DEFINING THE ABILITY OF SNAT NANOTHERAPEUTIC TO INHIBIT SARS-COV-2 INFECTION AND ALTER THE HOST MICRONOME TO INDUCE AN ANTIVIRAL CELLULAR ENVIRONMENT

By

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ABSTRACT

MicroRNAs (miRNAs) are regulatory elements that canonically bind to target mRNAs, reducing translation and protein output. They are non-coding strands of RNA, roughly 18-25 nucleotides long, and are found in plants, animals, and some viruses. During viral infection, they can be helpful or harmful to the host, affecting the cellular environment, immunity, and viral life cycle. Understanding miRNA interactions within the host and how they impact viral infections can inform therapeutic and diagnostic innovation. Nanomedicines have broad applications in the treatment of viral infections. They are less than 100 nm, and formulations include base materials such as metals, liposomes, or polymers. The nanoparticle alone may have therapeutic properties or be a carrier for therapeutic agents. Compared to traditional antivirals, nanodrugs are designed for greater bioavailability, specificity, stability, and safety. Smart Nano-enabled Antiviral

Therapeutic (SNAT) is a nanodrug that alleviated SARS-CoV-2 pathology in a preclinical study using a hamster model. It is comprised of taxoid (Tx)-decorated amino (NH2)-functionalized near-atomic size positively charged silver nanoparticles (Tx–[NH2-AgNPs]) and is delivered in aerosolized form. Herein, the molecular mechanism by which SNAT exerts antiviral effects in hamsters is determined. Results from molecular biology and virology techniques suggest SNAT binds to the S2 subunit of the viral spike (S) protein of SARS-CoV-2 to interfere with viral infectivity. Next generation sequencing (NGS) and bioinformatics data reveal SNAT-induced changes in miRNA expression with antiviral implications in vivo. In addition, infected hamsters given SNAT treatment express interleukin-6 (IL-6), a cytokine upregulated during SARS-CoV-2 infection, at the same level as uninfected controls. These findings illustrate the potential use of next-generation sequencing in the evaluation of nanotherapeutics and provide valuable insights into the antiviral molecular mechanism of SNAT. The results demonstrate the two-pronged approach by which SNAT induces antiviral effects against SARS-CoV-2; directly binding SARS-CoV-2 and neutralizing viral infection, and indirectly orchestrating a cellular environment capable of thwarting viral infection. In conclusion, our findings suggest that SNAT may serve as a novel antiviral therapeutic against SARS-CoV-2 infection.

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A549: Adenocarcinomic human alveolar basal epithelial cells

aa: Amino acid

ACE2: Angiotensin-converting enzyme 2

Ag^0: Elemental silver

AgNP: Silver nanoparticle

AGO: Argonaute

ANOVA: Analysis of variance

APOBEC3H: Apolipoprotein B mRNA-editing enzyme catalytic polypeptide 3H

ARDS: Acute respiratory distress syndrome

ASO: Antisense oligonucleotide

bp: Base pairs

BSA: Bovine serum albumin

BSL-3: Biosafety level 3

C group: Control group (healthy)

CAAR: Chimeric autoantibody receptor

Caco-2: Cancer coli-2

CAGR: Compound annual growth rate

Calu-3 cells: Cultured human airway epithelial cells

CAPA: COVID-19-associated pulmonary aspergillosis

CAR: Chimeric Antigen Receptor

cccDNA: Covalently closed circular DNA

CCR5: C-C chemokine receptor type 5

CD4+: Cluster of differentiation 4 positive

CD8+: Cluster of differentiation 8 positive

CDS: Coding sequence

circ-RNA: Circular RNA

CLDN1: Claudin-1

CMP: Comprehensive metabolic panel

COVID-19: Coronavirus Disease 2019

CPM: Counts per million

CREB: cAMP-response element binding protein

CREBBP: CREB-binding protein

CRISPR: Clustered regularly interspaced palindromic repeat

CRP: C-reactive protein

CTNNB1: Catenin Beta 1

CVB3: Coxsackievirus B3

CYLD: Cylindromatosis gene

DE: Differentially expressed

DLS: Dynamic light scattering

DMEM: Dulbecco modified Eagle medium

DNA: Deoxyribonucleic acid

dsRNA: Double stranded RNA

EBV: Epstein Barr Virus

ECU: East Carolina University

EDS: Energy dispersive spectroscopy

eIF4G: Eukaryotic translation initiation factor 4G

ELISA: Enzyme-Linked Immunosorbent Assay

EMA: European Medicines Agency

FDA: Food and Drug Administration

gB: Glycoprotein B

GO: Gene ontology

H&E: Hematoxylin and eosin

HBV: Hepatitis B Virus

HBx: Hepatitis B virus X protein

HCMV: Human Cytomegalovirus

HCV: Hepatitis C Virus

HDD: Hydrodynamic diameter

HEK-293: Human embryonic kidney 293

His-Tag: Polyhistidine tag

HIV: Human Immunodeficiency Virus

HMVEC-d: Human Dermal Microvascular Endothelial Cells

HNRNPA1L2: Heterogeneous nuclear ribonucleoprotein A1 like 2

HOXB8: Homeobox protein Hox-B8

HPC: Hematopoietic Progenitor Cell

HR-TEM: High resolution transmission electron microscopy

HRP: Horseradish peroxidase

hsa-miR: Homo sapiens microRNA

HSV: Herpes Simplex Virus

HUVEC: Human umbilical vein endothelial cells

I: Infected group (moderate-severe)

IAV: Influenza A Virus

IFITM1: Interferon-Induced Transmembrane Protein 1

IFN-γ: Interferon gamma

IFN: Interferon

IHC: Immunohistochemical

IKKγ: Inhibitor of κB kinase (IKK) gamma

IL-1a: Interleukin 1a

IL-1β: Interleukin 1 beta

IL-2: Interleukin 2

IL-6: Interleukin 6

ILC: Innate Lymphoid Cell

IRF3: Interferon regulatory factor 3

ISG: Interferon-stimulated gene

IV: Intravenous

JAK/STAT: Janus kinase/signal transducers and activators of transcription

K-S test: Kolmogorov–Smirnov test

KSHV: Kaposi sarcoma herpesvirus

LANA: Latency-associated nuclear antigen

LCMV: Lymphocytic Choriomeningitis

Let-7: Lethal-7

LMP-1: Latency-associated membrane protein 1

lnc-RNA: Long non-coding RNAs

LOS: Length of hospital stay

M protein: Membrane protein

MAP2K3: Mitogen-activated protein kinase kinase 3

MAPK: Mitogen activated protein kinases

MCMV: Murine Cytomegalovirus

miR-NC: Negative control miR

miRNA: MicroRNA

MK2: Mitogen-activated protein kinase-activated protein kinase 2

MOI: Multiplicity of infection

mRNA: Messenger RNA

MS: Multiple sclerosis

ncRNA: Non-coding RNAs

NF-κB: Nuclear Factor Kappa B

NGS: Next generation sequencing

NH2-AgNPs: Amino-functionalized silver nanoparticles

NIH: National Institute of Health

NK: Natural Killer

NOT/CCR4/CAF1: Negative on TATA/Chemokine Receptor 4/Chromatin Assembly Factor 1

NP: Nanoparticle

NS1: Non-structural protein 1

NS3: Nonstructural 3

NS5A: Nonstructural 5A

NS5B: Nonstructural 5B

NSP10: Non-structural protein 10

NTR: Non-Translated Region

ORF: Open Reading Frame

PABP: Poly-A Binding Protein

PB1: Polymerase basic protein 1

PBS: Phosphate buffered saline

PCA: Principal component analysis

PCAF: p300/CBP-associated factor

PCR: Polymerase Chain Reaction

Pol II: RNA Polymerase II

PP2A: Protein phosphatase 2A

pre-miRNA: Precursor miRNA

pri-miRNA: Primary miRNA

RA: Rheumatoid Arthritis

RBD: Receptor-binding domain

RISC: RNA-induced silencing complex

RNA: Ribonucleic acid

RNAi: RNA interference

RSV: Respiratory Syncytial Virus

RT-qPCR: Reverse transcription-quantitative polymerase chain reaction

RTA: Replication and Transcription Activator

Rtl1/Peg11: Retrotransposon Gag Like 1/ Paternally expressed gene 11

RV: Rotavirus

S protein: Spike protein

SARS-CoV-2: Severe acute respiratory syndrome coronavirus 2

SD: Standard deviation

SERBP1: SERPINE1 MRNA Binding Protein 1

SIDT1: SID1 transmembrane family member 1

SLIT2: Slit Guidance Ligand 2

SNAT: Smart Nano-enabled Antiviral Therapeutic

SOCS1: Suppressor of cytokine signaling-1

SOCS3: Suppressor of cytokine signaling-3

ssRNA: Single stranded RNA

STEM: Scanning transmission electron microscopy

SV40: Simian virus 40

T group: Treatment group

TAR: Transactivating response

TCM: Traditional Chinese Medicine

TEM: Transmission electron microscopy

TLR3: Toll Like Receptor 3

TLR: Toll Like Receptor

TMPRSS2: Transmembrane serine protease 2

TRIM27: Tripartite motif-containing protein 27

Tukey HSD post-hoc test: Tukey's Honest Significant Difference

TUSC2: Tumor Suppressor Candidate 2

Tx: Docetaxel

USD: US Dollar

UTR: Untranslated region

UV: Ultraviolet

V-miR: Viral miRNA

VOC: Variant of concern

VSV: Vesicular Stomatitis Virus

WHO: World Health Organization

Wnt: Wingless-related integration site

WT: Wild-type

WTA: Whole Transcriptome Assay

YTHDF2: YTH N6-methyladenosine RNA binding protein 2

CHAPTER 1: REVIEW OF LITERATURE

MICRORNAS: SMALL BUT KEY PLAYERS IN VIRAL INFECTIONS AND IMMUNE RESPONSES TO VIRAL PATHOGENS

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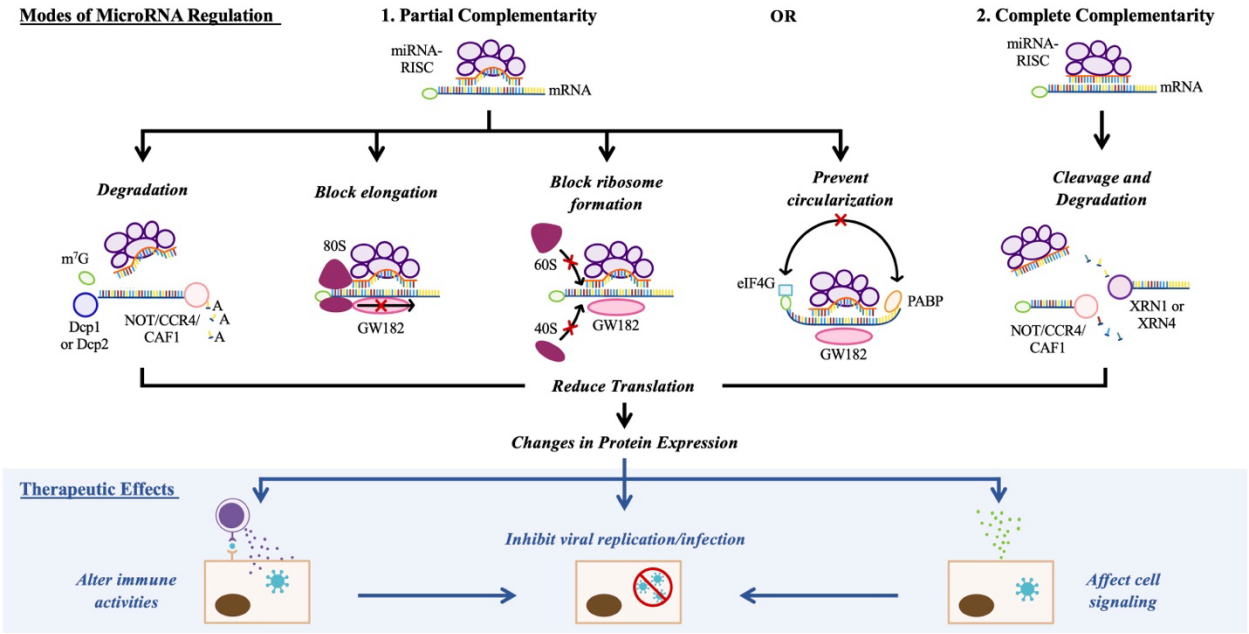
Simple Summary: Viral outbreaks continue to be an obstacle to human health, with the Severe Acute Respiratory Syndrome-related Coronavirus (SARS-CoV-2) pandemic shedding light on the current vulnerabilities of our healthcare system. Understanding viral pathology is paramount in developing methods to combat infection. The study of microRNAs (miRNAs) is relatively new to the world of virology research. These small strands of nucleotides are a versatile tool in the regulation of gene expression. Various miRNAs have roles in immune development, immune and inflammatory responses, and viral infections. Changes in miRNA expression can be a double-edged sword, used to alter cell activities to help the host fight infections or taken advantage of by viruses to enhance infection. Uncovering these interactions and their implications can provide direction for therapeutic and diagnostic advancement. Further, miRNAs may have the potential to predict the severity of viral infection and possible health outcomes, as well as track disease progression to inform treatment options. miRNA technology is also anticipated to be highly marketable and has already entered the realm of commercial biopharmaceuticals. Continuing to elucidate the functions of miRNA during infection and the therapeutic potential of these molecules will contribute to new strategies in the battle against current and future viral pathogens.

Abstract: Since the discovery of microRNAs (miRNAs) in C. elegans in 1993, the field of miRNA research has grown steeply. These single-stranded non-coding RNA molecules canonically work at the post-transcriptional phase to regulate protein expression. miRNAs are known to regulate viral infection and the ensuing host immune response. Evolving research suggests miRNAs are assets in the discovery and investigation of therapeutics and diagnostics. In this review, we succinctly summarize the latest findings in (i) mechanisms underpinning miRNA

regulation of viral infection, (ii) miRNA regulation of host immune response to viral pathogens, (iii) miRNA-based diagnostics and therapeutics targeting viral pathogens and challenges, and (iv) miRNA patents and the market landscape. Our findings show the differential expression of miRNA may serve as a prognostic biomarker for viral infections in regard to predicting the severity or adverse health effects associated with viral diseases. While there is huge market potential for miRNA technology, the novel approach of using miRNA mimics to enhance antiviral activity or antagonists to inhibit pro-viral miRNAs has been an ongoing research endeavor. Significant hurdles remain in terms of miRNA delivery, stability, efficacy, safety/tolerability, and specificity. Addressing these challenges may pave a path for harnessing the full potential of miRNAs in modern medicine.

Keywords: microRNA; miRNA; viral infection; miRNA therapeutics; miRNA diagnostics

Graphical Abstract:



1. Introduction

The central dogma of biology posits a deceivingly straightforward process. It describes how DNA is transcribed into RNA, which is then translated into protein - or not. In fact, only 1-2% of the transcribed genome encodes proteins in mammals (Chan, J. J. & Tay, 2018). Most of the DNA in eukaryotic cells is non-coding.

MicroRNA (miRNA) is one of the many types of non-coding RNA found in eukaryotes (Cable et al., 2021). Despite their average length of a mere ~22 base pairs, these small strands of nucleotides are predicted to regulate more than 60% of all human protein-coding genes (Friedman et al., 2008). They typically work at the post-transcriptional level to enhance or repress the translation of mRNA, though some evidence suggests they may also have regulatory functions at the transcriptional level (Catalanotto et al., 2016). But how does something so small make such a big impact? The minute size of miRNA contributes to its ability as a strong regulatory element. Functionally, the short sequence may allow room for multiple miRNAs to bind to a single messenger RNA (mRNA) and synergistically repress translation (Ding, J. et al., 2014). On the other hand, the target sequence in mRNA is also short and may be common between different groups of mRNAs (Correia de Sousa et al., 2019). Thus, a single miRNA has the potential to bind to many types of mRNAs. The regulatory effect of miRNA is further amplified by its ability to be released for reuse after its target has been degraded (Catalanotto et al., 2016)**.** These traits allow miRNAs to act alone or in combination to rapidly suppress gene expression and protein production.

Because of its key role in regulation, the miRNA biogenesis pathway is highly conserved between species (Rupaimoole & Slack, 2017). It begins in the nucleus, where the corresponding gene is transcribed into primary miRNA (pri-miRNA) (Rupaimoole & Slack, 2017). This pri-miRNA is then modified, and the resulting precursor miRNA (pre-miRNA) is transported to the cytoplasm via nuclear pores (Correia de Sousa et al., 2019; Rupaimoole & Slack, 2017). Once in the cytosol, precursor miRNA is processed, and the mature miRNA duplex binds to the RNA-induced silencing complex (RISC) (Correia de Sousa et al., 2019). The Argonaute (AGO) protein family within RISC, along with various cofactors, unwind the miRNA duplex and select one of the strands (Correia de Sousa et al., 2019). The AGO protein is largely responsible for transporting the complex to a mRNA with a short complimentary sequence (Rupaimoole & Slack, 2017). In animals, this sequence is often in the 3' untranslated region (UTR) of the mRNA with a perfect or near-perfect ~7 nucleotide base pairing with the seed sequence in miRNA (Bartel, 2004). This interaction typically triggers the mRNA degradation pathway to downregulate protein production (Guo, H. et al., 2010). In some instances, miRNA can alternatively interact with the 5' UTR or coding sequence (CDS) of mRNA to affect stability or translation (Akula et al., 2022a; Friedrich et al., 2020; Lin, Y. et al., 2019; Liu, Z. et al., 2021; Panigrahi et al., 2022).

Translation may be directly repressed by miRNA through decreased ribosomal interaction with the mRNA (Guo, H. et al., 2010). More often, the target mRNA is destabilized, accelerating the standard degradation process (Guo, H. et al., 2010). In rare cases where the base pairing is much more extensive, AGO may directly cleave mRNA, making it susceptible to rapid degradation (Catalanotto et al., 2016; Guo, H. et al., 2010). In contrast, miRNA has been shown to upregulate

translation in some cases (Valinezhad Orang et al., 2014; Zhang, Xiaorong et al., 2014). It may also directly regulate transcription via promotor interactions or indirectly by targeting the mRNA of transcriptional regulators (Catalanotto et al., 2016; Xiao, M. et al., 2017). Additionally, miRNA can bind to non-coding RNAs, including long non-coding RNA (lnc-RNA), circular RNA (circ-RNA), and other miRNAs (Hill & Tran, 2021).

The role of miRNAs in human health continues to be an area of active research. Since the discovery of miRNA in *C. elegans* in 1993 and mammalian miRNA in 2000, the field of miRNA research has grown dramatically (Rupaimoole & Slack, 2017). In 2017 alone, there were ~11,000 studies on miRNA, with more than 6,000 of these studies on miRNA diagnostics and therapeutics combined (Bonneau et al., 2019). There is evidence of miRNA involvement in many medical conditions, including cardiovascular disease (Siasos et al., 2020), various cancers (Hill & Tran, 2021), and infections (Akula et al., 2022a; Hussein & Akula, 2017a; Hussein & Akula, 2017b; Hussein et al., 2019; Kataria et al., 2022; Yuan et al., 2021). This review is aimed at succinctly summarizing the latest findings in (i) mechanisms underpinning miRNA regulation of viral infection and the (ii) host immune response to viral pathogens, (iii) miRNA diagnostics and therapeutics targeting viral pathogens, and (iv) miRNA market landscape.

2. Modes of miRNA-Mediated Gene Regulation

Gene regulation by miRNA-RISC may occur through one of the following mechanisms: (i) site-specific cleavage, (ii) mRNA degradation, and (iii) translation inhibition (Gu, S. & Kay, 2010; Park & Shin, 2014). Site-specific cleavage occurs when there is a near-perfect match of miRNA to the target RNA. This process is called RNA interference (RNAi). In contrast, miRNA-directed

mRNA degradation and translation inhibition are the non-cleavage modes of miRNA regulation. As early as 2004, it was demonstrated by the Bartel lab that miR-196 can effectively regulate gene expression by directed cleavage of HOXB8 mRNA (Yekta et al., 2004). Such miRNA cleavage activity utilizes AGO proteins (Faehnle & Joshua-Tor, 2007). miRNA-directed endonucleolytic cleavage of target mRNAs is more common in plants than animals but has a critical function (Gu, K. et al., 2018; Jones-Rhoades et al., 2006). In animals, cleavage of miRNA targets is a poorly understood concept, partly due to a lack of effort to investigate this phenomenon. Recent studies have demonstrated several animal miRNAs to target mRNA by cleavage, and a few examples of such miRNAs are Let-7 (targeting TUSC2) (Xu, K. et al., 2016), miR-92 (targeting SERBP1) (Barbato et al., 2022), miR-127 and miR-136 (targeting Rtl1/Peg11) (Davis et al., 2005).

