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1 In Silico Drug Repurposing for SARS-CoV-2 Main Proteinase and 2 Spike Proteins

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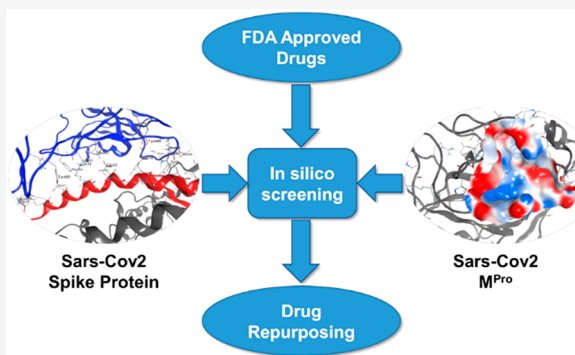
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Supporting Information

4 **ABSTRACT:** The pandemic caused by SARS-CoV-2 is currently
5 representing a major health and economic threat to humanity. So far,
6 no specific treatment to this viral infection has been developed and the
7 emergency still requires an efficient intervention. In this work, we used
8 virtual screening to facilitate drug repurposing against SARS-CoV-2,
9 targeting viral main proteinase and spike protein with 3000 existing
10 drugs. We used a protocol based on a docking step followed by a short
11 molecular dynamic simulation and rescoring by the Nwat-MMGBSA
12 approach. Our results provide suggestions for prioritizing *in vitro* and/or
13 *in vivo* tests of already available compounds.

14 **KEYWORDS:** SARS-CoV-2, COVID-19, virtual screening, main proteinase, 3CL^{pro}, spike protein, molecular dynamics, MM-GBSA,
15 drug repurposing



16 ■ INTRODUCTION

17 The outbreak of a novel β -coronavirus, SARS-CoV-2, is
18 currently a pandemic threat, with already more than 23 million
19 confirmed cases and more than 800 000 deaths all over the
20 world, according to the World Health Organization (data of
21 August 2020, <https://covid19.who.int>). Unfortunately,
22 although many clinical and preclinical studies are ongoing, to
23 date there is not a validated treatment to this infection.

24 As for other known coronaviruses, such as SARS-CoV and
25 MERS-CoV, the SARS-CoV-2 entry into host cells is mediated
26 by its transmembrane spike glycoprotein (S-protein). This is a
27 trimeric protein belonging to the class I fusion proteins, whose
28 structure for SARS-CoV-2 has been partially resolved by cryo-
29 electron microscopy (code PDB 6VXX and 6VSB).^{1,2} The S-
30 protein is divided into two functional subunits: the S₁ subunit,
31 which contains the receptor binding domain (RBD) responsible
32 for the interaction with host cell's receptors, and the S₂ subunit,
33 which is implicated in the fusion of the viral and cellular
34 membranes.

35 Recent works showed that SARS-CoV-2 S-protein is able to
36 bind the human angiotensin-converting enzyme 2 (hACE2),^{3–5}
37 explaining the symptoms linked to the SARS-CoV-2 infection
38 (COVID-19), since hACE2 is widely expressed in endothelial
39 cells from small and large arteries, in lung alveolar epithelial cells,
40 but also in the heart, kidney, testis, and gastrointestinal
41 system.^{6,7} Moreover, the crystallographic structure of the RBD
42 in complex with the hACE2 has been recently resolved (PDB
43 code 6MOJ),⁴ giving molecular details about this interaction
44 (Figure 2). The binding to the host cell receptor triggers a series

of conformational changes which allow the fusion with the host
cell and the entry of the virus.¹

In addition, a recognized target for coronaviruses treatments
is the main proteinase M^{Pro}, also known as 3CL^{Pro}.^{8,9} This
protein processes the polyprotein 1ab into mature nonstructural
proteins that are essential for viral replication¹⁰ and is rather
conserved among coronaviruses. Moreover, human proteases
with the same specificity have not been discovered so far, making
M^{Pro} an ideal target to treat coronavirus infections. The crystal
structure of the SARS-CoV-2 M^{Pro} in complex with a covalent
peptidomimetic inhibitor (PDB code 6LU7¹¹) was made
available. Additionally, the Zhang group, developer of the
popular homology-modeling software I-TASSER,¹² made
available 24 3D structural models¹³ of proteins in the SARS-
CoV-2 genome.¹⁴ Among these, the model of the M^{Pro} (code
QHD43415) was made available before the release of the crystal
and was characterized by a very high reliability score (TM-score
= 0.96).

It is clear that both spike and M^{Pro} proteins represent potential
targets for anti-SARS-CoV-2 drugs: on one side, hampering the
interaction between hACE2 and the viral RBD will block the
entry of the virus into the human cells. On the other side,

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67 inhibiting the viral proteases, as done with many antiviral drugs
68 currently used in the therapy of HIV infection,¹⁵ will interfere
69 with the viral replication.

70 However, the experimental procedure to conceive a new drug
71 is long (up to decades) and expensive (up to several millions of
72 dollars). Such a time and resources price is not affordable in the
73 current emergency situation; therefore, a promising alternative
74 consists in a drug repurposing investigation exploiting *in silico*
75 techniques, such as Virtual Screening (VS), which already
76 proved to be able to identify active molecules against a
77 target.^{16,17}

78 Within this context and aiming to give our contribution to the
79 current sanitary crisis, we designed a VS campaign of currently
80 worldwide approved drugs. Despite the fact that similar studies
81 have been recently published,^{18,19} in this work we independently
82 screened more than 3000 molecules against the two SARS-CoV-
83 2 proteins mentioned above to provide information useful for a
84 multiple treatment approach. In addition, we applied a solid VS
85 procedure we recently developed and which was shown to be
86 successful in discriminating active from inactive compounds
87 within the screening of classical small molecules and protein–
88 protein interaction inhibitors.²⁰

89 ■ METHODS

90 Receptor Preparation

91 Receptor models for the SARS-CoV-2 M^{pro} were prepared
92 starting from both the 6LU7 crystal structure and the
93 QHD43415 I-Tasser model. This choice was made to take
94 binding site flexibility into account through an ensemble
95 docking approach²¹ but without the need to perform time-
96 consuming molecular dynamic (MD) simulations to generate
97 reliable conformational ensembles. The two M^{pro} models were
98 prepared using the MOE2019 software,²² with the following
99 protocol:

100 6LU7: all water molecules were deleted. The covalently
101 bound peptidomimetic ligand was then unbound from Cys145,
102 and the α,β double bond of the ligand, that behaves as a Michael
103 acceptor, was restored. The Structure Preparation module of
104 MOE was used to correct PDB inconsistencies and to assign the
105 protonation state at pH = 7.0. The default Amber10EHT force
106 field, coupled to the Born solvation model was assigned to the
107 system. The ligand was then minimized, keeping the receptor
108 constrained. Then, the receptor was minimized by applying
109 backbone restraints and keeping the ligand constrained. Finally,
110 the complex was minimized in two separate steps, first by
111 keeping backbone restraints, second by removing all restraints.
112 All minimizations were performed up to a gradient of 0.1 kcal
113 mol⁻¹ Å⁻². The receptor and the ligand were then saved for
114 future use.

