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Current Opinion in Pharmacology

RNAi therapeutics: an antiviral strategy for human infections

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Gene silencing induced by RNAi represents a promising antiviral development strategy. This review will summarise the current state of RNAi therapeutics for treating acute and chronic human virus infections. The gene silencing pathways exploited by RNAi therapeutics will be described and include both classic RNAi, inducing cytoplasmic mRNA degradation post-transcription and novel RNAi, mediating epigenetic modifications at the transcription level in the nucleus. Finally, the challenge of delivering gene modifications via RNAi will be discussed, along with the unique characteristics of respiratory versus systemic administration routes to highlight recent advances and future potential of RNAi antiviral treatment strategies.

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Current Opinion in Pharmacology 2020, **54**:121–129

This review comes from a themed issue on **Anti-infectives**

Edited by **Leonor Huerta Hernández**

For a complete overview see the Issue and the Editorial

Available online 7th November 2020

https://doi.org/10.1016/j.coph.2020.09.011

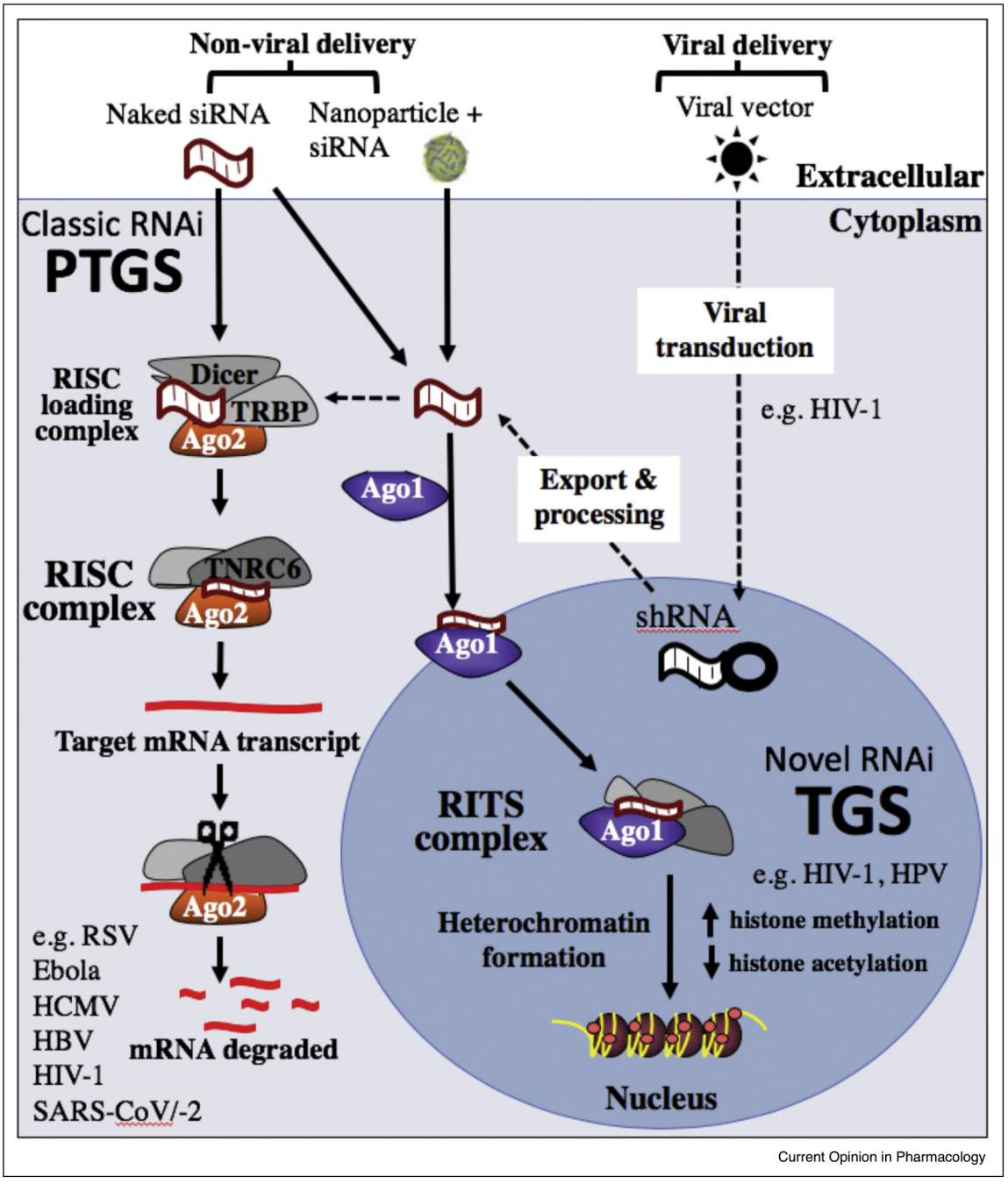
Introduction

RNA interference (RNAi) is based on the fundamental principle of short RNA sequences, typically 19–23 nucleotides, being complementary to a gene sequence and mediating-specific silencing of the targeted sequence. RNAi molecules include short interfering (si)RNA, short hairpin (sh)RNA and micro (mi)RNA, however this review will focus solely on advances in si/shRNA therapeutics. The mechanisms that generate RNAi gene silencing can be divided broadly into two pathways. The first gene silencing pathway is classic RNAi, where cytoplasmic messenger RNA (mRNA) sequences are targeted by complementary siRNA and then transiently degraded by the slicing activity of the Argonaute 2 (Ago2) protein in the RNA-induced silencing complex (RISC) [1]. In 1998, Fire and Mello discovered this conserved biological gene silencing process in the nematode, *Caenorhabiditis elegans*, which they termed RNAi [2], and for which they received a Noble prize award in 2006. The second gene silencing pathway is novel RNAi, also termed transcriptional gene silencing or epigenetic silencing, where Argonaute 1 (Ago1) protein enables complementary siRNA to enter the nucleus to target the gene promoter region and form the RNA-induced transcriptional silencing (RITS) complex, which induces heritable, repressive epigenetic modifications that silence downstream gene expression (Figure 1) [3]. This conserved biological gene silencing pathway was discovered in 1989 by Matzke in plants [4] and later in humans by Morris in 2004 [5]. Although classic RNAi is the most studied strategy for antiviral development, the novel RNAi pathway is rapidly gaining traction in this field, particularly in the chronic infections of human immunodeficiency virus type 1 (HIV-1) and human papillomavirus (HPV) (Figure 1 and Table 1).

There are currently no approved RNAi antiviral therapeutics. However, an exciting new age of RNAi therapy began in August 2018, with the first RNAi therapeutic, patisiran (Onpattro, Alnylam Pharmaceuticals) a liver targeting siRNA, which was approved by the US Food and Drug Administration (FDA) for treatment of hereditary transthyretin amyloidosis (hATTR) [6]. Following this in November 2019, a second RNAi therapeutic was approved, givosiran (Givlaari, Alnylam), to treat a rare genetic condition called acute hepatic porphyria [7]. The most recent progress was in February 2020 when the siRNA therapeutic STP705, developed by Sirnaomics, was granted orphan drug designation by the FDA for treatment of hepatocellular carcinoma [8]. Success of patisiran, givosiran and multiple other RNAi therapeutic candidates currently in clinical trials, highlights the enormous potential for therapeutic use in all forms of human disease (reviewed in Ref. [9••]), including known and emerging virus infections.

