A Chimeric Virus-Mouse Model System for Evaluating the Function and Inhibition of Papain-like Proteases of Emerging Coronaviruses

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Abstract

To combat emerging coronaviruses, developing safe and efficient platforms to evaluate viral protease activities and the efficacy of protease inhibitors is a high priority. Here we exploit a biosafety level 2 (BSL-2) chimeric Sindbis virus system to evaluate protease activities and the efficacy of inhibitors directed against the papain-like protease (PLpro) of Severe Acute Respiratory Syndrome coronavirus (SARS-CoV), a biosafety level 3 (BSL-3) pathogen. We engineered Sindbis virus to co-express PLpro and a substrate, murine interferon stimulated gene 15 (ISG15), and found that PLpro mediates removal of ISG15 (deISGylation) from cellular proteins. Mutation of the catalytic cysteine residue of PLpro or addition of a PLpro inhibitor blocked deISGylation in virus-infected cells. Thus, deISGylation is a marker of PLpro activity. Infection of Interferon-alpha/beta receptor knockout (IFNAR−/−) mice with these chimeric viruses revealed that PLpro deISGylation activity removed the ISG15-mediated protection during viral infection. Importantly, administration of a PLpro inhibitor protected these mice from lethal infection demonstrating the efficacy of a coronavirus protease inhibitor in a mouse model. However, this PLpro inhibitor was not sufficient to protect mice from lethal infection with SARS-CoV MA15, suggesting that further optimization of the delivery and stability of PLpro inhibitors is needed. We extended the chimeric virus platform to evaluate papain-like protease/deISGylating activity of Middle East Respiratory Syndrome Coronavirus (MERS-CoV), to provide a small animal model to evaluate PLpro protease inhibitors to this recently emerged pathogen. This platform has the potential to be universally adaptable to other viral and cellular enzymes that have deISGylating activity.
Importance

Evaluating viral protease inhibitors in a small animal model is a critical step in the pathway toward antiviral drug development. We modified a biosafety level 2 chimeric virus system to facilitate evaluation of inhibitors directed against highly pathogenic coronaviruses. We used this system to demonstrate the \textit{in vivo} efficacy of an inhibitor of the papain-like protease of Severe Acute Respiratory Syndrome Coronavirus. Furthermore, we demonstrate that the chimeric virus system can be adapted to study the proteases of emerging human pathogens such as Middle East Respiratory Syndrome Coronavirus. This system provides an important tool to rapidly assess the efficacy of protease inhibitors targeting existing and emerging human pathogens as well as other enzymes capable of removing ISG15 from cellular proteins.
Emerging coronaviruses (CoVs) are now recognized for their life-threatening potential. The outbreak of Severe Acute Respiratory Syndrome coronavirus (SARS-CoV) that occurred a decade ago resulted in over 8000 infected people with 10% mortality (1). A recently emerged coronavirus, designated Middle East Respiratory Syndrome coronavirus (MERS-CoV), has infected over 837 people, with 291 deaths as of July 24, 2014 (2). Epidemiologic studies implicate animal reservoirs as the source for emerging coronaviruses. By identifying a SARS-like CoV from Chinese horseshoe bats and analyzing the mutations in the spike glycoprotein, first in intermediate hosts and then in humans, researchers were able to document the evolution of an emerging CoV (3). The footprint for the evolution of MERS-CoV is not yet clear. MERS-CoV has about 80% genome sequence identity to the bat coronaviruses HKU4 and HKU5 (4, 5). In addition, infectious MERS-CoV has been isolated from the respiratory tract of young camels (6–8) and there is accumulating evidence that adult camels have specific antibodies to MERS-CoV, consistent with endemic infection in the camel population (9, 10). Currently, it is unclear if the human cases of MERS are from sporadic introduction from animal reservoirs with limited human to human transmission or if there is ongoing transmission of MERS-CoV in asymptomatic humans or intermediate hosts (11–13). It is clear that CoVs have zoonotic potential for crossing the species-barrier and emerging into the human population to cause lethal disease. Viral proteases are logical targets for antiviral drug development, and protease inhibitors have been identified to block the papain-like protease (PLpro) domain of SARS-CoV (14).
PLpro is encoded in the viral replicase polyprotein and is critical for processing the polyprotein to generate a functional replicase complex. Structural and enzymatic studies revealed that PLpro is also a viral deubiquitinase (DUB), which can cleave ubiquitin (Ub) or ubiquitin-like molecules, such as interferon stimulated gene 15 (ISG15), from substrate proteins (15–17). Moreover, the catalysis-dependent interferon antagonism of PLpro implies that it may be involved in evading host innate immunity (18, 19). High-throughput screening efforts led to the identification of small molecule inhibitors directed against the viral papain-like protease domain, and synthetic medicinal chemistry and structure-activity relationship studies have produced compounds that inhibit replication of SARS-CoV in cell culture (14, 20). However, one of the challenges for preclinical, antiviral drug development is the availability of a small animal model for emerging CoVs. For SARS-CoV, infection of mouse-adapted strains in mice (21, 22) or transgenic mice expressing the receptor (Angiotensin-converting enzyme 2, ACE2) (23, 24), may serve as model systems for evaluating the efficacy of therapeutics. However, these studies must be performed in biosafety level 3 laboratories (BSL-3) with select agent status. For MERS-CoV, although rhesus macaques can be infected (25, 26), less expensive animal models such as mice and hamster are not susceptible to natural infection (27, 28). Dipeptidyl peptidase 4 (DPP4) was identified as the receptor for MERS-CoV in human and bat cells (29). Recently, novel model systems were generated for MERS-CoV infection by infecting mice with recombinant adenovirus expressing the human DPP4 receptor, which renders them susceptible to infection under BSL-3 conditions (30) and by generating recombinant HKU5-expressing the SARS-CoV spike protein (31). Development of additional
affordable and adaptable small animal models is needed to evaluate antivirals against existing and potentially emerging coronaviruses.

