

1 **Efficacy of Host Cell Serine Protease Inhibitor MM3122 against SARS-CoV-2 for**  
2 **Treatment and Prevention of COVID-19**

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24 **ABSTRACT**

25 We have developed a novel class of peptidomimetic inhibitors targeting several host cell  
26 human serine proteases including transmembrane protease serine 2 (TMPRSS2), matriptase and  
27 hepsin. TMPRSS2 is a membrane associated protease which is highly expressed in the upper  
28 and lower respiratory tract and is utilized by SARS-CoV-2 and other viruses to proteolytically  
29 process their glycoproteins, enabling host cell receptor binding, entry, replication, and  
30 dissemination of new virion particles. We have previously shown that compound MM3122  
31 exhibited sub nanomolar potency against all three proteases and displayed potent antiviral effects  
32 against SARS-CoV-2 in a cell-viability assay. Herein, we demonstrate that MM3122 potently  
33 inhibits viral replication in human lung epithelial cells and is also effective against the EG.5.1  
34 variant of SARS-CoV-2. Further, we have evaluated MM3122 in a mouse model of COVID-19  
35 and have demonstrated that MM3122 administered intraperitoneally (IP) before (prophylactic) or  
36 after (therapeutic) SARS-CoV-2 infection had significant protective effects against weight loss  
37 and lung congestion, and reduced pathology. Amelioration of COVID-19 disease was associated  
38 with a reduction in pro-inflammatory cytokines and chemokines production after SARS-CoV-2  
39 infection. Prophylactic, but not therapeutic, administration of MM3122 also reduced virus titers in  
40 the lungs of SARS-CoV-2 infected mice. Therefore, MM3122 is a promising lead candidate small  
41 molecule drug for the treatment and prevention of infections caused by SARS-CoV-2 and other  
42 coronaviruses.

43

44 **IMPORTANCE**

45 SARS-CoV-2 and other emerging RNA coronaviruses are a present and future threat in  
46 causing widespread endemic and pandemic infection and disease. In this paper, we have shown  
47 that the novel host-cell protease inhibitor, MM3122, blocks SARS-CoV-2 viral replication and is  
48 efficacious as both a prophylactic and therapeutic drug for the treatment of COVID-19 in mice.  
49 Targeting host proteins and pathways in antiviral therapy is an underexplored area of research

50 but this approach promises to avoid drug resistance by the virus, which is common in current  
51 antiviral treatments.

52

## 53 **INTRODUCTION**

54 The COVID-19 pandemic, caused by the Severe Acute Respiratory Syndrome Coronavirus 2  
55 (SARS-CoV-2), has illuminated the devastating impact of new emerging viral diseases on both  
56 the global economy and health of populations<sup>1</sup>. It has affected all aspects of human life and  
57 highlighted the threat of future outbreaks with other respiratory viruses for which currently  
58 available drugs will be ineffective. Incredibly, vaccines as well as several new drug candidates  
59 targeting this virus were developed at unprecedented speed because of early countermeasure  
60 work that focused on paradigm pathogens within the coronavirus family<sup>2-4</sup>. This was made  
61 possible by the combined efforts of scientists worldwide who elucidated details about the makeup  
62 and pathogenesis of SARS-CoV-2 infection. This work also resulted in the identification of several  
63 potential therapeutic targets such as the viral entry receptor, angiotensin converting enzyme 2  
64 (ACE2), and host cell proteases, such as transmembrane protease serine 2 (TMPRSS2) and  
65 cathepsin L1 (CTSL1), required for entry, replication, and release of the virus<sup>5-8</sup>.

66 TMPRSS2<sup>9-11</sup> has a trypsin-like serine protease domain and belongs to the family of Type II  
67 Transmembrane Serine Protease (TTSP) proteolytic enzymes with reported physiological roles  
68 in cancer and many other diseases<sup>12-15</sup>. TMPRSS2 has previously been shown to be important in  
69 other coronavirus infections caused by SARS-CoV-1<sup>16, 17</sup>, HKU-1<sup>18</sup>, MERS-CoV<sup>19</sup>, and others<sup>20,</sup>  
70 <sup>21</sup>. Furthermore, both TMPRSS2 and the other TTSPs, matriptase (ST14)<sup>22, 23</sup> and HAT (human  
71 airway trypsin-like protease, TMPRSS11D)<sup>24, 25</sup>, have been demonstrated to proteolytically  
72 process the hemagglutinin (HA) protein on the surface of some influenza A viruses and SARS-  
73 CoV-1<sup>17</sup>, allowing viral cell adhesion and entry in these infections<sup>24, 26-31</sup>. Additionally, TMPRSS2  
74 was found to support replication of other respiratory viruses including human para-influenza virus

75 type 1, 2 and mouse Sendai virus<sup>32</sup>. This makes TMPRSS2 an excellent target for the  
76 development of a broadly acting antiviral inhibitor<sup>33</sup> against diverse respiratory viruses<sup>24, 34-39</sup>.

77 We recently reported on the discovery and development of a new class of peptidomimetic  
78 TMPRSS2 inhibitors<sup>39</sup>. These inhibitors were rationally designed based on the peptide substrate  
79 sequence specificity of TMPRSS2<sup>15</sup> and molecular docking studies using the X-ray structure of  
80 TMPRSS2 bound to another inhibitor nafamostat<sup>40, 41</sup>. These inhibitors, including MM3122 and  
81 MM3144, are significantly more potent than another reported TMPRSS2 inhibitor Camostat<sup>41</sup>,  
82 suggesting improved efficacy *in vivo*. Unlike MM3122, which has the desired selectivity for  
83 TMPRSS2 over the coagulation serine proteases thrombin and Factor Xa, MM3144 does not.  
84 This compound was subsequently reported as a matriptase and TMPRSS2 inhibitor by another  
85 group as N-0386<sup>42</sup>. We tested the *in vitro* and *in vivo* efficacy of the most promising compound at  
86 the time, MM3122, in Calu-3 human lung epithelial cells and in a mouse model of SARS-CoV-2.  
87 Overall, we showed potent antiviral efficacy of MM3122 against XBB.1.5 and EG.5.1 variant of  
88 SARS-CoV-2 *in vitro* and amelioration of COVID-19 disease *in vivo*.

