Can RNAi be used as a weapon against COVID-19/SARS-CoV-2?

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Abstract

Can RNA interference be used as a diagnostic and therapeutic for COVID-19? Can host or viral encoded miRNA or siRNA be used as a vaccine against SARS-CoV-2? RNAi has been used as a platform to make attenuated viral vaccines where the viral genome is engineered and modified to contain miRNA or siRNA binding sites [50]. One of these examples was the creation of self-attenuating Influenza A virus strain that expressed an siRNA from the NS segment (for wild type nonstructural protein NS1) that targets the ORF of the nucleoprotein [NP] segment just at a single site [51]. Intranasal administration of five chemically modified miRNA mimics corresponding to highly expressed miRNAs in respiratory epithelial cells synergistically suppressed H1N1 replication in mice. MicroRNA 122 is another most common example in RNAi literature, anti-miR against mir-122 is effective to lower the hepatitis C virus and miR-122 inhibition by anti-miR122 also reduces serum cholesterol levels [40,52]. RNAi patents and clinical trials for liver cancer, breast cancer, lymphoma, melanoma, IAV, are just a few examples and there are hopes for COVID-19 soon. How about the plant-based diet and plant microRNAs, can we use them against COVID-19 like infections? This mini-review discusses different types of non-coding small RNA molecules, their biogenesis, the role of innate immune response and the competition of proviral and antiviral proteins, and how RNAi can be alone or as a combination of COVID-19 intervention drugs and vaccines used as a therapeutic along with a little emphasis on plant-based miRNAs to prevent future pandemics like COVID-19?

Keywords: RNAi, Non-Coding Small RNAs, *miRNA*, *siRNA*, Drosha, Dicer, Argonaute Complex, COVID-19, Pattern Recognition Receptors, Interferon, IFN-regulatory factors, IFN-stimulatory genes, Cytokine storm, Vaccine, Plant miRNA

Introduction

RNAi means active cell response to foreign RNA [1]. RNAi is a major defense mechanism against alien nucleic acids of bacteria, viruses, and phages; RNAi is present in diverse organisms like fungi, plants, algae [2], invertebrates, and vertebrates including mammals. RNAi is known for at least the last two decades, is a double-stranded short interfering RNA (siRNA) or microRNA (miRNA) of approximately 21-23 nucleotides that can target the degradation or prevent the translation of an mRNA which contains complementarity to it [3].

Review

What is RNA Interference?

RNA interference(RNAi) is not interference but is a process where

noncoding RNA regulates gene expression, protein expression, RNA splicing, RNA silencing, chromatin structure, modifications, and segregation [3]. RNAi plays a big role in various biological phenomena like cell proliferation, cell death, fat metabolism in flies, neuronal patterning in nematodes, regulation of hematopoietic cells, and differentiation in mammals, root, stem, and leaves formation in plants [4], etc.

What are the different types of Small RNA Molecules?

Based upon the whole genome and transcriptome sequencing projects, and RNAi experiments, there are three main types of small RNAs: microRNA or miRNA, small interfering RNA or siRNA, & piwi-associated RNA or piRNA.

1.miRNA: They are 20-25 nucleotides long. Gene regulatory

network depicts miRNAs as endogenous, processed from larger dsRNA precursors, imperfect stem-loop structures, or partially double-stranded structures [3,5,6]. The miRNAs bind mainly 3'UTR by Watson-Crick base pairing; the region 2-3 bases 5' of miRNA that has exact complementarity are the seed sequence, and binding of miRNA results in a translational arrest, deadenylation, and degradation of the transcript.

2. siRNA: These were considered primarily exogenous in origin but recently endogenous siRNAs have been discovered [6,18]. Exogenous siRNAs are 20-25 nucleotides long, their main goal is to defend the genomic integrity; they are derived directly from viruses, transposons, repeat-associated transcripts, centromere [3], or by transgene trigger; and are excised from long, fully complementary double-stranded RNAs. Endogenous siRNAs are 20-23 nucleotides long and are synthesized from double-stranded RNAs such as hairpins with a long, perfect stem or sense-antisense transcript hybrids. New members of endogenous siRNA also include trans-acting siRNA (tasiRNAs), repeat-associated siRNAs, scan RNA (scnRNAs), long small interfering RNAs (lsiRNAs) [7,8]. Both miRNA and siRNA have been found in a single-stranded form with their effector complexes [3].

3. piRNA: The piRNAs are single-stranded RNAs, derived without dicer cleavage from distinct piRNA clusters. These were discovered as 25-33 nucleotide small RNAs, transcribed from repetitive elements of transposons. They stabilize germline cells by repressing transposon elements particularly in spermatogenesis [6,8,9]. They are named as piRNAs because they bind PIWI [P element-induced wimpy testes] proteins to execute their jobs in the nucleus and cytoplasm [86].

Both miRNA and siRNAs bind to the members of the Ago clade of Argonaute proteins whereas piRNA bind to members of PIWI clade [3].

What led to the serendipitous discovery of RNAi?

