mutations that compromise their function (108). A secondary advantage of targeting highly conserved regions is that the same RNAi target may be effective against multiple subtypes, serotypes, or genotypes, as demonstrated with HIV and HBV (98,109). However, as mentioned earlier, direct mutation of the viral target is not always necessary for viral escape, and targeting a highly conserved element does not guarantee long-term inhibition without escape (89).

Another strategy to reduce escape is to target multiple factors with RNAi. Such a combinatorial RNAi approach can result in synergistic suppression of viral replication, and it has, for example, also been demonstrated to delay the onset of escape in HIV (110). A multi-target approach has also been combined with the previously mentioned two strategies of targeting highly conserved viral factors and less mutable host factors to inhibit HIV and HCV replication (67,111). It has been computationally estimated that at least four RNAi targets would be required to successfully circumvent viral escape (42,112), yet maintaining expression and activity of multiple siRNAs is not trivial and can be problematic. For example, it was shown *in vivo* that high levels of exogenous siRNAs can competitively interfere with the endogenous activity of miRNAs and the natural function of the RNAi pathway (113). Additionally, competition among the various exogenous siRNAs themselves for RNAi machinery may lower the overall benefit attained from using multiple siRNAs and bias the interference towards a single siRNA that is selectively incorporated into RISC (114), thus eliminating any advantage gained from a combinatorial approach.

In the case of chronic infections or prophylactic prevention of infection, in which sustained and long-term expression is desired, production of multiple siRNAs from a single vector or plasmid can be difficult to engineer and maintain. Several strategies to achieve this have been pursued, each with its own advantages and shortcomings. The most straightforward is to include repeated expression cassettes in a single backbone, as was done for up to seven shRNA cassettes, each targeting a different HIV gene and driven by a RNA Polymerase III (Pol III) H1 promoter (112). While this strategy is elegant in the ease of its design, it suffers from difficulties in implementing the therapy, as repeated sequences can be eliminated by recombination during transduction of host cells, especially when mediated by retroviral vectors (115,116). In an effort to reduce repeated sequences, alternative promoters for siRNA production have been used in place of the traditionally used H1 and U6 Pol III promoters, including the 7SK Pol III and U1, TRE, and CMV Pol II promoters (116,117). While these options reduce the levels of repeated sequence present in any single construct, each promoter may require fine-tuning for optimized expression in various hosts and tissues (118,119) and thus complicate therapy design.

Finally, multiple siRNAs can be expressed from a single promoter using long hairpin RNA (lhRNA) or polycistronic miRNA expression strategies, in which a number of siRNAs can be excised and processed from a single mRNA precursor (120,121). These strategies reduce the likelihood of cassette deletion, but the activity of each siRNA relies on efficient processing, which can actually vary with its specific placement within the extended RNA product. In addition, for a therapy with advantages in ease and speed of design, such elegant combinations may involve considerable engineering that could slow development.

An alternative delivery strategy for combinatorial therapy was recently investigated *in silico* (122), in which the individual components of the combination were either equally distributed among all cells in the population or compartmentalized into different cell subpopulations. Depending on the efficacy of the therapy and the relative fitness of resistant mutants, the compartmentalized strategy could provide therapeutic benefits similar to those of an equally distributed combination therapy, yet it avoids the extra engineering required for sustained combinatorial RNAi induction and potentially circumvents the risk of overwhelming the RNAi pathway with numerous therapeutic siRNAs in a single cell.

As an alternate strategy to combat resistance, it has been shown in some studies that evolution of resistance to specific siRNAs is due to specific mutations at particular base positions in the virus, and combinatorial delivery of multiple siRNAs whose sequences correspond to the parent virus and the most likely escape mutants could preemptively reduce the risk of viral escape (123). However, not all variant siRNAs are effective in inhibiting replication of the corresponding mutant virus, most likely due to changes in the thermodynamic characteristics of the siRNA (124). Furthermore, it is unclear whether this is the best strategy if the mutants do not exist at the start of treatment, and the corresponding siRNAs could thus compete with more effective siRNAs targeting wild-type strains.

