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REVIEW



# Broad-spectrum coronavirus antiviral drug discovery

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**ABSTRACT**

**Introduction**: The highly pathogenic coronaviruses severe acute respiratory syndrome coronavirus (SARS-CoV) and Middle East respiratory syndrome coronavirus (MERS-CoV) are lethal zoonotic viruses that have emerged into human populations these past 15 years. These coronaviruses are associated with novel respiratory syndromes that spread from person-to-person via close contact, resulting in high morbidity and mortality caused by the progression to Acute Respiratory Distress Syndrome (ARDS).

**Areas covered**: The risks of re-emergence of SARS-CoV from bat reservoir hosts, the persistence of MERS-CoV circulation, and the potential for future emergence of novel coronaviruses indicate antiviral drug discovery will require activity against multiple coronaviruses. In this review, approaches that antagonize viral nonstructural proteins, neutralize structural proteins, or modulate essential host elements of viral infection with varying levels of efficacy in models of highly pathogenic coronavirus disease are discussed.

**Expert opinion**: Treatment of SARS and MERS in outbreak settings has focused on therapeutics with general antiviral activity and good safety profiles rather than efficacy data provided by cellular, rodent, or nonhuman primate models of highly pathogenic coronavirus infection. Based on lessons learned from SARS and MERS outbreaks, lack of drugs capable of pan-coronavirus antiviral activity increases the vulnerability of public health systems to a highly pathogenic coronavirus pandemic.

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## 1. Introduction

Outbreaks of severe acute respiratory syndrome (SARS, 2002–2004 [1,2]) and Middle East respiratory syndrome (MERS, 2012-current [3]) in the last two decades are a significant threat to global public health. SARS and MERS represent a new class of public health concern that may continue to emerge into human populations: respiratory syndromes caused by coronaviruses (CoVs) that are transmitted from person-to-person via close contact, resulting in high morbidity and mortality in infected individuals. Although SARS and MERS initially present as mild, influenza-like illnesses with fever, dyspnea, and cough, progression to more severe symptoms is characterized by an atypical interstitial pneumonia and diffuse alveolar damage. Both SARS-CoV and MERS-CoV are capable of causing acute respiratory distress syndrome (ARDS), the most severe form of acute lung injury where alveolar inflammation, pneumonia, and hypoxic lung conditions lead to respiratory failure, multiple organ disease, and death in 50% of ARDS patients [4]. The total confirmed number of patients infected with highly pathogenic CoVs is relatively low (approximately 10,000 cases of both SARS and MERS since 2002), but CoVs are of particular concern due to high case fatality rates, lack of proven therapeutics, as well as the demonstrated ability of these pathogens to seed outbreaks that rapidly cross geographic and geopolitical borders into other countries and continents [5,6].

### 1.1. *Coronaviridae* phylogeny and emergence

Highly pathogenic coronaviruses SARS-CoV and MERS-CoV recently emerged into human populations, but other human coronaviruses (HCoVs) including HCoV-OC43, HCoV-229E, HCoV-NL63, and HCoV-HKU1 are estimated to have circulated in human populations for hundreds of years, causing mild respiratory illness to which approximately 5–30% of 'common colds' are attributed [7,8]. Within the *Coronaviridae* family (order Nidovirales) four genera are recognized: alphacoronavirus, betacoronavirus, gammacoronavirus, and deltacoronavirus. The six HCoVs (Table 1) currently identified belong to the genera alphacoronavirus (HCoV-229E and HCoV-NL63) and betacoronavirus (SARS-CoV, MERS-CoV, HCoV-OC43, and HCoV-HKU1). Gammacoronaviruses and deltacoronaviruses have no known viruses that infect humans, but contain important agricultural pathogens of livestock. Epizootic coronaviruses in animals cause a wide range of disease signs resulting from respiratory, enteric, and neurological tissue tropism. Although HCoVs cause primarily respiratory symptoms, the potential for a wide range of severe disease symptoms in humans caused by infection by future emergent coronaviruses cannot be excluded. Despite the severity and diversity of coronavirus disease signs and symptoms affecting a large number of important livestock species as well as humans, there are no proven therapies that specifically target CoVs.

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**Article Highlights**

- Broad-spectrum drugs targeting coronaviruses must have efficacy against known highly pathogenic human coronaviruses SARS-CoV and MERS-CoV, but also have activity against additional novel coronaviruses that may emerge in the future.
- Conventional approaches identifying adaptive-based therapeutics like vaccines and monoclonal antibodies against coronaviruses target antigens that are not conserved and are unlikely to retain therapeutic efficacy against diverse coronavirus pathogens.
- Reverse genetics approaches that generate novel coronaviruses currently circulating in bats are an innovative but under-utilized resource to provide additional zoonotic and pre-emergent virus diversity to *in vitro* and *in vivo* drug discovery platforms.
- Many of the treatments used in SARS or MERS patients in outbreak situations were not based on clear *in vitro* and *in vivo* model evidence of efficacy, and meta-analyses of treatments failed to show effective therapeutic regimens.
- Development of a drug discovery pipeline consisting of *in vitro* and *in vivo* models of coronavirus infection is needed to identify antivirals targeting essential mechanisms of infection.

This box summarizes key points contained in the article.

In addition to CoVs known to cause disease in humans and livestock, a large number of highly diverse coronaviruses have been identified based on sequences collected from sampling bat species. Bat coronavirus (BatCoV) sequences recovered from sampling sites on different continents (Asia, Europe, Africa, North America) over the last decade contain putative BatCoVs from diverse branches of the betacoronavirus and alphacoronavirus phylogenetic tree [9–12]. Importantly, the two coronaviruses that cause the most severe disease in humans, SARS-CoV and MERS-CoV, emerged from BatCoVs that were not previously recognized to infect humans or animals other than bats [12–14]. Recent studies suggest that BatCoV-SHC014 and BatCoV-WIV1 are genetically similar to SARS-CoV and enter cells using human receptors [10,15,16]. Similarly, BatCoV-HKU4 and BatCoV-HKU5 are MERS-like BatCoVs that may also be circulating in bat populations, and some MERS-like BatCoVs may also be able to recognize human host cell receptors [17–19]. Such BatCoVs are now called 'pre-emergent', because they may have the potential to emerge into human populations. Importantly, therapeutics that rely on host memory responses to target CoV infection are often not effective against pre-emergent BatCoVs that differ antigenically from known HCoVs, highlighting the need for pan-coronavirus therapeutics that target conserved mechanisms utilized by HCoVs and BatCoVs [15].

SARS-CoV and MERS-CoV likely evolved from BatCoVs that infected other intermediate host animals in closer proximity to humans, resulting in SARS and MERS outbreaks (Figure 1) [20,21]. SARS-CoV was detected in small animals like civets and raccoon dogs that were present in live-animal markets [20]. Evolution of SARS-CoV evidenced by genomic sequence differences between zoonotic SARS-CoV strains infecting civets and epidemic SARS-CoV isolates likely resulted from viral adaptation, which is thought to be required for emergent CoVs to become transmissible from person-to-person [22,23]. MERS-CoV has been identified in dromedary camels, and is now known to be endemic in camel populations in the Middle East and Sub-Saharan Africa.