The goal of our study was to develop a biosafety level 2 system to evaluate inhibitors of the papain-like proteases of highly pathogenic emerging coronaviruses such as SARS-CoV and MERS-CoV. We were inspired by the fundamental work of Frias-Stahli et al., who first demonstrated that a chimeric Sindbis virus system could be used to evaluate the potential protease activity of a BSL-4 pathogen, Crimean Congo Hemorrhagic Fever virus (36). This chimeric virus system is based on the use of the positive-strand RNA virus, Sindbis virus (SV), a BSL-2 pathogen that is rapidly cleared by the immune system after infection in mice. Lenschow and co-workers showed that the interferon response, particularly interferon stimulated gene 15 (ISG15), is critical for efficient clearance of SV (35). Consequently, SV infection of interferon receptor knockout mice (IFNR−/−), which are unable to signal the induction of interferon stimulated genes, results in a lethal infection. However, if the gene for ISG15 is inserted into and expressed by SV, then infection with the chimeric virus induces an antiviral state and mice are protected from lethal infection. To induce the antiviral state, ISG15 must be conjugated to host cell proteins, a process termed ISGylation (40). The removal of ISG15 by deISGylating enzymes such as the L protease of CCHFV results in the abrogation of the protection mediated by ISG15 and mice succumb to infection. Thus, deISGylating enzymes can be used to “toggle off” the effect of ISG15 in this system. Given that the PLpros of CoVs not only function to process the viral polyprotein but also possess deISGylating activity (15, 37), we reasoned that the chimeric Sindbis-ISG15-Protease (SIP)
system could be exploited as a surrogate system to evaluate enzymatic activity and inhibition of CoV PLpros. Here we demonstrate the utility of the chimeric SIP system for evaluating the deISGylating activities of PLpros from SARS-CoV and MERS-CoV and the efficacy of a SARS-CoV PLpro inhibitor.

Materials and Methods

Cells, virus and plasmids. Baby hamster kidney cell line (BHK-21) and Vero-E6 cells were cultured in Dulbecco’s modified Eagle’s medium containing 10% (vol/vol) fetal calf serum (FCS), supplemented with penicillin (100 U/ml) and streptomycin (100μg/ml). Stocks of the recombinant mouse-adapted SARS-CoV (rMA15) were propagated and titrated on Vero-E6 cells. The virus was cryopreserved at -80°C until use as described below. PcDNA3-6×myc-mISG15 was kindly provided by Min-Jung Kim (Pohang University of Science and Technology, Pohang, Republic of Korea). PcDNA3-Ube1L, pcDNA3-UbcH8 and pcDNA3-Herc5 were kind gifts from Robert M. Krug (University of Texas). pcDNA-MERS-PLpro and its catalytic mutant (C1592A) were generated in our lab as described (38).

PLpro inhibitors 3e and 3h. The synthesis and characterization of these inhibitors were described in Baez-Santos et al (39). For mice administration, inhibitor 3e was formulated with 5% DMSO, 25% polyethylene glycol (PEG400) and 70% PBS (vol/vol/vol).

