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Cell-based antiviral screening against coronaviruses: Developing virus-specific and broad-spectrum inhibitors

Andy Kilianski and Susan C. Baker

Department of Microbiology and Immunology, Loyola University Chicago Stritch School of Medicine, Maywood, IL 60153

Abstract

To combat the public health threat from emerging coronaviruses (CoV), the development of antiviral therapies with either virus-specific or pan-CoV activities is necessary. An important step in antiviral drug development is the screening of potential inhibitors in cell-based systems. The recent emergence of the Middle East respiratory syndrome (MERS)-CoV necessitates adapting methods that have been used to identify antivirals against the severe, acute respiratory syndrome (SARS)-CoV and developing new approaches to more efficiently screen antiviral drugs. In this article we review cell-based assays using infectious virus (BSL-3) and surrogate assays (BSL-2) that can be implemented to accelerate antiviral development against MERS-CoV and future emergent coronaviruses. This paper forms part of a series of invited articles in *Antiviral Research* on “From SARS to MERS: 10 years of research on highly pathogenic human coronaviruses.”

Keywords

SARS-CoV; MERS-CoV; entry inhibitors; replicase inhibitors; antivirals; pGlo

1.1 - Introduction

The emergence of zoonotic coronaviruses with human to human transmission potential has highlighted the need for effective antivirals against coronavirus replication (introduced in Hilgenfeld & Peiris, 2013). Since the identification of the Middle East respiratory syndrome coronavirus (MERS-CoV), there have been 150 confirmed cases and 64 deaths; a fatality/case ratio of over 40% (World Health Organization (WHO), 2013). Currently it is unclear if there are additional asymptomatic cases of infection with MERS-CoV. MERS is reminiscent of the outbreak of severe acute respiratory syndrome (SARS) in the Guangdong province in China in 2002-2003 (reviewed in Cheng, Chan, To, & Yuen, 2013).

The causative agent of SARS, the severe acute respiratory syndrome coronavirus (SARS-CoV) likely emerged from a bat reservoir and was transmitted through an intermediate reservoir (civet cats), before obtaining the spike mutations to efficiently infect humans and cause severe respiratory disease (reviewed Li, 2013). Recently, a SARS-like bat coronavirus was isolated that can directly infect human cells (X. Y. Ge et al., 2013), suggesting that

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Corresponding Author: Dr. Susan C. Baker, Dept. Microbiology and Immunology, Loyola University Chicago Stritch School of Medicine, 2160 S. First Ave., Bldg. 105 Rm 3929, Maywood, IL 60153, Ph: 708-216-6910 sbaker1@lumc.edu.

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emerging coronaviruses may be transmitted directly from bats to humans. MERS-CoV is capable of infecting bat and human cells directly; as both the bat and human forms of the receptor, dipeptidyl peptidase IV, support viral entry (Muller et al., 2012; Raj et al., 2013). Additionally, a fragment of the RNA-dependent RNA polymerase gene that is genetically identical to MERS-CoV was detected in one Egyptian Tomb bat (Memish ZA, Mishra N, Olival KJ, Fagbo SF, Kapoor V, Epstein JH, et al., 2013) and dromedary camels were found to have neutralizing antibodies against MERS-CoV, perhaps linking an endemic source of the virus and an intermediate host to human MERS-CoV infections (Perera et al., 2013; Reusken et al., 2013).

Since the outbreak of SARS-CoV, there have been extensive efforts on antiviral drug development, but no FDA approved antiviral drugs or vaccines exist against any human coronaviruses (reviewed in Barnard & Kumaki, 2011). However, much has been learned from studies evaluating inhibitors directed against SARS-CoV that can be applied to new emergent coronaviruses. Understanding the experimental methods and results of studies performed to screen for SARS-CoV antivirals can guide the current work to develop drugs against MERS-CoV or to ultimately identify pan-coronavirus inhibitors. Cell-based assays are of great importance when developing effective antivirals, as these assays can identify possible antivirals from candidates initially selected from *in vitro* screens. The focus of this review will be to outline potential drug targets in the coronavirus life cycle, describe cell-based assays used to test antivirals against SARS-CoV, highlight novel techniques used to evaluate potential antivirals against MERS-CoV and discuss the challenges facing anti-coronaviral drug development.