**Table 1.** Human coronavirus *in vitro* properties.

| Human Coronavirus | Genus | Genogroup | Receptor | Protease | Human Target Cells | Cell Lines |
|---|---|---|---|---|---|---|
| HCoV-OC43 | betacoronavirus | 2A | O-acetylated Sialic Aicd (Protein Receptor Unknown) | Cathepsin L, TMPRSS2 | Upper Respiratory Tract | BS-C-1, RD, HRT-18, Huh-7 |
| HCoV-229E | alphacoronavirus | 1B | APN | Cathepsin L, TMPRSS2 | Upper Respiratory Tract | WI-38, MRC-5, L-132, Huh-7 |
| HCoV-HKU1 | betacoronavirus | 2A | O-acetylated Sialic Aicd (Protein Receptor Unknown) | Cathepsin L, TMPRSS2 | Upper Respiratory Tract | NR* |
| HCoV-NL63 | alphacoronavirus | 1B | ACE2 | NR | Upper Respiratory Tract | tMK, Vero, LLC-MK2, CaCo-2 |
| SARS-CoV | betacoronavirus | 2B | ACE2 | Cathepsin L, Elastase, TMPRSS2, TMPRSS11a, HAT, Trypsin | Lower Respiratory Tract | BGM, COS, CV-1, FRhK, LLC-MK2, MA-104, MEK, pCMK, Vero, Vero E6, HEK-293, HepG2, Huh-7, RK-13 |
| MERS-CoV | betacoronavirus | 2C | DPP4 | Cathepsins, TMPRSS2, Furin | Lower Respiratory Tract | Caco-2, Calu-3, HFL, Huh-7, HEK, His-1, LLC-MK2, Vero, Vero-E6, CL-1, PK-15 |

*NR – not reported

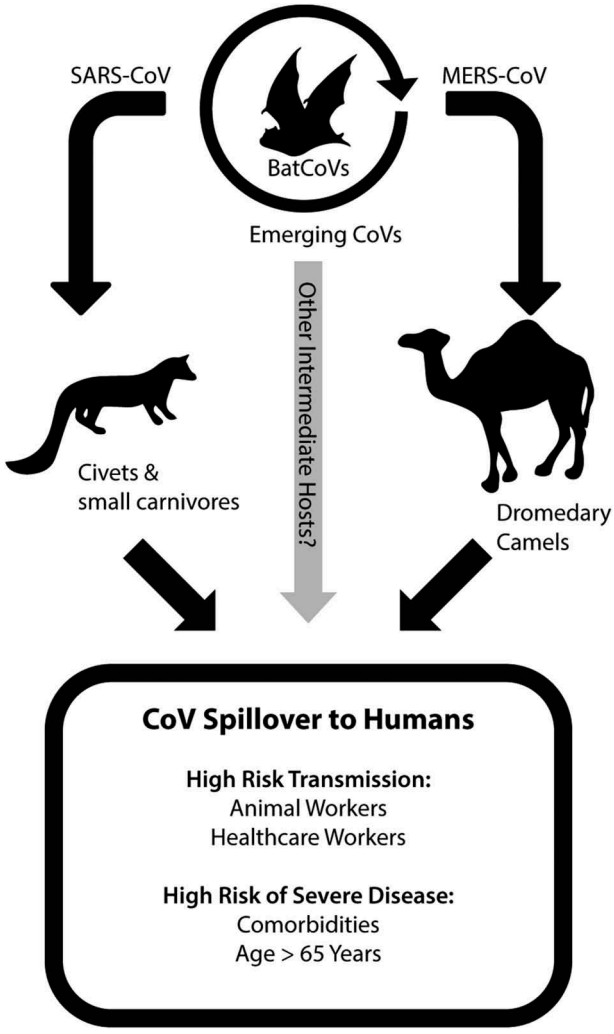


Figure 1. Coronavirus emergence from zoonotic reservoirs.

Emergence of coronaviruses into human populations, including highly pathogenic viruses like SARS-CoV and MERS-CoV, has occurred by spillover from bat reservoir hosts into intermediate hosts. The intermediate hosts during the 2003 SARS-CoV epidemic included civets and other small carnivore species located in wet animal markets. MERS-CoV has been identified in dromedary camels, and is particularly associated with active infection of juvenile camels. Novel emerging CoVs may occur in the future via infection from bat populations into other intermediate animal hosts. Additional evidence from BatCoVs indicates that pre-emergent CoVs with the ability to directly infect human cells may be poised for emergence into human populations. Based on prior research from SARS and MERS outbreaks, animal workers that have contact with intermediate animal host species and health-care workers that are exposed to nosocomial CoV infections are at increased risk of highly pathogenic coronavirus transmission. More severe disease in SARS and MERS cases resulted in patients that were over the age of 65 or had comorbidities such as obesity, heart disease, diabetes, renal disease, or hypertension.

### 1.2. Epidemiological features of CoV outbreaks

Research on coronavirus-specific antiviral drugs has focused primarily on highly pathogenic coronaviruses SARS-CoV and MERS-CoV due to the major potential consequences of pandemics resulting from these pathogens. SARS-CoV and MERS-CoV did not transmit as efficiently from person-to-person compared to other respiratory pathogens like seasonal influenza, but mortality in patients of SARS (approximately 10%) and MERS (approximately 35%) greatly exceeded typical seasonal influenza case-fatality rates (2.4 deaths per 100,000 cases) [24]. Air travel facilitated these CoVs in seeding outbreaks in regions distant from initial localized viral spread: SARS-CoV emerged in the Guangdong province of southeastern China in late 2002, and then spread rapidly to other parts of the world, with outbreaks in major cities including Beijing, Hong Kong, Singapore, and Toronto, resulting in one of the first pandemics of the twenty-first century [5]. Since MERS-CoV emerged in 2012, MERS cases have been exported from the Middle East to Europe, North America, Africa, and South East Asia, including a major outbreak of 186 people in the Republic of Korea in 2015 [25]. Superspreaders (individuals that transmit SARS-CoV or MERS-CoV to a large number of people) played an important role in initiating and perpetuating CoV outbreaks: the 2015 MERS-CoV outbreak in the Republic of Korea started from a single traveler case, and just five cases were responsible for more than 80% of the transmission events [25]. SARS-CoV and MERS-CoV were transmitted by close contact, with known outbreaks occurring in hotels, apartment buildings, and hospitals or health-care centers. Health-care workers, in particular, were at risk for infection by SARS-CoV and MERS-CoV at high rates [5,26]. In addition, animal workers were more likely to come into contact with CoV infected animals, and a large percentage of MERS patients had contact with intermediate host camels [26,27]. Analysis of severe SARS or MERS disease identified disproportionately high case-fatality rates in elderly patients (age > 65 years) and patients with pre-existing comorbidities including diabetes, heart disease, hypertension, and renal disease [4,26,28]. Based on these epidemiological considerations, pan-coronavirus therapeutics are needed to i) protect populations with occupational risk for transmission of CoVs, ii) protect populations with susceptibility to severe disease from CoVs, iii) work in concert with public health measures like quarantine and contact tracing, and iv) be rapidly deployable to geographically distant regions from local HCoV epidemics.

## 2. *In vitro* systems for pan-coronavirus drug discovery

### 2.1. Reverse genetics systems

Advances in the study of highly pathogenic coronaviruses and potential pan-coronavirus drug candidates partially depend on the technology to genetically manipulate CoVs to probe mechanisms of viral pathogenesis and antiviral drug activity. Reverse genetics systems synthetically generate viruses from known viral sequences [29]. In situations where clinical isolates of infectious material are unavailable due to restriction for collecting patient samples, shipping infectious materials, or availability of containment laboratories, reverse genetics systems provide essential research materials for studies on viral pathogenesis and model development. Prior to the SARS pandemic, robust reverse genetics systems to manipulate the genomes of CoVs had already been developed by systematic assembly of cDNA cassettes into full-length infectious clones, allowing precise and targeted genetic manipulation of viral genes [30,31]. Infectious clones allow the creation of near-homogenous viral stocks, whereas traditional viral stocks are prepared by amplification of infectious material in cell culture over many passages. Strategies to build reverse genetics systems were rapidly applied to both SARS-CoV and MERS-CoV within the first year of identification of these viruses [32,33].