SIP viruses. The Sindbis virus vector, dsTE12Q, was kindly provided by Dr. Deborah Lenschow (Washington University in St. Louis). To generate the chimeric Sindbis virus
expressing ISG15 and PLpro, the DNA fragment of ISG15-IRES-PLpro comprising the murine ISG15 cDNA (1~465 nt), hepatitis C virus internal ribosome entry site (HCV-IRES: 40-371 nt of genome of HCV 2b genotype), and PLpro in frame with a V5 epitope tag at the C-terminus (available upon request), were generated by synthesis or two-step overlapping PCR and subsequently cloned into the BstE II restriction site of dsTE12Q vector. The insert DNAs of each chimeric virus were generated as follows:

1) SIP-SWT and SIP-SMT. A DNA sequence comprising the ISG15-IRES-PLpro (amino acids 1599-1855 in pp1a of SARS-CoV, accession number AY278241) in frame with V5 epitope tag was codon-optimized, synthesized, and subcloned into the pUC57 vector (Genscript, NJ). A catalytically inactive mutant of PLpro (cysteine 1651 to alanine) was generated as described previously (19) by site-directed mutagenesis PCR using primers (available upon request). The DNAs of interest were cut from recombinant pUC57 plasmids and cloned into the TE12Q vector, and designated SIP-SWT and SIP-SMT, respectively.

2) SIP-MWT and SIP-MMT. A DNA sequence comprising of ISG15-IRES-PLpro (amino acids 1483-1802 in pp1a of MERS-CoV, accession number AFS88944) was generated by two-step overlapping PCR using primers (available upon request). Briefly, the fragment of ISG15-IRES was amplified from the recombinant pUC57 plasmid described above, and the fragment of MERS-CoV PLpro or its catalytic mutant in frame with V5 epitope was amplified from the plasmids of pcDNA-MERS-PLpro or its catalytic mutant (cysteine 1592 to alanine), respectively. The fragment of ISG15-IRES-PLpro was generated by PCR amplification using primers (available upon request) and cloned into the BstE II restriction site of the TE12Q vector.
vector, and designated SIP-MWT and SIP-MMT, respectively. All constructs were verified by sequencing and linearized by digestion with Xho I restriction enzyme. The viral RNA was synthesized by *in vitro* transcription reaction following the manufacturer’s instructions (Ambion) and the RNA was subsequently electroporated into the BHK-21 cells with 3 pulses at conditions of 850V, 25μF in a 0.4cm cuvette cap (Bio-Rad). Viral supernatants were harvested at 16~24 hours (hrs) post electroporation and the titers were determined by standard plaque assay on the BHK-21 cells.

**Western blotting.** Cell lysates were separated in a 10% SDS-PAGE gel and transferred to PVDF membrane in transfer buffer (25mM Tris, 192mM glycine, 20% methanol) for 1 hr at 65V. The membrane was blocked using 5% dried skim milk in TBST buffer (0.9% NaCl, 10mM Tris-HCl, pH7.5, 0.1% Tween 20) for 2 hrs at room temperature and subsequently incubated with primary antibodies for overnight at 4℃. The mouse anti-myc tag monoclonal antibody (MBL) was used to detect the myc-ISG15 and the ISGylated proteins. ISG15 was detected using rabbit anti-ISG15 polyclonal antibodies (kind gift of the Lenschow lab). The expression of PLpro and β-actin were detected using mouse anti-V5 (Invitrogen) and anti-beta actin (Ambion) monoclonal antibodies, respectively. HRP-conjugated goat-anti mouse (Southern Biotech) was used as the secondary antibody and detected by using Western Lighting Chemiluminescence Reagent Plus (PerkinElmer) and visualized using a FluoroChem E Imager.

**Viral growth kinetics.** To analyze the replication of SIP viruses, the viral growth kinetics assays were performed. Briefly, 10^5 BHK-21 cells per well in 24-well plate were infected with
each SIP virus at a multiplicity of infection (MOI) of 5 and the cell supernatants were collected at indicated time points. The viral titers of the supernatants were determined by standard plaque assay on the BHK-21 cells.