115 4MDS: the crystal structure of SARS-CoV 3CL^{pro} protei-
116 nase,²³ a close homologue of SARS-CoV-2 M^{pro}, in complex with
117 a carboxamide inhibitor was also modeled to be used as an
118 additional reference; this was done because no specific SARS-
119 Cov2M^{pro} noncovalent inhibitors were published at the time of
120 this screening.²⁴ The system was prepared for calculations as
121 follow: the PDB was corrected and protonated at pH = 7.0 using
122 MOE as stated above. The ligand was minimized, keeping the
123 receptor constrained, using the MMFF94x force field coupled
124 with the Born solvation model. The receptor was then
125 minimized, keeping the ligand constrained, using Amber10-
126 EHT+Born. Finally, the complex was minimized in two steps, as
127 described above.

QHD43415: the I-TASSER model QHD43415_5¹³ was
128 superposed to 4MDS (prepared as previously described) in
129 order to precisely define the binding site. Since we observed that
130 the 4MDS ligand also fitted QHD43415_5, the ligand was
131 transferred, and the complex was prepared as described for
132 4MDS. The resulting structure was used for docking.
133

The RBD of the S-protein was obtained by the recently
134 resolved X-ray structure of the complex between the SARS-
135 CoV-2 RBD and the human ACE2 (code PDB 6M0J).⁴ After the
136 deletion of this latter, the RBD has been protonated at
137 physiological conditions using the H++ server.²⁵
138

RBD binding site definition

In order to determine the RBD residues playing the most
140 important role in the binding to ACE2 (hot spots), the complex
141 between RBD and ACE2 has been initially protonated as the
142 single RBD. Successively, it has been submitted to a molecular
143 dynamics (MD) simulation using the AMBER18²⁶ package and
144 the ff14SB²⁷ force field. The system has been neutralized by
145 adding the proper number of Na⁺ ions and solvated adding a
146 cubic box of TIP3P water up to a distance of 10 Å from the
147 solute. The system has been relaxed by optimizing the geometry
148 of hydrogens, ions, and water molecules (1000 cycles of steepest
149 descent and 4000 cycles of conjugated gradient). The solvent
150 box has been equilibrated at 300 K by 100 ps of NVT (constant
151 volume and temperature) and 100 ps of NPT (constant pressure
152 and temperature) simulation. Then, a minimization of side
153 chains, water, and ions (2500 cycles of steepest descent and
154 2500 cycles of conjugated gradient) and a global minimization
155 (2500 cycles of steepest descent and 2500 cycles of conjugated
156 gradient) were performed with a restraint of 10 kcal/mol applied
157 on the backbone atoms. Successively the system has been heated
158 up to 300 K in 6 steps of 20 ps each ($\Delta T = 50$ K) during which
159 the backbone restraints were reduced progressively from 10 to 5
160 kcal/mol. The systems were then equilibrated for 100 ps in the
161 NVT ensemble and for 200 ps in the NPT ensemble keeping a 5
162 kcal/mol restraint on the backbone atoms. This was followed by
163 a 4 steps NPT equilibration during which the restraints were
164 progressively reduced to 1 kcal/mol. Finally, after a 500 ps
165 unrestrained NPT equilibration, a production run of 20 ns was
166 performed. During the whole simulation, an electrostatic cutoff
167 of 8 Å, a time step of 2 fs, and the SHAKE algorithm were
168 applied.²⁸ The root mean squared deviation (RMSD) of the
169 backbone atoms using the X-ray structure as reference was used
170 as a metric of simulation convergence (Figure S1). Hydrogen
171 bonds (H-bond) analysis was performed on the last 10 ns of the
172 simulation using the cpptraj module of AmberTools and using a
173 donor–acceptor distance cutoff of 3.5 Å and a donor–donor
174 hydrogen-acceptor angle cutoff of 150 deg (Table S2).
175

After having defined as interfacial those RBD residues whose
176 difference in the solvent accessible area when going from the
177 complex to the isolated state was greater than 0.75 Å, an *in silico*
178 alanine scanning was performed on the last 10 ns of the
179 production run. The mutated complexes have been built using
180 PyMol,²⁹ and the alanine scanning was run with the Amber
181 mmpbsa.py code on one frame every 100 ps and by choosing the
182 GB-Neck2³⁰ as implicit solvent model (igb = 8), the mbondi2 as
183 radii set, and a salt concentration of 0.15 M. The $\Delta\Delta G$ was
184 calculated as the difference between the ΔG of the mutated
185 system and the one of the native system (Table S1). The
186 residues giving $\Delta\Delta G$ greater than 2.5 kcal/mol were considered
187 as hot spots and were used to define the RBD potential binding
188 site for small molecules.
189

190 Database Preparation

191 Two separate databases were downloaded from the correspond-
192 ing sources^{31,32} and merged. The database was then checked and
193 redundant molecules, identified by CAS number, were removed.
194 The database was then processed by MOE in order to build the
195 3D structures and to minimize the geometry of each molecule.
196 The *wash* function of the MOE database tool was used with the
197 MMFF94x+Born force field, requesting the dominant proton-
198 ation state at pH = 7.0 and preserving existing chirality. The
199 final database, consisting of 3118 unique molecules, was saved in
200 SDF format.

201 Virtual Screening

202 The Virtual Screening (VS) was done according to our recently
203 developed protocol.²⁰ This is applied using a set of scripts
204 (available for download as [Supporting Information](#) within ref
205 20) that does the following steps automatically: (1) Preparation
206 of the screening library, including the generation of tautomers,
207 alternative protonation states, stereoisomers and ring con-
208 formers, if requested. (2) Docking of all molecules using
209 PLANTS.³³ (3) Analysis of results. (4) Parameterization of
210 docked ligands selected for rescoring. (5) Molecular dynamics
211 of complexes selected for rescoring, using Amber.²⁶ Rescoring
212 using the Nwat-MMGBSA method.^{20,34,35} All dockings were
213 performed by PLANTS, requesting a search speed = 1
214 (maximum accuracy) and the ChemPLP scoring function.³⁶
215 Only the principal tautomer and protonation state predicted at
216 pH = 7 were considered for the docking. The following receptor-
217 specific parameters were also set up: 6LU7: binding site center
218 (b.s.c.; x,y,z) = -10.2858, 12.3088, 69.3271; binding site radius
219 (b.s.r.; Å) = 16. QHD43415: b.s.c. = -15.124, 15.0521,
220 -24.6152; b.s.r. = 14. RBD-BS1: b.s.c. = -38.621, 39.731,
221 1.564; b.s.r. = 17. RBD-BS2: b.s.c. = -36.355, 20.471, 2.322;
222 b.s.r. = 17.

223 Nwat-MMGBSA rescoring was requested for the top 2% of
224 compounds (about 60 molecules for each target). Rescoring
225 consists in performing a short MD simulation (about 2.5 ns,
226 including 1.5 ns of equilibration and 1 ns of production),
227 followed by calculation of binding energy by MMGBSA.³⁷ In a
228 previous work we demonstrated that longer MD simulations are
229 not necessary for this purpose.²⁰ Nwat-MMGBSA binding
230 energies were computed by including no explicit waters (Nwat =
231 0, corresponding to standard MMGBSA calculations) or by
232 selecting a certain number of explicit waters to be included in the
233 calculation (Nwat = 10, 20, 30, 60, and 100).

234 The same protocol was applied to 4MDS also, since the
235 binding energy computed for the 4MDS crystallographic ligand
236 was used as a reference. Analogously, the binding energy of the
237 6LU7 ligand (whose covalent bond was broken as described
238 above), was computed as a reference.

