A review paper on Developing Novel Antiviral Strategies against Pathogenic RNA Viruses

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Department of Mathematics and Natural Sciences

Brac University

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Declaration

It is hereby declared that

1. The thesis submitted is my own original work while completing degree at Brac University.
2. The thesis does not contain material previously published or written by a third party, except where this is appropriately cited through full and accurate referencing.
3. The thesis does not contain material which has been accepted, or submitted, for any other degree or diploma at a university or other institution.
4. I have acknowledged all main sources of help.

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Approval

The project titled “A review paper on Developing Novel Antiviral Strategies against Pathogenic RNA Viruses” submitted by Numia Nawar (16136010)

Of Spring, 2021 has been accepted as satisfactory in partial fulfillment of the requirement for the degree of Bachelor of Science in Biotechnology on April 20, 2021.

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Abstract

Human disease causing RNA viruses are a major concern for people worldwide as many of the diseases caused by pathogenic RNA viruses pose great threat to health and exhibit significant mortality. Some of the dangerous RNA viruses that infect human include Orthomyxoviruses, polio virus, HIV, HCV, Ebola virus and coronavirus. Many of the diseases caused by these viruses can be treated by means of vaccination or antiviral drugs. However, the need for annual booster doses for vaccines as well as side effects and limitations of the approved antivirals necessitate the development of more effective strategies. In addition to the aforementioned limitations, the emergence of drug resistance has accelerated the urgent need for novel antiviral strategies. This review gives a brief outline of the possible strategies that may be implemented in order to develop novel antivirals.

Keywords: pathogenic RNA viruses; side effects; disease and mortality; T20; vaccines; drug resistance; novel antiviral strategies

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List of Acronyms

HA	hemagglutinin
NA	neuraminidase
RdRP	RNA dependent RNA polymerase
NP	nuceleoproteins
HSPG	Heparan-sulfate proteoglycans
VAL	viral attachment ligands
RSV	Respiratory Syncytial Virus
SALPs	synthetic anti-lipopolysaccharide peptides
RVFV	Rift Valley Fever Virus
ACE2	angiotensin-converting enzyme 2
MAbs	monoclonal antibodies
RBD	receptor-binding domain
RBM	receptor binding motif
NRTI	nucleotide reverse transcriptase inhibitors
NNRTI	non-nucleotide reverse transcriptase inhibitors
CRISPR	Clustered regularly interspaced short palindromic repeats

Chapter 1: Introduction

As the name suggests, RNA viruses possess RNA as their genetic material to replicate inside host cell which is in fact a contributing factor to their high level of virulence.

The error prone proof reading ability of RNA dependent RNA polymerase and reverse transcriptase enzyme renders RNA viruses with significantly high mutation rates that play a huge role in the rapid evolution of these viruses.(1) The high evolutionary rate coupled with other selective pressures have made it possible for pathogenic viruses to cause many global pandemics during the past centuries which include Spanish flu (year: 1918; 20-50 million deaths), (2) Swine flu (year: 2009; 12,469 deaths), (3) and the ongoing COVID 19 (year: 2019; 1,768,844 deaths). (4) RNA viruses can be of five types depending on their genetic material – plus stranded RNA viruses, negative stranded RNA viruses, double stranded RNA viruses and retroviruses. All of these viruses synthesize mRNA in the host cell using host cell machineries which encodes all the necessary information for viral protein synthesis (both structural and functional). RNA viruses make multiple mRNAs that overcome the monocistronic mRNA in eukaryotes and promotes multiple protein synthesis. (5) As with other types of viruses, the RNA virus has six steps in their lifecycle – Attachment, penetration, uncoating, biosynthesis, assembly and lastly, release. (6) In order to develop new strategies, it is essential to study and analyze these steps so that it would be possible to manipulate their system for our use. The following diagram gives an overview of the RNA virus lifecycle.

