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Review

Natural Products with Potential to Treat RNA Virus Pathogens Including SARS-CoV-2

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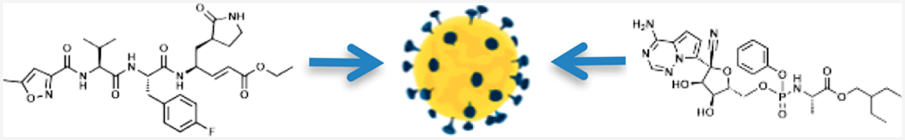

ABSTRACT: Three families of RNA viruses, the *Coronaviridae*, *Flaviviridae*, and *Filoviridae*, collectively have great potential to cause epidemic disease in human populations. The current SARS-CoV-2 (*Coronaviridae*) responsible for the COVID-19 pandemic underscores the lack of effective medications currently available to treat these classes of viral pathogens. Similarly, the *Flaviviridae*, which includes such viruses as Dengue, West Nile, and Zika, and the *Filoviridae*, with the Ebola-type viruses, as examples, all lack effective therapeutics. In this review, we present fundamental information concerning the biology of these three virus families, including their genomic makeup, mode of infection of human cells, and key proteins that may offer targeted therapies. Further, we present the natural products and their derivatives that have documented activities to these viral and host proteins, offering hope for future mechanism-based antiviral therapeutics. By arranging these potential protein targets and their natural product inhibitors by target type across these three families of virus, new insights are developed, and crossover treatment strategies are suggested. Hence, natural products, as is the case for other therapeutic areas, continue to be a promising source of structurally diverse new anti-RNA virus therapeutics.

INTRODUCTION

Deadly outbreaks caused by viruses have occurred periodically throughout human history. Several endemic infections evolved into pandemics that threatened the entire global population. The last pandemic of the 19th century, known as La Grippe or the Russian Flu, occurred between 1889 and 1890 and caused approximately one million deaths.[1] This outbreak was caused by an influenza A virus, subtype H2N2.[2] The 1918 Spanish flu epidemic arrived on the heels of World War I and killed mainly small children, young adults, and the elderly.[3] It is generally felt that this was the deadliest pandemic ever recorded, with an estimated 50 million fatalities. The Spanish flu epidemic was also caused by an influenza virus, subtype H1N1. Other notable pandemics are the 1957 and 1968 influenza infections. These were widespread, and the 1957 influenza epidemic killed roughly one million people. Thanks to extensive research and development spanning decades, pandemics caused by influenza viruses are now able to be abated and at times prevented by vaccines, although few truly effective drug treatments are available.

For decades, natural products (NPs) have been an inspiration for the development of new pharmaceuticals; approximately 50% of all small-molecule drugs have an NP derivation or inspiration.[4] Due to the vast diversity of natural products in nature made by microbes, plants, and fungi, these compounds have been a useful reservoir of structural information from which to draw in drug discovery campaigns. As Frank Lovering laid out in his treatise on stereochemical complexity in drug development, the complexity that natural products tend to possess is a testament to their usefulness in this area.[5,6] As we search for new answers to the threat of global pandemics, where better to begin looking than to nature?

The human immunodeficiency virus (HIV) has been endemic for the last 40 years. It was present in human populations at a low rate since the 1950s, but in the late 1970s and early 1980s, more cases of immunodeficiency occurred with an unknown cause. The human immunodeficiency virus was identified in 1983 by Luc Montagnier (Pasteur Institute, Paris, France) and Robert Gallo (NIH, Bethesda, Maryland, USA).[7−11] HIV infections led to the acquired immune deficiency syndrome (AIDS) epidemic that claimed approximately 30 million lives worldwide.[12] There is currently no cure for AIDS, but disease progression can be mitigated with a variety of antiviral treatments, where roughly 23 million

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Table 1. Viruses Discussed in This Review and Associated Information

virus	taxonomy	emergence	source/transmission	type	genome size	treatments
SARS-CoV	order: Nidovirales, family: *Coronaviridae*, genus: Betacoronavirus	Foshan, China 2002	bats → intermediate mammal host → human	positive-sense RNA virus	~30 kb	no
SARS-CoV-2	order: Nidovirales, family: *Coronaviridae*, genus: Betacoronavirus	Wuhan, China 2019	bats → intermediate mammal host → human	positive-sense RNA virus	~30 kb	no
MERS-CoV	order: Nidovirales, family: *Coronaviridae*, genus: Betacoronavirus	Jeddah, Saudi Arabia 2012	bats → intermediate mammal host → human	positive-sense RNA virus	~30 kb	no
Ebola virus	order: Mononegavirales, family: *Filoviridae*, genus: Ebolavirus	Sudan and Zaire 1976	bats → humans; mammal hosts → humans; humans → humans	negative-sense RNA virus	19 kb	vaccine: rVSV-ZEBOV
Marburg virus	order: Mononegavirales, family: *Filoviridae*, genus: Marburgvirus	Marburg, Germany 1967	bats → humans; mammal hosts → humans; humans → humans	negative-sense RNA virus	19 kb	no
Dengue	order: Amarillovirales, family: *Flaviviridae*, genus: Flavivirus	endemic since WWII, subtropics and tropics where the *aedes sp* mosquito exist	primates → mosquito vector → humans	positive-sense RNA virus	10.7 kb	no
West Nile	order: Amarillovirales, family: *Flaviviridae*, genus: Flavivirus	Africa 1937, United States 1999	birds → mosquito vector → human	positive-sense RNA virus	11 kb	no
Zika	order: Amarillovirales, family: *Flaviviridae*, genus: Flavivirus		unknown → mosquito vector → humans	positive-sense RNA virus	11 kb	vaccines in development, no other treatments available
tick-borne encephalitis virus	order: Amarillovirales, family: *Flaviviridae*, genus: Flavivirus	virus isolated in 1937, endemic in Russia, Europe	ticks → humans	positive-sense RNA virus	11 kb	vaccine available, but not outside of endemic areas
Japanese encephalitis virus	order: Amarillovirales, family: *Flaviviridae*, genus: Flavivirus	Southeast Asia 1930s	unknown → mosquito vector → humans	positive-sense RNA virus	10.9 kb	vaccine available, but no other pharmaeutical treatments available

treatments are prescribed each year. The most common of these treatments are integrase inhibitors such as dolutegravir and raltegravir. Another common drug class is the nucleoside/nucleotide reverse transcriptase inhibitors (NRTIs). The combination of dolutegravir/rilpivirine, a non-nucleoside reverse transcriptase inhibitor, can be given as a once-daily tablet, which improves compliance.[13] One reason why it has been so difficult to make an AIDS vaccine is that the virus infects the very cells of the immune system that a vaccine should induce; hence, HIV is a good example of why vaccines cannot always be developed, and thus antiviral treatments are needed.

The term hepatitis is used to describe the infection of the liver by any of the hepatitis A through E viruses. However, hepatitis viruses are fairly heterogeneous; hepatitis (Hep) A is an unenveloped symmetrical RNA virus, whereas Hep B is a double-stranded DNA virus. The most common strategy for controlling different hepatitis viruses has been through the development of vaccines (e.g., to Hep A and B), though there are some drug treatments as well. Vaccines are under development for Hep C and Hep E, and Hep D occurs only as a co-infectant with Hep B. Hence, immunization against Hep B also provides protection against Hep D.[14] Antiviral therapeutics have also been developed for treating Hep C[15] and are highly effective such that the virus can be completely eradicated.[16]

In this review, we focus on three virus families, the *Coronaviridae*, *Flaviviridae*, and *Filoviridae*, because these three families have been responsible for causing a number of endemic and pandemic outbreaks, including the current COVID-19 pandemic (Table 1). Examples belonging to the *Coronaviridae* are SARS-CoV-1, SARS-CoV-2, and MERS-CoV. The family *Flaviviridae* contains the Dengue, Yellow fever, Japanese encephalitis virus, Zika, and tick-borne encephalitis (TBV) viruses. Lastly, the filoviruses considered in this review include those causing Ebola and Marburg diseases. Unfortunately, many of these viruses have inadequate or completely absent treatment options. Because of this deficiency, we have focused this review on identifying potential druggable protein targets that have been or could be used in the development of small-molecule treatments, with an emphasis on natural products and their derivatives.

VIRAL LIFE CYCLES

Coronaviridae. The current outbreak of SARS-CoV-2 represents the third documented spillover of an animal coronavirus to humans in the last 20 years that has resulted in a major epidemic of global proportions.[17] As of this writing, the virus has infected worldwide almost 65 million people and claimed over 1.5 million lives.[18] The *Coronaviridae* Study Group of the International Committee on Taxonomy of Viruses has assessed the placement of this current human pathogen within the *Coronaviridae* family. They have

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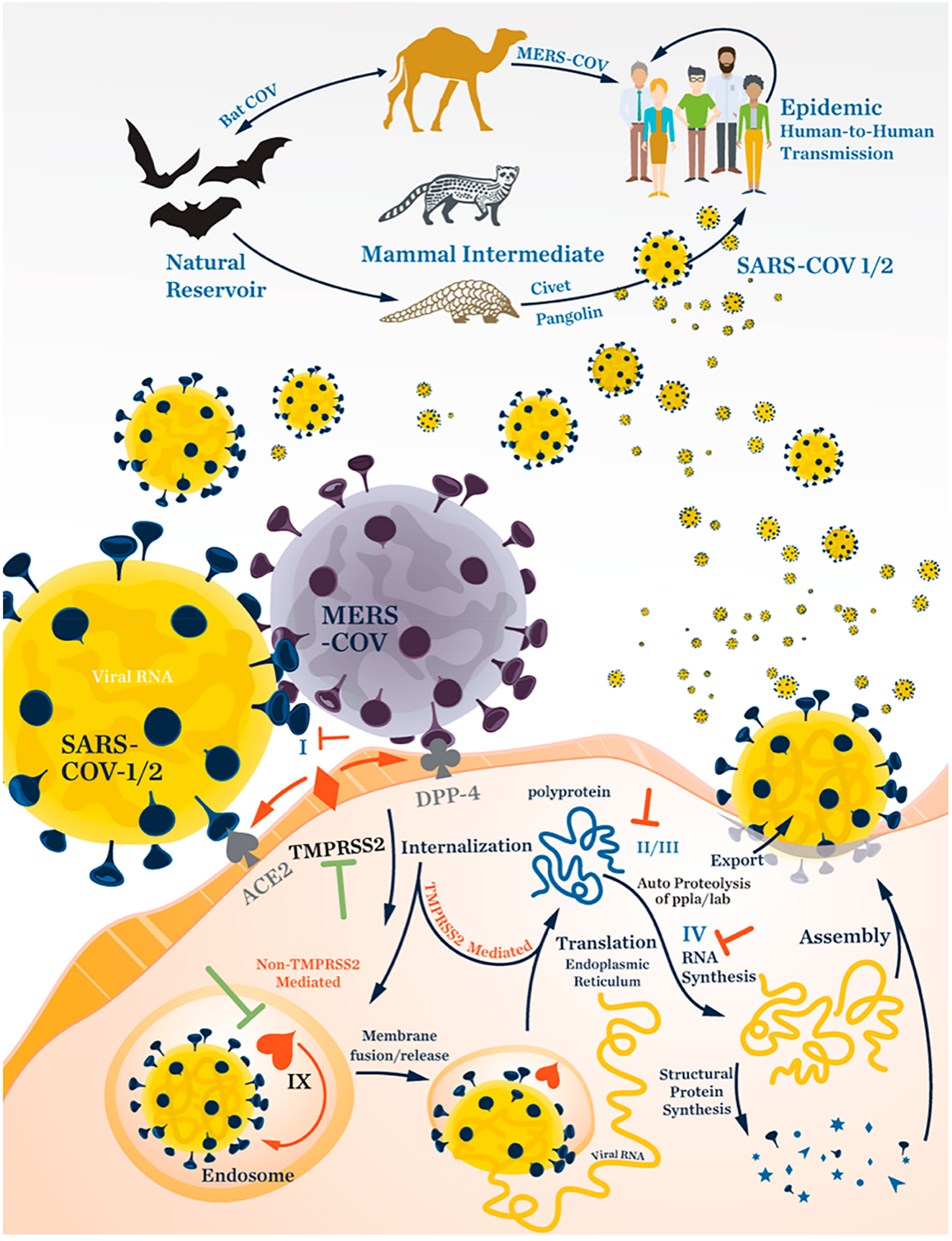

Figure 1. Life cycle of coronaviruses as represented by the SARS-CoV-1/2 and MERS-CoV viruses. The Roman numerals in the figure refer to entries in Table 2. The "T" symbol indicates a target with potential for developing an inhibitor.

