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Emerging paramyxoviruses: molecular mechanisms and antiviral strategies

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In recent years, several paramyxoviruses have emerged to infect humans, including previously unidentified zoonoses. Hendra and Nipah viruses (henipaviruses within this family) were first identified in the 1990s in Australia, Malaysia and Singapore, causing epidemics with high mortality and morbidity rates in affected animals and humans. Other paramyxoviruses, such as Menangle virus, Tioman virus, human metapneumovirus and avian paramyxovirus 1, which cause less morbidity in humans, have also been recently identified. Although the Paramyxoviridae family of viruses has been previously recognised as biomedically and veterinarily important, the recent emergence of these paramyxoviruses has focused our attention on this family. Antiviral drugs can be designed to target specific important determinants of the viral life cycle. Therefore, identifying and understanding the mechanistic underpinnings of viral entry, replication, assembly and budding will be critical in the development of antiviral therapeutic agents. This review focuses on the molecular mechanisms discovered and the antiviral strategies pursued in recent years for emerging paramyxoviruses, with particular emphasis on viral entry and exit mechanisms.

Globalisation and human encroachment into native wildlife habitats will probably continue to cause an increase in emerging zoonotic viral diseases. In recent years, members of the Paramyxoviridae viral family have caused some of the deadliest emerging zoonoses. The Paramyxoviridae family comprises important old and new human and animal viral pathogens, and Nipah (NiV) and Hendra (HeV) viruses make up the new *Henipavirus* genus within this family (Refs 1, 2, 3). HeV was first identified in 1994 in Australia, and NiV was discovered in 1998 in Malaysia and Singapore; both caused epidemics that concerned national and international authorities because of the high mortality and morbidity rates in affected animals and humans (Refs 4, 5). For most paramyxoviruses, the host range is narrow and cross-species transmission events are rare; hence, the recent emergence of the henipaviruses with high virulence and a broad host range is alarming.

Other paramyxoviruses, with lower mortality rates or fewer incidents in humans, have also emerged in recent years, including Menangle virus, Tioman virus, avian paramyxovirus 1

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and human metapneumovirus (HMPV). Nonetheless, the incidence of HMPV in human populations approaches 100%, and causes 5–20% of young children to be hospitalised with respiratory tract infections (reviewed in Ref. 6). In addition, although other emerging paramyxoviruses such as the Beilong or J viruses have not been reported to cross species from their putative rodent reservoirs, the ability of Beilong virus to cross-contaminate human cell cultures from rodent cell cultures in the same laboratory raises the spectre of zoonotic spread to humans (Refs 7, 8, 9).

Therefore, understanding the mechanistic underpinnings of viral entry, replication and assembly of these emerging paramyxoviruses is of critical importance. This review focuses primarily on henipaviruses because most recent molecular and mechanistic studies that inform potential antiviral strategies have been directed against this most lethal group of paramyxoviruses. We do not cover vaccine approaches, because they have been recently reviewed elsewhere (Refs 10,11, 12).

The Paramyxoviridae family

The Paramyxoviridae family has been divided into two subfamilies: Paramyxovirinae and Pneumovirinae (Fig. 1). The Paramyxovirinae subfamily comprises five genera: *Respirovirus*, *Rubulavirus*, *Avulavirus*, *Morbillivirus* and *Henipavirus*. This subfamily includes the important measles, mumps, Newcastle disease and parainfluenza viruses, as well as HeV and NiV, although some of the emerging Paramyxovirinae members (e.g. Menangle, Tioman, Beilong and J) do not formally cluster into these five main genera. Some viruses within this subfamily have caused important human diseases for millennia. For example, reports of symptoms such as those caused by the measles virus date back to the seventh century. Although the measles virus has now been eradicated from most developed countries through vaccination, it still produces a significant number of deaths globally, with 197 000 deaths reported in 2007 (Ref. 13).

The second subfamily, the Pneumovirinae, consists of two genera: *Pneumovirus* and *Metapneumovirus* (Fig. 1). This subfamily also includes important old and new human and animal pathogens, such as the human and bovine respiratory syncytial viruses (RSVs) that specifically affect bovine, caprine and ovine species, and the human and avian metapneumoviruses, among others. Human RSV (HRSV) is an important pathogen within this subfamily, causing 64 million infections and 160 000 deaths, primarily infant, per year (Ref. 14).

The emerging *Henipavirus* genus

HeV and NiV have been classified in a new genus because their genomic lengths and protein homologies are sufficiently different from extant genera of paramyxoviruses (Ref. 4). Their particularly broad tropism and extreme virulence compared with other paramyxoviruses also sets them apart. The henipaviruses naturally infect flying foxes (bats of the genus *Pteropus*), and transmit to humans either by an intermediate host, usually horses for HeV and swine for NiV, or directly from bat to human or from human to human, as reported for post-2004 epidemics for NiV in Bangladesh (Refs 1, 15, 16, 17).

HeV has reportedly caused the death of dozens of horses and three humans in Australia, through several outbreaks since 1994 (Refs 5, 18, 19, 20, 21). By contrast, NiV has caused the death of almost 200 humans and high numbers of animals, with 1.1 million pigs culled in the first 1998 Malaysian epidemic alone (Ref. 4). Since then, flying foxes seropositive for NiV have been detected in Cambodia, Thailand, India, and as far west as Madagascar and Ghana in West Africa (Refs 22, 23). NiV causes respiratory and neurological symptoms that often lead to encephalitis and mortality rates from 40% to 92% in humans (Refs 2, 24, 25). Additionally, NiV can spread efficiently and cause morbidity in economically important livestock (Ref. 24).

As a result of their high virulence and the absence of therapeutics or vaccines to control them, henipaviruses are classified as Biosafety Level 4 pathogens, and NiV is classified as a Category C Priority Pathogen by the US NIAID Biodefense Research Agenda for its bio- and agro-terrorism potential (Ref. 24). These characteristics of the henipaviruses underscore the need for research and treatment development against these perilous pathogens.

