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Small RNAs to treat human immunodeficiency virus type 1 infection by gene therapy

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Current drug therapies for human immunodeficiency virus type 1 (HIV) infection are effective in preventing progression to acquired immune deficiency syndrome but do not eliminate the infection and are associated with unwanted side effects. A potential alternative is to modify the genome of patient cells via gene therapy to confer HIV resistance to these cells. Small RNAs are the largest and most diverse group of anti-HIV genes that have been developed for engineering HIV resistant cells. In this review, we summarize progress on the three major classes of anti-HIV RNAs including short hairpin RNAs that use the RNA interference pathway, RNA decoys and aptamers that bind specifically to a protein or RNA as well as ribozymes that mediate cleavage of specific targets. We also review methods used for the delivery of these genes into the genome of patient cells and provide some perspectives on the future of small RNAs in HIV therapy.

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Introduction

Current treatment for human immunodeficiency virus type 1 (HIV) infection consists of using combination antiretroviral therapy (cART) that inhibits different steps of the viral replication cycle. Although there has been extensive progress in the development of cART over the years, supplying these drugs to infected individuals places a significant financial burden on the healthcare system. Also, these therapies induce unwanted side effects and patients must be compliant with the drugs during their entire lives as cART does not eliminate HIV infection [1].

Gene therapy is an alternative strategy that could replace cART to control HIV infection with minimal side effects and without the requirement of daily drug administration. The idea is to genetically modify an infected individual's cells so that they become resistant to HIV infection. Both $CD4^+$ T lymphocytes, the main target cells for HIV replication, and hematopoietic stem cells (HSCs), the progenitors of all blood cells, could be modified. An advantage of using $CD4^+$ T cells is that the transplant procedure is comparatively simple and should an unexpected toxicity arise in clinical trials, the cells could be more easily removed from the patient. However, as a therapy, modified $CD4^+$ T cells would need to be given repeatedly while modifying HSCs would provide long-term control of HIV replication following just one transplant. Proof of principle that this procedure could work comes from the success of clearing the virus in the body of Timothy Brown, known as the 'Berlin Patient'. This was accomplished following an HSC transplant from a donor that was homozygous for a 32-bp deletion in the gene encoding the HIV CCR5 co-receptor (CCR5Δ32/Δ32) to treat his acute myeloid leukemia [2]. Homozygosity for this mutation confers resistance to HIV infection and viral rebound following the discontinuation of antiretroviral therapy in 2007 has not been detected in Timothy Brown to date. Although allogeneic HSC transplants with or without resistant donor cells have been evaluated in several additional individuals to produce a similar outcome [3,4], further success with this method has only recently been achieved with the 'London Patient', leading to the clearance of the virus in the absence of cART for 18 months [5••]. This exact situation cannot be replicated for every person infected with HIV as homozygosity for this mutation is rare [6] and HSC transplant between individuals carries a lot of potential risks, such as graft versus host disease. Therefore, recreating the phenotype of HIV resistance by modifying an infected person's own cells with gene therapy represents a potentially safe and effective procedure to create a functional cure that mimics the situation of Timothy Brown and the London patient.

To directly mimic the procedure used for Timothy Brown and the London patient, several gene editing technologies have been designed to alter the CCR5 gene in patient cells [7–10]. While these strategies may work

for some infected individuals, results from the 'Essen Patient', who received a similar transplant as Timothy Brown and the London patient, demonstrated that viral rebound can occur due to the presence of minority variants that can use the CXCR4 co-receptor for entry [11,12•]. Therefore, CCR5 modification alone may not be sufficient for all patients and it is unknown how many patients harbor replication competent CXCR4 using viruses. An alternative is to insert antiviral genes coding for proteins, peptides and RNAs into patient cells. Advantages of RNAs for use in gene therapy are that they are typically transcribed from very small genes and they have limited potential to elicit innate and adaptive immune responses compared to proteins and peptides. While cells modified with antiviral RNAs may not be able to completely eliminate the virus, they could be used to remove the necessity of chronic drug administration, resulting in what is often referred to as a functional HIV cure. With advances in our understanding of RNA biology has also come advances in various RNA therapeutic technologies. Here, we review different classes of small RNAs that have been developed for HIV gene therapy including short hairpin RNAs (shRNAs), aptamers and ribozymes as well as delivery vectors used to insert them into patient cells.