**DeISGylation assay.** To determine the deISGylating activity of PLpro, BHK-21 cells in 24-well plate were transfected with 0.25 μg pcDNA3-myc-ISG15 and 0.125 μg each of ISGylating enzymes expression plasmids (pcDNA3-Ube1L, pcDNA3-UbcH8, and pcDNA3-Herc5) by Lipofectamine 2000 following the manufacturer’s instructions. At 6 hrs post-transfection, the medium was removed and replaced by mock or viral inoculums of SIP virus at MOI of 5. After 1 hr inoculation at 37°C, the inoculums were replaced by fresh DMEM containing 1% FCS. Cell lysates were prepared at 18 hrs post infection using 100 μL lysis buffer [4% SDS, 3% dithiothreitol (DTT), and 65mM Tris, pH 6.8] and analyzed by Western blotting.

To determine the effect of PLpro inhibitors on the deISGylating activity, BHK-21 cells were subjected to transfection and infection as described above, followed by the addition of fresh 1% FCS DMEM media containing the inhibitor at final concentration of 50 μM or a serial dilution. After 17 hrs of treatment (18 hrs post infection), cell lysates were prepared and analyzed by western blotting as described above.

**Infection of mice with SIP viruses.** Type 1 Interferon receptors knockout (IFNAR−/−) mice on the C57BL/6 background were initially obtained from Dr. Deborah Lenschow (Washington University in St. Louis). Mice were bred and maintained at Loyola University Chicago in accordance with all federal and university guidelines. Seven to eight-week-old male IFNAR−/− mice were infected subcutaneously in the left hind footpad with 6×10^6 PFU of SIP virus diluted...
in 25 μL DMEM medium followed by daily body weight monitoring. When the weight loss of an infected mouse was more than 25% of the initial body weight, the mouse was humanely euthanized. The survival rate was calculated by counting the dead or euthanized mice number and analyzed by log-rank test with GraphPad Prism software.

To determine if the PLpro inhibitor can block the function of PLpro in vivo, we tested PLpro 3e inhibitor in mice infected with SIP viruses. For each injection, 50 μg per dose of administered intraperitoneally (i.p.) at 0 and 2 day post infection (d.p.i). The weight loss of mice was monitored daily and survival rate was analyzed as described above.

**Infection of mice with SARS-CoV MA15 virus.** All viral and animal experiments were performed in a class II biological safety cabinet in a certified biosafety level 3 laboratory containing redundant exhaust fans, and workers wore personnel protective equipment, including Tyvek suits, hoods, and high-efficiency particle arrestor-filtered powered air-purifying respirators (PAPRs).

Eight-week old BALB/c mice were purchased from Harlan Laboratories and housed in accordance with all UNC-Chapel Hill Institutional Animal Care and Use Committee (IACUC) guidelines. Mice were administered either intranasally (i.n.) a single dose of 20 μg Poly (I:C), or i.p. 50 μg per dose of inhibitor 3e or vehicle. Mice were anesthetized with a mixture of ketamine-xylazine and were infected i.n. either with the $2 \times 10^4$ PFU rMA15 virus or with phosphate-buffered saline (PBS) in a dose of 50 μL. Infected animals were monitored daily for weight loss and sacrificed upon approaching 80% of their starting body weight or manifesting severe clinical symptoms, according to the IACUC guidelines. The lung tissues of infected
mice were collected at 4 d.p.i. and the viral titers were determined by plaque assay on Vero-E6 cells.