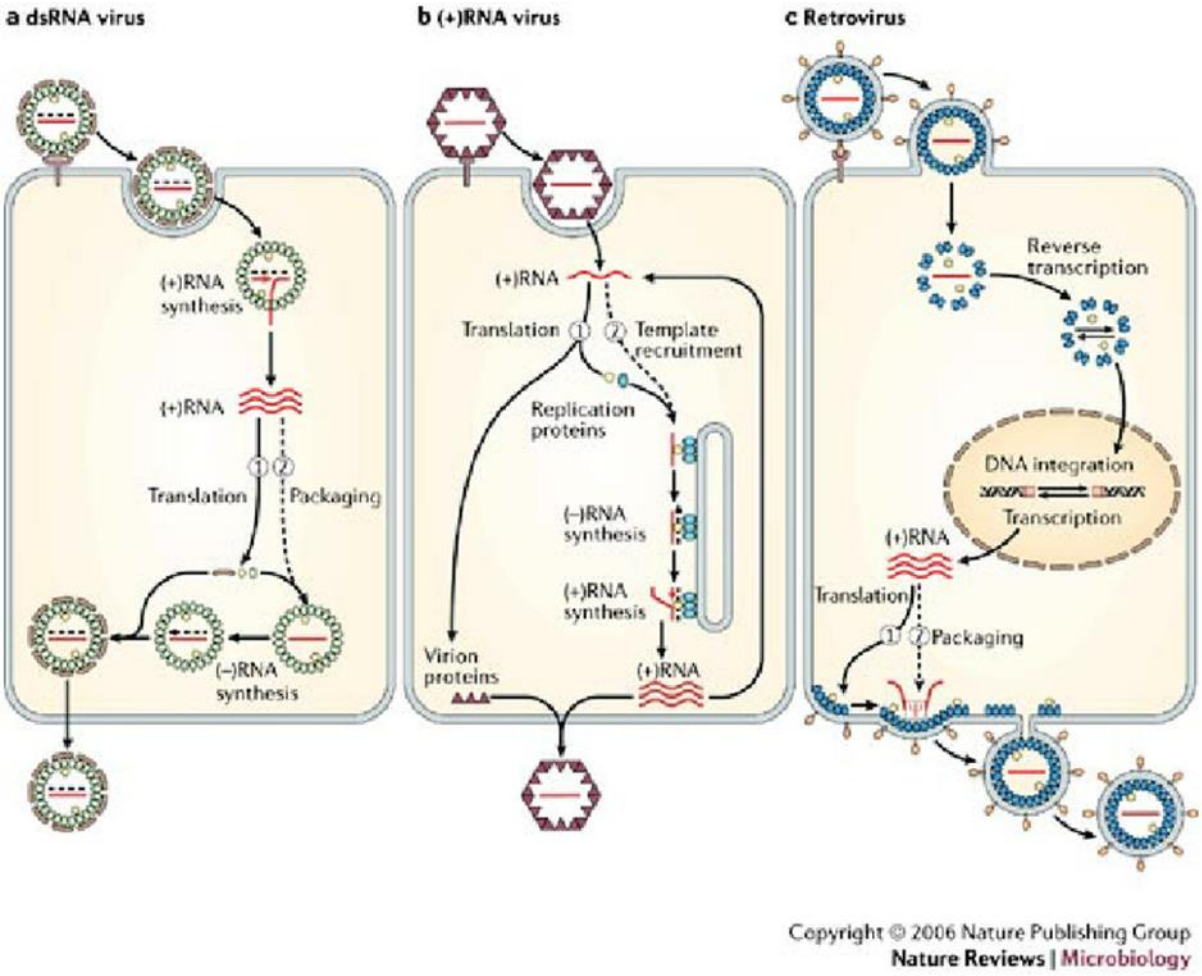

Figure 1: The life cycle of RNA virus (source: https://www.researchgate.net/figure/Replication-strategies-of-different-kinds-of-RNA-viruses-are-shown-a-replication-of_fig5_288827106)

RNA virus utilizes the host cell's receptor to attach and uses ion gradients to enter into the targeted cell. Components on the surface of viral capsid or surface glycoptotein/glyclipid in case of enveloped viruses assist in viral entry. For example, two glycoproteins - hemagglutinin (HA) and neuraminidase (NA), along with an essential ion channel protein, M2, exists on the virion surface of Influenza A virus. (7) The conserved domain or region of the surface proteins of RNA viruses or monoclonal antibodies that bind to viral binding receptors can be good antiviral targets. Upon entering the host cell, the virus particle uncoats its RNA for replication and transcription. Two enzymes – RNA dependent RNA polymerase

(RdRP) and reverse transcriptase in case of retroviruses catalyze the replication and transcription of viral genome using the host cell machineries. The multiple domains of RdRP can also be a promising target which can halt the further replication of the virus. Viral nuceleoproteins (NP) play the major role in viral assembly as well as replication. Analogues involving competitive binding with NP can disrupt the binding and translocation of RNA, which can work as an antiviral strategy. More possible virus based as well as host based novel approaches for developing novel antivirals are discussed in this review.

Chapter 2: Antiviral strategies targeting viral entry

Viral entry involves the attachment of viral surface proteins into specific host receptors. After the adsorption of viral particle, ion channels facilitate the endocytosis of viral particle by the means of proton gradient. To this date, quite a number of antiviral drugs have been used to block viral entry of which a popular example is the M2 blockers of IAV - amantadine and its methyl derivative rimantadine. (8)

Upon binding of Adamantane derivatives to the N-terminal channel lumen of the M2 pore, a positive electrostatic potential is produced in the channel lumen by the amino group. As a result, the generated electrostatic repulsion of protons prevents virus uncoating. However, drug-resistant virus variants are emerging with selective pressure induced mutation rendering amantadine and rimantadine ineffective in clinical treatment. Some of the alternative approaches to target viral entry are stated below.

2.1 Compounds to target mutated M2 ion channel

The most prevalent mutation in IVA M2 ion channel is S31N mutation. (9) Even the most prescribed drug targeting M2 ion channel named amantadine and rimantadine exhibits decreased inhibition of this target, indicating a greater chance of resistance. However, a compound, M2WJ332, with its adamantane ring in approximately the same location as in amantadine/rimantadine, but with a more stabilized ammonium group which mimics the intermediates in the conduction of protons to His37. This provides access of newly designed drugs to the highly conserved region adjacent to H37 and W41. Besides this, some benzyl-substituted amantadine derivatives were also found to inhibit S31N and wild-type M2 channel, among which 4-(adamantan-1-ylaminomethyl)-benzene-1,3-diol showed the most potent dual inhibitory effect. (10) It can be said that testing of different compounds having m2 channel inhibiting property should be tested via NMR and MD simulation and their structure based drug designing to target conserved regions of this channel can be a good target.

2.2 Inhibition of cell adhesion

Another approach for preventing viral multiplication is inhibiting the adhesion of viral particle to cell surface. This can be achieved by designing nanoparticles targeting cell surface proteins or synthesizing peptide molecules. A few examples are stated below:

2.2.1 Nanoparticles targeting cell surface proteins

Heparan-sulfate proteoglycans (HSPG) are present in almost every eukaryotic cell and can be a highly conserved target of viral attachment ligands (VAL). (11) Virucidal assays, electron microscopy images, and molecular dynamics simulations show that nanoparticles with long and flexible linkers mimicking HSPG, allows strong multivalent attachment of the analogues with VAL repeating units that generate enough force to cause irreversible viral deformation. Respiratory Syncytial Virus (RSV), Dengue and Lenti virus have exhibited susceptibility

towards the mentioned nanoparticles. Unlike Virustatic substances, which cause reversible stabilization of viral particle in a non-infectious state and may lose effect upon dilution, VAL targeting nanoparticles distort viral particles irreversibly. Moreover, they have been found to show no cytotoxicity like antiviral drugs which damages the host cell in the process. While almost all known HSPG-mimicking nanoparticles, polymers and dendrimers display short linkers to expose sulfonate groups to the viral ligands (i.e.: gold nanoparticles coated with 3-mercaptoethylsulfonate and heparin), ligands containing undecanesulfonic acid (MUS) coated nanoparticles have long linkers that irreversibly inhibit HSPG dependent viruses.

