1   **Characterization of raloxifene as potential pharmacological agent against SARS-CoV-2**

2   **and its variants**

3

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21   *Running title: Raloxifene as new pharmacological agent against COVID-19*

22

23   The new coronavirus that emerged, called SARS-CoV-2, is the causative agent of the COVID-19

24   pandemic. The identification of potential drug candidates that can rapidly enter clinical trials for the

25   prevention and treatment of COVID-19 is an urgent need, despite the recent introduction of several

26   new vaccines for the prevention and protection of this infectious disease, which in many cases

27   becomes severe. Drug repurposing (DR), a process for studying existing pharmaceutical products for

28   new therapeutic indications, represents one of the most effective potential strategies employed to

29   increase the success rate in the development of new drug therapies. We identified raloxifene, a known

30   Selective Estrogen Receptor Modulator (SERM), as a potential pharmacological agent for the

31   treatment of COVID-19 patients. Following a virtual screening campaign on the most relevant viral

32   protein targets, in this work we report the results of the first pharmacological characterization of

33   raloxifene in relevant cellular models of COVID-19 infection. The results obtained on all the most

34   common viral variants originating in Europe, United Kingdom, Brazil, South Africa and India,

35   currently in circulation, are also reported, confirming the efficacy of raloxifene and, consequently,

36   the relevance of the proposed approach.

37 Taken together, all the information gathered supports the clinical development of raloxifene and

38 confirms that the drug can be proposed as a viable new option to fight the pandemic in at least some

39 patient populations. The results obtained so far have paved the way for a first clinical study to test the

40 safety and efficacy of raloxifene, just concluded in patients with mild to moderate COVID-19.

41

42 **Keywords:** COVID-19, drug repurposing, raloxifene, estrogen receptors**,** antiviral activity, Vero E6

43 cells, Calu-3 cells, SARS-CoV-2 viral variants.

44

45 **Introduction**

46

47 Coronaviruses are the causative agent of multiple respiratory and intestinal infection in humans and

48 animals [1-3]. Unlike most other human coronaviruses, which only rarely cause severe disease and

49 death [4], SARS-CoV-2 is able to cause severe acute respiratory illness, multi-organ failure and death,

50 sharing common pathogenetic mechanisms with SARS-CoV and MERS-CoV betacoronavirus [5].

51 Common symptoms of COVID-19 include fever, sore throat, fatigue, cough, shortness of breath and

52 dyspnea that may eventually progress towards acute respiratory distress syndrome, with the

53 involvement of other systems/organs (e.g., heart, liver and kidneys) [6, 7] and up to the death in the

54 most critical cases. About 80% of patients have mild to moderate disease, 14% have severe disease,

55 and 6% become critical (namely, they develop respiratory failure, septic shock, and/or multiple organ

56 dysfunction/failure) (www.ecdc.europa.eu – fifth update, 2 March 2020). As of September 6[th,] 2021,

57 SARS-CoV-2 infection led to more than 4,5 million deaths worldwide (https://covid19.who.int/). To

58 date, notwithstanding the advent of vaccine programs and constant social distancing interventions, it

59 is believed that the virus is likely or very likely to become endemic [8, 9]. In addition, the emerging

60 of SARS-CoV-2 variants raises great concern for vaccine efficacy, reinfection events and increased

61 transmissibility and disease severity. As the virus started to spread around the world, a mutated spike

62 SARS-CoV-2 variant (D614G) emerged and was associated with increased infectivity, becoming the

63 predominant variant in Europe and worldwide without any increase in disease severity [10-13]. In

64 recent months, other variants were defined as "variants of concern" (VOC). The most relevant also

65 from a clinical point of view are: B.1.1.7 (UK), B.1.351 (South African), B.1.1.28 (Brazilian P.1),

66 B.1.427 and B.1.429 (Californian, also named CAL.20C), characterized by increased transmissibility,

67 immune evasion and higher virulence [14-20]. As of May 11th, 2021, the so called "Indian" Delta

68 variant (Delta B.1.617.2) was added to the WHO list of VOC; this variant seems to be able to escape

69　adaptive immunity induced by prior wild type infection roughly half of the time and to be more

70　infectious (around 60%) than wild type SARS-CoV-2 [21]. The growing relevance of the rapidly

71　emerging SARS-CoV-2 variants deserves further investigations, and new impetus will have to be

72　given to research to increase the availability of broad-spectrum drugs or vaccines for long-term

73　prevention, treatment and control of COVID-19, with the final goal to find new interventions and

74　cures to complement vaccine programs. To identify potential therapeutic targets, one of the main

75　studied mechanisms is the virus entry machinery, and several preclinical and clinical trials are

76　ongoing to find new inhibitors of clinical relevance [22]. The entry machinery involves two key host

77　proteins: the angiotensin-converting enzyme 2 (ACE2) and the cell surface transmembrane protease

78　serine 2 (TMPRSS2) [23, 24]. In addition, also Neuropilin1 (NRP-1) has been recognized as an

79　important receptor whose inhibition reduces SARS-CoV-2 entry and infectivity [25, 26].

80　Moreover, recent evidence shows that Nuclear Receptors (NRs), and in particular the sex hormone

81　receptors, like estrogen and androgen receptors, could be involved in the outcome of COVID-19.

