

1 **Inhibition mechanism and antiviral activity of an α -ketoamide based SARS-CoV-2 main
2 protease inhibitor**

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33 **Abstract**

34

35 SARS-CoV-2 has demonstrated extraordinary ability to evade antibody immunity by
36 antigenic drift. Small molecule drugs may provide effective therapy while being part of a
37 solution to circumvent SARS-CoV-2 immune escape. In this study we report an α -ketoamide
38 based peptidomimetic inhibitor of SARS-CoV-2 main protease (M^{pro}), RAY1216. Enzyme
39 inhibition kinetic analysis established that RAY1216 is a slow-tight inhibitor with a K_i of 8.6
40 nM; RAY1216 has a drug-target residence time of 104 min compared to 9 min of PF-
41 07321332 (nirmatrelvir), the antiviral component in Paxlovid, suggesting that RAY1216 is
42 approximately 12 times slower to dissociate from the protease-inhibitor complex compared to
43 PF-07321332. Crystal structure of SARS-CoV-2 M^{pro} :RAY1216 complex demonstrates that
44 RAY1216 is covalently attached to the catalytic Cys145 through the α -ketoamide warhead;
45 more extensive interactions are identified between bound RAY1216 and M^{pro} active site
46 compared to PF-07321332, consistent with a more stable acyl-enzyme inhibition complex for
47 RAY1216. In cell culture and human ACE2 transgenic mouse models, RAY1216
48 demonstrates comparable antiviral activities towards different SARS-CoV-2 virus variants
49 compared to PF-07321332. Improvement in pharmacokinetics has been observed for
50 RAY1216 over PF-07321332 in various animal models, which may allow RAY1216 to be
51 used without ritonavir. RAY1216 is currently undergoing phase III clinical trials
52 (<https://clinicaltrials.gov/ct2/show/NCT05620160>) to test real-world therapeutic efficacy
53 against COVID-19.

54

55 **Introduction**

56

57 SARS-CoV-2 has become established in the human population through the coronavirus
58 disease 2019 (COVID-19) pandemic and is likely to remain in circulation. Owing to
59 multinational efforts, vaccines were rapidly rolled out in the early stage of the pandemic and
60 proved successful in saving lives. However, likely due to population immune pressures
61 established by infections and vaccinations, SARS-CoV-2 Omicron variants with highly
62 mutated spike proteins quickly emerged (Tian et al., 2022). Rapid emergence of highly
63 mutated variants has demonstrated the virus's extraordinary capacity to escape humoral
64 immunity, representing a great challenge to vaccines and therapeutic antibodies (Cox et al.,
65 2022; Harvey et al., 2021).

66

67 A number of small molecule SARS-CoV-2 therapeutics have been developed (Fenton
68 and Keam, 2022). This therapeutic strategy may be part of a solution to combat SARS-CoV-2
69 immune escape. Of note, the orally available drugs molnupiravir and Paxlovid have been
70 approved for COVID-19 treatment after being validated through clinical trials. Molnupiravir
71 (LAGEVRIO, also known as EIDD-2801) is a prodrug of N-hydroxycytidine; this mutagenic
72 ribonucleoside is a broad-spectrum antiviral agent targeting the viral RNA polymerase by
73 lethal mutagenesis. However, this molecule has also been shown to be mutagenic to the host
74 (Zhou et al., 2021). Paxlovid is a combination of PF-07321332 (nirmatrelvir) and ritonavir.
75 PF-07321332 is a peptidomimetic that selectively inhibits the SARS-CoV-2 main protease
76 (M^{pro} , also known as 3C-like (3CL) protease) (Owen et al., 2021; Zhao et al., 2022), while
77 ritonavir is a cytochrome P450 inhibitor that functions to slow down cytochrome P450-
78 mediated metabolism of PF-07321332 to improve bioavailability. However, the usage of
79 ritonavir limits the clinical application range of Paxlovid due to the drug-drug interaction,
80 which may cause potential safety issues. Therefore, our original goal is to aim for a drug
81 candidate endowed with a longer half-life while maintaining good enzyme inhibitory potency
82 as demonstrated by PF-07321332. We expect that such a newly designed M^{pro} inhibitor may
83 possess prolonged pharmacokinetic stability in human, which can hopefully avoid the usage
84 of ritonavir. The drug target of PF-07321332, M^{pro} , plays a role in the viral polyprotein pp1a
85 and pp1ab processing that is essential in the SARS-CoV-2 life cycle (Ziebuhr et al., 2000).
86 The M^{pro} gene has been observed to be relatively conserved among various SARS-CoV-2
87 variants, therefore M^{pro} represents a promising target for drug development for SARS-CoV-2.
88

89 Other than PF-07321332, multiple series of SARS-CoV-2 M^{pro} inhibitors have been
90 developed or discovered (Boras et al., 2021; Breidenbach et al., 2021; Dai et al., 2020;
91 Drayman et al., 2021; Gao et al., 2022; Jin et al., 2020; Kitamura et al., 2022; Ma et al., 2020;
92 Ma et al., 2021; Owen et al., 2021; Qiao et al., 2021; Quan et al., 2022; Unoh et al., 2022;
93 Zaidman et al., 2021; Zhang et al., 2020; Zhu et al., 2020b). With a few exceptions
94 (Breidenbach et al., 2021; Gao et al., 2022; Jin et al., 2020; Unoh et al., 2022; Zaidman et al.,
95 2021), the majority of these molecules are peptidomimetics which often exhibit poor
96 pharmacokinetic (PK) properties. In this study, we report a further peptidomimetic M^{pro}
97 inhibitor - RAY1216 currently in phase III clinical trial. Inspired by the successful HCV
98 protease inhibitor discovery program reported for telaprevir (Chen and Tan, 2005; Kwong et
99 al., 2011; Yip et al., 2004a; Yip et al., 2004b), RAY1216 was developed to feature an α -
100 ketoamide warhead and incorporates chemical moieties known to confer selectivity towards
101 coronavirus M^{pro}. Here we characterize in detail the kinetics of SARS-CoV-2 M^{pro} inhibition
102 by RAY1216 and determine the crystal structure of the covalent adduct with SARS-CoV-2
103 M^{pro}. Further, the antiviral activity, protection against SARS-CoV-2 variants in animal
104 models, and PK properties are reported, and compared to those of PF-07321332.
105

106 **Structure of RAY1216**

107

108 RAY1216 (**Fig. 1**) was developed via multiple rounds of optimization conducted at P1,
109 P2, P3, and P4 moieties and finally the covalent warhead was changed from nitrile to α -
110 ketoamide moiety. The details of the structure–activity relationship (SAR) optimizations will
111 be further disclosed in a separate report. RAY1216 was chemically synthesized (**Fig. S1**) and
112 the identity of the product is confirmed by NMR (**Fig. S2-S4**). The inhibitor features a
113 cyclopentyl substituted α -ketoamide warhead, a pyroglutamine with a pyrrolidinone
114 sidechain at P1 (this moiety is known to mimic glutamine, which dominates in the P1
115 position of coronavirus M^{pro} recognition sequences (Xiong et al., 2021)), a P2
116 cyclopentylproline, a P3 cyclohexylglycine and a P4 tri-fluoroacetamide (**Fig. 1**). The
117 absolute configuration of synthesized RAY1216 was confirmed by X-ray crystallography
118 (**Fig. S5**).
119

120 **In vitro inhibition of M^{pro} by RAY1216 compared to PF-07321332**

121

122 We used a fluorescence resonance energy transfer (FRET)-based peptide cleavage assay
123 (Grum-Tokars et al., 2008) to monitor SARS-CoV-2 M^{pro} activity (**Fig. S6-7**) and we

124 estimated a K_M of 31 μM and a k_{cat} of 0.12 s^{-1} for M^{pro} (**Fig. S6-8** and **Table S2-S3**). To
125 compare inhibition by RAY1216 and PF-07321332, M^{pro} (final concentration 80 nM as
126 determined by the Bradford assay) was added to a solution of substrate (20 μM) and inhibitor
127 (maximum concentration 444 nM, 2:3 dilution series down to 17 nM) in the assay buffer. The
128 increase in fluorescence intensity was monitored in real time over a period of one hour.
129 Representative replicates for RAY1216 or PF-07321332 are shown in **Fig. 2** (also see **Fig. S8**
130 and **S9**). Both compounds displayed a gradual onset of inhibitory activity; an initial relatively
131 uninhibited phase in product formation is followed by a gradual approach to pseudo-
132 equilibrium (“slow binding” inhibition (Morrison, 1982; Morrison and Walsh, 1988)).
133 Compound concentrations significantly lower than the nominal enzyme concentration caused
134 a prominent inhibitory effect (“tight binding” inhibition (Cha, 1975; 1976; Cha et al., 1975)).
135 The time course of the assay in the absence of inhibitors ([I] = 0) was markedly nonlinear due
136 to substrate depletion. Under these particular experimental conditions, the classic algebraic
137 “ k_{obs} ” methods of enzyme kinetic analysis (Copeland, 2013) cannot be utilized. Instead,
138 combined progress curves obtained at various inhibitor concentrations were fit globally to a
139 system of first-order ordinary differential equations (ODE) solved by the software package
140 DynaFit (Kuzmic, 1996; 2009).

141 The data vs. model overlay plots in **Fig. 2a** and **Fig. 2b** illustrate that the overall
142 inhibitory potencies of RAY1216 and PF-07321332 are very similar. Note that at the three
143 highest inhibitor concentrations ([I] = 444, 296, and 198 nM) the reaction progress curves
144 become nearly horizontal at the end of the assay both in **Fig. 2a** and **Fig. 2b**. However, also
145 note that the approach to the quasi steady-state is markedly slower for RAY1216 when
146 compared with PF-07321332. This fundamental difference between the two compounds is
147 made most clearly visible in the instantaneous rate plots shown in **Fig. 2c** and **Fig. 2d**,
148 respectively. For example, at the highest inhibitor concentration ([I] = 444 nM, bottom curve
149 shown in red in **Fig. 2c**) it takes approximately 20 minutes for the enzyme to become fully
150 inhibited by RAY1216. In contrast, it takes less than one minute for the enzyme to become
151 fully inhibited by PF-07321332 under identical conditions. Note in **Fig. 2c** and **Fig. 2d** that
152 the reaction rate does not decrease to zero even at inhibitor concentrations significantly
153 higher than the enzyme concentration. This demonstrates the effective kinetic reversibility of
154 the observed enzyme–inhibitor interactions despite the fact that the crystal structure shows a
155 covalent binding mode (see below). Thus, RAY1216 appears to be an example of a
156 “reversible covalent” inhibitor (Bradshaw et al., 2015). Since the equilibrium dissociation
157 constants $K_i = k_d / k_a$ for the two compounds are similar (**Table 1**), while it takes very much

158 longer for RAY1216 to fully associate with the enzyme, it necessarily means that not only the
159 association rate constant but also the dissociation rate constant is very much lower for
160 RAY1216, in comparison with PF-07321332. In that sense, RAY1216 could be described as
161 a "slow-on, slow-off" inhibitor, whereas PF-07321332 inhibition of M^{pro} is "fast-on, fast-off".

162 The results of a comprehensive kinetic analysis using multiple replicates ($n = 3$, for each
163 inhibitor) are summarized in **Table 1** (see **Table S3-S4** for detailed analysis), where k_a is the
164 association rate constant and k_d is the dissociation rate constant. The inhibition constant K_i
165 and the drug-target residence time (t_{res}) were computed from these primary regression
166 parameters using the usual formulas (Copeland et al., 2006), while assuming that both
167 inhibitors are kinetically competitive with the fluorogenic peptide substrate (see *Methods* for
168 details). The results summarized in **Table 1** indicate that RAY1216 has a more than an order
169 of magnitude (12×) lower dissociation rate constant in comparison with PF-07321332. Thus,
170 the drug-target residence time for RAY1216 is measured in hours (1.7 hr), instead of in
171 minutes (9 min) in the case of PF-07321332. At the same time, the equilibrium binding
172 affinity of RAY1216 (8.4 nM) measured by the inhibition constant K_i is only approximately
173 two-fold lower than that of PF-07321332. Note that $K_i = (3.8 \pm 0.2)$ nM reported here for PF-
174 07321332 is in good agreement with $K_i = 3.1$ (1.5–6.8) nM previously reported by Pfizer
175 (Owen et al., 2021). The observed enzyme inhibition kinetics, in particular the drug-target
176 residence time results listed in **Table 1**, is consistent with slow-tight inhibition of M^{pro} by
177 RAY1216, suggesting that RAY1216 forms a more stable enzyme-inhibitor complex (E-I)
178 than that formed by PF-07321332.
179

180 **Structure of RAY1216 bound to SARS-CoV-2 M^{pro}**

181

182 To further understand the activity of RAY1216, we soaked SARS-CoV-2 M^{pro} crystals
183 with 6 mM RAY1216 in crystallization solution and the structure of RAY1216 bound to M^{pro}
184 at 2.0 Å resolution was determined by X-ray diffraction (**Fig. 3a** and **Table S5**). We
185 identified unambiguous electron density consistent with RAY1216 molecules in both active
186 sites of M^{pro} dimer (**Fig. 3a** and **Fig. S10**) and the dimer appears to be largely symmetric
187 (**Fig. 3a** and **Fig. S11**). The electron density shows that RAY1216 is covalently attached to
188 M^{pro} via a thiohemiketal bond formed between the Sγ sulfur of the catalytic Cys145 and the
189 α-keto carbon of the RAY1216 warhead (**Fig. 3b** and **Fig. S10**). The α-ketoamide warhead at
190 the inhibitor P1' position is able to interact with the M^{pro} active site through a number of
191 potential hydrogen bonds: the oxyanion (or hydroxyl) group of the thiohemiketal accepts a

192 hydrogen bond from His41; and the warhead amide oxygen is within hydrogen bond
193 accepting distance of the backbone amides of Gly143, Ser144 and Cys145 which form the
194 canonical cysteine protease “oxyanion hole” (**Fig. 3c**). These interactions are consistent with
195 the proposal that the α -ketoamide represents a superior warhead through its ability to engage
196 two hydrogen bonding interactions to the target protease catalytic center, rather than just one
197 (Zhang *et al.*, 2020), as seen for aldehyde (Zhang *et al.*, 2020; Zhu *et al.*, 2011) or Michael
198 acceptor (Tan *et al.*, 2013; Zhang *et al.*, 2020) warheads. The cyclopentyl substituent on the
199 warhead amide is well defined by the electron density (**Fig. 3b** and **Fig. S10**) and is situated
200 4.2 Å from the sidechain of M^{pro} Leu27, demonstrating a hydrophobic contact between the
201 cyclopentyl moiety and the aliphatic Leu27 sidechain (**Fig. 3c**).
202

203 In the P2 position of RAY1216, the peptide bond is stabilized within the
204 cyclopentylproline moiety previously utilized at the P2 position of telaprevir (Lin *et al.*, 2006;
205 Qiao *et al.*, 2021). Electron density shows that the hydrophobic cyclopentyl ring slots snugly
206 into the groove between M49 and M165 (**Fig. 3b-c** and **Fig. S10**). Plasticity has been
207 observed for the S2 substrate binding pocket which accommodates the P2 moiety upon
208 inhibitor binding (**Fig. S11**) (Kneller *et al.*, 2020). It has been shown that S2 pockets in
209 coronavirus M^{pro} have a strong preference towards hydrophobic amino acids, particularly
210 leucine (Rut *et al.*, 2021; Sacco *et al.*, 2020; Xiong *et al.*, 2021). It has also been shown in a
211 separate study that dimethylcyclopropylproline and cyclopentylproline, used in boceprevir
212 and telaprevir respectively (**Fig. 1**), when incorporated in α -ketoamide inhibitors, can each
213 occupy the S2 pocket with similar potencies (Qiao *et al.*, 2021).
214

215 The P3 moiety of RAY1216 features a cyclohexyl group that extends towards the
216 exterior of the active site without making any direct contacts with M^{pro} (**Fig. 3c**). The density
217 for the cyclohexyl *para*-carbon positioned furthest from the active site cavity is weak (**Fig.**
218 **3b**), suggesting that the cyclohexyl group remains relatively flexible within the inhibitor-
219 enzyme complex. Nevertheless, it has been reported that substituents at P3 position can affect
220 both drug potency and pharmacokinetic properties (Owen *et al.*, 2021; Qiao *et al.*, 2021).
221

222 RAY1216 and PF-07321332 share the same γ -lactam and tri-fluoroacetamide moieties at
223 P1 and P4 respectively. The P1 γ -lactam is known as an optimal fragment for viral protease
224 inhibition as it mimics glutamine and has been proven to be responsible for potent inhibitory
225 activity against a variety of enzymes with specificity towards native substrates with a P1
226 glutamine (Dragovich *et al.*, 1999; Owen *et al.*, 2021; Qiao *et al.*, 2021). In the RAY1216:

227 M^{pro} complex, the γ -lactam nitrogen donates potential hydrogen bonds to the backbone
228 carbonyl oxygen of Phe140 (3.19 Å), to the carboxylate of Glu166 (3.17 Å), and to the
229 sidechain hydroxyl of Ser1 from the second monomer of the M^{pro} dimer (**Fig. 3c**). The γ
230 lactam carbonyl oxygen accepts a hydrogen bond (2.54 Å) from the imidazole of His163
231 (**Fig. 3c**). These interactions have also been observed in the complex formed between PF-
232 07321332 and M^{pro} (**Fig. 3d**) (Owen *et al.*, 2021). Clear electron density is observed for the
233 P4 tri-fluoroacetamide capping moiety in the RAY1216:M^{pro} complex structure (**Fig. 3b** and
234 **Fig.S10**), it contacts Leu167 sidechain and accepts a hydrogen bond from Gln192 amide
235 (**Fig. 3c**). Equivalent interactions have been observed in the PF-07321332:M^{pro} complex
236 structure (**Fig. 3d**) (Owen *et al.*, 2021). In summary, despite differences in the P1' warhead,
237 P2 bicycloproline and P3 substituent structures, interactions mediated by the P1 γ -lactam and
238 P4 tri-fluoroacetamide moieties are largely maintained between RAY1216 and PF-07321332.
239

240 Antiviral activities of RAY1216 in cell culture and mouse models

241

242 Based on the encouraging *in vitro* activity of RAY1216, we next sought to investigate
243 inhibitory activity of RAY1216 towards SARS-CoV-2 infection in cell and mouse model.
244 The 50% cytotoxic concentration (CC₅₀) of RAY1216 was determined to be 511 μ M for
245 VeroE6 cells (**Fig. S12**). In virus inhibition assays the half-maximal effective concentration
246 (EC₅₀) values for RAY1216 against different SARS-CoV-2 variants are 95 nM (WT), 130
247 nM (Alpha), 277 nM (Beta), 97 nM (Delta), 86 nM (Omicron BA.1) and 158 nM (Omicron
248 BA.5), respectively (**Fig. 4a**). The corresponding selectivity indices (SI, CC₅₀/EC₅₀) are
249 ~5380 (WT), ~3930 (Alpha), ~1850 (Beta), ~5270 (Delta), ~5940 (Omicron BA.1) and
250 ~3230 (Omicron BA.5), respectively (**Table 2 and Fig. S13**).
251

252 We further characterized the protective effect of RAY1216 against virus infection in a
253 human ACE2 transgenic mouse model (Bao *et al.*, 2020). Mice were intranasally challenged
254 with lethal doses (10⁵ PFU) of SARS-CoV-2 (Delta variant) and the protective effect of
255 RAY1216 was assessed. The mortality of the mice in the untreated virus-infected group was
256 100% at 5 days post-infection. RAY1216 administered at three different doses (600
257 mg/kg/day, 300 mg/kg/day and 150 mg/kg/day) was able to protect mice infected with
258 SARS-CoV-2 by 100%, 43% and 14%, respectively (**Fig. 4b**). This result suggests that
259 treatment with RAY1216 effectively prolonged survival of SARS-CoV-2 infected mice (**Fig.**
260 **4c**). To examine effect of RAY1216 on lung virus titre and pathology, a separate set of
261 experiments was performed with a non-lethal dose of virus inoculum (10^{3.5} PFU). RAY1216

