1. Introduction
Antiretroviral drugs have made a significant impact on HIV/AIDS disease progression and have significantly extended the life expectancy of HIV-infected individuals, particularly when used in combination therapies such as HAART (highly active antiretroviral therapy). However, despite this success, recent reports indicate that HIV morbidity and mortality remain at epidemic proportions. It is estimated that over 33 million people are currently infected with the virus worldwide, while sub-Saharan Africa accounted for approximately 70% of all infected individuals and AIDS-related deaths in 2009 (UNAIDS, 2010). Issues of drug resistance, drug toxicity, correct patient compliance and the inability to remove latent reservoirs of infection remain significant problems to overcome. The need to develop novel and improved therapeutic strategies against HIV therefore remains an important medical objective. Gene-based therapies hold much promise as alternative treatment strategies for HIV/AIDS. Anti-viral gene therapies aim to provide a once-off, long-lasting treatment against the virus and thereby address some of the shortcomings associated with current antiretroviral therapies.

A gene therapy against HIV offers several unique advantages, including the sustained inhibition of viral replication and the removal of virus from cellular reservoirs. Moreover, by improving specificity, the common toxic side effects associated with current antiretroviral regimens can be diminished. A number of different RNA-based and protein-based gene therapy strategies have been explored and some have reached phase 1 and 2 clinical trials. Our research focuses on the development of RNA-based antiviral strategies and in particular, those that utilise gene expression strategies based on RNA interference (RNAi). In this chapter, we examine basic concepts and review recent advances in the development of expressed RNAi-based systems against HIV, with a focus on progress in construct and target design. We also discuss topics related to the use of RNAi-based strategies, including appropriate construct expression, target specificity, viral escape mutations and effective construct delivery. We aim to identify desirable properties of an RNAi-based anti-HIV therapy and highlight the future developments that are required to make this approach a reality.

2. RNA interference
RNA interference (RNAi) is a gene silencing phenomenon in which RNA molecules act to silence the expression of particular genes at a post-transcriptional level in the cell. RNAi has
become a popular tool in the development of antiviral therapeutics as the potent silencing mechanism can be redirected against viral genes to inhibit viral replication. RNAi was first described by Fire et al. (Fire et al., 1998) and has since been demonstrated in a number of different organisms including yeast, plants and animals. The mediators of RNAi are short 21- and 22-nucleotide (nt) RNAs known as small interfering RNAs (siRNAs) or microRNAs (miRNAs) and are derived from longer double stranded (ds) RNAs. SiRNAs/miRNAs direct the silencing of complementary gene transcripts in a sequence-specific manner. RNAi was first described in mammalian cells in 2001 (Elbashir et al., 2001a) and subsequent research has moved swiftly to reveal a number of pathway components and mechanisms. Mammalian RNAi is now emerging as a complex network with several alternative RNA forms and levels of regulatory interactions (Brevig and Esquela-Kerscher, 2009); (Ding et al., 2009). RNAi–based therapeutics, however, still make use of the central RNAi pathway involved in miRNA biogenesis (Figure 1). Our discussion of RNAi-based strategies begins here with a description of this major RNAi pathway and how it can be redirected to inhibit viral replication.

The mammalian RNAi pathway mediates gene silencing through the generation of miRNAs. MiRNA regulation of genes is both essential and ubiquitous and has been implicated in the regulation of developmental timing, cellular differentiation, apoptosis, cell proliferation and organ development (Bartel, 2004). MiRNAs are expressed from non-protein-coding genes in intergenic or intronic regions as single or polycistronic transcripts by RNA polymerase II (Lee et al., 2004); (Cullen, 2004). MiRNA transcripts are usually several kilobases long and fold back upon themselves to form characteristic hairpin structures known as primary-microRNAs (pri-miRNAs) with flanking sequences, a partially duplexed stem and a terminal loop. Pri-miRNAs are processed in the RNAi pathway in two successive enzymatic steps (Lee et al., 2002b) to produce mature miRNAs from the double-stranded stem region. The first processing step occurs in the nucleus where the pri-miRNA is cleaved asymmetrically by the “microprocessor” complex to produce a shorter ~ 70 nt hairpin known as a precursor-microRNA (pre-miRNA) with a 2 nt overhang at the 3’ hydroxyl end. The microprocessor complex includes two essential proteins, namely, the RNase III enzyme Drosha and the DGCR8 (DiGeorge critical region 8) protein (Han et al., 2004). The pre-miRNA is exported from the nucleus to the cytoplasm by the nuclear karyopherin Exportin-5 (Exp-5) in a Ran-GTP-dependant manner (Yi et al., 2003); (Lund et al., 2004). In the second processing step, the pre-miRNA is cleaved asymmetrically by another RNase III enzyme, Dicer (Paddison et al., 2002) to produce staggered ~22 base pair (bp) miRNA/miRNA* duplex with 2 nt overhangs at each 3’ hydroxyl end. Dicer is thought to form a complex with TRBP (TAR RNA-binding protein) (Chendrimada et al., 2005) and PACT (protein activator of protein kinase PKR) (Lee et al., 2006).

One strand of the miRNA/miRNA* duplex is selected as the mature miRNA or guide strand and loaded into the RNA-induced silencing complex (RISC) (Martinez et al., 2002a). The guide strand (miRNA) is typically selected from the duplex as a result of weaker 5’ end pairing, while the remaining passenger strand (miRNA*) is degraded (Khvorova et al., 2003); (Schwarz et al., 2003). In certain cases, both strands of the duplex may be capable of RISC incorporation. RISC facilitates sequence-specific gene silencing and is directed by the guide sequence to complementary regions in the 3’ untranslated regions of target messenger RNAs. RISC-targeting results in the cleavage, degradation or translational suppression of a gene transcript, depending on the level of total complementation between the miRNA and
Fig. 1. The mammalian microRNA biogenesis pathway which mediates RNAi. Artificial antiviral constructs can be introduced at points 1, 2 and 3 of the pathway and processed to inhibit viral replication.
target sequence (Hutvagner and Zamore, 2002); (Zeng et al., 2003). Guide strands with near-perfect complementation tend to mediate cleavage of the target by activating the core catalytic Argonaute-2 (Ago-2) protein of RISC. Target cleavage is also a typical characteristic of siRNA-mediated gene silencing. Most miRNAs exhibit incomplete complementation, resulting in translational suppression. Complete complementation, however, is still required for the seed region of the miRNA (5’ position 2-7) for effective target knockdown (Brennecke et al., 2005) and a single mismatch in this region can prevent silencing (Elbashir et al., 2001b).