shRNAs

shRNAs can be used as anti-HIV genes by exploiting the RNA interference (RNAi) pathway to target viral RNA or mRNAs coding for cellular factors that the virus uses such as CCR5 [13,14]. This mechanism is called post-transcriptional gene silencing (PTGS). In the cell, shRNAs are cleaved by Dicer into ~21 base pair small interfering RNAs (siRNAs), which are split up into the guide and passenger strands. The passenger stand is degraded while the guide strand is loaded into the RNA induced silencing complex (RISC) to mediate gene silencing by complementarity to a section of a given mRNA. For anti-HIV shRNAs, the guide strand will be complementary to the viral RNA or to a cellular mRNA that is crucial to the viral replication cycle. From this point, a component of RISC called Argonaute (Ago) 2 will induce cleavage of this mRNA (Figure 1a,b).

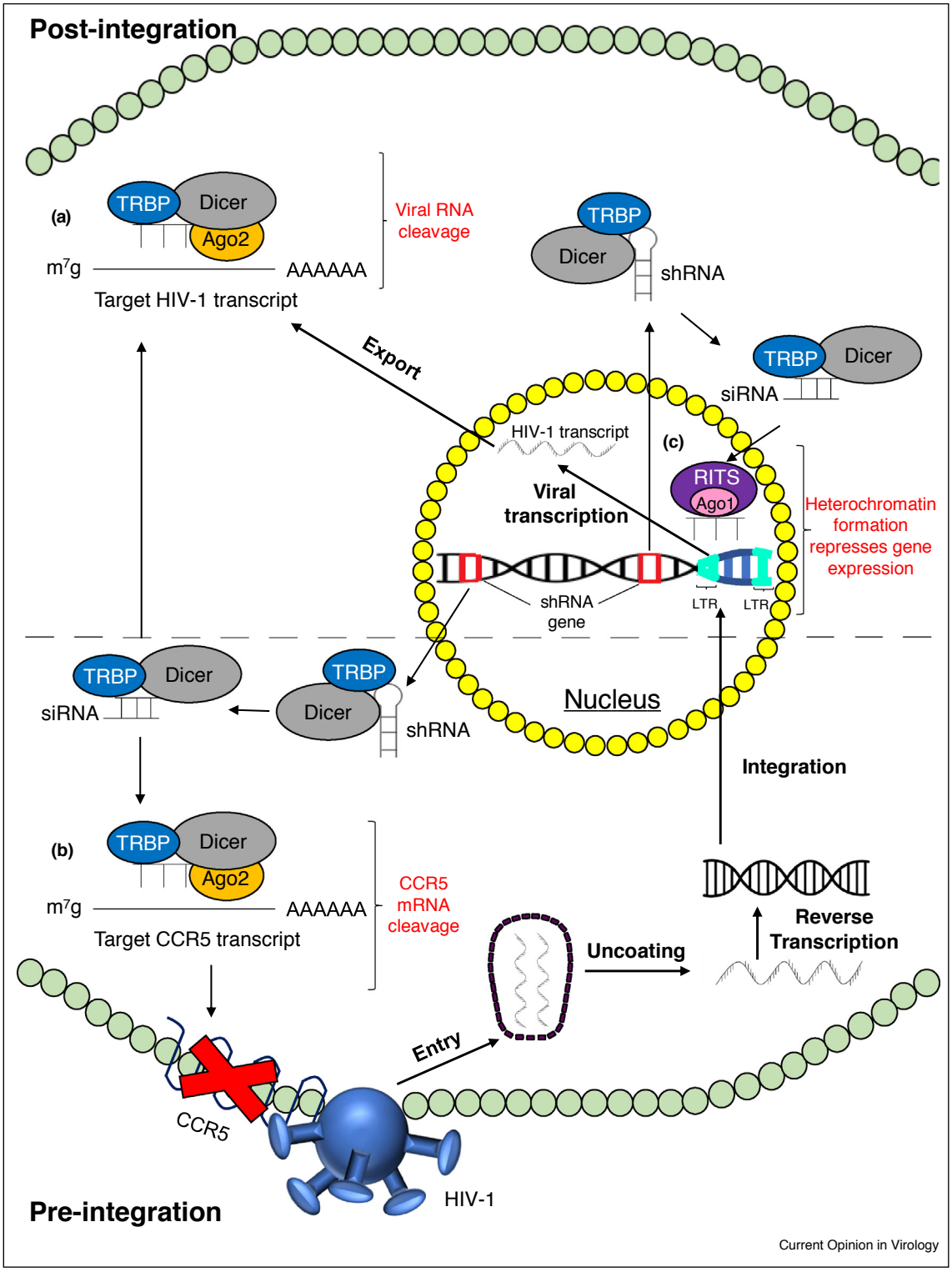
Several anti-HIV shRNAs have been designed to target HIV RNA, but resistance quickly occurs when a single molecule is used [15]. This is due to the substantial specificity of RNAi where one or two nucleotide mutations in the target site abolish the antiviral capabilities of the shRNA. An extensive screen of 86 different shRNAs yielded several shRNAs targeting highly conserved HIV sequences that inhibited viral production by greater than 75% and inhibited viral replication during long-term culture [16]. Also, co-expression of three shRNAs prevented virus escape, was safe and inhibited HIV replication in a mouse model [17]. In another screen, 96 target shRNAs were tested and 65 of them inhibited viral production by greater than 75%. The most potent targeted the long terminal repeat (LTR), *env*, *tat* or *gag* regions of HIV RNA [18]. Our lab identified a conserved target site in the *gag* coding sequence that was accessible to inhibition by an shRNA, which inhibited HIV strains from diverse subtypes. We also tested different stem lengths of this shRNA and found that a stem length of 20 or 21 base pairs was optimal for inhibition of HIV production [19•,20,21]. Another shRNA targeting a region overlapping both the *tat* and *rev* reading frames has been identified and is currently in clinical development [22,23] (Table 1). To address the potential for shRNAs to cause toxicity through saturation of the RNAi pathway, Dicer-independent or Ago shRNAs have also been designed to target HIV RNA [24]. However, in all cases, these shorter shRNAs were less potent compared to their target matched shRNAs and further optimization in their design is warranted.

