

1 **A simplified cell-based assay to identify coronavirus 3CL protease inhibitors**

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20

21 **Abstract**

22 We describe a mammalian cell-based assay capable of identifying coronavirus 3CL protease
23 (3CLpro) inhibitors without requiring the use of live virus. By enabling the facile testing of compounds
24 across a range of coronavirus 3CLpro enzymes, including the one from SARS-CoV-2, we are able to
25 quickly identify compounds with broad or narrow spectra of activity. We further demonstrate the
26 utility of our approach by performing a curated compound screen along with structure-activity
27 profiling of a series of small molecules to identify compounds with antiviral activity. Throughout these
28 studies, we observed concordance between data emerging from this assay and from live virus
29 assays. By democratizing the testing of 3CL inhibitors to enable screening in the majority of
30 laboratories rather than the few with extensive biosafety infrastructure, we hope to expedite the
31 search for coronavirus 3CL protease inhibitors, to address the current epidemic and future ones that
32 will inevitably arise.

33 **Introduction**

34 The outbreak of a novel coronavirus (SARS-CoV-2) infection of the past several months has
35 paralyzed countries around the world^{1,2}. This crisis is further exacerbated by the dearth of approved
36 therapeutics, leaving physicians with few proven treatment options. In the past two decades, the
37 world has already suffered from two other major coronavirus outbreaks, Severe Acute Respiratory
38 Syndrome (SARS) and Middle East Respiratory Syndrome (MERS), suggesting that coronaviruses
39 represent a real and ever-present threat to global health that must be addressed³. Yet, even if
40 therapeutics against the existing epidemic strains are identified, there are several hundred other
41 coronaviruses in active circulation within animal populations, many with the theoretical potential to
42 infect humans. To help identify therapeutics for the current epidemic along with preparing for the
43 next, there is a need for readily deployable small molecule screening assays that enable the
44 identification of therapeutics that are broad-acting across a large collection of coronavirus strains.

45
46 During coronavirus infection, the RNA genome is delivered into cells and translated into a pair of
47 polyproteins⁴. These polyproteins are then processed by a set of virally encoded proteases, of which
48 the three-chymotrypsin-like protease (3CLpro) performs the majority of cleavage events⁴. As a result
49 of its essential role in viral replication and high degree of conservation across all coronaviruses,
50 3CLpro enzymes represent important targets for therapeutic drug development^{5,6}. Previous work
51 expressing a variety of viral proteases within yeast and mammalian cellular systems have shown
52 that protease expression can lead to profound cellular toxicity, which can be rescued by the addition
53 of protease inhibitors⁷⁻¹². We hypothesized that if the expression of coronavirus 3CLpro enzymes
54 within mammalian cells leads to a similar toxic phenotype, this could form the basis of an easily
55 implemented mammalian cell-based assay to evaluate protease inhibitors. While multiple assays
56 exist to evaluate protease inhibitors, an assay of the nature we envisioned has clear advantages, as
57 it requires minimal upfront cost or effort, is accessible to many biomedical research labs, does not
58 involve the use of live virus, and requires no specialized reporter to read out protease activity. In
59 contrast, *in vitro* protease assays using purified protein have formed the backbone of inhibitor

60 screening, but require upfront efforts to isolate the pure protease and are not conducted under
61 physiologic cellular conditions^{13,14}. In addition, if one desires to identify broad-acting coronavirus
62 inhibitors, one must purify and identify experimental conditions suitable for testing each protease *in*
63 *vitro*. An alternative approach for identifying protease inhibitors is the use of live virus which is
64 performed under more biologically relevant conditions, assuming relevant host cell systems can be
65 identified, but requires intensive safety training and specialized biosafety protocols¹⁵. For many
66 coronaviruses, no live virus assay exists, limiting the ability to test compounds within mammalian cell
67 systems to a small subset of all coronaviruses¹⁶. Furthermore, compounds with activity against live
68 virus may function through a number of mechanisms other than protease inhibition which cannot be
69 readily determined, and may lead to undesired off-target activities which are not realized until much
70 later in the drug development process^{17,18}.

71
72 Here, we report a mammalian cell-based assay to identify coronavirus 3CLpro inhibitors that does
73 not require the use of live virus. We demonstrate the utility of the assay using the SARS-CoV-2
74 3CLpro, with EC₅₀ values obtained from the assay showing good concordance with traditional live
75 virus testing for multiple compounds. We next establish the generality of the approach by testing a
76 diverse set of 3CLpro enzymes. Finally, we perform a small molecule screen, along with structure-
77 activity profiling of a set of compounds to find those with enhanced antiviral activity. The presented
78 data support the use of our assay system for the discovery of small molecule 3CLpro inhibitors and
79 the rapid characterization of their activity across multiple coronaviruses to identify those with broad
80 inhibitory activity.

81

82

83 **Results**

84 **Expression of the SARS-CoV-2 3CLpro in HEK293T cells results in cell toxicity and can be**
85 **used as a readout of drug activity**

86 Prior work has shown that the exogenous expression of viral proteases within cellular systems can
87 result in growth suppression or cell death. Motivated by this work, we sought to determine the effect
88 of expressing the SARS-CoV-2 3CLpro in HEK293T cells. Utilizing a cost-effective crystal-violet-
89 based approach to quantify cell abundance, we observed that expression of SARS-CoV-2 3CLpro
90 results in significant growth inhibition as compared to the expression of a control protein, enhanced
91 yellow fluorescent protein (EYFP) (Fig. 1a-b)¹⁹. This suppression of growth was dependent upon the
92 catalytic function of the enzyme, as mutating cysteine 145, which is essential for the enzyme's
93 peptidase activity, abolished the growth defect (Fig. 1a-b). We sought to determine if the observed
94 growth defect could be rescued by incubating cells with GC376, a feline coronavirus inhibitor that
95 was recently reported to have activity against SARS-CoV-2 3CLpro²⁰. In comparison to untreated
96 control cells, the addition of GC376 led to a robust increase in cell growth (Fig. 1c-d).

97

98 **Compound rescue of transfected 3CLpro cytotoxicity is similar to results obtained with live** 99 **virus**

100 We next tested if this transfection-based assay could be used to determine compound EC₅₀ values
101 and whether the values showed any correlation with those obtained with live virus. After incubating
102 SARS-CoV-2 3CLpro transfected cells with a range of GC376 concentrations, we calculated an EC₅₀
103 of 3.30 μM, which is similar to published values reported using live virus on Vero E6 (EC₅₀ 2.2 μM,
104 0.9 μM, 0.18 μM, 4.48 μM) and Vero 76 cells (EC₅₀ 3.37 μM) (Fig. 2a and Table 1)²⁰⁻²⁴. We
105 investigated the assay's tolerance to deviation by varying the amount of plasmid transfected or the
106 number of cells seeded into wells containing compound (Supplementary Fig. 1). In all cases, the
107 assay was robust to variation, delivering a similar EC₅₀ for GC376 across all conditions. We also
108 tested an orthogonal method of quantifying cell abundance based on fluorescence microscopy and
109 observed agreement with the results obtained with crystal violet staining (Supplementary Fig. 2).
110 This suggests that the assay system is robust to variation and provides consistent results.

111

112 We next conducted dose-response profiling for two additional SARS-CoV-2 3CLpro inhibitors,
113 compound 4 and compound 11a, and observed reversal of the toxic effect of the protease in a dose-
114 dependent manner (Fig. 2b-c)^{25,26}. In agreement with the results obtained with GC376, the EC₅₀
115 value for compound 4 was comparable to those obtained with live virus, 0.98 μM and 3.023 μM,
116 respectively (Table 1)²⁴. Unexpectedly, we calculated an EC₅₀ of 6.89 μM for 11a, which is
117 approximately 10-fold higher than the literature reported value of 0.53 μM, based on viral plaque
118 assay²⁶. We have noticed that literature reported EC₅₀ values from live virus testing could range over
119 an order of magnitude depending on the exact method employed, as is the case for GC376 (Table
120 1). To resolve this discrepancy between the transfection-based approach and the live virus assay,
121 we conducted live virus testing of 11a using the commonly employed readout of cytopathic effect in
122 Vero E6 cells and observed closer concordance with our transfection-based results (Supplementary
123 Figure 3 and Table 1)^{20,24,27}. To measure the toxicity of each compound, we exposed EYFP-
124 transfected cells to each molecule and determined CC₅₀ values (Fig. 2). We also calculated the
125 selectivity index (SI) for each compound tested in this study (Supplementary Table 1).