1.2 - Druggable targets of coronaviruses

The coronavirus genome encodes many druggable targets, and these targets are highlighted in their role in the replication cycle life cycle (Figure 1). Human dipeptidyl peptidase IV (DDP4, CD26) has been discovered as the receptor for MERS-CoV (Raj et al., 2013), the receptor-binding domain (RBD) of the spike protein has been identified and structurally characterized (Y. Chen et al., 2013; Du et al., 2013; Mou et al., 2013) and the crystal structure of the complex between DPP4 and the RBD has been determined (Lu et al., 2013; Wang et al., 2013 and reviewed in Li, 2013). The interactions between viral glycoproteins and receptors have been targeted in other viruses, including SARS-CoV. Coronaviruses can enter cells through receptor mediated endocytosis or by membrane fusion with the plasma membrane. Endocytosis of the receptor-virus complex can occur, and upon acidification of the endosome, the host protease cathepsin L is activated and can cleave the viral spike protein to initiate viral fusion. The coronaviral spike can also be activated by extracellular proteases (trypsin) or proteases present on the cell surface (type II transmembrane serine protease or TMPRSS2), and this cleavage allows coronaviruses to enter cells in an cathepsin-independent manner (Glowacka et al., 2011; Matsuyama et al., 2010; Shulla et al., 2011 and reviewed in Simmons, Zmora, Gierer, Heurich, & Pohlmann, 2013). Upon viral entry and fusion of the viral and host cell membranes, the positive sense RNA genome, which is 5' methyl-capped and poly-adenylated, is translated in the cytoplasm. This translation yields two large polyproteins, pp1a and pp1b, which are then cleaved into 16 non-structural proteins by the papain-like protease, encoded within nsp3, and the 3C-like protease, encoded by nsp5. The proteases are drug targets, as the proteolysis of the nonstructural proteins is required for replication of the virus. Further, the papain-like protease of SARS-CoV and other coronaviruses has been shown to antagonize host innate immune responses, so inhibiting the papain-like protease will stop viral replication and may prevent antagonism of host innate immune responses (Barretto et al., 2005; Z. Chen et al., 2007; Devaraj et al., 2007; Frieman, Ratia, Johnston, Mesecar, & Baric, 2009; Sun et al.,

2012). Successful inhibitors have been generated against both SARS-CoV PL^{pro} and 3CL^{pro}.

To generate more genome copies and subgenomic mRNAs for synthesis of structural genes, the viral genome must be replicated by a series of enzymes that comprise the membrane-associated replication and transcription complex (RTC). The ADP-ribose-1''-phosphatase (nsp3), primase (nsp8), RNA-dependent RNA polymerase (RdRp, nsp12), helicase (nsp13), exonuclease and N7 methyltransferase (nsp14), endoribonuclease (nsp15), and 2' O-methyltransferase (nsp16) are all proteins that have enzymatic activity that can be targeted by antivirals. In fact, inhibitors have been identified that can block the activity of SARS-CoV RdRp, helicase, and 2' O-methyltransferase. After replication of the genome and generation of subgenomic mRNAs (sgmRNAs), structural and accessory proteins are translated from these sgmRNAs, assembly of the virion occurs at the endoplasmic reticulum-Golgi intermediate compartment (ERGIC), and the virion egresses through the exosomal pathway. Assembly and egress mechanisms have been targeted for inhibition in other viruses, but this strategy has not been explored for the development of coronavirus antivirals.

1.3 - Cell-Based Screens for SARS-CoV Antivirals

1.3.1 SARS-CoV Entry Inhibitor Screens

Viral glycoprotein binding with its cognate receptor and the spike protein mediating viral envelope fusion with cellular membranes are necessary for infection. These steps in infection have been successfully targeted in other viruses, with two FDA approved antivirals targeting HIV-1 entry in clinical use (reviewed in Henrich & Kuritzkes, 2013). The antiviral Maraviroc is a small-molecule CCR5 antagonist that inhibits the HIV-1 glycoprotein from binding to its receptor CCR5. Using a different mechanism, the antiviral Enfuvirtide inhibits viral fusion by interrupting the interaction between heptad repeat regions within the HIV-1 glycoprotein gp41. Partially based on the success of this strategy, both small-molecule and peptide inhibitors have been identified that target the entry of SARS-CoV. Here, we will review and describe the studies that used cell-based screening methods to identify these inhibitors.

The ability to evaluate of SARS-CoV entry specific inhibitors advanced with the demonstration that pseudotyped virion particles incorporating the S protein from SARS-CoV were competent for entry (Fukushi et al., 2005; Giroglou et al., 2004; Hofmann et al., 2004; Moore et al., 2004). In contrast to the previous cell-based screens based on virally induced CPE, pseudotyped lentiviral virions delivering a genome expressing GFP, luciferase, or other reporters allow for quantitative measurement and evaluation of coronaviral entry inhibitors. These assays can be used in BSL-2 laboratories and do not involve work with infectious CoVs. Using these techniques, the first demonstration of SARS-CoV entry inhibition was achieved using pieces of the SARS-CoV S or peptides designed to interact with the S, with the goal of both approaches to block viral membrane fusion intermediates (Yuan et al., 2004; Zhu et al., 2004). These studies demonstrated that small peptides that can bind to the heptad repeat regions of the SARS-CoV S (parallel in mechanism to FDA approved HIV entry inhibitor Enfuvirtide) could inhibit viral entry/fusion.