Multiple mechanisms are in place to regulate the miRNA-directed non-cleavage mode of regulating mRNA expression. The key event for all these activities is interactions between the scaffold protein, GW182, and any of the AGO proteins. The most common pathway is GW182-mediated deadenylation, followed by de-capping and mRNA degradation via NOT/CCR4/CAF1 complexes (Mauri et al., 2017). Other pathways include GW182 competing with eIF4G in association with poly-A binding protein (PABP) to prevent the circularization required for efficient translation of target mRNA (Braun et al., 2013; Naeli et al., 2023); GW182 preventing the formation of a functional 80S ribosome crucial to the translational process of the target transcript (Fukaya & Tomari, 2012; Gu, S. & Kay, 2010); or GW182/AGO complex-induced ribosomal stalling that induces a translation elongation block (Zhang, Kai et al., 2018). Such diversity in miRNA-mediated silencing mechanisms provides life with enhanced capacity for

gene regulation but also poses challenges to a complete understanding of the biology of miRNAs.

Apart from plant and animal miRNAs, there are also miRNAs encoded by viruses (v-miRs). More than 250 v-miRs have been reported to play crucial roles in virus pathogenesis (Mishra, Richa et al., 2020). Once again, perfect complementarity results in mRNA site-specific cleavage, as observed in simian virus 40 (SV40) encoded miR-S1-5p and miR-S1-3p, which target the mRNA coding for a protein known as Large T antigen (Tokorodani et al., 2020). In contrast, imperfect/partial complementarity with the target mRNA leads to translational repression via non-cleavage mode, as reported for EBV-encoded miR-BART-1p, miR-BART16, and miR-BART17-5p, which target latency-associated membrane protein LMP-1 (Lo et al., 2007). Viral miRNAs, perhaps, have evolved cleavage or non-cleavage modes of regulating mRNA expressions based on necessity. For example, if only one miRNA is required to regulate a key mRNA for viral replication, it may follow the cleavage mode as in the case of SV40 miR-S1-5p (Tokorodani et al., 2020). If there are multiple v-miRs regulating one viral mRNA, as in the case of EBV-encoded miR-BART-1p, miR-BART16, and miR-BART17-5p, it may follow a non-cleavage mode (Lo et al., 2007). Recent studies have also determined the ability of v-miRs to regulate cellular genes critical to their survival (Li, F. et al., 2022; Panigrahi et al., 2022).

3. Host Response to Viral Pathogens

The ability to defend against harmful agents is an evolutionary necessity (Netea et al., 2019). Even bacteria have developed a method to protect themselves against viral infection, using the clustered regularly interspaced palindromic repeat (CRISPR)/Cas system (Moineau et al., 2010).

Humans, however, have a more complex strategy with many components. Elements of the immune system are distinguished historically as innate or adaptive (Varadé et al., 2020). Though classically defined as separate, recent research indicates crosstalk between these two branches (Varadé et al., 2020). In addition, it suggests communication between the immune system, nervous system, endocrine system, and microbiome (Varadé et al., 2020).

Innate immunity is often referred to as the first line of defense and recognizes conserved antigens, including common motifs in pathogens (Weizman et al., 2017). It includes physical barriers, such as skin and the mucosal lining of the respiratory and digestive tracts (Varadé et al., 2020). At birth, infants are exposed to the external environment for the first time and must be able to survive interactions with other organisms, including viruses (Yu et al., 2018). Human breast milk helps strengthen a newborn's immune system and delivers ~1400 miRNAs, including many linked to immune system maturation and viral defenses (Yi & Kim, 2021).

When a novel pathogen breaches the physical barrier of the skin or mucous membranes, the innate immune response is nonspecific and quick, mounting a defense in just minutes to hours (Netea et al., 2019). Activation of adaptive immune cells occurs during the first exposure to a pathogen. When the body reencounters the same virus, its memory bank of immune cells developed during the first exposure quickly identifies the markers and responds efficiently (Netea et al., 2019). While the innate immune response still occurs, the resident adaptive immune cells assemble more rapidly from memory to provide a strong wave of protection (Netea et al., 2019).

4. miRNA and Immunity

The impact of miRNA on the immune system is observed before birth and has dynamic effects throughout the human lifetime (Gong et al., 2017; Kim, C. et al., 2021). For example, miR-181a has many targets involved in immune cell processes (Cichocki et al., 2011; Jensen et al., 2013; Kim, C. et al., 2021; Xie et al., 2013). It is involved in T cell development, homeostasis, activation, and proliferation (Kim, C. et al., 2021). In addition, miR-181a-5p may play a role in B cell development from precursors in bone marrow, and miR-181 in Natural Killer (NK) cells promotes development from Hematopoietic Progenitor Cells (HPCs) (Cichocki et al., 2011; Jensen et al., 2013). miR-181a has been implicated in inhibiting the production of IL-1a and other inflammatory factors in macrophages and monocytes (Xie et al., 2013). It also regulates monocyte-derived dendritic cell activation and release of inflammatory cytokines (Lim et al., 2020). This one type of miRNA has many roles in immune cell development, differentiation, expansion, activation, and effector functions.

Several other miRNAs influence similar processes. For example, the differential expression of miR-126, miR-146a, miR-150, and miR-17-92 is involved in the regulation of T cell development in the thymus (Emamgolizadeh Gurt Tapeh et al., 2020). During myelopoiesis, expression levels of miR-125b and miR-10a decrease with differentiation, and the corresponding increased expression levels of their target proteins may explain how key physiological differences develop between immune cells (Rajasekhar et al., 2018). For example, miR-125b has many targets, including several transcription factors involved in B cell and T cell differentiation (Rajasekhar et al., 2018). On the other hand, miR-10a targets transcription factors required for monocytopoeisis and megakaryocyte differentiation (Rajasekhar et al., 2018). In mice, miR-142

has a role in maintaining cell levels of type 1 Innate Lymphoid Cells (ILCs), NK cell survival, and response to cytokines (Berrien-Elliott et al., 2019). This may contribute to the greater susceptibility of miR-142-deficient mice to Murine Cytomegalovirus (MCMV) infection compared to wild-type counterparts (Berrien-Elliott et al., 2019). An overview of the roles of miRNA in general immunity is provided in **Figure 1**. The roles of miRNA in immune cell responses to viral pathogens will be explored in-depth in the following sections.

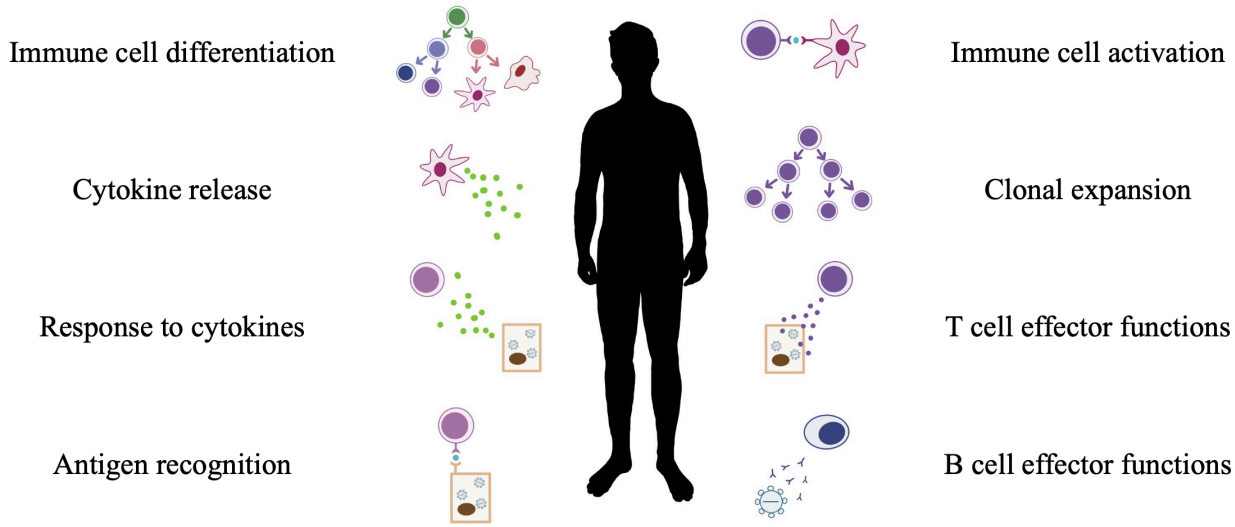


Figure 1. Summary of miRNA regulated processes in immunity.

miRNAs add a new layer to the complexity of the immune system. The immune system is constantly in a dynamic yet delicate balance. miRNAs may provide insight into diseases of the immune system and possible treatments. This includes autoimmune diseases such as multiple sclerosis (MS) and Rheumatoid Arthritis (RA) (Mirzaei et al., 2021), as well as allergic reactions (Cañas et al., 2021) and cancers such as leukemia (Wang, Xiao-Shuang et al., 2012). Population differences in miRNA expression may also inform differences in immunity (Rotival et al., 2020).

With roles in both development and function, miRNAs help moderate immune responses and maintain immune cell homeostasis.

5. Pro-viral miRNA Regulation of Viral Infection

The host response to a viral pathogen is triggered at the point of virus binding and interaction with the cell. The viral entry process involves intricate interactions with host cell-specific receptor molecules. Interactions with these appropriate receptors trigger entry of the viral pathogen. For example, SARS-CoV-2 (RNA virus) and Kaposi's sarcoma-associated herpesvirus (KSHV; DNA virus) interact with ACE2 and integrins expressed on the host cell surface, respectively (Safdar et al., 2023; van der Meulen et al., 2021). Such interactions allow SARS and KSHV to be internalized, or in other words, establish infection. This can activate cell signaling pathways to induce cellular miRNA production and create an antiviral or pro-viral environment (Trobaugh & Klimstra, 2016).

Viruses may also encode their own miRNA, known as v-miRs. The site of viral genome replication influences the method of v-miR biogenesis. Some viral genomes, such as SARS, end up in cytoplasm, while others, like KSHV, end up in the nucleus for replication. Viruses that replicate in the cytoplasm cannot access host miRNA biogenesis machinery in the nucleus (Mishra, Richa et al., 2020). However, cytoplasmic translocation of Drosha can occur during viral infection, allowing for the processing of miRNA encoded in the viral genome (Nanbo et al., 2021). Retroviruses such as Human Immunodeficiency Virus (HIV) integrate their genomes into host chromosomes via reverse transcription (Nanbo et al., 2021). Since their replication occurs in the nucleus, miRNA production can occur following canonical host pathways (Nanbo et al.,

2021). Similarly, DNA viruses that replicate in the nucleus have access to host miRNA biogenesis machinery for v-miR production (Mishra, Richa et al., 2020).

Viral pathogens use miRNAs to their advantage in several ways. v-miRs can target host (cellular) or viral mRNA directly, binding to the 3' or 5' UTR (**Figure 2**) (Liu, Z. et al., 2021). When canonically binding to the 3'UTR, they typically destabilize the transcript and trigger the degradation pathway of cellular mRNAs (Wang, Jie et al., 2022). v-miRs have also been shown to bind to the 5'UTR, preventing translation without altering mRNA levels in the cell (Pandeya et al., 2022; Zhao et al., 2020). Alternatively, v-miRs binding to the 5'UTR can increase mRNA stability, preventing degradation and upregulating gene expression (Liao, Y. et al., 2021; Panigrahi et al., 2022). Likewise, binding to the 5' non-translated region (NTR) can increase RNA viral genome stability (Trobaugh & Klimstra, 2016). v-miRs can target unique binding sites or act as functional mimics of host miRNAs by binding to the same site as corresponding endogenous miRNAs (Haneklaus et al., 2012).

Viruses take advantage of pre-existing machinery within infected cells to promote viral processes. Some viruses, such as Hepatitis C Virus (HCV), do not encode viral miRNA but can regulate endogenous miRNA levels (Balasubramaniam et al., 2018; Li, H. et al., 2016; Pascut et al., 2021). Viruses may induce the synthesis of host miRNAs when they are recognized upon entry (Sharma et al., 2021). They encode proteins that degrade a host miRNA or upregulate it (Jiang et al., 2020; Qiao et al., 2021; Rosato et al., 2012). Under certain circumstances, viruses exploit differential miRNA profiles between host cells to have tissue-specific effects depending on local expression levels (Li, F. et al., 2022; Panigrahi et al., 2022). This is especially important

for viruses that target specific tissues, like hepatitis viruses in the liver, as well as viruses with latency phases in specific tissues, like herpesviruses in neurons (Liao, T. et al., 2021; Panigrahi et al., 2022; Sun et al., 2021). Viruses can also utilize host secretory pathways and the exosomal release of miRNAs for uptake by other cells (Liao, T. et al., 2021; Nahand et al., 2020; Naqvi, 2020; Zhao et al., 2020).

Viruses can further manipulate cellular functions through v-miRs or modulation of host miRNA levels by regulating the transcription of specific genes. This includes targeting a transcription factor (Fan et al., 2021), enhancer (Li, F. et al., 2022; Liu, Z. et al., 2021), repressor (Wang, Jie et al., 2022), chromatin modifier (Xing et al., 2019), or another regulator within the cell (Othumpangat et al., 2021). In some cases, miRNAs may regulate signaling pathways within the cell to increase or decrease transcription of stimulated genes (Bouvet et al., 2021; Zhang, Qian et al., 2021; Zhao et al., 2020). miRNAs can also be used by the virus to regulate viral gene expression and transcription of the viral genome (Akula et al., 2022a; Wakabayashi et al., 2019). v-miRs or host miRs may be utilized to promote viral replication but may alternatively downregulate it to maintain chronicity or latency (Sun et al., 2021; Xing et al., 2019; Zhao et al., 2020). The use of miRNA gives viruses the ability to control gene expression and manipulate host cell activities to their benefit. miRNAs have been implicated in many processes during infection, including viral entry, viral replication, latency and reactivation, immune evasion, immune suppression or overactivation, inflammation, host cell survival, tumorigenesis, and more (Akula et al., 2022a; Arisan et al., 2022; Aydemir et al., 2021; Bouvet et al., 2021; Fan et al., 2021; Hancock et al., 2020; Li, F. et al., 2022; Liao, T. et al., 2021; Liu, Z. et al., 2021; Madrid-Elena et al., 2022; Melaiu et al., 2020; Mishra, Ritu & Banerjea, 2021; Othumpangat et al., 2021;

Pandeya et al., 2022; Panigrahi et al., 2022; Qiao et al., 2021; Sharma et al., 2021; Sun et al., 2021; Wakabayashi et al., 2019; Wang, Jie et al., 2022; Xing et al., 2019; Xu, D. et al., 2019; Zhang, Fangyi et al., 2019; Zhang, Qian et al., 2021; Zhao et al., 2020). See **Table 1** and **Table 2** for recent research developments in miRNA regulation of viral infection.

It is important to note that miRNAs predicted to be encoded by RNA viruses have been somewhat controversial (Li, X. & Zou, 2019). There is concern that the excision of miRNA or miRNA-like fragments could result in cleavage of the viral genome and hinder viral replication (Li, X. & Zou, 2019). It has been suggested that the HIV encodes a transactivating response (TAR) motif that includes miRNA precursors that can be produced without cleavage of the viral genome (Harwig et al., 2016; Komori et al., 2020). However, the exact mechanism of this non-canonical pathway of miRNA production is still under investigation (Harwig et al., 2016; Komori et al., 2020). That said, v-miR-TAR has been detected in the sera of HIV-infected patients along with HIV-encoded v-miR-88 and v-miR-199 (Bernard et al., 2014). Dengue virus, Ebola virus, H5N1 Influenza virus, and SARS-CoV-2 have also been reported to encode functional miRNA-like small RNAs (Li, X. & Zou, 2019; Liu, Z. et al., 2021).

The discovery of virally encoded miRNA and the ability of viruses to manipulate host miRNA has the potential to elucidate new viral functions and host-virus interactions. However, some of the viral miRNA characterized in publications and their putative targets are only computer-predicted (Aydemir et al., 2021; Liu, Z. et al., 2021). The production of these miRNAs and their interactions with the targets need to be verified through *in vitro* and *in vivo* studies. In addition, the outcome of these interactions should be confirmed at the cellar and organismal level. For

example, miR-mRNA interactions predicted to decrease gene expression by degradation of mRNA should reflect this in both attenuated mRNA transcript levels and reduced protein output (Pandeya et al., 2022). Furthermore, researchers may consider expanding on the local or systemic effects of viral miRNA packaged into exosomes and virus-induced changes in exosomal host miRNA levels (Akula et al., 2022a; Nahand et al., 2020; Zabrodskaya et al., 2022). They should also critically evaluate if changes in host miRNA levels are due to viral manipulation or if they are a potential defensive response to infection.

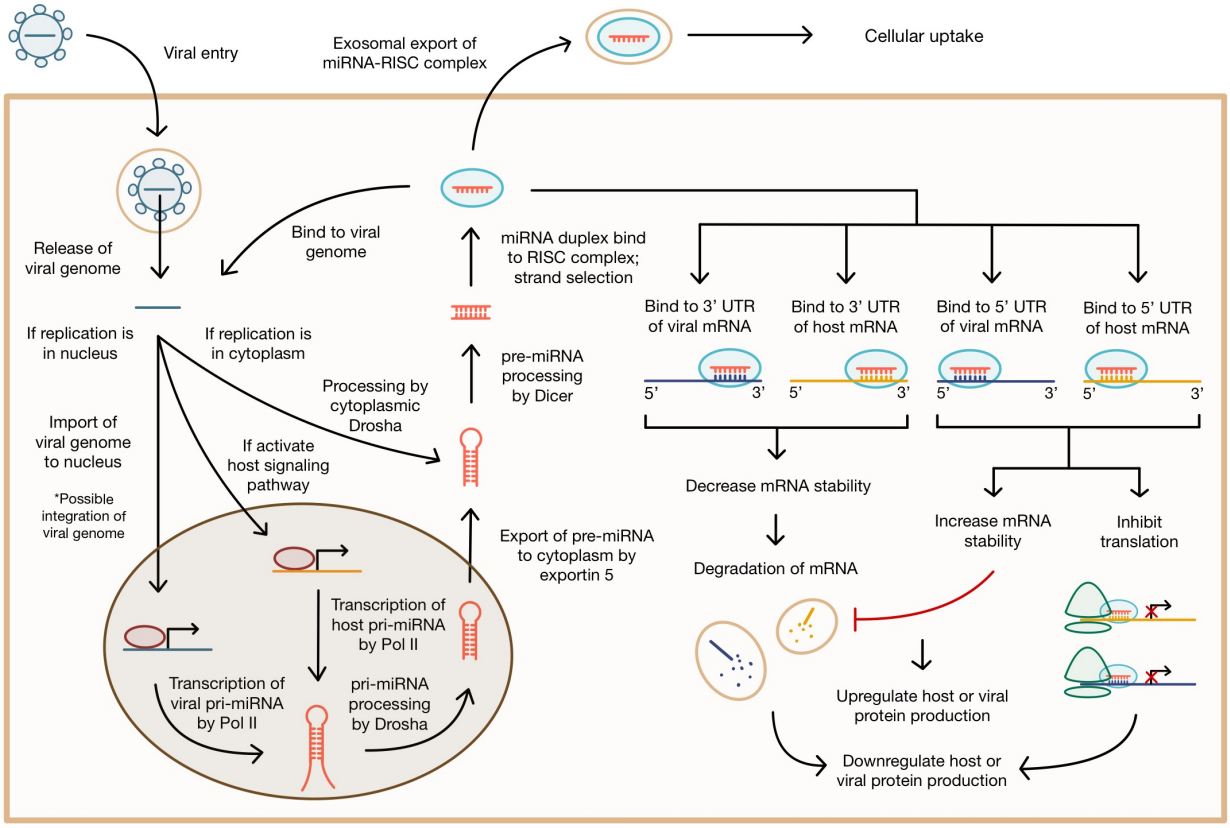


Figure 2. Biogenesis of miRNA during viral infection and possible effects. miRNA may be encoded by the host or virus. Viruses may upregulate or downregulate endogenous miRNA. miRNA may bind the viral genome, mRNA, or be packaged in an exosome for export to other cells. The binding site of miRNA at the 3' or 5' UTR of its mRNA target may alter stability of mRNA or inhibit translation.