126

127 We hypothesized that the assay would be able to distinguish between compounds that are only
128 active on the purified SARS-CoV-2 3CLpro and those that are able to inhibit the live virus through
129 protease inhibition. In general, we observe concordance between compounds showing activity within
130 this transfection-based 3CL assay and live virus studies (Supplementary Fig. 4a-e)¹³. However,
131 within the assay, we did not observe activity for ebselen, a small molecule with demonstrated activity
132 against the SARS-CoV-2 3CLpro *in vitro* and activity against the SARS-CoV-2 live virus
133 (Supplementary Fig. 4f). We suggest that this may be due to ebselen targeting more than 3CLpro
134 within the live virus assay, which is in line with work showing that ebselen is highly reactive and
135 readily forms selenosulfide bonds with numerous proteins including the SARS-CoV-2 papain-like
136 protease (PLP)^{18,28,29}.

137

138 **Demonstrating assay compatibility across a range of coronavirus 3CLpro enzymes**

139 We hypothesized that this assay may be used to study other coronavirus 3CLpros to enable users to
140 identify broad-acting inhibitors, as constructs containing other 3CLpro enzymes could be readily
141 synthesized. To test the assay's generality, we created expression constructs for 3CL proteases
142 from five other coronaviruses (SARS-CoV, MERS-CoV, Bat-CoV-HKU9, HCoV-NL63 and IBV) with
143 variable amino acid identity compared with SARS-CoV-2 3CLpro (Supplementary Fig. 5a). For each
144 of these proteases, we confirmed that expression in mammalian cells resulted in toxicity that is
145 dependent upon the enzyme's catalytic activity (Supplementary Fig. 5b). Next, we tested GC376,
146 compound 4, and 11a across this panel of proteases. GC376, a drug originally identified for use
147 against the Feline Infectious Peritonitis virus, showed $EC_{50} < 10 \mu\text{M}$ for the most, but not all of
148 proteases tested³⁰. Unexpectedly, compound 4, which was originally designed as a SARS-CoV
149 3CLpro inhibitor showed particular potency against IBV 3CLpro ($EC_{50} = 0.058 \mu\text{M}$) along with broad
150 activity ($EC_{50} < 10 \mu\text{M}$) for all other 3CL proteases tested. In contrast to GC376 and compound 4, 11a
151 had a relatively narrow activity spectrum with $EC_{50} < 10 \mu\text{M}$ against only SARS-CoV and SARS-CoV-
152 2 3CLpro enzymes (Fig. 3). Of note, in all cases where previous live virus data was available, the
153 EC_{50} values obtained from this transfection-based assay were similar (Table 1).

154

155 **Rapid testing to identify SARS-CoV-2-3CL protease inhibitors**

156 Having further determined the assay's ability to examine the effects of active individual compounds,
157 we sought to determine its suitability for small molecule screening. Before performing the screen, we
158 optimized the testing parameters to ensure suitable performance characteristics (Supplementary Fig.
159 6 and Methods)³¹. We compiled a collection of 162 diverse protease inhibitors, along with
160 compounds with reported *in vitro* activity against 3CLpro enzymes or structural similarity to known
161 3CLpro inhibitors (Supplementary Table 2 and Supplementary Table 3). Of the nearly 200
162 compounds tested, two potent hits were identified, GC373 and GRL-0496 (Fig. 4a, Supplementary
163 Table 2)³².

164

165 We noted that GC373 is structurally similar to its prodrug GC376, except for the change of the
166 bisulfide salt adduct to an aldehyde warhead^{22,33}. Additional testing of GC373 revealed it to have a
167 similar EC₅₀ as GC376 in both the transfection assay and when tested against live SARS-CoV-2
168 virus, suggesting that the differences in structure has a minimal effect on their potency
169 (Supplementary Fig. 7 and Table 1), although solubility may be affected³³. The other hit from the
170 screen, GRL-0496, shares structural similarity to several other compounds within the library, one of
171 which is a previously reported 3CLpro inhibitor (MAC-5576) that failed to show activity against
172 SARS-CoV-2 in a live virus assay^{24,34}. Additional testing of GRL-0496 revealed it to have an EC₅₀ of
173 5.05 μM against SARS-CoV-2 3CLpro within our transfection-based assay (Fig. 4c). To verify GRL-
174 0496's activity, we tested it against live SARS-CoV-2 virus, and confirmed its potency (EC₅₀ = 9.12
175 μM) (Fig. 4d). We next tested GRL-0496 against the full panel of 3CLpro enzymes we had previously
176 examined and observed a narrow range of activity, with EC₅₀ <10 μM only observed against SARS-
177 CoV 3CLpro and SARS-CoV-2 3CLpro, in agreement with previous live virus testing (Supplementary
178 Fig. 8 and Table 1)²⁷. We also tested GC373 against the full panel of 3CLpro enzymes and observed
179 concordance with GC376, with SARS-CoV 3CLpro, MERS-CoV 3CL pro, and IBV 3CL pro
180 demonstrating an EC₅₀ <10 μM (Supplementary Fig. 8).

181

182 **Discussion**

183 Given the potential for protease inhibitors in the treatment of viral illnesses, small molecule inhibitors
184 of coronavirus 3CL proteases represent a promising avenue for treating infections caused by this
185 large family of viruses. Here, we present a simplified assay to identify candidate inhibitors under
186 physiologic cellular conditions. This approach presents significant advantages over other methods to
187 detect 3CL protease inhibitory activity with its ease of use and ability to be performed with equipment
188 and reagents commonly available to many biomedical research laboratories. While conventional
189 methods for identifying 3CL protease inhibitors make use of *in vitro* purified protease, the isolation of
190 sufficiently pure enzyme in its native state can be costly and labor intensive. Furthermore, assays
191 using purified protease fail to consider cell permeability and the influence of the extracellular and

192 intracellular milieu on compound activity. In comparison to live virus-based assays, the outlined
193 approach does not require extensive biosafety containment. These data also suggest that the
194 approach described here is applicable to a number of coronaviruses for which live virus assays may
195 not be available or would be deemed ethically challenging to be performed even with extensive
196 biosafety infrastructure^{35,36}. Finally, because the phenotype assayed within this approach is driven
197 solely by protease activity, it may enable the distinction between compounds with multiple biological
198 targets and subsequent potential for off-target toxicity from those that function primarily as 3CLpro
199 inhibitors.

200

201 A number of surrogate assays that aim to identify molecules with activity against proteins encoded
202 by SARS-CoV-2 have recently been reported³⁷⁻³⁹. These assays were developed to provide
203 physiologically meaningful molecule testing without needing to use live SARS-CoV-2 cultures in
204 order to allow for rapid testing and widespread adoption to labs without necessary safety
205 infrastructure. The aforementioned assays have focused primarily on identifying neutralizing
206 antibodies targeted at the SARS-CoV-2 spike (S) protein. To our knowledge, a reporter-free
207 surrogate assay to identify coronavirus 3CL protease inhibitors validated with multiple known
208 coronavirus 3CL protease inhibitors has not been reported.

209

210 Within the literature, EC₅₀ values obtained for a 3CLpro inhibitor against live virus can show a broad
211 range of potency, with some compounds demonstrating EC₅₀ values across multiple orders of
212 magnitude (Table 1). These differences appear to be driven by variation in experimental setup such
213 as cell line used, assay readout, incubation period, and initial concentration of virus added. While we
214 have observed agreement between the EC₅₀ values obtained from the described transfection-based
215 method and those reported in the literature, given the differences in EC₅₀ across assays, we suggest
216 caution when comparing results across studies. By developing this transfection-based 3CLpro
217 testing platform, we hope to facilitate the discovery of new coronavirus inhibitors while also
218 facilitating the comparison of existing inhibitors within a single simplified assay system. Furthermore,

219 we propose that this cellular protease assay system could be industrialized to screen and optimize a
220 large number of compounds to discover potential treatments for future viral pandemics.

