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Hendra and Nipah Infection: Pathology, Models and Potential Therapies

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Abstract

The *Paramyxoviridae* family comprises of several genera that contain emerging or re-emerging threats for human and animal health with no real specific effective treatment available. Hendra and Nipah virus are members of a newly identified genus of emerging paramyxoviruses, *Henipavirus*. Since their discovery in the 1990s, henipaviruses outbreaks have been associated with high economic and public health threat potential. When compared to other paramyxoviruses, henipaviruses appear to have unique characteristics. Henipaviruses are zoonotic paramyxoviruses with a broader tropism than most other paramyxoviruses, and can cause severe acute encephalitis with unique features among viral encephalitides. There are currently no approved effective prophylactic or therapeutic treatments for henipavirus infections. Although ribavirin was empirically used and seemed beneficial during the biggest outbreak caused by one of these viruses, the Nipah virus, its efficacy is disputed in light of its lack of efficacy in several animal models of henipavirus infection. Nevertheless, because of its highly pathogenic nature, much effort has been spent in developing anti-henipavirus therapeutics. In this review we describe the unique features of henipavirus infections and the different strategies and animal models that have been developed so far in order to identify and test potential drugs to prevent or treat henipavirus infections. Some of these components have the potential to be broad-spectrum antivirals as they target effectors of viral pathogenecity common to other viruses. We will focus on small molecules or biologics, rather than vaccine strategies, that have been developed as anti-henipaviral therapeutics.

Keywords

Henipavirus; Nipah; Hendra; Inhibitors; Antivirals; Pathology; Animal Models; High-throughput screening

HENIPAVIRUSES

Natural History

Hendra (HeV) and Nipah (NiV) viruses are named after the places where they had first emerged in North West Australia (1994) and Malaysia (1998), respectively. To date, HeV has caused at least 13 outbreaks of fatal respiratory infections in horses in Australia, and

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infected at least 7 people, killing 4: a horse trainer (1994), a farmer (1995) and 2 veterinarians (2008 & 2009) (source: [1]). After the malaysian outbreak, NiV has caused at least 6 outbreaks in Bangladesh (2001, 2003, 2004, 2005, 2007 and 2008) and 2 in India (2001 and 2007) (for review see [2–4]). The malaysian outbreak killed 106 people out of 276 infected patients (38.5%) and subsequent bangladeshi and indian outbreaks had a case fatality rate of 67–92%, although each outbreak had a fewer number of infected cases (12–36 people infected) (reviewed in [5, 6]).

Both HeV and NiV are very likely to be transmitted to their amplifying and/or dead-end host by bats belonging to the genus *Pteropus*. The *Pteropus* genus contains several species (e.g. flying foxes and fruit bats) shown to be infected or to be seropositive for NiV and HeV, indicating that these viruses commonly infect them. Viruses have been detected in their urine, saliva and naso-pharyngeal secretions as well as placenta and uterine discharges, suggesting that viruses are most likely shed through these fluids. Overlap of *Pteropus* spp. natural habitat goes from North Australia and Oceania to East Africa (Madagascar) through South (East) Asia (reviewed in [2, 7–9]. Surprisingly, antibodies to HeV and NiV, and RNA of closely related viruses were detected in the continental african bat species *Eidolon helvum* sampled from their habitats in West Africa (Ghana) [10, 11]. This extends the range of henipavirus reservoir animals by thousand miles, and renders future outbreaks very likely over a much wider area than anticipated (see [12]).

NiV and HeV outbreaks differed by their amplifying hosts, pigs and horses respectively, their morbidity and mortality rates and their pathogenic features. First, although causing respiratory infections in horses, HeV was not shown to be airborne or readily transmitted between horses [13], and the human infections likely occurred through direct exposure to infected secretions and tissues during nursing of moribund or necropsy of dead horses [14], rendering the extensive spread of HeV unlikely. On the contrary, NiV infected a wider range of mammalian species (pigs, dogs, goats and cats [15]) and is extremely contagious between pigs, and readily transmitted to humans [16]. Moreover, unlike the malaysian outbreak [17], bangladeshi and indian outbreaks didn't involve amplifying hosts and most likely occurred through direct transmission from bats to humans [2, 3]. Subsequent human-to-human transmission was also documented [9, 18]. Second, almost all animals infected by HeV and NiV have high morbidity and mortality, except for pigs in which the mortality is surprisingly low. Finally, both HeV and NiV caused severe febrile encephalitis in humans with unusual multifocal neurological signs, when compared with other encephalitis caused by viruses (reviewed in [19]). Details of NiV pathogenesis are described later in this review.

One other particular feature of NiV infection is that it can lead to relapsed (~10% of survivors of acute encephalitis) or late onset (~5% of patients who had no neurological symptoms) encephalitis up to several years after initial infection, with a fatality rate of about 18% [20–22]. HeV also appears to cause similar relapsed encephalitis [23, 24]. Whether this follows a mechanism similar to the subacute sclerosing paraencephalitis (SSPE) caused by measles virus (MeV) remains to be determined, although the progression of SSPE is more insidious and progressive and does not show cerebrospinal fluid (CSF) pleocytosis [20, 22]. It is also not known whether relapsing NiV encephalitis is associated with the extraordinarily high anti-virus antibody titers or dysfunctional viral matrix protein mutations that are typically found in patients with MeV-associated SSPE.

Classification

NiV and HeV were identified as *Mononegavirales* from the family *Paramyxoviridae*, sub-family *Paramyxovirinae*. Due to similarities, HeV was first classified within morbilliviruses and named *Equine morbillivirus* [14]. After the identification and characterization of NiV [25], the new genus *Henipavirus* was created in 2002 to accommodate them because of their

higher relatedness and uncommon features amongst *Paramyxovirinae* [26, 27]. First, paramyxoviruses tend to be host specific and non-zoonotic, though a number of emerging and reemerging paramyxoviruses have been associated with high fatality rates when transmitted to humans (reviewed in [28]). Some exceptions include the canine distemper virus (CDV), which can infect a wide range of hosts [29], and the emerging Menangle virus that is likely zoonotic as it appears to have been transmitted between fruit bats, pigs and humans [30]. Of note, though nowadays sustained within the human population, MeV is thought to be of a zoonotic (ruminants) origin (see [31]). Second, though most paramyxoviruses tend to cause only mild respiratory syndromes in immunocompetent hosts, henipaviruses (HNV) are highly neurovirulent. Other neurovirulent paramyxoviruses are the morbilliviruses (e.g. MeV, mumps, CDV, discussed in [32]) and potentially the recently identified Tioman virus [33], which is classified as a *Rubulavirus*.

The unusually wide range of susceptible hosts, high virulence and effective zoonotic transmissibility set HNV apart from other paramyxoviruses. NiV and HeV are possible agricultural (>1 million pigs culled during the malaysian outbreak [34]) and public health threats. This and the lack of prophylactic or therapeutic treatments have led to their designation in the United States of America (USA) as select agents and category C “priority pathogens”. HNV are also internationally classified as Risk Group (RG)-4 pathogens due to their high virulence, and thus have to be handled in high containment biosafety level (BSL)-4 laboratories (see [35]). Although this hampers efforts to thoroughly study the biology and pathogenesis of these viruses, considerable advances have been made in the last 15 years. Indeed, although much of HNV’s biology remains unknown, its ability to grow to high titers *in vitro* has allowed for identification of specific features. The development of various animal models and the careful pathological studies on infected patients, coupled with various pseudotyped and minigenome systems, have revealed insights regarding the replication kinetics, host cell tropism, and systemic nature of HNV infection. All these advances triggered the development of many potential antiviral therapies against these deadly viruses.

Virion Structure

The genome of HNV consists of a non-segmented negative strand RNA and resembles those of other paramyxoviruses (e.g. *Respirovirus* and *Morbillivirus* genera) [25, 27, 36], although it is ~15% longer than most. It contains 6 open reading frames (ORF) bordered by genus-specific 55 nt 3′ leader and a 33 nt 5′ trailer sequences. The HNV ORF encode for 6 viral structural proteins: the nucleocapsid (N), phosphoprotein (P), matrix (M), fusion protein (F), attachment protein (G) and large polymerase protein (L) (Fig. (1A)). Three supposedly non-structural proteins are also encoded by ORF-P: the V and W proteins after co-transcriptional mRNA editing of ORF-P and the C protein after translation from an alternative start codon in P-mRNA. However, the C protein has been detected in virions of HNV [27] and at least one other paramyxovirus, Sendai virus (SeV) [37]. Most of the extra length in the HNV genome comes from the non-coding regions interspacing each ORF; the 3′ untranslated regions in each transcription unit are unusually long.

HNV are pleomorphic enveloped viruses with a ribonucleoprotein (RNP) core that can be wound up in complex tertiary structures that comes across as “herringbone” like structures under electron microscopy (EM) [38, 39]. The M protein underlies the lipid envelope from which protrude the two surface glycoproteins, F and G. The F and G proteins are thought to be associated on the surface of the virion (see [40]), and can be co-immunoprecipitated when pseudotyped onto virus-like particles (VLP) [41, 42]. The attachment and entry process is discussed below and represented in Fig (1B). From what is known for other paramyxoviruses, once in the cytoplasm the nucleoprotein (N)-attached RNA-dependant-RNA-Polymerase (L) transcribes the negative genomic RNA (vRNA (−)) into ORF-specific

mRNAs and antigenomic RNA (cRNA (+)), which serve as a template for both translation and replication into genomic RNA (vRNA (−)), respectively (see [43]). The P protein acts as a cofactor of the L protein. Like other paramyxoviruses, the HNV life cycle is thought to be cytoplasmic and virions assemble at the cell surface. The F, G and P proteins are the best-characterized HNV-proteins to date and, as discussed below, despite similarities with the corresponding homologs from other paramyxoviruses, some of their features seem unique to HNV. The F and G proteins are co-ordinately responsible for membrane fusion and viral entry, and thus, most of the antiviral therapies developed to date have focused on blocking their activities thus preventing infection.

The Attachment Protein - G

The HNV-G protein is an oligomeric type II transmembrane glycoprotein present at the surface of the virions and mediates initial attachment to the cognate cell-surface receptor (Fig (1B)). The G protein is composed of a N-terminal cytoplasmic tail, a hydrophobic transmembrane domain, an extracellular stem region and a large globular C-terminal head. The HNV-G globular head forms a six-bladed β-propeller structure shared with other paramyxoviruses [44, 45], and the stalk domain is likely responsible for the higher ordered oligomeric structure that forms the functional HNV-G spike on virions. HNV-G uses a protein-based receptor, but has neither hemagglutinin (responsible for virus attachment to sialic acid receptors and named after its ability to agglutinate red blood cells) nor neuraminidase (cleavage of sialic acid residues from the carbohydrate moieties of glycoproteins) activity [46], like pneumoviruses [43].

