

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24

Dual inhibition of vacuolar ATPase and TMPRSS2 is required for complete blockade of SARS-CoV-2 entry into cells

Simoun Icho^{1,2}, Edurne Rujas^{1,3,4,5}, Krithika Muthuraman^{1,2}, John Tam¹, Huazhu Liang^{1,2}, Shelby Harms⁶, Mingmin Liao⁶, Darryl Falzarano⁶, Jean-Philippe Julien^{1,2,7}, Roman A. Melnyk^{1,2,*}

¹Molecular Medicine Program, The Hospital for Sick Children Research Institute, 686 Bay Street Toronto, ON, Canada, M5G 0A4; ²Department of Biochemistry, University of Toronto, 1 King’s College Circle Toronto, ON, Canada, M5S 1A8; ³Pharmacokinetic, Nanotechnology and Gene Therapy Group, Faculty of Pharmacy, University of the Basque Country UPV/EHU, 01006 Vitoria, Spain; ⁴Ikerbasque, Basque Foundation for Science, 48013 Bilbao, Spain; ⁵Biofisika Institute (CSIC, UPV/EHU), 48080 Bilbao, Spain; ⁶Vaccine and Infectious Disease Organization, University of Saskatchewan, 120 Veterinary Road Saskatoon, SK, Canada, S7N 5E3; ⁷Department of Immunology, University of Toronto, 1 King’s College Circle Toronto, ON, Canada, M5S 1A8.

*Corresponding author
Roman A. Melnyk
Associate Professor of Biochemistry and Senior Scientist
University of Toronto and The Hospital for Sick Children,
686 Bay Street, Toronto, ON M5G 0A4
416-813-7654 x328557
roman.melnyk@sickkids.ca

25 **Abstract**

26 An essential step in the infection life cycle of the severe acute respiratory syndrome
27 coronavirus 2 (SARS-CoV-2) is the proteolytic activation of the viral spike (S) protein, which
28 enables membrane fusion and entry into the host cell. Two distinct classes of host proteases have
29 been implicated in the S protein activation step: cell-surface serine proteases, such as the cell-
30 surface transmembrane protease, serine 2 (TMPRSS2), and endosomal cathepsins, leading to
31 entry through either the cell-surface route or the endosomal route, respectively. In cells
32 expressing TMPRSS2, inhibiting endosomal proteases using non-specific cathepsin inhibitors
33 such as E64d or lysosomotropic compounds such as hydroxychloroquine fails to prevent viral
34 entry, suggesting that the endosomal route of entry is unimportant; however, mechanism-based
35 toxicities and poor efficacy of these compounds confound our understanding of the importance
36 of the endosomal route of entry. Here, to identify better pharmacological agents to elucidate the
37 role of the endosomal route of entry, we profiled a panel of molecules identified through a high
38 throughput screen that inhibit endosomal pH and/or maturation through different mechanisms.
39 Among the three distinct classes of inhibitors, we found that inhibiting vacuolar-ATPase using
40 the macrolide bafilomycin A1 was the only agent able to potently block viral entry without
41 associated cellular toxicity. Using both pseudotyped and authentic virus, we showed that
42 bafilomycin A1 inhibits SARS-CoV-2 infection both in the absence and presence of TMPRSS2.
43 Moreover, synergy was observed upon combining bafilomycin A1 with Camostat, a TMPRSS2
44 inhibitor, in neutralizing SARS-CoV-2 entry into TMPRSS2-expressing cells. Overall, this study
45 highlights the importance of the endosomal route of entry for SARS-CoV-2 and provides a
46 rationale for the generation of successful intervention strategies against this virus that combine
47 inhibitors of both entry pathways.

48 **Introduction**

49 In December 2019 a novel coronavirus, severe acute respiratory syndrome coronavirus 2
50 (SARS-CoV-2), emerged causing a global public health crisis [1-4]. The virus quickly spread
51 across the world infecting, as of February 2022, over 430 million people across 220 countries
52 and territories and leading to more than 5.9 million deaths [5]. In early 2021, several vaccines
53 were granted Emergency Use Authorization or approval by the U.S. Food and Drug
54 Administration for their ability to induce an immune response and decrease SARS-CoV-2
55 infection. However, the emergence of several variants of concern with an altered antigenic
56 profile, a higher infectivity, and a greater likelihood of death threaten the protective efficacy of
57 current vaccines [6-9]. In fact, several studies have already shown a reduction in neutralization
58 potency against these variants by convalescent serum and most monoclonal antibody therapies
59 [10, 11]. Hence, antiviral drugs are urgently needed to provide alternative therapeutic options but
60 also as a safeguard against the emergence of new variants that are poorly managed by current
61 vaccines.

62 The life cycle of SARS-CoV-2 provides numerous potential avenues for therapeutic
63 intervention. Pfizer's Paxlovid and Merck's molnupiravir are two remarkable examples of
64 antivirals that effectively stop viral replication by targeting viral proteins and significantly
65 reducing the risk of hospitalization or death [12, 13]. The SARS-CoV-2 replication cycle starts
66 with the spike (S) protein binding to the cell-surface receptor angiotensin converting enzyme 2
67 (ACE2) [14, 15]. Then, a cell-surface transmembrane protease, serine 2 (TMPRSS2) cleaves the
68 S protein at the S1/S2 junction and activates the S2 domain [16-19]. The activated S2 domain
69 brings the viral membrane into close proximity to the cell membrane and initiates membrane
70 fusion, which ultimately leads to the release of the viral genome into the host cell [20].
71 Alternatively, upon ACE2 attachment the virus is endocytosed into endosomes where low-pH-
72 activated proteases such as cathepsin B and cathepsin L activate the S2 domain, which similarly
73 results in viral genome release but in this case through endosomal membrane fusion [21]. Once
74 the viral genome enters the host cytosol it begins propagating [20]. Therefore, targeting any of
75 these steps within the life cycle of SARS-CoV-2 would lead to neutralization of the virus.

76 Even though TMPRSS2 is expressed on lung and intestinal epithelial cells and, to a lesser
77 extent, in the kidney, heart, adipose, and reproductive tissues, it is not found in other cells
78 susceptible to SARS-CoV-2 infection [22, 23]. Several studies have shown that inhibition of

79 TMPRSS2 activity negatively affects the capacity of SARS-CoV-2 to infect host cells. However,
80 the reported level of attenuation achieved by antivirals targeting TMPRSS2 vary depending on
81 which cell line is used or which variant of SARS-CoV-2 is being tested [16, 24]. More
82 specifically, in the absence of TMPRSS2 expression or in the case of the Omicron variant,
83 antivirals targeting TMPRSS2 are ineffective [16, 25]. Further, Gunst *et al.* demonstrated in a
84 randomised double-blind clinical trial that Camostat, a specific TMPRSS2 inhibitor, did not
85 reduce the time for clinical improvement, progression to intensive care unit admission, or
86 mortality [26]. Therefore, it is unlikely that a monotherapy strategy based on the use of antiviral
87 drugs solely targeting TMPRSS2 will effectively stop viral replication.

88 Modulating endosomal pH using small molecules represents another attractive strategy
89 that may be employed against SARS-CoV-2. The acidification of endosomes by vacuolar
90 ATPases (V-ATPases) is required for activation of cathepsin proteases, which are ubiquitous
91 across all cells [27, 28]. Although cathepsins represent attractive targets in SARS-CoV-2
92 infection, potent and/or specific inhibitors of cathepsin B or cathepsin L proteases are not
93 available [17-19]. Furthermore, coronaviruses have been shown to utilize endosomal proteases
94 other than cathepsins for viral activity [29, 30]. Therefore, inhibiting endosomal acidification
95 may represent an optimal strategy to fully neutralize SARS-CoV-2 infection. In fact, recent
96 studies have shown that inhibition of endosomal acidification by using chloroquine or
97 hydroxychloroquine is effective at neutralizing SARS-CoV-2 infection [31]. Yet, the data cannot
98 be replicated in TMPRSS2-expressing cells or translated to the clinic [32-34]. Since both
99 chloroquine and hydroxychloroquine are cationic amphiphilic compounds capable of inducing
100 phospholipidosis, it is unclear which mechanism is operant when neutralizing SARS-CoV-2
101 infections *in vitro* [35]. By specifically targeting V-ATPases, it may be possible both to
102 neutralize SARS-CoV-2 infection and mitigate off-target effects.