221

222 **Methods**

223 **Cell Lines and Cell Culture**

224 HEK293T and HEK293 cells used in this study were obtained from ATCC. Cells were maintained at
225 37°C in a humidified atmosphere with 5% CO₂. HEK293T and HEK293 cells were grown in
226 Dulbecco's Modified Eagle Medium (DMEM, Invitrogen) which was supplemented with 10% fetal
227 bovine serum (Gibco) and penicillin-streptomycin (Invitrogen). HEK293T and HEK293 cells were
228 confirmed to be free of mycoplasma contamination with the Agilent MycoSensor PCR Assay Kit.
229 To obtain HEK293 cells stably expressing EYFP, cells were co-transfected with EYFP plasmids
230 harboring the *piggyBac* transposon (pPB bsr2-EYFP) (Yusa et al., 2009) and pCMV-mPBase
231 (mammalian codon-optimized PBase) encoding a piggyBac transposase using Lipofectamine 2000
232 (Invitrogen) according to the manufacturer's instructions. One day after transfection, the transfected
233 cells were selected with 10 µg/mL of blasticidin (Invitrogen).

234

235 **Transfections and Drug Selections**

236 24 h prior to transfection, 293T cells were seeded at 65-75% confluency into 24-well plates coated
237 for 30 min with a 1 mg/mL solution of poly-D-lysine (MP Biomedicals Inc.) and washed with PBS
238 (Gibco) once prior to media addition. The next day, 500 ng of 3CLpro expression plasmid, unless
239 otherwise stated, was incubated with Opti-MEM and Lipofectamine 2000 for 30 min at room
240 temperature prior to dribbling on cells, as per manufacturer's protocol. For plating into drug
241 conditions, 20 h after transfection, cells were washed once with PBS and 200 µL Trypsin-EDTA
242 0.25% (Gibco) was added to cells to release them from the plate. Trypsinized cell slurry was pipetted
243 up and down repeatedly to ensure a single cell suspension. 96-well plates were coated with poly-D-
244 lysine, either coated manually with 1 µg/mL poly-D-lysine in PBS solution for 30 min or purchased
245 pre-coated with poly-D-lysine (Corning). Wells were filled with 100 µL of media ± drug and 1 µg/mL

246 puromycin and were seeded with 9 μ L of trypsinized cell slurry. For higher throughput experiments,
247 multiple individually transfected wells of a 24-well plate were combined after trypsinization and prior
248 to seeding in drug. After seeding into wells containing drug and puromycin, cells were incubated for
249 48 h unless otherwise specified.

250

251 **Plasmids**

252 All vectors used in this study were cloned into the pLEX307 backbone (Addgene #41392) using
253 Gateway LR II Clonase Enzyme mix (Invitrogen). 3CL proteases used in this study were generated
254 using gene fragments ordered from Twist Biosciences. Inactive 3CL proteases were generated by
255 site directed mutagenesis of the essential catalytic cysteine. DNA was transformed into NEB 10-beta
256 high efficiency competent cells. Sanger sequencing to verify proper inserts were done for all
257 plasmids used in this study (Genewiz).

258

259 Plasmid DNA was isolated using standard miniprep buffers (Omega Biotek) and silica membrane
260 columns (Biobasic). To reduce batch-to-batch variability between plasmid DNA isolations and its
261 subsequent impact on transfection efficiency, multiple plasmid DNA extractions were conducted in
262 parallel, diluted to 50 ng/ μ L and pooled together.

263

264 **Crystal Violet Staining and Quantification**

265 The crystal violet staining protocol was adapted from Feoktistova et. al.¹⁹ Briefly, after compound
266 incubation with 3CLpro expressing cells in 96-well plates, the medium was discarded and cells were
267 washed once with PBS. Cells were incubated with 50 μ L of crystal violet staining solution (0.5%
268 crystal violet in 80% water and 20% methanol) and rocked gently for 30 min. The staining solution
269 was removed and cells were washed four times with water using a multichannel pipette. Stained
270 cells were left to dry for \geq 4 h on the laboratory bench or within the chemical hood. The crystal violet
271 staining solution was eluted by the addition of 200 μ L of methanol over the course of 30 min with
272 gentle rocking. Plates were sealed with parafilm to mitigate methanol evaporation. 100 μ L of eluted

273 stain from each well was transferred to a new 96-well plate for reading in a Tecan Infinite F50 plate
274 reader. Absorbance was measured at 595 nm twice and values were averaged between replicate
275 measurements. Blank wells were included in each batch of experiments, and absorbance values
276 were normalized by background levels of staining from blank wells.

277

278 **Statistical Analysis of Dose Response Curves**

279 For analysis of crystal violet staining experiments, relative growth was calculated from background
280 normalized absorbance values. Test wells containing drug were divided by average background
281 normalized values from wells where cells were expressing protease and exposed to vehicle, when
282 available. Otherwise, values were normalized by values from protease-expressing cells exposed to
283 the lowest concentration of drug included in the dose-response curve. When there were significant
284 deviations from protease-expressing cells exposed to no drug and protease-expressing cells
285 exposed to lowest concentrations of drug included in the dose-response curve, experiments were
286 repeated with normalization by protease-expressing cells exposed to no drug. CC_{50} values were
287 calculated in Prism using the nonlinear regression functionality and derived from dose-response
288 curves with EYFP transfected cells. A nonlinear curve fitting function accounting for variable curve
289 slopes was calculated by plotting the normalized response as a function of $\log(\text{compound})$. Similarly,
290 EC_{50} values were calculated in GraphPad Prism also using the nonlinear regression functionality. A
291 nonlinear curve fitting function measuring the stimulatory response of a compound as a function of
292 an unnormalized response was used to calculate the EC_{50} . All reported values were confirmed to not
293 have ambiguous curve fitting. The 95% confidence interval of EC_{50} calculations was also calculated
294 and included.

295

296 For analysis of live virus experiments, EC_{50} values were determined by fitting a nonlinear curve to
297 the data with the assumption of a normalized response (GraphPad Prism). Cells were confirmed as
298 mycoplasma negative prior to use.

299

300 **Compound Screening**

301 For screening condition optimization, we measured the Z-Factor for replicates of positive controls
302 GC376, tested at 50 μ M, and compound 4, tested at 20 μ M. Replicate measurements were recorded
303 for DMSO negative controls and positive control compounds after 48, 72, and 96 h of incubation with
304 drug. Background normalized crystal violet absorbance values at each timepoint were collected.

305

306 During the drug screen, within each of the four plates screened, two positive controls wells were
307 included to ensure assay reliability, along with several wells with the negative control 0.1% DMSO
308 condition. All compounds were screened at 10 μ M resuspended in DMSO (Fisher Scientific).

309 For hit selection, we employed a robust z-score method. We first normalized data using a robust z-
310 score that uses median and median absolute deviation (MAD) instead of mean and standard
311 deviation. We then used a threshold of 3.5 MAD to determine which drugs rescued the cytotoxicity
312 imposed by expression of the viral protease⁴⁰.

313

314 **Live Virus Assay**

315 The SARS-CoV-2 strain 2019-nCoV/USA_WA1/2020 was grown and titered in Vero-E6 cells. One
316 day before the experiment, Vero-E6 cells were seeded at 30,000 cells/well in 96 well-plates. Serial
317 dilutions of the test compound were prepared in media (EMEM + 10% FCS +
318 penicillin/streptomycin), pipetted onto cells, and virus was subsequently added to each well at an
319 MOI of 0.2. Cells were incubated at in a humidified environment at 37 °C with 5% CO₂ for 72 h after
320 addition of virus. Cytopathic effect was scored by independent, blinded researchers. The reported
321 cytopathic effect value represents the average from two independent reviewers. Percent Inhibition
322 was calculated by comparison to control wells with no inhibitor added. All live virus experiments were
323 conducted in a biosafety level 3 lab.

