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Antiviral activity of natural phenolic compounds in complex at an allosteric site of SARS-CoV-2 papain-like protease

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81 **Abstract**

82 SARS-CoV-2 papain-like protease (PLpro) covers multiple functions. Beside the
83 cysteine-protease activity, facilitating cleavage of the viral polypeptide chain, PLpro
84 has the additional and vital function of removing ubiquitin and ISG15 (Interferon-
85 stimulated gene 15) from host-cell proteins to support coronaviruses in evading the
86 host's innate immune responses. We identified three phenolic compounds bound to
87 PLpro, preventing essential molecular interactions to ISG15 by screening a natural
88 compound library. The compounds identified by X-ray screening and complexed to
89 PLpro demonstrate clear inhibition of PLpro in a deISGylation activity assay. Two
90 compounds exhibit distinct antiviral activity in Vero cell line assays and one
91 inhibited a cytopathic effect in non-cytotoxic concentration ranges. In the context of
92 increasing PLpro mutations in the evolving new variants of SARS-CoV-2, the
93 natural compounds we identified may also reinstate the antiviral immune response
94 processes of the host that are down-regulated in COVID-19 infections.

95

96 **Introduction**

97 The coronavirus disease COVID-19, caused by SARS-CoV-2, remains devastating
98 with high numbers of infections and deaths¹. Several approved and highly effective
99 vaccines against COVID-19 were developed worldwide in only a year. However,
100 these vaccines are not uniformly available around the world, and consequently new
101 SARS-CoV-2 variants have already emerged which may impact the effectiveness of
102 the available vaccines in the future². In parallel, more efforts are needed to identify
103 and optimize alternative treatments for patients infected by SARS-CoV-2, who do
104 not respond to or cannot tolerate vaccines³. Hence, research is ongoing at a rapid
105 pace to identify effective drug candidates by applying complementary strategies.

106 One approach, we followed recently, is the massive X-ray crystallographic
107 screening for inhibitors of SARS-CoV-2 main protease Mpro, an essential protein in
108 the viral replication process and hence an important drug target⁴. We identified six
109 compounds inhibiting Mpro that showed antiviral activity and these compounds are
110 currently approaching the step of pre-clinical investigations⁴.

111 The positive-sense single stranded RNA genome of coronaviruses encodes
112 16 nonstructural polyproteins (nsps 1-16). The Papain-like protease (PLpro) is a
113 domain that is part of the nsp3 gene, the largest mature SARS-CoV-2 protein⁵.
114 PLpro is required to recognize and cleave the motif LXGG within preprocessed
115 polyproteins between nsp1/2, nsp2/3 and nsp3/4 into functional units for initiation,
116 replication and transcription of the viral genome^{6,7}. Apart from the proteolytic
117 activity, PLpro can also bind and cleave ubiquitin chains or ISG15 (interferon-
118 stimulated gene product 15) from ubiquitinated or ISGylated proteins⁸. The
119 deubiquitinase (DUB), as well as deISGylating activities are vital for the
120 coronaviruses to antagonize the host immune responses. It has been shown that
121 mono-ubiquitination at the endoplasmic reticulum (ER) membranes regulate
122 endocytosis, and vesicle trafficking and thus is important for coronavirus
123 propagation⁹. On the other hand, ISG15 causes metabolic pathway modifications
124 towards excessive inflammatory and autoimmune responses due to interferon
125 system dysregulations^{10,11}. Target proteins for the coronavirus, such as IRF3
126 (Interferon regulatory factor 3) need to conjugate with ISG15 to be correctly
127 phosphorylated for their entry into nucleus where they are required for downstream
128 signalling events for e.g., *via* the IFN- β pathway¹² to elicit an antiviral immune
129 response. Therefore, the cysteine protease activity, together with the deubiquitinase
130 and deISGylating activity of PLpro undoubtedly makes this enzyme a very

131 promising target for drug discovery investigations^{20,21}. Furthermore, the importance
132 of PLpro as a drug target was highlighted in several recent studies¹³⁻²² that have
133 identified novel and unexpected mutations in the PLpro domain of the nsp3 gene
134 from the SARS-CoV-2 variants of concern (VOC), currently circulating in different
135 parts of the world^{23,24}.

136 The crystal structure of SARS-CoV PLpro, in complex with Lys48-linked
137 di-ubiquitin²⁵ (PDB code 5E6J), clearly evokes a mechanism of DUB action in
138 which the LXGG residues at the C-terminal of the ubiquitin molecule bind in a cleft
139 (Ub S1 proximal binding site) located close to the catalytic active site, allowing
140 efficient cleavage. In addition, the recently obtained crystal structure of SARS-
141 CoV-2 PLpro, in complex with mouse-ISG15²⁶, demonstrates that the N-terminal
142 region of ISG15 interacts with PLpro at a binding site termed as ISG15/Ub S2 distal
143 binding site. Compared to the crystal structure of ISG15 (PDB code 5TLA), the N-
144 terminal part of the ISG15 molecule in the complex structure of SARS-CoV-2
145 PLpro is rotated by about 90° and coordinates with the S2-helix. These different
146 binding events explain and highlight why SARS-CoV-2 PLpro and SARS-CoV
147 PLpro, despite sharing 83% sequence identity, show different substrate preferences.
148 Recently data were published showing higher affinity and specificity of SARS-
149 CoV-2 PLpro to ISG15, whereas SARS-CoV PLpro preferentially cleaves ubiquitin
150 chains, which may be associated to the substantial higher morbidity and mortality of
151 SARS-CoV-2 in comparison to SARS-CoV infections²⁷.

152 Until now several high throughput assay screening (HTS) activities,
153 focussed on identifying potential inhibitors of PLpro using repurposing compound
154 libraries were initiated. Unfortunately, these were not very successful in obtaining
155 hits of compounds that could be further developed to obtain an effective antiviral

156 drug²⁷. Hence, we set out a different strategy to explore a unique library consisting
157 of 500 natural compounds assembled and characterized by the Molecular Bank,
158 ICCBS, Karachi, Pakistan, by structure-based drug design ([https://iccs.edu/page-](https://iccs.edu/page-mol-bank)
159 [mol-bank](https://iccs.edu/page-mol-bank)). Natural compounds of the ICCBS Molecular Bank extracted from plants
160 present characteristics such as high chemical diversity, medically relevant anti-
161 tumor, anti-oxidant, anti-inflammatory and importantly antiviral action with
162 typically milder or no side effects, in addition to lower cost of production as
163 compared to most available drugs on the market²⁸. A number of these compounds
164 also have a long history of use as drug molecules to treat distinct human diseases,
165 including viral infections, such as Hepatitis C virus infection²⁹. Recent reports also
166 demonstrate the potential use of plant molecules and their secondary metabolites
167 against SARS-CoV-2, and other human coronaviruses³⁰⁻³² by applying *in vitro* and
168 *in silico* approaches. However, to our knowledge, a systematic screening of natural
169 products by structure-based drug discovery providing direct experimental data about
170 complex formation was not available to date.

171 Our high throughput screening by X-ray crystallography identified three
172 natural compounds bound to PLpro. All three compounds, 4-(2-
173 hydroxyethyl)phenol (YRL), 4-hydroxybenzaldehyde (HBA) and methyl 3, 4-
174 dihydroxybenzoate (HE9) are phenol derivatives, classified as polyphenols, an
175 important and major class of bioactive compounds present in plants. This vast group
176 of bioactive compounds is divided into six major classes: hydroxybenzoic acids,
177 hydroxycinnamic acids, flavonoids, stilbenes, and lignans. In addition to the well-
178 known anti-oxidant and anti-inflammatory activities of phenol derivatives, several
179 studies have reported their anti-viral potential against Epstein-Barr virus^{33,34},

180 enterovirus 71³⁵, herpes simplex virus (HSV)³⁶, influenza virus³⁷, and other viruses
181 causing respiratory tract-related infections³⁸.

182 Interestingly, all three compounds YRL, HBA and HE9 bind at the same,
183 and yet unexplored ISG15/Ub-S2 allosteric binding site in the thumb region of
184 PLpro by forming specific interactions and clearly inhibit PLpro in deISGylation
185 activity assays. None of these lead compounds are cytotoxic in cellular cytotoxicity
186 assays and therefore could be promising lead compounds with the potential to be
187 developed as specific coronaviral PLpro inhibitors. Significantly, two of them
188 exhibit antiviral activity and one inhibits cytopathic effects in the range of 60-80%
189 in a non-cytotoxic concentration range up to 100 μ M in cellular assays. These three
190 natural phenolic compounds undoubtedly provide a scaffold as antiviral drugs for
191 further development and optimization towards the prevention and/or reduction of
192 SARS-CoV-2 viral replication, and to reinstate and support the innate immune
193 response of the host in parallel.