103 In this study, we set out to investigate the importance of the endosomal pathway for
104 SARS-CoV-2 entry using a collection of small molecule inhibitors of endosomal acidification
105 identified from a high throughput screen. We employed inhibitors using three distinct
106 mechanisms: (1) Lysosomotropic compounds, (2) Proton shuttle compounds, and (3) A direct V-
107 ATPase inhibitor. Our results indicate that widely used non-specific inhibitors of endosomal
108 acidification are toxic at concentrations at which they neutralize the pH, which both confounds
109 interpretation of the mechanism of SARS-CoV-2 inhibition and further portends clinical

110 complications. In contrast, we found bafilomycin A1 to be uniquely potent and safe at inhibiting
111 endosomal acidification and used it to investigate how infection by different variants of SARS-
112 CoV-2 can be blocked by a safe and potent host-targeted mechanism inhibitor. Further, the
113 combination of bafilomycin A1 and Camostat completely and synergistically neutralized SARS-
114 CoV-2 entry into TMPRSS2-expressing cells without confounding cytotoxicity, opening the
115 possibility of a new therapeutic modality that could be both effective and tolerable.

116 **Results**

117 **Inhibition of endosomal acidification blocks viral entry**

118 To address the importance of endosomal route of entry for SARS-CoV-2 and identify
119 diverse inhibitors of this pathway we developed a robust fluorescence-based high throughput
120 assay endosomal acidification (Supplemental Figure 1A). We screened the MicroSource
121 Spectrum collection of small molecules consisting of 2,360 approved drugs and
122 pharmacologically active molecules with known targets and properties. The top 29 compounds
123 (by percent inhibition) were initially selected for follow-up and tested across a 10-point dose
124 titration for both inhibition of endosomal acidification and compound-mediated toxicity at 24
125 hours (Supplemental Figure 2A). Among confirmed active compounds that dose dependently
126 inhibited endosomal acidification, in all cases there was overlapping cellular toxicity seen at
127 equivalent doses (Supplemental Figure 2B). To explore this further and determine whether this
128 was a general feature of inhibitors of endosomal acidification, we selected a subset of
129 compounds representing three distinct mechanisms of endosomal deacidification: (1)
130 Lysosomotropic compounds amodiaquine, chloroquine, and quinacrine; (2) Proton shuttle
131 compounds niclosamide and oxyclozanide; and (3) the direct V-ATPase inhibitor bafilomycin
132 A1 (**Figure 1A**). Each of the six compounds were tested for their ability to prevent endosomal
133 acidification, viral entry, and cell viability. Antiviral activity was assessed initially using a
134 pseudotyped virus approach in which the SARS-CoV-2 S protein was pseudotyped onto a human
135 immunodeficiency virus-1 (HIV-1) backbone along with a luciferase reporter gene to measure
136 viral infection (**Figure 1B**) [36]. Inhibition of the pseudoviral particle (PsV) by each compound
137 was tested on HeLa cells expressing ACE2 (HeLa-ACE2). In parallel, each compound was also
138 tested for its ability to inhibit endosomal acidification and its compound-mediated toxicity on
139 HeLa-ACE2 cells over the same dose ranges.

140 A reduction in cell infection titers as well as inhibition of endosomal acidification was
141 observed with the three lysosomotropic compounds at a similar concentration range (**Figure 1C**).
142 Specifically, the half-maximal inhibitory concentration (IC_{50}) obtained for amodiaquine,
143 chloroquine, and quinacrine was 2.5, 2.6, and 0.9 μ M, respectively. However, cell viability
144 significantly decreased as cell infection titers was reduced. The half-cytotoxic concentration
145 (CC_{50}) measured for amodiaquine, chloroquine, and quinacrine was 34.7, 25.7, and 1.7 μ M,
146 respectively, demonstrating a narrow window in which the compounds can be efficacious against

147 PsVs without causing toxicity. Similarly, incubation of cells with niclosamide, a compound
148 which shuttles protons out of acidified vesicles, at concentrations higher than 1 μM resulted in
149 no measurable infection and efficient endosomal acidification inhibition. However, at those
150 concentrations, cell viability was severely affected (CC_{50} of 0.4 μM). Oxyclozanide, on the other
151 hand, did not alter infection levels nor endosomal acidification at concentrations up to 40 μM but
152 was toxic to cells with a CC_{50} of 18.7 μM (**Figure 1C**). Uniquely, Bafilomycin A1 potently
153 neutralized PsV entry into HeLa-ACE2 cells with an IC_{50} of 0.4 nM and inhibited endosomal
154 acidification with an IC_{50} of 0.9 nM without displaying any associated cell cytotoxicity (**Figure**
155 **1C**). These findings highlight fundamental differences among inhibitors of endosomal
156 acidification on compound potency and compound-mediated cellular toxicity.

157

158 **Inhibition of either the cell-surface or endosomal entry mechanism attenuates viral entry**

159 In view of its uniquely potent and safe viral inhibition profile, we next sought to use
160 bafilomycin A1 to investigate the relative importance of endosomal activation relative to cell-
161 surface activation [37]. A recent report by Hoffmann *et al.* concluded that endosomal
162 acidification inhibitors, such as chloroquine, are incapable of neutralizing viral entry into cells
163 expressing TMPRSS2 calling into question the importance of the endosomal pathway for viral
164 propagation [32]. For this purpose, we assessed the capacity of bafilomycin A1, alongside the
165 control compound Camostat, to neutralize PsVs on cells expressing ACE2 and TMPRSS2 (Vero-
166 TMPRSS2) and cells expressing ACE2 but lacking TMPRSS2 (HeLa-ACE2) (**Figure 2A**).
167 Consistent with previous reports, Camostat inhibited PsV entry into Vero-TMPRSS2 cells (IC_{50}
168 of 252 nM) but was unable to inhibit PsV entry into HeLa-ACE2 cells lacking TMPRSS2 [32].
169 By contrast, bafilomycin A1 potently inhibited PsV entry into HeLa-ACE2 cells with an IC_{50} of
170 0.4 nM and retained its neutralization potency when tested on Vero-TMPRSS2. In fact,
171 bafilomycin A1 was 26-fold more potent than Camostat in stopping viral entry despite the
172 presence of TMPRSS2. These findings are contrary from those reported previously for
173 lysosomotropic molecules, which are suggested to be ineffective in TMPRSS2-positive cells,
174 further highlighting the differences between endosomal inhibitors with distinct mechanisms of
175 action [32].

176 To demonstrate that these observations were not cell-dependent, due to TMPRSS2
177 overexpression, or PsV-specific, we next assayed compound-induced neutralization in Vero

178 cells, which naturally lack TMPRSS2 expression, and Calu-3 cells, which endogenously express
179 TMPRSS2, using authentic SARS-CoV-2 viral particles. Importantly, the above results were
180 reproduced with authentic SARS-CoV-2 viral particles (**Figure 2B**). Consistent with what has
181 been reported previously, Camostat significantly reduced SARS-CoV-2 infection titers in
182 TMPRSS2-expressing Calu-3 cells but not in TMPRSS2-lacking Vero cells [32, 38].
183 Bafilomycin A1, on the other hand, significantly reduced SARS-CoV-2 infection titers in both
184 Vero and Calu-3 cells. Overall, while inhibition of either entry route effectively inhibits viral
185 infection, inhibition of endosomal acidification via V-ATPase inhibition alone is effective at
186 neutralizing SARS-CoV-2 entry irrespective of TMPRSS2 expression.

187

188 **Neutralization of SARS-CoV-2 Variants of Concern by Camostat and bafilomycin A1**

189 Next, we tested whether inhibition of SARS-CoV-2 cell entry by Camostat and
190 bafilomycin A1 is affected by the different variants of the S protein that have emerged, since
191 viral entry efficiency is different across different variants [39]. Specifically, we compared the
192 IC₅₀ values of the two compounds against pseudoviruses containing four distinct S proteins:
193 D614G (Wildtype) , B.1.1.7 (i.e., Alpha), B.1.351 (i.e., Beta), B.1.1.529 (i.e., Omicron).
194 Camostat inhibited the entry of wildtype, Alpha, and Beta variants into Vero-TMPRSS2 cells;
195 however, was unable to inhibit the entry of the Omicron variant into Vero-TMPRSS2 cells nor,
196 as expected, stop viral infection of any of the variants when tested on HeLa-ACE2 cells lacking
197 TMPRSS2 expression. These findings reinforce the notion that the relative importance of each
198 entry pathway differs among variants and that the Omicron variant relies more heavily on the
199 endosomal route for entry into cells [25]. Consistent with this, bafilomycin A1 inhibited viral
200 entry of all the variants, including the Omicron variant, in both HeLa-ACE2 cells and Vero-
201 TMPRSS2 cells (**Figure 3B**).