An important consideration specifically in antiviral RNAi therapies is that viruses have evolved to mutate their

Figure 1



RNAi pathways.
Both RNAi pathways can be mediated by viral or non-viral delivery of RNA sequences. Classic RNAi or post-transcriptional gene silencing (PTGS) occurs via RISC machinery initiating specific mRNA cleavage in the cytoplasm. Novel RNAi or transcriptional gene silencing (TGS) occurs in the nucleus via the RITS complex initiating repressive epigenetic modifications. Ago1, Argonaute 1; Ago2, Argonaute 2; shRNA, short hairpin RNA; RISC, RNA induced silencing complex; RITS, RNA induced transcriptional silencing complex; TRBP, transactivating response (TAR) RNA-binding protein; TNRC6, trinucleotide repeat containing 6 protein.

genomic sequence in order to evade host immune responses. This is one challenge for developing effective antivirals and vaccines, particularly for RNA viruses, for example, Human immunodeficiency virus type 1 (HIV-1) and hepatitis C virus (HCV), which have high mutation rates. Although early RNAi therapeutics relied on a single siRNA sequence to induce effective gene silencing, more recent RNAi therapies overcome this virus feature by

Table 1

Recent antiviral RNAi therapeutic development

Virus Infection	RNAi therapeutic	RNAi pathway and target	Delivery method	Stage/company	Reference
Acute					
RSV	ALN-RSV01	PTGS N protein	Naked siRNA	Phase IIb clinical trials (Alnylam)	[13,15]
RSV	TRIM25/HRSV-F, RIG-I/ HRSV-N	PTGS F & N protein, TRIM25, RIG-I	Naked siRNA	*In vitro*	[16•]
Ebola	TKM-130803; siLpol-2, siVP35-2	PTGS L polymerase, VP35	Lipid NP	*In vivo* macaque Phase II clinical trials (Tekmira/Arbutus)	[18] [7]
SARS-CoV	siSC2, siSC5	PTGS-Spike protein, ORF1b		*In vivo* macaque	[21]
SARS-CoV-2	VIR-2703 (ALN-COV)	PTGS		*In vitro* (Alnylam/Vir)	[22]
Bornavirus	TD-Borna	PTGS	Naked siRNA	In vitro	[24]
Chronic					
HIV-1	LVsh5 (Cal-1)	PTGS HIV CCR5 (& C46 fusion inhibitor)	LV shRNA	*In vivo* mouse *In vivo* macaques Phase I/II (Calimmune Inc. /CSL)	[25] [26] [28•]
HIV-1	PromA, 143	TGS-Promoter NF-kB & AP-1/COUP-TF	Naked siRNA, LV shRNA	*In vitro* *In vivo* mouse	[17•,29] [32,33,34•]
HIV-1	LTR-362, Tat/Rev, (& gp120 aptamer A-1)	TGS-Promoter, PTGS-Tat,Rev (gp120)	27 mer Dicer substrate & gp120 aptamer	*In vivo* mouse	[38]
HIV-1	HIV-1C	TGS- Promoter NF-kB sites	Naked siRNA	*In vitro*	[46]
SIV	SIV2A	TGS Promoter	LV shRNA	*In vitro*	[47]
HPV	HPV16	TGS-Promoter	Naked siRNA	*In vitro*	[48]
HPV	Si/PNPs@ HeLa	PTGS (& anti-tumour drug)	Membrane camouflaged NP	*In vitro* *In vivo* mouse	[40••]
HPV	Bcl2, BIRC5	PTGS	Magnetic Fe_3O_4 NP	*In vitro*	[39]
HBV	ARC-520, ARO-HBV/ JNJ-3989	PTGS All viral RNA	Cholesterol siRNA & NAG-MLP	*In vivo* mouse/NHP Phase IIb clinical trials (Arrowhead/Janssen)	[41] [42,44]
HBV	AB-729	PTGS	GalNAc conjugated siRNA	Phase I clinical trial (Arbutus)	[45]
HCMV	siUL54B, siUL97A, siUL122B	PTGS	Naked siRNA	*In vitro*	[49]
EBV	E1(T2)	PTGS-EBNA1	Naked siRNA	*In vitro*	[50]