Molecular advancements in emerging paramyxovirus biology: implications for drug development

The development of antiviral therapeutic agents for other viral infections has been facilitated by elucidation of the molecular mechanisms

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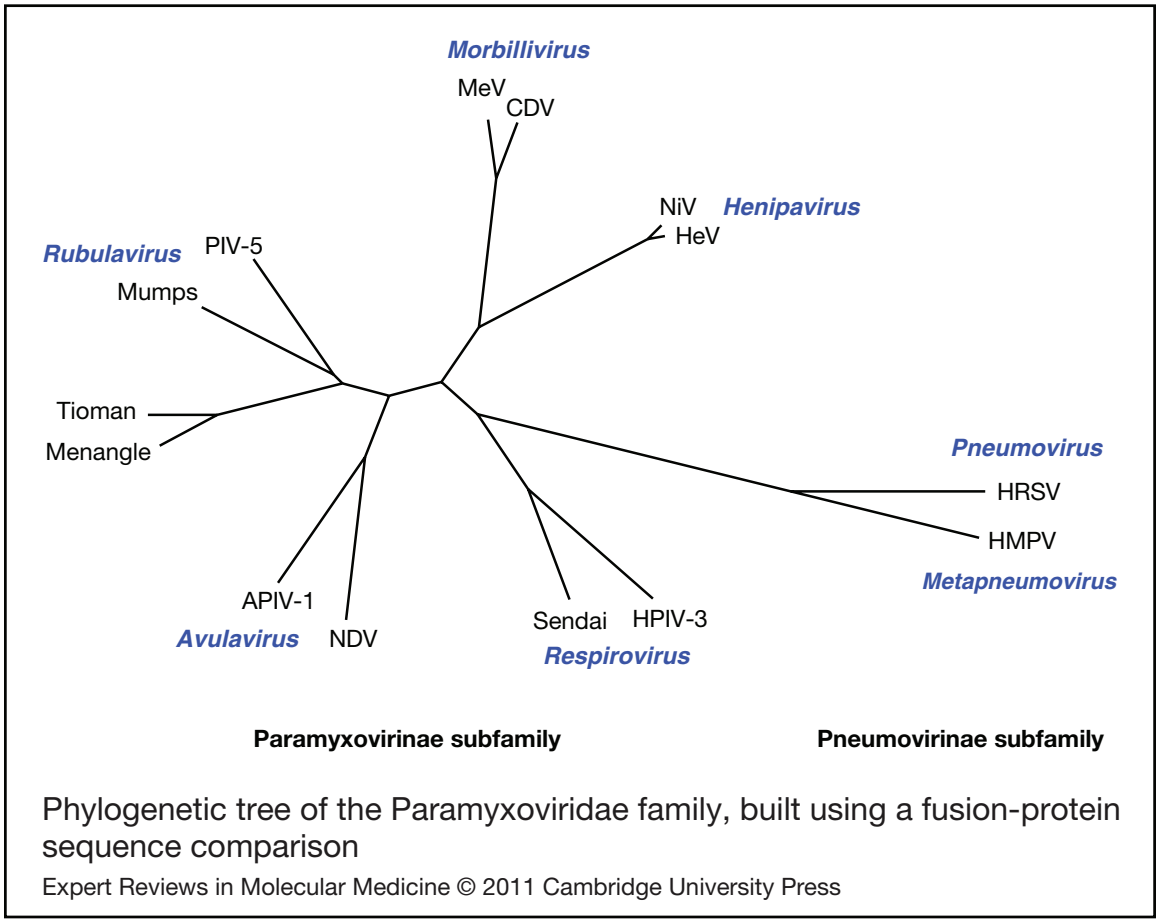

Figure 1. Phylogenetic tree of the Paramyxoviridae family, built using a fusion-protein sequence comparison. The tree was generated from a COBALT (NCBI) multiple fusion-protein sequence alignment, by the fast minimum evolution method, and visualised using the Fig Tree program (http://www.ncbi.nlm.nih.gov/tools/cobalt/cobalt.cgi?CMD=Get&cobaltRID=M93UBRKP212&SEQ_NUMBER=14&UNIQ_OBJ_NAME=A_CobaltResults_1PjIvj_2LC4_3DdVPpg5IK_GTJe2_NDktU&link_loc=FromRes, http://www.ncbi.nlm.nih.gov/blast/treeview/treeView.cgi). Representative members of each genus of the Paramyxovirinae and Pneumovirinae subfamilies are shown (genera are shown in blue type). Abbreviations: APIV-1, avian parainfluenza virus 1; CDV, canine distemper virus; HeV, Hendra virus; HMPV, human metapneumovirus; HPIV-3, human parainfluenza virus 3; HRSV, human respiratory syncytial virus; MeV, measles virus; NDV, Newcastle disease virus; NiV, Nipah virus; PIV-5, parainfluenza virus 5.

underlying various steps of their viral life cycles. As an example, insights into the life cycle of human immunodeficiency virus 1 (HIV-1) have led to approved antiretroviral drugs that target distinct steps: co-receptor antagonists and fusion inhibitors target viral entry, nucleoside and non-nucleoside inhibitors target viral reverse transcriptase, integrase inhibitors target integration, and protease inhibitors target viral maturation (reviewed in Refs 26, 27). Among the emerging paramyxoviruses, the henipaviruses have been studied most extensively because of their relatively high morbidity rates. Recent discoveries have shed light on the molecular mechanisms underpinning several steps of their life cycle, including host receptor usage, membrane fusion and viral entry, viral replication, interferon (IFN) responses, assembly and budding, and each step represents a potential target for the development of antiviral drugs (Fig. 2). These research advances and antiviral therapeutic strategies are discussed here, with most focus on the viral entry and assembly steps, carried out by the fusion, attachment and matrix viral proteins. The molecular mechanisms and antiviral approaches

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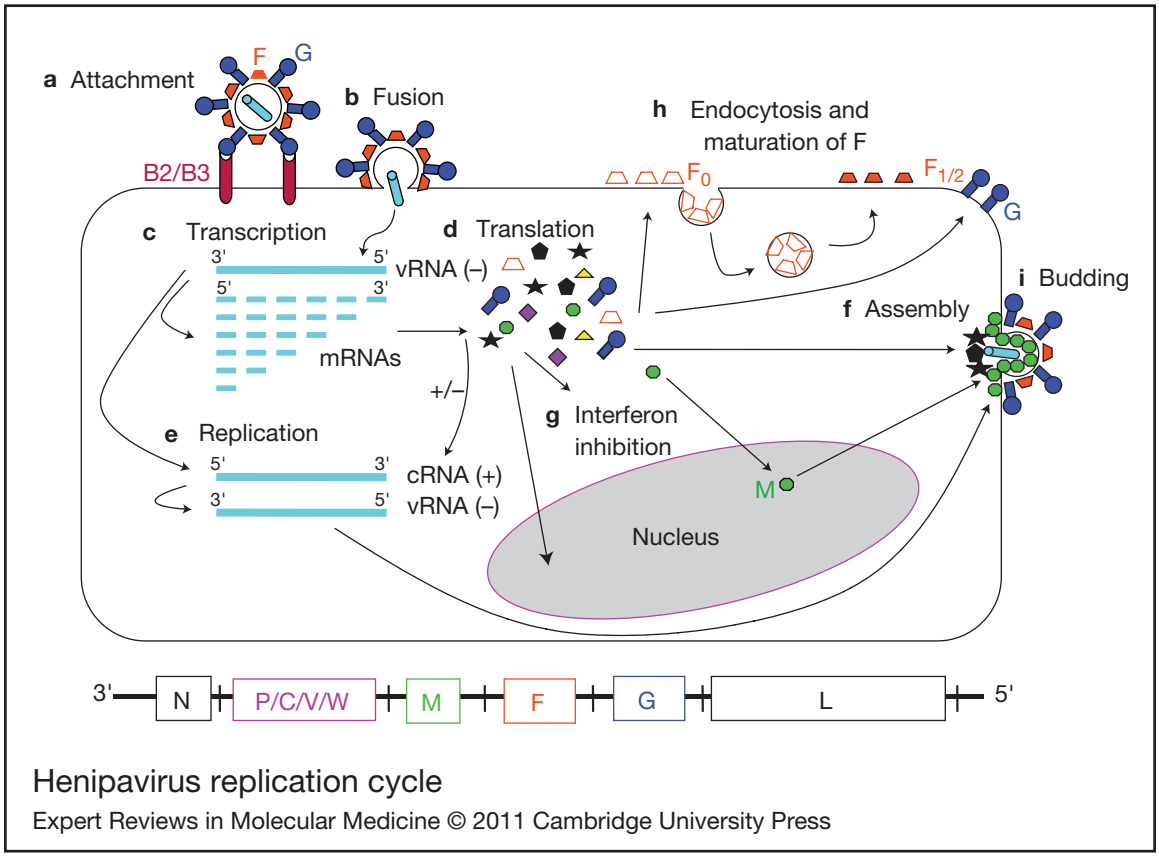