239 ■ RESULTS AND DISCUSSION

240 Virtual Screening on M^{Pro}

241 The VS campaign on SARS-CoV-2 M^{Pro} was conducted on two
242 different models (e.g., 6LU7 and QHD43415) to take binding
243 site flexibility into account through an ensemble docking
244 approach, increasing the solidity of the procedure with respect
245 to previous VSs on the same protein.

246 The results of the VS campaign are summarized in [Table 1](#) and
247 [Table 2](#), respectively, while [Tables S2 and S3](#), Supporting
248 Information, report compounds selected by docking but that
249 failed during the MD/Nwat-MMGBSA rescoring step. Results

Table 1. Results of the VS Campaign on the Crystal Structure of SARS-CoV-2 M^{Pro} (6LU7)^a

Drug Name	Dock score	Nwat-MMGBSA ^b
Angiotensin II	-124.4	-120.3 ± 10.1
GHRP-2	-132.6	-106.0 ± 8.3
Indinavir	-122.4	-86.5 ± 5.7
Polymyxin B	-107.9	-84.2 ± 8.3
Fexofenadine	-107.8	-77.0 ± 7.8
Atazanavir	-109.6	-73.0 ± 7.6
Cobicistat	-124.3	-72.8 ± 8.3
Aliskiren	-109.9	-70.9 ± 6.5
Lercanidipine	-106.6	-67.4 ± 8.4
Darunavir	-108.1	-66.6 ± 6.8
Montelukast	-112.8	-54.9 ± 6.8
Latanoprost	-108.5	-52.5 ± 4.2
Octenidine	-114.0	-50.8 ± 4.9
Velpatasvir	-108.4	-46.5 ± 8.1
Tyloxapol	-112.3	-42.5 ± 6.5
Salvianolic acid B	-124.4	-41.1 ± 11.0
Nilotinib	-106.6	-40.1 ± 8.6
Siponimod	-105.9	-38.5 ± 6.0
Travoprost	-114.9	-35.6 ± 6.1
Vitamin A Palmitate	-107.6	-35.5 ± 6.1
Penfluridol	-110.1	-30.2 ± 7.3
Clindamycin	-106.2	-20.5 ± 15.4
Ledipasvir	-109.6	-20.1 ± 7.8
Elbasvir	-106.3	-19.8 ± 9.9

^aTop 2% of compounds selected from the docking of 3118 FDA approved drugs and rescored by Nwat-MMGBSA (Nwat = 30) are shown. Compounds that ranked better than the reference are highlighted in bold. The 6LU7 crystallographic ligand of the SARS-CoV-2 main protease (6LU7) was used as the reference. Docking and Nwat-MMGBSA scores are -132.7 and -70.6 ± 8.0 kcal/mol, respectively. ^bNwat-MMGBSA rescoring was done considering 30 explicit water molecules around the ligand (Nwat = 30).

of Nwat-MMGBSA rescoring, using 30 explicit waters, are the
250 only reported, since Nwat = 30 was considered a reasonable
251 value in previous publications.^{20,34} 252

253 As can be observed, the protease inhibitors indinavir and
254 atazanavir, currently used to treat HIV infections, have been
255 selected by both models. Conversely, the protease inhibitor
256 lopinavir is top ranked for the QHD43415 model only, while it
257 failed during MD for 6LU7 ([Table S2](#), SI), probably due to steric
258 clashes originating from a position of the side chains in the
259 crystal structure not favorable for a stable binding of this
260 compound. [Figure 1](#) shows the predicted binding mode for
261 lopinavir, that anchors to the M^{Pro} binding site by multiple H-
262 bonds. The first H-bond is observed between the catalytic His41
263 residue and the aryloxyacetyl amido carbonyl (H-bond length =
264 2.1 Å). Additional H-bonds are observed with Glu166 and the
265 hydroxyl group at C-4, that acts both as a donor and an acceptor
266 (lop-O(H)⋯(H)N-Glu166 and lop-OH⋯O=C-Glu166 dis-
267 tances = 1.8 Å and 2.2, respectively). Finally, a dual H-bond is
268 observed between the side chain of Gln189 and both the
269 butanamido NH (lop-NH⋯O=C-Gln189 = 1.9 Å) and the 2-
270 oxo-1,3-diazinanyl carbonyl (lop-C=O⋯H₂N-Gln189 = 2.0
271 Å).

272 In addition, within an *in vitro* study against SARS-CoV-2, it
273 has been shown that lopinavir has an estimated 50% effective
274 concentration (EC₅₀) of 26.63 μM in Vero E6 cells.³⁸ 274

275 Other HIV protease inhibitors such as darunavir and ritonavir
276 were selected by one of the models, but failed for the other. 276

Table 2. Results of the VS Campaign on the Homology Model of SARS-CoV-2 M^{pro} (QHD43415)^{a,b}

Drug Name	Dock score	Nwat-MMGBSA ^{c,d}
Casposfungin	-108.3	-97.9 ± 12.4
Lopinavir	-106.5	-89.9 ± 5.9
Atazanavir	-109.9	-86.0 ± 7.0
GHRP-2	-116.7	-79.2 ± 11.1
Indinavir	-105.4	-78.6 ± 6.5
Angiotensin II	-125.7	-75.7 ± 9.2
Dehydroandrographolide Succinate	-99.4	-61.1
Ritonavir	-112.3	-58.3 ± 7.8
Azilsartan medoxomil	-102.1	-54.4
Salvianolic acid B	-116.0	-51.0 ± 7.7
Vilanterol	-100.7	-50.9
Elbasvir	-110.2	-48.0 ± 7.7
Clindamycin	-99.6	-47.8
Montelukast	-110.1	-47.5 ± 6.9
Latanoprost	-101.0	-46.8
Cobicistat	-119.3	-45.4 ± 11.6
Octenidine	-104.8	-43.6
Mupirocin	-98.1	-42.3
Tyloxapod	-105.5	-41.1 ± 8.3
Echinacoside	-103.1	-40.0
Salmeterol Xinafoate	-105.3	-37.9 ± 7.3
Ledipasvir	-101.5	-37.3
Thonzonium Bromide	-99.3	-36.7
Lomitapide	-98.1	-34.2
Travoprost	-99.2	-34.0
Itraconazole	-100.2	-32.6
Penfluridol	-106.2	-31.8 ± 9.6
Cisatracurium besylate	-100.3	-23.6
Retinol palmitate	-100.1	-21.8
Terfenadine	-98.1	-17.7

^aThe homology model of SARS-CoV-2 M^{pro} was made available by the Zhang group at <https://zhanglab.cmb.med.umich.edu/C-I-TASSER/2019-nCov/> ^bTop 2% of compounds selected from the docking of 3118 FDA approved drugs and rescored by Nwat-MMGBSA (Nwat = 30) are shown. Compounds that ranked better than the reference are highlighted in bold. The 4MDS crystallographic ligand in complex with SARS-CoV 3CLpro, a close homologue of SARS-CoV-2 M^{pro}, was used to compute reference scorings. Docking and Nwat-MMGBSA scores are -96.4 and -59.8 ± 5.3 kcal/mol, respectively. ^cNwat-MMGBSA rescoring was done considering 30 explicit water molecules around the ligand (Nwat = 30).