using multiple siRNA sequences, in a process called multiplexing. Combining siRNAs to simultaneously target several virus and/or host targets builds in redundancy to limit the opportunity for diminished effect due to mutation in a target site. Another way to address this virus phenomena is to use novel RNAi that acts via the nuclear, transcriptional gene silencing pathway to target the virus promoter, which in theory has less opportunity for effective mutations to arise, as the driver of gene expression is silenced upstream of the error-prone transcription process [10]. Interestingly, some viruses also generate viral RNA (viRNA), which are ~22 nt, and specific RNA viruses can also encode proteins that suppress RNAi (reviewed in Ref. [11]), such as Ebola VP30, 35 and 40 [12]. This review will focus solely on exogenous siRNAs and describe the recent advances in antiviral RNAi therapeutics, using both classic and novel RNAi pathways, in acute and chronic human virus infections. We will also discuss the challenges of RNAi delivery and innovative ways nano-bioengineering has transformed this field.

Acute virus infections

An acute virus infection is defined by a relatively short infection duration, with dysequilibrium between the host and virus, which results in the virus being rapidly cleared by the host, or the host succumbing to the virus infection. Because of the short, acute infection duration, all RNAi therapeutics developed for acute infections have utilised the transient, classic RNAi silencing mechanism (Figure 1 and Table 1). It is important to note that some virus infections may have both an acute and chronic stage of infection.

Respiratory syncytial virus (RSV)

The first RNAi therapeutic to enter human proof of concept clinical trials was ALN-RSV01, developed by Alnylam Pharmaceuticals [13]. ALN-RSV01 is a single siRNA targeting mRNA of the RSV non-structural, nucleocapsid (N) protein [14]. RSV causes severe lower respiratory tract disease, with complications including bronchiolitis and pneumonia. ALN-RSV01 progressed successfully through to Phase IIb trials using a respiratory administration route for delivery of naked siRNA [15]. However, efficacy endpoints were not met and indicate the requirement for further optimisation of siRNA stability, potentially using chemically modified siRNA and a nanocarrier to enhance delivery.

A more recent bispecific approach to simultaneously target virus and host using a single siRNA sequence was reported by the Martinez group [16•]. This study used monospecific siRNAs, ALN-RSV01 (re-named HRSV-N) and siRNA targeting the fusion (F) glycoprotein mRNA, HRSV-F [16•]. Degradation of RSV F glycoprotein mRNA renders the virus unable to fuse with the host cell membrane and virus entry is effectively blocked. This study also investigated siRNA silencing of host intracellular immune signalling molecules, tripartite motif-containing protein 25 (TRIM25) and retinoic acid-inducible gene-I (RIG-1). Using an innovative method to simultaneously target virus and host using a single siRNA, the authors designed bispecific siRNAs targeting: F and TRIM25 (TRIM25/HRSV-F), in which the F target matched 21/21 nucleotides and TRIM25 target matched 15/21 nucleotides; or N and RIG-I (RIG-I/HRSV-N), in which the N target matched 20/21 nucleotides and RIG-I target matched 18/21 nucleotides [16•]. While siRNA HRSV-N was still demonstrated to be the best candidate for silencing expression of all viral proteins and host innate immune response [16•], the bispecific approach is an important advancement which would reduce the cost of current Good Manufacturing Practice (cGMP) for siRNA progressing into clinical trials. The bispecific approach may not be feasible for novel RNAi, in which transcriptional gene silencing is ablated by one or more mismatches in the target sequence [17•].

Ebolavirus (EBOV)

EBOV is a severe, haemorrhagic fever, with case fatality rates varying between 25–90 %. Tekmira Pharmaceuticals (now called Arbutus Biopharma) have a track record in developing RNAi therapeutics to target Ebolavirus Zaire and Kikwit strains. During the West-African Ebola virus outbreak in 2014–2016, they rapidly developed TKM-130803, a 3rd generation combination RNAi therapeutic that inhibits Ebola-Zaire (Makona) virus replication via siLpol-2 RNA targeting L polymerase and siVP35-2 RNA targeting Viral Protein 35 [18]. The TKM-130803 formulation comprises of two siRNA molecules in equal ratio, delivered intravenously in a lipid nanoparticle ~80 nm in diameter at a dose of 0.3 mg/kg/day [19]. A single arm Phase II trial in patients with high-grade Ebolavirus disease was discontinued during the outbreak in Sierra Leone in 2015, due to low probability of therapeutic benefit. However, recent pharmacokinetics (PK) analysis of the Phase II study, demonstrate the importance of collecting samples during an outbreak to enable PK modelling for adjusting dose and delivery regimens [19]. Arbutus Biopharma is now pursuing an RNAi therapeutic targeting Hepatitis B virus as described below.