RNAi was first discovered in plants when enhancing the color of petunias via plasmid containing *chalcone synthase* gene led to co-silencing of its transgene and homologous gene which resulted in totally white or patterned flowers with white or pale non-clonal sectors on a wild-type pigmented background [10]. During the same time, Dougherty's lab discovered the Tobacco Etch Virus (TEV) coat protein antisense RNA induced resistance against its infection in *Nicotiana tabacum* plants [11]. Another milestone in RNAi was made by Andrew Fire and Craig C. Mello in *Caenorhabditis elegans*, double-stranded RNA but not the single-stranded RNA against *unc-22*, *unc-54*, *fem-1*, and *hlh-1* genes successfully silenced the targeted genes which led them to 2006 Nobel Prize in Physiology or Medicine [12-14]. But all of this started from the discoveries of small temporal RNAs by Lee et al., 1993 [15] and Reinhart et al., 2000 [16]. The word RNA made a different meaning after Dr. Lee's discovery of the *lin-4* gene that codes a precursor of 61 nucleotides to make 22 nt miRNA. This 22 nt miRNA represses *lin-14* by complementarily binding at 7 sites of its 3'UTR [16,17]. The expression of *lin-14* is required to transit *C.*

elegans from the first larval stage to the second larval stage. From *lin-4* to *let-7* in *C. elegans*, *Drosophila*, soon non-coding RNAs were discovered in humans, other bilateral animals, fungi, algae, plants and certain DNA viruses [10].

How are small RNA molecules made?

They are derived from larger RNA precursors, imperfect hairpin loop structure in case of miRNA, and perfect double-stranded RNA in case of siRNA. Both miRNA and siRNA genes at first are transcribed by RNA polymerase II and are known as primary miRNAs or pri-miRNAs or pri-siRNAs [18]. There are three major steps of biogenesis of mature and processed small RNA molecules (Figure 1).

1. The pri-miRNAs are processed into precursor miRNAs (pre-miRNAs) by the Microprocessor complex, consisting of the ribonuclease RNase III Drosha and DiGeorge Syndrome Critical Region 8 (DGCR8).
2. After synthesis of pre-miRNAs takes place in the nucleus they are exported into the cytoplasm by the Exportin 5 protein, where they are processed by a specialized RNase III-like enzyme named Dicer into smaller dsRNA molecules. Each pre-miRNA has two strands; the antisense strand to the targeted mRNA is referred to as the guide strand and its base-paired sense strand known as the passenger strand [19,20].
3. Both pre-miRNA strands are transferred by Dicer and its cofactors TRBP (TAR RNA binding protein) and PACT (Protein kinase RNA activator) into an Argonaute (AGO2 in mammals) containing RISC complex. This complex formation first destroys the passenger strand and then only the guide strand in RISC-complex is targeted at the mRNA, which can degrade the target mRNA or destabilize it to block the translation [19].

Dicer is not always required for the processing of pre-miRNA. There are two ways of miRNA processing- canonical or non-canonical. Canonical miRNAs processing depends upon the dicer and non-canonical miRNA does not require Dicer and Microprocessor complex. For example, the mirtron pathway, which is found in *D. melanogaster* and *C. elegans*, produces pre-miRNAs by the processing of introns by spliceosomes and debranching enzymes (not by dicer) in the nucleus, is an example of the non-canonical pathway [19]. The target mRNA recognition is initiated by a short nucleotide stretch at the 5' end of the miRNA (position 2-8), the so-called seed sequence, accompanied by various degrees of base pairing at the 3' end [19]. In contrast to miRNA, siRNA target recognition requires base pairing of entire small RNA and subsequent target cleavage by AGO2. Guide pre-miRNA targets the RISC complex to 3'UTR or other parts of mRNA results in translational inhibition, mRNA cleavage, de-adenylation, or histone and DNA methylation [3].

RNA interference as a vaccine

We do not live in 1,000 AD anymore where a charlatan would

Figure 1. Non-Coding Small RNA Biogenesis and Viral Interference.

Three major steps, Step I Droscha processing, Step II Dicer cleavage, & Step III Argonaut and RISC complex Formation. Examples of Viral interference: Adenovirus VA1 RNA prevents maturation of miRNA by saturating exportin 5 and dicer [40]. B2 protein of Flock House virus (FHV) binds long and short dsRNAs in a sequence-independent manner to inhibit Dicer cleavage [27,28], vaccinia virus and small flavivirus sRNAs sequester dicer, and ago2 to prevent mature miRNA formation [23]. TBSV p19 protein, CMV 2b protein and EBV inhibit loading of miRNA into RISC complex [23]. HVS, HCMV, MCMV, Gamma-Herpesviruses destroy mature miRNAs [23] (Fig 1 is made by the help of 3, 19, 23, 43, and 73).

make a cut in the skin and transfers smallpox scabs from a patient into a normal human to immunize against smallpox. Our vast information on immunology and a spectrum of lab tools are at an edge in a way that we should be able to temporarily provide some kind of vaccine against any outbreak at any time.

The first experiment where RNAi was used as therapeutics was to prevent hepatitis. When mouse hepatocytes were injected with Fas siRNA they were resistant to Fas-induced apoptosis and hepatocyte necrosis upon exposing cells to Fas antibody or concanavalin-A (ConA). These experiments demonstrated the therapeutic value of Fas siRNA for preventing liver insults induced by viral, autoimmune hepatitis, and liver disorders [21,22]. Can we use small ncRNA molecules as a treatment against viral infections? But we first start with what happens in the waging battle between virus and host?

Telltale of Virus vs Host RNAi

How do cells distinguish between self and viral DS-, SS-DNA, or RNA and their modified forms?