82　These receptors regulate the viral entry protein expression and activity [27-29]. Additionally, a

83　protective effect of estrogens in the progression of COVID-19 infection has been associated with their

84　role in regulation of innate and adaptive immune responses, as well as in the control of the cytokine

85　storm [30-34], whereas activation of androgen receptors seems to correlate with the worse COVID-

86　19 clinical outcome observed in men compared to women [29, 35-37].

87　Recently, several molecules with potential efficacy against SARS-CoV-2 were selected from an

88　extensive virtual screening campaign based on the EXaSCale smArt pLatform Against paThogEns

89　[38], a powerful tool for repurposing of drugs and compounds in new indications [39-42] for

90　immediate response and quick identification of effective treatments, useful during pandemic

91　situations. So, in the context of the Horizon 2020 project EXSCALATE4CoV, raloxifene, a well-

92　known SERM (Selective Estrogen Receptor Modulator) [43-47] was selected through an integrated

93　approach of drug repurposing and *in silico* screening on SARS-CoV-2 target proteins, an approach

94　that, combined with the scientific rationale and literature evidence that support a potential antiviral

95　and protective action of SERMs in COVID-19, led the molecule to be selected as clinical candidate

96　for studies in mild to moderate COVID-19 patients [38].

97　Raloxifene is a drug registered in Europe and US for the treatment and prevention of osteoporosis in

98　postmenopausal women, and for the reduction of the risk of invasive breast cancer in postmenopausal

99　women [48, 49]. It is known to act as an agonist in the bone, liver and cardiovascular system and as

100　antagonist in human breast and uterine tissues [50-52], and tissue specificity is relevant for its use in

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101 postmenopausal osteoporosis and prevention of breast cancer without increase of risk of endometrial

102 cancer, differently from the behavior of other SERMs like tamoxifen [53, 54]. The drug has also been

103 studied in men for uses such as for treatment of schizophrenia, prostate cancer and osteoporosis [55-

104 57]. Recently, raloxifene has been also characterized in viral infections. It was found active against

105 Ebola virus [58, 59], Hepatitis C virus [60, 61], Hepatitis B virus [62], and Zika virus [63]. Further,

106 it showed efficacy in human female cells from nasal epithelium, against the Influenza Virus A [64],

107 and as adjuvant antiviral treatment of chronic hepatitis C (CHC) in postmenopausal women [65].

108 These observations, together with raloxifene activity on the Estrogen Receptor (ER) pathways,

109 highlight a possible relationship between clinical outcome and sex and age of patients with viral

110 infections.

111 In this paper, we report for the first time a full characterization of the antiviral activity of raloxifene

112 in two different well-established cellular contexts (Vero E6 and Calu-3) and we tested the potential

113 influence of the most common COVID-19 variants on raloxifene biological activity. Raloxifene *in*

114 *vitro* activity was high and consistent in the different cell lines tested, preserved in all main VOCs of

115 clinical relevance.

116 Repurposing and *in silico*/experimental synergy are powerful useful approaches in case of pandemic

117 infections by viruses and other pathogens, where an immediate response and the swift identification

118 of effective treatments are of paramount importance. Taken together, the collected evidence confirms

119 the potential of raloxifene as a promising agent with the potential to control COVID-19 infection with

120 pleiotropic mechanisms supporting the rationale for the ongoing clinical investigation (study

121 RLX0120, EudraCT Nr: 2020-003936-25) for the treatment of mild to moderate COVID-19 patients.

122

123 **Materials and methods**

124

125 **System Biology Screening**

126

127 First, we isolated 12 genes identified by The Host Genetic Initiative

128 (https://www.covid19hg.org/results/r3/) as relevant for the infection and present in the data release

129 number 3 (July 2020), namely: ANKRD32, CDRT4, PSMD13, ERO1L, LZTFL1, XCR1, FYCO1,

130 IFNAR2, CXCR6, CCR9, AP000295.9, AK5. Based on a lookup of previous GWAS results in the

131 GWAS ATLAS database (a database of publicly available GWAS summary statistics), these genes

132 are considered primarily implicated in immunological phenotypes. Then, we looked at the human-

4

133   SARS-CoV-2 interactome network as published [66], and extracted all the human genes included in

134   the set. A list combining the two dataset was used as seeding for a BioGrid search by mean of

135   Cytoscape v.3.8.0; the resulting enriched functional network connected those human proteins, known

136   to directly bind SARS-CoV-2 proteins, with the human gene products involved in the host pathology.

137   Subsequently, we screened 8721 Scopus-derived documents, referred to raloxifene, for the presence

138   of at least one of the proteins/genes included in the Cytoscape-generated network; this allowed to

139   isolate 600 papers, which were manually examined and annotated for enriched human gene ontology

140   according to BiNGO v.3.5.0.

141

142   **Cells**

143

144   African green monkey kidney Vero E6 cell line was obtained from American Type Culture Collection

145   (ATCC, Manassas, VA, USA) and maintained in Dulbecco's Modified Eagle Medium (DMEM;

146   Gibco, Thermo-Fisher, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS;

147   Gibco, Thermo-Fisher) at 37°C in a humidified atmosphere of 5% CO_2.

148   Calu-3 (human, Caucasian, lung, adenocarcinoma) cell line was obtained from ATCC and maintained

149   in Minimum Essential Medium (MEM; Gibco, Thermo-Fisher) supplemented with 10% fetal bovine

150   serum (FBS; Gibco, Thermo-Fisher) at 37°C in a humidified atmosphere of 5% CO_2.