In addition to peptide studies based on the amino acid sequence of the SARS-CoV S, compound screens were performed against SARS-CoV entry. Using a commercial screen of diverse compounds (50,240 compound screen from ChemBridge) a small-molecule inhibitor was found to inhibit SARS-CoV entry based first on virally induced CPE reduction and then prevention of pseudotyped virion entry (Kao et al., 2004). Shortly after the demonstration

that small-molecule compounds could target SARS-CoV entry, Yi et al demonstrated that natural compound screens could also be used to inhibit SARS-CoV entry (Yi et al., 2004). They showed inhibition by small-molecules in a pseudotyped virion screen and identified two candidates that inhibited entry in the micromolar range. Utilizing a luciferase expressing lentiviral vector pseudotyped with S protein, molecules were screened based on luciferase expression levels of transduced cells. Two molecules, TGG and leuteolin (screened from a Chinese herbal library) were shown to have antiviral activity in the pseudotype assay and confirmed with live virus using an MTT colorimetric assay for cytotoxicity. These initial studies have evolved into three classes of inhibitor studies; small peptides that block viral fusion by interacting with SARS-CoV S, inhibition of cellular proteases that cleave the viral glycoprotein to mediate entry, and small chemical compounds that interact with the SARS-CoV S and inhibit entry.

The peptide studies have advanced to identify necessary peptides with very high affinity interaction and potent inhibition (Liu et al., 2009; Ni et al., 2005; Struck, Axmann, Pfefferle, Drosten, & Meyer, 2012; Ujike et al., 2008; Zheng et al., 2005). Zheng et al. developed 20-mer peptides corresponding to small fragments of the SARS-CoV S protein and identified three synthetic peptides with inhibitory properties. These peptides also had additive inhibitory effects when pre-incubated in combination with cells prior to infection. To elucidate the mechanism of inhibition, the authors used molecular modeling and speculated that these 20-mer peptides interacted with faces of SARS-CoV S that are required for interaction between the monomers of S to form functional, trimeric S. Inhibition at these interfaces has not been previously observed, and suggested a novel targeting strategy for prevention of S mediated viral entry.

In addition to peptides designed to bind to the S protein, Han et al took the opposite approach and designed peptides that corresponded to residues critical for entry mediated by ACE2 (Han, Penn-Nicholson, & Cho, 2006). Using a pseudotyped MuLV and HeLa cells expressing wild-type or alanine point mutants within ACE2, they determined that many of the charged residues within alpha helices 1 and 2 (residues 22-57) have inhibitory effects on viral entry. Importantly, their studies determined that these effects could only be seen with a 20 minute adsorption, and that the commonly used 60 minute adsorption time led to a high background and couldn't properly identify inhibitors in the pseudotype assay. Based on these studies, the authors created peptides corresponding to the amino acids from residues 22-57 and determined that these peptides had specific inhibitory activities against SARS-CoV pseudotype entry, but not entry of VSV-G pseudotypes. Overall, peptides corresponding to SARS-CoV S or ACE2 are effective at limiting entry of pseudotyped and wild-type SARS-CoV; however, none of these inhibitors have been tested further in animal models.

The inhibition of cellular factors required for SARS-CoV entry are attractive targets, as they could be less susceptible to viral escape due to mutations within the viral glycoprotein. Catalytic mutants of ACE2 had no effect on viral entry, suggesting that the enzymatic activity of coronaviral receptors is not required for entry (Moore et al., 2004). The SARS-CoV S protein requires cleavage either extracellularly by trypsin or TMPRSS2 or within the endosomal compartment by cathepsin L (Figure 1). Simmons et al. determined that cathepsin L mediates SARS-CoV S protein cleavage and that it can be pharmacologically targeted to prevent SARS-CoV S cleavage and entry (Simmons et al., 2005). In this study, a unique virus-virus membrane fusion assay was utilized. This assay involves two pseudotyped virions, one decorated with SARS-CoV S and the Avian sarcoma leukosis virus (ASLV) envelope protein, and one containing ACE2 and a luciferase expression construct. These particles can be induced to undergo fusion *in vitro*, and particles able to enter cells containing the ASLV receptor and express luciferase must have undergone viral membrane fusion upon mixing of the two pseudoparticle populations. If cathepsin L is responsible for S

cleavage within endosomes, then inhibition of purified cathepsin L *in vitro* will prevent viral-viral membrane fusion and no pseudoparticle expressed luciferase will be detected. The authors used this assay to demonstrate cathepsin L activation of SARS-CoV S and also that other endosomal cathepsins were not responsible for mediating SARS-CoV entry. Cathepsin L has been the continued target of antiviral development not only for SARS-CoV but other enveloped viruses that have a cleavage requirement within their glycoproteins, such as Ebola virus (Shah et al., 2010).