Severe acute respiratory syndrome (SARS)-coronaviruses (CoVs)

SARS-CoV and SARS-CoV-2 are severe respiratory illness that can result in life-threatening complications, such as viral pneumonia and acute respiratory distress syndrome (ARDS). The current SARS-CoV-2 pandemic has reignited RNAi therapeutic development for coronavirus treatment, reviewed by Liu *et al.*, who presents a comprehensive summary of all patents relating to RNAi therapeutics targeting the original SARS-CoV [20••]. One of these RNAi therapeutics progressing to *in vivo* macaque studies used combined siRNA sequences, siSC2 and siSC5, targeting the Spike protein-coding and ORF1b (NSP12) regions of SARS-CoV, respectively, and demonstrated significant virus suppression [21]. Following the WHO pandemic declaration in March 2020, Alnylam and Vir announced a joint collaboration to screen 350 siRNAs targeting SARS-CoV-2 and host receptors ACE2 and TMPRSS (Table 1) [22]. Alnylam is a leader in RNAi therapeutics and had previously developed the antiviral siRNA ALN-RSV01 to target lung infection. Considering the likelihood of future emerging SARS-CoV a highly conserved, 'universal' RNAi therapeutic, targeting all prevalent betacoronaviruses, warrants urgent investigation.

Bornavirus

Bornavirus infections have been reported to cause fatal human encephalitis [23]. There are currently only two antiviral agents for bornavirus, ribavirin and favipiravir (T-705). A study by Teng *et al.*, identified two siRNAs; one targeting N mRNA, which is essential for bornavirus genome encapsidation, and another targeting L mRNA, which is an RNA-dependent RNA polymerase essential for viral RNA synthesis. These two siRNAs were combined in a TD-Borna cocktail and significantly reduced N and L mRNA in 293T cells (~70%), with increased reduction in virus expression when used in combination with T-705 [24].

Chronic virus infections

A chronic virus infection is one that persists in the human body over a long period. Some virus infections persist lifelong. For this reason, chronic infections may benefit from long-term/permanent RNAi. This may be in the form of novel RNAi or transcriptional gene silencing to induce

heritable repressive epigenetic modifications, or lentivirus vector delivery of classic or novel RNAi to ensure constitutive expression of the gene modification due to lentivirus integrate into the host genome. Some chronic virus infections naturally enter a latent phase and have the ability to reactivate, such as DNA herpesviruses and the RNA virus HIV-1.

Human immunodeficiency virus type 1 (HIV-1)

In the case of HIV-1, highly effective antiretroviral therapy (ART) has been developed that can control the latent virus reservoir, however this requires daily, life-long treatment and is only available to ~17 million of the ~38 million people living with HIV/AIDS. For this reason, ART is not a cure and alternate approaches are being developed. The most advanced RNAi therapeutic targeting HIV-1 infection is LVsh5/C46, termed Cal-1, which is a lentiviral vector containing a shRNA CCR5 and a C46 fusion peptide inhibitor. Cal-1 has been comprehensively characterised both *in vitro* and *in vivo* in two different animal models (mouse and macaques) and induces robust silencing of HIV-1 virus replication [25–27]. Cal-1 is currently in Phase I/II clinical trials to assess efficacy in HIV-1 patients with high-risk lymphoma [28•].