262 (600 mg/kg/day and 300 mg/kg/day) decreased viral titres in lungs significantly compared
263 with the infection-only group (**Fig. 4d**). Compared to the infection-only group, RAY1216
264 (600 mg/kg/day) was able to reduce lung virus titre by more than 1 Log unit. This effect may
265 be slightly weaker for RAY1216 compared to PF-07321332 under the same experimental set-
266 up (**Fig. 4d and e**), but the difference is not statistically significant. Lung histopathology of
267 infected mice, compared to that of infected mice treated by RAY1216, shows that RAY1216
268 administered at 600 mg/kg/day and 300 mg/kg/day reduced virus induced pathology (**Fig. 4f**).
269 RAY1216 administered at a dose of 600 mg/kg/day provided a similar level of protection
270 against lung tissue inflammation injury to that observed with PF-07321332 (**Fig. 4f**).
271

272 **Pharmacokinetics of RAY1216**

273

274 Pharmacokinetics (PK) can significantly influence drug therapeutic efficacy. We next
275 examined pharmacokinetics of RAY1216 and PF-07321332 in head-to-head experiments.
276 RAY1216 and PF-07321332 show comparable *in-vitro* stabilities in plasmata of various
277 different animal species (**Table S6**), based on this result, we investigated *in-vivo* PK
278 properties of RAY1216 and compared with those of PF-07321332 in mice, rats and
279 cynomolgus macaques (**Table 3**). Following intravenous (IV) administration, RAY1216 has
280 plasma clearance (Cl) rates in the range of 10 – 22.5 mL/min/kg (compared to 23.4 – 30.2
281 mL/min/kg for PF-07321332) and elimination half-lives in the range of 0.9 - 3.8 h (compared
282 to 0.3 – 0.7 h for PF-07321332) among different animals. Following oral (PO)
283 administration, RAY1216 has elimination half-lives ranging between 2.6 – 14.9 h (compared
284 to 1.1-1.4 h for PF-07321332) among the different animal models. These characteristics
285 represent an improvement over PF-07321332, which demonstrates faster plasma clearance
286 and shorter elimination half-lives under equivalent conditions across all the animal models
287 tested. These *in vitro* and *in vivo* data indicate that RAY1216 may have promising human PK
288 profile. Indeed, it has been noted in a number of studies that α -ketoamides appear to possess
289 superior chemical and metabolic stability, particularly comparing to aldehyde based
290 peptidomimetics (Robello et al., 2021).
291

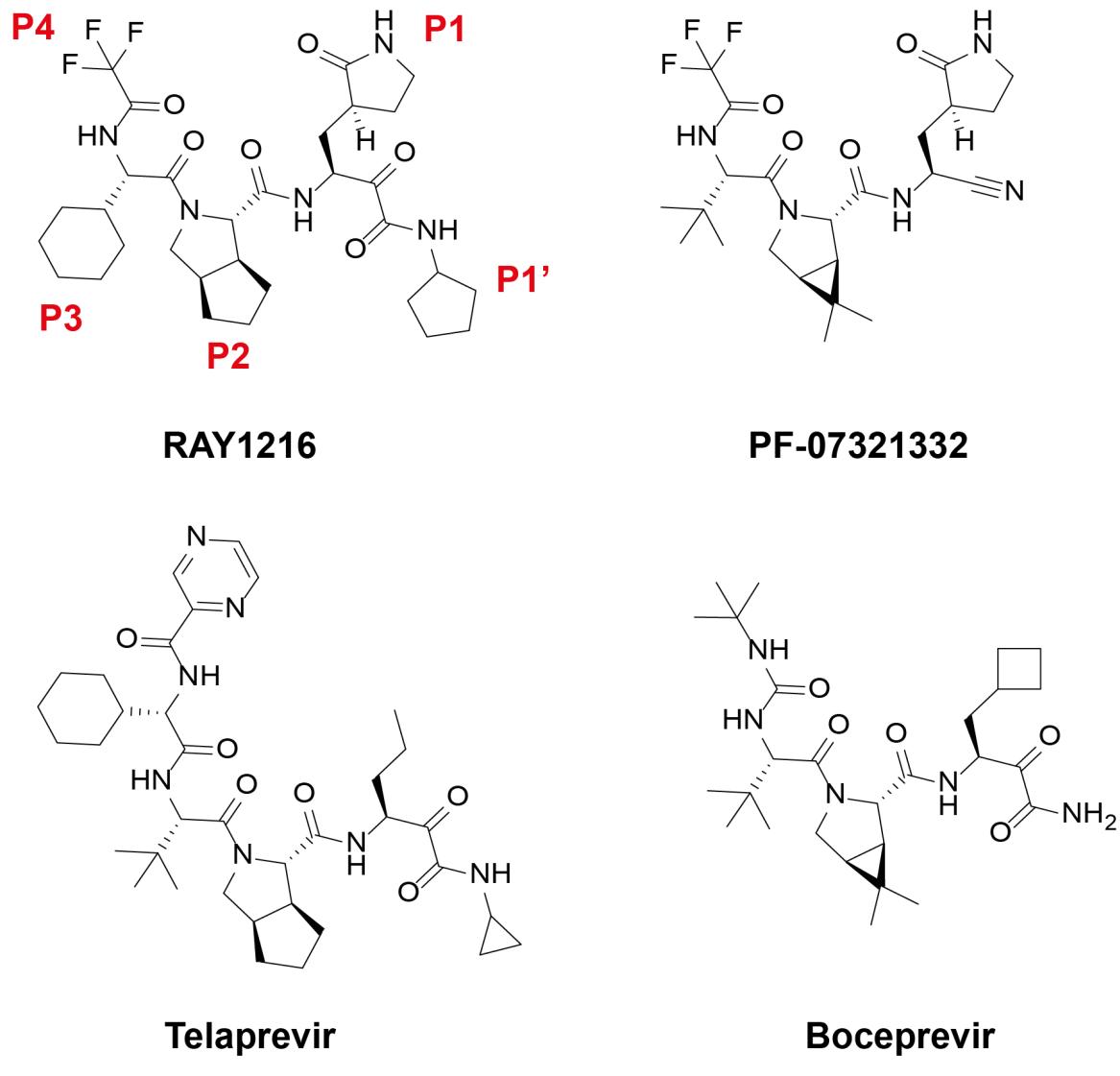
292 In mouse pharmacokinetics experiments, RAY1216 also exhibits improvements when
293 compared to PF-07321332, in area under the curve from time 0 extrapolated to last
294 (AUC_{0~last}) for serum drug concentration (**Fig. 5**). Based on EC₅₀/EC₉₀ values determined
295 using VeroE6 cells, a single IV dose of 3 mg kg⁻¹ maintained the RAY1216 plasma
296 concentration above EC₅₀ and EC₉₀ for 4 hours and 8 hours respectively. A single PO dose of

297 10 mg kg⁻¹ maintained the RAY1216 plasma concentration above EC₅₀ and EC₉₀ for 6 hours
298 and 10 hours respectively. Both parameters represent a marked improvement over those
299 obtained for PF-07321332, the active anti-viral component in Paxlovid.
300

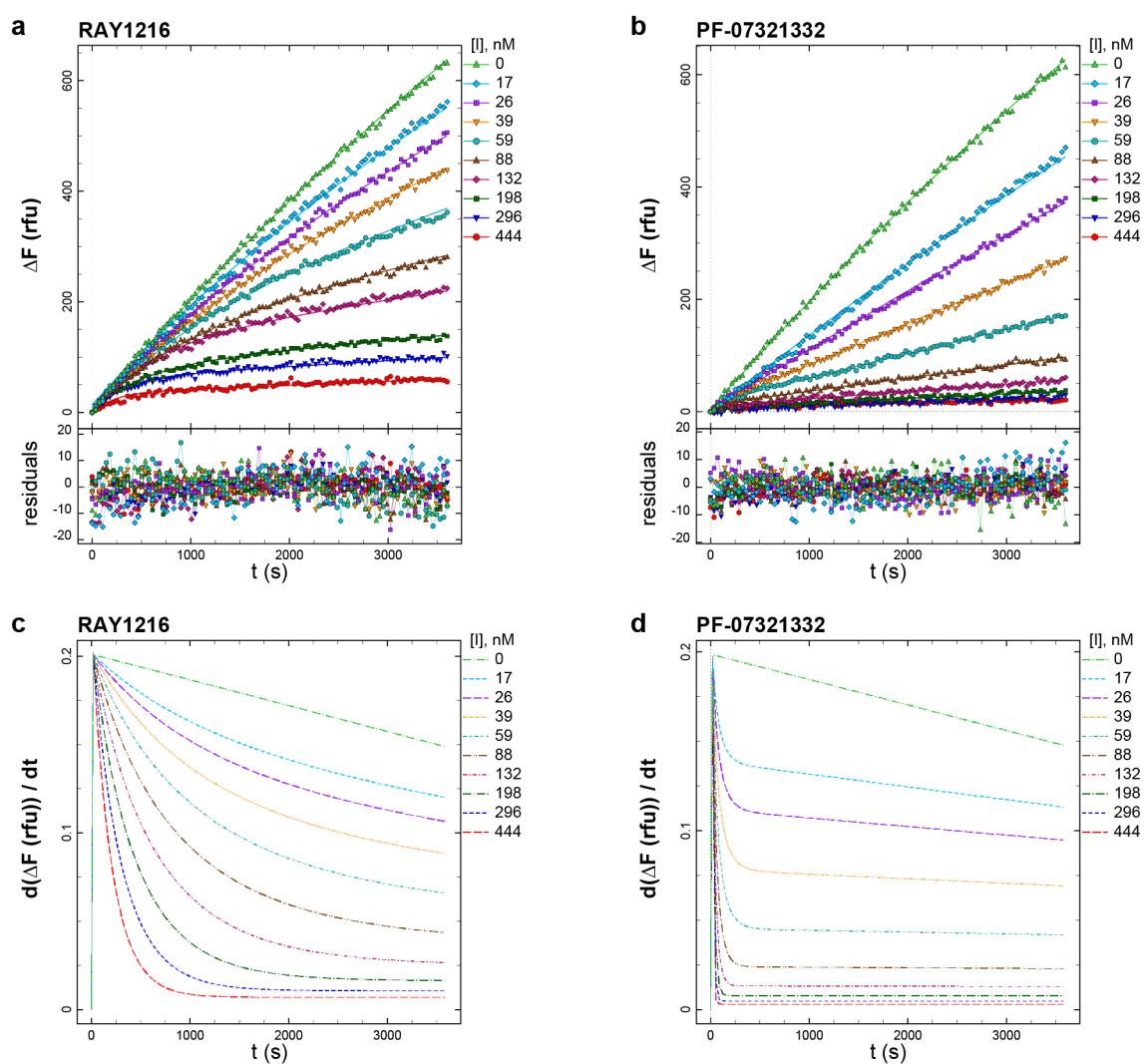
301 Discussion

303 In this study, we characterized inhibition of SARS-CoV-2 M^{pro} by RAY1216, a
304 peptidomimetic inhibitor. This compound features a cyclopentyl-substituted α -ketoamide
305 warhead, a P1 pyroglutamine (known to confer selectivity towards CoV M^{pro}), a P2
306 cyclopentylproline as originally utilized in the anti-HCV drug telaprevir (Yip *et al.*, 2004a), a
307 P3 cyclohexylglycine, and finally a P4 tri-fluoroacetamide as utilized in PF-07321332. We
308 found more extensive interactions between the covalently attached drug and the SARS-CoV-
309 2 M^{pro} enzyme in the crystal structure of the RAY1216:M^{pro} acyl enzyme complex, compared
310 with that of PF-07321332. In enzyme inhibition assays we found that RAY1216 inhibits M^{pro}
311 via a slow-tight mechanism, with an approximately 12-fold longer drug-target residence time.
312 These inhibition characteristics suggest that RAY1216 forms a more stable acyl-enzyme
313 adduct when compared with PF-07321332. It has recently emerged that drug-target residence
314 time is an important parameter to optimise for drug efficacy (Copeland *et al.*, 2006; Dahl and
315 Akerud, 2013; Lu and Tonge, 2010). In pharmacokinetic studies, RAY1216 showed
316 improved elimination half-lives compared to PF-07321332. This may allow its use without
317 ritonavir which is known to have significant unwanted drug-drug interactions.
318

319 In summary, RAY1216 possesses superior drug-target residence time and
320 pharmacokinetic properties when compared with PF-07321332 (nirmatrelvir), the active anti-
321 viral component in Paxlovid. On the other hand, PF-07321332 is slightly favoured over
322 RAY1216 in reducing mouse lung viral titre. The real-world efficacy of RAY1216 as a
323 potential therapeutic for the treatment of COVID-19 in humans will be revealed by phase III
324 clinical trial, which is currently ongoing.
325



326
327
328 **Fig 1. Chemical structures of RAY1216 and related anti-viral protease inhibitors.**
329



330
331
332 **Fig. 2 SARS-CoV-2 M^{pro} inhibition by RAY1216 and PF-07321332.** a, Progress curves of
333 M^{pro} inhibition (80 nM M^{pro}, 20 μM substrate) at different RAY1216 concentrations (data
334 points), reactions were started without preincubation. Progress curves are fit in DynaFit
335 (Kuzmic, 1996; 2009) using ODE method (lines) and residuals of the fits are shown. b,
336 Progress curves of M^{pro} inhibition by PF- 07321332 under the same experimental conditions
337 and they are fit in DynaFit using the same analysis procedure. c and d, Instantaneous reaction
338 rates derived from the fits to the progress curves. See *Methods* for mathematical and
339 statistical details of data-analytic procedures.
340

parameter	unit	RAY1216	PF-07321332	ratio RAY/PF
k_a	$\mu\text{M}^{-1} \text{s}^{-1}$	0.019 ± 0.001	0.49 ± 0.04	0.04
k_d	s^{-1}	0.000161 ± 0.000008	0.0018 ± 0.0002	0.09
$K_i = k_d/k_a$	nM	8.4 ± 0.2	3.8 ± 0.2	2.2
t_{res}	min	104 ± 5	9 ± 1	11.5

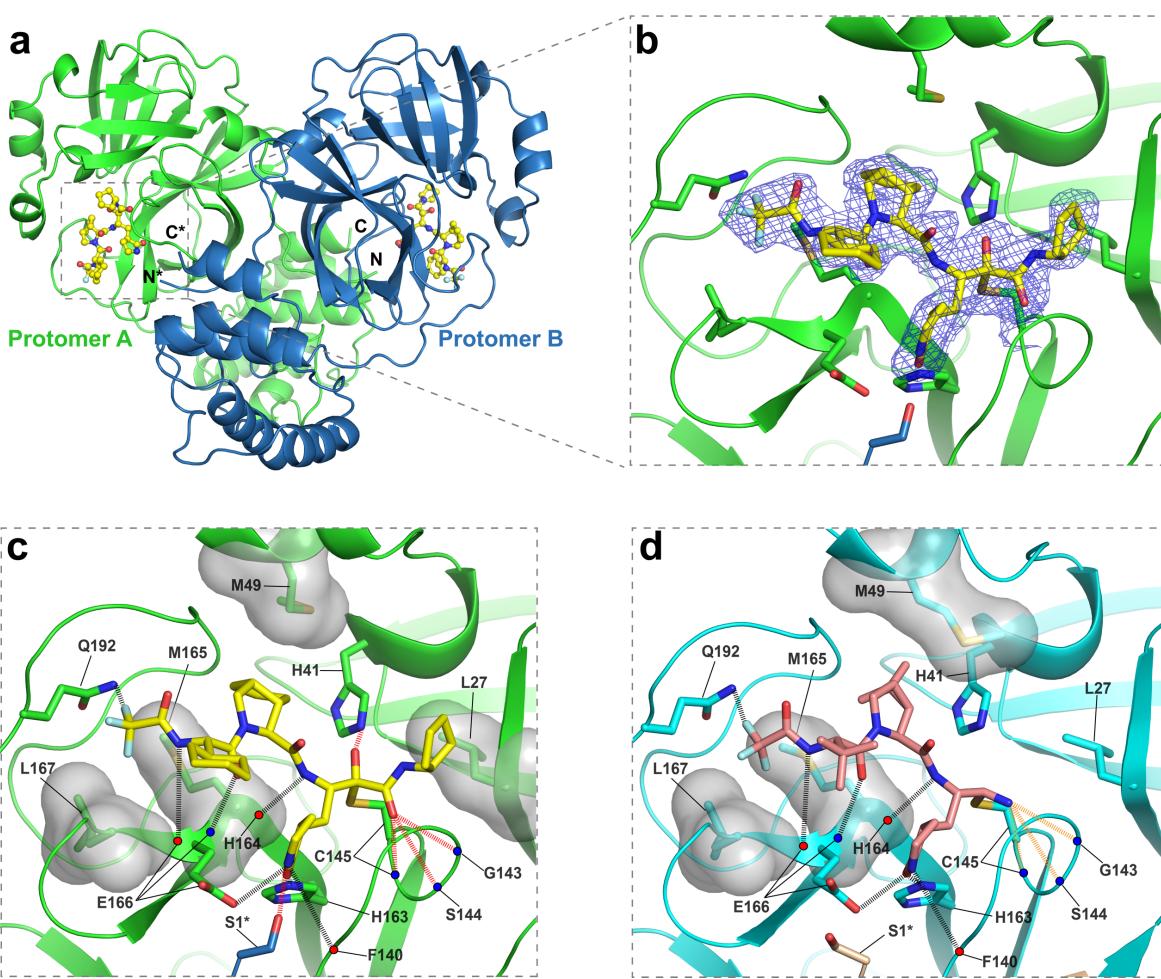
341

342 **Table 1. Kinetic parameters of M^{Pro} inhibition by RAY1216 and PF-07321332 as**
343 **determined by ODE method in DynaFit.** Mean and standard deviation from replicates ($n =$
344 3) are reported. See *Methods* for mathematical and statistical details of data-analytic
345 procedures.