151

152   **Virus**

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154   Different SARS-CoV-2 variants isolated from COVID-19 patients' respiratory samples were used.

155   The identity of each variant was verified by metagenomic sequencing. Genomic data of SARS-CoV-

156   2, belonging to the B.1 lineage, are available at EBI (under study accession number: PRJEB38101)

157   [67, 68].

158   Below the list of the viral strains used to assess the activity of raloxifene against viral variants:

159   -   Human 2019-nCoV strain 2019-nCoV/Italy-INMI1, clade V (Ref-SKU: 008V-03893, EVAg

160   portal), and isolated in January 2020 from a chinese patient (control infection) (named Wuhan)

161   -   SARS-CoV-2 isolate SARS-CoV-2/Human/ITA/PAVIA10734/2020, clade G, D614G (S)

162   (Ref-SKU: 008V-04005, EVAg portal), named D614G, isolated in Lombardy in February

163   2020

164   -   SARS-CoV-2 isolate hCoV-19/Italy/LAZ-INMI-82isl/2020, clade GV, A222V, D614G (S)

165   (Ref-SKU: 008V-04048, EVAg portal), named GV and representig the dominant strain

166   circulating in Europe from April to December 2020.

167   -   SARS-CoV-2 variant VOC 202012/01, isolate hCoV-19/Italy/CAM-INMI-118isl/2020, clade

168   GR, Δ69-70, Δ144, N501Y, A570D, D614G, P681H, T716I (S) (Ref-SKU: 008V-04050,

169   EVAg portal), named VOC B.1.1.7 and representing the variant of major concern from UK

170   -   SARS-CoV-2 variant GR/501Y.V3, isolate hCoV-19/Italy/LAZ-INMI-216isl/2021, clade

171   GR, PANGO lineage P.1, K417T, E484K, N501Y (S) (Ref-SKU: 008V-04101, EVAg portal),

172   named VOC P1 representing the variant of major concern from Brazil

173   -   SARS-CoV-2 variant VOC SA/B.1.351, obtained by GHSAG (Public Health England),

174   named B.1.351 and representing the variant of major concern from South Africa

175   -   SARS-CoV-2 variant VOC G Delta /B1.617.2 isolate hCoV-19/Italy/LAZ-INMI-648/2021

176   (EPI_ISL_2000624) , named VOC B.1.617.2 representing the variant of major concern from

177   India

178 All the infection experiments were performed in a biosafety level-3 (BLS-3) laboratory at a

179 multiplicity of infection (MOI) of 0.05.

180

181 **Cell viability studies of raloxifene**

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183 Cells were seeded into 24-well plates (2.5×10^4 cells/well) in DMEM supplemented with 10% FBS,

184 and treated with different doses of raloxifene (1.25, 2.5, 5, 10, 15, 20, 25 and 30 µM) at 37°C for 48

185 h. Cell viability was estimated by measuring the ATP levels using CellTiter-Glo (Promega, Madison,

186 WI, USA).

187

188 **Evaluation of antiviral efficacy of raloxifene**

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190 Cells were infected at 37°C for 1 h with the SARS-CoV-2 isolate at a MOI of 0.05. Infection was

191 carried out in DMEM without FBS. Then, the virus was removed and cells washed with warm

192 phosphate buffered saline (PBS) and cultured with medium containing 2% FBS in the presence or in

193 the absence of raloxifene. The compound was used at the concentration of 1.25, 2.5, 5, 10 and 15

194 µM and both cells and supernatants were collected for further analysis 48 h post infection (p.i).

195

Plaque Assay

Cells were seeded at a density of 5×10^5 cells/well in a 12-well plate and incubated at 37°C for 24 h. Supernatants from infected cells were serially diluted in DMEM without FBS and added to the cells. After 1 h incubation, media were removed and cells washed with warm PBS. Then cells were covered with an overlay consisting of DMEM with 0.4% SeaPlaque (Lonza, Basel, Switzerland). The plates were further incubated at 37°C for 48 h. Cells were fixed with 10% formaldehyde at room temperature for 3 h. Formaldehyde was aspirated and the agarose overlay was removed. Cells were then stained with crystal violet (1% CV w/v in a 20% ethanol solution), and viral titer (PFU/mL) of SARS-CoV-2 was determined by counting the number of plaques.

Viral RNA extraction and quantitative real-time RT-PCR (qRT-PCR)

RNA was extracted from clarified cell culture supernatants (16,000 g x 10 min) and from infected cells using QIAamp Viral RNA Mini Kit and RNeasy Plus mini kit (Qiagen, Hilden, Germany), respectively, according to the manufacturer's instructions.

RNA was eluted in 30 µl of RNase-free water and stored at -80 °C till use. The qRT-PCR was carried-out following previously described procedures with minor modifications [69]. Briefly, reverse transcription and amplification of the S gene were performed using the one-step QuantiFast Sybr Green RT-PCR mix (Qiagen) as follows: 50 ºC for 10 min, 95 ºC for 5 min; 95 ºC for 10 sec, 60 ºC for 30 sec (40 cycles) (primers: RBD-qF1: 5'-CAATGGTTTAACAGGCACAGG-3' and RBD-qR1: 5'-CTCAAGTGTCTGTGGATCACG-3). A standard curve was generated by determination of copy numbers derived from serial dilutions (10^3-10^9 copies) of a pGEM T-easy vector (Promega, Madison, WI, USA) containing the receptor binding domain of the S gene (primers: RBD-F: 5'-GCTGGATCCCCTAATATTACAAACTTGTGCC-3'; RBD-R: 5'-TGCCTCGAGCTCAAGTGTCTGTGGATCAC-3').