In addition to reconstructing epidemic strains of CoVs, reverse genetic systems allow targeting of mutations to specific viral genes and assembly of viruses when infectious

material is not available. As an example, the ability to isolate mutations in particular genes was applied to studies of the spike (S) glycoprotein of SARS-CoV, while maintaining the isogenic background of the viral replicase and other structural proteins. Mutations from zoonotic, early, middle, and late epidemic strains of the SARS-CoV outbreak were inserted into the S glycoprotein of the epidemic strain of SARS-CoV (Urbani) to determine the effect of evolution on viral entry into human cells as well as viral pathogenesis in rodent and primate models of disease [34–36]. By targeting mutations to a specific viral gene, reverse genetics systems allow researchers to probe cause-and-effect relationships of host pathogenic responses to viral genetic changes. In addition, reverse genetics techniques were utilized to study pre-emergent BatCoV strains: recombinant versions of BatCoV-HKU3, BatCoV-WIV1, and BatCoV-SHC014 (SARS-CoV-like), as well as BatCoV-HKU5 (MERS-CoV-like) viruses, were generated and used for *in vitro* and *in vivo* models of emerging coronavirus disease [15,16,37,38]. Panels of zoonotic, epidemic, and pre-emergent viruses synthesized by reverse genetics techniques encompass a diverse array for use in high-throughput platforms for the discovery of countermeasures that are effective against the broadest range of CoVs without being reliant on procuring clinical isolates.

### 2.2. Cell-based systems

Like all other viruses, coronaviruses require host cell machinery to replicate their genomes, produce progeny virions, and cause disease. Cell lines require expression of the host cell receptor as well as expression of necessary proteases to facilitate viral entry, although additional host factors may also be important for infection. The S glycoprotein of coronaviruses, the main determinant of host cell attachment and viral entry, is not well conserved between HCoVs. Most human coronaviruses use different host cell receptors for viral entry, and may also require different host cell proteases that allow fusion of viral and cellular membranes (Table 1) [39]. Although all known HCoVs have viral tropism targeted at the human respiratory tract, lung cell lines infected by a broad range of HCoVs have not been defined. A key feature of SARS-CoV and MERS-CoV is that highly pathogenic coronaviruses grow to higher viral titer on a wider range of cell lines than the other mildly pathogenic coronaviruses HCoV-OC43, HCoV-229E, HCoV-NL63 and HCoV-HKU1 [40–44]. High throughput approaches to screen compound libraries for targeted activity against coronaviruses have been underdeveloped and limited in the number of viral strains used [45–52]. Infection of panels of cell lines from various animal species with HCoVs and BatCoVs informs on the potential host range of the pathogen, and may help to identify susceptible mammalian host involved in viral spread. However, productive infection of cell lines does not always translate to recapitulation of pathogenesis in the same animal model that the cell lines are derived from, which may be due to receptor availability in live animals or other biological and immunological factors during infection.

Infection of pseudostratified airway epithelium cultures from primary cells of the lung provides a cell culture model that simulates infection of cells in a more complex environment more closely resembling the human respiratory tract. Known as Human Airway Epithelia (HAE) or Normal Human Bronchial Epithelia (NHBE) cells, these cultures can be infected with all of the HCoVs identified thus far, including SARS-CoV and MERS-CoV, providing a potential platform to screen novel CoVs for emergence into human populations [42,53–56]. However, several limitations are associated with HAEs including difficulty in collection due to the scarcity of donors and difficulty in maintenance because of limited capacity for cell divisions. HAEs may be sourced from donors with a preexisting disease state, which could influence viral pathogenesis. In addition, because of the genetic variability of donors, HAEs cultures often differ in expression levels of genes crucial to infectivity, including the various host receptors for HCoVs, which leads to high variability in the infectivity of these cultures. Importantly, these *in vitro* methods fail to capture more complex viral interactions that occur with an intact immune system including infiltration of proinflammatory cells that may promote and contribute to ARDS in the most severe forms of SARS and MERS. Organ-on-a-chip models in development may provide the next generation of *in vitro* models that could capture these critical interactions between respiratory cells and immune cells, but infection of these novel culture systems has not been reported with coronaviruses [57].

## 3. *In vivo* systems for pan-coronavirus drug discovery

Based on the results from *in vitro* screening methods, potential new pan-coronavirus drugs that successfully target HCoVs require additional evaluation in animal species that model viral infection on an organismal scale. Due to urgent public health need for effective treatments against SARS-CoV and MERS-CoV, development of animal models of CoV infection emphasized these pathogens [58,59]. Reproducible models of highly pathogenic coronavirus infection in common laboratory animal species have utility not only in development and testing of pan-coronavirus drugs, but also in elucidating mechanisms of viral replication or disease pathogenesis. Desirable qualities for animal models of SARS-CoV and MERS-CoV include recapitulation of severe disease symptoms seen in SARS and MERS patients, and lethality caused by fulminant viral infection of the lung as indicated by high viral titers, inflammatory infiltrates, and aberrant cell signaling programs. Although therapeutic efficacy against viral transmission is essential to disrupting SARS or MERS outbreaks, current animal model development has focused on disease resulting from relevant infection routes (i.e. intranasal) over directly developing models of CoVs transmission.

### 3.1. Small animal models for pan-coronavirus drug discovery

Following the emergence of SARS-CoV in 2003, small animal model development was initiated by inoculating animals with human epidemic isolates of SARS-CoV that replicated in mice, hamsters, guinea pigs, and ferrets, but only ferrets exhibited disease signs resulting from infection (Table 2) [60–63]. SARS-CoV replicated in laboratory strains of mice, but did not cause

Table 2. *In vivo* models of SARS-CoV.

| Severe Acute Respiratory Syndrome Coronavirus (SARS-CoV) | | | | | | |
|---|---|---|---|---|---|---|
| **Human Clinical Disease** | | SARS manifests in humans as a severe atypical pneumonia associated with diffuse alveolar damage. SARS-CoV incubation period is 2–10 days. Initial flu-like symptoms of fever, cough, and shortness of breath may progress to pneumonia, and in severe cases infection may lead to Acute Respiratory Distress Syndrome (ARDS), the most severe form of acute lung injury. Pathological findings include the deposition of alveolar exudates and formation of hyalin membranes in severe cases. Overall mortality rate for SARS-CoV in reported cases was approximately 10%, with more severe disease in patients over age 65. | | | | |
| | | Viral Strain | Route of Infection | Replication | Disease signs, severity, and other comments | Reference |
| **Non Human Primate Models** | | | | | | |
| | African Green Monkey | Urbani | IN/IT | Yes | No clinical disease signs. Higher viral replication in lungs than Rhesus or Cynomolgus Macaques. Interstitial pneumonitis observed. No lethal disease observed. | [83,86] |
| | Rhesus Macaque | Urbani | IN/IT | Yes | No clinical disease signs. Alveolar pneumonitis observed, but less pathology and viral replication when directly compared to African Green Monkeys. | [83] |
| | Cynomolgus Macaque | Urbani | IN/IT | Yes | No clinical disease signs. Alveolar pneumonia observed, but less pathology and viral replication when directly compared to African Green Monkeys. | [83,85,86] |
| | Common Marmoset | Urbani | IT | Yes | No clinical disease signs. Mild/moderate pathology: Interstitial pneumonitis, bronchiolitis, and occasion edema. | [84] |
| **Small Animal Models** | | | | | | |
| | Mouse Wild Type | Urbani | IN | Yes | Viral replication in the lungs, but no clinical disease signs. | [60] |
| | Mouse Adapted Virus | MA15* | IN | Yes | Lethal disease is dependent on genetic background of mice and age. BALB/c: >20% weight loss, mortality in young and old mice C57BL6J: ~15% weight loss and recovery in 10 week old animals, increasing weight loss and mortality in older mice; Age dependent and dose dependent responses; Other laboratory and wild-derived mouse strains evaluated include: 129, A/J, NOD, NZO, CAST, PWK, and WSB | [64,68] |
| | Hamster | Urbani | IN | Yes | No weight loss, no clinical disease signs, no mortality | [61] |
| | Ferret | HKU39849 | IN | Yes | No lethal disease, but disease signs observed include fever and sneezing. Observed lung pathology is consistent with interstitial pneumonia and replicates severe lung disease. SARS-CoV infected ferrets transmit virus to co-housed naïve ferrets. | [63] |
| | Guinea Pig | Frankfurt | IP | Yes | No clinical disease signs; minor replication and pathological changes in lungs. Route of infection is not relevant to human SARS-CoV transmission. | [62] |