Several shRNAs have also been designed to target cellular factors that are critical to the viral replication cycle and CCR5 is the most studied target [25–27] (Figure 1b). Indeed, people with non-functional CCR5 mutants have no apparent phenotype other than being resistant to HIV infection [28,29]. After initial concerns to achieve high efficacy without cytotoxicity, several shRNAs targeting CCR5 were identified that could drastically reduce CCR5 expression without causing cytotoxicity [25]. However, targeting the CCR5 mRNA alone could lead to the rebound of CXCR4 using virus that may be present as minority variants [11,12•].

To overcome this problem several shRNAs targeting CXCR4 have also been designed [26,30]. However, the CXCR4 receptor is responsible for localizing the HSCs to the bone marrow [31]. Targeting CXCR4 causes HSCs to accumulate in the blood, which would prevent appropriate maturation and differentiation, as illustrated by using a CXCR4 antagonist called AMD3100, which was later developed for use in HSC mobilization to the blood [31–33]. Therefore, interfering with either the expression or the functionality of CXCR4, will likely be harmful to patients in the long-term. Another strategy is to combine CCR5 shRNAs with other antiviral genes that could target all HIV strains [34••]. Currently the CCR5 shRNA expressed from the H1 promoter is being evaluated in a clinical trial in combination with a peptide inhibitor of viral fusion [35,36] (Table 1).

Interestingly, targeting proviral DNA by shRNAs also leads to viral suppression by inducing transcriptional gene silencing (TGS), a mechanism distinct from PTGS. In this case, the RNA-induced transcriptional silencing (RITS) complex is formed with Ago1, which prompts heterochromatin formation and transcriptional repression [37] (Figure 1c). An shRNA targeting the tandem NF-κB motifs in the HIV promoter inhibited virus replication

Figure 1



Mechanisms of shRNAs targeting HIV replication.
shRNAs are transcribed from a gene inserted into a cell's chromosome and exported to the cytoplasm where they bind to Dicer and TRBP. Dicer cleaves the loop off the shRNA to generate an siRNA which associates with Ago2. The guide strand, that is complementary to a specific target, is then used to direct Ago2 to cleave that target RNA sequence. **(a)** shRNA targeting an HIV RNA transcript. **(b)** shRNA targeting the cellular CCR5 mRNA transcript. **(c)** shRNAs targeting DNA in the HIV LTR promoter for transcriptional gene silencing. In this case the siRNA generated by Dicer