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Table 2. Validated and Potential Drug Targets and Known NP Inhibitors against *Coronaviridae*[a]

	number	target protein	function	natural product inhibitors	NP-inspired inhibitors
viral proteins	I	spike glycoprotein	host cell recognition	griffithsin (**2**), emodin (**3**)	EK1 and other spike fragments
	II	main protease (Mpro/3CLpro)	viral protein maturation	herbacetin (**13**), rhoifolin (**15**), and other flavonoids	peptidomimetics
	III	papain-like protease (PLpro)	viral protein maturation	tomentin B (**16**), hirsutenone (**17**), tanshinones (**19**), psoralidin (**18**), and others	inhibitors needed
	IV	RNA-dependent RNA polymerase (RdRp)	viral RNA replication	inhibitors needed	favipiravir (**22**), remdesivir (**1**), ribavirin (**23**), galidesivir (**24**), β-D-N4-hydroxycytidine (**25**)
	V	NSP1	obstructs host cell protein synthesis and innate immune response	inhibitors needed	inhibitors needed
	VI	NSP13	viral helicase	myricetin (**26**), scutellarein (**27**)	inhibitors needed
	VII	Orf8b	obstructs innate immune response	inhibitors needed	inhibitors needed
host proteins	VIII	TMPRSS2	cell membrane protease, cleaves viral spike protein facilitating membrane fusion and entry	aprotinin (**34**)	inhibitors needed
	IX	cathepsin L	endosomal protease, cleaves spike protein facilitating membrane fusion	gallinamide A (**35**), nicolaidesin C (**36**), grassypeptolide (**37**), E-64, leupeptin (**38**)	other reviews

[a]Roman numbering corresponds to numbering in Figure 1. Numbers in parentheses correspond to structures in Figures 5−11.

recognized this virus as forming a sister clade to the prototype human and bat severe acute respiratory syndrome coronaviruses (SARS-CoVs) and designated it as SARS-CoV-2.[19] Coronaviruses are enveloped, positive-sense RNA viruses that are distributed broadly among humans, other mammals, and birds and are known to cause respiratory, enteric, hepatic, and neurologic diseases.[20,21] The *Coronaviridae* family of viruses contains the largest known RNA genomes, 30−32 kb,[22] and is divided into four genera (alpha, beta, gamma, and delta coronaviruses). While most coronavirus infections cause common cold symptoms in humans, SARS-CoV, MERS-CoV, and SARS-CoV-2, the three coronaviruses covered in this review, have the potential to cause more serious disease, including death. These three viruses have emerged relatively recently, all in the 21st century, and have been the focus of extensive research in an urgent response to their global occurrence and impact. SARS-CoV appeared first in late 2002 and was characterized by rapid human-to-human transmission with various degrees of morbidity and mortality ranging from moderate to high. The zoonotic origin of these outbreaks was linked to bat coronaviruses (>90% sequence identity) with possible intermediate transmission through other mammals such as civets[20] or pangolins.[23] These infections tended to disproportionately affect older populations, with SARS mortality reaching 50% of those infected over 60 years of age.[20] To date there are no clinically approved treatments for these viruses, and therefore we provide this review with the goal of providing insight into these viruses, their vulnerabilities, and past and ongoing natural product-based drug discovery efforts to curb these deadly pathogens.

Coronaviruses infect their hosts through a number of pathways, exploiting host receptors and transmembrane proteases to facilitate their entry into cells and delivery of viral RNA into the cytoplasm (Figure 1, Table 2). In overview, the viral spike glycoprotein (**S**) facilitates entry into target cells by binding to cellular receptors and is subsequently primed by host cellular proteases that promote fusion of viral and cellular membranes and internalization. Trimers of the **S** protein form peplomers embedded in the viral envelope, giving the virus its characteristic crown-like morphology. Due to their highly homologous **S** proteins (96% sequence identity), both SARS-CoV and SARS-CoV-2 make use of the mammalian angiotensin converting enzyme 2 (ACE2) as a means of initial contact.[20,24,25] The MERS-CoV and SARS-CoV-1/2 spike proteins share a high degree of structural similarity in their core subdomains, but are notably divergent in the receptor-binding subdomain. For this reason, MERS-CoV utilizes a different surface protein for entry, namely, the dipeptidyl peptidase 4 receptor (**DPP-4** or **CD26**).[26] It has been posited that the more widespread distribution of SARS-CoV-2 is facilitated by a stronger binding affinity to the ACE2 receptor.[27] Once contact is made, host proteases TMPRSS2/4 (colocalized with ACE2 or DPP-4 on the cell surface) or endosomal cathepsin L cleaves at two or more sites on the **S** protein to facilitate a conformational rearrangement and fusion with the host membrane, releasing viral RNA into the host cytoplasm.[24,28−30] Mechanistically, it appears that the coronavirus **S** protein undergoes a receptor-mediated conformational change that reveals cryptic cleavage sites within the viral envelope glycoprotein.[31] Proteolysis by host proteases is then necessary to fully activate the viral glycoprotein's membrane-fusion potential. Inhibiting activity of either host protease partially blocks infection, and inhibiting both efficiently prevents cell entry and replication *in vitro*.[29,32] Temporary blockage of these host proteases by small molecules is nonlethal to mammalian cells, and thus they are active targets of investigation for prophylactic treatment. Following entry, the replicase gene is translated by host machinery and cotranslationally processed by the **Mpro** and **PLpro** into 16 nonstructural proteins encoded in orf1a/1ab. These proteins are assembled into the replicase−transcriptase complex to create an environment suitable for viral RNA synthesis and are responsible for RNA replication and transcription of the genomic and subgenomic RNAs. A comprehensive list of these proteins and their known functions has been assembled by Fehr and Perlman.[33] Viral RNA synthesis by the replicase complex produces RNAs that serve as mRNAs for production of the structural and accessory proteins. This process is currently the focus of active drug discovery efforts to prevent viral replication. For example, remdesivir (**1**) (Gilead Sciences; Figure 7) is a promising candidate targeting the RNA-dependent RNA polymerase (**RdRp**).[34,35]

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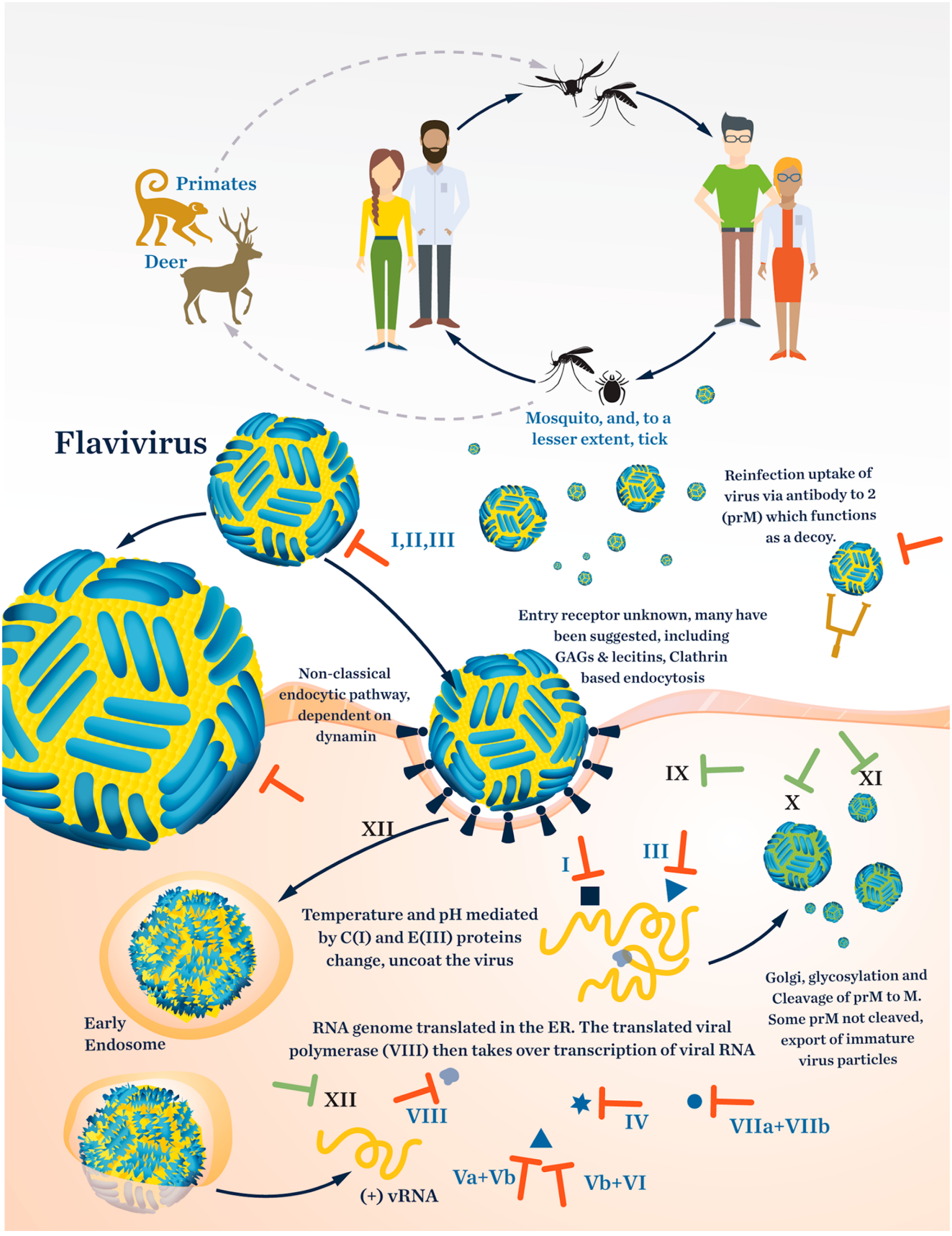

Figure 2. Life cycle of *Flaviviridae* as represented by the dengue virus. The Roman numerals in the figure refer to entries in Table 3. The "T" symbol indicates a target with potential for developing an inhibitor.

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Table 3. Validated and Potential Drug Targets in *Flaviviridae* and NP Inhibitors (Dengue Virus Shown as Example)[a]

	number	target protein	function	natural product inhibitors	NP-inspired inhibitors
viral proteins	I	capsid protein	binds to viral nucleotide strand, forms viral capsid	nordihydroguaiaretic acid (NDGA) (**4**)	inhibitors needed
	II	prM/M protein	membrane protein	inhibitors needed	ectodomain of M protein, decanoyl-Arg-Val-Lys-Arg-CMK
	III	E protein	envelope protein	inhibitors needed	heparan sulfate mimic PG545 (**5**)
	IV	NS1	Antihost factor glycoprotein	Castanospermine (**32**)	Heparan sulfate mimic PG545, Celgosivir (**33**)
	Va	NS2A	membrane protein, interacts with calmodulin (Ca-influx)	inhibitors needed	inhibitors needed
	Vb	NS2B	NS3 cofactor/part of the replication complex	inhibitors needed	inhibitors needed
	VI	NS3	protease/helicase	ganoderma lucidum triterpenoid, ganodermanontriol (**20**)	ivermectin (**21**)
	VIIa	NS4A	ER membrane protein/also part of replication complex	inhibitors needed	inhibitors needed
	VIIb	NS4B	ER membrane protein/also part of replication complex	inhibitors needed	inhibitors needed
	VIII	NS5	RdRp/methyltrans ferase/helicase	mycophenolic acid (**28**)	ribavirin, ZX-2401
host proteins	IX	calmodulin	Ca-influx. interacts with NS2A	inhibitors needed	inhibitors needed
	X	signal peptidase/ Signalase	cleaves signal peptides, in Dengue four cleavages (prM, E, NS1, and NS4B)	cavinafungin (**44**)	inhibitors needed
	XI	Aalpha-glucosidase	aids in ER release of glycoproteins E and NS 1	nojirimycin (**45**), deoxynojirimycin (**46**)	CM-10-18 (deoxynojirimycin analogue)
	XII	80S ribosome	translation of viral polypeptide	inhibitors needed	geneticin (neomycin analogue) (**47**)

[a]Roman numbering corresponds to numbering in Figure 2. Numbers in parentheses correspond to structures in Figures 5−11.

The main pillars of drug discovery against these recently emergent coronaviruses have been inhibition of viral entry or prevention of replication once infection has occurred. There are several routes by which the virus can escape the cell, including exocytosis or by inducing apoptosis, and this makes the targeting of packaging or export mechanisms much more challenging drug targets. However, by targeting the spike glycoprotein, host recognition by the virus is prevented and entry nullified. Alternatively, inhibition of polyprotein processing by the viral proteases or targeting the **RdRp** can partly or wholly prevent replication. A list of repurposed or investigational agents against SARS-CoV-2 was recently reported,[36] and several studies have been published identifying new inhibitors of SARS-CoV and MERS-CoV.[37,38]

Flaviviridae. Several well-known endemics are caused by flaviviruses, for example, West Nile, Dengue, Zika, Japanese encephalitis (JEV), and tick-borne encephalitis virus (TBV). Most *Flaviviridae* infections involve an intermediate host before transmission to humans. West Nile, Zika, Dengue, and JEV viruses are all transmitted by mosquitoes, whereas TBV involves spread by ticks; humans are typically the end host. Intermediate animal hosts include monkeys, birds, and pigs, with transfers to mosquitoes, which pass the infection to humans (Figure 2, Table 3). However, Dengue is common in human populations around the world, with around 4.2 million cases reported to WHO in 2019; however the true number of infections is estimated to be much higher. Because of its prevalence, human to mosquito to human transmission of Dengue is the most common route (Figure 2).[39] The tick-borne flaviviruses are commonly transmitted from deer and sheep to ticks and then to humans.[40] Because there are many different flaviviruses that can cause potential pandemics, after a brief review of the diversity of disease-causing *Flaviviridae*, this review focuses on Dengue virus. This is appropriate and representative, as all flaviviruses have similar genome sizes (10−11 kb) as well as similar gene organization in their positive RNA strand genomes.