NR, not reported; IN, intranasal; IT, intratracheal; IP intraperitoneal;
*Other mouse-adapted SARS-CoV strains exist, with similar published phenotypes [55]
Urbani (GenBank Accession AY278741)
BJ01 (GenBank Accession AY278488)
GZ01 (GenBank Accession AY278489)
MA15 (GenBank Accession DQ497008)
HKU39849 (GenBank Accession AY278491)
Frankfurt (GenBank Accession AY291315)

disease signs, and virus was rapidly cleared from the lung in these models [60]. Serial passage of SARS-CoV in the lungs of mice by multiple research groups resulted in mouse-adapted SARS-CoV strains that caused lethal lung disease in wild-type mouse intranasal (IN) infection models [64,65]. Mouse-adapted SARS-CoV MA15 is the best characterized small animal model of CoV infection, and has been used to test several pan-coronavirus drug candidates [66,67]. The benefit of mouse-adapted models of SARS-CoV in wild-type inbred mouse strains includes reproducible susceptibility to disease assayed by survival, weight loss, and whole body plethysmography of individual mice as well as quantification of infiltrating cells, viral titers, histopathology, and transcriptomics and proteomics changes in target organs. To evaluate pathogenesis of emergent viruses *in vivo*, zoonotic SARS-CoV and pre-emergent BatCoV mutations have been incorporated into the SARS-CoV MA15 backbone, providing novel animal models for viruses that have the potential to emerge into humans [15,35]. Additional valuable avenues of research on variables known to impact severe CoV disease in the MA15 models of SARS-CoV include age, dose, and host genetic contributions to disease phenotypes [68,69]. The greatest limitations of SARS-CoV mouse-adapted models for drug discovery are the incorporation of mutations in the SARS-CoV genome (particularly for testing antiviral drugs that target viral genes with mutations) and acknowledged differences between mouse and human immune responses.

Unlike SARS-CoV, human clinical strains of MERS-CoV (Table 3) did not replicate in mice, hamsters, or ferrets, and further studies of the host receptor identified critical amino acid residue differences between the MERS-CoV receptor, DPP4, in laboratory animal model species that prevented entry into cells compared to human DPP4 [70–72]. MERS-CoV infection of rabbits resulted in viral replication in the upper respiratory tract, but no clinical disease signs that reflect more severe MERS-CoV disease symptoms were reported, although the model has been used for limited testing of MERS-CoV antiviral therapeutics [73,74]. However, due to the utility of the mouse-adapted SARS-CoV model, a mouse model continued to be pursued, and adenovirus-vectored transient expression of the human DPP4 receptor in mice and subsequent replication of MERS-CoV in these mice determined that MERS-CoV replication was dependent on human DPP4 expression in rodents [75]. Transgenic expression of human DPP4 in mice allowed MERS-CoV replication in mice, but resulted in lethal brain disease not representative of MERS-CoV infection in humans [76,77]. Replacing mouse DPP4 with the expression of human DPP4 in mice resulted in a humanized DPP4 mouse model that allows MERS-CoV replication within the lung and some MERS-associated lung pathology, but no lethal disease [78]. Similarly, knock-in expression of the human DPP4 exons 10–12 in mice allowed viral replication but no overt MERS disease signs [79]. Serial passage of MERS-CoV in the lungs of these mice resulted in a MERS-MA virus that caused lethal disease in mice, including weight loss and severe lung pathology [79]. Identification of amino acids in mouse DPP4 that prevent entry of MERS-CoV into mouse cells led to the rational design of the mouse DPP4 gene edited by CRISPR/Cas9 to express two human DPP4 mutations (288/330 DPP4) [80]. 288/330 DPP4 mice supported viral replication without severe disease or lethality, but serial passage of MERS-CoV (generating a mouse-adapted virus called MERS-15) produced lethal disease in the 288/330 DPP4 mice [80]. Although many of the same metrics of disease to MA15-SARS-CoV are available in the MERS-15 or MERS-MA models for drug discovery of coronavirus antivirals, an additional drawback is the requirement for both mouse-adapted virus and a modified rodent host. Despite these limitations, mouse models of adapted SARS-CoV and MERS-CoV are currently the best-developed models of highly pathogenic coronavirus infection available for pan-coronavirus drug discovery.

### 3.2. Primate models for pan-coronavirus discovery

Small animal models have been more thoroughly developed as models of SARS-CoV and MERS-CoV infection, due to ease of manipulation with rodents and increased costs and ethical concerns associated with nonhuman primates (NHPs). However, NHP model development of highly pathogenic coronavirus infections is pivotal in the evaluation of pan-coronavirus therapeutics, because host immune responses from NHPs share greater homology with humans compared to rodents, and may more accurately indicate immunological biomarkers of severe disease needed to evaluate pan-coronavirus therapeutics. Disease signs are observed in NHP models of infection without adaptation of CoVs required in rodent models of SARS-CoV and MERS-CoV. Both SARS-CoV and MERS-CoV isolates from humans replicate in NHPs, indicating conservation of important aspects to coronavirus-induced diseases including respiratory tract biology, receptor homology, and pattern of expression of host receptor and proteases.

SARS-CoV infection of common laboratory primate species by the IT route including African green monkeys, rhesus macaques, cynomolgus macaques, and common marmosets, resulted in disease signs with differing degrees of severity, but none were reflective of the lethal SARS disease seen in humans (Table 2) [81–85]. Commonly reported disease signs included lethargy and increased respiratory rates following SARS-CoV infection in multiple NHP models of infection, but other acute signs of illness including fever or dyspnea were infrequently reported. The most severe disease phenotypes were observed in the histopathology of the lungs at acute times post-infection (3–6 days) with typical findings of pulmonary lesions and pneumonitis and occasional observations of diffuse alveolar damage [86]. Although none of the NHP species that were infected with SARS-CoV developed lethal respiratory disease reflective of SARS patients, NHP models did recapitulate enhanced disease in aged NHPs, including aberrant innate immune signaling programs [87]. However, lack of emulation of human SARS disease was never resolved in an NHP model that could be used for consistent evaluation of therapeutic candidates against SARS-CoV.