Viruses contain different types of the genome like long dsDNA, dsRNA, 5'triphosphate, or 5'-diphosphate dsRNA, ssRNA, CpG motifs in ssDNA, and short dsDNA with guanosine-containing

overhangs [19,23,24]. The host cell can distinguish itself from nonself-genomic material. Cells have evolved a system of identifying own nucleic acid material from the foreign one by modifying it, for example, 2'O-methylation at the N1 position of capped RNA eliminates recognition by RIGI and IFIT1, but if 2'-O-methyl groups or other modifications such as pseudouridine are added at internal positions of RNA that prevents recognition by TLR7, TLR8, and MDA5 [24]. The C5 methylation of CpG motifs in DNA abolishes TLR9 recognition [24]. Vaccinia virus expresses an enzyme poly-A polymerase known as VP55, which is not only responsible for polyadenylation of viral mRNAs but also cellular miRNAs that results in degradation of mature miRNAs to allow viral sustenance, maybe that is how cellular RNAs and modified miRNAs were evolved for 2'-O-methylation to avoid degradation [40,41,42,45].

The immune response to viral genomes depends upon their structure, concentration or availability, localization-inside, or outside the cell [24]. There are different types of cell receptors to recognize different nucleic acid molecules. These are categorized into first-line and second-line nucleic acid receptors. The pattern recognition receptors (PRRs) of the Toll-like receptor (TLR3, TLR7, TLR8, & TLR9); the RIGI-like receptor (RLR) family of RNA sensors (also known as DDX58);

melanoma differentiation-associated gene 5 (MDA5; also known as IFIH1); the DNA sensors absent in melanoma 2 (AIM2) and cyclic GMP-AMP synthetase (cGAS) come under first-line receptors category [19,23,24] (Figure 2). These first-line receptors instigate a cascade of signaling events that involve MYD88, TRIF, IRAKS, TRAFs, transcription factors like, nuclear factor- κ B (NF κ B) and IFN-regulatory factor 3 (IRF3), IRF7, and are dominated by type I interferon response and IFN-stimulatory genes (ISGs) [24] (Figure 2).

After Interferon is expressed; it is translocated across the cell membrane; it can signal in an autocrine or a paracrine way via the interferon- α/β receptor consisting of two sub-

nits, IFNAR-1 (IFNAR; IFN receptor) and IFNAR2, this leads to phosphorylation of STAT transcription factors through the JAK-STAT pathway. STAT1 and STAT2 now heterodimerize and translocate to the nucleus to activate a broad range of ISGs (**Figure 2**). Second-line category receptors comprise nucleic acid receptors with direct antiviral activity, for example, double-stranded RNA (dsRNA)-activated protein kinase R (PKR; also known as eIF2AK2), 2'5'oligoadenylate synthetase 1 (OAS1), adenosine deaminase acting on RNA 1 (ADAR1) [24], PKR, OAS1, and ADAE1 are also known as ISGs. The major difference between first-line and second-line nucleic acid receptors is that the second line receptors do not induce an immune

Figure 2: Innate Immune Response and the Role of miRNAs.

Viruses activate innate immune response by the pattern recognition receptors (PRRs) like Toll-like receptors, (TLRs) RIG-like receptors (RLRs), NOD-like receptors (NLRs), and C-type lectin receptors (CLRs), only TLRs and RLRs are shown above. Endosomal TLR3s recognize double-stranded RNA, Poly-IC, viral RNA, siRNA, and endogenous mRNA; TLR7 and 8 recognize single-stranded RNA; TLR9 binds to CpG-ODN [44]. TLRs activate the signal pathway that starts from adaptor proteins like TRIF/MyD88 then through IRAK-TRAF6 to transcription factors like IRF3/IRF7/NF- κ B/AP-1 which translocate to nucleus and cause type I IFN and subsequent ISGs expression. Secreted type I IFNs can translocate across the cell membrane in an autocrine or a paracrine fashion through IFN- α/β receptor, this activates the phosphorylation of STAT1 and STAT2 via JAK family kinases, JAK-1 and TYK2. Phosphorylated STATs heterodimerize and translocate to the nucleus to activate a broad range expression of ISGs [19,24]. Besides TLRs, RIG-1 and MDA5 detect dsRNA in the cytosol and activate MAVS to induce type I IFN production via IRF3-IRF7; MDA5 also activates apoptosis and signals NLRP3 inflammasome formation which causes pyroptosis [19,24]. The second line receptors PKR, OAS, IFIT1 directly destroy viral RNA by cleavage, modification, or translational inhibition. Red arrows represent inhibition by miRNAs. Human Influenza type A (NS1) inhibits phosphorylation of IRF3. Viral proteins B2, NS1, VP35, N1, and viral miRNAs miR-485, miR-342 inhibit the IFN-response pathway. MiR-144 is a proviral and miR-146a is an antiviral for HCV. miR-146a of EV71 suppresses IRAK1 and TRAF6 [23]. MiR-144 is a positive regulator of RNA viruses by targeting TRAF-6, miR-485 reduces the expression of Interferon and IL-6, miR-485 targets RIG-1 3' UTR, miR-155, miR-223, and miR-136 are induced by RIG-1/JNK/NF κ B pathway and inhibit replication of VSV [23], let-7 inhibits IL-6 translation. MiR-466i acts on IFN- α and let-7b on IFN- β . Adenovirus VA1 RNA inhibits PKR or OAS1 degradation [40]. Fig2 is inspired from ref 19, 24, 40, 59 & 61, 82.

response by transcription factors or cytokines but directly destroy viral RNA by cleavage, modification, or translational inhibition [24]. Different TLRs responses lead to different outcomes, for example, TLR7 and TLR9 on plasmacytoid dendritic cells lead to type I IFN production while TLR8 on myeloid cells release IL-12 [24] (Figure 2). Overall excitement of innate immune response causes a storm of cytokines, chemokines, antiviral proteins that directly or indirectly, with or without adaptive immune response, cause the end of the viral realm by preventing viral replication, transcription, translation, virus assembly which ultimately leads to cell death including apoptosis, necroptosis, and pyroptosis.

