Abstract

The current emergency of the novel coronavirus SARS-CoV2 urged the need for broad-spectrum antiviral drugs as the first line of treatment. Coronavirus are a large family of viruses that already challenged humanity in at least two other previous outbreaks and are likely to be a constant threat for the future. In this work we developed a pipeline based on in silico docking of known drugs on SARS-CoV1/2 RNA-dependent RNA polymerase combined with in vitro antiviral assays on both SARS-CoV2 and the common cold human coronavirus HCoV-OC43. Results showed that certain drugs displayed activity for both viruses at a similar inhibitory concentration, while others were specific. In particular, the antipsychotic drug lurasidone and the antiviral drug elbasvir showed promising activity in the low micromolar range against both viruses with good selectivity index.

1. Introduction

The growth in human and animal population density through urbanization and agricultural development, combined with increased mobility and commercial transportation, land perturbation and climate change, all have an impact on virus emergence and epidemiology. Over the past decades, emerging zoonotic RNA viruses continuously gripped the world’s attention, either briefly (like the severe acute respiratory syndrome HCoV-HKU1); the latter includes SARS-CoV1 and the Middle East respiratory syndrome coronavirus (MERS-CoV). A new previously unknown coronavirus, named SARS-CoV2, was discovered in December 2019 in Wuhan (Hubei province of China) and sequenced by January 2020 (Lu et al., 2020). SARS-CoV2 is associated with an ongoing Public Health Emergency of International Concern (PHEIC, on January 30th, 2020).

CoVs consist of a large and diverse family of viruses that cause multiple respiratory, gastrointestinal and neurologic diseases of varying severity, including the common cold, bronchiolitis, and pneumonia (Weiss and Leibowitz, 2011). The CoV family is divided into four genera (alpha, beta, gamma, and delta) and thus far human CoV are limited to the alpha (HCoV-229E and HCoV-NL63) and beta genera (HCoV-OC43, HCoV-HKU1); the latter includes SARS-CoV1 and the Middle East respiratory syndrome coronavirus (MERS-CoV). A previously unknown coronavirus, named SARS-CoV2, was discovered in December 2019 in Wuhan (Hubei province of China) and sequenced by January 2020 (Lu et al., 2020). SARS-CoV2 is associated with an ongoing outbreak of atypical pneumonia (COVID-19), and was declared as 'Public Health Emergency of International Concern' on January 30th, 2020.

Research paper

Combined in silico and in vitro approaches identified the antipsychotic drug lurasidone and the antiviral drug elbasvir as SARS-CoV2 and HCoV-OC43 inhibitors

Mario Milani a,b,1, Manuela Donalisio c,1, Rafaela Milan Bonotto d, Edoardo Schneider e, Irene Arduino c, Francesco Boni a,b, David Lembo c, Alessandro Marcello d,*, Eloise Mastrangelo a,b,***

a CNR-IBF, Istituto di Biofisica, Via Celoria 26, I-20133, Milano, Italy
b Dipartimento di Bioscienze, Università di Milano, Via Celoria 26, I-20133, Milano, Italy
 c Dipartimento di Scienze Cliniche e Biologiche, Università di Torino, Regione Gonzole, 10, I-10043, Orbassano, Turin, Italy
 d Laboratory of Molecular Virology, International Centre for Genetic Engineering and Biotechnology, Padriciano 99, I-34149, Trieste, Italy
 e High Throughput Screening Facility of the International Centre for Genetic Engineering and Biotechnology, Padriciano 99, I-34149, Trieste, Italy

** Corresponding author. International Centre for Genetic Engineering and Biotechnology, Padriciano 99, 34149, Trieste, Italy.
*** High Throughput Screening Facility of the International Centre for Genetic Engineering and Biotechnology, Padriciano 99, I-34149, Trieste, Italy.
2020 by the World Health Organization (www.who.int).

Currently, for the COVID19 outbreak, many known drugs are under clinical investigation (Kupferschmidt, 2020) (Magro, 2020), following different general principles and mechanisms of action: 1. the control of cytokine storms due to the hyper-reaction of the immune system against the virus (e.g. corticosteroids (Salton et al., 2020)); 2. the control of coagulopathy (e.g. heparin) 3. the inhibition of viral RNA dependent RNA polymerase (RdRp; e.g. produgs favipiravir and remdesivir); 4. the inhibition of viral entry (e.g. hydroxychloroquine); 5. the inhibition of the viral main protease (e.g. lopinavir and ritonavir); 6. the inhibition of viral attachment (the viral receptor ACE2 antagonist losartan).

Despite their species diversity, CoVs share key genomic elements that are essential for viral replication, suggesting the possibility to design broad spectrum therapeutic agents to address the current epidemic and manage possible future outbreaks. The target considered in this work to identify new inhibitors is the highly conserved RdRp, that plays a crucial role in CoV replication cycle, catalyzing the synthesis of new viral RNA (Te Velthuis et al., 2012). The cryo-EM structure of SARS-CoV1 and SARS-CoV2 RdRp, bound to nsp7 and nsp8 co-factors, have been recently solved (PDB-id: 6NUR (Kirchdoerfer and Ward, 2019), and 6M71 (Gao et al., 2020), respectively). The two proteins share a sequence identity of 96% (98% conservative substitution) and a structural r.m.s.d. of 0.54 Å (considering 788 C).

