A new mouse-adapted strain of SARS-CoV as a lethal model for evaluating antiviral agents in vitro and in vivo
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ARTICLE INFO
Article history:
Received 5 June 2009
Returned to author for revision 26 June 2009
Accepted 16 September 2009
Available online 22 October 2009

Keywords:
SARS-CoV
Lethal
IL-6
Ribavirin
Ampligen™
UDA
Protease inhibitor
Cytokine
Chemokine
Mouse

A B S T R A C T
Severe acute respiratory syndrome (SARS) is a highly lethal emerging disease caused by coronavirus SARS-CoV. New lethal animal models for SARS were needed to facilitate antiviral research. We adapted and characterized a new strain of SARS-CoV (strain v2163) that was highly lethal in 5- to 6-week-old BALB/c mice. It had nine mutations affecting 10 amino acid residues. Strain v2163 increased IL-1α, IL-6, MIP-1α, MCP-1, and RANTES in mice, and high IL-6 expression correlated with mortality. The infection largely mimicked human disease, but lung pathology lacked hyaline membrane formation. In vitro efficacy against v2163 was shown with known inhibitors of SARS-CoV replication. In v2163-infected mice, Ampligen™ was fully protective, stinging nettle lectin (UDA) was partially protective, ribavirin was disputable and possibly exacerbated disease, and EP128533 was inactive. Ribavirin, UDA, and Ampligen™ decreased IL-6 expression. Strain v2163 provided a valuable model for anti-SARS research.

Introduction
Severe acute respiratory syndrome (SARS) emerged in 2002 in the Guandong province of southern China as a new infectious respiratory disease characterized by influenza-like symptoms and signs, but with a very high mortality rate (Ksiazek et al., 2003; Peiris et al., 2003b). A novel coronavirus termed SARS-CoV was identified as the etiological agent (Drosten et al., 2003a; Drosten et al., 2003b). The World Health Organization (WHO) reported 8098 SARS cases from November 2002 to July 2003, and approximately 10% of the patients died (http://who.int/csr/sars/country/table2003_09_23/en/). Subsequent outbreaks including two laboratory events occurred, but they were rapidly contained. These outbreaks demonstrated the need for strategies to treat sporadic cases of SARS disease, including economical lethal rodent models that would simulate human SARS disease.

Studies on the molecular evolution of SARS-CoV suggested that the virus emerged from non-human sources (Guan et al., 2003), although the natural reservoir of the virus has not been positively identified. A current hypothesis is that the horseshoe bat is the main natural reservoir of SARS-CoV-like viruses (Hon et al., 2008; Li et al., 2005b). A number of SARS-CoV-like viruses have been isolated from bats, although the full lineage for the ancestors of SARS-CoV remains uncharacterized (Hon et al., 2008). Virus transmission to humans may have occurred when civet cats, infected by bats, were traded on Chinese wet markets (Guan et al., 2003).

Severe SARS-CoV infections in humans are characterized by diffuse alveolar damage and sparse inflammatory infiltrates (Chen and Subbarao, 2007; Franks et al., 2003; Nicholls et al., 2003). Hyaline membrane formation is a hallmark of SARS-CoV lung damage in humans (Hsiao et al., 2005). In addition to the hyaline membrane formation in severe SARS cases, findings could include serous, fibrinous, and hemorrhagic inflammation in pulmonary alveoli with capillary engorgement, including micro-thrombosis in some of these capillaries (Lang et al., 2003). Pulmonary alveoli are thickened with interstitial mononuclear inflammatory infiltrates. Desquamation of pneumocytes can be detected along with fibrinoid materials, and erythrocytes in alveolar spaces (Lang et al., 2003). Cell types in the respiratory tract in which SARS-CoV antigen has been detected include the following: alveolar epithelial cells (primarily type II pneumocytes), bronchial epithelial cells, and alveolar macrophages (Ding et al., 2004; Ye et al., 2007). These cell types all have the...
angiotensin-converting enzyme 2 (ACE2), which has been identified as one of the receptors to which SARS-CoV binds in order to be taken up into host cells (Li et al., 2005a; 2003).

A number of animal species have been proposed to model SARS infections in humans, including macaques (Fouchier et al., 2003), marmosets (Greenough et al., 2005), mice (Glass et al., 2004; Roberts et al., 2005), golden Syrian hamsters (Roberts et al., 2006), rats (Nagata et al., 2007), cats (Martina et al., 2003; van den Brand et al., 2008), and ferrets (Chu et al., 2008; Martina et al., 2003; Subbarao and Roberts, 2006; van den Brand et al., 2008). The utility of various animal models has been extensively reviewed by Roberts and Roberts, 2006; van den Brand et al., 2008). The utility of various animal models has been extensively reviewed by Roberts and Roberts, 2006; van den Brand et al., 2008).

Since early in the SARS outbreak, there has been a concerted effort by many laboratories to evaluate vaccines (Du et al., 2009) or to develop clinically approved drugs for efficacy against SARS-CoV to rapidly provide a treatment for SARS infections in humans (Tong, 2009a,b). To date, there is no approved agent for treating SARS-CoV and no agent has reached human clinical trials (Wong and Yuen, 2008). This is due to lack of animal model testing among other reasons. Ribavirin had been used to treat SARS infections in 2003 and no agent has reached human clinical trials (Wong and Yuen, 2007). Ribavirin had been used to treat SARS infections in 2003 and resulted in a highly lethal strain we designated as v2163. The v2163 virus caused severe signs of illness in 5- to 6-week-old BALB/c mice including ruffled fur and lethargy within 3–4 days and death by day 6. Another lethal mouse-adapted strain designated as MA15 (Roberts et al., 2007) was tested in parallel for comparison. Both mouse-adapted strains caused substantial weight loss and produced high virus titers in the lungs of infected mice (Table 1). In contrast, SARS-CoV strain Urbani-infected mice had no significant weight loss compared with uninfected controls, although they had moderate virus titers in the lungs 3 days post-virus exposure as shown previously by Barnard et al. (2006a,b). Lung virus titers at 3 days post-virus inoculation (d.p.i.) were higher in v2163-infected mice than in MA15-infected mice. Lung titers did not appear dependent on the level of virus inoculum, but did differ depending on the virus strain. The v2163 virus and MA15 virus persisted in the lungs for 6 days, while Urbani strain was below detection limit by day 6.

### Results

#### Morbidity and virus titers in mice

The Urbani strain of SARS-CoV, which is not lethal to BALB/c mice, was serially passaged 25 times in BALB/c mice at 3-day intervals and resulted in a highly lethal strain we designated as v2163. The v2163 virus caused severe signs of illness in 5- to 6-week-old BALB/c mice including ruffled fur and lethargy within 3–4 days and death by day 6. Another lethal mouse-adapted strain designated as MA15 (Roberts et al., 2007) was tested in parallel for comparison. Both mouse-adapted strains caused substantial weight loss and produced high virus titers in the lungs of infected mice (Table 1). In contrast, SARS-CoV strain Urbani-infected mice had no significant weight loss compared with uninfected controls, although they had moderate virus titers in the lungs 3 days post-virus exposure as shown previously by Barnard et al. (2006a,b). Lung virus titers at 3 days post-virus inoculation (d.p.i.) were higher in v2163-infected mice than in MA15-infected mice. Lung titers did not appear dependent on the level of virus inoculum, but did differ depending on the virus strain. The v2163 virus and MA15 virus persisted in the lungs for 6 days, while Urbani strain was below detection limit by day 6.
Mortality and recovery in mice

Infection with v2163 resulted in 100% of the 5- to 6-week-old mice losing 20% of their initial weight, and 70–100% of mice dying depending on the infectious dose (Table 1). Nearly all MA15-infected mice lost more than 20% of their initial weight when infected with 10^4.5 or 10^5.5 CCID₅₀ (Table 1), confirming results observed in 6- to 8-week-old mice by Roberts et al. (2007). However, the v2163 strain killed more mice than strain MA15 regardless of the inoculum used (Table 1). The v2163 virus was lethal at concentrations as low as 10^3.5 CCID₅₀ per mouse. In a replicate experiment, results were similar to those in Table 1, except 6 of 10 mice receiving 10^4.5 CCID₅₀ of MA15 died (Table 2). However, the MA15-infected mice that died had a mean day of death (MDD) of 13.5 ± 6.9 days, compared with 5.9 ± 1.4 MDD for the corresponding v2163-infected group (Table 2). Overall, death of 6-week-old mice infected with v2163 was significantly greater (P < 0.001) than MA15-infected mice (Fig. 1). Recovery from severe illness was more likely for MA15-infected mice than for v2163-infected mice, and v2163 was lethal at a lower infectious dose. The average LD₅₀ for v2163 was 10^3.3 ± 0.3 CCID₅₀ per mouse. These data were unique because, by special provision, animals were not euthanized during severe illness but allowed to die or recover spontaneously.