Dengue is the most prevalent mosquito-borne viral disease in the world.[41] The dengue virus consists of four serotypes, which introduces considerable complication in the disease and its clinical course. Infection with one serotype does not confer immunity to another serotype. Rather, infection with a different serotype can create an antibody-dependent enhancement of the disease. This makes it very difficult to develop a universal vaccine for all the Dengue serotypes[41] and makes imperative the development of small-molecule therapeutics.

West Nile virus infections first appeared in the United States in 1999. The most common route of human infection is by a mosquito bite with the natural reservoir mainly being in birds. Since 1999 an estimated 7 million humans have been infected in the United States; this makes the West Nile virus the most common mosquito-borne virus infection and also the virus that is responsible for the most cases of viral encephalitis in the country.[42] According to the CDC there are no currently approved treatments for West Nile virus, other than standard supportive care.[43]

The Zika virus was first isolated from a monkey in Uganda in 1947, and transmission to humans was first detected in 1952. So far, only 12 cases in Puerto Rico and one case in Virginia have been recorded in the United States (CDC.gov). It is, like many other *Flaviviridae*, transmitted by mosquitoes and is widespread in Africa and Asia Pacific countries. In 2015, reports of the virus spreading to Brazil started circulating, and several thousand babies were born with microcephaly.[44] The virus has since spread through South America and the Caribbean.

The Japanese Encephalitis Virus (JEV) was first discovered in the 1870s in Japan. It is widespread throughout Asia with about 69 000 cases per year.[45] Several different genotypes and the unpredictable spread of the JEV makes developing specific pharmaceutical treatments very attractive. Several vaccines exist against different genotypes, and one manufactured by IXIARO must be given as two doses with 28 days between followed by a yearly booster.[46] This elaborate vaccination

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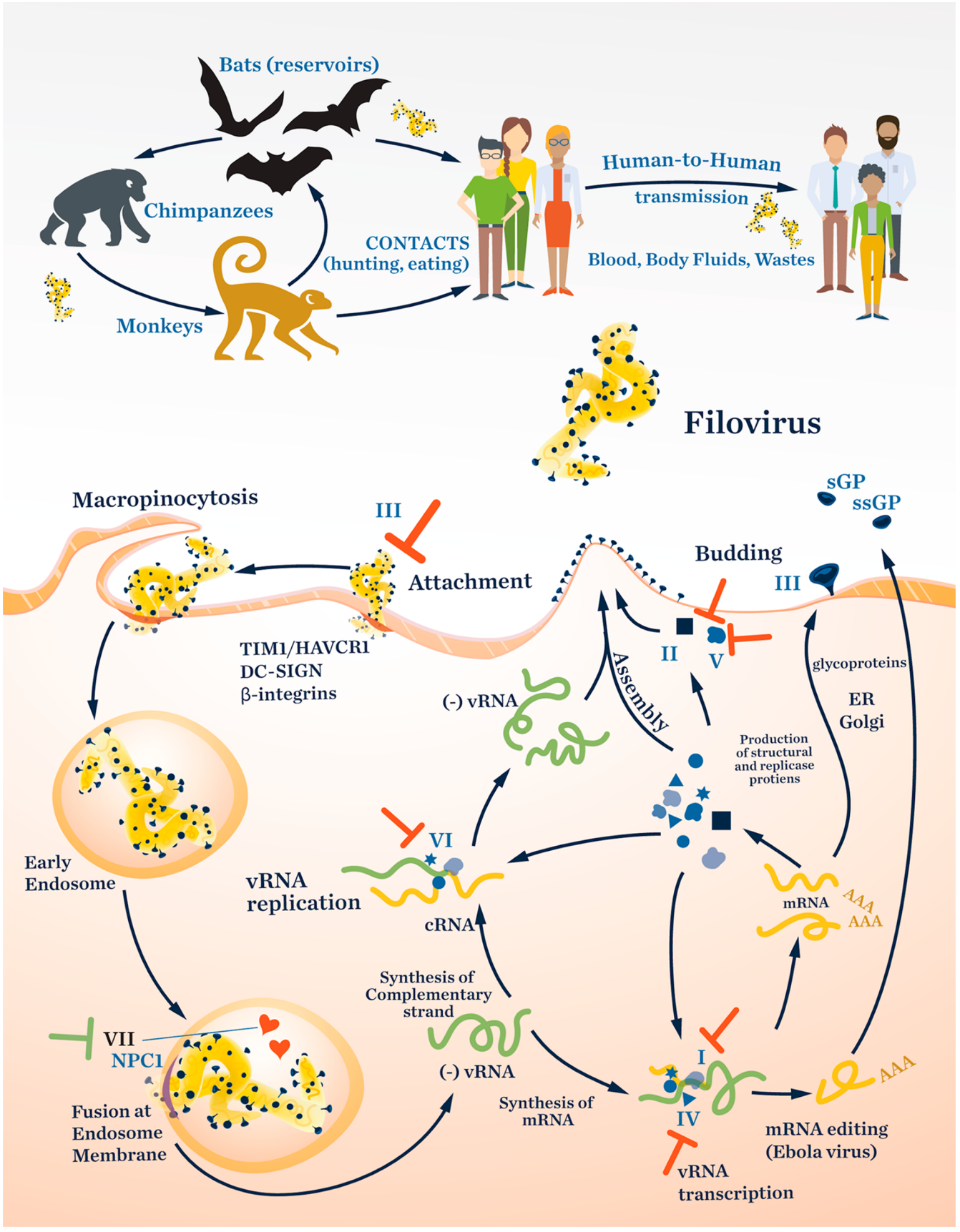

Figure 3. Life cycle of filoviruses as represented by the Ebola virus. The Roman numerals in the figure refer to the entries in Table 4. The "T" symbol indicates a target with potential for developing an inhibitor.

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Table 4. Validated and Potential Drug Targets in *Filoviridae* and NP Inhibitors[a]

	number	target protein	function	natural product inhibitors	NP-inspired inhibitors
viral proteins	**I**	VP35	polymerase cofactor responsible for transcription and replication with L protein	myricetin (**26**), epigallocatechin gallate (**31**)	inhibitors needed
	II	VP40	matrix protein involved in budding	inhibitors needed	inhibitors needed
	III	GP	host cell recognition	ellagic acid (**6**), gallic acid (**7**), cyanovirin-N (**11**)	triterpenoid derivatives (**8**, **9**), a borneol derivative (**10**)
	IV	VP30	transcriptional activator	inhibitors needed	inhibitors needed
	V	VP24	structural protein, important role in nucleocapsid formation	inhibitors needed	inhibitors needed
	VI	L protein	RNA-dependent RNA polymerase	inhibitors needed	favipiravir (**22**), remdesivir (**1**), BCX4430 (**30**)
host proteins	**VII**	cathepsin B/ cathepsin L	endosomal protease, cleaves spike protein facilitating membrane fusion and entry	gallinamide A (**35**), tokaramide A (**39**), miraziridine A (**40**)	dichlorobenzyl aloperine dihydrochloride (**41**), E-64d (**42**), CA074 (**43**)

[a]Roman numbering corresponds to numbering in Figure 3. Numbers in parentheses corresponds to structures in Figures 5−11.

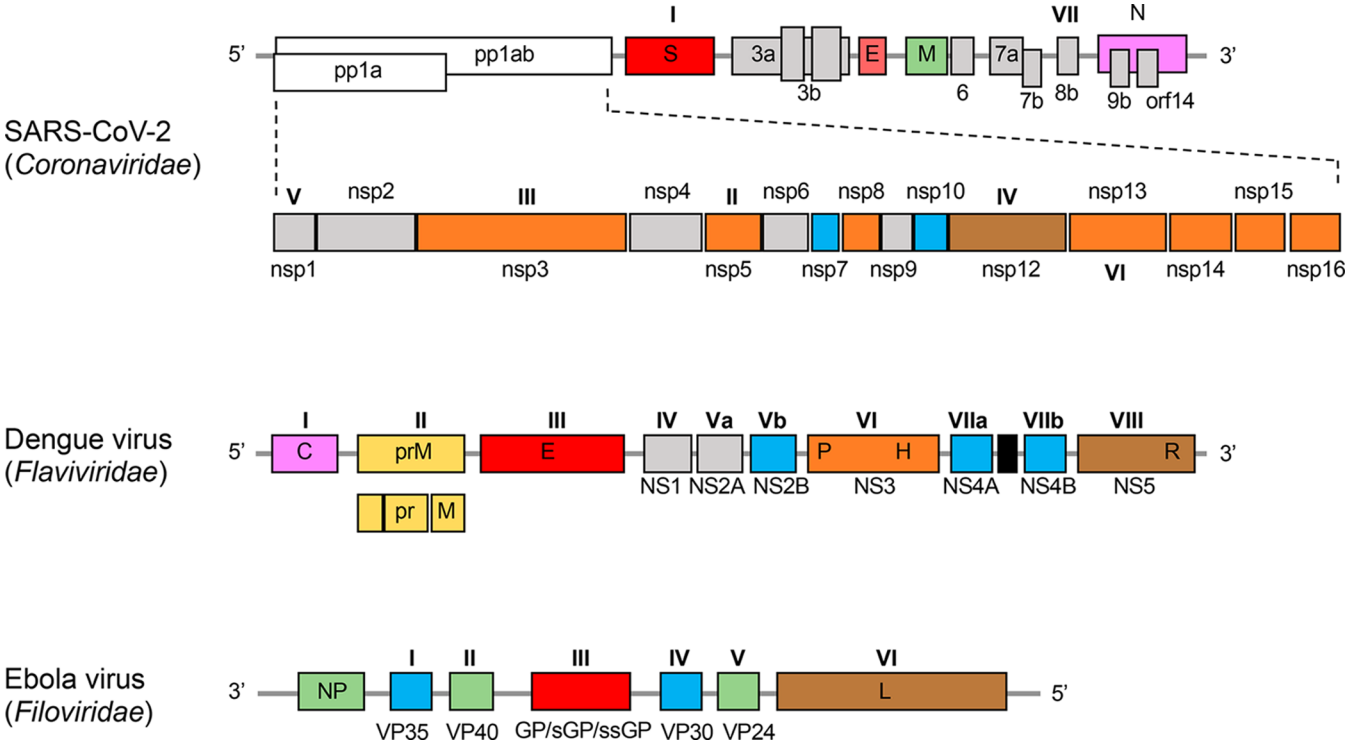

Figure 4. Schematic diagrams for the genomes of coronavirus SARS-CoV-2 (*Coronaviridae*), Dengue virus (*Flaviviridae*), and Ebola virus (*Filoviridae*). The genomes of *Coronaviridae* and *Flaviviridae* are composed of single-strand positive-sense RNA, whereas those of the *Filoviridae* are composed of a single strand of negative-sense RNA. Genes labeled by Roman numerals encode potential druggable protein targets mentioned in this review and accord with the same Roman numerals used in Figures 1−3 and Tables 2−4. Capsid protein (pink), spike protein (red), polymerase (brown), enzyme (orange), structural protein (green), envelope protein (yellow), cofactor/activator (blue), others/undefined protein (gray).

schedule can be prohibitive for travelers, and thus there is an unmet medical need for specific agents to treat JEV infections.

Several flaviviruses are transmitted via ticks, an example being tick-borne encephalitis (TBE). TBE is endemic to Eurasia and is becoming more widespread. A recent review provides details on the viral life cycle and viral and host proteins involved in transmission.[47]

Viruses of the family *Flaviviridae* have a positive RNA strand that can, upon entry into the cell, immediately hijack the ribosome for translation. The size of the RNA genome varies from about 10.6 to 10.9 kb. The RNA encodes for 10 proteins, three of which are structural and seven are nonstructural (Figure 4). The orfs for the structural proteins contain the nucleocapsid protein (**C**), the envelope protein (**M**), and the major spike (envelope) glycoprotein (**E**) of the capsid. The seven remaining orfs encode for nonstructural proteins (**NSP**).