277 Indeed, darunavir scored rather well within 6LU7 screening, 278 while ritonavir was high-ranked by QHD43415 although the 279 Nwat-MMGBSA score was slightly lower than the chosen 280 thresholds for both compounds. Interestingly, similar results 281 were also obtained by another group using artificial 282 intelligence.³⁹ However, ritonavir alone showed an EC₅₀ greater 283 than 100 μM in Vero E6 cells.³⁸

284 Although some clinical studies on the use of HIV protease 285 inhibitors in COVID-19 were already terminated when this 286 screening was made, their results were not already available 287 (<https://clinicaltrials.gov/ct2/results?cond=COVID-19>). 288 Now, a publication showing that a combination of lopinavir and 289 ritonavir succeeded in alleviating symptoms and shortening the 290 hospitalization in patients with mild to moderate COVID-19, 291 especially when used in association with ribavirin.⁴⁰ Never- 292 theless, it should also be noted that no benefits were observed for 293 hospitalized patients with severe COVID-19 when treated with 294 lopinavir-ritonavir, beyond standard care,⁴¹ suggesting that the

treatment is only effective when given at an early stage of the 295 disease. Cobicistat, another drug that is approved for the 296 treatment of HIV infection, has been selected by the VS on the 297 6LU7 receptor model as a potential M^{pro} inhibitor, even if its 298 main mechanism is claimed to be the inhibition of CYP3A.⁴² At 299 the moment, a combination of darunavir and cobicistat is under 300 clinical evaluation for COVID-19, but preliminary results on 301 efficacy and safety are not encouraging. However, final results 302 are expected for August 31st, 2020.⁴³ 303

Some drugs already approved for the treatment of hepatitis C 304 were also identified. These includes elbasvir,⁴⁴ ledipasvir,⁴⁵ and 305 velpatasvir,⁴⁶ that were also identified in other *in silico* 306 screenings.^{19,47} Notably, clinical trials to evaluate the efficacy 307 of ledipasvir on COVID-19 are currently ongoing ([https://](https://clinicaltrials.gov/ct2/results?cond=COVID-19) 308 clinicaltrials.gov/ct2/results?cond=COVID-19). 309

Interestingly, angiotensin II and GHRP-2 are selected in both 310 screenings. Since the M^{pro} catalytic activity is to cleave the 311 polypeptide, a peptide of about 15 amino acids, it is plausible that 312 peptides, such as angiotensin II and GHRP-2 of 8 and 6 amino 313 acids, respectively, can fit into the M^{pro} active site. Although the 314 use of these hormones might not be indicated for the treatment 315 of SARS-CoV-2, their structures might be used as templates for 316 further drug development. Similarly, the antibiotic polymyxin B 317 was also picked as a high-rank hit, although by the 6LU7 model 318 only, probably due to its peptide nature. Interestingly, 319 polymyxin B was top-ranked within the S-protein screening 320 also, as discussed later in this article. Another lipopeptide, 321 caspofungin, has also been identified, but by the QHD43415 322 model only. Caspofungin is an antifungal drug specifically used 323 in HIV-infected individuals.⁴⁹ It was also identified in another 324 independent study as an inhibitor of SARS-CoV-2 replication,⁵⁰ 325 even if the Nsp12 polymerase has been claimed as the target. 326

A hit that might be worthy of attention, although only 327 identified by the QHD43415 model, is dehydroandrographolide 328 succinate (DAS). DAS is a natural product extracted from 329 *Andrographis paniculate*, well-known by traditional Chinese 330 medicine.⁵¹ Indeed, while the herb has long been used to treat 331 cold and fever, purified andrographolides, including DAS and 332 analogues, have been prepared and used to treat respiratory 333 diseases.^{52,53} Antibacterial, antiviral, anti-inflammatory, and 334 immune-stimulatory activities were claimed for DAS,⁵⁴ 335 including the inhibition of HIV and H5N1 viruses *in vitro*.^{55,56} 336

Several other compounds were selected by initial docking but 337 failed during the MD simulation phase (Tables S2 and S3). Such 338 a failure might be due to several causes, among all poor 339 parametrization (the BCC charge parametrization method,⁵⁷ 340 instead of the more rigorous RESP method,⁵⁸ was chosen for 341 time constraints). However, the reason for the failure might also 342 be due to severe steric clashes or mispositioning during the 343 docking stage. Thus, although some good hits might be found 344 within the “F” series, these selections are to be considered as the 345 least reliable. 346

Virtual Screening on Spike Protein 347

When this work was realized, no S-protein RBD-targeting small 348 molecule was known either for SARS-CoV-2 or other 349 coronaviruses. Conversely, few antibodies recognizing the 350 RBD^{59–62} and a recombinant ACE2 enzyme⁶³ are reported. In 351 addition, a 23-mer peptide derived from the ACE2 showed an 352 affinity of 47 nM toward the SARS-CoV-2 RBD,⁶⁴ and a few 353 other peptides were developed to inhibit the interactions of the 354 S2 subunit during the fusion process in both SARS-CoV and 355 SARS-CoV-2.⁶⁵ Nonetheless, considering that several com- 356

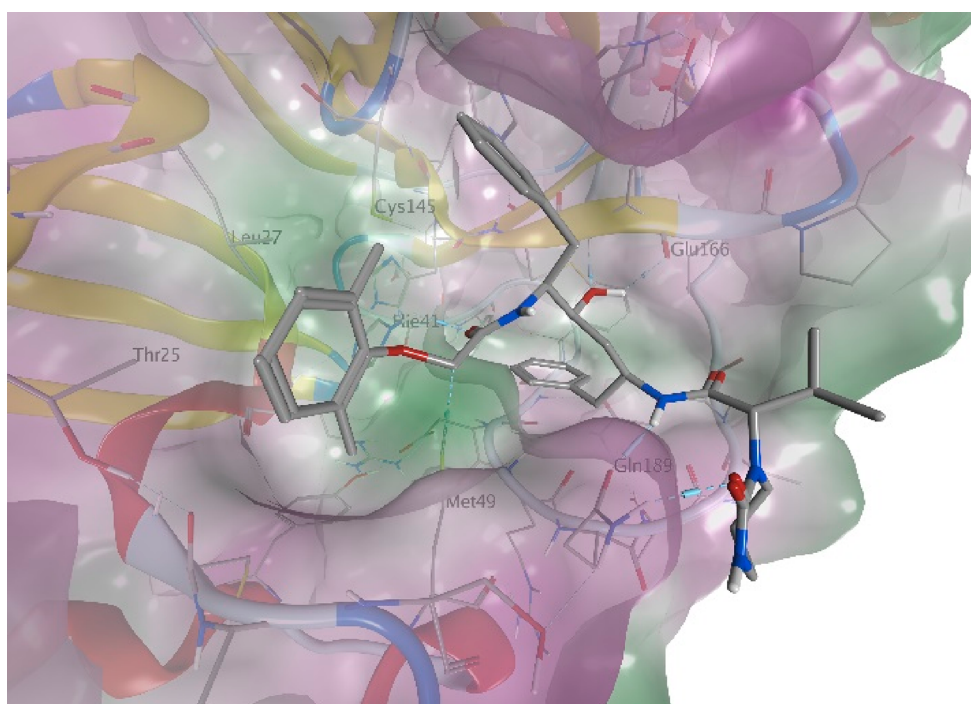


Figure 1. Predicted binding mode of lopinavir to M^{pro} . The model was obtained by performing a cluster analysis of the MD trajectory of the docking pose, followed by a backbone-restrained geometry minimization of the main cluster using MOE.