To redirect the RNAi silencing pathway to silence viral genes, artificial anti-viral siRNAs and miRNAs can be introduced into the cell to enter different points of the RNAi pathway (Figure 1: 1, 2, 3). Antiviral guide sequences are designed to be complementary to viral transcripts and can be incorporated into various forms of artificial RNAi intermediates including pri-miRNAs, pre-miRNAs, and miRNAs. Once in the pathway, these anti-viral intermediates are processed to give therapeutic guide sequences which act to suppress viral gene expression and inhibit viral replication. RNAi strategies have been used against incoming viral RNA to prevent integration, but it appears as if the strength of RNAi remains in its role in post-transcriptional gene silencing (PTGS). Incoming viral RNA appears to be unsuitable for targeting as it is bound by several proteins and transcribed within a short time frame, which may limit its susceptibility to RNAi. A number of different antiviral constructs and targeting and delivery strategies have been investigated against HIV with varying success and are discussed in the following sections.

3. Antiviral RNAi constructs

The potential application of RNAi for the treatment of HIV was recognised shortly after the first application of RNAi modalities in mammalian cells (Capodici et al., 2002; Coburn and Cullen, 2002; Jacque et al., 2002; Lee et al., 2002a; Martinez et al., 2002b). However, it soon became apparent that RNAi therapies fall into two broad groups: those that are expressed in the cell and those that are not. The basic forms of antiviral RNAi constructs are shown in Figure 2. There are specific advantages and drawbacks associated with both synthetic and expressed constructs with regard to delivery, duration of inhibition and dose control (Table 1), but which type of anti-viral construct is best suited for the treatment of HIV?

3.1 Non-expressed, synthetic RNAi constructs

Small interfering RNAs (siRNAs) are the most common form of non-expressed RNAi constructs. Initial studies of RNAi induction in mammalian cells showed that siRNAs can be used as powerful tools for artificial gene silencing. Despite the relative potency of siRNAs, their use in a permanent therapeutic application is limited by the lack of continued expression (Tuschl and Borkhardt, 2002). Although, this feature can be useful for particular applications where topical administration is possible and doses can be more easily controlled. An siRNA (ALN-RSV01) against nucleocapsid expression of Respiratory Syncytial Virus (RSV) was successfully delivered to healthy individuals in the form of a nasal spray in a randomized, double-blind, placebo-controlled clinical trial (Devincenzo et al., 2010). The treatment was shown to decrease the number of infected subjects by 38%, independently of other factors like pre-existing RSV antibody and intranasal pro-inflammatory cytokines. While siRNAs may not be suitable for once-off gene therapies, this example demonstrates how novel delivery methods can enable successful siRNA use in a therapeutic setting.
Fig. 2. Various forms of therapeutic constructs used to trigger antiviral RNAi. Each construct generates antiviral siRNA or miRNA guide sequences (green, yellow, red and blue) which initiate silencing of complementary viral targets. Constructs may be non-expressed or expressed in a singular or multiple format (combinatorial-RNAi).
<table>
<thead>
<tr>
<th>Non-Expressed Constructs</th>
<th>Expressed Constructs</th>
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<tbody>
<tr>
<td>Small interfering RNAs (siRNAs)</td>
<td>Short hairpin RNAs (shRNAs) Primary microRNA mimics (shRNA-miRs)</td>
</tr>
<tr>
<td>Multiple siRNAs</td>
<td>Multiple shRNA cassettes Extended short hairpins (e-shRNAs) Long hairpin RNAs (lhRNAs, dlhRNAs) Polycistronic microRNA mimics</td>
</tr>
<tr>
<td>No. Therapeutic effects are transient. Multiple or continuous treatments are required.</td>
<td>Yes. Auto-expression sustains the supply of anti-viral effectors.</td>
</tr>
<tr>
<td>No. SiRNAs are chemically synthesised.</td>
<td>Yes, but this is dependent on the type of promoter selected. Cell-specific or inducible promoters are favourable.</td>
</tr>
<tr>
<td>No. SiRNAs are suitable substrates for direct association with RISC.</td>
<td>Yes. Anti-viral siRNA/miRNA guides must be processed from RNA precursors by Drosha and/or Dicer.</td>
</tr>
<tr>
<td>Less likely as processing and export enzymes are not utilised.</td>
<td>A serious concern related to over-expression of artificial constructs. Choice of expression system is critical.</td>
</tr>
<tr>
<td>Doses can be more easily predicted and controlled as construct expression is not a factor.</td>
<td>Exact expression levels under specific promoters can only be determined empirically and may vary with time and genetic background.</td>
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<tr>
<td>SiRNAs can be administered directly. Chemical modifications can be included for enhanced absorption or targeting to a specific tissue.</td>
<td>More complex. Viral vectors are often used for transduction, but issues with safety and efficacy persist. Cell-based delivery systems appear promising.</td>
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Table 1. A comparison of non-expressed and expressed antiviral RNAi constructs.

### 3.1.1 siRNAs

SiRNA duplexes have been shown to effectively silence a number of HIV target genes. SiRNAs against mRNAs of the Gag protein and CD4 cellular receptor have been shown to inhibit post-integrative expression events with a four-fold reduction in viral entry and a 47% decrease in p24 expression in HIV cell culture challenge assays (Novina et al., 2002). SiRNAs against tat and rev transcripts specifically inhibited protein function and viral replication in human T cell lines and primary lymphocytes (Coburn and Cullen, 2002). SiRNA duplexes against the long terminal repeat (LTR) and accessory genes *vif* and *nef* were shown to be effective at inhibiting viral production from infectious molecular clones by 30 to 50 fold in 24 hours (Jacque et al., 2002).
Exogenous siRNAs in the form of duplexes were found to be the most effective at redirecting the silencing mechanism against both endogenous and transfected genes (Elbashir et al., 2001a), while the characteristic 2 nt overhangs at both the 5' and 3' ends are an essential feature (Caplen et al., 2001). Artificial siRNAs are selected for RISC incorporation more directly and there is no need for Drosha or Dicer processing (Figure 1, point 1). This can prevent saturation of the RNAi pathway components and interference with the essential miRNA biogenesis. The use of short siRNA duplexes is preferable over longer dsRNAs which were previously used, as interferon responses in the cell can be avoided. The most effective synthetic siRNA duplexes are designed to be about 19 – 21 nts in length with 3’ overhangs, but it has also been shown that shorter siRNAs of only 16 nts may trigger more potent RNAi as a result of higher RISC-loading capacity (Chu and Rana, 2008).

