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Proposing an RNA Interference (RNAi)-based Treatment for Human Immunodeficiency Virus (HIV) Infection by Analyzing the Post-Transcriptional Gene Targeting of SARS-CoV-2, Hepatitis C Virus, and A549 Lung Cancer Cells

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ABSTRACT

Human Immunodeficiency Virus (HIV) is a retrovirus that infects $CD4^+$ T cell lymphocytes in humans, leading to the development of Acquired Immunodeficiency Syndrome (AIDS) if left untreated. While current treatment methods, including antiretroviral combination treatments, effectively limit HIV replication, HIV can evade these treatments due to its high mutation rate. Long-term antiretroviral treatment can also be toxic to patients, meaning patients would benefit from a new mechanism of HIV treatment. RNA interference (RNAi) is an antiviral pathway found in mammals, plants, and insects that involves a small-interfering RNA that is incorporated into a protein complex called the RNA-induced Silencing Complex (RISC). This complex binds to and cleaves viral mRNAs, reducing viral gene expression. RNAi is a promising method of treating HIV/AIDS, since it has been found to adapt to and target highly-conserved sequences in Hepatitis C Virus, A549 lung cancer, and SARS-CoV-2 in mammalian cells. Patisiran is an FDA-approved RNAi treatment for use in humans, making an HIV RNAi treatment plausible. However, an RNAi treatment for HIV has not yet been designed or developed. This paper aims to propose a comprehensive potential RNAi treatment for HIV. RNAi may effectively inhibit HIV replication by containing two siRNAs with the sequences 5'-UUAAUACUGACGCUCUCGC-3' and 5'-UGUAUUGAUAGAUAACUAU-3' that target the highly-conserved p17 and Reverse Transcriptase genes, respectively, delivered within a solid lipid nanoparticle composed of equimolar amounts of DOTAP and DODMA cationic lipids and containing LFA-1 antibody on the surface for receptor-mediated endocytosis, and cotransported with Rev and GagPol HIV proteins to limit the anti-RNAi function of HIV's RRE and TAR.

KEYWORDS

RNA interference • HIV • virus replication • siRNA • target specificity • coevolution • endocytosis • off-target effects

Introduction

Human Immunodeficiency Virus (HIV) has been a consistently-spreading concern for at least the past 40 years (Fischer et al., 2021). HIV-1 is the virus responsible for causing HIV, and primarily infects $CD4^+$ T cell lymphocytes in the human host, a class of white blood cells that is highly active in defending against infection. As HIV progresses, more and more $CD4^+$ T cells become infected and the number of $CD4^+$ cells in the body greatly decreases, weakening the immune system of the host and progressing the disease into Acquired Immunodeficiency Syndrome (AIDS) (Bobbin et al., 2015; Boden et al., 2003; Fischer et al., 2021; Kim et al., 2009; Kretova et al., 2017; Schopman et al., 2012). The World Health Organization determined that nearly 56 to 100 million people have been infected with HIV-1, and HIV/AIDS has claimed about 25 to 42 million lives (Fischer et al., 2021). Currently, nearly 45 million people are suffering from HIV/AIDS, making finding an effective treatment a major priority across the world (Fischer et al., 2021; Kretova et al., 2017; Schopman et al., 2012).

However, HIV-1's mode of infection makes it tricky to create an effective treatment. HIV-1 is a retrovirus, and embeds its genome into the host, making it difficult to remove the infection from the body (Bobbin et al., 2015; Boden et al., 2003; Fischer et al., 2021; Kim et al., 2009; Kretova et al., 2017; Schopman et al., 2012). Current methods of treating HIV/AIDS include antiretroviral therapy, which are proteins that inhibit the pathway that converts HIV's RNA genome into DNA and inserts it into the human host. However, antiretroviral therapy comes with a cost, which can become expensive with the combinatorial mixture of multiple brands of antiretroviral drugs. The lifetime cost of antiretroviral therapy for an HIV patient who began treatment at 35 years old can reach $195,000, not including doctor visits or other forms of treatment (Schackman et al., 2015, p. 6). In addition, if resistance is developed to certain combinations of antiretrovirals, newer drugs or different combinations of antiretrovirals will need to be used, which will drive lifetime prices up even more. Since 2012, the average cost of brand-name antiretroviral medicines has increased 34%, which is more than three times the rate of inflation. In addition, treatment regimens for newly-diagnosed HIV patients can cost at least $36,000 per year with the current treatments available, which can be difficult to afford even with insurance for many patients (McCann et al., 2020, p. 603). While antiretroviral therapy has been effective at reducing HIV-1 proliferation, it is an unsustainable treatment since it causes toxic side-effects in long-term treatment, and drives HIV-1 mutation against the antiretroviral treatment, rendering it ineffective in the long-term, along with its high lifetime cost to the patient, compromising their quality of life (Bobbin et al, 2015; Kim et al., 2009; Kretova et al., 2017; McCann et al., 2020; Schackman et al., 2015).