The exploration of libraries of molecules already in use as human drugs and well characterized in terms of human metabolism might allow the identification of antivirals that could be, in principle, rapidly tested in patients. Accordingly, we chose to analyze in silico the public database of approved/investigational drugs (DrugBank library, https://go.drugbank.com/), targeting a wide region around the active site of SARS-CoV1 RdRp. The computational work allowed the selection of 13 commercially available compounds with predicted high affinity for the protein and favorable solubility properties. These potential inhibitors (together with suramin, known to inhibit several RNA viruses) have been tested in cell-based assays against SARS-CoV2 and HCoV-Oc43 (Su et al., 2016), revealing moderate to high antiviral activities for seven of them. Our results confirm antiviral properties already described for some of the selected compounds, and, more importantly, show new interesting properties for the compounds lurasidone and elbasvir as beta-CoV inhibitors.

2. Materials and Methods

2.1. In silico docking

The virtual Library of DrugBank (https://go.drugbank.com/) employed for the docking analysis includes FDA-approved drugs as well as experimental drugs going through the FDA approval process. Starting from the 2D sdf structures of the library (7180 molecules), we filtered out all the molecules with Mw ≥ 900 Da (keeping 7025 molecules) and then added explicit hydrogens (at pH 7.4) with the program Open Babel (O’Boyle et al., 2011). Next, we used the program Molconvert (https://chemaxon.com/) to obtain a low energy 3D conformer for most of the molecules (6996 compounds) that were finally transformed into the AutoDock4 pdbqt format (adding charges and defining rotational freedom) with the AutoDockTools package (http://mgltools.scripps.edu/).

The atomic coordinates of SARS-CoV1 RdRp (PDB-id: 6NUR) bound to NS7 and NS8 co-factors, were chosen as docking model for CoV polymerase. Hydrogen atoms and Kollman charges (Singh and Kollman, 1984) were added using the program Python Molecule Viewer 1.5.4 (MGL-tools package http://mgltools.scripps.edu/). The protein model was then used to build a discrete grid within a box of dimensions 22.5 × 26.3 × 22.5 Å³ (program AutoGrid (Goodford, 1985)) as the explored volume for both the AutoDock4.2 and AutoDock Vina searches. The grid was centered near the side chain of Lys545, to include a wide region around the protein active site. During the computational analysis, the protein was constrained as rigid, whereas the small molecules were free to move. The in silico screen was divided into two runs: a fast procedure using the program AutoDock Vina (Trott and Olson, 2009) for the selection of the best compounds, followed by a more accurate screen using the program AutoDock4.2 (Morris et al., 2009). The AutoDock Vina docking search (energy range = 4; numModes = 4; exhaustiveness = 10) produced a ranked list of all compounds, with predicted binding free energy values (ΔG) ranging between −0.9 kcal/mol and −8.9 kcal/mol. The best 118 compounds (~2% of the library, ΔG between −8.9 and −7.6 kcal/mol) were further analyzed using AutoDock4.2 (Morris et al., 2009), with 80 hybrid GA-LS genetic algorithm runs (ga_num_evals = 1750000, ga_pop_size = 150). Among the molecules with higher predicted affinity for RdRp (ΔG values varying between −4.67 and −11.7 kcal/mol), 13 FDA approved drugs were selected, taking into account commercial availability and solubility (as suggested by the theoretical logP values in the DrugBank library) properties, for in vitro assays. To confirm the binding of the 13 selected compounds to the recently released SARS-CoV2 RdRp we performed an additional in silico docking using as model the structure of Gao et al. (PDB-id: 6m71 (Gao et al., 2020)) with 300 hybrid GA-LS genetic algorithm runs. Furthermore, since among such drugs were present known inhibitors of viral protease we investigated in silico their binding affinity for CoV main protease (PDB-id: 6LU7 (Jin et al., 2020)). Briefly, we explored with AutoDock4.2 a region of 15 × 22.5 × 22.5 Å³ (after mutating the active site Cys145 to Ala) centered between the side chains of Asn142 and Gln189 to cover the whole cavity in front of the protease active site. We used the same docking procedure as described for the RdRp.

2.2. SARS-CoV2 cell based assays

2.2.1. Cell lines and viruses

Vero E6 cells (ATCC-1586), the human hepatocarcinoma HuH7 cells kindly provided by Ralf Bartenschlager (University of Heidelberg, Germany) and HuH 7 engineering by lentivirus transduction to overexpress the human ACE2 (HuH7-hACE2) were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS, Gibco). Working stocks of SARS-CoV2 ICGEB-FVG_5 isolated in Trieste, Italy, were routinely propagated and titrated on Vero E6 cells (Licastro et al., 2020).

2.2.2. Compounds preparation

Compounds were prepared in 2-fold serial dilutions (8 points dilutions) in DMSO, and then diluted 16x in PBS in an intermediate plate. Finally, compounds were transferred to the 96 well assay plate containing cells and virus medium (6x in grown medium, final dilution 100x).

2.2.3. High Content Assay

HuH-7-hACE2 cells were seeded in a 96 wells’ plate, at 8 × 10³ cells/well density and incubated at 37 °C overnight. Cells were treated with serial dilution of the compounds and then infected with SARS-CoV2 at 0.1 MOI. Controls included: positive controls like infected cells treated with 50 μM of Hydroxychloroquine as well as non-infected cells treated with vehicle (1% DMSO), and negative controls such as infected cells treated with vehicle. Plates were incubated for 20 h at 37 °C, and then fixed with 4% PFA (Paraformaldehyde) for 20 min at room temperature and washed twice with PBS 1X. Cells were treated with 0.1% of Triton-X for 15 min, followed incubation of 30 min in blocking buffer (PBS 1X containing 1% of bovine serum albumin-BSA). Then, a primary recombinant monoclonal Spike antibody (CR3022) was diluted in blocking buffer and incubate for 2 h at 37 °C (Rajasekharan et al., 2020). Cells were washed 2 times in PBS 1X and incubated with the secondary antibody AlexaFluor488-conjugated goat anti-mouse IgG (Cat No. A-11001, Thermo-Scientific) plus 4′,6-diamidino-2-phenylindole (DAPI) for 1 h at 37 °C. Each plate was washed twice with PBS 1X. All plates were filled up with 150 μl of PBS/well. Digital images were
acquired using a high content imaging system, the Operetta (PerkinElmer). The digital images were taken from 9 different fields of each well at 20× magnification. Total number of cells and the number of infected cells were analyzed using Columbus Image Data Storage and Analysis System (PerkinElmer).