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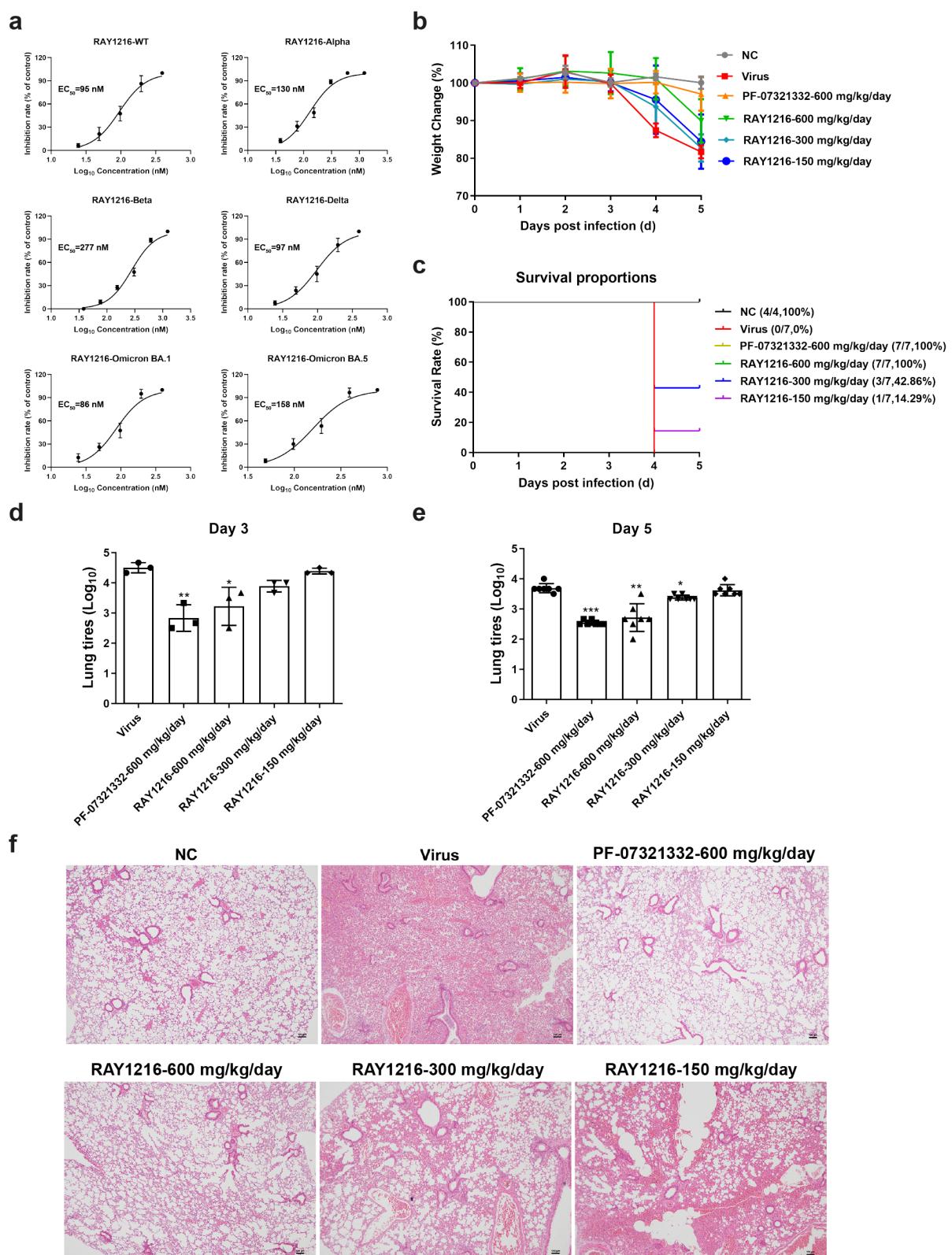
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351 **Fig. 3 Crystal structure of RAY1216 in complex with SARS-CoV-2 M^{pro}.** a, Cartoon
352 representation of the dimeric M^{pro} bound to RAY1216. Promoter A is in green, protomer B is
353 in blue and RAY1216 is shown as yellow ball-and-stick models in active sites of both M^{pro}
354 protomers. b, A zoom-in view of the RAY1216 bound active site of protomer A. 2Fo-Fc
355 density map (blue mesh, contoured at 1.3σ) is shown around bound RAY1216 and the
356 catalytic Cys145 side chain (also see Fig. S10 for omit map densities). Clear electron density
357 is observed for the thiohemiketal bond formed between the bound RAY1216 α-keto carbon
358 and the catalytic Cys145 sulfur. c, Same view as in b showing detailed interactions between
359 RAY1216 and active site of M^{pro}. Selected sidechains of interacting residues are shown,
360 backbone carbonyl and amide are represented as red and blue dots. d, Detailed interactions
361 between PF-07321332 and active site of M^{pro} (based on PDB: 7RFW (Owen *et al.*, 2021)) are
362 shown in the same view as in c. In c and d, Molecular surfaces of selected residues involved
363 in hydrophobic contacts with bound inhibitors are shown. Hydrogen bonds are shown in
364 dashed lines. Extra hydrogen bonds formed by RAY1216 to M^{pro} or hydrogen bonds of

365 different properties to M^{pro} between RAY1216 and PF-07321332 are highlighted with
366 colours.
367



368

369 **Fig 4. Antiviral activities of RAY1216 in cell culture and animal model. a,** Inhibition of
 370 SARS-CoV-2 wildtype ancestral strain and variants in cell culture. Protection of Vero E6 cell
 371 from cytopathic effect (CPE) of SARS-CoV-2 virus infection was assessed by MTT cell

372 viability assay (mean ± SD, $n = 3$). Virus inhibition titres are estimated from dose response
373 curves of cell survival vs RAY1216 concentration. **b** and **c**, body weight change (mean ± SD)
374 and survival rates of ACE2 transgenic C57BL/6 mice infected with SARS-CoV-2 after
375 receiving indicated daily oral doses of RAY1216, PF-07321332 or PBS control ($n = 7$). **d** and
376 **e**, SARS-CoV-2 virus titres (mean ± SD) in mouse lung tissues at 3 d.p.i. ($n = 3$) and 5 d.p.i
377 ($n = 7$) after receiving indicated daily doses of RAY1216 or PF-07321332. **f**, Comparison of
378 virus induced histology changes in mouse lung tissues after receiving indicated oral daily
379 doses of RAY1216, PF-07321332 ($n = 3$). Histology examples of no virus (NC) and virus
380 (virus) controls are included for comparison.
381
382

SARS-CoV-2 variant	RAY1216 (nM)		PF-07321332 (nM)	
	EC ₅₀	EC ₉₀	EC ₅₀	EC ₉₀
WT	95	228	94	193
Alpha	130	349	72	205
Beta	277	685	145	328
Delta	97	251	71	177
Omicron BA.1	86	204	107	215
Omicron BA.5	158	359	111	266

383 **Table 2. Antiviral activities of RAY1216 compared to PF-07321332 in cell culture.**
384
385

Compound	Species	dose (mg/kg)	C _{max} (nM)	T _{max} (h)	AUC _{0-last} (nM·h)	Cl (mL/min/kg)	Vd _{ss} (L/kg)	T _{1/2} (h)	oral F (%)
RAY1216	mouse	3.0 (IV)	--	--	7789	10	1.4	3.8	--
		10 (PO)	1287	2.0	5698	--	--	2.6	22
	rat	2.0 (IV)	--	--	4505	12.5	1.1	2.2	--
		10 (PO)	916	0.9	7429	--	--	4.3	33
	cynomolgus	1.0 (IV)	--	--	1157	22.5	1.0	0.9	--
PF-07321332	macaque	5.0 (PO)	102	1.5	458	--	--	14.9	8
	mouse	3.0 (IV)	--	--	3327	30.2	0.46	0.7	--
		10 (PO)	2715	0.3	3577	--	--	1.1	33
	rat	2.0 (IV)	--	--	1600	42	1.2	0.8	--
		10 (PO)	2173	0.4	2384	--	--	1.0	30
	cynomolgus	1.0 (IV)	--	--	1511	23.4	0.5	0.3	--
	macaque	5.0 (PO)	601	1.5	793	--	--	1.4	11

386

387 **Table 3. Compound pharmacokinetics parameters in different animal species.**

388 **C_{max}:** the maximum observed concentration of the drug collected in bodily material from subjects in a clinical study

389 **T_{max}:** the time it takes to reach the maximum concentration or time to C_{max}

390 **AUC:** “Area Under the Curve” and represents the total exposure of the drug experienced by the subject in a clinical study

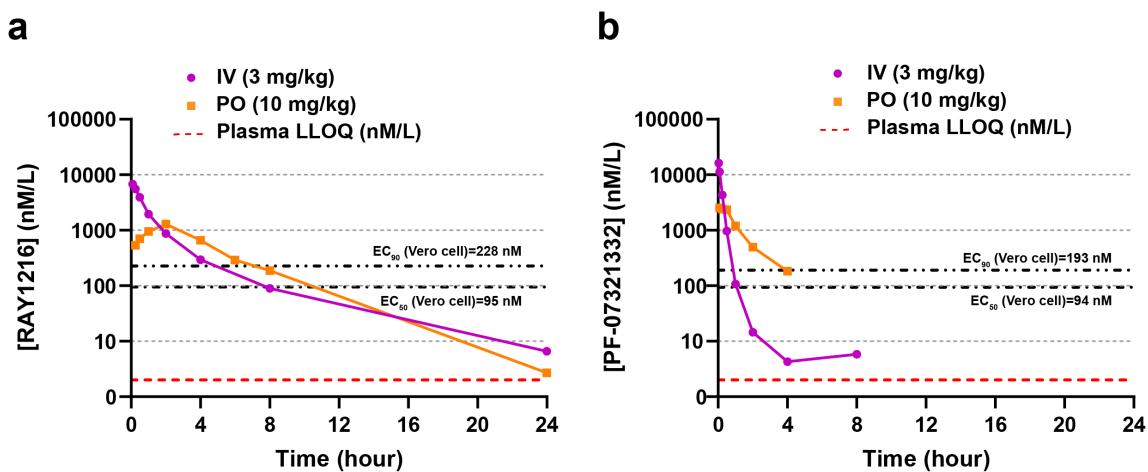
391 **Cl:** total plasma clearance

392 **Vd_{ss}:** Steady state volume of distribution

393 **T_{1/2}:** Half-time is the time it takes for half the drug concentration to be eliminated

394 **oral (F%):** Oral bioavailability

395



396

397 **Fig 5. Plasma concentrations of RAY1216 and PF-07321332 after intravenous**
398 **injection (IV) dosing and gavage (PO) dosing in mice.** Red dashed line represents the
399 lower limit of quantitation (LLOQ: 2 nM/L) of plasma drug concentration. EC₅₀ and EC₉₀
400 values against SARS-CoV-2 WT strain determined using Vero E6 cell are indicated by black
401 dashed lines.

402

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404 **References**

- 405
- 406 Bao, L., Deng, W., Huang, B., Gao, H., Liu, J., Ren, L., Wei, Q., Yu, P., Xu, Y., Qi, F., et al. (2020). The
407 pathogenicity of SARS-CoV-2 in hACE2 transgenic mice. *Nature* 583, 830-833. 10.1038/s41586-
408 020-2312-y.
- 409 Bates, D.M., and Watts, D.G. (1988). Nonlinear regression analysis and its applications (Wiley).
- 410 Boras, B., Jones, R.M., Anson, B.J., Arenson, D., Aschenbrenner, L., Bakowski, M.A., Beutler, N.,
411 Binder, J., Chen, E., Eng, H., et al. (2021). Preclinical characterization of an intravenous
412 coronavirus 3CL protease inhibitor for the potential treatment of COVID19. *Nat Commun* 12,
413 6055. 10.1038/s41467-021-26239-2.
- 414 Bradshaw, J.M., McFarland, J.M., Paavilainen, V.O., Bisconte, A., Tam, D., Phan, V.T., Romanov, S.,
415 Finkle, D., Shu, J., Patel, V., et al. (2015). Prolonged and tunable residence time using reversible
416 covalent kinase inhibitors. *Nat Chem Biol* 11, 525-531. 10.1038/nchembio.1817.
- 417 Breidenbach, J., Lemke, C., Pillaiyar, T., Schakel, L., Al Hamwi, G., Diett, M., Gedschold, R., Geiger,
418 N., Lopez, V., Mirza, S., et al. (2021). Targeting the Main Protease of SARS-CoV-2: From the
419 Establishment of High Throughput Screening to the Design of Tailored Inhibitors. *Angew Chem
420 Int Ed Engl* 60, 10423-10429. 10.1002/anie.202016961.
- 421 Cha, S. (1975). Tight-binding inhibitors—I: Kinetic behavior. *Biochemical Pharmacology* 24, 2177-
422 2185. [https://doi.org/10.1016/0006-2952\(75\)90050-7](https://doi.org/10.1016/0006-2952(75)90050-7).
- 423 Cha, S. (1976). Tight-binding inhibitors—III: A new approach for the determination of competition
424 between tight-binding inhibitors and substrates—inhibition of adenosine deaminase by
425 coformycin. *Biochemical Pharmacology* 25, 2695-2702. [https://doi.org/10.1016/0006-2952\(76\)90259-8](https://doi.org/10.1016/0006-
426 2952(76)90259-8).
- 427 Cha, S., Agarwal, R.P., and Parks, R.E. (1975). Tight-binding inhibitors—II: Non-steady state nature of
428 inhibition of milk xanthine oxidase by allopurinol and alloxanthine and of human erythrocytic
429 adenosine deaminase by coformycin. *Biochemical Pharmacology* 24, 2187-2197.
430 [https://doi.org/10.1016/0006-2952\(75\)90051-9](https://doi.org/10.1016/0006-2952(75)90051-9).
- 431 Chen, S.H., and Tan, S.L. (2005). Discovery of small-molecule inhibitors of HCV NS3-4A protease as
432 potential therapeutic agents against HCV infection. *Curr Med Chem* 12, 2317-2342.
433 10.2174/0929867054864769.
- 434 Copeland, R.A. (2013). Evaluation of enzyme inhibitors in drug discovery : a guide for medicinal
435 chemists and pharmacologists, 2nd Edition (Wiley).
- 436 Copeland, R.A., Pompliano, D.L., and Meek, T.D. (2006). Drug-target residence time and its
437 implications for lead optimization. *Nat Rev Drug Discov* 5, 730-739. 10.1038/nrd2082.
- 438 Cox, M., Peacock, T.P., Harvey, W.T., Hughes, J., Wright, D.W., Consortium, C.-G.U., Willett, B.J.,
439 Thomson, E., Gupta, R.K., Peacock, S.J., et al. (2022). SARS-CoV-2 variant evasion of
440 monoclonal antibodies based on in vitro studies. *Nat Rev Microbiol*, 1-13. 10.1038/s41579-022-
441 00809-7.
- 442 Dahl, G., and Akerud, T. (2013). Pharmacokinetics and the drug-target residence time concept. *Drug
443 Discov Today* 18, 697-707. 10.1016/j.drudis.2013.02.010.

- 444 Dai, W., Zhang, B., Jiang, X.M., Su, H., Li, J., Zhao, Y., Xie, X., Jin, Z., Peng, J., Liu, F., et al. (2020).
445 Structure-based design of antiviral drug candidates targeting the SARS-CoV-2 main protease.
446 Science 368, 1331-1335. 10.1126/science.abb4489.
- 447 Dragovich, P.S., Prins, T.J., Zhou, R., Webber, S.E., Marakovits, J.T., Fuhrman, S.A., Patick, A.K.,
448 Matthews, D.A., Lee, C.A., Ford, C.E., et al. (1999). Structure-based design, synthesis, and
449 biological evaluation of irreversible human rhinovirus 3C protease inhibitors. 4. Incorporation of
450 P1 lactam moieties as L-glutamine replacements. J Med Chem 42, 1213-1224.
451 10.1021/jm9805384.
- 452 Drayman, N., DeMarco, J.K., Jones, K.A., Azizi, S.A., Froggatt, H.M., Tan, K., Maltseva, N.I., Chen,
453 S., Nicolaescu, V., Dvorkin, S., et al. (2021). Masitinib is a broad coronavirus 3CL inhibitor that
454 blocks replication of SARS-CoV-2. Science 373, 931-936. 10.1126/science.abg5827.
- 455 Emsley, P., and Cowtan, K. (2004). Coot: model-building tools for molecular graphics. Acta Crystallogr
456 D Biol Crystallogr 60, 2126-2132. 10.1107/S0907444904019158.
- 457 Fenton, C., and Keam, S.J. (2022). Emerging small molecule antivirals may fit neatly into COVID-19
458 treatment. Drugs Ther Perspect 38, 112-126. 10.1007/s40267-022-00897-8.
- 459 Fersht, A. (1999). Structure and mechanism in protein science : a guide to enzyme catalysis and
460 protein folding (W.H. Freeman).
- 461 Gao, S., Sylvester, K., Song, L., Claff, T., Jing, L., Woodson, M., Weisse, R.H., Cheng, Y., Schakel, L.,
462 Petry, M., et al. (2022). Discovery and Crystallographic Studies of Trisubstituted Piperazine
463 Derivatives as Non-Covalent SARS-CoV-2 Main Protease Inhibitors with High Target Specificity
464 and Low Toxicity. J Med Chem 65, 13343-13364. 10.1021/acs.jmedchem.2c01146.
- 465 Grum-Tokars, V., Ratia, K., Begaye, A., Baker, S.C., and Mesecar, A.D. (2008). Evaluating the 3C-like
466 protease activity of SARS-CoV: recommendations for standardized assays for drug
467 discovery. Virus Res 133, 63-73. 10.1016/j.virusres.2007.02.015.
- 468 Harvey, W.T., Carabelli, A.M., Jackson, B., Gupta, R.K., Thomson, E.C., Harrison, E.M., Ludden, C.,
469 Reeve, R., Rambaut, A., Peacock, S.J., and Robertson, D.L. (2021). SARS-CoV-2 variants, spike
470 mutations and immune escape. Nature Reviews Microbiology 19, 409-424. 10.1038/s41579-021-
471 00573-0.
- 472 Jin, Z., Du, X., Xu, Y., Deng, Y., Liu, M., Zhao, Y., Zhang, B., Li, X., Zhang, L., Peng, C., et al. (2020).
473 Structure of M(pro) from SARS-CoV-2 and discovery of its inhibitors. Nature 582, 289-293.
474 10.1038/s41586-020-2223-y.
- 475 Johnson, K.A. (2009). Fitting enzyme kinetic data with KinTek Global Kinetic Explorer. Methods
476 Enzymol 467, 601-626. 10.1016/S0076-6879(09)67023-3.
- 477 Kabsch, W. (2010). XDS. Acta Crystallogr D Biol Crystallogr 66, 125-132.
478 10.1107/S0907444909047337.
- 479 Kitamura, N., Sacco, M.D., Ma, C., Hu, Y., Townsend, J.A., Meng, X., Zhang, F., Zhang, X., Ba, M.,
480 Szeto, T., et al. (2022). Expedited Approach toward the Rational Design of Noncovalent SARS-
481 CoV-2 Main Protease Inhibitors. J Med Chem 65, 2848-2865. 10.1021/acs.jmedchem.1c00509.
- 482 Kneller, D.W., Phillips, G., O'Neill, H.M., Jedrzejczak, R., Stols, L., Langan, P., Joachimiak, A., Coates,
483 L., and Kovalevsky, A. (2020). Structural plasticity of SARS-CoV-2 3CL M(pro) active site cavity

- 484 revealed by room temperature X-ray crystallography. *Nat Commun* 11, 3202. 10.1038/s41467-
485 020-16954-7.
- 486 Kuzmic, P. (1996). Program DYNAFIT for the analysis of enzyme kinetic data: application to HIV
487 proteinase. *Anal Biochem* 237, 260-273. 10.1006/abio.1996.0238.
- 488 Kuzmic, P. (2009). DynaFit--a software package for enzymology. *Methods Enzymol* 467, 247-280.
489 10.1016/S0076-6879(09)67010-5.
- 490 Kwong, A.D., Kauffman, R.S., Hurter, P., and Mueller, P. (2011). Discovery and development of
491 telaprevir: an NS3-4A protease inhibitor for treating genotype 1 chronic hepatitis C virus. *Nat*
492 *Biotechnol* 29, 993-1003. 10.1038/nbt.2020.
- 493 Lin, C., Kwong, A.D., and Perni, R.B. (2006). Discovery and development of VX-950, a novel,
494 covalent, and reversible inhibitor of hepatitis C virus NS3.4A serine protease. *Infect Disord Drug*
495 *Targets* 6, 3-16. 10.2174/187152606776056706.
- 496 Liu, Y., Kati, W., Chen, C.M., Tripathi, R., Molla, A., and Kohlbrenner, W. (1999). Use of a fluorescence
497 plate reader for measuring kinetic parameters with inner filter effect correction. *Anal Biochem*
498 267, 331-335. 10.1006/abio.1998.3014.
- 499 Lu, H., and Tonge, P.J. (2010). Drug-target residence time: critical information for lead optimization.
500 *Curr Opin Chem Biol* 14, 467-474. 10.1016/j.cbpa.2010.06.176.
- 501 Ma, C., Sacco, M.D., Hurst, B., Townsend, J.A., Hu, Y., Szeto, T., Zhang, X., Tarbet, B., Marty, M.T.,
502 Chen, Y., and Wang, J. (2020). Boceprevir, GC-376, and calpain inhibitors II, XII inhibit SARS-
503 CoV-2 viral replication by targeting the viral main protease. *Cell Res* 30, 678-692.
504 10.1038/s41422-020-0356-z.
- 505 Ma, C., Xia, Z., Sacco, M.D., Hu, Y., Townsend, J.A., Meng, X., Choza, J., Tan, H., Jang, J., Gongora,
506 M.V., et al. (2021). Discovery of Di- and Trihaloacetamides as Covalent SARS-CoV-2 Main
507 Protease Inhibitors with High Target Specificity. *J Am Chem Soc* 143, 20697-20709.
508 10.1021/jacs.1c08060.
- 509 Ma, Q., Lei, B., Chen, R., Liu, B., Lu, W., Jiang, H., Chen, Z., Guo, X., Wang, Y., Zhang, L., et al.
510 (2022). Liushen Capsules, a promising clinical candidate for COVID-19, alleviates SARS-CoV-2-
511 induced pulmonary in vivo and inhibits the proliferation of the variant virus strains in vitro. *Chin*
512 *Med* 17, 40. 10.1186/s13020-022-00598-4.
- 513 McCoy, A.J., Grosse-Kunstleve, R.W., Adams, P.D., Winn, M.D., Storoni, L.C., and Read, R.J. (2007).
514 Phaser crystallographic software. *J Appl Crystallogr* 40, 658-674. 10.1107/S0021889807021206.
- 515 Morrison, J.F. (1982). The slow-binding and slow, tight-binding inhibition of enzyme-catalysed
516 reactions. *Trends in Biochemical Sciences* 7, 102-105. [https://doi.org/10.1016/0968-0004\(82\)90157-8](https://doi.org/10.1016/0968-0004(82)90157-8).
- 517 Morrison, J.F., and Walsh, C.T. (1988). The Behavior and Significance of Slow-Binding Enzyme
518 Inhibitors. In *Advances in Enzymology and Related Areas of Molecular Biology*, pp. 201-301.
519 <https://doi.org/10.1002/9780470123072.ch5>.
- 520 Murshudov, G.N., Vagin, A.A., and Dodson, E.J. (1997). Refinement of macromolecular structures by
521 the maximum-likelihood method. *Acta Crystallogr D Biol Crystallogr* 53, 240-255.
522 10.1107/S0907444996012255.