324

325 **Microscopy**

326 Cells plated on a 96-well plate (Greiner Bio-One™) and washed once immediately before imaging.
327 EYFP fluorescence imaging was performed using an Axio Observer 7 microscope (Zeiss) equipped
328 with a Plan-Apochromat 10X objective (0.45 N.A.) with 1-by-1 pixel binning. Optical Illumination bias
329 was empirically derived by sampling background areas and subsequently used to flatten images.
330 After a global background subtraction, cell density was calculated based on area of EYFP intensity.

331

332 **Compounds and Chemical Synthesis**

333 GC376 was purchased from Aobious. Myrecetin, rupintrivir, grazoprevir, saquinavir, fosamprenavir,
334 indinavir, apigenin, quercetin, famotidine, MDL28170, bicailein, betrixaban, and amentoflavone were
335 purchased from Fisher Scientific. Tipranavir was purchased from Cayman Chemical. MAC5576,
336 MAC22272, MAC8120, MAC30731, BTB07404, BTB07408, MWP00332, BTB07417, MWP00508,
337 MWP00333, BTB07407, SPB08384, SPB06613, SPB06636, SPB06591, SPB06593, MWP00709,
338 CC42746, BTB07789, BTB07420, MWP00710, BTB07421, SCR00533, and SEW03089 were
339 purchased from Maybridge. GRL-0496 and GRL-0617 were purchased from Focus Biomolecules.
340 AZVIII-40A (1,2-Benzisothiazol-3(2H)-one) was purchased from Alfa Aesar. Other protease inhibitors
341 in table below were purchased from TargetMol.

342

CAS Number	Drug Name	CAS Number	Drug Name
1226781-44-7	Omarigliptin	134381-21-8	Epoxomicin
79183-19-0	Apoptosis Activator 2	110044-82-1	MG101
3731-52-0	Picolamine	125697-93-0	lavendustin C
541-91-3	Muscone	729607-74-3	BMS707035
60-23-1	2-Aminoethanethiol	630420-16-5	Asunaprevir
51146-56-6	Dexibuprofen	76684-89-4	Loxistatin Acid
3416-24-8	Glucosamine	1025015-40-0	GK921
56974-61-9	Gabexate mesylate	292632-98-5	L-685,458
7481-89-2	Zalcitabine	202138-50-9	Tenofovir Disoproxil Fumarate
2016-88-8	Amiloride hydrochloride	937174-76-0	GSK690693
945667-22-1	Saxagliptin hydrate	1256388-51-8	Ledipasvir
668270-12-0	Linagliptin	960374-59-8	ONX0914
486460-32-6	Sitagliptin	1401223-22-0	PI1840
136-77-6	Hexylresorcinol	13552-72-2	(+)-Isocorydine hydrochloride

497-76-7	Arbutin	697797-51-6	UAMC 00039 dihydrochloride
908-54-3	Diminazene Aceturate	1402727-29-0	PE859
6419-36-9	3-Pyridylacetic acid hydrochloride	847925-91-1	RO4929097
81110-73-8	Racecadotril	254750-02-2	Emricasan
50924-49-7	Mizoribine	161314-82-5	CGS 27023A
7414-83-7	Sodium etidronate	150080-09-4	Talabostat mesylate
103-16-2	Monobenzone	1441674-54-9	Ledipasvir acetone
1180-71-8	Limonin	130370-60-4	Batimastat
472-15-1	Betulinic acid	54857-86-2	TOFA
329-98-6	PMSF	1009734-33-1	HZ1157
42017-89-0	Fenofibric acid	188062-50-2	Abacavir sulfate
196597-26-9	Ramelteon	127373-66-4	Sivelestat
155213-67-5	Ritonavir	1132935-63-7	Dasabuvir
850649-62-6	Alogliptin Benzoate	20575-57-9	Calycosin
179324-69-7	Bortezomib	673-22-3	4-Methoxysalicylaldehyde
546-88-3	Acetohydroxamic acid	111-20-6	Sebacic acid
129618-40-2	Nevirapine	53936-56-4	Deoxyarbutin
192725-17-0	Lopinavir	490-78-8	2-5-dihydroxyacetophenone
39809-25-1	Penciclovir	29700-22-9	Oxyresveratrol
916170-19-9	AOB2796	88321-09-9	Aloxistatin
176161-24-3	Maribavir	864953-29-7	Fostemsavir
1029877-94-8	Trelagliptin succinate	519055-62-0	Tasisulam
1201902-80-8	MLN9708	425386-60-3	Semagacestat
354812-17-2	SC514	35943-35-2	Triciribine
1072833-77-2	Ixazomib	331862-41-0	IMR-1A
871038-72-1	Raltegravir potassium	310456-65-6	IMR1
863329-66-2	PSI6206	210344-98-2	Z-IETD-FMK
82009-34-5	Cilastatin	1624602-30-7	VR23
480-18-2	Taxifolin	161814-49-9	Amprenavir
82956-11-4	Nafamostat mesylate	1312782-34-5	AA26-9
1009119-65-6	Daclatasvir dihydrochloride	1051375-16-6	Dolutegravir
635728-49-3	Darunavir Ethanolate	1026785-59-0	Lomibuvir
142880-36-2	Ilomastat	112246-15-8	Ginsenoside Rh2
697761-98-1	Elvitegravir	256477-09-5	UK371804
1051375-19-9	Dolutegravir sodium	147859-80-1	CA-074 methyl ester
84687-43-4	Astragaloside IV	1404437-62-2	ML281
7770-78-7	Arctigenin	591778-68-6	CP 640186
83-48-7	Stigmasterol	5631-68-5	Hydroumbellic acid
478-01-3	Nobiletin	831-61-8	Ethyl gallate
34157-83-0	Celastrol	2469-34-3	Senegenin
29031-19-4	Glucosamine sulfate	28831-65-4	lithospermic acid

27409-30-9	Picoside I	209984-56-5	Dibenzazepine
848141-11-7	Alvelestat	209984-57-6	LY411575
128-53-0	N-Ethylmaleimide	1221573-85-8	Paritaprevir
208255-80-5	DAPT	1190307-88-0	Sofosbuvir
865759-25-7	Trelagliptin	1421438-81-4	Crenigacestat
187389-52-2	Z-VAD(OMe)-FMK	1338225-97-0	Doravirine
514-10-3	Abietic Acid	847499-27-8	Delanzomib
229975-97-7	Atazanavir sulfate	25406-64-8	Morrionside
136470-78-5	Abacavir	20633-67-4	Calycosin-7-O-beta-D-glucoside
354813-19-7	Balicatib	59870-68-7	Glabridin
868540-17-4	Carfilzomib	58749-22-7	Licochalcone A
198904-31-3	Atazanavir	1377049-84-7	Velpatasvir
274901-16-5	Vildagliptin	402957-28-2	Telaprevir
244767-67-7	Dapivirine	603139-19-1	Odanacetib
292605-14-2	SB-3CT	206361-99-1	Darunavir
179461-52-0	PD 151746	850876-88-9	Danoprevir
315183-21-2	PAC1	159989-65-8	Nelfinavir Mesylate
59721-29-8	Camostat mesilate	935888-69-0	Oprozomib
154598-52-4	Efavirenz	30827-99-7	AEBSF hydrochloride
1192224-24-0	Des(benzylpyridyl) Atazanavi	273404-37-8	Belnacasan
1194044-20-6	LY2811376	210344-95-9	Z-DEVD-FMK
313967-18-9	FLI06	197855-65-5	Z-FA-FMK
218156-96-8	SRPIN340	149488-17-5	Trovirdine
7497-07-6	NSC 405020	133407-82-6	MG132
103476-89-7	Leupeptin Hemisulfate	1051375-10-0	Cabotegravir
57-11-4	Stearic acid	1146699-66-2	Avagacestat

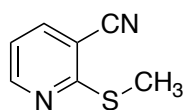
343

344 All other compounds used in the study were synthesized and quality checked according to the

345 following protocols.

346

347



348 2-(methylthio)nicotinonitrile (xx-1) The title compound was prepared according to a published

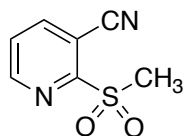
349 procedure; spectral data are in agreement with literature values⁴¹.