Table 1

Anti-HIV RNAs in clinical studies

Antiviral gene/gene combination	Cells modified	Delivery vector	Status	Ref
tat/rev-specific shRNA, CCR5-specific ribozyme, TAR decoy	HSCs	Lentivirus (HIV)	NCT01961063, NCT02337985, NCT00569985	[23]
CCR5-specific shRNA, fusion inhibiting peptide (C46)	HSCs and $CD4^+$ T cells	Lentivirus (HIV)	NCT03593187, NCT02390297, NCT01734850	–
CCR5-specific shRNA, TAR decoy, chimeric TRIM5α (HRH)	HSCs	Lentivirus (HIV)	NCT02797470	–
vpr/tat-specific ribozyme	$CD4^+$ T cells	Murine retrovirus	Completed	[82]
	Syngeneic $CD4^+$ T cells	Murine retrovirus	Completed	[83]
	HSCs	Murine retrovirus	Completed	[71,84]
	HSCs	Murine retrovirus	Completed	[64]
RRE-decoy	HSCs	Murine retrovirus	Completed	[85]

NCT, National Clinical Trials (ClinicalTrials.gov); Ref, Reference.

through TGS for one year following transduction of a T cell line [38] and was able to reduce HIV replication in macrophages differentiated from pluripotent stem cells transduced with the shRNA [39•]. Furthermore, the combination of this shRNA with an shRNA targeting a sequence upstream in the HIV promoter, containing AP-1 and COUP-TF binding sites, showed a reduced reactivation of the provirus in a latently infected T cell line following treatment with several known latency reactivating agents [40].

RNA decoys and aptamers

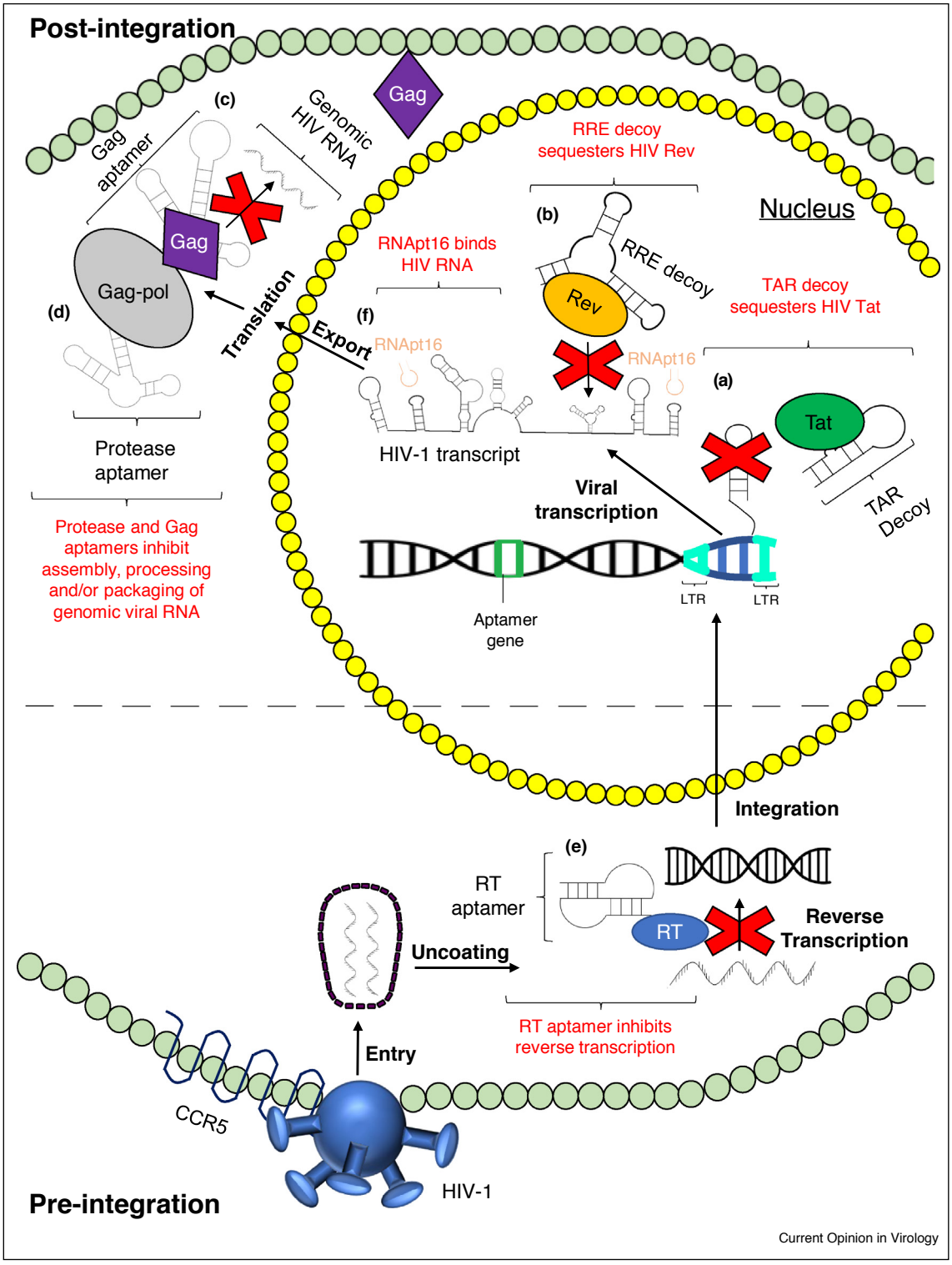
RNA decoys and aptamers are RNAs that bind to a target protein or nucleic acid due to their three-dimensional structure and/or sequence [41]. RNA decoys mimic natural RNA structures that bind to a viral protein, whereas RNA aptamers are synthesized and selected for their interaction properties. The binding interactions of both structures with viral components is used to interfere with the viral replication cycle. RNA decoys have been developed to mimic the HIV transactivation response (TAR) element [42] and the Rev response element (RRE) [43], to inhibit the function of the HIV Tat and Rev proteins, respectively. The selection of RNA aptamers has lead to inhibitors which interact with the HIV protease [44], reverse transcriptase (RT) [45•], integrase [46•], matrix and nucleocapsid proteins [47], as well as with the untranslated region (UTR) of HIV RNA [48].