Figure 2. Henipavirus replication cycle. After attachment to the ephrinB2/B3 receptor (a) and fusion (b), the virus enters the cell. The negative RNA genome [vRNA(−)] is a template for transcription of viral mRNAs following a 3′ to 5′ attenuation gradient from N to L (c). N and L are depicted on the henipavirus genomic RNA, represented in its 3′ to 5′ orientation, at the bottom of the figure. mRNAs are translated into proteins (d), while the vRNA(−) is also a template for cRNA(+), which in turn is a template for vRNA(−) genomes during replication (e). New vRNA(−) genomes will be incorporated into new virions during viral assembly (f). Following translation (d), various viral proteins function in interferon (IFN) signalling pathways (g), and the precursor fusion protein (F_0) will be endocytosed and matured ($F_{1/2}$) (h). Assembly (f) and budding (i) are orchestrated primarily by the M (matrix) protein, and N, P, C, M, F (fusion) and G (attachment) proteins are incorporated into virions.

that target the functions of other nonstructural paramyxovirus proteins, particularly the gene products P, V, C and W, have been previously reviewed in detail elsewhere (Refs 11, 28, 29, 30).

In general, after virus binding to the host cell receptor, paramyxoviruses require the cooperation of their separate attachment and fusion transmembrane glycoproteins (reviewed in Refs 31, 32, 33, 34). However, how the attachment glycoprotein activates the fusion protein, or how the fusion protein senses that it is the right time and place for carrying out its host–virus membrane fusion function, is still a matter of intense investigation. The regulation of the molecular choreography that leads to productive membrane fusion provides a particularly fertile area for the development of therapeutics that can thwart this process.

Molecular mechanisms and antiviral strategies targeting the attachment glycoprotein

The paramyxovirus attachment proteins are type II transmembrane proteins on the surface of virions that mediate attachment of the virus to the cell-surface receptor. This interaction between the viral attachment protein and the host receptor has an important role in determining cell tropism.

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There are several conserved features among all known paramyxovirus attachment proteins (G, H or HN). They contain a head domain linked to the viral membrane by a stalk domain, and a cytoplasmic tail that is intraviral, or intracellular when the proteins are expressed at the cell surface (Fig. 3). The globular head of HeV-G and NiV-G (HNV-G) has a six-bladed β-propeller structure common to the head domains of multiple paramyxovirus attachment proteins (Refs 37, 38). The oligomeric structure of HNV-G (dimers of dimers) (Ref. 39) is also thought to resemble that of the attachment glycoproteins of other Paramyxovirinae (Refs 33, 40), and it is likely that a finely balanced stoichiometry is required for optimal fusion because endogenous lectins such as galectin-1 (see below) that cause inappropriate oligomerisation of henipavirus envelope proteins can be detrimental to the fusion process (Ref. 41).

Emerging paramyxovirus receptors

The host receptors for Menangle virus, Tioman virus, HMPV, and Beilong or J viruses, which are considered as emerging paramyxoviruses with lower morbidities in humans, are unknown (reviewed in Ref. 6). By contrast, the receptors for the henipaviruses were discovered in 2005 and 2006 to be ephrinB2 and ephrinB3, respectively (Refs 42, 43, 44). These transmembrane proteins are receptor tyrosine kinases that interact with their endogenous receptors on opposing cells and have critical roles in cell–cell signalling, particularly during angiogenic and neuronal development (Ref. 45). The distribution of ephrinB2 and ephrinB3 is consistent with the respiratory and neurological symptoms of henipavirus infections, because ephrinB2 and ephrinB3 are highly expressed in endothelial cells that line the microvasculature and in neurons (Refs 42, 43, 44). In the central nervous system, ephrinB3 but not ephrinB2 is expressed in the brain stem, and ephrinB3-mediated entry might account for the brain stem dysfunction that is the ultimate cause of death from NiV encephalitis (Refs 44, 46). The identification of NiV and HeV receptors greatly facilitates the rational development of strategies and therapeutics that block virus–receptor binding.

Mechanisms of fusion triggering by the attachment protein

With very few exceptions, the attachment protein of paramyxoviruses is essential for viral entry (Fig. 3). Even for HRSV, whose attachment protein is not required for membrane fusion, fusion is enhanced in the presence of the attachment glycoprotein. Interestingly, HMPV membrane fusion, and sometimes replication, is not enhanced by the presence of the attachment protein (reviewed in Refs 31, 33). Thus, the specific role of the attachment protein in promoting viral entry is a subject of intense study (reviewed in Refs 32, 33, 47).