The chemical synthesis of siRNA duplexes is conceptually simple, as are the methods of electroporation, microinjection and liposome-mediated transfections for siRNA introduction into cells. The continuous need for these methods in large-scale siRNA use, however, can become labour-intensive. Coupled with the transient nature of siRNA-induced gene silencing (maximum ~ 1 week), the advantages of an expressed siRNA construct in therapeutic applications are clear. Nevertheless, novel delivery mechanisms for synthetic siRNAs have been developed for HIV. Specifically, studies in humanized mouse models of HIV using anti-gp120 aptamer-siRNA chimeras have demonstrated their potential as specific antiviral agents (Neff et al., 2011); (MacRae et al., 2008); (Zhou and Rossi, 2010). Similarly, siRNAs conjugated to single-chained monoclonal antibody fragments targeted to T-cell or lymphocyte-specific receptors have shown promise in vivo (Kim et al., 2010; Kumar et al., 2008). However, these methods are in the earlier stages of development and there are still several delivery and potency hurdles which must be overcome. The focus of this review will be on expressed RNAi modalities, where siRNA duplexes are expressed from plasmid DNA vectors with lasting effects on gene silencing.

### 3.2 Expressed RNAi constructs

SiRNAs that are generated from expression cassettes have the advantage of sustained production which makes them suitable for long-term, once-off therapeutic applications. Recent developments in construct design and delivery methods have shown much promise for the advancement of siRNA expression systems against HIV. In earlier siRNA expression studies, linear cassettes were constructed to code for both sense and antisense sequences of the siRNA duplex under the control of separate promoters and termination signals (Lee et al., 2002a); (Miyagishi and Taira, 2002). Expressed siRNAs then associate post-transcriptionally to form a duplex with 2 – 4 nt uridine overhangs. This system was found to be effective against HIV-1 sequences and siRNAs targeting a highly-accessible region of the rev transcript were found to inhibit viral transcript expression (Lee et al., 2002a). However, the reliability of correct siRNA duplex association in vivo is questionable. Most siRNA expression constructs are now designed to generate mimics of RNAi intermediates in the form of siRNA or miRNA hairpin precursors which are processed by Drosha and/or Dicer enzyme complexes (Figure 2). This is preferable for more reliable siRNA processing in a manner that is regulated by the RNAi pathway, but inappropriate expression levels can lead to saturation of critical RNAi components.

The choice of promoter is therefore critical in achieving suitable levels of construct expression. Polymerase III (pol III) promoter sequences, like human U6 snRNA (small
nuclear RNA U6) or H1 (human RNase P H1), are commonly used to drive efficient expression of short downstream sequences and often feature in short hairpin expression cassettes. They have been well characterised in earlier ribozyme expression studies (Good et al., 1997) and are suitable to drive nuclear expression in a wide range of human cell types (Paul et al., 2002). However, the high level of constitutive expression from pol III promoters can be undesired in a long-term therapeutic treatment. Polymerase II (pol II) promoters, like the human cytomegalovirus (CMV) promoter, are now being favoured for lower and potentially regulatable expression of pri- and pre-mRNA mimics. They allow for safer tissue-specific expression of constructs with tighter in vivo regulation (Cullen, 2005); (Giering et al., 2008). Other types of inducible promoters have also been investigated for regulated expression in the presence of an activator molecule (Jacque et al., 2002), which would satisfy the need for greater control of construct expression in therapeutic applications.

3.2.1 Short hairpin RNAs

Short hairpin RNAs (shRNAs) were developed for the expression of siRNA duplexes (Paul et al., 2002). shRNAs are essentially mimics of precursor-miRNAs that are processed by Dicer to produce staggered siRNA duplexes. ShRNAs typically have short, completely complementary stem regions of about 19 -29 base pairs (bp), a 2 nt 3’ overhang and one of several commonly used terminal loops. Synthetic shRNAs have been shown to trigger more effective gene silencing than siRNA duplexes with the same guide sequences (Siolas et al., 2005). The association of shRNAs with Dicer may result in more effective loading of guide sequences onto RISC, as Dicer forms part of the RISC-loading complex (RLC) (MacRae et al., 2008).

A variety of different guide sequences can be expressed from shRNAs. Sequence composition of individual guides can affect the processing efficiency, but common shRNA formats generally give high levels of expression. An shRNA against the HIV-1 transactivator (Tat) protein gene was incorporated into an H1-driven expression cassette and delivered to cells through the use of a recombinant AAV (adeno-associated virus) DNA vector (Boden et al., 2003). In a cell culture challenge assay with the infectious molecular clone HIV-1NL4.3, HIV-1 p24 antigen levels were decreased by 97% 48 hours post-transfection in cells expressing the shtat compared to control cells. The high mutability of HIV, however, severely hinders the potency of silencing by a single shRNA in a long-term application. In cells stably expressing shtat, HIV-1 replication was reduced by 95% in the first three weeks, but had again risen by day 25 as a result of a nonsynonymous mutation in the targeted region.

The silencing efficacy of an shRNA mostly depends on the level of conservation of the HIV target sequence and shRNAs with equal processing do not necessarily result in the same level of HIV inhibition. ShRNAs against the viral integrase sequence (shIN) and the U3 region of the viral genome required for integration (shU3) showed a more potent inhibitory effect on HIV-1 replication than shtat in shRNA-transduced MT-4 or primary CD4+ T cells (Nishitsuji et al., 2006). In p24 viral replication assays, shint produced a ~ 4 fold reduction in p24 production, while shtat resulted in a ~2.5 fold reduction four days post-infection. In contrast, a similar hairpin against the U5 region of the viral genome resulted in weak inhibition, possibly due to high GC content. At 10 days postinfection, viral replication was again detected in the shTat-transformed MT-4 cells, while HIV-1 replication was undetectable for up to 1 month postinfection, in cells that received shIN or shU3. While this is an improvement, the use of single shRNAs is still unsuitable for long-term HIV suppression.
The choice of stem and loop structures must also be carefully considered in shRNA design. A completely duplexed stem assists in preserving shRNA structure and can be useful for the prevention of 3′ – 5′ exonuclease attack (Paul et al., 2002), but is not a necessity and the high level of duplex stability may also interfere with strand selection. While typical stem lengths of about 19 bp are effective, longer 29 bp stems can be more potent triggers of RNAi with more effective processing, suggesting that Dicer requires a minimum stem length for efficient cleavage (Siolas et al., 2005). Loop sizes are more variable and can be anywhere between 3 and 9 nts in size. A recent investigation has confirmed that loop sequences are indeed critical in determining shRNA function against HIV-1 sequences (Schopman et al., 2010). ShRNAs with sub-optimal loop sequences (Brummelkamp et al., 2002) can be slightly altered to increase RNA activity by up to 7 fold. The size of optimal loops appears to be between 7 and 10 nts, while decreasing loops to 5 nts or less appears to be detrimental to RNAi activity. Particular loop structures, especially those derived from pri-miRNAs, can enhance processing of weak shRNAs. The importance of loop structure may be attributed to Dicer co-factors, like the KH-type splicing regulatory protein (KSRP), which binds to the terminal loop and affects processing (Vermeulen et al., 2005); (Trabucchi et al., 2009).