Age variability

The animal experiment was repeated comparing 5- to 6-week-old mice with 10- to 11-week-old mice (Table 2). The older mice infected with MA15 lost more weight by 4 d.p.i. than corresponding v2163-infected mice, and their MDD was 2–3 days later than v2163-infected mice. Mortality in 10- to 11-week mice was high for both mouse-adapted virus strains using 10^5.5 and 10^6.5 CCID₅₀ inocula. However, only 10% of the MA15-infected mice receiving the 10^3.5 inoculum died, while 100% of the corresponding v2163-infected mice died. No mice died from Urbani infection. Interestingly, in one separate age experiment, only 20% (2/10) of mice 3–4 weeks old died from a 10^4.5–4.5 CCID₅₀ v2163 infection, while 100% of 7- to 8-week-old mice died (data not shown). This supported the notion that SARS-CoV was more lethal in older hosts.

Genome sequence

The v2163 virus had nine mutations, one of which affected two reading frames, resulting in changes to 10 amino acid residues compared with the wild-type Urbani. Four mutations occurred in the spike protein (S) region (Fig. 2). A putative G-A mutation was observed in base pair 22722 with some replicates, but it was not found in RNA from infected mouse lungs. The mutations were compared to the MA15 lethal strain (Roberts et al., 2007), revealing interesting similarities and differences in the mutation spectra associated with mouse adaptation in young BALB/c mice (Fig. 2). In the structural genes, the Y436H mutation in the v2163 spike region was conserved in strain MA15, and the M mutation was in a similar location on both lethal strains (S6L for v2163, E11K in MA15). In the replicase genes, the nsp9 mutation (E4185D) was located near an nsp9 mutation (T4184A) found in strain MA15, and both lethal strains had an nsp13 mutation.

Cytokines and chemokines

The mouse-adapted strains of SARS-CoV elicited expression of cytokines IL-1α and IL-6, and chemokines MIP-1α, MCP-1, and RANTES in the lungs of mice 3 days after infection as measured with the multiplex ELISA assay (Fig. 3). Levels of IL-1β, IL-2, IL-3, IL-4, IL-5, IL-9, IL-10, TNF-α, and GM-CSF were below the minimum detection level for all test groups (<38 pg/ml of lung homogenate). Low levels of IFN-γ were observed with both mouse-adapted strains but not detected with the Urbani strain. Two out of 10 v2163-inoculated mice showed low cytokine response, and were excluded from cytokine analysis because they also had little or no virus detected in their lungs.
Fig. 3. Average cytokines detected in BALB/c mice lungs 3 days after infection with various strains of SARS-CoV (n ≥ 8). Black bars are v2163, gray bars are MA15, striped bars are Urbani, and white bars are uninfected controls. ⁎p<0.05, ⁎⁎p<0.01, ⁎⁎⁎p<0.001 MA15 compared with Urbani. aLower limit of detection. bThe v2163 and MA15 averages included some extrapolated values that were minimum estimates.

Fig. 4. Histological slides showing representative lung pathology. (a) Urbani-infected lung with no significant changes (40×). (b) MA15-infected lung with small numbers of foamy macrophages that widen scattered alveolar septae (40×). (c) MA15-infected lung with rare individual bronchiolar lining cells that were swollen and hypereosinophilic, and scattered alveolar septae widened by foamy macrophages (40×). (d) v2163-infected lung with moderate numbers of neutrophils and edema fluid surrounding several large vessels (40×). (e) v2163-infected lung with small numbers of alveolar macrophages throughout airspaces (60×). (f) Uninfected lung with no significant changes (40×).
The MA15 virus stimulated significantly more MIP-1α and RANTES compared with Urbani (P<0.05), and the v2163 virus caused significantly greater MCP-1 response than Urbani (P<0.01). A significantly higher IL-6 response compared with the wild-type Urbani strain was observed from both v2163 and MA15 mouse-adapted strains (P<0.001 and P<0.05, respectively). The v2163-infected mice expressed 6-fold more IL-6 than the MA15-infected mice. At the infection level used in this experiment, 100% of v2163-infected mice died and no MA15-infected mice died, showing a correlation between IL-6 concentrations in the lungs and mortality.

Histopathology

Three lung samples for each virus or control were evaluated for pathological changes 3 days after inoculation by a board certified veterinary pathologist (Fig. 4). Lungs of mice inoculated with the Urbani strain of SARS-CoV showed no significant changes, and sham-infected controls showed no marked changes except some scattered dense granulomas in one of three lungs. All three MA15-infected lungs showed small numbers of foamy macrophages and small numbers of neutrophils. Alveolar septae were seen in two of the three MA15-infected lungs. One MA15-infected lung had rare individual bronchiolar lining cells that were swollen and hypereosinophilic and degenerate. Pathological changes were observed in two of the three v2163-infected lungs; one had moderate numbers of neutrophils, edema fluid surrounding several large vessels, and rare granulomas containing refractile central fragments of foreign material replacing groups of alveoli. Another v2163-infected lung showed small numbers of alveolar macrophages throughout airspaces. In general, lungs infected with MA15 or v2163 showed acute to subacute alveolitis with some perivascular edema in some sections (Fig. 4). Differences between the two mouse-adapted strains were minimal. Hyaline membrane formation, as occurs in human SARS patients (Roberts et al., 2005), was not observed.

In vivo efficacy of drugs against v2163 infection

To demonstrate the utility of the lethal mouse model for antiviral drug testing, we evaluated the known antiviral agents ribavirin, an antiviral nucleoside analog (Sidwell et al., 1972), and Ampligen™ (poly I:poly C₃₇U), a synthetic dsRNA polymer that induces interferon production (Barnard et al., 2006a; Carter et al., 1972; Gowen et al., 2007). We also evaluated the novel drugs EP128533, a dipeptidyl glutaminyl fluoromethylketone protease inhibitor (Zhang et al., 2006), and UDA, a lectin that impedes viral fusion and egress (Keyaerts et al., 2007). Ampligen™ (10 mg/kg/day) afforded mice complete protection against death, reduced virus titers in the lungs, and significantly reduced lung scores and weight loss (Table 3). UDA treatment of 5 mg/kg/day resulted in 50% protection from death up to 10 days after infection (Table 3), but no reduction in lung virus titer. A replicate 21-day study showed 40% protection and no measurable

Table 3

Survival, mean day of death, lung scores, weight change, and virus titers in BALB/c mice infected with mouse-adapted SARS-CoV v2163 and treated with ribavirin, UDA, EP128533, or Ampligen™.