350 ^1H NMR (500 MHz, CDCl_3) δ 8.60 (dd, $J = 5.0, 1.8$ Hz, 1H), 7.79 (dd, $J = 7.7, 1.8$ Hz, 1H), 7.07
351 (dd, $J = 7.7, 4.9$ Hz, 1H), 2.64 (s, 3H).

352 ^{13}C NMR (126 MHz, CDCl_3) δ 163.7, 152.2, 140.6, 118.4, 115.7, 107.5, 13.4.

353 HRMS High accuracy (ASAP): Calculated for $\text{C}_7\text{H}_6\text{N}_2\text{S}$ (M+H) $^+$: 151.0330; found: 151.0327.

354



356 2-(methylsulfonyl)nicotinonitrile (xx-2) The title compound was prepared from xx-1 according to
357 a modified procedure from the literature⁴².

358

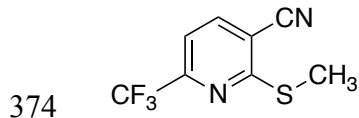
359 Pyridine xx-1 (87 mg, 0.58 mmol, 1 equiv) was weighed into 50 mL round bottom flask equipped
360 with a stir bar. The solid was dissolved in 10 mL of anhydrous MeOH, followed by portion-wise
361 (usually 3 portions) additions of mCPBA (500 mg, 2.9 mmol, 5 equiv). Substrate conversion was
362 monitored via TLC analysis (70% EtOAc:Hex to 100% EtOAc), with up to an additional 5 equiv
363 of mCPBA added if necessary. Upon complete conversion of starting material, the reaction was
364 quenched with 20 mL of saturated aqueous NaHCO_3 , diluted with an additional 20 mL of DCM.
365 The layers were separated and the organic layer was further washed (2x) with 10 mL of of
366 saturated aqueous NaHCO_3 . The organic layer was then dried *in vacuo* and purified via silica
367 gel column chromatography (100% EtOAc to 5% MeOH:EtOAc) to yield 51 mg (48% yield) of
368 the desired sulfone as a white solid.

369 ^1H NMR (400 MHz, CDCl_3) δ 8.86 (dd, $J = 4.8, 1.6$ Hz, 1H), 8.26 (dd, $J = 7.9, 1.6$ Hz, 1H), 7.70
370 (dd, $J = 7.9, 4.8$ Hz, 1H), 3.39 (s, 3H).

371 ^{13}C NMR (101 MHz, CDCl_3) δ 159.8, 151.8, 143.8, 126.8, 113.2, 107.4, 40.1.

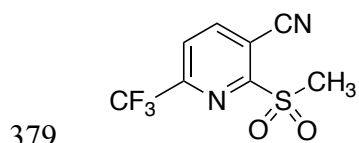
372 HRMS High accuracy (ASAP): Calculated for $\text{C}_7\text{H}_6\text{N}_2\text{O}_2\text{S}$ (M+H) $^+$: 183.0228; found: 183.0223.

373



375 2-(methylthio)-6-(trifluoromethyl)nicotinonitrile (xx-3) The title compound was prepared following
376 the published procedure, the product (yellow solid) was carried towards the synthesis of xx-4
377 without further purification⁴¹.

378



380 2-(methylsulfonyl)-6-(trifluoromethyl)nicotinonitrile (xx-4) The title compound was prepared from
381 xx-3 according to a modified procedure from the literature⁴².

382

383 Pyridine xx-2 (105 mg, 0.50 mmol, 1 equiv) was weighed into 50 mL round bottom flask
384 equipped with a stir bar. The solid was dissolved in 10 mL of anhydrous MeOH, followed by
385 portion-wise (usually 3 portions) additions of mCPBA (0.86 mg, 5 mmol, 10 equiv). Substrate
386 conversion was monitored via TLC analysis (40% EtOAc:Hex to 60% EtOAc). Upon complete
387 conversion of starting material, the reaction was quenched with 20 mL of saturated aqueous
388 NaHCO₃, diluted with an additional 20 mL of DCM. The layers were separated and the organic
389 layer was further washed (2x) with 10 mL of saturated aqueous NaHCO₃. The organic layer
390 was then dried *in vacuo* and purified via silica gel column chromatography (40% to 80%
391 EtOAc:Hex) to yield 38 mg (30% yield) of the desired sulfone as a white solid.

392 ¹H NMR (400 MHz, CDCl₃) δ 8.49 (dd, *J* = 8.1, 0.7 Hz, 1H), 8.06 (d, *J* = 8.1 Hz, 1H).

393 ¹³C NMR (101 MHz, CDCl₃) δ 160.4, 149.7 (q, *J* = 37.6 Hz), 146.0, 123.7 (q, *J* = 2.3 Hz), 120.0
394 (q, *J* = 275.5 Hz), 112.1, 109.7, 39.7.

395 ^{19}F NMR (376 MHz, CDCl_3) δ -68.28.

396 HRMS High accuracy (ASAP): Calculated for $\text{C}_8\text{H}_5\text{F}_3\text{N}_2\text{O}_2\text{S}$ ($\text{M}+\text{H}$) $^+$: 251.0102; found: 251.0104.

397

398 Compound 11a was synthesized according to the specified protocol in Dai. et. al. 2020²⁶. Compound

399 11a was confirmed by LCMS with m/z = 453 ($\text{M}+1$) and 451 ($\text{M}-1$).

400

401 Compound 4 was synthesized according to the procedure described in Yang et. al 2006²⁵. AZVIII-

402 34D was formed as a byproduct (15%) in the synthesis of Compound 4 and was isolated by RP

403 HPLC. ^1H NMR (400 MHz, CDCl_3) 7.88 (s, 1H), 7.44-7.34 (m, 5H), 6.80 (d, 1H, J = 15.4 Hz), 6.69 (s,

404 1H), 6.16-5.87 (m, 2H), 5.12 (s, 2H), 4.76 (s, 1H), 4.42 (s, 1H), 4.31-3.99 (m, 4H), 3.39 (s, 2H), 2.40-

405 1.45 (m, 8H), 1.29–1.20 (m, 12H), 1.13-1.02 (m, 3H), 1.00 – 0.89 (m, 6H). MS $\text{M}+\text{H}$ = 631.

406

407 AZVIII-38, AZVIII-30, and AZVIII-42 were synthesized according the procedures described in Mou

408 et. al. 2008⁴³. AZVIII-37A was synthesized as described in Prior et al. 2013. AZVIII-33B was

409 synthesized according to the method described in Amblard et. al. 2018⁴⁴.

410

411 AZVIII-41A was synthesized according to the procedure described for synthesizing AZVIII-33B in

412 Amblard et. al. 2018 and substituting Z-Leu-OH for BOC-Leu-OH⁴⁴. ^1H NMR (400 MHz, CDCl_3) 7.77

413 (s, 1H), 7.41-7.27 (m, 5H), 6.80 (dd, 1H, J = 5.5, 15.6 Hz), 5.98-5.84 (m, 2H), 5.15-5.03 (m, 2H),

414 4.55 (bd s, 1H), 4.27 (bd s, 1H), 4.23-4.07 (m, 3H), 3.38-3.23 (m, 2H), 2.56–1.40 (m, 8H), 1.33 –

415 1.20 (m, 3H), 0.95 (s, 6H). MS $\text{M}+\text{H}$ = 474.

