



Prospect of Nucleic - Acid Based Immune System - RNAi as Potent Antiviral Agents

Andreas Soejitno¹, Prichilia Sarah Permadi¹, Desak Made Wihandani²

¹Undergraduate 4th Semester Faculty of Medicine, Udayana University, Denpasar, Indonesia

²Department of Biochemistry and Biomolecular, Faculty of Medicine, Udayana University, Denpasar, Indonesia

Discovery and Brief Description of RNAi

RNAi, also known as RNA silencing or post-transcriptional gene silencing is a mechanism of inactivating specific target gene expressions by reducing their rates of transcription, stability of target mRNAs, or the translational process of those mRNAs, thereby promoting degradation of the related RNAs.¹ Critical event leading up to the discovery of RNAi in mammals is investigated by Guo and Kemphues² who successfully inhibit gene expression of nematode *C.elegans* using sense or antisense single strand RNA (ssRNA). Later, Fire et.al³ discovered that double-stranded RNA (dsRNA) is more potent than sense or antisense ssRNA in silencing gene expression of the same species; this mechanism was named RNA interference.⁴

During inhibition of a specific gene product, dsRNA plays a major role as an intermediate to the triggering process of silencing phenomena.^{1,4,5} Many research performed to reveal how dsRNA trigger the gene silencing. It is reported that dsRNA could cause homologous genes silencing in transgenic plants which subsequently resist viral infection (so-called cosuppression).⁶⁻⁸ Therefore, RNA silencing can be triggered by viruses or transposons that generate dsRNA during their replication or – as an alternative – by introducing synthetic dsRNA.^{4,9,10} In brief, RNAi machinery uses dsRNA as a guide to target and degrade specific cellular or viral RNAs.¹¹

Scientists have discovered ways to control RNAi in manipulating gene expression

of various biological systems, including viruses. Many investigations have proven the enormous therapeutic potential of RNAi in preventing the establishment¹², reducing replicative activity¹³, or even promoting viral clearance^{4,5} including: human immunodeficiency virus type 1 (HIV-1)¹²⁻¹⁶, hepatitis virus (B, C)¹⁷⁻¹⁹, human papillomavirus (HPV)²⁰, Rous sarcoma virus¹³, poliovirus²¹, respiratory syncytial virus²², and also influenza virus⁴ (this discussion focused on RNAi as therapeutic agents for HIV-1 and hepatitis virus). It is essential to discuss the recent progress of RNAi in response to their molecular mechanism of viral gene silencing, major obstacles, and future directions toward their optimum application.

Molecular Principles of Gene Silencing

The molecular pathway underlying the work of RNAi is relatively simple. Firstly, dsRNAs are introduced into cells either with the use of plasmid and virus vector-based cassette or by using non-viral delivery strategies such as integration with a cholesterol-lipoprotein complex (common in targeting liver infected by hepatitis virus) or encapsulated in stable nucleic acid lipid particles (SNALPs)²⁴. dsRNA can be produced via bidirectional transcription, transcription of an inverted repeat (hairpin sequence), or physically introduced into feeding dsRNA-expressing bacteria.^{1,25}

After intracellular entry, long dsRNAs are then cleaved into small fragments called short interfering RNAs (siRNAs) by

the action of a 218-kDa dsRNA-specific endonuclease (RNase type III) known as Dicer.^{1,4,5,9,11} The resultant siRNAs are 21 to 25 nucleotides in length, double-stranded, and have 3' overhangs of 2 nucleotides.^{1,4,5} These siRNAs in turn are incorporated into a complex of nuclease (Argonaute subunits; in humans only Ago2 possesses an active catalytic domain for cleavage activity) known as RNA-induced silencing complex (RISC) by the help of dsRNA-binding protein R2D2.²⁵ siRNAs unwind in ATP-dependent manner to activate the RISC.¹¹ The unwound antisense siRNA act as a guide to direct RISC toward homologous target RNA (i.e. viral mRNAs or genomic RNA itself) which later undergo endonucleolytic cleavage by Slicer enzyme along with the role of Dicer.^{4,5}