NS1, in at least the Zika virus, appears to ubiquitinate Arg63 of the metalloproteinase 9 (**MMP9**) to increase its stability. **MMP9** degrades structural proteins and tight junctions between cells such that the virus can invade tissues more easily.[48] Involvement of **MMP9** in West Nile infection has also been reported, but there is no experimental data to support **NS1** ubiquitination. Instead, some evidence exists that the interferon pathway is downregulated by this protein.[49] The Dengue virus **NS1** was examined by Glasner[50] and was reported to break down extracellular glycocalyx and cause cell-intrinsic vascular leakage. Regardless of the mechanism of action of **NS1**, it appears to be an important virulence factor that may be targeted for potential drug development. The **NS2** orf encodes for two proteins; **NS2A** is a transmembrane protein and in Dengue has been shown to bind directly to calmodulin, the protein involved in calcium influx.[51] There

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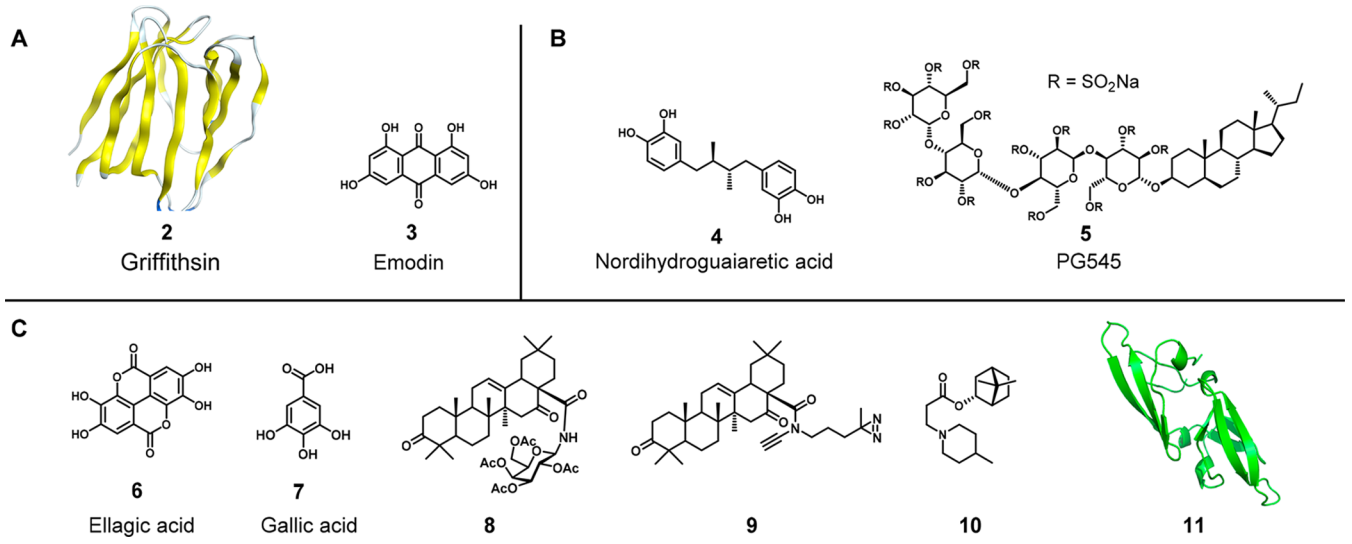

Figure 5. Inhibitors of viral structural proteins of (A) *Coronaviridae*, (B) *Flaviviridae*, or (C) *Filoviridae*. The following are natural products: griffithsin (PDB: 2GTY), emodin, nordihydroguaiaretic acid, ellagic acid, gallic acid, cyanovirin-N (**11**, PDB: 2EZM), and natural product derivatives: PG545, **8**, **9**, **10**.

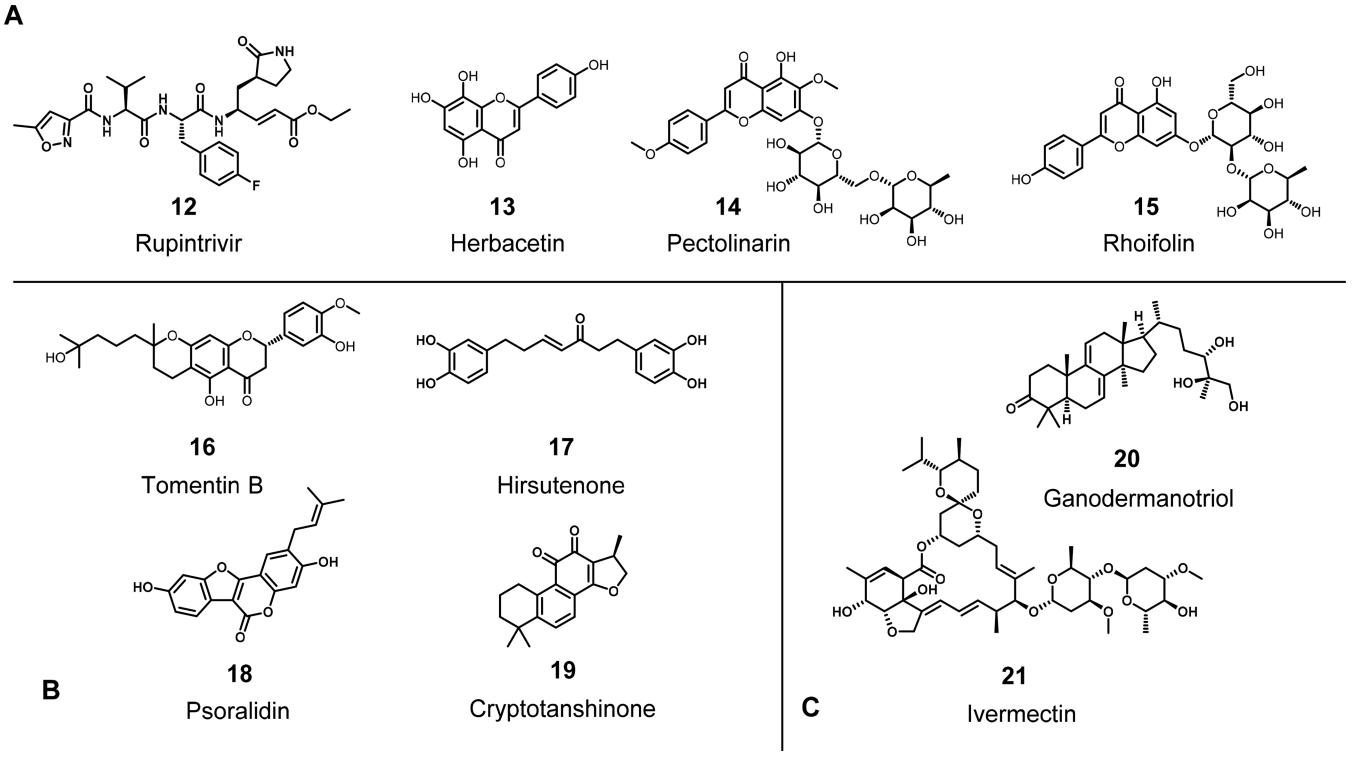

Figure 6. Inhibitors of viral proteases 3CLpro (A), PLpro (B), or NS3 (C). The following are natural products: herbacetin, pectolinarin, rhoifolin, tomentin B, hirsutenone, cryptotanshinone, psoralidin, ganodermanotriol, ivermectin, and natural product derivatives: rupintrivir.

have been no efforts to date to develop inhibitors of **NS2A**; however, a known calmodulin inhibitor has been shown to inhibit the dengue virus.[51,52] The **NS2B** protein is a hydrophobic protein that serves as a cofactor to the **NS3** protease.[53] **NS2B** has not been validated as an independent drug target, but only when in combination with **NS3**.[54] The Dengue virus **NS4** orf encodes for a membrane protein in the endoplasmic reticulum membrane. It consists of **NS4A** (127 aa) and **NS4B** (248 aa). These two are connected by a 23-residue linker from the C terminal of **NS4A**.[47] After integration into the membrane, the 23 aa linker is cleaved by a host signal peptidase (signalase). **NS4** has 11 helices, and the C terminal containing helices 9 and 9′ flip from the ER membrane to the cell membrane, where it interacts with the RNA-dependent RNA polymerase (**RdRp**) **NS5**, also a key drug target in *Flaviviridae*.[55,56]

Filoviridae. Filoviruses, such as Ebola virus (EBOV) and Marburg virus (MARV), are known as zoonotic pathogens that cause rare yet severe diseases affecting humans and other primates. Ebola virus disease (EVD) was first discovered in 1976 as a result of consecutive outbreaks in two areas of central Africa, in South Sudan and Zaire.[57] EVD is often a

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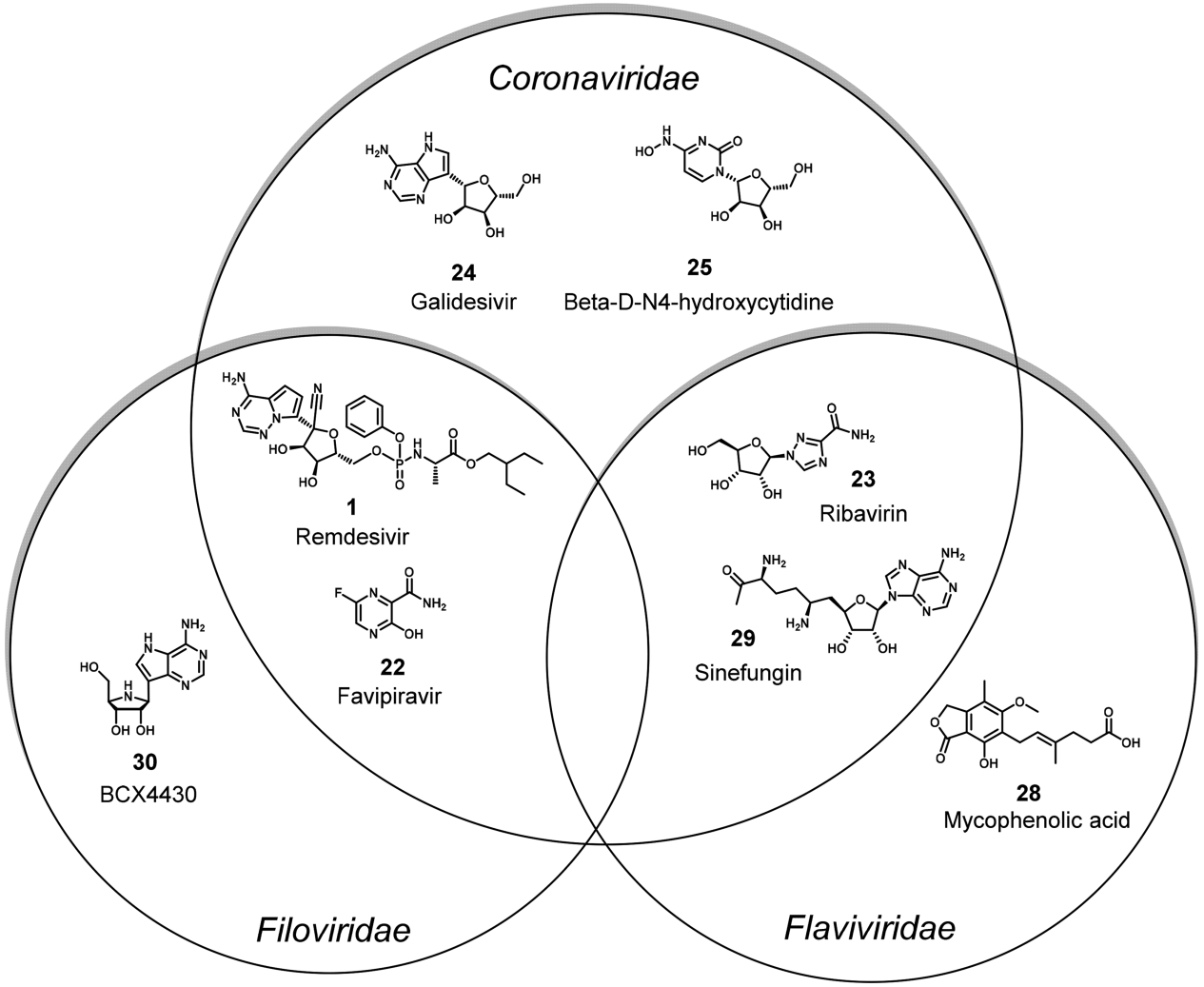

Figure 7. Inhibitors of viral replicase complex component **RdRp**. Shown here are compounds that are inhibitors of *Coronaviridae*, *Flaviviridae*, *Filoviridae*, or multiple families. The following are natural products: mycophenolic acid and sinefungin, and natural product derivatives: Remdesivir, Favipiravir, Ribavirin, Galdesivir, *β*-D-N4-hydroxycytidine, BCX4430.

deadly disease, and typical symptoms include fever, headache, vomiting, and diarrhea. Some people in severe cases can suffer internal and external bleeding. It was later discovered that these outbreaks were caused by genetically distinct EBOVs, *Sudan ebolavirus* and *Zaire ebolavirus*. Many outbreaks of EVD have occurred since 1976, primarily in Africa.[58] Today, there are five characterized species of the genus *Ebolavirus*: *Sudan ebolavirus* (SUDV), *Zaire ebolavirus* (EBOV), *Reston ebolavirus* (RESTV), *Taï Forest ebolavirus* (TAFV), and *Bundibugyo ebolavirus* (BDBV). RESTV is the only known filovirus that does not cause severe disease in humans; however, it can be fatal in monkeys and pigs.[59,60]

Marburg virus disease (MVD) was first observed in 1967 in Marburg, Germany, and is caused by the filovirus *Marburg Marburgvirus* (MARV). The symptoms of MVD are similar to those of EVD.[61] MVD appears in sporadic outbreaks in African countries as well.[62] The family of *Filoviridae* is a member of the order *Mononegavirales* and generally has a filamentous morphology (about 1000 nm in length with a diameter of 80 nm). It has been reported that fruit bats serve as a wildlife reservoir in nature for EBOV and MARV.[63,64] Bats carrying the virus transmit it to other animals such as monkeys[65] and chimpanzees,[66] as well as to humans. Human-to-human transmission can occur through contact with a patient's blood and bodily fluids.[67] In December 2019, the first vaccine, rVSV-ZEBOV, was approved for the prevention of EVD by the Food and Drug Administration (FDA) in the United States,[68] and other vaccines are now in development.[69]