Raging battle of proviral versus antiviral factors

From first-line to second-line nucleic acid receptors as mentioned above, then to IRF3/IRF7, STAT1 or STAT2, NF- κ B,

signaling transcription factors cascade to finally type I and type III interferon response, and expression of interferon stimulatory genes, anywhere in these steps host and virus deploy an arm of miRNAs on this raging war. The host makes miRNAs to destroy viruses and viruses make viral own miRNAs, and virulence factors (VSRs) to degrade host miRNAs and deploy host proteins for its multiplication [23]. On the other hand, host miRNAs destroy viral proviral factors which are important for viral replication. The virus is extremely clever, it can also use its genomic RNA as a sponge or sequester of endogenous host miRNA molecules, to help in its replication, these RNA sequences that share binding sites with host miRNA are known as competitive viral and host RNAs (cvhRNA) [25]. Host miRNA can also act as proviral factors and inhibit antiviral host factors, for example, miR-122, that would facilitate viral replication. Here are a few more examples and also see Table 1.

Table1. Examples of Host and Viral miRNAs involved in viral infections.

Type of Virus	MiRNA (Host-made)	Viral Replication
Hepatitis C (HCV)	miR-196, miR-296, miR-351, miR-431, miR-448	Inhibits
Vesicular stomatitis (VSV)	miR-155 & miR-223	Inhibits
Epstein-Barr (EBV)	miR-155	Inhibits
Dengue virus2 (DENV2)	miR-150	Inhibits
Enterovirus 71 (EV71)	miR-296-5p, miR-23b	Inhibits
Coxsackievirus B3 (CVB3)	miR-342-5p	Inhibits
Herpes simplex type 1 (HSV-1)	miR-138	Inhibits
DENV	miR-548g-3p	Inhibits
Hepatitis C (HCV)	miR-122	Helps
Bovine viral diarrhea virus (BVDV)	miR-17, Let-7	Helps
Eastern equine encephalitis virus (EEEV)	miR-142-3p	Helps/Inhibits

2.Host miRNAs can degrade host proviral factors that help viral replication

Virus	MiRNA	Host Protein
DENV2	miR-223	downregulates the microtubule destabilizing protein stathmin 1 (STMN1)
Herpesviruses and alphaviruses	miR-199a-3p	downregulation of ERK/MAPK, oxidative stress and PI3K/AKT pathway
WNV	miR-532-5p	downregulates SESTD1 (SEC14 and spectrin domains 1) and TAB3 (TGF-beta activated kinase 1/MAP3K7 binding protein 3) mRNAs

3.Viral miRNAs

Virus	Non-coding RNA	Function
γ -herpesvirus saimiri (HVS)	miR-HSURs	Degrades miR-27
Human cytomegalovirus (HCMV)), Epstein-Barr virus (EBV)	miRNA decay element (miRDE) (BARTs)	degrades miR-17 Cancer development
Herpes simplex virus 1	miR-H2-3p	Viral latency
West Nile virus Kunjin (WNVkun)	Kun-miR-1	Viral replication enhancement
HIV-1	HIV1-miR-H1	Cellular apoptosis induction Viral protein R (Vpr) stabilization

From Bruscella et al., 2017 and Girardi et al., 2018.

Many viral dsRNA-binding proteins bind viral dsRNA to hide it from dsRNA sensors of the host, so that the first step of invasion is successful, for example, B2 protein of Nodamura virus, NS1, the nonstructural protein of influenza A virus, the 3A protein of human enterovirus 71 (HEV71), VP35 of Ebola virus, SARS coronavirus N protein, and yellow fever virus capsid protein, these dsRNA binding proteins are both viral suppressors of RNAi and interferon response pathway [19] (Figure 2). Human Influenza type A nonstructural protein (NS1) inhibits phosphorylation of IRF3 and its nuclear translocation to prevent transcription of interferon stimulatory genes [23,26]. The B2 protein of Flock House virus (FHV) binds long and short dsRNAs in a sequence-independent manner to inhibit Dicer cleavage (Figure 1) [27,28]. These examples are evidence that viral proteins hide their genome from host recognition; viral proteins inhibit both IFN response pathway and RNAi [19,23].