Pol III promoters like U6 or H1 are well suited for the constitutive expression of shRNAs in a range of cell types. The pol III termination signal consists of a short stretch of uridine residues which are cleaved at the termination site after two residues. This is ideal for the generation of a 3′ UU overhang in the hairpin, which is important for the efficiency and the specificity of siRNA processing by Dicer. Robust shRNA expression from pol III promoters can be detrimental for a therapeutic application. High levels of sustained expression can lead to cytotoxicity and even to a lethal saturation of the RNAi pathway. The long-term effects of robust shRNA expression were investigated in the livers of adult mice and found to cause liver injury, organ failure and death within one month (Grimm et al., 2006). Morbidity was associated with the downregulation of natural liver miRNAs, which suggested that competition exists for components of the RNAi pathway such as Exportin-5.

In a more recent publication, Ago-2 (Slicer) was identified as the primary rate-limiting determinant of both in vitro and in vivo RNAi efficacy, toxicity, and persistence (Grimm et al., 2010). Ago/shRNA coexpression studies have shown that increased Ago-2 and Exp-5 expression can rescue long-term U6-driven shRNA expression in adult mice with enhanced silencing of exogenous and endogenous hepatic targets, reduced hepatotoxicity, and extended RNAi stability of more than 3 months. The benefits of using a weaker promoter were demonstrated in this study where in vivo toxicity was alleviated, allowing for sustained target silencing of over a year. Overall, shRNAs are very potent gene-silencing moieties, but their safe and effective use in anti-HIV gene therapies is dependent on appropriate promoter and target selection.

### 3.2.2 Mimics of microRNA precursors

There seem to be several advantages in creating antiviral constructs with properties that are similar to endogenous miRNA precursors. This includes the incorporation of mismatches into the stem region, the use of longer stems and different terminal loops. Enhanced silencing has been observed for siRNAs derived from hairpins based on precursor-miRNAs (pre-miRNAs). SiRNAs against the HIV-1 tat gene were placed into the natural pre-miR-30 backbone and found to be 80% more effective at reducing HIV replication than the same guide expressed from a conventional shRNA (Boden et al., 2004). Hairpins based on primary-microRNAs (pri-miRNAs) with pol II promoters have also been shown to induce
potent, stable and regulatable gene silencing in vivo, even when present as a single copy in the genome (Dickins et al., 2005). These pri-miR mimics have been described as second-generation shRNAs and termed shRNA-miRs (Silva et al., 2005).

Artificial miRNAs not only show a greater inhibitory efficacy against HIV targets when compared to conventional shRNAs (Liu et al., 2008), but may also be better at suppressing imperfect HIV-1 targets (Liu et al., 2009a). This enhanced silencing ability has been attributed to more efficient processing in the RNAi pathway by both Drosha and Dicer enzyme complexes. Pri-miRNA mimics may also be subjected to regulatory mechanisms and other important components of the RNAi pathway, unlike substrate mimics introduced further on in the pathway (Obernosterer et al., 2006). In addition, there may be functional differences between RISC-siRNA and RISC-miRNA with respect to Ago protein association.

As might be implied from nature, it is necessary to maintain several key elements of natural pri-miRNA structures for effective processing of artificial miRNAs. It has been suggested that a large terminal loop (≥10 nts), a stem between 26 and 40 bp and at least 40 nts of non-structured flanking RNA sequences are required for efficient processing by Drosha (Zeng et al., 2005). Single-stranded flanking sequences may form part of the Drosha-RNA interface (Zeng and Cullen, 2005) and it seems logical to preserve the natural flanking sequences in the use of miRNA precursors as scaffolds. Preservation of natural loop sequences also appears to be desirable and has been shown to rescue the inhibitory potential of weakly functioning shRNAs (Schopman et al., 2010).

In comparisons of the silencing ability of shRNA and artificial microRNA constructs with similar guide strands, shRNAs were generally found to produce a more potent silencing effect (Boudreau et al., 2008). This has, however, been attributed to a higher level of expression both in vivo and in vitro. As already discussed, higher expression of shRNAs is undesirable in a therapeutic setting. Artificial miRNAs with a lower expression are processed more efficiently in the RNAi pathway and cause less of a bottleneck which can lead to saturation toxicity. SiRNAs expressed from a microRNA backbone do not appear to show the same level of inhibitory competition for nuclear export by Exportin-5 and incorporation into RISC (Castanotto et al., 2007). Pri-miRNA mimics therefore appear to be safer option for therapeutic use and show less disruption of natural microRNA biogenesis (Boudreau et al., 2009). Pri-miRNAs, however, do not show consistent processing over a range of different guide sequences, as can be observed for shRNAs. Pri-miRNAs therefore appear to be a more favourable expression format for siRNAs, but sufficient processing of guide sequences must be assessed empirically.

3.3 Combinatorial RNAi constructs

Despite the potency of RNAi against HIV targets in short-term studies, the sustained inhibition of viral replication is not possible with a single siRNA construct. Viral escape mutations arise readily in response to the strong selective pressure of effective RNAi constructs. SiRNAs directed against the viral nef gene and introduced into human T cells by retroviral transduction successfully inhibit viral replication at first, but after several weeks of culture RNAi-resistant viruses developed (Gregory et al., 2004). Viral mutations included nucleotide substitutions or deletions in the Nef gene that modified or deleted the siRNA-Nef target sequence. Similarly, expressed shRNAs targeting the HIV-1 tat gene soon give rise to a viral quasispecies harbouring a point mutation in the shRNA target region which abolishes antiviral activity of tat shRNA (Boden et al., 2003). Silent mutations in protein-
coding sequences can also occur. Viral escape mutations are, however, not limited to point mutations in the siRNA target sequences. Mutations can occur in other regions of the genome that alter the local RNA secondary structure of the target site and diminish siRNA binding (Westerhout et al., 2005).