Figure 3 shows the generalized life cycle of filoviruses.[62] The filovirus infects its host by the **GP** protein binding to several host molecules on the cell membrane, including the T-cell immunoglobulin mucin receptor 1 (TIM1), also known as hepatitis A virus cellular receptor 1 (HAVCR1), C-type lectins such as dendritic cell-specific intercellular adhesion molecule 3-grabbing nonintegrin (DC-SIGN), and *β*-integrins.[70−72] The virus is then incorporated into the cell through macropinocytosis.[73] Next, two host cell cysteine proteases, cathepsin B and cathepsin L, cleave the surface **GP** protein, and the processed **GP** binds to the Niemann−Pick C1 (NPC1) cholesterol transporter.[74,75] This interaction leads to the fusion of the virus with the endosomal membrane and release of the viral RNA into the cytoplasm for transcription and replication in the inclusion body. Viral mRNAs produced by an **RdRp** are translated into proteins associated with **VP30**, **VP35**, and **L** by host ribosomes (primary transcription). The synthesized viral proteins are used in secondary transcription and vRNA

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replication. Following transcription, the **GP** protein, a type I transmembrane glycoprotein, is produced as a precursor protein known as $\mathbf{GP_0}$; this is then cleaved post-translationally by a furin-like protease to yield the ectodomains $\mathbf{GP_1}$ and $\mathbf{GP_2}$. These proteins form dimers, which in turn form trimers to produce the mature and functional heterotrimeric **GP**.[76,77] The mature **GP** protein is transported to the cell membrane in secretory vesicles. vRNA is replicated through a complementary positive sense RNA (cRNA) along with **VP35** and **L**.[78] After translation, viral proteins are assembled to form the nucleocapsid, then transported to the plasma membrane. Finally, budding to form new virus particles is mediated by **VP40** and **GP** at the membrane (Figure 3, Table 4).[79]

Genome Structures. Schematic diagrams for the genomes of coronaviruses (*Coronaviridae*), Dengue virus (*Flaviviridae*), and Ebola virus (*Filoviridae*) are provided in Figure 4. The genes labeled by Roman numerals are encoding for potential druggable protein targets and accord with the same Roman numerals in Figures 2−4 and Tables 1−3. The coronavirus genome is one of the larger known viral genomes at over 30 kb, with the viral replicase gene cassette comprising roughly two-thirds of these nucleotides (orf1a/orf1ab); the remaining one-third contains structural and accessory proteins.[33,80,81] The replicase is translated as two large polyproteins 1a and 1ab, which are autoproteolytically cleaved[22] into the 16 proteins that form the replicase/transcriptase complex (Figures 1 and 4A). The family of *Flaviviridae* viruses, characterized by a positive-sense RNA single-strand, upon cellular recognition, immediately hijacks the host's ribosome to translate its viral RNA. *Flaviviridae* RNA genomes vary in size from about 10.6 to 10.9 kb. The RNA encodes for 10 proteins: three structural and seven nonstructural (Figure 4B). The orfs for the structural proteins contain the nucleocapsid protein (**C**), the envelope protein (**M**), and the major spike (envelope) glycoprotein (**E**) of the capsid. The seven remaining orfs encode for nonstructural proteins (**NS**). The *Filoviridae* genome is a single-strand negative-sense RNA approximately 19 kb in length.[82,83] Seven viral proteins are encoded in this genome: the nucleoprotein (**NP**), a polymerase cofactor protein (**VP35**), a matrix protein (**VP40**), a glycoprotein (**GP**), a transcriptional activator (**VP30**), a nucleocapsid-associated protein (**VP24**), and an **RdRp** (**L**) (Figure 4C).[62,78] EBOV produces some secreted **GP**s, soluble **GP** (**sGP**) and small soluble **GP** (**ssGP**), via a transcriptional editing event derived from the same **GP** gene, whereas MARV does not. It has been reported that **sGP** may act as a decoy antigen to disturb the host immune response as a result of developing antibodies against **GP**s.[76]

In this review, we discuss both validated as well as prospective protein targets under investigation for the potential treatment of *Coronaviridae*, *Flaviviridae*, and *Filoviridae* viral infections. We focus this analysis on natural products and NP-like or inspired inhibitors of viral infection or replication and modulators of immune evasion. We opted to organize our treatment of potential therapeutic targets for the three classes of RNA viruses by biochemical target rather than by virus. The arrangement by virus family was rejected, as very similar targets would contribute to repetitive discussions of the same agents. Additionally, we conceived that insights would be gained by comparison and contrast of the same or similar targets in different virus families and their therapeutic modulators. Finally, therapeutics effective against a given target in one family may have as yet unrealized application to a comparable target in another viral family. For this combination of reasons, we have chosen to arrange and present the natural product inhibitors of these RNA-containing viruses by respective biochemical target. Reviews covering synthetic or other natural product inhibitors have appeared elsewhere and are largely excluded from this review, as are compounds reported with only *in silico* data that have not been further validated.[37,84−86] In addition to the more well-studied targets, we also discuss prospective targets based on recent reports characterizing some of these lesser-known viral proteins and their functions.

Protein Targets and Drug Development. Across viral diversity there are several biochemical themes and mechanisms that are conserved. For example, different families of viruses exploit different host surface proteins to gain entry into target cells, and the general mechanisms employed show great similarity to one another. Similarly, the viral replication cycle has points of commonality across different viral families, including (1) exploiting host proteins to gain entry into cells, (2) commandeering a host's protein manufacturing machinery, (3) manipulating a host's innate immune system to evade detection, and (4) siphoning the host's resources to replicate, mature, and export new virions so as to propagate infection. The proteins responsible for carrying out these biochemical steps, both those of viral and host origin, are presented in some detail and then used to organize this perspective review by target across viral families so as to emphasize similarities and differences. In so doing, we present new connections and present a fresh approach for potential antiviral drug discovery.

As we search for new answers to the threat of global pandemics, where better to begin looking than to nature? In this discussion, we will review natural products and natural product-inspired compounds that have activities reported against viruses of the *Coronaviridae*, *Flaviviridae*, and *Filoviridae* outlined above. We have chosen to focus on these families of RNA viruses because of their potential to cause pandemics in human populations. Here we reveal gaps and shortcomings in past drug development efforts and hope to inspire a renewed vigor in antiviral natural product discovery. Generally, the individual protein targets that we discuss are homologous between viral families; however that is not to say that every compound discussed herein will be universally applicable. Additional investigation and careful structural analysis of each protein target should be pursued in any drug development campaign.

A. VIRAL PROTEINS

Viruses have many nuanced mechanisms for promoting infection and propagation. They exploit host receptors and proteases to enter undetected into cells, then suppress the innate immune response, and ultimately manipulate the host to manufacture new virions for export, all with only a handful of viral proteins at their disposal. There are four main classes of proteins that cover the majority of druggable space of these viral genomes: structural proteins such as the spike protein, nonstructural proteins such as viral proteases, proteins involved in replication, and accessory proteins that perform a variety of functions nonessential for replication but important for propagation. This latter class of protein may prove to be useful targets as well.

Structural Proteins. Structural proteins make up a smaller portion of the viral genome; however it is well known that the spike glycoproteins facilitate host recognition and endocytosis. Therefore, spike proteins have been an active target for drug

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discovery and have focused on disrupting binding to host receptors to prevent entry into cells. Indeed, a number of vaccine and convalescent plasma efforts have focused on developing antibodies against spike proteins.[57,87] However, in this section we will review small-molecule NPs and their inspired agents that inhibit the function of these receptor-binding proteins and thus block viral uptake into cells (Figure 5).

The *Coronaviridae* have five structural proteins in their genomes including an **S** protein responsible for cellular recognition, a small envelope (**E**) protein that is essential for proper virion assembly in most coronaviruses and is implicated in inducing apoptosis, a membrane (**M**) protein that comprises the majority of the virus envelope, an RNA-binding nucleocapsid (**N**) protein that complexes with genome RNA to form the viral capsid and is also known to be an interferon antagonist, and in some betacoronaviruses, a hemagglutinin-esterase (**HE**) protein that forms a second, smaller spike on the envelope. This second spike protein may serve to enhance virus attachment via binding to sialic acid-containing molecules. These proteins provide a vessel for the viral RNA and perform critical functions during assembly, though they are seldom the target of drug discovery efforts because of the late-stage nature of their existence in viral replication. After the emergence of SARS in 2002, there has been an ongoing effort to develop inhibitors for these viruses with an emphasis on preventing viral entry via inhibition of **S** binding. Inhibitors of this spike−receptor binding interaction have largely been based on peptide fragments of the **S** protein[88,89] or variations thereof.[90] Although these showed efficacy *in vitro*, the translation of these peptides into the clinic may be arduous, and as of yet, none have been approved for treatment for coronavirus infections.

Two natural products, griffithsin (**2**) and emodin (**3**), have been validated as disruptors of viral receptor binding to date, inhibiting SARS-CoV **S** at 48 nM and 200 μM respectively.[91,92] Compound **2**, a small protein isolated from the red algae *Griffithsia*, has demonstrated broad-spectrum ability to bind to viral glycoproteins and prevent binding to cellular receptors including HIV and EBOV with minimal toxicity.[93] The activity of **2** appears to be due to several binding sites for monosaccharides such as mannose and glucose, and it is currently under development for use in the clinic. Compound **3** is a small plant-derived anthraquinone that was found to specifically inhibit **S** protein interaction of SARS-CoV1/2 with host-derived ACE2 in a dose-dependent manner.

Because there are many *Flaviviridae* that pose a risk of endemic disease, we opted to use the Dengue virus as a proxy for all *Flaviviridae* (Figure 2, Table 3), as discussing all members of this family would become highly repetitive. In general, the *Flaviviridae* have three structural proteins, the capsid (**C**), **M**, and **E** proteins (Figure 2, Table 3). The **C** protein forms the capsid that becomes unraveled inside the endosome during endocytosis. Few efforts have been directed toward inhibiting **C**. One example is the dissociation of **C** from lipid droplets by the compound nordihydroguaiaretic acid (**4**) isolated from the creosote brush. Fat metabolism and lipid droplets play a role in Dengue viral replication.[94] The **M** protein is initially translated as a pro-protein (**prM**), and the precursor portion is cleaved in the trans Golgi network by a host furin protease. However, the precursor portion is often not cleaved, and many immature viral particles are secreted. It remains to be determined if furin is a good drug target; however, Imran et al. developed a peptide that inhibits furin cleavage of **prM**, and Braun and Sauter in their 2019 review argue that a furin inhibitor could be useful for both combating infectious diseases as well as cancer.[95] The major envelope protein (**E**) is essential for the virus to enter the cell via clathrin-mediated endocytosis. There are several host receptor candidates, but one suggestion appears to be the glycosaminoglycan (GAG) receptor. The **E** protein binds to GAGs, some of which contain heparin sulfates, and the heparin sulfate mimic PG545 (**5**) disrupts that binding by acting as a decoy receptor.[96]

The structural proteins in *Filoviridae* include the nucleoprotein (**NP**), which encapsulates the viral genome, glycoprotein (**GP**), nucleocapsid-associated protein (**VP24**), and matrix protein (**VP40**). The **GP** is an essential spike protein that, like many other viruses, interacts with a receptor expressed on the host cell to facilitate entry. A screening program evaluated 373 extracts from 128 traditional Chinese medicines for activity as EBOV inhibitors. The extract of *Rhodiola rosea* (Crassulaceae) was identified as a specific inhibitor at 12.5 μg/mL using a pseudotyped EBOV screening test.[97] Furthermore, ellagic acid (**6**) and gallic acid (**7**) of *R. rosea* were the most effective compounds in this assay system. Compound **6** had an IC_{50} value of 1.4 and 6.4 μM against EBOV and MARV pseudovirions, respectively, while **7** showed an approximately 4-fold lesser activity against each pseudovirion. It should be noted that **6** and **7** are often classified as pan-assay interference compounds (PAINS) in that they give false-positive results in a wide range of biological screening assays. The mechanism by which these compounds (**6** and **7**) block viral entry is not clear; however, it was suggested that they act at a similar postbinding step in the endosome as the cathepsin B inhibitor CA074 or as the entry inhibitor benztropine that binds to the EBOV-**GP** and interferes with **GP**-mediated fusion in the endosome.[98]

Two triterpenoid derivatives (**8** and **9**), derived from the naturally occurring oleanane-type triterpene echinocystic acid, were found to inhibit EBOV−host fusion.[99] The IC_{50} values for **8** and **9** against the EBOV pseudotyped viruses were 59.2 and 467.3 nM, respectively, as evaluated in A549 and 293T cells. Heptad repeat-2 (HR2), a prevalent heptad repeat sequence comprising an α-helical coil in the **GP**, was identified as a site accessible to these triterpenoid analogues and results in antagonizing EBOV−cell fusion. This results from interacting with the hydrophobic helix and blocking of the HR1-HR2 interaction critical to common trimer-of-hairpins formation. In addition, compounds **8** and **9** were able to inhibit the infection of MARV using a Marburg pseudoparticle entry assay with IC_{50} values of 2.29 and 5.52 μM, respectively.