Combinatorial strategies can also be extended to encompass other antiviral therapies. A combination of novel RNA-based gene therapies that includes RNAi, a ribozyme, and a RNA decoy is currently being tested in an important clinical trial for HIV (66). By analogy, a coxsackievirus therapy combining RNAi with an antibody that inhibits viral uptake has been tested *in vitro* (125). Combinations of RNAi and conventional therapies have also been tested *in vitro* and *in vivo*. RNAi targeting HCV was successfully combined with the traditional interferon therapy to inhibit HCV replication synergistically *in vivo* (107). Such combinations can also be advantageous in contending with existing resistance. In another example, RNAi was combined with a nucleoside reverse transcription inhibitor (NRTI) to inhibit HIV mixtures that contain wild

type and NRTI-resistant strains (126), and this may be a particularly fruitful line of application, as the resistance mechanisms to first line antiretrovirals are often well documented (9). RNAi combined with a NRTI was also shown to effectively suppress viral replication of both wild type and RNAi-resistant virus using low NRTI concentrations that alone were insufficient to inhibit wild type replication (89). Thus, RNAi can serve to lower the dosage needed for conventional therapies, decreasing the likelihood of side effects and improving the likelihood of continued patient compliance that is required to avoid the development of resistance to existing therapies.

RNAi Suppressors and Hijackers

While the natural role of RNAi as an antiviral mechanism in many organisms is considered advantageous for translating RNAi into a general antiviral therapy, this advantage can also lead to complications, as many viruses that infect these organisms have evolved mechanism to suppress RNAi or hijack the pathway for their own uses. Viral suppressors of RNAi were first discovered in plant viruses (127,128) and have since been identified in viruses infecting invertebrate and vertebrate animals (129–133), though the extent to which RNAi plays a natural role in antiviral defense in mammals and the extent to which viral proteins truly suppress this innate antiviral RNAi have been questioned (134). Nonetheless, viruses of certain hosts have evolved a variety of mechanisms to subvert innate antiviral RNAi (135–137), and it is possible that sustained RNAi treatment in these hosts may force even more evolution of the suppressors to subvert both innate and therapeutic RNAi. As final examples, it may be challenging to use RNAi as a therapy against Ebola, HIV, and La Crosse virus, in particular, because these viruses have documented RNAi suppressors that function in their mammalian hosts or mosquito vectors (129,130,133), and RNAi in conjunction with another therapy that inhibits the suppressor function may be a promising strategy.

Finally, HIV and HSV, notorious for their capacity to form latent infections, have been shown to use endogenous miRNAs or virus-derived miRNAs to modulate viral gene expression during latency (138,139). Epstein-Barr virus (EBV), human cytomegalovirus (HCMV), and SV40 have also been shown to generate viral miRNAs that are thought to play a role in the development and maintenance of latency for EBV and HCMV (140,141) and immune evasion in SV40 (142). While elucidation of such mechanisms for innate RNAi suppression, immune evasion, and latency can help to guide the development of new therapy strategies, the ability of viruses to inhibit the RNAi pathway and hijack RNAi resources for regulation of viral gene expression could limit the number of genes that could reasonably be targeted by a RNAi therapy before overwhelming this pathway.

ROOM FOR IMPROVEMENT

RNAi therapies present considerable therapeutic opportunities, and several RNAi therapies have reached the clinic in the relatively short time this class of therapeutics has been under development. To date, four antiviral RNAi therapies have entered into clinical trials (Table II), for respiratory syncytial virus (RSV), HBV, HCV, and HIV (66,143–145). These trials represent major advances in the antiviral RNAi therapy field, and they can also provide insights that may accelerate future clinical RNAi efforts.