Another approach to control the latent HIV reservoir is the 'block and lock' strategy, which utilises novel RNAi therapeutics targeting the HIV-1 promoter, PromA and 143, to block virus replication and lock the virus in a deep latent state via repressive epigenetic modifications resulting from transcriptional gene silencing. This strategy has also been comprehensively characterised *in vitro* [17•,29–31] and *in vivo* [32,33,34•] and is currently being combined with LVsh5 from Cal-1 to assess the combined effect in humanized mouse models. The block and lock strategy to control HIV is reviewed in Refs. [35,36] and an animated infographic provides a graphic description [37].

A further HIV-1 combination gene therapy that has recently shown promising *in vivo* mouse model data uses an RNAi therapeutic targeting the HIV-1 promoter, LTR-362, RNAi therapeutics targeting HIV-1 Tat and Rev mRNA, and the anti-HIV-1 gp120 aptamer, A-1 [38].

Human papillomavirus (HPV)

HPV causes a range of malignancies including cervical, anal and oropharynx. Recent advances in delivery of RNAi therapeutics targeting HPV-associated malignancies involve bioengineering of nanoparticles. One study reported the development of polyethyleneimine (PEI)-modified magnetic Fe_3O_4 nanoparticles to deliver therapeutic siRNAs targeting Bcl2 mRNA and Baculoviral IAP repeat-containing 5 (BIRC5) mRNA in oral cancer cells, with significant gene silencing of both targets [39]. Another recent study demonstrated the benefits of an innovative biomimetic dual drug delivery system (Si/PNPs@HeLa) [40••]. This delivery system uses HeLa cell membranes to camouflage lactic-co-glycolic-acid (PLGA) nanoparticles, co-loaded with siRNA targeting E7 and chemotherapy drug, paclitaxel (PTX). Efficient *in vitro* gene silencing was reported in HeLa cells, as well as a synergistic anti-tumour effect in HeLa tumour-bearing mice *in vivo*, with a tumour volume inhibition rate of ~84% [40••]. This study demonstrates a novel dual-drug delivery system for cervical cancer treatment via combined chemo-gene therapy.

Hepatitis B virus (HBV)

HBV infects ~300 million people globally and is the leading cause of liver cirrhosis, liver failure and hepatocellular carcinoma. With no curative therapy available, RNAi therapeutics have been pursued as a potential functional cure. The RNAi therapeutic ARO-520, developed by Arrowhead Pharmaceuticals, comprises a combination of siHBV-74 and siHBV-77 in equimolar ratio, conjugated to liver-tropic cholesterol and is co-delivered with an excipient, hepatocyte-targeted, *N*-acetylgalactosamine-conjugated melittin-like peptide (NAG-MLP), that enables endosomal escape following cell entry [41]. ARO-HBV targets episomal covalently closed circular (ccc)DNA, due to the siRNA target sites being in the overlapping 3'end of all cccDNA HBV transcripts, and not integrated HBV DNA which do not contain the target sites. The importance of targeting both cccDNA and integrated HPV DNA became evident from Phase II clinical trial data, which demonstrated that Hepatitis B surface antigen (HBsAg) was reduced in some siRNA treated cells, presumable those containing cccDNA, but was still present in high levels in other cells, possibly those containing integrated HBV DNA [42]. This study highlights the importance of integrated HBV DNA as a target for future RNAi therapeutics. Arrowhead has teamed up with Janssen (JNJ) to develop a triple combination therapy using i) RNAi therapeutic ARO-HBV (now called JNJ-3989, which degrades all viral RNA with the addition of siHBV-75 targeting integrated HBV DNA) [41], ii) the JNJ therapeutic, JNJ-6379, a N capsid assembly modulator (CAM-N) [43] and iii) a nucleos(t)ide analogue (NA); tenofovir disoproxil (TDF) or entecavir (ETV). Recent data from a Phase 2b clinical trial assessing safety and efficacy of the triple combination therapy was presented at the American Association for the Study of Liver Diseases (AASLD) meeting in November 2019 and demonstrated the therapy was well tolerated and all chronic hepatitis B patients achieved robust reductions in HBV DNA, HBV RNA and HBsAg [44].