2.2.4. Cytotoxicity assay
The cytotoxicity assay was conducted with Alamar Blue (Invitrogen) as recommended by the manufacturer’s protocol. Huh7-hACE2 cells were seeded at 8 × 10⁴ cells per well in a 96 well plate, and incubated at 37°C overnight. Then 50 μL of compound at the indicated concentrations were added to 150 μL of medium (final 200 μL). Plates were incubating at 37°C for 20 h and then the colorimetric reagent was added (20 μL for 8 h). Measurements from compound treated cells were normalized against those from untreated cells.

2.2.5. Virus yield reduction assay
Huh7-hACE2 were seeded into a 12-well plate 24 h prior infection. Cells were infected with SARS-CoV2 at MOI 0.1 and simultaneously treated in 2-fold serial dilutions of compound. After 1 h infection, cells were washed with PBS 1x, and incubated in medium containing 2% FBS and the indicated compounds. Cell culture supernatants were collected after 20 h, virus titers were determined in duplicate by a plaque assay in Vero E6 cells. Values of virus titer (Log pfu/ml) from each sample were calculated and reported. To obtained the % of inhibition, values of infected-treated samples were normalized with values of the not-treated infected samples control. The half maximal effective concentrations (EC₅₀) were calculate using GraphPad Prism Version 7.

2.2.6. Data normalization and analysis
Infection ration was defined as ratio between (i) the total number of infected cells, and (ii) the total number of cells. Data were normalized with the negative (DMSO-treated, infected cells) and positive (50 μM Hydroxychloroquine, treated infected cells) controls. Percentage inhibition was calculated based on infection ratio values with the formula: [(1-(infection ratio samples – Average (Av) infection ratio of positive control)/(Av. infection ratio of negative control – Av. infection ratio of positive control))x100. Percentage of nuclei was calculated from values of cell number with the formula: (Cell number test sample/Av. cell number of positive control) × 100. Values were plotted against dilutions expressed as antilog. The half maximal effective concentration (EC₅₀) and the half maximum cytotoxic concentration (CC₅₀) were calculated using GraphPad Prism Version 7.

2.3. HCoV-OC43 cell-based assays

2.3.1. Reagents
Dimethyl sulfoxide (DMSO) was purchased from Sigma-Aldrich (Saint Louis, MO). The mouse anti-coronavirus monoclonal antibody MAB9013 was purchased from Merck (Darmstadt, Germany). The secondary antibody peroxidase-conjugated AffiniPure F(ab')2 Fragment Goat Anti-Mouse IgG (H + L) was purchased from Jackson ImmunoResearch Laboratories Inc. (West Grove, PA, USA).

2.3.2. Cell lines and viruses
Human lung fibroblast cells MRC-5 (ATCC® CCL-171) were propagated in Dulbecco’s Modified Eagle Medium (DMEM; Sigma, St. Louis, MO, USA) supplemented with 1% (v/v) penicillin/streptomycin solution (Euroclone, Milan, Italy) and heat inactivated, 10% (v/v) fetal bovine serum (Sigma). Human coronavirus strain OC43 (HCoV-OC43) (ATCC® VR-1558) was purchased from ATCC (American Type Culture Collection, Rockville, MD, USA). The virus was propagated in MRC-5 cells at 33°C in a humidified 5% CO₂ atmosphere, and titrated by standard plaque method on MRC-5 cells, as described elsewhere (Marcello et al., 2020); titers were expressed in terms of plaque forming units per ml (PFU/ml).

2.3.3. Cell viability assay
Cell viability was measured using the MTS assay, as described elsewhere (Lembo et al., 2014). MRC-5 cells were seeded at a density of 2 × 10⁴ cells/well in 96-well plates and treated the next day with compounds at concentrations ranging from 1000 to 0.05 μM, under the same experimental conditions described for the antiviral assays. Treatment of control wells with equal volumes of DMSO was performed in order to rule out the possibility of any cytotoxic effect ascribable to the solvent. After 20 h of incubation, cell viability was determined using the Cell Titer 96 Proliferation Assay Kit (Promega, Madison, WI, USA) according to the manufacturer’s instructions. Absorbances were measured using a Microplate Reader (Model 680, Bio-Rad Laboratories, Hercules, CA, USA) at 490 nm. The effect on cell viability at different concentrations of compounds was expressed as a percentage, by comparing absorbances of treated cells with those of cells incubated with culture medium and equal volumes of DMSO. The 50% cytotoxic concentrations (CC₅₀) and standard deviation (SD) values were determined using GraphPad Prism 5.0 software (GraphPad Software, San Diego, CA).

2.3.4. Antiviral assay
The antiviral activity was determined by focus reduction assay. MRC-5 cells were seeded at 2 × 10⁴ cells/well density, in 96-well plates and incubated at 37°C overnight. The next day, the medium was removed from the plates and infection was performed with ca. 40 PFU of a stock of HCoV-OC43 (MOI 0.02 PFU/cells) in presence of serial dilutions of compounds, ranging from 100 to 0.005 μM. Control wells were infected in presence of equal volumes of DMSO. After 20 h of incubation at 33°C in a humidified 5% CO₂ atmosphere, cells were fixed with cold acetone-methanol (50:50) and subjected to indirect immunostaining by using an anti-coronavirus monoclonal antibody (MAB9013). The number of immunostained foci was counted, and the percent inhibition of virus infectivity was determined by comparing the number of foci in treated wells with the number in untreated control wells. The focus reduction assays were conducted in three independent experiments. Where possible, half-maximal antiviral effective concentration (EC₅₀) and SD values were calculated by regression analysis using the software GraphPad Prism 5.0 (GraphPad Software, San Diego, CA) by fitting a variable slope-sigmoidal dose–response curve.