202

203 **Camostat and bafilomycin A1 synergistically inhibit SARS-CoV-2 entry**

204 Camostat has been shown to reach maximal blood concentrations of ~200 nM upon oral
205 dosing [24, 26]. Given the complete lack of protection seen for Camostat on cells lacking
206 TMPRSS2, and only partial protection seen in cells expressing TMPRSS2 (**Figure 2A**), it would
207 be predicted that Camostat would not provide complete blockade of viral entry *in vivo*. Previous
208 studies on the related virus SARS-CoV found that simultaneous inhibition of the endosomal- and

209 cell-surface-entry route was required for complete inhibition of viral entry [40]. Therefore, we
210 hypothesized that a combination of Camostat with bafilomycin A1 would inhibit both entry
211 pathways and thus lead to the complete inhibition of SARS-CoV-2 entry. To this end, we tested
212 two doses of bafilomycin A1 that inhibit viral entry (*viz.* 8.3 and 16.7 nM) in combination with a
213 range of Camostat doses covering a full dose titration on Vero-TMPRSS2 cells and measured
214 PsV entry (**Figure 4A**). Across all doses of Camostat, both doses of bafilomycin A1 significantly
215 increased the inhibition of PsV entry into TMPRSS2-expressing cells as compared to Camostat
216 alone.

217 The addition of 8.33 nM bafilomycin A1 to 104 nM of Camostat drastically improved
218 neutralization of SARS-CoV-2 beyond what would be expected if the two compounds inhibited
219 additively (**Figure 4B**). In view of these results, we explored whether the simultaneous inhibition
220 of the two entry mechanisms of SARS-CoV-2 using Camostat and bafilomycin A1 could
221 synergistically inhibit viral entry. To assess this and following a checkerboard assay format, we
222 employed the Zero Interaction Potency (ZIP) model which combines the Bliss independence
223 model and the Loewe additivity model [41]. The Bliss independence model assumes each
224 compound elicits its effect independently, while the Loewe additivity model assumes both
225 compounds are the same and thus the activity must be double when two compounds are
226 combined at equal ratios. Hence, combined, the ZIP model identifies potential synergy between
227 two compounds by comparing the change in potency of each compound with different
228 combinations. A ZIP synergy score >10 has been proposed as a threshold indicative of
229 synergistic inhibition by two compounds [41]. Using this analysis, we observed that a
230 combination of 104 nM of Camostat and either 2.08 nM, 4.16 nM, 8.33 nM, or 16.67 nM of
231 bafilomycin A1 had a synergy score of 23.2, 28.5, 34.7, and 15.6, respectively. At 8.33 nM
232 bafilomycin A1, the synergy score was >16 as the concentration of Camostat increased from 104
233 nM to 833 nM indicating that this combination is highly synergistic at inhibiting PsV entry into
234 Vero-TMPRSS2 cells (**Figure 4D**). In addition, the data shows that neither Camostat nor
235 bafilomycin A1 alone fully inhibited PsV entry even at the highest concentrations tested (3333
236 nM and 66.7 nM, respectively) but, when combined, the two compounds provided complete
237 inhibition (**Figure 4C**). Importantly, none of the combinations had a toxic effect on Vero-
238 TMPRSS2 cells (**Figure 4E**). Taken together, by simultaneously blocking without cytotoxicity

239 both entry mechanisms using Camostat and bafilomycin A1, SARS-CoV-2 is completely
240 inhibited in a synergistic manner from infecting mammalian cells.
241

242 **Discussion**

243 The prospect of novel SARS-CoV-2 variants of concern emerging that are more
244 transmissible and/or are resistant to the antibodies raised by natural infection, through
245 vaccination or developed as therapeutics underscores the need for developing antivirals acting
246 through distinct mechanisms [6-9]. Promising phase III clinical trial data from Pfizer's Paxlovid
247 and Merck's Molnupiravir have demonstrated the potential of small molecule therapeutics
248 against SARS-CoV-2 [12, 13]; however, it remains to be seen whether resistance against these
249 virus targeted compounds will emerge [42]. An alternative strategy to block the virus lifecycle is
250 to target the host factors and host mechanisms that are required for SARS-CoV-2 infection. This
251 strategy has the added benefit of being resilient to SARS-CoV-2 mutations since the likelihood
252 of drug resistance against a host-dependent mechanism is negligible without substantial changes
253 to the S protein structure and function [43]. Here, we focused on the major categories of host
254 proteases implicated in the two distinct viral entry routes used by SARS-CoV-2: (1) The cell-
255 surface route (mediated by TMPRSS2 found in some but not all cells), and, (2) the endosomal
256 route (primarily mediated by cathepsin B and cathepsin L, found in all cells) [28]. Because
257 endosomal cathepsins require the low pH of acidified endosomal compartments for activation, it
258 has been recognized that agents that raise the pH of endosomes can indirectly inhibit both
259 cathepsin B and cathepsin L [27].

260 In the present study, we screened a panel of prototypic inhibitors of endosomal
261 acidification that act through three distinct mechanisms to identify the most efficacious means to
262 inhibit viral entry across multiple cellular contexts. Given recent reports of confounding
263 toxicities for a subset of molecules that inhibit endosomal pH as part of their mechanism [35],
264 together with a lack of clinical efficacy seen for molecules like hydroxychloroquine (a
265 lysosomotropic molecule) [33, 34], we measured in parallel the extent to which each of the
266 molecules tested induced cellular toxicity. We found that both lysosomotropic compounds
267 (amodiaquine, chloroquine, and quinacrine) and a compound capable of shuttling protons out of
268 the endosome (niclosamide) were cytotoxic at doses perceived to be effective at neutralizing
269 viral entry. By contrast, we found that inhibition of endosomal acidification through a specific V-
270 ATPase inhibitor (bafilomycin A1) was safe and effective at neutralizing SARS-CoV-2 entry
271 into cells. The larger safety window of bafilomycin A1 minimally suggests that not all modes of
272 inhibiting endosomal acidification are equal but more importantly demonstrates that the

273 concomitant cytotoxicity of all other compounds confounds their further use to understand how
274 SARS-CoV-2 is neutralized. Importantly, in support of this mechanism being important for viral
275 entry, Daniloski *et al.*, and others, used CRISPR (clustered regulatory interspaced short
276 palindromic repeats) knockout screens to demonstrate that the disruption of the V-ATPase or
277 endosomal acidification pathway results in protection against SARS-CoV-2 infection of
278 mammalian cells [44, 45].

279 In contrast to Camostat, which only protects cells expressing TMPRSS2, we found that
280 bafilomycin A1 was active in all cell lines tested irrespective of TMPRSS2 expression. These
281 results emphasize the importance of the endosomal pathway in the cell entry of SARS-CoV-2, a
282 mechanism previously suggested not to be important in the presence of TMPRSS2 [32]. These
283 contradictory findings might be due, at least in part, to the cytotoxicity associated with
284 compounds such as chloroquine or hydroxychloroquine that have been previously used to
285 elucidate the importance of endosomal acidification for the entry of SARS-CoV-2. Indeed, their
286 compound-mediated toxicity and different modes of inhibiting SARS-CoV-2 propagation makes
287 it difficult to delineate the importance of endosomal acidification on viral entry [46-48]. Further,
288 recent work by Tummino *et al.* demonstrated that cationic amphiphilic compounds, such as
289 chloroquine, induce phospholipidosis at efficacious doses underscoring the need for careful
290 assessment of off-target toxicity of compounds with multiple modes of action [35]. Taken
291 together, safely and specifically inhibiting endosomal acidification using bafilomycin A1 blocks
292 viral entry into mammalian cells irrespective of TMPRSS2 expression.

293 A recent report has demonstrated a decreased reliance on TMPRSS2 and a concurrent
294 increased dependence on the endosomal route of entry by the Omicron variant, as compared to
295 other variants such as Delta [25]. This shift highlights both the importance of the endosomal
296 route of entry and the need for an antiviral drug strategy that will remain effective as SARS-
297 CoV-2 continues to mutate and evolve. In line with these findings, Camostat was able to inhibit
298 most of the tested variants with a similar efficacy as compared to the wildtype virus using Vero-
299 TMPRSS2 cells but was unable to inhibit viral entry of the Omicron variant. More importantly,
300 we found that bafilomycin A1 inhibited all tested variants with a similar efficacy as compared to
301 the wildtype pseudovirus, and did so irrespective of TMPRSS2 expression. Therefore, targeting
302 endosomal acidification specifically appears to be the strategy by which multiple variants of
303 concern are potentially inhibited.