89

## 90 RESULTS

91 **MM3122 inhibits authentic SARS-CoV-2 replication in human lung epithelial cells.** The  
92 ability of MM3122, a TMPRSS2 inhibitor, to inhibit wild-type (wt) SARS-CoV-2 infection and  
93 replication was assessed on Calu-3 cells, a human lung epithelial cell line. At a 0.03  $\mu$ M  
94 concentration of MM3122, virus replication was completely inhibited, and no infectious virus was  
95 detected in the supernatant of the treated and SARS-CoV-2 infected cells (**Fig 1**). The inhibitory  
96 concentration ( $IC_{50}$ ) of MM3122 was  $\sim$ 0.01-0.02  $\mu$ M against the authentic wt SARS-CoV-2 virus.  
97 This is greater than 50 times more potent than Remdesivir which had an  $IC_{50}$  of  $\sim$ 1  $\mu$ M, an RNA-  
98 dependent RNA polymerase inhibitor developed for other viral infections that is one of the FDA-  
99 approved drugs approved to treat SARS-CoV-2 infected patients<sup>43</sup>. MM3122 was also tested for  
100 its activity against the EG.5.1 variant of SARS-CoV-2. Similar to wt SARS-CoV-2, we observed

101 robust inhibition of the virus with an  $IC_{50}$  of  $\sim 0.05$ - $0.1$   $\mu M$ . Taken together, these studies  
102 demonstrate the high potential for small molecule TMPRSS2 inhibitors to inhibit replication of  
103 SARS-CoV-2 and several of its many variants.

104 **MM3122 is a multi-targeted serine protease inhibitor with activity against some**  
105 **cathepsins.** To determine the target specificity of MM3122 for TMPRSS2, we tested for its  
106 inhibitory activity against 53 serine and cysteine proteases (**Table 1**). In our previous paper<sup>39</sup>, we  
107 profiled MM3122 for its inhibition of hepatocyte growth factor activator (HGFA), matriptase,  
108 hepsin, thrombin, and Factor Xa. For the 47 other proteases we contracted Reaction Biology Co.  
109 (Malvern, PA) to determine the  $IC_{50}$  values of MM3122 against a large panel of serine and cysteine  
110 proteases of high importance. A previous group had reported the selectivity profile for Camostat  
111 and Nafamostat against these same proteases<sup>44</sup> which is also shown in **Table 1**. In addition to  
112 TMPRSS2, MM3122 has potent activity (0.01 to 10 nM) against only 7 other proteases,  
113 matriptase, hepsin, matriptase-2, plasma kallikrein, trypsin, tryptase b2 and tryptase g1. It has  
114 moderate inhibitory activity (10 nM to 1  $\mu M$ ) against HGFA, Factor Xa, kallikrein 1 (KLK1), KLK5,  
115 KLK14, plasmin, and proteinase K and surprisingly against the cysteine protease cathepsin S with  
116 an  $IC_{50}$  of 590 nM. Furthermore, MM3122 also inhibited the other cysteine proteases cathepsin  
117 C, cathepsin L and papain, with  $IC_{50}$ s of 1.4  $\mu M$ , 12.8  $\mu M$ , and 1.1  $\mu M$ . Comparing the selectivity  
118 profile of MM3122 to that of Camostat and Nafamostat reveals that the cysteine protease activity  
119 is absent in the latter. Otherwise, the profiles are generally similar with some exceptions, notably  
120 decreased activity of MM3122 against thrombin, plasmin, Factor VIIA and XIA, KLK12, KLK13  
121 and KLK14, urokinase, matriptase-2, trypsin and the 2 tryptases. We also found that MM3122  
122 does not inhibit furin or the SARS-CoV-2 proteases,  $M_{pro}$  and  $PL_{pro}$ .

123 **MM3122 ameliorates SARS-CoV-2 induced disease in mice.** To assess the antiviral activity  
124 of MM3122 *in vivo*, four groups of mice were treated with 50 mg/kg or 100 mg/kg MM3122 30  
125 minutes prior to (prophylactic) and 24 hours after (therapeutic) intranasal infection with the MA10  
126 strain of SARS-CoV-2. A mock infected and vehicle treated group were included as controls.

127 Intranasal inoculation of vehicle treated mice with SARS-CoV-2 resulted in significant weight loss  
128 compared to mock infected animals (**Fig 2A**). Importantly, none of the MM3122 groups lost any  
129 significant amount of body weight. Five days after infection, lung congestion was assessed using  
130 an independently developed scoring system as described in the Methods. Vehicle-treated mice  
131 that were infected with SARS-CoV-2 MA10 demonstrated evidence of congestion with scores  
132 ranging from 1-3. These scores were reduced to 0 for infected mice that received 50 and 100  
133 mg/kg MM3122 prophylactically ( $P < 0.01$ ), and 0.5 for mice that received 50 and 100 mg/kg  
134 MM3122 therapeutically. Mice that received MM3122 prior to infection also had reduced virus  
135 titers (5000-10,000-fold) compared to vehicle treated and infected animals (**Fig 2B**). However,  
136 mice that received MM3122 24 h after infection had similar amounts of virus in the lungs compared  
137 to the vehicle treated animals. Finally, we performed pathological analysis of the lungs of MM3122  
138 and control treated mice. Global pneumonia, used to assess the % of lung affected, was between  
139 3 (>50%) and 4 (>80%) for the SARS-CoV-2 infected and vehicle treated mice. This score was  
140 reduced to 1.2 ( $P < 0.05$ ) and 1.6 in the mice that received 50 mg/kg and 100 mg/kg of MM3122  
141 prior to virus infection. No change in the global pneumonia score was observed for the mice that  
142 received 50 (score = 3) and 100 (score = 3.2) mg/kg of MM3122 after virus infection. Similarly,  
143 the bronchointerstitial pneumonia score was reduced in the animals that received MM3122 prior  
144 to infection (1.2 and 1.5 for 50 mg/kg ( $P < 0.05$ ) and 100 mg/kg respectively) but not after SARS-  
145 CoV-2 infection. Finally, vasculitis and endotheliitis replicated control baseline levels (average  
146 score = 0.3) in the prophylactically treated group (score = 0.4 and 0.3 for 50 mg/kg and 100 mg/kg  
147 respectively). A reduction in score was also observed for the mice that received MM3122 after  
148 infection (score = 1.2 and 1.8 for 50 mg/kg and 100 mg/kg respectively), but this was not  
149 statistically significant compared to the infected and vehicle control treated animals (score = 2.8).