RNA interference (RNAi) is a promising method to inhibit HIV-1 replication and proliferation. RNAi is a gene-silencing mechanism that is found in many different organisms, ranging from plants, insects, and even mammals (Bobbin et al., 2015; Cui et al., 2015; Fischer et al., 2021; Kim et al., 2009; Kretova et al., 2017; Schopman et al., 2012; Sklan & Glenn, 2007; Yung et al., 2016). It limits viral replication by inhibiting expression of viral genes using complementary base pairing with viral messenger RNA (mRNA) transcripts, and cleaving the mRNA using the RNAi-

induced silencing complex (RISC). RISC is a combination of a cleaving protein and a small interfering RNA (siRNA) complementary to the host genome, produced when Dicer, a nuclease, cleaves the viral double-stranded RNA (dsRNA) genome into short sequences (Bobbin et al., 2015; Cui et al., 2015; Fischer et al., 2021; Kim et al., 2009; Kretova et al., 2017; Schopman et al., 2012; Yung et al., 2016). While the RNAi pathway is highly-conserved and effective against antiviral treatment in plants, insects, nematodes, and fungi, its prevalence in mammals is relatively low (Cui et al., 2015; Fischer et al., 2021; Kretova et al., 2017; Schopman et al., 2012; Meister, G. & Tuschl, T., 2004).

However, an RNAi treatment for HIV has not yet been designed or developed. This paper aims to consolidate research on RNAi's mechanism of viral inhibition, possible target sequences in the HIV genome, delivery mechanisms for RNAi treatment into the body, and how to counter HIV RNAi suppressors that could hamper RNAi treatment, in order to propose a comprehensive potential RNAi-based treatment for HIV/AIDS.

Antiretroviral Treatments Cannot Effectively Cure HIV/AIDS

Because HIV-1 can form a latent reservoir of viruses in resting $CD4^+$ cells and can rapidly evolve against antiretroviral treatments that become toxic after long-term usage, current antiretroviral treatments are not effective enough at mitigating the replication of HIV-1, and a new mechanism of treatment may benefit HIV-1 patients.

In a review examining possible approaches to treating HIV-1 infection, Bobbin et al. (2015) reported that HIV-1 preferentially infects human $CD4^+$ T-lymphocytes, leading to a depletion of $CD4^+$ cells and the clinical progression to acquired immunodeficiency syndrome (AIDS) (p. 1). $CD4^+$ T cells are an integral part of the human immune system, and their depletion can compromise the host's ability to fight off pathogens. Current methods of treatment include administering antiretrovirals, a class of drugs meant to inhibit HIV-1 genome insertion into the host genome, in order to slow down the replication of HIV-1.

However, simply preventing HIV-1 replication in $CD4^+$ T cells is not enough to cure HIV/AIDS infection. Bobbin et al. also reported the current method of treatment, which involves a cocktail of three antiretroviral drugs to leverage combinatorial treatment in order to effectively suppress viral replication by limiting evasive mutation, which would reduce HIV-1 replication and improve the patient's condition by slowing the progression to AIDS. However, Bobbin et al. reported that patients treated with combinatorial antiretrovirals can still develop chronic inflammation and death due to non-AIDS-related complications. Antiretroviral treatments are also unable to rid the patient of HIV-1 viruses, since antiretroviral treatment cannot target latent viral reservoirs which are not replicating, resulting in lifelong antiretroviral treatment for HIV-1-infected patients (p. 1). The ineffectiveness and complications of antiretroviral treatment make it more of a compromise rather than a corrective treatment for HIV-1-infected individuals. Bobbin et al. also states that long-term treatment can result in toxic side effects, requiring alternative treatment after several years of treatment.

In a 2021 review on the similarities and differences between the physiology and mechanisms of infection between SARS-CoV-2 and HIV-1, Fischer et al. also discussed latent reservoirs as an obstacle in developing an HIV treatment. According