2.3.5. Virus yield reduction assay
MRC5 cells were seeded in 24-well plates at a density of 2 × 10⁵ cells/well and grown overnight at 37°C. The next day, infection was performed with HCoV-OC43 at a MOI of 0.02 PFU/cells in the presence of serial dilutions of compound, ranging from 100 to 0.006 μM. Following adsorption at 33°C for 1 h, the virus inoculum was removed and cells were grown in presence of compound. Supernatants were harvested and pooled as appropriate 24 h after infection and cell-free virus infectivity titers were determined by focus reduction assay in MRC5 cell monolayers. The end-point of the assay was the effective concentration of compound that reduced virus yield by 50% (EC₅₀) compared to untreated virus controls.

2.4. Statistical analyses
All data were analyzed using GraphPad Prism (GraphPad Software, San Diego, CA). All results are presented as means ± standard deviations. The p value was calculated by comparing between % inhibition of infected-treated samples with % inhibition of control infected not-treated samples and one-tailed Student’s T-test was used to compare groups. Significance was reported for p-value < 0.05 (*), <0.01 (**) and <0.001 (***)
3. Results

3.1. In silico docking of approved drugs

For the purpose of known drugs repurposing, a total of 6996 molecules were analyzed from the DrugBank library (https://go.drugbank.com/) to target a wide region (−13,300 Å²) around the active site of SARS-CoV1 RdRp (PDB-id: 6NUR (Kirchdoerfer and Ward, 2019)). The in silico screening was divided into two runs: a fast procedure for the selection of the best 2% of the library (118 compounds), with predicted binding free energy values (ΔG) from −8.9 to −7.6 kcal/mol, followed by a more accurate analysis with AutoDock4.2. In this way we ranked the 118 known drugs based on the lower ΔG value among the 80 poses tested for every compound (between −11.7 kcal/mol (predicted Ki = 2.7 nM) and −4.67 kcal/mol (predicted Ki = 377.6 μM)). The list of the first best 60 compounds is reported in supplemental material (Table S1).

From our list, a reasonable number of compounds was selected for cell-based assays (Table 1), taking into account commercial availability and solubility properties. Such compounds were also submitted to an additional screening (with 300 hybrid GA-LS genetic algorithm runs) using the SARS-CoV2 RdRp structures (PDB-id: 6m71) and the scored Ki of number and of conformations clustered around the one with lower ΔG are reported on Table 1. Suramin was added to the list, since it was already known to inhibit several RNA viruses such as flavivirus (Basavarajacharya and Vasudevan, 2014) (Albulusecu et al., 2017), norovirus (Mastrangelo et al., 2012, 2014), but also chikungunya and Ebola viruses (Hené et al., 2016).

The best in silico docking pose of lurasidone and elbasvir, in the RdRp active site, is reported in Fig. 1A. The protein region explored is located between thumb, fingers and palm domains and would host growing dsRNA during polymerase activity (Fig. 1A). Such region defines a wide, complex and variable hydrophilic protein surface, and it is therefore able to host very different types of ligands. In Fig. 1B-E we report the details of the lurasidone and elbasvir best docking poses, between the thumb and fingers domains, and the schematic view of ligand-protein interactions, respectively.

Since, among the selected compounds, known inhibitors of viral proteases (like simeprevir and grazoprevir) were also present, we performed an additional in silico analysis targeting the active site of main protease (PDB-id: 6LU7 (Jin et al., 2020)) obtaining the results listed in Table 1. Eight of the selected compounds showed predicted binding affinity for the protease lower than 50 nM suggesting that such known drugs could be in principle active against multiple targets.

### 3.2. Antiviral activity against SARS-CoV2 and HCoV-OC43

The antiviral activity of the selected compounds was assessed against two pathogenic CoVs strains: SARS-CoV2 and HCoV-OC43 (Table 2 and 3, respectively).

#### 3.2.1. Antiviral activity against SARS-CoV2

A High Content Assay (HCA) has been developed to test antiviral drugs against SARS-CoV2 in vitro. Since a preliminary characterization showed sub-optimal infection of HuH7 cells, as also reported elsewhere (Ogando et al., 2020), we decided to engineer HuH7 cells carrying the human ACE2 receptor. The engineered HuH7-hACE2 cell line supports a level of SARS-CoV2 infection suitable for analysis. The assay on HuH7-hACE2 was based on immunofluorescence to quantify the number of infected Spike-positive cells and the number of nuclei to assess cell viability, as shown in Fig. 2.

The panel of compounds was tested in dose response, and the assay was validated using Hydroxychloroquine as reference compound. Results are reported in Table 2, Fig. 3 and Fig. S2.

All 14 compounds were tested from a starting concentration of 100 μM in 2-fold dilutions. 11 compounds showed activity at least in one tested concentration. Lurasidone and elbasvir showed the best outcomes, with EC50 in the micromolar range (18 μM and 23 μM, respectively) and cytotoxicity >1000 μM. Ponatinib and venetoclax reached the lowest EC50 (1.1 μM and 6.2 μM, respectively) against SARS-CoV2, although the elevated cytotoxicity (CC50 = 8.7 μM and 22.0 μM, respectively) indicated poor selectivity index for both compounds (calculated as the ratio of the CC50 and the EC50 values).