First, initial success in translating therapies to the clinic may come from synthetic siRNAs as the therapeutic effector, at least for acute infections, as they may pose fewer risks compared to their gene-encoded counterparts. Such an approach was successfully used to protect patients from RSV infection during a clinical trial (143). On the other hand, it is desirable to treat a chronic infection such as HIV with sustained RNAi from gene-modified cells, as was recently utilized by DiGiusto and colleagues (66). Practically speaking, the more advanced drug development process for synthetic siRNAs compared to gene-encoded shRNAs may result in faster clinical evaluation of siRNAs in the short term; however, sustained expression will be necessary for a long-term solution to chronic infections such

Table II Antiviral RNAi Clinical Trials with Virus Name, Target Gene(s), Mode of RNAi Induction, Delivery, Stage of Trial, Sponsors and Year the Trial was Started

Virus	Target	Delivery and mode of induction	Stage	Sponsors	Year started	Reference
RSV	Nucleocapsid	siRNA nasal spray	Phase II	Alnylam	2008	(143)
HBV	Four different HBV targets	Intravenous injection of liposome-encapsulated shRNA expression plasmid	Phase I	Nucleonics	2007	(144)
HCV	miR-122	Subcutaneous injections of LNA	Phase II	Santaris	2010	(219)
HIV	tat/rev	*Ex vivo* transduction by retroviral vector	Phase I	City of Hope, Benitec	2007	(66)

as HIV. Taken together, RNAi stands to make a considerable impact in the treatment of both acute and chronic viral infections.

Additionally, as several large pharmaceutical companies exit the human RNAi therapy field (146), treating non-human hosts such as livestock may represent a test bed that provides insights to aid longer-term human therapeutic development. RNAi in non-human hosts will likely also face fewer regulatory limitations, such that therapies may be commercialized faster and alleviate concerns about viability. In a recent example, chickens were prophylactically treated to induce RNAi targeting Marek's disease virus (MDV) and shown to have increased resistance to infection (119). Such a strategy could potentially be used to combat H5N1, a particularly lethal strain of influenza that is usually transmitted to humans via close contact with poultry. Similarly, RNAi was prophylactically induced in pigs to target foot and mouth disease virus (FMDV), which also infects humans (81). Given some anatomical and physiological similarities between pigs and humans, a great deal could be learned about efficacy, delivery and long-term viability by conducting such smaller studies on non-human hosts and translating these results to humans.

There are also a number of therapeutic RNAi applications for invertebrates that could have significant impacts on public health. Mosquitoes serve as vectors for a number of tropical viruses, including Dengue virus, O'nyong nyong virus (ONNV), WNV, and yellow fever virus (YFV). Priming mosquitoes with ONNV dsRNA was shown to decrease the spread of the virus in the insect host (147), and this strategy could serve as a general mechanism to control the transmission of additional vector-borne viruses. More recently, dengue virus-resistant transgenic mosquitoes were created using inverted repeat RNA expression cassettes that specifically activate in the mosquito midgut after a blood meal (64). These mosquitoes demonstrated significantly reduced transmission of dengue virus, though the stability of RNAi expression over multiple generations decreased (148). Further research into population replacement strategies could make this elegant strategy viable (149).

CONCLUSION

Since the mechanism of RNAi was first elucidated in *C. elegans*, its use to treat viral infections has itself spread like an epidemic. The ability to design a therapy based on simple sequence information and thermodynamic guidelines has the potential to accelerate therapeutic development. Furthermore, as the number of virus-specific RNAi computational design tools grows, target prediction based on viral constraints, such as sequence conservation, will improve. Delivery remains a challenge for all RNAi therapy applications, but progress in targeting and transduction will progressively alleviate these shortcomings. Furthermore, computational efforts have elucidated important therapy parameters for clinical success, such as the number of RNAi targets and the efficiency of transduction, and can help define targets for further improvements. Finally, while RNAi targeting human viruses importantly have entered the clinic, the treatment of livestock and vectors using antiviral RNAi should not be overlooked. Not only may these applications be easier to commercialize due to lower regulatory barriers, the results could also be generalized and translated into clinical and public health benefits for people.

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