MERS-CoV infection of nonhuman primate models was reported, with the best characterized NHP models of MERS-CoV infection in rhesus macaques and common marmosets (Table 3) [88–91]. Administering MERS-CoV via the IT route to either rhesus macaques or common marmosets resulted in mild disease with very few observable disease phenotypes [89,91]. However, infecting rhesus macaques or common marmosets by multiple concurrent routes (IN, IT, oral, and ocular) resulted in moderate disease in rhesus macaques, but more

Table 3. *In vivo* models of MERS-CoV.

| Middle East Respiratory Syndrome Coronavirus (MERS-CoV) | | | | | | |
|---|---|---|---|---|---|---|
| **Human Clinical Disease** | MERS-CoV manifests in humans as a severe atypical pneumonia associated with diffuse alveolar damage. This is likely to include the deposition of alveolar exudates and formation of hyalin membranes in severe cases. Incubation period of MERS-CoV is 2–14 days. Initial symptoms may include fever and influenza-like illness followed by a progression to lower respiratory symptoms including dyspnea, cough, and pneumonia. MERS-CoV infection may lead to Acute Respiratory Distress Syndrome (ARDS), the most severe form of acute lung injury. Clinical MERS symptoms may occur for weeks following infection, including persistence of viral RNA in the lower respiratory tract. Healthcare workers and animal workers (particularly handlers of camels) are at increased occupational risk of exposure. Overall mortality rate for MERS-CoV in reported cases is approximately 30–40%, with more severe disease in patients over age 65 or with preexisting co-morbidities. Asymptomatic or subclinical MERS-CoV infection, particularly among healthy individuals may occur. | | | | | |
| | | Viral Strain | Route of Infection | Replication | Disease signs, severity, and other comments | Reference |
| **Non Human Primate Models** | | | | | | |
| | Rhesus Macaque | EMC/2012 | Multiple Routes* | Yes | Disease signs include elevated temperature, loss of appetite, hunched posture, cough lasting for several days. Pathology indicates interstitial pneumonia. No lethal disease. | [88] |
| | Rhesus Macaque | JOR/2012 | IT | ND | Few clinical disease signs observed. Mild pathology of interstitial pneumonia. No lethal disease. | [89] |
| | Common Marmoset | EMC/2012 | Multiple Routes* | Yes | Moderate to severe disease with disease signs including loss appetite and lethargy. High viral load in the lung of all animals, some with viremia. Interstitial pneumonia observed. | [90] |
| | Common Marmoset | EMC/2012, JOR/2012 | IT | ND | Mild to moderate non-lethal disease with few clinical signs. Viral replication in lungs and interstitial pneumonia observed. Similar phenotypes with EMC and Jordan isolates | [91] |
| **Small Animal Models** | | | | | | |
| | Mouse Wild Type | EMC/2012 | IN | No | No viral replication or disease signs were observed in BALB/c, 129Sv/Ev or STAT-/- mice. Other immune deficient mice are not susceptible to MERS-CoV (RAG-/-, SCID-/-, Myd88-/-). The mouse DPP4 receptor does not allow viral replication. | [71] |
| | Mouse hDPP4 Transgenic | EMC/2012 | IN | Yes | Transient expression of the human DPP4 (hDPP4) receptor in mice by Ad5-hDPP4 allows vial replication in C57Bl6/J and BALB/c mice, but only minor clinical disease signs. Global constitutive expression of hDPP4 in transgenic mice results in lethal disease with minor lung pathology, but high viral replication in the CNS and lethal neuropathology not associated with human disease. Expression of hDPP4 in place of the mouse DPP4 ORF results in non-lethal disease characterized by viral replication and lung pathology. | [75–78] |
| | Mouse Adapted Virus | EMC/2012 | IN | Yes | Editing of mouse DPP4 by CRISPR/Cas9 generated 288/330 hDPP4 mice resulting in viral replication in the lungs of mice. Serial passage of MERS-CoV 15 times in the lung of 288/330 hDPP4 mice resulted in MERS-15 virus that was lethal in about 40% of mice infected. Knock In (KI) expression of hDPP4 exons 10–12 in mice resulted in viral replication with no clinical disease signs following MERS-CoV infection. Serial passage of MERS-CoV 30 times in the lung of KI DPP4 mice resulted in a lethal mouse adapted strain, MERS-MA. | [79,80] |
| | Hamster | EMC/2012 | IT | No | No disease signs or viral replication observed, the hamster DPP4 receptor does not allow viral replication | [70] |
| | Ferret | EMC/2012 | IN/IT | No | No disease signs or viral replication observed, the ferret DPP4 receptor does not allow viral replication | [72] |
| | Rabbit | EMC/2012, England-2 | IN/IT | Yes | Viral replication observed from nasal swabs, MERS-CoV detected in lungs by RT-qPCR, ISH, and IHC. Similar phenotypes with EMC and England isolates | [73,74] |

NR, not reported; ND, not detected; IN, intranasal; IT, intratracheal;
* Multiple Routes indicates combined Ocular, Oral, IN and IT inoculation
EMC/2012 (GenBank Accession JX869059)
JOR/2012 (GenBank Accession KC776174)
England-2 (GenBank Accession KM015348)

severe disease in marmosets [88,90]. Infecting NHPs by multiple routes likely caused a systemic infection potentially not representative of human MERS-CoV infection. Although the marmoset is currently the best developed NHP model of MERS-CoV disease, discrepancies in disease severity of marmosets infected by multiple routes and marmosets infected by the IT route illuminate potential difficulties with using this model for drug development studies. Small size and fragility of marmosets precluded serial blood draws on multiple days following infection, and may confound experimental outcomes resulting from MERS-CoV infection or treatment [92]. Absence of reproducible clinical disease signs like fever, respiratory distress, or lethality that recapitulated human symptoms of SARS or MERS indicates that currently developed models present significant challenges to testing of pan-coronavirus antivirals in NHP models of infection.

## 4. Pan-coronavirus antivirals

Pan-coronavirus antivirals must target viral or host factors that are i) highly conserved among known CoVs, ii) essential to viral replication or viral pathogenesis by known CoVs, and iii) likely to be conserved and essential in emerging CoVs. Inhibiting highly conserved mechanisms involved in the coronavirus lifecycle is likely to result in a reduction of viral titers, alteration of host responses, and/or amelioration of disease signs. SARS-CoV and MERS-CoV are known threats to global health, but other novel coronaviruses may emerge in the future complicating drug design if antiviral targets are too specific to known viral strains. Unlike with influenza viruses, specific antiviral drugs like oseltamivir and zanamivir targeting coronaviruses are not yet available, but several promising candidates have been recently described in the literature. The most conserved proteins among CoVs are nonstructural proteins (nsps) involved in essential functions of the viral lifecycle. The structural proteins that make up the virion are less conserved than nsps, and accessory proteins are only functionally conserved among very closely related viruses (Figure 2). In addition to potential lack of conservation between known and emerging HCoVs, targeting viral proteins can be problematic for drug discovery due to viral escape by mutation. Alternatively, antivirals that target conserved host factors utilized during the viral life cycle may also be potential pan-coronavirus antiviral therapeutics, but have the disadvantage of potential off-target effects.

### 4.1. Targeting CoV nonstructural proteins

Coronavirus nsps are highly conserved components of the coronavirus lifecycle that mediate viral replication including 3C-like protease (3CLpro), papain-like Protease (PLpro), and RNA-dependent RNA polymerase (RdRp). The CoV RdRp replicates the viral RNA genome and generates viral RNA transcripts, essential functions that cannot be performed by cellular polymerases. Another essential element of the CoV lifecycle is proteolytic processing of viral polyproteins into functional nsps by two viral proteases, the 3CLpro and PLpro. In addition to polymerase and protease functions, other essential functions performed by the nsps of CoVs include immune antagonism, double membrane vesicle organization, scaffolding for replication complex formation, nucleic acid binding, helicase activity, and viral RNA proofreading which may be future targets of coronavirus specific antiviral drug discovery [93].