194

195 **Results and Discussion**

196 **X-ray screening of a natural compound library identifies three allosteric** 197 **inhibitors of SARS-CoV-2 PLpro**

198 We initiated a structure-based drug discovery approach to identify potential
199 inhibitors for PLpro by X-ray screening of 500 compounds from the ICCBS
200 Molecular Bank. SARS-CoV-2 PLpro was expressed recombinantly in *Escherichia*
201 *coli*, and purified to homogeneity as a monomer (see materials and methods). Wild-
202 type enzyme crystals were obtained in a stable and reproducible condition and
203 diffracted X-rays to a high resolution of 1.42 Å. Data collection and refinement
204 statistics are summarized in the Table 1. The electron density maps obtained for the

205 wild-type enzyme allowed the elucidation of all 315 amino acid residues, the zinc
206 ion and 529 solvent water molecules. Further, a glycerol molecule from the
207 cryoprotectant, used for freezing crystals, could be modelled in the electron density
208 map, as well as a phosphate and two chloride ions.

209 PLpro folds with a right-handed architecture consisting of thumb, palm, and
210 fingers domains with a catalytic triad consisting of Cys111-His272-Asp286 and a
211 N-terminal ubiquitin-like domain (Figure 1). Four cysteine side chains coordinate a
212 zinc ion, constituting a ‘zinc finger motif’ that is essential for structural stability and
213 protease activity of the enzyme³⁹. The overall structure of SARS-CoV-2 PLpro is
214 homologous to SARS-CoV PLpro (PDB code 2FE8) that shares a sequence identity
215 of 83% with a r.m.s.d. of 0.58Å for 260 equivalent C α atoms and also to MERS-
216 CoV PLpro (PDB code 4RNA) despite a lower sequence identity of 29% and a
217 corresponding r.m.s.d. of 1.83Å for 258 equivalent C α atoms (Figure S4 & S8). The
218 most structurally dynamic regions are the ubiquitin-fold like, and the zinc fingers
219 domains. The catalytic active site region is conformationally well conserved among
220 the different coronaviral PLpro enzymes. The access to the active site is regulated
221 via a flexible loop named “blocking loop 2” (BL2, Figure 1), as this loop changes
222 from an ‘open’ to a ‘closed’ conformation in the context of substrate binding⁴⁰. A
223 number of known PLpro inhibitors bind at this site, including the high affinity
224 inhibitor GRL0617, and structural variations have been observed in this loop among
225 different PLpro enzymes⁴¹.

226 Crystals of SARS-CoV-2 PLpro, in complex with the three natural
227 compounds, were obtained by co-crystallization using the vapour diffusion method
228 in a screening approach utilizing 500 molecules from a library of natural
229 compounds. Crystals were grown in the same condition as for the native PLpro and

230 diffraction datasets were collected in the resolution range of 1.7-1.9 Å. Over 2,000
231 crystals were harvested, and multiple datasets for each compound were collected
232 that resulted in ~2500 diffraction datasets (Figure S12). PLpro structures complexed
233 with inhibitor compounds were solved using the ligand-free PLpro (PDB code
234 7NFV) as reference model for consistent indexing of datasets with a previously
235 established automatic pipeline⁴ (see materials and methods). Data collection and
236 refinement statistics are summarized in Table 1. The three complex structures
237 obtained superimpose well with the ligand-free structure 7NFV with a r.m.s.d. of
238 0.26 Å (298 Cα atoms) to 7OFS, r.m.s.d. of 0.07 Å (283 Cα atoms) to 7OFU and
239 r.m.s.d. of 0.33 Å (299 Cα atoms) to 7OFT, respectively. The three compounds bind
240 at the ISG15/Ub S2 allosteric site, near the thumb region of PLpro (Figure 1) that is
241 located about 30 Å apart to the active site residue Cys 111. The interaction between
242 these allosteric inhibitors and PLpro are formed *via* hydrogen bonds, hydrophobic,
243 and π -stacking interactions (Figure S5, S6 and S7).

244 **Molecular basis of inhibition by the three allosteric inhibitors of SARS-CoV-2** 245 **PLpro**

246 The natural compound 4-(2-hydroxyethyl)phenol (YRL), isolated from
247 *Lawsonia alba*, is a well-known antioxidant and an anti-arrhythmia agent
248 (https://pubchem.ncbi.nlm.nih.gov/compound/2-_4-Hydroxyphenyl_ethanol). YRL
249 binds to PLpro at the ISG15/Ub S2 allosteric binding site in a hydrophobic cavity
250 with a predicted binding energy of -7.17 kcal/mol (calculated using Prodigy⁴²). The
251 benzene core is covered by hydrophobic interactions with side chains of Val 11, Val
252 57, Pro 59, Tyr 72 and Leu 80. The main-chain nitrogen atom of Leu 80 is hydrogen
253 bonded *via* a water molecule to the hydroxyethyl substituent of YRL. Interestingly,
254 the hydroxyethyl substituent is observed with two alternative conformations and is

refined to equal occupancy in the complex structure. One conformation forms a hydrogen bond to carbonyl backbone of Asp 76, the alternative hydroxyl to the carbonyl of Thr 74. Both alternative hydroxyl groups replace water molecules in the ligand-free enzyme and have contacts to solvent water molecules at the entry of the binding pocket. The phenolic hydroxyl is hydrogen bonded to carbonyl oxygen of Val 57 at the bottom of the binding pocket to complete the interaction of the ligand YRL in the PLpro-YRL complex structure (Figure S5).

The second compound, 4-hydroxybenzaldehyde (HBA), isolated from *Acalypha torta*, is a well-known anti-tumor agent.^{43,44} The calculated binding energy for the interaction of the HBA ligand to PLpro is -6.97 kcal/mol. The benzene core and the phenolic hydroxyl is observed in the same position and, thus, has similar interaction to PLpro as described above for YRL (Figure S6). The distance of the phenolic hydroxyl of both compounds to the C α of Val 11 indicates a C-H $\cdots$ O hydrogen bond (3.4 Å). The aldehyde substituent has weak water contacts at the entrance of the binding pocket. The remarkable structural change in PLpro to accommodate these two compounds is that the side chain of Leu 80 has to tilt away in the complex PLpro structures (Figure S13).

The third compound, methyl 3,4-dihydroxybenzoate (HE9), isolated from *Tagetes patula* (marigold), is a major diphenol found in green tea with antioxidant and anti-inflammatory effects⁴⁵ (https://pubchem.ncbi.nlm.nih.gov/compound/Methyl-3_4-dihydroxybenzoate). The calculated binding energy for this interaction is -6.15 kcal/mol. HE9 binds at the surface of PLpro adjacent to the binding cavity with ligands HBA and YRL. The interaction to PLpro is formed by hydrogen bonds of the dihydroxyphenol edge to the side chain of Glu 70. Hydrophobic interactions are observed including the π -

stacking with the imidazole of His 73 and contacts of the benzene core to the side chain of Phe69 (Figure S7). The extraction, isolation and purification of the three compounds HBA, YRL and HE9 are presented in the supplementary notes 1,2 and 3. The NMR spectra of HBA, YRL and HE9 are shown in the supplementary figures S1, S2 and S3 respectively.

We determined the binding constants for the ligands HBA and HE9 using their quenching effect on the fluorescence for PLpro applying the nanoDSF (nano Differential Scanning Fluorimetry) method⁴⁶. This resulted in K_d values of approximately 400 μ M for HBA and 1 mM for HE9 (depending on the emission wavelength used, Figure S15). YRL showed a high intrinsic fluorescence intensity and therefore could not be used in a fluorescence titration experiment. Since YRL is structurally very homologous to HBA and it binds in the same pocket of PLpro, we assume a similar binding affinity.

The described interaction networks of the three compounds involve amino acid residues Phe 69, Glu 70 and His 73 that have been previously shown to interact with ISG15 and Lys48di-Ub molecules^{25,26}. Crystal structures of SARS-CoV PLpro, in complex with Lys48di-Ub (5E6J), and SARS-CoV-2 PLpro in complex with mouse-ISG15 (6YVA) supported by molecular dynamics simulations clearly reveal the hydrophobic interactions between these amino acid residues in PLpro with either ISG15 or Lys48-di ubiquitin molecules²⁶. A superimposition of the PLpro+inhibitor complex structures with PLpro+ISG15 complex (Figure 2a), shows that the binding of the natural compounds clearly disrupts and prevents the binding of ISG15 to PLpro. Critical residues Ser 22, Met 23 and Glu 27 located in the binding surface of ISG15 are no longer available to form interactions with PLpro (Figure 2b) upon binding of these natural products.

305 PLpro enzymes share the same core residue, SARS-CoV Phe 70 and SARS-
306 CoV-2 Phe 69 at the ISG15 binding site. A mutation of this residue in PLpro to
307 alanine decreased the enzymatic activity, and also resulted in a slower reaction with
308 ISG15, as compared to the wild type enzyme²⁶. In MERS-CoV PLpro, Phe 69 is
309 replaced by a lysine residue (F69K) and His 73 by a glycine residue (H73G). These
310 variations might account for the different substrate preferences among SARS-CoV
311 and SARS-CoV-2 and MERS PLpro. It can be seen from a superimposition of the
312 crystal structure of the MERS-CoV PLpro+ISG15 (6BI8), with the SARS-CoV-2
313 PLpro-HE9 complex structure (7OFU), that F69K and H73G substitutions confer
314 different surface properties for the interaction with ISG15 (Figures S10a, b).