304 Here, we show that a combination of Camostat and bafilomycin A1 fully inhibit viral
305 entry in TMPRSS2-expressing cells in a synergistic manner and without cytotoxicity. In a recent
306 clinical trial, Camostat was shown to be tolerable at oral doses which can achieve a blood
307 concentration of ~200 nM [26]. Unfortunately, Camostat on its own was shown to be ineffective
308 at protecting against SARS-CoV-2 infections and bafilomycin A1 has yet to be progressed to a
309 clinical trial as a single compound treatment. Our study identifies a new modality that should be
310 explored in future experiments where a cell-surface inhibitor and endosomal route inhibitor are
311 simultaneously used to protect against SARS-CoV-2 infections. Supporting this idea, Yuan *et al.*
312 demonstrate the power of using two inhibitors targeting different mechanisms of the SARS-CoV-
313 2 life cycle to synergistically inhibit SARS-CoV-2 infection *in vivo* [49]. Importantly, inhibition
314 of the endosomal route of entry, using bafilomycin A1, equally neutralized all variants tested.
315 Our results suggest that the use of such inhibitors targeted at host-dependent mechanisms may be
316 resilient against emerging variants of concern, and capable of broadly blocking viral entry into
317 mammalian cells

318 **Materials And Methods**

319

320 **High Throughput Screen of Endosomal Acidification**

321 Inhibition of endosomal acidification was measured using LysoTracker Red DND-99
322 (ThermoFisher). Vero cells (ATCC) were seeded in 96-well clear CellBind plates (Sigma
323 Aldrich) 24 hours prior to the experiment at a density of 40,000 cells/well in a 100 μ L volume of
324 complete DMEM supplemented with 10% inactive FBS and 1% of penicillin/streptomycin. On
325 the day of the experiment, the media was replaced with 100 μ L of serum free DMEM. A volume
326 of 0.4 μ L of each compound from the Spectrum Collection library (MicroSource), consisting of
327 2,560 individual compounds formatted as 10 mM solutions in DMSO, was incubate with the
328 cells at 37°C for 2 hours. Following this, 0.1 μ M final concentration of LysoTracker Red DND-
329 99 (ThermoFisher) was added to each well and incubated for 30 minutes at 37°C. The cell media
330 was then replaced with 100 μ L of FluoroBrite DMEM (ThermoFisher). Fluorescence at
331 excitation/emission 574/594 nm was measured using an Envision plate reader (Perkin Elmer).
332 Data was plotted using Prism 9 (GraphPad).

333

334 **Pseudovirus production and Neutralization assay**

335 HIV-1-derived viral particles were pseudotyped with full-length SARS-CoV-2 spike (S)
336 protein as described previously [50]. Briefly, plasmids expressing a lentiviral backbone encoding
337 the luciferase reporter gene (BEI NR52516), the HIV structural and regulatory proteins Tat (BEI
338 NR52518), Gag-pol (BEI NR52517) and Rev (BEI NR52519), and the full-length SARS-CoV-2
339 S protein were co-transfected into human kidney HEK293T cells (ATCC, CRL-3216) using the
340 BioT transfection reagent (Bioland Scientific) following the manufacturer's instructions. In order
341 to test the effect of the S protein mutation D614G that renders a more infectious virus [8], the
342 plasmid encoding the S protein containing the D614G mutation (kindly provided by D.R.
343 Burton; The Scripps Research Institute) was used instead of the plasmid encoding the wild-type
344 SARS-CoV-2 S protein. Similarly, the wild-type S plasmid was substituted with plasmids
345 codifying for the B.1.117 or B.1.351 SARS-CoV-2 S proteins (kindly provided by David Ho,
346 Columbia University) or B.1.1.529 (synthesized and cloned by GeneArt, LifeTechnologies into
347 pcDNA3.4 expression vector) to generate the corresponding SARS-CoV-2 pseudoviral particle
348 (PsV) variants. Transfected cells were incubated at 37°C and after 24 hours, 5 mM sodium

349 butyrate was added to the media. Cells were incubated for an additional 24-30 hours at 30°C,
350 after which PsVs were harvested, passed through 0.45 µm pore sterile filters, and finally
351 concentrated using a 100K Amicon Ultra 2.0 Centrifugal Filter Units (Merck Millipore Amicon).

352 Neutralization was determined in a single-cycle neutralization assay using HeLa cells
353 expressing ACE2 (HeLa-ACE2), kindly provided by D.R. Burton; The Scripps Research
354 Institute, and Vero E6 cells constitutively expressing the transmembrane protease, serine 2
355 (Vero-TMPRSS2), obtained from the Centre For AIDS Reagents (National Institute for
356 Biological Standards and Control) [51, 52]. Cells were seeded in 96-well clear CellBind plates
357 (Sigma Aldrich) 24 hours prior to the experiment at a density of 10,000 cells/well in a 100 µL
358 volume. On the day of the experiment, the sample compounds were serially diluted in complete
359 DMEM media (cDMEM^{2%}) that contained 2% inactive fetal bovine serum (FBS) and 50 µg/mL
360 of gentamicin. Media from the cell culture was replaced with 100 µL of fresh cDMEM^{2%} media
361 and 50 µL of the previously diluted sample compounds were added to each well and incubated
362 for 1 hour at 37°C. After incubation, 50 µL of PsVs was added to each well and incubated for 48-
363 60 hours in the presence of 10 µg/mL of polybrene (Sigma Aldrich). Unless stated otherwise,
364 PsV bearing the SARS-CoV-2 S protein (BEI NR52310) was used. PsV entry level was calculated
365 based on luminescence in relative light units (RLUs). For that, 130 µL of supernatant was
366 aspirated from each well to leave approximately 50 µL of media. 50 µL Britelite plus reagent
367 (PerkinElmer) was added to each condition and incubated for 2 minutes at room temperature.
368 100 µL volume was transferred to a 96-well white plate (Sigma-Aldrich) and luminescence was
369 read using a Synergy Neo2 Multi-Mode Assay Microplate Reader (Biotek Instruments). Data
370 was plotted and half-maximal inhibitory concentration (IC₅₀) values were calculated using Prism
371 9 (GraphPad).

372 In order to confirm that the reduced luminescence was not related to cell toxicity, HeLa-
373 ACE2 and Vero-TMPRSS2 cell viability upon incubation with serial dilutions of the sample
374 compounds were tested in parallel to the neutralization assay. Hence, following the above-
375 mentioned protocol, 10,000 cells/well of pre-seeded cells were co-cultured with the same serial
376 dilutions of the sample compounds at 37°C for 48-60 hours. Cell viability was monitored by
377 adding 50 µL of CellTiter-Glo reagent (Promega) to 200 µL of media containing cells. After a
378 10-minute incubation, 100 µL volume was transferred to a 96-well white plate (Sigma-Aldrich)
379 to measure luminescence using a Synergy Neo2 Multi-Mode Assay Microplate Reader (Biotek

380 Instruments). Data was plotted and half-cytotoxic concentration (CC_{50}) values were calculated
381 using Prims 9 (GraphPad).

382 Inhibition of endosomal acidification was measured using LysoTracker Red DND-99
383 (ThermoFisher). HeLa-ACE2 and Vero-TMPRSS2 cells were prepared following the above-
384 mentioned protocol, where 10,000 cells/well of pre-seeded cells were co-cultured with the same
385 serial dilutions of the sample compounds at 37°C for 2 hours. Following this, 0.1 μ M final
386 concentration of LysoTracker Red DND-99 (ThermoFisher) was added to each well and
387 incubated for 30 minutes at 37°C. The cell media was then replaced with 100 μ L of FluoroBrite
388 DMEM (ThermoFisher). Fluorescence was measured using a Synergy Neo2 Multi-Mode Assay
389 Microplate Reader (Biotek Instruments) while employing a Texas Red filter. Data was plotted
390 and IC_{50} values were calculated using Prism 9 (GraphPad).

391

392 **Authentic SARS-CoV-2 Neutralization and Titer Assay**

393 Authentic SARS-CoV-2 experiments were conducted using Vero'76 (ATCC, CRL-1587)
394 or Calu-3 (ATCC, HTB-55) cells. Cells were seeded and grown in complete DMEM media
395 (cDMEM) that contained 10% inactive FBS and 1% of penicillin/streptomycin overnight at 37°C
396 to approximately 90% confluence in 96-well plates. SARS-CoV-2 [SARS-CoV-
397 2/Canada/ON/VIDO-01/2020/Vero'76/p.2 (Seq. available at GISAID – EPI_ISL_425177)] virus
398 was diluted in complete DMEM supplemented with 2% inactive FBS and 1% of
399 penicillin/streptomycin to obtain a multiplicity of infection (MOI) of 0.1 (approximately 2000
400 $TCID_{50}$ /well). Two test compounds, Camostat and bafilomycin A1, were prepared to a 3-fold
401 dilution in DMEM supplemented with 2% inactive FBS. Cells were incubated with the test
402 compounds for 1 hour prior to infection at which point 50 μ L of viral inoculum was added to
403 each well. After 1 hour of incubation with the viral inoculum, the inoculum and compound
404 mixture were removed and fresh compounds were added to the plate. The plates were incubated
405 for 48 hours at 37°C. At 24 hours, plates were assessed for contamination. Media alone and cell-
406 alone control wells (without viral infection and without compound treatment) were used as
407 controls for virus replication.