- 524 Owen, D.R., Allerton, C.M.N., Anderson, A.S., Aschenbrenner, L., Avery, M., Berritt, S., Boras, B.,
525 Cardin, R.D., Carlo, A., Coffman, K.J., et al. (2021). An oral SARS-CoV-2 M(pro) inhibitor clinical
526 candidate for the treatment of COVID-19. *Science* 374, 1586-1593. 10.1126/science.abl4784.
- 527 Park, K.I., Park, H.S., Kang, S.R., Nagappan, A., Lee, D.H., Kim, J.A., Han, D.Y., and Kim, G.S.
528 (2011). Korean Scutellaria baicalensis water extract inhibits cell cycle G1/S transition by
529 suppressing cyclin D1 expression and matrix-metalloproteinase-2 activity in human lung cancer
530 cells. *J Ethnopharmacol* 133, 634-641. 10.1016/j.jep.2010.10.057.
- 531 Qiao, J., Li, Y.S., Zeng, R., Liu, F.L., Luo, R.H., Huang, C., Wang, Y.F., Zhang, J., Quan, B., Shen, C.,
532 et al. (2021). SARS-CoV-2 M(pro) inhibitors with antiviral activity in a transgenic mouse model.
533 *Science* 371, 1374-1378. 10.1126/science.abf1611.
- 534 Quan, B.X., Shuai, H., Xia, A.J., Hou, Y., Zeng, R., Liu, X.L., Lin, G.F., Qiao, J.X., Li, W.P., Wang, F.L.,
535 et al. (2022). An orally available M(pro) inhibitor is effective against wild-type SARS-CoV-2 and
536 variants including Omicron. *Nat Microbiol* 7, 716-725. 10.1038/s41564-022-01119-7.
- 537 Robello, M., Barresi, E., Baglini, E., Salerno, S., Taliani, S., and Settimo, F.D. (2021). The Alpha Keto
538 Amide Moiety as a Privileged Motif in Medicinal Chemistry: Current Insights and Emerging
539 Opportunities. *J Med Chem* 64, 3508-3545. 10.1021/acs.jmedchem.0c01808.
- 540 Rut, W., Groborz, K., Zhang, L., Sun, X., Zmudzinski, M., Pawlik, B., Wang, X., Jochmans, D., Neyts,
541 J., Mlynarski, W., et al. (2021). SARS-CoV-2 M(pro) inhibitors and activity-based probes for
542 patient-sample imaging. *Nat Chem Biol* 17, 222-228. 10.1038/s41589-020-00689-z.
- 543 Sacco, M.D., Ma, C., Lagarias, P., Gao, A., Townsend, J.A., Meng, X., Dube, P., Zhang, X., Hu, Y.,
544 Kitamura, N., et al. (2020). Structure and inhibition of the SARS-CoV-2 main protease reveal
545 strategy for developing dual inhibitors against M(pro) and cathepsin L. *Sci Adv* 6.
546 10.1126/sciadv.abe0751.
- 547 Sheldrick, G.M. (2015a). Crystal structure refinement with SHELXL. *Acta Crystallogr C Struct Chem*
548 71, 3-8. 10.1107/S2053229614024218.
- 549 Sheldrick, G.M. (2015b). SHELXT - integrated space-group and crystal-structure determination. *Acta
550 Crystallogr A Found Adv* 71, 3-8. 10.1107/S2053273314026370.
- 551 Tan, J., George, S., Kusov, Y., Perbandt, M., Anemüller, S., Mesters, J.R., Norder, H., Coutard, B.,
552 Lacroix, C., Leyssen, P., et al. (2013). 3C protease of enterovirus 68: structure-based design of
553 Michael acceptor inhibitors and their broad-spectrum antiviral effects against picornaviruses. *J
554 Virol* 87, 4339-4351. 10.1128/jvi.01123-12.
- 555 Tian, D., Sun, Y., Xu, H., and Ye, Q. (2022). The emergence and epidemic characteristics of the highly
556 mutated SARS-CoV-2 Omicron variant. *J Med Virol* 94, 2376-2383. 10.1002/jmv.27643.
- 557 Unoh, Y., Uehara, S., Nakahara, K., Nobori, H., Yamatsu, Y., Yamamoto, S., Maruyama, Y., Taoda, Y.,
558 Kasamatsu, K., Suto, T., et al. (2022). Discovery of S-217622, a Noncovalent Oral SARS-CoV-2
559 3CL Protease Inhibitor Clinical Candidate for Treating COVID-19. *J Med Chem* 65, 6499-6512.
560 10.1021/acs.jmedchem.2c00117.
- 561 Watts, D.G. (1994). Parameter estimates from nonlinear models. *Methods Enzymol* 240, 23-36.
562 10.1016/s0076-6879(94)40041-5.
- 563 Winn, M.D., Ballard, C.C., Cowtan, K.D., Dodson, E.J., Emsley, P., Evans, P.R., Keegan, R.M.,

- 564 Krissinel, E.B., Leslie, A.G., McCoy, A., et al. (2011). Overview of the CCP4 suite and current
565 developments. *Acta Crystallogr D Biol Crystallogr* 67, 235-242. 10.1107/S0907444910045749.
- 566 Xiong, M., Su, H., Zhao, W., Xie, H., Shao, Q., and Xu, Y. (2021). What coronavirus 3C-like protease
567 tells us: From structure, substrate selectivity, to inhibitor design. *Med Res Rev* 41, 1965-1998.
568 10.1002/med.21783.
- 569 Yip, Y., Victor, F., Lamar, J., Johnson, R., Wang, Q.M., Barket, D., Glass, J., Jin, L., Liu, L., Venable,
570 D., et al. (2004a). Discovery of a novel bicycloproline P2 bearing peptidyl alpha-ketoamide
571 LY514962 as HCV protease inhibitor. *Bioorg Med Chem Lett* 14, 251-256.
572 10.1016/j.bmcl.2003.09.074.
- 573 Yip, Y., Victor, F., Lamar, J., Johnson, R., Wang, Q.M., Glass, J.I., Yumibe, N., Wakulchik, M., Munroe,
574 J., and Chen, S.H. (2004b). P4 and P1' optimization of bicycloproline P2 bearing tetrapeptidyl
575 alpha-ketoamides as HCV protease inhibitors. *Bioorg Med Chem Lett* 14, 5007-5011.
576 10.1016/j.bmcl.2004.07.007.
- 577 Zaidman, D., Gehrtz, P., Filep, M., Fearon, D., Gabizon, R., Douangamath, A., Prilusky, J.,
578 Duberstein, S., Cohen, G., Owen, C.D., et al. (2021). An automatic pipeline for the design of
579 irreversible derivatives identifies a potent SARS-CoV-2 M(pro) inhibitor. *Cell Chem Biol* 28, 1795-
580 1806 e1795. 10.1016/j.chembiol.2021.05.018.
- 581 Zhang, L., Lin, D., Sun, X., Curth, U., Drosten, C., Sauerhering, L., Becker, S., Rox, K., and
582 Hilgenfeld, R. (2020). Crystal structure of SARS-CoV-2 main protease provides a basis for
583 design of improved α-ketoamide inhibitors. *Science* 368, 409-412. 10.1126/science.abb3405.
- 584 Zhao, Y., Fang, C., Zhang, Q., Zhang, R., Zhao, X., Duan, Y., Wang, H., Zhu, Y., Feng, L., Zhao, J., et
585 al. (2022). Crystal structure of SARS-CoV-2 main protease in complex with protease inhibitor PF-
586 07321332. *Protein Cell* 13, 689-693. 10.1007/s13238-021-00883-2.
- 587 Zhou, S., Hill, C.S., Sarkar, S., Tse, L.V., Woodburn, B.M.D., Schinazi, R.F., Sheahan, T.P., Baric,
588 R.S., Heise, M.T., and Swanstrom, R. (2021). β-d-N4-hydroxycytidine Inhibits SARS-CoV-2
589 Through Lethal Mutagenesis But Is Also Mutagenic To Mammalian Cells. *J Infect Dis* 224, 415-
590 419. 10.1093/infdis/jiab247.
- 591 Zhu, L., George, S., Schmidt, M.F., Al-Gharabli, S.I., Rademann, J., and Hilgenfeld, R. (2011). Peptide
592 aldehyde inhibitors challenge the substrate specificity of the SARS-coronavirus main protease.
593 *Antiviral Res* 92, 204-212. 10.1016/j.antiviral.2011.08.001.
- 594 Zhu, N., Zhang, D., Wang, W., Li, X., Yang, B., Song, J., Zhao, X., Huang, B., Shi, W., Lu, R., et al.
595 (2020a). A Novel Coronavirus from Patients with Pneumonia in China, 2019. *New England
596 Journal of Medicine* 382, 727-733. 10.1056/NEJMoa2001017.
- 597 Zhu, W., Xu, M., Chen, C.Z., Guo, H., Shen, M., Hu, X., Shinn, P., Klumpp-Thomas, C., Michael, S.G.,
598 and Zheng, W. (2020b). Identification of SARS-CoV-2 3CL Protease Inhibitors by a Quantitative
599 High-Throughput Screening. *ACS Pharmacol Transl Sci* 3, 1008-1016.
600 10.1021/acsptsci.0c00108.
- 601 Ziebuhr, J., Snijder, E.J., and Gorbalenya, A.E. (2000). Virus-encoded proteinases and proteolytic
602 processing in the Nidovirales. *J Gen Virol* 81, 853-879. 10.1099/0022-1317-81-4-853.
- 603

604 **Author contributions**

605 Z.Y., X.X. and X.C. conceived the study under the direction of N.Z.; X.C., J.H., H.L., C.L.
606 and S-H. C. provided the M^{pro} inhibitors, collected chemical characterization data and *in-vivo*
607 and *in-vitro* pharmacokinetics data. X.H. expressed, purified M^{pro} and performed enzyme
608 kinetics assays under the supervision of X.X.; Q.M. performed virus inhibition assays in cell
609 culture and animal models and prepared figures with assistance from B.L., H.J., W.Z., C.Y.,
610 S.W. and under the supervision of Z.Y.; P.K. and X.X. analysed enzyme kinetics data and
611 prepared figures; X.H. and B.Z. obtained M^{pro} crystals and performed crystal soaking
612 experiments under the supervision of X.X.; J.X., X.H., B.Z., Y.S., Y.G. performed M^{pro}
613 crystal diffraction experiments and collected diffraction data under the supervision of Y.X.
614 and J.L.; X.H determined the M^{pro} crystal structures and built molecular models with
615 assistance from C.N., L.X. and under the supervision of X.X.; H.X. and X.X. analysed the
616 M^{pro} crystal structures and prepared figures; with input from all authors, X.X., P.K., X.H.,
617 Q.M. and X.C. wrote the initial draft which was reviewed and edited by Z.Y., S-H.C., Z.X.,
618 J.S. T.Z., J.H. and W.D.; N.Z., X.X. and Z.Y. acquired funding and supervised the research.
619

620 **Competing interests**

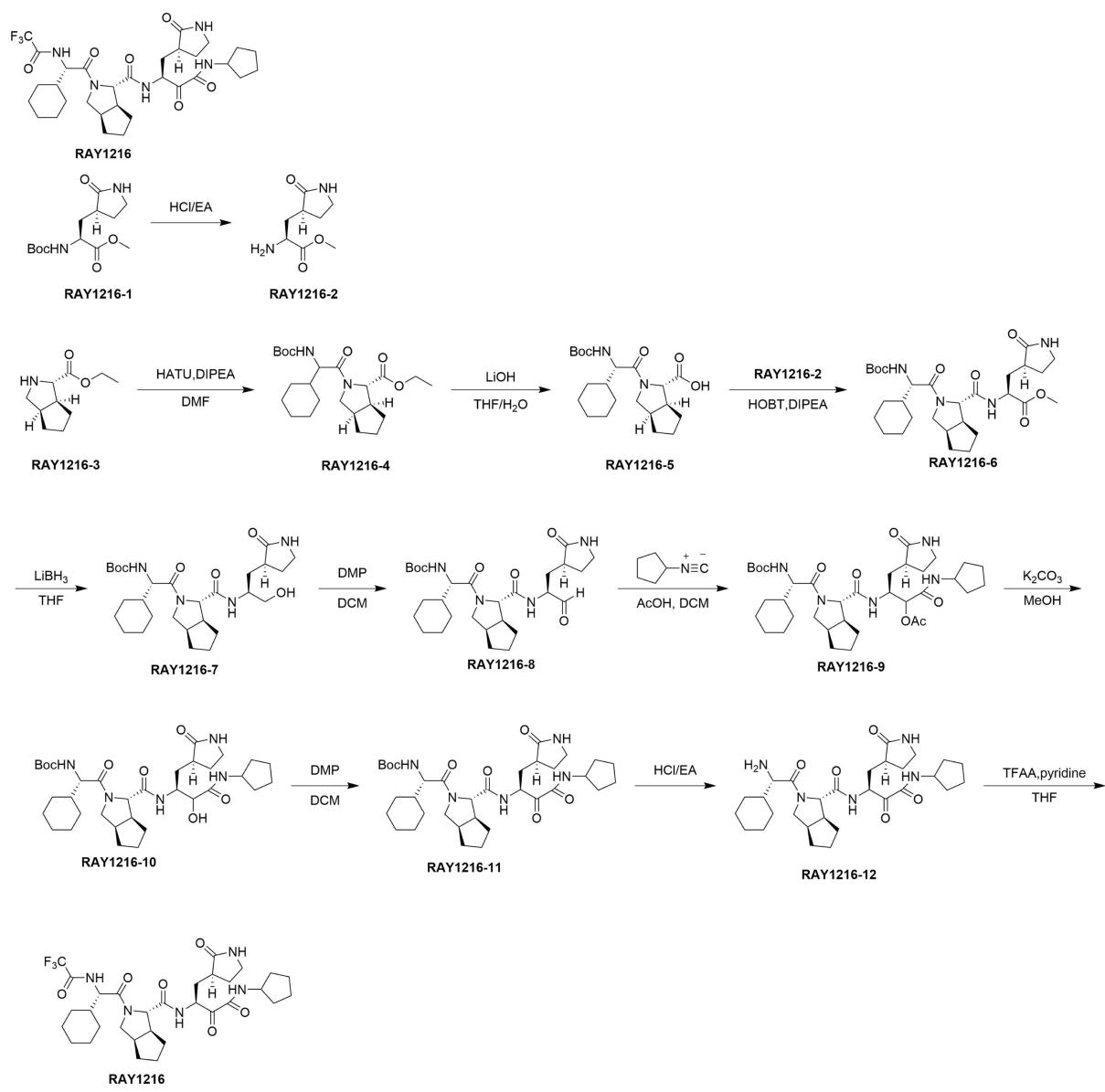
621 X.C., J.H., H.L. and C.L. are employees of Guangdong Raynovent Biotech Co., Ltd, which
622 holds the patent of RAY1216.
623

624 **Acknowledgement**

625 We thank the staffs at beamline BL19U1 of Shanghai Synchrotron Radiation Facility for
626 assistance on data collection. This work was supported by National Multidisciplinary
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628 Guangdong Science and Technology Foundation (2022B1111060003 to Z.Y.); Guangzhou
629 Science and Technology Planning Project (2022B01W0001 and 202102100003 to Z.Y.);
630 Emergency Key Program of Guangzhou Laboratory (EKGPG21-06 to X.X.); R&D Program of
631 Guangzhou Laboratory (SRPG22-002 and SRPG22-003 to X.X.; TL22-13 to Z.Y.). Natural
632 Science Fund of Guangdong Province (2021A1515011289 to X.X.). X.X. acknowledges
633 Start-up grants from the Chinese Academy of Sciences.
634

635 **Supporting Information**

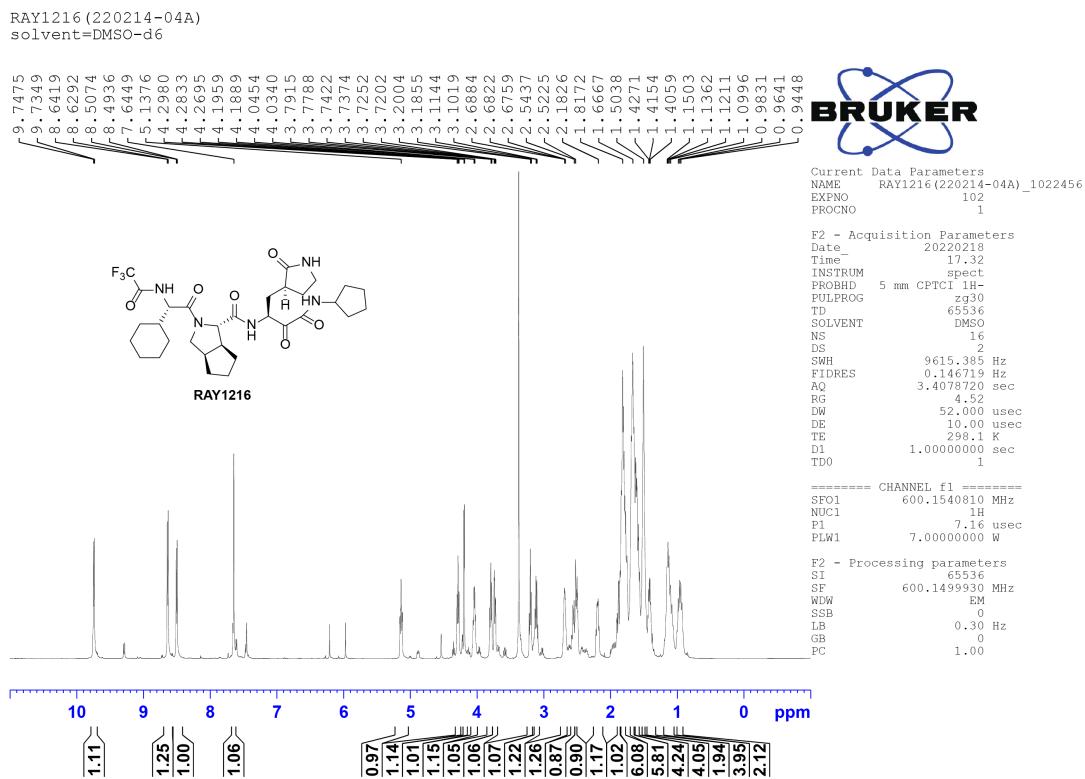
636



637

638 **Fig. S1. Synthesis of RAY1216.**

639



640

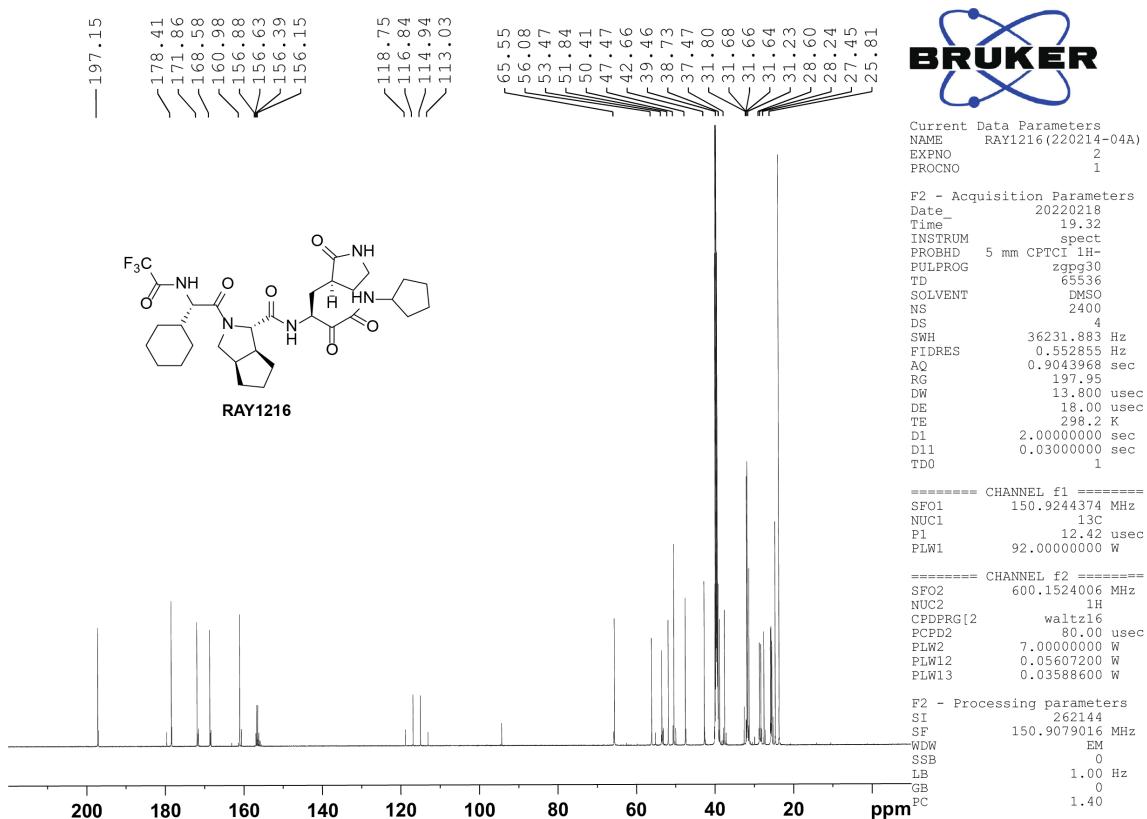
641 **Fig. S2. ^1H NMR spectra of RAY1216 in DMSO-d6.**