357 pounds that are currently being tested, some with positive
358 results, were identified by our VS campaign on M^{pro} , the results
359 reported hereafter can also represent a step toward the
360 treatment against SARS-CoV-2.

361 RBD Binding Sites Definition

362 As for most of the protein–protein interaction (PPIs)
363 interfaces,⁶⁶ the one between hACE2 and RBD is quite
364 extended. However, it is possible to target only the residues
365 making the major contribution to the binding free energy
366 between the two proteins (hot spots). In light of this, an alanine
367 scanning analysis was performed by individually mutating the
368 RBD residues at the interface with hACE2 (see [Methods](#), [Figure](#)
369 [2](#) and [Table S4](#)), in order to determine which are the RBD hot

spot residues. This allowed us to define two clusters of hot spots
([Figures 2](#) and [3](#)), in agreement with a recently published
preprint article.⁶⁷ More in detail, the first cluster (cluster 1)
involves Leu455, Phe456, Phe486, Asn487, Tyr489, and
Gln493, while the second one (cluster 2) includes Tyr449,
Gln498, Thr500, Asn501, and Tyr505 ([Figures 2](#) and [3](#)). It has
to be noted that the found hot spots are not included in the
observed mutations found so far,⁶⁸ suggesting that they can be
safely targeted by potential inhibitors of the ACE2-RBD
interaction.

In the second cluster we can find Gln498: when it is mutated
to alanine, the complex ΔG has a loss of ~ 9 kcal/mol, indicating
that this residue is fundamental for the interaction with hACE2.
Indeed, the Gln498 side chain is involved in a highly stable H-
bond (occupancy $>80\%$, [Table S2](#)) with the hACE2 Asp38 side
chain, and for the remaining $\sim 20\%$ it interacts with the hACE2
Lys353 side chain.

Since cluster 1 and 2 are well separated and localized at the
two extremities of the RBD interface to hACE2, it is possible to
determine two distinct binding sites to target by VS (see
[Methods](#)), called BS1 and BS2, respectively, in the following
discussion. Working with two different possible RBD binding
sites represents an advantage as compared to other similar
studies, where a single but larger binding site on the RBD was
defined.^{18,69} Indeed, as previously said, most of the currently
available drugs are small molecules which are able to interact
with a limited surface and only with a few residues. Therefore,
selecting two binding sites corresponding to specific hot spot
clusters makes the docking pose search more efficient, by
limiting the search space. In addition, it allows us to discard
molecules which are predicted to strongly interact with the
protein target but on a protein region without hot spots, not
assuring the PPI inhibition.

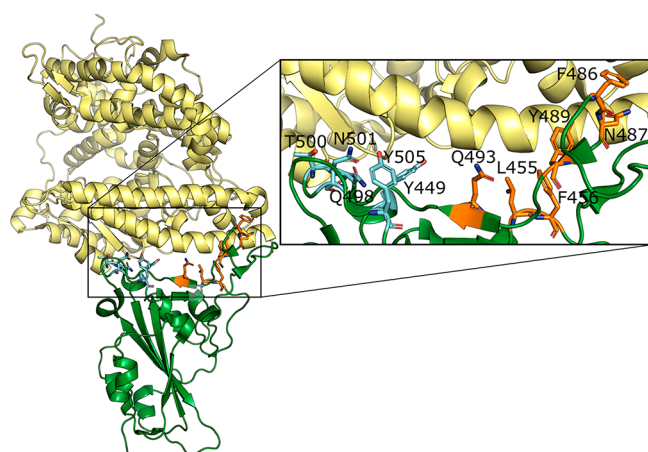


Figure 2. Complex between hACE2 (yellow) and SARS-CoV-2 RBD (green) from the X-ray structure (PDB code 6M0J). The hot spot residues are represented in sticks and labeled in the inset. Hot spots of cluster 1 are represented in orange, while those of cluster 2 are represented in cyan.

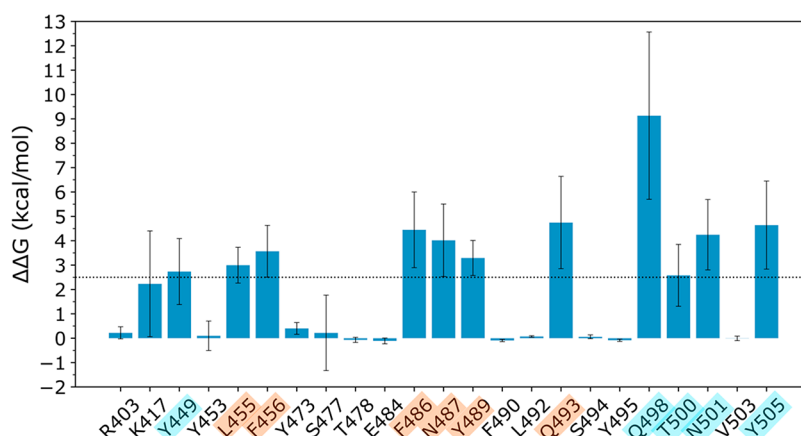


Figure 3. Difference in the binding free energy between the mutated system and the native one computed on the last 10 ns of the MD simulation of the hACE2-RBD complex. All the residues for which the $\Delta\Delta G$ is greater than the threshold (2.5 kcal/mol) have been considered as hot spots. The hot spots of cluster 1 are highlighted in orange and those of cluster 2 in cyan.

Table 3. Results of the VS Campaign on the Crystal Structure of SARS-CoV-2 S-Protein RBD Binding Site 1^a