2.2.2 Inhibitory peptide molecule

It has been found that synthetic anti-lipopolysaccharide (LPS) peptides (SALPs), bind to heparan sulfate moieties on the plasma membrane. This new class of peptides depends on the LPS-binding domain of the Limulus anti-LPS factor, neutralizing LPS and blocking its immunopathological consequences in vitro and in vivo. (12) Positively charged SALPs bind to negatively charged heparan sulfate (HS). Heparan sulfate works as a docking molecule for many enveloped viruses by binding viral gp120. Thus, peptide molecules binding to HS moieties can be a possible antiviral option. Experiments and studies have shown two SALPs, Pep19-2.5 (GCKKYRRFRWKFKGKFWFWG) and Pep19-4 (GKKYRRFRWKFKFGKWFWFG), inhibit the attachment and infection in case of HSV, HCV and HIV-1.

	Pep19-2.5		Pep19-4	
Virus	**IC50 (µg/mL)**	**IC50 (µM)**	**IC50 (µg/mL)**	**IC50 (µM)**
HIV-1_{BaL}	**8.0**	**2.9**	**22**	**8.0**
HIV-1_{NL4-3}	**16**	**5.9**	**10**	**3.6**
HSV-1	**0.42**	**0.15**	**1.8**	**0.7**
HCVpp	**40**	**15**	**37**	**14**

Table 1: Half Maximal Inhibitory Concentrations (IC50) for Synthetic Anti-Lipopolysaccharide Peptide–Mediated Inhibition of Studied viruses. Abbreviations: HCV, hepatitis C virus; HIV-1, human immunodeficiency virus type 1; HSV-1, herpes simplex virus 1

Synthesized peptides analogous to specific regions of fusion protein also display an effective inhibition of viral entry (13). Studies have shown that peptides synthesized by targeting Gc domain in classII fusion protein of Rift Valley Fever Virus (RVFV) effectively inhibited certain strains of RVFV along with few other viruses. Two synthetic peptides, RVFV-6 and RVFV-10 significantly inhibited pathogenic RVFV strain, RVFV-ZH501 while RVFV-6 also inhibited additional diverse viruses such as hantavirus (ANDV) and Ebola virus (EBOV).

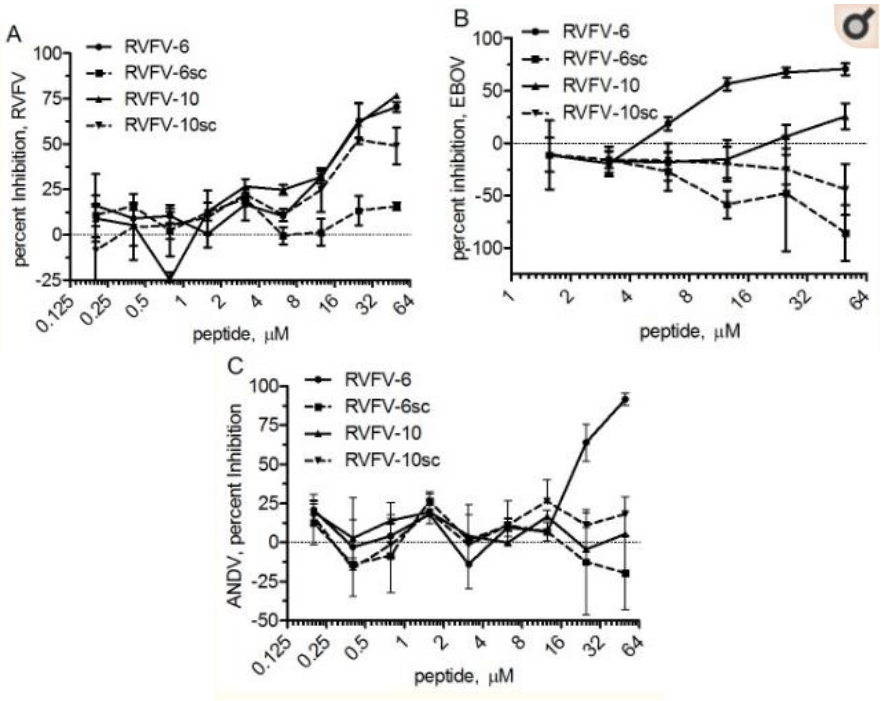

Figure 2: A. Inhibition of RVFV by synthesized peptides RVFV-6 and RVFV-10; B. Inhibition of EBOV by RVFV-6 and RVFV-10; C. Inhibition of ANDV by RVFV-6 and RVFV-10

Another strategy to inhibit viral binding to host receptor can be the synthesis of decoy protein receptor. This strategy was studied in case of the recent COVID 19 outbreak by assessing the effect of engineered human ACE2 to optimize binding to the spike protein of SARS coronavirus 2 (14). The spike (S) protein of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) binds to angiotensin-converting enzyme 2 (ACE2) on host cells for entry. A synthesized soluble ACE2 decoy receptor with greater binding affinity to spike protein can be good therapeutic option. Through deep mutagenesis approach, determination of the enrichment or depletion of 2340 coding mutations in the library was conducted by the comparison of the frequencies of transcripts in the sorted populations to sequence frequencies in the naïve plasmid library. A mutant variant, sACE2.v2, was purified as it showed avid binding with viral spike protein along with lesser immunogenicity due to similarity with the native ACE2. The 8His-tagged analyte of the mentioned variant,

sACE2.v2-8h outcompeted the wild-type sACE2-8h in terms of binding with sACE2-IgG1, also displaying a slower off-rate compared to the wild type. In order to address the lower yield of sACE2.v2, another mutant with reverted mutations to the wild type, sACE2.v2.4 was designed. It showed higher yield along with tight nanomolar binding to the RBD of SARS-CoV-2 spike protein.

2.3 Synthesis of Neutralizing Antibodies

The synthesis of neutralizing MAbs can be another viable approach to halt viral entry. A study showed that ZMapp, a cocktail of three monoclonal antibodies (MAbs; c2G4, c4G7, and c13C6) against the ebolavirus (EBOV) glycoprotein (GP), exhibits promising therapeutic potential to combat outbreaks of EBOV, as occurred in West Africa in 2014(15). Cryo-Electron Tomography results showed that all three antibodies bind avidly to EBOV-Makona GP, since no unbound GP classes were resolved for any of these complexes during 3D averaging.