Western blot analysis

Western blot was carried-out following previously described procedures with minor modifications [70]. Protein samples (30 µg) obtained from lysis in RIPA buffer (Cell Signaling Technology, Danvers, MA, USA) of infected cells were separated on 10% SDS-PAGE and then transferred onto

7

228 polyvinylidene difluoride (PVDF) membranes (Millipore, Sigma, Burlington, MA, USA). After

229 being blocked with 3% BSA in TBS buffer containing 0.05% Tween 20, the blot was probed with a

230 human serum (1:1000 dilution) containing IgG to the SARS-CoV-2 nucleoprotein (NP) and with

231 mouse anti-human GAPDH monoclonal antibody (G-9: Santa Cruz Biotechnology, Dallas, TX,

232 USA). The antigen-antibody complexes were detected using peroxidase-conjugated goat anti-human

233 or goat anti-mouse IgG (Sigma) and revealed using the enhanced chemiluminescence (ECL) system

234 (Santa Cruz Biotechnology).

235

236 **Evaluation of antiviral efficacy of raloxifene on SARS-CoV2 variants**

237

238 Vero E6 cells were infected at 37°C for 1 h with the SARS-CoV-2 strains indicated in the Virus

239 section at a MOI of 0.05 in 96 well plates. Infection was carried out in MEM (Sigma) without FBS

240 (Gibco). Then, the virus was removed and cells washed with warm phosphate buffered saline (PBS)

241 and cultured with medium containing 2% FBS in the presence or absence of raloxifene at different

242 doses (0.23, 0.47, 0.94, 1.88, 3.75, 7.5, 15 μM) at 37°C and 5% CO2 up to 72 h. To determine antiviral

243 efficacy of raloxifene, cell viability and viral induced CPE were mesured in not infected and infected

244 cells treated with serial dilution of the drug, staining the cells with a solution of Crystal Violet

245 (Diapath) and 2% formaldehyde. After 30 min, the fixing solution was removed by washing with tap

246 water, and cell viability was measured by a photometer at 595 nm (Synergy™ HTX Multi-Mode

247 Microplate Reader, Biotek, Winooski, VT, USA).

248 The percentage of viable cells for each condition was calculated compared to infected-not-treated (set

249 as 0%) and not-infected-not-treated cells (set as 100%). The effect of raloxifene on cell viability was

250 also checked by crystal violet/ 2% formaldheyde staining in each experiment performed for SARS-

251 CoV-2 variants study.

252

253 **Data analysis**

254

255 The half-cytotoxic concentration (CC_{50}) and the half-maximal inhibitory concentration (IC_{50}) for

256 raloxifene were calculated from concentration-effect-curves after non-linear regression analysis using

257 GraphPad Prism8. The selectivity index (SI) for raloxifene was calculated as the ratio of CC_{50} over

258 IC_{50} [71].

259

260 **Statistical analysis**

261

262 Data for the *in vitro* experiments performed were analyzed for statistical significance using the 1-way

263 ANOVA, and the Bonferroni post-test was used to compare data. Differences were considered

264 significant when $p < 0.05$. Statistical tests were performed using GraphPad Prism 8.

265

266 **Results**

267

268 *In vitro effects of raloxifene on metabolism and SARS-CoV-2 infection in different cell lines*

269 At first, a standard assay was carried out to measure the activity of raloxifene on cell metabolism. To

270 this end, Vero E6 cells were cultured for 48 h in the absence or presence of different drug

271 concentrations (range from 1.25 µM to 30 µM). As shown in Figure 1A, raloxifene-treated Vero E6

272 cells showed a normal surface-adherent phenotype until the concentration of 15 µM. A drug-

273 dependent cytopathic effect was evident at concentration of 20 µM, involving the entire monolayer

274 at a concentration of 25 µM and 30 µM. At the same time, raloxifene shows a slight effect on the

275 extent of cellular ATP accumulation at a concentration ranging from 1.25 µM to 15 µM (87% and

276 70%, respectively). At higher doses, raloxifene showed a dose-dependent effect on ATP

277 accumulation, reaching 56%, 35% and 0.6% at 20 µM, 25 µM and 30 µM (Figure 1B). The CC_{50} of

278 raloxifene in Vero E6 cells was determined to be 18.4 µM.