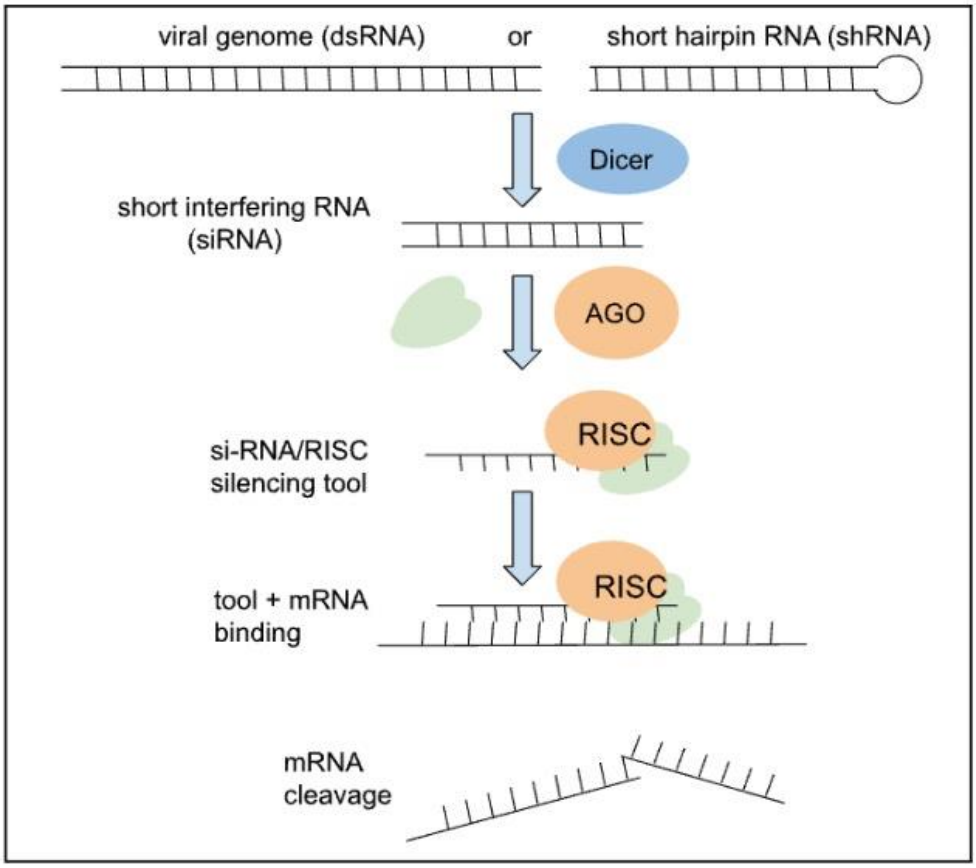

Figure 1. RNAi Silencing Mechanism.

to Fischer et al., one of the unique qualities of HIV-1 is that it forms a latent reservoir of cells in the body after infection when infected $CD4^+$ T cells convert into a resting state that prevents viral expression. This halts viral expression and HIV-1 replication, creating a latent reservoir of infected cells that can eventually be reactivated (p. 1094). The latent HIV-1 viruses in resting memory-state $CD4^+$ cells make it difficult to access the source of HIV-1 infection and completely clear out HIV-1 using standard treatments, which results in prolonged infection and worsening symptoms.

However, simply administered consistent antiretroviral treatment for a prolonged time period is ineffective and potentially dangerous. In an ultra-deep sequencing analysis of HIV-1 sequences obtained from Russian HIV patients, Kretova et al. (2017) reported that although antiretroviral treatment shows temporary success at slowing HIV-1 infection, it is ineffective in the long-term, exerting high toxicity and inducing selection pressure, resulting in drug-resistant mutants for nearly every antiretroviral currently available (p. 330). The ineffectiveness of long-term antiretroviral treatment was also iterated by Kim et al. (2009), who stated that the toxicity and drug resistance prevalent in lifelong antiretroviral treatment supports the development of novel therapeutic options for HIV treatment (p. 370).

While antiretrovirals are the most popular method of treating HIV currently, they are unable to completely clear HIV-1 from latent reservoirs in the infected host,

and lifelong antiretroviral treatment to suppress symptoms results in toxicity, chronic inflammation, and drug resistance, which warrant development of a novel mechanism of treatment.

How HIV-1 Evades Conventional Treatment Methods

Because HIV-1 has an error-prone Reverse Transcriptase protein and develops insertion-deletion mutations from immune pressure, HIV-1 has a very high mutation rate that allows it to quickly evolve against immune antibodies and vaccine treatment, requiring an adaptive treatment such as RNAi.

Fischer et al. (2021) reported research by Mansky and Temin (1995) that stated how HIV-1 is able to consistently evade the immune system. Mansky and Temin found that HIV-1's Reverse Transcriptase (RT) protein, which is part of the Pol polyprotein, is an RNA-dependent DNA polymerase that does not have the ability to proofread errors it makes during reverse transcription (p. 1094). The lack of proofreading activity of HIV-1 reverse transcriptase, a DNA polymerase that helps incorporate the viral HIV-1 RNA genome into the human host DNA genome, results in many mistakes in transcription per cycle of replication. This results in HIV-1 accumulating mutations and evolving at a high rate, since the myriad of random mutations allow it to respond to selective pressure from the immune system. According to research by Korber et al. (2017), Liu et al. (2013), and Gao et al. (2014) as reviewed by Fischer et al. (2021), HIV-1 evolves due to constant pressure from T cells and B cells in the immune system, changing and adapting to defend against the infective parts of the virus (p. 1094). Although the immune system's T and B cells can co-evolve with HIV-1, HIV-1 is able to consistently evolve to evade the immune system, resulting in prolonged infection if left untreated.

In addition, HIV-1 can evolve against antibody and antiretroviral treatments as well. To verify these claims, Fischer et al. used the Los Alamos HIV-1 database curated reference set to make an HIV consensus sequence to compare actual HIV-1 sequences to, and conducted a mutational analysis of the HIV-1 genome using 3,903 HIV-1 sequences, comparing them to the consensus sequence (Fischer et al., 2021, p. 1096). Fischer et al.'s analysis revealed that the Env protein, the primary surface protein on the HIV-1 viral capsid, has a high density of mutations. This makes it difficult to target using antibodies in the body or in clinical vaccines, since Env can selectively mutate against the antibodies, resulting in an arms race between HIV and antibody treatments. HIV-1 has additional methods of mutating in order to evade selective pressure according to Kretova et al. (2017), including recombination between DNA copies after reverse transcription to increase genetic diversity and the high replication volume of HIV-1, creating large variant populations (p. 330). Kretova et al. emphasizes that HIV-1's rapid replication also allows mutations to accumulate quickly, which would make advantageous mutations more frequent in the population.