HNV use the proteins ephrin-B2 as an attachment-receptor [47, 48] and ephrin-B3 as an alternate receptor [49]. Moreover, whereas morbilliviruses can use only species-specific signaling lymphocyte activation molecules (SLAM/CD150) as cell entry receptors [50], HNV can use ephrin-B2 and -B3 from a wide range of species [51], which likely accounts for HNV's unusually wide host range. Ephrin-B2 and -B3 are cell surface ligands that bind to cognate EphB receptors. Ephrin-B ligands and EphB receptors are highly conserved throughout evolution and belong to a large family of receptor tyrosine kinases involved in many physiological and pathological pathways (reviewed in [52–54]). The specific expression of ephrin-B2, particularly in neurons and microvascular endothelial cells of particular organs [55], as well as ephrin-B3, localized to certain areas of the brain and the brain stem [52, 53], correlates with the known cellular tropism of HNV infections and contributes to the pathophysiology of henipavirus infections [56, 57]. As for any viral infection, inhibiting the attachment of HNV to their cellular targets is of particular relevance to strategies for preventing infection and disease. This can be achieved using antibodies or small molecule inhibitors.

The Fusion Protein - F

Paramyxoviral F proteins are oligomeric type I fusion proteins (see [58]) composed of a N-terminal extracytoplasmic domain and a C-terminal transmembrane and cytoplasmic domain [59]. Fusion proteins are produced as inactive precursors (F_0) that need proteolytic cleavage to become an active heterodimer consisting of two disulphide-linked subunits, F_1 and F_2. The extracellular domain of the F_1 subunit comprises of two putative α-helical regions (heptad repeats, HR) called HR1 and HR2 and also referred to as HRN and HRC, respectively. Receptor binding to G triggers a conformational cascade that results in eventual fusion of the virus and cell membranes. This conformational cascade is complex and the details are a subject of intense study by multiple groups (for review see [58, 60–62]). The salient points involve the triggering of F following receptor binding to G. F triggering involves a conformational change that allows the insertion of the hydrophobic N-terminus of F_1 into the target-cell membrane [41, 63]. At this point, the two alpha helical repeats (HR1

and HR2) are fully formed, and membrane fusion occurs after a subsequent conformational change, which involve the HR1 and HR2 domains folding and zipping up with each other. Since F actually exists as a trimer, this results in the formation of a trimer-of-hairpins structure, termed a six-helix bundle (6HB) (see [6, 64, 65]). The same steps take place in cell-cell fusion (syncytia formation). Each of the aforementioned steps presents distinct F conformations that can be potential targets for inhibitors. Our increasing knowledge of the mechanisms underlying viral-cell fusion has triggered the development of many strategies aimed at preventing the proteolytical activation of the fusion protein, its blockade in a pre- or post-fusion step, and inhibition of the 6HB formation, the critical step that physically drives membrane fusion.

HNV-F_0 is proteolytically activated in a unique fashion compared to other paramyxoviruses. In general, paramyxoviral-F_0 are either cleaved in the secretory pathway compartments (*trans*-Golgi) by the ubiquitous intra-cellular subtilisin-like proteases like Furin, resulting in systemic infections (e.g. MeV), or extracellular - often tissue specific - trypsin-like proteases like tryptase Clara and mini-plasmin, resulting in localized infections (e.g. SeV) (see [40]). However, HNV-F_0 is cleaved by the papain-like lysosomal endopeptidase, Cathepsin-L, only after endocytosis of the neosynthetized membrane-expressed F_0 [66, 67] (represented in Fig. (1B)). Processing of HNV-F_0 by a ubiquitous protease gives a rational for the systemic nature of HNV infection, and may lead to the development of specific protease-inhibitors.

The P Gene and its Products

Many viruses have countermeasures to antagonize the interferon (IFN) induction and signaling pathways that are part of our innate immune defenses (see [68]). In the subfamily *Paramyxovirinae* the P gene encodes for the proteins having anti-IFN activities, and it is unusually long in HNV (see [69]). As aforementioned, four proteins can be translated from the HNV-P gene: P, C, V and W [27, 70, 71]. P, V and W share a common N-terminal domain but have different C-terminal domains. Although the HNV-P gene encodes 4 different proteins (P, C, V and W) by overlapping reading frames, the P protein sequence varies greatly among paramyxoviruses whereas C protein is more conserved and V is the most conserved one [72].

The P protein is a structural protein described as a co-factor for the activity of the RNA replicase/transcriptase L (see [43]). A study of the paramyxovirus simian virus 5 (SV5) showed that the P protein also has a role in the inhibition of apoptosis, IFN-beta synthesis and proinflammatory cytokine secretion [73]. This can in part come from the regulation of the viral transcription levels and prevention of aberrant transcription. Indeed, aberrant transcripts have been shown to activate the retinoic acid inducible gene I (RIG-I) RNA helicase pathway [74]. Interestingly, as for other paramyxoviruses [75–78] the HNV-C but also -V and -W proteins have also been shown to limit genome replication [79]. Additionally, these proteins have key roles in the anti-IFN response. HNV-P, -V, -W and -C proteins have all been shown to exhibit anti-IFN activities through inhibition of at least the Janus kinases/signal transducers and activators of transcription (JAK/STATs) and Toll-like receptor (TLR)-3 (recognizing dsRNA) pathways (reviewed in [69] and [80]). Surprisingly, the W protein shows a very strong IFN-inhibitory activity, attributed to its atypical nuclear localization [81, 82]. Restoring or inducing the interferon response has proven to be effective in systems where its response is blocked or ineffective such as cancer and some infectious diseases [83], and thus, as such, may help in the treatment of HNV infection [84].

HIGH-THROUGHPUT SCREENING TECHNIQUES DEVELOPED

High-throughput screening (HTS) is a powerful way to rapidly identify potent inhibitory molecules against pathogens among a large library of candidate compounds. It requires the use of scalable, sensitive and reliable assays mimicking the targeted pathways, which potentially allow for the identification of compounds with a broad antiviral spectrum or targeting multiple steps of the viral life cycle. However, since NiV and HeV require BSL-4 containment, HTS using live NiV or HeV remains impractical, and surrogate assays for the study of HNV biology and HTS at less than BSL-4 conditions have been developed.

Cell-Cell Fusion Assay

NiV envelope-mediated fusion can be studied in the absence of any virus. This approach relies on the fact that transfection of plasmids leading to the expression of NiV-F and -G in “effector” cells mixed with permissive “target” cells, expressing the receptors ephrin-B2 and/or -B3, can trigger cell-cell fusion, resulting in the formation of large syncytia [85, 86]. Of note, F and G expression in effector cells has also been achieved via infection with a recombinant vaccinia vector encoding these proteins [85, 86]. Various reporter systems can be used to quantify cytoplasmic mixing, and hence fusion, between effector and target cells. This approach also helped to confirm the receptor used by HNV [47, 48] and has been applied to screen for attachment and fusion inhibitors, by monitoring the reduction in the amount or size of syncytia (i.e. fusion) observed [87, 88]. However, cell-cell fusion geometric and kinetic constraints are most likely different from the one found in virus-cell fusion. Indeed, a recent study by Pernet *et al.* reported differencies between the mechanisms of viral entry and cell-cell fusion [89]. In this study, most inhibitors that block infection with a live virus had no effect on syncytia formation mediated by the same envelope glycoproteins. Thus, understanding the mechanistic differences between cell-cell fusion and virus-cell fusion is critical for discerning relevant hits that may come out of a cell-cell fusion HTS.

Pseudotyping

A common approach to study viral entry at less than BSL-4 relies on the pseudotyping of a RG-2 virus with the envelope-glycoproteins of the RG-4 virus under study [90, 91]. Thus, NiV-F and -G pseudotyped vesicular stomatitis (pVSV) [48, 49] reporter viruses have been used to probe NiV entry mechanisms. This approach reflects native HNV entry and compares well with plaque reduction neutralization tests (PRNT) using live NiV to detect serum neutralizing antibodies to the HNV’s surface glycoproteins, F and G [92, 93]. Soluble envelope glycoproteins coupled to microspheres also appear to be a potent tool to assess inhibition of G-ephrin–B2 interaction in the total absence of any virus [94]. These safe, rapid and sensitive strategies provide a valuable tool to study receptor interaction and entry of HNV and test for potential attachment and entry inhibitors. The use of surrogate reporter viruses has been improved with the simulation of multicycle replication of pVSV through infection of cells expressing HeV-F/G for transcomplementation of the neosynthesized particles [95]. This approach allows for the screening of compounds that may target mechanisms beyond attachment and entry only. However, VSV though belonging to the *Mononegavirales* order is a *Rhabdoviridae* with a distinctive rod/bullet-shape. Thus, use of this backbone virus may not fully recapitulate conformational needs imposed by the native envelope structure of live pleomorphic HNV. A more “native” way of examining HNV entry at less than BSL-4 conditions would be helpful for confirmatory screen of entry inhibitors before moving on to using live virus.

Virus-Like Particles

Some viral structural proteins are capable of self-assembly into particles that resemble the native virus, but lack the viral genome, called VLP. These non-replicating and non-pathogenic VLP have been largely employed as new vaccine approaches (see [96]). As for many viruses [97], the matrix protein of NiV (NiV-M), alone or in combination with its fusion (NiV-F) and receptor-binding (NiV-G) proteins, efficiently buds and forms VLP that are physically, morphologically and biologically similar to NiV virions [42, 98–100]. These observations gave insight into the mechanisms of NiV assembly and egress, and identification of sequences essential for viral release may suggest novel strategies for antiviral therapeutics. As an example, interference with the “late” domains of viral budding proteins could be of therapeutic interest, though it has been reported for some other *Mononegavirales* (e.g. the *Filoviridae* Ebola virus and the *Rhabdoviridae* VSV and rabies virus) that disruption of late domains have a profound but not an absolute impact on budding [101–103]. However, classical late domains have not been unambiguously identified in HNV-M, and it appears that HNV budding does not even use the same endosomal sorting complex required for transport (ESCRT)-dependent pathways required for other viruses with classical late domains [98, 100] (for review see [104–106]). Nevertheless, development of a HTS assay for HNV budding would be an important milestone that might reveal something about the basic biology of HNV budding in addition to the potential of obtaining novel inhibitors of budding.