The known HCV inhibitors elbasvir, simeprevir and grazoprevir showed EC50 values of 23 μM, 9.3 μM and 16 μM, respectively. However, their activity as HCV inhibitors is in the low nanomolar range and simeprevir showed an unfavorable CC50 of 47.5 μM. The antiviral activity of suramin was confirmed, with an EC50 of 64 μM, similarly to previous reports (Salgado-Benvindo et al., 2020). A weak activity was detected with compounds irinotecan, teniposide and carbadoxalone, all with an EC50 around 50–100 μM. In conclusion, our data showed in vitro activity for most of the compounds selected in silico against SARS-CoV1/2 RdRp. Among all, lurasidone grazoprevir and elbasvir showed the best antiviral profile against SARS-CoV2 (Fig. 3).

Next, to validate the antiviral activity of grazoprevir, lurasidone, elbasvir these compounds were further analyzed by virus yield reduction assay, a stringent test that measures the ability of a compound to inhibit multiple cycles of viral replication and limit the production of infectious viral particles. Values of EC50 were obtained based on virus titration and normalized with the not-treated control. The three compounds confirmed their activity, showing EC50 values similar to those observed
in the high content screening. Grazoprevir demonstrated the highest reduction of virus titer, with a reduction of 3.9 logs at the highest concentration tested, while lurasidone and elbasvir showed a log reduction of 1.3 and 0.6 compared to the not-treated infected control, respectively (Table 4).

3.2.2. Antiviral activity against HCoV-OC43

In order to evaluate the anti-HCoV-OC43 activity of the selected compounds, focus reduction assays were performed on MRC-5 cells, as described in the Materials and Methods section and elsewhere (Marcello et al., 2020). Results on antiviral activity and cell toxicity are reported in Table 3. The figure was prepared using PyMOL (The PyMOL Molecular Graphics System, Version 2.0 Schrödinger, LLC).

### Table 2
HCA for the in silico selected compounds against SARS-CoV2.

| Compound     | EC_{50} \( (\mu M) \) (mean ± SD) | CC_{50} \( (\mu M) \) (mean ± SD) |
|--------------|----------------------------------|----------------------------------|
| Alectinib    | n.a.                             | n.a.                             |
| Tedizolid    | n.a.                             | n.a.                             |
| Ponatinib    | 1.1 ± 0.2                        | 8.7 ± 3.8                        |
| Carbenoxolone| 66 ± 11                           | >100                             |
| Lurasidone   | 18.0 ± 4.6                       | >1000                            |
| Gefapertin   | n.a.                             | >1000                            |
| Elbasvir     | 23.0 ± 3.6                       | >1000                            |
| Temiposide   | 97.0 ± 0.7                       | >1000                            |
| Venetoclax   | 6.2 ± 0.6                        | 22.0 ± 0.2                       |
| Irinotecan   | 85.2 ± 17.0                      | >1000                            |
| Simeprevir   | 9.3 ± 2.0                        | 47.5 ± 41.0                      |
| Grazoprevir  | 16.0 ± 5.7                       | 118 ± 6                          |
| Natamycin    | 24.3 ± 4.4                       | 35.9 ± 13.4                      |
| Suramin      | 64.0 ± 6.6                       | >100                             |

n.a. not assessable.

\( ^{a} \) Half maximal effective concentration.

\( ^{b} \) Standard deviation.

\( ^{c} \) Half maximal cytotoxic concentration.

### Table 3
Anti-HCoV-OC43 activity of the selected compounds.

| Compound     | EC_{50} \( (\mu M) \) (mean ± SD) | CC_{50} \( (\mu M) \) (mean ± SD) |
|--------------|----------------------------------|----------------------------------|
| Alectinib    | 0.6 ± 0.2                        | >1000                            |
| Tedizolid    | 94.0 ± 25                        | >1000                            |
| Ponatinib    | 0.10 ± 0.05                      | 3.1 ± 0.5                        |
| Carbenoxolone| 45.7 ± 8.0                       | 251 ± 17                         |
| Lurasidone   | 1.1 ± 0.4                        | >1000                            |
| Gefapertin   | n.a.                             | >1000                            |
| Elbasvir     | 1.5 ± 0.5                        | >1000                            |
| Temiposide   | n.a.                             | 701 ± 281                        |
| Venetoclax   | 3.5 ± 0.5                        | 10.6 ± 1.5                       |
| Irinotecan   | n.a.                             | 63.4 ± 11.4                      |
| Simeprevir   | 2.1 ± 0.6                        | 11.1 ± 2.3                       |
| Grazoprevir  | 11.0 ± 1.1                       | 70.0 ± 12.8                      |
| Natamycin    | n.a.                             | 47.3 ± 4.4                       |
| Suramin      | 19.3 ± 4.3                       | >1000                            |

n.a. not assessable.

\( ^{a} \) Half maximal effective concentration.

\( ^{b} \) Standard deviation.

\( ^{c} \) Half maximal cytotoxic concentration.

Among the tested compounds, alectinib showed the strongest inhibitory activity against HCoV-OC43, with an EC_{50} in the low micromolar range (0.6 \( \mu M \)). Lurasidone and elbasvir also exerted high antiviral activity against HCoV-OC43, exhibiting EC_{50} in the low micromolar range: 1.1 \( \mu M \) and 1.5 \( \mu M \), respectively. A moderate antiviral activity was shown by tedizolid, carbenoxolone and suramin, with EC_{50} ranging from 11.0 \( \mu M \) to 94.0 \( \mu M \). The aforementioned compounds’ antiviral effect was not a consequence of cytotoxicity, since none of the screened compounds significantly reduced cell viability at any concentration used in the antiviral assays (i.e. up to 100 \( \mu M \)), exhibiting CC_{50}.
values higher than 1000 μM. By contrast, the remaining compounds did not exhibit interesting features as anti-HCoV-OC43 molecules, due to either no antiviral activity (cefoperazone, teniposide, irinotecan, natalmycin), or low-moderate selectivity index (ponatinib, venetoclax, simeprevir, grazoprevir). In summary, these data showed that alectinib, lurasidone and elbasvir were endowed with strong anti-HCoV-OC43 activity (Fig. 4), with minimal toxicity and selectivity indexes higher than 600.