The key to successful HIV inhibition lies in the targeting of several highly conserved regions simultaneously in a combinatorial approach (co-RNAi). This strategy has been used in a number of conventional drug regimens and aims to reduce the emergence of viral escape mutants by inhibiting multiple HIV targets. Expressed shRNA and shRNA-miR constructs can be adapted to produce multiple siRNAs and combined in single plasmid vectors. RNAi constructs can also be more easily adapted than small molecules in response to viral evolution. Several studies have investigated viral mutation pathways in response to particular therapeutic stimuli in order to identify and block anticipated escape paths. Interestingly, viral escape paths against shRNA therapy differ to those triggered by drug therapy (Applegate et al., 2010).

### 3.3.1 Multiple short hairpin RNAs

Multiple short hairpins can be used against HIV simply through the use of multiple vectors or through the design of consecutive shRNA constructs. The simultaneous use of two separate hairpins against the CCR5 and CXCR4 cellular receptors has been shown to protect transduced primary macrophages against HIV infection (Lee et al., 2003). The consistent delivery and expression of two separate shRNAs in an equal ratio is not precise using a simultaneous approach and multiple shRNA constructs are preferable for more controlled expression levels. A bi-specific construct containing a U6-driven shRNA against CXCR4 and an H1-driven shRNA against the CCR5 has been shown to effectively downregulate both targets simultaneously (Anderson and Akkina, 2005). When the siRNA expressing transduced cells were challenged with X4 and R5 tropic HIV-1, they demonstrated marked viral resistance. Targeting of three different HIV regions is even more favourable for effective coRNAi and has been demonstrated using a multi-shRNA construct. Three H1-driven shRNAs against two pol and one gag sequence were successfully used to create an additive inhibition of viral production and delay viral escape (ter Brake et al., 2006).

Combining multiple shRNAs with the same construct structure can be problematic. The use of repeated promoter sequences can lead to rearrangements and deletions of whole transcriptional units as a result of recombination in lentiviral delivery vectors. To prevent this, non-identical pol III promoters U6, H1, and 7SK and the polymerase II U1 promoter can be used to drive simultaneous expression in a multi-shRNA cassette which can inhibit HIV without viral escape (ter Brake et al., 2008). However, equivalent expression of each siRNA is not guaranteed and high expression levels of several anti-viral guides still occurs which can lead to even more serious saturation toxicity (McIntyre et al., 2009). ShRNAs, even in a multiple format, are therefore not necessarily the most preferable expression systems for therapeutic applications.

### 3.3.2 Long hairpin RNAs

To avoid issues associated with the toxicity of multiple promoter-driven constructs, several adjacent siRNA sequences can be incorporated into single long hairpin constructs (lhRNAs) under the control of one promoter. Consecutive Dicer cleavage is required
along the length of the hairpin to release individual siRNA duplexes (Paddison et al., 2002). A modified long hairpin against a 50 nt region of the integrase gene effectively suppressed both wild-type and shint-resistant viral strains (Nishitsuji et al., 2006). A U6-driven long hairpin RNA spanning a possible 60 bp of a 5’LTR target region has shown silencing of respective target sequences and inhibition of HIV replication (Barichievy et al., 2007). The greatest silencing in this format was observed for the target corresponding to the base of the hairpin stem.

In a more pre-meditated approach, several well-characterised shRNA sequences can be concatenated into a single long or extended shRNA (e-shRNA). E-shRNAs were designed with two siRNAs against nef and pol HIV-1 sequences which were efficiently processed and showed viral inhibition (Liu et al., 2007). The position of the two siRNAs was found to be critical for the generation of functional siRNAs. In a further step, the generation of three siRNAs from a single U6-driven hairpin was investigated against tat, rev and vif (Saayman et al., 2008). All sequences were capable of target silencing depending on their position within the hairpin and processing efficiency decreased from the stem of the hairpin towards the terminal loop. Spacing between the siRNA sequences within the duplex stem region can also affect processing efficiency. E-shRNAs can be extended to include a maximum of 3 siRNAs with an optimal length of 66 bp. Further stem extension results in a loss of RNAi activity (Liu et al., 2009a). A size limit of 80 bp has also been suggested and the incorporation of G:U wobbles may have several advantages related to hairpin expression (Sano et al., 2008).

A further advancement which circumvents the length limitation of lhRNAs is the use of a long hairpin concatenation. A recent study has shown that four functional anti-HIV siRNAs can be derived from a single Pol III-generated transcript comprising two adjacent long hairpin RNA precursors (Saayman et al., 2010). Two active anti-HIV siRNAs were engineered into each of two lhRNAs, which were arranged in tandem to create a double long hairpin (dlhRNA) expression cassette. Each hairpin component was found to generate two of four unique siRNA sequences (tat, nef, LTR and int) and thereby mediate dual-targeting. Processing of the individual siRNAs was found to be affected by both internal ordering and spacing between siRNAs. An inverse correlation between siRNA silencing potency and increased spacing was observed, while processing at the 3’ position of each lhRNA was more variable. Optimal siRNA processing was found to occur when only one mismatched base pair was placed between each siRNA in accordance with predicted Dicer cleavage intervals. Effective multiple processing was achieved by manipulating the order of the siRNA-encoding sequences to create an optimized combinatorial dlhRNA expression cassette. Despite the use of a pol III promoter, expression potency of the individual guides is diluted and therefore less likely to cause toxic saturation. This work has highlighted the versatility of dlhRNAs and shown that they are a promising construct form for effective silencing of multiple HIV targets.