Table 1. Pro-viral miRNAs involved in infection by RNA viruses IAV, SARS-CoV-2, HIV-1, and HCV. hsa-miRs are encoded by the human host and may be manipulated by viruses. The miRs without hsa notation are virally encoded.

Virus Name	miRNA	Target(s)	Viral effect	Viral significance	Ref
Influenza A Virus (IAV)	hsa-miR-132-3p	IRF1	Upregulate miR-132-3p to inhibit Type I IFN production and downregulate ISGs	Immune evasion, host cell survival, viral replication	(Zhang, Fangyi et al., 2019)
	put-hsa-miR-34	STAT3	Downregulate STAT3/IL-6 mediated antiviral response and upregulate NF-κB pathway	Viral replication, prevent immune and inflammatory responses	(Othumpangat et al., 2021)
Severe Acute Respiratory Syndrome-related Coronavirus (SARS-CoV-2)	MR-147-3p	EXOC7; RAD9A; TFE3	Downregulate exocytosis; cell death and apoptosis; lipid and glucose metabolism and TGF-β induced transcription	Exocytosis; host cell survival; metabolism and transcription of host genes	(Liu, Z. et al., 2021)
	MR359-5p	FOXO3; GCPR1	Downregulate autophagy and dysregulate oxidative damage responses; bind 5'UTR to upregulate GPCR1 and viral propagation	Host cell survival; viral pathogenesis	(Liu, Z. et al., 2021)
	hsa-miR-148a; hsa-miR-590	USP33; IRF9	Higher exosomal loading of miR-148a and miR-590 in infected cells downregulates USP33 and IRF9 in macrophages to upregulate NF-kB, TNFα and IFNβ pathways	Hyperinflammation	(Mishra, Ritu & Banerjea, 2021)
	hsa-miR-150-5p	Viral nsp10 gene	Downregulate miR-150-5p to prevent decreased viral gene expression	Translational efficiency, viral replication, immune evasion	(Akula et al., 2022a)
Human Immunodeficiency Virus 1 (HIV-1)	hsa-miR-144	Nrf2	Upregulate miR-144 to downregulate antioxidant response and impair alveolar macrophage phagocytosis	Immune evasion	(Fan et al., 2021)
	hsa-miR-210-5p	TGIF2	Induce miR-210-5p production to downregulate TGIF2 and promote G2 cell cycle arrest	Cell cycle arrest	(Qiao et al., 2021)
Hepatitis C Virus (HCV)	hsa-miR-122	TLR7	Induce host miRNA and exosomal transport to macrophages to activate TLR7, inducing NF-κB pathway and upregulating B cell activating factor (BAFF)	Autoimmune response	(Liao, T. et al., 2021)
	hsa-miR-122	HCV genome	Liver specific miRNA increases stability of viral genome and promotes viral translation	Viral replication, gene expression	(Panigrahi et al., 2022)

Table 2. Pro-viral miRNAs involved in infection by DNA viruses EBV, HSV-1, HCMV, HBV. The hsa-miRs are encoded by the human host and may be manipulated by viruses. The miRs without hsa notation are virally encoded.

Virus Name	miRNA	Target(s)	Viral effect	Viral significance	Ref
Epstein Barr Virus (EBV)	miR-BART3, miR-BART19	RIG-1	Downregulate PRR and Type I IFN production in B cells	Innate immune evasion	(Bouvet et al., 2021)
	miR-BART1; miR-BART3	IRF-9; JAK1	Downregulate JAK/STAT pathway response to Type I IFN and ISGs	Innate immune evasion	(Bouvet et al., 2021)
	miR-BART11; miR-BART17-3p	FOXP1; PBRM1	Downregulate repressors of PD-L1 transcription to prevent T cell cytotoxic activity against infected cells	Immune evasion	(Wang, Jie et al., 2022)
	miR-BHRF1-1	p53 gene	Downregulate p53 to decrease cell cycle arrest, prevent apoptosis, and induce proliferation	Cell survival, proliferation, tumorigenesis	(Xu, D. et al., 2019)
Herpes Simplex Virus (HSV-1)	hsa-miR-138	HSV-1 ICP0, Oct-1, Foxc1	High expression of miR-138 in neurons allows cell specific repression of viral gene expression, transcription, and replication	Latency	(Sun et al., 2021)
	hsa-miR-24	STING	Induce production of miR-24 to downregulate STING pathway and decrease IFN production	Immune evasion	(Sharma et al., 2021)
Human Cytomegalovirus (HCMV)	miR-US33as-5p	IFNAR1	Downregulate IFN activation of Jak/STAT pathway and transcription of ISGs	Immune evasion	(Zhang, Qian et al., 2021)
	miR-US5-2; miR-UL22A	NAB1; SMAD3	Upregulate TGF-β production to decrease CD34+ HPC proliferation and myelopoiesis; Downregulate TGF-β stimulated genes	Myelosuppression Latency and reactivation	(Hancock et al., 2020)
	miR-UL148D	ERN1	Downregulate JNK signaling pathway and ER stress induced apoptosis	Host cell survival	(Pandeya et al., 2022)
	miR-UL59, UL70-3p, US4-5p, US5-1, US22-5p, US25-2-5p, US29-5p, US33-5p	ERAP1	Viral miRNAs preferentially bind different genetic variants of ERAP1 to downregulate MHC class I antigen processing	Immune evasion	(Melaiu et al., 2020)
Hepatitis B Virus (HBV)	hsa-miRNA-548ah	HDAC4	Promotes miRNA-548ah expression, downregulating HDAC4 to reduce histone interactions with viral cccDNA	Viral replication, viral transcription	(Xing et al., 2019)
	HBV-miR-3	SOCS5	Upregulate JAK/STAT pathway and ISGs in hepatocytes; Exosomal HBV-miR-3 triggers macrophage polarization to M1 and promotes EGFR to increase IL-6 secretion	Innate immune activation, maintain chronic infection	(Zhao et al., 2020)
	hsa-miR-192-3p	ZNF143	Upregulate miR-192-3p in hepatocytes to downregulate ZNF143/Akt/mTOR signaling, enhance viral replication	Viral transcription, viral replication	(Li, F. et al., 2022)

6. miRNA Regulation of the Antiviral Host Response

Viral pathogens manipulate host miRNA for a reason: they are master regulators of immunity. Host miRNA may target viral gene expression directly or indirectly to prevent viral replication (Liao, Y. et al., 2021; McCaskill et al., 2017). Binding to the 3' NTR (non-translated region) of an RNA viral genome can inhibit translation and, thus, replication in some cases (Trobaugh & Klimstra, 2016). They are involved in the intracellular antiviral response upon infection, including the regulation of pathways for interferon production or transcription of interferon-stimulated genes (ISGs) (Singaravelu et al., 2019). miRNAs play a role in immune cell activation, effector functions, and memory development, as well as antigen presentation of infected cells (Dudda et al., 2013; Goncalves-Alves et al., 2019; Khurana et al., 2020; Zawislak et al., 2013). They may mediate the susceptibility of host cells to infection through the regulation of endocytic or secretory pathways (Guo, L. et al., 2018; He et al., 2019; Khurana et al., 2020). In addition, host cells have defenses against viral miRNAs (Tagawa et al., 2018). **Table 3** provides a summary of antiviral miRNAs, their targets, and their role in viral infection.

6.1. miRNA in the Intracellular Antiviral Response

Cells have many mechanisms in place to guard against viral pathogens. When infection occurs, cells display different miRNA profiles compared to healthy or resistant cells (Lodge et al., 2020; Pierce et al., 2020). While some miRNAs may be manipulated by the virus for pro-viral functions, others have antiviral effects (Li, F. et al., 2022; Liao, Y. et al., 2021; Smith et al., 2017).

Host miRNAs may target viral genes directly to downregulate expression. For instance, hsa-miR-3145 has been shown to silence the viral *PB1* gene in H5N1, H1N1, and H3N2 subtypes of Influenza A Virus (IAV) (Liao, Y. et al., 2021). The miRNA-mediated downregulation of the PB1 protein prevents effective viral transcription and replication in A549 cells (Liao, Y. et al., 2021). Type I IFN can upregulate hsa-miR-1307 during H1N1 infection of A549 cells to inhibit the expression of its target, the viral *NS1* gene (Liao, Y. et al., 2021). NS1 protein has been implicated in creating a favorable environment for viral replication through G0/G1 cell cycle arrest of infected cells (Liao, Y. et al., 2021). By downregulating NS1, miR-1307-3p is reported to be an effective inhibitor of IAV replication (Liao, Y. et al., 2021). Similarly, hsa-miR-150-5p targets the *nsp10*-coding strand in the SARS-CoV-2 genome (Akula et al., 2022a). The *nsp10* gene product is important in facilitating viral replication and immune evasion (Akula et al., 2022a). As seen in Table 1, hsa-miR-150-5p downregulation in moderate-severe patients during SARS-CoV-2 infection may have pro-viral consequences (Akula et al., 2022a).

Host miRNA may impair viral infection by interfering with viral entry. For example, during KSHV infection of human endothelial cells (HMVEC-d), hsa-miR-36 is upregulated shortly after infection and targets IFITM1, an interferon-induced transmembrane protein upregulated by KSHV that helps facilitate viral entry (Hussein & Akula, 2017a). In this case, cells directly combat manipulation by the virus. During SARS-CoV-2 infection, resistant cell lines have higher expression of several miRNAs that target host cell receptor proteins involved in viral entry when compared to susceptible cell lines (Pierce et al., 2020). This includes ACE2, predicted to be targeted by miR-9-5p and miR-218-5p, as well as TMPRSS2 targeted by let-7d-5p, miR-494-3p, miR-382-3p, let-7e-5p, miR-181c-5p, and miR-452-5p (Pierce et al., 2020). During SARS-CoV-

2 infection, hsa-miR-1827 and hsa-miR-1277-5p are predicted to target proteins involved in viral entry and antigen presentation of the viral spike protein in host cells (Khurana et al., 2020). A miRNA implicated in HCV viral entry is miR-182, which targets a tight junction protein CLDN1 that aids in the internalization of the virus (Riad et al., 2018). mir-182 expression in infected Huh7 cells decreased viral load compared to mir-155, a known inhibitor of CLDN1 that contrastingly increased viral load (Riad et al., 2018).

Host cells also have methods to contend with virally encoded miRNA. Recent evidence suggests that some host circular RNAs act as sponges or decoys to prevent the binding of viral miRNAs to their targets (Qu et al., 2021; Tagawa et al., 2018). For instance, hsa_circ_0001400 is induced during KSHV infection of HUVEC cells (Tagawa et al., 2018). This circ-RNA is predicted to have a binding site for KSHV-encoded miR-K12-10b and was shown to decrease the expression of viral *RTA* and *LANA* genes (Tagawa et al., 2018). However, this effect may be due to interactions between the circ-RNA and human mRNA encoding transcription factors involved in chromatin modification instead (Tagawa et al., 2018). Host cells can alternatively regulate their own miRNA to further antiviral cellular functions. During IAV infection of A549 cells, the intronic circ-RNA AIVR is upregulated and functions as a miRNA sponge in the cytoplasm that sequesters mir-330-3p (Qu et al., 2021). mir-330-3p downregulates CREBBP, a protein involved in enhancing IFN-β production (Qu et al., 2021). The circ-RNA AIVR downregulates mir-330-3p activity to increase the expression of this antiviral factor (Qu et al., 2021).

miRNAs can be released via exocytosis and exert antiviral effects in other cells. During HIV infection, intestinal epithelial cell TLR3 activation can induce the production of miRNAs that

can restrict HIV (Guo, L. et al., 2018). This includes miR-17 and miR-20, which downregulate the expression of p300/CBP-associated factor (PCAF), an HIV protein cofactor (Guo, L. et al., 2018). In addition, miR-28 targets the HIV transcript, and miR-29 family members interfere with HIV replication (Guo, L. et al., 2018). The uptake of these exosomal miRNAs, along with other antiviral elements were able to induce the production of HIV restriction factors in macrophages (Guo, L. et al., 2018).

6.2. miRNA in Cellular Signaling Pathways During Viral Infection

Cellular signaling pathways involved in the antiviral response can also be regulated by host miRNA. The Wnt, IFN, MAPK, and NF-κB pathways have all been implicated in antiviral responses (Barbu et al., 2020). The Wnt pathway is associated with cell survival and proliferation and is activated during Rotavirus infection (RV) (Banerjee et al., 2022). hsa-miR-192 and hsa-miR-215 target the frizzled receptors, while hsa-miR-181a directly targets β-catenin (CTNNB1) in this pathway (Banerjee et al., 2022). These host miRNAs are downregulated during RV infection to promote Wnt/β-catenin signaling and survival of infected cells (Banerjee et al., 2022). However, combined overexpression of miR-192 and miR-215 in RV-infected Caco2 cells inhibited RV replication (Banerjee et al., 2022). The Wnt pathway is also targeted and downregulated by the miR-34 family, which has been shown to repress flavivirus replication and enhance the interferon response in infected cells (Smith et al., 2017).

The interferon pathway is a key player in intercellular signaling and production of antiviral effector proteins. The miR-183 cluster, consisting of miR-96, miR-182, and miR-183, promotes IFN signaling and production and was shown to decrease vesicular stomatitis virus production in

infected HepG2 cells (Singaravelu et al., 2019). Type I and III IFNs typically activate the JAK/STAT pathway with downstream activation of STAT1, a transcription factor regulating ISGs, but miR-183 can upregulate STAT1 mRNA without immune stimulation (Singaravelu et al., 2019). miR-183 also downregulates PP2A and TRIM27, two repressors of IRF3 activation, to increase Type I and III IFN production (Singaravelu et al., 2019). Influenza A virus has been shown to downregulate the miR-30 family due to its antiviral effects (Lin, X. et al., 2019). miR-30 family members downregulate SOCS1 and SOCS3, inhibitors of the IFN/JAK/STAT pathway (Lin, X. et al., 2019).

The p38 MAPK pathway can be activated by many stimuli, including signaling via cytokines or viral recognition (McCaskill et al., 2017). This pathway is upregulated in IAV infection, but suppression of p38 MAPK or the downstream MK2 protein can inhibit viral replication of IAV and Respiratory Syncytial Virus (RSV) (McCaskill et al., 2017). miR-124a, miR-744, and miR-24 have broad antiviral effects against IAV and RSV, including downregulation of MK2 and decreased activation of p38 MAPK (McCaskill et al., 2017). During coxsackievirus (CVB3) infection of HeLa cells, miR-21 was shown to be upregulated, resulting in downregulation of its target MAP2K3 and suppression of the p38 MAPK signaling pathway (He et al., 2019). This inhibited viral progeny release and decreased apoptosis (He et al., 2019).

The NF-κB pathway has many roles in innate and adaptive immunity (Barbu et al., 2020). During Human Cytomegalovirus (HCMV) infection of neural precursor cells (NPCs), mir-221 is upregulated (Yan et al., 2019). mir-221 directly targets and downregulates SOCS1, an inhibitor of cytokine signaling (Yan et al., 2019). This promotes the phosphorylation and activation of

NF-κB as well as the Type I IFN signaling pathway to increase inflammation and decrease HCMV replication (Yan et al., 2019).

6.3. miRNA in Immune Cell Response to Viral Pathogens

The signaling molecules released by infected cells can be regulated by miRNA and may recruit immune cells. miRNA may, in turn, regulate immune cell effector functions to fight viral infection. In pNK cells, miR-362-5p is upregulated and targets CYLD, an inhibitor of NF-κB signaling (Ni et al., 2015). miR-362-5p upregulation of the NF-κB pathway resulted in enhanced effector functions of NK cells (Ni et al., 2015). In MCMV infection of mice, cytokines can upregulate miR-155 in NK cells and are required for effective NK cell expansion (Zawislak et al., 2013). miR-155 targeted Noxa and SOCS1 in these infected NK cells (Zawislak et al., 2013). In CD8+ T cells, miR-155 is expressed highly in effector cells and is upregulated to a lesser extent in memory cells when compared to naïve counterparts (Dudda et al., 2013). Like NK cells, miR-155 targeted SOCS1 and was needed for robust expansion and cytokine signaling of CD8+ T cells during Lymphocytic Choriomeningitis (LCMV) infection of mice (Dudda et al., 2013). During Vesicular Stomatitis Virus (VSV) infection of mice, miR-155 was needed for activation and proliferation of CD4+ T helper cells as well as IL-2 and IFN-γ production (Goncalves-Alves et al., 2019). miR-155 activity in CD4+ cells was also needed for activation of B cells for antibody production (Goncalves-Alves et al., 2019).

During HIV infection, IL-1β-induced miR-103/107 expression in macrophages downregulates CCR5 and prevents viral entry in these cells (Lodge et al., 2020). miR-103 is upregulated in CD4+ T cells in elite controllers of HIV, which may help to explain how these individuals

maintain undetectable viral load during infection (Lodge et al., 2020). Other miRNAs have been implicated in effector functions of immune cells, but have not yet been characterized in the context of viral infection (Baumjohann & Heissmeyer, 2018; Cho et al., 2016; Emamgolizadeh Gurt Tapeh et al., 2020; Grimaldi et al., 2021; King et al., 2022; Nanbakhsh & Malarkannan, 2021; Wigton & Ansel, 2021).

6.4. Considerations

Antiviral responses are not limited to immune cells. Most cells have internal defenses such as those described above that assist in the immune response. Understanding the role of miRNA in these processes and signaling pathways is complicated by the cell-specific expression and effects of miRNA. Variations between cell types can make it difficult to determine the practical applicability of in vitro research to the human body. In addition, some miRNAs can be released via exocytosis, but not all. miRNAs are not randomly incorporated into exosomes, though the sorting process is still under investigation (Garcia-Martin et al., 2022; Groot & Lee, 2020; Liu, X. et al., 2021). Determining which miRNAs can be released via exocytosis is crucial for ascertaining potential effects. Furthermore, the same miRNA may exert antiviral effects against one virus but serve as pro-viral for another. For example, the IFITM1 protein can enhance viral replication of KSHV, Epstein Barr Virus (EBV), and Herpes Simplex Virus 2 (HSV-2) but is critical in preventing viral entry of many RNA viruses (Hussein & Akula, 2017a).

As previously discussed, hsa-miR-36 targets IFITM1 and was demonstrated to be upregulated during KSHV infection and have antiviral effects against KSHV, EBV, and HSV-2 (Hussein & Akula, 2017a). Yet there are no publications available on PubMed to date indicating modified

expression of miR-36 during IAV, HIV, HCV, West Nile Virus, or Dengue Virus infection. miRNA regulation of viral processes is well studied, but knowledge of the mechanisms by which cells regulate antiviral miRNA in response to specific viruses is limited. The antiviral functions of miRNAs during infection are depicted in **Figure 3**.

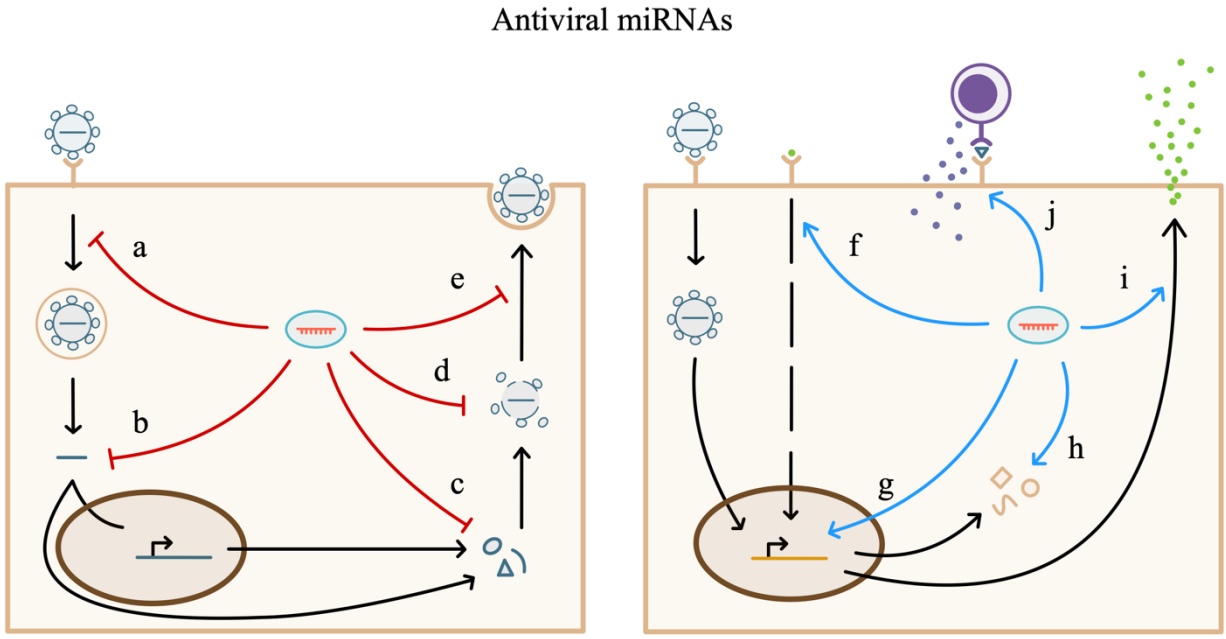


Figure 3. Antiviral miRNAs inhibit viral replication (**left**) including (a) viral entry, (b) transcription of the viral genome, (c) viral protein production, (d) viral assembly, and (e) viral escape. Antiviral miRNAs enhance antiviral cellular processes (**right**) including (f) cell signaling pathways (g) transcription of ISGs, (h) antiviral cellular protein production, (i) interferon release, and (j) immune cell recognition. Pro-viral miRNAs have opposing effects. 4

Table 3. Summary of antiviral miRNAs involved in infection by viruses IAV, SARS-CoV-2, HIV-1, HCV, HCMV, KSHV, RSV, VSV, and CVB3. The hsa-miRs are encoded by the human host. Targets are cellular protein transcripts unless otherwise stated.