408 The plate of infected cells that had been exposed to the test compounds was examined for
409 cytopathic effect (CPE) and cytotoxicity (if noticeable) under a microscope at 48 hours. At 48
410 hours, 100 μ L of supernatant from each well was transferred into 96-well rounded-bottom plates.

411 Viral titration by the median tissue culture infectious dose (TCID₅₀) assay of the supernatant was
412 carried out by a serial 7-fold dilution. These dilutions were used to infect pre-seeded cells, as
413 described for the initial infection. Cells were observed for CPE at 1, 3 and 5 days after the
414 infection. The Spearman-Kärber algorithm were used to calculate TCID₅₀ titers which were used
415 to calculate IC₅₀ values for each compound using Prims 9 (GraphPad).

416

417 **Pseudovirus Neutralization Synergy Assay**

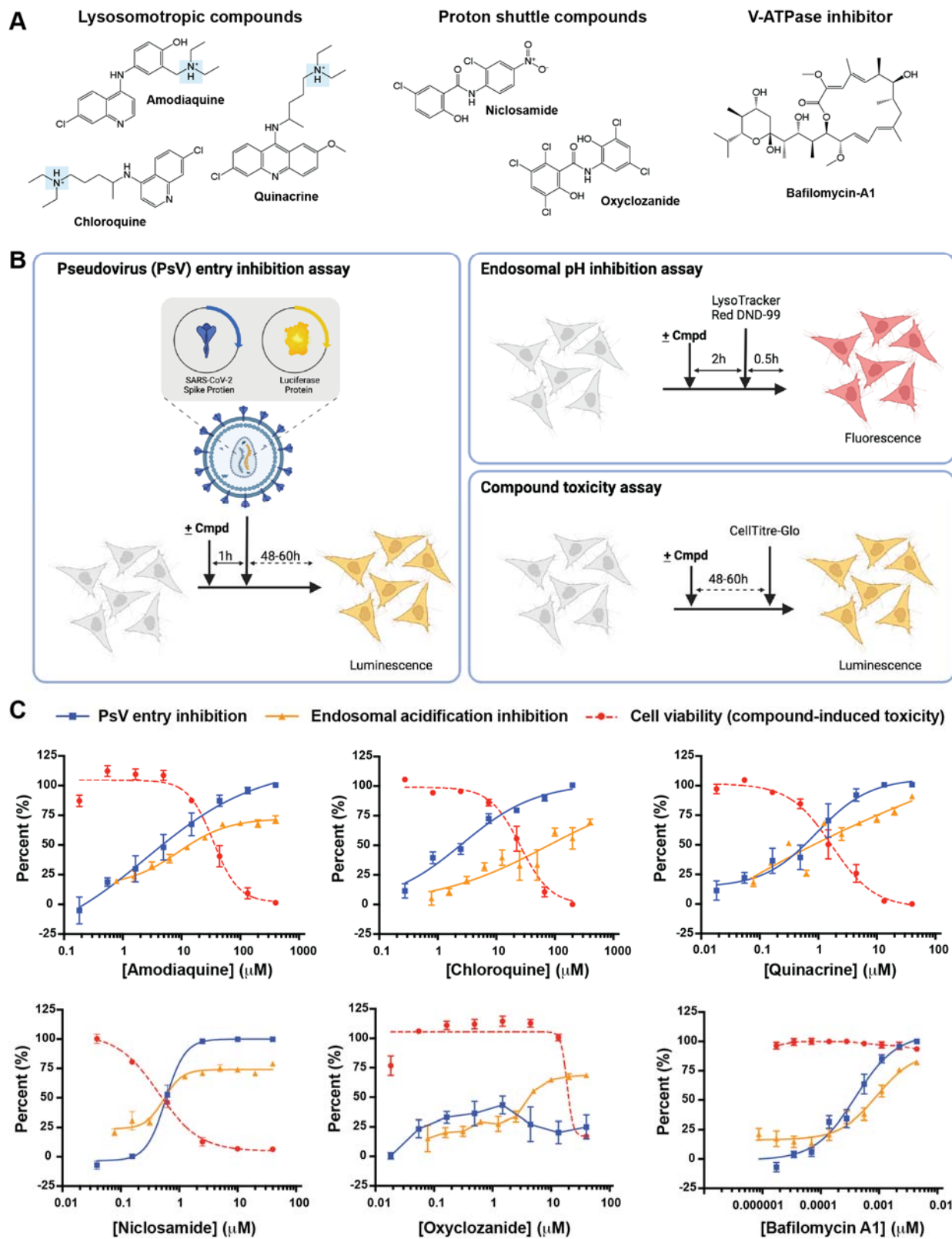
418 Synergistic neutralization by a combination of two compounds was defined as an increase
419 in the inhibition of PsV entry upon the combination of two different compounds in comparison
420 to the sum of the expected inhibition of both molecules or double the concentration of the best
421 molecule in the *in vitro* assay [41]. Hence, different ratios of the two compounds were mixed and
422 serial dilutions were prepared in a total volume of 50 µL for each condition using a checkerboard
423 assay. The assay was performed using Vero-TMPRSS2 cells following the above protocol. The
424 level of inhibition of each condition was calculated and compared to the expected values using
425 the Zero Interaction Potency (ZIP) synergy score model [41]. Synergy scores were assigned for
426 each condition and values above 10 were interpreted as a synergistic effect.

427 In order to confirm that a combination of two compounds did not also lead to increased cell
428 toxicity Vero-TMPRSS2 cell viability was measured using CellTiter-Glo reagent (Promega).
429 Vero-TMPRSS2 cells were seeded overnight at 10,000 cells/well in 96-well clear CellBind
430 plates (Sigma Aldrich). Media was exchanged with 100 µL of serum-free DMEM containing 1%
431 penicillin-streptomycin. An Agilent Bravo liquid handler was used to deliver 0.27 µL of
432 bafilomycin A1 then 0.27 µL of Camostat to the cell plates. The cell plates were incubated at
433 37°C for 48-60 hours before 50 µL of CellTiter-Glo reagent (Promega) was added. Cell plates
434 were gently mixed at room temperature for 10 minutes then 100 µL of the mixture was
435 transferred to 96-well white plates to measure luminescence using a SpectraMax M5 (Molecular
436 Devices). Data was plotted using Prism 9 (GraphPad).