We posit that HNV-VLP are more likely to reflect the biological properties of their live-virus counterparts and permit a more biologically relevant analysis of entry, uncoating, and even budding kinetics. As such, Wolf *et al.* engineered a codon-optimized fusion protein between NiV-M and the reporter β-lactamase enzyme (NiV-βla-M) [42]. This assay is amenable for efficient and high-throughput enzymatic and fluorescent screenings using NiV-(βla-M/F/G)–VLP under BSL-2 conditions. The cardinal advantage of this system is the codon-optimization and catalytic enhancement of the βla gene itself, which results in higher sensitivity than currently used forms of βla (which is of bacterial orgin). In principle, this will also allow for HTS of budding inhibitors. A sensitive and high-throughput assay for viral budding has been a relative limitation for studies into HNV pathogenesis and therapeutics.

Reverse Genetic Techniques

Reverse genetic techniques that allow for the generation of viruses from DNA and manipulation of the virus genes within the complete virus genome were first applied to a negative-stranded RNA virus in 1994 (see [107]). It has been largely employed from that time onwards to different viruses from the *Mononegavirale* order (i.e. *Rhabdo*-, *Filo*-, *Bunya*-, *Borna*- and *Paramyxoviridae*). It has been a powerful discovery tool for pathogenecity studies and drug development. Halpin *et al.* were the first to use reverse genetic techniques to study NiV-genome replication under BSL-2 containment [108]. The reporter gene coding the chloramphenicol acetyltransferase (CAT) protein was placed under the control of putative NiV-N gene transcription motifs and flanked with the NiV genomic termini. After infection of cells with a recombinant vaccinia virus expressing T7 RNA polymerase followed by cotransfection of the minigenome construct with NiV-N, -P and -L encoding plasmids, strong CAT expression, directly reflecting the extent of viral replication, was measured. This approach confirmed that NiV-genome size conforms to the rule of 6, that NiV uses a replication strategy similar to those of other Paramyxoviruses, and that NiV-N, -P and -L are sufficient for efficient NiV transcription and replication. Furthermore, these proteins can recognize cis-acting sequences in the genomic termini of the closely related HeV but not of MeV [108], emphasizing the relatedness of NiV and HeV. This approach was subsequently used to understand the promoter structure and promoter-polymerase

interactions at the molecular level [109], which could potentially lead to the development of targeted strategies to combat NiV infection. This approach also showed that the C, V and W proteins of NiV, MeV and human parainfluenzavirus (HPIV)-3 can to some extent cross inhibit minigenome replication of these respective viruses, with only HPIV-3–C protein able to inhibit all minigenomes replication [79].

Most of reported minigenome rescue systems for negative sense viruses necessitate the introduction of the exogenous T7 RNA polymerase (see [107]). Refinement of the NiV minigenome technology came from the use of the endogenous RNA polymerase I (PolI)-driven replication [110]. This helpervirus-free RNA PolI-driven transcription approach had effectively been applied for most negative-sense RNA viruses. By downscaling this system to 96-well plates, Freiberg *et al.* showed proof-of-concept of a HTS system for potential antivirals compounds that interfere with the NiV transcription and replication processes [110]. Analysis of 80 samples from the National Cancer Institute (NCI) Diversity Set Library gave two potentials hits that reduced minigenome activity.

Reverse genetic has been successfully applied to generate full length NiV. Indeed, Yoneda *et al.* described the successful rescue of a recombinant enhanced green fluorescent protein (eGFP)-expressing NiV from cDNA after cotransfection with plasmids coding NiV-N, -P, and L [111]. Pre-infection of transfected cells with a recombinant vaccinia virus expressing the T7 RNA polymerase allowed for the transcription of the cDNA. The rescued NiV recapitulated the phenotypes of the parent virus in various cell lines *in vitro* and in an *in vivo* golden hamster model [111]. Although Aljofan *et al.* developed a two step (BSL-4 then BSL-2) HTS assay based on an immunoplaque assay and using enzyme linked immunodetection in a microtitre plate format [112, 113], live or full length replicating recombinant NiV still necessitate high biocontainment.

PATHOLOGY IN HUMANS

We will first review the clinical and pathological manifestation of HNV disease, so we can better judge what animal model best reflects the human disease when using them to judge the *in vivo* efficacy of candidate anti-HNV therapeutics. Henipavirus symptoms and pathology in human have been more extensively described for NiV than for HeV due to the highest incidence of NiV infection, but the pathologies share similar features (reviewed in [22, 24]).

Clinical Hallmarks of HNV Disease

NiV-infected patients displayed rapid onset of symptoms within 7–10 days of infection. Aseptic meningitis and encephalitis with fever, headache and vomiting, and reduced level of consciousness were most common. NiV-infected patients had characteristic brain magnetic resonance imaging (MRI) scan findings and CSF pleocytosis with elevated protein levels [114, 115]. Thrombocytopenia (30% of patients), elevated serum levels of alanine aminotransferase (ALAT) (33% of patients) and aspartate aminotransferase (ASAT) (42% of patients) with normal blood urea, creatinine and electrolyte levels were the major clinico-hematological features. Most patients also suffered segmental myoclonus, hypotonia or areflexia, hypertension and tachycardia and presented with a modified Glasgow Coma Scale score < 14 (mean score of 7.5 at nadir) indicative of a severe brain-stem impairment [115, 116]. MRI of the brain showed widespread focal lesions mainly in the white matter, and abnormal electroencephalograms correlated with the severity of the neurological involvement in the disease, but not with myoclonus or focal lesions on MRI [115]. Whereas electroencephalic changes can be attributed to any type of viral encephalitis, the multifocal lesions in the brain and segmental myoclonus appear unique to NiV infection [115].

Older age, electroencephalographic changes, evidence of brain-stem involvement (segmental myoclonus, seizures, areflexia), high levels of ALAT and thrombocytopenia at admission were indicators of poor prognostic for survival [115]. Anti-HeV antibodies (Ab) were detected in the serum of 76% and the CSF of 31% of the patients tested, with no correlation with the outcome of the disease. However, a positive linear correlation between the detection of virus in the CSF, and thus in the brain, and the age of the patients was observed and associated with a poor survival prognostic [114]. There was no correlation with pleocytosis or virus isolation from urine and throat and nasal swabs [17, 114, 115]. Virus isolation from these fluids indicates that NiV has a short incubation period and promptly spreads systemically. The acute lethality of NiV also suggests it has potent mechanisms to overcome initial innate immune responses. HeV infection resembles neurological NiV infection with perhaps more obvious respiratory symptoms [117]. Respiratory distress was observed in approximately 25% of patients during the malaysian NiV outbreak and had a higher incidence during bangladeshi and indian outbreaks [118] (reviewed in [7]).

Finally, HNV infection has an unusual propensity to cause relapsing or late-onset encephalitis, up to 53 months after infection (reviewed in [19, 22, 24]). MRI monitoring of surviving patients with a relapse did not indicate a progression of the encephalitis and suggest that NiV remained quiescent until onset of relapsing or late-onset symptoms [20, 22]. A reduction of the incidence of the above-mentioned symptoms, observed during acute infection, was observed. Instead, seizures and focal cortical signs, different MRI results, involving mainly the gray matter, with focal cerebral inflammation rather than disseminated microinfarctions were observed. Finally, persistent neuronal damages were more important in patients who had a relapse, reflecting a higher degree of irreversible neuronal damage [115]. Together with the absence of the virus in CSF, these observations suggest that the pathophysiology of acute and relapsed or late-onset encephalitis differs, which necessitates further investigation [115].

Pathology

Autopsy revealed widespread vasculitis, thrombosis, ischemia, endothelial cell destruction and focal perivascular necrosis in small vessels in the lung, heart, spleen and kidney, with most damages observed in the vessels of the central nervous system (CNS) [20, 115, 119]. Syncytial giant-cell formation, non-specific for NiV infection, was observed in the epithelium of multiple organs. However, it was noted that the microvascular endothelial cells are a major target of NiV infection. As such, a unique feature of NiV infection amongst viral encephalitides is the presence of endothelial cells syncytia in the microvasculature of several organs, vasculitis, and viral inclusions [56] (reviewed in [19, 22]). Immunohistology demonstrated the presence of viral antigens in all the affected organs, indicating active replication of the virus in these systems. Of note, vasculitis and perivenous demyelination was not observed in patients who died from late-onset NiV encephalitis [20, 22]. Again, this observation underscores the differences between the pathogenesis of acute and relapsed or late onset encephalitis suggesting that different therapeutic approaches may thus be necessary.

ANIMAL MODELS

Animal models have been pivotal in the understanding of human diseases, including viral pathogenesis, and the development of therapeutics and vaccines. Animal models have to recapitulate most, if not all, of the clinical signs described in humans. Because clinical trials are not practical for highly pathogenic agents, the US Food and Drugs Administration (FDA) agreed that vaccines and therapeutics might bypass human efficacy testing if proof of

efficacy has been demonstrated in at least two well-characterized animal models that closely resemble human pathogenesis (the so-called “two animals” rule) [120].

Natural Hosts

During NiV outbreaks, several mammalian species were also infected but the absence of neutralizing antibodies in non-infected animals from these species indicated that they were, as humans, “dead-end hosts” and clinical and pathological responses appeared to be species-specific. Horses, bats and pigs, the natural amplifying hosts of HNV, have been used to study natural biology of these viruses.

In experimental NiV infections, oronasally and subcutaneously infected **pigs** showed mostly age-dependant respiratory syndromes with a broad spectrum of other symptoms [121]. Eventhough virtually all pigs can be infected, the morbidity was low and the mortality rate of only 1–5%, with some sudden deaths occurring with no premonitory clinical symptoms observed (reviewed in [4, 122, 123]). However, CNS involvement in pigs is rare, yet NiV was detected into the CNS of many animals. Pigs do not appear to shed viruses in urine, but viruses can be detected in oronasal secretions for almost 3 weeks (reviewed in [4, 7, 123]). Interestingly, a study noted bacterial infection in the CSF of 5/6 of the infected animals, suggesting a possible immunosuppression [124]. Finally, a preliminary study showed that swine can be experimentally infected with HeV through oro-nasal or nasal inoculation and shed the virus through multiple routes [125].

Experimental infection of **horses** confirmed the severe respiratory disease observed in the field, and that HeV is not readily transmitted between horses [123]. Subcutaneously and oronasally HeV-infected [13] or NiV-infected [126] **bats** showed only subclinical symptoms and seroconvertion, which is consistent with bats being the natural reservoir for HNV. Although there is evidence of NiV contamination on partially eaten fruits in the field [2], HeV was only detected in placenta and fetal fluids whereas NiV was detected in urine of experimentally infected bats (see [4, 123]).