Cleavage of target RNA begins at a single site 10 nucleotides upstream of the 5'-most residue of the siRNA-target RNA duplex.^{1,4} A perfect degradation of target RNA can be achieved if RNAi-mRNA complex (RISC) is highly organized in a sequence-specific pattern, thus promoting natural endogenous degradation by Slicer. However, in case RISC possesses any mismatch in nucleotide sequences (several nucleotides may not have equal base pair), the target mRNA would still exist but the tRNA in ribosome will be unable to translate the codon sequences into expected amino acids, thus aborting the translational process.^{1,12} These two mechanisms obstruct the protein synthesis in a gene construction, hence silencing its final end-product.



Mechanism of RNAi as a Potent Antiviral Agent

Many infectious diseases still can not be eradicated safely, completely, and efficiently; especially certain viral pathogens such as HIV-1 and hepatitis B, C (HBV, HCV); HIV-1 has a high rate of mutation and complexity (also related with toxicity) to HAART regimen, while acute/subacute liver failure and hepatocellular carcinoma induced by HBV and HCV are still unable to be resolved completely by conventional therapy. Yet, RNAi offers a promising therapeutic application as antiviral since the RNA targets are exogenous and can be inhibited without affecting cellular function.⁴

Several molecular pathways in disrupting HIV-1 infection have been elucidated. To date, RNAi can be used to silence the expression of CCR5 and CXCR4 coreceptor that is involved in the entry process of HIV-1 to host cell. Martinez et.al (2002)²⁶ transfected siRNAs-specific for CCR5 and CXCR4 gene expression in HIV-1+ cells and found siRNA that target those chemokine receptors could effectively inhibit surface protein expression and their function as HIV-coreceptors. The inhibitory effect of RNAi does not overlap (i.e. specific to certain coreceptor) with the blockage percentage of CXCR4 and CCR5 reaching 63% and 48%, respectively. Although not curing, this finding is worthwhile because