315 Cleavage of polyubiquitin chains by SARS-CoV-2 PLpro is significantly
316 enhanced when a longer ubiquitin chain is used. This demonstrates that either Ub or
317 ISG15 molecules bind not only to the Ub-S1 binding site but also to the Ub-S2 site,
318 facilitated by the conserved S2 helix in PLpro, being important for the enzymatic
319 activity⁴⁷. Superimposition of crystal structures of PLpro in complex with Lys48
320 linked di-ubiquitin (5E6J) with the PLpro-HE9 complex (7OFU) showed that key
321 residues involved in ubiquitination, Lys 11 and Lys 48 in the S2-Ub binding site,
322 are no longer available for binding either to ubiquitin or ISG15 (Figure S9 a, b).
323 Hence, a clear molecular basis for the inhibition emerges from the three PLpro
324 inhibitor complex structures, showing that PLpro-ISG15 interactions are affected
325 upon the binding of the three phenolic natural products.

326 ***In vitro* enzymatic assays to monitor the inhibition of SARS-CoV-2 PLpro**

327 Fluorescence activity assays were carried out to assess the inhibitory effect of the
328 three compounds (HE9, YRL and HBA), co-crystallized with the wild type SARS-
329 CoV-2 PLpro. A catalytically inactive PLpro mutant (C111S) was used as a control

330 applying ISG15-Rhodamine and Ub-Rhodamine as substrates. Wild type PLpro
331 (WT PLpro) at 10nM concentration represents 100% of deISGylation activity and
332 the three natural compounds YRL, HBA and HE9 at 50 μ M show clear inhibition of
333 PLpro enzymatic activity using ISG15-Rhodamine as the substrate. In particular the
334 two compounds, HBA and YRL significantly decreased PLpro activity by
335 approximately 73% and 70% respectively, followed by HE9 inhibiting to
336 approximately 55% in a deISGylation assay (Figure S11); while the inhibition was
337 not as pronounced applying Ub-Rhodamine as substrate (Figure S16). It can be
338 rationalized that the binding of the natural compounds at the S2 helix region clearly
339 prevents the essential PLpro/ISG15 molecular interactions required for the
340 deISGylation mechanism of PLpro.

341 Further, we determined the inhibition efficacy by performing *in vitro* IC₅₀ assays,
342 which demonstrated efficient inhibition of the three compounds namely HE9
343 (methyl 3,4-dihydroxybenzoate), HBA (p-hydroxybenzaldehyde) and YRL (4-(2-
344 hydroxyethyl)phenol), in a concentration range of 3.76 ± 1.13 , 3.99 ± 1.33 and
345 6.68 ± 1.20 μ M respectively. The compound, GRL0617 (5-Amino-2-methyl-N-
346 [(1R)-1-(1-naphthalenyl) ethyl] benzamide), a known inhibitor of PLpro was used
347 as a control (Figure 3a). To characterize the specificity of the three compounds
348 towards PLpro, enzymatic inhibition assays with the SARS CoV-2 main protease
349 (Mpro) and applying the three compounds were also performed, considering the
350 same PLpro protocol with incubation time up to 6 hours. Results clearly
351 demonstrated no inhibitory effect of Mpro activity in the presence of the three
352 natural compounds when compared to the known Mpro inhibitor GC-376, as shown
353 in Figure S14. Antiviral activities for these natural phenolic compounds, either in
354 crude or purified form, were reported previously^{35,38} and can now be related to

355 PLpro inhibition, as shown in our activity assays. However, we cannot exclude the
356 interaction of these compounds with other vital cellular target proteins. Further, it
357 has recently been demonstrated that the PLpro minimal domain is unable to cleave
358 the Nsp1/2 fusion protein and it has been demonstrated that the full-length Nsp3
359 core protein is required to represent the PLpro peptidase activity, which needs to be
360 considered in terms of drug discovery investigations⁴⁸.

361 **Cellular assays to monitor the inhibition of SARS-Co-V-2 PLpro**

362 Considering the observed inhibitory synergic effect, combined with the
363 molecular regulative antiviral homeostasis function of ISG15 in the human host, we
364 investigated the inhibitory efficacy of the compounds HE9, HBA, and YRL towards
365 viral replication and the cytopathic effect in living cells using Vero cell line assays.
366 Two distinct approaches were applied, qRT-PCR reaction as previously
367 described^{4,25} and CellTiter-Glo assay, a luciferase reporter assay to determine the
368 ATP level present in viable cells⁴⁹. Screening experiments started at 5mM of the
369 three compounds and used a 10-point, 1:10 dilution series with infections being
370 performed at a multiplicity of infection (MOI) value of 0.01. The two compounds
371 HE9 and YRL showed a reduction of viral RNA (vRNA) replication with IC₅₀
372 values of 0.13μM and 1μM respectively, with no associated cell toxicity at 100μM
373 (Figure 4a). Cell viability experiments were performed simultaneously under the
374 same conditions in the absence of virus and revealed no effects on cell viability at
375 concentrations where the compounds showed antiviral activity (Figure 4b).

376 The compound HE9 significantly reduced viral RNA (vRNA) replication
377 among the three compounds studied and was further evaluated to determine the
378 effective concentrations that can reduce not only vRNA levels but also SARS-CoV-
379 2 virus infectious particles applying a cytopathic effect (CPE) inhibition assay

380 (Figure 4c). An active compound was the one which exhibited a CPE inhibition of >
381 50% without compromising cell viability. We were unable to fit a sigmoidal curve
382 to the data for the compounds HBA and YRL. Importantly, treatment of the cells
383 with the compound HE9 reduced the viral replication and showed an ability to
384 inhibit CPE with $IC_{50} = 10\mu M$ (Figure 4c). These results from the cellular assays
385 are in line with the *in vitro* enzymatic studies using deISGylation assays and clearly
386 demonstrate that the compound HE9 is a potential inhibitor of PLpro, which can
387 protect the host cells from the viral CPE.

388 PLpro from SARS-CoV, MERS-CoV and other coronaviruses are able to
389 inactivate the components of type I interferon partly mediated by their
390 deubiquitination and deISGylation functions^{5,25}. A unique feature of Vero cells is
391 that they are interferon-deficient lacking the production of interferons type I (IFN),
392 the antiviral signaling proteins typically produced by mammalian cells⁵⁰ and known
393 to strongly express ISG15. Thereby, the cellular viability, the inhibition of viral
394 replication in a micromolar range and the effective inhibition of the cytopathic
395 effect in the presence of HE9 in Vero cells were modulated via an alternative
396 cellular pathway during the infection by SARS-CoV-2.

397 Recent studies utilizing similar cell-based protocols to assess the replication
398 of SARS-CoV-2 with the Vero cell line have reported a stable viral replication
399 curve between 24 and 40 h post infection, followed by an evident declining of
400 replication and a strong cytopathic effect on the cellular viability⁵¹. This observation
401 was not seen in our study when the HE9 compound was titrated in the same Vero
402 cells line. In the same study, a significant inhibition of the viral replication was
403 detected when Vero cells were infected with SARS-CoV-2 and treated
404 independently with IFN- β 1a and IFN- α (type I IFN). Moreover, treatment of un-

405 infected Vero cells with human interferon type III (IFN- λ 1) stimulated endogenous
406 cellular expression of other ISGs, such as MxA (myxovirus resistance protein),
407 PKR (protein kinase R), OAS-1 (2',5'-oligoadenylate synthetase), SOCS-1
408 (Suppressor Of Cytokine Signaling 1) and Rig-1 (Retinoic acid-inducible gene
409 I)^{52,53}, which suggest that Vero cells, even devoid of type I IFNs, can elicit
410 functional IFN III responses⁵⁴. Thus, the compound HE9 could indirectly attenuate
411 the viral activity by reversing the host deubiquitination events linked to the IFN
412 response deficiency in Vero cells.

413 **Conclusions**

414 We have identified three phenolic compounds that inhibit PLpro by binding
415 at an allosteric S2 site, an interaction and binding region for the ISG15 molecule.
416 All three compounds show inhibition to PLpro in a deISGylation assay and
417 demonstrate no inhibition in an enzymatic activity assay performed with the main
418 protease, Mpro. Interestingly, two compounds exhibit distinct antiviral activity in
419 Vero cell line assays and one compound additionally inhibited a cytopathic effect in
420 non-cytotoxic concentration ranges. The binding affinities for the three compounds
421 are in the lower micromolar range, indicating the compounds are weak binders to
422 PLpro, but certainly provide valuable starting scaffolds as lead compounds targeting
423 an allosteric binding site in PLpro. Molecular docking studies with the three
424 phenolic compounds either covalently linked or extended with the
425 thiosemicarbazone structures exhibit an increase of predicted binding energies by
426 0.8 to 1.8 kcal/mol in comparison to the unextended initial compounds⁵⁵. Thus, the
427 observed binding affinities and specificities of the three compounds can be
428 improved by a systematic SAR (structure-activity relationship) analysis.