### 3.3.3 Polycistronic primary microRNA mimics

Safe and controlled expression of siRNAs is a particular concern in coRNAi. Once again, a logical way of doing this is to mimic mammalian microRNA expression systems. MiRNAs are often expressed as pol II-driven polycistronic units in the cell and multiple siRNAs can be expressed in a similar fashion. In some systems, effective singular miRNA mimics, like those based on the pri-miR30 backbone, were simply incorporated in tandem under the control of a single pol II promoter to express two or three artificial guides (Han et al., 2006).
(Ely et al., 2009). Different pri-miRNA backbones have different expression aptitudes for individual sequences and the ordering of pri-miRNA expression units can affect both expression and silencing abilities. The preservation of natural pri-miRNA structural elements is still required in multiple constructs with a minimum of 22 nt of natural flanking sequence required at the 5′ arm and at least 15 nt at the 3′ arm (Zeng and Cullen, 2005). In addition to this, extra restriction sequences must often be included to create the tandem format. This can be very useful for creating modular pri-miRNA units that can be exchanged as required. On the other hand, extra artificial and repetitive natural flanking sequences in the expressed transcript can interact in an unexpected fashion to form undesirable secondary structures which prevent or alter processing of the intended guide sequences.

Multiple guide sequences can be incorporated into other natural miRNA precursor forms. In an earlier example, the BIC non-coding RNA with its embedded miR-155 miRNA precursor was used as a scaffold for construction of the SIBR vector (Chung et al., 2006). Synthetic miRNA sequences were incorporated into a modified miR-155 stem-loop, along with flanking sequences from the third exon of the BIC transcript, which proved to be sufficient for the expression of miR-155. It was found that two artificial miRNAs could be expressed from a single polycistronic transcript to give effective inhibition of targets without a decrease in the efficacy of individual target suppression. Alternatively, up to 8 tandem copies of the same artificial miRNA can be expressed from the SIBR vector in tandem for enhanced expression, but this is not a favourable option for HIV inhibition where strong silencing of a single target should be avoided.

A simpler approach for polycistronic design is to mimic entire naturally occurring polycistronic pri-miRNA units. Multiple effective siRNAs can be inserted into a naturally occurring polycistronic scaffold and expressed from a single promoter sequence. The mir-17-92 polycistron has been successfully used as a scaffold for four siRNAs against rev/tat, gag, pol and leader HIV sequences (Liu et al., 2008). In this example, each siRNA sequence was initially incorporated into an individual pri-miRNA structure with about 40 nts of flanking sequences and assessed. In doing so, the passenger strand was altered with the use of predictive secondary structure software to maintain all mismatches, bulges and thermodynamic stability as far as possible. Positioning of guide sequences in each pri-miRNA hairpin was found to be crucial for optimal processing. Individual hairpins showed moderate anti-HIV activity, but co-expression of two or more hairpins in a polycistronic format gave greatly enhanced silencing from each individual pri-miRNA component. Antiviral siRNAs have also been engineered into the tri-cistronic miR-106b cluster (Aagaard et al., 2008) to produce 3 siRNAs against tat/rev, tat and rev. In both of these examples, polycistronic expression systems appear to have an intrinsic inhibitory activity greater than that of conventional shRNA constructs or individual hairpins.

In all examples of mimic design, it appears that the preservation of key structural elements is crucial for effective processing and inhibitory function. Although the predictive software for this purpose is of a very high standard, folded structures and sequence interactions in vivo can never be guaranteed. This is of particular concern when modular pri-miRNA units are being combined in a novel way. Guide sequence expression from pri-miRNA mimics is also variable and can depend on both the anti-viral sequence and backbone used. Transposition of 19 nt siRNAs from shRNA expression systems into pri-miRNA units can be tricky as miRNA sequences can be up to 24 nts in length. Only one guide can be used per pri-miRNA hairpin, which means that combinatorial constructs will inevitably contain quite a lot of extra, non-guide sequence. Construct size can be a limiting factor for insertion into
viral vectors, but polycistronic pri-miRNA units are still generally within an acceptable size range. In general, the use of pri-miRNA mimics requires more planning and testing of individual components, while the final construct behaviour can only really be observed experimentally. This makes polycistronic miRNA expression systems more labour intensive, but thorough testing should be part of any therapeutic strategy. The extra input may be well worthwhile if the potential advantages of combined HIV targeting at an appropriate expression level with regulated and efficient processing can be realised.

3.3.4 Therapeutic constructs

Overall, it appears as if dlhRNAs or polycistronic mimics appear to possess the best combinations of desirable properties for a therapeutic RNAi application. Developments are, however, still required before these constructs can be implemented in a clinical setting. Expression systems can be further optimised to give restricted expression in target cell populations and therefore reduce the risk of unwanted off target effects (OTEs). More specific expression can be achieved through the use of a haematopoietic or T-cell-specific promoter (Liu et al., 2008). The WAS promoter, for example, is active in human hematopoietic precursor cells (CD34+), T lymphocytes, B cells and dendritic cells, but not in non-haematopoietic cells and may be an excellent candidate (Charrier et al., 2007). Expression could ideally be further restricted to HIV infected cells by using the HIV-1 LTR promoter to express the miRNA polycistron only in the presence of the viral Tat protein (Unwalla et al., 2004). Furthermore, RNAi activators are probably best used in combination with other types of RNA- or protein-based anti-HIV constructs in a therapeutic application to mediate an even more potent viral inhibition that does not rely on a single genetic mechanism. A polycistronic miRNA mimic, for example, can be combined with a TAR decoy for enhanced viral inhibition. ShRNAs can also be applied therapeutically in combination with other RNA-based constructs, for example, an anti-CCR5 ribozyme and a TAR decoy for greater protection against from HIV-1 challenge (Wilson et al., 2003). It therefore seems that the best therapeutic approaches involve the use of combinations of both RNAi triggers and different types of inhibitory mechanisms, while maintaining natural RNAi processing and overall cellular function as far as possible.

4. RNAi target selection

A critical factor in the success of any RNAi-inducing therapeutic strategy is the choice of target sequence. Highly effective therapeutic effectors can be rendered ineffective in a clinical setting if careful consideration is not given to the long-term targeting strategy. SiRNAs have been designed against most regions of HIV-encoded RNAs, including tat, rev, gag, pol, nef, vif, env, vpr, and the long terminal repeat (Figure 2). However, there is no single Achilles heel in the HIV genome and targeting of several highly conserved regions across multiple viral strains is a requirement for a clinically relevant RNAi-based therapy. In silico approaches for target identification are therefore crucial, although targeting strategies must still be experimentally validated. Highly conserved HIV sequences are rare. In an extensive study of siRNA target prediction for optimal design of siRNAs, highly conserved sequences were analysed from the Los Alamos HIV Sequence Database covering 495 divergent strains of subtype M (Naito et al., 2007). Of the 4 million potential 21-mer siRNA target sites, only 5.2 % showed a level of conservation greater than 50%. Highly conserved (> 80%) siRNA target sequences are very rare (< 1 %) and
only about 14% of these rare potential sites correspond to functional siRNA predictions. This finding has called for serious reconsideration of the clinical potential of numerous previous studies which generally target regions outside of this highly conserved category.