During HIV replication, the viral Tat protein binds to TAR, which results in an increased rate of transcriptional initiation and elongation by the formation of an active transcription complex with RNA polymerase II [49]. By creating an RNA decoy modeled after TAR, Tat will be sequestered away from the HIV promoter and the transcription of HIV provirus will be inhibited (Figure 2a). Since Tat accumulates preferentially in the nucleolus, a chimeric molecule was created, which includes the TAR motif conjugated to the U16 small nucleolar RNA backbone and showed improvement at inhibiting HIV replication [42]. The U16 backbone helps localize the TAR decoy to the nucleolus where it can more effectively sequester Tat and the U16TAR decoy has reached clinical development along with the shRNA targeting the *tat* and *rev* reading frames [23] (Table 1). Similarly, RRE decoys have been designed to sequester the viral Rev protein. Rev binds to the RRE RNA present in the unspliced and incompletely spliced HIV transcripts, and is needed for their nuclear export [50]. RRE decoys sequester Rev away from viral transcripts, which inhibits their nuclear export (Figure 2b). A U16RRE decoy was also more effective than RRE alone against HIV production [51].

RNA aptamers are generated by systematic evolution of ligands by exponential enrichment (SELEX). SELEX involves generating a library of random RNAs that are selected for their affinity to bind to a particular target. After multiple rounds of amplification, a group of high-binding affinity RNAs are identified [41]. An RNA aptamer selected to bind Rev inhibits HIV replication by interfering with both Rev–RRE and Rev–Rev interactions [52]. The HIV Gag polyprotein and its mature matrix, nucleocapsid and capsid structural proteins are integral components of the HIV particle and are therefore excellent targets for antiviral molecules. An aptamer targeting Gag inhibited viral replication through binding to both the matrix and nucleocapsid regions and acts by competition with the viral genomic RNA for Gag binding [47] (Figure 2c). The HIV protease in the Pol region of

(Figure 1 Legend Continued) localizes with Ago1 to the nucleus and mediates heterochromatin formation. A line is drawn through the cell to distinguish between shRNAs that act at pre-integration and post-integration steps in the viral replication cycle.

