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REVIEW

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Structural and functional similarities in bunyaviruses: Perspectives for pan-bunya antivirals

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Summary

The order of *Bunyavirales* includes numerous (re)emerging viruses that collectively have a major impact on human and animal health worldwide. There are no vaccines for human use or antiviral drugs available to prevent or treat infections with any of these viruses. The development of efficacious and safe drugs and vaccines is a pressing matter. Ideally, such antivirals possess pan-bunyavirus antiviral activity, allowing the containment of every bunya-related threat. The fact that many bunyaviruses need to be handled in laboratories with biosafety level 3 or 4, the great variety of species and the frequent emergence of novel species complicate such efforts. We here examined the potential druggable targets of bunyaviruses, together with the level of conservation of their biological functions, structure, and genetic similarity by means of heatmap analysis. In the light of this, we revised the available models and tools currently available, pointing out directions for antiviral drug discovery.

KEYWORDS

antiviral drugs, *Bunyavirales*, endonuclease, nucleoprotein, phylogenetic analysis, viral polymerase

1 | INTRODUCTION

Currently, two out of eight "prioritized infectious diseases" by the World Health Organization belong to the order of the *Bunyavirales* (previously the family *Bunyaviridae*), ie, Crimean-Congo hemorrhagic fever virus (CCHFV) and Rift Valley fever virus (RVFV).[1] The frequent report of novel bunyaviruses, detected in humans, animals, and plants together with the high genetic diversity among its species, led to a reclassification by the International Committee on Taxonomy of Viruses.[2] This illustrates the ubiquity, the variety of hosts, and the epidemic potential of viruses in the *Bunyavirales* order, which is now a collection of nine viral families, comprising 13 genera (Figure S1). The scope of this review includes the four genera that have been associated with human disease, ie, *Orthohantaviruses*, *Orthonairoviruses*, *Orthobunyaviruses*, and *Phleboviruses* (Figure 1).

Many of these viruses need to be handled in biosafety level (BSL)-3 or -4 laboratories, which complicates the research. Yet, it is important to step up to the challenge since these emerging human viruses are now spread over more than 80 countries in Europe, Asia, Middle East, America, and Africa.[3] The great diversity and continuous emergence of new bunyaviral species that cause severe disease make it unfeasible to develop drugs or vaccines for every single virus.

List of abbreviations: AKAV, Akabane orthobunyaviruses; ANDV, Andes virus; BMP, bismonoacylglycero-phosphate; BSL, biosafety level; BUNV, Bunyamwera virus; CCHFV, Crimean-Congo hemorrhagic fever virus; cRNA, complementary RNA; DC-SIGN, dendritic cell–specific ICAM-grabbing nonintegrin; eIF4F, eukaryotic initiation factor 4F; EREV, Erve virus; HAZV, Hazara virus; HPS, hantavirus pulmonary syndrome; IFNAR$^{-/-}$, lack of alpha/beta interferon receptor; IMPDH, inosine monophosphate dehydrogenase enzyme; IRF-3, IFN regulatory factor-3; KUPV, Kupe virus; LACV, La Crosse virus; LASV, Lassa virus; LEAV, Leanyer virus; L-segment, large segment; MPRLV, Maporal virus; M-segment, middle-sized segment; N-protein, nucleoprotein; P bodies, processing bodies; PTV, Punta Toro virus; PUUV, Puumala virus; RdRp, RNA-dependent RNA polymerase; RVFV, Rift Valley fever virus; SBV, Schmallenberg virus; SFTSV, severe fever with thrombocytopenia syndrome virus; SNV, Sin Nombre virus; S-segment, small segment; UUKV, Uukuniemi virus; vRNA, viral RNA

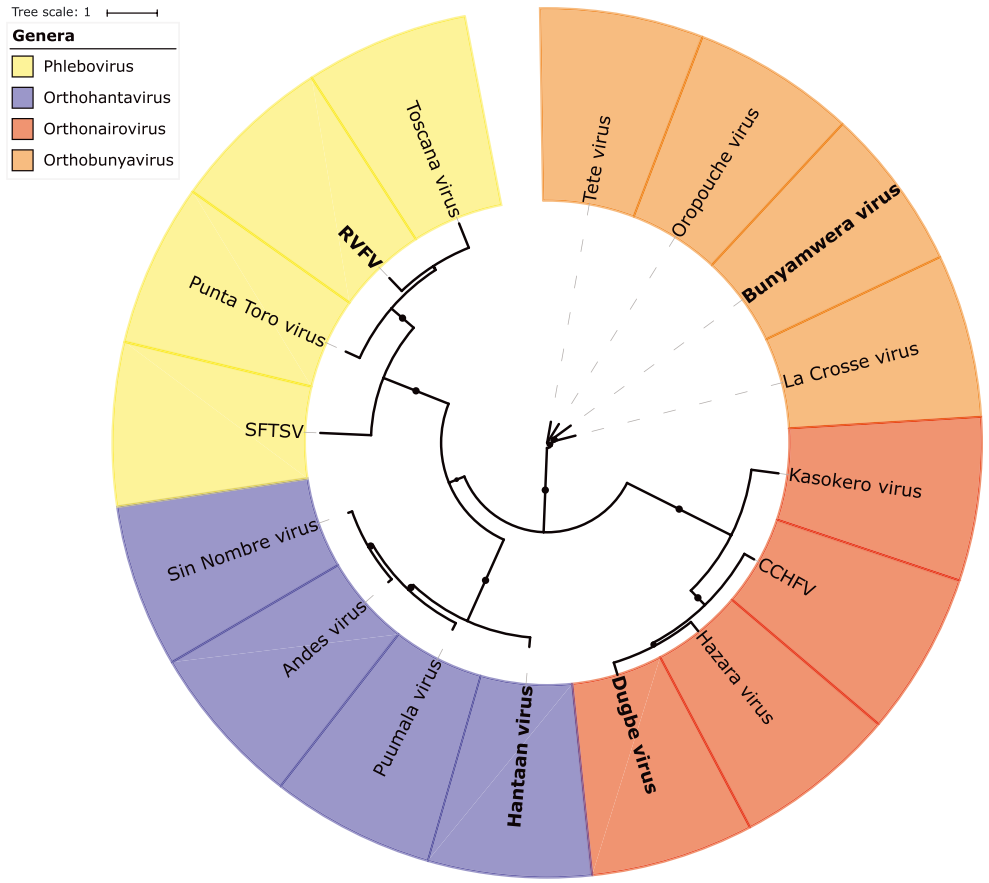

FIGURE 1 Phylogenetic tree constructed with amino acid sequences of L-proteins of bunyaviruses from the orthohantaviruses, orthonairoviruses, orthobunyaviruses, and phleboviruses genera. Names in bold indicate the type of species. Crimean-Congo hemorrhagic fever virus (CCHFV), severe fever with thrombocytopenia syndrome virus (SFTSV), Rift Valley fever virus (RVFV)

Therefore, comprehensive efforts towards the development of antiviral drugs with an extended efficacy against an entire virus family or even virus order need to be made. These offer protection against not only the (re)emerging viruses of today but also from the pandemic threats of tomorrow. To aid in such efforts, we here review the available knowledge of potential druggable targets in the replication cycle of these viruses together with current available tools and models.