Endosomal proteases are not the only proteases that are able to activate SARS-CoV S. TMPRSS2 can activate S at the host cell plasma membrane, allowing SARS-CoV to enter without utilizing cathepsin L (Figure 1). Cell-cell fusion assays have been used to identify and characterize plasma membrane surface factors, and can be used to investigate cell surface fusion inhibitors. Cells expressing SARS-CoV S and a T7 polymerase are plated with cells expressing ACE2 and a luciferase or GFP reporter plasmid driven by a T7-dependent promoter. Upon receptor interaction and fusion, the cytoplasm from both cell types mixes, allowing for the transcription and translation of the luciferase or GFP (Shulla et al., 2011; Simmons et al., 2005). Pseudotyped virions were used by Kawase et al in their work identifying commercially available protease inhibitors targeting TMPRSS2 (Kawase, Shirato, van der Hoek, Taguchi, & Matsuyama, 2012). Inhibiting only serine proteases, which includes TMPRSS2, partially inhibited the entry of pseudotyped particles; however, also inhibiting cysteine proteases (which includes cathepsins) fully inhibited the entry of both pseudoparticles and authentic SARS-CoV. Targeting these host cell factors has been shown to be an effective way to prevent viral fusion and entry in cell culture.

Finally, small-molecule inhibitors have been screened and developed to target SARS-CoV entry. Recently, Adedeji et al used a SARS-CoV S pseudotyped HIV-Luc virus to screen a 3,000 compound library (Adedeji et al., 2013). Forty four hits were taken from this library and analyzed for specificity against SARS-CoV entry as compared to inhibition of a VSV-G enveloped HIV-Luc pseudovirus. Out of the initial 44 compounds, only 3 were found to be specific for inhibition of SARS-CoV. These counter screens highlight the importance of identifying specific compounds before undergoing further analysis of positive hits in initial screens. Further *in vitro* analysis was performed and the three compounds were identified to separately inhibit cathepsin L activity, inhibit interaction between S and ACE2, and inhibit a fusion step downstream of S-ACE2 recognition. The feasibility of chemical compound inhibition of virus entry is an established strategy to develop efficacious antivirals, especially for HIV; however, small compounds remain a relatively unexplored strategy with respect to coronaviruses. Overall, targeting viral entry of coronaviruses (especially SARS-CoV) has been demonstrated using specific cell-based assays outlined here. These strategies can be utilized for the evaluation of peptides or chemical inhibitors of S protein-receptor interaction, S-mediated fusion, or host cell factors necessary for mediating MERS-CoV and other emerging coronavirus entry.

1.3.2 - SARS-CoV Protease and Replicase Inhibitor Screens

After entry and fusion, the SARS-CoV genome is translated into two large polyproteins from ORF1a and ORF1b. For replication to continue, these polyproteins must be cleaved by viral proteases, either 1 or 2 papain-like proteases (designated PL^{pro} or PLP1/PLP2) and the 3C-like protease (3CL^{pro} or M^{pro} for main protease). This step in the replicative process has been the target of the most extensive work done to identify inhibitors for SARS-CoV replication (reviewed in Barnard & Kumaki, 2011). SARS-CoV PL^{pro} is responsible for three cleavages while SARS-CoV 3CL^{pro} cleaves the polyprotein at 11 sites (Figure 1). Because the use of proteases is conserved across coronaviruses, both PL^{pro} and 3CL^{pro} are considered attractive targets for antiviral drug development. Much of the antiviral work

performed with the coronaviral proteases has been done using *in vitro*, cell free conditions, and this work is crucial in laying the foundation for continued antiviral development (reviewed by Mesecar, Hilgenfeld, and their colleagues in this series). In this section, we will continue to focus on highlighting important cell based assays and techniques to efficiently identify and validate antiviral candidates against coronavirus replication.