Virus Name	miRNA	Target(s)	Antiviral effect	Viral significance	Ref
Influenza A Virus (IAV)	hsa-miR-3145	Viral *PB1* gene	Downregulate viral *PB1* protein expression	Inhibit viral replication	(Liao, Y. et al., 2021)
	hsa-miR-1307	Viral *NS1* gene	Prevent induction of cell cycle arrest	Prevent favorable environment for virus	(Liao, Y. et al., 2021)

	hsa-miR-24, hsa-miR-124a; hsa-miR-744	MAPK14; Myc	Suppress downstream p38 MAPK expression and activation	Inhibit viral replication	(McCaskill et al., 2017)
Severe Acute Respiratory Syndrome-related Coronavirus (SARS-CoV-2)	hsa-miR-150-5p	Viral *nsp10* gene	Downregulate activation of downstream elements nsp14 and nsp16	Decrease translation efficiency, immune evasion, viral replication	(Akula et al., 2022a)
	hsa-miR-9-5p, hsa-miR-218-5p	ACE2	Downregulate host cell receptor for virus	Prevent viral entry	(Pierce et al., 2020)
	hsa-let-7d-5p, hsa-miR-494-3p, hsa-miR-382-3p, hsa-let-7e-5p, hsa-miR-181c-5p, hsa-miR-452-5p	TMPRSS2	Downregulate host cell receptor for virus	Prevent viral entry	(Pierce et al., 2020)
	hsa-miR-1827	CTSV	Downregulate host protein that regulates virus entry	Prevent viral entry	(Khurana et al., 2020)
	hsa-miR-1277-5p	CANX	Downregulate host protein that stabilizes S protein for folding	Antigen presentation	(Khurana et al., 2020)
Human Immunodeficiency Virus 1 (HIV-1)	hsa-miR-17, hsa-miR-20	PCAF	Downregulate cellular cofactor of the HIV Tat protein	Prevent viral gene expression	(Guo, L. et al., 2018)
	hsa-miR-28, hsa-miR-29a	HIV mRNA	Downregulate viral protein production	Prevent viral replication	(Guo, L. et al., 2018)
Hepatitis C Virus (HCV)	hsa-miR-182	CLDN1	Downregulate host protein involved in internalization of virus	Prevent viral entry	(Riad et al., 2018)
Human Cytomegalovirus (HCMV)	hsa-mir-221	SOCS1	Downregulate inhibitor of NF-κB phosphorylation and activation	Promote cytokine signaling	(Yan et al., 2019)
Kaposi's sarcoma-associated herpesvirus (KSHV)	hsa-miR-36	IFITM1	Downregulate cellular transmembrane protein	Prevent viral entry	(Hussein & Akula, 2017a)
Respiratory Syncytial Virus (RSV)	hsa-miR-24, hsa-miR-124a; hsa-miR-744	MAPK14; Myc	Suppress downstream p38 MAPK expression and activation	Inhibit viral replication	(McCaskill et al., 2017)
Vesicular Stomatitis Virus (VSV)	hsa-miR-183 cluster	PP2A, TRIM27; STAT1	Downregulate negative regulators of IRF phosphorylation; upregulate STAT1	Promote interferon production	(Singaravelu et al., 2019)
Coxsackievirus (CVB3)	hsa-miR-21	MAP2K3	Suppress of the P38 MAPK signaling pathway	Inhibit viral release	(He et al., 2019)

7. miRNA Diagnostics and Therapeutics Targeting Viral Pathogens

7.1. miRNA Diagnostics

Many studies have investigated the modulation of miRNA profiles during viral infections and have indicated possible biomarkers for diagnostics (Chen, Z. et al., 2016; Fernández-Moreno et al., 2021; Loureiro et al., 2020; Su, B. et al., 2018). The principle of using miRNAs as diagnostics is based on the circulating levels of miRNAs in biological fluids. Current studies suggest that the expression of circulating miRNA can indicate the severity of viral infections such as SARS-CoV-2 (Akula et al., 2022a). In addition, miRNA profiles have the potential to predict health consequences related to viral infection. For example, levels of circulatory EBV miRNAs may predict nasopharyngeal carcinoma and a plasma miRNA panel could be utilized to screen for Hepatitis B Virus (HBV) related hepatocellular carcinoma (Zhang, Gaohong et al., 2015; Zhou, J. et al., 2011). miRNAs may also be used to track disease progression and predict treatment outcomes using agents that do not target miRNA directly (Ceccarelli et al., 2017; Chen, J. et al., 2013; Chockalingam et al., 2016; Sabbatinelli et al., 2021).

7.2. miRNA Based Therapeutics

Because of its important role in the regulation of viral infection, miRNA has remarkable potential for antiviral therapeutics. Therapies could function by mimicking antiviral miRNA or antagonizing pro-viral miRNA (Bonneau et al., 2019; Hussein & Akula, 2017a; Li, W. et al., 2016; Zhang, Fuming et al., 2019). The HCV drug miravirsen was the first antimiR-based agent administered to patients and is currently undergoing phase 2 clinical trials (Bonneau et al., 2019; Janssen et al., 2013). It is an antisense oligonucleotide (ASO) that targets miR-122 and has been

shown to reduce HCV RNA levels in a dose-dependent and prolonged manner in a phase 2 clinical trial (Janssen et al., 2013). There was also no evidence of dose-limiting toxicity, and no patients in the trial discontinued treatment due to adverse events (Janssen et al., 2013). A different study indicates that miravirsen can effectively be used alone against HCV *in vitro*, including variants resistant to direct-acting antivirals, and has additive antiviral effects when used in combination with current NS3, NS5B, and NS5A inhibitors (Ottosen et al., 2015). RG-101 was another antimiR targeting miR-122 shown to decrease HCV RNA levels (Stelma et al., 2017; Winkle et al., 2021). However, this clinical trials was stopped due to elevated bilirubin levels in the blood (Stelma et al., 2017; Winkle et al., 2021).

There are current studies investigating the antiviral potential of other miRNAs related to HCV infection, as well as HSV, SARS-CoV-2, IAV, HIV, and other viruses that have not yet reached clinical trials (Akula et al., 2022a; Chakraborty et al., 2021; Hussein & Akula, 2017a; Karothia et al., 2020; Li, W. et al., 2016; Ma et al., 2014; Panda et al., 2022; Sadegh Ehdaei et al., 2021; Shabani et al., 2019; Shafaati et al., 2021; Zhang, Fangyi et al., 2019). Future antiviral miRNA-based therapies may include miRNA mimics, which have been used successfully in clinical trials against cancer but have not reached clinical trials in antiviral therapy (Winkle et al., 2021). Furthermore, artificial miRNA sponges and miRNA masking ASOs have been proposed as possible methods to sequester miRNA or mask the binding site of miRNA on its target, respectively (Winkle et al., 2021).

In addition to therapies based on human miRNA, there are some studies investigating the use of plant miRNA for antiviral therapeutics. Treatment with honeysuckle extract has been shown to

increase miRNA let-7a levels during Enterovirus infection leading to a decrease viral replication *in vitro* and in mice (Lee et al., 2021). The honeysuckle-encoded atypical microRNA2911 has been studied in the context of IAV and SARS-CoV-2 infection and has predicted binding sites in both viral genomes (Zhou, L. et al., 2020; Zhou, Z. et al., 2015; Zhou, Z. et al., 2020). It was shown to decrease viral replication against IAV H1N1, H5N1, and H7N9 in mice (Zhou, Z. et al., 2015). SARS-CoV-2 patients taking routine antiviral therapy given a honeysuckle decoction daily had absorbed miR2911 detected in serum, decreased viral replication, and were more likely to test negative seven days after diagnosis compared to infected individuals given routine antiviral therapy and a Traditional Chinese Medicine (TCM) mixture without miR2911 (Zhou, L. et al., 2020). However, the authors do not disclose the ingredients of the TCM mixture, nor do they include a patient group given antiviral therapy without honeysuckle decoction or TCM (Zhou, L. et al., 2020). A later study found that patients with a SIDT1 polymorphism had decreased absorption of miR-2911, and the honeysuckle decoction failed to inhibit SARS-CoV-2 replication (Zhou, Z. et al., 2020). This does not contradict the previous findings concerning the antiviral effects of miR-2911. Instead, it underscores an important consideration when evaluating oral treatments and absorption of dietary miRNAs.

Chimeric Antigen Receptor (CAR)-T cell therapy has shown promise in treating cancers and has recently been studied as a potential therapy for autoimmune diseases and viral infections (Zmievskaya et al., 2021). This form of immunotherapy uses human T cells engineered to produce CARs with the ability to bind to specific proteins and target cancerous or diseased cells (Schaible et al., 2023). The application of CAR-T cells in infectious diseases is currently under investigation, with promising results in treating HBV, HCV, HCMV, HIV, and SARS-CoV-2

(Zmievskaya et al., 2021). In addition, the chimeric autoantibody receptor (CAAR) T cells have been used to treat autoimmune diseases by targeting B cells with specificity for autoantigen receptors (Zmievskaya et al., 2021). Since miRNAs regulate cellular activities without complete gene knock-out, they can be used to fine-tune the design of CAR-T cells and help mitigate their limitations. For example, miR-155 upregulation in CAR-T cells has been shown to promote T cell function, survival, and infiltration (Renrick et al., 2021; Schaible et al., 2023; Zhang, Jing et al., 2022). Similarly, upregulation of miR-H18 in CAR-T cells has been shown to enhance cytotoxic activity (Wirges et al., 2022); miR-17-19 has been used to promote the persistence of effector T cells (Schaible et al., 2023); and overexpression of miR-27a-5p may enhance infiltration of tissues (Zhang, Jing et al., 2022). Furthermore, overexpression of miR-143 promotes memory T cell formation for enhanced specificity and reduced toxicity in CAR-T therapy (Schaible et al., 2023). This research reveals miRNA as a crucial tool for the improvement of current therapeutics.

7.3. Challenges and Future Considerations

Several miRNAs have been implicated in the regulation of viral infection. However, in many cases their exact mechanisms have yet to be elucidated (Arisan et al., 2022; Aydemir et al., 2021). Computational approaches may be used to predict miRNA targets but can have high false-positive rates (Mohebbi et al., 2021). Novel algorithms continue to be developed in efforts to improve these predictions (Ding, Y. et al., 2022; Liu, B. et al., 2021; McGeary et al., 2019; Mohebbi et al., 2021; Uthayopas et al., 2021; Wang, Shu-Hao et al., 2022; Wang, Xin-Fei et al., 2022). Predicting miRNA binding sites can cut down on research costs by narrowing potential

targets, but they need to be experimentally validated using *in vitro* and *in vivo* models (Mohebbi et al., 2021).

Different strains of viruses can cause similar changes in miRNA levels, with some variations partly due to minor changes to their antigenicity and replication. For example, the Mayinga, Makona, and Reston strains of the Ebola virus share 82% early-phase miRNAs and 90% late-phase miRNAs, and only seemed to selectively modulate 1 out of 5 miRNAs (Diallo et al., 2021). Another study found that only 5 out of 25 differentially expressed miRNAs were similarly modulated across four Lyssavirus strains (Farr et al., 2021). A viral protein from Epstein Barr Virus (EBV) has also been shown to distinctly modulate the expression of 3 miRNAs between M81 and B95.8 strains (Müller Coan et al., 2022). An *in silico* study on SARS-CoV-2 found redundancy in predicted pre-miRNAs encoded in the viral genomes of different strains, with six unique pre-miRNAs between the Reference (Wuhan), Delta, and Omicron strains (Saini et al., 2023). These subtle variations could be used to explain differences in the pathology of different strains or to identify individual strains for diagnostic purposes. Alternatively, targeting miRNAs shared across multiple strains could be beneficial when developing therapeutics with broad applications.

miRNA-based diagnostics rely on characteristic miRNA profiles for given diseases (Bonneau et al., 2019). However, miRNA profiles naturally show variation between individuals, and not every virus causes a characteristic upregulation of a tissue-specific miRNA such as miR-122 in HCV (Panigrahi et al., 2022; Rotival et al., 2020). Diagnostic tools scanning for multiple dysregulated miRNAs are currently being developed for non-viral diseases, but similar panels

could be used for viral infections (Bonneau et al., 2019). Testing for virally encoded miRNAs may be even more effective in diagnosing viral infections than running an endogenous miRNA panel in some cases (Zhang, Gaohong et al., 2015). However, not all viruses are known to encode miRNAs, and some predicted v-miRs are still under investigation (Liu, Z. et al., 2021; Nanbo et al., 2021).

miRNA-based therapeutics have the capacity to combat viral infections. However, they face three main challenges: delivery, specificity, and tolerance (Winkle et al., 2021). For miRNA therapeutics to exert any effect, they must remain stable and be delivered efficiently to the desired cell type (Winkle et al., 2021). Extracellular miRNAs have a relatively short half-life in mouse serum ranging from ~1.5 hours to more than 13 hours depending on the sequence (Coenen-Stass et al., 2019). Because miRNA is naturally occurring in cells, there are degradation mechanisms already in place (Winkle et al., 2021). This can be beneficial when considering toxicity, but chemical modifications may be needed to extend the half-life of miRNA-based therapeutics (Coenen-Stass et al., 2019; Winkle et al., 2021). RNA is negatively charged and does not passively diffuse across cell membranes, so delivery methods must facilitate cell entry (Winkle et al., 2021). Exosomes, liposomes, antibody conjugates, viral vectors, and various nanoparticles have been studied as delivery vehicles for RNA-based therapeutics (Traber & Yu, 2023). Exosomes are particularly enticing as delivery vehicles of miRNA because they are natural, can protect the contents from enzymatic degradation, fuse directly with the cell membrane, are unlikely to evoke an immune response, can contain multiple compounds for combined therapeutic effects, and could be modified to have enhanced uptake by targeted cell types (Winkle et al., 2021).

Some miRNAs can bind to multiple targets with similar seed sequences (Winkle et al., 2021). Therefore, specificity is essential in preventing off-target effects. Targeting genes unique to the virus using miRNA-like sequences may allow for direct and specific inhibition of viral gene expression. Furthermore, researchers may consider adding an element to control for tissue specificity. For example, the injection of artificial pri-miRNA transcripts based on human pri-miR-31 with liver-specific promotors targeted the X gene sequence in HBV circular DNA and was shown to decrease viral replication in mice (Maepa et al., 2017). Virus-specific considerations also need to be accounted for. For example, treatment of HBV infection is made difficult by the persistence of viral covalently closed circular DNA (cccDNA), which was combated by targeting the open reading frame (ORF) encoding the HBx protein that maintains the cccDNA (Maepa et al., 2017; van den Berg et al., 2020). On the other hand, some miRNA-based therapies may be enhanced by less specificity, such as a miRNA sponge that could sequester multiple types of pro-viral miRNA (Jie et al., 2022; Qu et al., 2021).

For miRNA-based therapies to be safe and effective, they must be tolerated well by the body (Winkle et al., 2021). This includes using safe delivery vehicles that do not mount an immune response as well as limiting off-target toxicity (Winkle et al., 2021). In addition, the miRNA therapeutic itself should not have cytotoxic effects or induce immune-related adverse events (Winkle et al., 2021). Immunogenicity can be reduced by avoiding GU-rich sequences which are recognized by TLRs, using dsRNA rather than ssRNA to reduce the likelihood of an immune response, using sequences less than 21 bp for ssRNA to minimize activation of TLRs, and adding modifications to prevent interactions with proteins such as TLRs or degrading enzymes

(Winkle et al., 2021). Any chemical modifications added to enhance miRNA-based therapeutics should emulate natural RNA to prevent increased risk of off-target effects (Traber & Yu, 2023).

In miRNA-based therapeutics, researchers must be wary of varying effects in different cell types, particularly when manipulating levels of miRNA with multiple targets (Winkle et al., 2021). This is highly relevant when considering possible side effects. For example, miravirsen targets miR-122 in HCV patients and lowers HCV replication but can also lower cholesterol levels (Janssen et al., 2013). However, less specificity may not be a disadvantage in some cases and may lead to miRNA-based therapies with broad antiviral effects that have the potential to treat multiple viruses (Hussein & Akula, 2017a; McCaskill et al., 2017). Further advances in miRNA therapeutics should aim to improve delivery, stability, tolerability, and specificity for appropriately targeted and prolonged effects that minimize toxicity and do not evoke an immunogenic response (Chakraborty et al., 2021; Winkle et al., 2021). Nutritional uptake of miRNAs provides another avenue for immune modulation and may be of interest to researchers investigating therapeutics or possible active ingredients in homeopathic remedies (Zempleni et al., 2017; Zhou, Z. et al., 2015). In addition, diagnostic and therapeutic research should consider population differences in miRNA expression related to immunity and the immune response to viral infections (Rotival et al., 2020). This may provide insight into how to make therapeutics based on resistant populations or avoid sampling biases in diagnostic devices (Bellini et al., 2022; Da Luz et al., 2014).

8. miRNA Market Landscape

With several potential applications of miRNAs, the global miRNA market size has been increasing at a rapid pace. In 2022, it was valued at around 1,230.3 million USD and is expected

to grow at a CAGR (compound annual growth rate) of 19.9% from 2022 to 2032 (*MicroRNA (miRNA) Market By Products (Instruments, Kits, Reagents & Consumables), By Research & Tools (qRT-PCR, Biomarkers), By Application (Cancer, Infectious Diseases, Immunological Disorder), By End-use, and By Region Forecast to 2032.*2023). Among the various subspecialties, oncology has the largest market share at 33%, followed by infectious diseases (*MicroRNA Market Size, Share & Trends Analysis Report By Products & Services (Profiling, Localization, & Quantification), By Application (Cancer, Neurological Disease,) By End Use, By Region, And Segment Forecasts, 2023 - 2030.*2021). In terms of applications, the disease diagnostics segment accounted for the largest market share of 54% in 2019. miRNAs are among the most promising biopharmaceutical candidates making their way into the commercial world for the development of future medications (*MicroRNA Market (By Products: Instruments, Consumables; By Services: Service Type, Specimen; By Application: Cancer, Infectious Diseases Immunological Disorder, Cardiovascular Disease, Neurological Disease, Others; By End Use: Biotechnology & Pharmaceutical Companies, Academic & Government Research Institutes, Other end-users) - Global Industry Analysis, Size, Share, Growth, Trends, Regional Outlook, and Forecast 2023-2032.*2023). Examples of a few companies that hold the market share for miRNA applications are Qiagen, Thermo Fisher Scientific, PerkinElmer, Illumina, Takara Bio, Mirna Therapeutics, miRagen Therapeutics, Regulus Therapeutics, and Santaris Pharma (Chakraborty et al., 2021).

9. Conclusions

This review offered an overview of miRNA involvement in immune processes related to viral infection as well as significant advancements and potential applications of miRNA in the field of

viral diagnostics and therapeutics. The differential expression of miRNA profiles can act as potential biomarkers for future diagnostic purposes, to predict the severity of viral infections, or to assess adverse health effects associated with viral diseases. miRNA-based diagnostic tools are useful in detecting and measuring specific miRNAs indicative of viral infections. The novel approach of using miRNA mimics to enhance antiviral activity or antagonists to inhibit pro-viral miRNAs is still under investigation but is showing promise. Plant miRNA has also been studied for use in viral treatments but requires further clinical experiments.