2 | PARTICLE STRUCTURE AND VIRAL PROTEINS

2.1 | Virion and genome

Bunyaviruses have spherical 80- to 120-nm-sized enveloped virions. Their lipid bilayer envelop is covered with capsomers consisting of transmembrane glycoproteins (Gc and Gn). The genome exists out of negative sense single-stranded [(−)ss]RNA, which is trisegmented in all human pathogenic genera. These RNA segments, together with oligomers of N-proteins, form looped RNP. A single L-protein is bound to each of the RNA segments (Figure 2).

The large segment (L segment) encodes the RNA-dependent RNA polymerase (RdRp), or L-protein, which is responsible for the

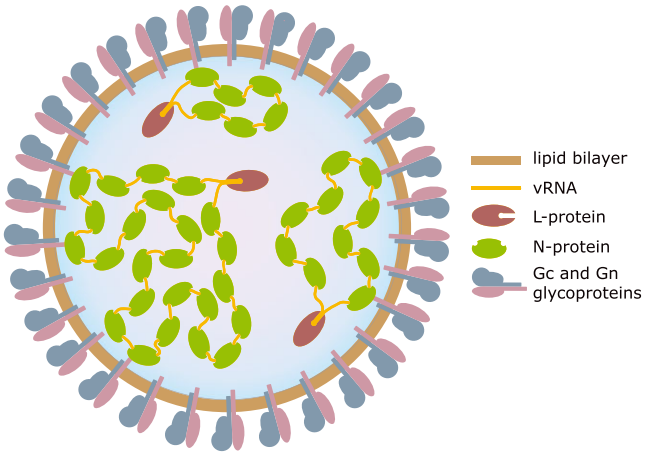

FIGURE 2 Bunyaviral particle. A schematic illustration of the virion showing the structural proteins (Gn, Gc, L, and N), the vRNA genome (L, M, and S segments), and the RNP complexes

production of complementary RNA (cRNA) and viral RNA (vRNA). The middle-sized segment (M segment) encodes for the Gc and Gn glycoproteins. Depending on the species, the M segment also codes for the NSm protein. The small segment (S segment) contains the transcript for the nucleoprotein (N-protein), which, like all above-

mentioned proteins, is encoded in the (−)sense. The S segment further codes for the nonstructural protein NSs in either negative or positive orientation (as in phleboviruses and orthobunyaviruses, respectively) but can also be lacking (as is the case in some orthohantaviruses.[4,5]

2.2 | L-protein

The L-protein is responsible for transcription of the (−)ssRNA and replication of new vRNA. Both these processes occur in the cytosol where newly translated L-proteins can interact with either additional L-proteins to facilitate replication or with newly synthesized vRNA to be assembled into new viral particles.[5] While the size of the L-proteins may vary between families, three subdomains, ie, the finger, palm, and thumb can be distinguished in all L-proteins. The relative orientation of these three subdomains defines the catalytic cavity, which can be attuned to facilitate different stages of replication.[6] All L-proteins recognize highly conserved complementary 3′ and 5′ extremities of the genome segments that either form a double stranded "pan-handle" bound by the L-protein or both ends are bound separately to the L-protein.[7,8] These 5′ and 3′ UTR are acting as promotor and transcription termination signal for the viral polymerase.[9,10] Similar regions were found in influenza viruses, where they also contribute to the activation of several polymerase functions.[11] The endonuclease and RdRp domains of the L-protein are present throughout the bunyaviruses and share functional characteristics and structural similarities with other (−)stranded segmented viruses such as arenaviruses and orthomyxoviruses (Figure 3A).[12]

The endonuclease domain is responsible for cap snatching, which initiates transcription in all orthomyxoviruses, arenaviruses, and bunyaviruses. The endonuclease cleaves the 5′-end of host mRNAs to acquire primers for viral mRNA transcription. The manganese-dependent endonuclease domain of bunyaviruses is located at the N-terminus of the L-protein, with exception of the nairoviruses where it is located more downstream (Figure 3A).[13,14] The aa similarity of the endonuclease domain of species within the four studied bunyaviral families is 50% to 80% conserved, whereas between families, this is 30% to 45%. The degree of similarity with the arena and influenza