Some of the first SARS-CoV PL^{pro} inhibitors discovered were first screened against purified protein *in vitro* and then further characterized in infection and cell lysate assays (Chou et al., 2008; Ratia et al., 2008). To determine if the inhibitors characterized were specific for SARS-CoV PL^{pro}, Ratia et al utilized a cell lysate experiment that combined purified SARS-CoV PL^{pro} with cell lysates, a tagged ubiquitin-vinyl sulfone, and the inhibitor. In this experiment, cell lysates were incubated with the tagged ubiquitin-vinyl sulfone, and any deubiquitinating enzyme will be covalently linked via the vinyl sulfone linkage to the tagged ubiquitin. These lysates can then be analyzed by western blot to determine if deubiquitinating enzyme binding to the ubiquitin vinyl-sulfone is affected in the presence of inhibitors. In the presence of the specific SARS-CoV inhibitor, only SARS-CoV PL^{pro} binding to the tagged ubiquitin vinyl sulfone was shown to be decreased. While not strictly a cell-based method, this is a useful technique for determining the specificity of inhibitors of papain-like protease activity.

Frieman et al took an entirely cell-based approach in their screening, identification, and mechanistic evaluation of a small compound library against SARS-CoV PL^{pro} (Frieman et al., 2011). The expression and activity of SARS-CoV PL^{pro} within *S. cerevisiae* led to a slow growth phenotype, and the basis for the screen was that inhibition of PL^{pro} activity by small compounds could reverse this slow growth phenotype. Growth was measured by optical density, and from a 2000 compound library provided by the NIH, the authors generated candidate compounds that were then analyzed for their effect on PL^{pro} expression or toxicity within *S. cerevisiae*. Based on these results, the authors further screened the compounds in VeroE6 and MA104 cells for activity against SARS-CoV replication. These compounds were also tested against three different activities of SARS-CoV papain-like protease. First, the drugs were tested for their ability to inhibit cleavage of a synthetic PL^{pro} polyprotein substrate, nsp2-3, by SARS-CoV PL^{pro}. This assay uses a tagged nsp2-3 and the cleavage product, which is dependent of PL^{pro} catalytic activity, and can be visualized by western blot. The second assay uses the deubiquitinating activity of PL^{pro} as a readout for PL^{pro} activity and inhibition. Cells are transfected with PL^{pro} and a tagged ubiquitin and then treated with inhibitors. PL^{pro} will cleave the ubiquitin molecules, resulting in a decreased smear of ubiquitinated proteins and an increased smear if the inhibitors are blocking PL^{pro} deubiquitinating activity. Finally, the authors assessed the ability of inhibitors to block the interferon antagonism properties of PL^{pro} using an IFNP luciferase assay. Cells transfected with either poly(I:C) or a constitutively active form of RIG-I, an IFNP luciferase reporter, and PL^{pro}, and then were treated with inhibitors. PL^{pro} inhibits IFNP promoter activation in response to stimulus, so if PL^{pro} inhibitors block the ability of PL^{pro} to inhibit IFNP activation, an increase in IFNP promoter activity will be seen. These cell based screening techniques are good tools to assess the mechanism of inhibitors and how they inhibit papain-like proteases within cells. However, these assays are not amenable to a higher-throughput analysis of compounds, and do not offer the specific quantitation of PL^{pro} catalytic activity within cells, which is critical when attempting to optimize small compounds and build structure activity relationships.

SARS-CoV 3CL^{pro} has also been the target of extensive efforts to generate specific antivirals. Modeling of SARS-CoV 3CL^{pro} based on the structures of transmissible gastroenteritis virus (TGEV) and HCoV-229E 3CL^{pro} presented the likelihood of structural conservation. Rhinoviral 3C^{pro} inhibitors bound efficiently to HCoV-229E 3CL^{pro}, and were

later shown to form complexes with SARS-CoV 3CL^{pro} (Anand, Ziebuhr, Wadhwani, Mesters, & Hilgenfeld, 2003; Yang et al., 2003). Based on this biochemical characterization, and the enzymatic properties of proteases, much of the early work on 3CL^{pro} inhibitors was performed *in vitro* in cell-free conditions. For example, Blanchard et al used purified SARS-CoV 3CL^{pro} and a FRET-based *in vitro* assay to screen a large compound library of compounds for 3CL^{pro} inhibition (Blanchard et al., 2004). Multiple studies have used a live infection approach based on cell cytotoxicity, and then worked backwards from the hits to identify the mechanism of action of the identified inhibitors (Kao et al., 2004; Wu et al., 2004). Experiments like these are critical for the identification of lead compounds; however, cell-based assays designed to measure protease activity are needed to identify if these compounds could be efficacious in the context of viral infection.