When a virus enters the host cell, along with the signal cascade to the interferon response, transcription of antiviral siRNA and miRNAs, and suppressors of host RNAi armamentarium are all in high alert. But viruses can use these antivirals as proviral factors; many viral RNAs contain common binding sites of miRNAs with the host RNA. For example, liver-specific miR-122 helps stabilize, replication, and transcription of hepatitis C virus (HCV) RNA; miR-122 binds at 5'-UTR of HCV RNA genome stimulates viral translation, protects the genome from XRN-1 mediated degradation; miR-122 also competes with cellular poly(rC)-binding protein 2 (PCBP2) binding to the HCV RNA genome and thereby promotes replication and packaging of HCV [23,29-32] (Figure 2, Table 1). The miRNA profiling of HCV by NanoString nCounter miRNA expression assays in Huh7.5.1 cells and primary human hepatocytes found major miRNA candidates out of which three proviral and 9 antiviral miRNAs showed interaction with HCV RNA, the top hits miR-25, miR130-a/b, let-7a were downregulated by the virus [33,34]. Bovine viral diarrhea virus (BVDV) of pestiviruses hijacks miR-17 and let-7 for their genome stability and replication [35], miR-10a star strand (miR-10a-3p) directly targets CVB3 3D-coding sequence to help its replication [36]. Host miRNAs like miR-141 in enterovirus (EV)71-infected cells [37], miR-485-5p in Newcastle disease virus (NDV) and the H5N1 strain of influenza virus [38], miR-144 in influenza-A virus (IAV) [39] were proviral factors or helped in their viral replication by competing with important host proteins for translation or immune response. MiR-144 is a positive regulator of RNA viruses; it attenuates IRF-7 mediated immune response by targeting TRAF-6 (Figure 2). MiR-485 results in the reduction of IFN and IL6 during infection by NDV or IAV [40] (Figure 2) and helps in viral replication. Herpes simplex virus (HSV-1) miR-H11 can be degraded by vaccinia virus protein VP55 results in lower viral DNA synthesis, restriction of viral spreading, and low viral yields, the mechanism is still unclear [42]. MiR-199a-3p against herpesvirus (a, b, g), miR-34, miR-15, & miR-517 against flaviviruses, suggests some strong antiviral miRNA therapeutic candidates [40].

Can RNAi be used as a diagnostic and therapeutic? What do we need in our quiver to be prepared for an outbreak in the future?

The above examples are evidence of fierce competition between virus and host. Can we learn and use some of these tricks against viral infections? Can we use host or viral miRNA or siRNA or antisense RNAs as an attenuated vaccine? Can we use RNAi therapeutics as intervention drugs against viral infections? Can RNAi be used as a gene therapy against viral infections?

The global antisense & RNAi therapeutics market size is expected to reach USD 1.81 billion by 2025 [47], right now it is about 1.2 billion [47]. RNAi with miRNAs and siRNAs have been successfully used in both diagnostics as signature biomarkers for a particular disease, foreign infection, and the progression of the disease and as a prognostic marker [47-53]. The clinical studies database clinicaltrials.gov includes phase 4 trials with selective miRNAs as biomarkers. These trials have assessed or are actively recruiting patients to examine the profiles of these ncRNA transcripts in a range of health conditions such as diabetes, coronary heart disease, breast cancer, lupus, epilepsy, depressive disorder, stroke, Addison's disease, influenza, liver disease, and even toxic exposure to agents such as acetaminophen [48]. There is a great need and competition in RNAi as clinical intervention drugs. In 2018, the FDA approved the siRNA drug, Patisiran, for a rare polyneuropathy caused by hereditary transthyretin-mediated (hATTR) amyloidosis. It works by binding and degrading the messenger RNA transcript for transthyretin [54,55]. Many miRNA drugs like MRG110 against miR-92 antiangiogenic miRNA that prevent wound repair in diabetics [56]; MRG-201 for miR-29 to treat keloid and scar tissue formations; MRG-106 for miR-155 in T-cell lymphoma patients are under phase 1 or phase 2 trials [48]. Regulus has announced new miRNA drug RGLS5579 in 2019, this miRNA targets miR-10b for glioblastoma multiforme treatment [48]. First siRNA treatment in human started with ALN-RSV01, a 19 bp RNA duplex with two [2'-deoxy] thymidine overhangs on both 3' ends to prevent its nuclease degradation, a single site siRNA targeted to the nucleocapsid gene of RSV. The ALN-RSV01 relieves bronchiolitis obliterans when administered by intranasal spray. This drug is made by Alnylam Pharmaceuticals and has reduced a 38% decrease in the number of infected people [62,63]. At least eight anti-HBV siRNAs are in clinical trials [62]. The first synthetic miR-34 was developed in 2013 for the treatment of hepatocellular carcinoma [63]. There has been a success in generating synthetic miRNAs, packing in liposomes, and transfected into the mononuclear cells of peripheral blood. These protocols enhance TNF- α that favors the innate immune response. The PR8-amiR-93NP virus was generated by inserting an expression cassette for miR-93 between viral genes encoding non-structural proteins in an attenuated Influenza virus (IV), and this miRNA specifically targets the nucleoproteins of the IV. This vaccine, administered intranasally, conferred

immunity against several heterologous viral strains [63,70]. New attenuated vaccines are in trials containing attenuated viruses that are loaded with an expression cassette encoding a synthetic designed miRNA that targets the structural protein of the virus.

How about RNAi against COVID-19, Status Quo

Coronaviruses are enveloped, non-segmented viruses that contain positive-sense single-stranded RNA; the viral genome is in the size from 26 to 32 kilobases, the largest known viral RNA genome [57]. Most of the cells containing cell membrane-bound angiotensin-converting enzyme 2 (ACE2) and associated proteases, transmembrane protease serine 2 (TMPRSS2), and Cathepsin L (CTSL), are identified as the mediators of SARS-CoV2 cellular entry [58,59]. Single-cell RNA sequencing datasets (sc-RNA-seq) from healthy donors by the Human Cell Atlas consortium and multiple published and unpublished datasets in multiple human tissues showed that ACE2 expression was low in all datasets but was expressed in cells from multiple tissues, like, airways, cornea, esophagus, ileum, colon, liver, gallbladder, heart, kidney and testis. TMPRSS2 had a broader distribution. Both ACE2 and TMPRSS2 were co-expressed in respiratory tree, cornea, esophagus, ileum, colon, gallbladder and common bile duct cells [58,59], but ACE2 and TMPRSS2 were highly expressed in a nasal goblet and ciliated cells. These cells are the locus and reservoir of viral outbreak and transmission through nasal infectious droplets and perhaps intranasal spray or vaccine can be effective in stymying its spread. These datasets expression studies also suggest that once SARS-CoV-2 has entered the respiratory tract, it can attack any cell because most of the cells contain ACE2 or TMPRSS2 and some cells contain both. Upon looking at the genes co-expressed with ACE2 across all cells within the lung epithelial cell dataset, the interesting finding is that genes that perform innate and antiviral immune functions like IDO1, IRAK3, NOS2, TNFSF10, OAS1, and MX1 are co-expressed with ACE2 [58].