Several studies in various paramyxoviruses implicate the attachment glycoprotein stalk domain in interaction with and triggering of the fusion glycoprotein, which is the ultimate protein that mediates membrane fusion (Refs 35, 48, 49, 50, 51, 52, 53). Biochemical and biophysical studies suggest that a receptor-induced conformational change in NiV-G, which involves crucial residues at the base of the NiV-G head domain and the presence of an intact stalk domain, is important for allosteric triggering of the fusion protein (Ref. 35). Although no dramatic differences were found between the apo- and receptor-bound structures of NiV-G (Refs 38, 54), the stalk domain was not apparent in any of these structures. Perhaps the presence of the stalk allows for proper disassembly of higher-ordered oligomers on receptor binding, which might lead to the exposure of neo-epitopes that functionally trigger the fusion protein. Although the specifics of how HNV-G triggers its own fusion protein are beyond the scope of this review, it is likely that this triggering process is finely tuned (Ref. 35) and therefore vulnerable to disruption. A better understanding of this triggering process might lead to therapeutics that target conserved features, which might limit the development of resistance. For example, anti-HNV-G antibodies that recognise conserved neo-epitopes exposed after receptor binding might be good candidates for passive immunisation strategies (Ref. 35).

Antiviral strategies that target the attachment protein

There have been several monoclonal antibodies (mAbs) produced against NiV-G and HeV-G, with a range of in vitro neutralisation activities (IC_{50} of ~40–600 ng/ml) (Refs 35, 55, 56, 57). One of these human mAbs (m102.4), which engages the receptor-binding site in NiV- or

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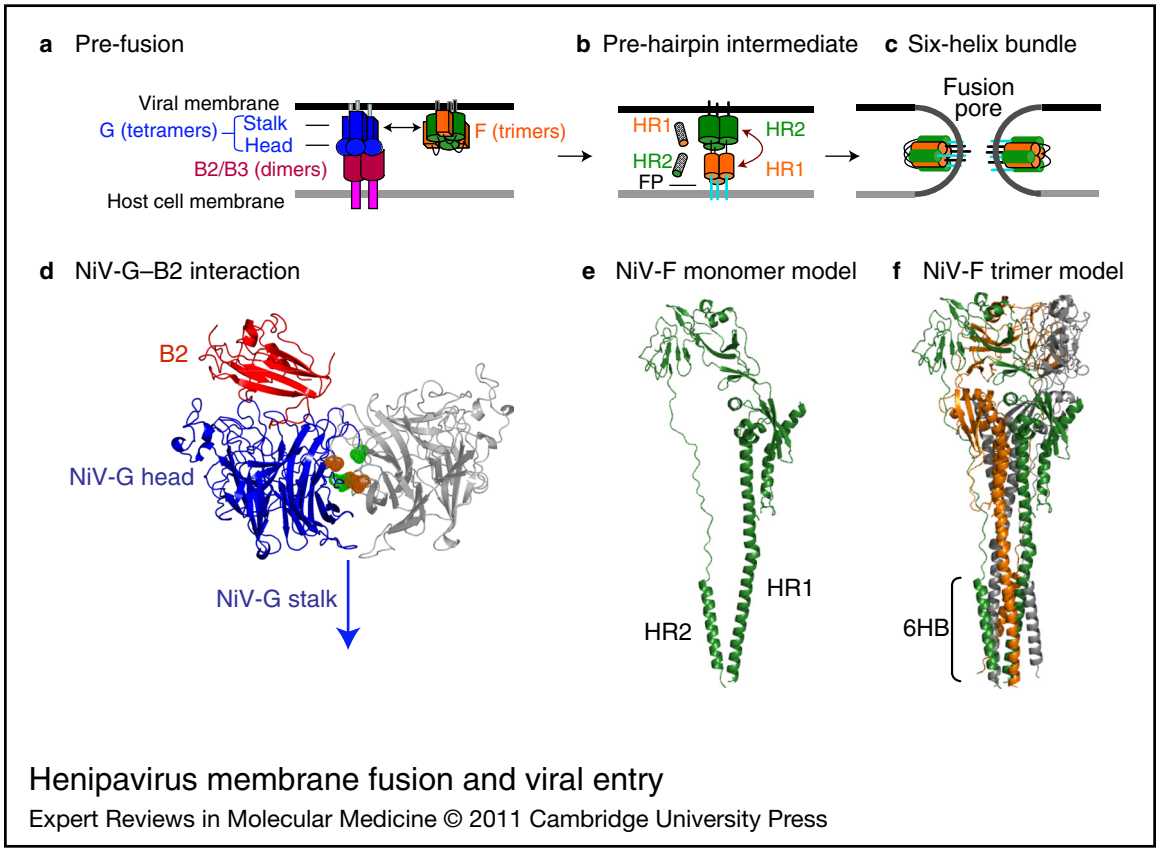

Figure 3. Henipavirus membrane fusion and viral entry. The attachment and membrane fusion steps necessary for viral entry [steps (a) and (b) from Fig. 2] are depicted here in greater detail in three major stages. (a–c) The fusion protein F is depicted in its pre-fusion, pre-hairpin intermediate and post-fusion forms. (a) EphrinB2 or ephrinB3 binding to NiV-G initiates a conformational cascade in F. (b) After F is triggered, it forms a pre-hairpin intermediate, in which the fusion peptide (FP) is harpooned into the host cell membrane. The pre-hairpin intermediate can be captured by peptides that mimic the NiV HR1 (orange-ended cylinder) or HR2 (green-ended cylinder) regions and bind the F HR2 or HR1 regions, respectively. (c) The HR1 and HR2 regions in the pre-hairpin intermediate coalesce to form the six-helix bundle conformation, bringing the viral and cell membranes together and facilitating viral–host membrane fusion and viral entry. (d) Ribbon structure of the monomer of NiV-G (blue) head domain (pdb code 2VSM) and its interaction with its ephrinB2 receptor (red), drawn using PYMOL (http://www.pymol.org) and modelled by aligning the G–B2 monomer with each monomer of the HPIV-3 haemagglutinin–neuraminidase dimer (pdb code 1V2I) similarly to Ref. 35. The second monomer is shown in grey. According to this model, the flexible region in the NiV-G ectodomain (green and orange) might interact with the same region in another monomer and might be involved in receptor-induced G-mediated NiV-F triggering (Ref. 35). (e) Representation of the structure of the NiV-F protein modelled using the HPIV-3-F crystal structure (pdb code 1ztm) by the Phyre threading program, as performed in Ref. 36. (f) Representation of the trimer of NiV-F monomers from (e), also modelled using the HPIV-3-F crystal structure as in Ref. 36. Abbreviations: HR1, heptad repeat 1; HR2, heptad repeat 2; HPIV-3, human parainfluenza virus 3; NiV-G, Nipah virus attachment protein.

HeV-G, appears to be protective in a lethal challenge ferret model when administered intravenously 10 h post-infection but not 24 h pre-infection (Ref. 55). This difference could be due to the relatively low serum stability of m102.4 when administered intravenously, but nevertheless bodes well for the development of m102.4 as a post-exposure therapeutic in resource-sufficient settings. In comparison, palivizumab (Synagis®, MedImmune Inc.), a mAb therapeutic approved by the US Food and Drug Administration (FDA) that targets the fusion protein of HRSV, has an in vitro IC_{50} of 363.7 ng/ml (Ref. 58) and monthly administration

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(for HRSV prophylaxis) by intramuscular injections can maintain serum concentrations of 100-fold (>40 μg/ml) above its in vitro IC_{50} in most patients (Ref. 59). It would be interesting to see whether intramuscular injection will increase the effective half-life of m102.4 in vivo.