| Treatment                      | Survivors/total | Mean day of death | Lung score Mean day of death | Weight change (g) | Lung titer 3 d.p.i. (Log CCID50/g) | Lung titer 6 d.p.i. (Log CCID50/g) |
|--------------------------------|-----------------|-------------------|------------------------------|-------------------|-----------------------------------|-----------------------------------|
| Normal saline                  | 0/10            | 6.2±1.7           | 3.2±0.58                     | −2.8±0.40         | 7.0±0.11                          | 5.9±1.4                           |
| Ribavirin (75 mg/kg/day)       | 3/10            | 4.5±0.84          | 2.5±0.94                     | −2.0±0.07         | 7.0±0.24                          | 7.0±0.82                          |
| Ampligen™ (10 mg/kg/day)      | 10/10***        | >14***            | 0.90±0.83*                   | 0.40±0.14***      | 7.6±0.52                          | 4.3±0.16                          |
| UDA (5 mg/kg/day)             | 5/10            | 6.6±2.0           | 2.17±0.29                    | −2.3±0.14         | 7.32±0.7                          | 6.6±0.93                          |
| Cremaphor®                    | 1/15            | 4.3±1.3           | NT                          | −2.6±0.35         | 8.1±0.23                          | NT                                |
| EP128533™ (30 mg/kg/day)      | 0/10            | 4.5±1.4           | NT                          | −3.2±0.28         | 8.2±0.14                          | NT                                |

⁎⁎⁎P<0.05, **P<0.01 compared with saline or cremaphor control.

NT = not tested, all mice died before day 6.

Table 4

Lung IL-6 levels, virus titer, and pathology in BALB/c mice 3 and 6 days after infection with SARS-CoV strain v2163 and treatment with Ampligen™, ribavirin, UDA, or EP128533.

| Treatment          | Daya | IL-6 (pg/mL) | Lung titer (Log CCID50/g) | Summary of histopathology (inclusive) |
|--------------------|------|--------------|---------------------------|--------------------------------------|
|                    |      |              |                           | Neutrophilsb | Histiocytesc | Widened alveolid | Degenerate/necroticd |
| Saline, uninfected | 3    | 2200±670     | <2.8                      | 0          | 0            | 0                | 0                    |
| Saline             | 3    | 3880±2210    | 7.7±0.63                  | 3          | 3            | 2                | 3                    |
| Ribavirin (75 mg/kg/day) | 3    | 2230±610a   | 7.7±0.21                  | 2          | 2            | 2                | 1                    |
| Ampligen™ (10 mg/kg/day) | 3    | 1650±380h   | 8.0±0.39                  | 3          | 3            | 3                | 0                    |
| UDA (5 mg/kg/day)  | 3    | 3330±1340c  | 7.9±0.84                  | 3          | 3            | 3                | 2                    |
| EP128533™ (30 mg/kg/day) | 3    | 4690±1520  | 8.2±0.14                  | 0          | 1            | 0                | 1                    |
| Cremaphor control | 3    | 5000±1990   | 8.1±0.23                  | 1          | 1            | 1                | 1                    |
| Saline control     | 6    | 2140±560    | 6.8±0.85                  | 2          | 2            | 2                | 1                    |
| Ribavirin (75 mg/kg/day) | 6    | 2320±820  | 5.0±1.7                   | 0          | 2            | 0                | 0                    |
| Ampligen™ (10 mg/kg/day) | 6    | 1590±380  | 6.2±1.5                   | 3          | 3            | 3                | 0                    |
| UDA (5 mg/kg/day)  | 6    | 2530±1760  | 7.0±0.32                  | 3          | 3            | 3                | 0                    |
| EP128533™ (30 mg/kg/day) | 6    | nt          | nt                        | nt         | nt           | nt               | nt                   |
| Cremaphor control | 6    | nt          | nt                        | nt         | nt           | nt               | nt                   |

⁎P<0.05, compared to untreated control (excluding 655 pg/mL outlier in PSS day 3).

NT = not tested because all mice from this group died before day 6.

Days post-inoculation.

Small to moderate numbers of neutrophils in alveoli or surrounding scattered vessels.

Small to moderate numbers of histiocytes in alveoli.

Widened groups of alveolar septae.

Terminal bronchioles contain luminal degenerate and necrotic cellular debris, or are lined by individual degenerate or necrotic epithelial cells and/or necrotic cell debris.
toxicity with 15 mg/kg/day of UDA (data not shown). Protease EP128533 was inactive in BALB/c mice.

Ribavirin treatment of 75 mg/kg/day did not significantly protect mice from death (Table 3). Virus lung titers from mice treated with ribavirin were no different than those detected from control mice on 3 d.p.i., but at 6 d.p.i. the titers in ribavirin-treated mice were higher than those detected in the control group, suggesting that ribavirin prolonged lung infection, although this difference was not statistically significant (Table 3). Gross pathology (lung scores) at day 6 revealed substantial lung discoloration in ribavirin-treated mice. The MDD in mice treated with ribavirin was reduced (Table 3), but the survivor curve was statistically similar to that of PSS controls (Fig. 5). In the second experiment, virus titers were lower than controls in ribavirin-treated mice on day 6 (Table 4), but 50% of that group also died by 6 d.p.i. (data not shown).

The IL-6 concentration in the lungs on 3 d.p.i. was significantly lower \( (P<0.05) \) in mice receiving Ampligen™ or ribavirin treatment, and slightly lower with UDA compared with controls (Table 4). EP128533-treated mice showed no difference in IL-6 compared with controls. The uninfected control group had IL-6 levels of 2200± 670 pg/mL, which could be a result of stress induced by handling mice during daily weights and twice daily treatments. Therefore, 2200 pg/mL was considered the baseline IL-6 concentration in this study.

Histopathology of mice with varied antiviral treatments showed neutrophil infiltration and degenerate cells (Table 4). Lesions observed by a board certified pathologist were summarized in tabular form, and all lesions observed were included. Lungs in mice treated with a protease inhibitor, EP128533, and cremaphor solvent control appeared to have less overall lesions on 3 d.p.i. than lungs of mice in other treatment groups. Ribavirin-treated lungs had fewer overall lesions than those of other groups on 6 d.p.i. Macrophage infiltration, lymphocyte infiltration, and edema were not detected in infected lungs in this experiment, and no hyaline membrane formation was observed. Histological changes were similar between Ampligen™ treated groups and untreated control groups (Table 4).

In vitro efficacy testing with strain v2163

The v2163 strain was tested in vitro for sensitivity to multiple antiviral compounds. Compounds were chosen based on their mode of action or purported activity in previous literature. Vero-76 cells were treated with multiple concentrations of each compound then infected with virus, and the 50% effective concentration (EC50), the 50% cytotoxic concentration (IC50), and the selective index (SI) for each compound were calculated (Table 5). Compounds with a high selective index were considered active. The antiviral activity of drugs against v2163 was similar to that of the same compounds against the Urbani strain \( (P>0.05) \). Infergen™, a consensus interferon-α (Kumaki et al., 2008), was shown to be highly active, with a 90% effective concentration of <0.65 μg/mL against both strains and SI values >490. Ribavirin, promazine, an antipsychotic drug (Barnard et al., 2008), and calpain inhibitor VI (Barnard et al., 2004) showed no appreciable activity above their cytotoxic concentration (Table 5). EP128533 (Zhang et al., 2006) and UDA were moderately active. Calpain inhibitor IV (Barnard et al., 2004) was slightly active against both SARS-CoV v2163 and Urbani strains, with a 90% effective concentration of 12 and 6.4 μg/mL and SI values of 3.9 and 8.1, respectively. The v2163 strain appeared less sensitive to calpain

![Image](54x533 to 283x741)