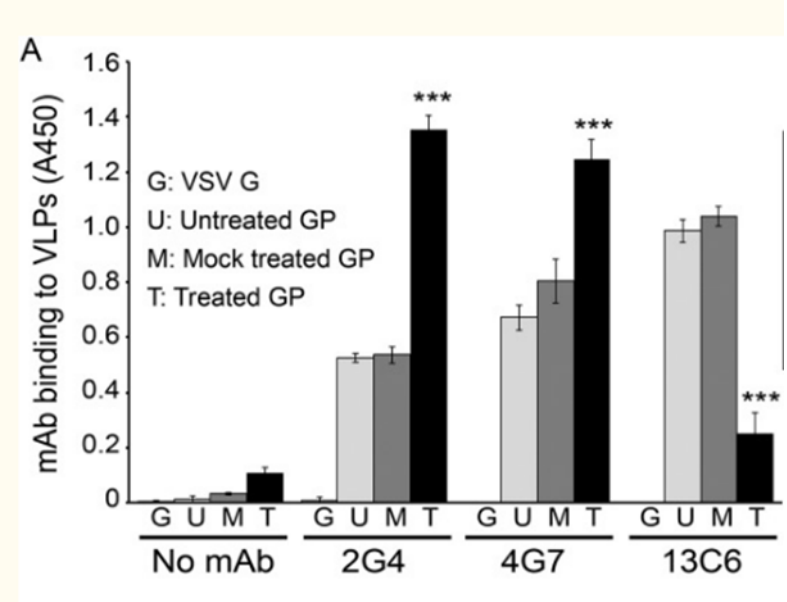

Figure 3: VLPs bearing EBOV-Makona GP were untreated, mock treated, or (thermolysin) treated and then bound to high-bind ELISA

Moreover, two other MAbs, mAb100 and mAb114, isolated from a 1995 Kikwit Ebola survivor, showed neutralization of Ebola virus by preventing the exposure of the RBD to protease cleavage and binding of cathepsin-cleaved GP to its receptor NPC1 respectively(16). Unlike antibodies such as KZ52 and 13C6, mAb100 and mAb114 has much slower off-rate and 10 to 40 fold tighter binding affinity to GP at neutral and low pH. Recently a report showed that severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), which caused the latest global pandemic can be blocked by a humanized monoclonal antibody from entering the host cell (17). The spike protein of SARS-CoV-2 and SARS-CoV uses human angiotensin-converting enzyme 2 (hACE2) to enter host cells. The homotrimeric S-mediated virus-receptor engages through the receptor-binding domain (RBD) after the fusion virus-host membrane and enters the host cell. A number of neutralizing antibodies characterized by recent studies include CR3022, 47D11, S309. However, a more potent therapeutic antibody, H014, can cross-neutralize can cross-neutralize SARS-CoV-2 and SARS-CoV infections by blocking attachment of the virus to the host cell with greater effectiveness. While the targeted

site of the conserved epitopes of the previously mentioned nAbs span only a portion of RBM (Receptor Binding Motif), H014 recognizes a conformational epitope on one full side of the open RBD (Receptor Binding Domain), which involves protein-protein interaction, distinct from RBM. Moreover, H014 exhibited wider cross neutralizing ability by possessing indistinguishable affinity towards both wild type and mutant RBD, while outcompeting or blocking ACE2 in terms of binding to RBDs of SARS-CoV-2 and SARS-CoV respectively.

Chapter 3: Antiviral strategies targeting viral biosynthesis

Viral biosynthesis includes the replication of viral genome and the transcription of viral mRNA which encodes the structural and non-structural proteins for the virus to propagate and function. Many nucleotide and non-nucleotide reverse transcriptase inhibitors have been administered for the treatment of HIV that include Abacavir, Emtricitabin, Stavudine, Tenofovir, Zidovudine etc. among nucleotide reverse transcriptase inhibitors (NRTI) and Efavirenz, Nevirapine, Etravirine among non-nucleotide reverse transcriptase inhibitors (NNRTI) [(18)]. However, emerging point mutations in response to selective pressure have rendered HIV resistance against the mentioned antiviral drugs. While reverse transcriptase inhibitors have been used as inhibitors, not much antiviral drugs have been used as polymerase inhibitors against other RNA viruses such as influenza virus or Ebola virus. Favipiravir (T-705 ribofuranosyl-5′-triphosphate) however showed antiviral activity against influenza virus by halting RNA polymerase activity as nucleoside inhibitor [(19)]. Some of the novel antiviral strategies that can be implemented by targeting viral biosynthesis are discussed in this section.

3.1 Use of small molecules as biosynthesis inhibitors:

Small molecules or protein analogues that compete with substrates or block the structurally equivalent site of RdRp in RNA viruses can be a possible strategy in developing antiviral drugs. Despite the sequence diversity of RdRp across virus families, these viral RdRps share a structurally homologous core that can be used as a target. Nucleoside inhibitors work by chain termination which involves the antivirals to phosphorylate into triphosphate form. Recently studied nucleoside analog, 4′-azidocytidine (4′N3-C, R1479) and its 2′-monofluoro- and 2′-difluoro-modified analogs (2′F-4′N3-C and 2′diF-4′N3-C) have exhibited antiviral activity against paramyxoviruses (Nipah virus, Hendra virus, measles virus, and human parainfluenza virus 3) and filoviruses (Ebola virus, Sudan virus, and Ravn virus) [(20)]. Both 2′diF-4′N3-C and 2′F-4′N3-C compared to R1479 showed greater antiviral activity in case of paramyxoviruses, where R1479 and 2′diF-4′N3-C inhibited filoviruses effectively.