150 **MM3122 reduces inflammatory cytokine and chemokine production after SARS-CoV-2**  
151 **infection.** Weight loss and severe disease after SARS-CoV-2 infection is associated with  
152 exacerbated inflammatory responses resulting in lung congestion and immunopathology. To

153 assess inflammation, cytokine and chemokine concentrations were quantified in lung tissue  
154 homogenates using a mouse cytokine 23-plex assay. Compared to uninfected and vehicle control  
155 animals, the levels of IL-6, KC, G-CSF, CCL2, CCL3, CCL4, IL1 alpha, IL-12p40 and CCL5 were  
156 increased three-fold or more five days after infection with the MA10 strain of SARS-CoV-2 (**Fig**  
157 **3**). Prophylactic and therapeutic treatment with MM3122 significantly ( $P < 0.001$ ) reduced the  
158 amount of IL-6, KC, G-CSF, CCL2 and CCL3 in the lungs of these mice. Smaller reductions in  
159 cytokine and chemokine productions were observed for CCL4, IL-1 alpha, IL-12p40, and CCL5.  
160 Combined, these data show that the TMPRSS2 inhibitor MM3122 reduces virus titers and  
161 inflammation after SARS-CoV-2 infection.

162

## 163 **DISCUSSION**

164 The COVID-19 pandemic began 4 years ago and is still a major economic catastrophe and  
165 medical problem worldwide costing over \$14 trillion in economic losses in the US<sup>45</sup> and resulting  
166 in excess mortality rates estimated at exceed 24 million people worldwide through early 2023<sup>46</sup>.  
167 While highly efficacious vaccines have saved millions of lives globally<sup>47</sup>, they are not 100%  
168 effective, even less so for variants, and a large percentage of the population refuses to be  
169 vaccinated so there is a dire need for new drugs to prevent and treat this life-threatening disease.  
170 There are some small molecule FDA-approved drugs to treat COVID-19<sup>1</sup> including the viral  
171 polymerase inhibitors remdesivir and molnupiravir, as well as SARS-CoV-2 Mpro protease  
172 inhibitor nirmatrelvir, sold under the brand name Paxlovid. With the exception immunomodulatory  
173 treatments for COVID-19 such as the JAK1/JAK2 inhibitor baricitinib<sup>48-50</sup>, which target the  
174 symptoms of infection and not viral pathogenesis directly, there are no approved antiviral drugs,  
175 which target the host and confer protection against multiple different viruses from diverse viral  
176 families. Concomitantly, others, and we have reported on the first potential drugs, targeting the  
177 TMPRSS2 and matriptase, to treat SARS-CoV-2 and COVID-19<sup>39, 42</sup>. TMPRSS2, and other host  
178 cell transmembrane proteases<sup>51</sup> including matriptase are essential for viral entry of many



179 respiratory RNA viruses into the lung tissue. In this communication, we have demonstrated that  
180 one lead candidate drug that we have developed, MM3122, exhibited significant protective effects  
181 against weight loss, lung congestion (gross lung discoloration), and inflammation administered  
182 prophylactically at both low and high doses, in aged mice infected with mouse-adapted SARS-  
183 CoV-2. These effects were less pronounced in the therapeutic groups. Interestingly, these  
184 protections did not fully correspond with protection from viral replication, as titers were lower or  
185 absent in the prophylactic group but were not significantly different from the infected vehicle  
186 control in the therapeutic group. These protective effects of COVID-19 in the lung can potentially  
187 be explained by inhibition of the proteolytic activation of other substrates by TMPRSS2,  
188 matriptase, and hepsin or the other proteases, which MM3122 targets such as tryptase (**Table 1**).  
189 In summary, these promising results suggest MM3122 is a potential clinical candidate for the  
190 treatment and prevention of diseases including COVID-19, caused from infection by SARS-CoV-  
191 2 and other coronaviruses.

192 **Limitations of the study.** We note several limitations of our study. (a) The *in vivo* efficacy of  
193 MM3122 was not tested against more recent variants of SARS-CoV-2 in mice or in Syrian  
194 hamsters. For the latter, we will need to perform PK studies, which is beyond the scope of this  
195 study. Also, the more recent variants of SARS-CoV-2 are attenuated compared to MA10 strain of  
196 SARS-CoV-2, creating challenges in observing effects on weight loss and disease of MM3122.  
197 Also, given that MM3122 has similar activity against EG.5.1 *in vitro*, we do not expect differences  
198 *in vivo*. (b) We did not evaluate the efficacy of MM3122 in male mice. Males are considered more  
199 susceptible to SARS-CoV-2 infection and therefore we expect more disease in the untreated  
200 animals with continued amelioration of disease in the MM3122 treated animals. (c) To increase  
201 efficacy, we are currently working on developing improved compounds with longer half-life and  
202 higher compound exposure compared to MM3122.<sup>39</sup> One potential way to achieve increased  
203 efficacy with MM3122 would be to develop formulations for inhalation or nebulization as  
204 alternative administration routes, which would deliver compound directly to the respiratory tract at



205 the primary site of viral entry and the infection. In summary, these studies demonstrate that  
206 MM3122 is effective in inhibiting SARS-CoV-2 replication *in vitro* and that administration of  
207 MM3122 *in vivo* reduces COVID-19 disease in mice.