#### 4.1.1. GS-5734

GS-5734 is a small molecule nucleoside analog that has demonstrated antiviral activity *in vitro* against several viral families of emerging infectious diseases including *Filoviridae, Pneumoviridae, Paramyxoviridae*, and *Coronaviridae* [45,66,94]. Efficacy of GS-5734 in post-exposure treatment of Ebola virus-infected nonhuman primates led to GS-5734 inclusion in an experimental therapy for an infant survivor of Ebola virus disease [45,95]. These encouraging results demonstrated that GS-5734 may be an acceptable therapeutic intervention to lethal viral disease, even days after viral exposure, and tolerated by patients with viral diseases that were previously treated primarily with supportive care. Based on activity against MERS-CoV within a larger panel targeting lethal viruses from multiple viral families, additional studies demonstrated that GS-5734 decreased viral titers and viral RNA in *in vitro* models

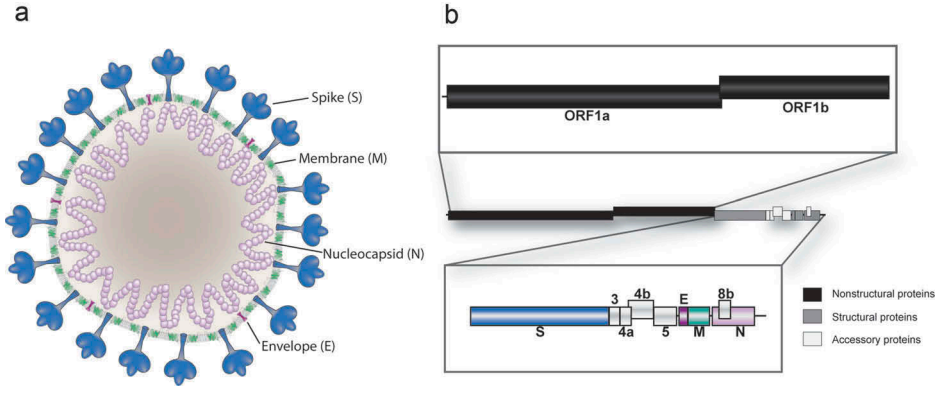


Figure 2. Coronavirus virion structure and genomic organization.

As an example of coronavirus virion (A) and genome (B) structure, a schematic of MERS-CoV (GenBank JX869059) is provided. Virions exist as enveloped viral particles, with the Spike (S), Membrane (M), and Envelope (E) proteins decorating the outside of the membrane. Coronaviruses in genogroup 2a have an additional structural protein hemagglutinin esterase (HE), which has been omitted from this discussion. Inside of the virion, the Nucleocapsid (N) protein encapsidates the viral genome. The viral genome is composed of + sense, single-stranded RNA. At the 5′ end of the genome, a single polyprotein open reading frame encodes the more highly conserved nonstructural proteins (ORF1a, ORF1b). At the 3′ end of the genome, the functionally conserved structural proteins that make up the virion are interspersed with virus-specific accessory proteins (ORF3, ORF4a, ORF4b, ORF5, and ORF 8b). Accessory proteins are conserved between very closely related viruses like BatCoV-HKU4, BatCoV-HKU5, and MERS-CoV. There is no conservation of accessory proteins between known HCoVs.

of both SARS-CoV and MERS-CoV infection of HAEs [66]. Additionally, GS-5734 had similar effects against other diverse CoVs including HCoV-NL63 and Mouse Hepatitis Virus (MHV, betacoronavirus group 2a) [66,96]. Importantly, GS-5734 inhibited replication of pre-emergent BatCoVs including BatCoV-HKU5, BatCoV-HKU3, BatCoV-SHC014, and BatCoV-WIV1 [66]. Activity *in vivo* against CoVs was supported by ameliorated disease signs (weight loss, lung viral titers) in MA15 SARS-CoV infected mice treated prophylactically or therapeutically with GS-5734 [66]. Although viral resistance to GS-5734 was shown experimentally *in vitro*, mutations to conserved motifs in SARS-CoV and MHV resulted in decreased viral fitness *in vitro* and *in vivo* [96]. Altogether, *in vitro* and *in vivo* data support GS-5734 development as a potential pan-coronavirus antiviral based on results against several CoVs, including highly pathogenic CoVs and potentially emergent BatCoVs.

#### 4.1.2. Lopinavir–Ritonavir

Lopinavir–ritonavir was initially developed as an HIV-1 protease inhibitor but *in vitro* activity also targeted SARS-CoV nonstructural protein 3CLpro [97]. During the SARS-CoV epidemic, lopinavir–ritonavir combination therapy with ribavirin in SARS patients was associated with decreased viral load and decreased adverse clinical outcomes of death or ARDS when compared with historical control cases [98]. Shortly after the emergence of MERS, high throughput screening approaches of known antiviral compounds identified lopinavir activity against MERS-CoV *in vitro* [51]. Oral treatment with lopinavir-ritonavir in the marmoset model of MERS-CoV infection resulted in modest improvements in MERS disease signs, including decreased pulmonary infiltrates identified by chest x-ray, decreased interstitial pneumonia, and decreased weight loss [92]. MERS patient case reports of treatment regimens including lopinavir-ritonavir were associated with positive disease outcomes including defervescence, viral clearance from serum and sputum, and survival [99,100]. Based on *in vitro* and *in vivo* activity against MERS-CoV, a clinical trial has been designed using combination of lopinavir-ritonavir and IFN-β1b therapies in hospitalized MERS patients in Saudi Arabia [101].

#### 4.1.3. Ribavirin

Ribavirin is a guanosine analog with *in vitro* activity against a large number of highly lethal emerging viruses. Mechanistically, ribavirin inhibits RNA synthesis by viral RdRp as well as inhibits mRNA capping. However, studies demonstrated that while SARS-CoV, MERS-CoV, and HCoV-OC43 were sensitive to ribavirin *in vitro*, doses that significantly inhibited CoV replication exceeded ribavirin concentrations attainable by typical human regimens [46,102–104]. Recently, it was demonstrated that excision of ribavirin nucleoside analogs by conserved coronavirus proofreading mechanisms likely accounted for decreased *in vitro* efficacy of ribavirin than expected [105]. Additional *in vivo* testing of ribavirin in mouse models found limited activity against MA15 SARS-CoV by ribavirin alone, and suggested that ribavirin treatment enhanced SARS disease signs [65,106]. However, combination treatment of ribavirin and type I Interferons in primate models improved MERS disease signs [107]. Ribavirin has been given as part of treatment regimens for SARS and MERS patients, but meta-analyses of case studies have found limited (if any) efficacy of ribavirin in treating patients with highly pathogenic coronavirus respiratory syndromes [108,109].

### 4.2. Targeting CoV structural and accessory proteins

Coronavirus structural proteins compose the virion, including the Spike (S) glycoprotein, Envelope (E) protein, Membrane (M) protein, and the Nucleopcapsid (N) protein (Figure 2). These proteins also perform important functions in the viral life cycle: S is the main determinant of cell tropism, host range, and viral entry; E facilitates viral assembly and release, and has viroporin activity; M maintains the membrane structure of the virion; and N encapsidates the viral RNA genome. Although most of these functions are essential to viral infection, CoVs tolerated E deletion and remained replication competent, but viral fitness was impaired [110]. Unfortunately, while structural protein functions are similar between CoVs, protein identity is less conserved than with nsps, making the development of pan-coronavirus therapeutics directly targeting structural proteins problematic. Genes encoding structural and accessory proteins are interspersed at the 3′ end of the coronavirus RNA genome (Figure 2). Deletion of accessory protein genes using reverse genetics systems demonstrated that these proteins were not essential for viral replication, but impacted viral replication or viral fitness *in vitro* and *in vivo* [111,112]. However, unlike nonstructural proteins or structural proteins, significant variation in number, function, and sequence of accessory proteins between closely related viruses makes accessory proteins poor targets for pan-coronavirus therapeutic approaches.