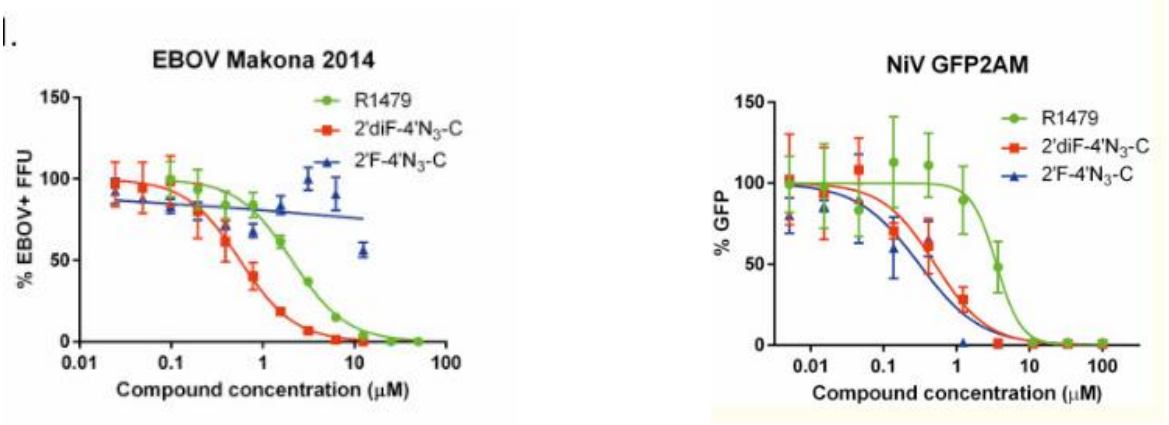

Figure 4: left: Representative dose-response curves of the same compounds against wild-type EBOV (2014 Makona variant) using a focus forming unit (FFU) assay.

Right: Representative dose-response curves of the same compounds against Nipah virus (NiV-GFP2AM) using a Green Fluorescent Protein (GFP) assay.

While nucleoside inhibitors often cause off-target side effects, making non-nucleoside inhibitors a better option as they bind to the allosteric pockets of RdRp and inhibit conformational changes that are required for viral RNA transcription. Such a non- nucleoside small molecule called Entrectinib (RAI-13) has been successfully identified as a potent inhibitor of both Human norovirus (hNV) and dengue virus (DENV) [(21)]. RAI-13 has been shown to bind tightly with the RdRp binding sites of both hNV and DENV. More promisingly, 2 μM concentration of RAI-13 inhibited HCV infection by 95%. Another small protein targeting the Nucleoprotein (NP) of Ebola virus has been identified as novel inhibitor of EBOV genome replication [(22)]. The two binding sites in the hydrophobic pocket on NP (which either binds intramolecularly with a flexible arm of NP or with an NP binding peptide of VP35) control the binding of NP and release of RNA and oligomerisation – essential to viral replication. NP has been shown to be a viable target for small molecule inhibitors in case of other negative stranded RNA viruses as well. From the results of virtual Screening, an active compound named MCCB4 (hydrophobic molecule containing an ene-thiazolidinedione group) have been predicted to bind to hotspots 1 and 2 within the hydrophobic pocket on NP structure.

3.2 CRISPR-Cas based antiviral strategies

Clustered regularly interspaced short palindromic repeats (CRISPR) and associated proteins (Cas) render archaea and bacteria adaptive immunity against exogenous DNA elements. This CRISPR-Cas system can be turned into an effective gene editing tool as a protective strategy against human pathogenic viruses. According to a recent study, CRISPR-Cas system has been extensively used against HIV infection [(23)].

3.2.1. CRISPR-Cas9 cleavage of the HIV genome

Two approaches have been proposed in this regard, the first one involves the Cas9 nuclease and gRNAs with sequence complementarity to HIV to be introduced in infected cells to attack the established proviral DNA. The second approach involves Cas9 and the gRNAs to be stably introduced in uninfected cells and should await and immediately attack the reverse transcribed viral DNA that is produced upon a future infection. The promising target for CRISPR-Cas in HIV is the LTR (Long Terminal Repeat) domain. Present in both 5' and 3' of proviral genome, gRNAs can be targeted for these two sites. gRNA along with Cas protein can simultaneously cleave at highly conserved sequences in both LTRs and the chromosomal termini will subsequently ligate to result in the complete deletion of the bulk of the HIV-1 genome through high frequency hypermutation. Two gRNAs rather than one displayed more effective result in this case and simultaneous cleavage is recommended as fewer cleaving will result in dsDNA break that introduces mutation prone NHEJ repair pathway.

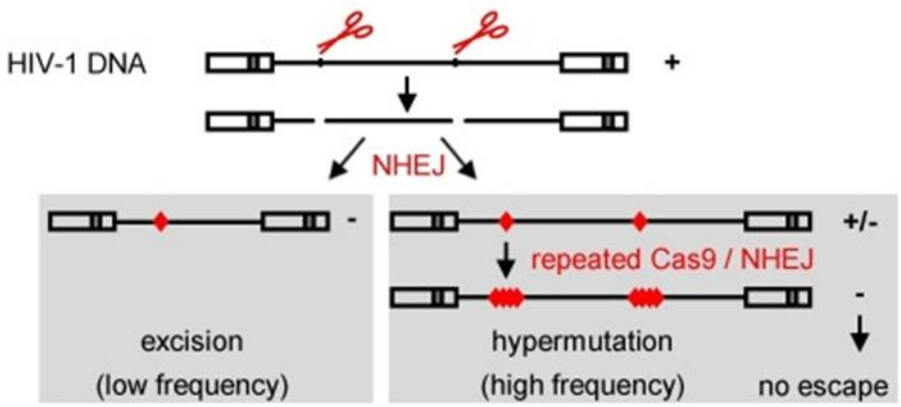

Figure 5: Left panel: cleavage at both sites by gRNAs targeting protein-encoding HIV sequences results in DNA excision. Right panel: repeated attack by CRISPR-Cas9 results in hypermutation of the viral DNA that causes gradual disappearance of the wild type or mutated variants at the expense of major indels and multiple-nucleotide substitutions at both targets.

Another study showed that Cas9 from Francisella novicida (FnCas9) tagged with RNA-targeting guide RNA (rgRNA) can bind to highly conserved HCV 5′ and a portion of 3' untranslated region (UTR) and cleave HCV RNA, resulting in translational inhibition [24]. FnCas9 cleavage is proven to be PAM independent, which makes it more flexible in targeting.

3.2.2 Combinatorial CRISPR-Cas9 attack along with RNAi antiviral

CRISPR-Cas9 in combination with the sequence-specific attack mediated by RNAi antivirals resulted in the reduced level of viral replication and increased threshold for viral escape. RNAi-inducing short hairpin RNA (shRNA) along with CRISPR-Cas9 proved to inhibit HIV replication more durably than monotherapies [23].