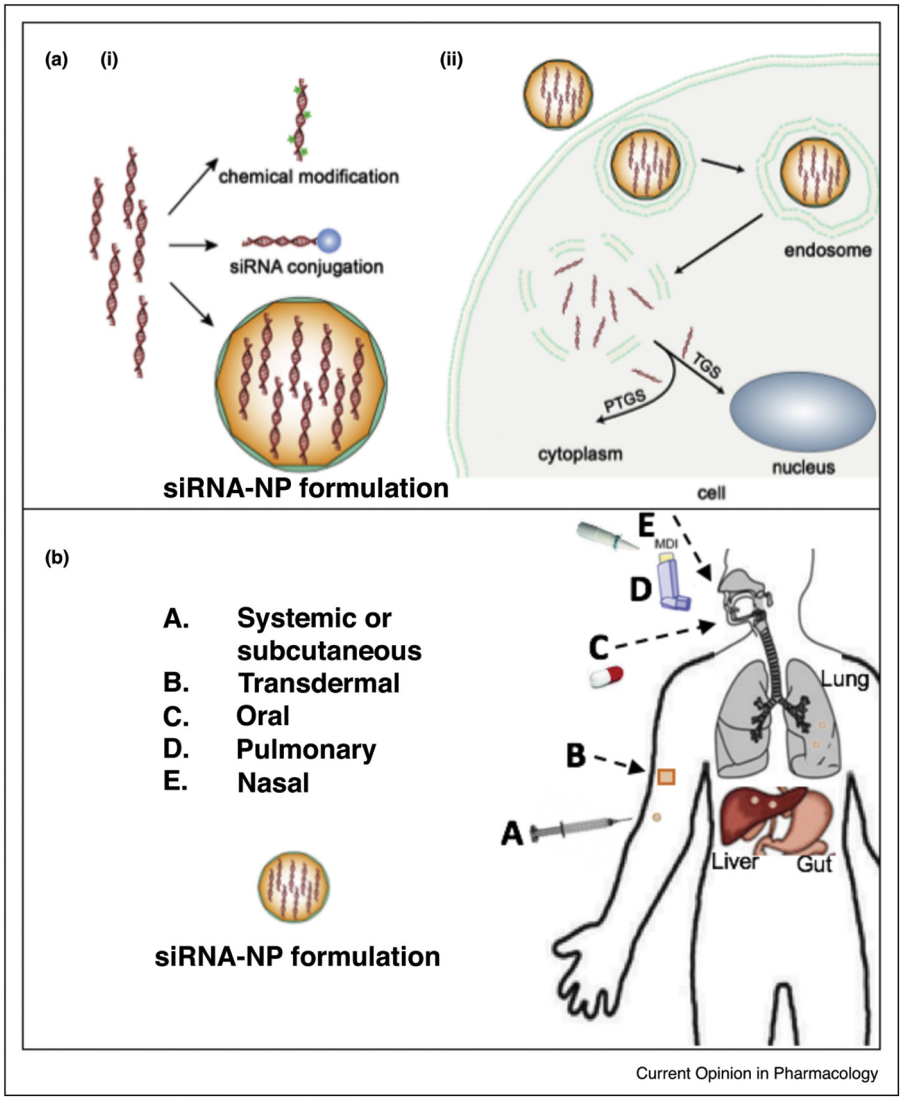
An alternate RNAi therapeutic developed by Arbutus Biopharma (previously Tekmira) is AB-729-001, which targets HBV mRNA and is conjugated to *N*-acetylgalctosamine (GalNAc) for targeted delivery to hepatocytes. Arbutus Biopharma announced positive preliminary results from an early first in human Phase I clinical trial in March 2020, with treatment being well tolerated and

showing ~1 log reductions in HBsAg levels at 12 weeks [45].