429 In summary, the high resolution PLpro complex structures with phenolic

430 natural compounds YRL, HBA and HE9 complemented by enzymatic and cellular
431 assays, provided a molecular basis to understand the inhibitory mechanism, a route
432 to develop effective PLpro inhibitors of substrates binding to the PLpro S2 helix
433 binding pocket and shed light on the mode of ISGylation of COVID-19 viral
434 proteins as a new approach for preventing their interaction with human host cellular
435 pathways. We believe that this approach to inhibit PLpro may hinder and reduce the
436 viral ability to perform deISGylation in post COVID-19 viral complications, as well
437 as providing more ISG15 within the lung tissues for the modulation of
438 cytokine/chemokine production, to support the repair of the respiratory epithelium
439 within COVID-19 infections¹¹.

440

441 **Material and Methods**

442 **Cloning, protein overexpression and purification of SARS-CoV-2 PLpro**

443 A fragment of SARS-CoV-2 ORF pp1a/ab encoding the PLpro domain and
444 corresponding to amino acids 746-1060 of non-structural protein 3
445 (YP_009742610.1) was cloned into pETM11(EMBL), which encodes N-terminal
446 hexa-his tag followed by a tobacco etch virus (TEV) protease cleavage site. After
447 cleavage by TEV protease extra two amino acids (GA) are left on the N-terminal of
448 PLpro construct. The plasmid encoding the desired construct was transformed into
449 *E. coli* Rosetta (DE3) cells (Merck, Germany) to perform expression via
450 autoinduction medium, essentially as described before⁵⁶ and using kanamycin for
451 selection. An overnight cell culture was diluted and incubated in autoinduction
452 medium containing 0.5 g L⁻¹ β -D-glucose and 2 g L⁻¹ lactose under constant
453 shaking for 4 h at 37 °C and then in the presence of 100 μ M ZnCl₂ additionally
454 over-night at 18 °C. Subsequently, cells were harvested by centrifugation and

455 disrupted by sonication in lysis buffer (50 mM NaH₂PO₄, 150 mM NaCl and
456 10 mM imidazole, pH 7.2).

457 Cell extracts were maintained at 4 °C and centrifuged at 12,000 × g for 1 h.
458 The clear supernatant was incubated with Ni-NTA affinity resin (Thermo Fisher
459 Scientific, USA). PLpro was eluted by gravity flow using lysis buffer supplemented
460 with 300 mM imidazole and subsequently incubated with TEV protease at a molar
461 ratio of 20:1 in the presence of 1 mM DTT. Cleavage was performed during
462 dialysis against 50 mM Tris, 150 mM NaCl and 1 mM DTT adjusted to pH 7.3 and
463 for 14 h at 8 °C. After removing protease and the cleaved off tag by affinity
464 chromatography, PLpro was purified to homogeneity using size-exclusion
465 chromatography, i.e. a HiLoad 16/600 Superdex 75 column connected to an ÄKTA
466 purifier (GE Healthcare, GB) equilibrated with 50 mM Tris, 150 mM NaCl and
467 1 mM TCEP at pH 7.5. Purity and integrity of the protein were verified via SDS
468 polyacrylamide gel electrophoresis and DLS (Dynamic Light Scattering). The
469 concentration of PLpro with a calculated molecular weight of 35760 Da ($\epsilon_{\text{calculated}} =$
470 $45270 \text{ M}^{-1} \text{ cm}^{-1}$) was adjusted to 20 mg mL⁻¹ in preparation for vapor diffusion
471 crystallization trials.

472

473 **Crystallization of ligand free SARS-CoV-2 PLpro and complexes with** 474 **compounds**

475 Initial crystallization screening experiments were performed using the sitting-drop
476 vapour diffusion method utilizing the Oryx4 robot (Douglas Instruments) with the
477 SWISSCI 96-well plates. Wizard™ Classic 1, 2, 3 and 4, JCSG+, PACT
478 crystallization formulations were tried for initial screening experiments.
479 Crystallization was performed with a protein:reservoir ratio of 2:1 at 4 °C and 20 °C.

480 Initial hits were obtained from the Wizard screen, condition G11 (0.1M acetate
481 buffer pH 4.5, 0.8M NaH₂PO₄/1.2M K₂HPO₄) at 4 °C. Further optimization was
482 done by changing the buffer to 0.1M Tris-HCl pH = 8.0 and including 10% glycerol
483 that resulted in 0.2-0.3mm bipyramidal crystals. These crystals diffracted X-rays to
484 a resolution of 1.42 Å.

485 PLpro complex crystals with compounds were grown by the co-
486 crystallization method using the same condition as summarized above. 100 nL
487 droplets of 10 mM compound solutions in DMSO from the Sadia Molecular Bank,
488 Karachi library of natural compounds were applied onto a 96-well SWISSCI plate
489 and the compounds were dried in vacuum before the addition of 200 nL of (20
490 mg/mL) PLpro protein solution and 100 nL of the crystallization condition (0.1 M
491 Tris-HCl buffer pH 8.0, 0.8 M NaH₂PO₄/1.2 M K₂HPO₄ and 10% glycerol). The
492 drops were equilibrated in a sitting drop vapour diffusion setup with 80 µl of
493 reservoir solution. The plates were incubated at 4 °C and crystals appeared in 2 days
494 and grew reproducibly to dimensions of approx. 0.2 × 0.3 × 0.2 mm³ in 4 days.
495 Crystals were manually harvested directly from the drop and flash-frozen in liquid
496 nitrogen for diffraction data collection.

497 **Data collection, structure solution and refinement**

498 Diffraction data from the ligand-free and complex PLpro crystals were collected at
499 beamline P11, PETRA III / DESY, Hamburg. All datasets of ligand-free PLpro
500 were processed using the program XDS⁵⁷ with a reference dataset to ensure
501 consistent indexing. From a total of 64 complete datasets, the strongest were
502 selected based on $(I/\sigma)^{\text{asymptotic}}$ greater than 20⁵⁸. These 25 datasets were then
503 subjected to iterative merging using CODGAS⁵⁹ run with standard parameters. The
504 best merged datasets was further manually filtered leading to the final dataset that

505 contained five datasets. These were scaled with XSCALE⁵⁷ and final merging and
506 resolution cut-off was applied using AIMLESS⁶⁰. Structure solution was achieved
507 by molecular replacement method with PHASER⁶¹ using the PLpro coordinates
508 with PDB code 7JRN as search model. Successive rounds of manual building with
509 the program COOT⁶² and refinement with PHENIX⁶³, the addition of phosphate,
510 glycerol, chloride ions and water solvent molecules to the model, followed by a
511 final round of TLS refinement completed the structure refinement at a resolution of
512 1.42 Å.

513 An automatic data processing pipeline, hit finding, clustering⁶⁴, PanDDA analysis⁶⁵
514 and refinement protocols as described previously⁴ were used for the structure
515 solution and analysis of PLpro in complex with the natural compounds from the
516 library consisting of 500 compounds. Data processing with XDS resulted in 1469
517 datasets and includes more than one dataset per compound. Data quality indicators
518 CC_{1/2} and Wilson B-factors are plotted as shown in Figure S7. Final rounds of
519 manual refinement with either Refmac⁶⁶ or Phenix⁶⁷ together with manual model
520 building applying COOT resulted in the final refined structures. Data collection and
521 refinement statistics for PLpro and complexes are summarized in Table 1,
522 supplementary information. All figures were prepared using PyMol⁶⁸

523 **SARS-CoV-2 PLpro inhibition assays and IC₅₀ determination**

524 Activity assays were performed for SARS-CoV-2 PLpro native and mutant enzyme
525 (PLpro C111S mutant) to determine the deISGylation and deubiquitination
526 activities effected by the three natural compounds, following previously published
527 protocols^{26,27,47}. The assays were performed with a total reaction volume of 100 µL
528 in non-binding, black bottom, 96-well plate and reactions were measured on a
529 Tecan Infinite M plus plate reader (Tecan Group Ltd, Switzerland) using optical

530 settings specific for ISG15-Rhodamine (UbiQ-127, UbiQ Bio) and Ubiquitin-
531 Rhodamine (UbiQ-126, UbiQ Bio). ISG15-Rhodamine and Ub-Rhodamine are
532 fluorogenic substrates that contain the cleavage sequence RLRGG recognized by
533 PLpro at the C-terminus. The cleavage of the amide bond between the terminal
534 glycine residue and the rhodamine110 fluorophore releases the fluorescent Rh110-
535 morpholinecarbonyl that results in an increase of fluorescence intensity, measured
536 as RFU (Relative Fluorescence Unit). The fluorophore has an excitation and
537 emission at 492nm and 525nm respectively. The ISG15 substrate (UbiQ-127) and
538 Ub substrate (UbiQ-126) were used at a final concentration of 100nM and the
539 concentration of PLpro was 10nM in the assay. Relevant substrate and positive
540 controls (GRL0617) was used throughout the assay. SARS-CoV-2 PLpro native,
541 mutant and substrates were diluted in assay buffer (20 mM Tris-HCl pH 7.5, 150
542 mM NaCl, 1 mM TCEP) and reactions were started upon addition of PLpro in a
543 final volume of 100 μ L and measured at 25 $^{\circ}$ C. The putative inhibitor compounds
544 were incubated with PLpro enzyme at 10 $^{\circ}$ C for six hours. Inhibition kinetics were
545 measured in triplicates over 60 minutes with one read per minute in two
546 independent experiments. Measured fluorescence values were blank corrected with
547 buffer containing either the ISG15-Rhodamine or the Ub-Rhodamine substrates,
548 respectively.