279 Next, to assess the antiviral activity of raloxifene, Vero E6 cells were infected with SARS-CoV-2

280 (B.1 lineage) at a MOI of 0.05 [67]. Specifically, Vero E6 were infected with SARS-CoV-2, and 1 h

281 later cultured in the absence or presence of different raloxifene concentrations (range from 1.25 µM

282 to 15 µM). Raloxifene efficiently inhibits viral replication. In particular, viral genome copy numbers

283 evaluated on supernatants collected at 48 h p.i. by qRT-PCR, showed a significant reduction of the

284 virus yield already at 2.5 µM raloxifene concentration (2.9-fold reduction), with a maximal reduction

285 at 15 µM (1400-fold reduction) (Figure 1C). Raloxifene also displayed a dose-dependent inhibition

286 of viral replication in Vero E6 cells, as determined by infectious viral titers, exhibiting a 70%

287 reduction of viral titer at a concentration of 5 µM, with 94% to 100% inhibition at 10 µM and 15 µM,

288 respectively (Figure 1D). Raloxifene efficacy was then confirmed at intracellular level. Quantification

289 of viral RNA in SARS-CoV-2-infected cells showed a significant reduction of intracellular SARS-

290 CoV-2 genome copy number already at 10 µM and a 99-fold reduction at 15 µM (Figure 1E).

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291   Accordingly, western blot (WB) analysis showed a dose-dependent inhibition of SARS-CoV-2 upon

292   raloxifene treatment with 43% reduction of NP viral protein expression at a concentration of 5 µM,

293   with 65% and 97% reduction at 10 µM and 15 µM, respectively (Figure 1F). Raloxifene IC_{50} value

294   was calculated to be 3.3 µM. SI was then calculated and found to be 5.6. We then tested the raloxifene

295   activity on Calu-3 cells as a model of human pulmonary cell line. The CC_{50} value was determined, as

296   above, and found to be 24.4 µM (Figure 2A). Next, Calu-3 cells were infected as described above.

297   Supernatants were collected at 48 h p.i., and tested for viral genome copy numbers by qRT-PCR. As

298   shown in Figure 2B, the treatment significantly reduced the virus yield. In particular, raloxifene

299   displayed a dose-dependent inhibition of viral replication, as determined by infectious viral titers,

300   exhibiting a 67% reduction of viral titer at a concentration as low as 10 µM, with 96% and 98%

301   inhibition at drug concentrations of 15 µM and 25 µM, respectively. The efficacy of the treatment

302   was confirmed at intracellular level by qRT-PCR and WB on NP (Figures 2C-E). The IC_{50} was

303   calculated and found to be 9 µM. SI was then calculated and found to be 2.7.

304
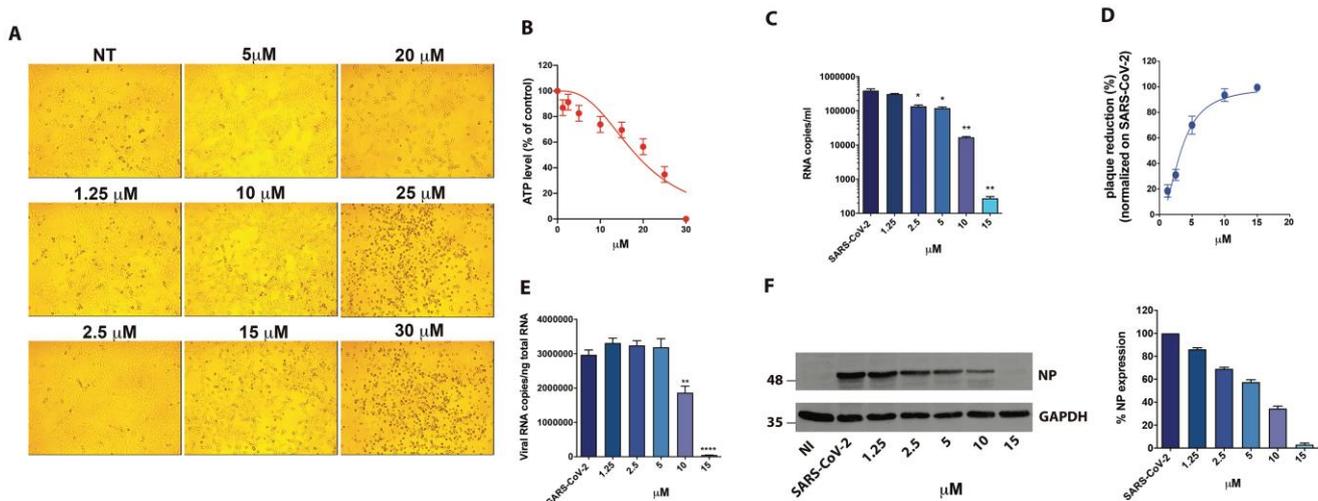
305   **Figure 1.** *Effect of Raloxifene on Vero E6 cells.* Vero E6 cells were cultured for 48 h in the absence or in the presence

306   of raloxifene at different concentrations. (**A**) 10× bright-field images of Vero E6 cells after incubation for 48 h at 37°C

307   with the indicated raloxifene concentrations. (**B**) CellTiter-Glo was used to measure the antimetabolic effect of raloxifene.

308   (**C-F**) Vero E6 cells were infected with SARS-CoV-2 and cultured in the absence or in the presence of different doses of

309   raloxifene. (**C**) Viral yield in cell supernatants was quantitated by qRT-PCR. (**D**) Viral titer in cell supernatants was

310   evaluated by plaque assay and plotted as percentage of plaque reduction compared to SARS-CoV-2. (**E**) Quantitation of

311   SARS-CoV-2 genomes at the intracellular level by qRT-PCR. (**F**) NP expression in infected cells was analyzed by western

312   blot (left panel). Densitometric analysis of western blot is shown in the right panel. Graph represents the percentage of

313   NP expression. Data are representative of two independent experiments with similar results. All the experiments were

314 performed at least in three independent replicates and pictures shown are representative. Data are presented as the mean