208

## 209 **MATERIALS AND METHODS**

210 **Cells and Viruses.** Vero cells expressing human angiotensin converting enzyme 2 (ACE2)  
211 and transmembrane protease serine 2 (TMPRSS2) (Vero-hACE2-hTMPRSS2<sup>52, 53</sup>, gift from Adrian  
212 Creanga and Barney Graham, NIH) were cultured at 37°C in Dulbecco's Modified Eagle medium  
213 (DMEM) supplemented with 10% fetal bovine serum (FBS), 10 mM HEPES (pH 7.3), 100 U/mL of  
214 Penicillin, 100 µg/mL of Streptomycin, and 10 µg/mL of puromycin. Vero cells expressing  
215 TMPRSS2 (Vero-hTMPRSS2)<sup>53</sup> were cultured at 37°C in DMEM supplemented with 10% fetal  
216 bovine serum (FBS), 10 mM HEPES (pH 7.3), 100 U/mL of Penicillin, 100µg/mL of Streptomycin,  
217 and 5 µg/mL of blasticidin. Calu-3 cells were cultured in DMEM media supplemented with 1.0 mM  
218 sodium pyruvate, non-essential amino-acids (NEAA), 100 U/mL of penicillin, 100 µg/mL  
219 streptomycin, 2.0 mM L-glutamine, 10 mM HEPES, and 10% Fetal Bovine Serum (FBS).

220 The Lineage A variant of SARS-CoV-2 (WA1/2020), or the XBB.1.5 and E.G.5.1 (from Mehul  
221 Suthar) variants of SARS-CoV-2 were propagated on Vero-hTMPRSS2 cells. The virus stocks  
222 were subjected to next-generation sequencing, and the S protein sequences were identical to the  
223 original isolates. The infectious virus titer was determined by plaque and focus-forming assay on  
224 Vero-hACE2-hTMPRSS2 or Vero-hTMPRSS2 cells.

225 Baric laboratory-generated stock of SARS-CoV-2 MA10, a mouse-adapted virulent mutant  
226 generated from a recombinantly derived synthesized sequence of the Washington strain that  
227 causes severe acute and chronic disease in mice<sup>54, 55</sup>. Virus was maintained at low passage (P2-  
228 P3) to prevent the accumulation of additional potentially confounding mutations.

229 **Drug Preparation and Administration.** MM3122<sup>39</sup> was freshly prepared at 8 mg/mL in 5%  
230 DMSO in PBS. MM3122 was administered intraperitoneal (IP) at 50 and 100 mg/kg bodyweight in

231 100 $\mu$ L volumes to mice starting at 30 minutes before infection (prophylactic treatment) or 24 hours  
232 after infection (day 1, therapeutic treatment). Subsequent doses were administered at  
233 approximately the same times each day post-infection.

234 **SARS-CoV-2 challenge studies.** All studies with mice were conducted under the University  
235 of North Carolina IACUC approval (20-114). Aged (11- to 12-month-old) female BALB/c mice  
236 obtained from Envigo (retired breeders) were acclimated for 7 days in the Biosafety laboratory  
237 level 3 prior to any experimentation. Food and water were provided ad libitum, and the animal  
238 room maintained a 12-hour light/dark cycle. Prior to inoculation with SARS-CoV-2, animals were  
239 anesthetized intraperitoneal with a combination of 50 mg/kg Ketamine and 15 mg/kg Xylazine in  
240 50  $\mu$ L, and infected intranasally with 1,000 PFU of sequence- and titer-verified SARS-CoV-2 MA10  
241 in 50  $\mu$ L PBS. Mice were monitored daily for weight loss and disease. At 5 days post-infection,  
242 mice were euthanized following sedation by isoflurane and thoracotomy, and lungs were collected  
243 for assessments of virus titer, inflammatory cytokines and chemokine levels, histological analysis,  
244 and lung congestion score. Lung congestion score was measured using an independently defined  
245 scale of 0-4 (0: no congestion; 1: one lobe involved; 2: two lobes involved; 3: three lobes involved;  
246 4: all four lobes involved; all scores have 0.5-point intervals). Lung sections used in all experiments  
247 for various assessments were as follows: lower right lobe, histology; upper right lobe, RNA; left  
248 lobe and central lobe, virus titer. Prior to virus titration and cytokine analysis, the lung lobes were  
249 homogenized with glass beads in 1.0 mL PBS, clarified by centrifugation and stored at -80°C.  
250 Infectious virus titers were quantified by plaque assay on Vero E6 cells and calculated at PFU/mL  
251 of homogenized tissue.

252 **Cytokine Analysis.** Homogenized lung samples were subjected to cytokine and chemokine  
253 analysis using the Bio-Rad Mouse Cytokine 23-plex assay (Cat # M60009RDPD) per the  
254 manufacturer's protocol. Cytokine assays were performed in non-inactivated samples at BSL3.  
255 The data were analyzed on a Luminex MAGPIX machine, and cytokine concentrations in the lung  
256 homogenates were extrapolated using the provided standards.

257 **Histological analysis.** Lung tissues from the lower right lobe were fixed for a minimum of 7  
258 days in 10% formalin, paraffin embedded, sectioned, and stained with hematoxylin and eosin  
259 (H&E). H&E sections were submitted for graded blindly for vasculitis/endotheliitis,  
260 Bronchointerstitial pneumonia, and global pneumonia severity score by a board-certified veterinary  
261 pathologist. Details on the histology scoring system are provided in the Supplementary Material.