549 IC₅₀ determination was performed with ISG15-Rhodamine as the substrate at a
550 concentration of 250nM. The assays were performed as described above, however a
551 gradient concentration of all three natural compounds and GRL-0617 were used in
552 the concentration ranging from 2 μ M to 50 μ M in reaction mixture prior to
553 incubation. The IC₅₀ values were calculated by the dose-response-inhibition
554 function after the normalization of the enzymatic activity values. Microsoft Excel

555 and GraphPad Prism (version 8.3.1) were used for analyzing the results and
556 preparation of corresponding figures.

557

558 **Cytotoxicity assays**

559 Vero cell lines (ATCC® CCL-81™) were cultivated in Dulbecco's modified
560 Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). The
561 cells were seeded in 96-well plates at a density of 3.5×10^4 cells/well, following 24
562 hours incubation at 37°C and 5% CO₂ atmosphere. The cell culture media was
563 changed and ten-fold serial dilutions of the compounds were added. Cell viability
564 following 72 h treatment of cells with the respective compounds was determined via
565 CellTiter-Glo® Luminescent Cell Viability Assay (Promega), following
566 manufacturer's instructions. Luminescent signal was recorded using a CLARIOstar
567 multi-mode microplate reader (BMG Labtech, Germany). Data were obtained from
568 three independent replicates in three biological experiments. Samples deemed to be
569 technical failures and extreme outlier were removed. Wells containing only culture
570 medium served as a control to determine the assay background.

571

572 **Antiviral activity assay**

573 Vero cell lines (ATCC® CCL-81™) cultivated in DMEM supplemented with 10%
574 FBS was seeded in 96-well plates at a density of 3.5×10^4 cells/well, following 24
575 hours incubation at 37°C and 5% CO₂ atmosphere. The cell culture media was
576 changed and tenfold serial dilution of the compounds were added to the cells. The
577 assays were performed as published previously⁴. Briefly, after 1 h incubation,
578 SARS-CoV-2 strain⁶⁹, diluted in DMEM with 2.5% FBS, was added to the cells at a
579 MOI of 0.01 and allowed absorption for 1 h. The viral inoculum was removed, and

580 cells were gently washed with phosphate-buffered saline (PBS) without calcium and
581 magnesium. Fresh DMEM with 2.5% FBS containing the compounds was added
582 back onto the cells. Cell culture supernatant was harvest 42 h post-infection and
583 viral RNA was purified using MagMAX™ Viral/Pathogen Nucleic Acid Isolation
584 Kit (Thermo Fisher Scientific). The samples were processed using the semi-
585 automated NucliSENS® easyMag® platform (bioMérieux, Lyon, France),
586 following the manufacturer's' instructions. All SARS-CoV-2 infections were
587 performed in a biosafety level 3 laboratory at the Institute of Biomedical Sciences,
588 University of São Paulo, Brazil. The viral titers were determined by the qRT-PCR
589 method using AgPath-ID™ One-Step RT-PCR Kit (Thermo Fisher Scientific) and a
590 sequence of primers and probe for the E gene⁷⁰. The viral titers were calculated
591 using a standard curve generated with serial dilutions of a template known
592 concentration and expressed in TCID₅₀/mL. Infected cells with the addition of 0.5%
593 DMSO were used as control. IC₅₀-values were calculated by fitting the data using
594 GraphPad Prism version 8.00 (GraphPad Software, La Jolla California USA). Data
595 were obtained from four independent replicates in two biological experiments.
596 Samples deemed to be technical failures and extreme outlier were removed.

597 **Cytopathic effect inhibition**

598 Vero cell lines (ATCC® CCL-81™) cultivated in DMEM supplemented with 10%
599 FBS were seeded in 96-well plates at a density of 3.5×10^4 cells/well, following 24
600 hours incubation at 37°C and 5% CO₂ atmosphere. The cell culture media was
601 changed and tenfold serial dilution of the compounds were added to the cells. The
602 cells were infected at MOI 0.01 and the cytopathic effect (CPE) inhibition following
603 42 h treatment of cells with the respective compounds was determined via
604 CellTiter-Glo® Luminescent Cell Viability Assay (Promega). Luminescent signal

605 was recorded using a CLARIOstar multi-mode microplate reader (BMG Labtech,
606 Germany). Data were obtained from three independent replicates in one biological
607 experiment. Samples deemed to be technical failures and extreme outlier were
608 removed.

609 The luminescent-based assay measures the inhibition of SARS-CoV2–induced
610 cytopathic effect (CPE) in Vero cell line (ATCC® CCL-81™)⁴⁹. Percent cytopathic
611 effect (CPE) inhibition was defined as $[(\text{test compound} - \text{virus control})/(\text{cell control}$
612 $- \text{virus control})] * 100$. IC₅₀ values were fitted by sigmoidal function using GraphPad
613 Prism version 8.00 (GraphPad Software, La Jolla California USA).

614

615 **Nano Differential Scanning Fluorimetry**

616 nDSF measurements were performed applying a Nanotemper Prometheus NT.48
617 fluorimeter (Nanotemper) operated by PR.ThermControl software and using
618 Prometheus Premium grade capillaries (Nanotemper). The excitation power was
619 adjusted to obtain fluorescence signals above 2000 RFU for a wavelength range of
620 330 and 350 nm. For all measurements a PLpro concentration of 50 μM in the
621 buffer consisting of 20 mM Tris-HCl, 150 mM NaCl, 0.5 mM TCEP, pH 8.0 was
622 used and varying ligand concentrations. For the ligand HE9 0.5% DMSO was added
623 to ensure solubility. For the fluorescence titrations 1:1 dilution series with 16 points
624 of ligands was designed and after the corresponding protein solutions were added.
625 Ligand concentrations range from 20 mM to 610 nM for HBA and 5 mM to 153 nM
626 for HE9. After incubation of 30 min, the solutions were transferred to capillaries
627 and utilized for the measurements. Data were analysed and visualized applying self-
628 written python scripts using the Python modules Numpy, Matplotlib, Scipy and
629 Pandas and a publicly available SPC data analysis platform.⁴⁶ The fluorescence

values F vs. the ligand concentration $[L]_0$ of HBA and HE9 were fitted with a simple 1:1 binding model using the equations 1 and 2 below:

$$F([L]_0) = F_{upper} + (F_{upper} - F_{lower}) * (1 - a([L]_0)) \quad (1)$$

$$a([L]_0) = ([P]_0 - K_d - [L]_0 + \sqrt{([P]_0 + [L]_0 + K_d)^2 - 4 * [P]_0 * [L]_0}) / (2 * [P]_0) \quad (2)$$

SARS-CoV-2 Mpro inhibition assays and IC₅₀ determination

Activity assays were performed for SARS-CoV-2 Mpro^{14,71} utilizing the three natural compounds, aiming to characterize the specificity of the compounds towards PLpro. The assays were performed applying a total reaction volume of 50 μ L using non-binding, black bottom, 96-well plates and the relative fluorescence was measured utilizing a Tecan Infinite M plus plate reader (Tecan Group Ltd, Switzerland) using optical settings specific for the substrate 2-AbzSAVLQSGTyr(3-NO₂)R-OH (Biotrend). The corresponding fluorophore has an excitation and emission at 355nm and 460nm wavelength respectively. The substrate and Mpro were used at a final concentration of 5 μ M and 75nM respectively. The known Mpro inhibitor (GC-376) was used as positive control throughout the assay. SARS-CoV-2 Mpro and substrates were diluted in assay buffer (20 mM Tris-HCl pH 7.3, 150 mM NaCl, 1 mM EDTA, 1mM DTT) and reactions were initiated upon addition of Mpro in a final volume of 50 μ L and were measured at 25 °C. The three compounds were incubated prior to the experiments with Mpro at 10 °C for six hours, as accomplished also for the PLpro activity assays. Fluorescence values were measured for 15 minutes with one read out per minute.

IC₅₀ determination was performed applying the same substrate at a concentration of 5 μ M and the corresponding assays were performed as described above. A

657 concentration gradient in a range of 1nM to 150 μ M was used for all three natural
658 compounds and GC-376. The IC₅₀ values were calculated applying a dose-response-
659 inhibition function after normalization of the enzymatic activity values. Microsoft
660 Excel and the software Origin (OriginLab) were used for analyzing the data
661 obtained to prepare the corresponding figures.