Results

Exploiting Chimeric Sindbis virus to evaluate CoV PLpro activity. The goal of our study was to establish a BSL-2 mouse model system to assess the function of CoV PLpros and to evaluate PLpro inhibitors *in vivo*. To do this, we modified a chimeric Sindbis virus that co-expresses ISG15 and a viral OTU domain deISGylating enzyme (36). To modify the system to allow expression of the larger Ubiquitin Specific Protease (USP) family enzyme from SARS-CoV, we replaced the original EMCV IRES with the IRES from HCV. A dicistronic DNA fragment containing ISG15 cDNA followed by the HCV-IRES and subsequent PLpro coding region was synthesized and inserted into the BstE II site of recombinant Sindbis genome, namely S\textsubscript{indbis-}ISG15-IRES-PLpro (SIP). Four chimeric Sindbis viruses expressing either wild-type (WT) or catalytic cysteine mutant PLpros (MT) from SARS-CoV and MERS-CoV were generated, and named as SIP-SWT, SIP-SMT, SIP-MWT, and SIP-MMT, respectively (Fig. 1A). The expression of ISG15 and PLpro was detected by immunoblotting lysates prepared from virus-infected cells (Fig. 1B). Analysis of viral growth kinetics revealed that the four SIP viruses replicate with similar kinetics and to high titer in BHK-21 cells (Fig. 1C). Furthermore, these viruses were stable upon passage in BHK-21 cells, which was in contrast to chimeric viruses containing larger insertions that had an EMCV IRES (Data not shown).
**PLpros expressed by chimeric Sindbis virus have deISGylating activity.** To determine whether PLpros expressed from Sindbis virus is able to cleave ISG15, BHK-21 cells were transfected with plasmids expressing ISGylation substrates and enzymes (myc tagged ISG15, Ube1L, UbcH8, and Herc5) and subsequently infected with SIP-WT or SIP-MT viruses. Western blot results show that cellular proteins were ISGylated in mock-infected cells transfected with ISGylation machinery plasmids. In contrast, the level of ISGylated proteins was significantly reduced in cells infected with SIP-WT but not SIP-MT viruses (Fig. 2). The decreased level of ISGylated proteins in SIP-WT infected cells is not due to virus induced cell death or lack of host translation in the context of a Sindbis virus infection as the SIP-MT grows to similar levels as the SIP-WT (Fig. 1C), and cellular proteins are ISGylated in SIP-MT infected cells. These results indicate that both SARS-CoV PLpro and MERS-CoV PLpro exhibit broad deISGylating activity, even when expressed in the context of Sindbis virus infection. These results extend the work of Frias-Staheli et al. (36) and show that viral USP-type enzymes, like the viral OTU-type enzyme used in their study, can function as deISGylating proteases in the context of Sindbis virus infection.

**PLpro disrupts ISG15-mediated protection in mice.** To determine whether co-expression of PLpro removes the protective effect of ISG15 during Sindbis virus infection, we infected IFNAR\(^{-/-}\) mice with the SIP-SWT or SIP-SMT and monitored for weight loss and survival. Mice exhibiting greater than 25% weight loss were humanely euthanized in accordance with animal care guidelines. As expected, the mice infected with SIP-SWT virus lost more body weight...
than mice infected with SIP-SMT virus at day 2~5 post infection (d.p.i) (Fig. 3A). SIP-SWT infection results in over 80% mortality, which is significantly higher than the mortality observed in mice infected with the SIP-SMT virus (26.7%) (P=0.0005; Fig. 3B). Moreover, we found the SIP virus expressing wild-type PLpro from MERS-CoV (SIP-MWT), but not catalytic inactive cysteine mutant of MERS-CoV PLpro (SIP-MMT) is able to inhibit the ISG15 function. The weight loss at 4~5 d.p.i and the mortality of mice infected with SIP-MWT were significant more than those of SIP-MMT infected-mice (Fig. 3C and 3D). These results indicate that PLpros of SARS-CoV and MERS-CoV are capable of disrupting the ISG15-mediated protection in IFNAR−/− mice, suggesting its role in antagonizing the innate immune response. In our system, the SIP virus infected-mice approached the maximum weight loss at 5~6 d.p.i and then either recovered or succumbed to infection, whereas in Frias-Staheli’s work the majority of chimeric virus infected-mice succumbed to infection at 8~12 d.p.i. The differential survival time may due to the different genetic background of mouse strains (129/SV/Pas in their work) or the expressions of different proteases. Next, we wanted to determine if this chimeric Sindbis-mouse system could be used for evaluating small molecule inhibitors directed against the PLpro domain.

An Inhibitor of SARS-CoV PLpro specifically blocks delSGylating activity. We reported the identification of small molecule compounds that block PLpro activity in vitro and block the replication of SARS-CoV in cell culture (14, 20). We recently designed and evaluated the biological activity of a second-generation of SARS-CoV PLpro inhibitors in vitro (39). One of
these compounds, 3e, inhibits SARS-CoV PLpro with a potency of 390 nM and has an antiviral potency of 8.3 μM against SARS-CoV in Vero-E6 cells. Importantly, this compound is not cytotoxic, does not bind to human serum albumin and has increased metabolic stability compared to other compounds evaluated (39). Therefore, to determine if compound 3e blocked PLpro activity in the context of the chimeric SIP virus, we exploited the deISGylation assay in cell culture. We found that the cells treated with compound 3e, but not the control compound 3h (Fig. 4A), a structural homolog of 3e with a higher IC₅₀ (600 nM) and no antiviral activity (39), showed an increase in the level of ISGylated proteins in SIP-WT virus-infected cells (Fig. 4B). This indicates that the deISGylating activity of PLpro was blocked by compound 3e. We also found that the deISGylating activity of PLpro was inhibited by compound 3e in a dose-dependent manner (data not shown). To assess the specificity of compound 3e, we tested the activity of a cellular deISGylating enzyme USP18 (also known as Ubp43) in the presence of 3e. Western blot results revealed that the level of ISGylated proteins in USP18-transfected cells was significantly decreased compared to the control; and there was no observable change in level of ISGylation in the transfected cells treated with compound 3e (Fig. 4C). This result indicates that compound 3e does not inhibit the deISGylating activity of USP18. Taken together, these results suggest that compound 3e specifically blocks SARS-CoV PLpro deISGylating activity in cell culture during replication of the SIP virus.