Highly conserved target sites are essential in a therapeutic application to successfully inhibit mixed and fluctuating viral populations. Ideal target regions of the HIV genome include essential regulatory regions of viral gene expression. Among these are the primer activation signal (PAS), primer binding site (PBS), packaging signal (ψ), central polypurine tract (cPPT), central termination sequence (CTS) and 3’ polypurine tract (3’ PPT). These regions are conserved at the nucleotide sequence level, presumably to conserve secondary RNA structures which are important for viral fitness. Other highly conserved potential siRNA target sites include the packaging signal, TAR/polyA and regions in protease and integrase protein codes. It does not seem possible for a single siRNA to target all known HIV-strains, but this work by Naito et al. suggests that it is theoretically possible to target >99% of circulating subtype M strains with escape resistance by combining only two siRNAs against highly conserved viral sequences.

In other previous studies, it has been suggested that four conserved sequences will be sufficient to inhibit several hundred circulating viral stains (Leonard and Schaffer, 2005). It is, however, unlikely that sequences across several viral strains will harbour sufficient sequence identity to be effectively targeted by the same set of four shRNAs. It has since been proposed that more than four shRNAs should be utilised in therapeutic design such that each viral strain will be effectively targeted by at least four shRNAs (McIntyre et al., 2011). ShRNA combinations of seven H1-driven expression units were found to provide up to 87% coverage for all known HIV strains and 100% coverage of clade B subtypes. Position within a specific (1–7) multi-shRNA cassette generally had little effect on the suppressive activity of individual shRNAs when expressed in isolation, but when shRNA expression was simultaneous, expression decreased for shRNAs in position 3–7. The effective and equal expression of 6 or 7 tandem shRNAs is a challenge and the use of so many pol III promoter units poses a substantially higher risk of toxic saturation of the endogenous RNAi pathway. However, the possibility of inhibiting multiple viral strains simultaneously is a tempting motivation for further development of both multi-shRNA and larger polycistronic mimic expression systems.

As an alternative to highly mutable viral sequences, host dependency factors (HDFs) encoded by the cell can also be targeted to further inhibit viral replication. The CD4 receptor required for viral entry is an obvious choice, but is also present on other host cells in which silencing of CD4 may result in undesirable side-effects. The CCR5 and CXCR4 co-receptors are more attractive targets for silencing and have been investigated (Novina et al., 2002, Song et al., 2003). There are however many other host factors involved in HIV replication, such as those required for Tat binding to TAR (cyclin T1 and CDK9) and those that bind to the LTR to control gene expression (NF-κB, SP1, LBP, and LEF). SiRNAs against the NF-κB p65 subunit resulted in decreased viral replication (Surabhi and Gaynor, 2002). Large screen studies have also revealed numerous other potential targets (Brass et al., 2008). Cellular targeting is promising, but must be used with caution as the inhibition of cellular proteins can have widespread effects on cellular function with undesirable side effects.

5. Safety & toxicity of RNAi activators

A primary concern with the use of RNAi-based strategies is that of safety and the specificity of the inhibitory effect in vivo. The presence of double stranded RNA (dsRNA) can activate
cellular defence mechanisms which lead to a non-specific halt in translation and cell death. DsRNA induces an interferon type 1 (IFN-1) response in the cell which in turn activates the transcription of other immune effector molecules, IFN stimulated genes (ISGs) and Dicer-related pathways (de Veer et al., 2005); (Karpala et al., 2005). DsRNA can also activate the retinoic-acid inducible gene-1 (RIG-I) and members of the oligoadenylate synthetase (OAS) receptor family which catalyze the synthesis of 2’-5’ oligoadenylates to activate a latent cellular endoribonuclease (RNASEL), which in turn cleaves cellular and viral mRNAs. A key effector molecule is the dsRNA-responsive protein kinase receptor (PKR) which functions to block translation of both viral and cellular proteins. PKR activation is typically induced by long dsRNA molecules (>30 nts), but can also be induced by exogenously introduced short 19–29 nt dsRNAs. SiRNAs and shRNAs can induce an IFN response in cells through toll-like receptors (TLRs), particularly TLR3 (Kariko et al., 2004). Ironically, these immune responses play a role in viral defense systems of the cell, but can create issues for the introduction of artificial anti-viral constructs. However, siRNA sequences tend to be only weak inducers of the IFN response and the use of siRNA expression systems can be effective in avoiding an immune response (Robbins et al., 2006).

Another major concern with RNAi activators is the unintentional suppression of cellular transcripts with partial sequence complementation described as off-target effects (OTEs). As the seed region (position 2-7 nt) is the most crucial determinant of target specificity, it seems probable that several cellular transcripts will be susceptible to such a short region of sequence complementation. Some microarray studies have shown that even targets with one or two base pair mismatches with an siRNA can be affected (Jackson et al., 2003). The use of multiple guide sequences is required for effective long-term viral inhibition, but this increases the number of potential OTEs. In a number of studies, cellular toxicity has not been observed, suggesting that OTEs may not necessarily have a significant impact on cellular function (Liu et al., 2008). Nonetheless, extensive attempts should be made to predict potential OTEs before clinical application.

Saturation of the endogenous RNAi pathway with highly expressed RNAi-activators, like pol III-driven shRNAs, can have potentially lethal toxic side effects (Grimm et al., 2006). As already mentioned, polycistronic pri-miRNA mimics can be useful in avoiding competition with the components of the pathway through reduced expression levels and more regulated processing of guide sequences. The natural properties of these mimics may also be useful in avoiding immune stimulation and careful target selection may reduce unwanted OTEs. Although, the complete reduction of OTEs is unlikely and extensive screening of in vivo cell expression patterns may be the only real way to assess the extent of undesirable effects.