437

438
439

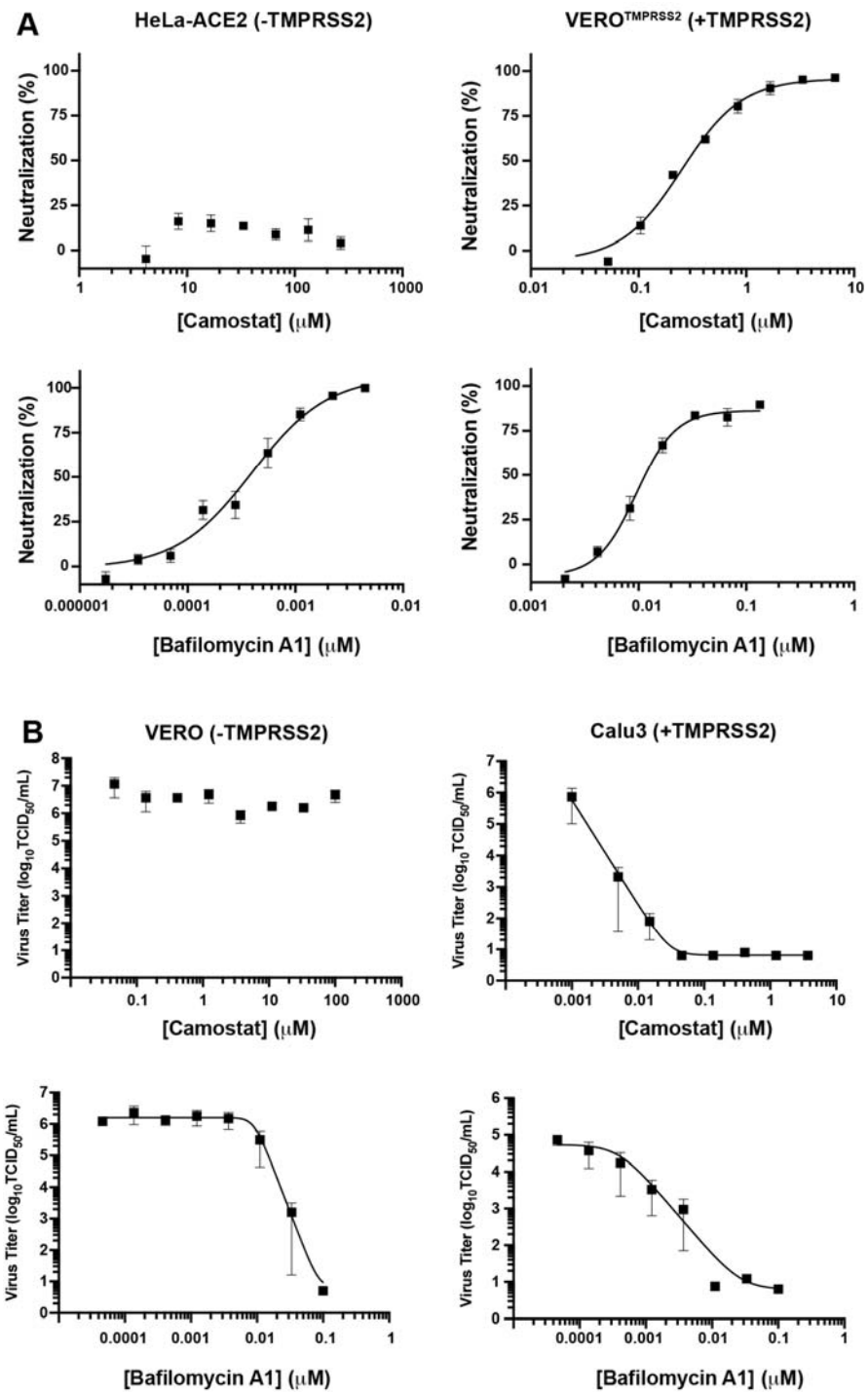
Figure 1



440
441

442
443

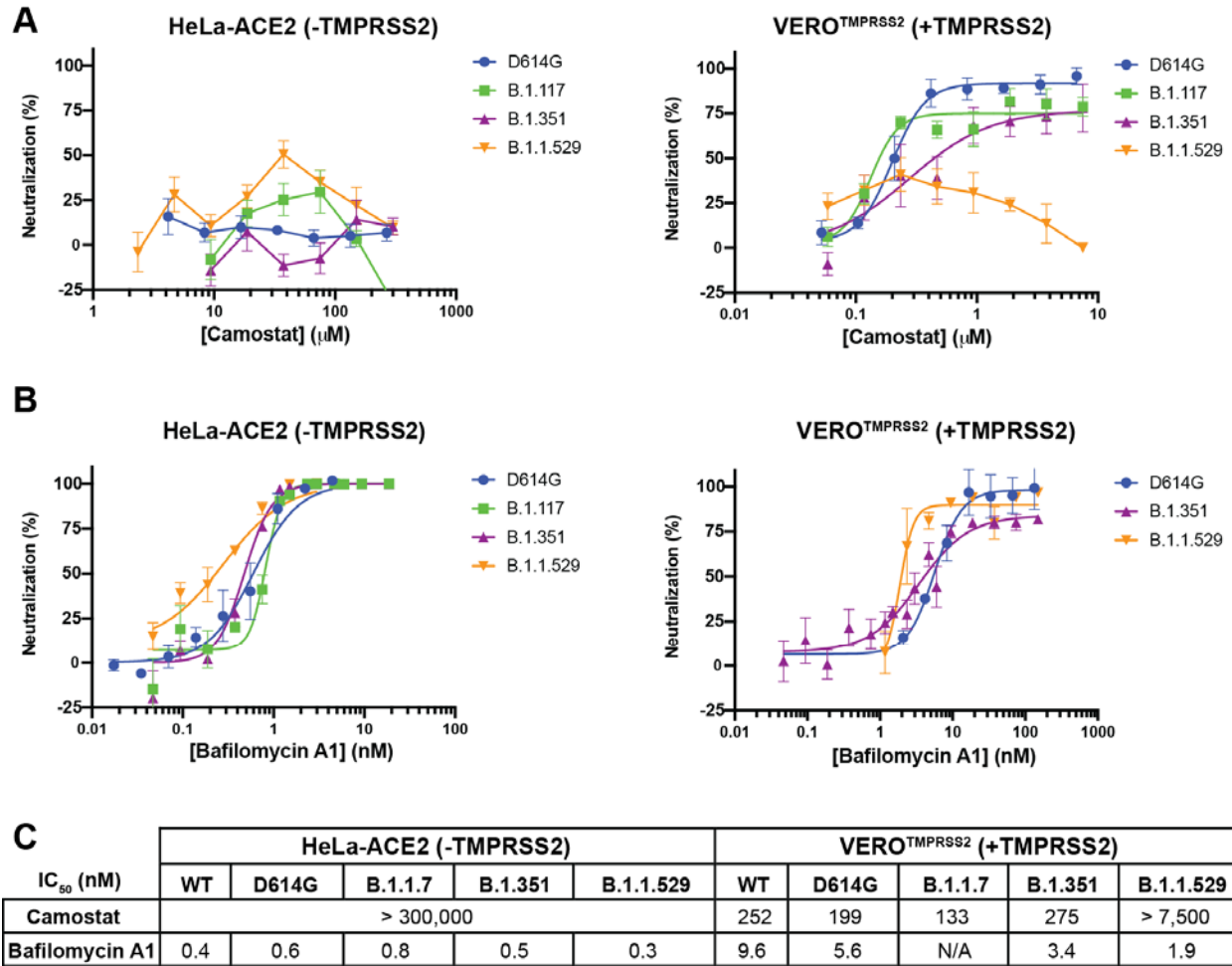
Figure 2



444
445

446
447
448

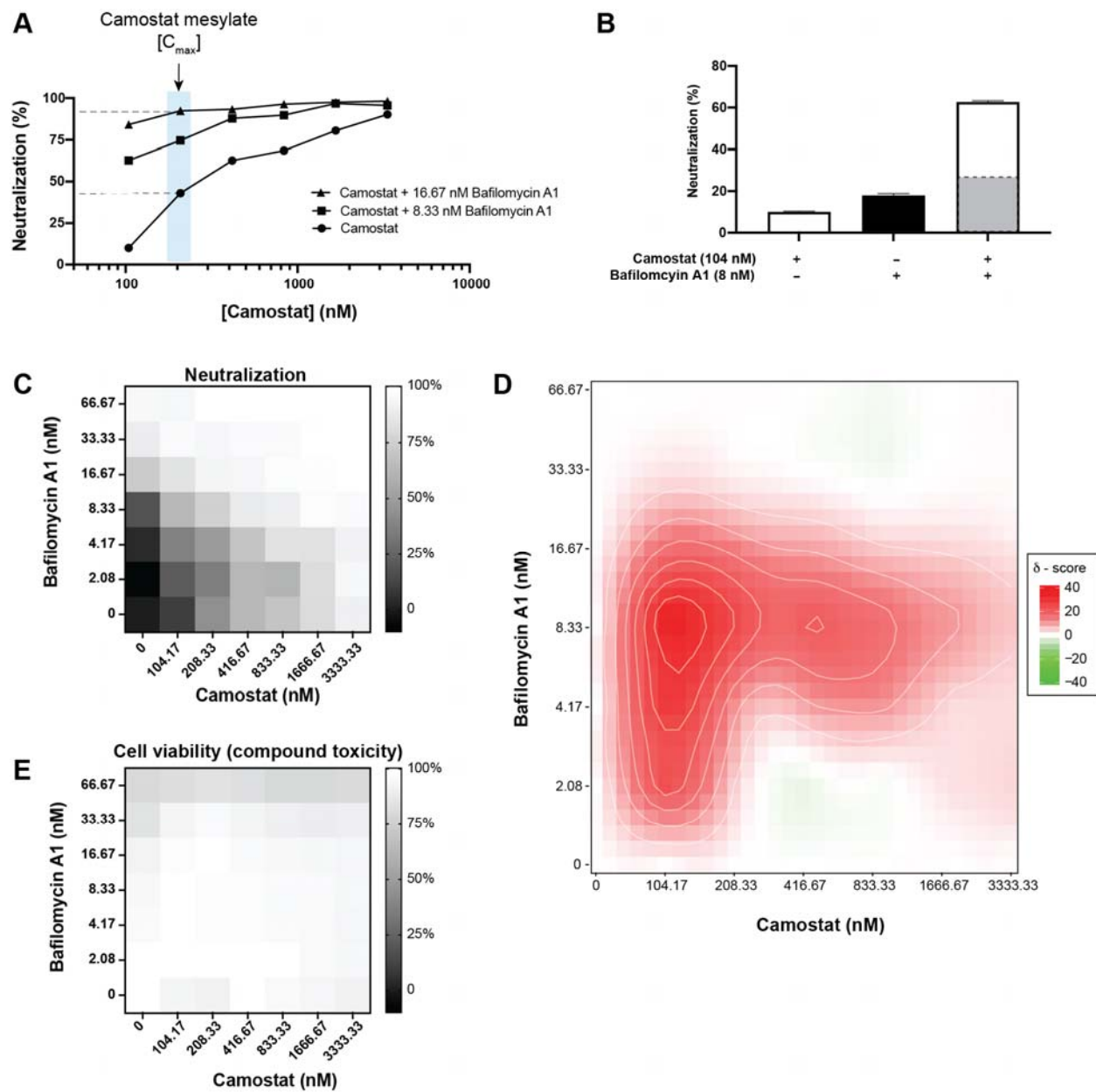
Figure 3



449
450
451

452
453

Figure 4



454
455

456 **REFERENCES**

- 457
- 458 1. Coronaviridae Study Group of the International Committee on Taxonomy of, V., *The*
459 *species Severe acute respiratory syndrome-related coronavirus: classifying 2019-nCoV*
460 *and naming it SARS-CoV-2*. *Nat Microbiol*, 2020. **5**(4): p. 536-544.
 - 461 2. Zhou, P., et al., *A pneumonia outbreak associated with a new coronavirus of probable*
462 *bat origin*. *Nature*, 2020. **579**(7798): p. 270-273.
 - 463 3. Zhu, N., et al., *A Novel Coronavirus from Patients with Pneumonia in China, 2019*. *N Engl*
464 *J Med*, 2020. **382**(8): p. 727-733.
 - 465 4. Wu, F., et al., *A new coronavirus associated with human respiratory disease in China*.
466 *Nature*, 2020. **579**(7798): p. 265-269.
 - 467 5. *Coronavirus disease (COVI-19) pandemic*. 2021; Available from:
468 <https://www.who.int/emergencies/diseases/novel-coronavirus-2019>.
 - 469 6. Planas, D., et al., *Reduced sensitivity of SARS-CoV-2 variant Delta to antibody*
470 *neutralization*. *Nature*, 2021.
 - 471 7. Yurkovetskiy, L., et al., *Structural and Functional Analysis of the D614G SARS-CoV-2 Spike*
472 *Protein Variant*. *Cell*, 2020. **183**(3): p. 739-751 e8.
 - 473 8. Korber, B., et al., *Tracking Changes in SARS-CoV-2 Spike: Evidence that D614G Increases*
474 *Infectivity of the COVID-19 Virus*. *Cell*, 2020. **182**(4): p. 812-827 e19.
 - 475 9. Davies, N.G., et al., *Increased mortality in community-tested cases of SARS-CoV-2 lineage*
476 *B.1.1.7*. *Nature*, 2021. **593**(7858): p. 270-274.
 - 477 10. Bates, T.A., et al., *Neutralization of SARS-CoV-2 variants by convalescent and BNT162b2*
478 *vaccinated serum*. *Nature Communications*, 2021. **12**(1): p. 5135.
 - 479 11. Kumar, S., A. Chandele, and A. Sharma, *Current status of therapeutic monoclonal*
480 *antibodies against SARS-CoV-2*. *PLOS Pathogens*, 2021. **17**(9): p. e1009885.
 - 481 12. *Pfizer's Novel COVID-19 Oral Antiviral Treatment Candidate Reduced Risk of*
482 *Hospitalization or Death by 89% in Interim Analysis of Phase 2/3 EPIC-HR Study*. 2021,
483 Pfizer.
 - 484 13. *Merck and Ridgeback's Investigational Oral Antiviral Molnupiravir Reduced the Risk of*
485 *Hospitalization or Death by Approximately 50 Percent Compared to Placebo for Patients*
486 *with Mild or Moderate COVID-19 in Positive Interim Analysis of Phase 3 Study*. 2021,
487 Merck.
 - 488 14. Wang, Q., et al., *Structural and Functional Basis of SARS-CoV-2 Entry by Using Human*
489 *ACE2*. *Cell*, 2020. **181**(4): p. 894-904 e9.
 - 490 15. Shang, J., et al., *Structural basis of receptor recognition by SARS-CoV-2*. *Nature*, 2020.
491 **581**(7807): p. 221-224.
 - 492 16. Hoffmann, M., et al., *SARS-CoV-2 Cell Entry Depends on ACE2 and TMPRSS2 and Is*
493 *Blocked by a Clinically Proven Protease Inhibitor*. *Cell*, 2020. **181**(2): p. 271-280 e8.
 - 494 17. Zhao, M.M., et al., *Cathepsin L plays a key role in SARS-CoV-2 infection in humans and*
495 *humanized mice and is a promising target for new drug development*. *Signal Transduct*
496 *Target Ther*, 2021. **6**(1): p. 134.
 - 497 18. Pišlar, A., et al., *The role of cysteine peptidases in coronavirus cell entry and replication:*
498 *The therapeutic potential of cathepsin inhibitors*. *PLOS Pathogens*, 2020. **16**(11): p.