SARS-CoV PLpro protease inhibitor 3e protects mice from lethal SIP virus infection. To
determine whether the PLpro inhibitor was effective in a small animal model, we injected IFNAR−/− mice subcutaneously with SIP-WT and administered intraperitoneally (i.p.) compound 3e (50μg/dose) at 0 and 2 d.p.i. Although we found there is no significant weight loss between 3e and vehicle treated mice at early time points except at 5 d.p.i. (P<0.01), the majority of the 3e treated-mice (64.7%) recovered from infection before approaching the weight loss of euthanasia. In contrast, SIP-WT infected mice administered the vehicle control exhibited significantly higher mortality (31.6% survival rate, P=0.021; Fig. 5). The effect of compound 3e was specific to SARS-CoV PLpro activity as it had no significant effect on the pathogenesis of Sindbis virus infection of IFNAR−/− mice and there was no evidence of weight loss in mice injected with the compound 3e alone (data not shown). These results demonstrate the protective effect of compound 3e during SIP-WT virus infection.

**Evaluation of inhibitor 3e in SARS-CoV MA15 infected-mice.** To extend our studies to a respiratory tract model system, we evaluated 3e using the established SARS-CoV-MA15 intranasal infection model (21). Wild-type Balb/c mice were pretreated intranasally with 20μg single dose of Poly (I:C) as a positive control or 1 dose of compound 3e (50μg/dose) at 4 h prior to infection. Over all, mice were administered 50μg/dose of 3e twice a day on Day 0 (4h post infection), 1 and 2 post infection, and the mice were monitored for weight loss through day 4 post infection, and virus titers in lungs were assessed on day 4 post infection. Mice treated with compound 3e were not protected from virus induced weight loss, or virus replication, whereas the Poly (I:C) treated mice were completely protected from weight loss,
and the virus titers on Day 4 showed significant reduction in virus replication, compared to untreated virus control (Fig. 6). The results suggest that although protease inhibitor 3e was capable of protecting the majority of mice from lethal systemic SIP virus infection, the inhibitor is either not sufficiently stable or bioavailable in the respiratory tract to reduce the replication and pathogenesis of a respiratory tract infection with SARS-CoV MA15 in mice.

Discussion

This work establishes a chimeric Sindbis virus-mouse model system for assessing deISGylating activity of SARS-CoV and MERS-CoV PLpros and for evaluating SARS-CoV PLpro inhibitors in BSL-2 containment. To develop this model system, we exploited a chimeric Sindbis system pioneered by Frias-Staheli and coworkers who first showed that viral proteases with deISGylating activity could remove the protective antiviral state induced by ISG15 (36). We extended their studies by: 1) modifying the chimeric virus to express either SARS-CoV PLpro or MERS-CoV PLpro under translational control of the HCV-IRES, this shortened IRES enabled insertion of larger CoV USP-like proteases; and 2) showing that SARS-CoV PLpro inhibitors could be evaluated in SIP virus-infected IFNAR−/− mice. This is the first demonstration of the efficacy and specificity of an inhibitor that targets a viral papain-like cysteine protease (PLpro) in a virus-infected animal. This is important because there are over 100 cellular DUBS and previously it was unclear if the PLpro inhibitor was sufficiently specific to alter protease/deISGylating activity in an infected animal. In general, this chimeric Sindbis-protease system enables the study of enzymes with deISGylating activity, and
Establishes a BSL-2 model that can be used to evaluate the efficacy of small molecule inhibitors to existing and emerging coronaviruses *in vivo*. We hypothesize that the multifunctionality of CoV PLpros as proteases, deubiquitinating and deISGylating enzymes is important in viral pathogenesis, especially in antagonizing the innate immune response. ISG15 functions as an antiviral molecule through ISGylation of host substrates and by eliciting cytokine activity (39). Mice lacking ISG15 are more susceptible to lethal infection with Sindbis virus, herpesvirus and influenza virus (35, 41). The role of ISG15 in CoV pathogenesis is not yet clear. Ma and co-workers showed that murine coronavirus infection of USP18-deficient mice, where ISGylation levels are high, resulted in lower viral titers and prolonged survival compared to wild type mice, suggesting that high levels of ISGylation may delay CoV replication and pathogenesis (42). However, USP18 also mediates ISGylation-independent dendritic cells maturation (43), thus the loss of USP18 function may affect the kinetics of the immune response to viral infection. In the present study, we directly shown that CoV PLpros are capable of disrupting the protective effect of ISG15 *in vivo*, suggesting that PLpros have evolved an ISGylation antagonism mechanism to promote viral replication.