Drug name	Dock score	Nwat-MMGBSA ^b	Drug name	Dock score	Nwat-MMGBSA ^b
Polymyxin B	-107.6	-152.1 ± 11.5	Ginsenoside Rb1	-96.2	-64.2 ± 10.9
Colistin	-101.7	-149.4 ± 12.5	Somatostatin	-98.6	-61.2 ± 10.2
Daptomycin	-95.2	-137.8 ± 13.1	Ledipasvir	-97.4	-60.8 ± 8.5
Oritavancin	-93.6	-126.8 ± 13.3	Zafirlukast	-91.0	-57.7 ± 6.0
Thymopentin	-92.4	-121.9 ± 14.6	Latanoprost	-98.0	-56.5 ± 7.4
Terlipressin	-103.7	-118.0 ± 9.6	Fexofenadine	-91.0	-53.3 ± 17.9
Lypressin	-103.2	-111.3 ± 12.6	Velpatasvir	-91.3	-53.1 ± 8.7
Vancomycin	-96.2	-104.6 ± 17.9	Nebivolol	-90.9	-52.2 ± 7.5
Leuprolide	-110.7	-101.3 ± 10.8	Azelnidipine	-91.1	-51.6 ± 7.6
Alarelin	-104.6	-98.3 ± 8.9	Astemizole	-91.9	-51.1 ± 5.5
Deferoxamine	-90.8	-97.4 ± 9.0	Pranlukast	-91.5	-50.3 ± 5.6
Bacitracin	-93.9	-97.0 ± 11.8	Travoprost	-89.9	-49.1 ± 8.5
Senenoside B	-91.3	-94.9 ± 8.9	Vilazodone	-97.0	-48.6 ± 5.9
Angiotensin II human	-104.2	-94.8 ± 11.6	Acidinium	-90.0	-48.5 ± 6.4
Salvianolic acid B	-104.5	-93.9 ± 10.2	Octenidine	-102.4	-48.1 ± 9.5
Gonadorelin	-104.2	-93.5 ± 8.7	Elbasvir	-97.4	-47.8 ± 10.0
Nafarelin	-111.8	-90.3 ± 11.5	L-Ascorbyl 6-palmitate	-90.8	-47.7 ± 8.0
Amphotericin B	-108.0	-89.2 ± 11.8	Silodosin	-90.3	-47.1 ± 9.2
Madecassoside	-96.0	-88.5 ± 10.2	Ponatinib	-96.6	-44.6 ± 7.7
Micafungin	-95.6	-86.0 ± 12.4	Ebastine	-95.2	-44.3 ± 7.9
Mupirocin	-91.4	-82.1 ± 7.0	Vitamin K2	-95.7	-41.5 ± 6.1
Goserelin	-107.9	-81.0 ± 12.7	Posaconazole	-99.5	-32.6 ± 7.7
Nystatin	-102.2	-78.8 ± 12.8	Penfluridol	-90.3	-31.5 ± 9.1
Echinacoside	-93.2	-71.9 ± 9.5	Vitamin A	-96.6	-31.1 ± 8.1
Dalbavancin	-90.7	-69.7 ± 12.5	Lapatinib	-100.8	-31.1 ± 7.2
Tyloxapol	-106.6	-68.5 ± 7.7	Behenic alcohol	-93.4	-28.8 ± 7.2
Icatibant	-115.8	-67.9 ± 10.4	Gefarnate	-89.8	-26.2 ± 10.4
Landiolo	-91.6	-67.3 ± 12.4	Azilsartan	-90.2	-24.5 ± 12.4
Venetoclax	-92.5	-66.9 ± 7.3			
Vilanterol	-95.1	-65.5 ± 8.7			
Montelukast	-97.6	-65.3 ± 11.9			
Salmeterol	-100.4	-64.6 ± 7.5			

^aTop 2% of compounds selected from the docking of 3118 FDA approved drugs and rescored by Nwat-MMGBSA (Nwat = 60) are shown ranked by Nwat-MMGBSA scores. ^bEnergy obtained by using Nwat = 60, ± standard deviation.

403 Virtual Screening on RBD-BS1

404 The results of the VS are reported in Table 3, after the deletion
405 of those molecules which, although being part of the top 2%
406 during the docking step, left the binding during the MD
407 simulation (namely, GHRP-2, cobicistat, oxytocin, and vitamin
408 B12). Although different Nwat values have been evaluated, we
409 will limit our discussion to the results provided by Nwat-

MMGBSA with Nwat = 60, since this value resulted to be
appropriate when dealing with PPI inhibitors.^{20,34}

It is not surprising that the best ranked ligands are peptide-like
molecules, since these are usually larger than small molecules
and allow a better interaction with the PPI binding partner. Most
of the top ranked peptide-like molecules, such as the polymyxin
B, colistin, and daptomycin, are currently used as antibiotics
because of their ability in disrupting the bacterial membrane.

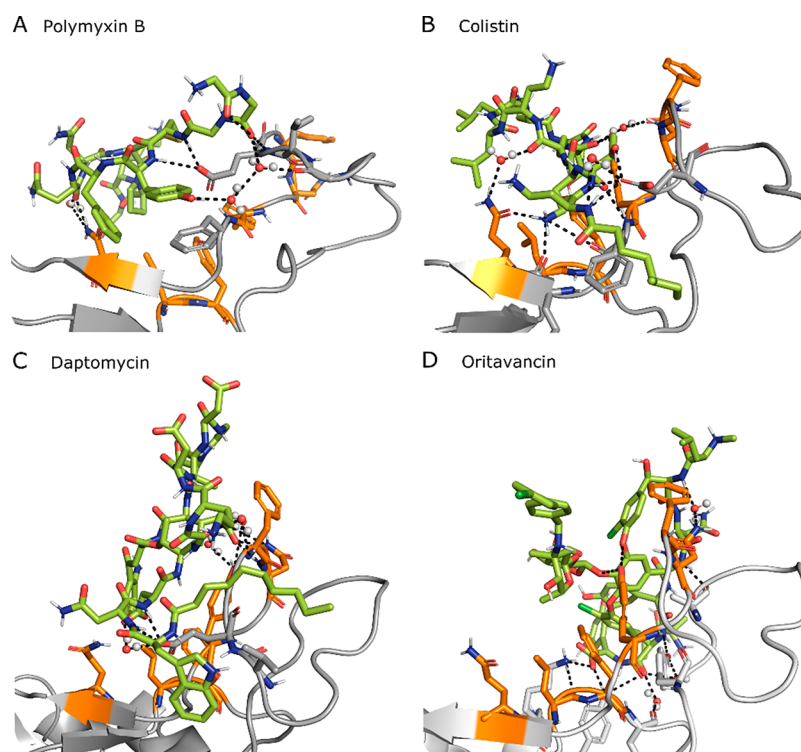


Figure 4. Snapshot of the MD simulation between RBD (gray) and one of the top four ligands (green). RBD BS1 hot spots are highlighted in orange. Direct and water (spheres) mediated H-bonds are also displayed as dashed lines. Additional RBD residues interacting with the ligand are displayed as sticks.

418 Among these, polymyxin B has been tested within a compas-
419 sionate use protocol for patients with an immediately life-
420 threatening condition.⁷⁰

421 Others compounds identified herein as potential binders of
422 the S-protein are terlipressin and lypressin, analogs of vaso-
423 pressin and used against hypotension. We can also find
424 hormone-peptides, such as alarelin or leuporelin, which belong
425 to the gonadotropin-releasing hormone family, or somatostatin,
426 an endocrine system regulator. Furthermore, we can observe the
427 presence of peptidomimetics, such as icatibant, which acts as an
428 antagonist of B2 bradykinine receptors. This last compound was
429 also identified by an independent study as a potential disruptor
430 of the Spike-ACE2 PPI.⁷¹

431 The only nonpeptide molecules found in the top 2% are large
432 compounds (molecular weight >500 g/mol) rich in H-bond
433 acceptor and donor atoms. This is not surprising, indeed it has
434 been suggested that the SARS-CoV-2 binds to the host heparan
435 sulfate chains of the heparan sulfate proteoglycan receptor also,
436 initiating the internalization.⁷² In addition, it has been recently
437 shown that the RBD can bind to the heparin^{73–75} and that an
438 octosaccharide sequence strongly inhibits this interaction (IC_{50}
439 = 38 nM).⁷³ In our VS campaign, we found the salvianolic acid B
440 (also found in M^{pro} screening), used as antioxidant, antifungal
441 drugs (amphotericin B, micafungin, nystatin, micafungin), the
442 madecassoside and ginsenoside Rb1, molecules with anti-
443 inflammatory properties, and the tensioactive tyloxapol in the
444 top 2%. Interestingly, a combination of amphotericin B and
445 deoxycholate was shown to have an effect in decreasing the
446 infectivity of transmissible gastroenteritis coronavirus.⁷⁶ We also
447 found at #11 deferoxamine, a chelating agent under clinical
448 study against COVID-19 (ClinicalTrials.gov Identifier:
449 NCT04333550). However, this molecule is claimed as
450 responsible of chelating the iron whose dissociation from

heme is increased by SARS-CoV-2, causing oxidative stress and
451 damage to the lung.⁷⁷ The top 2% also contains antivirals such as
452 ledipasvir and elbasvir, used against hepatitis C. However, their
453 mechanism of action involves the inhibition of viral proteins.
454 Additionally, both elbasvir and ledipasvir were identified on the
455 same target in another independent study, using a different
456 computational protocol.^{47,48}