416

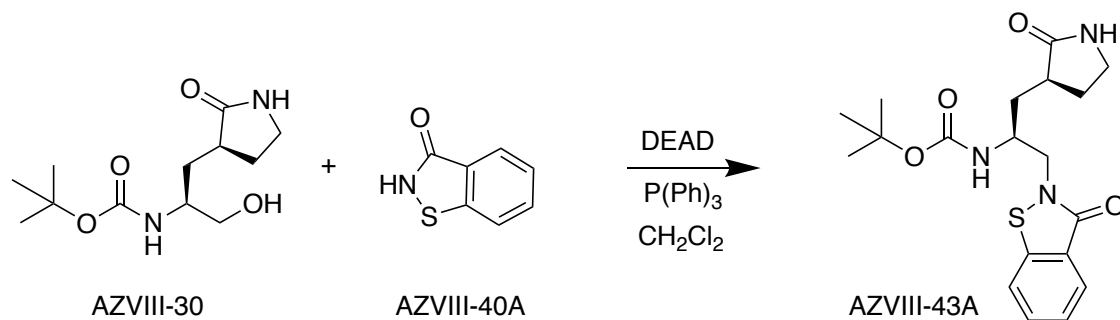
417 AZVII-43A was synthesized by treatment of a dichloromethane solution of AZVIII-30 and AZVIII-40A

418 with triphenyl phosphine and diethyl azodicarboxylate. ^1H NMR (400 MHz, CDCl_3) 7.91 (d, 1H, J =

419 8.0), 7.76 (d, 1H, J = 8.0), 7.55-7.34 (m, 2H), 6.16 (bd s, 1H), 5.29 (bd s, 1H), 4.73-4.39 (m, 2H),

420 4.35-3.96 (m, 1H), 3.58-3.16 (m, 2H), 3.79-3.43 (m, 2H), 3.39-3.91 (m, 1H), 1.98-1.77 (m, 1H), 1.73-

421 1.57 (m, 1H), 1.42 (s, 9H). MS $\text{M}+\text{H}$ = 392.



422

423

424 AZVIII-44B, AZVIII-44D, AZVIII-44E, AZVIII-44H, AZVIII-49C, and AZVIII-49F were synthesized by

425 treatment of the appropriate arylalkylamine or heteroarylalkylamine with the corresponding

426 arylsulfonyl chloride or heteroarylsulfonyl chloride and diisopropylethylamine in dichloromethane.

427 AZVIII-57D was synthesized by treatment of AZVIII-44D with methyl bromoacetate and potassium

428 carbonate in dimethylformamide to give the corresponding ester which was reduced to the

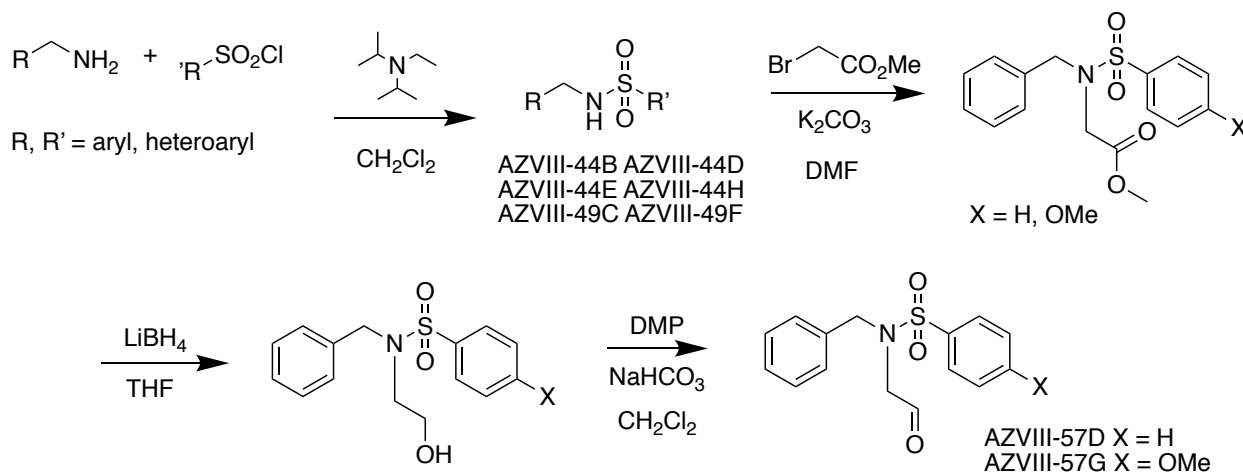
429 corresponding alcohol by treatment with lithium borohydride in tetrahydrofuran. The alcohol was

430 then treated with 1,1,1-tris(acetyloxy)-1,1-dihydro-1,2-benziodoxol-3-(1*H*)-one and sodium

431 bicarbonate in dichloromethane to give to the corresponding aldehyde AZVIII-57D. AZVIII-57G was

432 synthesized from *N*-benzyl-4-methoxybenzenesulfonamide according to the procedure used to

433 synthesize AZVIII-57D.



434

435 GC373 was synthesized from GC376 by converting the bisulfite group to an aldehyde group by

436 treatment with aqueous sodium bicarbonate. To a solution of 5.31 mg of GC376 in 200 μL H_2O , 2 μL

437 of saturated NaHCO₃ was added. The cloudy mixture was extracted with CH₂Cl₂, dried over Na₂SO₄,
438 and concentrated in vacuo to give GC373 as a colorless oil (4.0 mg).

439 **Data availability**

440 All reagents generated in this study are without restriction. Plasmids generated in this study will be
441 deposited to Addgene. Source data for all figures are provided with this manuscript online. All
442 statistics were performed using Prism v.8.4.2.

443

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453

454 **Author contributions**

455 S.J.R., S.I., and A.C conceived the project. S.J.R., S.I., B.R.S., D.D.H., and A.C. planned and
456 designed the experiments. S.J.R. performed crystal violet-based assays. S.J.R. and S.K. performed
457 the HEK293-EYFP based assays. S.J.R., S.I., and S.J.H. cloned plasmids. A.Z. and H.L.
458 synthesized compounds and provided compound structure information for synthesized compounds.
459 N.E.S.T. and T.R. synthesized compounds NT-1-21, NT-1-24, and NT-1-32. M.S.N. and Y.H.
460 conducted the live virus assays. S.K. and H.Y. performed imaging and created the HEK293-EYFP
461 cell line. S.J.R. and S.M. conducted data analysis. S.J.R, S.I., S.J.H, and A.C. wrote the manuscript
462 with input from all authors.

463

464 **Competing interests**

465 S.I., H.L., A.Z., B.R.S., A.C., and D.D.H. are inventors on a patent application submitted based on
466 some of the molecules described in this work. B.R.S. is an inventor on additional patents and patent
467 applications related to small molecule therapeutics, and co-founded and serves as a consultant to
468 Inzen Therapeutics and Nevrox Limited.