642

643

644

RAY1216 (220214-04A)
solvent=DMSO-d6



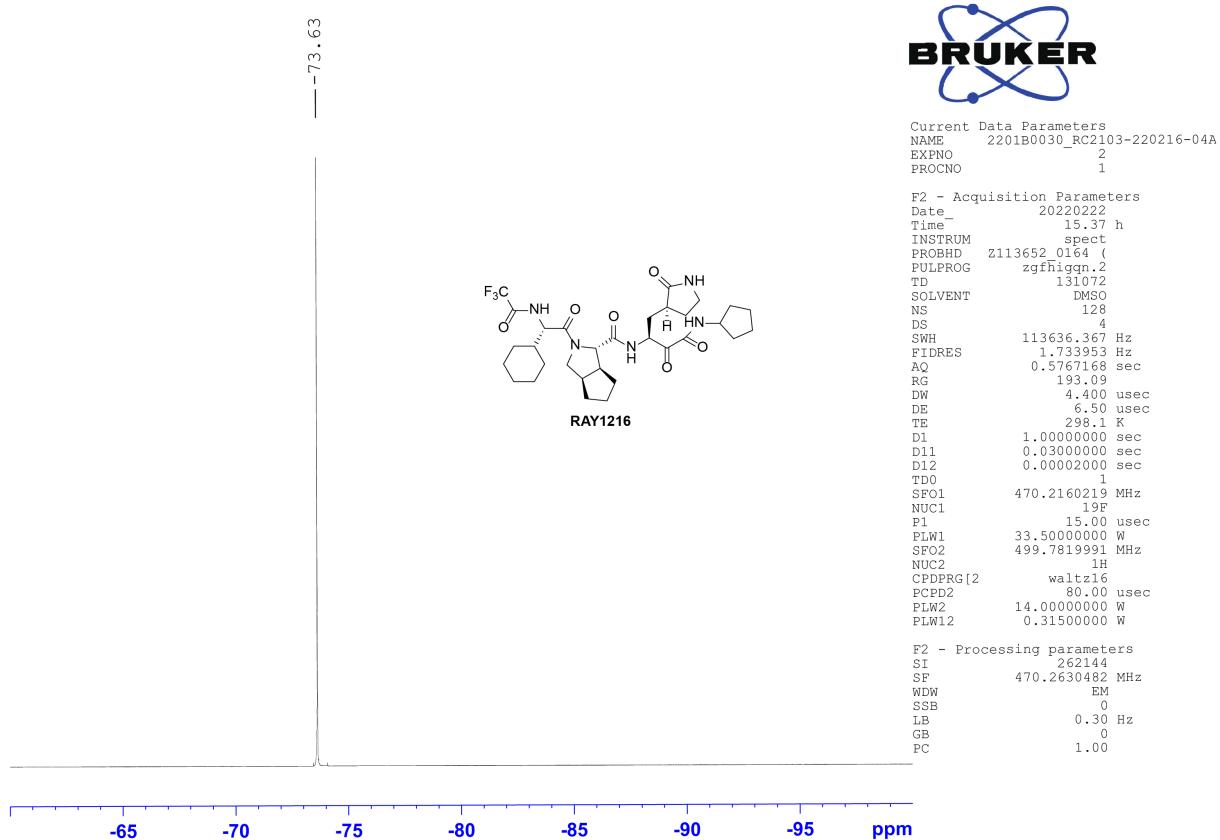
645 Fig. S3. ¹³C NMR spectrum of RAY1216 in DMSO-d6.

646

647

648

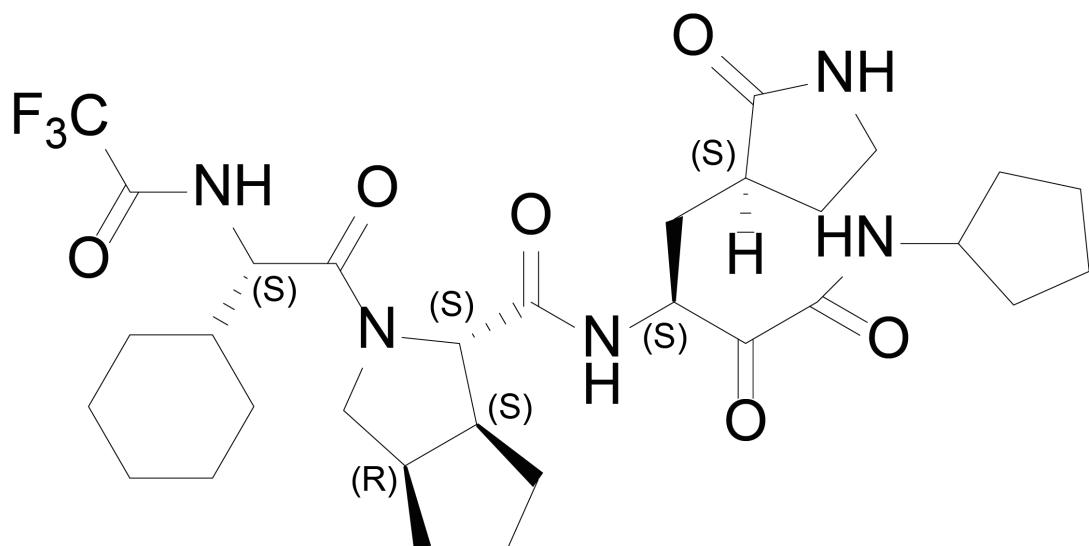
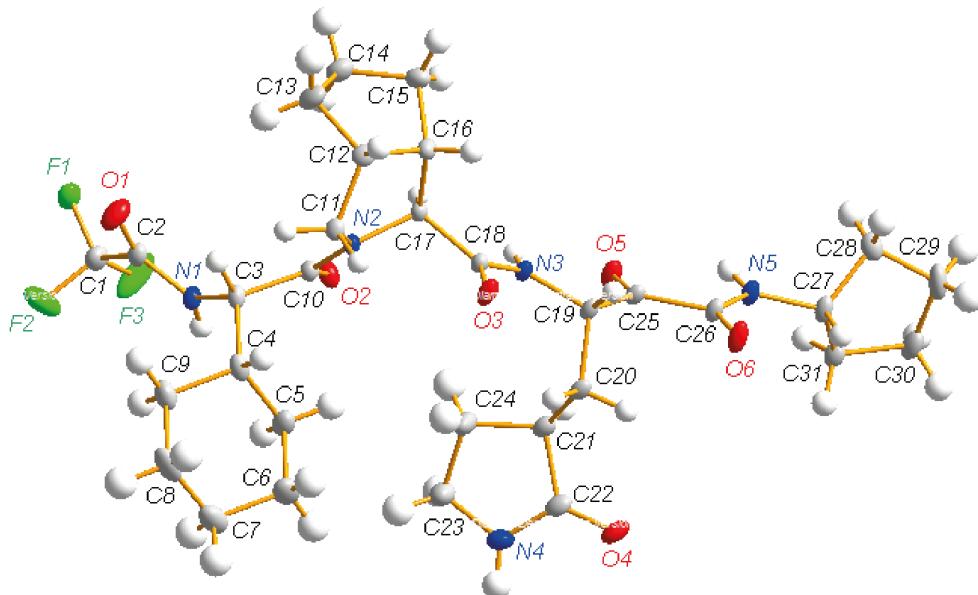
RAY1216 (220216-04A)
solvent=DMSO-d6



649 Fig. S4. ¹⁹F NMR spectrum of RAY1216 in DMSO-d6.

650

651



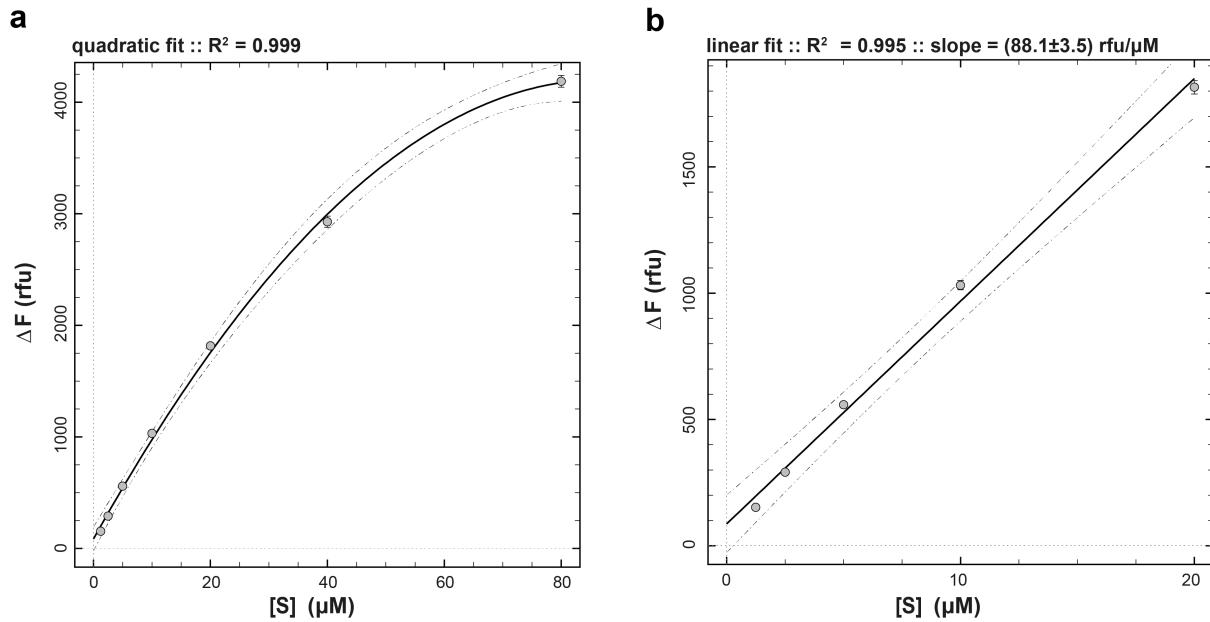
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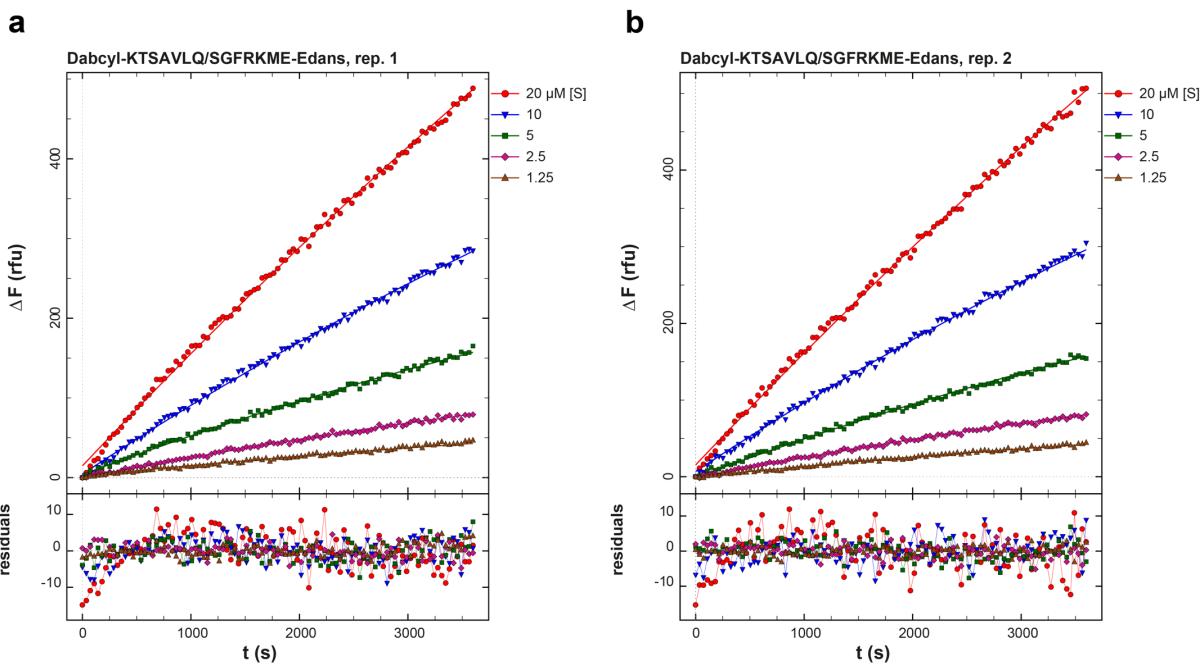
653 **Fig. S5. Single crystal X-ray structure of RAY1216.**

654

Molecule	RAY1216
Radiation Type	CuK α ($\lambda=1.54184\text{ \AA}$)
Crystal size (mm³)	0.16 × 0.18 × 0.22
Crystal system	monoclinic
Space group	<i>P</i> 2 ₁
cell dimensions	
a, b, c (Å)	10.6833 9.8921 15.3665
α, β, γ (°)	90.00 91.11 90.00
Cell volume (Å³)	1623.63 (2)
Cell formula units Z	2
Crystal density _{calc} (g/cm³)	1.309
Crystal F (000)	680
Absorption coefficient (μ/mm^{-1})	0.862
Index ranges	-12 ≤ <i>h</i> ≤ 12, -11 ≤ <i>k</i> ≤ 11, -18 ≤ <i>l</i> ≤ 18
Cell measurement temperature (K)	149.9 (8)
2θ range for data collection (°)	5.752 to 133.106
Goodness-of-fit on F²	1.041
Final R indexes [$I >=2\sigma(I)$]	$R_1=0.0351$, $wR_2=0.0914$
Final R indexes [all data]	$R_1=0.0358$, $wR_2=0.0921$
Largest diff. peak/hole/e Å⁻³	0.39/-0.31
Reflections collected/unique	40929/5638 [$R_{\text{int}} = 0.0391$]
Flack parameter	0.10(5)

655 **Table S1. Data collection and statistics of single crystal X-ray structure of RAY1216.**
656
657





Replicate 1

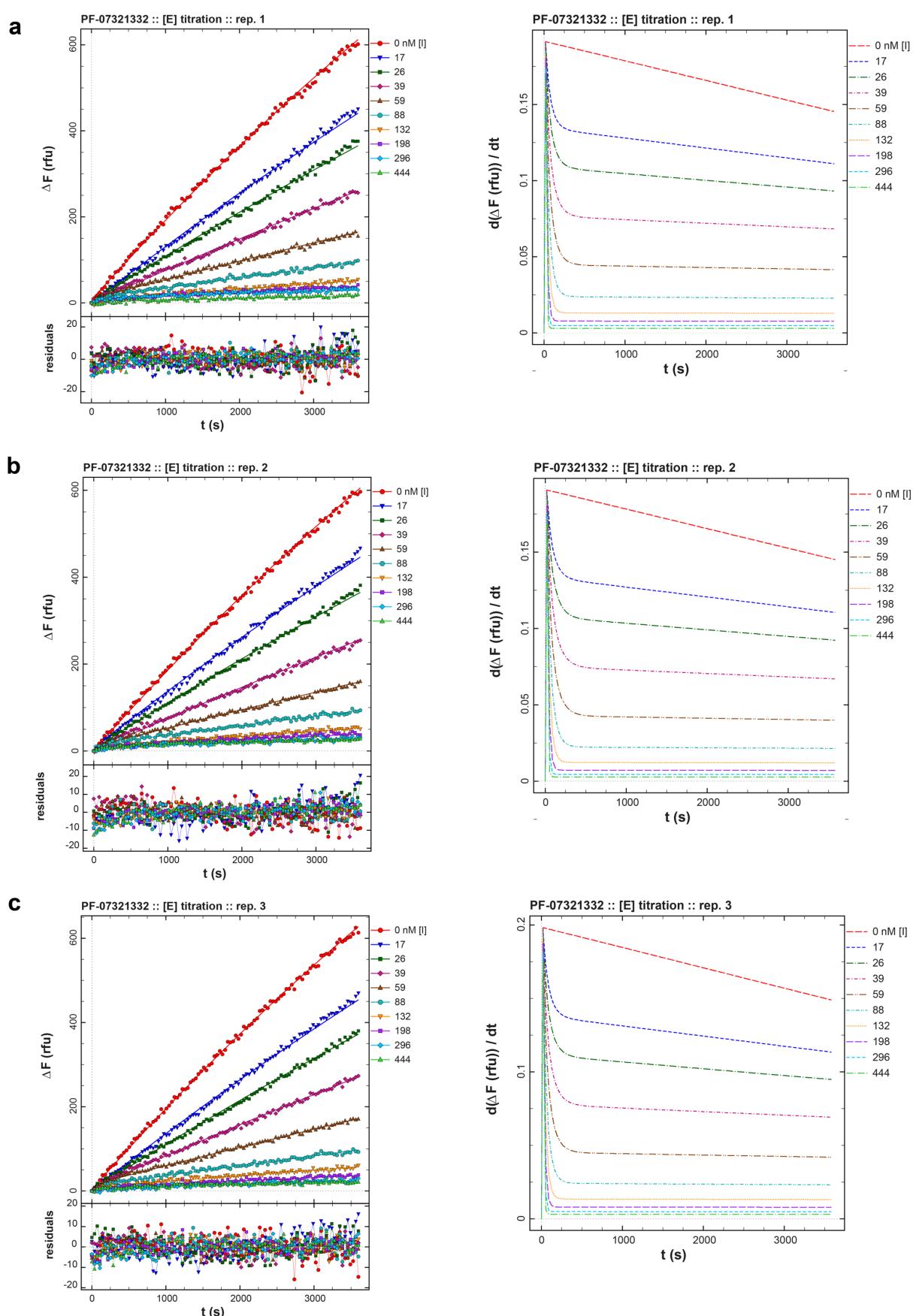
#	parameter	initial	final ± std.err.	low	high
1	k_{dS} , s ⁻¹	10	31.09 ± 0.82	27.4	35.67
2	k_{dP} , s ⁻¹	0.1	0.05246 ± 0.00097	0.04808	0.05787
3	F_0 # 1, rfu	0	14.82 ± 0.72		
4	F_0 # 2, rfu	0	4.44 ± 0.53		
5	F_0 # 3, rfu	0	3.88 ± 0.5		
6	F_0 # 4, rfu	0	-0.74 ± 0.41		
7	F_0 # 5, rfu	0	1.77 ± 0.36		

Replicate 2

#	parameter	initial	final ± std.err.	low	high
1	k_{dS} , s ⁻¹	10	30.82 ± 0.81	27.16	35.36
2	k_{dP} , s ⁻¹	0.1	0.0542 ± 0.0010	0.0497	0.0598
3	F_0 # 1, rfu	0	15.33 ± 0.75		
4	F_0 # 2, rfu	0	6.83 ± 0.54		
5	F_0 # 3, rfu	0	-1.22 ± 0.52		
6	F_0 # 4, rfu	0	-2.02 ± 0.43		
7	F_0 # 5, rfu	0	-0.41 ± 0.37		

671 **Table S2. Results of fit of substrate-only progress curves to the Michaelis-Menten**
672 **reaction model, replicate 1 and 2.** Columns labeled as “low” and “high” contain the lower
673 and upper limits, respectively, of non-symmetrical confidence intervals obtained by the
674 profile-t method of Bates and Watts (Bates and Watts, 1988; Watts, 1994) while using the
675 empirical value of $\Delta SSQ = 5\%$ according to # parameter initial the previously suggested
676 method (Johnson, 2009).