To measure 3CL^{pro} inhibitors in a cell-based assay, Lin et al. created a *cis* cleavage luciferase assay (Lin et al., 2004). This involved the creation of an expression construct that encoded for SARS-CoV 3CL^{pro} in frame with an inserted 3CL^{pro} cleavage site (SAVLQSGFRK) and a downstream luciferase construct. The rationale for this design was the knowledge that when luciferase is fused to larger proteins, its activity is substantially decreased. Upon the *cis* cleavage of the 3CL^{pro}-luciferase polyprotein, the liberated luciferase should give an increased signal. The authors demonstrated that this luciferase construct was not very active when translationally fused with a mutated 3CL^{pro} cleavage site. When the cleavage site was intact they witnessed a 5-25 fold increase in luciferase activity. This assay was the first demonstration of a quantitative readout for 3CL^{pro} activity when the protease is transiently expressed within cells.

Replicons have been developed for SARS-CoV and have been used as a platform for screening anti-coronaviral compounds (Almazan et al., 2006; F. Ge, Luo, Liew, & Hung, 2007; F. Ge, Xiong, Lin, Zhang, & Zhang, 2008). These replicons contain all of ORF1a/b from SARS-CoV in addition to the N gene (required for replication) and a reporter cassette. The N gene and reporter are both transcribed off of separate sub-genomic messenger RNAs, similar to the replication of the virus (Figure 1), meaning that the reporter can only be synthesized when the replicon is replicating correctly. These SARS-CoV replicons can be either transfected or incorporated into stable cell lines for antiviral screening. While these replicons are useful, this approach does not address the specificity of compounds screened. To determine mechanisms of action, the candidate compounds must be screened against replicase proteins in another system.

Antivirals have also been developed against the SARS-CoV helicase (Adedeji et al., 2012; Tanner et al., 2005; Yu et al., 2012), polymerase (Ahn et al., 2011; te Velthuis et al., 2010), and 2'-O-methyltransferase (Ke et al., 2012). A previous study by van Hemert et al. established a protocol to isolate and monitor the activity SARS-CoV replication complexes *in vitro* (van Hemert et al., 2008). The study performed by te Velthuis et al. used this protocol and showed that zinc-ionophores inhibited the synthesis of RNA by SARS-CoV RdRp. This method allows for the study of cell-produced replication complexes *in vitro*.

1.4 - Applications and development of cell-based screens for MERS-CoV antiviral screening

Since the outbreak of MERS-CoV, there has been adaptation of methods used to screen for SARS-CoV inhibitors to MERS-CoV and the development of novel methods to identify potential antiviral inhibitors against MERS-CoV (Table 1). The entry of some coronaviruses, including SARS-CoV, is known to be mediated by TMPRSS2 (Figure 1). To determine if TMPRSS2 inhibition is a viable strategy to prevent MERS-CoV entry, Shirato et al targeted TMPRSS2 activity with camostat and assessed the ability of MERS-CoV to

cause syncytia and to enter cells using qRT-PCR for viral RNA. They demonstrated that inhibition of TMPRSS2 activity could inhibit MERS-CoV syncytia formation and that camostat, when combined with an inhibitor of cathepsin L (Figure 1) potentially inhibited MERS-CoV entry into cells (Shirato, Kawase, & Matsuyama, 2013). These data represent the first chemical inhibition of MERS-CoV entry using infectious MERS-CoV by targeting host proteases. Targeting both the MERS-CoV S and receptor (CD26/DPP4) have been shown to be effective approaches, as Gao et al designed viral fusion peptide inhibitors against the heptad repeat region HR2 of the MERS-CoV S and Ohnuma et al created monoclonal antibodies against CD26 that were able to block MERS-CoV entry (Gao et al., 2013; Ohnuma et al., 2013).

To understand how MERS-CoV infection influences and interacts with host cells, Josset et al infected a lung epithelial cell line, Calu3, with MERS-CoV and SARS-CoV and analyzed the host transcriptome (Josset et al., 2013). This approach identified host factors to which inhibitory compounds exist, that could be exploited as antiviral therapeutics. To validate these putative inhibitors, the authors infected cells with MERS-CoV and showed that the host kinase inhibitor SB203580 had inhibitory effects on both SARS-CoV and MERS-CoV replication. This approach is useful for identifying host cell factors beneficial for replication, determining if they are druggable targets, and identifying existing inhibitors to these targets.

Based on the knowledge that cyclophilins play an important role in coronaviral infections (Pfefferle et al., 2011), de Wilde et al. used cyclosporine A to determine if cytotoxicity as a result of MERS-CoV infection is a possible readout for antiviral screening (de Wilde et al., 2013). Cyclosporin A was able to prevent MERS-CoV cytopathic effect in both Vero and Huh7 cells, verifying CPE as a read out of MERS-CoV infection, and a possible way to screen antiviral compounds against MERS-CoV. To aid in the study of MERS-CoV biology and antiviral screening, reverse genetics systems for MERS-CoV have been developed by two groups (Almazan et al., 2013; Scobey et al., 2013). The use of reverse genetics will allow for the creation of viruses expressing reporters as readouts of viral replication, and can be used to screen antiviral compounds in a more quantitative fashion. This was demonstrated by Scobey et al, who engineered a MERS-CoV encoding an RFP gene that is expressed during viral replication.