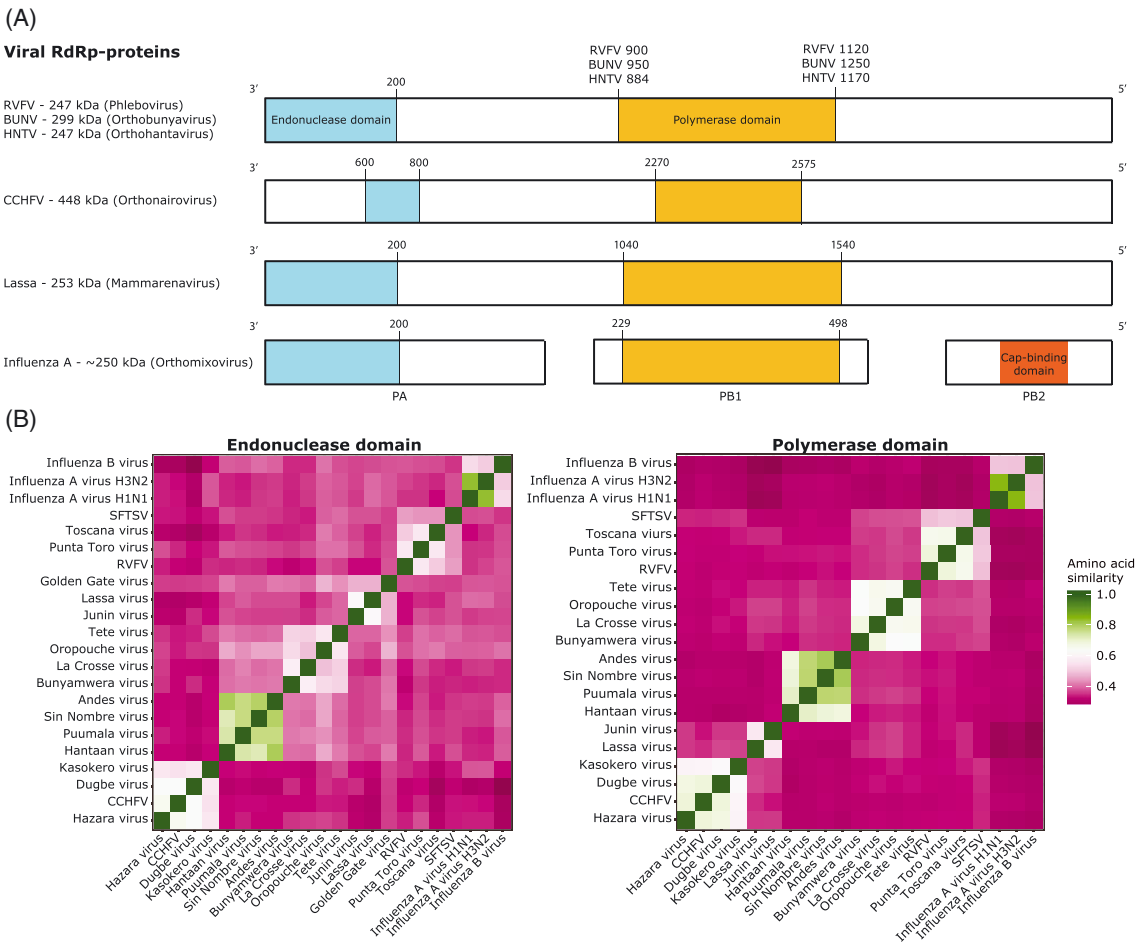

FIGURE 3 A, Comparison of L-proteins of segmented (−)ssRNA viruses. The proteins are represented as bars and domains of interest are the endonuclease domain (in blue) and polymerase domain (in yellow). Numbers above the bars indicate the area of the region that has been used in the comparison. B, Heatmaps of the amino acid similarity of endonuclease domains (left) and polymerase domains (right) between (−)ssRNA viruses. Rift Valley fever virus (RVFV), Bunyamwera virus (BUNV), Hantaan virus (HNTV), Crimean-Congo hemorrhagic fever virus (CCHFV), severe fever with thrombocytopenia syndrome virus (SFTSV)

domains is 28% to 45% and 22% to 38%, respectively (Figures 3B and S2). However, within the active site of these domains, there are motifs that are highly conserved (H..E/D..PD..E/D).[15] Their crucial function and conserved regions makes such domains a suitable target for antiviral endeavors. Successes have already been achieved against the endonuclease of influenza, confirming this enzyme is indeed a druggable target.[16] Such compounds include endonuclease inhibitors pimodivir/JNJ872 (formally VX-787), which is active against a diverse panel of influenza A virus strains and influenza JNJ5806 (formally AL-794) (J&J/Alios) and S-033188 (Shionogi).[17] This not only shows that the endonuclease is a viable drug target but its conservation among strains and species may also indicate possibilities for the development of pan-bunya inhibitors. However, it is not self-evident that such compounds will be equipotent against all bunyaviruses, since subtle differences remain.

A second highly conserved domain among (−)ssRNA viruses is the active RdRp domain in the central region of the L-proteins of bunyaviruses and arenaviruses and in the PB1 subunit of orthomyxoviruses. While the aa similarity of this region between bunyavirus families is below 50% (Figures 3B and S2), there are highly conserved motifs (preA/F..A..B..C..D..E..H) that extend into arenaviruses and influenza viruses.[5] This could make it a target for broad-acting antivirals. In fact, some polymerase-targeting broad-acting antiviral drugs have been reported. Galidesivir (BCX4430, BioCryst Pharmaceuticals) is an adenosine nucleoside RdRp-inhibitor currently being developed against filoviruses (e.g., Ebola virus and Marburg virus), also exerts activity against coronaviruses, picornaviruses, paramyxoviruses, flaviviruses, orthomyxoviruses, arenaviruses, and bunyaviruses.[18] GS-5734, a monophosphoramidate prodrug of an adenosine analogue, is being developed as anti-filovirus drug. Its acting range includes also flaviviruses and paramyxoviruses, but not bunyaviruses or arenaviruses.[19] Also favipiravir/T-705 exerts a wide range of anti-RNA–virus inhibition, including some minor activity against bunyaviruses.[20] The antiviral activity of favipiravir is discussed in more detail in Section 4. The similarities between the polymerases of bunyaviruses, arenaviruses, and influenza viruses, ie, their common motifs and similar architecture, could point in the direction of a common ancestor.[6] From an antiviral prospective, this could be a very interesting notion, since a single drug might be able to target them all.

2.3 | N-protein

The N-protein is a variable and multifunctional entity of all bunyaviruses (Figure 4). Their main function, the encapsulation of

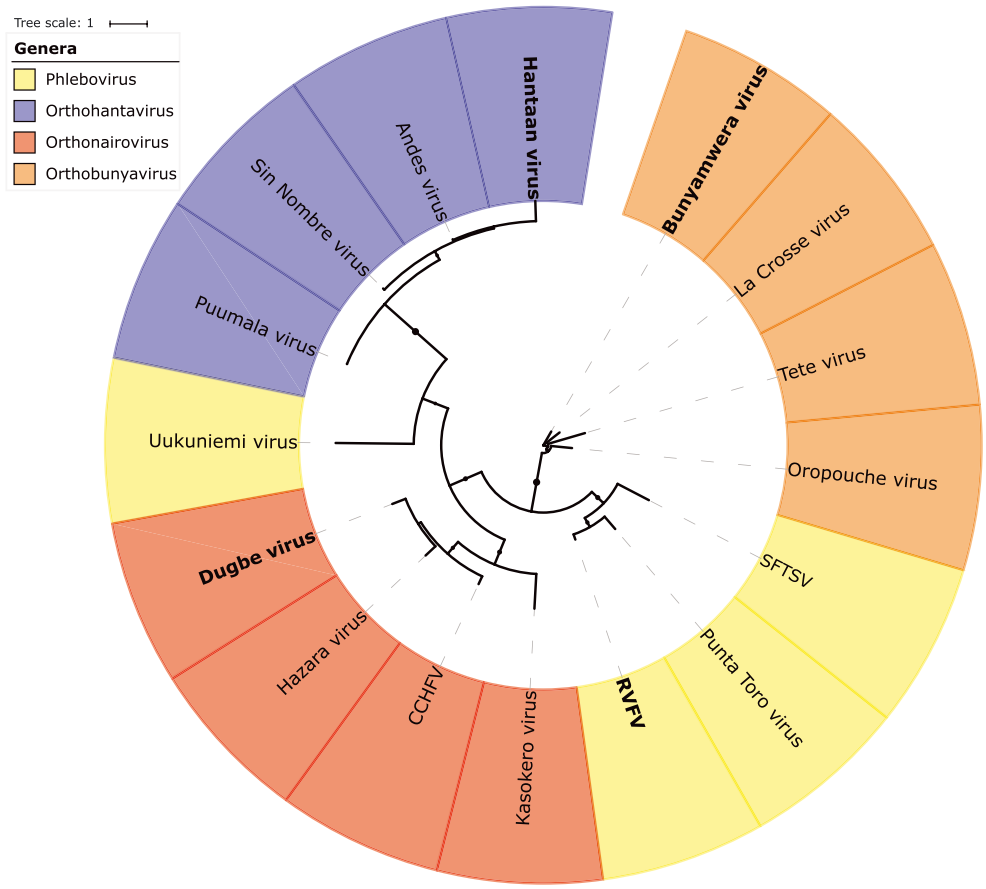

FIGURE 4 Phylogenetic tree constructed with amino acid sequences of N-proteins of bunyaviruses from the orthohantaviruses, orthonairoviruses, orthobunyaviruses, and phleboviruses genera. Names in bold indicate the type species. Severe fever with thrombocytopenia syndrome virus (SFTSV), Rift Valley fever virus (RVFV), Crimean-Congo hemorrhagic fever virus (CCHFV)