Inflammatory cytokine storm is the hallmark of COVID-19 which leads to plasma leakage, vascular permeability, and disseminated vascular coagulation and uncontrolled inflammatory response that account for life-threatening respiratory symptoms [60]. The plasma concentrations of IL-1 β , IL-1 α , IL-7, IL-8, IL-9, IL-10, basic FGF, G-CSF, GM-CSF, IFN- γ , IP-10, MCP-1, MIP-1 α , MIP-1 β , PDGF, TNF α , and VEGF were higher in both ICU (intensive care unit) patients and non-ICU patients than in healthy adults. Moreover, when comparing ICU and non-ICU patients, plasma concentrations of IL-2, IL-7, IL-10, G-CSF, IP-10, MCP-1, MIP-1 α , and TNF α were higher in ICU patients than non-ICU patients, evidence that the cytokine storm is correlated with disease severity [61] (Figure 2). Selected drugs like tocilizumab-sarilumab, baricitinib-fedratinib, and fingolimod are in consideration to manage this storm [60].

Academic laboratories, biotech companies, and the pharmaceutical industries are all involved in the clinical research efforts against COVID-19. There is no cure for COVID-19 ex-

cept for a few therapeutic drugs that are available for other viruses, e.g., chloroquine and hydroxychloroquine, anti-viral remdesivir, Kaletra which is a combination of lopinavir and ritonavir that works against HIV, favipiravir, and arbidol [65]. In one of the tissue culture experiments for testing efficacy of remdesivir, after 23 passages in Calu-3 2B4 cells, SARS CoV viral RNA-dependent RNA polymerase enzyme mutations at F480L and V557L showed resistance to remdesivir even though these mutations did not affect the pathogenicity of remdesivir in mice [66]. TMPRSS2 cleaves and activates SARS-CoV-2 spike protein and helps in the viral entrance. Camostat mesylate and K11777, a cysteine protease inhibitor inhibits the enzymatic activity of TMPRSS2 [65] (Figure 2).

Monoclonal antibodies represent a major class of passive-immunotherapy treatment against viral infections. Convalescent plasma has been successfully used in various infections like SARS, MERs, influenza, and was started in the Spanish flu pandemic [67]. The different convalescent blood products to achieve artificial acquired passive immunity have been categorized into 4 types (i) convalescent whole blood (CWB), convalescent plasma (CP) or convalescent serum (CS); [ii] pooled human immunoglobulin (Ig) for intravenous or intramuscular administration; [iii] high-titer human Ig; and (iv) polyclonal or monoclonal antibodies [67]. Antibodies from a patient recovering from a viral infection can be transferred or can be manufactured in the lab. The specific monoclonal antibody raised against the receptor-binding domain in spike (S) protein can stop the viral entry. A pair of antibodies isolated from the patient's convalescent plasma-B38 and H4 block the binding between virus S-protein RBD and cellular receptor ACE2 [68]. The human 47D11 derived from SARS and SARS-CoV-2 and reformatted to a fully human immunoglobulin is the first monoclonal against spike RBD that cross-neutralizes SARS and SARS-CoV-2 in a mechanism independent of receptor-binding inhibition [69]. Companies that are in process of COVID-19 vaccine are Moderna, Inovio, the University of Oxford in England, the University of Queensland in Australia, J&J, Sanofi, etc.

Are there any RNAi therapies for COVID-19?

There are three major steps of COVID-19 infection- viral entry, viral replication, viral maturation, and release. We can design small non-coding RNA molecules to halt this virus at any of these steps. We already have drugs, vaccines, antibodies, as patent or in clinical trials for all of these steps. How about RNAi? Cells affected by human coronavirus activate signal cascades that increase NF- κ B1 mRNA and miR-9 expression, miR-9 reduces the translation of NF- κ B and can be competed away by OC43 and which allows NF κ B translation [63], so miR-9 is a candidate or RNAi since it is induced by the coronavirus to inhibit NF κ B pathway. SARS-HCoV mainly infects bronchioalveolar stem cells (BASCs) where they induce overexpression of miR-574-5p and miR-214. Some proteins of the SARS-HCoV viral nucleocapsid downregulate miR-224 and miR-98 expression

in BASCs and that control several stages of their differentiation as well as pro-inflammatory cytokine production [63,64]. So we know miR-9, miR-574-5p, miR-214, miR-224, and miR-98 are important targets of coronaviruses and can be used as RNAi therapeutics.

A total of 188 patents are directly associated with anti-SARS and anti-MERS vaccines with a demonstrated immune response [71] (Table 2). Based upon *clinicaltrials.gov* out of 127 potential COVID-19 vaccines ten are in phase III trial. SARS-CoV-2 mRNA-1273 vaccine which is in phase trial III has shown a safe and strong response in older adults. This vaccine is co-developed by NIAID and Moderna (87). This vaccine contains mRNA-1273 that codes for two prolines substituted at the top of the central helix full length SARS-CoV-2 spike glycoprotein trimer, S-2P. The mRNA is encapsulated in lipid nanoparticles. Other few examples of COVID-19 vaccines are given in Table 2.