According to Kretova et al., HIV-1's mutation rate is so high that even RNAi's specific targeting could potentially be hampered. However, the host's immune system also plays a role in HIV-1's high mutation rate. The effect of the host immune system in administering selective pressure for HIV-1 comes in the form of APOBEC3G proteins, which modify viral sequences to hopefully cause detrimental mutations to arise. Kretova et al. reported

that the host protein APOBEC3G (apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3G, A3G) causes single nucleotide mutations from cytosine to uracil during reverse transcription, resulting in guanine to adenine mutations in the complementary DNA (cDNA), resulting in accelerated mutation in the HIV-1 genome and possibly impacting an RNAi-based treatment approach (p. 330). However, since HIV-1's replication rate is so high, it can develop advantageous sequences regardless of APOBEC3G using hypermutation. This results in multiple different viable sequences of HIV-1 in the body and a constantly changing gene sequence, which would make a stagnant treatment ineffective at inhibiting HIV.

The coevolution between HIV and the body is further described by Ferree and Barbash (2007), Anderson and May (1982), and Ebert (2008), as reviewed by Palmer et al. (2018). Ferree and Barbash (2007) emphasized how host-virus conflict can cause coevolution between the virus and the infected patient. Anderson and May (1982) and Ebert (2008) stated that host-parasite coevolution could cause balancing or directional selection during evolution, which can help the virus evade immune suppression (p. 1586). Palmer et al. (2018) stated that due to RNAi's mechanism of silencing gene expression of target genes, target genes may evolve to avoid detection and silencing by RNAi, setting up coevolution between the target genes and the host's RNAi machinery.

Viral mutation is expected against selective pressure such as the immune system, in order for the virus to maintain replication and spread among hosts. However, HIV-1 mutates especially well, due to its reverse transcription errors, genetic recombination, APOBEC3G-induced hypermutation, and coevolution with the host's immune system, making it difficult to control for the immune system and for conventional antibody and antiretroviral treatments.

RNAi Can Evolve to Specifically Silence HIV-1 Target Genes

Because RNAi is a specific, adaptive mechanism of reducing foreign gene expression, it is able to specifically target viral gene sequences and evolve to match the changing viral sequence of HIV-1, overcoming its high mutation rate and effectively preventing off-target side effects on the recipient.

Palmer et al. (2018) analyzed the rate of adaptive evolution of various antiviral and control proteins in several insect species using the MK framework designed by McDonald and Kreitman (1991), which used data on polymorphism and divergence in HIV-1 variants to determine nonsynonymous fixations, or advantageous mutations that represent positive selection. These nonsynonymous fixations were used to determine the proportion of advantageous nonsynonymous mutations that were a result of adaptation (pp. 1587-88). Palmer et al. determined the proportion of adaptive, selected-for mutations in genes to determine which genes are able to selectively mutate more than other genes, and used DFE-alpha and SnIPRE algorithms to reduce the effect of deleterious, random mutations on polymorphism data. Palmer et al. found that genes associated with the RNAi pathway had significantly higher rates of adaptive evolution than position-matched control genes. Specifically, Palmer et al. found that *D. pseudoobscura* had 0.16 nonsynonymous substitutions per synonymous (random) mutation (p. 1589). RNAi-associated genes display adaptive divergence in insect species, which indicates that advantageous

mutations develop at high rates in RNAi genes. It is possible that homologous RNAi pathways in mammals can adapt to viruses during infection as well.

Palmer et al. also compared rates of adaptive evolution among various types of RNAi as well in various insect and nematode species by conducting a meta-analysis of the proportion of adaptive nonsynonymous substitutions, pooling polymorphism and divergence data together, and found that the piRNA ($p = 0.001$), viRNA (exo-siRNA) ($p = 0.002$), and endo-siRNA ($p = 0.004$) pathways had significantly more adaptive substitutions than the control genes. While viRNA and piRNA did not differ significantly ($p = 0.07$), Palmer et al. found that both viRNA and piRNA had more adaptive substitutions than the endo-siRNA ($p = 0.01$) and miRNA pathways ($p = 0.001$) (pp. 1591-92). Since viRNA and piRNA both primarily have extracellular and viral targets, while endo-siRNA and miRNA are used internally within the cell, the viRNA and piRNA pathways are better suited for host-virus coevolution through adaptive substitution, and would be able to adapt to the changing sequence of HIV-1.

Another method of limiting evasive mutation against RNAi treatment would be to target multiple target sequences at once, according to Boden et al. (2003) and Das et al. (2004), as reviewed by Bobbin et al. (2015), since targeting multiple genes would work synergistically to possibly reduce treatment dosage and/or frequency, or make combinatorial antiretroviral treatment more effective (p. 9). Targeting multiple sequences at once can also limit evasive mutation, since HIV-1 would have to coordinate multiple successful mutations to overcome a single dose of treatment.