### Table 5

| Virus strain | Compound       | Inoculum | Visual assay EC50 (μg/mL) | IC50 (μg/mL) | SI | Neutral red assay EC50 (μg/mL) | IC50 (μg/mL) | SI | Virus yield reduction EC50 (μg/mL) | SI |
|-------------|----------------|----------|---------------------------|-------------|----|-------------------------------|-------------|----|----------------------------------|----|
| v2163       | Ribavirin      | 10^{7.2} | 66 ± 4.3                  | >1000       | 15 | 80 ± 7.4                      | 200 ± 92    | 2.5 | 83 ± 63                         | 2.4 |
| Urbani      | Ribavirin      | 10^{9}   | 63 ± 36                   | >1000       | 16 | 86 ± 50                       | 99 ± 51     | 1.7 | 104 ± 84                        | 1.4 |
| v2163       | Promazine      | 10^{9}   | 2.6 ± 1.1                 | 6.4 ± 2.3   | 2.5 | 8.0 ± 7.2                    | 7.3 ± 4.2   |    | 3.9 ± 2.2                       | 1.9 |
| Urbani      | Promazine      | 10^{9}   | 2.1 ± 1.0                 | 7.9 ± 3.4   | 3.8 | 3.4 ± 2.1                    | 8.1 ± 3.0   | 2.4 | 3.1 ± 2.1                       | 2.6 |
| v2163       | Calpain VI inhibitor | 10^{9} | 14 ± 5.4                  | >37         | 2.6 | 17 ± 7.3                    | 37 ± 5.1    | 2.1 | 24 ± 7.9                       | 1.5 |
| Urbani      | Calpain VI inhibitor | 10^{9} | 13 ± 2.9                  | >37         | 2.8 | 14 ± 10                      | 27 ± 4.4    | 1.9 | 15 ± 1.0                       | 1.7 |
| v2163       | Calpain IV inhibitor | 10^{9} | 4.1 ± 3.5                 | >56         | 14 | 9.5 ± 9.2                    | 48 ± 19     | 5.1 | 12 ± 2.6                       | 3.9 |
| Urbani      | Calpain IV inhibitor | 10^{9} | 0.87 ± 0.13               | >56         | 64 | 4.3 ± 4.5                    | 52 ± 18     | 12 | 6.4 ± 2.6                       | 8.1 |
| v2163       | Infergen™      | 10^{9}   | <0.00012                  | >0.32       | >2700 | <0.00011               | >0.32     | >2800 | <0.00065                  | >490 |
| Urbani      | Infergen™      | 10^{9}   | <0.00092                  | >0.32       | >1100 | <0.31                   | >0.32     | >1000 | <0.05                        | >490 |
| v2163       | Ampligen™      | 10^{9}   | >240                      | >240        | 1.0 | >240                          | >240        | 1.0 | >240                           | 1.0 |
| Urbani      | Ampligen™      | 10^{9}   | >240                      | >240        | 1.0 | >240                          | >240        | 1.0 | >240                           | 1.0 |
| v2163       | UDA            | 10^{7}   | 0.70 ± 0.22               | >10         | 14 | 0.86 ± 0.39                 | 21 ± 9.5    | 24 | 1.1 ± 19                       | 19  |
| Urbani      | UDA            | 10^{7}   | 0.62 ± 0.26               | >10         | 16 | 0.76 ± 0.22                 | 21 ± 9.5    | 27 | 0.95 ± 0.36                    | 22 |
| v2163       | EP128533       | 10^{9}   | 1.4 ± 1.1                 | >100        | >71 | 1.6 ± 1.6                  | >100        | >61 | 12 ± 9.4                       | >8.5 |
| Urbani      | EP128533       | 10^{9}   | 0.56 ± 0.57               | >100        | >180 | 1.3 ± 2.1                | >100        | >75 | 5 ± 3.7                        | >19  |

* Average CCID_{50} virus per well from three replicates.
* b 50% effective antiviral concentration.
* c 50% cell inhibitory concentration of drug without virus.
* d In vitro 90% effective antiviral concentration by virus yield assay.
* e Selective index: SI = IC50/EC50, and virus yield SI = IC50/EC50.
* f Two replicates only.
inhibitor IV and EP128533 than strain Urbani, although the differences were not statistically significant.

Discussion

We have developed a new mouse-adapted strain called v2163 that causes increased morbidity and mortality in BALB/c mice, which the parent Urbani strain does not do. Strain v2163 was derived from the Urbani strain of SARS-CoV by serial passage in BALB/c mice. Mice infected with the v2163 strain were less likely to survive SARS-CoV infection than Urbani-infected or MA15-infected mice. The MA15 strain appeared less lethal in the current study than shown by Roberts et al. (2007). However, in the study previously reported, death was based on 20% weight loss. When 20% weight loss was equated to mortality in the current work, the results for MA15 were closely similar to previous reports (Table 1). Further, the prior finding that an inoculum of 10^3.9 CCID_{50} of MA15 per mouse was sublethal (Roberts et al., 2007) was confirmed in the current work. The reduced lethality of strain MA15 in the current experiments may also be attributed to the use of 5- to 6-week-old mice rather than 6- to 8-week-old mice. In the current study, 5- to 6-week-old mice were much less susceptible to MA15 than were 10- to 11-week-old mice (Table 2). SARS-CoV is known to be more fatal in aged patients than in younger patients (Chen and Subbarao, 2007). Age-related susceptibility to SARS-CoV in mice has been shown previously with 4-week-old versus 6-month-old mice (Nagata et al., 2008), with 12- to 14-month-old mice (Roberts et al., 2005), and between 10-week-old and 1-year-old mice (Rockx et al., 2007). The lethality of the v2163 virus in younger mice is an advantage for its use as a convenient model for evaluation of potential antiviral treatments.

The v2163 genome contained nine mutations compared with the parent Urbani strain, and these resulted in 10 amino acid residue changes since by 26133 affects two reading frames. Although the v2163 strain was developed independently, its genome shared mutations with the previously created mouse-adapted MA15 strain (Fig. 2). The spike mutation Y436H was common to both lethal strains, and is within the receptor-binding motif (Roberts et al., 2007). The Y436H mutation has been shown to enhance infectivity of recombinant viruses in mice (Becker et al., 2008), presumably by providing a more favorable interaction with the mAACE2 receptor for docking and entry. The v2163 strain had three mutations in the 5 (spike) region that were not present in MA15, suggesting that the increased virulence observed with v2163 potentially may be due to binding properties. Moreover, Y442F is a contact interface residue site that engages the ACE2 receptor, and passage of civet strain S216 K479N in human airway epithelial cells selected for a similar change at this position, shown to enhance SZ16 S interaction with the hACE2 receptor (Sheahan et al., 2008). Further, in separate work, a Y442L mutation was seen on a third mouse-adapted strain (MA20) in the same amino acid residue as the Y442F mutation of v2163 (Frielan et al., publication in preparation). Interestingly, the T1181L and N1169D alterations are in the heptad repeat elements in S2 where they could promote fusion and entry or host range expansion (McKoy and Baric, 2008).

Mutations outside of the spike region also appeared to be important. The mutation in the M glycoprotein gene (S6L) is near the MA15 E11K mutation that has been associated with increased production of virus particles from infected cells, presumably by promoting more efficient assembly and release, although the exact mechanism is unknown (Pacini et al., 2008). The nsp9 E4185D mutation is located near the nsp9 T4184A mutation of MA15, and likely confers a similar phenotypic difference, presumably increasing virulence. Both lethal strains had an nsp13 mutation, although it is not known if they confer similar changes to the protein structure. The fact that independent selections resulted in similar mutation profiles in target genes supports their role in increased replication and/or pathogenesis. Elucidation of the effects of these specific mutations may be key to understanding the genotypic variations that lead to more lethal phenotypes of SARS-CoV and coronaviruses in general. Such knowledge could allow rapid synthesis of chimeric viruses for use in evaluating and developing effective antiviral treatments.