Kononova et al. reported that an *N*-heterocyclic borneol derivative exhibited antiviral activity via inhibition of MARV-**GP**-dependent virus entry.[100] Borneol is a bicyclic monoterpenoid found in essential oils of numerous medicinal plants. The *N*-heterocyclic bornyl ester **10**, containing a methylpiperidine moiety, showed an IC_{50} value of 4 μM using a MARV **GP**-mediated VSIV pseudotype system.

The natural product cyanovirin-N (**11**) is an 11 kDa protein that was isolated from cultures of the cyanobacterium *Nostoc ellipsosporum*.[101] It has been reported that **11** inhibits HIV infection by binding to the surface envelope glycoprotein (gp120) through an interaction between **11** and high-mannose oligosaccharides on gp120. In 2003, Barrientos et al. reported that **11** also binds with high specificity to the EBOV **GP** and

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shows antiviral activity *in vitro* with an EC_{50} value of 100 nM.[102] Similar to its inhibitory mechanism of HIV infection, oligosaccharide-mediated **11**−**GP** interaction plays an important role in the inhibition of EBOV infection.

Proteases. Although there are only one to three proteases in each of the virus families we discuss here, they are absolutely essential for replication. Importantly, after initial translation of the viral RNA into large polyproteins, these proteases, from within the polyprotein, fold into active form and cleave the polyprotein into its individual components.[16] These cleaved proteins in turn form the replicase complex that allows the virus to reproduce. Inhibition of these proteases has proven to be an effective way to mitigate replication and spread of the viruses. Because they are so few and so essential, a large amount of effort has gone into drug discovery efforts to screen and generate new protease inhibitors (Figure 6). In this section we cover recent reports of viral protease inhibitor discovery and outline suggestions for future success in this area. Interestingly, the filoviruses do not possess their own proteases and rely completely on the host proteases for replication.

Coronaviruses, with the exception of SARS-CoV-1/2, possess three proteases including two papain-like proteases and a chymotrypsin-like protease (**3CLpro** or **Mpro**); the latter protease does not have a known human homologue. SARS-CoV-1/2 lack one of the papain-like proteases, although this does not appear to have any impact on its ability to replicate and is likely redundant in other coronaviruses. These proteases are responsible for viral protein maturation after translation and have been at the center of drug discovery efforts against coronaviruses. That they are each essential for viral replication makes them especially attractive for targeting with small molecules. A large body of work exists in the literature of synthetic compounds targeting these proteases and has been reviewed previously.[36,37,103] Because of their inherent peptidase activity, much effort has been put toward designing peptidomimetic inhibitors of these proteases. The main protease sequence is quite conserved across the *Coronaviridae* family and beyond, and inhibitors designed for **3CLpro** have found use against other viruses as well. Extensive structure−activity relationships have been established for this enzyme, and several crystal structures have been reported with bound inhibitors (PDB: 1UK4, 4YOG, 6Y2F, and others). One of the most important features of these inhibitors is the absolute requirement for a Gln or Gln-like residue at P1 followed by a generally hydrophobic side chain at P2; the latter pocket has been shown to be quite flexible. Interestingly, this specificity is not seen in any human enzyme, thus making the **3CLpro** a prime target for peptidomimetic inhibition without competing off-target effects. A number of compounds with micromolar to nanomolar *in vitro* activity are depicted in Figure 6, most notably the approved drug rupintrivir (**12**), which was developed earlier to target the rhinovirus **3C** protease. Some natural products, mainly flavonoids, have been reported to have mild inhibitory activity against **3CLpro** as well including herbacetin (**13**), pectolinarin (**14**), and rhoifolin (**15**) with IC_{50} values of 33, 38, and 27 μm, respectively.[104] Interestingly, there have been very few efforts to screen natural products against this promising target.[105] However, there have been several screening studies that have identified natural product inhibitors of *Coronaviridae* propagation without identifying a target; some of these may target the **3CLpro** protease, and we will cover these in a later section.

The papain-like protease cleaves the viral polyprotein at fewer sites than the main protease, though inhibition is still effective at preventing replication. As a whole, this protease is less sought after as a drug target due to closer homology to human enzymes that may lead to off-target effects. As a result, there has been little development of peptidomimetic molecules designed against this protease. In contrast, there have been a number of reports of natural products inhibiting its activity including tomentin B at 6.1 μm[106] (**16**), hirsutenone at 4.1 μm[107] (**17**), tanshinones from 14.4 to 226.7 μm[108] (**18**), psoralidin at 4.2 μm[109] (**19**), and others depicted in Figure 6.[110] Many of these phytochemicals were identified from extract screening, and like the **3CLpro**, there has not been a large-scale effort to explore a wider diversity of natural product scaffolds against it. With most compounds reported eliciting low micromolar activity and inherently nonspecific nature of planar, aromatic phytochemicals, there remains much to be desired. Nevertheless, these studies may provide a good starting point for a medicinal chemistry campaign.

The *Flaviviridae* genome is smaller than the *Coronaviridae* and only contains one orf that encodes for a protease, the **NS3** orf (Figure 4B). **NS3** belongs to the S7 serine protease family and requires **NS2b** as a cofactor for full activity.[111] The **NS2B-NS3** protease was virtually screened, and triterpenoids from *Ganoderma lucidum* were suggested to be active. When tested in an *in vitro* Dengue virus inhibition assay, ganodermanontriol (**20**) (Figure 6) was found to inhibit the virus by 25% at 25 μM.[112] The **NS2B-NS3** complex is crucial for cleavage of the polypeptide as well as providing helicase activity during replication. Ivermectin (a mixture of two analogs, only one shown, compound **21**) (Figure 6), isolated from *Streptomyces avermitilis*, is a well-known antiparasitic drug. By molecular modeling it was determined that it could also bind effectively to the helicase portion of **NS3** where the single-stranded RNA is bound. Ivermectin (**21**) has been found to reduce SARS-CoV-2 viral loads by 5000-fold in Vero cells treated at a high dose (2.5 μg/mL, >50 times the dose used in humans to treat onchocerchiasis).[166] Various reports of the prophylactic or early stage treatment with ivermectin are appearing in Rxiv form from India and South America, with indications that the target is helicase as found in Dengue virus; however, there are cautionary notes about its premature widespread use.[167,168] Nevertheless, an approved clinical treatment protocol has appeared in Peru (the I-MASK+ protocol; see https://covid19criticalcare.com/). Compound **20** also inhibits Dengue helicase. **NS2B-NS3** is an important target, and continued investigation and identification of inhibitors for the **NS2B-NS3** complex is warranted.

Replicase/Transcriptase Complex Proteins. The viral replicase complex consists of several key proteins including the **RdRp** and helicase that are solely responsible for generating new genomic as well as subgenomic viral RNAs; these in turn are responsible for the production of structural proteins that assemble into new virions. Several critical features exist in this assortment of proteins, especially the **RdRp**, that can be targeted with small molecules to disrupt replication, and considerable effort has been expended to discover inhibitors of **RdRp** activity. Approved drugs such as favipiravir (**22**), ribavirin (**23**), and remdesivir (**1**) were all developed to target this polymerase, and its highly conserved nature across viral families has made it a promising target for drug development. In this section we discuss the various NP-inspired **RdRp** inhibitors that have been discovered to date (Figure 7).

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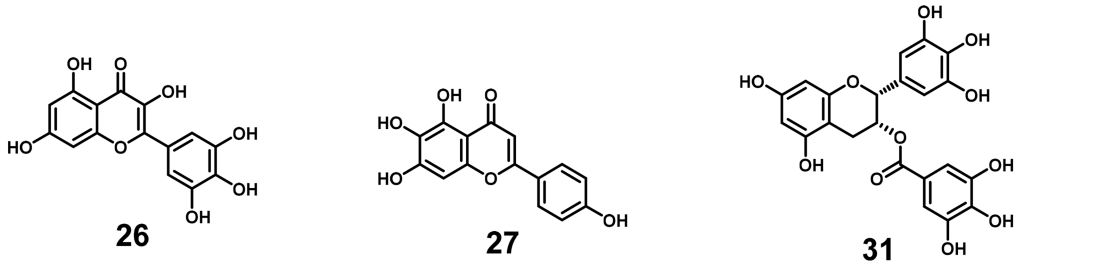

Figure 8. Inhibitors of viral helicase. All of these are natural products.

In the *Coronaviridae*, the majority of the known effective inhibitors of **RdRp** are derived from its natural nucleotide substrates. These NP-inspired **RdRp** inhibitors include favipiravir (**22**), remdesivir (**1**), ribavirin (**23**), galdesivir (**24**), and β-D-N4-hydroxycytidine (**25**).[36,113] While these nucleotide analogues were developed as inhibitors of **RdRp** activity in other viruses, they also have activity to these more newly emergent coronaviruses. *Coronaviridae* helicase **NSP13** is also instrumental for viral replication, and two flavonoid natural products, myricetin (**26**) and scutellarein (**27**), have shown inhibitory activity with IC_{50} values of 2.71 and 0.86 μM, respectively (Figure 8).[114]

In the *Flaviviridae*, the **NS2B-NS3** complex confers helicase activity as well as protease activity; the **NS5** protein has been designated as the **RdRp**. **NS5** is indirectly inhibited by mycophenolic acid (MPA) (**28**) (Figure 7), a natural product originally isolated from *Penicillium glaucum* in 1896. MPA as well as **23** inhibit inosine monophosphate dehydrogenase and, in so doing, limit the amount of guanosine available to the RNA polymerase for RNA synthesis.[115] Several synthetic compounds have been designed as direct inhibitors of **NS5**.[116] The **NS4** orf produces two full-length membrane proteins, **NS4A** and **NS4B**. They are linked by a 23-residue C-terminal region of **NS4A**, and the full-length **NS4** is cleaved by the **NS2B-NS3** protease into **NS4A** and **2K-NS4B**. The 2k fragment is a signal peptide that traffics **NS4B** to the endoplasmic reticulum (ER). **NS4A** also inserts into the ER membrane and is quite hydrophobic due to its eight transmembrane regions. **NS4A** stabilizes the membrane-associated replication machinery.[51] Reddey et al. have identified several possible inhibitors to both **NS4A** and **NS4B**.[51]

Methyltransferase (MTase) is an important enzyme for replication of *Coronaviridae* and *Flaviviridae* and is crucial for their RNA cap formation. The natural product sinefungin (**29**) was first isolated as an antibiotic from a strain of *Streptomyces griseolus* in 1973.[117] This compound has a potent interaction with the MTases of SARS-CoV-2,[118] ZIKV,[119] and DENV (Figure 7).[120]

Natural products have inspired the discovery of additional **L** protein (e.g., RNA-dependent RNA polymerase; see Table 4, Figure 4) inhibitors. Compound **22** (Figure 7) mimics the structure of nucleic acids and has a broad-spectrum activity against a wide variety of both negative-strand and positive-strand RNA viruses. It is first converted to its phosphoribosyl derivative and subsequently to the triphosphate before it inhibits the RNA polymerase, principally through direct competition with GTP.[121] Oestereich et al. reported that **22** suppressed replication of EBOV in cell cultures with IC_{50} and IC_{90} values of 67 μM and 110 μM, respectively.[122] Remdesivir (**1**), a prodrug, is also converted to its triphosphate metabolite and interferes with viral **RdRp** activity. In cell-based assays, it has a potency against a broad range of filoviruses including MARV and several variants of EBOV.[123] Compound **1** also inhibited EBOV replication in multiple relevant human cell types with EC_{50} values of 0.06−0.14 μM. Another synthetic adenosine analogue, BCX4430 (**30**), inhibits RNA polymerase function by inducing RNA chain termination, which occurs two bases after the incorporation of **30** monophosphate, perhaps as a result of inhibitory stereochemical distortions of the nascent RNA chain.[124] Moderate antiviral activity of **30** has been reported against members of the *Filoviridae*; EC_{50} values were 11.8 μM for EBOV, 3.4 μM for SUDV, and 4.4−6.7 μM for MARV, respectively. Weak activity against positive-sense RNA viruses has also been reported: DENV-2 (EC_{50} 32.8 μM), SARS-CoV (EC_{50} 57.7 μM), and MERS-CoV (EC_{50} 68.4 μM).

Daino et al. have tested the extract from *Limonium morisianum* (Plumbaginaceae) in a fluorescence-based **rVP35**-dsRNA interaction assay.[125] The extract was shown to inhibit **VP35**-dsRNA binding at a concentration of 19 μg/mL, and two flavonoid compounds, myricetin (**26**) and (−)-epigallocatechin-3-*O*-gallate (**31**), were identified as the active inhibitors (IC_{50} values were 2.7 and 43.5 μM respectively). In addition, molecular docking studies revealed that **26** binds to the highly conserved region of the **VP35** RNA binding pocket. However, compounds **26** and **31** may also be considered PAINS due to their widespread biological activity.

Accessory Proteins. Accessory proteins are typically not considered essential for replication but play other roles in the viral life cycle such as immune evasion, targeting degradation of host RNAs, and inducing apoptosis. Some viruses have few accessory proteins, whereas others have many, such as the coronaviruses. Several have been characterized and their biochemical function is known; for others they remain to be investigated. In this section we cover a collection of known accessory proteins and their functions and discuss opportunities for drug discovery.