Figure 2



Mechanisms of RNA decoys and aptamers targeting HIV replication.
Decoys and aptamers bind to their targets through their specific three-dimensional structure. Illustrated are decoys and aptamers that are transcribed from a gene inserted into a cell's chromosome. **(a)** TAR decoy that sequesters Tat away from viral TAR. **(b)** RRE decoy, which sequesters Rev away from viral RRE. **(c)** Gag aptamer, which competes with genomic viral RNA for binding to Gag. **(d)** Protease aptamer, which inhibits the Gag and Gag-Pol polyproteins from being cleaved by HIV protease. **(e)** RT aptamer that inhibits reverse transcription. **(f)** The RNApt16

Gag-Pol cleaves the Gag and Gag-Pol polyproteins to produce their mature components (Figure 2d) and three protease aptamers had antiviral efficacy comparable to the shRNA targeting the *tat/rev* reading frame [44]. In addition, RT aptamers inhibited viral production after transfection as well as replication in T cells (Figure 2e), suggesting that they could inhibit both reverse transcription following entry and virus production by targeting Gag and Gag-Pol during encapsidation [45•,53]. Recently, aptamers have also been designed against HIV integrase. These integrase aptamers were conjugated to the stem of the *tat/rev* shRNA to give rise to an active anti-HIV combination, where the aptamer replaces the loop of the shRNA and is therefore released following Dicer cleavage of the shRNA stem [46•]. Aptamers also target the UTR RNA of HIV transcripts. For example, a 16 nucleotide aptamer (RNApt16) binds within the poly(A) domain [48] (Figure 2f). It likely inhibits HIV replication by interfering with 3′ end polyadenylation of HIV transcripts but it may also interfere with reverse transcription or translation.

Ribozymes

Ribozymes are a class of RNAs that have catalytic activity and are involved in various processes in different organisms, such as cleaving pre-tRNAs to generate functional tRNAs [54], self-cleaving satellite RNAs [55] and self-splicing introns [56]. Several small self-cleaving ribozymes have been engineered to cleave an RNA target in *trans*, including the hammerhead, hairpin and hepatitis delta virus (HDV) ribozymes [57–59]. As with shRNAs, specificity of the cleavage target for the ribozymes is mediated by antisense binding and several have been designed to target HIV RNA or cellular mRNAs (Figure 3a,b). A potential advantage of ribozymes over shRNAs is that they act without cellular proteins and therefore have low potential to cause toxicity through saturation of cellular pathways. Their main disadvantage is that they are generally much less potent compared to shRNAs.