vRNA and cRNA, is conserved throughout all (−)ssRNA viruses. They do so by forming ribonucleoprotein complexes, thereby protecting the viral genomic material against host defense mechanisms. Structural analysis shows that there is limited overall aa similarity among N-proteins, both between viral families and genera, but also between bunyaviruses and the other (−)ssRNA viruses.

The Bunyamwera virus (BUNV) N- and C-terminal aa's protrude outward from the protein core domain and are able to interact with neighboring N-proteins, causing head-to-tail interactions.[21] This way, these proteins can form ring-shaped tetramers. This multimerization might be a flexible feature though, since it was observed that Schmallenberg virus (SBV) N-proteins form both tetrameric and hexameric multimers.[22] N-N-protein interactions were also found in BUNV and Leanyer virus (LEAV), suggesting this is at least a genus-wide conserved feature.[23,24] The structure of LEAV shows two distinct domains together forming the core domain: an N-lobe and C-lobe (Figure 5A). Between these two lobes, a positively charged cleft is formed that can hold 11 nt of ssRNA, which together form filamentous RNPs. The ssRNA is bound to the LEAV N-protein through hydrogen bonds to the backbone and maybe also to the bases. On the surface of the N-protein, there is a hydrophobic region that is suggested to be involved in N-N-protein interactions.[24]

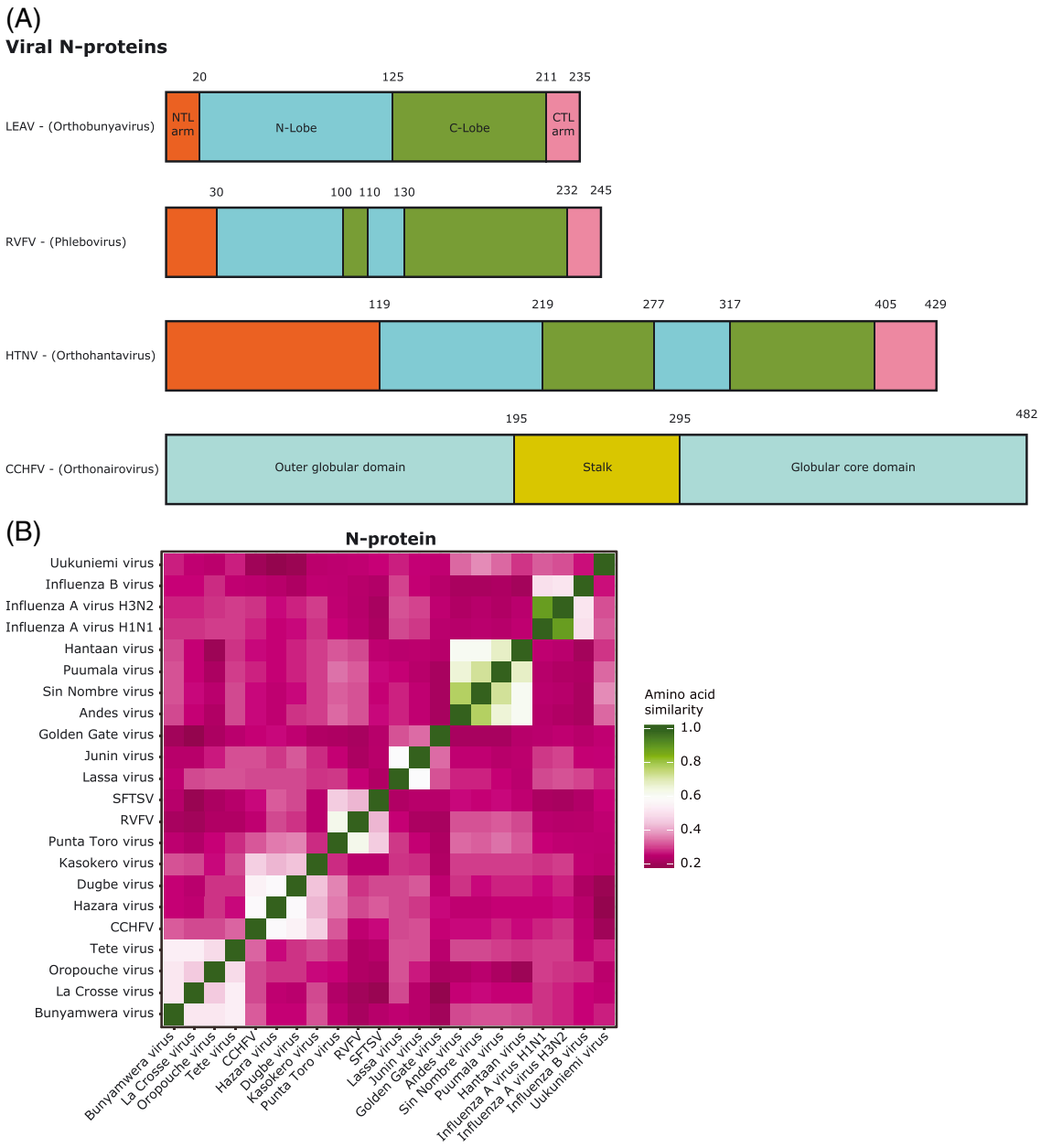

FIGURE 5 Comparison of N-proteins of segmented (−)ssRNA viruses. A, Domain distribution comparison between bunyaviruses. The proteins are represented as bars. B, Heatmap of the amino acid similarity of N-proteins between (−)ssRNA viruses. Leanyer virus (LEAV), Rift Valley fever virus (RVFV), Hantaan virus (HNTV), Crimean-Congo hemorrhagic fever virus (CCHFV), severe fever with thrombocytopenia syndrome virus (SFTSV)