Showing inhibitory effects using the infectious virus is the critical step in developing antivirals, but screening large compound libraries or determining inhibitor mechanisms using infectious virus is not practical. Many approaches rely on using infectious MERS-CoV as a way to determine if compounds had inhibitory effects. As MERS-CoV is highly pathogenic and is transmittable from human to human, all assays must be performed in a BSL-3 laboratory. So far, two approaches have been developed for assaying antiviral compounds at either the entry or viral protease stage of the MERS-CoV lifecycle without using infectious MERS-CoV. The first approach involves the familiar pseudotype assay that has been used to evaluate SARS-CoV entry inhibitors. Gierer et al. used VSV-luciferase pseudotyped with the MERS-CoV S to determine that MERS-CoV does not utilize any other coronaviral receptors, that TMPRSS2 and endosomal cathepsins facilitate viral entry, and that the S protein can be neutralized with MERS-CoV infected patient serum (Gierer et al., 2013). Zhao et al. also used pseudotyped virions, but for their experiments they utilized an HIV-luciferase virus pseudotyped with MERS-CoV S (Zhao et al., 2013). They used this system to also show that the S could be neutralized by antibodies and that a small-molecule inhibitor of HIV entry had inhibitory properties on the transduction of their pseudovirions. Gao et al used pseudotyped virions to determine that HR targeting peptides inhibit viral entry (Gao et al., 2013).

A novel, cell-based approach to assay for coronaviral protease activity was developed by Kilianski et al. and used to determine if previously identified SARS-CoV protease inhibitors had activity against MERS-CoV proteases (Kilianski, Mielech, Deng, & Baker, 2013). These experiments utilized a circularly permuted luciferase construct with an inserted cleavage site corresponding to either the papain-like or 3C-like protease recognition sites. Expression vectors for both MERS-CoV PL^{pro} and 3CL^{pro} were able to cleave their respective biosensors when transfected together. An endpoint dual-luciferase assay with renilla luciferase as a transfection control and a live cell assay using a cell permeable luciferase substrate both showed luciferase activation when the MERS-CoV proteases were present. These assays were used to test previously identified SARS-CoV PL^{pro} and 3CL^{pro} inhibitors. The PL^{pro} inhibitor had no effect on the activity of MERS-CoV PL^{pro}, while the 3CL^{pro} inhibitor CE-5 did show inhibitory activity against MERS-CoV 3CL^{pro}. This was the first demonstration of an efficacious antiviral compound specific to a MERS-CoV enzyme, and will lead to SARS-CoV specific compounds being further tested. The applications of this assay are not limited to MERS-CoV, as the viral papain-like protease from SARS-CoV also activated the luciferase construct. This cell-based biosensor assay is especially useful as it can be used at BSL-2 level to study the effects of compound inhibitors on coronaviral proteases in the context of a host cell.

1.5 - Current State of Coronaviral Drug Development

There are many challenges currently facing scientists who are developing coronaviral, and especially MERS-CoV, antiviral drugs. As highlighted above, there is a need for validated cell-based assays that can be used to accelerate antiviral compound discovery in the face of emerging coronavirus infections (Table 1). One issue with MERS-CoV antiviral design is that no suitable animal model exists. Rhesus macaques can be infected with MERS-CoV, exhibit symptoms, and respond to therapies (de Wit et al., 2013b; Falzarano et al., 2013; Munster, de Wit, & Feldmann, 2013); however, the symptoms are transient and do not completely reflect the severity of the disease in humans. Unlike SARS-CoV, de Wit et al. demonstrated that MERS-CoV does not establish a productive infection in Syrian hamsters (de Wit et al., 2013a). To date, there have been no published observations of MERS-CoV infection in small animal models, such as mice or ferrets. In addition, more work needs to be done to develop broad-spectrum inhibitors that would work against the common human coronaviral pathogens like HCoV-NL63, HCoV-229E, HCoV-OC43, or HCoV-HKU1. Development of inhibitors that also target these common viruses would allow for clinical trials on the effectiveness of antiviral drugs against endemic and possible emergent coronaviruses.