In a 2012 study on inhibiting HIV-1 replication *in vitro* using RNAi, Schopman et al. applied SOLiD deep sequencing to find endogenously-produced miRNAs and examine their effectiveness. Schopman et al. transfected pLA1 plasmids containing the HIV-1 genome into human embryonic kidney 293T cells to produce live HIV-1 viruses, which were extracted and used to infect SupT1 suspension T cells. Total RNA was extracted 48 hours post infection for gene expression analysis of SupT1 T cells (pp. 415-416). Schopman et al. found 25,981 HIV-1-specific reads in HIV-1-infected SupT1 T cells, making up 1% of all small RNAs in the cell. These small RNAs ranged between 15-22 nucleotides long and were mostly around 18 nucleotides long (pp. 417-18). Since siRNAs are usually at least 15 nucleotides long, these complementary small RNA sequences could represent activation of the siRNA RNAi pathway.

Shawan et al. (2021) used sequencing data as well to analyze possible antiviral involvement of RNAi, although for SARS-CoV-2 infection instead of HIV-1. Shawan et al. used the GenBank database to find possible siRNA sequences for RdRp, the target gene for SARS-CoV-2 inhibition, to propose an RNAi-based treatment for SARS-CoV-2. Shawan et al. used various siRNA design algorithms including siRNA prediction software, RdRp target prediction algorithms, RNAi docking models, and computer simulations of RNAi to determine a SARS-CoV-2 target sequence for RNAi inhibition. Among many viable siRNA sequences generated, the sequence guide strand: 5′ -UAGUACUACAGAUAGAGACAC -3′ ; passenger strand: 5′ -GUCUCUAUCUGUAGUACUAUG-3′ was the most optimal, stable sequence which targeted the most exposed part of the RdRp gene among other sites. (p. 13). Although the designed siRNA was not validated *in vitro*, the systematic design

and testing process for determining the optimal siRNA sequence indicates how specific and targeted the RNAi pathway can be to a specific target site.

RNAi's advantages, including adapting to mutations and targeting specific sections of the genome, has led to its recognition as a treatment for humans, including the development of an RNAi treatment for polyneuropathy. Patisiran (Onpattro) utilizes siRNA to knockdown the expression levels of transthyretin, and is the first siRNA-based therapy approved by the United States Food and Drug Administration (USFDA) for the treatment of polyneuropathy. Patisiran's approval as an siRNA remedy for humans establishes precedent for the development of RNAi treatment for other human diseases as well, including viral infection. Clinical trials evaluating the effectiveness of Patisiran for patients with hereditary transthyretin amyloidosis found a significant effect on improving polyneuropathy symptoms, according to Adams et al. (2018), the researchers responsible for Phase 3 clinical trials of Patisiran. Adams et al. randomly assigned 225 patients to receive either Patisiran treatment or a placebo treatment intravenously every 3 weeks (148 to the patisiran group and 77 to the placebo group). The modified Neuropathy Impairment Score+7 (mNIS+7) and the Norfolk Quality of Life-Diabetic Neuropathy questionnaire (NQoL-DN) were recorded before and after treatment to determine the effect of Patisiran on polyneuropathy-related symptoms. In the Patisiran group, the mean mNIS+7 changed from 80.9±41.5 to 74.9±43.2 after 18 months of treatment, while in the placebo group, the mean mNIS+7 changed from 74.6±37.0 to 102.6±39.6 ($p < 0.001$). The same trend was found for the NQoL-DN, where for the Patisiran group, the mean NQoL-DN improved from 59.6±28.2 to 52.9±30.0, while in the placebo group, the mean NQoL-DN worsened from 55.5±24.3 to 69.9±27.0 ($p < 0.001$) after 18 months (p. 11). In the relatively short time span of 18 months, Patisiran was able to significantly improve symptoms of polyneuropathy in live patients with hereditary transthyretin amyloidosis and represents the potential siRNA treatment has as a medical treatment for humans for other diseases as well, such as HIV.

Determining the Ideal HIV-1 Target Sequence

Because parts of the p17 and Reverse Transcriptase genes of the HIV-1 genome have been found to be resistant to mutation and are highly-conserved, RNAi can inhibit HIV-1 gene expression and replication long-term since RNAi's specific gene targeting is most effective at inhibiting gene expression of precise, stagnant sequences.

Even though RNAi is shown to adapt to its viral target's mutations, RNAi is only effective if the right target sequence is chosen from the viral genome. Torrecilla et al. (2016) analyzed the use of RNAi to silence Hepatitis C Virus (HCV) replication in Huh-7 mammalian cells and determined that the Internal Ribosome Entry Site (IRES) gene was an ideal target for RNAi-based suppression due to its functional role in the cell in translation and replication, along with its high sequence conservation, which would limit treatment-resistant mutants from developing (p. 808). Since RNAi treatment depends on complementary target binding, it is important that chosen targets are resistant to mutation for RNAi treatment to be effective.