Multiple cytokines and chemokines expressed in the lungs of BALB/c mice 3 d.p.i. infected with mouse-adapted SARS-CoV were significantly higher than those found in lungs of mice infected with the Urbani strain of SARS-CoV (Fig. 3). IL-6 and MCP-1 were more strongly expressed in v2163-infected mice than in MA15-infected mice. IL-6 and MCP-1 have been observed in human SARS patients (Chen and Subbarao, 2007), and one human study determined that IL-6, IL-8, and MCP-1 indicated a high risk of death (Jiang et al., 2005). Therefore, MCP-1 and IL-6 are likely good early indicators of disease outcome in mice. In contrast, cytokines MIP-1α and RANTES were more elevated in MA15-infected groups than v2163-infected groups. Since MA15-infected mice had lower mortality than v2163-infected mice, MIP-1α and RANTES may not be good indicators of disease outcome. When comparing the mouse cytokine profile to that detected in human SARS patients, the majority of human studies showed the same profile as seen in mice for IL-2, IL-4, IL-6, IL-10, IFN-γ, TNF-α, IP-10, and MCP-1 (Cameron et al., 2008; Chen and Subbarao, 2007; Hsu et al., 2004; Zhu, 2004) (Table 6). In contrast, IL-1β was not elevated in mouse lungs or in human lungs, but with serum three of five studies reviewed showed elevated IL-1β in human patients. RANTES was elevated in mouse lungs but not in the human sera. However, uninfected controls had elevated RANTES levels in mice as well, so the response was not considered important. IFN-γ was detected in lethally infected mouse lungs but not in human lungs. Nevertheless, the IFN-γ findings were considered consistent with human data since the IFN-γ level in mice was low, and IFN-γ has been found in human serum in some studies but not others. The two mouse-adapted SARS-CoV strains in this study both resulted in similar cytokine profiles (Fig. 3).

IL-6 levels were of particular interest. The increase in IL-6 corresponded directly with an increase in mortality in the respective

| Cytokine    | Lungs of lethally-infected mice | Human lungsa (number of studies) | Human serumb (number of studies reviewed) |
|-------------|--------------------------------|---------------------------------|------------------------------------------|
| IL-1α       | +                              | ND                              | ND                                       |
| IL-1β       | 0                              | 0 (1)                           | 0 (2)                                    |
| IL-2        | 0                              | 0 (1)                           | 0 (5)                                    |
| IL-3        | 0                              | 0 (1)                           | 0 (5)                                    |
| IL-4        | 0                              | ND                              | ND                                       |
| IL-5        | 0                              | ND                              | ND                                       |
| IL-6        | +                              | + (1)                           | 0 (2)                                    |
| IL-9        | 0                              | ND                              | ND                                       |
| IL-10       | 0                              | 0 (1)                           | 0 (6)                                    |
| IFN-γ       | +                              | 0 (1)                           | + (1)                                    |
| TNF-α       | 0                              | 0 (1)                           | 0 (7)                                    |
| GM-CSF      | 0                              | ND                              | ND                                       |
| MCP-1       | +                              | + (1)                           | 0 (1)                                    |
| MIP-1α      | +                              | ND                              | ND                                       |
| RANTES      | +                              | ND                              | 0 (4)                                    |
| IP-10       | +                              | + (2)                           | + (5)                                    |

a = cytokine elevated, 0 = cytokine not elevated, ND = not determined.

b Sources: Hsueh et al. (2004); Cameron et al. (2008); review of Chen and Subbarao (2007); review of Zhu (2004).
experimental groups. Mice infected with the wild-type Urbani strain showed no signs of disease or death, and they had no IL-6 detected in the lungs, while v2163-infected mice expressed high levels of IL-6. In one replicate study, 100% of v2163-infected mice died, and the average IL-6 level for lung homogenates in that group was 2750 pg/mL with only one mouse lower than 2700 pg/mL. The corresponding MA15-infected group in the same study had 100% recovery after severe illness, and those mice had an average IL-6 concentration of only 520 pg/mL with no sample higher than 900 pg/mL. Similarly, one clinical study found that patients with the poorest outcomes also had high levels of IL-6 secreting cells (Jones et al., 2004). In the current work, infected mice receiving PSS, cremaphor, and EP128533 groups showed higher IL-6 concentrations than the ribavirin, Ampligen™, and UDA groups (Table 4). The lower IL-6 concentration on 3 d.p.i. coincided with treatments that afforded at least some protection against death in the survival study (Table 3).

The role of IL-6 in SARS-CoV infection merits further investigation. IL-6 is critical in B-cell maturation and acute-phase responses (Kopf et al., 1994), and it is critical in coordination of immune response (Gowen et al., 2006). Conversely, a study reported by Hegde, Pahne, and Smola-Hess (2004) suggests that IL-6 also has immunosuppressive properties. However, high expression of IL-6 may exacerbate disease. In SARS patients, damage to the lungs seems to be primarily by viral destruction of alveolar and bronchial epithelial cells and macrophages (Chen and Subbarao, 2007). However, elevated IL-6 levels and concomitant lung damage continued even after peak virus production, which suggests that IL-6 contributes to disease progression (Wang et al., 2004). Furthermore, a SARS patient study showed altered liver function, leukopenia, lymphopenia, thrombocytopenia, and subsequent respiratory distress syndrome, suggesting that there was inflammatory damage resulting from SARS-CoV infection (Pereira et al., 2003a). Studies with other viruses also suggest that excess production of IL-6 may exacerbate disease. For example, a study of herpes simplex virus (HSV) suggested that exuberant production of IL-6 in neonates may explain why sepsis syndrome is more common with HSV infection in neonates than in adults (Kurt-Jones et al., 2005). A study of phlebovirus infection also proposed that IL-6 contributes to pathogenesis (Gowen et al., 2006). Regardless of the cause–effect relationship, we found that IL-6 is strongly expressed in the lungs during infection with mouse-adapted SARS-CoV virus and may be an effective predictor of disease outcome.

The histopathology of SARS is difficult to distinguish because most lesions observed with SARS can be found with many infections. The main distinguishing histopathological factors in human SARS disease are consolidation, edema, and hyaline membrane formation (Guo et al., 2008). Also, inflammatory infiltrates, usually composed of mostly macrophages and lymphocytes, are disproportionately low in number with respect to the alveolar damage, and sometimes absent (Chen and Subbarao, 2007; Cheung et al., 2004; Guo et al., 2008). In the current study, lungs infected with mouse-adapted strains of SARS-CoV showed acute to subacute alveolitis with some perivascular edema in some sections. Mice lungs had slight to moderate neutrophil and macrophage infiltration, but lymphocytes were not observed. Erythrocyte infiltration and some fibrous material were observed with v2163 in some experiments not shown. No hyaline membrane was formed in the mouse lungs. Since lung pathology differed from human disease, and since mice groups that died from infection had similar pathology to that of treated mice that survived infection (Tables 3 and 4), we submit that histopathology will not be an important marker in this SARS model.

SARS infects multiple tissues in humans, including spleen, intestines, kidneys, liver, adrenal, parathyroid, pituitary, cerebrum, and pancreas and neurons of CNS (Chen and Subbarao, 2007; Guo et al., 2008). With v2163, infectious virus was consistently found in the lung and snout of v2163 inoculated mice on days 3 and 6 post-inoculation, but was only rarely seen in serum. Virus was not recovered from kidney, brain, spleen, intestine, liver, or heart of v2163-infected mice with limits of detection of 2.1 log CCID50 for kidney; 1.1 log CCID50 for brain, spleen, and heart; and 4.1 log CCID50 for liver and intestine (data not shown). With MA15, Roberts et al. (2007) reported low titers of infectious virus found in lung, brain, spleen, and liver of mice infected with MA15.