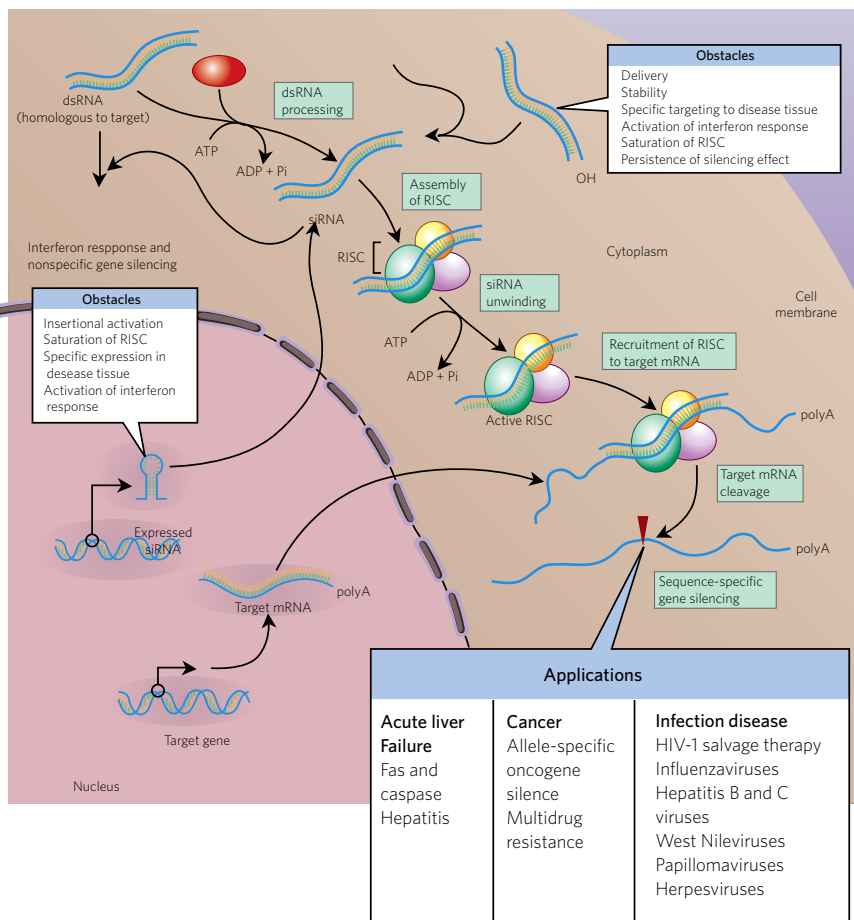


Figure 1. Mechanism of Gene Silencing by RNA interference. (ADP = adenosine diphosphate, Pi = inorganic phosphate, P = phosphate, OH = hydroxyl) (cited with permission from Stevenson).⁴

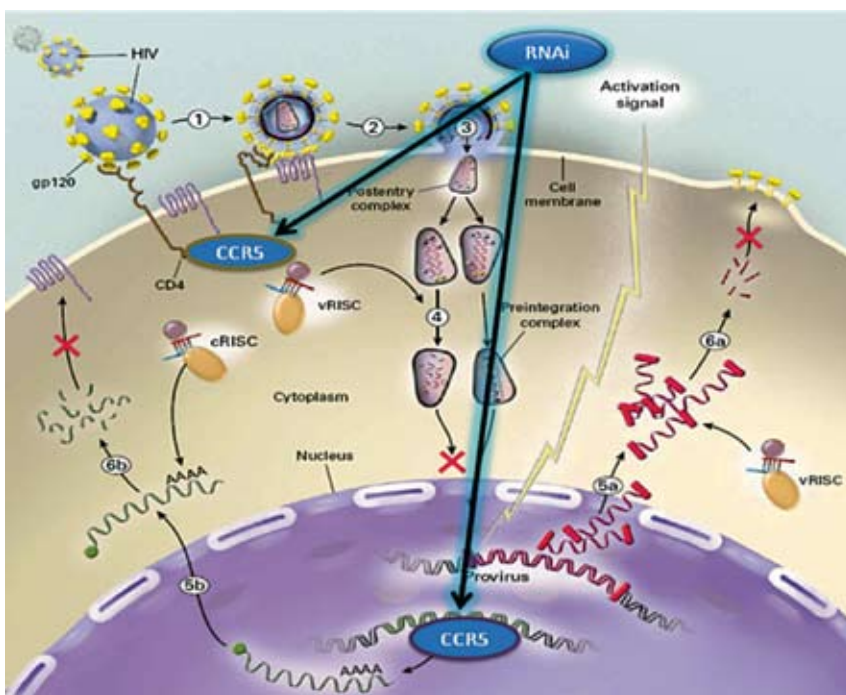


Figure 2. Inhibitory mechanism of RNAi to CCR5 coreceptor expression in CD4⁺ T cells.

individuals with \pm 50% decrease in CCR5 surface expression have lower plasma viral load and a substantially prolonged course of disease.²⁷ RNAi through virus-specific RNA-inducing silencing complex (vRISC) could also be used to target deletion of 32-bp homozygote gene located in CCR5 gene (CCR5 Δ 32).²⁸ If successfully performed, this deletion would cause zero expression of CCR5 coreceptor in CD4⁺ T cells, thereby inducing high resistance to HIV-1 infection. Furthermore, a reduction in viral replication rate was observed by the decrease of p24 intracellular antigen in HIV-1-infected cells.²⁷

RNAi inhibits the expression of DC-SIGN, a specific dendritic cell which internalizes HIV-1 and introduces HIV-1 to CD4⁺ T lymphocytes via CD80-CD86 interaction-activating MAPK pathway in the lymph nodes as viral replicating sites. RNAi targets DC-SIGN's mRNA for gene encoding CD40, CD80 and CD86 expressions, therefore inhibiting the p38 MAPK



signalling pathway which in turn, reduces co-stimulatory molecules expression and viral replication.^{29,30}