Cats were found to be seropositive in the field [127], hence the cat has been evaluated as a model of HNV infection. Oronasal and subcutaneous administration of HeV produce an infection in cats that resembles horse infection, with fever and a severe respiratory syndrome due to a generalized vascular disease [121, 128, 129]. Experiments showed that HeV was readily transmitted between cats and that their urine may transmit the disease to horses [13]. Subcutaneous NiV-infection of cats triggered similar symptoms and virus was detected in placenta, fetal tissues, and uterine fluids (see [4, 123, 130]). While the cat is a consistent model of HNV infection, it only recapitulates the pulmonary syndrome with no symptoms in the CNS, despite the detection of NiV in the brain [131], thus mostly recapitulating the (viral tropism and) pathology observed in horse or pig.

Laboratory Animals Models

Mouse is an inexpensive and well-known animal model commonly used for a wide range of disease studies. However, rodents (like rabbits, chicken and dogs) do not appear to be susceptible to HNV infections although neutralizing antibodies can be detected in most inoculated animals [132, 133]. Interestingly, some rodent cells expressing the HNV receptor ephrin-B2 (i.e. the mouse microvessel endothelium cells (MMEC) [113] and the rat epithelial cell lines L2 and 4/4RM4 [111]) can nevertheless support NiV replication, albeit with a lower efficiency than human cell lines. Mouse ephrin-B2 can clearly be used by HNV for entry [51], and mice can die after intracranial injection of NiV or HeV (discussed in [7]), suggesting that the lack of productive infection in murine models is likely to due to some post-entry restriction and/or the inability of HNV to antagonize murine specific components

of the innate or intrinsic immunity. Uncovering the level of HNV restriction in murine models may inform future development of therapeutics that target HNV's anti-innate immune responses. However, alternative animal models - natural and experimental hosts - also need to be evaluated to study henipavirus biology and pathogenesis and identify therapeutics.

Guinea pig has been shown to be a reliable experimental model of HNV infection and transmission, due to the shedding of virus in urine, placenta and fetal fluids of infected animals (reviewed in [4, 123, 134]). Subcutaneous HeV injection induced generalized vascular disease without pulmonary edema [128] whereas injection of a higher dose more closely recapitulated human disease, with even encephalitis lesions [135]. Interestingly, intradermal injection did not induce the disease and intranasal injection only triggered seroconvertion. A study with NiV reported similarities with the human disease, yet with reduced pulmonary symptoms, after intraperitoneal injection [136].

The **golden hamster** is another reliable model that recapitulates most of the symptoms (e.g. vasculitis, thrombosis and syncytia in blood vessels) including acute encephalitis observed in humans either after intranasal or intraperitoneal inoculation [133, 137]. Death in lethally challenged hamsters usually occurs between 6 to 10 days after infection. Pattern of the disease is however different than the one observed in lung and kidney of NiV- and HeV-infected humans and horses, respectively. The severity of the disease is also route-of-infection and dose dependant. Also, eventhough virus can be detected in urine of infected animals [133, 137], no transmission was observed to non-infected animals housed in the same cage as infected ones [137]. Interestingly, whereas NiV is more virulent in older pigs, HeV is less virulent in older hamsters.

Finally, the **ferret** is an emerging model of human respiratory diseases also employed for toxicology and safety assessment studies and is used as a model of morbilliviruses [138] and influenza [139] infection. Thus, it was evaluated as a model for NiV infection and appears to very closely recapitulate the disease observed in humans [134, 140, 141]. Oronasally infected ferrets, develop clinical symptoms rapidly (6–10 days) including severe respiratory and neurological disease such as pneumonia, hind limb paresis, tremor, myoclonus and depression. Systemic vasculitis and pin-point hemorrhage due to endothelial infection and focal necrosis and syncytia, with presence of viral antigens and/or infectious virus, were observed in multiple organs. Lungs and lymphoid tissues appeared to be the sites of extensive virus replication. Some ferrets had nonsuppurative meningitis and NiV was isolated from their brains. However, fewer lesions were observed in the brain of infected ferrets than in human brains, but these ferrets were euthanized at a time when survival was considered too unlikely [134, 140, 141]. Finally, positive pharyngeal and rectal swabs suggested viral shedding, but transmission to non-infected cage-mates ferrets has not been demonstrated.

The **embryonated chicken egg** constitutes a surrogate to the handling of live animals in BSL-4 facilities and as been used as a model to study the neuronal and vascular tropism of NiV [142]. NiV injection into the yolk sac triggered a uniform infection with severe pathology within the CNS and rapid mortality, whereas its injection into the allantoic sac showed varying levels of infection and a lower death rate.

All theses different models highlight that endothelial cells are a common target among species, whereas the neuronal, epithelial, and immunological (lymphoid cells) tropism can differ. Indeed, endothelial cells from the blood vessels, CNS and upper and lower respiratory tracts are consistent targets of HNV infection, which is consistent with the expression of the HNV-receptor, ephrin-B2. Urinary tract and kidney are also consistent

targets of infection, as well as placenta and fetus (observed in bats, guinea pigs and cats), and could have a role in the vertical and probably oronasal horizontal transmission and "spill-over" of the HNV. Finally, lymphoid cells and tissues can be targeted by HNV and suggest the possibility of an immunosuppression caused by HNV infection, as observed for other paramyxoviruses like morbilliviruses [143]. This hypothesis may be supported by the detection of CSF bacterial infection of experimentally infected pigs, the possibility to infect pigs' peripheral blood mononuclear cells (PBMC), and the lymphoid organ tropism and necrosis observed in infected animals [124]. However, in humans the CSF was aseptic and the disease too acute to determine whether immunosuppression can occur in all hosts. Lastly, if non-human primates are susceptible to HNV infection and reproduce the pathogenesis observed in humans, it would be an optimal and relevant model to test the efficacy of anti-HNV therapeutics. Although non-human primate models are restricted by the availability of adequate BSL-4 facilities, Marianneau *et al.* reported that intravenous infection of Squirrel Monkeys with NiV shows some similarities with NiV infection in humans [144].

ANTIVIRAL STRATEGIES

Table (1) summarizes the molecules active against HNV infection *in vitro* and the way they have been identified.

Attachment Competitors

A common strategy to prevent infection of target cells is to prohibit protein-protein interactions involved in viral attachment and entry processes. The identification of the HNV receptors [47–49] has facilitated the development of different strategies that target viral entry. Use of soluble proteins derived either from the cellular HNV-receptors or from HNV-G is an attractive approach. Indeed, the picomolar affinity of soluble NiV-G for ephrin-B2 (Kd = 0.06 nM) [49] is higher than the nanomolar affinity observed for most antibody-based therapeutics, including the anti-RSV Ab Synagis™ (palivizumab) (see [145]). As such, soluble forms of HNV-G proteins have been generated and are potent (cross-)inhibitors of HNV entry in permissive cells [48, 146]. Although foreign viral glycoproteins are likely to be immunogenic and will generate antibody responses that will compromise their eventual effectiveness at blocking acute viral infection, perhaps this may be exactly what "the doctor" ordered: passive "immunotherapy" to block immediate acute viral replication while eliciting humoral responses to HNV-G that will block longer term viral replication and help control the disease. Thus, it may be considered useful as a "therapeutic" vaccine. Fusion of HNV-G to the Fc region of the human immunoglobulin (Ig) G1 has been reported [48, 49, 57], and may be of interest in *in vivo* settings, as Fc-fusion proteins are known to be more stable and have longer half-lifes and bioavailability.

Soluble forms of ephrin-B2 or -B3 or of the receptors EphB2, B3 or B4 can also inhibit NiV-pseudotyped–VSV entry [48, 49, 57] *in vitro*. However, these are unlikely to become viable therapeutics as the use of soluble versions of the ephrin-B2 or -B3 ligands or the EphB2, B3 or B4 receptors are more likely to induce aberrant signaling across the ephrin-B–EphB axis and lead to unintended toxicities [52].

Fusion Inhibitors: Peptides

Progress made in the study of class I fusion proteins, particularly gp41 of the human immunodeficiency virus (HIV), but also the fusion proteins of other paramyxoviruses, showed that peptidic sequences from either viral HR domains can inhibit fusion, likely by inhibiting the 6HB formed by trimers of the HR1 and HR2 domains "zipping" up on themselves. The 6HB formation is the critical phase that physically drives membrane fusion

[147] and HR2-derived peptides are the most studied (and consistent) inhibitors of fusion (see [148]). The most advanced clinically effective inhibitors are the anti-HIV T-20 (also called DP-178, Enfuvirtide or Fuzeon™) and T-1249 peptides derived from the HR2 domain of gp41 HIV-fusion protein [149–152]. For paramyxoviral-fusion inhibition, peptides derived from either HR of SV5 [153], MeV [154, 155], RSV [155, 156], Newcastle disease virus (NDV) [157, 158], HPIV-2 and -3 [155, 159], and SeV [160] have a proven efficacy at least *in vitro*.

For HNV, Bossart *et al.* showed that peptides derived from the HR2 region of both NiV- and HeV-F were potent fusion inhibitors [85, 86] *in vitro*. Second generation version of these peptides, based on shorter (36 aa) peptides either capped or made with the addition of the nontoxic water-soluble polymer polyethylene glycol (PEG) at the N- or C-terminus, had increased solubility and *in vivo* half-lifes [161] (see [162]). The N-terminally PEGylated version of NiV's HR2-C–terminal region peptide (called FC2 in the publication) showed IC_{50} values for both NiV and HeV ranging from 0.46 to 9.71 nM depending on the cell line or assay (live virus or cell-cell fusion) used. C-terminally PEGylated peptides were consistently less effective with IC_{50} ranging from 11.94 to 147.2 nM.