6. RNAi towards the clinic

A number of RNAi-based effectors have reached clinical trials, but safe and effective expression and delivery of RNAi constructs remains an obstacle for most therapeutic approaches. Recent developments have shown much promise in addressing common delivery issues. Novel nanotechnologies have been used for the delivery of exogenous siRNAs (Davis et al., 2010), while lentiviral vectors that are stably transduced with an extended hairpin expression cassette have been shown to durably inhibit HIV-1 in T-cells (Liu et al., 2009b). Finer details of lentiviral optimisation are now being elucidated and it seems that unique strategies are required for shRNA and miRNA expressing vectors (Schopman et al., 2010).
Cell-based delivery appears to be the most promising approach for the development of a realistic therapeutic strategy (Figure 3). Essentially, haematopoietic stem cells (HSCs) are collected from suitable donor candidates and transduced ex vivo with anti-HIV expression constructs. Lentiviral vectors are preferable for this purpose as they can mediate integration of therapeutic constructs into the cellular genome even in non-dividing cells for long-term construct expression (Naldini et al., 1996). Transduced HSCs are then infused into an HIV-infected patient where they can give rise to HIV-resistant cell populations. This method allows for controlled transduction of target cell populations where aberrant integrative events may be detected and eliminated ex vivo.

Cell-based delivery may involve allogeneic or autologous cell transplantation. In a pivotal allogeneic study, replicating HIV remained undetected in a recipient patient 20 months after transplantation and termination of HAART (Hutter et al., 2009). In this case, HSCs were obtained from a donor homozygous for a naturally occurring HIV-resistant phenotype and successfully transplanted into an HIV-infected patient with acute myeloid leukemia following myeloablative therapy. Cells with the Δ32CCR5 mutation harbour a 32 bp deletion in the gene for chemokine receptor 5 and are protected from infection by R5-tropic viral subtypes. This strategy proved to be effective for treating leukaemia and preventing viral replication. Notorious HIV-reservoirs, like the intestinal lamina propria, remained HIV free 159 days after transplantation. While this approach seems ideal for effective HIV inhibition in vivo, homozygous donors for the CCR5 mutation are rare, occurring in only ~1% of the white population. Nonetheless, this promising strategy can be adapted for the delivery of HSCs with an artificial HIV-resistant genotype.

Autologous cell-based approaches do not require matching of HLA genotypes and avoid the host-versus-graft complications. In a recent RNA-based example, a ribozyme (OZ1) against the tat-vpr region of the HIV genome was delivered to patients through transduced autologous CD34+ hematopoietic progenitor cells (Mitsuyasu, 2009). Progenitor cells were transduced with a murine retroviral vector encoding the OZ1 ribozyme. The cell-delivery method was assessed through a randomised, double-blind, placebo-controlled phase 2 gene transfer clinical trial with 74 HIV-1 infected individuals. The OZ1 group showed significantly lower viral loads after 40 weeks and significantly higher CD4+ lymphocyte counts through 100 weeks. This study demonstrated that cell-delivered gene transfer can be both a safe and effective therapeutic strategy.

In a more recent clinical trial, three RNA-based anti-HIV constructs were introduced into patients undergoing transplantation for AIDS-related lymphoma (DiGiusto et al., 2010). HIV-infected individuals represent a unique and ethically-sound research group where marrow ablation can be performed prior to transplantation. A tat/rev short hairpin, TAR decoy and CCR5 ribozyme combination construct was used to modify the patient’s own CD34+ cells through lentiviral transduction. Transduced cells showed no difference in haematopoietic potential compared to non-transduced cells in in vitro analysis and were successfully engrafted in four patients. Expression of the anti-HIV moieties was initially as high as 22 %, but declined to ~1% over four weeks of cell culture. Persistent siRNA expression was observed at low levels for up to 24 months in multiple lineages. No short-term toxicity was associated with the infusion of the genetically modified cells, and observed toxicities were instead related to the standard autologous hematopoietic cell transplantation (HCT) procedure.
This study has demonstrated a viable approach for effective therapeutic expression of RNAi-based constructs against HIV-1. Persistent, constitutive shRNA expression over 24 months was not found to be toxic to peripheral blood cells and there was no evidence for lineage-specific toxicity. An artificial, anti-HIV expression vector can therefore be stably expressed in human blood cells without significant toxic side effects. The method of ex vivo lentiviral transduction and autologous cell-based gene transfer has been demonstrated as a safe and effective means of construct expression. The long term inhibitory effect on viral replication and evolution in the absence of anti-retroviral drugs remains to be seen. The demonstration of sustained anti-viral siRNA expression, however, has moved us one step closer to the realisation of a clinically applicable once-off treatment against HIV-1 infection. Further improvements in transduction and transplantation procedures are likely to yield even more favourable results for the therapeutic application of RNAi.
7. Conclusion

A genetic approach using expressed RNAi modalities offers the possibility of a once-off treatment against HIV with permanent and sustained viral inhibition and without common toxic side effects associated with current drug regimens. In this chapter, we have discussed the pros and cons of several RNAi-inducers that can be used to inhibit HIV replication. To summarise, an ideal RNAi-based gene therapy against HIV will make use of a combination of effective siRNA sequences in a single expression vector against at least four, but preferably six or seven, highly conserved viral target sequences or host dependency factors. This will provide potent silencing of target sequences across all known viral strains and prevent the emergence of viral escape mutants. Each siRNA sequence needs to be expressed at an equivalent and appropriate level under the control of a regulatable or HIV-inducible pol II promoter to avoid biased targeting and prevent the toxic and potentially lethal competition-based saturation of the natural RNAi pathway. The use of polycistronic pri-miRNA mimic expression systems appears most favourable and the preservation of natural structural motifs appears to enhance processing and silencing capabilities, as well as avoid activation of the innate immune system, which may otherwise occur with the introduction of exogenous constructs. Off-target effects should be modelled as far as possible before therapeutic testing and should be limited to non-significant effects. The most suitable delivery method to date appears to be through autologous cell-based gene transfer transplantation in a myeloablated recipient background with ex vivo lentiviral transduction of the patient's own haematopoietic progenitor cells. This is quite a comprehensive list of desirable properties for an ideal RNAi-based therapy against HIV and is the result of intensive research over the past decade. Further developments are necessary for the realisation of a safe and effective genetic therapy against HIV, but in light of the research presented here, we are moving closer.

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9. References


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Like any other book on the subject of HIV/AIDS, this book is not a substitute or exhausting the subject in question. It aims at complementing what is already in circulation and adds value to clarification of certain concepts to create more room for reasoning and being part of the solution to this global pandemic. It is further expected to complement a wide range of studies done on this subject, and provide a platform for the more updated information on this subject. It is the hope of the authors that the book will provide the readers with more knowledge and skills to do more to reduce HIV transmission and improve the quality of life of those that are infected or affected by HIV/AIDS.

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