Another HIV-1 inhibitory mechanism is through NF- κ B inactivation, the transcription factor family which consists of five structural proteins (c-Rel/p65, RelA, RelB, p50/p105 and p52/p100) and plays major role in host immune responses. NF- κ B inactivation via silencing of interference sites on p65 subunit will abort the binding process to HIV-1 LTR proximal promoter via hindrance to gp120 in signaling to p56^{lck} tyrosine kinase, which is required by the virus to begin the transcriptional process, after internalization in the host's cell cytoplasm.³⁰⁻³²

HBV and HCV can be inhibited by the application of RNAi in a similar fashion to HIV-1. HBV possesses the life cycle and genome structure which are both susceptible to specific siRNAs. HBV has a

open reading frames (ORFs) and mRNAs within the genome can be targeted by siRNAs. This includes the 3.5 kb pregenomic RNA (serves as the template for HBV DNA replication, also encodes the viral core and polymerase protein), 2.4 kb and 2.1 kb mRNA (encodes the viral envelope proteins), and 0.7 kb mRNA (encodes the viral X protein).

From the clinical perspective, decrease in HBV replication by siRNA has been confirmed. A reduction of HBV DNA-RNA and HBsAg-HBcAg as many as 77–92% and 85–99% is achieved when siRNAs target C, S, P, and X genes by using plasmid vector in the Huh7 or HepG2.2.15 cells.¹⁸ Whereas a reductive stage of more than 90% reduction of HBV DNA-RNA and up to 100% reduction of HBsAg-HBcAg can be obtained when the plasmid vector was substituted with adenovirus.¹⁹ The most effective sequence, which targeted a region of the surface and overlap-

property is distinct from anti-HBV nucleoside or nucleoside analogues, which act on the viral DNA polymerase to have their therapeutic effect. Efficacy of surface ORF-targeted siRNAs was confirmed in other studies^{36,37} and improved in viral replication decrement by repeated siRNA transfection of cells in culture was also reported.³⁸ In addition, RNAi also able to reduce the incidence of acute/subacute liver failure by preventing apoptosis of hepatocytes through inhibition of cell death receptors expression.⁴ The siRNAs targeted to Fas RNA to the liver of mice were shown successfully inhibit Fas expression and protect mice from hepatitis.³⁹ Similarly, siRNAs that target CASP RNA (encoding caspase 8) can prevent acute liver failure induced by Fas activation.⁴⁰

Major Obstacles and Future Directions to Therapy

The studies of the effect of RNA silencing on viral replication in mammalian cells pose several barriers which can be divided into two groups: philosophical and technical. Philosophical barriers comprise critical questions regarding intrinsic therapeutic characterization of RNAi that is not totally elucidated by recent studies. For instance, can RNAi target the incoming viral RNA when in transit to the nucleus while it is still associated with nucleocapsid proteins?

This is crucial since certain viral infections can be blocked during this phase to prevent further dissemination, while viruses genomes are often protected by a proteinaceous structure (dsRNA viruses), nucleoproteins and matrix layers (negative-stranded RNA viruses), or by association with cellular membranes during replication (positive-stranded RNA viruses).⁵

Another important question is about the duration and amount of RNAi should be administered to exert optimal effects. The ability of siRNA-transfected cells to resist virus infection was maintained for several days.^{12,21} However, it could either indicate that the interference ability persists for a few days or as a caused of the slow-released siRNAs characteristic.⁵ Secondly, the amplification system (a catalytic activity in which small amount of dsRNAs could exert plenty of siRNAs)^{2,4} has been proven to be RdRP-dependent, whereas recent investigation failed to identify RdRP in humans.¹

The delivery mechanism probably becomes the biggest technical problem. To date, dsRNAs can be delivered to cells by