Several challenges, as outlined above, need to be addressed to fully harness the potential of miRNA in medicine. Delivery methods must be optimized to ensure efficient and well-targeted delivery of miRNA to the cells of interest. Specificity is key for avoiding off-target effects, and methods for enhancing tissue specificity and targeting viral genes are still being explored. The cell-specific nature of miRNA expression adds complexity to this process as well. Additionally, miRNA-based therapeutics should be well tolerated by the body, and efforts must be made to overcome cytotoxicity and possible immune-related adverse effects. Treatment methods should consider the pros and cons of the administration route and absorption of miRNA. Researchers need to be conscious of miRNA's ability to act as an antiviral or pro-viral element when considering therapeutic uses. Population variations in miRNA expression levels warrant further study as they may shed light on differences in immunity and treatment viability.

miRNA technology offers an exciting new approach for future diagnostics and therapeutics against viral infections. Further investigation should attempt to decipher the potential effects of virally encoded miRNAs on the host system. Endogenous miRNAs with antiviral properties

should continue to be evaluated for their therapeutic potential. The journey toward refining recently discovered techniques to address existing challenges and ensure their safety, efficacy, and clinical applicability is a long one. Continued advancements in this field show great promise for the future of miRNA-based medicine.

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CHAPTER 2: BODY OF THESIS

EVALUATING MOLECULAR MECHANISM OF VIRAL INHIBITION OF AEROSOLIZED SMART NANO-ENABLED ANTIVIRAL THERAPEUTIC (SNAT) ON SARS-COV-2 INFECTED HAMSTERS

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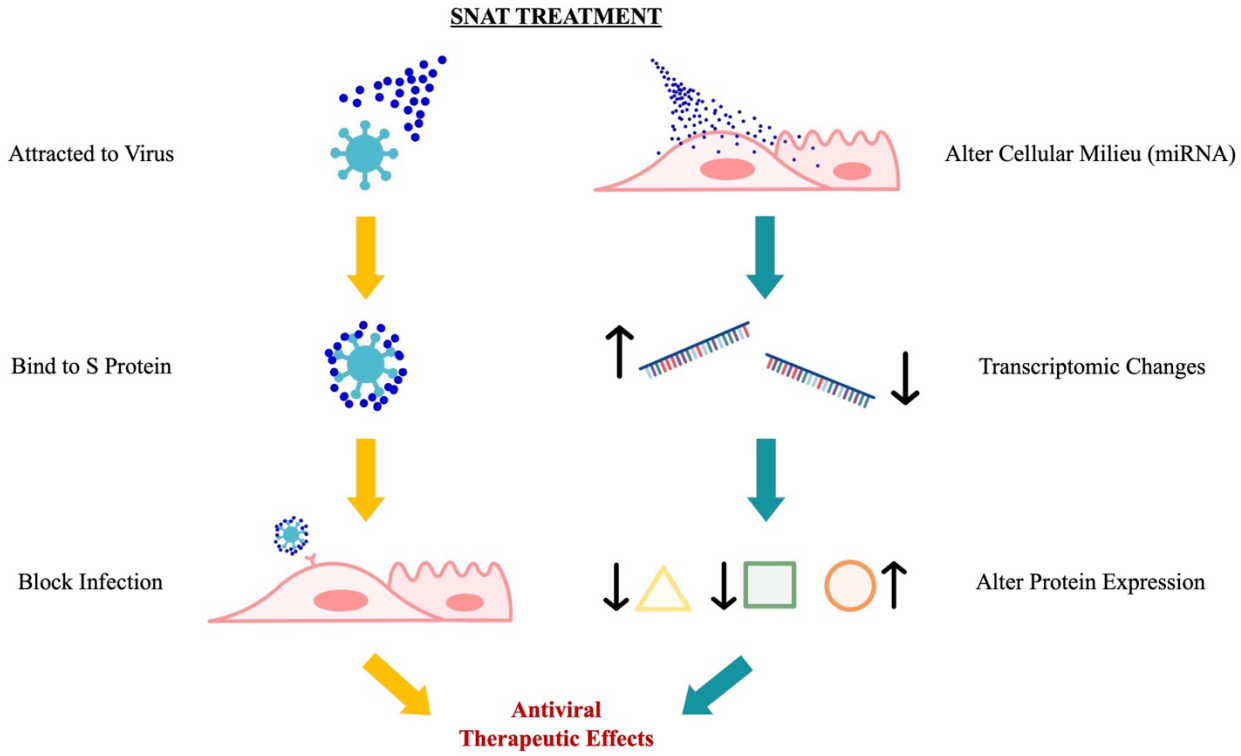
Abstract:

Purpose: Smart Nano-enabled Antiviral Therapeutic (SNAT) is a promising nanodrug that previously demonstrated efficacy in preclinical studies to alleviate SARS-CoV-2 pathology in hamsters. SNAT is comprised of taxoid (Tx)-decorated amino (NH2)-functionalized near-atomic size positively charged silver nanoparticles (Tx–[NH2-AgNPs]). Herein, we aimed to elucidate the molecular mechanism of viral inhibition and safety of aerosolized SNAT treatment in SARS-CoV-2 infected hamsters using complementary biophysical and molecular techniques. **Methods:** High resolution transmission electron microscopy (HR-TEM) and energy dispersive spectroscopy (EDS) analyses were used to investigate plausibleinteractions between SNAT and intact SARS-CoV-2 or recombinant Spike (S) protein. ELISA was used to confirm interactions between SNAT and SARS-CoV-2 encoded S protein and its subunits. In vitro, SARS-CoV-2 infected Calu-3 cells were used to determine if different concentrations of SNAT could lower viral infectivity. Extraction-free whole transcriptome assay was used to detect changes in circulatory micronome in golden Syrian hamsters treated with two doses of intranasally delivered SNAT (10 μg/ml of 2 ml each administered 24 h apart). Physiologically relevant targets of circulatory microRNAs (miRNAs) were verified using immunohistochemsitry (IHC) and Western blotting. **Results:** HR-TEM coupled with EDS and ELISA analyses showed SNAT binds to the SARS-CoV-2 virus by interacting with intact spike (S) protein; specifically to its S2 subunit. SNAT treatment ≥1 μg/ml significantly lowered SARS-CoV-2 infections of Calu-3 cells. Uninfected hamsters treated with SNAT had altered circulatory concentrations of 18 miRNAs (8 miRNAs upregulated, 10 downregulated) on day 3 post-treatment compared to uninfected controls. SNAT-induced downregulation of miR-141-3p and miR-200b-3p may reduce viral replication and inflammation by targeting Ythdf2 and Slit2, respectively. Further,

SNAT treatment significantly lowered IL-6 expression in infected hamster lungs compared to untreated infected hamsters. **Conclusion:** We demonstrate that SNAT binds directly to SARS-CoV-2 via the S protein to prevent viral entry and propose a model by which SNAT alters the cellular miRNA-directed milieu to promote antiviral cellular processes and neutralize infection. Our results provide promising insights into the use of low-dose intranasally delivered SNAT in treating SARS-CoV-2 infections.

Keywords: nanotechnology; nanomedicine; miRNA; microRNA; transcriptome; virus

Graphical Abstract:



1. Introduction

In the quest to identify effective treatments for COVID-19, researchers have been exploring the potential of nanomedicines, also called nanodrugs. Nanodrugs are drug delivery systems that utilize nanoparticles to enhance the therapeutic properties of drugs, including their stability, bioavailability, and targeted delivery (Duan et al., 2021; Kianpour et al., 2022; Wilson & Mukundan Geetha, 2022). Nanodrugs are also being studied as treatments for cancers, microbial infections, and other diseases (Guan et al., 2023; Pokhrel et al., 2022b; Souri et al., 2023; Su, W. et al., 2023; Tang et al., 2021). In 1995, the anticancer drug called Doxil became the first Food and Drug Administration (FDA) approved nanodrug on the market. Interest in the field continues to grow, with approximately 100 nanomedicines approved by the FDA and European Medicines Agency (EMA) on the market in 2021 (Shan et al., 2022; Thapa & Kim, 2023). There are many types of nanocarriers for drugs, including liposomes, nanocrystals, and inorganic nanoparticles (Farjadian et al., 2019). The nanoparticle chosen may inherently possess therapeutic properties, be coated with a therapeutic, or have a combination of the two (Farjadian et al., 2019).

Nanotechnology has contributed to several advances in antiviral treatments, addressing some of the limitations of current strategies. Compared to traditional antivirals, nanomedicines can have improved safety and efficacy, and they serve as an alternate therapy to prevent drug resistance (Chakravarty & Vora, 2021; Farjadian et al., 2019; Rudramurthy et al., 2016). Nanoparticles can be modified to carry chemotherapeutic drugs on their surface, some of which have already been used to treat viral infections, enhancing their delivery and effectiveness (Jeon et al., 2023). Their small size imparts a large surface area to volume ratio, improving their bioavailability and potency (Chakravarty & Vora, 2021). The greatest concern surrounding nanomedicine is

potential off-target cytotoxicity. Because small particles <150 nm have the potential to enter cells, there is potential for cytotoxic effects (Onoue et al., 2014). In addition, the biological and environmental effects of nanoparticle exposure are still under investigation (Reagen & Zhao, 2022). However, design elements such as surface coatings can be included in the engineering of nanodrugs to limit toxicity, and preclinical *in vitro/in vivo* studies serve as a key step to evaluate cytotoxicity during drug development (Bonamy et al., 2023; Wolfram et al., 2015).

Smart Nano-enabled Antiviral Therapeutic, or SNAT, is a novel nanomedicine that has been shown to have antiviral effects against SARS-CoV-2 in an *in vivo* preclinical study (Pokhrel et al., 2022a). It is comprised of positively charged silver nanoparticles (AgNPs) that are taxoid (Tx)-decorated and amino (NH_2)-functionalized (Tx–[NH_2-AgNPs]) (Pokhrel et al., 2022a). The "seed" NH_2-AgNPs are spherical with a mean TEM diameter of 5.8 nm ± 2.8 nm (S.D.) and are collectively embedded within Tx molecules in solution (Pokhrel et al., 2022a). This study involved cytotoxicity assays and an infection experiment using male golden Syrian hamsters (*Mesocricetus auratus*) (Pokhrel et al., 2022a). We demonstrated that administration of inhaled SNAT at 2h and 48h post-infection with SARS-CoV-2 could significantly reduce virus titer, improve lung health, and counteract virus-induced body weight loss in hamsters (Pokhrel et al., 2022a). However, the molecular mechanisms underlying SNAT inhibition of SARS-CoV-2 in hamsters were unclear. Therefore, in this study, we designed experiments to uncover molecular mechanisms by which SNAT may alleviate SARS-CoV-2-induced pathology using a combination of virology, next-generation sequencing (NGS), and biophysical and biochemical approaches.

Previous research has indicated that AgNPs can bind to viral surface proteins, potentially interfering with viral entry or hampering the structural integrity of the virus (Jeremiah et al., 2020). Evidence also suggests that nanoparticles may impact the cellular environment, such as reducing inflammation or altering the transcriptome (Atherton et al., 2022; Chen, M. et al., 2023). Thus, we investigated two plausible mechanisms by which SNAT may interfere with SARS-CoV-2 infection: (i) by binding with the virus to directly hinder viral infection of cells; and (ii) by altering the cellular microRNA-directed milieu to alleviate virus-induced inflammation and pathology.

2. Materials and Methods

2.1 Cells

Vero and human lung adenocarcinoma-derived Calu-3 cells were propagated in Dulbecco modified Eagle medium (DMEM) (Invitrogen, Carlsbad, CA) containing 10% charcoal-stripped fetal bovine serum, L-glutamine, and antibiotics as per standard laboratory protocol (Akula et al., 2022a).

2.2 Virus

All work pertaining to the use of SARS-CoV-2 was performed in BSL-3 laboratory. SARS-CoV-2 (isolate USA-WA1/2020) purchased from bei RESOURCES (Manassas, VA) was propagated in Vero cells as per standard procedures (Akula et al., 2022a). SARS-CoV-2 virus was propagated in Vero cells, purified, and concentrated using a 10–30% sucrose gradient in an ultracentrifuge (Beckman L8-55), and the yield was titrated as per standard protocols (Akula et al., 2022a). The following research was approved by the Office of Prospective Health/Biological

Safety for the use of biohazardous agent (SARS-CoV-2) and the registration number is 20–01 (title: Host response to COVID-19 infection in Eastern North Carolina). *In vitro* effects of different concentrations of SNAT on SARS-CoV-2 infection of cells were performed by viral titration as per standard laboratory procedures (Pokhrel et al., 2022a).

2.3 SNAT Synthesis and Characterization

The details of SNAT (Tx–[NH_2-AgNPs]) synthesis were previously described by our group (Pokhrel et al., 2022a). Briefly, SNAT was synthesized using the "seed" of NH_2-AgNPs (International Patent Application No. PCT/US2020/014343; US Patent Application No. 17/759,260; European Regional Phase Application No. 21744759.8). One-pot design synthesis was used, including $UV_{254\ nm}$ irradiation for six hours followed by heating at 95°C for 45 mins, then KBH_4 reduction, and cooling at room temperature overnight, followed by Docetaxel addition (1 µg/ml) and warming at 60°C with gentle stirring for 12 h, leading to the formation of SNAT. SNAT was purified using the dialysis method (Spectra/Por 3.5kD dialysis kit).

2.4 Transmission Electron Microscopy (TEM):

Multimethod complementary techniques allowed a detailed characterization of SNAT. To this end, we used electron microscopy (TEM, Philips EM 420; HR-TEM, Titan 80-300 TEM) coupled with energy dispersive spectroscopy (EDS) detector, dynamic light scattering (DLS; Malvern Zetasizer Nano ZS90), UV-Vis spectrophotometer (Hach DR6000), and other physicochemical analyses (pH, electrical conductance). Potential stabilities of SNAT and seed NH_2-AgNPs were also measured as change in hydrodynamic diameter (HDD) and zeta potential

as a function of time (0-3 years) at room temperature (25°C) using DLS and UV-Vis spectrophotometer. To characterize SNAT interaction with SARS-CoV-2 and S proteins, HR-TEM (Titan 80-300 TEM) coupled with EDS detector was used (CAMCOR, Oregon). 50µl of heat killed SARS-CoV-2 (isolate USA-WA1/2020; ATCC, Manassas, VA) or recombinant HEK-293-derived SARS-CoV-2 spike (S) protein (R&D Systems Inc., Minneapolis, MN) were mixed with 10 µg/ml SNAT (50 µl), gently shaken and incubated at 37°C for 30 min, and samples were drop casted onto holey carbon film-coated copper grid and air dried before imaging and EDS elemental mapping occurred. Images were captured in brightfield or STEM mode at 80 kV and magnification of over 200kx.

2.5 Enzyme-Linked Immunosorbent Assay (ELISA)

ELISA was conducted sequentially by immobilizing 0.1 µg/ml of various recombinant viral proteins, blocking with human BSA, incubating with different concentrations of SNAT, incubating with primary antibodies from bei Resources (Manassas, VA): SARS-CoV-2 spike S1 subunit mouse antibody (for S1 subunit), SARS-CoV-2 spike RBD mouse antibody (for RBD domain); from Thermo Fisher Scientific (Waltham, MA): SARS-CoV-2 spike S2 subunit mouse antibody (for S2 subunit and full-length S protein), and rabbit antibodies KSHV gB (Akula et al., 2002), incubating with HRP conjugated appropriate secondary antibodies, and addition of substrate as per earlier studies (Walker et al., 2014). The results were read at 450nm. Data are the means ± SD (error bars) of three experiments.

2.6 Infection Assay Conducted Using Calu-3 Cells

SNAT at different concentrations was mixed with 1 multiplicity of infection (MOI) of SARS-CoV-2 for 30 min at 37˚C prior to infecting Calu-3 cells as per earlier studies for 2 h at 37˚C. Unadsorbed virus particles were removed by washing cells twice with DMEM and further incubating them with infection medium at 37˚C. On the 3rd day post-infection, supernatant was collected, and the virus concentration in the supernatant was determined by RT-qPCR using the 2019-nCoV RUO kit as per the manufacturer protocols (Integrated DNA Technologies, Coralville, IA).

2.7 SNAT Administration and Experiment

Each hamster was treated with two doses of 10 µg/ml SNAT (2 ml/dose; 1st dose given 2 h post-infection followed by 2nd dose at 24 h post-infection) in a modified nose-only inhalation exposure equipment. Briefly, the nebulizer (UNOSEK nebulizer) was connected to the modified SCIREQ nose-only exposure chamber that was conveniently fit inside the BSL-3 biosafety cabinet. The key features of the nebulizer are as follows: atomization rate: ≥ 0.2 ml/min; aerosol size range: 0.5–5 µm; mean aerosol diameter: 2.81 ± 0.14 µm; and drug delivery rate: 75.7%; capacity: 8 ml max, 0.2 ml/min. This nebulizer uses compressed air technology that allows for a high nebulization rate. Our Animal Use Protocol entitled, “Effect of SNAT on SARS-CoV-2 infection of hamsters” (AUP #K177) was reviewed by ECU Institution’s Animal Care and Use Committee (approval date: 11/12/2020). The study involved three groups (n = 4 per group) of male Syrian hamsters (*Mesocricetus auratus*) aged 6–8 weeks and procured from Jackson Laboratory. For studies involving next-generation sequencing (NGS), the three groups were (i) untreated; (ii) SNAT treated; and (iii) phosphate buffered saline (PBS) treated on day 1 and

second dose on day 2. A simplified schematic showing the study design is depicted in **Table 1**. On day 3, the animals were anesthetized by inhalation of vaporized isoflurane using an IMPAC6 veterinary anesthesia machine prior to euthanizing animals.

Table 1. Experimental design. SNAT dose: Nebulized using a nose-only inhalation exposure equipment for 20 minutes at an atomization rate of ≥0.2 ml/min. SARS-CoV-2 dose: Intranasal route (20 µl per nostril) of viable $8x10^4$ $TCID_{50}$ virus particles.

Functional Biology Studies

NGS Studies

Group	Day 1	Day 2	Day 3
Uninfected (n=4)			✕
Uninfected + Saline (n=4)	💧	💧	✕
Uninfected + SNAT (n=4)	SNAT	SNAT	✕
Infected (n=4)	Infection		✕
Infected + SNAT (n=4)	Infection + SNAT	SNAT	✕

Key:
- 💧 Saline
- SNAT
- Infection
- ✕ Sacrifice

2.8 Preparation of Lung Tissues for Histology

Lung tissues collected on day 3 post-infection were fixed in 10% neutral buffered formalin, trimmed, processed, embedded in paraffin, cut at 5 – 6 µm, and stained with hematoxylin and eosin (H&E) (Pokhrel et al., 2022a).

2.9 RT-qPCR to Monitor Expression of Hamster IL-6

RNA was extracted from the hamster lung specimens using miRNeasy Serum/Plasma Advanced kit (Qiagen, Germantown, MD). The RNA concentrations were measured with a NanoDrop ND-2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Only the RNA samples with 260/280 ratios of 1.8 to 2.0 were used in the study. We performed RT-qPCR using specific primers (Yang et al., 2021) to IL-6 as per earlier studies (Akula et al., 2022b).

2.10 Immunohistochemistry (IHC)

Lung sections from SNAT-treated hamsters were processed for immunohistochemistry. Rabbit primary antibody to IL-6 (1:500 dilution; Abcam, Waltham, MA), 1:500 dilution) was used for immunohistochemical staining. The sections were incubated with the primary antibodies overnight at 4°C and developed using ImmPRESS Excel Staining Kit (MP-7601, Vector Laboratories) following manufacturer's instructions. All slides were imaged using the slide scanner Philips IntelliSite Ultra-Fast Scanner. Representative images were then extracted and shown at 40x magnification. Three 40x images were selected from each group and 10 subsections of equal size were taken from each to control for white balance, for a total of 30 subsections used per group for quantification. Quantification of IL-6 immunoreactivity was performed using NIH ImageJ software and presented as optical density, which is proportional to stain intensity. Paired t-tests were performed between each group to test for significance at $p \leq 0.05$.