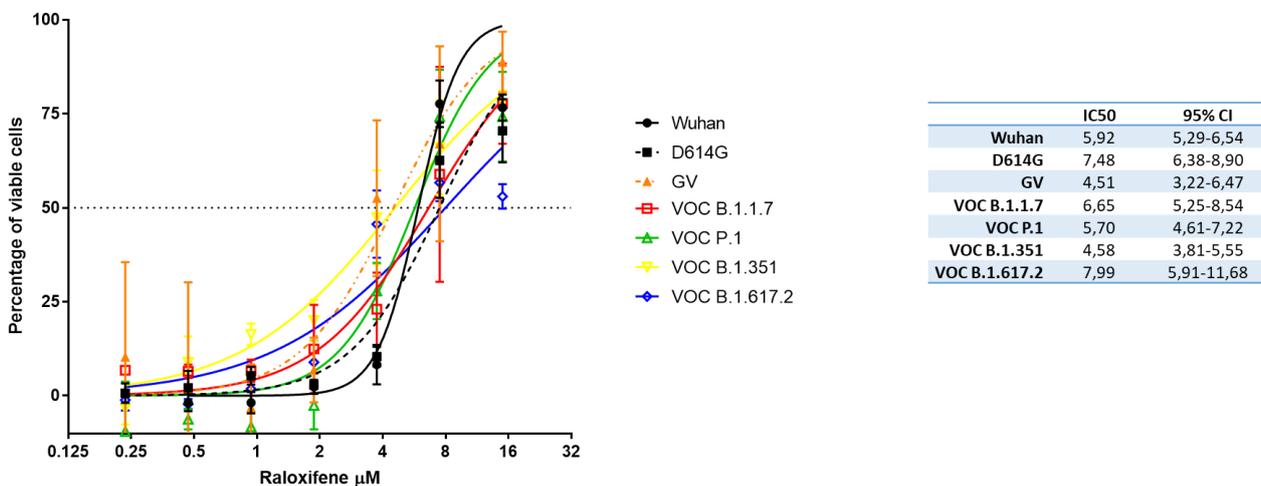315 $\pm$ standard error of the mean *, P < 0.05; **, P < 0.01; ****, P < 0.0001.
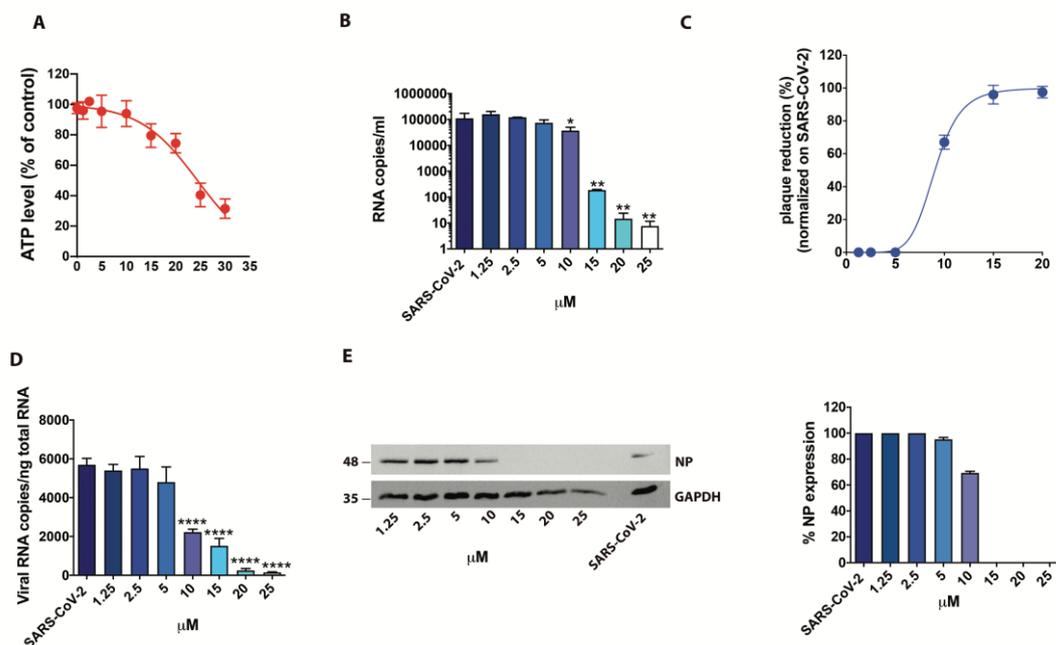
316

317 **Figure 2.** *Effect of Raloxifene on Calu-3 cells.* (**A**) Calu-3 cells were cultured for 48 h in absence or in the presence of

318 raloxifene at different concentrations. CellTiter-Glo was used to measure, the antimetabolic effect of raloxifene. (**B-E**)

319 Cells were infected with SARS-CoV-2 and cultured in the absence or in the presence of different doses of raloxifene. (**B**)

320 Viral yield in cell supernatants was quantitated by qRT-PCR. (**C**) Viral titer in cell supernatants was evaluated by plaque

321 assay and plotted as percentage of plaque reduction compared to SARS-CoV-2. (**D**) Quantitation of SARS-CoV-2

322 genomes at the intracellular level by qRT-PCR. (**E**) Nucleocapsid (NP) protein expression in infected cells was analyzed

323 by western blot (left panel). Densitometric analyses of western blot results are shown. Graph represents the percentage of

324 NP protein expression. Data are representative of two independent experiments with similar results. All the experiments

325 were performed at least in three independent replicates and pictures shown are representative. Data are presented as the

326 mean + standard error of the mean *, P < 0.05; **, P < 0.01; ****, P < 0.0001.