262 **MM3122 in vitro inhibition assays.** Calu-3 cells ( $5 \times 10^5$  cells/well) were seeded in 24-well  
263 culture plates in infection medium (DMEM + 1.0 mM Sodium pyruvate, NEAA, 100 U/mL of  
264 penicillin, 100  $\mu$ g/mL streptomycin, 2.0 mM L-glutamine, 10 mM HEPES, and 2% FBS) and  
265 incubated overnight at 37°C and 5% CO<sub>2</sub>. After 24 h, media was removed and fresh 250  $\mu$ L media  
266 was added to each well containing MM3122 or Remdesivir starting at 60  $\mu$ M concentration and  
267 diluted 3-fold to 20, 6, 2, 0.6 and 0.2  $\mu$ M. Media alone, and DMSO were included as negative  
268 controls. Next, the cells were transferred to the BSL3 laboratory and 250  $\mu$ L of media containing  
269 4,000 PFU of SARS-CoV-2 was added for 1 h at 37°C and 5% CO<sub>2</sub>. Note, that the final  
270 concentration of MM3122 and Remdesivir is 30, 10, 3, 1, 0.3, and 0.1  $\mu$ M. After 1 h, the virus  
271 inoculum was removed, the cells were washed twice with infection media and fresh infection media  
272 containing MM3122, Remdesivir or DMSO was added to each well. At 48 h post-infection, culture  
273 supernatant is collected and used to quantify virus titers by plaque assay as described below.

274 **Virus titration assays.** Plaque assays were performed on Vero-hACE2-hTRMPSS2 cells in  
275 24-well plates. Lung tissue homogenates or nasal washes were diluted serially by 10-fold, starting  
276 at 1:10, in cell infection medium (DMEM + 100 U/mL of penicillin, 100  $\mu$ g/mL streptomycin, and 2%  
277 FBS). Two hundred and fifty microliters of the diluted virus were added to a single well per dilution  
278 per sample. After 1 h at 37°C, the inoculum was aspirated, the cells were washed with PBS, and  
279 a 1% methylcellulose overlay in MEM supplemented with 2% FBS was added. Seventy-two to  
280 ninety-six hours after virus inoculation, dependent on the virus strain, the cells were fixed with 4%  
281 formalin and the monolayer was stained with crystal violet (0.5% w/v in 25% methanol in water)

282 for 30 min at 20°C. The number of plaques were counted and used to calculate the plaque forming  
283 units/mL (PFU/mL).

284

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293

## 294 **AUTHOR CONTRIBUTIONS**

295 T.L.B. performed the *in vitro* inhibition studies on Calu-3 cells. E.J.F., S.R.L., K.G., R.S.B,  
296 R.L.G. performed all the *in vivo* mouse studies with MM3122 and SARS-CoV-2. B.V.T performed  
297 the histology experiments. M.M. synthesized the MM3122 compound. T.L.B., A.C.M.B., R.L.G.,  
298 R.S.B., J.W.J. analyzed the data. A.C.M.B. performed the statistical analysis. A.C.M.B. had  
299 unrestricted access to all the data. A.C.M.B, J.W.J. provided key reagents, supervised  
300 experiments, and acquired funding. A.C.M.B. and J.W.J. wrote the manuscript and all authors  
301 reviewed and edited the final version. All authors agreed to submit the manuscript, read, and  
302 approved the final draft, and take full responsibility of its content.

303

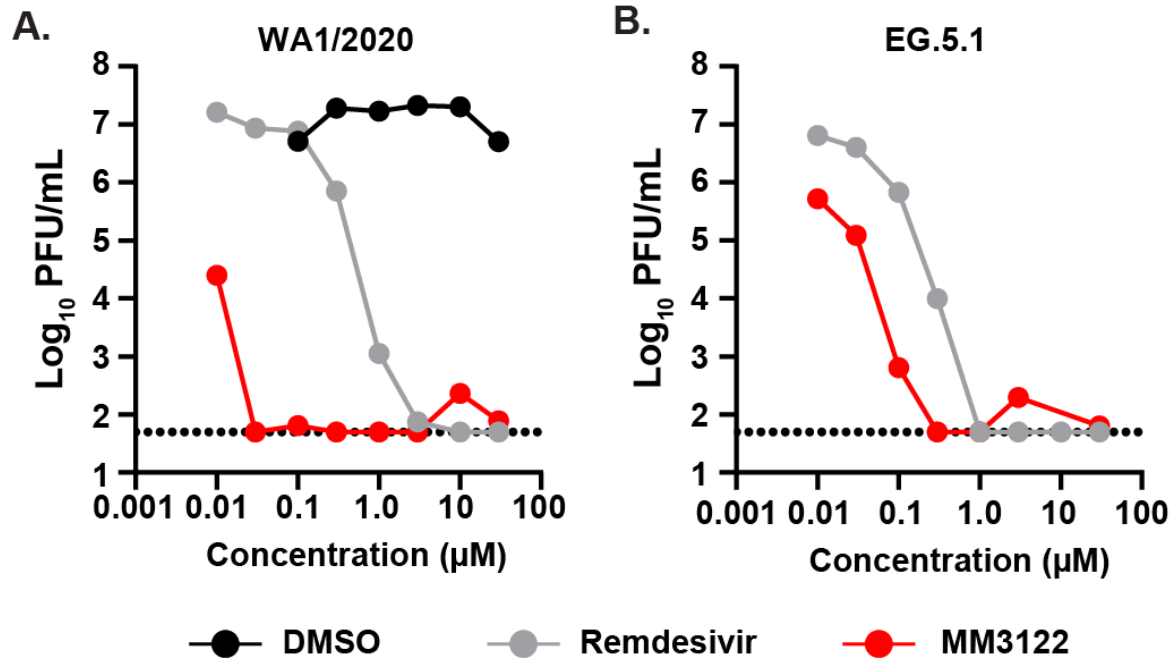
## 304 **DECLARATION OF INTERESTS**

305 The Boon laboratory has received unrelated funding support in sponsored research  
306 agreements from AI Therapeutics, GreenLight Biosciences Inc., Moderna Inc., and Nano targeting  
307 & Therapy Biopharma Inc. The Boon laboratory has received funding support from AbbVie Inc.,

308 for the commercial development of SARS-CoV-2 mAb. R.S.B. is a member of the advisory board  
309 of VaxArt and Invivyd and has collaborations with Takeda, Janssen Pharmaceuticals, Pfizer,  
310 Moderna, Ridgeback Biosciences, and Gilead that are unrelated to this work. J.W.J. has a patent  
311 application covering the MM3122 compound. R.S.B. and S.R.L. hold a patent on the MA10 strain  
312 of SARS-CoV-2.