The typical two lobed structure of N-proteins was also observed in phleboviruses, but no protruding C-terminal arm observed. These rather appear to be folded back against the protein.[25] Just as in orthobunyaviruses, a strong hydrophobic region was found, which allows interaction with other N-proteins.[26] Even in the sequence dissimilar Uukuniemi virus (UUKV), such N-N interactions have been observed.[27] The hydrophobic region is highly conserved among phleboviruses and is found in a pocket at the N-lobe surface. This allows the typical ring-shaped oligomers to form, but these show variability in size and shape. This indicates structural flexibility of N-protein complexes in combination with RNA. The ring shape was also seen in TOSV N-protein oligomers, but on introducing RNA to such oligomers, a helical RNP was observed.[28] The binding of RNA in this ring- or helical-shaped manner allows it to be concealed. Binding of RNA occurs at the interface of the N-protein N- and C-lobe where a cleft is formed containing a positively charged patch that allows, in case of RVFV, the binding of six RNA bases. Such a hydrophobic patch is conserved among phleboviruses.[25]

While the N-protein of hantaviruses has almost twice the molecular weight as the same protein in orthobunyaviruses and phleboviruses, similar structural properties are observed. There are the N- and C- terminal arms and subsequent binding pockets that together regulate N-N-protein interactions, and there is the hydrophobic cleft at the interface of the N- and C-lobes to accommodate RNA.[29] On top of these similarities, there are some additional functionalities attributed to hantaviral N-proteins. It has been demonstrated that trimers of the protein can substitute the eukaryotic initiation factor 4F (eIF4F) cap-binding complex, which is responsible for translation initiation. By taking over the helicase function of eIF4A subunits, binding to initiation complexes like eIF4G and binding to 5′ m7G caps just like eIF4E, it makes eIF4F obsolete.[30] The N-protein facilitates the association of ribosomal protein S19 and the viral mRNA 5′ UTR allowing ribosomes to be loaded onto the mRNA.[31] Furthermore, N-proteins are able to interfere with cellular mRNA decay by associating with processing bodies (P bodies), which are hot spots for cap snatching.[32] Besides these translational-enhancing properties, the hantavirus N-protein has also been found to interfere in host innate immune responses.[33]

Nairovirus N-proteins do not share the common N- and C-lobe structure described above. Their N-proteins have a racket shape that consists of a globular (or head) domain and a stalk domain.[34,35] The globular domain is structurally very similar to the Lassa virus (LASV) N-protein N-terminal domain, but despite the similarity, they may not have equivalent functions, since the CCHFV- N-protein lacks the 3′-5′ exoribonuclease functionality attributed to the LASV N-protein.[35,36] The stalk domain can alter its relative position, thereby altering the function of the protein. One main function is again the binding of RNA, which seems to drastically modify its structure, and the interaction with the globular domain of neighboring N-proteins and thereby form circular oligomers.[37] Three positively charged regions (two on the globular domain, one on the stalk) are present and may play a role in RNA binding, which is in contrast to other bunyaviral families with their clear RNA-binding cleft.

However, the RNA-binding domains overlap with other functional sites; thus, it seems likely that in an RNA-bound state, the other abilities are inhibited.[37] It was shown that CCHFV N-protein allows cap-independent translation of viral RNA by interacting with eIF4G, but details about these interactions are lacking.[38] The structure of the N-protein of CCHFV seems to be shared by all nairoviruses, since structures of HAZV, Kupe virus (KUPV), and Erve virus (EREV) showed very similar structures, although functionality might slightly vary.[39]

Our own aa similarity comparison of the N-proteins shows there is very limited similarity between viral families; this is substantial within families (Figure 5B) (Supporting Information). Within hantaviruses, the sequence is especially conserved, from 62% up to 78%. We observed that UUKV N-protein shares lower sequence similarity with other phleboviruses. Lower aa identity of UUKV was reported earlier.[26] Whereas the similarity between families is low, their structural and functional comparability seems, with exception of nairoviruses, much higher. The numerous sites for interaction of the N-protein with other viral proteins, RNA, and host factors, together with their crucial functions in the viral replication cycle, could possibly make good antiviral targets.[40] This is supported by findings of small molecule targeting N-proteins in other (−)ssRNA viruses. Inhibitors against conserved epitopes of the N-protein of influenza (eg, nucleozin) and RSV (eg, RSV604 and AstraZeneca) have been discovered.[41,42] Antiviral effect against influenza was observed by targeting N-protein with the nonsteroidal antiinflammatory drug naproxen. It binds the highly conserved RNA binding groove of the N-protein, demonstrating that this is indeed a druggable target and thus could also be a potential target in bunyaviruses.[43]

2.4 | Glycoproteins

The viral envelope is covered by oligomers of membrane glycoproteins (Gn and Gc), which are derived through endoproteolytic cleavage of the RNA M segment–encoded polyprotein.[44] In CCHFV, this polyprotein is cotranslationally cleaved into the PreGn and PreGc precursors, which are then cleaved by SKI-1 and SKI-1–like proteases to generate the N-termini of Gn and Gc, respectively.[45] The outward-directed domains are variable between families and species and are responsible for the attachment to host cell. This variability is reflected in the wide range of host cell receptors that have been described (see Section 3) and could make it difficult to use these domains as a target for broad-acting antivirals. Moreover, viruses have shown that they can easily mutate their receptor-binding interface to escape the drug pressure and pay little to no cost in fitness to do so. This would quickly lead to the emergence of drug resistant variants, thus rendering antivirals targeting these domains ineffective. The domain that is directed inwards into the virion is able to interact with the N-proteins of the RNP or directly with the RNA.[46,47] The disruption of these critical interactions could interfere with the viral replication cycle, making them potentially good antiviral targets.

2.5 | Nonstructural proteins

Bunyaviruses use their nonstructural proteins in strategies to interact with apoptotic pathways and to interfere with the host innate immune system. Unlike the structural proteins, these proteins are not conserved throughout the viral order. The nonstructural proteins can be lacking entirely, such is the case in most hantaviruses. The NSs (encoded on the S segment) and NSm (encoded on the M segment) can both be encoded in the (−)sense as in orthobunyaviruses, or the NSs can be encoded in the (+)sense, as is the case for the phenuiviruses.

2.6 | NSs protein

The main purpose of the NSs protein is to interfere with the host IFN-mediated immunity; it therefore is a major virulent factor. The NSs of phleboviruses and orthobunyaviruses is transported to the nucleus of infected cells and prevents the transcription of IFN by suppressing IFN regulatory factor-3 (IRF-3). The NSs of RVFV induces a characteristic filament formation in infected cell nuclei. The structure of an RVFV NSs protein was reported, showing a stable core domain that might contain all essential determinants for nuclear translocation and filament formation. While this core domain has only a 15% to 30% sequence identity between phleboviruses, these all have a similar core domain fold, while N- and C-terminal regions are highly variable.[48] In the orthobunyaviruses BUNV and La Crosse virus (LACV), IRF-3 is suppressed through interfering with the assembly and/or leading to degradation of the host RNA polymerase II complex, which has also been seen in RVFV.[49,50] The S segment of some hantaviruses carried by voles and lemmings of the northern hemisphere and American mice and rats have an ORF for an NSs protein, which is not found in the S segments of hantaviruses associated with mice and rats in Europe and Asia. These NSs proteins seem to be able to inhibit the expression of the IFN-β gene, although in a weaker fashion than BUNV and RVFV.[51] The NSs of CCHFV (orthonairovirus) is an inducer of apoptosis through disruption of the mitochondria and activation of caspases.[52]

2.7 | NSm protein

RVFV NSm is thought to interfere with apoptotic executive caspase activity by inhibiting upstream initiator caspases.[53] However, for most bunyaviruses, the function of NSm is still uncertain.