2.11 Whole Transcriptome Assay

We determined the circulating miRNA profile in the SNAT-treated hamster plasma specimens by performing the HTG EdgeSeq miRNA Whole Transcriptome Assay (miRNA WTA) (HTG Molecular Diagnostics, Inc. Tucson, AZ, USA). The HTG EdgeSeq miRNA WTA allows for measuring the expression of 234 hamster miRNA transcripts using next-generation sequencing (NGS). The miRNA expression profile was determined in 20 µl of plasma samples as described previously in our earlier studies (Akula et al., 2022b). We limited the batch effects using the following checklist while performing NGS: samples were randomized in a balanced manner; samples were always used in duplicates; correlation of all sample pairs was confirmed;

normalization was performed to transform data in such a way as to make data from different replicates and experimental conditions directly comparable; and a multivariate method of analysis (principal component analysis-PCA) performed to detect any possible batch effects. PCA was performed based on the Scree plot (eigenvalues vs. components).

2.12 Dual-luciferase Reporter Assay

The 3'-UTR (with wild-type and mutant binding sites for miR-141-3p) of Ythdf2 (accession number: XM_040756461), and the 3'-UTR (with wild-type and mutant binding sites for miR-200b-3p) of Slit2 (accession number: XM_040747064), respectively, was PCR amplified and then cloned into XhoI/XbaI site located at 3'-UTR of pmirGLO dual-luciferase vector (Promega, Madison, WI, USA). The 3'-UTR of Ythdf2, and Slit2 with mutations to binding sites (MUT1 and 2) were generated by site-directed mutagenesis as per standard protocols (Akula et al., 2022b). HEK-293T cells were plated onto 6-well plates. At 24 h post-plating, HEK-293T cells were co-transfected with 3'-UTR luciferase reporter plasmid and miR-mimic (or control mimic) using FuGene HD (Promega). At 48 h post-transfection, Renilla luciferase activity was measured using a dual luciferase reporter assay system (Promega) as per manufacturer's recommendations. The oligos tested in this study are provided in **Supplementary Table 1 (Table S1)**.

2.13 Western Blotting

All buffers used in this project were made with water that was endotoxin and pyrogen free. Western blotting was conducted as per earlier studies using the following primary antibodies to IL-6 (rabbit polyclonal antibodies; Abcam, Cambridge, UK), Slit2 (rabbit Slit2 polyclonal

antibodies; Cell Signaling, Danvers, MA), Ythdf2 (rabbit polyclonal antibodies; Cell Signaling, Danvers, MA), and human β-actin (Cell Signaling, Danvers, MA). The bands were scanned, and the band intensities were assessed using the ImageQuaNT software (Molecular Dynamics).

3. Results

3.1 Characterization of SNAT

Transmission electron microscopy (TEM) was used to determine the particle size of SNAT. The amino-functionalized silver nanoparticles (NH_2-AgNPs) had a mean TEM diameter of 5.8 nm ± 2.8 nm (S.D.). SNAT has a spherical morphology with a core composed of an elemental/metallic silver (Ag^0) surface functionalized with cationic NH_2-functional group **(Fig. 1A, B)**. Surface decorating with docetaxel (Tx) led to the formation of Tx–[NH_2-AgNPs], i.e., SNAT, whereby two or more individual NH_2-AgNPs (blue arrow) self-assembled presenting a near triangular architecture collectively embedded within Tx molecules **(red arrow; Fig. 1C)**. The average hydrodynamic diameters (HDDs) for NH_2-AgNPs and SNAT were similar: 4.3 nm and 5.0 nm, respectively, suggesting that Tx binding did not influence the original TEM particle size of seed NH_2-AgNPs. Both NH_2-AgNPs and SNAT had high positive mean surface zeta potentials: +41 mV and +22 mV, respectively, and were highly stable for over 3 years at room temperature (25°C) as the standard deviation for HDD was within ±1 nm and within ±3 mV for zeta potential over the 3-year period (Pokhrel et al., 2022a). The blue-shift of 40 nm and red-shift of 14 nm as demonstrated by the UV–Vis spectra suggest a strong electrostatic binding of anionic Tx with cationic NH_2-groups on the surface of AgNPs **(Fig. 1D)**. The representative EDS spectra demonstrate that the SNAT is composed of Ag, C, O, and N. The dialysis-purified SNAT and

NH_2-AgNP formulations had an alkaline pH (7.5–8.5) and low electrical conductance of 0.120 mS/cm. We used this SNAT in the entire study to define the mechanism by which it may function as a therapeutic against SARS-CoV-2.

3.2 SNAT Directly Interacts with SARS-CoV-2

In our previous work, we found that SNAT (10 μg/ml) treatment neutralized SARS-CoV-2 infection *in vitro,* while the NH_2-AgNPs (10 μg/ml) or Tx (1 μg/ml) alone could not.(Pokhrel et al., 2022a) Additionally, we showed that SNAT significantly reduces SARS-CoV-2 load in hamsters (Pokhrel et al., 2022a), but the mechanism of action is not clearly understood. There are two ways by which a therapeutic may lower viral infections: (i) by directly binding the virus and neutralizing infection; and (ii) by altering the miRNA-directed cellular milieu to alleviate viral pathogenesis. First, we tested if SNAT could directly bind with the virus employing HR-TEM and EDS. HR-TEM coupled with EDS analyses confirmed that intact SARS-CoV-2 virus electrostatically interacts with several SNAT particles (shown as bright white electron-dense dots in **Fig. 2A**). EDS mapping confirmed the several electron-dense SNAT observed atop and around the virus particles to be composed of elemental Ag^0 along with C, O, and N (**Fig. 2B**). Significant amounts of C, N, and O signals were also observed for the virus particle (**Fig. 2B**). Interestingly, HR-TEM images depict SNAT particles to physically associate with SARS-CoV-2 encoded S proteins (**Fig. 2C**) while EDS confirmed elemental/metallic Ag^0 signature for SNAT interacting with S proteins (**Fig. 2D**). SNAT did not physically interact with Kaposi sarcoma-associated herpesvirus (KSHV) encoded glycoprotein B, a functional homologue of SARS-CoV-2 encoded S protein (data not shown). The results confirm that SNAT specifically interacts with SARS-CoV-2 and the S protein.

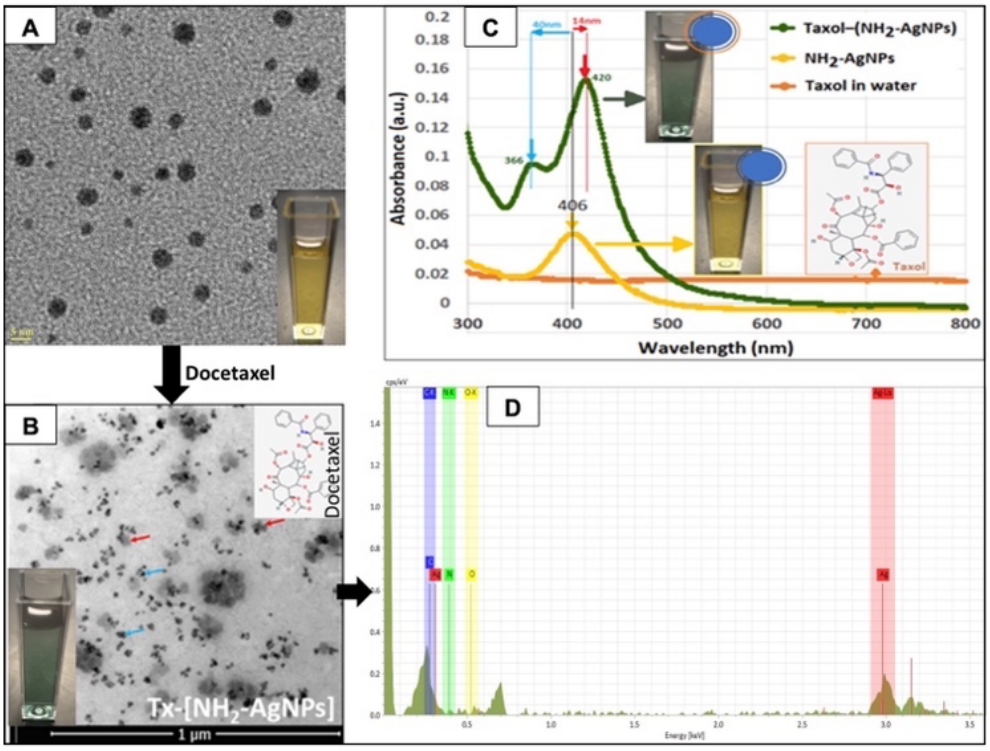


Figure 1. Characterization of SNAT. (A) Transmission Electron Microscopy (TEM) micrographs of amino-functionalized silver nanoparticles (NH_2-AgNPs) showing spherical particle morphology of elemental/ metallic silver (Ag^0) with mean particle diameter of 5.8 nm ± 2.8 nm (N=807). Inset photo shows yellow color suspension of NH_2-AgNPs. **(B)** Electron micrographs of surface Taxol (Tx)-decorated (shown with red arrow) amino-functionalized silver nanoparticles (Tx–[NH_2-AgNPs]) showing two or more individual NH_2-AgNPs (shown with blue arrow), each about 5 nm diameter, self-assembled forming a somewhat triangular nanoparticle architecture that is collectively embedded within Tx molecules. Inset photo shows light blue/green color suspension of Tx–[NH_2-AgNPs]. **(C)** UV–Vis spectra of docetaxel (Tx)-decorated amino-functionalized silver nanoparticles (Tx–[NH_2-AgNPs]) (green spectrum/red arrow; (blue) green suspension), NH_2-AgNPs alone (yellow spectrum, yellow suspension), and Tx alone (in sterile Milli-Q water) (orange spectrum showing a flat line; inset to the right showing molecular structure of Tx). The "seed" of NH_2-AgNPs alone had a maximum absorbance (λmax) at 406 nm, which red-shifted to 420 nm (a change of 14 nm) and blue shifted to 366 nm (a change of 40 nm) upon direct surface binding with Tx molecules. **(D)** Representative EDS spectra of Tx–[NH_2-AgNPs].

To further confirm the ability of SNAT to interact with SARS-CoV-2 encoded S protein, we performed ELISA. SARS-CoV-2 encoded S protein (1273 aa) comprises of N-terminal signal peptide (1-13 aa), S1 subunit containing the receptor binding domain (RBD (14 – 685 aa), and S2 subunit (686 – 1273 aa) (Cheng et al., 2023; Schroeder & Bieneman, 2022). The rationale for this experiment was that if SNAT is bound to the S protein, it may hinder the ability of

antibodies to bind to the protein. We tested recombinant full-length S protein-His tag, SARS-CoV-2 Spike S1-His Tag, SARS-CoV-2 Spike RBD-His Tag, SARSCoV-2 Spike S2-His Tag protein, and unrelated KSHV gB,(Hussein et al., 2016) in this assay (**Fig. 2E**). Our results demonstrated the ability of SNAT to physically interact with the full-length S protein and the S2 subunit contained within it compared to the S1 subunit and RBD domain of S protein, BSA, or KSHV gB (**Fig. 2E**). If this is true, SARS-CoV-2 infection of cells in the presence of SNAT must be significantly lowered. Hence, we tested the ability of different concentrations of SNAT to inhibit SARS-CoV-2 infection of Calu-3 cells. Interestingly, SNAT at ≥1 µg/ml could significantly lower SARS-CoV-2 infection *in vitro* **(Fig. 2F)**. We observed a maximum of 55±7.6% drop in viral infectivity in the presence of SNAT when compared to the vehicle (PBS) or untreated cells. Taken together, our results indicate SNAT can neutralize viral infection by physically interacting with S2 subunit of SARS-CoV-2 encoded S protein.

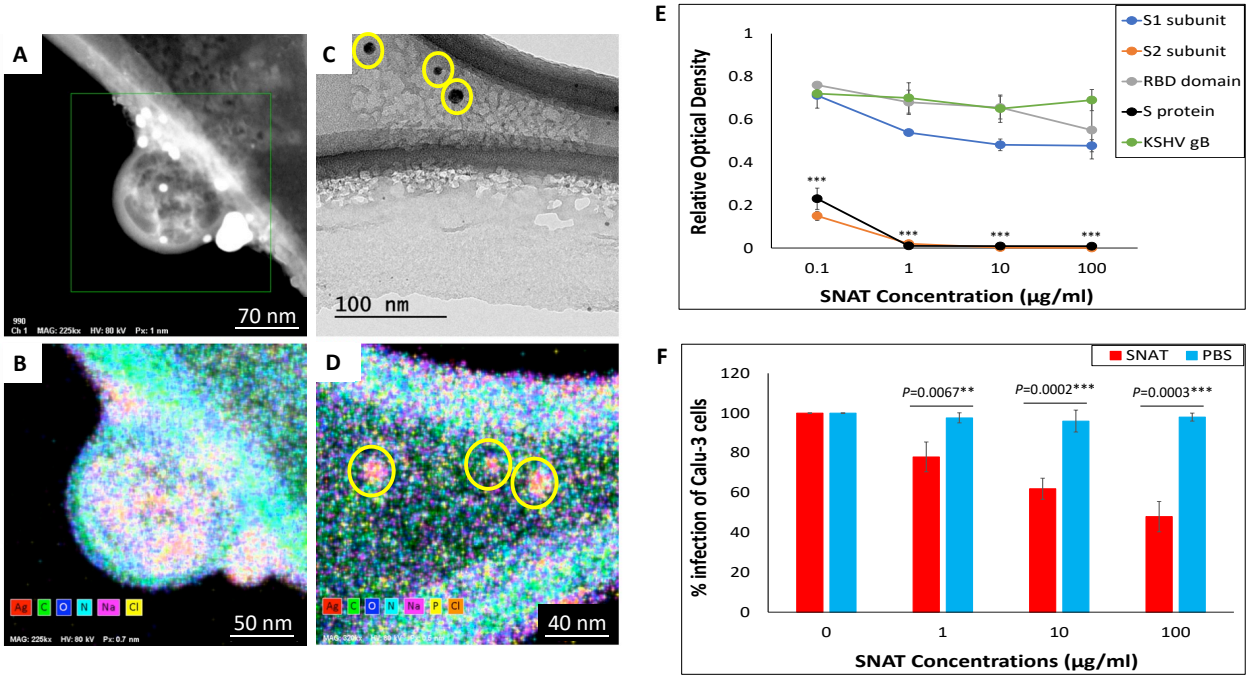


Figure 2. SNAT interactions with SARS-CoV-2 virus and viral S protein. (**A**) HR-TEM and energy dispersive spectroscopy (EDS) analyses of electrostatic interaction between SARS-CoV-2 virus and SNAT particles. (**B**) EDS mapping of SNAT chemical composition. (**C**) HR-TEM image of SNAT particles physically associating with SARS-CoV-2 encoded S proteins. (**D**) EDS shows

elemental/metallic Ag^0 signature for SNAT interacting with S proteins. (**E**) ELISA assay of recombinant full-length S protein-His tag, SARS-CoV-2 Spike S1-His Tag, SARS-CoV-2 Spike RBD-His Tag, SARSCoV-2 Spike S2-His Tag protein, and unrelated KSHV gB across varying concentrations SNAT (*** indicates $p < 0.0005$). (**F**) SARS-CoV-2 infection assay using different concentrations of SNAT or PBS treatment.

3.3 SNAT-Induced Differential Expression of Circulatory miRNAs in Hamsters

We assessed the potential direct effects of SNAT treatment on hamsters by screening for changes in the circulatory micronome using the extraction-free whole transcriptome assay. We observed intranasal SNAT treatment to significantly alter 18 miRNAs in hamsters on day 3 post-treatment. A total of 8 miRNAs were up-regulated, while 10 miRNAs were down-regulated by SNAT treatment when compared to untreated control hamsters (**Fig. 3A, B**). Principal component analysis (PCA) showed that the miRNA expression profiles in SNAT-treated hamsters and untreated hamsters were segregated into distinct clusters along principal component 1 and principal component 2, explaining 56% and 14% of the total variances (**Fig. 3C**). Administration of PBS to hamsters did not significantly alter the circulatory micronome when compared to those that were untreated (**Fig. 4**). PCA plot showed no separation between the groups (PBS treated and untreated), meaning there was no distinct clustering of the points for the two groups, and the points from the two groups appeared to be evenly intermixed. The scree plots are provided in **Supplementary Figure 1 (Fig. S1)**, depicting principal components and their eigenvalues.

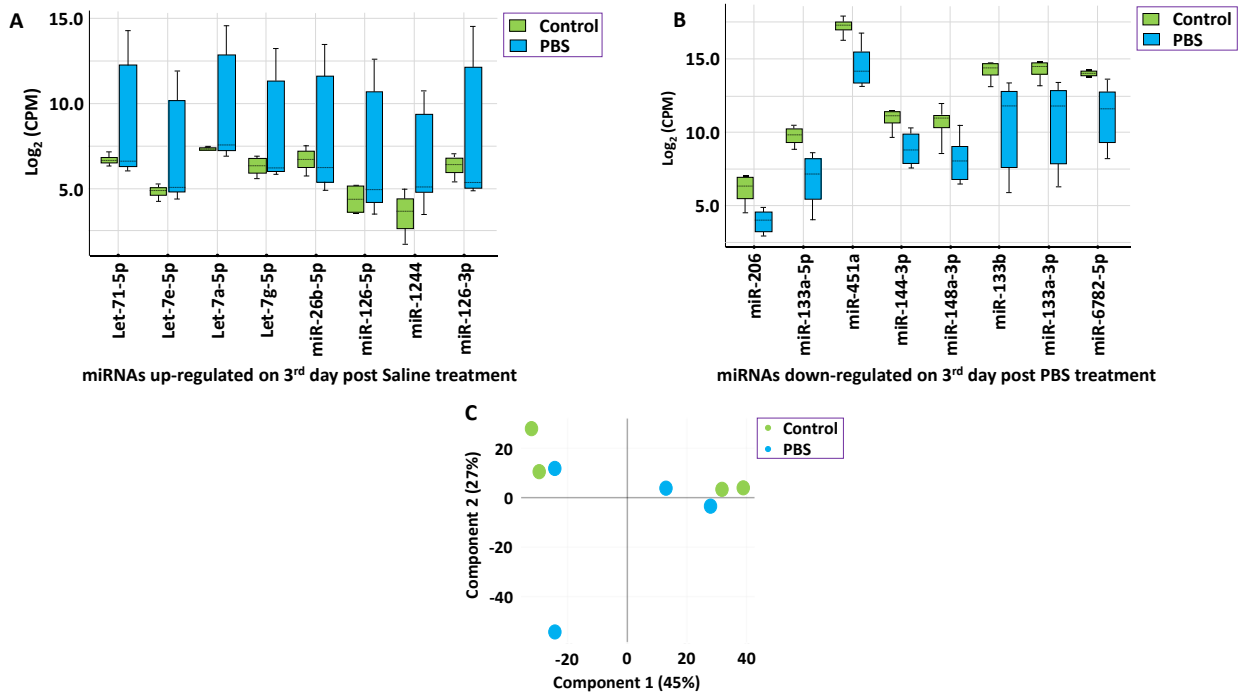


Figure 3. (A) upregulated and **(B)** downregulated expression of miRNAs in untreated uninfected hamsters (green) compared to uninfected hamsters treated with SNAT (red). **(C)** PCA plot.