313 **FIGURE AND FIGURE LEGENDS**

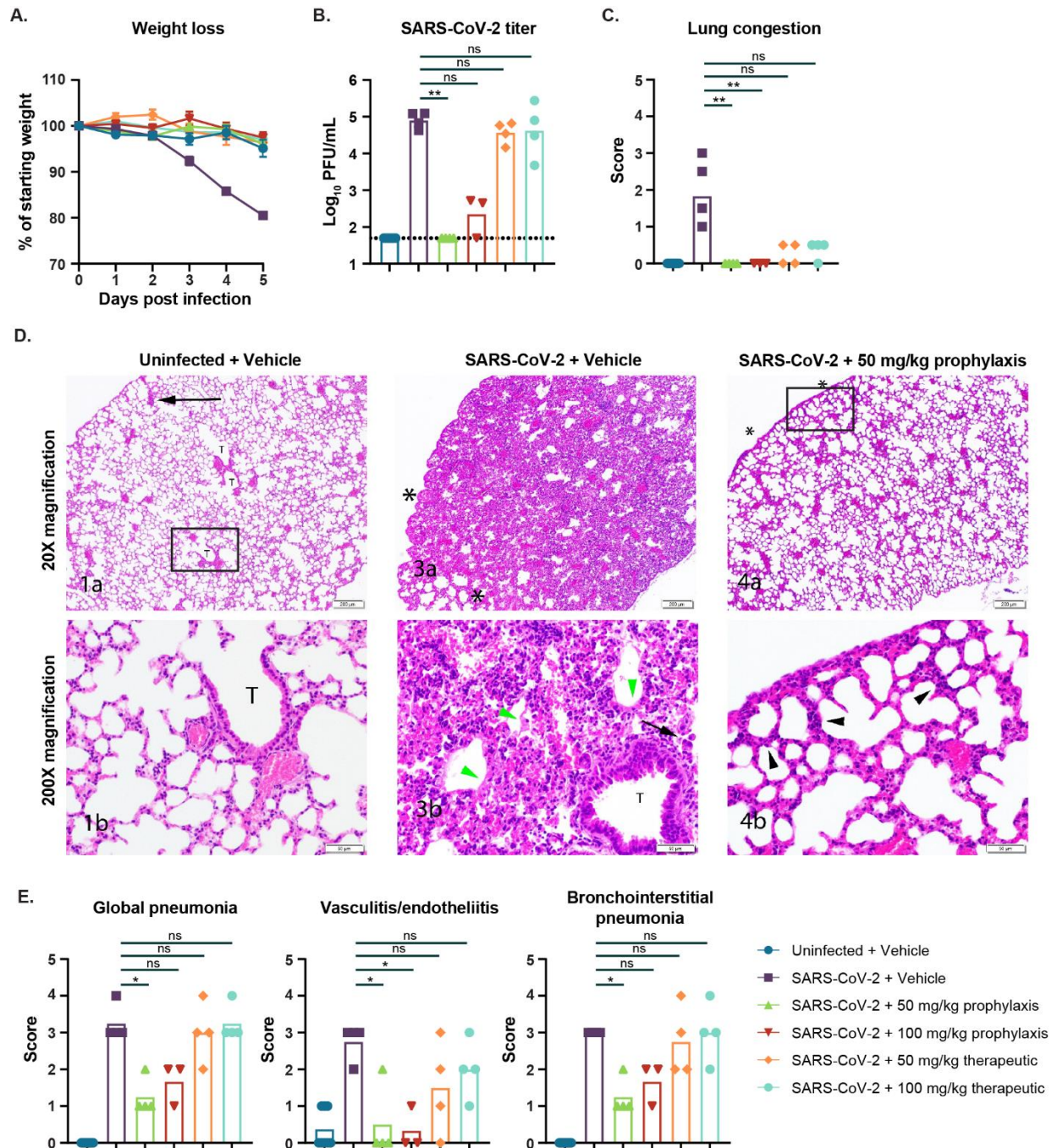
314



315

316 **Figure 1: The TMPRSS2 inhibitor MM3122 inhibits replication of authentic SARS-CoV-2 and**  
317 **variants.** Calu-3 cells, plated in 24-well plates, were infected with 4,000 PFU of A) WA1/2020  
318 and B) EG.5.1 strains of SARS-CoV-2 for one hour at 37°C. After two washes, the cells were  
319 incubated for 48 hours in media with different concentrations of MM3122 (red symbol), Remdesivir  
320 (grey symbols), or DMSO (black symbols) as the positive and negative controls respectively.  
321 Infectious virus titer in the supernatant of the wells 48 hours after infection with SARS-CoV-2. The  
322 results are the average of 3 independently repeated assays. The dotted line is the limit of  
323 detection.