The antiviral activity of alectinib, lurasidone, and elbasvir was further validated by means of virus yield reduction assay. All compounds were found able to effectively reduce HCoV-OC43 yield on MRC-5 cell cultures in a dose-dependent manner (Table 4). Specifically, an EC_{50} of 1.0 μM was calculated for alectinib, 8.3 μM for lurasidone, and 1.3 μM for elbasvir.

4. Discussion

An accurate in silico docking search within a wide region around the SARS-CoV1/2 RdRp active site, allowed us to select 13 known drugs from the DrugBank library to be experimentally tested. We added suramin to the list, a well-known compound able to inhibit different RNA viruses (Mastrangelo et al., 2012) (De Clercq, 1979) (Albulescu et al., 2015).

To test the in vitro antiviral activity of the selected compounds, cell-based assays were established for SARS-CoV2 and HCoV-OC43 using Huh7-hACE2 and MRC-5 cells, respectively. Huh7 cells have already been widely used for screening purposes, being considered suitable model for image processing and being able to support infection of several viruses. Since preliminary analysis and previous published data indicated a limited infection capacity of SARS-CoV2 in Huh7 cells (Ogando et al., 2020), we engineered the human ACE2 receptor in these cells to increase infection as previously proposed (Wang et al., 2020)
unrelated protein, the main protease of SARS-CoV2 (Table 1).

This explanation is supported by the different kind of ligands, with a preferential affinity for large compounds possessing polar/charged moieties and planar aromatic groups: i.e. compounds that generally mimic RNA backbone and bases. In other words, the in silico docking on RdRp not only selects compounds potentially capable of interfering with the polymerase activity but could also act as a molecular filter for the selection of properties generally favorable for protein binding. This explanation is supported by the predicted high affinity of most of the selected compounds for another unrelated protein, the main protease of SARS-CoV2 (Table 1).

Among the tested compounds lurasidone and elbasvir displayed higher activity and lower cytotoxicity against both SARS-CoV2 and HCoV-OC43 strains. Lurasidone lead to complete inhibition of both strains with EC_{50} values in the micromolar range (18 and 1.1 μM, respectively) and favorable selectivity indexes. Lurasidone is an antipsychotic drug for treatment of acute depression and schizophrenia, known to bind with a low nanomolar affinity to Dopamine-2, 5-HT1A, 5-HT2A, and 5-HT7 receptors, and with slightly lower affinity to alpha-2C adrenergic receptors (Greenberg and Citrome, 2017). Lurasidone was already identified as a potential inhibitor of SARS-CoV2 main protease (Elmezayen et al., 2020); (Shamsi et al., 2020), and in our in silico analysis it showed good predicted binding affinity for both RdRp and the main protease. Furthermore, in a very recent paper, it has been reported that lurasidone and its derivatives displayed in silico binding affinity against five proteins (Mpro, PLpro, Spro, helicase and RdRp (Thurakkal et al., 2021)). Meanwhile, a homologous molecule known as ziprasidone, has been shown to lower ACE2 expression in Vero cells and inhibits the entry of a pseudotyped retrovirus exposing the SARS-CoV2 spike protein. Such results indicate that ziprasidone could act against SARS-CoV2 by affecting the ACE2 receptor (Massignan et al., 2020). Since SARS-CoV2 and HCoV-OC43 share a high level of protein sequence conservation (Vijgen et al., 2005) we hypothesize that mechanisms of action of lurasidone against these viruses might be the same.

Elbasvir inhibited SARS-CoV2 and HCoV-OC43 with EC_{50} values in the micromolar range (about 23 and 1.5 μM, respectively). Previous in vitro studies predicted elbasvir as a high affinity ligand for the RdRp, the papain-like protease and the helicase of SARS-CoV2 (Balasubramaniam and Shmookler Reis, 2020), whereas our in silico investigation suggested a preferential binding for the main protease (predicted Kd = 1.2 nM). In combination with grazoprevir (Zepatier), elbasvir has been shown to increase 25-fold remdesivir’s apparent potency in preventing SARS-CoV2 replication (Nguyenla et al., 2020). Elbasvir is an inhibitor of the HCV NSSA protein that has no homologues in coronaviruses: in light of our and previous work results, it has the potential to inhibit different viral proteins.

Table 4

| Compound | Virus titer reduction Log_{10} (mean ± SD)^a | EC_{50}b (μM) | CC_{50}b (μM) |
|----------|----------------------------------------|--------------|--------------|
| SARS-CoV2 |                                        |              |              |
| Grazoprevir | 3.9 ± 0.07 (100 μM) | 3.3 ± 0.9 | 118 ± 6 |
| Lurasidone | 1.3 ± 0.3 (100 μM)   | 6.4 ± 3.9   | >1000        |
| Elbasvir   | 0.6 ± 0.2 (100 μM)   | 14.4 ± 7.9  | >1000        |
| HCoV-OC43 |                                        |              |              |
| Alectinib  | 2.9 ± 0.1 (33 μM)    | 1.0 ± 0.1   | >1000        |
| Lurasidone | 6.3 ± 1.5 (100 μM)   | 8.3 ± 2.0   | >1000        |
| Elbasvir   | 1.4 ± 0.2 (100 μM)   | 1.3 ± 0.4   | >1000        |

Virus titer reduction is the difference between log_{10} values of PFU (SARS-CoV2) or FFU/mL (HCoV-OC43) from infected untreated controls and infected-treated at the highest tested dose. Mean virus titer for untreated control was 9.8 × 10^6 pfu/mL and 3 × 10^6 FFU/mL for SARS-CoV2 and HCoV-OC43, respectively.

a Standard deviation.

b Half maximal effective concentration obtained by virus yield reduction assay.

c Half maximal cytotoxic concentration obtained by Alamar Blue (SARS-CoV2) and MTS (HCoV-OC43) assays.