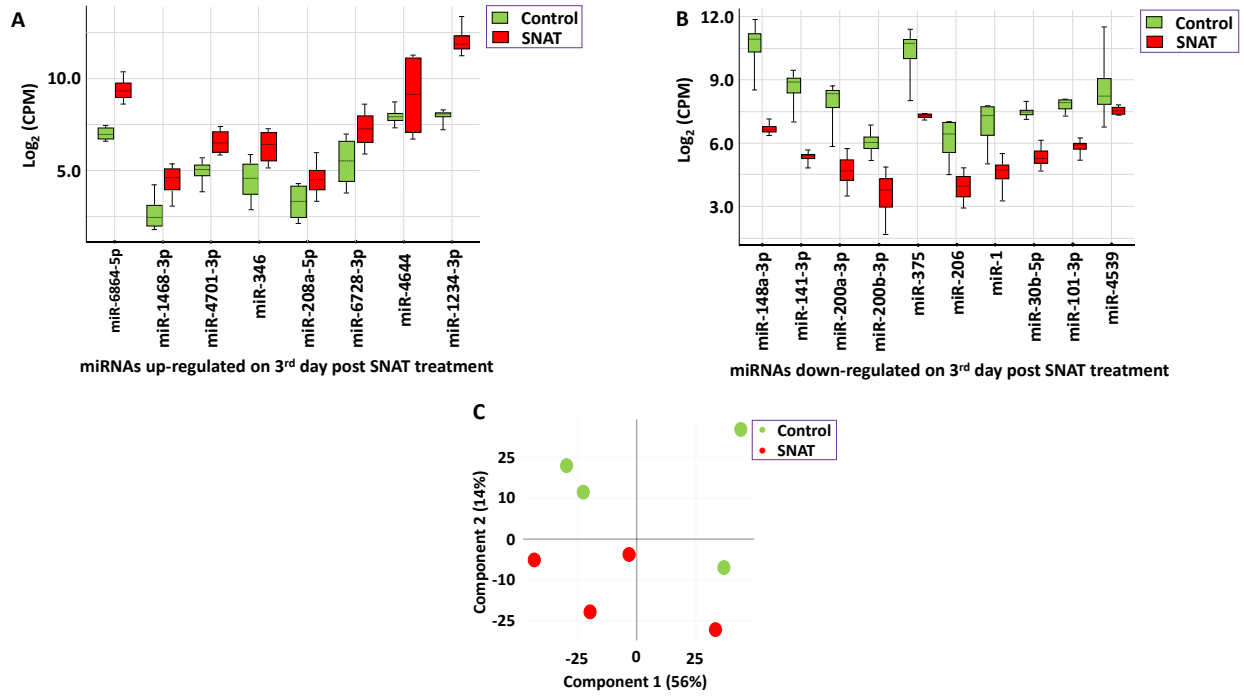


Figure 4. (A) upregulated and **(B)** downregulated expression of miRNAs in untreated uninfected hamsters (green) compared to uninfected hamsters treated with saline (blue). **(C)** PCA plot.

3.4 SNAT Promotes Recovery from SARS-CoV-2 Viral Infection by Altering Select Circulatory miRNAs in Hamsters

Of the multiple circulatory miRNA expressions that were altered, we focused on the seven most physiologically relevant miRNAs as depicted in **Fig. 5**. Upregulation of miR-6864-5p and miR-1234-3p along with the downregulation of miR-141-3p, miR-200a-3p, and miR-30b-5p could potentially lower SARS-CoV-2 replication (Ghezelbash et al., 2023; Karim et al., 2022; Luong et al., 2018; Qiu et al., 2017; Xu, J. et al., 2021). Enhanced miR-1234-3p expression accompanied by a decrease in miR-200b-3p can potentially reduce inflammation (Chan, A. C. et al., 2009; Ghezelbash et al., 2023; Luong et al., 2018; Xiao, K. et al., 2023; Ye et al., 2010). A significant decline in the expression of miR-148a-3p and miR-200a-3p may promote healing processes (Kato et al., 2013; Kim, H. et al., 2018; Kook et al., 2021; Lao & Barron, 2023; Zheng et al., 2023). The overall result of these changes to the miRNA expression profile observed in SNAT-treated hamsters is decreased viral load and inflammation while promoting the healing process; all of which support recovery from SARS-CoV-2 infection.

The rationale for probing for the expression of IL-6 (downstream target of miR-200b-3p), Ythdf2 (target of miR-141-3p), and Slit2 (target of miR-200b-3p) in SNAT treated hamster lungs are: (i) SARS-CoV-2 infection is known to increase expression of IL-6 in hamsters as well as in humans (Francis et al., 2021); (ii) our luciferase reporter assay confirmed Ythdf2 and Slit2 to be direct targets of miR-141-3p and miR-200b-3p, respectively **(Supplementary Fig. S2**). The active miR-141-3p and miR-200b binding sites in 3'-UTR of Ythdf2 and Slit2 mRNA, respectively, is provided in **Supplementary Fig. S4**.

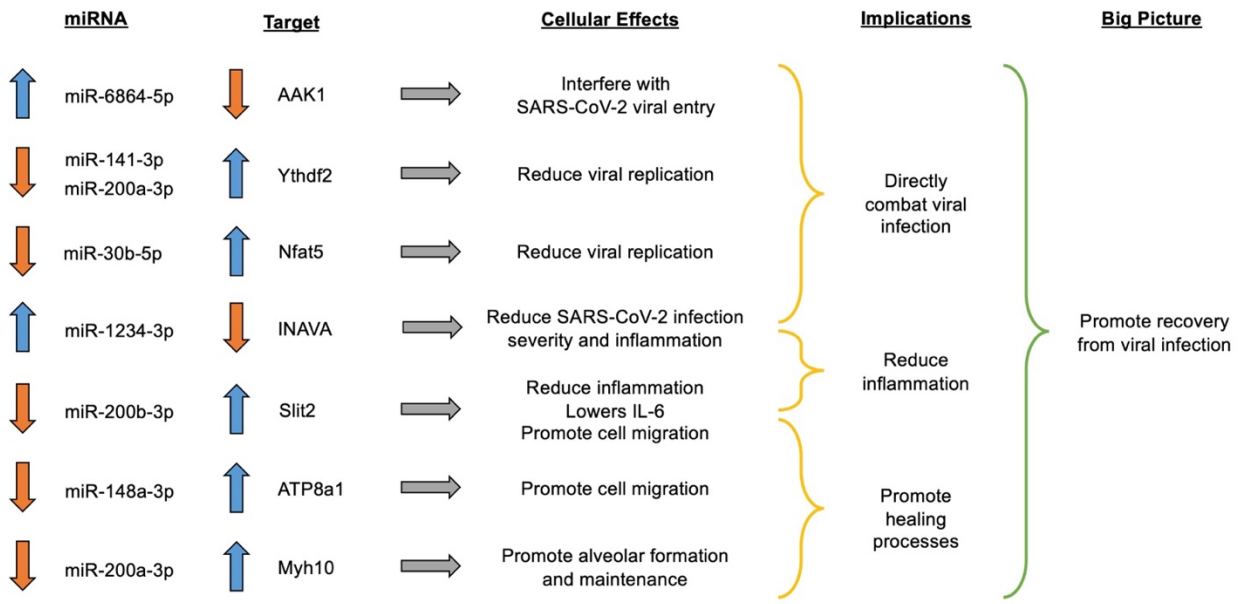


Figure 5. Differentially expressed miRNAs in hamsters treated with SNAT and potential effects of altered expression of physiologically relevant targets.

The immunohistochemistry results indicated no significant difference in IL-6 expression in the lungs that were uninfected and treated with PBS or uninfected and treated with SNAT (**Fig. 6A-B**). SNAT did not seem to have any significant adverse effects on the lung tissues when analyzed by H&E staining **(Supplementary Fig. S3)**. We also tested IL-6 expression in lungs derived from SARS-CoV-2 infected hamsters and SARS-CoV-2 infected hamsters treated with SNAT, 3 days post-infection. The IL-6 expression in lungs from infected hamsters was significantly elevated (**Fig. 6D**) compared to uninfected hamsters treated with PBS or uninfected hamsters treated with SNAT (**Fig. 6A-B**). Also, SNAT-treated SARS-CoV-2 infected hamsters expressed significantly lower levels of IL-6 (**Fig. 6C**) compared to SARS-CoV-2 infected hamsters that did not receive this treatment **(Fig. 6D)**. Raw data corresponding to relative IL-6 levels is provided for comparisons (**Fig. 6E**).

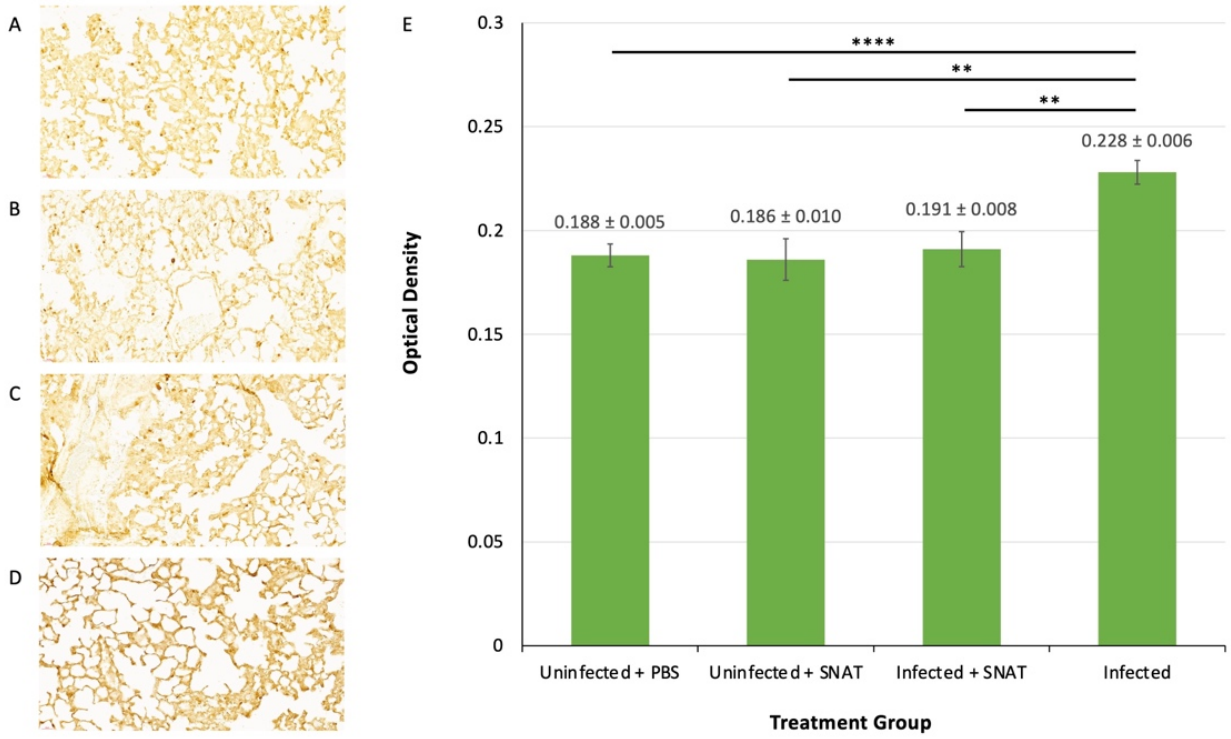


Figure 6. Immunohistochemical analysis of IL-6 expression in hamster lung. H DAB stain for IL-6 in uninfected hamsters treated with PBS (**A**) or SNAT (**B**) and hamsters infected with SARS-CoV-2 treated with SNAT (**C**) or untreated (**D**) at 40x magnification. IHC quantification of IL-6 for each group using optical density with standard error bars. Significance determined by p value < 0.05 using two tailed paired t-test (** indicates $p < 0.01$; **** indicates $p < .0001$; no asterisk indicates no significant difference between groups).

To further confirm the effects of SNAT on hamster lungs, we performed Western blotting using lysates derived from the lung specimens below. Our data demonstrate the ability of SNAT to significantly lower IL-6 expression compared to hamsters that were infected with SARS-CoV-2; while increasing the expression of Ythdf2 and Slit2 when compared to uninfected controls (**Fig. 7**). These data confirm our model proposed in **Fig. 5**.

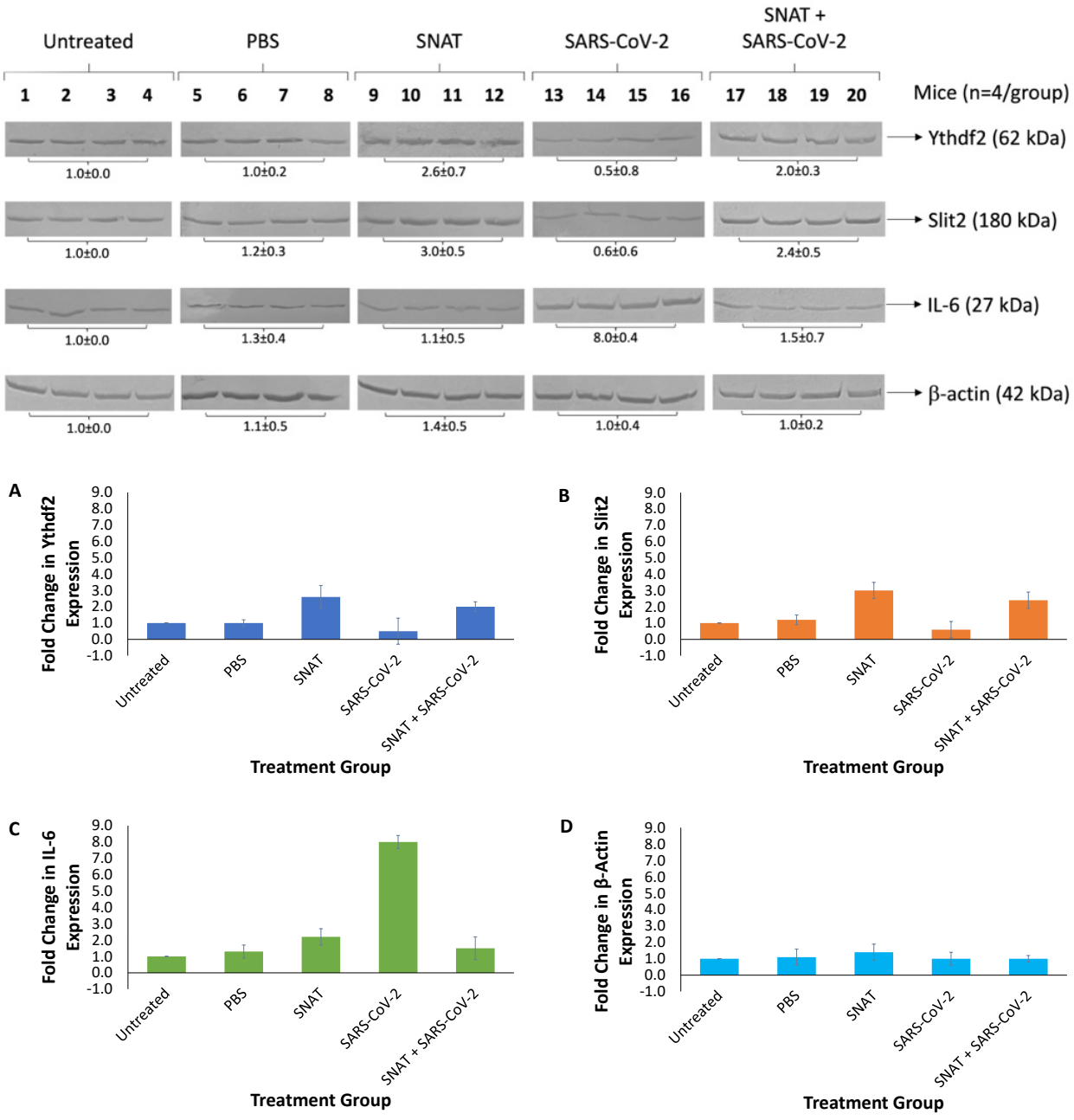


Figure 7. Western blot results and graphical representation of protein expression of (**A**) Ythdf2, (**B**) Slit2, (**C**) IL-6, and (**D**) β-actin in uninfected hamsters untreated (n=4), treated with PBS (n=4), treated with SNAT (n=4), untreated infected hamsters (n=4), or infected hamsters treated with SNAT. β-actin was used as a control and showed no significant difference in expression between groups.

4. Discussion

SNAT was previously shown to combat SARS-CoV-2 infection by lowering viral titer, improving lung health, and reversing virus-induced weight loss in golden Syrian hamsters (Pokhrel et al., 2022a). Here we elucidated potential mechanisms underlying SNAT inhibition of SARS-CoV-2 infection in hamsters. We determined two potential explanations for its antiviral properties: SNAT could be directly interacting with the virus to prevent infection of cells and/or could be affecting the global transcriptome of the host to indirectly interfere with viral pathogenesis.

High-resolution TEM imaging revealed that SNAT directly binds to SARS-CoV-2 to block SARS-CoV-2 infection (**Fig. 2**). To investigate how this occurs, we focused our efforts on S protein because (i) S protein is a glycosylated protein that is expressed on the viral envelope (Nguyen et al., 2021) and (ii) S protein plays a crucial role in the virus entry process (Lavie et al., 2022). We did not focus on the M protein that is also expressed on the viral envelope because it plays a role in the viral assembly and not in the entry process (Cao et al., 2022; Dolan et al., 2023). Targeting the S protein with therapeutics may prevent viral entry and reduce infectivity. The S protein is comprised of two subunits, with S1 predicted to house the receptor binding domain (RBD) and S2 predicted to play a role in viral fusion (Pawlowski, 2021).

Our ELISA assays confirmed that SNAT selectively binds to the S2 subunit contained within the S protein (**Fig. 2E**). Though the SARS-CoV-2 virus has an overall positive charge, the S protein is net negative, with 99 cationic and 111 anionic amino acid residues (Pawlowski, 2021). The S2

subunit has a net negative charge, with 14 positive and 18 negative amino acids (Pawlowski, 2021). This suggests electrostatic interactions with the positively charged SNAT particles may occur. In contrast, the S1 subunit of the S protein is positive, with 7 cationic amino acid residues in the RBD resulting in electrostatic attractions with the 28 anionic residues in the ACE2 receptor (Pawlowski, 2021). We hypothesize that the excess negative charge at the S2 subunit of S protein is crucial to the binding of SNAT, which interferes with viral entry at the receptor interface.

Disulfide bonds between the S protein and ACE2 receptor have been implicated in viral binding and AgNPs have been shown to disrupt disulfide bonds (Jeremiah et al., 2020). Furthermore, AgNPs preferentially bind to viral surface proteins rich in sulfhydryl groups (Jeremiah et al., 2020). The S proteins of SARS-CoV-2 variants contain 30 conserved cysteine residues on the ectodomain that form specific disulfide bonds within the protein (Yao et al., 2023). This suggests that SNAT may disrupt disulfide bridges between the S protein and ACE2 to impede viral binding, as well as impair the structural stability of the S protein across multiple variants of SARS-CoV-2.

SNAT can also affect the global transcriptome of the host by altering miRNA levels (**Fig. 3, 4**). Therefore, we chose to examine the effects of SNAT alone on the cellular milieu *in vivo*. We only used SNAT and its vehicle PBS in this assay as there is no report on the effects of SNAT or similar therapeutics on the micronome in hamsters and mice models. We deliberately avoided the use of SARS-CoV-2 as there are multiple papers to show how the virus alters miRNA profiles in

various rodent models including hamster (Kim, W. R. et al., 2020; McDonald et al., 2021). Our extraction-free whole transcriptome assay revealed significant differences in the expression levels of 8 physiologically relevant circulatory miRNAs (**Fig. 5**). Using miRBD and miRBase, we investigated potential targets of these miRNAs and their cellular functions. These results informed our predictive model (**Fig. 5**) that supports the ability of SNAT to promote recovery from viral infection by reducing inflammation, promoting healing processes, and inhibiting viral replication.

Western blotting experiments were performed to verify the effects of SNAT treatment on protein expression in hamsters. Our results indicated that SNAT-induced upregulation of miR-141-3p decreased the expression of target protein Ythdf2 (**Fig. 7**). Decreased Ythdf2 has been implicated in increased lentiviral protein expression and production of lentiviruses *in vitro* (Lao & Barron, 2023). Additionally, Ythdf2 is involved in the negative regulation of the innate immune response by RNA degradation of IKKγ and p65 transcripts, and silencing of Ythdf2 resulted in reduced viral replication of vesicular stomatitis virus (VSV) *in vivo* (Xu, J. et al., 2021). Since the innate immune response is non-specific, it is plausible that downregulation of Ythdf2 could increase cellular expression of these antiviral transcripts and bolster the innate immune response during SARS-CoV-2 infection.

Our results suggested that SNAT-induced downregulation of miR-200b-3p increased the expression of target protein Slit2, which is significantly downregulated by SARS-CoV-2 infection (**Fig. 7**). Slit2 is involved in inflammatory processes and has been demonstrated to

reduce IL-6 levels via the NF-κB pathway (Ahirwar et al., 2021). This may help explain lower IL-6 expression in lungs derived from infected hamsters treated with SNAT compared to untreated infected hamsters (**Fig. 6**). In addition, decreased inflammation may contribute to reduced lung injury as observed in SARS-CoV-2 infected hamsters treated with SNAT compared to infected hamsters without treatment (Pokhrel et al., 2022a).