324

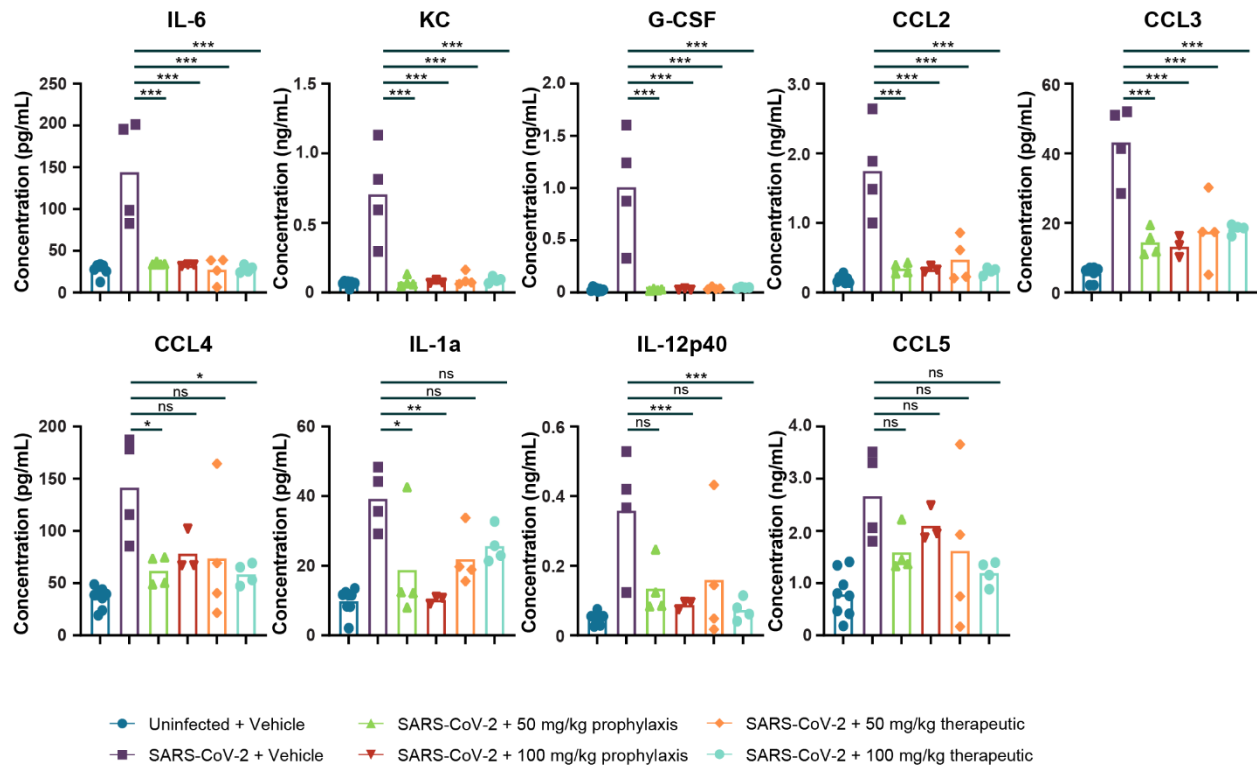
Figure 2

325 **Figure 2: MM3122 protects mice against SARS-CoV-2 disease in mice.** Eleven- to twelve-

326 month-old female mice received MM3122 30 minutes before and 24 hours after intranasal



327 inoculation with 1,000 PFU of MA10. **(A)** Weight loss was measured daily for five days. **(B)**  
328 Infectious virus titer in left lung lobe 5 days after infection. **(C)** Lung congestion score 5 days after  
329 infection. **(D)** Representative images and magnifications of H&E sections of lungs from uninfected  
330 and vehicle control treated mice (left panel), SARS-CoV-2 infected, and vehicle control treated  
331 mice (middle panels), and MM3122 treated and SARS-CoV-2 infected mice (right panels). **(E)**  
332 Global pneumonia, vasculitis and endotheliitis, and bronchointerstitial pathology scores of these  
333 same animals. All data was analyzed by a non-parametric one-way ANOVA (Kruskal-Wallis) with  
334 multiple comparisons corrects against the SARS-CoV-2 + vehicle group. The dotted line is the  
335 limit of detection. Each data point is an individual mouse, and the data are from a single  
336 experiment with 3-4 mice per group. \*\* =  $P < 0.01$ , ns = not significant.



337

Figure 3

338 **Figure 3: Prophylactic and therapeutic administration of MM3122 reduces inflammation in**  
339 **mice.** Eleven- to twelve-month-old female mice received MM3122 30 minutes before and 24  
340 hours after intranasal inoculation with 1,000 PFU of SARS-CoV-2 MA10. Cytokine and chemokine  
341 concentrations in left lung lobe homogenates collected from these same animals. Data was  
342 analyzed by a parametric ordinary one-way ANOVA with multiple comparisons corrected against  
343 the SARS-CoV-2 + vehicle group. Each data point is an individual mouse, and the data are from  
344 a single experiment with 3-4 mice per group. \* =  $P < 0.05$ , \*\* =  $P < 0.01$ , \*\*\* =  $P < 0.001$ , ns = not  
345 significant.

346 **Table 1: Protease selectivity profile of MM3122, Camostat and Nafamostat tested against**  
 347 **a panel of 53 serine and cysteine proteases.**