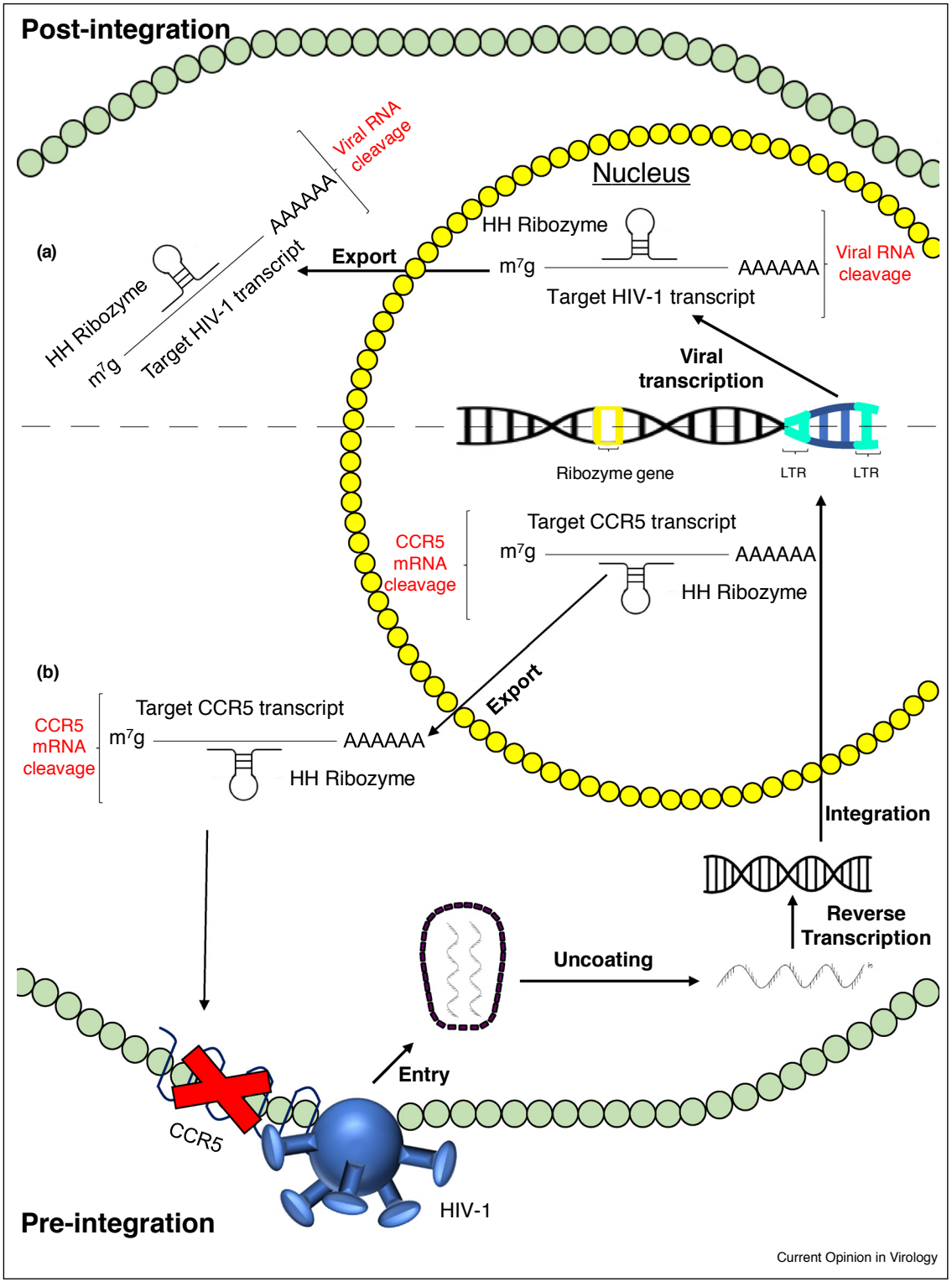
Ribozyme targets can either be viral or cellular factors and include *vpr/tat* [60], *gag* [19•], UTR [61], *pol* [62] and CCR5 [63]. A phase II clinical trial with the *vpr/tat*-specific ribozyme (Table 1) showed that the ribozyme was quantifiable in cells of all enlisted participants at week 1, but decreased to 29% of patients at week 28 [64]. No severe adverse effects occurred in association to ribozyme expression, but only a moderate antiviral effect could be observed. The lack of efficacy could also be attributed to the low engraftment of $CD34^+$ cells expressing the ribozyme. Another ribozyme targeting the CCR5 mRNA has entered clinical development along with the U16TAR decoy and *tat/rev* targeting shRNA [23] (Table 1). This clinical trial also showed that treatment of HIV by gene therapy is safe, but again, expression of the therapeutic molecules severely decreased through time. Indeed, 23% of $CD34^+$ cells expressed the combination of molecules after one week, which decreased to 1% after 4 weeks. While progress in the development of ribozymes as therapeutics has been hampered by poor catalytic activity in cells, further improvements are ongoing by using multimeric or chimeric ribozymes [65,66], the more stable HDV ribozymes [19•,67], RNase P ribozymes [68] or by direct intracellular evolution of more efficient trans-cleaving ribozymes [69]. Their incorporation in new combinations will help develop more effective RNA gene therapies [58].