662 **Data availability**

663 Coordinates and structure factors were deposited in the Protein Data Bank PDB,
664 with codes: 7NFV (PLpro), 7OFS (PLpro in complex with YRL, 4-(2-
665 hydroxyethyl)phenol), 7OFT (PLpro in complex with HBA, p-
666 hydroxybenzaldehyde) and 7OFU (PLpro in complex with HE9, 3, 4-
667 dihydroxybenzoic acid, methyl ester).

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689 VS, HB, EES, HNC, ARP, AM, CW and CB designed research. VS, HB, PRP,
690 EES, HNC, CW and CB wrote manuscript. HH, NW, SF, BNF, MW, ALCB, ARM,
691 MW participated in sample preparation. VS performed crystallization experiments.
692 VS, SeG, PR, JL, OY, SS, JH, HA, NU, MP, MS, FHK, WE, JS, SF, MG, FT,
693 MaG performed X-ray data collection. VS, TJL, HG, SeG, PR, WE, JS, FHK, DT
694 and WH performed X-ray data analysis. KL, LB, CS, HH, RS, LG, MD, PM
695 performed X-ray data management. VS, HB, PRP, PR performed and analyzed
696 PLpro and Mpro enzymatic activity assays. EES, RRG, EDC, DBLO and ELD
697 performed and analyzed antiviral activity assays. MGA, SN, ASG performed and
698 analysed nanoDSF binding studies.

699 **COMPETING INTERESTS**

700 The authors declare no competing interests.

701 **ADDITIONAL INFORMATION**

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704

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917 **Figure 1:**
918 **Crystal structures of SARS-CoV-2 PLpro complexes with the three natural**
919 **compounds**
920 PLpro domains are depicted in a right-handed architecture, ubiquitin-fold
921 like (blue), thumb (green), palm (salmon pink) and fingers (light orange).
922 Catalytic active site residues Cys 111, His 272 and Asp 286 are represented
923 as sticks and a zinc ion in the fingers domain is shown as a grey sphere. The
924 flexible blocking loop (BL2 loop) that changes conformation in the context
925 of substrate binding is shown in blue. YRL (green spheres), HBA (yellow
926 spheres) and HE9 (pink spheres) compounds bind at the allosteric site that is
927 located about 30 Å apart to the active site. S2 helix involved in the
928 interaction of the ISG15 molecule is indicated. The inset shows an enlarged
929 view of the two compounds HBA and YRL in the binding site.
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Figure 2:

Interaction of the ISG15 molecule to PLpro is disrupted by the binding of the three natural compounds

a. Superposition of the crystal structures of SARS-CoV-2 PLpro-C111S in complex with mouse-ISG15 (PDB code 6YVA, ISG15 molecule in blue) with SARS-CoV-2 PLpro+HE9 (PDB code 7OFU, in grey surface representation). The three compounds YRL, HBA and HE9 are depicted as spheres.

b. Close-up view of the ISG15 binding site. ISG15 molecule is shown as a cartoon representation (blue) with the interacting residues Ser 22, Met 23 and Glu 27 in sticks. The bound inhibitor compounds (spheres) clearly prevent the binding of the ISG15 molecule to the S2 binding site of PLpro.

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Figure 3:

Inhibition of PLpro by the three natural compounds in deISGylation assay with ISG15-Rh substrate

- a. IC₅₀ determination was performed with ISG15-Rhodamine as the substrate at a concentration of 250nM. A gradient concentration of all three compounds YRL, HBA, HE9 and the inhibitor GRL-0617 as a control in the range from 2 μ M to 50 μ M was used in the reaction mixture. IC₅₀ values were calculated by fitting the data to a sigmoidal dose-response-inhibition function and are presented in the log scale for interpolation. Individual data points represent the mean of normalized relative fluorescence unit per min $\pm$ SD from triplicates.
- b. Summary of the inhibition profiles for the three natural compounds YRL, HBA, HE9 and the control compound GRL-0617 (TTT) obtained from enzyme activity assays and cell line antiviral assays.

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Figure 4:
Effect of the natural compounds on SARS-CoV-2 loading in Vero cells.

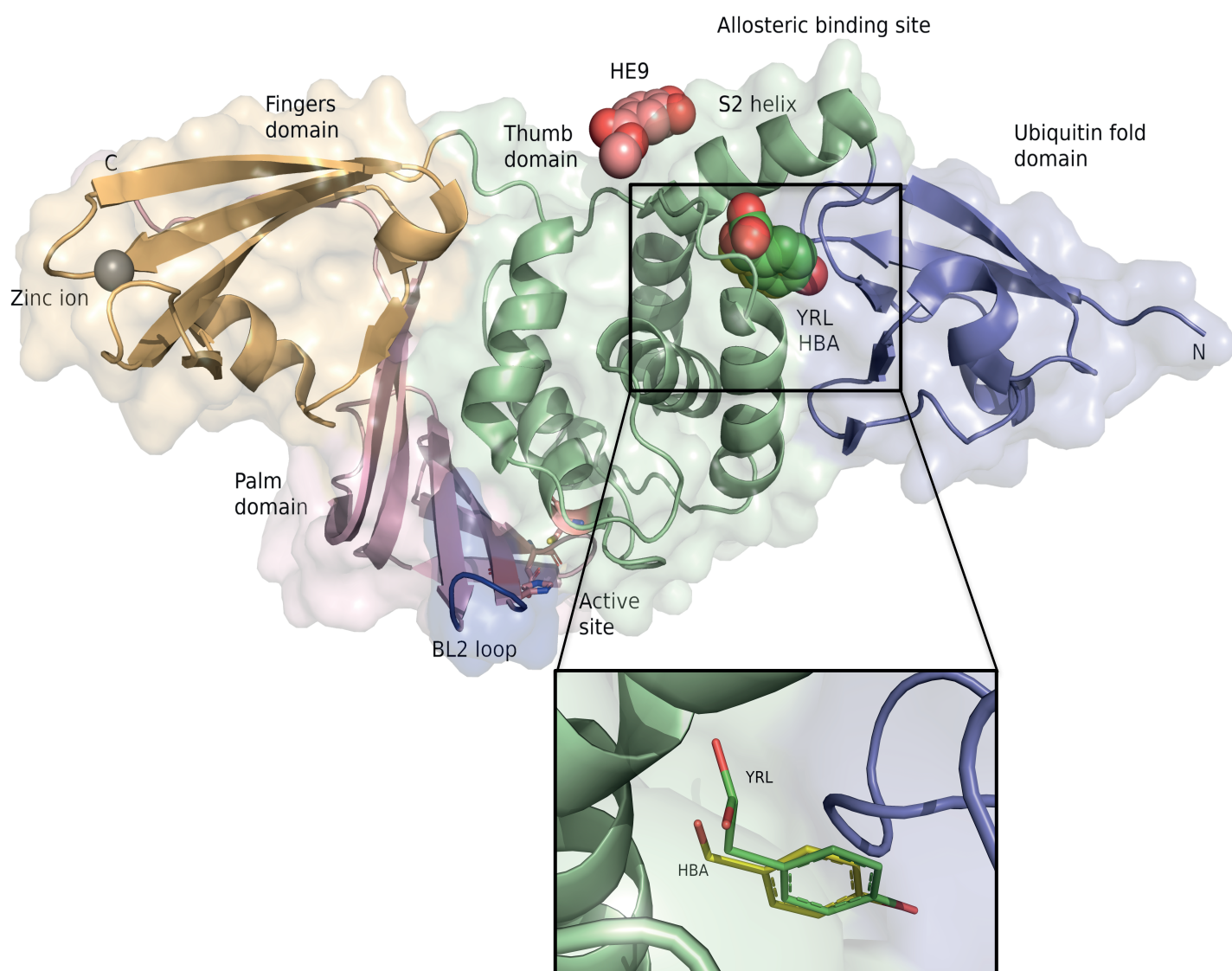
- a. The viral titer and cell viability were quantified by qRT-PCR (●) and CellTiter-Glo luminescence method (■), respectively. IC₅₀- and R-squared values for viral titers are shown. IC₅₀-values were calculated by fitting the data to the sigmoidal function as previously described⁴. Compounds concentrations are presented in log scale for interpolation. HE9 was diluted to a stock concentration of 100 mM in DMSO, while YRL was diluted in sterile water to a 50 mM stock concentration. All compounds were stored at -20°C. Individual data points represent means ± SD from four independent replicates in two biological experiments. Values were plotted in a line graph with error bars displaying standard deviation.
- b. Cell viability in the presence of the three compounds was determined by CellTiter-Glo luminescence method. Individual data points from three independent replicates in three biological experiments.
- c. CPE inhibition was determined by CellTiter-Glo luminescence method. IC₅₀- and R-squared values are shown. IC₅₀-values were calculated by fitting the data to the sigmoidal function. Individual data points represent means ± SD from three independent replicates in one biological experiment. Values were plotted in a line graph with error bars displaying standard deviation.