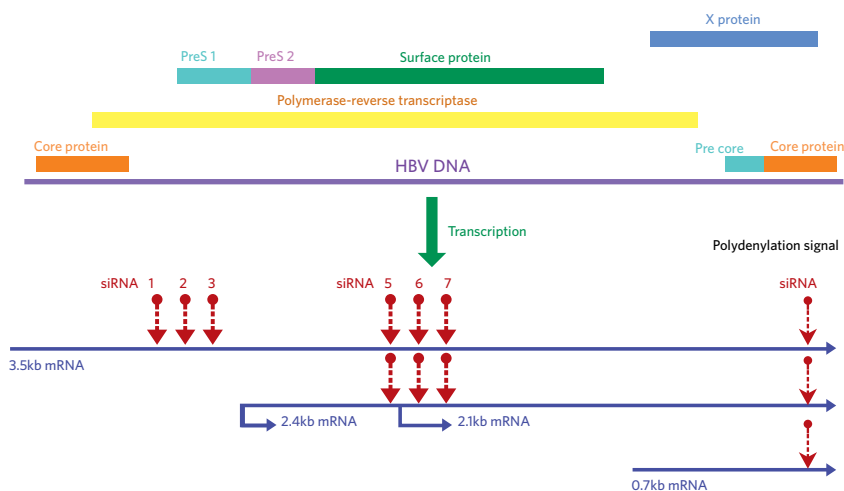


Figure 3. A schematic diagram depicting the location of potentially siRNAs targeting in association with viral open reading frames and viral mRNAs within the HBV genome.¹⁷

compact genome with a partially double-stranded DNA of approximately 3200 bases in length and contains four open reading frames (ORFs) that encode precore/core, polymerase, surface and HBVx (HBx) proteins.¹⁷ The core and polymerase genes are essential for viral DNA replication and encodes the viral capsid protein, known as hepatitis B virus core antigen (HBcAg). While HBV life cycle is characterized by the synthesis of a 3 kb partially double-stranded, relaxed-circular DNA (rcDNA) genome which has important roles in the entry, uncoating, and delivery of the viral genome into the cell nucleus. HBV viral

ping polymerase ORF, inhibited HBV surface antigen secretion by 94% in transfected cultured cells, and 85% in vivo in the murine hydrodynamic tail vein injection (MHI) model. Inhibitory effects were observed in normal (C57BL/6) as well as immunocompromised mice, which indicate that the expressed shRNAs have a direct effect that is not dependent on an antigen-dependent immune response.^{33,34}

siRNA duplex that targeted sequence nucleotides 9-27 from the surface ORF initiation codon was found to be particularly effective against HBV without a requirement for HBV DNA synthesis.³⁵ This



vectors or as artificial siRNAs.^{9,23} However, there are concerns regarding the hazards that could be arise when inserting foreign vector sequences into chromosomal DNA, such as insertional activation or inactivation of cellular genes.⁴ Furthermore, intravenous administration requires siRNAs that is resistant to nucleases.

These problems perhaps could be resolved by the use of synthetic siRNAs and conjugated carrier such as cholesterol conjugates or SNALPs.²³ In addition to the potential harm of using integrated vectors to genome, insertion of dsRNA more than

500 bp can trigger the activation of interferons, despite there is no evidence that this activation could interfere the extent of RNA silencing.⁴¹

Lastly, it is important to consider the viral escape possibility after RNAi therapy. This is crucial since mismatch potential (the presence of a single or multiple uncomplement base siRNA with target RNA) of RNAi machinery is not well-tolerated.⁵ The tolerance of RNAi machinery to mismatches is critical to ensure that the ability of the virus to escape inhibition is blunted. Therefore, it is recommended to target

multiple viral genes by RNAi to reduce the chances of a virus escaping RNAi repression through spontaneous mutation.⁴

Conclusions

RNAi is a potent antiviral agent which is compatible to nearly most of labile pathogens that has not been able to be eradicated yet. Given the need for therapeutic machinery that is able to maintain pace with the high mutation rate of viruses such as HIV, it is wise to expect RNAi-based therapeutic potential nearly in the future contemporary medicine. ■

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