Schopman et al. (2012) used the QuickAlign tool of the Los Alamos HIV sequence database to compare miRNA

candidate sequences to the HIV target sequences of 371 HIV subtypes, and noticed high sequence conservation in sequences associated with endogenous HIV-1-induced miRNA formation due to HIV-1 infection, specifically for vmiRNA-43/9175 (95% conserved) and vmiRNA/2413 (99% conserved) (p. 419-20). Schopman et al. used eight HIV-1-matching reads including vmiRNA-43/9175 and vmiRNA/9175 to produce viral siRNAs to analyze whether they could significantly inhibit HIV replication, using CA-p24 concentration to indicate viral load. Compared to the positive control siNef, which potently inhibited HIV's Nef protein, the designed siRNAs also significantly inhibited HIV replication. The cleavage of viral mRNAs of the pLAI plasmid using the siRNAs was confirmed using 50-RACE PCR(pp. 422-23). Since the highly-conserved vmiRNA target sequences in HIV-1 strains were highly conserved at levels up to 99% and displayed potent inhibition, highly-conserved target sequences may be effective targets for HIV-1 RNAi therapy.

The conservation of sequences in the HIV genome was explored by Kretova et al. (2017), who used ultra-deep sequencing to determine the conservation of various targets of the HIV-1 genome in strains in Russia, and found a highly-conserved region within the Reverse Transcriptase (RT) gene, a crucial gene for HIV proliferation. Specifically, the IVIYQYMD stretch in the RT gene, which contains the M184 residue that forms the dNTP-binding pocket necessary for appending DNA nucleotides and lengthening the DNA transcript of the HIV genome during reverse transcription, is highly-conserved (p. 333). Targeting this specific section of the RT gene using RNAi may hinder RT's ability to produce and insert DNA copies of the HIV genome into the host, effectively preventing HIV infection and replication. While analyzing sequence conservation in various Russian strains of HIV, Kretova et al. also analyzed HIV-1 sequences in patients being treated with lamivudine and emtricitabine antiretrovirals, since they are known to cause mutations in the IVIYQYMD target of the RT gene, and found that mutation in Residue M184 yielded diminished function of RT and diminished replication of HIV-1 (p. 333). The conservation of M184 and its ineffectiveness in mutant forms makes the IVIYQYMD stretch of RT an effective target for RNAi silencing and HIV-1 inhibition.

Kretova et al. then analyzed the conservation of possible HIV-1 target genes around the world by using the Nucleotide BLAST search tool to analyze the conservation percentage of 5000 different HIV-1 sequences for six HIV-1 target genes, and found that the most highly-conserved sequences in samples around the world were of the center of the p17 gene (99% conservation) and the IVIYQYMD stretch of the RT gene (96.7%). The HIV-1 sequences for these sections of the HIV-1 genome were conserved in many countries, including the United States, Russia, Kenya, China, Germany, Uganda, and Cameroon, among many others (p. 336-39). The worldwide sequence conservation in these two sections of the HIV-1 genome, along with their important functional role in the replication of HIV-1, makes both of them ideal targets to inhibit HIV-1 replication and stop the progression of HIV. The target sequences of RT and p17 and corresponding siRNA sequences are listed in Table 1.

Table 1. HIV Target Genes and Corresponding siRNA Sequences

Target Gene	Target Sequence (5'-3')	19-nt siRNA Sequence (5'-3')
RT	ATAGTTATCTATCAATACA	UGUAUUGAUAGAUAACUAU
p17	GCGAGAGCGTCAGTATTAA	UUAAUACUGACGCUCUCGC

Optimizing Delivery of RNAi Treatment into CD4$^+$ T Cell Lymphocytes

Because lipid nanoparticles can effectively bind to oligonucleotides, enter specific target cells, and protect them from DNA nucleases in the interior of the cell, cationic lipid nanoparticles containing LFA-1 antibody on the exterior can be used to deliver RNAi treatment directly to the source of the infection, within the nucleus of CD4$^+$ T cell lymphocytes.

Torrecilla et al. (2015) produced solid lipid nanoparticles (SLN), which are small, spherical, hollow particles composed of a lipid bilayer similar to cell membranes, in order to transport short hairpin RNA (shRNA) siRNA precursors into cells for RNAi inhibition of Hepatitis C Virus replication. Torrecilla et al. constructed the SLNs using the solvent emulsification evaporation technique, involving mixing aqueous-phase quaternary amine cationic lipid (DOTAP) with Tween 80 (0.1%, w/v), lipid Precirol ATO 5, dichloromethane (5%, w/v), and emulsifying this mixture by sonication. Torrecilla et al. then removed the dichloromethane by evaporation and kept the solution in vacuum conditions for 15 minutes to obtain the nanoparticles (p. 182). This method of DOTAP assembly into nanocarriers was used in Torrecilla et al. (2016)'s paper to produce Huh-7-specific nanoparticles for IRES target gene inhibition using shRNA, adding on protamine (P) and hyaluronic acid (HA) to the surface of the SLN. shRNA-SLN ratios of 1:2 and 1:5 by weight were prepared to determine the optimal ratio for shRNA-SLN binding (p. 809) RNAi small RNA components, including both shRNA and siRNA, can be transported into cells using this method of DOTAP cationic lipid-containing nanoparticles.