The M segment can encode more nonstructural proteins: a mucin-like domain and GP38.[45] RVFV M segment encodes the 14-kDa cytosolic protein and P78/NSm-GN. P78 has no association with virulence in mice but appears to play an important role in infectivity of mosquitos.[54]

While the nonstructural proteins are very variable between, and even within, bunyaviral families, interfering with this viral inhibition of the innate immune response is a proposed antiviral strategy, although examples of such molecules in clinical development are still lacking.[55,56]

3 | REPLICATION CYCLE

3.1 | Viral attachment and entry

The replication cycle of the *Bunyavirales* is in general very similar among its members (Figure 6). Bunyaviruses may, depending on the family, viral species, or type of host cell, use different cellular targets for attachment and employ different host entry pathways. For the *Phenuiviridae* family and a variety of bunyaviruses, the most widely described attachment factor is dendritic cell-specific ICAM-grabbing nonintegrin (DC-SIGN). Mutations or knock down of these molecules result in lower infection rates in vitro.[57-59] A second attachment factor in these viruses is L-SIGN, another human C-type lectin, which shares a 77% aa sequence homology to DC-SIGN.[60] Even though these factors have not been extensively studied in other bunyaviruses, they are involved in the infection pathway of other viruses such as dengue, Lassa, and hepatitis C virus.[61-63] It was recently shown that heparan sulfate proteoglycan is also an important attachment factor for the SBV and Akabene orthobunyaviruses (AKAV).[64] Hantaviruses use integrin as receptor for cell entry: β_1 integrin by nonpathogenic hantaviruses and β_3 integrin by the pathogenic species in vitro.[65]

Cell entry varies between bunyaviruses, with clathrin-mediated, calveolin-mediated, and independent endocytosis all being described in one or more bunyaviruses, depending also on the host cell type.[66,67] Envelope fusion with the endosomal membrane is thought to be triggered by the acidification of the late endosome, which initiate a conformational change of the viral particle glycoproteins. However, it was recently shown that also potassium channels play a crucial role in this step and thus could be potential druggable targets.[58,68-71] In UUKV, this is promoted by the presence of the late endosomal phospholipid bis(monoacylglycero)-phosphate (BMP).[72] Essential histidine residues have been identified in the RVFV Gc-protein by mutational analysis, which serve as sensors for acidification in endosomal lumen and often define the optimal pH value for virus fusion.[73,74] Hantaviruses depend heavily on cholesterol for membrane fusion.[75] The Puumala virus (PUUV) Gc has shown to be the membrane fusion effector, and it presents a class II membrane fusion protein fold in its postfusion conformation.[76,77] Such a fold was also observed in RVFV.[78]

3.2 | Transcription and translation of bunyaviral proteins and genome replication

The L-protein becomes active once it is released in the cytosol and starts by transcribing the three segments of the (−)sense genome. This process is preceded by cap snatching, where a fragment of 12- to 18-nt-long 5′-capped primer is stolen from host cell mRNA to initiate the synthesis of viral mRNA's, which will be translated into new viral proteins.[79] Translation of the L-, N-, and Ns proteins occurs in the cytosol by free ribosomes, while the viral glycoproteins are produced by the ER-membrane bound ribosomes.[80] For the replication of the genome, cRNA, i.e. a (+)sense copy of the genome segments, is created by de

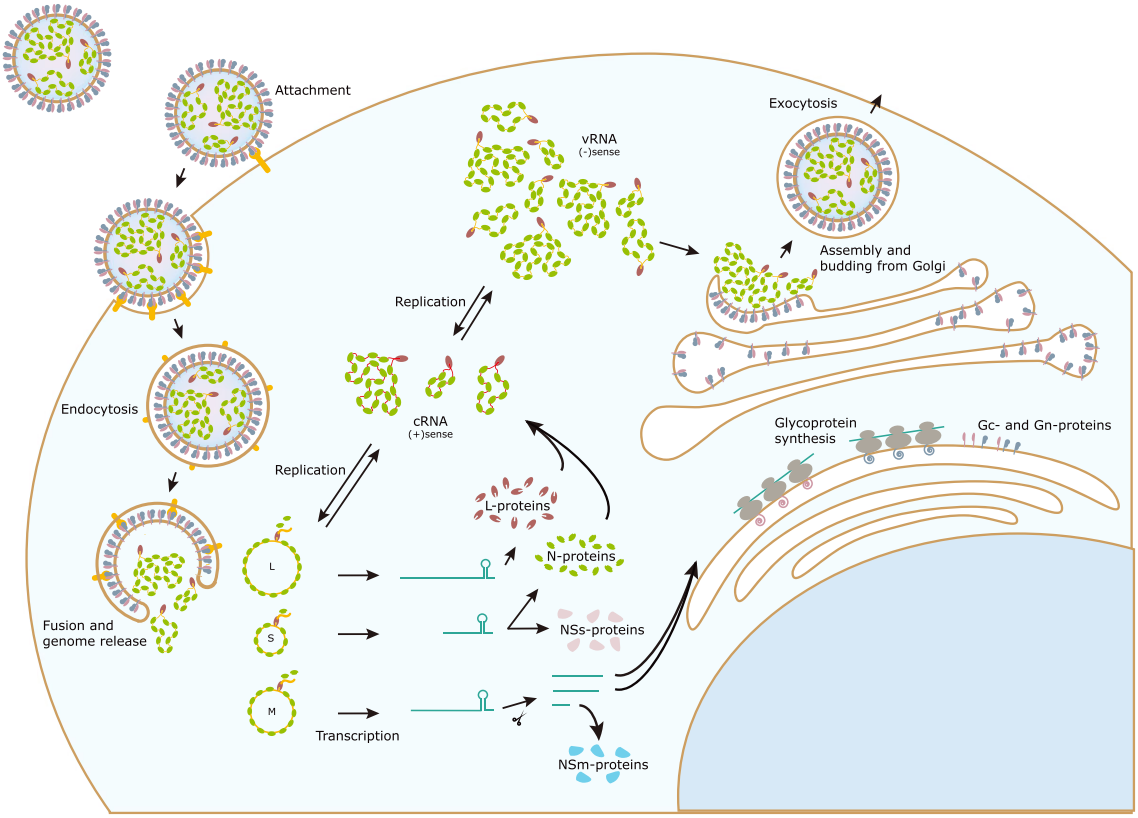

FIGURE 6 Replication cycle of bunyaviruses. Infection starts with attachment of the virus to the host cell receptors, a process mediated by the viral glycoproteins and endocytosis. Due to a pH drop, the endosomal and viral membrane will fuse, and the genome is released into the cytosol. The viral L-protein catalyzes primary transcription of viral mRNAs, which will translate into new structural proteins and (depending on the species) nonstructural proteins. The three negative-sense vRNA segments are converted into positive-sense cRNA that will serve as template for novel RNPs. These will be transported to the Golgi complex where it will interact with newly translated glycoproteins to commence assembly processes. The new viral particle will bud from the Golgi and will be transported out of the cell

novo synthesis, which is suggested to be initiated by an interaction between two L-proteins.[5] If this is indeed the case, targeting this critical interaction could be an efficient way to inhibit bunyavirus replication.