457 Unexpectedly, among the best 60 RBD ligands there is
458 salmeterol and vilanterol, which are agonists of β_2 -adrenergic
459 receptors, a class of molecules which showed a minor
460 amplification of the viral phenotype in a recent preprinted
461 study.⁷⁸ Conversely, another antiasthmatic drug, montelukast,
462 has been shown to cause the disruption of the viral integrity of
463 the Zika virus;⁷⁹ thus, its presence in the top 2% (#39) enhances
464 the interest of this compound against SARS-CoV-2 also.
465

466 In addition, we also found a few beta-adrenergic blockers,
467 namely landiolol and nebivolol; this class of molecules, although
468 it is not known to bind to the S-protein, has been hypothesized
469 to be able to decrease the SARS-CoV-2 entry into the cells by
470 downregulating ACE2 receptors.^{78,80}

471 Peptides are usually highly flexible and require an extensive
472 conformational sampling before the VS procedure. However,
473 most of the peptides herein considered are partially cyclic: this
474 creates a structural constraint, making feasible and globally
475 reliable their docking to RBD.

476 The best scored compounds interact with the RBD through
477 hydrophobic interactions and stable direct and water-mediated
478 H-bond with BS1 hot spots and neighboring residues, creating a
479 stable network of interactions, as shown in Figure 4 for the four
480 top-ranked ligands. For example, polymyxin B can create direct
481 H-bonds with Glu484, Phe486, Asn487, Tyr489, and Gln493
482 and water-mediated H-bonds with Asn487, Glu484, and

Table 4. Results of the VS Campaign on the Crystal Structure of SARS-CoV-2 S-Protein RBD Binding Site 2⁴

Drug name	Dock score	Nwat-MMGBSA (nwat = 60) ± standard deviation	Drug name	Dock score	Nwat-MMGBSA (nwat = 60) ± standard deviation
Polymyxin B	-99.4	-164.3 ± 11.3	Elbasvir	-108.7	-73.4 ± 7.3
Thymopentin	-97.7	-154.5 ± 12.9	Manidipine	-92.6	-72.3 ± 6.4
Icatibant	-107.6	-143.1 ± 12.0	Ginsenoside Rb1	-93.8	-72.3 ± 10.8
Octreotide	-94.6	-127.2 ± 10.9	Lercanidipine	-95.5	-71.3 ± 6.5
Oritavancin	-98.3	-123.6 ± 14.1	Atazanavir	-98.1	-70.8 ± 7.4
Nystatin	-110.8	-123.2 ± 10.5	Cobicistat	-100.3	-69.5 ± 8.7
Terlipressin	-98.2	-122.8 ± 10.7	Montelukast	-100.8	-67.5 ± 7.6
Salvianolic acid B	-112.0	-121.6 ± 10.6	Vitamin B12	-93.5	-65.9 ± 11.7
Echinacoside	-104.6	-113.3 ± 8.2	Tyloxapol	-104.1	-64.5 ± 7.1
Bleomycin	-103.4	-110.1 ± 15.3	Micafungin	-95.4	-63.2 ± 12.7
Angiotensin II human	-100.3	-107.3 ± 12.1	Salmeterol	-99.8	-62.8 ± 8.6
Nafarelin	-121.9	-106.4 ± 10.6	Zafirlukast	-94.6	-61.8 ± 5.8
Leuprorelin	-114.5	-106.2 ± 9.8	Labetalol	-91.8	-61.4 ± 5.9
Senoside B	-91.9	-99.1 ± 10.6	Indinavir	-105.0	-60.0 ± 8.7
Aliskiren	-99.6	-96.3 ± 6.7	Latanoaprost	-94.7	-57.2 ± 6.5
Caspofungin	-99.1	-95.4 ± 14.3	Amphotericin B	-132.7	-57.0 ± 7.8
Alarelin	-103.7	-94.6 ± 10.3	Ombitasvir	-94.3	-53.2 ± 12.6
GHRP-2	-104.9	-93.8 ± 9.9	Tocofersolan	-91.6	-52.5 ± 6.8
Lentinan	-96.5	-93.4 ± 12.5	Haloperidol	-91.9	-52.5 ± 9.2
Leuprolide	-109.6	-93.4 ± 11.1	Tafuprost	-94.3	-51.6 ± 6.3
Hederacoside C	-98.5	-89.1 ± 10.1	Itraconazole	-96.0	-46.5 ± 7.3
Gonadorelin	-111.4	-88.8 ± 13.0	Avanafil	-96.7	-46.2 ± 5.8
Pneumocandin	-95.3	-86.4 ± 11.4	Ledipasvir	-92.6	-43.4 ± 8.2
Daptomycin	-94.4	-85.4 ± 18.5	Octenidine	-99.1	-43.2 ± 9.1
NAD+	-96.9	-83.6 ± 33.4	Thonzonium	-92.4	-41.0 ± 8.1
Deferoxamine	-97.2	-83.3 ± 8.5	Fulvestrant	-96.5	-40.9 ± 7.1
Goserelin	-99.2	-80.4 ± 10.9	Gefarnate	-91.7	-39.3 ± 6.6
Neohesperidin	-94.2	-79.8 ± 8.0	Clindamycin	-91.9	-33.4 ± 7.8
Gramicidin	-98.5	-79.3 ± 11.8			
Somatostatin	-110.7	-77.2 ± 10.7			
Vilanterol	-96.3	-75.5 ± 6.3			
Desmopressin	-95.1	-74.9 ± 11.7			

⁴Top 2% of compounds selected from the docking of 3118 FDA approved drugs and rescored by Nwat-MMGBSA (Nwat = 60) are shown ranked by Nwat-MMGBSA scores.

483 Phe490. In addition, we can observe hydrophobic interactions
484 between polymyxin B and Val483 and Phe486.

485 Virtual Screening on RBD-BS2

486 Similar results were obtained for the BS2, as shown in Table 4. In
487 this case also, the ligands which left the binding site during the
488 MD simulation, namely colistin, ritonavir, salmeterol, dalba-
489 vancin, and atazanavir, were removed from the list. It is
490 interesting to notice that colistin was the second best ligand for
491 BS1; conversely for BS2, even if the docking procedure ranked
492 this ligand in the top 2%, it could not maintain the favorable
493 interactions during the MD simulation. This highlights the
494 importance of performing MD simulations on the complexes
495 obtained by the docking procedure and provides a further
496 confirmation of the quality of our protocol.²⁰ In addition,
497 although the amino acid composition of the two binding sites is
498 quite similar (Figure 2), their conformational organization is
499 specific and exploitable for further studies on the development of
500 new potential inhibitors of the RBD-hACE2 interaction.