Interestingly, a peptide derived from the HR2 domain of HPIV-3–F inhibits heterotypic HNV-glycoproteins–mediated cell-cell fusion, even more effectively than the homotypic HNV-HR2–derived peptides, while it has no effect on another paramyxovirus (NDV) fusion [95, 161, 163, 164]. This phenomenon of heterotypic inhibition by peptides derived from the HR2 domain of HPIV-3 was previously reported for MeV and RSV and was not reciprocal [155]. However the IC_{50} to attain heterotypic inhibition was higher than for homotypic inhibition. HR2-derived peptides from either NiV- or HeV-F proteins were found to equally inhibit the other henipavirus but had no effect on fusion mediated by heterotypic fusion proteins (MeV- or CDV-F) [86]. The unusual capability of peptides derived from the HR2 domain of HPIV-3 might suggest a new inhibitory mechanism, though it was suggested that it might rely on a faster kinetic of association [164]. Moreover, it is assumed that these peptides have a helical structure in solution analogous to the HR region from which they are derived and bind to their complementary HR region thus preventing 6HB formation and membrane fusion. However a recent study suggested that these peptides may have several sets of structures in solution and that larger alpha-helical structures (increased helicity) may relate to a greater efficacy [152]. None of the aforementioned peptides has been evaluated *in vivo* to protect from HNV infection but are very promising in light of the proven clinical efficacy of the HIV fusion peptide inhibitors.

The use of peptide inhibitors of fusion is very promising but drawbacks have already been reported. Clinical studies with T-20 showed its lack of oral availability and its sensitivity to proteolysis. Moreover, it was observed that resistant, but also T-20 dependant, virus strains can emerge [165, 166]. The second-generation fusion-inhibitor T-1249 appears to be more effective than T-20 and acts also against T-20–resistant viruses. However, resistance can be developed against T-1249 as well [167, 168]. Besides, the therapeutic window of HR-derived peptides is transient and is available only after the virus is bound to the cell, when the fusion process itself has been triggered. Fusion-peptide–based inhibition would most probably benefit from the combination with other strategies that target entry. For example, in HIV, entry inhibitors have shown to be highly synergistic when used in combination with T-20 [169–173].

To increase the bioavailability and *in vivo* half-life of fusion-peptide–based inhibitors, numerous improvements in the pharmacology of therapeutic molecules, such as PEGylation [162] or use of albumin as a drug carrier [174], have been tested and new inhibitors are being designed [175]. Similarly, it was described that NiV-F–HR2 fused to the Fc region of

human IgG1 also inhibits cell fusion at similar concentration as the free peptide [176]. Fc-fusion has been shown to enhance the *in vivo* plasma half-life of therapeutic proteins and has been successfully employed in cytokine-based therapies (for a review see [177]). As an example, the tumor necrosis factor (TNF) receptor linked to the Fc fragment of a human IgG1 (etanercept, enbrel®), as an extended half-life of ~100 hours [178]. It also allows for dimeric forms of the fused-protein, which may enhance the avidity of the HR2-derived peptide to bind to its complementary HR domain.

Fusion Inhibitors: Small Molecules

Characterization of the conformational cascades that lead to fusion, and structural analysis of the putative fusion intermediates, can lead to the design of nonpeptidic small molecule inhibitors that target vulnerable or "druggable" regions in the fusion proteins [150, 179]. Class I membrane fusion proteins [58] of several unrelated viruses (e.g. retroviruses, paramyxoviruses, filoviruses) share several common features [59, 180] that may facilitate the development of small molecules that target similar or conserved structures exposed during the fusion cascade. This could potentially lead to the development of a broad spectrum antiviral. Computer models show that effective anti-fusion molecules dock into a cavity present on the N-terminus of HR1 surface and thus interfere with the proper folding of HR to form the critical 6HB that drives membrane fusion [179, 180]. As such, the first non-peptidic HIV-fusion inhibitor (ADS-J1) was identified by the use of conformation specific Ab and molecular docking techniques testing for 20,000 organic molecules [181, 182].

Based on the structural similarities of MeV- and HNV-F proteins, Niedermeier *et al*. tested MeV inhibitors and a library of quinolone derivatives, and identified quinolone-based small molecule inhibitors of HNV fusion [88]. Molecular docking simulations suggest that they bind to the conserved pocket found in the HR1 trimer that is formed during the fusion cascade. Some molecules, not the most active against MeV, showed strong inhibitory potential in cell-cell fusion or live virus entry assays against HNV. Among them, molecules named 19 and 20 showed an IC_{50} of 1.5 and 3 μM, respectively, in a cell-cell fusion assay and an IC_{50} of 4 and 2 μM, respectively, in live virus entry inhibition assays [88]. However, the low therapeutic index (> 13 and 3.3 for the compounds 19 and 20, respectively) and micromolar IC_{50} of these compounds suggest that much optimization is required even before animal testing can begin.

Passive Immunotherapy

Passive immunization has a long record of proven efficacy against numerous infectious diseases (see [183]). Even in the absence of specific monoclonal antibodies (MAb), hyperimmune serum (pooled IgG from convalescent sera) is still used to treat acute viral diseases for which there are no other treatment alternatives [184–186]. Guillaume *et al.* showed the possibility of passive immunization (IP 1h before and 24h after lethal challenge) of hamsters receiving serums from animals vaccinated against NiV-F or -G, or a mixture of both [187] (see Table (2)). However, the detection of antibodies directed against other NiV-proteins indicated that viral replication had occurred. Thus, with the intention to attain sterilizing immunity Guillaume *et al.* subsequently developed murine MAb (mMAb) [188]. Prophylactic injection of mMAb, 24h before and 1h after lethal injection of NiV triggered a sterilizing immunity. However, mMAb-injection at lower concentrations or after infection protected animals to a lesser extent and did not result in sterilizing immunity, as revealed by the detection of anti-NiV–N Ab. Anti-G or -F mMAbs could protect 50% of the animals if injected up to 24h and 96h p.i., respectively, and delayed the mean time of death (11 to 21 days). However, only anti-F mMAb protected 100% of the animals when given as soon as 1h post-infection (p.i.). Interestingly, anti-NiV–F mMAb also neutralized HeV infection *in*

vitro, whereas anti-NiV–G did not, perhaps suggesting that the HNV-F proteins are more conserved or susceptible to Ab neutralization. Altogether these results suggest a greater potential for anti-F MAb to treat both infections [188]. Identification of potent neutralizing mMAb is important to characterize the best epitopes but does not represent a class of therapeutics usable in humans unless the mMAbs are subsequently humanized and further enhanced (for a review see [189, 190]).

Panning of a naïve human antibody library using a soluble form of the HeV-G protein as an antigen allowed for the identification of 7 Fabs efficiently neutralizing cell-cell fusion and live virus entry in Vero cells [191, 192]. Among them, antibodies called m101 showed a strong neutralization of HeV-G–mediated cell fusion and m102 and m106 displayed cross-reactivity to inhibit both HeV- and NiV-G–mediated cell fusion. The conversion of m101 (HeV-specific) and m102 (cross-reacting) to IgG1 increased their IC_{50} to approximately 1 μg/ml and complete neutralization was achieved at 12.5 μg/ml. Affinity maturation (light-chain shuffling and heavy-chain variable domain random mutagenesis) of m102 and conversion to IgG1 greatly increased the cross-reactivity and inhibitory potential of the resulting m102.4 Ab, with inhibitory concentrations between 0.04–0.6 μg/ml [87] (Table (1)). Finally, m102.4 proved very effective at protecting ferrets in a post-challenge setting after the injection of 50 mg per ferret 10h after oronasal infection [141] (Table (2)). Experiments showed that this human MAb (hMAb) recognizes conformation dependant epitopes, bind at the base and in the first portion of the globular head of G and specifically inhibit Ephrin-B2–binding of different HNV isolates [191].

Altogether these results show that passive immunotherapy might be used as a prophylactic or rather therapeutic treatment for people who are exposed to HNV infection. To date, the only MAb approved by the US Food and Drug Administration (FDA) for clinical use (palivizumab; MEDI-493) treats RSV infection in children [193]. Nonetheless, RNA viruses are well known to mutate easily in order to develop resistance against selective pressures they may encounter. Thus, further identification of highly conserved epitopes and structures are of particular importance.

Although effective, current technologies may not allow passive immunization to meet the criteria for widespread use, at least not in developing countries. However, the development of potent neutralizing hMAb like m102.4 is still very encouraging [141], and may prove useful in accidental exposures in a laboratory for example, or even used for prophylatic protection of front-line responders to future HNV outbreaks.

Ribavirin

Ribavirin is a purine nucleoside analog used as a broad spectrum antiviral against many viruses, particularly RSV and hepatitis C virus, or when no other treatment is known [194, 195]. Facing the high mortality rate of NiV infection during the malaysian outbreak, the efficacy of ribavirin was evaluated in an open-label trial directly in the field [116]. In this non-randomized, unblinded limited clinical trial, the mortality rate in the treated group was reduced by 36% [116] (see Table (2)). However, the number of relapsed encephalitis in patients who received ribavirin compared to untreated patients was not described, and may have been informative. Among all the patients (treated or not) who recovered, 15% had persistent neurologic deficits. There appeared to be a trend towards a reduction in neurological deficits in survivors who received ribavirin although this trend did not reach statistical significance. The inhibitory effect of ribavirin on HNV replication *in vitro* has consistently been described, whether added before, during or after infection [84, 95, 112, 196, 197]. In contrast, the *in vivo* efficacy of ribavirin as a prophylactic or therapeutic treatment against HNV infections has not been borne out by animal challenge studies [84, 197].

The evaluation of ribavirin as a treatment of experimental HNV infection in the hamster model is summarized in Table (2). In general, ribavirin itself or related analogs (6-aza-uridine) did not protect animals from a lethal HNV challenge, regardless of the route or dose of ribavirin administered or the amount of viruses used (Table (2) and references therein). However, death was usually delayed by 1–4 days, which may reflect on ribavirin's inability to cross the blood-brain barrier (BBB) and thus protect animals from later complications such as encephalitis. Indeed, ribavirin has been described to improve survival of hamster models of (MeV-induced) SSPE only when administered intracranially, and not intraperitoneally [198]. This route of injection should be considered in subsequent evaluations of the protective effect of ribavirin, and may even be considered under extenuating circumstances in humans, as intra-thecal administration of therapeutic drugs are not uncommon. Nevertheless, it should be kept in mind that in animal experiments the challenging dose and route of infection are undoubtly different than in natural infections, which may confound the interpretation of ribavirin's effectiveness. Finally, although Geroges-Courbot *et al.* [84] also tested other ribavirin-like compounds with OMP-carboxylase inhibitory activity such as EICAR and pyrazofurin, toxicity or other problems prevented testing the efficacy of these compounds *in vivo*.