The positive charge of DOTAP allows it to bind to negatively-charged siRNA well, and the nanoparticle is able to effectively protect the shRNA from nuclease degradation as verified by gel electrophoresis, according to Torrecilla et al. (2015) (pp. 183-84). Torrecilla et al. (2016) analyzed IRES expression levels when Huh-7 cells were treated with shRNA delivered alone (naked shRNA) compared to shRNA delivered within the HA-containing SLN (HA-SLN), and were able to effectively inhibit IRES expression, as measured by fluorescence levels of a green fluorescence protein (GFP) expression reporter. Huh-7 cells treated with naked shRNA showed <3% inhibition, while IRES was significantly inhibited in a dose-dependent manner from 4-50% inhibition for doses ranging from 1.5-3.0 μg of HA-SLN. The 1:5 ratio of shRNA-SLN displayed significantly greater inhibition than 1:2 shRNA-SLN ($p < 0.01$) (p. 811). The significant levels of knockdown of IRES indicate the effectiveness of DOTAP solid lipid nanoparticles in transporting and delivering oligonucleotides into cells for RNAi antiviral inhibition.

Similar findings were found by Yung et al. (2016), who created a delivery mechanism using SLNs in order to delivery antiMiR-21 (AM-21), an miRNA that targeted miR-21 in A549 lung cancer cells to inhibit A549 replication. Contrary to Torrecilla et al. (2015)'s use of only DOTAP for the SLN, Yung et al. (2016) used a combination of DOTAP and tertiary amine cationic lipid (DODMA) to produce the SLN, utilizing the advantages of both cationic lipids to optimize oligonucleotide delivery. In addition, Yung et al. claimed that DOTAP and DODMA have previously been used in clinical trials and are readily available, making them clinically efficient to approve and produce (p. 654). When determining the ratio of quaternary amine cationic lipids (DOTAP) and tertiary amine cationic lipids (DODMA), Yung et al. found that an equimolar composition of DOTAP and DODMA was most effective at minimizing SLN particle size, to about 120 nm (pp. 655-56). Smaller particle sizes for the SLN delivery particle would improve internalization of the oligonucleotide into the cell, improving treatment efficacy.

This equimolar formation, named QTsome, was able to successfully reduce miR-21 expression as measured by RT-qPCR of miR-21-associated tumor suppressor genes DDAH1, PTEN, and RECK in A549 lung cancer cells (Yung et al., 2016, p. 657). Since QTsome and AM-21 (QT/AM-21) were able to silence miR-21 and upregulate the tumor suppressor genes that miR-21 inhibits in a dose-dependent manner, QT/AM-21 displayed effectiveness at tumor suppression. Yung et al. found that administration of naked AM-21 had no effect on A549 cells, underlining the importance of the QTsome delivery mechanism for effective treatment. These results were validated in a live A549 xenograft mouse model by inoculating female athymic nude (T-cell-lacking) mice with 1,000,000 cells/mouse to produce A549 xenograft mice, and administering QT/AM-21 treatment by tail vein injection to mice when A549 tumors reached 80 mm^3 (p. 655). Yung et al. found that QT/AM-21 was effective at inhibiting A549 tumor activity in an *in vivo* model of A549 xenograft mice as well in a dose-dependent manner, with strongest inhibition at 1 mg/kg QT/AM-21. Tumor sizes ended at 816, 618, and 172 mm^3 for the untreated, 0.5 mg/kg, and 1 mg/kg QT/AM-21 groups, respectively. The survival times of the mice also varied in a dose-dependent manner, with QT/AM-21 increasing lifespan to 24 days and 33 days for the 0.5 mg/kg and 1 mg/kg QT/AM-21 groups, respectively, compared to the 21 days for the control mice (p. 658).

In Torrecilla et al. (2016)'s study inhibiting Hepatitis C Virus replication using shRNA, uptake of the shRNA-containing SLN was boosted using hyaluronic acid (HA) on the surface of the SLN (HA-SLN), which targeted CD44 receptors for receptor-mediated endocytosis. Torrecilla et al. found that blocking CD44 receptors reduced HA-SLN uptake into Huh-7 cells (p. 815). Targeting possible surface receptors on $CD4^+$ T cells could improve the SLN uptake and treatment efficacy of an RNAi treatment for HIV. Bobbin et al. (2015) similarly stated the importance of using targeted lipid nanoparticles containing attached proteins and conjugates in order to overcome the cell membrane and endosomes within the cell to ensure cellular delivery (p. 11)