Ribavirin, Ampligen™, EP128533, and UDA treatments were selected to assess the new v2163 strain of SARS-CoV as a model for in vivo evaluation of potential antiviral drugs. These drugs where chosen to represent a variety of modes of action.

Ribavirin is a known antiviral compound (Sidwell et al., 1972) with multiple modes of action, most of which stem from inhibition of inosine monophosphate dehydrogenase (IMPDH) leading to depressed intracellular GTP levels (Streeter et al., 1973). We had previously found in a mouse virus lung replication model of SARS-CoV that ribavirin extended the time that virus could be detected in the lungs of mice (Barnard et al., 2006b), and this was also observed in one trial of the current study (Table 3). The prolonged high lung titers observed suggested that ribavirin may contribute to the pathogenesis of SARS-CoV by prolonging and/or enhancing viral replication in the lungs, allowing continual stimulation of the inflammatory response, which may contribute to pathogenesis (Perlman and Dandekar, 2005). Also, ribavirin is known to be somewhat toxic, causing hemolytic anemia and other adverse side effects (Wong and Yuen, 2008), so ribavirin would presumably exacerbate disease if there were no concomitant antiviral effect. Interestingly, exacerbation of viral disease by ribavirin has also been shown with West Nile virus, resulting in increased mortality in hamsters (Morrey et al., 2004) and possibly in human patients (Chowers et al., 2001). In contrast, the second experiment in the current work showed significantly decreased IL-6 concentrations on 3 d.p.i. and somewhat decreased virus titers in ribavirin-treated animals on 6 d.p.i., suggesting some effect of ribavirin in mice (Table 4). The IL-6 reduction with ribavirin was interesting in light of previous reports that ribavirin inhibited IL-6 production in respiratory epithelia cells infected with RSV virus (Jiang, Kunimoto, and Patel, 1998) and endothelial cells infected with dengue virus (Huang et al., 2000). However, IL-6 was not reduced by ribavirin in activated T-cells (Hultgren et al., 1998), and ribavirin treatment did not reduce SARS-CoV viral load or IL-6 in human SARS patients (Wang et al., 2005). Ribavirin has not been shown to be effective against SARS in humans. In 2006, systematic review and comprehensive summary of treatments used for SARS patients found that 26 studies with ribavirin were inconclusive and that four showed possible harm (Stockman, Bellamy, and Garner, 2006). However, a 2008 review stated that ribavirin treatment combined with protease inhibitors could be useful clinically, although randomized controlled trials were still needed (Wong and Yuen, 2008). Another study showed synergy with ribavirin in combination with IFN-β in vitro (Morgenstern et al., 2005). Additional research is required to draw more certain conclusions about ribavirin in mice, including age-related studies and drug combination experiments using the lethal v2163 virus as a model.
secreted by infected cells and stimulate expression of potent antiviral proteins (Sadler and Williams, 2008; Samuel, 2001). However, IFN pathways are blocked in cells infected with SARS-CoV (Friedman, Heise, and Baric, 2008), thus making the host more susceptible to infection. We believe that exogenous stimulation of IFN pathways with Ampligen™ overcomes the evasion of virus sensing and signaling pathways. Furthermore, Kuri et al. (2009) suggested that “priming” with small amounts of IFN helps the cells themselves restore their normal IFN response to SARS-CoV. In the current study, Ampligen™ appeared not to affect virus replication initially, but led to more rapid decline of virus in the lungs compared with untreated animals by 6 d.p.i. (Table 3). The study showed that Ampligen™ protected against death and gross damage to the lungs in the presence of lethal SARS-CoV.

Stinging nettle lectin (Urtica dioica agglutinin, UDA) is an N-acetyl glucosamine-specific lectin that was reported as efficacious against HIV (De Clercq, 2000). More recently, UDA was reported to inhibit coronaviruses in vitro with some selectivity (van der Meer et al., 2007a). Keyaerts et al. (2007) showed that UDA was a potent and selective inhibitor of SARS-CoV strain Frankfur1-1. Plant lectins like UDA probably target viral attachment and fusion, and exocytosis or egress of the virus from the cell (Balzarini, 2007; van der Meer et al., 2007b; Keyaerts et al., 2007). The reduction of IL-6 in lungs at 3 d.p.i. along with 50% survival of mice in this study provide evidence to support further investigation of UDA treatment regimens as potential antiviral therapies.

Zhang et al. (2006) reported the design and synthesis of a SARS-CoV protease inhibitor EP128533, 4-(Z-Val-amido)-6-fluoro-5-oxo-hexanoic acid dimethylamide, chemical formula C21H30FN3O5 and molecular weight 423. It was shown to inhibit replication of SARS-CoV strains FFM1 and 6109 in Vero and CaCo2 cells. In the current study, Ampligen™ was active not to affect virus replication initially, but led to more rapid decline of virus in the lungs compared with untreated animals by 6 d.p.i. (Table 3). The study showed that Ampligen™ protected against death and gross damage to the lungs in the presence of lethal SARS-CoV.

In conclusion, the new v2163 mouse-adapted strain of SARS-CoV was highly virulent in BALB/c mice 5–6 weeks old, even with infectious doses as low as 10^{3.5} CCID_{50} per mouse. Infection with v2163 caused signs of disease, a strong immunological response, and lung pathology. The v2163 infection largely simulated human disease, except that hyaline membrane formation was not detected in the lungs, and infectious virus was not found in tissues outside of the respiratory tract, as is characteristic in human infection (Chen and Subbarao, 2007). Mutations in the virus were consistent with those seen in another mouse-adapted strain, and these may be useful in identifying genotypic markers for lethality. The v2163 strain propagated well in cell culture and was shown to be suitable for in vitro screening of potential antiviral compounds against SARS-CoV. Strain v2163 was used effectively as an in vivo model to evaluate SARS-CoV therapies, showing that Ampligen™ pretreatment was active against lethal SARS-CoV infection, and that UDA may have antiviral effects as well. This new model will facilitate identification of valuable antiviral compounds against SARS-CoV and against future emerging pathogens related to SARS-CoV.

Materials and methods

Mice, cells, and media

Female BALB/c mice were obtained from Charles River Laboratories (Wilmington, MA). They were maintained on Wayne Lab Blox and tap water ad libitum. They were quarantined for ≥24 h prior to use. Cells used were Vero-76 (ATCC CRL1587), Vero-E6 (ATCC CRL1586), and Vero (ATCC CCL81) maintained in MEM supplemented with 10% fetal bovine serum (FBS) and no antibiotics. For virus titer tests, MEM with 2% FBS and 50 μg/mL gentamicin was used.

Test compounds

Ribavirin was provided by ICN Pharmaceuticals (Costa Mesa, CA). Calpain inhibitor IV and calpain inhibitor VI were obtained from CalBiochem (San Diego, CA). Infergen™ was interferon alfacon-1 lot 002586 kindly provided by InterMune, Inc. (Brisbane, CA) in 27 μg/mL solution and held frozen until tested. Ampligen™ from Hemispherx Biopharma, Inc. was obtained through the NIAID Antiviral Substances Program. Promazine HCl was obtained from Sigma Chemical Corporation (St. Louis, MO). EP128533 was obtained from Epicept Corporation (San Diego, CA) and through the NIAID Antiviral Substances Program. Stinging nettle lectin (UDA) was obtained from EY Laboratories, Inc. (San Mateo, CA). Compounds were suspended in DMSO or purified water at 20 mM, then diluted in minimal essential medium (MEM) as needed for in vitro assays. Compounds for in vivo assays were diluted in saline, except protease inhibitor EP128533, which was diluted in a cremophor vehicle (10% cremophor, 10% ethanol, 80% purified water).