Protease	MM3122 IC <sub>50</sub> (M)	Camostat IC <sub>50</sub> (M)	Nafamostat IC <sub>50</sub> (M)	Protease	MM3122 IC <sub>50</sub> (M)	Camostat IC <sub>50</sub> (M)	Nafamostat IC <sub>50</sub> (M)
<b>Serine Proteases</b>				<b>Cysteine Proteases</b>			
HGFA	3.20E-08	>2.00E-05	1.58E-07	Cathepsin B	>2.00E-05	>2.00E-05	>2.00E-05
Matriptase	3.10E-10	7.00E-09	5.00E-11	Cathepsin C	1.393E-06	>2.00E-05	>2.00E-05
Hepsin	1.90E-10	7.00E-09	9.00E-10	Cathepsin H	>2.00E-05	>2.00E-05	>2.00E-05
Factor Xa	7.00E-07	>2.00E-05	4.57E-06	Cathepsin K	>2.00E-05	>2.00E-05	>2.00E-05
Thrombin	>2.00E-05	>2.00E-05	5.02E-06	Cathepsin L	1.28E-05	>2.00E-05	>2.00E-05
TMPRSS2	3.40E-10	1.50E-08	1.40E-10	Cathepsin S	5.857E-07	>2.00E-05	>2.00E-05
FVIIa	1.21E-05	3.55E-06	2.75E-07	Cathepsin V	>2.00E-05	>2.00E-05	>2.00E-05
FXa	1.08E-07	9.91E-06	1.11E-06	SARS-CoV-2-Mpro	>2.00E-05	ND	ND
FXIa	1.76E-06	3.46E-09	8.56E-10	SARS-CoV-2-Plpro	>2.00E-05	ND	ND
Kallikrein 1	1.29E-07	>1.00E-05	2.39E-06	Papain	1.13E-06	>2.00E-05	>2.00E-05
Kallikrein 5	6.81E-07	1.01E-06	6.37E-07	Calpain 1	>2.00E-05	>2.00E-05	>2.00E-05
Kallikrein 7	>2.00E-05	>2.00E-05	>2.00E-05	Caspase 1	>2.00E-05	>2.00E-05	>2.00E-05
Kallikrein 12	>2.00E-05	1.31E-06	3.59E-07	Caspase 2	>2.00E-05	>2.00E-05	>2.00E-05
Kallikrein 13	1.14E-05	8.45E-07	3.02E-07	Caspase 3	>2.00E-05	>2.00E-05	>2.00E-05
Kallikrein 14	6.09E-07	9.99E-07	2.15E-09	Caspase 4	>2.00E-05	>2.00E-05	>2.00E-05
Matriptase-2	2.03E-09	7.80E-09	<5.08E-10	Caspase 5	>2.00E-05	>2.00E-05	>2.00E-05
Plasma Kallikrein	8.06E-09	8.36E-10	<5.08E-10	Caspase 6	>2.00E-05	>2.00E-05	>2.00E-05
Plasmin	7.40E-08	5.62E-09	1.04E-09	Caspase 7	>2.00E-05	>2.00E-05	>2.00E-05
Proteinase A	1.45E-06	>2.00E-05	>2.00E-05	Caspase 8	>2.00E-05	>2.00E-05	>2.00E-05
Proteinase K	1.13E-07	>2.00E-05	>2.00E-05	Caspase 9	>2.00E-05	>2.00E-05	>2.00E-05
Trypsin	<1.02E-09	5.24E-10	<5.08E-10	Caspase 10	>2.00E-05	>2.00E-05	>2.00E-05
Chymotrypsin	>2.00E-05	>2.00E-05	>2.00E-05	Caspase 11	>2.00E-05	>2.00E-05	>2.00E-05
Tryptase b2	3.24E-09	<5.08E-10	<5.08E-10	Caspase 14	>2.00E-05	>2.00E-05	>2.00E-05
Tryptase g1	4.30E-09	<5.08E-10	<5.08E-10				
Urokinase	1.29E-05	1.64E-08	<5.08E-10				
Elastase	>2.00E-05	>2.00E-05	>2.00E-05				
Chymase	>2.00E-05	>2.00E-05	>2.00E-05				
Furin	>2.00E-05	ND	ND				

348

349 Green is IC<sub>50</sub> from 0.05 to 10 nM, blue is 10 nM to 1 μM, and yellow is 1 to 100 μM.

350 **SUPPLEMENTARY INFORMATION**

351 **Scoring criteria for Global pneumonia severity.**

<b>Grade</b>	<b>Criteria</b>
Normal = 0	The lung is considered to be within normal limits, under the conditions of the study and considering the age, sex, and strain of the animal
Minimal = 1	≤ 25% of lung affected
Mild = 2	26-50% of lung affected
Moderate = 3	51-79% of lung affected
Marked = 4	≥ 80% of lung affected

352

353

354 **Scoring criteria for Vasculitis / Endotheliitis.**

<b>Grade</b>	<b>Criteria</b>
Normal = 0	No significant microscopic changes present above background lesions if any.
Minimal = 1	Multifocal, scattered, individual infiltrates of inflammatory cells are present along the surface, below the endothelium, or within the tunica media or tunica adventitia of rare vessels.
Mild = 2	Infiltrates of small numbers of individual to occasional focal aggregates of inflammatory cells are present along the surface, below the endothelium, or within the tunica media or tunica adventitia of multiple vessels. There is rare leukocytoclastic vasculitis.
Moderate = 3	Infiltrates of variable numbers of inflammatory cells are present along the surface, below the endothelium, or within the tunica media or tunica adventitia of the majority of vessels. Focal aggregates are common within the endothelium narrowing the lumen and there is occasional leukocytoclastic vasculitis.
Marked = 4	Infiltrates of focally large numbers of inflammatory cells are present along the surface, below the endothelium, or within the tunica media or tunica adventitia of almost all vessels. Large aggregates are common within the endothelium narrowing the lumen and there is common leukocytoclastic vasculitis.

355

356 **Scoring criteria for Bronchointerstitial pneumonia.**

<b>Grade</b>	<b>Criteria</b>
Normal = 0	No significant microscopic changes present above background lesions, if any.
Minimal = 1	Affected alveolar septa are visibly but minimally thickened and there are only small amounts of hyaline membrane formation, edema, fibrin deposition, or hemorrhage within bronchioles and alveolar airways, with little loss of total airway space.
Mild = 2	Affected alveolar septa are mildly thickened and hyaline membrane formation, edema, fibrin deposition, or hemorrhage are commonly observed within alveolar spaces and bronchioles. Focal areas of atelectasis and consolidation may be present.
Moderate = 3	Infiltrates of inflammatory cells are present within the septa and spaces of most alveoli. Affected alveolar septa are moderately thickened, and hyaline membrane formation, edema, fibrin deposition, or hemorrhage are frequently observed within alveolar spaces or bronchioles along with prominent loss of airspace. Multifocal and regional areas of atelectasis and consolidation are present.
Marked = 4	Infiltrates of large numbers of inflammatory cells are present within the septa and spaces of most alveoli. Affected alveolar septa are markedly thickened, and hyaline membrane formation, edema, fibrin deposition, or hemorrhage within alveolar airways and bronchioles are frequently observed. Large areas of atelectasis and consolidation are present.

357

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