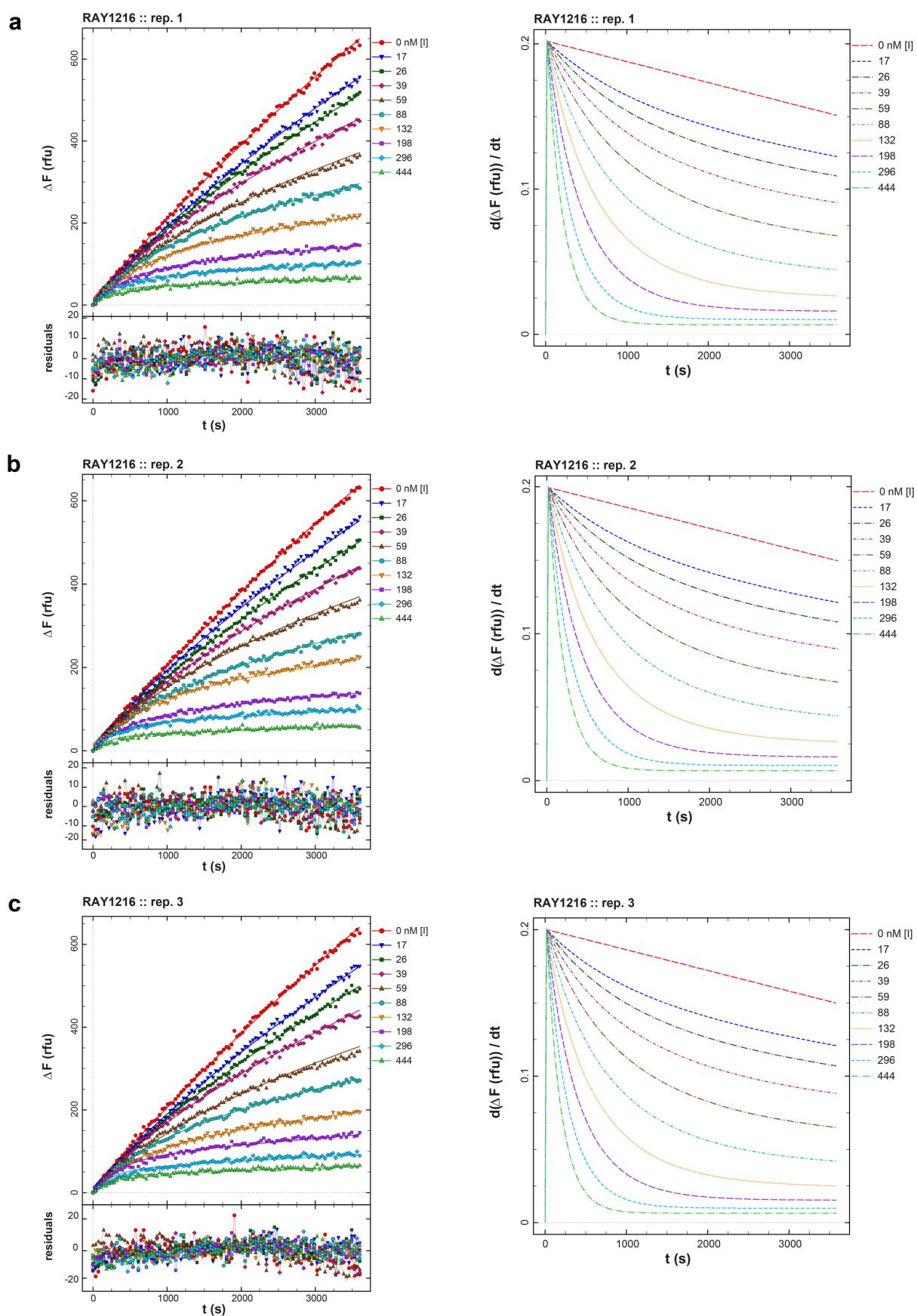
677



679 **Fig. S8. Inhibition of M^{pro} by PF-07321332.** Left panels, Replicated (a-c) PF-07321332
680 inhibition progress curves (dots) are overlaid with best-fit model curves corresponding to the
681 reaction mechanism in **Scheme 2** and represented by **Eq.6-Eq.11**; the residuals of the fits are
682 shown. Right panels, corresponding plots of instantaneous reaction rates for the 3 replicates.
683

parameter	rep. 1	rep. 2	rep. 3	mean ± stdev	cv(%)	note
$k_{as}, \mu\text{M}^{-1} \text{s}^{-1}$	1	1	1			fixed
k_{ds}, s^{-1}	31	31	31			fixed
k_{dp}, s^{-1}	0.112	0.112	0.119	0.115 ± 0.004	3.7	
$k_a, \mu\text{M}^{-1} \text{s}^{-1}$	0.53	0.46	0.48	0.49 ± 0.04	7.4	
k_d, s^{-1}	0.002	0.0016	0.0018	0.0018 ± 0.0002	10.7	
[E], nM	49.5	49.5	48.2	49.1 ± 0.7	1.5	
F_0 # 1, rfu	7	1.8	3.9			
F_0 # 2, rfu	-4	3.6	-2.6			
F_0 # 3, rfu	-3.5	-0.7	-4.7			
F_0 # 4, rfu	-7.4	-7.6	-2			
F_0 # 5, rfu	1.1	0.7	5.4			
F_0 # 6, rfu	4.4	6.1	4.3			
F_0 # 7, rfu	0.9	3.7	3.7			
F_0 # 8, rfu	6	9	4.3			
F_0 # 9, rfu	10.1	8.5	3.5			
F_0 # 10, rfu	2.8	12.6	7.2			
k_{cat}, s^{-1}	0.112	0.112	0.119	0.115 ± 0.004	3.7	$= k_{dp}$
$K_M, \mu\text{M}$	31.112	31.112	31.119	31.115 ± 0.004	0.01	$= (k_{dp} + k_{ds})/k_{as}$
$k_{cat}/K_M, \text{M}^{-1} \text{s}^{-1}$	3610	3600	3840	3680 ± 140	3.8	
K_i, nM	3.86	3.6	3.87	3.78 ± 0.16	4.1	$= k_d/k_a$
t_{res}, min	8.2	10.2	9	9.1 ± 1.0	10.7	$= 1/k_d$
% enzyme activity	61.9	61.9	60.3	61.3 ± 0.9	1.5	$= 100 \times [E] / 80.0$

684 **Table S3. Results of fit from global analysis of PF-07321332 inhibition data.** Note that
685 both active enzyme concentration [E] (nominal concentration 80.0 nM) and the turnover
686 number k_{dp} were treated as adjustable parameters. “stdev” is the standard deviation from
687 replicates ($n = 3$) and “cv(%)” is the corresponding coefficient of variation, $cv = 100 \times$
688 stdev/mean . For details see *Methods* section.
689



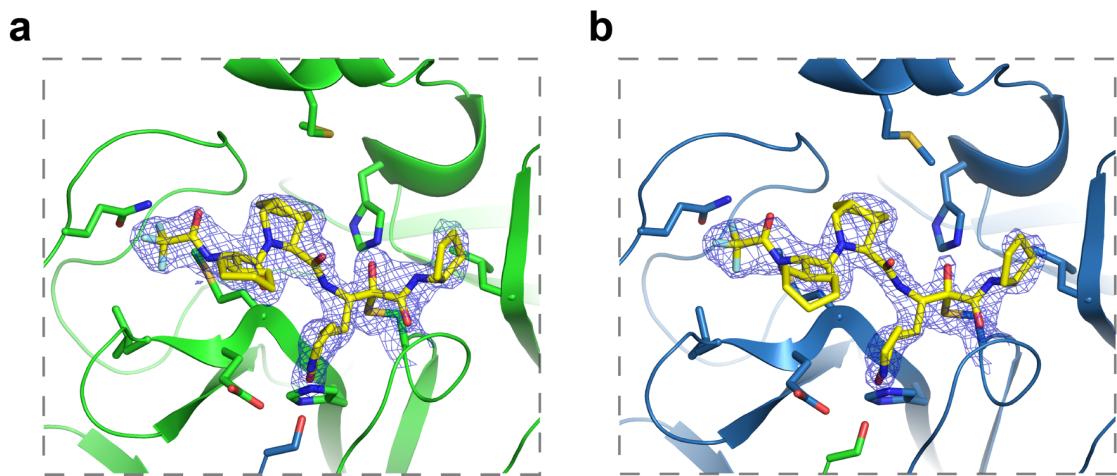
691 **Fig. S9. Inhibition of M^{pro} by RAY1216.** Left panels, Replicated (a-c) RAY1216 inhibition
692 progress curves (dots) are overlaid with best-fit model curves corresponding to the reaction
693 mechanism in **Scheme 2** and represented by **Eq.6-Eq.11**; the residuals of the fits are shown.
694 Right panels, corresponding plots of instantaneous reaction rates for the 3 replicates.
695

parameter	rep. 1	rep. 2	rep. 3	mean ± stdev	cv(%)	note
$k_{as}, \mu M^{-1} s^{-1}$	1	1	1			fixed
k_{ds}, s^{-1}	31	31	31			fixed
k_{dp}, s^{-1}	0.11	0.11	0.11			fixed
$k_a, \mu M^{-1} s^{-1}$	0.0183	0.0187	0.0208	0.0193 ± 0.0014	7.1	
k_d, s^{-1}	0.000153	0.000161	0.000169	0.000161 ± 0.000008	4.9	
[E], nM	53.3	52.7	52.8	52.9 ± 0.3	0.6	
$F_0 \# 1, rfu$	15.9	10.3	12.7			
$F_0 \# 2, rfu$	11.1	14.7	12.0			
$F_0 \# 3, rfu$	10.2	5.3	7.9			
$F_0 \# 4, rfu$	8.5	2.6	8.7			
$F_0 \# 5, rfu$	-2.0	2.0	-3.5			
$F_0 \# 6, rfu$	5.4	-0.9	3.3			
$F_0 \# 7, rfu$	7.2	13.9	2.8			
$F_0 \# 8, rfu$	1.1	-1.4	9.4			
$F_0 \# 9, rfu$	4.7	5.1	4.6			
$F_0 \# 10, rfu$	0.7	-3.2	5.1			
K_i, nM	8.4	8.6	8.1	8.4 ± 0.2	2.9	= k_d/k_a
t_{res}, min	109	104	99	104 ± 5	4.9	=1/ k_d
% enzyme activity	66.6	65.9	66.0	66.2 ± 0.4	0.6	=100 × [E] / 80.0

696 **Table S4. Results of fit from global analysis of RAY1216 inhibition data.** Note that active
697 enzyme concentration [E] (nominal concentration 80.0 nM) was treated as adjustable
698 parameters while the turnover number k_{dp} was fixed. “stdev” is the standard deviation from
699 replicates ($n = 3$) and “cv(%)” is the corresponding coefficient of variation, $cv = 100 \times$
700 stdev/mean. For details see *Methods* section.
701

	Protein/Ligand	M ^{pro} free enzyme (PDB: 8IGO)	M ^{pro} +RAY1216 (PDB: 8IGN)
	wavelength (Å)	0.97849	0.97853
	Space group	C 2	P 1
	cell dimensions		
	a, b, c (Å)	97.39 82.49 51.42	55.83 60.94 64.04
	α, β, γ (°)	90.00 115.52 90.00	80.03 68.34 70.67
Data collection	Resolution (Å)	43.46-2.00 (2.05-2.00)	59.42-2.00 (2.05-2.00)
	R _{merge}	0.031 (0.327)	0.067 (0.504)
	No. Reflections (total)	156563 (11225)	168870 (11201)
	No. Reflections (unique)	23876 (1817)	48627 (3545)
	//σ//	31.6 (5.1)	9.9 (2.1)
	Completeness (%)	96.2 (98.8)	97.5 (96)
	Multiplicity	6.6 (6.2)	3.5 (3.2)
	No. Reflections	23851 (2089)	47154 (4676)
	R _{work} /R _{free}	0.2059/0.2491	0.1862/0.2312
	No. atoms		
	Protein	2314	4654
	Inhibitor	-	90
	Water	100	255
Refinement	B-factors		
	Protein	43.06	38.84
	Inhibitor	-	37.25
	Water	44.64	45.39
	R.m.s deviations		
	Bond lengths(Å)	0.008	0.0066
	Bond angles(°)	0.87	1.478
	MolProbity score	1.41	1.94
	Clashscore	4.15	6.02
	Poor rotamers (%)	0	2.13
Validation	Ramchandran plot		
	Favored (%)	96.66	94.82
	Allowed (%)	3.34	5.18
	Disallowed (%)	0	0

702 **Table S5. Data collection and refinement statistics of M^{pro} crystal structures.**
 703



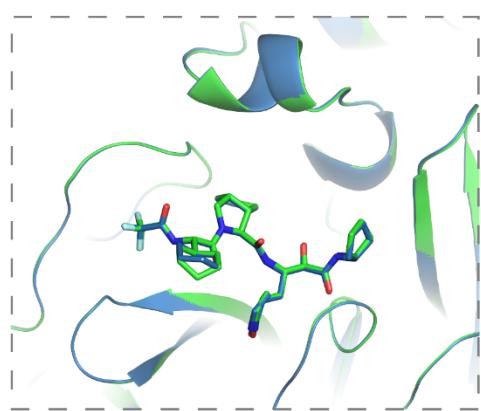
704
705

706 **Fig S10. Simulated-annealing 2mFo-DFc composite omit map densities showing bound**
707 **RAY1216 and covalent linkages between RAY1216 and Cys145.** The simulated-annealing
708 composite omit map for the M^{pro} dimer was calculated by omitting bound RAY1216 and
709 Cys145 in both monomers. Shown densities are contoured at 1.1σ , models are colored
710 according to **Fig. 2.** **a**, density in protomer A. **b**, density in protomer B.

711

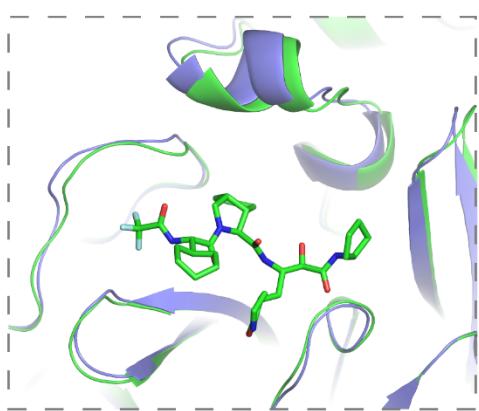
712

a



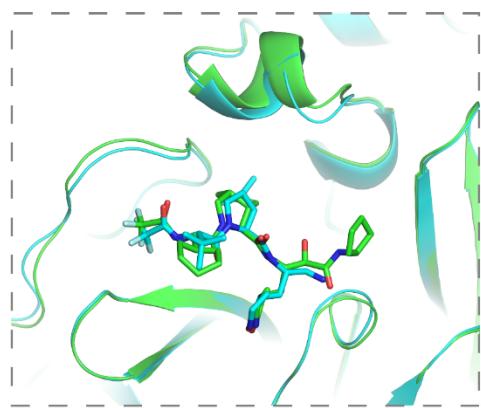
RAY1216 Protomer A
RAY1216 Protomer B

b



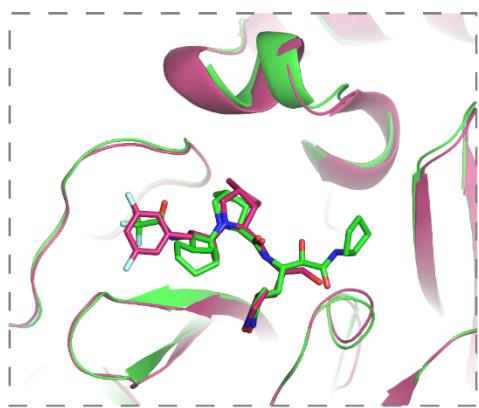
RAY1216 Protomer A
apo Protomer A

c



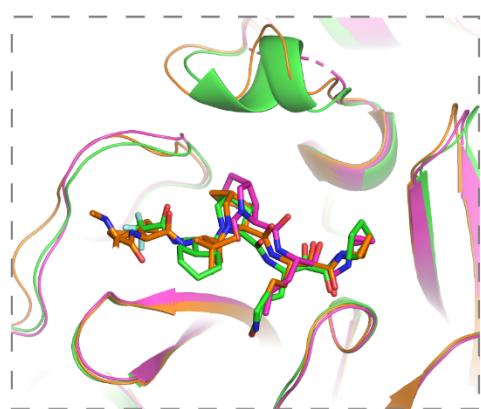
RAY1216 Protomer A
PF-07321332 Protomer A

d



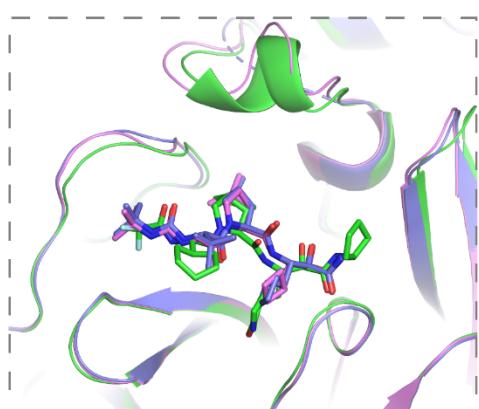
RAY1216 Protomer A
MI-23 Protomer A

e



RAY1216 Protomer A
Telaprevir Protomer A
Telaprevir Protomer B

f

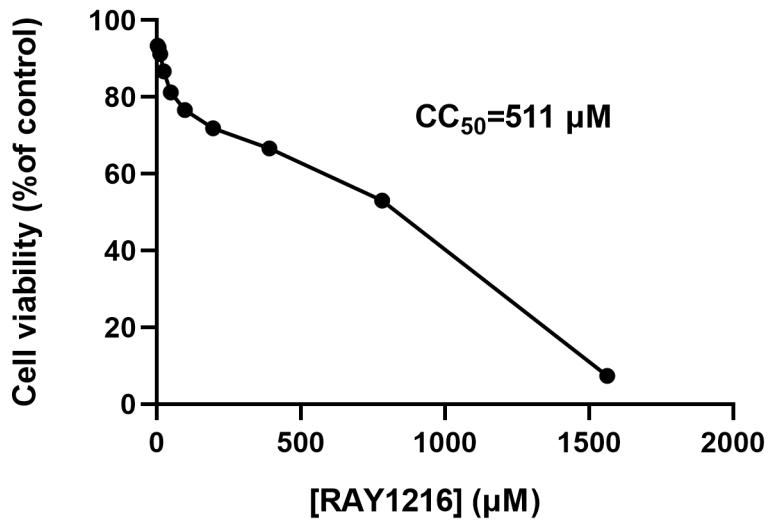


RAY1216 Protomer A
Boceprevir Protomer A
Boceprevir Protomer B

713

714 **Fig S11. Comparison of active site structures of M^{pro} in different inhibitor complexes**
715 **shows active site structural plasticity.** Structures of boceprevir (PDB: 7com (Qiao *et al.*,
716 2021)), MI-23 (PDB: 7d3i (Qiao *et al.*, 2021)), PF-07321332 (PDB: 7rfw (Owen *et al.*,
717 2021)), telaprevir (PDB: 7c7p (Qiao *et al.*, 2021)) in complex with M^{pro} are compared with
718 RAY1216:M^{pro} structures.