There are anti-siRNA patents against four major structural proteins, spike (S) protein, membrane (M) protein, nucleocapsid (N) protein, and envelope (E) proteins of SARS virus, some of them are more than 70% effective. One of the patent applications, CN1569233 discloses siRNAs that target SARS genes encoding RNA-dependent RNA polymerase, helicase, N protein, and proteolytic enzymes (Table 2). These siRNAs were able to inhibit or kill 50–90% of the SARS virus BJ01 strain, with the proteolytic enzyme-targeting siRNAs being the most effective [71]. RNA aptamers, ribozymes, and antisense oligonucleotides have been developed to fight against SARS by reducing its severity, preventing its multiplication steps, detecting, or diagnosing it accurately.

Since human adeno-associated virus (AAV) is a frequent cause of upper respiratory infections, AAV is designed to deliver genes, miRNAs, and siRNAs in the lungs. AAV is about 4.7 kb and has a positive or negative-sensed single-stranded DNA. Because of AAV's low immune response, it can target both dividing and quiescent cells, makes AAV an attractive vector for gene delivery. Recombinant AAV (rAAV) is constructed by replacing the viral structural (cap) and packaging (rep) genes with the desired transgene along with promoter and polyadenylation sequences [72,73] (Figure 1).

SARS-CoV-2s 3' region of about 10,000 kb encodes for the S, E, M, and N functional proteins, specific miRNAs or siRNAs can be designed to target the sequences for degradation by RISC complex [73]. It can be designed as a vaccine or an intervention drug. There is another great scientific adventure on the horizon, synthesis of asymmetrical siRNAs to target the 5' and 3' UTRs and 10 viral ORFs of the ~29 kb SARS-CoV-2 RNA against viral infection [74,75].

But there is another twist to this story

Our diet has many functions including, sustenance, immunomodulatory [81], neuro-modulatory, cardiovascular regulation, auto-immune disorders, and viral infections [79]. What we eat controls our health, personality, and our chance of survival; our genes, miRNAs, transposon elements, proteins,

etc., work in synchrony to determine our surrender or victory over known human medical conditions and infections. Since the discovery of rice miR-168a by Zhang et al., 2012, plant-microRNAs have been a hot topic of discussion. The rice miR-168a targets mammalian LDLRAP1 which increases LDL levels in plasma [76]. This has set out scientists all over the world to investigate diet plant miRNAs for the cure and prevention of human medical conditions. How do plant-based diet and plant microRNAs contribute to human well-being?

Plants produce miRNAs that regulate virus replication. MiR-2911 from *Lonicera japonica*, and *Lonicera periclymenum* target and inhibit Influenza A virus (H1N1, H5N1, H7N9) replication in mice [77,78]. The miR159 present in plants including, *Arabidopsis thaliana*, *Glycine max*, and *Brassica oleracea*, has a role in breast cancer suppression by targeting the TCF-7 gene [79]. MiR-156a from green vegetables acts as a vasoprotective molecule by targeting the junction adhesive molecule-A (JAM-A), it reduces cytokine-induced monocytes adhesion in endothelial cells [79]. American sweetgum green fruits are used for making an antifu tincture. The key ingredient in Tamiflu® is oseltamivir phosphate, of which shikimic acid is a precursor, and it is present in green seeds of American sweetgum [80]. We need to do more experiments on deciduous trees of the eastern seaboard to investigate their medicinal importance.

Future Prospective

We are still far from fully understanding the molecular mechanisms of networking pathways run by non-coding RNAs in different cells, cell types, plants, and animals, and how they benefit or destroy foreign invaders. There are already many anti-miRNA or anti-siRNA patents and clinical trials against a range of health conditions such as diabetes, coronary heart disease, breast cancer, lupus, epilepsy, depressive disorder, stroke, Addison's disease, influenza, liver disease, and even toxic exposure to agents such as acetaminophen [48]. We already have patents and clinical trials against Influenza, HBV, HCV, RSV, HIV, and SARS-CoV and SARS-CoV-2. For example, ALN-RSV01, against RSV, administered by intranasal spray, is made by Alnylam pharmaceuticals, has reduced 38% infected patients [62,63]. Alnylam also has 350 siRNAs in a trial against SARS-CoV-2; Sirnaomica has siRNA formulation via nebulizer Table 2 [85]. The PR8-amiR-93NP virus particle is designed against the Influenza virus and its heterologous strains [73,70]. RG101 is N-acetylgalactosamine conjugated antisense oligonucleotide which improves its delivery in hepatocytes for sequestration of miR-122 to reduce the HBV burden and relief from its symptoms in patients [40]. These examples are evidence of tremendous hard work by the scientific workforce in the direction of improving healthcare using RNAi and we hope for the SARS-CoV-2 also.

Small non-coding RNAs like miRNA, siRNA, and anti-sense RNAs are very interesting molecules because they induce low immunogenicity, and show cross-species conservation and can be tested in different animal models. But there are

Table 2. Drugs, RNA therapies, and other treatments for SARS-CoV-2.