501 Globally, the top 2% of ligands binding to BS2 is similar in
502 composition to the one binding to BS1: most of the ligands are
503 peptide-like molecules known to be antibiotics, antifungals,
504 peptide hormones, and pressure regulators. As noticed for BS1,
505 we can also find molecules containing both large hydrophobic
506 groups and H-bond donors and acceptors, such as echinacoside
507 and aliskiren (Table 4). The best ranked ligand poses show a

tight network of direct and water-mediated H-bonds with both
the hot spot residues and the neighboring ones, in addition to
additional hydrophobic interactions (Figure 5). For example,
the best ranked molecule, which is polymyxin B also in this case,
creates direct H-bonds with Arg403, Tyr449, Gly496, the most
relevant hot spot Gln498, and Asn501, together with water-
mediated H-bonds with Glu406, Tyr449, Tyr453, Gln493,
Gln498, and Thr500. Except for polymyxin B, the ranking is
quite different from that for BS1, with molecules which were not
present in the top 10 for BS1 being ranked in the top positions
for BS2, such as icatibant (#3) and octeotide (#4).

Thymopentin is the only linear peptide found in the top 2%
docked molecules for both BS. In order to verify if the docked
conformation properly took into account for the peptide
flexibility and its accessible conformations, we predicted the
3D structure of the peptide using the PEPFOLD3⁸¹ server, and
the best model has a backbone RMSD of 1.3 Å (Figure S2) from
the thymopentin docked to RBD and ranked second in the VS
campaign targeting RBD BS2. It should be underscored that
thymopentin is an immunostimulant peptide applied in
numerous clinical studies during the AIDS pandemic between
1983 and 1985.^{82,83} Therefore, together with the good binding
to the SARS-CoV-2 S-protein RBD shown within this VS
campaign, a potential immunostimulant effect of this peptide

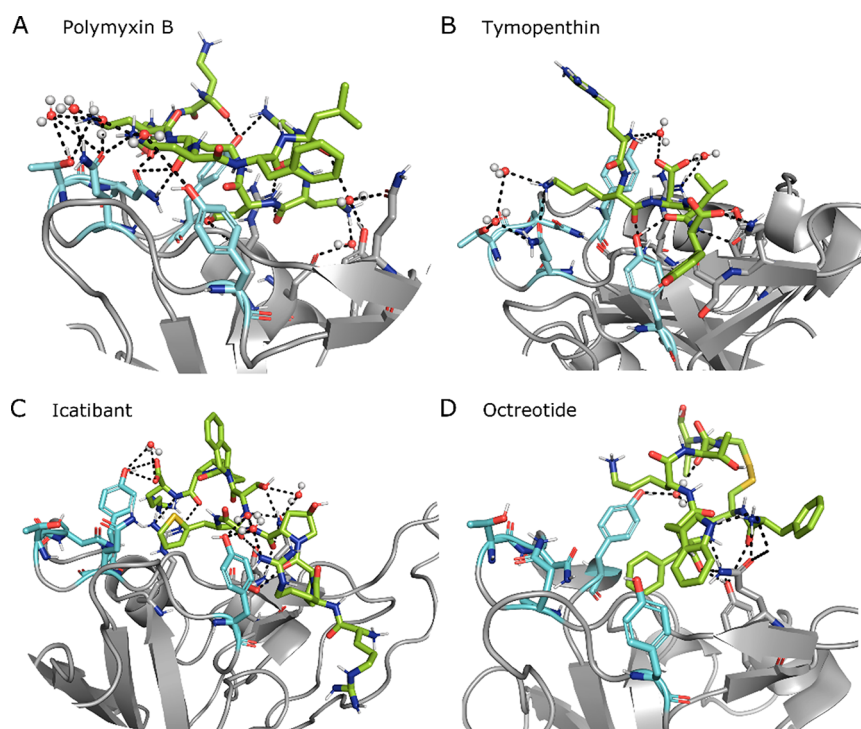


Figure 5. Snapshot of the MD simulation between RBD (gray) and one of the top four ligands (green). RBD BS2 hot spots are highlighted in cyan. Direct and water (spheres) mediated H-bonds are also displayed as dashed lines. Additional RBD residues interacting with the ligand are displayed as sticks.

could be helpful in enhancing the immune response to the viral infection.

CONCLUSIONS

SARS-CoV-2 currently represents a major threat to human health, having caused hundreds of thousands of deaths in a few months. At the moment a cure against this pandemic infection is still lacking, together with a vaccine against this virus. In order to rapidly face the emergency, testing the efficacy against SARS-CoV-2 of drugs already approved for the treatment of other diseases (drug repurposing) is a good option. Indeed, it has the advantage of exploiting molecules which have already been tested in terms of toxicity and which are usually easy to purchase for clinical tests and patient administration. Therefore, positive results from this kind of procedure can speed up the process of finding a treatment against SARS-CoV-2. However, the number of approved drugs by the major jurisdictions is huge and directly performing either *in vitro* or *in vivo* studies on all of them would be time-consuming; thus a fundamental contribution to accelerate the screening can come from *in silico* techniques.

Within this context, we proposed a multiple VS campaign aimed to prioritize the testing against SARS-CoV-2 of already approved molecules. More in detail, we performed 4 independent VS procedures of more than 3000 approved drugs using two different SARS-CoV-2 proteins: the main proteinase M^{Pro} and the RBD of the S-protein. Inhibiting the former would block the viral replication, while targeting the S-protein domain (i.e., RBD) would hamper the viral entry into the human cells. We applied an advanced VS procedure, which already proved to better discriminate between active and inactive compounds on multiple systems, compared to standard docking procedures.²⁰

The VS campaign against M^{Pro} ranked in the top 2% of inhibitors of the HIV protease, such as indinavir, atazanavir, and

lopinavir, which recently proved to be able to alleviate the symptoms of mild-to-moderate SARS-CoV-2 infection in combination with ritonavir.⁴⁰

The VS campaign on Spike protein RBD indicated that peptides or peptidomimetics actually used as antibiotics (i.e., polymyxin B, colistin, and daptomycin), pressure regulators (i.e., terlipressin and lypressin), hormone-peptides (i.e., alarelin and leuporelin), and immunostimulants, such as the thymopentin, could be evaluated against SARS-Cov-2 also. Currently, there are not clinical studies on molecules known to specifically disrupt the interaction between the human ACE2 and the RBD; however, a few peptides were designed with this aim and successfully tested *in vitro*, validating our hypothesis that peptide-based molecules can be adapted to inhibit the ACE2-RBD interaction.

In conclusion, together with providing a good starting point for future *in vitro* and *in vivo* investigations on the resulting top compounds, the results of this extensive VS can support the design of selective and specific molecules to treat SARS-CoV-2 infection by targeting different viral proteins.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acs.jproteome.0c00383>.

Figure S1. RMSD of the RBD-hACE2 complex backbone atoms from the X-ray structure during the MD simulation. Table S1. Difference in the binding free energies between the mutated and the native RBD-hACE2 complex obtained from the alanine scanning. Table S2. Compounds selected by docking on M^{Pro} (6LU7 model) that failed during the MD/Nwat-MMGBSA rescoring step. Table S3. Compounds selected by docking on M^{Pro}

(QHD43415 homology model) that failed during the MD/Nwat-MMGBSA rescoring step. Table S4. Hydrogen bonds between RBD and hACE2 during the last half of the 20 ns MD simulation. Figure S2. Superposition of thymopentin docked to RBD BS2 and the PEPFOLD3 structure prediction. (PDF)

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript. All the authors contributed equally.

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ABBREVIATIONS

VS, virtual screening; MD, molecular dynamics; RBD, receptor binding domain; ACE2, angiotensin converting enzyme 2; H-bond, hydrogen bond

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