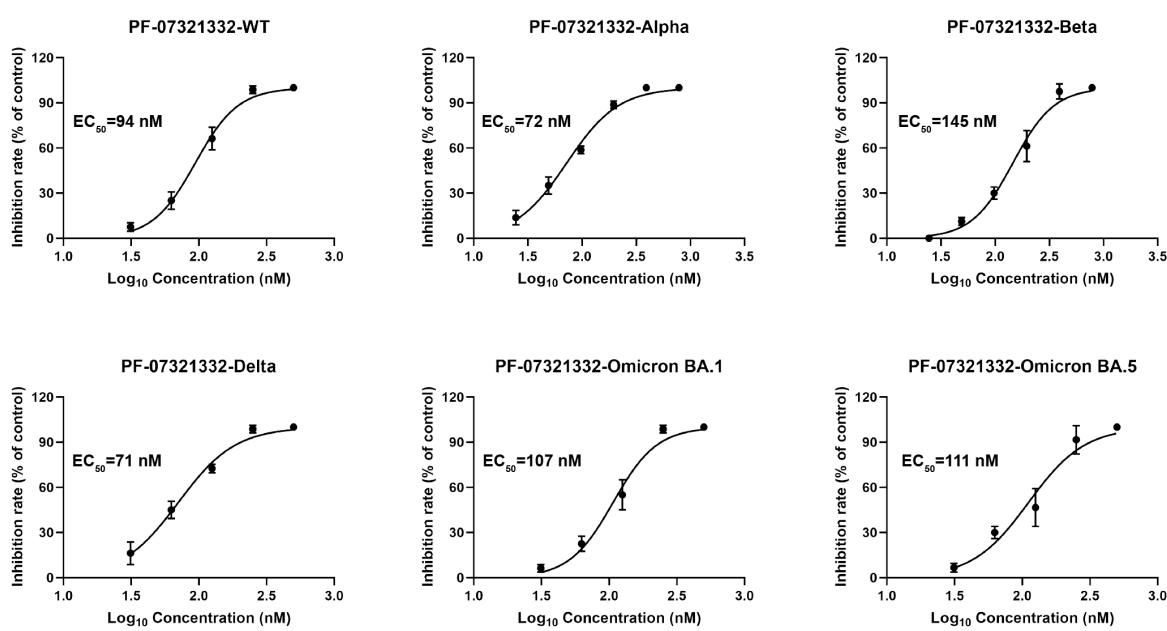
719



720

721 **Fig. S12. Cytotoxicity of RAY1216 on Vero E6 cells.**

722



723
724 Fig. S13. Dose-response curves (mean ± SD, n = 3) of wildtype (WT)
725 and variant SARS-CoV-2 strains by PF-07321332 in Vero E6 cell using MTT cell viability assay.
726

Animal species	Plasma stability (%2h)	
	RAY1216	PF-07321332
mouse	85	91.4
rat	85	84.3
dog	83	74.6
cynomolgus macaque	81	99.6
human	110	74.6

727 **Table S6. *In-vitro* plasma stability data.**

728

729 **Materials and Methods**

730

731 **Synthesis of RAY1216**

732 Step 1: Synthesis of the RAY1216-2 hydrochloride salt

733 To a solution of RAY1216-1 (500 mg, 1.75 mmol) in ethyl acetate (5 mL) at 20 °C HCl/EA
734 (10 mL, 4 M) was added. The resulting mixture was stirred for 2 h at 20 °C. Solvent was
735 removed to afford the residue as unpurified RAY1216-2 hydrochloride salt. ¹H NMR (400
736 MHz, CD₃OD) δ = 4.28 - 4.20 (m, 1H), 3.91 - 3.81 (m, 3H), 3.45 - 3.35 (m, 2H), 2.86 - 2.74
737 (m, 1H), 2.48 - 2.36 (m, 1H), 2.29 - 2.19 (m, 1H), 2.02 - 1.94 (m, 1H), 1.93 - 1.80 (m, 1H).

738 Step 2: Synthesis of RAY1216-4

739 To a solution of Boc-L-cyclohexylglycine (1 g, 3.89 mmol) in N,N-dimethylformamide (10
740 mL) 2-(7-azobenzotriazole)-N,N-tetramethylurea hexafluorophosphate (1.77 g, 4.66 mmol)
741 was added. The resulting mixture was stirred for 0.5 h, to which diisopropylethylamine (1.26
742 g, 9.72 mmol) and RAY1216-3 hydrochloride salt (1.02 g, 4.66 mmol) were added. The
743 resulting mixture was stirred for 16 h at 20 °C. The reaction mixture was added to methyl
744 tert-butyl ether (50 mL), water (20 mL) and washed with 3% citric acid (20 mL×2) and brine
745 (20 mL). The combined organic phase was dried over anhydrous sodium sulfate. Solvent was
746 removed and the residue was purified by silica gel column chromatography (petroleum ether:
747 ethyl acetate = 3:1) to afford RAY1216-4. ¹H NMR (400MHz, CDCl₃) δ = 5.22 - 5.11 (m,
748 1H), 4.36 (d, *J*=3.9 Hz, 1H), 4.27 (dd, *J*=6.9, 9.3 Hz, 1H), 4.21 - 4.12 (m, 2H), 3.83 (dd,
749 *J*=7.8, 10.4 Hz, 1H), 3.70 (br dd, *J*=3.6, 10.4 Hz, 1H), 2.81 - 2.61 (m, 2H), 1.82 - 1.70 (m,
750 6H), 1.68 - 1.61 (m, 4H), 1.56 - 1.48 (m, 2H), 1.46 - 1.38 (m, 9H), 1.29 - 1.22 (m, 4H), 1.21 -
751 0.98 (m, 4H).

752 Step 3: Synthesis of RAY1216-5

753 To a THF (14 mL) solution of RAY1216-4 (1.41g, 3.34 mmol), LiOH•H₂O (280.03 mg, 6.67
754 mmol) in water (5 mL) was added. The resulting mixture was stirred for 16 h at 20 °C. Citric
755 acid was added to the reaction mixture to 3%. Solvent was removed and residue was
756 extracted with ethyl acetate (50 mL), washed with brine (30 mL). The combined organic
757 phase was dried over anhydrous sodium sulfate. Solvent was removed to afford the residue as
758 unpurified RAY1216-5. ¹H NMR (400MHz, DMSO-d₆) δ = 12.58 - 12.23 (m, 1H), 6.92 -
759 6.82 (m, 1H), 4.11 - 3.94 (m, 2H), 3.82 - 3.76 (m, 1H), 3.72 - 3.62 (m, 1H), 2.73 - 2.64 (m,
760 1H), 2.62 - 2.55 (m, 1H), 1.92 - 1.42 (m, 12H), 1.40 - 1.32 (m, 9H), 1.18 - 1.06 (m, 3H), 1.00
761 - 0.81 (m, 2H).

762 Step 4: Synthesis of RAY1216-6

763 To a 2-butanone (7 mL) solution of RAY1216-5 (650 mg, 1.65 mmol), 1-
764 hydroxybenzotriazole (222.63 mg, 1.65 mmol), 1-(3-dimethylaminopropyl)-3-
765 ethylcarbodiimide hydrochloride (379.03 mg, 1.98 mmol), diisopropylethylamine (638.84
766 mg, 4.94 mmol) were added. The resulting mixture was stirred for 0.5 h at 20 °C, before
767 RAY1216-2 hydrochloride salt (366.88 mg, 1.65 mmol) was added. The resulting mixture
768 was stirred for 16 h at 20 °C. The reaction mixture was diluted with water (20 mL) and

769 extracted with dichloromethane: methanol (30 mL×2, 10:1), the combined organic phase was
770 washed with 3% citric acid (20 mL×2), brine (30 mL). The organic phase was dried over
771 anhydrous sodium sulfate. Solvent was removed and the residue was purified by silica gel
772 column chromatography (dichloromethane: methanol = 20: 1) to afford RAY1216-6. ¹H
773 NMR (400 MHz, CDCl₃) δ = 7.49 - 7.42 (m, 1H), 6.23 - 6.05 (m, 1H), 5.28 - 5.17 (m, 1H),
774 4.64 - 4.51 (m, 1H), 4.43 - 4.24 (m, 2H), 3.92 - 3.81 (m, 1H), 3.78 - 3.70 (m, 3H), 3.39 - 3.27
775 (m, 2H), 2.94 - 2.75 (m, 2H), 2.57 - 2.36 (m, 2H), 2.24 - 2.07 (m, 1H), 1.94 - 1.50 (m, 14H),
776 1.49 - 1.41 (m, 9H), 1.27 - 0.95 (m, 6H).

777 Step 5: Synthesis of RAY1216-7

778 To a THF (31 mL) solution of RAY1216-6 (3.10 g, 5.51 mmol) at 0 °C, lithium borohydride
779 (240.02 mg, 11.02 mmol) was added. Temperature of the mixture was allowed to warm to
780 20 °C slowly and stirred for 2 h at 20 °C. The mixture was diluted with water (10 mL) and
781 ethyl acetate (20 mL) and stirred for 10 min, white solid was collected as crude target product
782 RAY1216-7 by filtration. LCMS (m/z) 535.4 [M+1]⁺.

783 Step 6: Synthesis of RAY1216-8

784 To a DCM (10 mL) solution of compound RAY1216-7 (0.5 g, 935.13 μmol) Dess-Martin
785 periodinane (594.94 mg, 1.40 mmol) was added. The resulting mixture was stirred for 16 h.
786 The mixture was diluted with saturated sodium thiosulfate (15 mL) and saturated sodium
787 bicarbonate solution (15 mL) and stirred for 10 min. The aqueous layer was extracted with
788 DCM (50 mL×2). The combined organic phase was washed with brine (5 mL) and dried over
789 anhydrous sodium sulfate. Solvent was removed and to afford the residue as unpurified
790 RAY1216-8. LCMS (m/z) 533.4 [M+1]⁺.

791 Step 7: Synthesis of RAY1216-9

792 To a DCM (10 mL) solution of RAY1216-8 (436 mg, 818.52 μmol), acetic acid (58.98 mg,
793 982.22 mmol) and cyclopentyl isocyanide (94.44 mg, 982.22 μmol) were added. The
794 resulting mixture was stirred for 2 h at 25 °C. After addition of saturated ammonium chloride
795 solution (10 mL), the reaction mixture was stirred for 10 min and extracted with DCM (20
796 mL). The combined organic phase was washed with brine (5 mL). The organic phase was
797 dried over anhydrous sodium sulfate, solvent was removed and the residue was purified by
798 silica gel column chromatography (dichloromethane: methanol = 10:1) to afford RAY1216-9.
799 LCMS (m/z) 688.4 [M+1]⁺.

800 Step 8: Synthesis of RAY1216-10

801 To a MeOH (3 mL) solution of RAY1216-9 (190 mg, 276.22 μmol), K₂CO₃ (95.44 mg,
802 690.54 μmol) in water (5 mL) was added. The resulting mixture was stirred for 16 h at 20 °C.
803 The reaction mixture was diluted with 3% citric acid and extracted with DCM (40 mL× 3),
804 the combined organic phase was washed with brine (30 mL) and dried over anhydrous
805 sodium sulfate. Solvent was removed to afford the residue as unpurified crude RAY1216-10.

806 Step 9: Synthesis of RAY1216-11

807 To a DCM (10 mL) solution of RAY1216-10 (238.00 mg, 368.52 μmol), Dess-Martin
808 periodinane (203.19 mg, 479.08 μmol) was added. The resulting mixture was stirred for 16 h
809 at 20°C. The reaction mixture was diluted with saturated sodium thiosulfate (15 mL) and

810 saturated sodium bicarbonate solution (15 mL), extracted with DCM (50 mL×2), and washed
811 with brine (15 mL). The organic phase was dried over anhydrous sodium sulfate, solvent was
812 removed, and the residue was purified by silica gel column chromatography
813 (dichloromethane: methanol = 20:1) to afford RAY1216-11.

814 **Step 10: Synthesis of RAY1216-12**

815 To a THF (3 mL) solution of RAY1216-11 (125 mg, 194.16 μmol), HCl/EA (2.91 mL, 4 M)
816 was added. The resulting mixture was stirred for 1 h at 25 °C. Solvent was removed to afford
817 crude RAY1216-12.

818 **Step 11: Synthesis of RAY1216**

819 To a THF (3 mL) solution of RAY1216-12 (125 mg, 229.91 μmol) at 0 °C, TFAA (193.15
820 mg, 919.63 μmol) and pyridine (127.30 mg, 1.61 mmol) were added. The resulting mixture
821 was stirred for 16 h at 20 °C. The reaction mixture was diluted with water (20 mL) and
822 extracted with dichloromethane (30 mL× 2). The combined organic phase was washed with
823 3% citric acid (40 mL) and brine (40 mL×2). The organic phase was dried over anhydrous
824 sodium sulfate. Solvent was removed and the residue was purified by HPLC to afford
825 RAY1216. LC-MS (m/z) 640.0 [M+1]⁺.

826 ¹H NMR (400 MHz, DMSO-d6) δ ppm 9.75 d (J=7.5 Hz,1H) 8.64 d (J=7.5 Hz,1H), 8.50 d
827 (J=8.3 Hz,1H), 7.65 s (1H), 5.14 ddd (J=11.5, 8.3, 2.9 Hz,1H), 4.28 t (J=8.6 Hz,1H), 4.20 d
828 (J=4.2 Hz,1H), 4.04 m (1H), 3.76 AABB-d (J=10.2, 7.6, 3.0 Hz,2H), 3.20 - 3.11 m (2H), 2.69
829 m (1H), 2.55 m (1H), 2.50 m (1H), 2.20 - 1.69 m (2H), 1.88 - 1.60 m (2H), 1.83 m (1H), 1.80
830 -1.40 m (6H), 1.80 - 1.50 m (4H), 1.80-1.40 m (4H), 1.79-0.95 m (4H), 1.65 - 1.13 m (6H).
831 ¹³C NMR (400 MHz, DMSO-d6) δ ppm 115.9, 156.5, 56.1, 38.7, 28.2, 28.6, 25.4, 25.6, 25.8,
832 168.6, 53.5, 42.7, 24.6, 31.2, 31.66, 47.5, 65.5, 171.9, 51.8, 31.8, 37.5, 178.4, 39.5, 27.5,
833 197.2, 161.0, 50.4, 23.6, 31.64, 31.68.

834 **Crystallization of RAY1216 and X-ray diffraction**

835 RAY1216 powder (approximately 30 mg) was dissolved in isopropyl acetate (approximately
836 600 μl) with gentle stirring gently until the mixture was saturated. The solution was
837 transferred into a transparent 2ml MS sample vial after being filtered by syringe filter. The
838 sample vial was sealed with a parafilm and 5-10 holes on the parafilm were pierced by a
839 syringe needle. The MS vial was placed in a closed brown bottle which contained 0.1 - 0.2
840 cm level of n-hexane. Solutions were let stand at 20 - 30 °C for 48 hours. After granular
841 white crystals were observed in the MS vial, the isopropyl acetate was removed, and a single
842 crystal of RAY1216 was sealed with silicone grease and subjected to X-ray diffraction on a
843 Rigaku Oxford Diffraction XtaLAB Synergy-S four-circle diffractometer equipped with a
844 CuKα source (λ =1.54184 Å) and a HyPix-6000HE area detector.

845 **Data collection and structure determination**

846 40929 diffraction spots were collected by X-ray diffraction experiment, and 5638
847 independent diffraction dots were indexed and integrated (R_{int} =0.0391). Diffraction collection
848 range $2\theta = 5.752^\circ$ to 133.106° , diffraction index range $-12 \leq h \leq 12$, $-11 \leq k \leq 11$, $-18 \leq l \leq 18$.
849 Structure was determined by SHELXT (Sheldrick, 2015b) and refined by SHELXL (against

850 F²) (Sheldrick, 2015a). 406 parameters are participating in structural refinement. The final
851 result has a goodness-of-fit (s) = 1.041, R₁ = 0.0351, wR₂ = 0.0914. The residual electron
852 density values are 0.39 and -0.31 e Å⁻³. The data collection and structure refinement statistics
853 are summarized in **Table S1**.

854 **Recombinant protein production**

855 Based on a previous study (Zhang *et al.*, 2020), a construct encoding SARS-CoV-2 Mpro
856 (ORF1ab 3264-3569, GenBank code:MN908947.3) was subcloned into the pGEX-6p-1
857 vector between the BamHI and XhoI restriction sites with extra C-terminal extension
858 GPHHHHHHHHHH. The resulting construct was verified by DNA sequencing. The
859 construct plasmid was transformed into BL21 (DE3) *E. coli* cells (Vazyme, #C504-02/03)
860 and scale-up expression (~ 6 L) was started from a single colony in LB medium
861 supplemented with 100 µg/ml of ampicillin at 37 °C. The cells were induced with 0.5 mM
862 IPTG when the OD600 reached 0.8. Cells were allowed to grow post induction for 20 h at
863 16 °C.

864 The cells were harvested by centrifugation, and cell pellet was lysed in the lysis buffer
865 (20mM Tris, pH 7.8, 150mM NaCl, 10mM imidazole) by sonication on ice. Cell lysate was
866 cleared by high-speed centrifugation (20,000 × g at 4°C for 1h). The supernatant was mixed
867 with Ni-NTA resin for ~ 2 h at 4 °C on a shaker. The Ni-NTA resin was washed with two
868 buffers of different imidazole concentrations (20 mM Tris, pH 7.8, 150 mM NaCl, 20 mM/50
869 mM imidazole) each for over 30 resin volumes to remove contaminants. The target protein
870 was eluted by the elution buffer (20 mM Tris, pH 7.8, 150 mM NaCl, 500 mM imidazole).
871 400U Human rhinovirus (HRV) 3C enzyme was added into the eluted protein to remove the
872 C-terminal histidine tag and the mixture was dialysed at 4 °C overnight in dialysis buffer (20
873 mM Tris, pH 7.8, 150 mM NaCl, 1m M DTT) using a dialysis bag with MWCO (Molecular
874 Weight Cut Off) of 10 kDa. The dialysed mixture was reloaded onto the Ni-NTA resin and
875 His-tag-free target protein was collected from the flow-through.

876 Since the expressed M^{pro} contains the native M^{pro} cleavage sequence “SAVLQ/SGFRK”
877 found between Nsp4 and Nsp5 (M^{pro}) in the SARS-CoV-2 Nsp polyprotein (slash indicates
878 the M^{pro} cleavage site) near the N-terminus, M^{pro} auto-cleaving activity generates an authentic
879 N-terminus during protein expression. The HRV 3C enzyme recognition site has the
880 sequence (LEVLFQ/GP, slash indicates the HRV 3C enzyme cleavage site) after HRV 3C
881 cleavage, it will generate an authentic M^{pro} C-terminus (LEVLFQ/SAV, native M^{pro} C-
882 terminal recognition sequence, slash indicates the cleavage site). Purified M^{pro} with authentic
883 N- and C-termini was concentrated by a 10 kDa MWCO Amicon Ultra 50 centrifugal filters

884 (Merck Millipore) at 4 °C to ~ 10 mg/ml. Concentrated protein was either used for
885 crystallization without freezing or flash frozen in liquid nitrogen and stored under -80 °C.

886 **Enzyme activity assay**

887 The enzyme assays were performed in enzyme kinetics assay buffer (20 mM Tris pH 7.8, 150
888 mM NaCl, 1 mM DTT and 100 µg/ml bovine serum albumin) using Dabcyl-
889 KTSAVLQ/S^gGFRKME-Edans (Beyotime, #P9733, ‘/’ indicates the M^{pro} cleavage site) as the
890 substrate. Fluorescent signal by enzyme cleavage of the substrate was monitored on a
891 Molecular Devices FlexStation 3 reader with filters for excitation at 340 nm and emission at
892 490 nm at 20 °C.