A major advantage of the SIP virus system is that we were able to study the PLpros of pathogenic CoVs in mice in a BSL-2 environment. This is particularly important because of the limited number of small animal models currently available for the study of MERS-CoV PLpro inhibitors (30, 31). MERS-CoV enters cells by interaction with DPP4 and both human and bat DPP4 are functional receptors (29). In contrast, mice and rats are resistant to
infection (27, 28), likely because of differences in the portion of DPP4 that interacts with the receptor binding domain of the spike glycoprotein of MERS-CoV (44, 45). The development of mouse-adapted strains of MERS-CoV and the generation of transgenic mice expressing the human DPP4 receptor are aimed at providing critical tools needed for understanding pathogenesis and evaluating candidate vaccines, but this work will be performed in BSL-3 containment. In contrast, the SIP virus mouse model is used in BSL-2 containment, and experiments are cost effective for evaluating efficacy and toxicity of PLpro inhibitors. In addition, we envision expanding the SIP virus system to identify broad spectrum PLpro inhibitors that block a wide array of human and bat PLpro activity and protect mice from lethal viral infection. Using the SIP system, we can evaluate the efficacy of an inhibitor to a novel bat PLpro without the need for developing a transgenic mouse model expressing the receptor for the novel virus. An important caveat of the SIP virus system is that it is a systemic infection and lethality is due to transmission of the virus through the central nervous system (35). Thus, it is a very sensitive system for evaluating protease inhibitors and we demonstrate efficacy of protease inhibitor 3e, which was previously shown to block replication of SARS-CoV in cell culture (39). However, as we report here, protease inhibitors must also be evaluated in the context of a respiratory tract infection, such as with mouse adapted-SARS-CoV-MA15 (21, 22). We found that protease inhibitor 3e was not effective at blocking replication and pathogenesis of SARS-CoV-MA15 in the respiratory tract of infected mice. This lack of efficacy may be due to the relative instability of 3e (39), or limitations of delivery into the mucosal surfaces of the respiratory tract, the site of natural infection. Further work is required
to optimize PLpro inhibitors for bioavailability, stability and appropriate delivery to block replication and pathogenesis of coronavirus.

Papain-like proteases (PLpros or PLPs) are conserved in all coronaviruses and the goal of identifying a broad spectrum inhibitor would be to inhibit existing human and potential emerging CoVs. Several CoV PLPs have been identified as deISGylating enzymes, such as the PLpro domain of SARS-CoV (15) and the PLP2 domain of human coronavirus NL63 (HCoV-NL63) (19). The SIP system described here could be extended to study the function of the PLpro/PLP2 domain of endemic human coronaviruses such as HCoV-NL63, HCoV-HKU1, HCoV-229E and HCoV-OC43. HCoV-NL63 generally causes mild upper respiratory disease in adult but it can cause more severe respiratory disease in young children (46, 47). HCoV-HKU1 has been associated with pneumonia in the elderly (48). Currently there are no animal models for evaluating inhibitors to HCoV-NL63 or HCoV-HKU1. Other PLPs including those of bat coronaviruses are speculated to act as deISGylating enzymes as well since they share conserved catalytic elements and are predicted to recognize similar cleavage sites in the viral polyprotein. We are currently developing SIP viruses expressing the PLP2 domain of HCoV-NL63 and other CoVs with the long term goal of identifying broad spectrum PLpro inhibitors that could block replication of existing and emerging coronaviruses.

Overall, this study provides evidence of CoV PLpro deISGylating activity in the context of viral infection and establishes a BSL-2 animal model for evaluating PLpro inhibitors in a mouse model. The current studies are designed to facilitate antiviral drug development for existing
and emerging coronavirus infections and are a forerunner to the development of similar platforms aimed at testing inhibitors against other deISGylating enzymes in vivo.