Chloroquine

Chloroquine is a 9-aminoquinoline known since 1934 and has long been used in the treatment of Malaria. More recently, interest in chloroquine use as a drug against other infectious diseases has risen. Indeed, it has been observed that chloroquine is endosomotropic and can impair pH-dependant steps of the life cycle of many viruses (see [199]). Interestingly, chloroquine was identified as a potential inhibitor of NiV infection in a model of multicycle NiV infection [95]. In this assay, chloroquine reduced the number of syncytia and the spread of the virus. It was hypothesized that, as the processing of HNV-F necessitate an endosomal stage, chloroquine could prevent the pH-dependant proteolysis of F_0 leading to inactive fusion proteins at the surface of the viruses [66, 67]. Similarly to ribavirin, micromolar concentrations of chloroquine inhibit HNV infection *in vitro*, regardless of whether it was added before, during or after infection [95, 140, 197].

However, the efficacy of chloroquine has not been borne out in animal models. Orally administered chloroquine did not protect ferrets from a lethal NiV challenge [140], when used either as a prophylactic or therapeutic regimen. The failure of chloroquine in this study cannot be attributed to insufficient bioavailability as serum concentrations attained in treated ferrets ranged from 1.6–16.8 μM, which is consistent with the concentrations used *in vitro* to inhibit NiV infection (~1 μM) and in humans receiving chloroquine as a treatment for malaria (1.6–12.5 μM). Another study in the golden hamster model reported the same lack of efficacy of intraperitoneally administered chloroquine after challenge with either NiV or HeV [197]. Moreover, this study reported a high toxicity of chloroquine with skin reactions after SC injection of low doses (50 mg/kg/day) and death after intraperitoneal injection of high doses (100 and 150 mg/kg/day). Finally, no additive effect or synergism was observed when hamsters were treated with a combination of ribavirin and chloroquine, death occurring about at the same time as in the group treated with ribavirin alone [197].

As for ribavirin, the apparent lack of efficacy of chloroquine *in vivo* may be explained by the doses and route of infection or by the distribution of chloroquine into the brain. Indeed, chloroquine is administered orally to humans and has been shown to cross the BBB to some extent, but appears to be unevenly distributed in areas of the brain [200]. Other explanations involve the processing of F_0 by other proteases although it was shown that only Cathepsin-L, among the different proteases tested, efficiently cleaved F_0 *in vitro* [66, 67]. Also, the mechanism of action of chloroquine in viral replication is not well known and seems to vary according to the target virus. Discrepancy between *in vitro* and *in vivo* efficacy of

chloroquine has also been reported for viruses such as influenza [201], severe acute respiratory syndrome (SARS) [202], HIV [203] and Chikungunya [204]. Chloroquine also has immunomodulatory effects (see [199]) that may not favor host's immunological response against viral infections.

Interferon Response

Most of mammalian viruses have evolved mechanisms to inhibit IFN response in infected cells. Experience shows that restoring this response can be of therapeutic interest in viral and autoimmune diseases as well as in cancer therapy [205, 206]. HNV have been shown to have a broad anti-IFN armamentarium. Aljofan *et al.* also described that Vero cells, that are defective in IFN production, support highest levels of viral replication and are less sensitive to antiviral compared to cells that develop an IFN response [113]. Thus, stimulating or restoring the IFN response may prove very beneficial for the treatment of HNV infections.

Indeed, in a hamster model of NiV infection, Georges-Courbot *et al.* described the potent protection offered by poly(I):poly(C12U) (Ampligen®) [84]. Poly(I):poly(C12U) is a mismatched double-stranded RNA that is a strong inducer of the IFN production and activates the intracellular enzyme RNase-L. It had been evaluated for the treatment of HIV infection [207]. In lethally NiV-challenged hamsters, only 1 out of 6 animals treated with poly(I):poly(C12U) was euthanized as a result of a bad clinical condition, although much later (15d post challenge) than control animals died (5–9 days) (Table (2)). In this animal, viral RNA was detected in the various organs analyzed, and viruses were isolated from the brain. The other 5 surviving poly(I):poly(C12U)-treated animals were euthanized at day 30 post challenge and viral RNA, but no virus, was detected only in the liver of 3 of them. The protective effect of poly(I):poly(C12U), as well as direct IFN-alpha injection, was also reported in a murine model of SARS infection [202]. Altogether these results suggest that IFN therapy shows promises in the management of HNV infection, although its therapeutic window has to be determined in post challenge studies.

Inhibition of Viral Replication/Transcription

The C protein of paramyxoviruses is a multifunctional protein known to inhibit IFN signaling but also viral transcription, and studies of the HNV-C protein confirmed these phenotypes (reviewed in [69, 80]). Of note, not only the C protein but also the V and W proteins of NiV were shown to inhibit viral replication in a minigenome rescue system [79]. Moreover, it was shown that these proteins and the MeV-C and -V proteins not only displayed a homotypic potential to inhibit their corresponding minigenomes but also mutually displayed the capacity to inhibit heterotypic minigenome replication. Interestingly, whereas NiV- and MeV-derived C proteins were not able to inhibit HPIV-3–minigenome replication, the HPIV-3–C protein was able to inhibit minigenome-replication of various paramyxoviruses (NiV, MeV and HPIV-3) [79]. Another study showed that a N-terminally–truncated version of the HPIV-3–C protein (CNDelta25) was able to heterotypically inhibit RSV-genome replication [78]. Alike HR-derived domains from HPIV-3, it appears that the HPIV-3–C protein can heterotypically inhibit genome replication of several paramyxoviruses. These data provide a rational for the development of a new class of protein-based replication-inhibitors. It was speculated that this heterotypical capacity to cross-inhibit genomes replication may rely on an interaction with a common host-cell protein. If it is the case, this cellular-partner could be targeted to block genome replication.

An emerging way to inhibit viral replication and or transcription (or rather gene expression) is the use of small interfering RNA (siRNA) [208, 209]. As such, Mungall *et al.* evaluated the potential of this approach to block HNV infection [210]. siRNA molecules directed against the L and N genes were evaluated in a minigenome assay and validated in a viral

(live virus) assay. Interestingly, 2/4 anti-L siRNA inhibited minigenome replication but had no effect on viral replication. Similarly, 4/4 siRNA directed against the N gene inhibited minigenome replication but only 3 inhibited viral replication. This discrepancy between the two types of assay underscores the necessity to confirm the observations obtained using surrogate assays. Nevertheless, this is the first time siRNA prove to be effective against HNV infection, and the combination of two anti-N siRNA showed synergy. As hypothesized by Mungall *et al.*, refinement of this approach could come from the targeting of conserved regions like the common N-terminal region of the P, V and W proteins. As for any antiviral, resistance is likely to appear. The targeting of highly conserved regions and the potential synergism of multiple siRNA targeting different genes and/or regions may prevent the emergence of escape mutants or at least reduce the fitness of these mutants. However, appealing as they are, the use of siRNA against HNV-infection imply the use of gene-therapy–based delivery methods [208, 209], which, if achievable, will be at high costs incompatible with the economy of the countries that need it most.

Other Potential Drugs

Inhibitors of macropinocytosis—Recently, Pernet *et al.* reported that NiV can enter cells via macropinocytosis (for a review see [211]) after ephrin-B2 attachment and triggering [89]. Macropinocytosis of NiV appears to be dependant of the receptor tyrosine kinase-induced cascade mediated through the phosphorylation of the cytoplasmic domain of ephrin-B2, after NiV-G attachment. Interestingly, whereas the deletion of the cytoplasmic domain of ephrin-B2 affected live virus entry, it had not effect on cell-cell fusion mediated by transfected NiV-glycoproteins. Pernet *et al.* tested a set of inhibitors targeting different discrete steps involved in the macropinocytic pathway. Interestingly, with the exception of chloroquine, these drugs only inhibited live virus entry but not cell-cell fusion mediated by NiV-F and -G. Two of the strongest inhibitors identified are latrunculin A and the amiloride analogue EIPA (5-(N-ethyl-N-isopropyl)amiloride) [89]. Latrunculin A is an inhibitor of actin polymerisation and may prove hazardous *in vivo*. However, EIPA is a well-known affordable antihypertensive, affecting the cellular Na+/H+ exchange system, and provides a rationale for the development of future drugs.

Galectin-1—Our lab has shown that galectin-1 inhibits cell-cell fusion mediated by HNV envelope glycoproteins [212]. Galectin-1 is an endogenous beta-galactoside binding lectin secreted by multiple cell types, including endothelial cells, which are one of the primary targets of HNV infection. Galectin-1 appeared to bind to specific N-linked glycans on HNV-F and -G, causing the aberrant oligomerization of the envelope glycoproteins. This likely perturbs the appropriate stoichiometry of HNV-F and -G required to effect productive fusion. In addition, galectin-1 also induces IL-6 and TNF-alpha production in dendritic cells. Interestingly, high serum levels of proinflammatory cytokines such as IL-6 and TNF-alpha during the acute phase of Ebola infection has been associated with increased survival [213]. Thus, the direct anti-fusogenic effect of galectin-1 combined with its ability to increase secretion of proinflammatory cytokines such as IL-6 and TNF-alpha, warrants further investigation into the potential therapeutic value of galectin-1 during acute HNV infection.

LJ001—Our lab also recently reported on a new antiviral small molecule identified when screening for HNV fusion inhibitors [214]. This rhodanine derivative, termed LJ001, is active against virtually all enveloped viruses, including henipaviruses. LJ001 irreversibly targets and modifies the viral lipid membrane in a way that compromises its ability to fuse with the cell membrane. Its precise mechanism of action remains to be determined, but its apparent selective activity on viral membranes relies on the reparative biogenic capacity of metabolically active cellular plasma membranes compared to the lack of such regenerative

capacity on static viral membranes. Preliminary studies showed that therapeutic serum concentrations might be attainable if pharmacokinetic issues can be resolved.

Using the ***multicycle HTS assay*** to screen a library of 23,232 compounds, 11 strong potential HNV inhibitors, with IC_{50} values < 6 μM and no cytotoxicity, were identified concurrently with chloroquine [95]. These include triazines and derivatives as the antiprotozoal quinacrine and the anti-histamine clemastine. Due to the nature of the assay, these compounds have the potential to inhibit binding, fusion and/or F-processing before its incorporation in neosynthetized virions. The study focused on the effects of chloroquine but showed that clemastine was as effective as chloroquine to inhibit HNV infection *in vitro*. Of note, the antipsychotic drug, chlorpromazine, was also identified as an inhibitor and was previously described to alter the proteolytic activation of F [67]. However, though it displayed cytotoxicity and was not further evaluated, the identification of its precise mechanism of action may help in the design of new therapeutics.