Possible uptake mechanisms for targeted delivery of RNAi treatment to HIV-1-infected $CD4^+$ T cells were investigated in a study by Kim et al. (2009). Kim et al. created a neutral SLN containing Lymphocyte function-associated antigen-1 (LFA-1) antibody, an

antibody of the LFA-1 surface protein, to harness LFA-1-mediated endocytosis into $CD4^+$ cells (p. 370). Kim et al. tested the efficacy of the LFA-1 antibody-containing SLN (LA-SLN) by administering anti-CD4 siRNA to CD3+ T cells using LA-SLN, and observed CD4 silencing in a dose-dependent manner of up to 95% silencing, while anti-CD4 siRNA alone had negligible silencing. Although the LFA-1 receptor is typically used endogenously to activate naive $CD4^+$ T cells, Kim et al. found that even when naive T cells were exposed to LA-SLN, they showed no activation as measured by CD25 and CD69 activation markers (p. 371). To validate these results *in vivo*, Kim et al. injected anti-CCR5 siRNA using LA-SLN intravenously into bone marrow liver thymic (BLT) mice transplanted with CD34+ cells, and found using PCR that anti-CCR5 was detected in CD3+ T cells, CD19+ B cells, and immune monocytes, but not in brain cells or other mouse-derived (non-transplant) cells, showing how LFA-1 antibody allows LA-SLN to be internalized by a variety of immune cells, possibly defending against HIV-1 infection and replication in multiple types of cells. CCR5 levels were silenced in these cells for up to 10 days after injection, displaying the integrity of the LA-SLN delivery mechanism and its possible applications in an HIV RNAi treatment in humans.

Overall, SLNs containing equimolar DOTAP and DODMA with LFA-1-antibody on the surface may be an effective mechanism to deliver RNAi treatment to $CD4^+$ T cells and related immune cells for targeted inhibition of HIV replication.

How to Counter HIV-coded RNAi Suppressors

Because HIV-1 has evolved anti-RNAi mechanisms meant to inhibit RNAi gene silencing, including the RRE and TAR elements, RNAi treatment needs to be accompanied with Rev and GagPol HIV proteins in order to maintain effectiveness of RNAi and effectively stop infection of HIV-1 by inhibiting RRE and TAR activity.

Schopman et al. (2012) claimed that mammalian viruses usually express RNA-silencing suppressors (RSS) in response to the antiviral activation of RISC in the RNAi pathway. By inhibiting certain components of the host's RNAi pathway, the virus can continue to replicate and spread. Schopman et al. reported that virus-specific small RNAs in virus-infected mammalian cells were often undetectable due to low expression levels of viRNAs, which highlights the effect of RSSs on the development of an effective RNAi treatment (p. 414).

HIV-1 has multiple proteins that help maintain genomic integrity and replication ability. According to Fischer et al. (2021), the APOBEC-induced mutagenesis of HIV-1 cDNA during reverse transcription is an immune defense against HIV-1. Kretova et al. (2017) similarly stated the role of APOBEC3G in causing deleterious mutations in HIV-1 cDNA. However, Fischer et al. (2021) claimed that HIV-1 is able to defend against genetic modification using the Vif protein (p. 1096). HIV-1 also has defense mechanisms against RNAi as well, as predicted by Schopman et al. (2012).

Schopman et al. (2012) claimed that after infecting SupT1 suspension T cells with pLA1 plasmids containing the HIV-1 genome, the levels of Trans Activation Response (TAR) precursor increased significantly (p. 422; Daniels et al., 2015, p. 124). Schopman et al. reported that this could be due to TAR's role in suppressing miRNA activity of the miRNAs administered, since binding to Tat can inhibit the function of Dicer, a crucial

protein in the RNAi pathway responsible for converting viral dsRNA into siRNA precursors (p. 422). In a 2015 study on possible HIV suppressors of RNAi, Daniels et al. (2015) further asserted the role of TAR as an RNAi-antagonist. Daniels et al. claimed that TAR binds to TAR RNA Binding Protein (TRBP), preventing TRBP from combining with RISC and disrupting the RNAi pathway (p. 124). Due to its anti-RNAi function, TAR needs to be inhibited in order for a RNAi-based HIV treatment to be effective.

Daniels et al. (2015) explored the extent to which TAR and Rev-Response Element (RRE) are RSSs by expressing both TAR and RRE using anti-let7 miRNA with a enhanced green fluorescent protein (eGFP) into HeLa human cells, and found that RRE was able to suppress RNAi up to 67% while TAR had about 29% inhibition (p. 124). However, a lentivirus containing RRE, TAR, and overexpressed Rev and GagPol proteins showed no RNAi inhibition (p. 127). To effectively use RNAi to suppress HIV replication, target siRNAs need to be co-administered with Rev and GagPol proteins to limit the RSS activity of TAR and RRE.

Conclusion

HIV-1 contains certain sequences on the HIV Reverse Transcriptase and p17 genes which are highly conserved throughout various strains and generations of replication, and RNAi can be utilized to target and inhibit expression of these stagnant sequences to effectively inhibit HIV-1 replication long-term. Small interfering RNAs (siRNAs), the primary molecule involved in RNAi-mediated gene silencing, can be precision-delivered to $CD4^+$ T cells, the target cell of HIV infection, using lipid nanoparticles containing LFA-1 antibody to ensure that the treatment is selectively administered to T cells and related white blood cells only, and that the siRNA is internalized into the cell. However, HIV-1 contains RNAi suppressors that can inhibit RNAi activity, such as TAR and RRE RNA sequences, which can be limited by co-administering Rev and GagPol HIV proteins along with the Reverse Transcriptase-targeting and p17-targeting siRNAs. Future studies can test this formulation *in vitro* to determine optimal dosages and timings of treatment to determine whether RNAi can possibly be used in the future to silence HIV before it can become AIDS.

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