3.3 | Assembly and exocytosis

The newly formed glycoproteins instantly form oligomers in the ER membrane. From there, they target to the Golgi to assemble, allowing the new viral particles to bud from the Golgi membrane, which is a unique feature for the bunyaviruses since all other (−)ssRNA viruses bud from the cellular membrane.[81,82] This mechanism was targeted in RVFV by a small molecule, G202-0362, which seems to inhibit assembly and/or exocytosis by depolymerizing microtubules.[83]

In the replication complexes, the glycoprotein cytoplasmic tails, which are located on the inside of the virion after budding, are presented outwards towards the cytosol. This could be the bunyavirus alternative to matrix proteins, since the tail domain of the glycoproteins is able to interact with the RNP and initiate assembly of new viral particles.[46,84] The assembly of RVFV is induced by an interaction between the RNP N-proteins and the viral glycoproteins. This seems to be a nonselective process that results in particles carrying a random amount of genomic segments.[85] It is not evident that this mechanism is the same for all bunyaviruses. In CCHFV, the Gn cytosolic tail directly binds to the RNA to initiate assembly.[86] This could enable a more specific selection of genomic segments. Besides, it was shown that assembly mechanisms depend not only on the virus species but also on the type of cell that is infected.[87]

3.4 | Available model systems and tools for antiviral research

Since numerous bunyavirus species require BSL-3 and 4 facilities for handling, only a relatively limited number of research facilities are equipped to study these viruses.

3.5 | Biosafety and surrogate models

Working with a surrogate virus may be a good alternative. For several highly pathogenic bunyaviruses, such surrogates are described. The Hazara virus (HAZV) is a CCHFV-related orthonairovirus that is nonpathogenic to humans and can therefore be used both in in vitro assays to study the effect of antiviral agents and in mouse models at BSL-2.[88] A closer

alternative would be the CCHFV AP92 or AP92-like strains, which have a moderately reduced virulence compared with the wild-type virus. However, a first lethal case has been reported.[89,90] This, together with the lack of a detailed virulence and safety studies, makes that in most countries this is still classified as a BSL-4 pathogen. Unlike wild-type CCHFV, there are no reverse genetics systems available for Hazara or AP92.

For RVFV (a BSL-3 pathogen), the attenuated MP-12 strain (BSL-2) may be a suitable surrogate. MP-12 is a cell-culture–attenuated strain derived from the RVFV ZH548 strain. It replicates robustly in vitro and in vivo, making it a suitable research tool (Table 1).[91,92]

All orthohantaviruses are classified as either BSL-3 or -4 pathogens. The Maporal virus (MPRLV), a New World hantavirus, has been proposed as a potential surrogate, since there is currently no evidence that it can infect humans or cause disease.[93]

Most orthobunyaviruses are BSL-2 pathogens, because symptoms in humans are mostly limited to fever, headache, joint and muscle aches, nausea, and rash.[94] Many models and tools are available to study these viruses (Table 1).

Not all highly pathogenic bunyaviruses can currently be studied by using surrogates. For example, for severe fever with thrombocytopenia syndrome virus (SFTSV) (BSL-3), there are currently no attenuated strains or surrogates available.

The majority of bunyaviruses replicate in a wide variety of routinely used cell-culture systems, allowing to study their replication cycle (and inhibition thereof) in cell-based assays. The most widely used cell lines are Vero, A549, and BHK-21 (Table 1).

3.6 | Animal infection models

While many bunyaviruses are not (or only low) pathogenic in immunocompetent rodents, RVFV is highly pathogenic in immunocompetent mice and hamsters. The virulent RVFV-ZH548 strain is pathogenic in wild type mouse models, whereas the attenuated MP-12 strain only gives similar pathogenicity in mice that lack the alpha/beta interferon receptor (IFNAR$^{-/-}$) and STAT-1 KO mice.[95,96] This indicates that the virulent strain possesses viral factors to interfere with IFN-mediated innate immune response.

IFNAR$^{-/-}$ mice are also highly sensitive to CCHFV, and HAZV in which infection causes acute hepatitis and death of the animals within a few days after infection[88] CCHFV infection is robust in STAT-1 KO mice too.[97]

An alternative rodent model are Golden Syrian hamsters, which are susceptible to RVFV and Hantaviruses. When devoid of functional STAT-2, they are also highly susceptible to SFTSV, showing thrombocytopenia, typical marked systemic inflammation, and mortality.[98,99]

Hamsters develop a lethal Hantavirus pulmonary syndrome (HPS)-like disease when infected with ANDV, while infection with the Sin Nombre virus (SNV) results in an asymptomatic infection.[100,101] MPRLV, which does not cause disease in humans, causes partial lethality and HPS-like disease in these hamsters.[102] This makes the Syrian hamster model currently the best Hantavirus infection and HPS-disease model, making it well suited to study the antiviral efficacy of small molecule inhibitors.[103]

4 | CURRENTLY USED ANTIVIRAL COMPOUNDS IN BUNYAVIRAL INFECTIONS

4.1 | Ribavirin

Ribavirin is a purine nucleotide analog approved for the treatment of RSV and Lassa virus and was until 2014 routinely used for the treatment of infections with hepatitis C virus. In vitro antiviral activity against CCHFV has been reported, and the compound was shown to reduce CCHFV viral load and to slow down disease progression in an IFNAR$^{-/-}$ mouse infection model.[104] Its antiviral effect derives from multiple mechanisms of action.[105] The foremost antiviral mechanism against RNA viruses (described for flaviviruses and paramyxoviruses) is thought to be the inhibition of the inosine monophosphate dehydrogenase enzyme (IMPDH), resulting in the depletion of GTP pools.[106]

Ribavirin is currently the only drug used in a clinical setting against CCHFV infection in humans. Clinical benefits have been controversial, since randomized placebo–controlled clinical trials are not in line with the Declaration of Helsinki.[107] However, available studies indicate that ribavirin is effective during initial viremia when disease is still mild or moderate, but not once patients reach the hemorrhagic stage. Consequently, ribavirin could also be used as a (postexposure) prophylaxis for health care workers at risk of contracting CCHFV.[108,109] The blocking of the viral replication by ribavirin could explain why the drug is beneficial during the viremic phase and not in the later hemorrhagic phase, which is the result of released proinflammatory cytokines and the disruption of coagulation cascades.[110] Currently, two phase II trials have been enrolled to get more insight in the safety and efficacy of ribavirin (NCT00992693 & NCT02483260).