Through the ***chemiluminescent immunodetection HTS assay*** using live virus to screen a library of > 8,000 compounds, Aljofan *et al.* identified 28 potential novel antivirals with IC_{50} values < 2 μM [112, 113]. Due to the nature of the assay, using live virus infection, these compounds have the potential to inhibit any step of the viral life cycle. A panel of 9 of these antivirals was further evaluated, and the effectiveness of three has been reported. Indeed, Aljofan *et al*. reported the identification of brilliant green, gentian violet and gliotoxin as potential anti-HNV [215]. The effectiveness and potential mechanisms of action of these compounds is discussed in their publication. These molecules showed a broad range of activity by inhibiting not only live HNV and HNV-G–pseudotyped VSV but also the Influenza A H1N1 and HPIV-3 viruses. However, as they discuss, due to high cytotoxicity these compounds may rather be used as antiseptics and disinfectants rather than treatments. Nevertheless, gliotoxin is a polymerase inhibitor and the description of its mechanism of action may provide a rational for anti-HNV drugs that target its RNA polymerase activity [215]. Since mammalian cells do not generally have RNA-dependent RNA polymerase activity, targeting the RNA polymerase in negative-strand RNA viruses may provide an opportunity to develop virus-, rather than host-cell–specific drugs.

The thorough analysis of the mechanisms by which each of these reported potential hits inhibit HNV infection may facilitate the design of more effective molecules with high therapeutic indices.

PERSPECTIVES

Other Therapeutics

As described for filoviruses (see [216]) inhibition of budding is also an appealing strategy that has not been pursued for HNV. However, this relies on a more complete understanding of the HNV budding process, with the M protein having a central role. It appears that NiV-M does not bud using the classical ESCRT-dependent pathways that have been described for HIV and filoviruses [98, 100] (for review see [104–106]). So, much progress still needs to be made in this area of HNV biology. Finally, not only for M but for all viral-proteins, a better understanding of the atomic structures and functions of key protein-protein interactions involved in critical steps of the viral life cycle can serve as a basis for the development of small molecules that specifically target these essential interactions. Indeed, during the last decade, *in silico* drug screening has become increasingly sophisticated, and high-resolution 3D structures of multiple viral proteins may facilitate the identification of broad spectrum antivirals that target conserved domains in phylogenetically divergent viral proteins that share similar structures and functions [217, 218].

The potential effectiveness of anti-malarial drugs, like chloroquine, is very appealing because they are well characterized and studied, and they are available in developing countries. The demonstrated synergism of chloroquine with HIV-protease inhibitors led to the screening of other anti-HIV compounds among antimalarial drugs [219]. Mefloquine showed a marked synergy with several protease inhibitors *in vitro* but also *in vivo*, resulting in an increased distribution of the drugs in the brain. Again, these results underscore the necessity of evaluating combination therapies. Also, endosomal protease (e.g. cathepsin-L) inhibitors offers great promise as potential broad-spectrum antivirals as several priority pathogens (e.g. Ebola virus and the SARS coronavirus) also rely on these enzymes during their life cycle [220, 221].

Crossing the Blood-Brain Barrier

Last but not least, one of the major pathologies in HNV infection involves the CNS, which is protected by the blood-brain barrier (BBB). The BBB is a physiological barrier maintaining and regulating the homeostatic environment of the brain. The brain microvascular endothelial cells composing the BBB interact via tight junctions and the BBB is thus poorly permeable. Indeed, only a select group of molecules is allowed to diffuse (certain amino acids, glucose and most molecules of less than 500 Da) or cross the BBB via receptor-mediated active transcytosis (transferrin, lactoferrin and insulin) (reviewed in [222]). As such, and in order to avoid invasive direct intracerebral injection, if it appears effective, development of anti-HNV drugs needs to keep in mind that these molecules should be able to cross the BBB to control the onset of the neurological form of the disease.

As aforementioned, the BBB may be responsible for the relative ineffectiveness of ribavirin and chloroquine, at least in animal models. The efficacy of ribavirin in humans who survived NiV infection may come from the fact that these patients were treated before the onset of neurological disease. Indeed, a lower Glasgow Coma Scale score indicative of brainstem involvement and presence of virus in the CSF were of poor prognostic of survival even in treated patients [114, 116]. Recent data indicate that the complexation of ribavirin with alpha-cyclodextrin increases its anti-MeV activity *in vitro*, as well as in an *in vivo* murine model of MeV-induced encephalitis, due to higher quantities distributed to the brain [223–226]. Thus, the efficacy of this molecule should be evaluated in animal models of HNV infection. Though chloroquine is unevenly distributed in areas of the brain [200], it showed its potential to protect the BBB when given as an adjuvant in an anti-Glioblastoma multiform therapy [227]. However, during the latest (August 2009) HeV outbreak in Queensland, Australia, four potentially infected patients were treated 5 days with a combination of intravenous ribavirin and oral chloroquine (discussed in [197]). Serious side effects of the treatment were observed and one patient later died as a result of a confirmed HeV infection.

It is unlikely that HR-derived small peptides can permeate across the BBB, and their complexation with carriers or stealths (albumin, Ab-Fc fragments or PEG) is also likely to further reduce their ability to cross the BBB. Larger peptides as Ab do not effectively cross the BBB, though passive diffusion can be observed [228]. The protective effect of passive immunotherapy is, however, very promising and may depend on how much “head-start” the virus has before infusion of the neutralizing Ab. Nucleic acid drugs (e.g. siRNA) face the same problems of low permeability, although general nucleic-acid based activators of innate immunity such as poly(I):poly(C12U) that increase generalized interferon responses, can act at a distance, so to speak.

Numerous strategies have been developed to enhance the delivery of therapeutics across the BBB and alternative routes of delivery (nasal or intraperitoneal), as well as *in vitro* and *in silico* approaches to evaluate BBB penetration, are being developed [229, 230]. This bodes

well for the eventual development of strategies and therapeutics that can treat HNV and other viral encephalitides.

THE UTILITY OF ANTI-HNV THERAPEUTICS

In summary, much effort has been expended on the development of multiple therapeutic strategies that can counter HNV infection. It appears that the logistics and costs of developing imaginative therapeutics against a currently sporadic though deadly human disease are probably economically unrealistic, although these developments have been stimulated by the potential bioterrorism threat posed by these viruses [231], and will be of prime importance in the case of accidental exposure in laboratories studying these viruses, or to protect front-line responders to HNV outbreaks.

Nevertheless, despite the relative rare occurrences of HNV outbreaks, HNV are clearly zoonotic emerging viruses with apparently increasing “viral chatter” (intermittent transmissions into the human population) as defined by Nathan Wolfe and colleagues [31]. The Wolfe-Diamond scheme that describes how zoonotic pathogens adapt to become fully human diseases has 5 stages, where stage 1 classifies pathogens as being only in animal reservoirs (e.g. rabies), and stage 5 describes a fully human-adapted pathogen (e.g. MeV and HIV-1 group M). In this scheme, HNV likely rates as being in Stage 3, where limited outbreaks and occasional cycles of human-to-human transmission have been observed. This raises the spectre that HNV may become adapted enough to spread efficiently from human to human. If this occurs, our efforts to develop therapeutics against a pathogen that causes what is, in effect, an “orphan disease”, may one day be considered as forward looking.

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ABBREVIATIONS

6HB	Six-helix bundle
Ab	Antibody
BBB	Blood-brain barrier
BSL	Biosafety level
CDV	Canine distemper virus
CNS	Central nervous system
CSF	Cerebrospinal fluid
HeV	Hendra virus
HIV	Human immunodeficiency virus
hMAb	Human monoclonal antibody
HNV	Henipavirus
HPIV	Human parainfluenza virus
HR	Heptad repeat
HTS	High-throughput screening

IC_{50}	50% inhibitory concentration
IC_{90}	Concentration totally inhibiting detectable infection
IFN	Interferon
Mab	Monoclonal antibody
MeV	Measles virus
mMAb	Murine monoclonal antibody
MRI	Magnetic resonance imaging
NDV	Newcastle disease virus
NiV	Nipah virus
ORF	Open reading frame
pVSV	Pseudotyped vesicular stomatitis virus
RG	Risk group
RSV	Respiratory syncytial virus
SARS	Severe acute respiratory syndrome
SeV	Sendai virus
siRNA	Small interfering RNA
SSPE	Subacute sclerosing paraencephalitis
SV5	Simian virus 5
VLP	Virus-like particles
VSV	Vesicular stomatitis virus

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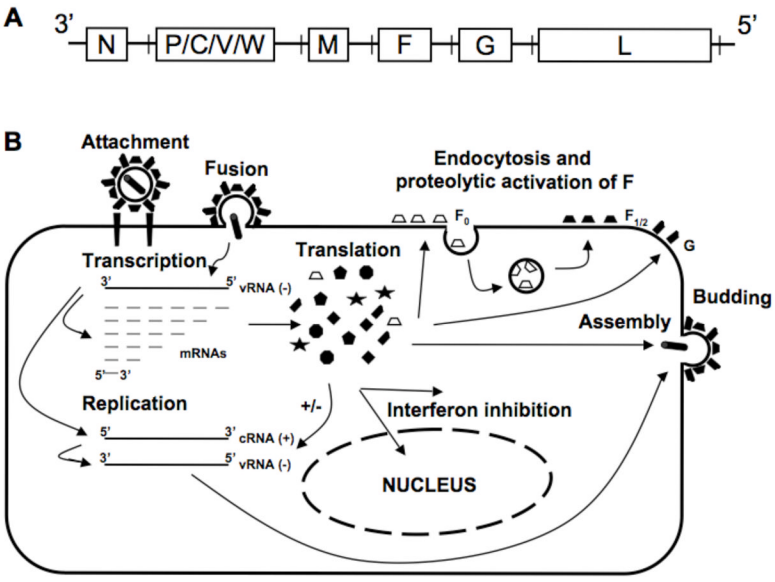

Fig. 1. Genomic organisation (A) and replication cycle of henipaviruses (B)

(A) The negative genomic RNA is represented in its 3′–5′ orientation. It is composed of 6 units (represented by boxes) encoding in order the nucleoprotein (N), phosphoprotein (P), matrix protein (M), fusion protein (F), attachment protein (G) and RNA polymerase (L). The gene start and stop signals are represented by vertical lines after each unit. The size of each domain is not drawn to scale but the untranslated 3′ regions (5′ ends of the gene units) are unusually long for a Paramyxovirus, but for the L gene. The P gene is the only polycistronic unit and encodes for the V and W proteins after insertion in the mRNA of one or two non-templated G, respectively, in a conserved RNA-editing site. The C protein is translated from an alternate reading frame present in any of the P-mRNA and has its stop codon upstream of the RNA-editing site. (B) The principal described events in the replication cycle of henipaviruses are summarized. After attachment and fusion, the negative genome (vRNA (−)) serves as a template for the transcription of viral mRNAs following a 3′–5′ gradient with significant attenuation at the M-F and G-L junctions, N being transcribed earlier and in greater quantities than L [196]. The vRNA (−) also serves as a template for the replication in cRNA (+), which in turn will be the template for the synthesis of the vRNA (−) that will be incorporated into neovirions. Following traduction of the viral mRNA, different roles of the viral proteins in the inhibition of the interferon signaling pathways (in the cytoplasm and nucleus), regulation of the genome replication, as well as the mechanism of F_0 proteolytic activation via the endosomal Cathepsin-L protein have been identifed. Orchestration of assembly and budding is attributed to the M protein (not represented), though the exact mechanism has not been described yet. The N, P, C, M, F, G and L proteins are incorporated into the virions. With the exception of traduction, assembly and budding, all of the represented steps have been evaluated as targets for the development of anti-henipaviral drugs.