Soluble ephrinB2 or ephrinB3, or soluble HNV-G proteins, have also been shown to block virus entry and cell–cell fusion (Refs 42, 43, 44, 60), although the likely interference with ephrinB function and the antigenicity of HNV-G itself limits the practical utility of these molecules as antivirals. However, the structure of the ephrinB2- or ephrinB3-bound HNV-G complex shows a large protein–protein interface and also a lock-and-key binding pocket that might be targeted by small-molecule therapeutics (Refs 37, 38). For example, Trp125 and Phe120 in the G–H loop of ephrinB2 interact differently with ephrinB4 than with HNV-G, suggesting a 'druggable' pocket to disrupt B2/B3–G interactions specifically (Ref. 61). A likely caveat to this approach is that a small molecule designed to fit the B2/B3–G binding pocket specifically might still not be able to overcome the strong avidity of oligomeric B2/B3–G interactions. For example, ephrinB2 binds to NiV-G with a subnanomolar affinity (K_d ~0.06 nM) (Ref. 44), suggesting that a drug would have to bind at picomolar concentrations or have a very slow off-rate to compete with B2–G interactions.

Molecular mechanisms and antiviral strategies targeting the fusion glycoprotein

The fusion (F) glycoproteins are synthesised as type I transmembrane trimeric precursors that are activated by protease cleavage into a metastable pre-fusion conformation, poised for enabling membrane fusion (Fig. 3). Cleavage generates a new hydrophobic N-terminus, the fusion peptide, which is buried in the metastable pre-fusion F conformation. On attachment-protein–receptor binding, the fusion cascade is triggered and the fusion peptide is harpooned into the target cell membrane in the pre-hairpin intermediate conformation (Fig. 3b). Two helical regions present in the pre-hairpin intermediate, HR1 and HR2, have high affinities for each other and coalesce to form the six-helix bundle (6HB), which brings the viral and target cell membranes together in close apposition, allowing virus–target-cell membrane fusion and viral entry.

Maturation of the fusion protein

Important differences in viral entry and membrane fusion mechanisms carried out by the F protein have been highlighted for the emerging paramyxoviruses (Refs 28, 31, 34). First, although many paramyxoviral F proteins are cleaved (once or twice) by furin-like cellular proteases during transport through the trans-Golgi network (Refs 62, 63, 64, 65, 66), HMPV and Sendai virus F proteins are cleaved by tissue-specific extracellular proteases such as mini-plasmin or tryptase Clara (Refs 67, 68), and cell-surface henipavirus F is cleaved by cathepsin L on endocytosis (Refs 69, 70, 71, 72). Specific inhibition of these proteases by antiviral compounds could be envisioned. For example, the lack of an acutely lethal phenotype in cathepsin-L-knockout mice suggests that short-term inhibition of cathepsin L in the context of a highly pathogenic virus infection might be a clinically viable option. Recently, a small-molecule oxocarbazate-specific inhibitor of cathepsin L was reported to be effective against Ebola and severe acute respiratory syndrome (SARS) viruses at subnanomolar concentrations in vitro (Ref. 73). Although Ebola and SARS viruses directly require cathepsin L cleavage during viral entry, this compound could also prove useful in treating henipavirus infections by preventing the generation of mature F protein. However, past in vitro versus in vivo discrepancies between drugs that indirectly inhibit cathepsin L cleavage have been observed. Chloroquine, normally used to treat malaria, has been shown to inhibit pseudotyped NiV entry, presumably by inhibiting endosomal acidification and indirectly cathepsin L activity (Ref. 74). However, chloroquine treatment did not prevent NiV infection or disease in ferrets (Ref. 75), and combined chloroquine and ribavirin treatments did not prevent death in a hamster model of NiV and HeV infection (Ref. 76). These in vitro versus in vivo discrepancies suggest that we need to improve our understanding of the role of endocytosis and cathepsin L cleavage in henipavirus infection.

N-glycans in henipavirus fusion protein, and galectin-1

Another characteristic of emerging paramyxoviral F proteins is their atypical use of *N*-glycans.

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For most paramyxovirus F proteins, specific *N*-glycans are necessary for proper protein folding and *N*-glycan removal is deleterious to the fusion process (Refs 77, 78). Surprisingly, removal of specific individual or multiple *N*-glycans from NiV- and HeV-F resulted in marked hyperfusogenicity manifested in fusion and viral entry assays (Refs 36, 79). However, *N*-glycan removal also increased the sensitivity of NiV-F to antibody neutralisation; it thus seems that *N*-glycans in henipavirus F are kept (at least partially) to serve as a shield against antibody neutralisation (Ref. 36).

NiV-F *N*-glycans were also found to mediate binding to galectin-1, an innate immune lectin with many functions that binds to specific galactose-containing carbohydrates on the surface of mammalian cells or pathogens (reviewed in Ref. 80). Galectin-1 inhibits NiV-mediated cell–cell fusion and syncytia formation, a hallmark of NiV pathogenicity (Ref. 41). Interestingly, the individual *N*-glycan in NiV-F (F3) whose removal resulted in the highest level of hyperfusogenicity also gave rise to the most optimal *N*-glycan moiety that mediates galectin-1 binding to NiV-F. Endogenous levels of galectin-1 in endothelial cells were sufficient to inhibit NiV-envelope-mediated syncytia, and galectin-1 binding to the F3 *N*-glycan in NiV-F inhibited maturation, mobility and triggering of the F protein (Ref. 81). Although it is unlikely that galectin-1 can be developed as an antiviral therapeutic because of its pleiotropic effects, these reports shed light on the innate immune defences based on recognition of pathogen-associated molecular patterns. Furthermore, 14 single-nucleotide polymorphisms have been identified in the genomic locus of galectin-1 (Ref. 82), which raises the intriguing possibility that genetic variability at this locus might contribute to the range in pathophysiology seen in henipavirus infections.