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Figure Legends

Figure 1. Generation of chimeric SIP viruses. (A) Schematic diagram of chimeric Sindbis viruses expressing ISG15, HCV-IRES and SARS-CoV PLpro or MERS-CoV PLpro. (B) Detection of PLpro and ISG15 from cells infected with SIP viruses. BHK-21 cells were mock infected or infected with SIP viruses as indicated at moi of 5, lysates prepared at 18 hrs post-infection, and proteins detected by immunoblotting. (C) Replication kinetics of SIP viruses. BHK-21 cells were infected SIP viruses as indicated at moi of 5 and supernatants were collected at indicated time points. Viral titer was representative of three independent experiments. Error bars represent SD.

Figure 2. PLpros expressed by chimeric Sindbis virus have deISGylating activity. BHK-21 cells were transfected with ISGylating machinery expression plasmids (myc-ISG15, Ube1L, UbCH8, and Herc5) and subsequently infected with SIP viruses as indicated. Cells lysate were probed with anti-myc to detect ISGylated proteins and unconjugated ISG15. The expression of PLpro and beta-actin as a loading control were detected with anti-V5 and anti-beta actin antibodies, respectively.

Figure 3. PLpros inhibit the ISG15-mediated antiviral effect in IFNAR−/− mice. Seven to eight-week old male IFNAR−/− mice were injected in footpad with the WT ( SWT or MWT) or MT ( SMT or MMT) SIP viruses at 6×10⁶ PFU and monitored for weight loss. Data are pooled from three independent experiments. Numbers of mice per group are indicated in parenthesis.
The statistical differences in body weight loss (A) and survival rate (B) were analyzed by Prism software with the two-way ANOVA test and the log-rank test, respectively. Error bars represent SEM. *, p<0.05; **, p<0.01; ***, p<0.001.

**Figure 4. Compound 3e blocks the delISGylating activity of SARS-CoV PLpro in cell culture.** (A) The structures of tested compounds. (B) BHK-21 cells were transfected with ISGylating machinery plasmids and subsequently infected with SIP-SWT virus as indicated followed by addition of 50 μM compound 3e or 3h; (C) BHK-21 cells co-transfected with ISGylating machinery plasmids and USP18 expression plasmids were treated with 50 μM compound 3e or DMSO. Cell lysates were immunoblotted with anti-myc antibody for detecting the ISGylated proteins. Expressions of PLpro, USP18, and beta-actin as loading control were detected with anti-V5, anti-HA, and anti-beta actin antibodies, respectively.

**Figure 5. Compound 3e blocks PLpro from disrupting ISG15-mediated protection in IFNAR−/− mice.** IFNAR−/− mice were infected subcutaneously with SIP-SWT virus at 0 d.p.i and administered i.p. 50 μg per mouse of compound 3e or vehicle only at 0 and 2 d.p.i. Mice were monitored for body weight loss (A) and survival (B). Data are pooled from three independent experiments. Total mouse number per group indicated in parenthesis. The statistical differences in weight loss and survival were analyzed by Prism software using the 2-way ANOVA test and the log-rank test, respectively. Error bars represent SEM. **, p<0.01.
Figure 6. Evaluation of PLpro inhibitor 3e in SARS-CoV MA15 mouse model. (A) Five eight-week old Balb/c mice of each group were administered either i.n. a single dose of 20 μg Poly (I:C), or i.p. 50 μg per dose of 3e or vehicle at 4 hrs prior to infection i.n. with MA15 strain virus 2×10⁴ PFU. Infected mice were further treated with 3e or vehicle for twice a day at 0, 1, and 2 d.p.i. Mice were monitored for weight loss and mortality. (B) Viral titer in lung was determined at 4 d.p.i. The statistical differences in weight loss and titer were analyzed by Prism software using the T-test. Error bars represent SEM.
A

Genomic Sub-Genomic Sub-Genomic

Non-structural Structural

B

SIP

kD mock SWT SMT MWT MMT

β-actin MPLpro SPLpro ISG15

C

Viral titer (log 10 PFU/ml)

3 4 5 6 7 8 9

Hours post infection

3 6 12 24 32
A

\[ \text{Compound } 3e \]

\[ \text{Compound } 3h \]

B

<table>
<thead>
<tr>
<th>Compound</th>
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C

- ISGylated proteins
- myc-ISG15
- PLpro
- β-Actin

- Compound 3e