3.2.3 Shock and kill therapy

Histone deacetylases (HDAC) and other chromatin modifying enzymes confer latency to HIV by associating with LTR promoter. This results in a more condensed chromatin structure that causes the transcriptional silencing of viral genome. Reactivation of viral gene expression by HDAC inhibitors that open the chromatin structure, protein kinase C activators that stimulate transcription via the NFκB pathway and proinflammatory cytokines that activate T cells may introduce a "shock and kill" approach to clear out the latent reservoir. Zinc finger and transcription activator-like effector (TALE) transcription factors can be designed to more specifically target HIV LTR promoter.

3.2.4 CRISPR-based transcription activators

dCas9-AD fusion protein can be employed by gRNA to a specific promoter or enhancer to activate transcription of latent viral genome. dCas9-AD fusion protein consists of an N-terminal nuclease-deficient dCas9 protein and a C-terminal activation domain (AD) derived from a viral or cellular transcription factor, which has the capability to recruit transcription-

activating and chromatin-remodeling factors. In another approach called the “synergistic activation mediator” (SAM) system, a modified gRNA with binding sites for the MS2 bacteriophage coat protein is used, which not only attract dCas9-AD but also MS2-AD fusion proteins. These multiplex systems can be deployed to activate HIV genome for antiviral targeting.

Chapter 4: Antiviral strategies targeting viral assembly

Assembly of viral proteins after viral biosynthesis can be utilized as an antiviral target by inactivating viral NP or synthesizing small peptide analogues for the binding site of NP or RNA polymerase. Both Nucleoprotein (NP) and RNA polymerase are important factors involved in viral packaging which makes them attractive targets.

4.1 Analogues that bind with Viral protein

Nucleoprotein and other essential viral nucleocapsid proteins play vital role in viral assembly by interacting with each other. For example, EBOV nucleocapsid is composed of the proteins NP, VP35, and VP24 which have been studied with mutational analyses, biochemical and bioinformatics approaches to demonstrate that the interaction between VP24 and NP is required for both nucleocapsid assembly and genome packaging [25]. Such proteins essential for genome packaging or viral assembly can be a promising target for analogues which are specifically designed to interact with the targeted region.

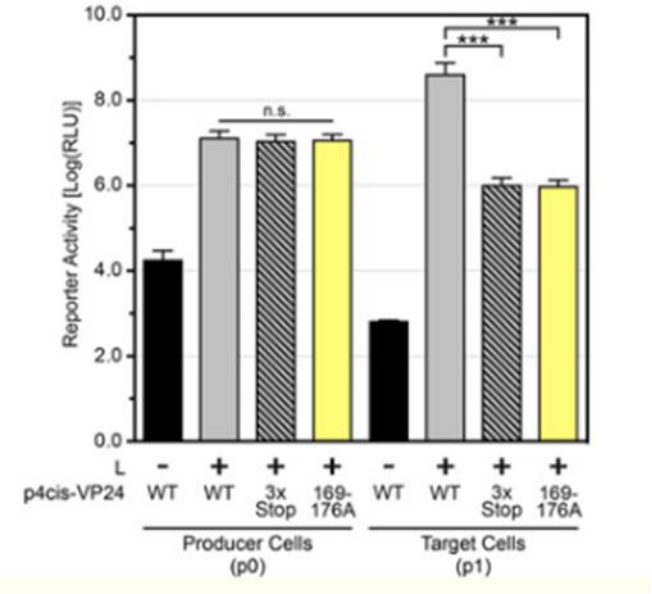

Figure 6: comparison of EBOV replication cycle in presence of wild type and mutant VP24

4.2 Synthesizing peptides that interfere with viral assembly

The assembly of RNA dependent RNA polymerase (RdRp) is essential for the replication and transcription of RNA viruses. Short peptides that interfere with the interaction between the RdRp subunits can significantly disrupt the assembly process of the polymerase [(26)]. For instance, the RdRp of influenza virus is assembled by the interaction between three heterotrimeric subunits PB1, PB2 and PA. A recent study showed that short peptides derived from amino acids 731–757 of PB1 (PB1731–757) can disrupt the interaction between the C-terminal part of PB1 (denoted as PB1c corresponding to PB1676–757) and the N-terminal part of PB2 (denoted as PB2n corresponding to PB21–40). It has also been found previously that a peptide derived from the N-terminal 25 amino acids of PB1 (denoted as PB11–25) can effectively inhibit viral pathogenicity by disrupting PA–PB1 interaction. Studies suggested that the PB1731–757 peptide preferably binds to PB1c and works as a competitor for PB2n in terms of binding with PB1c. Such short peptides can be an approach to inhibit viral replication and transcription, contributing to a novel antiviral development.

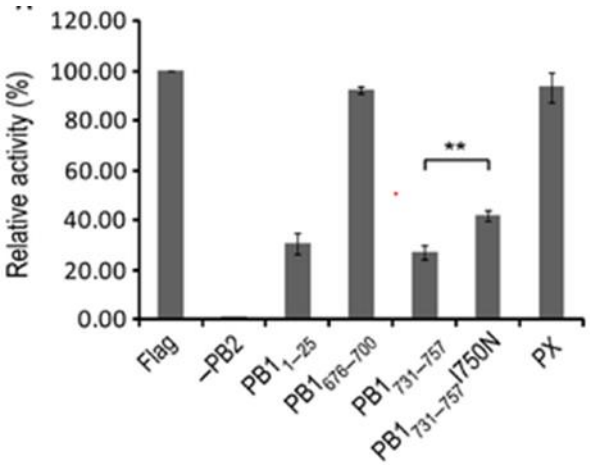

Figure 7: Effects of over-expressed short peptides on influenza A virus polymerase activity. The viral polymerase activity was assayed in a mini-replicon system with a PolI-driven plasmid expressing influenza virus-like RNA coding for firefly luciferase to detect viral polymerase activity.