Ribavirin has also been used occasionally in the treatment of HPS in humans, but due to the small numbers of studies, no defining conclusions can be drawn.[111] However, a significant reduction in mortality in rodents has been observed.[112,113]

4.2 | Favipiravir

Favipiravir acts as a nucleobase analog that functions in its active form (T-705-ribose-triphosphate) as a substrate by the RdRp and inhibits the RNA polymerase activity.[20] It was first described as antiviral drug against influenza, but in vitro and in vivo activity has also been demonstrated against a variety of other RNA viruses, such as arenaviruses, filoviruses, togaviruses, and bunyaviruses. In case of bunyaviruses, its in vitro activity is superior to ribavirin. Its ability to increase survival rates in PTV- and RVFV-infected hamsters and in SFTSV- and CCHFV-infected mice further proof the potency of favipiravir.[114]

Favipiravir has been approved in Japan for the use in patients infected with novel or reemerging influenza viruses. The drug has also been suggested for the treatment of Ebola infections, and clinical trials have been performed despite concerns of teratogenicity shown in animals.[115] These trials showed favipiravir to be well tolerated and decrease the mortality of patients with the low viral load.[116] Promising data obtained in preclinical experiments, together with positive

TABLE 1 Overview of viruses from the *Bunyavirales* order that are important human pathogens or are often used in antiviral research

Genus	Virus	BSL	Vector Transmission	In Vitro Systems	Reverse Genetics System	Animal Models	Ref
Orthohantavirus	Andes virus	3	Inhalation aerosolized excrement long-tailed pigmy rice rat (*Oligoryzomys fulvescens*) / human-to human transmission	Vero	Minireplicon system	Syrian hamster	100,112,118-120
	Hantaan virus	3	Inhalation aerosolized excrement striped filed mouse (*Apodemus agrarius*)	Vero	Minireplicon system	C57BL/6 mice BALB/c mice Syrian hamster	121-123
	Puumala virus	3	Inhalation aerosolized excrement bank vole (*Myodes glareolus*)	Vero	-	Syrian hamster	124,125
	Sin Nombre virus	3	Inhalation aerosolized excrement deer mouse (*Peromyscus maniculatus*)	Vero, HUVEC	-	Immunosuppressed Syrian hamsters	120,126,127
Orthonairovirus	CCHFV	4	Tick (*Hyalomma spp.*) / contact with infectious blood or body fluids	Vero, SW-13, Huh-7	Minireplicon system VLP system	129S6/SvEv STAT-1$^{-/-}$ mice IFNAR$^{-/-}$ mice	97,104,128,129
	Dugbe virus	3	Tick (*Amblyomma variegatum*)	BHK	-	129S6/SvEv mice	130
	Hazara virus	2	Ticks (*Ixodes*)	Vero, A549, SW-13	-	IFNAR$^{-/-}$ mice	88,131
Phlebovirus	RVFV	3	Mosquito (mainly *Aedes* and *Culex*) /contact with infectious blood or body fluids	Vero, A549, Hek293	Minireplicon system VLP system Rescue from plasmid	IFNAR$^{-/-}$ mice Syrian hamsters	91,92,98,132-134
	Punta Toro virus	2	Sandfly (*Phlebotominae*)	Vero, LLC-MK2, Hek293	-	C57BL/6 mice Syrian hamsters	135-137
	SFTSV	3	Tick (*Haemaphysalis longicornis*) /contact with infectious blood or body fluids	DH82, Vero, Huh-7	Minireplicon system	C57BL/6 mice IFNAR$^{-/-}$ mice	138-141
	Toscana virus	2	Sandfly (*Phlebotominae*)	Vero, HeLa, Hek293T	Minireplicon system	BALB/c mice	142,143
	Uukuniemi virus	2	Tick (*Ixodes ricinus*)	BHK, A549	Minireplicon system Rescue from plasmid	-	144-147
Orthobunyavirus	Bunyamwera virus	2	Mosquito (mainly *Aedes*)	Vero, BHK, A549	Minireplicon system	Swiss albino suckling mice 129S6/SvEv mice	148-150
	La Crosse virus	2	Mosquito (mainly *Aedes*)	Vero, RAW264.7	Minireplicon system	C57BL/6 mice	151,152
	Oropouche virus	3	Mosquito (mainly *Aedes* and *Culex*)	Vero, A549	Minireplicon system VLP system Rescue from plasmid	Swiss albino suckling mice Syrian hamster	153-157

Abbreviations: BHK, baby hamster kidney; CCHFV, Crimean-Congo hemorrhagic fever virus; Hek293, human embryonic kidney 293; HUH-7, human hepatoma-7; HUVEC, human umbilical vein endothelial cells; IFNAR, interferon α/β receptor; LLC-MK2, Lilly Laboratories Cell–Monkey Kidney 2; RVFV, Rift Valley fever virus; SFTSV, Severe fever with thrombocytopenia syndrome virus; STAT-1, signal transducer and activator of transcription 1; VLP, virus-like particle.

results from clinical trials against other RNA viruses resulted in clinical trials with favipiravir against SFTSV (UMIN000022398 & UMIN000029020), leading to a phase three clinical trial (JapicCTI-183872).[117]

4.3 | Perspectives

The order of the *Bunyavirales* with its nine viral families, various animal hosts, and wide range of transmission vectors have a major impact and form a severe threat to human health. There are no drugs for the prevention or treatment of bunyavirus infections, and current response to bunya-related disease is still largely insufficient. Even though bunyaviruses prove a tremendous challenge to the field of antiviral research, it is imperative to step up to the challenge. Antiviral therapy has progressed enormously in the last decades, and history has taught us that it is possible to achieve what was sometimes deemed impossible (eg, one pill a-day treatment regimen for HIV and a cure for a chronic HCV infection after only 12 wk of treatment). Thus, if sufficient effort is put forward by the scientific community, insight into bunyavirus biology is gained and new small molecule inhibitors will be identified that can potentially be developed as antiviral treatment. This holds truth even against a viral order as diverse as the *Bunyavirales*. The current paper provides an in-depth review of the biology of bunyavirus replication, and we expand on how certain viral proteins can be exploited for antiviral drug development, together with the level of conservation of their biological functions, structure, and genetic similarity.

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CONFLICT OF INTEREST

The authors have no competing interest.

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