Viruses

MA15 virus

The MA15 strain of SARS-CoV mouse-adapted virus was kindly provided by Dr. Kanta Subbarao, National Institute of Health, NIAID, designated “MA-15 3A-1 P1 in Vero cells 4/25/2005.” For cytokine and lung pathology, MA15 virus was infected into BALB/c mice, and then the mouse lung homogenates containing the virus were filtered and subcultured again in Vero (ATCC CCL-81) cell culture for 2 days. Cell culture homogenates were sonicated then frozen at −80 °C. For all other in vivo work, the MA15 virus was subcultured in Vero-E6 cells in MEM with 10% FBS, 1 mM sodium pyruvate, and 0.1 mM non-essential amino acids. After a 2-day incubation, cell culture homogenates were sonicated, centrifuged to clarify the solution, then aliquoted and held frozen at −80 °C. Subcultured stocks were verified by PCR using primers flanking characteristic mutations.
**Urbani virus**

Severe acute respiratory syndrome coronavirus (SARS-CoV), strain Urbani (200300592), was obtained from the Centers for Disease Control and Prevention (CDC, Atlanta, GA) and routinely passaged in Vero-76 cells.

**Mouse-adapted SARS-CoV v2163**

Three BALB/c mice were anesthetized with ketamine/xylazine mixture (approximately 100 mg/kg + 5 mg/kg, respectively) and infected with 50 or 100 μL of SARS-CoV Urbani virus solution by nasal inhalation. Three or 5 days after infection, mice were euthanized, and the lungs were removed and homogenized in 5 mL of MEM + 10% FBS. The homogenate was tested for virus titer by CPE endpoint dilution assay, then re-infected into a subsequent group of mice. These infection steps continued for 25 passages, 5 passages with the homogenate diluted 1:5, then 20 additional passages without dilution of the lung homogenate between groups. This was determined to be valid because control mice demonstrated no notable effect from inhalation of uninfected lung tissue. One interim passage, P-12, was plaque purified. Passages ceased when increased virus titer in the lungs and signs of illness were observed in the infected animals. Lung homogenate from passage 25 mice was filtered and subcultured in Vero-76 cells, then diluted and plated to isolate single plaques. Plaque purification in Vero-76 cells was repeated three times. Four isolated plaques were subcultured in Vero-76 cells then compared by infecting into BALB/c mice. The most virulent isolate was selected. Aliquots of the virus stock were incubated in MEM medium for 7 days and demonstrated no growth of extraneous bacteria or fungi. RNA was isolated from the virus stock using the RNeasy kit (Qiagen, Alameda, CA) and tested by qRT-PCR with two primer pairs to verify the presence of SARS-CoV sequences (accession #AY278741). The presence of SARS-CoV antigen in the virus stock was also demonstrated using the SARS-CoV Ag Detection kit (Wantai, Inc., China). The new virus stock was designated “SARS-CoV USU strain v2163” and was used for subsequent studies. It is hereafter referred to as v2163.

**Genome sequence**

Sequence analysis was then performed at the University of North Carolina. RNA was purified from the cell lysate of Vero-76 cells infected with SARS-CoV v2163 using the RNeasy kit (Qiagen, Alameda, CA) followed by first-strand cDNA synthesis by reverse transcription using Superscript II (Invitrogen, Carlsbad, CA) as per the manufacturers’ protocol. Reverse transcription was performed with an initial incubation of 65°C for 5 min then cooled on ice followed by a 50-min incubation at 42°C and a 5-min denaturation at 70°C. Two microliters of each cDNA reaction were amplified by PCR using Accuprime High Fidelity Taq Polymerase (Invitrogen, Carlsbad, CA). Fifteen pairs of primers were used to generate the overlapping PCR products spaced every 2.5 kb generating DNA fragments with 500 bp overlap at each end. Amplification products were visualized on agarose gels and purified by use of the QIAquick Gel Extraction Kit (Qiagen, Alameda, CA). PCR products were sequenced in both forward and reverse directions with primers spaced every 500 bp along the genome. Automated sequencing was performed utilizing the BigDye Terminator version 3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) as per manufacturer’s instructions on an ABI Prism 3730 DNA Analyzer (Applied Biosystems). Sequences were assembled and analyzed with Vector NTI and Auto Assembler DNA Sequence Assembly Software (ABI Prism; Applied Biosystems). When a mutation was identified in comparison with the SARS-CoV published sequence (Urbani accession #AY278741), independent RT-PCR reactions were run and subsequent RT-PCR products were sequenced through the region containing the putative mutation to confirm the mutation.

**Mouse virulence studies**

Mice 5–6 weeks old (14–16 g) were separated into groups of 10 mice for sacrifice on days 3 and 6 post-infection, and 9 or 10 mice for weight and survival data. Mice were anesthetized by 0.1 mL intraperitoneal injection of ketamine (100 mg/kg)/xylazine (5 mg/kg) and then infected intranasally with a 50 μL suspension of the virus. Mice were inoculated with approximately 10^5.5, 10^4.5, and 10^3.5 CCID50 of v2163 or MA15. Plaque assays were performed in Vero-76 cells on the actual inocula after infection and showed that the MA15 virus titer was equal or higher than that of the corresponding v2163 titer for both replicate experiments. As a wild-type control and for comparison, mice were inoculated with approximately 10^5 CCID50 of the Urbani SARS-CoV strain. Groups of 5 and 10 mice were sham infected with MEM as a negative control. The groups of 10 survival mice were weighed individually to the nearest 0.1 g each day, and observed daily for death. Animals were held until death naturally occurred or up to 21 d.p.i. Three days and 6 d.p.i., five survivors in each group were euthanized and the lungs were removed. Lungs were then diluted 1:20 in MEM + 10% FBS, homogenized, and held at −80°C for virus titer testing. Lung homogenates were later thawed and serially diluted in MEM +2% FBS in triplicate in 96-well plates to obtain CCID50 values by the endpoint dilution method described below for virus yield reduction assays. The mean day of death (MDD) was calculated using mice that died and excluding survivors. The study was repeated with 5- to 6-week-old mice and 10- to 11-week-old (17–21 g) mice (Table 2). Another study was performed as above, with just the 10^4.5 inoculum in BALB/c mice of various ages to determine the age-dependent susceptibility to lethal infection.

**Cytokine and chemokine analysis**

Groups of 15 mice (14–18 g) were inoculated intranasally with the three virus strains suspended in 50 μL of MEM solution, and controls were mock infected with saline. Inocula (CCID50 per mouse) were 4.1 ± 0.3 log CCID50 for Urbani, 4.2 ± 0.4 for v2163, and 4.0 ± 0.5 for MA15. On day 3 post-virus exposure, five surviving mice were euthanized and the lungs were harvested. Remaining mice were held for measurements of weight loss and death. Lung sections were saved for histopathology in formalin solution. The remaining lung section was diluted 1:20 in MEM solution and homogenized, then held frozen at −80°C. Samples were then tested for cytokines IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, TNF-α, GM-CSF, and IFN-γ, and for chemokines MIP-1α, MCP-1, and RANTES using the Q-Plex™ mouse cytokine array screen (Quansys Bioscience, Logan, UT), a quantitative ELISA-based test with multiple distinct capture antibodies absorbed to each well of a 96-well plate in a defined array. Cytokines were quantified by relative luminescence of each spot in the array, and values (pg/mL) were calculated using the concurrently-run Quansys Bioscience™ standard curve and the QuansysAnalysys™ software (version 3.4). In one replicate some MCP-1 and RANTES values exceeded the standard curve range and were estimated by extrapolation (Fig. 3). Two of 10 v2163-inoculated mice had abnormally low cytokines and were excluded as outliers because they had little or no virus detected in the lungs. The cytokine results of all mice from two experiments were combined for averages, standard deviation, and Kruskal–Wallis analysis of variance followed by Dunn’s multiple comparison test compared to Urbani-infected mice.