The goal of this review was to highlight not only the cell-based assays used for evaluation of coronavirus antivirals, but also to bring to light novel techniques being used by the field. Highlighting these methods and application of techniques will assist in generation of new assays and more rapid development of coronaviral inhibitors for future use. As the public health threat from MERS-CoV and future emergent coronaviruses continues to persist, adapting knowledge and methodology from SARS-CoV research while also developing novel methods to screen antivirals will be important for achieving the goal of creating broad-spectrum anti-coronaviral drugs that are approved for human use.

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Highlights

- Coronavirus entry, proteolytic processing and RNA synthesis enzymes are attractive targets for antivirals.
- Cell-based screens have been used to identify and evaluate antivirals against SARS-CoV and MERS-CoV.
- New approaches are available to characterize pan-coronavirus inhibitors against CoV proteases.
- Antivirals against SARS-CoV may have efficacy against MERS-CoV and other emerging coronaviruses.

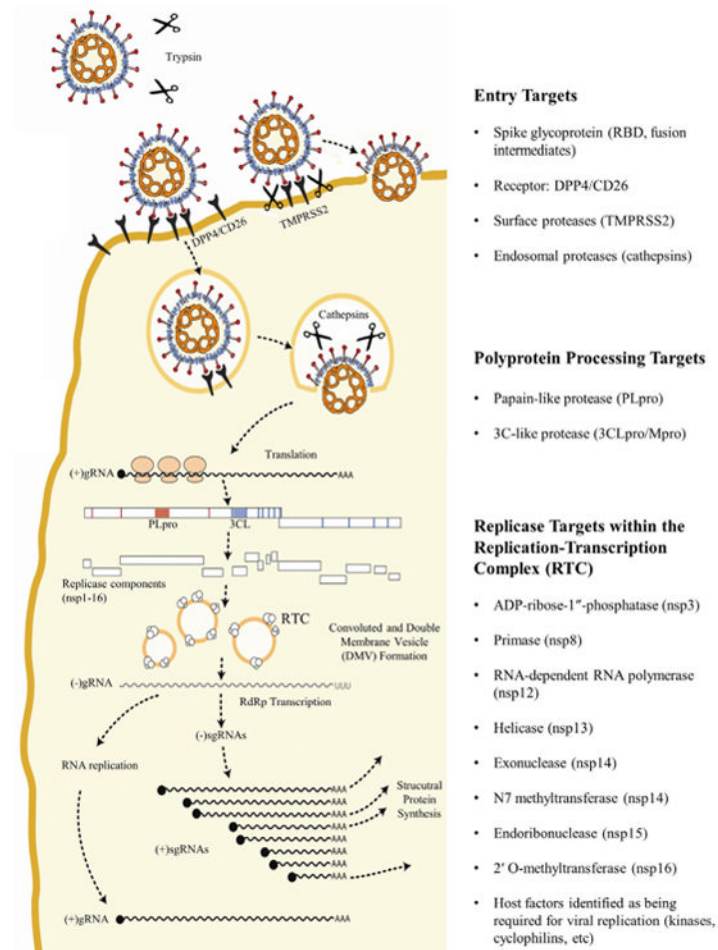


Figure 1. Coronavirus entry and RNA replication targets for antiviral drug development

Targets for viral entry include the viral spike-host receptor interaction, and host proteases that cleave the viral spike to mediate fusion. Viral replicase polyprotein processing can be targeted by inhibiting the papain-like or 3C-like proteases. The enzymatic activities of the replication-transcription complexes (RTCs) on convoluted membranes and double-membrane vesicles are also attractive targets for inhibitors.

Table 1
Cell-based assays used for antiviral drug development against MERS-CoV

Cell-based Assay	For evaluation of:	References
Infectious Virus (BSL-3)		
Cytopathic Effect	TMPRSS2 MERS-CoV S cleavage Cyclophilins required for MERS-CoV replication	Shirato, Kawase, & Matsuyama, 2013 de Wilde et al., 2013
Immunofluorescence	Blocking MERS-CoV S and CD26 interaction	Ohnuma et al., 2013
Viral Titer	Host kinases up-regulated by MERS-CoV	Josset et al., 2013
qRT-PCR	TMPRSS2 MERS-CoV S cleavage	Shirato, Kawase, & Matsuyama, 2013
Reporter Virus	Synthetic RFP expressing MERS-CoV for antiviral screening	Scobey et al., 2013
Surrogate Assays (BSL-2)		
Spike pseudotyped virus	Peptide blockage of HR2	Gao et al., 2013
	Neutralization with patient serum	Gierer et al., 2013
	Neutralization and entry inhibition by small molecules	Zhao et al., 2013
Protease cleavage bioassay	Small molecules targeting PLpro and 3CLpro	Kilianski et al., 2013