499 e1009013.

- 500 19. Hashimoto, R., et al., *Dual inhibition of TMPRSS2 and Cathepsin B prevents SARS-CoV-2*
501 *infection in iPS cells*. *Molecular Therapy - Nucleic Acids*, 2021. **26**: p. 1107-1114.
- 502 20. Bayati, A., et al., *SARS-CoV-2 infects cells after viral entry via clathrin-mediated*
503 *endocytosis*. *J Biol Chem*, 2021. **296**: p. 100306.
- 504 21. Cai, Y., et al., *Distinct conformational states of SARS-CoV-2 spike protein*. *Science*, 2020.
505 **369**(6511): p. 1586-1592.
- 506 22. Delorey, T.M., et al., *COVID-19 tissue atlases reveal SARS-CoV-2 pathology and cellular*
507 *targets*. *Nature*, 2021. **595**(7865): p. 107-113.
- 508 23. Murgolo, N., et al., *SARS-CoV-2 tropism, entry, replication, and propagation:*
509 *Considerations for drug discovery and development*. *PLoS Pathog*, 2021. **17**(2): p.
510 e1009225.
- 511 24. Hoffmann, M., et al., *Camostat mesylate inhibits SARS-CoV-2 activation by TMPRSS2-*
512 *related proteases and its metabolite GBPA exerts antiviral activity*. *EBioMedicine*, 2021.
513 **65**: p. 103255.
- 514 25. Meng, B., et al., *Altered TMPRSS2 usage by SARS-CoV-2 Omicron impacts tropism and*
515 *fusogenicity*. *Nature*, 2022.
- 516 26. Gunst, J.D., et al., *Efficacy of the TMPRSS2 inhibitor camostat mesilate in patients*
517 *hospitalized with Covid-19-a double-blind randomized controlled trial*. *EClinicalMedicine*,
518 2021. **35**: p. 100849.
- 519 27. Uhlman, A., et al., *Effects of Vacuolar H(+)-ATPase Inhibition on Activation of Cathepsin*
520 *B and Cathepsin L Secreted from MDA-MB231 Breast Cancer Cells*. *Cancer Microenviron*,
521 2017. **10**(1-3): p. 49-56.
- 522 28. Vidak, E., et al., *Cysteine Cathepsins and Their Extracellular Roles: Shaping the*
523 *Microenvironment*. *Cells*, 2019. **8**(3): p. 264.
- 524 29. Simmons, G., et al., *Different host cell proteases activate the SARS-coronavirus spike-*
525 *protein for cell-cell and virus-cell fusion*. *Virology*, 2011. **413**(2): p. 265-74.
- 526 30. Simmons, G., et al., *Proteolytic activation of the SARS-coronavirus spike protein: cutting*
527 *enzymes at the cutting edge of antiviral research*. *Antiviral Res*, 2013. **100**(3): p. 605-14.
- 528 31. Liu, J., et al., *Hydroxychloroquine, a less toxic derivative of chloroquine, is effective in*
529 *inhibiting SARS-CoV-2 infection in vitro*. *Cell Discov*, 2020. **6**(1): p. 16.
- 530 32. Hoffmann, M., et al., *Chloroquine does not inhibit infection of human lung cells with*
531 *SARS-CoV-2*. *Nature*, 2020. **585**(7826): p. 588-590.
- 532 33. Self, W.H., et al., *Effect of Hydroxychloroquine on Clinical Status at 14 Days in*
533 *Hospitalized Patients With COVID-19: A Randomized Clinical Trial*. *JAMA*, 2020. **324**(21):
534 p. 2165-2176.
- 535 34. Administration, U.S.F.a.D., *Coronavirus (COVID-19) Update: FDA Revokes Emergency Use*
536 *Authorization for Chloroquine and Hydroxychloroquine*, U.S.F.a.D. Administration, Editor.
537 2020.
- 538 35. Tummino, T.A., et al., *Drug-induced phospholipidosis confounds drug repurposing for*
539 *SARS-CoV-2*. *Science*, 2021. **373**(6554): p. 541-547.
- 540 36. Rujas, E., et al., *Multivalency transforms SARS-CoV-2 antibodies into ultrapotent*
541 *neutralizers*. *Nature Communications*, 2021. **12**(1): p. 3661.
- 542 37. Ou, X., et al., *Characterization of spike glycoprotein of SARS-CoV-2 on virus entry and its*
543 *immune cross-reactivity with SARS-CoV*. *Nat Commun*, 2020. **11**(1): p. 1620.