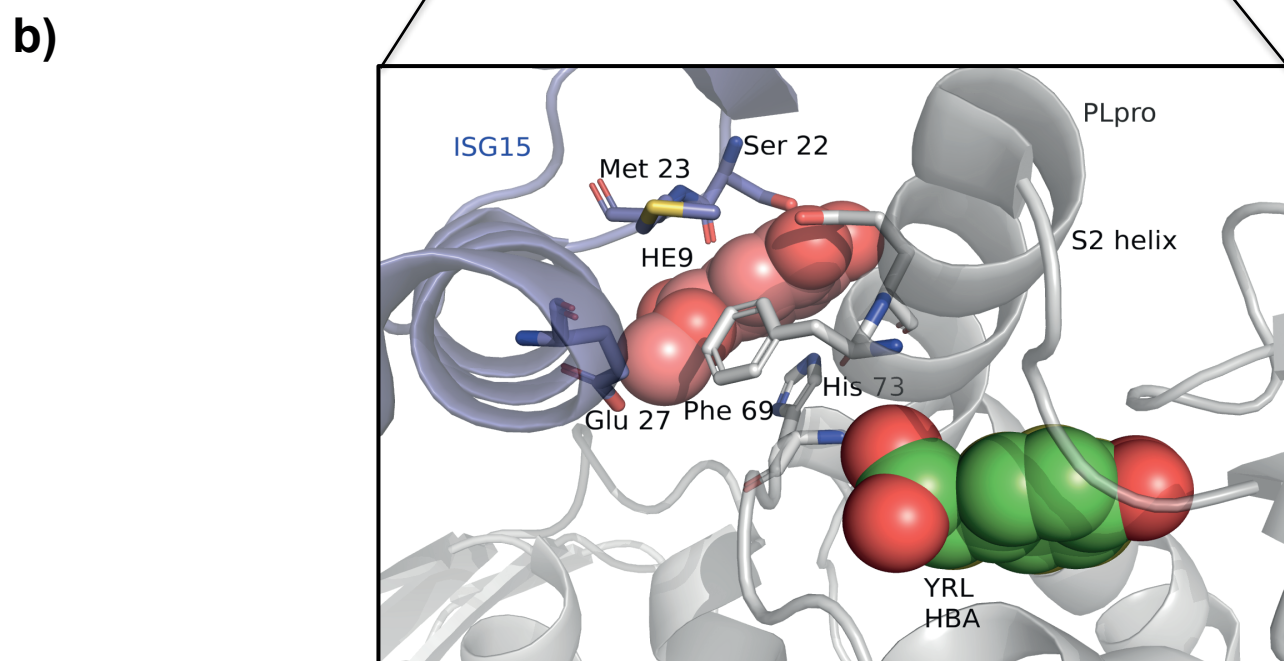
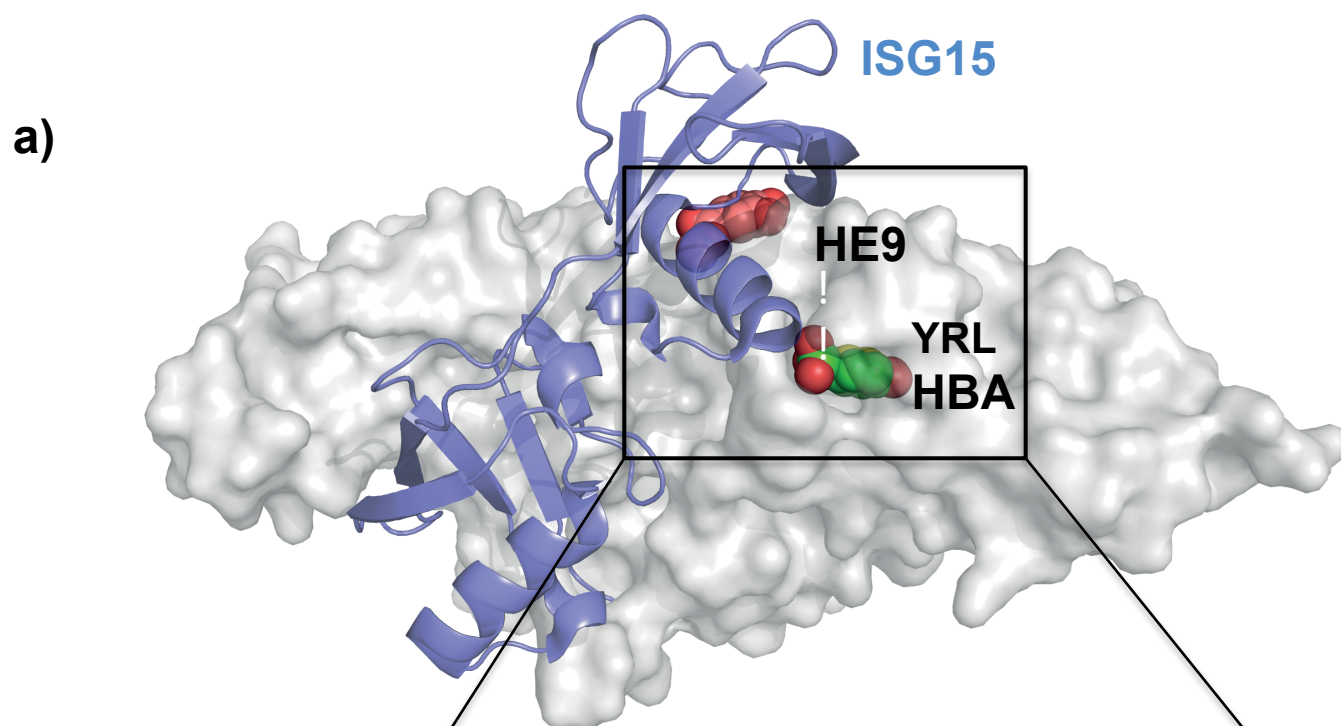
1008 **Table 1. Data collection and refinement statistics**

PDB code / in complex with	7NFV / native	7OFS / YRL	7OFU / HE9	7OFT / HBA
Resolution range (Å)	48.89 - 1.42 (1.47 - 1.42)	44.78 - 1.90 (1.97 - 1.90)	48.87 - 1.72 (1.78 - 1.72)	40.81 - 1.95 (2.02 - 1.95)
Space group	P 3 ₂ 2 1	P 3 ₂ 2 1	P 3 ₂ 2 1	P 3 ₂ 2 1
Unit cell a, b, c (Å) α,β,γ (°)	82.33, 82.33, 134.32 90, 90, 120	82.40, 82.40, 134.33 90, 90, 120	82.39, 82.39, 134.14, 90, 90, 120	81.61, 81.61, 134.37 90, 90, 120
Total number of reflections	5275155 (383126)	465619 (43757)	624464 (63178)	416072 (39883)
Unique number of reflections	99791 (9853)	42250 (4154)	56558 (5573)	38419 (3775)
Multiplicity	52.9 (38.9)	11.0 (10.5)	11.0 (11.3)	10.8 (10.6)
Completeness (%)	99.94 (99.53)	99.92 (99.90)	99.95 (99.82)	99.95 (99.87)
Mean I/sigma(I)	29.17 (0.68)	15.25 (0.86)	19.44 (0.87)	20.61 (1.31)
R-merge	0.07248 (6.281)	0.08749 (2.58)	0.07159 (2.692)	0.08102 (1.916)
CC 1/2	1 (0.418)	0.999 (0.505)	0.999 (0.453)	0.999 (0.667)
Refinement				
Reflections used	99737 (9810)	42226 (4150)	56587 (5565)	38403 (3766)
Reflections used for R-free	5022 (474)	2036 (187)	2834 (259)	1931 (187)
R-work	0.154 (0.330)	0.185(0.344)	0.175 (0.341)	0.181 (0.289)
R-free	0.171 (0.350)	0.214(0.368)	0.202 (0.386)	0.213 (0.356)
Protein atoms	2674	2548	2599	2545
Ligand atoms	0	26	46	20
Solvent atoms	550	205	328	270
RMS (bonds) Å	0.020	0.009	0.016	0.008
RMS (angles) °	2.30	1.23	1.933	1.23
Ramachandran favored (%)	96.49	97.44	96.45	97.12
Ramachandran allowed (%)	3.51	2.24	3.19	2.88
Ramachandran outliers (%)	0.00	0.32	0.35	0.00