Blocking the membrane fusion cascade

Blocking viral entry by trapping one of the fusion protein intermediates during the membrane fusion cascade is a therapeutic approach that has been pursued and used for class I fusion protein enveloped viruses. For example, enfuvirtide, sifuvirtide and their analogues are peptides that mimic the C-terminal heptad-repeat region (HR2) of class I fusion proteins, and are approved for HIV-1 treatment (reviewed in Refs 83, 84, 85). Because paramyxoviral F proteins undergo equivalent class I fusion protein conformational changes, including pre-hairpin intermediate formation (Refs 28, 31, 33, 34, 86), the paramyxovirus HR2 (also known as HRC) peptide has been used to trap the pre-hairpin intermediate (Refs 35, 36, 87–93) (Fig. 3b). Although a peptide mimicking the N-terminal HR1 also inhibits fusion, it is generally a less efficient inhibitor (Ref. 89), even when artificially trimerised to mimic the trimeric HR1 core (Ref. 35).

HR2 peptides

For the henipaviruses, the HR2 peptide has been shown to inhibit cell–cell membrane fusion and viral entry in a pseudotyped viral system at nanomolar concentrations (Refs 36, 88, 89, 91). Surprisingly, higher levels of inhibition of HeV fusion were observed when using a human parainfluenza virus 3 (HPIV-3)-F- versus a HeV-F-derived HR2 peptide, although the mechanism for this phenomenon is unclear (Ref. 92). Additionally, a second generation of capped and PEGylated HR2 peptides resulted in increased solubility in water, stability, synthesis yields and possibilities for their use as antiviral agents in vivo (Ref. 89). Another strategy for increasing HR2 peptide inhibition efficacy has been the addition of cholesterol to the peptide C-terminus. This approach probably brings the peptide into close proximity to the membrane site of action where fusion occurs, reducing the IC_{50} of HPIV-3-derived peptides on pseudotyped HeV and NiV infections from 10–100 nM to near 1 nM (Ref. 94). However, the IC_{50} values for inhibition of live HeV and NiV viruses in vitro were close to 100 nM, and relatively large amounts of HR2–cholesterol peptides (2 mg/kg) were needed to achieve ≤60% survival of hamsters infected with NiV, when used simultaneously to or before NiV infection. It is likely that large HR2 peptide amounts are needed in order to efficiently 'coat' the surfaces of target cells in the host (Ref. 95).

Anti-F mAbs

Another approach to inhibiting membrane fusion is the blocking of the fusion protein conformational changes required for the fusion cascade by the use of mAbs. Two anti-NiV-F antibodies have been reported to neutralise NiV

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and HeV in vitro (1.6–20 ng) and in a hamster model (180–520 μg/animal) (Ref. 96). Although the binding epitopes of these antibodies have not been characterised, their cross-reactivity suggests they might target a conserved region in HNV-F, which might limit the generation of escape variants. Moreover, antibodies that bind conformational epitopes critical for membrane fusion are highly desirable, because mutations that annul both mAb binding and the need of conformational changes would be relatively rare. Conformational mAbs against the henipaviruses that preferably bind hyper- or hypo-fusogenic mutants have been reported, but their neutralisation activities or their binding epitopes have not been shown (Ref. 88).

Small-molecule inhibitors

Quinolone derivatives designed based on structure similarities among paramyxovirus F proteins in their HR1/HR2-binding motifs were tested for inhibition of NiV- and measles-virus-induced cell fusion. Two of 18 compounds tested were moderately active as inhibitors of NiV-induced cell–cell fusion and NiV-infection-induced syncytia at an EC_{50} of 1–3 μM. These compounds also showed some cytotoxicity in Vero cells [CC_{50} of 10 to >20 μM using the MTT (cytotoxity) test], resulting in a selectivity index (SI; CC_{50}/IC_{50}) of ~13 for the compound with the lowest toxicity (Ref. 97). This SI is relatively poor for a lead compound but might be improved by further structure–activity relation analysis. Mutants that cause resistance to HR2 peptide binding have been detected for HIV (Refs 83, 84, 85), and similar mutants might occur after the use of these small-molecule inhibitors that target HR1–HR2 interactions.

Molecular mechanisms and antiviral strategies targeting the matrix protein

Paramyxoviral matrix (M) proteins are structural proteins that directly underlie the viral envelope, and are important for the assembly and budding of viral particles (Refs 98, 99). Infectious paramyxoviral particles form after all the structural viral components have assembled at selected sites on the cell membrane, and M proteins are known to organise the assembly process. The position of M proteins underneath the cellular plasma membrane allows them to interact with ribonucleoproteins [RNA genomes bound to nucleocapsid (N or NP) proteins] as well as viral glycoproteins through their cytoplasmic tails (Refs 98, 99). Recently, the atomic structure of the paramyxovirus HRSV M protein was solved and shown to contain two β-sheet-rich domains, joined by a short unstructured linker (Ref. 100). This structure is similar to that of the filovirus Ebola M (Ref. 101). The joined domains share an extensive positively charged surface, which probably binds to the negatively charged membrane phospholipid head groups (Ref. 100). For many paramyxoviruses, transient expression of M proteins alone, without the expression of other viral proteins, is sufficient to form and release viral-like particles (VLPs); this is the case for HPIV-1 (Ref. 102), Sendai virus (Ref. 103), Newcastle disease virus (Ref. 104), measles virus (Refs 105, 106) and NiV (Refs 107, 108). However, in some cases, M-dependent VLP production is enhanced in the presence of other viral proteins, such as the glycoproteins, the nucleocapsid protein or the C protein (reviewed in Ref. 98).

Antivirals against M

Because the M protein is crucial in paramyxoviral assembly and budding, antiviral agents that target important aspects of M-directed assembly and budding can be envisioned. For example, inhibition of Newcastle disease virus replication by targeting two distinct sites of the M gene using interfering RNA has been recently reported (Ref. 109). In addition, for simian virus 5, proteasome inhibitors and expression of dominant-negative VPS4(E228Q) ATPase blocked budding, probably because of the involvement of the ubiquitin–proteasome pathway in budding (Ref. 110). For NiV, a recent study showed that ubiquitin-regulated nuclear–cytoplasmic trafficking of NiV-M is important for viral budding (Ref. 111). Therefore, compounds that block M-ubiquitinating enzymes by depleting free ubiquitin in the cell (proteasome inhibitors), or that preferentially block nuclear import or export of NiV-M, could be potential antihenipavirus candidates (Fig. 2). Indeed, bortezomib, an FDA-approved proteasome inhibitor used for treating multiple myeloma, reduced viral titres significantly at an IC_{50} of 2.7 nM, 100-fold less than the achievable plasma concentration in humans (Ref. 111). Thus, this FDA-approved agent has the potential for being evaluated as an off-label use for henipavirus treatment. Understanding the cellular components that have important roles in viral assembly and release should also aid the discovery

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of novel drugs to target these steps of the life cycle of emerging paramyxoviruses.