#### 4.2.1. Monoclonal antibody therapeutics

Monoclonal antibodies (mAbs) have potential utility in combating highly pathogenic viral diseases, by prophylactic and therapeutic neutralization of structural proteins on virions. *In vitro* and *in vivo* approaches by multiple groups identified mAbs targeting either SARS-CoV or MERS-CoV that inhibited viral replication and ameliorated SARS and MERS disease in animal models [74,78,89,113]. As an example, antibodies generated against the S glycoprotein of MERS-CoV inhibited viral replication when administered 24 h prior to infection, as well as 24 h postinfection in a humanized DPP4 mouse model [78]. In general, mAbs that were effective against CoV infection in animal models targeted the highly variable S glycoprotein, but these mAbs lack cross-protection against other related CoVs [114]. Monoclonal antibodies developed against SARS-CoV, MERS-CoV, or other emerging CoVs may require separate formulations for each virus due to differences in the targeted antigen. For example, mAbs targeted against S from 2003 SARS-CoV isolates failed to neutralize closely related BatCoV-SHC014 and only some mAbs neutralized BatCoV-WIV1 [15,16]. In general, mAbs target specific epitopes, and viruses avoid neutralization by accruing mutations in the targeted epitope that allow viral escape from mAb therapy. Pre-clinical and clinical mAb formulations may include a cocktail of multiple mAbs that target different epitopes to ensure that viruses cannot escape neutralization. However, SARS-CoV S tolerated

mutations in multiple epitopes allowing escape from neutralization from multiple mAbs, and the introduction of mAb escape mutations enhanced pathogenesis of the virus in some animal models [115]. In sum, efficacious monoclonal antibody therapy against highly pathogenic coronaviruses may require several mAbs targeting conserved epitopes and rigorous testing is required to demonstrate that viral evasion of mAbs does not result in enhanced virulence.

#### 4.2.2. Coronavirus vaccines

Vaccines have long been considered the gold standard for infectious disease prevention and eradication targeted at human populations as well as conferring the benefits of long-lived immune protection for the individual. Zoonotic pathogens like coronaviruses emerge from animal reservoir species, thus vaccination strategies are unlikely to lead to eradication while the virus continues to circulate in reservoir hosts. One Health approaches to solving the problems of emerging infectious diseases consider the environment and animal health, as well as human health [116]. For example, vaccination strategies targeting the camel intermediate host of MERS-CoV have been developed, which may work to repress viral replication in camels, preventing MERS-CoV transmission to humans [117].

In human infections of highly pathogenic coronaviruses SARS-CoV and MERS-CoV, the most vulnerable populations are patients over the age of 65 and patients with comorbidities, and design of efficacious vaccines for patients in these groups is difficult. Vaccine formulations that have been developed against SARS-CoV not only fail to protect animal models of aged populations, but also result in immunopathology in younger populations, where SARS disease is enhanced in vaccinated groups that are subsequently challenged with SARS-CoV [118,119]. In addition, vaccines generate memory immune responses to specific pathogens, and no vaccine formulations have been developed that are effective against multiple CoVs. Due to the diversity of BatCoVs, it seems unlikely that current therapeutic strategies targeting specific SARS-CoV or MERS-CoV antigens will be efficacious against future coronaviruses that emerge into the human population. Vaccines formulated against the SARS-CoV epidemic antigens do not offer effective protection against SARS-like BatCoVs that are currently circulating in bat populations [15]. Rather, a modular vaccine platform that can be rapidly adjusted for newly emergent viral antigens in potentially pandemic CoVs may be able to provide emergency vaccine coverage against emergent viral strains.

### 4.3. Targeting host factors essential for CoV infection

#### 4.3.1. Host factor modulation

Antiviral compounds that specifically target viral proteins may result in viral escape by mutation in the targeted viral proteins, as has been described with monoclonal antibodies and GS-5734 [96,115]. However, targeting conserved host mechanisms utilized by multiple coronavirus as an essential part of the viral life cycle is an approach to pan-coronavirus drug development where viral escape by mutation is less likely. Several groups have attempted to inhibit host proteases (including furin, cathepsins, and TMPRSS2) that process viral S glycoproteins at the cell surface during viral entry [50,120–122]. However, due to variation in viral S glycoproteins among different CoVs and variation in the host proteases required for viral entry (Table 1), combinations of protease inhibitors would be required for pan-coronavirus treatment regimens, particularly for emergent novel CoVs where host protease requirements have not been evaluated. Additional host targets with less established mechanisms of activity include Cyclosporins, a class of cyclophilin inhibitors with antiviral activity against coronaviruses in addition to immunosuppressive properties [123]. Non-immunosuppressive derivatives of cyclosporins like alisporivir retained antiviral properties *in vitro* against coronaviruses including SARS-CoV, MERS-CoV, but were not effective against SARS-CoV in mouse models of infection [124].

#### 4.3.2. Host immune modulation

Interferons (IFNs) are a class of immunomodulatory compounds produced by host cells in response to detection of pathogen-specific motifs, resulting in IFN secretion that affects not only the stimulated cell, but also neighboring cells. Early in infection, IFN stimulation results in altered cellular transcriptional programs, leading to an antiviral state characterized by the activation of a large set of host genes with partially defined antiviral functions [125]. Based on these potentially beneficial immunomodulatory properties in the context of infections, IFNs have been used for the treatment of emerging viral infections where no specific antiviral drugs yet exist, with the greatest benefits resulting from administration very early following infection. For SARS-CoV and MERS-CoV, Type I IFNs were effective at decreasing viral replication *in vitro* and showed additional benefits in *in vivo* primate models of infection [103,104,107,126,127]. IFNs used in the treatment of SARS and MERS patients often occurred in combinatory therapies with other drugs including ribavirin and lopinavir-ritonavir, although potential beneficial effects of therapies were limited, potentially due to administration at later times postinfection [108]. Upstream stimulants of IFN induction, including polyI:C resulted in IFN signaling cascade activation, with demonstrated effectivity *in vitro* and *in vivo* against SARS-CoV and MERS-CoV [67,75]. Additional immunomodulatory compounds that regulate the expression of innate immune genes have been suggested as potential therapeutics for highly pathogenic coronaviruses; however, compounds that modulate the host response require significant testing in the most rigorous animal models before therapeutic applications could be pursued. For example, corticosteroids (methylprednisolone) were given as treatment during the SARS and MERS epidemics due to immunomodulatory effects that suppress inflammatory responses with no perceived benefit and possible deleterious effects [108,109].

## 5. Expert opinion

In response to outbreaks of previously unrecognized respiratory syndromes characterized by atypical pneumonia in 2003 and 2012, collaborative new research programs were quickly established that identified the etiologic agents involved as highly pathogenic coronaviruses SARS-CoV and MERS-CoV.

These coronaviruses may have the potential to cause devastating pandemics due to unique features in virus biology including rapid viral replication, broad host range, cross-species transmission, person-to-person transmission, and lack of herd immunity in human populations. SARS-CoV and MERS-CoV were contained by diligent enforcement of public health measures that limited viral spread to approximately 10,000 cases total for both SARS and MERS since 2003. However, the threat of SARS-CoV, MERS-CoV, or an as-yet unknown BatCoV that causes severe disease in humans makes antiviral therapeutics that broadly target coronaviruses a highly desirable commodity to ensure global public health. The current challenge is to produce medical countermeasures that can protect vulnerable populations against known coronaviruses like SARS-CoV and MERS-CoV, but that are also effective against novel highly pathogenic coronaviruses that may emerge from animal reservoir hosts.