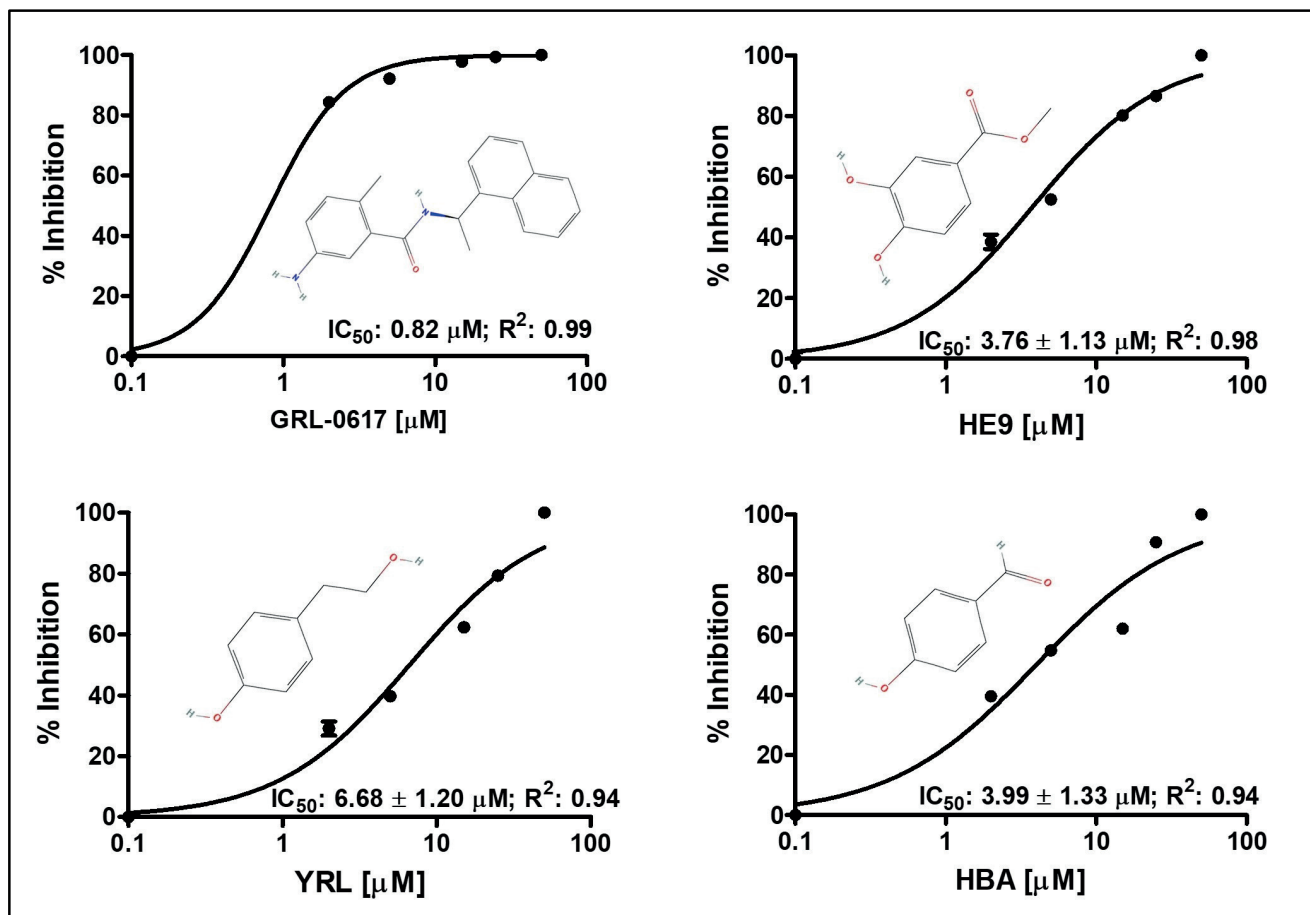
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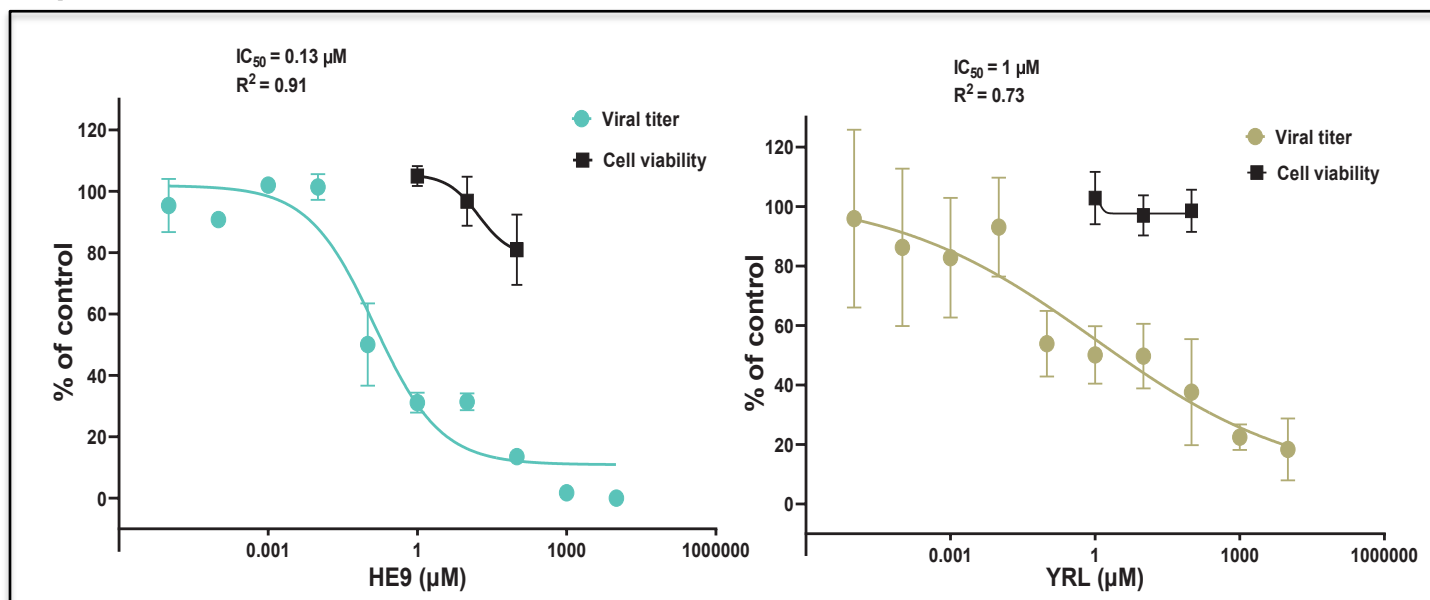
a)



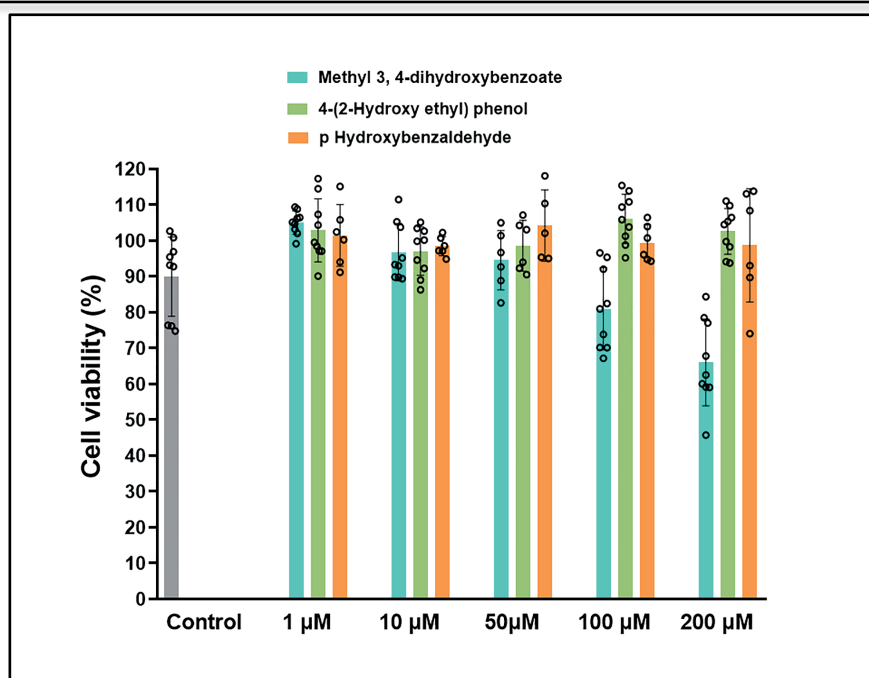
b)

PDB code	Ligand code	Empirical formula	MW	% Inhibition	IC_{50} (μM) Activity assay	IC_{50} (μM) Antiviral cell assay	IC_{50} (μM) CPE
7OFS	YRL	$\text{C}_8\text{H}_{10}\text{O}_2$	138.6	70	6.68 ± 1.20	1	-
7OFT	HBA	$\text{C}_7\text{H}_6\text{O}_2$	122.1	73	3.99 ± 1.33	-	-
7OFU	HE9	$\text{C}_8\text{H}_8\text{O}_4$	168.1	55	3.76 ± 1.13	0.13	10.37
-	TTT	$\text{C}_{20}\text{H}_{20}\text{N}_2\text{O}$	304.39	100	0.82	-	-

a)



b)



c)

