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Review

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

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ABSTRACT

To combat the public health threat from emerging coronaviruses (CoV), the development of antiviral therapies with either virus-specific or pan-coronaviral activities is necessary. An important step in antiviral drug development is the screening of potential inhibitors in cell-based systems. The recent emergence of Middle East respiratory syndrome coronavirus (MERS-CoV) necessitates adapting methods that have been used to identify antivirals against severe acute respiratory syndrome coronavirus (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."

Contents

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 and Peiris, 2013). Since the identification of 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, 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 et al., 2013).

The causative agent of SARS, 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 (Ge et al., 2013), suggesting that 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

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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 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 and 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.

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 (Fig. 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 (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 et al., 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 non-structural 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; Chen et al., 2007; Devaraj et al., 2007; Frieman et al., 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 accessary 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.

3. Cell-based screens for SARS-CoV antivirals

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 and 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