(Shamsi et al., 2020) (Yaron et al., 2020). Interestingly, a rather high percentage (>60%) of the selected compounds showed some in vitro activity against one or both of the tested CoV strains. A possible explanation for such a positive result is related to the characteristics of the protein region selected for the in silico docking. Such a portion of the protein is a wide, complex and rather hydrophilic surface with many conformational degrees of freedom, allowing it to adapt to the growing dsRNA during translation. Accordingly, the average crystallographic (or cryoEM) conformation of this region must be capable to accommodate different kind of ligands, with a preferential affinity for large compounds possessing polar/charged moieties and planar aromatic groups: i.e. compounds that generally mimic RNA backbone and bases. In other words, the in silico docking on RdRp not only selects compounds potentially capable of interfering with the polymerase activity but could also act as a molecular filter for the selection of properties generally favorable for protein binding. This explanation is supported by the predicted high affinity of most of the selected compounds for another unrelated protein, the main protease of SARS-CoV2 (Table 1).

Among the tested compounds lurasidone and elbasvir displayed
Fig. 4. Antiviral efficacy of the selected compounds against HCoV-OC43. The antiviral activity of compounds was evaluated by focus reduction assay, infecting MRC-5 cells in presence of increasing concentration of compounds. Cell viability assays were performed in the same conditions as for antiviral assays, in absence of viral inoculum. The percentage of infectivity inhibition (white dots) and the percentage of cell viability (blue triangles) were calculated by comparing treated and untreated wells. Error bars represent the standard deviation (SD) of 3 independent experiments. Treated and untreated samples for the infectivity inhibition curves were compared with one-tailed Student’s T test. * = p-value <0.05. ** = p-value <0.01. *** = p-value <0.001.

Alectinib inhibits the anaplastic lymphoma kinase (ALK) tyrosine kinase receptor in the nM range (Kinoshita et al., 2012), binding to the ATP binding site of the protein. Therefore, we can speculate that it might inhibit other host kinases essential for virus replication in lung epithelia and Vero E6 cells, but not in Huh7 cells. Grazoprevir inhibited SARS-CoV2 with an EC₅₀ around 16 μM (CC₅₀ value > 100 μM). Published computational studies suggested grazoprevir as a potential inhibitor of the nucleocapsid protein or the papain-like protease of SARS-CoV2 (Behera et al., 2020). Furthermore, in a very recent pre-print report (Bafna et al., 2020) it was shown that grazoprevir, together with simeprevir, synergizes with the viral polymerase inhibitor remdesivir, increasing its inhibitory activity as much as 10-fold. Grazoprevir is an inhibitor of HCV protease and it is often used for therapy in combination with elbasvir (in the drug named zepatier).

Simeprevir, another inhibitor of HCV protease, has been previously shown to inhibit SARS-CoV2 in synergy with remdesivir (Lo et al., 2020). In our experiments it showed a similar potency against both SARS-CoV2 (EC₅₀ about 9.3 μM) and HCoV-OC43 (EC₅₀ about 2.1 μM) but with low SI. From our docking results its effect is likely directed against the protease, but RdRp inhibition cannot be excluded.

ABT-199, also known as venetoclax, is a potent selective Bcl2 inhibitor, which induces the apoptosis pathway. An early work showed that Bcl2 expression prevents SARS-CoV1 induced apoptosis (Bordi et al., 2006). In addition, previous reports demonstrated that SARS-CoV1 7a protein was dependent on Bcl2 to induce apoptosis, suggesting Bcl2 as an important host factor for virus replication and pathogenesis (Tan et al., 2007). However, despite its good EC₅₀ (about 6.2 μM) against SARS-CoV2, venetoclax shows high toxicity in the tested cells.

Ponatinib, an oral drug for the treatment of chronic myeloid leukemia and Philadelphia chromosome-positive acute lymphoblastic leukemia, was already proposed as SARS-CoV2 inhibitor (Nguyen et al., 2020) (Sauvat et al., 2020) (Gordon et al., 2020), and it is shown here to inhibit SARS-CoV2 and HCoV-OC43 with a poor selective index.

All the other compounds, although some of them have been described in the literature as potential inhibitors of SARS-CoV2 (i.e. teniposide (Kadioglu et al., n.d.) and irinotecan (B, 2020)), did not show any relevant activity in either of the two viruses tested.

5. Conclusions

In our work we have: 1. excluded SARS-CoV2 antiviral activity for teniposide (Kadioglu et al., n.d.) and irinotecan (B, 2020), selected from previous experimental studies; 2. showed the ability of some of the already described anti-SARS-CoV2 compounds to inhibit also coronaviruses HCoV-OC43 causing the common cold (suramin, ponatinib - although with a low SI); and most importantly 3. showed the capability of some of the selected drugs to selectively inhibit HCoV-OC43 (alectinib) or SARS-CoV2 (grazoprevir) or be active against both CoV strains (lurasidone and elbasvir). The antiviral activity of lurasidone and elbasvir was confirmed by virus yield reduction assay for both viruses. Treatment of CoV infections with drugs that could inhibit different viral targets, as predicted for lurasidone and elbasvir, would be an effective way to lower chances of the emergence of drug resistant viral strains.