**Histopathology**

Groups of animals were infected as described for the cytokine assay above. After 3 days, lungs from sham controls and mice exposed to 10^5 CCID50 Urbani, 10^4 v2163, and 10^3 MA15 were formalin-fixed. Three lungs from each group were sectioned and stained with H&E.
stain and evaluated by a board certified veterinary pathologist for histopathological changes.

**In vivo efficacy of drugs against v2163 infection**

Groups of mice (16–19 g) were inoculated with 50 μL containing 10^{10–4.4} CCID_{50} of v2163 virus by the i.n. route. Groups of mice were administered 100 μL of the following drugs by the intraperitoneal (i.p.) route: ribavirin at 37.5 mg/kg twice a day 8 h apart for 4 days beginning 4 h prior to exposure to virus; Ampligen™ at 5 mg/kg/day for four treatments 12–16 h apart beginning 16 h before virus exposure; and physiological saline solution (PSS) as a placebo using the dosing regimen described for ribavirin. In separate studies, 16- to 18-g mice were treated with UDA at 30 mg/kg/day using the treatment regimen described above for ribavirin, and 18- to 20-g mice were treated with 30 mg/kg/day of EP128533 protease suspended in 10% cremaphor administered b.i.d. as above for 5 days beginning at time of infection. Mice for toxicity controls were treated with PSS or cremaphor diluent using the treatment regimens described above but without virus exposure. Mice were observed daily, and group weights were taken periodically throughout the test period. The average weight loss was calculated on 3 d.p.i., and significance between treatments was evaluated by one-way analysis of variance followed by Newman–Keuls multiple comparison test. Compound toxicity in uninfected mice was evaluated in terms of weight change and adverse events, and no drug toxicity was observed in this study.

On days 3 and 6 post-infection, three to five surviving mice from each treatment group were sacrificed. The remaining mice were held and observed for death up to day 14 post-virus exposure (10 days for UDA). Animals that lost greater than 30% of their initial body weight in this experiment were humanely euthanized and the day of euthanasia was designated as the day of death. Lungs from sacrificed mice were observed for gross pathology and discoloration and assigned a score ranging from 0 (normal appearing lung) to 4 (maximal plum coloration in 100% of lung). Mouse lung samples from each test group were pooled and homogenized in MEM solution and assayed in duplicate for infectious virus using the method described below for virus yield assays using triplicate wells of Vero-76 cells.

The procedures above were repeated in 15- to 18-g mice for IL-6, histopathology, and virus titers in lungs (Table 4), and multiple tissues on 3 and 6 d.p.i. Lung sections were placed in formalin and examined for histopathology as described above. Remaining lung portions were homogenized individually in 2 mL of MEM + 10% FBS and held frozen at −80 °C. Samples were thawed, mixed, and then tested for IL-6 levels using the eBioScience (San Diego, CA) IL-6 ELISA kit per manufacturer's instructions. Samples size was three per treatment group for histopathology and four or five for IL-6 and virus titer depending on number of survivors remaining. Values in pg/g of lung were converted to pg/mL corresponding with the 1:20 dilution used in the initial untreated mice (Fig. 3). A low outlier in saline day 3 (655 pg/mL) was included in results but excluded from statistical analysis. IL-6 levels in treated groups were compared to untreated controls by ANOVA with Newman–Keuls pairwise comparisons. Individual tissues were harvested with separate sterile instruments to ensure that each tissue was isolated from the lung during necropsy. Tissues were homogenized and titers were performed as described for lungs above.

**In vitro efficacy testing with strain v2163**

The antiviral activity of test compounds was evaluated using the v2163 mouse-adapted virus strain as an in vitro model compared with strain Urbani run concurrently. Antiviral activity was measured by inhibition of virus-induced cytopathic effect (CPE) (Sidwell and Huffman, 1971), neutral red (NR) dye uptake (McManus, 1976), and also by virus yield reduction assays. Vero-76 (ATCC CRL 1587) cells grown in MEM with 10% fetal bovine serum (FBS) were seeded into 96-well plates at a concentration of 2 × 10^{4} cells per well and grown overnight at 37 °C with 5% CO_{2}. Test compounds were dissolved in DMSO or water, then serially diluted in MEM by half-log dilutions in triplicate wells. Test wells were infected with a multiplicity of infection (MOI) of less than 0.007 CCID_{50} per cell, sufficient to cause near complete cytopathic effect (CPE) in 3 days, which was 1.6–2.1 log CCID_{50} per well for Urbani, and 1.0–2.4 log CCID_{50} per well for v2163. The test medium was MEM with 2% FBS and 50 μg/mL gentamicin. Toxicity of compounds was assayed in duplicate wells without virus. Plates were incubated as above for 3 days, then each well was read microscopically for CPE. Cells were incubated with 0.011% NR dye for 2 h at 37 °C. Free dye was removed from the wells, then theuptaken dye was eluted with 50% ethanol in Sorensen's citrate buffer for ~30 min. Absorbance was then measured as described by Finter (1969), with an Opsys MR microplate reader (Dynex, Chantilly, VA) at 540 and 405 nm. Absorbance values and visual CPE were expressed as percentages of cell controls and untreated virus controls, adjusted for toxicity. The 50% effective concentration (EC_{50}) and 50% inhibitory concentration (cytotoxic, IC_{50}) values were calculated by regression analysis.

Virus yield reduction assays were performed using the cell culture 50% infectious dose (CCID_{50}) assay essentially as described previously (Sme et al., 1992). Briefly, supernatants from each well were serially diluted in triplicate wells of 96-well plates containing Vero-76 cells. Plates were incubated for 6 days and then checked for virus-induced CPE. Quantitation of virus yield titers was by the end point method of Reed and Muench (1938). The EC_{50} value was calculated using linear regression to estimate the concentration necessary to inhibit virus yield by 90% or a one log_{10} decrease in virus titer. Results of at least three assays were averaged and activity with the two virus strains was compared by Student's t test.

**Statistical analyses**

Statistical analyses were performed using PRISM™ 5.01 for Windows, PRISM™ 4.0c for MAC (GraphPad Software, Inc., La Jolla, CA), and Microsoft Excel 11.5. Comparisons not otherwise detailed were performed by the Student’s two-tailed t test assuming normal distribution and equal variance, and more sophisticated analyses were performed if differences were observed. The Kolmogorov–Smirnov test (KS test) for normal distribution and Bartlett’s test for equal variance were performed as applicable. MDD, cytokine values, and gross lung score data were analyzed by Mann–Whitney pairwise comparisons or the Kruskal–Wallis test followed by Dunn’s multiple comparison test as applicable. Surviving animals were not included in MDD calculations. Raw survival numbers were compared by the Fisher exact test. Survivor curve analysis (as in Fig. 1) was done using the Kaplan–Meier method and a log rank test. When that analysis revealed significant differences among the treatment groups, pairwise comparisons of survivor curves were analyzed by the Gehan–Breslow–Wilcoxon test, and the relative significance was adjusted to a Bonferroni-corrected significance threshold for the number of treatment comparisons made. Differences in percent weight loss were tested by one-way ANOVA with Newman–Keuls multiple comparison test, assuming equal variance and normal distribution. For lung titer data, we performed a KS test for normality, on log-transformed values, then used non-parametric Kruskal–Wallis test with Dunn’s multiple comparison test for groups that were not normally distributed, and a one-way ANOVA with Newman–Keuls multiple comparison test for groups that were normally distributed. LD_{50} was calculated using Probit analysis (StatPlus: MAC 2009).

**Ethics and biosafety**

This study was conducted in accordance with the approval of the Institutional Animal Care and Use Committee of Utah State University dated September 21, 2004. The work was done in the AAALAC-
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