Group	Mechanism of Action	Examples
Viral entry inhibitors	Increase in endosomal pH that inhibits viral-cell fusion, proper genome release and efficient infection; Interferes with glycosylation of ACE2	Hydroxychloroquine, Chloroquine
Viral entry inhibitors	an inhibitor that may disrupt the binding of viral envelope protein to host cells and prevent viral entry to the target cell, inhibits intracellular vesicular trafficking	Arbidol
Inhibitors of viral RNA polymerase /RNA synthesis	Prodrug of Adenosine nucleotide analogue, inhibits viral RdRp	Remdesivir
Inhibitors of viral protein synthesis	Guanosine nucleotide analogue, prodrug, inhibits viral RdRp	Favipiravir
Inhibitors of viral protein synthesis	Protease inhibitor that may inhibit 3CLpro or PLpro	Lopinavir/ritonavir aka Kaletra
Viral Protease	protease inhibitors that may inhibit the viral proteases: 3CLpro or PLpro	
JAK signaling inhibitors	targeting IL-6, inhibiting IL6/JAK/STAT signaling, attenuate the host inflammatory response associated with massive pro-inflammatory cytokine and chemokine storm	Baricitinib-Fedratinib, Fingolimod
RNA Therapies		
RNA Therapies	Anti-miRNA, anti-siRNA, ribozymes, RNA aptamer, anti-sense oligos, recombinant AAV	
Patents	US20040192626, siRNA, microRNA, shRNA WO2004092383 WO2018227440 MicroRNA inhibitor microRNA200c-3p CN101173275 US20050004063 CN1569233	Various regions of SARS & MERS Various regions of SARS & MERS Applications of mir-200c-3p microRNA in treating lung injuries siRNAs targeting coronavirus proteins M, N, or E of SARS siRNAs targeting replicase and RdRp region of SARS siRNAs targeting RdRp, helicase, nucleoprotein N, and proteolytic enzymes of SARS
Companies		
Alnylam Pharmaceuticals	350 siRNAs in trial	Against SARS-CoV-2
Sirnaomica	siRNA formulation by a nebulizer	Against SARS-CoV-2
Other treatments		
mRNA-1273 Vaccine	prolines substituted SARS-CoV-2 spike glycoprotein trimer, S-2P (87)	Against SARS-CoV-2
BNT162B2 by Pfizer and Biontech	A nucleoside-modified mRNA expresses spike glycoprotein (88)	Against SARS-CoV-2
NVXCoV2373	spike (S) protein&saponin-based adjuvant (89)	Against SARS-CoV-2
JNJ-78436725 or Ad.26.COV2.S	adenovirus serotype 26 (Ad26) expressing the spike (S) (90)	Against SARS-CoV-2
MRT5500 by Sanofi	Spike glycoprotein S (91)	Against SARS-CoV-2
Convalescent Plasma	Convalescent blood products, pooled human immunoglobulins (IgG), mono or polyclonal antibodies	B38 and H4 (68) Human 47D11(69)

RdRp, RNA-dependent RNA polymerase; coronavirus main protease, (3CLpro), and papain-like protease (PLpro), Table2 adapted from refs 60, 65,68, 69, 71, 83, 84, 85, 87, 88, 89, 90, & 91.

challenges with design, delivery of live attenuated vaccines, miRNA drugs, and off-target effects. Their safety and toxicity remain a controversial issue.

We are writing so much about different miRNA as a pro- or anti-viral, but all of these mechanisms will vary from lab to lab, the results could be contradictory, but perhaps we can find

some commonalities in data that can lead us to therapeutical interventions and vaccines against COVID-19 and other viral infections. There is a big scope of plant miRNA therapeutics as a dietary supplement and future medicine.

List of abbreviations

RNAi: RNA interference
HVS: Herpesvirus saimiri
HCMV: Human cytomegalovirus
MCMV: Murine cytomegalovirus
TBSV: Tomato bushy stunt virus
EBV: Epstein-barr virus
CMV: Cucumber mosaic virus
CVB3: Coxsackievirus B3
FHV: Flock House virus
VSV-G: Vesicular stomatitis virus G protein
IV: Influenza virus
HCoV: Human coronavirus
RSV: Respiratory syncytial virus
HBV: Hepatitis B virus
DGCR8: DiGeorge Syndrome Critical Region 8
RIG-1: Retinoic acid inducible gene I
5'ppp: 5'-triphosphate
IFN: Interferon
IFIT1: IFN-induced protein with tetratricopeptide repeats 1
IFNAR: IFN receptor
STAT: Signal transducer and activation of transcription
ISG: Interferon stimulatory genes
ADAR1: Adenosine deaminase acting on RNA 1
dsRNA: Double-stranded RNA
ssRNA: Single-stranded RNA
RBD: Receptor binding domain
IFIT1: IFN-induced protein with tetratricopeptide repeats 1
MDA5: Melanoma differentiation associated gene 5
NP: Nucleocapsid protein
OAS1: 2'-5'-oligoadenylate synthetase 1
PKR: Protein kinase R
TLR: Toll-like receptor
TRAF6: Tumor necrosis factor receptor-associated factor 6
TRAM: TRIF-related adapter molecule
TRIF: TIR domain-containing adapter protein inducing IFN- β
MyD88: Myeloid differentiation primary response gene 88
MDA5: Melanoma differentiation-associated gene 5
IRAK4: Interleukin 1 receptor-associated kinase 4
IRF: Interferon regulatory factor
NLRP3: NOD-, LRR- and pyrin domain-containing 3

Competing interests

The authors declare that they have no competing interests.

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Collection and/or assembly of data	✓	--	--
Data analysis and interpretation	✓	✓	✓
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