Molecular mechanisms and antiviral strategies targeting the P, V and C proteins

IFNs are part of the innate immune system and constitute one of the first lines of defence against viral pathogens in mammals (Ref. 112) in the early virus–host battle that determines the establishment of an infection (Ref. 113). The P gene encodes for the P, C, V and W proteins, and in the subfamily Paramyxovirinae the P gene products generally have anti-IFN activities (see Ref. 28). In part, P gene antiviral activities are due to their effects in limiting the extent of viral genome replication, because aberrant transcripts activate the retinoic acid inducible gene I (RIG-I; DDX58) RNA helicase pathway, which activates IFN production (Ref. 114). For example, the simian virus 5 P protein (Ref. 115), Sendai C protein (Ref. 116), measles C protein (Ref. 117), J virus and Beilong virus C proteins (Ref. 114), HPIV-3 C protein (Ref. 114), and henipavirus C, V and W proteins (Ref. 118) have all been shown to inhibit viral genome replication. Recently, a study in golden hamsters showed that V and C proteins play key roles in NiV pathogenicity (Ref. 151). In addition, all the henipavirus P gene proteins have been shown to inhibit IFN signalling pathways (reviewed in Refs 119, 120).

Because restoring IFN responses has been successful in the treatment of cancer, autoimmune and infectious diseases (Refs 121, 122, 123), this type of approach might also be suitable against emerging paramyxovirus infections. One study showed that the IFN inducer poly(I)–poly(C12U) (Ampligen®, a mismatched double-stranded RNA) prevented death from NiV infection in a hamster model (Ref. 124). Ampligen was also observed to be effective against SARS-coronavirus infection in a mouse model (Ref. 125), and has shown positive effects in HIV-infected patients (Ref. 126). Congruent with these studies is the finding that NiV and HeV replicate more efficiently in Vero cells, which are defective in IFN responses, compared with other cell lines (Ref. 127). Therefore, stimulation of IFN production seems to be a promising treatment for henipavirus infections.

Broad-spectrum and other antiviral strategies

Most current antiviral drugs target differences between viral agents and hosts, such as specific viral protein moieties important for viral entry, replication, assembly, budding and so on, conferring specificity for the infected cells. However, targeting specific viral protein moieties is not always the best solution, because viral resistance by mutagenesis is very common when targeting single or even multiple viral proteins (Refs 128, 129). Thus strategies that target nonprotein determinants of important steps in the viral life cycle, particularly for a broad assortment of viruses, are highly desirable. For example, broad-spectrum compounds that target the viral membrane fluidity required for viral entry or exit, or RNA replication, have recently been explored.

LJ001, a viral membrane inhibitor

Recently, a high-throughput screening assay based on NiV/vesicular stomatitis virus (VSV)-pseudotype viral entry inhibition identified a small molecule that intercalates into and irreversibly damages viral membranes, but not cellular membranes, at low micromolar concentrations (Ref. 130). Studies with lipid biosynthesis inhibitors indicated that LJ001 exploits the differences between static viral membranes and biogenic cellular membranes with reparative capacity. LJ001, a rhodanine derivative, was effective against numerous enveloped viruses, but not against nonenveloped viruses, and showed no overt toxicity in vitro or in vivo, with an SI of >100. LJ001 inactivated virions while leaving envelope proteins functionally intact, inhibiting a post-binding but pre-fusion step (Ref. 130). Thus, LJ001 might represent a new class of broad-spectrum antivirals that target physiological rather than physical differences between viral and cellular lipid membranes. A potential mechanism of action would be disruption of the proper balance between saturated and unsaturated phospholipids that is required for the positive to negative membrane curvature transitions during the fusion process (reviewed in Ref. 131). Elucidating the exact mechanism by which LJ001 damages membranes will shed light on whether differences between viral and cellular membranes can be exploited by other chemotypes, and help refine medicinal chemistry efforts to improve bioavailability and in vivo efficacy.

Cationic compounds

In another study, a high-throughput screen based on live virus infection identified three compounds

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unsuitable for internal administration, but possibly suitable for topical applications (Ref. 132). These three compounds – gliotoxin, Gentian Violet and Brilliant Green – have been previously used as antibacterial and antifungal agents, and showed antiviral activity against NiV, HeV, VSV and HPIV-3. Additionally, gliotoxin inhibited influenza A, suggesting a broad-spectrum activity for this compound. Although the mode of action of these cationic compounds is not known, it has been proposed that they directly bind to and inhibit viral membranes (Ref. 132).

Calcium influx inhibitors

In a recent study that tested licensed pharmaceuticals against henipavirus replication in vitro, calcium chelators and compounds that released intracellular calcium stores, as well as calcium channel and calmodulin antagonists, inhibited henipavirus replication at the micromolar range (Ref. 133). However, the mechanism that links calcium influx to henipavirus replication is unknown, and in vivo assays have not been reported.

Ribavirin

Ribavirin is a broad-spectrum antiviral used particularly for HRSV and hepatitis C, and it is also used for RNA viruses for which there is no other available treatment (Refs 134, 135). It is a purine nucleoside analogue, and although its exact mechanism of inhibition of viral replication is not completely understood, it is known that ribavirin interferes with RNA metabolism, which is required for virus replication (Ref. 136). For the emerging paramyxoviruses, various results with ribavirin have been reported. In the first NiV outbreak in Malaysia in 1998–1999, a 36% reduction in mortality in humans was reported (Ref. 137). In addition, several studies have reported the inhibition of henipavirus replication by ribavirin in vitro (Refs 74, 76, 124, 138, 139). However, in vivo studies carried out in animal models have not yielded promising results with ribavirin (Refs 76, 124). The inability of ribavirin to cross the blood–brain barrier might account for its inadequacy in in vivo studies. It has been previously shown that ribavirin is effective in the brain only when administered intracranially (not by intraperitoneal injection) in a hamster model (Ref. 140). In the Malaysian epidemic, the effect of ribavirin in late-onset NiV encephalitis was not reported (Refs 137, 140). In addition, the complex molecular mechanisms of inhibition of viral replication by ribavirin, such as induction of error catastrophe (excessive RNA mutations) and depletion of intracellular GTP pools, might not allow the rapid design of more potent analogues (reviewed in Ref. 141).