4.3 Viral nucleoprotein inactivation

Viral nucleoprotein plays an irreplaceable role in the binding of RNA genome and associates with the RNA polymerase in a ribonucleoprotein complex (RNP) that is required for transcription and replication. Targeting the viral nucleoprotein can be a hot target for disrupting viral assembly, which can result in the inhibition of viral propagation. A few of the methods that can inactivate nucleoprotein are stated below-

4.3.1 Inactivating viral nucleoprotein with drugs

Studies have found that an anti-inflammatory drug, naproxen and a new class of drug exemplified by the compound nucleozin can target viral NP [(27) (28)]. These molecules function as inhibitors that stabilize interactions between NP monomers, resulting in non-functional aggregates. The RNA binding groove of NP is highly conserved and surface plasmon resonance (SPR) experiments showed that naproxen competes with RNA to bind tightly with the conserved groove of NP, resulting in the disruption of RNA-NP complex. On top of that,

Naproxen is more potent than COX-2 inhibitors such as nemesulide against influenza A virus and a more readily available safer pharmaceutical drug. Another class of drug, nucleozin and related compounds have been observed to work as "molecular staples" that stabilize NP-NP interaction by introducing inappropriate binding modes. This results in the formation of cytoplasmic aggregates of newly translated NP, consequently, blocking its nuclear import and inhibiting viral genome replication of the building blocks required for encapsidation. Administration of nucleozin in early hours of infection exhibited direct inhibition of the synthesis of viral proteins and all classes of RNA, while application in later steps only showed cytoplasmic aggregation. Presumably due to the alteration in vRNP trafficking, nucleozin also displayed inhibitory effect on viral budding, resulting in the production of smaller, fewer and defected viral particles.

4.3.2 Inactivating viral nucleoprotein via RNAi:

A study showed that short double-stranded RNA, designated 3p-mNP1496-siRNA, can work as an siRNA targeting influenza NP gene as well as serve as an agonist for RIG-I activation [(29)]. The 3p-mNP1496-siRNA has the same guide RNA that targets the influenza NP gene sequence but has been modified by adding a triphosphate group into the 5′ end of an RNA molecule. This renders the monofunctional siRNA with an additional function of increasing RIG-I and IFN-β expression both in vitro and in mice. Although 3p-mNP1496-siRNA is specific for IAV infection, similar synthesis of dualfunctional siRNA targeted towards other pathogenic viruses can contribute to the arsenal of tools in developing novel antivirals.

Chapter 5: Antiviral strategies targeting viral release

Although some drugs that target viral release are available which include widely used neuraminidase (NA) inhibitors such as oseltamivir, zanamivir, peramivir in case of influenza virus [30], the need for newer strategies have arisen due to the resistance, side effects and lower bioavailability of the drugs. Drug modification and combination therapies have shown some promising results in this regard.

5.1 Enhancing antiviral ability using modified drug

Multivalent drug conjugate comprising of commercial drug zanamivir covalently attached to poly-l-glutamine have shown 20,000-fold improvement in anti-influenza potency compared with the zanamivir parent against both wild and drug resistant mutant strains of human and avian influenza [31]. NAIs target the active site of influenza A and B neuraminidase (NA) enzyme, preventing cleavage of terminal sialic acid residues on the membrane of the infected cell and thus hampering viral propagation. Multivalent drug species exhibit better NA inhibition due to enhanced steric effects and increased affinity. They promote improved ligand-protein interaction due to the introduction if flexible linkers which increase effective ligand concentration and reduce steric obstacles.

	IC50 (nM of Zanamivir)				
Strain	1	2	3	4	5
A/Wuhan/359/95	(2.3 ± 1.6) × 104	(4.3 ± 0.2) × 105	(1.8 ± 0.5) × 102	21 ±7	(3.4 ± 1.0) × 102
A/Wuhan/359/95 E119V	(4.8 ± 1.5) × 104	(3.1 ± 0.1) × 105	(7.7 ± 5.1) × 102	51 ±5	(1.0 ± 0.6) × 103
A/turkey/MN/833/80	(2.1 ± 0.9) × 104	(4.3 ± 1.1) × 104	(1.2 ± 0.4) × 103	(1.8 ± 0.2) × 102	(9.6 ± 5.9) × 102
A/turkey/MN/833/80 E119D	(1.8 ± 0.8) × 107	>3.6 × 106	(6.2 ± 4.0) × 103	(1.7 ± 0.8) × 103	(2.6 ± 1.5) × 103
A/turkey/MN/833/80 E119G	n.d.	>3.6 × 106	(6.6 ± 3.3) × 103	(3.6 ± 1.3) × 103	(3.0 ± 1.2) × 104

Table 2: Antiviral Activities of Zanamivir (1), As Well As of Its Monomeric (2) and Polymeric (3-5) Derivatives, Against Human Wuhan and Avian Turkey/MN Influenza Strains, As Determined by the Plaque Reduction Assay.

5.2 Enhancing antiviral ability using combination therapy

Neuraminidase inhibitors (NAI) or polymerase inhibitors have been used to treat influenza till date but their prolonged use leaves us with the danger of encountering resistant strains. Therefore, the evaluation of drug combination treatment became mandatory. In vitro testing of endonuclease inhibitor baloxavir acid (BXA) combined with three NAIs (oseltamivir, zanamivir and peramivir) or two other polymerase inhibitors (favipiravir and ribavirin) have shown that combinations of BXA with NAIs had higher potency [(32)]. Combinations of other

polymerase inhibitors with NAIs, such as oseltamivir with favipiravir and peramivir–favipiravir or oseltamivir–favipiravir have exhibited various beneficial effects in controlling drug-resistant A (H1N1) strains.

Chapter 6: Antiviral strategies targeting host factors

Antiviral strategies involving host factors include targeting host proteins, host metabolism, host signaling pathway, host cell antiviral response etc. These host based approaches are stated below.

6.1 Targeting host proteins

Targeting host proteins such as molecular chaperon Hsp70, adaptor protein importin-α7 can be possible options [33] [34]. Hsp70 has been found to be a prerequisite for zika virus replication. Depending on different cell models, analogue compounds targeting a highly conserved site in Hsp70 may vary. Hsp70 is needed for multiple steps of flaviviral life cycle which include entry, viral replication, capsid interactions and production of infectious viral particles. As a result, Hsp70 inhibitors can block viral propagation both during and after infection, unlike entry inhibitors and polymerase inhibitors and have not exhibited any appreciable toxicity or drug resistance. Another host adaptor protein responsible for increasing the replication of human-type influenza virus is importin-α7. Experiments have shown that mice with deleted importin-α7 coding gene showed 100% survival rate. However, mutational analyses have shown that mutants resistant to dependency on NP with those in HA and NA can emerge with mutations in RNP along with those in HA and NA. This finding suggests that importin-α7 can be used a target combined with drugs targeting cell entry and NA inhibitors. Neverthless importin-α7 can be a viable target as resistance associated with this host factor results from five mutations

in four different gene segments unlike resistance to neuraminidase inhibitors that requires a single mutation.