327

328 *Raloxifene exerts antiviral activity on SARS-CoV-2 variants*

329

330 We then performed a systematic study of the antiviral efficacy of raloxifene on the most common

331 variants on Vero E6 cells (Figure 3). Different viral strains were used: the wild type isolated in

332 January 2020 from Chinese patient (named Wuhan), two different isolates for the D614G spike

333 variants representing the dominant strains circulating in Europe from April to December 2020 (named

11

334 GV and D614G) and the variants of major concern originated in UK, Brazil, South Africa and India
335 (named VOC B1.1.7, VOC P.1, VOC B1.351, and VOC B1.617.2, respectively). We first determined
336 the time-window in which CPE appeared for each variant. The CPE was evident at 48h for all the
337 tested strains but VOC B1.1.7 and VOC P.1 variant, for which evident CPE appeared later (56h and
338 72h, respectively). In parallel, uninfected cells were cultured in the presence of different doses of
339 raloxifene to evaluate possible cytotoxicity due to the treatment. In cells treated with drug at 15 μM,
340 we observed a reduced percentage of viable cells as revealed by crystal violet staining (82.9 +/-8.69%
341 and 76.3+/-5.84%, at 48 and 72h respectively, compared to 100% in untreated cells). No significant
342 effect on cell viability with lower dug concentrations (7.5 to 0.23 μM) was observed. To determine
343 antiviral efficacy of the drug, CPE was measured in infected cells treated with seven two-fold serial
344 dilutions of raloxifene (15 to 0.23 μM) using the time windows identified for each strain. The drug
345 was able to recover cell viability in Vero E6 cells infected with all the tested viral strains. The half-
346 maximal inhibitory concentration (IC$_{50}$) calculated on recovering of cell viability varied from 4.50 to
347 7.99 μM depending on the strain (Figure 3), showing a strong antiviral activity against all the variants
348 under investigation.

349

	IC50	95% CI
Wuhan	5,92	5,29-6,54
D614G	7,48	6,38-8,90
GV	4,51	3,22-6,47
VOC B.1.1.7	6,65	5,25-8,54
VOC P.1	5,70	4,61-7,22
VOC B.1.351	4,58	3,81-5,55
VOC B.1.617.2	7,99	5,91-11,68

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351 **Figure 3. Raloxifene reduces the cytopathic effect (CPE) induced by SARS-CoV-2 variants in Vero E6 cells.** The
352 graph shows the inhibition of CPE observed at different concentration of raloxifene. The IC50 calculated by non linear
353 regression are shown in the table. Percentage of viable cells calculated on not treated not infected = 100%; not treated
354 SARS-CoV-2 infected cells= 0%. Bars indicate SD.

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358 *System Biology screening to investigate poly-pharmacological effects of raloxifene against SARS-*

359 *CoV-2 infection*

360 Besides being able to directly bind viral proteins such as Spike, raloxifene may exert a range of other

361 modulatory effects on the infection by SARS-CoV-2. In a recent paper we reviewed the possible links

362 between ER modulation and host response to viral infections against different viruses, suggesting a

363 therapeutic potential for SERMs in the control of COVID-19 infection [38]. Aiming to strengthen the

364 above hypothesis we built a molecular network connecting the human-virus interactome and those

365 proteins known to be involved in the COVID-19 pathogenesis, as described in the Materials &

366 methods section. The resulting network was in turn used to generate a list of proteins; each member

367 of this list was used as a probe to screen all the papers on raloxifene nominating the proteins relevant

368 for the SARS-CoV-2 infection. In this way, three functional groups of human genes involved in the

369 biology of the viral infection and potentially modulated by raloxifene were identified:

370 1. A group of genes including those modulated by the raloxifene molecular target, specifically

371   ESR2, and connected to inflammation;

372 2. A group of genes including those expressed in the lungs which are modulated by the raloxifene

373   molecular target, specifically ESR; when deregulated, the corresponding genes cause severe

374   asthma, in agreement with the enrichment in this group of genes whose unfavorable variants cause

375   worse respiratory consequences, according to GWAS studies;

376 3. A group of genes directly modulated by the virus, both during the cell entry phase and the

377   replication phase, which also include proteins upstream or downstream of some raloxifene-

378   controlled pathways.

379 Taken all together, these results suggest a potential poly-pharmacological effect of raloxifene in

380 COVID-19 as anti-inflammatory, respiratory and antiviral.

381 **Effect on inflammation**: Looking at the genes identified as linked to the promotion of inflammation

382 by the virus, it is relevant to keep in mind that one of the clinically validated targets of the anti

383 COVID-19 therapy is the cytokine Interleukin-6 (IL-6) [72]. A downregulation of the inflammatory

384 signal and of the IL-6 expression was found with raloxifene in a clinical setting [73]. Besides IL-6,

385 other serum cytokines (i.e. TNF-alpha and TGF-beta1) involved in the cytokines storm due to SARS-

386 CoV-2 are regulated by raloxifene.

387 **Effect on respiratory response:** Genes regulating the production of nitric oxide are implicated in

388 the vascular and respiratory response to the viral infection. Treatment of rats with raloxifene was

389 shown to upregulate the expression of eNOS (NOS3) in rat thoracic aorta, after complexing with the

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390 ESR_2 expressed in lungs. This is expected to exert a potentially important vasculo-protective effect,

391 and eventually to contribute to clinical improvements in ARDS and pulmonary hypertension [74].