Delivery of anti-HIV RNAs for gene therapy

Delivery of genes to patient cells is typically accomplished with viral vectors and recombinant adeno-associated viruses (rAAV) have emerged as promising gene delivery vectors [70]. However, only retroviral vectors are able to provide permanent expression of the anti-HIV RNAs discussed in this review and they have been used to deliver anti-HIV RNAs in all clinical trials conducted to date (Table 1). While early clinical trials used murine leukemia virus-based gammaretroviral vectors [64,71], concerns about their oncogenicity [72,73] has led to the increased use of lentiviral vectors (LVs), which have reduced potential to integrate into oncogenic loci [74]. Additionally, LVs can transduce quiescent HSCs, eliminating the requirement for cytokine stimulation of patient cells for efficient gene delivery. The first HIV gene therapy clinical trials to use LVs involved autologous gene modified $CD4^+$ T cell transplants [75,76]. Despite moderate transduction efficiencies, gene-modified T cells were observed in some patients 1–5 years post infusion, albeit at very low percentages of total blood cells. Safety evaluations in these trials showed an enrichment for LV integration events in gene-rich [75] but non-oncogenic regions [76], and no replication-competent viruses were detected [75,77]. However, since the LV RNAs used in these studies contained wild-type 5′ and 3′ LTRs, they were packaged by viral components in HIV^+ cells to generate new LVs up to a year post-infusion [76]. Therefore, self-inactivating (SIN) transfer vectors have been designed with truncated 3′ LTRs to prevent post-transduction expression of the LV RNA and have been used in recent [23] and ongoing HIV gene therapy clinical trials (Table 1). Use of such vectors to transduce patient HSCs appears to generate comparable or higher levels of long-term gene-modified blood cells compared to earlier studies with non-SIN vector-transduced T cells [23]. Promising safety and efficacy profiles of lentiviral SIN vectors in other recent HSC gene therapy clinical trials highlight the

(Figure 2 Legend Continued) aptamer is a 16 nucleotide molecule that binds to the poly(A) domains of HIV transcripts. A line is drawn through the cell to distinguish between aptamers that act at pre-integration and post-integration steps in the viral replication cycle.

Figure 3



Ribozymes cleaving target RNAs necessary for the viral replication cycle.
Ribozymes are transcribed from a gene inserted into a cell's chromosome and cleave an RNA target in a sequence-specific manner. **(a)** Ribozymes that mediate cleavage of HIV transcripts. **(b)** Ribozymes that mediate cleavage of CCR5 mRNA transcripts. A line is drawn through the cell to distinguish between ribozymes that act at pre-integration and post-integration steps in the viral replication cycle.

potential of these vectors for future use in HIV gene therapy [78,79].

Conclusion/perspectives

Each anti-HIV RNA presents advantages and drawbacks for their use as therapeutic molecules in gene therapy. Although shRNAs are extremely potent, they can lead to sequence independent toxicity through saturation of the RNAi machinery [80,81]. To prevent the resulting disturbance of micro RNA (miRNA) mediated gene regulation, all current combination clinical trials employ only one shRNA with other antiviral genes (Table 1). An advantage of aptamers and ribozymes is that they do not use cellular proteins to inhibit HIV replication and therefore have less potential to cause toxicity. However, the main disadvantage is that they are generally much less potent and therefore may not reach sufficient inhibition of HIV replication when used alone. Nevertheless, they could become extremely useful by strengthening the efficacy of other molecules, in order to prevent the development of resistant virus when used in combination. In addition to the choice of molecules, the choice of different targets is also important for the development of successful combinations. To inhibit HIV replication, either viral or cellular elements used during the viral replication cycle must be targeted. While targeting viral elements more effectively inhibits the virus, it also comes with the development of viral resistance, as these elements will be under selective pressure. A successful curative strategy via gene therapy must therefore employ multiple types of antiviral molecules that target multiple viral elements involved in different stages of the replication cycle to avoid viral escape. Molecules targeting cellular elements like CCR5 offer a significant advantage since they are not under selective pressure but increase the risk that CXCR4 virus will emerge.

A major limitation for the selection of optimal combinations of anti-HIV RNAs is that various assays and conditions have been used in different studies to measure the effects of small RNAs on HIV production or replication. Even when similar assays have been used, differences in delivery methods, doses and cell types make it difficult to identify which molecules would be the most effective for use in combination therapy. Going forward there is a need for more direct comparisons of antiviral effects and potential toxicities of different anti-HIV RNAs both alone and in combination. Such studies will help identify optimal combinations that will be both safe and effective as well as being able to prevent the development of resistant virus.

Conflict of interest statement

AG, RJS and McGill University hold the US patent 9,932,364 issued April 3rd 2018 for one of the shRNA described in the text. The corresponding Canadian patent is under review. We have no affiliation with a financial or commercial entity for this patent.

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