While SARS-CoV and MERS-CoV were rapidly identified following clinical reports of novel atypical pneumonia, progress in developing effective antivirals for SARS-CoV and MERS-CoV has been impeded by several factors. A key finding from our review of the literature is that current animal models for highly pathogenic coronaviruses SARS-CoV and MERS-CoV are not adequate to support advanced development of antiviral therapeutics. MERS-CoV continues to circulate on the Arabian Peninsula, providing the opportunity to investigate some treatments of MERS in clinical trials. However, future emerging coronaviruses may require use of the FDA Animal Efficacy Rule for Investigational New Drugs (INDs). INDs must fulfill the Animal Efficacy Rule criteria of i) reasonable safety for initial use in humans, ii) pharmacological data that support reasonably well-understood mechanism of activity against the pathogen, and iii) efficacy in animal models with disease signs representative of clinical illness in humans (including one non-rodent model). While the vigorous pursuit of small animal models has been successful in generating rodent models that recapitulate severe SARS and MERS disease signs (including morbidity and mortality), progress in generating additional animal models has lagged, particularly in primate models of SARS-CoV or MERS-CoV infection. Past strategies for experimental treatment regimens primarily relied on combination therapies with approved drugs known to have acceptable safety profiles and broad-spectrum antiviral activity including IFNs, ribavirin, and corticosteroids. However, analyses of data returned from these treatments indicated that most regimens were not effective in treating SARS and MERS patients. With sufficient investment in the development of drug discovery pipeline model systems, pan-coronavirus targets based on supportive *in vitro* and *in vivo* evidence for effective treatments during the current MERS outbreaks and future outbreaks of emergent CoVs (Figure 3).

In addition, logistical challenges to drug development have hindered discovery of pan-coronavirus therapeutics. Availability of diverse coronavirus clinical isolates for building *in vitro* and *in vivo* systems of drug discovery is limited. Most of the emphasis of the discovery of coronavirus antivirals has been targeted toward the genus betacoronavirus, which includes SARS-CoV and MERS-CoV. However, while therapeutics that target known coronavirus threats to public health are of paramount importance, it is critical to note that future outbreaks of emerging highly pathogenic coronaviruses in humans could result from other coronavirus genera with unique tropism to different

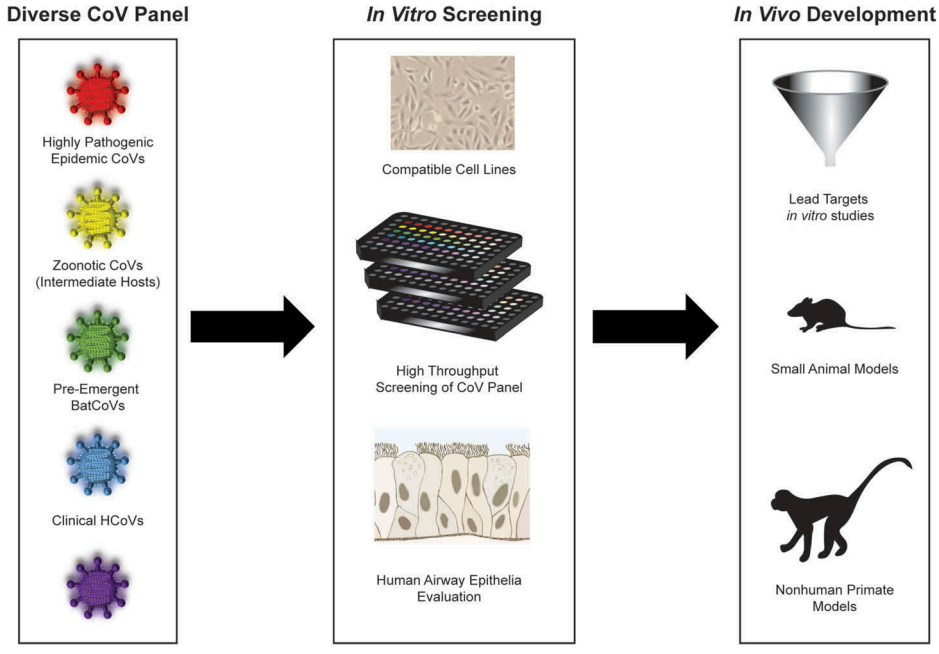


Figure 3. Pan-coronavirus drug discovery.

Currently, the state of pan-coronavirus drug discovery is not structured to provide adequate pre-clinical therapeutics to combat emerging CoV pathogens. A diverse array of coronaviruses is needed that includes epidemic isolates of SARS-CoV and MERS-CoV, zoonotic viruses isolated from intermediate reservoir hosts, pre-emergent CoVs from bats, and clinical isolates of mildly pathogenic HCoVs. *In vitro* testing in compatible cell lines uses high throughput screening to identify novel targets that mitigate replication of coronaviruses. Targets identified by *in vitro* methods can be confirmed using human airway epithelial cultures. Based on these results, lead targets will be tested in small animal models and nonhuman primate models of highly pathogenic coronavirus infections that recapitulate signs of human SARS or MERS patients. Our analysis identified several key weaknesses in both *in vitro* and *in vivo* models of highly pathogenic coronavirus virus infection impeding the identification of pan-coronavirus antiviral drugs.

tissues, different clinical signs and symptoms, and altered transmission profiles that cannot be captured by limiting drug discovery studies to the very few viral strains of SARS-CoV and MERS-CoV currently available to researchers. Currently, Biodefense and Emerging Infections Research Resources Repository (BEI Resources) is a source for a limited number of strains of SARS-CoV, MERS-CoV, and HCoV-NL63. Laboratory strains of HCoV-OC43 and HCoV-229E are available through the American Type Culture Collection, but strains of HCoV-HKU1 are not available through either resource. High throughput surrogate systems at biosafety level 2 are not well-developed and do not yet capture the phylogenetic diversity within the family *Coronaviridae*. Biosafety level 3 conditions are required for working with SARS-CoV, MERS-CoV, or pre-emergent zoonotic strains due to the severe disease these viruses cause, which restricts the number of laboratories that can safely perform screening for pan-coronavirus therapeutics.

In the last 15 years, two outbreaks of previously unknown highly pathogenic coronaviruses, SARS-CoV and MERS-CoV, have demonstrated that CoVs will continue to spill over into human populations, likely facilitated by interaction between infected animals and humans. Reverse genetics approaches have generated pre-emergent BatCoVs from sequence, especially those related to SARS-CoV and MERS-CoV. These particularly novel avenues of research have identified that BatCoV strains with similar pathogenic profiles to SARS-CoV or MERS-CoV continue to circulate within bat populations, indicating a continued vulnerability to highly pathogenic coronavirus emergence. While the next emerging coronavirus may be symptomatically or antigenically similar to SARS-CoV or MERS-CoV, the possibility exists that novel highly pathogenic coronaviruses may be poised for spillover into human populations, with potentially disastrous consequences. Currently, public health measures have been adequate to stymie the spread of SARS-CoV and MERS-CoV primarily due to disease surveillance coupled with viruses with limited person-to-person transmission. However, biological factors that increase cross-species transmission or facilitate person-to-person spread may lead to future coronavirus strains not capable of being contained by timely quarantine of infected individuals. Any increase in highly pathogenic CoV virulence, pathogenesis, or transmission would likely require a targeted medical countermeasure. Without strategic research programs that fill the gaps identified in our literature review, medical countermeasures that target highly pathogenic coronaviruses cannot be brought to market, leaving global public health vulnerable to this emerging threat.

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## Declaration of interest

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

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