392 Other compounds, e.g. Rho kinase (ROK) inhibitors, phosphodiesterase-5 inhibitors [75], omentin

393 [76] were also shown to activate the eNOS (NOS3) pathway, with protective effect for ARDS and

394 related inflammation in experimental models. In ARDS patients, the effects of inhaled nitric oxide on

395 the reduction of pulmonary blood pressure and on the improvement of oxygenation, offered the

396 rationale for a clinical trial in severe COVID-19 patients (NCT04388683).

397 **Effect on antiviral action**: Consistently with the identification of the genes from the GWAS study,

398 a direct antiviral action of raloxifene, in terms of inhibition of viral replication and/or infection, was

399 found in several different contexts like in vitro against EbolaVirus [58, 59, 77], against HBV [62],

400 and against HCV [60]; in human female cells from nasal epithelium against the Influenza Virus A

401 [64], and also in a randomized clinical trial on 123 postmenopausal women, against HCV [65].

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404 **Discussion**

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406 Raloxifene, a second generation SERM, was previously proposed as a potential candidate for the

407 treatment of COVID-19 patients due to the *in silico* predicted possibility to interfere with the viral

408 replication and disease progression with multiple mechanisms of action both ER dependent and

409 independent [38].

410 In this paper we report for the first time the *in vitro* characterization of the antiviral activity of

411 raloxifene against SARS-CoV-2 infection using two relevant experimental systems, Vero E6 monkey

412 kidney cells and human pulmonary Calu-3 cells. SARS-CoV-2 infected monkey Vero E6 cells are

413 commonly used to study coronavirus infection as they support viral replication to high titres and

414 highly express ACE-2 receptor [78-82] that plays an essential role for SARS-CoV-2 entry into the

415 cells [83]. SARS-CoV-2 infected human Calu-3 cells are a relevant and predictive model because of

416 airway epithelial origin [84]. In both assays the results confirmed that raloxifene blocks with high

417 efficiency SARS-CoV-2 replication.

418 The characterization was completed testing raloxifene also against all the most common circulating

419 SARS-CoV-2 variants of clinical relevance, confirming that it maintains a high and consistent

420 activity, thus reinforcing the interest on its potential clinical use as antiviral agent in COVID-19

421 patients.

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422 Raloxifene cytotoxicity was assessed with two independent assays: the first measuring the activity of
423 the compound on cell replication, the second on cellular metabolism. With both approaches we found
424 that the CC50 was attested at high micromolar range, which is far from the low micromolar range in
425 which the antiviral activity was observed. The selectivity index (SI) of the drug was superimposable
426 in the two experimental models in the range of 2 to 7. In general, the value of SI for a drug with direct
427 antiviral activity is greater than 1; the higher the SI value, the more effective and safer the drug is.
428 Some authors [85-87] report a limit value of SI = 4 to define a compound as a good compound with
429 direct antiviral activity. The SI value found in the models is therefore indicative of a molecule with a
430 significant antiviral activity and with an activity/toxicity profile consistent with a possible translation
431 to human clinical trials. In addition, raloxifene is a drug that has been used for a long time, and its
432 safety profile is supported by a huge volume of clinical data from long term treatments [88-90]. The
433 occurrence of thromboembolic events, even though rare, in patients treated with raloxifene has to be
434 regarded with particular caution due to the high risk of thromboembolic manifestations in COVID
435 patients. A short duration of treatment and the careful avoidance to treat patients with concomitant
436 risks of thromboembolic events are recommended.

437 Among SERMs raloxifene has a unique risk/benefit profile built on a large safety database not limited
438 to oncological patients, like for other SERMs, but on a large use in postmenopausal women for the
439 management of osteoporosis, including men treated for a variety of indications. The potential of
440 SERMs, and in particular of raloxifene, found a promising confirmation in a recent retrospective
441 study on a large population of cancer patients that demonstrated a protective effect on SARS-CoV-2
442 infection and a significant reduction of severity and duration in the subpopulation of patients treated
443 with raloxifene [91].

444 A system biology study was also conducted with the aim to match the available information on gene
445 and pathways regulated by raloxifene against a Cytoskape-generated human SARS-CoV-2
446 interactome network. The results of the study strongly support the concept that raloxifene may
447 positively influence the course of SARS-COV-2 infection by modulating three functional groups of
448 human genes, all of them playing a key role in the biology of the viral infection. The systematic data
449 analysis went further, confirming the putative antiviral activity, and also highlighting the potential of
450 raloxifene to exert both an antinflammatory action by downregulating the expression of key mediators
451 of the cytokine storm, and a vasculo-protective effect by upregulating eNOS expression (NOS3) [74].
452 These findings on one hand are in agreement with previous papers highlighting the protective effect
453 of estrogen signalling in the context of COVID-19 infection, and on the other hand confirm the

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454 specific characteristics of raloxifene as an ideal candidate to put to a test the hypothesis in the clinics

455 due to ist peculiar mechanism within the class of SERMs and its potential ability to exert a pleiotropic

456 effect by targeting viral and host targets with a key role in the disease progression and exacerbation.

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