Table 1

Candidate antivirals against *Henipavirus* infections

Target step	Drug	Discovery	Efficacy on live virus		Broad spectrum	Ref.
			*In vitro**	*In vivo***		
Replication/transcription	Ribavirin	Empirical	IC_{50}: ~4 μM (~1 μg/ml) IC_{90}: ~100 μM	No #	Yes	[84, 95, 112, 116, 196, 197]
	EICAR	Empirical	IC_{90}: 1 μg/ml	NT (Toxicity)	Yes	[84]
	6-aza-uridine	Empirical	IC_{90}: 0.25 μg/ml	No	Yes	[84]
	Pyrazofurin	Empirical	IC_{90}: 0.125 μg/ml	NT	Yes	[84]
	Gliotoxin	HTS live virus	IC_{50}: 159 nM (NiV) 579 nM (HeV)	NT	Yes	[215]
	NiV-C, -V and -W	Empirical minigenome	NT	NT	NT	[79]
	HPIV-3–C	Empirical minigenome	NT	NT	Heterotypic inhibition (HPIV-3, NiV, MeV, RSV)	[78, 79]
	siRNA (anti-L or -N)	Empirical minigenome	> 60% inhibition at 50 nM anti-N no anti-L effective	NT	Potentially (CR anti-N)	[210]
	2 candidate compounds	HTS minigenome	NT	NT	Potentially	[110]
F_0 cleavage	Chloroquine	Multicycle HTS pVSV	IC_{50}: ~1 μM IC_{90}: ~20–100 μM	No (Toxicity)	Yes	[89, 95, 140, 197]
	Chlorpromazine	Empirical + Multicycle HTS pVSV	IC_{90}: ≤ 25 μM	NT	Potentially	[67, 95]
Fusion	HNV-F HR2	Empirical cell-cell fusion	NT	NT	CR	[85, 86]
	2nd generation HR (N-PEG NiV HR2)	Empirical	IC_{50}: 0.46–2.05 nM	NT	CR	[161]
	HPIV-3–F HR	Empirical cell-cell fusion + pVSV	IC_{50}: 208 nM (NiV) 179 nM (HeV)	NT	Potentially (HPIV-3, NiV, HeV, MeV, RSV)	[95, 155, 163, 164]
	Quinolones derivatives (5 hits)	Empirical cell-cell fusion	2 tested (19 & 20) IC_{50}: 0.5–4 μM	NT	Potentially (NiV, HeV, MeV)	[88]
	mMAb *anti-NiV–F*	Empirical	IC_{90}: 1.6–425 ng	Yes	CR	[137, 188]
	Galectin-1	Empirical cell-cell fusion	NT	NT	Potentially (NiV, HeV, HPIV-3)	[212]
Attachment	Soluble proteins: Ephrin-B2, -B3 EphB3, B4	Empirical cell-cell fusion	IC_{50}: Ephrin-B2: <10μg/ml	NT	CR	[51, 146]

Target step	Drug	Discovery	Efficacy on live virus		Broad spectrum	Ref.
			In vitro[*]	*In vivo*[**]		
	HNV-G		Ephrin-B3: ≤25μg/ml EphB3 or B4: ≥100 μg/ml HeV-G: 13.20 μg/ml (NiV) 3.3 μg/ml (HeV)			
	mMAb *anti-NiV–G*	Empirical	IC_{90}: 0.27–2.34 ng	Yes	No	[137, 188]
	hMAb *anti-HeV–G*	Empirical	IC_{90}, m101: ≤12.5 μg/ml IC_{50}, m102.4: 0.04 μg/ml (NiV) 0.6 μg/ml (HeV)	Yes	m101: No m102.4: CR	[87, 192]
IFN inducer	Poly(I):poly(C12U)	Empirical live virus	IC_{90}: ≤6.25 μg/ml	Yes	Yes	[84]
Others	Macropinocytic inhibitors (>7)	Empirical	(e.g.) IC_{50}: Latrunculin A: < 2 μM EIPA: ~ 15 μM	NT	Yes	[89]
	12 hits, including: Triazines, Quinacrine, Clemastine	Multicycle HTS pVSV	NT	NT	Potentially	[95]
	28 hits, including: brilliant green (BG) gentian violet (GV)	HTS live virus	All IC_{50} ≤ 2 μM BG: ~0.2–0.8 μM GV: ~0.5–2.7 μM	NT (Toxicity)	Yes	[112, 113, 215]
	LJ001	HTS pVSV	IC_{50}: ~ 1 μM	NT	Yes	[214]

[*] May differ according to the assay (entry, viral production, RNA replication or plaque neutralization) used and the virus tested (NiV or HeV)

[**] See Table (2)

[#] Though mortality is reduced in humans, animal models show no efficacy, see table (2)

IFN: interferon; HR: heptad repeat-derived peptide; PEG: polyethylene glycol; m/hMAb: murine/human monoclonal antibodies; IgG1: immunoglobulin G1; pVSV: pseudotyped VSV; HTS: high-throughput screening; IC_{50}: 50% inhibitory concentration; IC_{90}: concentration totally inhibiting detectable infection; NT: not tested; PFU: plaque forming units; CR: cross reactive, inhibits both NiV and HeV; e.g.: exempli gratia; EICAR: 5-ethynyl imidazole analogue of ribavirin; EIPA: 5-(N-Ethyl-N-isopropyl)amiloride

Table 2

In vivo efficacy of antivirals on survival after *Henipavirus* infection

Drug *	Treatment			Virus		Effect	Ref.
	ROA	Time	Dose	Dose **	ROI		
Ribavirin (Human)	Oral or IV	After hospital admission	PSS ~2,500 ng/ml (6–9 days)	~Low	~ON	Mortality reduced (36%)	[116]
Ribavirin	SC	Just before infection	50 mg/kg/day	350 LD_{50}	IP	Survival increased (+1–2 days)	[84]
	IP	Just after infection	25 mg/kg b.i.d.	35 LD_{50}	IP	Survival increased (+2 days)	[84]
	IP	6h p.i.	30 mg/kg q.12h.	10,000 $TCID_{50}$ NiV or HeV	IP	Survival increased (+3 days, NiV) No effect on HeV	[197]
	IP	6h p.i.	50, 75 or 100 mg/kg q.12h.	10,000 $TCID_{50}$	IP	Survival increased 50 mg: +3 days 150 & 200 mg: +4 days but SE	[197]
6-aza-uridine	SC	Just before infection	175 mg/kg/day	350 LD_{50}	IP	Survival increased (+1–2 days)	[84]
Chloroquine	IP	6h p.i.	50 mg/kg q.d.	10,000 $TCID_{50}$ NiV or HeV	IP	No protection (NiV and HeV)	[197]
	IP	6h p.i.	50, 100 or 150 mg/kg q.d.	10,000 $TCID_{50}$	IP	No protection 100 & 150 mg: lethal	[197]
Chloroquine (Ferret)	IT	24h before and/or starts 10h p.i.	25 mg/kg q.d.	5,000 $TCID_{50}$	ON	No protection	[140]
Ribavirin + Chloroquine	IP	6h p.i.	30 mg/kg q.12h. 50 mg/kg q.d.	10,000 $TCID_{50}$ NiV or HeV	IP	No synergy ~ ribavirin alone	[197]
Murine antisera (anti-F, -G or -F/G)	IP	1h before + 24h p.i.	NA	1,000 pfu	IP	Total protection (all antisera)	[187]
mMAb	IP	1 day before + 1h p.i.	*Anti-NiV–G*: Nip GIP 1.7: 112 μg Nip 3B10: 100 μg *Anti-NiV–F*: Nip GIP 35: 180 μg Nip GIP 3: 520 μg	100 LD_{50}	IP	Protection: 100% 100% 100% 75%	[188]
mMAb	IP	1 day before + 1h p.i.	Nip GIP 1.7: 112–0.0012 μg Nip GIP 35: 180–0.0018 μg	100 LD_{50}	IP	100% protection down to 1.12 μg 100% protection only at 180 μg	[188]
mMAb	IP	1h or 1, 2, 3 or 4 days p.i.	Nip GIP 1.7: 112 μg Nip GIP 35: 180 μg	100 LD_{50}	IP	50% protection at 1 & 24h p.i. 100% protection at 1h p.i. 50% protection up to 4 dpi	[188]
mMAb	IP	1h before+ 24h p.i.	≠ *Anti-NiV–F* mMAbs 2.5–6 mg/kg	1,000 pfu HeV	IP	Total protection	[137]
hMAb: m102.4 (Ferret)	IV	24h before or 10h p.i.	50 mg	5,000 $TCID_{50}$	ON	Protection of 1/3 preinfused 3/3 postinfused	[141]
Poly(I):poly(C12U)	IP	Just after infection	3 mg/kg q.d. (10 days)	35 LD_{50}	IP	Protection 5/6 1/6 euthanized 15 dpi	[84]

[*] Otherwise mentioned, all the experiments were done in the hamster model

[**] Otherwise mentioned, all the experiments were done with NiV

ROA: route of administration; ROI: route of infection; PSS: pseudo-steady sate; SC: subcutaneous infusion via osmotic pump; ON: oronasal; IP: intraperitoneal; IT: intratracheal; IV: intravenous; t.i.d.: three times a day; b.i.d.: twice a day; q.d.: once a day; q.Nh.: every N hours; (d)p.i.: (days) post infection/challenge; LD_{50}: median lethal dose; $TCID_{50}$: 50% tissue culture infectious dose; pfu: plaque forming unit; m/hMAb: murine/human monoclonal antibody; SE: side effects