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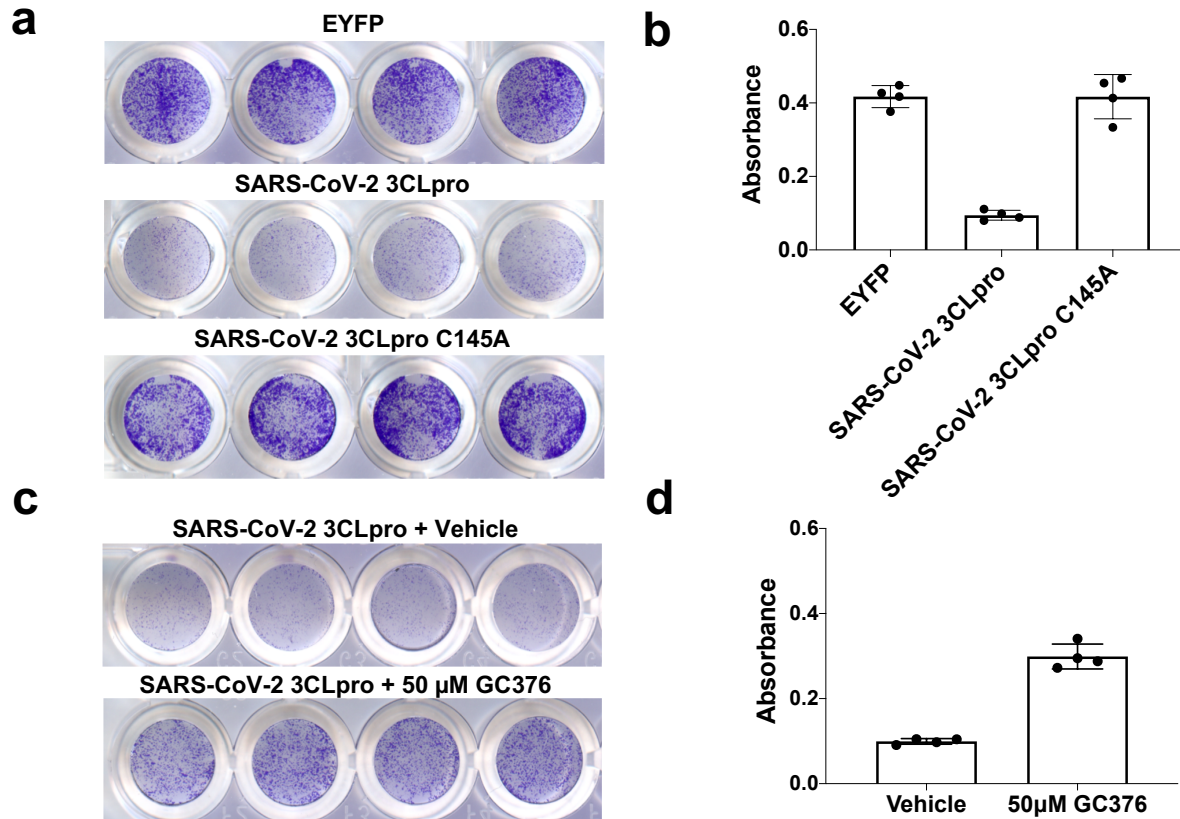
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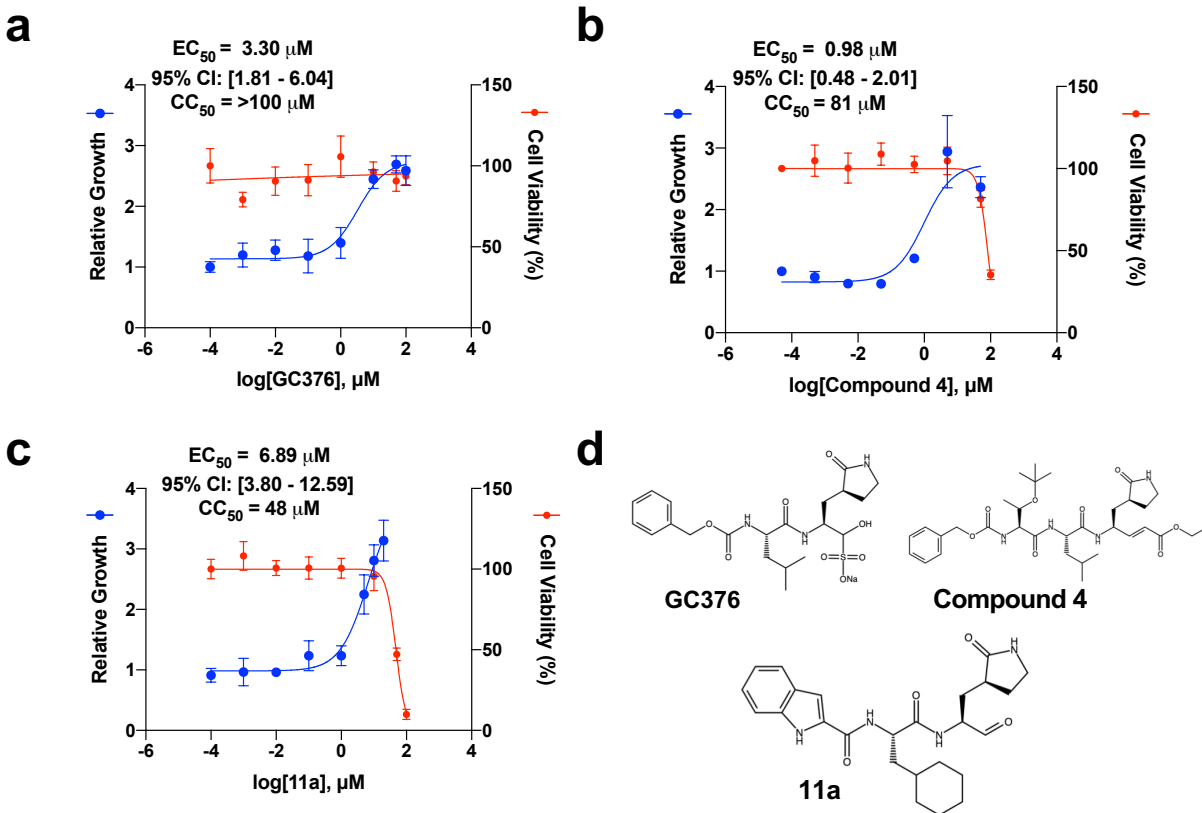
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572 **Fig. 1. Expression of SARS-CoV-2 3CLpro in HEK293T cells results in toxicity that can be**
573 **rescued by GC376. a.** SARS-CoV-2 3CL toxicity is dependent on protease activity and can be
574 visualized with crystal violet staining. **b.** Quantification of crystal violet staining in a. **c.** Treatment of
575 SARS-CoV-2 3CLpro expressing cells with protease inhibitor GC376 results in rescue of cytotoxicity.
576 **d.** Quantification of c. Data are shown as mean \pm s.d. for four technical replicates.