It's all in the delivery

Delivery remains a major technological challenge in the clinical translation of siRNA. Delivering naked siRNA systemically risks enzymatic cleavage and rapid clearance through liver accumulation and renal filtration [51]. Lentivirus vectors (LV) are commonly used (Table 1) but may suffer from reduced safety profile and achieve modest outcomes *in vivo*. In addition to the chemical modification of siRNA to improve nuclease resistance and decrease off-target effects and immune activation, alternative non-viral delivery methods based on nanomaterials are being investigated (Figure 2) and include polymer, lipid and inorganic [52] materials that serve to encapsulate, complex or conjugate with siRNA to improve their biodistribution, facilitate cell entry and endosomal escape. The FDA-approved RNAi therapeutic Patisiran (Alnylam) for polyneuropathy in hereditary ATTR amyloidosis is formulated in lipid nanoparticle [6], such as those used in TKM-Ebola (Table 1). The second FDA-approved RNAi therapeutic Givlaari (Alnylam) for acute hepatic porphyria is based on siRNA conjugated to *N*-acetyl-D-galactosamine (GalNAc) [7]. GalNAc-conjugated siRNA is also in Phase

Figure 2



RNA therapeutics and administration routes for nanoparticle delivery of RNAi cargo.
(a) (i) To improve delivery outcomes, siRNA can be chemically modified, conjugated to a macromolecule, or formulated within a NP. (ii) NP formulation (as well as chemically modified or conjugated siRNA, not shown) can facilitate cell entry and endosomal escape of siRNA leading to enhanced gene silencing. **(b)** Administration routes for the delivery of nanoparticle formulation with RNAi cargo.

I clinical trial for HBV (Table 1). Other promising carbohydrate-based nanoparticle platforms for siRNA include cyclodextrin [53], glycogen [54,55•], and chitosan [56].

Intravenously administered siRNA, even when formulated in nanoparticles, typically accumulate in the liver, limiting access to target sites, unless the liver itself is the target as is the case for the FDA-approved RNAi therapeutics and HBV [39,44]. Systemic RNA delivery to dendritic cells using lipid nanoparticles was recently developed for cancer immunotherapy [57]. A phase I trial of RNA-Lipid vaccines encoding four tumour antigens (NY-ESO-1, MAGE-A3, tyrosinase and TPTE) for intravenous administration recruited patients with advanced malignant melanoma [57].

RSV, SARS-CoV, and SARS-CoV-2 severely affect the respiratory system and could be ameliorated by local lung delivery of RNAi therapeutics via inhalation [58] (Figure 2). Inhalation therapy using liquid aerosol or dry powder formulations bypasses first pass metabolism and is a relatively non-invasive administration route that enables high local concentrations in the lungs [59•], hence the potential to reduce dosage and side effects compared to systemic delivery [60]. Nuclease activity is also lower in the alveolar region of the lung [60,61]. Alveolar macrophage clearance and mucociliary clearance, including the presence of lung surfactant, however, present barriers to effective pulmonary delivery. Ultimately, precise engineering of siRNA nanoformulations is needed to overcome or exploit barriers in the lung [62] or any other organs or target sites for siRNA therapeutics. Translational criteria include a fine control over nanocarriers properties including size, surface charge, encapsulation efficiency scalable manufacturing processes and adequate product stability.

Concluding remarks

RNAi therapeutic development has expanded exponentially in the 20 years since it was discovered, with two RNAi drugs approved for human treatment and many more in clinical trials. Antiviral RNAi therapeutic development has another layer of complexity due to rapid virus mutation in target sites and requires a multiplexed RNAi therapeutic approach. Despite this additional challenge, there are multiple antiviral RNAi therapeutics in Phase II clinical trials and together with advances in delivery technology, these will pave the way for breakthroughs that will directly benefit people impacted by virus infections worldwide.

Credit author statement

CA, CCJ, FCav and AK wrote the review. CA and YQ designed the figures. All authors contributed to the article and approved the submitted version.

Conflict of interest statement

GS is an employee of CSL. AK, CA and GS have patent applications for HIV-1 siRNA sequences. The remaining authors declare no conflict of interest.

Acknowledgement

This work was funded by a Australian NHMRC Project Grant. APP1128012.

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