Two notable accessory proteins from the *Coronaviridae* include **NSP1**[126] and **orf8b**,[127] and a comprehensive review of the full list of accessory proteins and their role in pathogenesis was reported in 2012.[128] Both of these proteins were found to take part in immune evasion by two distinct mechanisms. **NSP1** was reported to promote host mRNA degradation and thus suppress host gene expression, including proteins involved in the host innate immune system. Alternatively, **orf8b** has been observed to trigger intracellular stress pathways via

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formation of insoluble aggregates that induce ER stress, lysosomal damage, and subsequent activation of transcription factor EB involved in lysosomal biogenesis, leading to cell death. However, there have been no reports of inhibitors targeting these proteins to date, and clarification of the mechanisms by which these viruses evade immune detection is still needed.

The *Flaviviridae* **NS1** protein appears to be an anti-host factor; however, it has many attributed functions and mechanisms. As indicated above, the Zika virus **NS1** has been shown to stabilize metalloproteinase 9 (**MMP9**). A higher concentration of **MMP9** aids viral entry into cells by breaking down tight intercellular junctions and cellular structural proteins.[48] However, in the West Nile virus it appears that **NS1** downregulates interferon, and in Dengue it breaks down the glycocalyx. Another function appears to be as a GAG-binding protein. Regardless of mechanism, **NS1** inhibitors could be promising candidates for development. An effort to inhibit **NS1** binding was achieved with a synthetic heparan sulfate mimetic, **5**. This mimic blocked **NS1** binding completely and decreased viremia in mice.[96] **NS1** is also indirectly inhibited by the bicyclic alkaloid castanospermine

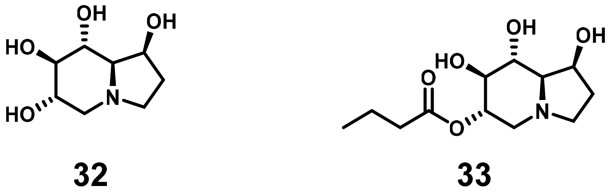

Figure 9. Inhibitors of selected accessory proteins. Celgosivir is a derivative of the natural product castanospermine.

(**32**) as well as its prodrug ester celgosivir (**33**) (Figure 9). These compounds interfere with the glycosylation of NS1 and create misfolding of the protein.[129,130]

B. HOST PROTEINS

Viruses often utilize host proteins, such as cathepsin L (**CatL**) or **TMPRSS2/4** in the case of SARS-CoV-1/2, for cellular recognition, entry, and translation. Thus, these host proteins can also be targeted to hinder viral infections. In this section we cover the host proteins of interest in antiviral drug discovery, their functions, and efforts to find suitable inhibitors among natural products and their derivatives. It should be noted that the compounds discussed in this section are preclinical agents, and toxicity and other side effects will need to be assessed when targeting host proteins.

Proteases. After receptor binding, host proteases either at the cell surface or in endosomes are responsible for critical cleavage events that induce fusion of the viral capsid with the cell or endosome membrane and the subsequent release of its genetic material into the cell cytoplasm. Across the three families of viruses in this review, it appears that only a few host proteases are implicated, none of which are completely essential for viability. There have been a number of drug discovery campaigns targeting these proteases in the context of other diseases and, more recently, a recognition that they may be viable antiviral drug targets as well. In this section, we will cover a number of inhibitors of host proteases that prevent viral entry by blocking processing of the spike glycoproteins and membrane fusion (Figure 10).

TMPRSS2 and **TMPRSS4** are transmembrane serine proteases located on the surface of human cells. They have been shown to cleave the coronavirus extracellular spike protein **S** after the virus has bound to surface **ACE2** receptors, and this allows viral fusion to the cell membrane. These proteases are essential for viral entry in several different tissues.[24,28] Despite the essentiality of this process, there are very few known inhibitors, and only one is a natural product. Aprotinin (**34**) is a small protein that is marketed as a bovine pancreatic trypsin inhibitor; it has also shown inhibitory activity against **TMPRSS2**.[131] In addition to these surface proteases, cathepsin L and B (**CatL/B**) promote membrane fusion of the virus via an endocytic pathway. Unlike **TMPRSS2/4**, there are a number of reviews describing the activity and role in human physiology and disease of **CatL** and **Cat-B**.[132,133] Furthermore, a number of natural products have been reported with inhibitory activity against these important enzymes, such as gallinamide A (**35**), nicolaiodesin C (**36**), grassypeptolide (**37**), and leupeptin (**38**), against **CatL**. The wide variety of natural products effective and selective against these proteases raises hopes that useful antiviral therapeutics may emerge from continued investigation.

CatL was also reported to be involved in the entry of JEV into cells.[134] It was shown that a **CatL** inhibitor could decrease shedding of the *Flaviviridae* anti-host factor protein **NS1** with a subsequent decrease in endothelial permeability. It would be worthwhile to continue to explore cathepsin L inhibitors for use in treatment of Dengue, JEV, and other *Flaviviridae* viruses.[135]

The mostly linear lipopeptide gallinamide A (**35**), isolated from a *Schizothrix* sp. cyanobacterium, selectively inhibited **CatL** with an IC_{50} value of 5.0 nM.[136] It was also shown that **35** was remarkably selective for **CatL**, as it was only modestly active to **CatB** with an IC_{50} value of 11.7 μM. From the marine sponge *Theonella* aff. *mirabilis*, tokaramide A (**39**) and miraziridine A (**40**) were isolated. These selectively inhibited **CatB** with IC_{50} values of 29 ng/mL and 1.4 μg/mL, respectively.[137−139] Zhang et al. reported a new aloperine derivative (**41**) that exhibited activity against EBOV and MARV with EC_{50} values of 4.8 and 7.1 μM, respectively. Aloperine was reported as a component of the seeds and leaves of *Sophora alopecuroides* (Fabaceae). Aloperine was shown to selectively inhibit **CatB** but had no activity toward **CatL**.[140] This remarkable selectivity of **41** for **CatB** over **CatL** was subsequently explained through a molecular docking analysis.

Chandran et al. measured the antiviral effects of the peptide derivatives E-64d (**42**) and CA074 (**43**) using EBOV. Vero cells were pretreated with **42** (300 μM) or **43** (80 μM) and exposed to EBOV. Yields of infectious EBOV progeny and expression of cell-associated GP_1 were markedly reduced, suggesting that EBOV multiplication in Vero cells is sensitive to these inhibitors of endosomal cysteine proteases in general and of **CatB** in particular.[75] Compound E-64d (**42**), also known as EST, is a synthetic analogue of E-64 that was first isolated from the extract of a solid media culture of *Aspergillus japonicus*.[141,142] Additionally, **43**, a new epoxysuccinyl peptide with a structure similar to **42**, was designed as a specific inhibitor of **CatB**.[143]

Furin is a protease located in the trans golgi apparatus. It belongs to the subtilisin-like pro-protein convertase family, and it aids in cleaving precursor proteins into mature proteins.

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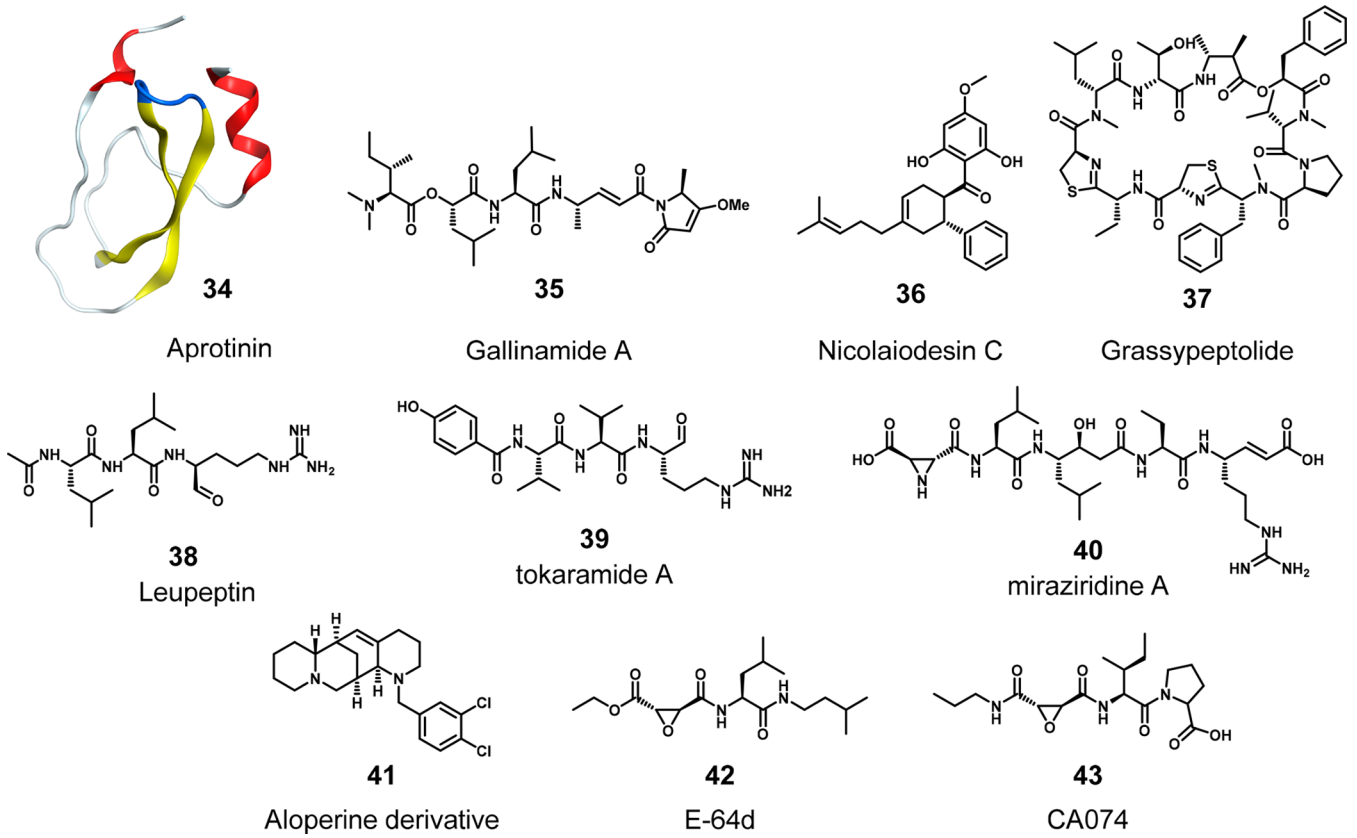

Figure 10. Inhibitors of host cysteine proteases cathepsin L/B or serine proteases TMPRSS2/4. The following are natural products: aprotinin, gallinamide A, nicolaiodesin C, grassypeptolide, leupeptin, tokoramide A, miraziridine A, E-64d, and natural product derivatives: **43**, CA074.

During viral infections, furin cleaves a precursor spike protein into mature spike **S** proteins.[95] As discussed above for the *Flaviviridae*, furin is also responsible for cleavage of the **prM** protein to the **M** protein; however, some **prM**-coated viral particles escape from the cell before furin cleavage. These immature virus particles are known to generate antibodies, and these antibodies are proposed to enhance the disease if an individual is infected with a different serotype of the Dengue virus.[144] Therefore, furin is not likely a good drug target, as it may increase the severity of subsequent infection with Dengue.

Others. There are a handful of other targets that show promise in additional areas of the viral life cycle and may be used in combination with direct inhibitors of viral proteases or **RdRp**. Here we discuss these targets and their potential for inhibition by natural products to hinder virus reproduction and distribution (Figure 11).

Calmodulin (calcium-modulated protein) in normal cells senses calcium levels and regulates calcium flux into the cells. During Dengue infection the viral protein **NS2A** interacts with calmodulin. A synthetic calmodulin inhibitor was shown to inhibit virus production,[52] suggesting that natural products that are calmodulin inhibitors could also be useful. In fact, plants and fungi have been a rich source of structurally diverse calmodulin inhibitors, and these should be further evaluated for their anti-Dengue effects.