Nanomedicines such as SNAT have many advantages over traditional antivirals. The method of drug administration or engineered coatings on the surface of the NP may allow targeting of specific cell types or tissues (Uskokovic, 2023). SNAT was delivered intranasally, allowing for more direct drug delivery than IV or oral alternatives to the lungs, an area of concern in respiratory illnesses such as SARS-CoV-2 infection (Jonigk et al., 2022; Pokhrel et al., 2022a). The size of the nanoparticle can also affect delivery. SNAT nanoparticles have a hydrodynamic diameter (HDD) of 5.0 nm, on average, within the size range of inhalable particles that localize to the tracheobronchial and alveolar regions of the human respiratory tract (Pokhrel et al., 2022a; Uskokovic, 2023).

In addition, spherical nanoparticles such as SNAT have more effective cellular uptake than non-spherical nanomaterials (rods, cylinders, and cubes) under 100 nm (Albanese et al., 2012; Pokhrel et al., 2022a). Nanodrugs can also have a longer half-life, which in combination with the size and targeted delivery, may reduce dosages (Chakravarty & Vora, 2021). In turn, this could result in reduced adverse effects and increased patient compliance compared to traditional regimens (Chakravarty & Vora, 2021). As little as only two doses of 10 μg/ml SNAT (2 ml/dose,

given 24 h apart) could demonstrate potent antiviral effects in hamsters infected with SARS-CoV-2 (Pokhrel et al., 2022a).

Other AgNPs have been used against SARS-CoV-2; however, the few in clinical trials focus on severe infection and preventative measures. For example, *Wieler et al.* used AgNPs as adjuvant treatment for SARS-CoV-2 infected patients (n=40) with virus-induced pneumonia and demonstrated a significant improvement in survival rate (Wieler et al., 2023). Yet, with intravenous administration across three consecutive days, this treatment requires consistent patient access to a healthcare facility (Wieler et al., 2023). This regimen is well suited for hospitalized patients with moderate to severe infections; however, it may not be conducive to outpatient treatment. In contrast, SNAT treatments can be administered at home with ease via a nebulizer (Pokhrel et al., 2022a).

Another study by *Almanza-Reyes et al.* utilized AgNPs in a nose rinse and mouthwash to prevent SARS-CoV-2 infection among high-risk healthcare workers (Almanza-Reyes et al., 2021). Treatment significantly reduced the incidence of infection with only 2 out of 114 participants in the experimental group compared to 33 out of 117 in the control group testing positive over 9 weeks (Almanza-Reyes et al., 2021). This method of administration targets the nasal and oral cavities, which are areas of entry for the virus (Almanza-Reyes et al., 2021; Jonigk et al., 2022). In contrast, SNAT targets the airways and penetrates into the lungs, making it better suited for patients with active infections. SNAT has not yet been studied in the context of prevention; however, this may be an interesting direction for future works.

Overall, our findings suggest that SNAT may serve as a safer antiviral therapeutic against SARS-CoV-2 infection. While *Wieler et al.* did not define the exact mechanism of their AgNPs, they hypothesized that they may interfere with viral binding and trafficking, binding to the viral genome to combat infection, and/or reducing inflammatory cytokines to prevent cytokine storms (Wieler et al., 2023). We have gone further to illuminate the mechanism of SNAT and its direct and indirect effects on viral infection, which may help inform future clinical studies. Both *Wieler et al.* and *Almanza-Reyes et al*. reported no adverse events from the use of their AgNPs (Almanza-Reyes et al., 2021; Wieler et al., 2023), a result consistent with our findings.

5. Conclusions

Herein, we propose a two-pronged approach by which SNAT inhibits SARS-CoV-2 infection and ensuing pathogenesis. By binding to the S2 subunit of the S protein, SNAT interferes with viral fusion to prevent infection of cells. It may also disrupt the stability of the S protein and impair viral binding to ACE2 receptors to prevent viral entry. In addition, SNAT treatment alters the cellular environment through transcriptomic changes to help neutralize infection. It downregulates miR-200b-3p to increase the expression of Slit2, lowering inflammation, including IL-6 expression. It also upregulates miR-141-3p to decrease the expression of Ythdf2 to reduce viral replication and enhance the innate immune response. The antiviral effects on the miRNA-directed milieu indicate broader applications of SNAT in viral respiratory infections. As research continues to progress in this field, nanomedicines such as SNAT hold promise for revolutionizing our ability to combat current and future viral outbreaks.

Ethics Approval and Informed Consent: This study was approved by the Office of Prospective Health/Biological Safety for the use of biohazardous agent (SARS-CoV-2) and the registration number is 20–01 (title: Host response to COVID-19 infection in Eastern North Carolina). Our Animal Use Protocol entitled, "Effect of SNAT on SARS-CoV-2 infection of hamsters" (AUP #K177) was reviewed by ECU Institution's Animal Care and Use Committee (approval date: 11/12/2020).

Author Contributions

S.M.A., P.P.C., and A.N.B. conceived and designed the study. A.N.B. conducted the experiments, analyzed the data, wrote the first draft of the manuscript. LRP designed, synthesized, purified, characterized, and patented the SNAT. J.F.W. actively participated in hamster and *in vitro* studies. S.G. and J.B.E. conducted Western blotting and immunohistochemistry experiments, respectively. All co-authors read and approved the manuscript.

Conflicts of interest

There are no conflicts to declare.

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CHAPTER 3: SUMMARY

MicroRNAs have many roles in viral infection and immunity, making them ideal candidates for diagnostic and therapeutic technologies. Circulating levels of miRNAs have the potential to indicate the presence of virus, predict patient outcomes, and evaluate effects of antiviral treatments. Modulation of specific miRNAs may disrupt viral replication and infectivity. Therefore, antiviral therapeutics can be used to antagonize pro-viral miRNAs or mimic antiviral miRNAs. Treatments not based on miRNA technology may still induce changes in miRNA levels to promote an antiviral cellular environment. Thus, differential expression of miRNAs may illuminate the mechanism by which antiviral therapeutics inhibit viral infection.

Smart Nano-enabled Antiviral Therapeutic (SNAT) alleviates SARS-CoV-2 pathology in vivo through a previously unknown mechanism. Our studies have determined SNAT directly binds to the S2 subunit of the viral S protein through electrostatic interactions, interfering with viral entry. We employed next generation sequencing to monitor the circulatory miRNA profile of hamsters treated with SNAT compared to untreated controls to elucidate its indirect effects as an antiviral therapeutic. The SNAT-induced changes in miRNA altered translation of physiologically relevant target mRNAs and their corresponding proteins. Overall, SNAT-induced changes in miRNA expression inhibited viral replication, reduced inflammation, and promoted healing processes. Our results allowed us to construct a holistic model explaining the antiviral effects of SNAT against SARS-CoV-2. Our studies validate the use of next-generation sequencing in the evaluation of medical treatments and illustrates the therapeutic potential of nanotechnology against viral infections.

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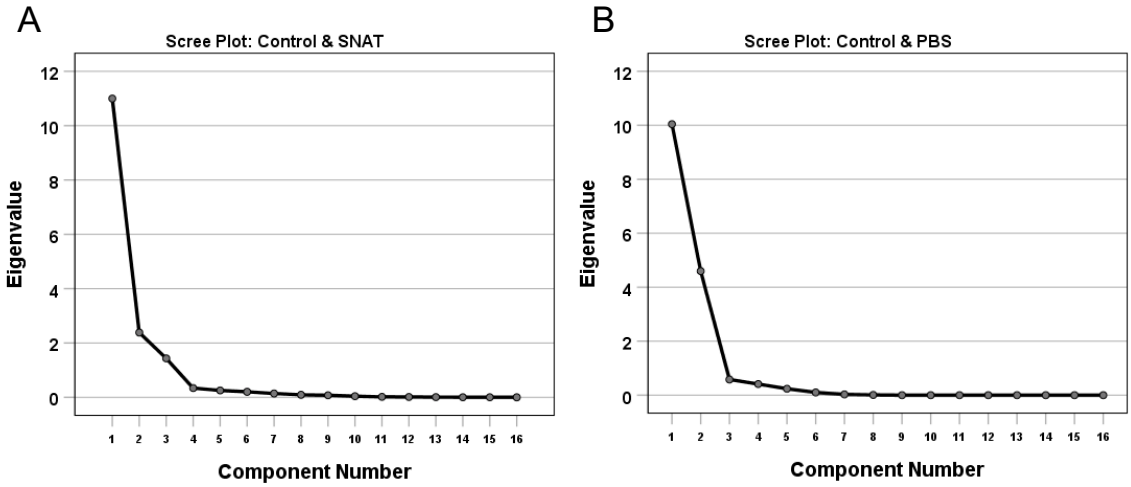
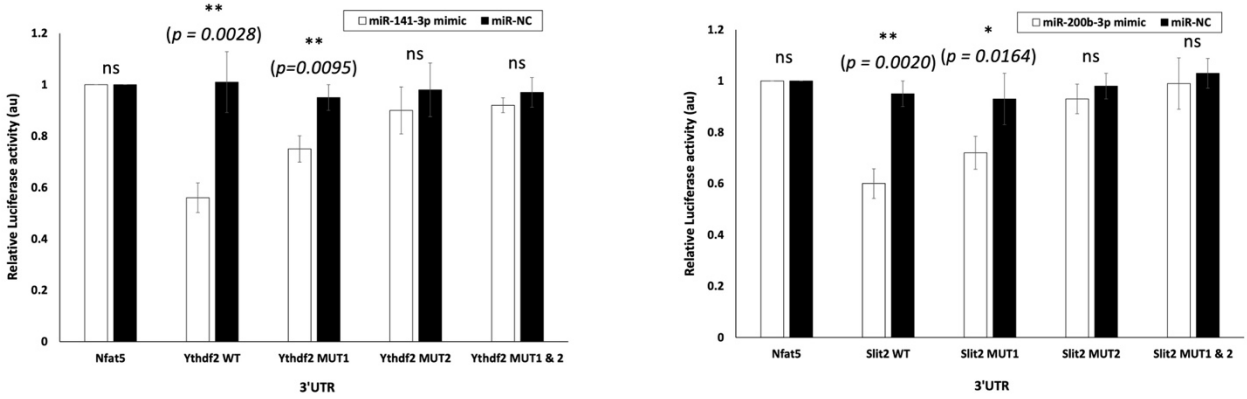
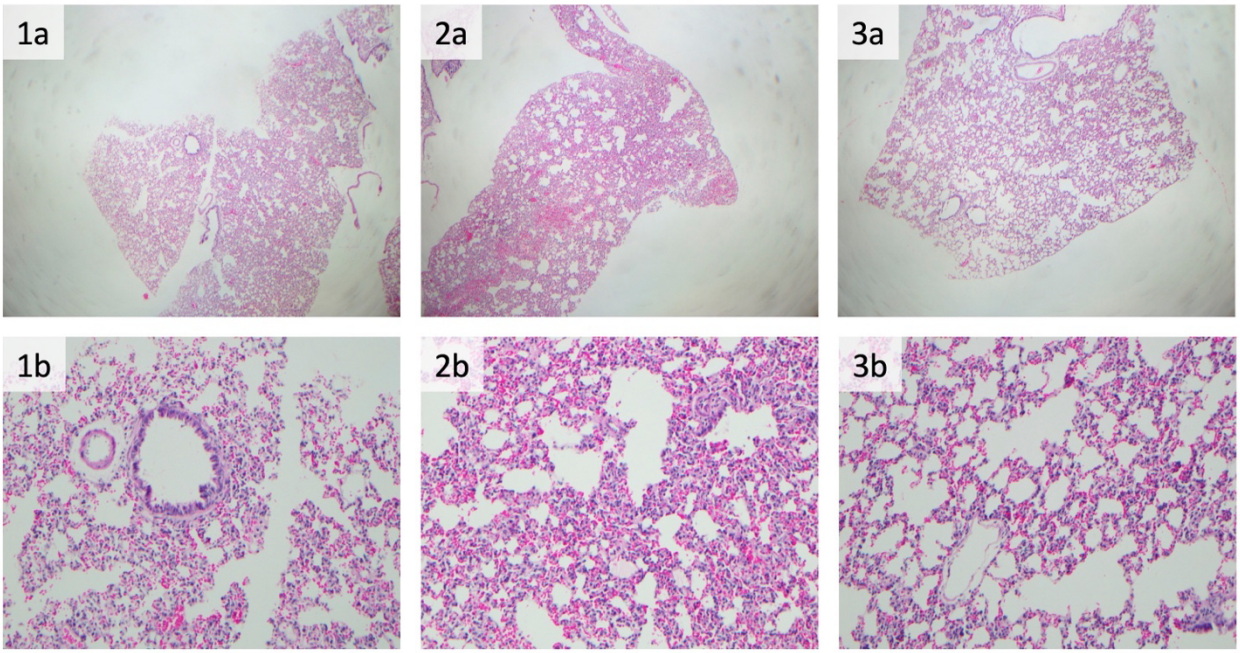
APPENDIX A: SUPPLEMENTAL FIGURES



Supplementary Figure S1. Scree plots showing eigenvalues for the principal components presented in Figures 3 and 4 in the main text. (**A-B**) Scree Plot showing eigenvalues on the Y-axis and the number of factors on the X-axis for transcriptome differences between miRNA expression in uninfected Control, SNAT treated, and PBS groups of golden Syrian hamster lungs.



Supplemental Figure S2: miR-200b-3p and miR-141-3p specifically binds and interact with Slit2 and Ythdf2, respectively. HEK293T cells were transfected with (i) Slit2 3′UTR, Ythdf2 3'UTR, or Nfat5 3′UTR (*Control*); (ii) co-transfected with vector encoding the above Slit2 3′UTR and miR-200b-3p mimic, co-transfected with Slit2 3′UTR and control mimic (miR-NC); (iii) co-transfected with vector encoding the above Ythdf2 3′UTR and miR-141-3p mimic, co-transfected with Ythdf2 3′UTR and control mimic (miR-NC). We also tested Slit2 and Ythdf2 MUT1 and MUT2 3′UTR in this study. Luciferase activity was monitored at 48 h post-transfection and was normalized against the effect of the miRNAs on the Firefly luciferase reporter alone. The relative luciferase activity for cells transfected with Slit2 3′UTR, Ythdf2 3'UTR, or Nfat5 3′UTR is considered as 1 au. The x-axis denotes the UTRs transfected, and y-axis indicates the relative luciferase activity. Bars represent average ± s.d. of three individual experiments. One-way ANOVA was performed compare between multiple groups. The p value and their significance is indicated; 'ns' denotes not significant at p=0.05.



Supplementary Figure S3: Hematoxylin and eosin (H&E) staining of the lungs of uninfected hamsters (1) control, (2) with SNAT treatment, or (3) with saline treatment at (a) 40X and (b) 200X magnification.

Supplementary TABLE 1: Oligos used in the Luciferase assay.

Name	Sequence
miR-141-3p mimic	UAACACUGUCUGGUAAAGAUGG
miR-200b-3p mimic	UAAUACUGCCUGGUAAUGAUGA
miR-NC	ucacaaccuccuagaaagaguaga
WT hamster Ythdf2 3'UTR sequence	CTCGAGtttttgtctaggagagtttaataacagtgtta
Hamster Ythdf2 3'UTR sequence Mutant-1	CTCGAGtacccaaccaggagagtttaataacagtgtta
Hamster Ythdf2 3'UTR sequence Mutant-2	CTCGAGtttttgtctaggagagtttaataaacccaacc
Hamster Ythdf2 3'UTR sequence Mutant-1&2	CTCGAGtacccaaccaggagagtttaataaacccaacc
WT hamster Slit2 3'UTR sequence	CTCGAGcgaaggtgtttatctaccactttccagtatta
Hamster Slit2 3'UTR sequence Mutant-1	CTCGAGcacccaaccttatctaccactttccagtatta
Hamster Slit2 3'UTR sequence Mutant-2	CTCGAGcgaaggtgtttatctaccactttcacccaacc
Hamster Slit2 3'UTR sequence Mutant-1&2	CTCGAGcacccaaccttatctaccactttcacccaacc

miRNA binding sequence

Mutated sequence

miR-141-3p : Ythdf2 interactions
Hamster Ythdf2: XM_040756461

Position 2573-2582 of Ythdf2 3'UTR	5'	G G A G A G T T T A A T A A C A G T G T T A 3'
miR-141-3p	3'	G G U A G A A A U G G U C U G U C A C A A U 5'

miR-200b-3p : Slit2 interactions
Hamster Slit2: XM_040747064

Position 7075-7082 of Slit2 3'UTR	5'	T A T C T A C C A C T T T C C A G T A T T A 3'
miR-200b-3p	3'	A G U A G U A A U G G U C C G U C A U A A U 5'

Supplementary Figure S4. RNA hybrid analysis shows miR-141-3p and miR-200b binding site in 3'-UTR of Ythdf2 and Slit2 mRNA. This is predicted using miRDB and TargetScan algorithms.

APPENDIX B: OTHER PUBLICATIONS

CELLULAR MIR-6741 AS A PROGNOSTIC BIOMARKER PREDICTING LENGTH OF HOSPITAL STAY AMONG COVID-19 PATIENTS

Shaw M. Akula[1,2]*, John F. Williams[1], Lok R. Pokhrel[3], **Anais Bauer[1]**, Smit Rajput[2], Paul P. Cook[2]

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PMID: 36560686 PMCID: PMC9781286 DOI: 10.3390/v14122681

APPENDIX C: ECU IACUC APPROVAL MEMO

Animal Care and Use Committee
003 Ed Warren Life Sciences Building | East Carolina University | Greenville NC 27834 – 4354
252-744-2436 office | 252-744-2355 fax

November 12, 2020

Shaw Akula, Ph.D.
Department of Microbiology and Immunology, ECU

Dear Dr. Akula:

Your Animal Use Protocol entitled, "Effect of SNAT on SARS-CoV-2 infection of hamsters." (AUP #K177) was reviewed by this institution's Animal Care and Use Committee on 11/12/2020. The following action was taken by the Committee:

"Approved as submitted"

****Please contact Aaron Hinkle prior to any hazard use****

A copy of the protocols is enclosed for your laboratory files. Please be reminded that all animal procedures must be conducted as described in the approved Animal Use Protocol. Modifications of these procedures cannot be performed without prior approval of the ACUC. The Animal Welfare Act and Public Health Service Guidelines require the ACUC to suspend activities not in accordance with approved procedures and report such activities to the responsible University Official (Vice Chancellor for Health Sciences or Vice Chancellor for Academic Affairs) and appropriate federal Agencies. **Please ensure that all personnel associated with this protocol have access to this approved copy of the AUP/Amendment and are familiar with its contents.**

Sincerely yours,

Susan McRae, Ph.D.
Chair, Animal Care and Use Committee

SM/GD

enclosure

APPENDIX D: BIOLOGICAL SAFETY MEMO

The Brody School of Medicine
Office of Prospective Health
East Carolina University
188 Warren Life Sciences Building ● Greenville, NC 27834
252-744-2070 office ● 252-744-2417 fax

Occupational Medicine
Employee Health

Radiation Safety

Infection Control

Biological Safety

TO: Dr. Shaw M. Akula
Department of Microbiology & Immunology

FROM: Chad Spruill
John Baumgartner
Biological Safety Officers

RE: Registration Final Approval

Date: November 12, 2020

Your Biological Safety Protocol, **Effect of SNAT on SARS-CoV-2 infection of hamsters** has received **final approval** to be conducted at Biosafety Level 2 and 3 and Animal Biosafety Level 3 in LSB Biocontainment facility, Brody 5N-99 and 5N-136 based on your registration/revisions submitted,

using: A. Biohazards

- ☒ Infectious Agent(s)
- ☐ Biotoxin(s)
- ☐ Allergen(s)
- ☐ Prion(s)
- ☒ Human blood, fluid, cells, tissue or cell cultures
- ☐ Transformed cells
- ☐ Other

and/or B. ☒ NIH Use of Recombinant DNA (or RNA) molecules, microorganisms use or breeding transgenic or techniques (plasmids, viral vectors, transfection); of transgenic animals or plants at NIH Category III-D.

This approval is effective for a period of 3 years and may be renewed with an updated registration if needed at that time. Your laboratory will be inspected periodically (every 1-3 years) depending upon the materials/techniques used.

Please notify the Animal Care staff before beginning work with Biohazard agents in animals. Also, please keep in mind all individuals who will be exposed to or handle human-derived biohazardous agents will be due for Blood Borne Pathogens refresher training annually.

Please do not hesitate to contact Biological Safety at 744-2070 if you have any questions, concerns, or need any additional information. Best wishes on your research.

cc: Dr. Daniel Martin, Chair, Biosafety Committee
Dr. Everett Pesci, Department Chair
Dr. Susan McRae, IACUC
Gaelle Deshayes, IACUC
Aaron Hinkle, Comparative Medicine