Chloroquine

Chloroquine (9-aminoquinoline) is used for the treatment of pathogens that require endosome acidification, such as malaria and pH-dependent viruses. Because the henipaviruses require endosomal cleavage of their F protein, it was not surprising that chloroquine was found to be a potential inhibitor of NiV infection in vitro (Refs 74, 75, 76). However, oral administration of chloroquine did not protect ferrets from lethal NiV infection (Ref. 75) even though effective serum chloroquine concentrations were achieved, and peritoneal administration of chloroquine alone or in combination with ribavirin did not protect hamsters from lethal NiV or HeV challenge (Refs 75, 76). As with ribavirin, the lack of in vivo success with chloroquine might be due to its inability to cross the blood–brain barrier or inadequate tissue distribution (Ref. 142), and to its effects on the immune system that might not favour the host (Ref. 143). In vitro versus in vivo discrepancies in choroquine treatment results have also been reported for influenza, SARS, HIV and chikungunya viruses (Ref. 143).

siRNA

An alternative way of inhibiting viral gene expression is by the use of small interfering RNA (siRNA) (Ref. 144). In one recent study, siRNA molecules directed against the L and N genes were tested against minigenome and live henipavirus replication in vitro (Ref. 145). Whereas some siRNA had effects on both minigenome and live virus replication, some had effects only on minigenome replication and some on neither. In addition, siRNA targeting more-conserved genome sequences, for instance in P, V or W, has been proposed (Ref. 145). Although somewhat promising, one disadvantage of this approach is the need for gene-therapy-based siRNA delivery methods, which might not be readily available.

Inhibitors of macropinocytosis

A recent report indicates that NiV can enter cells by macropinocytosis (Ref. 146). This type of entry

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Table 1. Effect of antiviral agents on emerging paramyxovirus infections

Target	Antiviral	Efficacy in vitro (on live virus)	Refs
Attachment	Soluble proteins: ephrinB2, B3	EphrinB2 IC_{50}: <10 μg/ml EphrinB3 IC_{50}: <25 μg/ml	60, 149
	EPhB3, B4	EPhB3, EPhB4 IC_{50}: >100 μg/ml	
	NiV-G HeV-G	NiV-G IC_{50}: 13.2 μg/ml HeV-G IC_{50}: 3.3 μg/ml	
	Mouse mAbs[a]: α-NiV-G Human mAbs[b]: α-HeV-G	IC_{90}: 0.27–2.34 ng IC_{90}, m101: <12.5 μg/ml IC_{50}, m102.4: 0.04 mg/ml (NiV) and 0.6 mg/ml (HeV)	96, 150 55
Fusion	Second-generation N-PEG NiV HR2	IC_{50}: 0.46–2.05 nM	89
	HPIV3-F HR2	IC_{50}: 208 nM (NiV) and 179 nM (HeV)	91, 92
	Mouse mAb: α-NiV-F	IC_{90}: 1.6–425.0 ng	96, 150
	Quinolone derivatives	IC_{50}: 0.5–4.0 μM	97
Matrix	Bortezomib	IC_{50}: 2.7 nM	111
IFN responses	Poly(I)–poly(C12U)[c]	IC_{90}: <6.25 μg/ml	124
Broad-spectrum and other antivirals	LJ001	IC_{50}: ~1 μM	130
	Ribavirin[d]	IC_{50}: ~4 μM (~1 μg/ml) IC_{90}: ~100 μM (~25 μg/ml)	6, 124
	Chloroquine[e]	IC_{50}: 1 μM IC_{90}: 20–100 μM	74 75, 76
	siRNA	>60% inhibition at 50 nM	145
	Macropinocytic inhibitors	Latrunculin A IC_{50}: <2 μM EIPA IC_{50}: ~15 μM	146
	Favipiravir	EC_{50}, HRSV: 260 μM	148

[a]100% protection in vivo at 100–112 μg.
[b]Human mAb m102.4: protection of 1/3 pre-infused and 3/3 post-infused ferrets at a dose of 50 mg.
[c]Protection of 5/6 animals, at a dose of 3 mg/kg once a day.
[d]Survival increased by 1–3 days, at a dose of 25–100 mg/kg.
[e]No protection at 50–150 mg/kg.
Abbreviations: EIPA, 5-(*N*-ethyl-*N*-isopropyl)-amiloride; HeV, Hendra virus; HR2, heptad repeat 2; IFN, interferon; mAb, monoclonal antibody; NiV, Nipah virus; PEG, polyethylene glycol; HRSV, human respiratory syncytial virus; siRNA, small interfering RNA.

pathway for NiV necessitates phosphorylation of the cytoplasmic domain of ephrinB2, after NiV-G attachment. Although it is not known whether this is a major pathway utilised for NiV entry, drugs that affect macropinocytosis, with the exception of chloroquine, affected NiV entry, but not cell–cell fusion (Ref. 146). Two of the strongest inhibitors of NiV entry were latrunculin A and the amiloride analogue 5-(*N*-ethyl-*N*-isopropyl)amiloride (EIPA). Although the first one is probably hazardous in vivo, EIPA is a commonly used antihypertensive agent, and can be evaluated for its in vivo efficacy in animal models of henipavirus infection.

Favipiravir (T-705)

Favipiravir is a compound with promising broad-spectrum antiviral activities. Host enzymes metabolise its precursor into a ribofuranosyltriphosphate derivative that selectively inhibits viral RNA-dependent RNA polymerases, for reasons not fully understood

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(reviewed in Ref. 147). Importantly, it does not inhibit host DNA or RNA synthesis, and is not cytotoxic to mammalian cells. In vivo experiments with T-705 against influenza virus, arenavirus, bunyaviruses, West Nile virus, yellow fever virus and foot-and-mouth disease virus have shown one or more of the following results: protection from death, reduction of viral loads and limitation of symptoms. In addition, protective effects of T-705 were observed when it was administered 1–7 days after virus inoculation (see Ref. 147). Although these pathogens were not paramyxoviruses, in vitro susceptibility of HRSV to T-705 has been observed (Ref. 148), suggesting that favipiravir might serve as an antiviral against emerging paramyxoviruses.

Future of antiviral strategies

The various antiviral strategies discussed in this review are summarised in Table 1. In general, a better understanding of the structures and functions of viral and host proteins involved in the viral life cycle (Fig. 2) will aid in the development of new antiviral therapeutics. In addition, animal model experiments that examine the potential antivirals arising from the in vitro studies described above are important – for example, because not all compounds can successfully cross the blood–brain barrier. Because the emerging virus entry mechanisms have been explored in greater detail than the assembly and budding mechanisms, further progress in the elucidation of these late (and other) steps of the viral life cycle is imperative. Prompt antiviral discovery and characterisation against emerging paramyxoviruses should be facilitated by the use of pseudotyped and reverse genetics viral systems.

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These review animal Henipavirus studies and Henipavirus antivirals, respectively.

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This covers other human-infecting paramyxoviruses, in addition to Hendra and Nipah viruses.

Features associated with this article

Figures

Figure 1. Phylogenetic tree of the Paramyxoviridae family, built using a fusion-protein sequence comparison.
Figure 2. Henipavirus replication cycle.
Figure 3. Henipavirus membrane fusion and viral entry.

Table

Table 1. Effect of antiviral agents on emerging paramyxovirus infections.

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