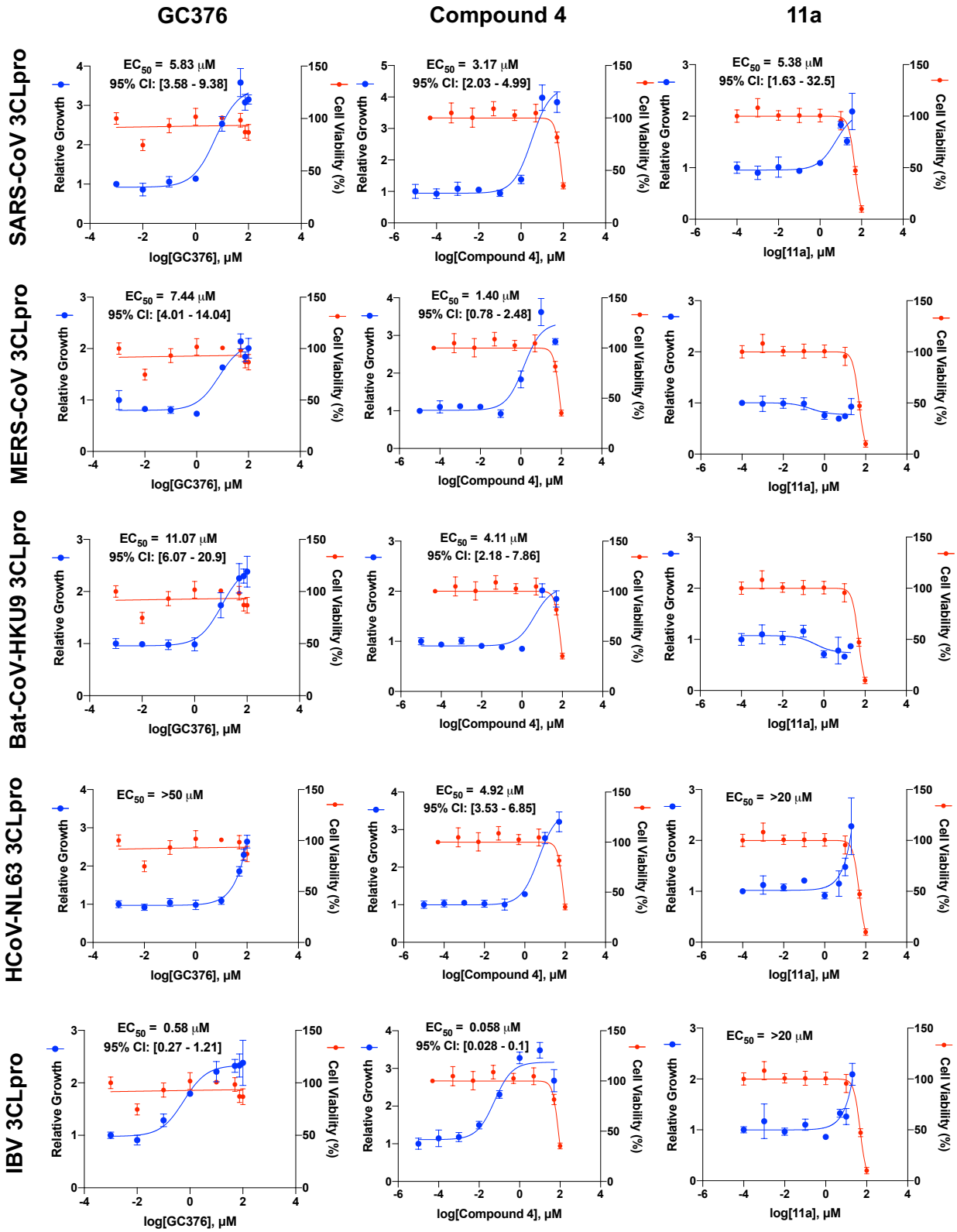
577

578 **Fig. 2. Dose response curves for SARS-CoV-2 3CLpro can be conducted with transfection-**
579 **based assays. a-c.** SARS-CoV-2 3CLpro can be inhibited by known 3CLpro inhibitors GC376,
580 compound 4, and 11a. The toxicity of each compound was determined by treating EYFP-transfected
581 cells with indicated concentrations of drug and is reported as Cell Viability. **d.** Chemical structures for
582 each of the compounds tested. EC₅₀ values are displayed as best-fit value alongside 95%
583 confidence interval. CC₅₀ values are displayed as best-fit value. Data are shown as mean ± s.d. for
584 four technical replicates.



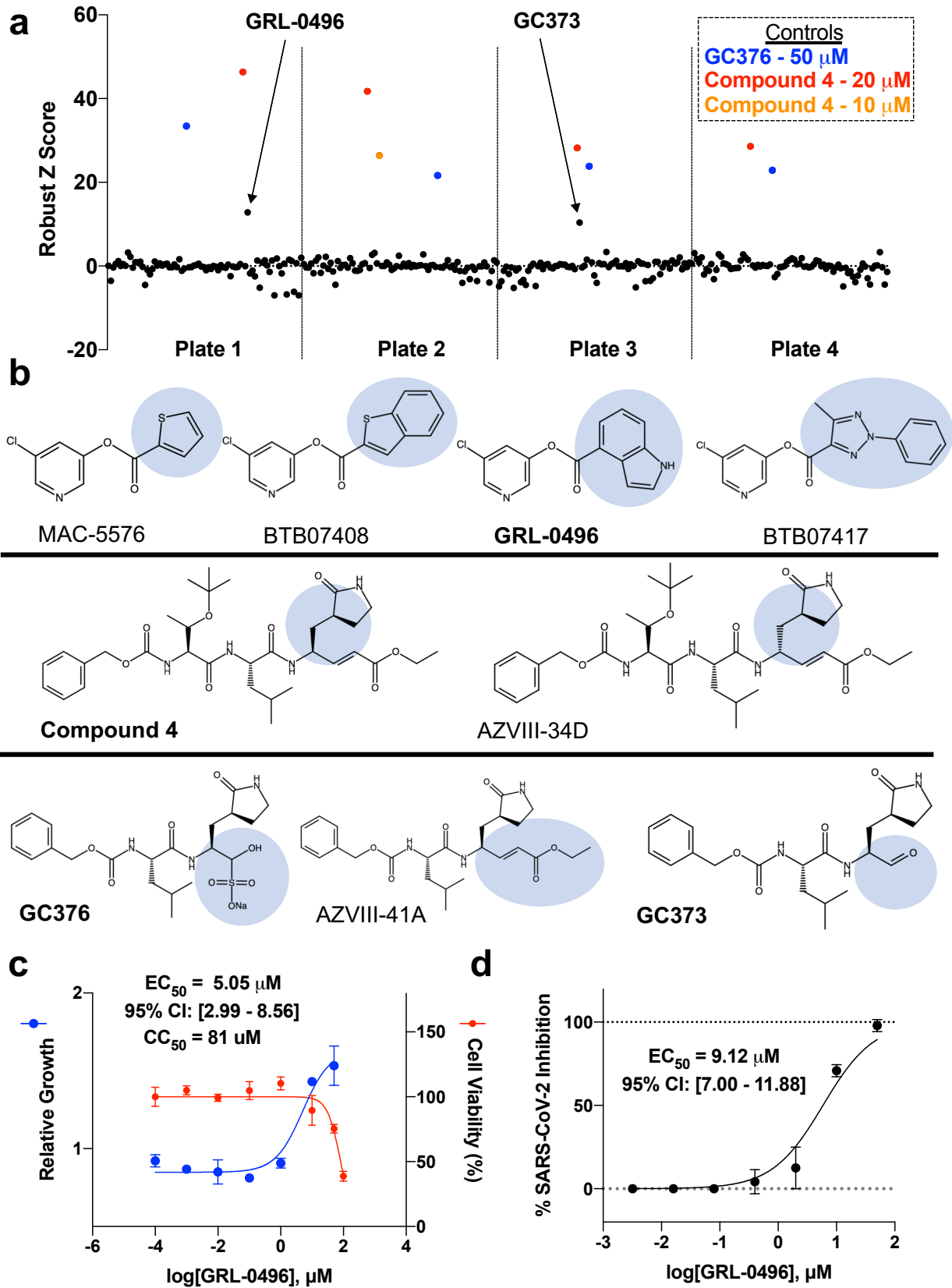
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586 **Fig. 3. The activity of GC376, compound 4, and 11a show variable effectiveness and potency**
587 **against the coronavirus 3CL proteases from SARS-CoV, MERS-CoV, Bat-CoV-HKU9, HCoV-**
588 **NL63, and IBV.** EC₅₀ values are displayed as best-fit value alongside 95% confidence interval. CC₅₀
589 values are displayed as best-fit value. Data are shown as mean ± s.d. for three or four technical
590 replicates.



592 **Fig. 4. Small-scale drug screen and structure-activity profiling at 10 μ M identify two**
593 **compounds, GC373 and GRL-0496, with activity against the SARS-CoV-2 3CLpro. a.**

594 Identification of hits from the drug screen and structure-activity profiling. Positive control compounds
595 were included in each plate and are highlighted. **b.** Compounds with structural similarity to known
596 inhibitors. Compounds in bold are molecules that show activity against the SARS-CoV-2 3CLpro at
597 10 μ M. **c.** Dose-response profiling and cytotoxicity determination of GRL-0496 against the SARS-
598 CoV-2 3CLpro. **d.** Live virus testing of GRL-0496 against SARS-CoV-2.
599 EC_{50} values are displayed as best-fit value alongside 95% confidence interval. The live virus assay
600 was conducted with two biological replicates, each with three technical replicates and the EC_{50} value
601 was derived from all replicates. CC_{50} values are displayed as best-fit value. Data are shown as mean
602 \pm s.d. for three or four technical replicates.



603

604 **Table 1. Comparison of literature reported live virus based EC₅₀ values compared to values**
 605 **generated during this study.** CPE = Cytopathic effect.

Protease	Drug	Calculated EC ₅₀ (μM)	Literature Reported Value (μM)	Method	Cell Line	Citation
SARS-CoV-2 3CLpro	GC376	3.3	3.37	CPE	Vero 76	Ma et. al.
			0.9	Plaque Assay	Vero E6	Vuong et. al.
			0.18	qPCR	Vero E6	Luan et. al.
			2.2	qPCR	Vero E6	Froggatt et. al.
			4.48	CPE	Vero E6	Iketani et. al.
SARS-CoV-2 3CLpro	11a	6.89	2.06	CPE	Vero E6	This study
			0.53	Plaque Assay	Vero E6	Dai et. al.
SARS-CoV-2 3CLpro	Compound 4	0.98	3.023	CPE	Vero E6	Iketani et. al.
SARS-CoV-2 3CLpro	GRL-0496	5.05	9.12	CPE	Vero E6	This study
SARS-CoV 3CLpro	GRL-0496	7.84	6.9	CPE	Vero E6	Ghosh et. al.
SARS-CoV-2 3CLpro	GC373	2.8	1.5	Plaque Assay	Vero E6	Vuong et. al.
			4.83	CPE	Vero E6	This Study

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