893 **Molar response coefficient of fluorescent product**

894 M^{pro} at a relatively high concentration (1.0 µM) was assayed with 1.25, 2.5, 5, 10, 20, 40, and
895 80 µM substrate at 37 °C. Under these experimental conditions the substrate was completely
896 converted into the fluorogenic product over a course of 20 minutes. The total observed
897 change in fluorescence intensity (ΔF), when plotted against the starting concentration of
898 substrate ([S]), displayed a significant involvement of the frequently seen Dabcyl-Edans
899 inner-filter effect (Liu et al., 1999). The dependence of ΔF on [S] follows a non-linear
900 quadratic function as illustrated in **Fig. S6a**. However, within the restricted range of substrate
901 concentrations (1.25 - 20 µM, **Fig. S6b**) the dependence of ΔF on [S] is approximately linear,
902 with the slope equal to the differential molar response coefficient of the product $r_P = (88.1 \pm$
903 3.5) rfu/µM. Thus, in all the following enzyme kinetic analyses we constrained the substrate
904 concentration accordingly ($[S] \leq 20 \mu\text{M}$) and treated the molar response coefficient as a fixed
905 parameter, set to the best-fit value of $r_P = 88 \text{ rfu}/\mu\text{M}$.

906

907 **Determination of the Michaelis constant, K_M**

908 Preliminary attempts to determine the Michaelis constant K_M by fitting initial rates vs [S] to
909 the Michaelis-Menten equation were found to be inaccurate due to the Dabcyl-Edans inner-
910 filter effect (Liu et al., 1999). Thus, substrate kinetic parameters were determined by the
911 global fit of reaction progress curves recorded at 1.25, 2.5, 5, 10, and 20 µM to the first-order
912 ordinary differential-equation (ODE) model corresponding to the reaction mechanism shown
913 in **Scheme 1**, using the software package DynaFit (Kuzmic, 1996; 2009). The DynaFit script
914 file is provided in the Supplementary Dataset.



916 **Scheme 1. Assumed Michaelis-Menten reaction mechanism of M^{pro} substrate hydrolysis.**

917 The mathematical model for the reaction progress curves is auto-generated by DynaFit
918 according to **Scheme 1** and is shown as **Eq.1**, where F is the fluorescence intensity at the
919 arbitrary reaction time t ; F_0 is the baseline offset or fluorescence intensity observed at time t
920 = 0; r_P = 88 rfu/ μ M is the molar response of the reaction product; and $[P]$ is the concentration
921 of the reaction product at time t . In its turn, the product concentration is computed by
922 numerically solving the ODE system represented by **Eq.1–Eq.5**.

923

$$F = F_0 + r_P [P] \quad (\text{Eq.1})$$

$$\frac{d[E]}{dt} = -k_{aS}[E][S] + k_{dS}[E.S] + k_{dP}[E.S] \quad (\text{Eq.2})$$

$$\frac{d[S]}{dt} = -k_{aS}[E][S] + k_{dS}[E.S] \quad (\text{Eq.3})$$

$$\frac{d[E.S]}{dt} = +k_{aS}[E][S] - k_{dS}[E.S] - k_{dP}[E.S] \quad (\text{Eq.4})$$

$$\frac{d[P]}{dt} = +k_{dP}[E.S] \quad (\text{Eq.5})$$

924

925

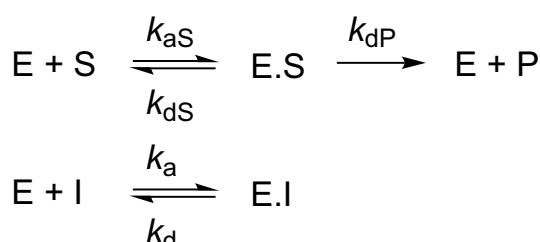
926 The enzyme–substrate association rate constant k_{aS} was fixed at the diffusion limited
927 constant value of $1.0 \mu\text{M}^{-1}\text{s}^{-1}$ (or $1 \times 10^6 \text{ M}^{-1}\text{s}^{-1}$) (Fersht, 1999), whereas the dissociation rate
928 constants k_{dS} and k_{dP} were treated as globally adjustable model parameters. Each individual
929 progress curve was associated with a locally optimized offset parameter F_0 . The experimental
930 data files (Km-R1-f.csv and Km-R2-f.csv) are provided as **Supplementary Datasets**. The
931 results of fit for both replicates are illustrated graphically in **Fig. S7**.

932 The best-fit values of adjustable model parameters are listed in **Table S2**. The average and
933 standard deviation from replicates ($n = 2$) of the dissociation rate constant is $k_{dS} = (31.0 \pm$
934 $0.2) \text{ s}^{-1}$. This particular value of k_{dS} is utilized in subsequent kinetic analyses of M^{pro}
935 inhibition as a fixed model parameter (see below). The corresponding average and standard
936 deviation of the Michaelis constant is $K_M \equiv (k_{dS} + k_{dP})/k_{aS} = (31.0 \pm 0.2) \mu\text{M}$. This value is
937 identical, within the specified experimental error, to a previously published value of $K_M =$
938 $(28.2 \pm 3.4) \mu\text{M}$ (Ma *et al.*, 2020).

939 Inhibition kinetics of PF-07321332 and active-site titration

940 The microscopic rates constants for association and dissociation of PF-07321332, as well as,
941 the concentration of M^{pro} active sites hence the enzyme’s turnover number k_{cat} , were
942 determined as follows. The enzyme (nominal concentration 80 nM) was assayed at varied

943 concentrations of the inhibitor (maximum 444 nM, 2/3 dilution series down to 17 nM, nine
944 concentrations plus control $[I] = 0$) in triplicate. The ten reaction progress curves from each
945 replicate were combined into a global dataset and fit to a differential-equation model
946 corresponding to the reaction mechanism shown in **Scheme 2** using the software package
947 DynaFit (Kuzmic, 1996; 2009). The requisite DynaFit script file and PF-07321332 inhibition
948 progress curve data are provided as **Supplementary Datasets**.



949

950 **Scheme 2. Proposed reaction and inhibition mechanisms of M^{pro}.**

$$\frac{d[E]}{dt} = -k_{aS}[E][S] + k_{dS}[E.S] + k_{dP}[E.S] - k_a[E][I] + k_d[E.I] \quad (\text{Eq.6})$$

$$\frac{d[S]}{dt} = -k_{aS}[E][S] + k_{dS}[E.S] \quad (\text{Eq.7})$$

$$\frac{d[E.S]}{dt} = +k_{aS}[E][S] - k_{dS}[E.S] - k_{dP}[E.S] \quad (\text{Eq.8})$$

$$\frac{d[P]}{dt} = +k_{dP}[E.S] \quad (\text{Eq.9})$$

$$\frac{d[I]}{dt} = -k_a[E][I] + k_d[E.I] \quad (\text{Eq.10})$$

$$\frac{d[E.I]}{dt} = +k_a[E][I] - k_d[E.I] \quad (\text{Eq.11})$$

951

952 To obtain kinetic parameters of PF-07321332 inhibition, the microscopic rate constants k_{aS}
953 and k_{dS} are fixed, whereas the turnover number rate constant $k_{\text{cat}} \equiv k_{dP}$ and the active enzyme
954 site concentration are treated as adjustable model parameters (**Table S3**) (marked by the
955 notation “??” in DynaFit script files). The combined progress curves were fit globally to
956 **Eq.1**, where the product concentration $[P]$ in this case is computed by numerically solving the
957 ODE system represented by **Eq.6–Eq.11**.

958 Thus, the complete list of globally optimized parameters (shared by all progress curves)
959 consists of k_{dP} , k_a , k_d , and $[E]$; the ten baseline offset values, F_0 , each specific to a particular
960 progress curve, were treated as locally adjustable model parameters. The overlay of
961 experimental data on the best-fit model curves and the corresponding plots of instantaneous
962 reaction rates are shown in **Fig. S8**. Numerical results are summarized in **Table S3**.

963 The results listed in **Table S3** show that all adjustable model parameters were obtained with
964 better than 11% reproducibility. The enzyme was approximately 61% active. The turnover
965 number $k_{\text{cat}} = 0.12 \text{ s}^{-1}$ compares well with $k_{\text{cat}} = 0.16 \text{ s}^{-1}$ previously reported (Ma *et al.*,
966 2020). Similarly, the inhibition constant $K_i \equiv k_d/k_a = (3.8 \pm 0.2) \text{ nM}$ determined here
967 compares well with the previously reported value of $K_i = 3.1 (1.5 - 6.8) \text{ nM}$ (Owen *et al.*,
968 2021). The PF-07321332 inhibitor associates relatively rapidly with the enzyme, with
969 bimolecular association rate constant equal to $k_a = 4.9 \times 10^5 \text{ M}^{-1}\text{s}^{-1}$. The drug-target
970 residence time $t_{\text{res}} \equiv 1/k_d$ is approximately 9 minutes.

971 **Inhibition kinetics of RAY1216**

972 Preliminary analysis of RAY1216 inhibition data revealed that it is possible to reliably deter-
973 mine either the turnover number $k_{\text{cat}} \equiv k_{\text{dP}}$, or the active enzyme concentration [E], but not
974 both. However, the precise values of these model parameters and in particular the active
975 enzyme concentration strongly influence the best-fit values of the inhibition rate constants k_a
976 and k_d . For this reason we have analysed the RAY1216 datasets while treating the
977 microscopic rate constant k_{dP} as a fixed parameter determined in the fit of the PF-07321332
978 data above (The RAY1216 and PF-07321332 experiments were carried out in parallel on the
979 same day using the same M^{pro} prep). (The “??” notation is deleted on the “ k_{dP} ” line in the
980 provided DynaFit script to fix “ k_{dP} ” for fitting the RAY1216 datasets.)

981 Thus, the complete list of globally optimized parameters (shared by all progress curves)
982 consists of k_a , k_d , and [E]; the ten baseline offset values, F_0 , each specific to a particular
983 progress curve, were again treated as locally adjustable model parameters. The overlay of
984 experimental data on the best-fit model curves and the corresponding plots of instantaneous
985 reaction rates are shown in **Fig. S9**. Note that, in comparison with **Fig. S8**, the instantaneous
986 rate plots show significantly longer time that is required for near-equilibrium to be
987 established, even at the highest inhibitor concentrations. This graphically illustrates that
988 RAY1216 is a “slow-on, slow-off” inhibitor of M^{pro} , while PF-07321332 is a “fast-on, fast-
989 off” inhibitor. Numerical results of fit are summarized in **Table S4**.

991 The results listed in **Table S4** show that all adjustable model parameters were obtained with
992 better than 5% reproducibility. The enzyme was apparently 66% active. This value differs
993 slightly from the 61% enzyme activity determined in the analysis of PF-07321332 data (see
994 **Table S3**). We observed that, throughout the working day, the enzymatic activity of M^{pro}
995 decreased slightly but systematically over time. RAY1216 associates relatively slowly with
996 the target enzyme, with bimolecular association rate constant equal to $k_a = 1.9 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$
997 The inhibition constant of RAY1216, $K_i = 8.4 \text{ nM}$, is 2.2 times higher than the K_i for PF-
998 07321332 determined here (**Table S3**). In contrast, the drug-target residence time is $104/9 =$
999 11.5 times longer.

1000 **Crystallization and crystal soaking with RAY1216 inhibitor**

1001 Apo M^{pro} crystals were crystallized by mixing 1 μ l of freshly purified M^{pro} (without freezing)
1002 at 10 mg/ml with 1 μ l crystallization solution (0.1 M MES monohydrate pH 6.5, 12% w/v
1003 Polyethylene glycol 20,000) using hanging drop vapor diffusion method at 16 °C.

1004 Crystals normally grew overnight. The apo crystals were flash frozen in cryoprotection
1005 solution (0.1 M MES monohydrate pH 6.5, 12% w/v polyethylene glycol 20,000, 40%
1006 glycerol) using liquid nitrogen. To obtain RAY1216 soaked crystals, apo crystals were
1007 transferred into the crystallization solution supplemented with 6.6 mM RAY1216 and 3%
1008 DMSO (from the RAY1216 solution). The crystals were soaked for ~10 min at 16 °C.

1009 Finally, crystals were briefly soaked in cryoprotection solution (0.1 M MES monohydrate pH
1010 6.5, 12% w/v polyethylene glycol 20,000, 40% glycerol) supplemented with 6.6 mM
1011 RAY1216 before being frozen in liquid nitrogen.

1012 **Data collection and structure determination**

1013 Single crystal X-ray diffraction data were collected on beamline BL19U1 at Shanghai
1014 Synchrotron Radiation Facility (SSRF) at 100 K using an Eiger X 16M hybrid-photon-
1015 counting (HPC) detector. Data integration and scaling were performed using the XDS
1016 software (BUILT 20220220) (Kabsch, 2010). Structures were determined by molecular
1017 replacement (MR) using the Phaser MR 2.8.3 (McCoy et al., 2007) program in CCP4 7.1.018
1018 (Winn et al., 2011), with a SARS-CoV-2 M^{pro} structure (Zhao et al., 2022) (PDB code:
1019 7VH8) as the search model. Iterative manual model building was carried out in Coot 0.9.6
1020 (Emsley and Cowtan, 2004). Final structures were refined with Refmac 5.8.0267 (Murshudov
1021 et al., 1997). The data collection and structure refinement statistics are summarized in **Table**
1022 **S5**.

1023 **Cell lines and virus strains**

1024 African green monkey kidney epithelial (Vero E6) cells were purchased from the American
1025 Type Culture Collection (ATCC), and cultured in Dulbecco's modified Eagle's medium
1026 (DMEM, Gibco, USA) supplemented with 10% fetal bovine serum (FBS, Gibco, USA), 100
1027 μ g/mL streptomycin (Gibco, USA), and 100 U/mL penicillin (Gibco, USA). SARS-CoV-2
1028 and its variants, namely Alpha (B.1.1.7), Beta (B.1.1.529), Delta (B.1.617.2), Omicron BA.1
1029 (B.1.1.529) and Omicron BA.5 (BA.5.2) were isolated from clinical samples and were
1030 deposited at the First Affiliated Hospital of Guangzhou Medical University. Viruses were
1031 propagated as previously described (Zhu et al., 2020a), the viruses were aliquoted and stored

1032 at -80 °C and the titres of cultured viruses were estimated as 50% tissue culture infective
1033 doses (TCID₅₀) using the Reed–Muench method.

1034 **Cytotoxicity and cytopathic effect (CPE) inhibition assays-SARS-CoV-2**

1035 The 50% toxicity concentration (CC₅₀) for RAY1216 in Vero E6 cells was determined using
1036 the MTT (3-(4,5-dimethylthiazolk-2-yl)-2,5-diphenyl tetrazolium bromide) assay (Park et al.,
1037 2011). Different dilutions of RAY1216 and PF-07321332 were incubated with Vero E6 ($5 \times$
1038 10⁴ cells/well) cells in 96-well plates for the cytotoxicity assay, and the concentration of
1039 RAY1216 and PF-07321332 causing 50% cell death were determined as the CC₅₀ value. The
1040 50% inhibition concentration (EC₅₀) of virus-induced cytopathic effect (CPE) was used to
1041 investigate the efficacy of RAY1216 and PF07321332 against SARS-CoV-2. A monolayer of
1042 Vero E6 cells was inoculated with 100 TCID₅₀ of SARS-CoV-2 wildtype or variant strains at
1043 37 °C for 2 h. The cells were incubated with different concentrations of RAY1216 and PF-
1044 07321332 after removal of the inoculum. Infected cells were observed under a microscope
1045 after 72 h of incubation to assess CPE. Dose response curves were plotted as CPE vs Log
1046 inhibitor concentrations. The selectivity indices (SI) were determined using the ratio of CC₅₀
1047 to EC₅₀.

1048 **Antiviral and anti-inflammatory activity of RAY1216 in mouse model**

1049 Antiviral studies using animals were approved by the Guangzhou Medical University Ethics
1050 Committee of Animal Experiments (IACUC certificate No.: GZL0008). All antiviral
1051 experiments using animals passed the ethical review and were performed in strict accordance
1052 with the National Research Council Criteria and the Chinese Animal Protection Act. Five-to
1053 six-week-old female ACE2 transgenic C57BL/6 mice (Bao *et al.*, 2020; Ma *et al.*, 2022)
1054 weighing 18–22 g were acquired from GemPharmatech Co., Ltd. (Jiangsu, China) and housed
1055 under specific pathogen-free (SPF) conditions at Guangzhou Customs District Technology
1056 Center Biosafety Level 3 (BSL-3) Laboratory. Mice were randomly divided into six groups
1057 ($n = 7$): the control group; SARS-CoV-2 virus (Delta variant (B.1.617.2)) infected group;
1058 treatment groups of three different RAY1216 concentrations (600 mg/kg/day, 300 mg/kg/day,
1059 150 mg/kg/day); and a PF-07321332 treatment group (600 mg/kg/day). Mice were
1060 anesthetized by inhalation of 5% isoflurane and each mouse was inoculated with 50 µl PBS
1061 containing a lethal dose of 10⁵ PFU SARS-CoV-2 (Delta variant) for the infected groups. For
1062 the control group, 50 µl PBS was administered intranasally. 2 hr after infection, the infected
1063 mice were intragastrically administered with RAY1216 (600 mg/kg/day, 300 mg/kg/day, 150
1064 mg/kg/day), PF-07321332 (600 mg/kg/day) or PBS daily for 5 days. Weight change and
1065 mortality of mice in each group was recorded daily for 5 days. To measure lung virus titres

1066 and to examine lung pathology, a separate set of experiments was performed under the same
1067 grouping and conditions except that each mouse was inoculated with a non-lethal dose of
1068 $10^{3.5}$ PFU SARS-CoV-2 (Delta variant) for the infected groups. At 3 and 5 days post
1069 infection, mice were sacrificed, and lung tissues were collected to measure virus titres and to
1070 examine lung pathology.

1071 ***In-vitro* plasma stability analysis**

1072 To assess *in-vitro* plasma stabilities of RAY1216 and PF-07321332, 2 μ M of the tested
1073 compounds were incubated in plasma from different CD-1 mouse, SD rat, beagle dog,
1074 cynomolgus monkey and human at 37 °C for 2 hr. 40 μ L of samples were added into 160 μ L
1075 of internal standard working solution (200 ng/mL tolbutamide in MeOH). The mixed solutions
1076 were vortex and centrifuged at 16000 g for 10 min at 2-8 °C. The supernatant was analysed by
1077 LC-MS/MS.

1078 **Pharmacokinetic studies**

1079 The pharmacokinetic (PK) studies using animals were approved by the WuXi AppTec Ethics
1080 Committee of Animal Experiments (IACUC certificate No.: NJ-20220531). All animals used
1081 in this study were male and chosen randomly. Pharmacokinetic properties of RAY1216 and
1082 PF-07321332 following a single intravenous injection (IV) or gavage (PO) administration
1083 were examined. Briefly, two groups were assigned for each animal species, male CD-1
1084 mouse (2 mice/group), male SD rat (3 rats/group) and male cynomolgus macaque (2
1085 cynomolgus macaques /group), respectively. The test compound was administered to each
1086 group at the indicated dose, orally or intravenously. The specific doses administered are
1087 shown in **Table 3**. Plasma samples of animals in IV groups were collected before
1088 administration (0), 0.083, 0.25, 0.5, 1, 2, 4, 8 and 24 hours after administration; samples of
1089 animals in PO groups were collected before administration (0), 0.25, 0.5, 1, 2, 4, 6, 8, and 24
1090 hours after administration.

1091 **LC-MS/MS analysis of plasma samples**

1092 Plasma samples were obtained by centrifugation and concentrations of the compounds in the
1093 serum were assessed by LC-MS/MS. On an ACQUITY UPLC System, ACQUITY UPLC
1094 Protein BEH C4 column (300Å, 1.7 μ m, 2.1 × 50 mm), ACQUITY UPLC HSS T3 column
1095 (1.8 μ m, 2.1 × 50 mm) and Phenomenex Kinetex C18 LC column (2.6 μ m, 100 Å, 2.1 × 50
1096 mm) were used for analyses of samples from mouse, rat and cynomolgus macaque,

1097 respectively. The pharmacokinetic parameters of RAY1216 and PF-07321332 in plasma were
1098 calculated using non-compartmental analysis as implemented in Phoenix WinNonlin software
1099 (version 8.3.4).