The normal function of signalase/signal peptidase is to cleave signal peptides when they are trafficked to the endoplasmic reticulum.[145] In Dengue, the host cell signalase is used to cleave the viral polyprotein between the **prM**, **E**, **NS1**, and **NS4B** proteins. In a phenotypic screen the fungal natural product cavinafungin (**44**) was shown to inhibit replication of all four serotypes of dengue virus, as well as Zika virus, with an IC_{50} in the low nanomolar range. Compound **44** was initially isolated in 2015 from the fungus *Colispora cavincola*, and subsequently, the antiviral target was identified as signalase by using a CRISPR/Cas9-based chemogenomic profiling where the subunits of the signal peptidase were identified.[146]

Alpha-glucosidase, located in the ER, adds *N*-linked sugars to proteins. Inhibitors that block this addition have been shown to decrease the production of several ER-budding viruses, such as Dengue and Japanese encephalitis virus.[147] In particular, these inhibitors affect the glycosylation of the **NS1** and the **E** proteins. Oral administration of glucose mimics such as nojirimycin (**45**) and its derivative 1-deoxynojirimycin (**46**) to mice was effective at inhibiting viral replication.[147] Compound **46** (duvoglustat or moranolin) was first reported in mulberry leaves.[148] Medicinal chemistry efforts starting with derivative CM-10-18 of deoxynojirimycin resulted in potent inhibitors of alpha-glucosidase. Moreover, they were shown to inhibit bovine viral diarrhea virus (a proxy for Dengue virus) at high nanomolar or low micromolar ranges while not showing any overt cytotoxicity.[149] Furthermore, miglitol (a derivative of 1-deoxynojirimycin) and acarbose, approved type-II diabetes drugs that target alpha-glucosidase, have also shown antiviral properties to Filoviruses and Flaviviruses.[150,151]

The 80S ribosome is a very general target that, when inhibited, blocks all translation including production of viral polyproteins. Geneticin (**47**), an analogue of neomycin, was tested for its ability to inhibit Dengue virus and found to inhibit the cytopathic effect resulting from Dengue virus infection with an EC_{50} of 3.0 μg/mL in BHK cells. Curiously,

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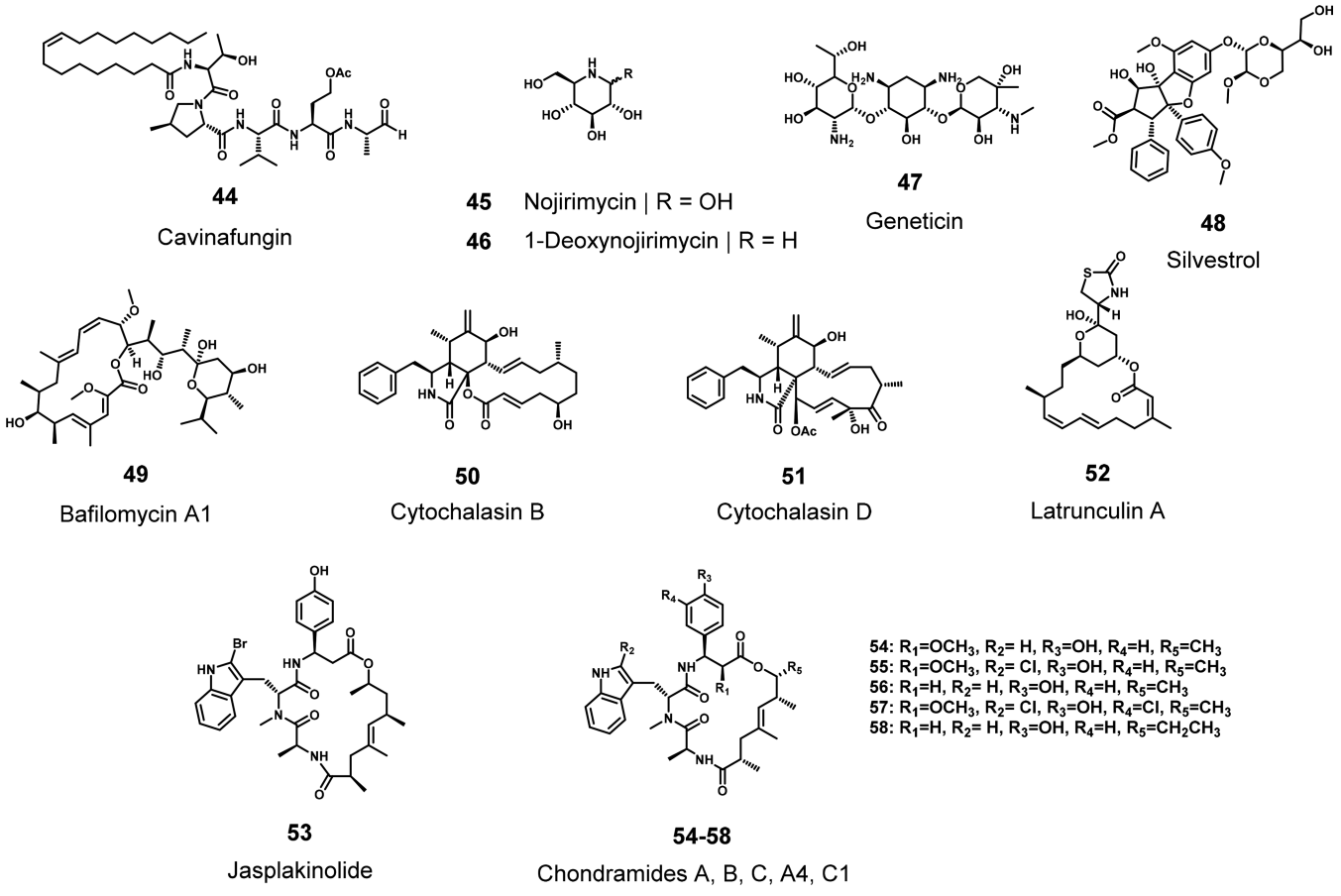

Figure 11. Inhibitors of other selected targets with activity to RNA viruses. All of these compounds are natural products.

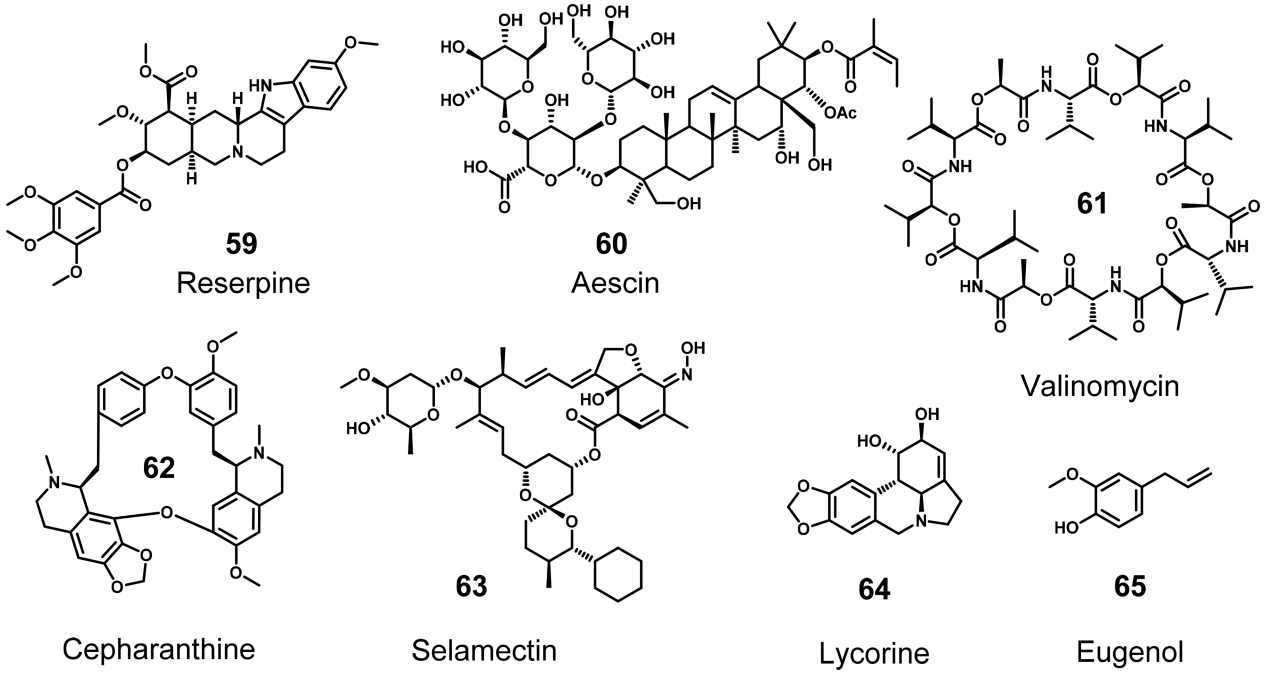

Figure 12. Inhibitors with demonstrated antiviral activity without a known target or mechanism of action. All of these compounds are natural products.

the closely related analogues kanamycin, gentamycin, and guanidylated geneticin showed no protective effect in this cytopathic assay.[152]

Silvestrol (**48**), isolated from the fruits and twigs of *Aglaia silvestris* (Meliaceae),[153] is a potent inhibitor of the ATP-dependent DEAD-box RNA helicase **eIF4A**. This helicase

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activity appears to be essential for 5′-cap-dependent translation of mRNAs with highly structured 5′-UTRs to enable binding of the translation preinitiation complex in eukaryotes.[154] Biedenkopf et al. reported inhibition of viral propagation by treatment with 10 nM silvestrol.

It has been suggested that EBOV **GP**-mediated entry and fusion requires acidification within the endosome. This acidification is produced by vacuolar ATPases that create a proton gradient. Yonezawa et al. pretreated target cells with the vacuolar ATPase inhibitor bafilomycin A1 (**49**), a macrolide antibiotic isolated from mycelia of *Streptomyces griseus*.[155] They evaluated the effects of incubation of **49** in a virion pseudotyped with EBOV **GP**.[156] As a result, treatment with compound **49** at 5−500 nM nearly completely blocked detection of viral entry and fusion mediated by EBOV **GP**.

Yonezawa et al. also reported that compounds that impair microfilament function inhibit EBOV **GP**-mediated entry and fusion. They demonstrated that cytochalasins B (**50**) and D (**51**), obtained from the molds *Helminthosporium dematiodeum* and *Metarrhizium anisopliae*,[157] latrunculin A (**52**) from the marine sponge *Latrunculia magnifica*,[158] and jasplakinolide (**53**) from the marine sponge *Jaspis johnstoni*[159] were all active inhibitors of EBOV cell entry. They suggested that microtubules and actin filaments play key roles in these antiviral events. Similarly, Beck et al. reported that the chondramides (**54**−**58**), antifungal and cytostatic depsipeptides, inhibited EBOV **GP**-mediated cell entry with IC_{50} values of 24−42 nM. The chondramides are known to exert modulatory effects on the actin cytoskeleton.[160] Chondramides A (**54**), B (**55**), C (**56**), and A4 (**57**) and propionyl chondramide C1 (**58**) were isolated from two myxobacterial strains, *Chondromyces crocatus* and *Chondromyces* sp. MSr9030.[161,162]

C. UNKNOWN TARGETS

During the course of drug discovery there have been a number of reports that do not specify a target or investigate a mechanism by which a compound acts. Although the compounds reported in this section were shown to have antiviral activity, the nature of their effect is unknown and requires more investigation (Figure 12). A small screening effort revealed several disparate micromolar inhibitors of SARS-CoV infection including the existing natural product drugs reserpine (**59**) (an indole alkaloid from Indian snakeroot *Rauvolfia serpentina*), aescin (**60**) (saponins from the horse chestnut *Aesculus hippocastanum*), and valinomycin (**61**) (a cyclic depsipeptide from *Streptomyces* spp.).[105] In a recent study, the FDA-approved cyclic alkaloid cepharanthine (**62**) and a veterinary product related to ivermectin (**21**), selamectin (**63**), were found to completely inhibit the cytopathic effects of betacoronaviruses in cell culture at 10 μM; however, no target nor mechanism was proposed.[163] The alkaloid lycorine (**64**) was also found to inhibit viral replication with an EC_{50} of 15.7 nM.[164] Eugenol (**65**), a ubiquitous phenolic compound in plants, was found to have activity against EBOV with an EC_{50} value of 1.3 μM.[165] Further exploration of the targets and mechanisms of these reported natural product inhibitors would be invaluable for future antiviral drug development.

SUMMARY AND OUTLOOK

Natural products have been underexplored for their potentially useful antiviral activity, especially to RNA viruses causing endemic and pandemic infections. Nevertheless, several useful compounds based on natural products have emerged from these efforts, most notably in the purine-based inhibitors of the viral replicase complex component, the RNA-dependent RNA polymerase (**RdRp**), and protease inhibitors, including those that target proteases that are virally encoded and those that are host derived. Another broad class of natural product with anti-RNA virus activity include those with polyphenolic structures; however, these are generally considered to be PAINS and nonselective to these viral targets. From a broad perspective, this review covers anti-RNA virus natural products that illustrate a large number of different molecular architectures, suggesting a variety of enzymatic protein targets and a range of inhibitory mechanisms. This foreshadows an even richer potential for the contribution that natural products can make to our antiviral pharmacopeia as more thorough and broader screening occurs in the future.

It is clear that human populations will continue to see more endemics and pandemics in the future, be they caused by viruses, bacteria or other infectious agents. Thus, it is simply common sense that we should put into place the infrastructure necessary to more rapidly develop treatments when future pandemics occur. One such recommendation is to create and maintain international compound libraries with substances that possess antiviral, antibacterial, or antiparasitic activity. These could be rapidly deployed into relevant biological screening systems as new pandemics arise. This resource could be internationally housed, and a logical entity might be the World Health Organization. But to accomplish this, new types of international treaties and agreements need to be drawn up in advance to cover the evolving concepts of intellectual property and inherent national ownership of genetic resources. Similarly, new international legislation needs to be written in advance of the next pandemic so as to cover the rights and responsibilities of international scientific teams so that they may form quickly and with a transparent sharing of data and results. Because the private sector is likely the segment of society that will bring new therapeutics to people, laws and policies that protect economic interests while simultaneously promoting openness and collaboration need to be put in place. Ultimately, the discovery and development of new pharmaceuticals from nature provides a justification for biodiversity preservation that is very understandable by the lay public and, thus, is ultimately good for human society, the planet, and the valuation of our rich biodiversity.

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Notes

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