6.2 Targeting host cell metabolism

Compounds that target host cellular enzymes that are used by viruses for propagation can be used as possible therapeitic measure. Pyrimidine synthesis pathway or the dephosphorylation mechanism of viral transcription factor VP30 are such metabolic targets. The NS1 protein of influenza virus is a major virulence factor contributing to its replication process by inhibiting host mRNA processing and export and enhancing viral gene expression. A host enzyme, dihydroorotate dehydrogenase (DHODH) is required for de novo pyrimidine biosynthesis in rapidly growing viral cells, where salvage biosynthesis is insufficient. A nontoxic quinoline carboxylic acid, Brequinar, is a proven DHODH inhibitor that has exhibited the capability of inhibiting viral repliction and NS1 transcription by blocking de novo pyrimidine synthesis and reverted the NS1-mediated mRNA export block [(35)]. Another compound with an amino-tetrahydrocarbazole scaffold, SW835, exhibited broad spectrum antiviral activity against EBOV, VSV and ZIKV by inhibiting DHODH and upregulating IRF1 [(36)]. Ebola virus genome transcription on the other hand, is mediated by the viral transcription factor VP30 in its unphosphorylated form. A study showed that the molecular mechanism involving the dephosphorylation depends on a phosphatase enzyme PP2A-B56, which binds to conserved motifs of VP30 [(37)]. Further research has to be conducted to ensure whether the inhibition of this phosphatase without any potential side-effects can be a feasible approach.

6.3 Targeting host signaling pathway

Host signalling pathways that are affected by the viral infection can serve as potential targets. By the help of RNAi, studies have shown that several genes in the PI3K-AKT-mTORs pathway aid viral replication process. Everolimus, an mTOR inhibitor, exhibited

antiviral activity against cowpox, dengue type 2, influenza A, rhino- and respiratory syncytial viruses [38]. PI3K-AKT-mTOR signalling pathway is initiated by ligand binding of receptor tyrosine kinases such as the insulin receptor, activation of PI3K and finally the phosphorylation of AKT1 or AKT2 into active form. Administration of everolimus at different concentrations showed significant reductions in lung haemorrhaging and lung viral titres at day 3 post-infection with A/Duck/MN/1525/81 (H5N1), but not day 6 postinfection. Everolimus also did not provide complete protection from death. Although everolimus showed weaker antiviral activity, it can be assesed that PI3K-AKT-mTOR can be a probable target. Two other receptor tyrosine kinase inhibitors (RTKI), AG879 and A9, can each effectively inhibit influenza virus replication at multiple steps of the virus life cycle, disrupting vRNP nuclear export, RNA synthesis, and virus release [39]. Another pathway that is stimulated in response to viral infection is the RIG-I like receptor pathway. Cytosolic RIG-I is stimulated by its interaction with viral RNA that contain short hairpin dsRNA and 5′ triphosphate (5′ppp) terminal structure and induces IRF, NF-κB, STAT, chemokines and pro-inflammatory cytokine genes. A short in vitro-synthesized 5′pppRNA derived from the highly conserved 5′ and 3′ UTRs of negative single strand RNA virus genomes activated the RIG-I signaling pathway and elicited a strong antiviral response against VSV, Dengue (DENV), Vaccinia viruses and H1N1 Influenza [40].

6.4 Targeting host antiviral response

Compounds and methods that induce host's natural immune response against viruses may pave ways to develop more novel antiviral strategies. A novel small molecule, ASN2 had proven to induce the expression of type I IFN and ISGs against multiple subtypes of influenza A virus effectively [41]. ASN2 displayed specific inhibitory effect on viral polymerase action and thus the interferon induction in possibly due to the loss of NS1 expression, which is responsible for the inhibition of IFN release. CRISPR-Cas9 based gene

editing can also be used to modify interferon (IFN-I)-inducible human restriction factors such as TRIM5α, which blocks infections caused by nonhuman retroviruses, such as N-MLV and EIAV, but not by HIV-1. CRISPR-Cas9 protocol can be used to modify TRIM5α by introducing therapeutic amino acid substitutions in Arg332 and Arg335 with homology-directed repair (HDR) to create a HIV-1-restrictive version [42]. gRNA complementary to the loci near potential targets for CRISPR-Cas9-mediated double-strand cleavage was designed and a HDR donor DNA was synthesized for the experiment. However, most of the resulting DNA contained single modified allele, where two modified alleles are required for diploid human chromosomes. Moreover, non- HDR corrected alleles showed many indels incorporated by NHEJ repair along with additional on-target indels. In order to obtain flawless modification of TRIM5 by HDR, marker-free system to encourage HDR and double-strand break generating Cpf1 instead of Cas9 as the enzyme can be used to induce bi-allelic modifications.

Chapter 7: Concluding remarks

In the recent years, the rapidly emerging drug resistant RNA viruses have plagued our minds with a fear of unexpected outbreaks of global pandemic. Recent approaches include the enhancement and modifications of entry based drugs and therapies, development of inhibitors against viral biosynthesis, assembly and release, modulation of host cell factors. Targeting of host cellular factors has become a preferred approach over the years as doing so has lesser possibility of conferring resistance. Use of small molecules, analogues, peptides and neutralizing antibodies are on constant development. Widely administered drugs are as well being tested for modification by the means of analyzing combinational effects and structure modification. Newer concepts such as gene editing and gene silencing are also being utilized in the concept of developing novel antiviral therapies. Indeed, the ways and possibilities paved by the ongoing research and studies can endow us with multifaceted strategies to encounter resistant and mutant strains. Even though some of the mentioned compounds and therapeutic methods have shown potential to fight viral infections, many of them need abundance of future research to come into reality. Nonetheless, we hope that with developing technologies and studies, mankind will be able to see the light of new and advanced antiviral strategies.

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