- 544 38. Li, K., et al., *The TMPRSS2 Inhibitor Nafamostat Reduces SARS-CoV-2 Pulmonary*
545 *Infection in Mouse Models of COVID-19*. mBio, 2021: p. e0097021.
- 546 39. Shuai, H., et al., *Attenuated replication and pathogenicity of SARS-CoV-2 B.1.1.529*
547 *Omicron*. Nature, 2022.
- 548 40. Zhou, Y., et al., *Protease inhibitors targeting coronavirus and filovirus entry*. Antiviral
549 Res, 2015. **116**: p. 76-84.
- 550 41. Ianevski, A., A.K. Giri, and T. Aittokallio, *SynergyFinder 2.0: visual analytics of multi-drug*
551 *combination synergies*. Nucleic Acids Res, 2020. **48**(W1): p. W488-W493.
- 552 42. Ledford, H., *COVID antiviral pills: what scientists still want to know*. Nature, 2021.
553 **599**(7885): p. 358-359.
- 554 43. Kaufmann, S.H.E., et al., *Host-directed therapies for bacterial and viral infections*. Nature
555 Reviews Drug Discovery, 2018. **17**(1): p. 35-56.
- 556 44. Daniloski, Z., et al., *Identification of Required Host Factors for SARS-CoV-2 Infection in*
557 *Human Cells*. Cell, 2021. **184**(1): p. 92-105 e16.
- 558 45. Zhu, Y., et al., *A genome-wide CRISPR screen identifies host factors that regulate SARS-*
559 *CoV-2 entry*. Nature Communications, 2021. **12**(1): p. 961.
- 560 46. Li, X., et al., *Is hydroxychloroquine beneficial for COVID-19 patients?* Cell Death &
561 Disease, 2020. **11**(7): p. 512.
- 562 47. Tichauer, J.E., D. Soto, and M. Andresen, *Characterization of the Modulatory Effect of*
563 *Hydroxychloroquine on ACE2 Activity: New Insights in relation to COVID-19*. BioMed
564 Research International, 2021. **2021**: p. 6614000.
- 565 48. Prabhakara, C., et al., *Strategies to target SARS-CoV-2 entry and infection using dual*
566 *mechanisms of inhibition by acidification inhibitors*. PLOS Pathogens, 2021. **17**(7): p.
567 e1009706.
- 568 49. Yuan, S., et al., *Clofazimine broadly inhibits coronaviruses including SARS-CoV-2*. Nature,
569 2021. **593**(7859): p. 418-423.
- 570 50. Crawford, K.H.D., et al., *Protocol and Reagents for Pseudotyping Lentiviral Particles with*
571 *SARS-CoV-2 Spike Protein for Neutralization Assays*. Viruses, 2020. **12**(5).
- 572 51. Matsuyama, S., et al., *Enhanced isolation of SARS-CoV-2 by TMPRSS2-expressing cells*.
573 Proc Natl Acad Sci U S A, 2020. **117**(13): p. 7001-7003.
- 574 52. Nao, N., et al., *Consensus and variations in cell line specificity among human*
575 *metapneumovirus strains*. PLoS One, 2019. **14**(4): p. e0215822.
- 576
- 577

578 **FIGURE LEGENDS**

579

580 **Figure 1. Identification of bafilomycin A1 as a safe and potent inhibitor of SARS-CoV-2**
581 **pseudovirus (PsV) entry into mammalian cells.** (A) Chemical structure of three groups of
582 endosomal acidification modifiers: lysosomotropic compounds (amodiaquine, chloroquine, and
583 quinacrine), proton shuttles (niclosamide and oxyclozanide), and a specific V-ATPase inhibitor
584 (bafilomycin A1). The highlighted tertiary ammonium cation of the lysosomotropic compounds
585 is a characteristic structure of phospholipidosis-causing compounds. (B) Assays testing
586 pseudoviral entry inhibition (left), endosomal acidification inhibition (top-right), and compound-
587 mediated cell cytotoxicity (bottom-right) of each compound. (C) Comparison of inhibition of
588 PsV entry, inhibition of endosomal acidification, and cell viability by each tested compound. All
589 experiments were conducted in triplicate, at the minimum, (n=3-14).

590

591 **Figure 2. Inhibition of endosomal acidification attenuates SARS-CoV-2 entry irrespective**
592 **of TMPRSS2 expression.** (A) Camostat and bafilomycin A1 were tested against SARS-CoV-2
593 PsVs using HeLa cells expressing ACE2 but lacking TMPRSS2 or Vero cells expressing both
594 ACE2 and TMPRSS2. All experiments were conducted in at least n=6 up to n=13 (n=6-13). (B)
595 Camostat and bafilomycin A1 were tested against authentic SARS-CoV-2 virus (MOI=0.1) using
596 Vero cells endogenously expressing ACE2 but lacking TMPRSS2 or Calu-3 cells endogenously
597 expressing both ACE2 and TMPRSS2. Data represents three to four independent experiments
598 each done in triplicate using SARS-CoV-2 [SARS-CoV-2/Canada/ON/VIDO-
599 01/2020/Vero'76/p.2 (Seq. available at GISAID – EPI_ISL_425177)] (n=3-4).

600

601 **Figure 3. Potency of host-dependent inhibition against different variants of SARS-CoV-2**
602 **remains unaffected.** Camostat (A) and Bafilomycin A1 (B) were tested against SARS-CoV-2
603 D641G, B.1.117 (i.e., Alpha), B.1.351 (i.e., Beta) or B.1.1.529 (i.e., Omicron) PsV variants
604 using HeLa cells expressing ACE2 but lacking TMPRSS2 or Vero cells expressing both ACE2
605 and TMPRSS2. All experiments were conducted in quadruplicates, at the minimum (n=4-5). (C)
606 The half-maximal inhibitory concentration (IC₅₀) of Camostat and bafilomycin A1 against
607 several SARS-CoV-2 PsVs variants using HeLa cells expressing ACE2 but lacking TMPRSS2 or
608 Vero cells expressing both ACE2 and TMPRSS2 was calculated using Prism 9 (GraphPad). All
609 experiments were conducted in quadruplicates, at the minimum (n=4-13).

610

611 **Figure 4. Host-dependent dual-entry inhibition safely and synergistically inhibits SARS-**
612 **CoV-2 pseudovirus entry into mammalian cells.** (A) A dose-dependent inhibition of SARS-
613 CoV-2 PsV entry into Vero cells expressing both ACE2 and TMPRSS2 was measured using
614 Camostat alone or Camostat in addition to either 8 nM or 17 nM bafilomycin A1. The blue box
615 highlights the maximal blood concentration Camostat was found to achieve in patients [26]. All
616 experiments were conducted in duplicate (n=2). (B) 104 nM Camostat and 8 nM bafilomycin A1
617 were tested individually or in combination against SARS-CoV-2 PsVs using Vero cells
618 expressing both ACE2 and TMPRSS2. The highlighted gray bar in the combination experiment
619 highlights the theoretical neutralization if the two compounds acted additively. All experiments
620 were conducted in duplicate (n=2). (C) A checkerboard assay using Camostat and bafilomycin
621 A1 was conducted against SARS-CoV-2 PsV using Vero cells expressing both ACE2 and
622 TMPRSS2. The experiments were conducted in duplicate (n=2). (D) A Zero Interaction Potency
623 (ZIP) score of the checkboard assay using Camostat and bafilomycin A1 from (C) was calculated

624 using SynergyFinder 2.0. (E) A checkerboard assay using Camostat and bafilomycin A1 at the
625 same concentrations as (C) on Vero cells expressing both ACE2 and TMPRSS2 was tested for
626 compound-mediated toxicity. The experiment was conducted in triplicate (n=3).
627

628 **ACKNOWLEDGEMENTS**

629

630 This research was funded (SI and RAM) from Fast Grants, part of the Emergent Ventures
631 Program at the Mercatus Centre at George Mason University, with support from Thistledown
632 Foundation. This research was supported by the European Union's Horizon 2020 research and
633 innovation program under Marie Skłodowska-Curie grant 790012 (ER). This work was further
634 supported by the CIFAR Azrieli Global Scholar program (JPJ), the Ontario Early Researcher
635 Award program (JPJ) and the Canada Research Chair program (JPJ). The Synergy Neo2 Multi-
636 Mode Assay Microplate Reader instrument was accessed at the Structural and Biophysical Core
637 Facility, The Hospital for Sick Children, supported by the Canada Foundation for Innovation and
638 Ontario Research Fund.