Of note, in previous works it was demonstrated that alectinib (Song et al., 2015) could penetrate the blood-brain barrier (BBB) exerting its activity in the central nervous system (CNS). Since HCoV-OC43, as other coronaviruses, is able to invade the CNS (Dubé et al., 2018), alectinib might be an interesting candidate for the treatment of HCoV-OC43 persistent infections in the brain. Moreover, the free levels of alectinib found in both plasma and cerebrospinal fluid are similar (Herden and Waller, 2018) and its EC₅₀ against HCoV-OC43 (0.6 μM) is lower than the maximum level attainable in human serum with daily recommended dosage (676 ng/mL corresponding to 1.4 μM) (Ly et al., 2018).

In conclusion, in this work we showed that lurasidone and elbasvir are not only potential drugs against SARS-CoV2, but that they can also inhibit the infection established by another beta-CoV, HCoV-OC43. Thus, our approach allowed the identification of lead-drugs for further in vitro and clinical investigation to contain the present outbreak. Furthermore, it could contribute to the identification of broad spectrum anti-CoV inhibitors/therapies that would allow for a rapid and effective reaction to future epidemics.

Funding

This work was funded by the #FarmaCovid crowdfunding initiative (https://www.gofundme.com) to MM, AM and EM; by SNAM Foundation, Beneficentia Stiftung and Generall Foundation to AM; by the Ricerca Locale (2019) grant from the University of Turin, to MD and DL.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

We would like to thank all the people that contributed to the
Antiviral Research 189 (2021) 105055

M. Milani et al.

Rothen, H.A., Stone, S., Natekar, J., Kumari, P., Arora, K., Kumar, M., 2020. The FDA-approved gold drug auranofin inhibits novel coronavirus (SARS-COV-2) replication and attenuates inflammation in human cells. Virology 547, 7–11. https://doi.org/10.1016/j.virol.2020.05.002.

Rut, W., Groborz, K., Zhang, L., Sun, X., Zmudzinski, M., Pawlik, B., Wang, X., Jochmann, D., Neyts, J., Mlynskis, W., Hilgenfeld, R., Drag, M., 2021. SARS-CoV-2 Mpro inhibitors and activity-based probes for patient-sample imaging. Nat. Chem. Biol. 17 (2), 222–228. https://doi.org/10.1038/s41589-020-00689-z.

Salgado-Benvindo, C., Thaler, M., Tan, A., Ogando, N.S., Breidenbeek, P.J., Ninaber, D.K., Wang, Y., Hiemstra, P.S., Snijder, E.J., Van Hennert, M.J., 2020. Suramin inhibits SARS-CoV-2 infection in cell culture by interfering with early steps of the replication cycle. Antimicrob. Agents Chemother. 64 (8) https://doi.org/10.1128/AAC.00900-20.

Salton, F., Confalonieri, P., Meduri, G.U., Santus, P., Harari, S., Scala, R., Lanini, S., Vertui, V., Oggioni, T., Caminati, A., Patruno, V., Tamburrini, M., Scartabellati, A., Parati, M., Villani, M., Radovanovic, D., Tomassetti, S., Ravaglia, C., Poletti, V., Confalonieri, M., 2020. Prolonged low-dose methylprednisolone in patients with severe COVID-19 pneumonia. Open Forum Infect. Dis. 7 (10) https://doi.org/10.1093/ofid/ofaa421.

Sanner, M.F., 1999. PyMol: a programming language for software integration and development. J. Mol. Graph. Model. 17 (1), 57–61. https://www.academia.edu/download/25505223/10.1.1.35.6459.pdf.

Sauvat, A., Ciccossanti, F., Colavita, F., Di Rienzo, M., Castilletti, C., Capobianchi, M.R., Kepp, O., Zitvogel, L., Fimia, G.M., Kroemer, G., 2020. On-target versus off-target effects of drugs inhibiting the replication of SARS-CoV-2. Cell Death Dis. 11 (8), 1–11. https://doi.org/10.1038/s41419-020-02842-x.

Shamsi, A., Mohammad, T., Anwar, S., AlAjmi, M.F., Hussain, A., Md Tabish, R., Islam, A., Md Imtaiyaz, H., 2020. Glecaprevir and Maraviroc are high-affinity inhibitors of SARS-CoV-2 main protease: possible implication in COVID-19 therapy, Bioisci. Rep. 40 (6) https://doi.org/10.1024/BSR20201256.

Singh, U.C., Kollman, P.A., 1984. An approach to computing electrostatic charges for proteins. J. Comput. Chem. 5 (2), 129–145. https://doi.org/10.1002/jcc.54005204.

Song, Z., Wang, M., Zhang, A., 2015. Alectinib: a novel second generation anaplastic lymphoma kinase (ALK) inhibitor for overcoming clinically-acquired resistance. Acta Pharm. Sin. B 5 (1), 34–37. https://doi.org/10.1016/j.apsb.2014.12.007. Chinese Academy of Medical Sciences.

Su, S., Feng, G., Shi, W., Liu, J., Lai, A.C.K., Zhou, J., Liu, W., Bi, Y., Gao, G.F., 2016. Epidemiology, genetic recombination, and pathogenesis of coronaviruses. Trends Microbiol. 24 (6), 490–502. https://doi.org/10.1016/j.tim.2016.03.003. Elsevier Ltd.

Tan, Y.-X., Tan, T.H.P., Lee, M.J.-R., Tham, P.-Y., Gunalan, V., Druce, J., Birch, C., Catton, M., Fu, N.Y., Yu, V.C., Tan, Y.-J., 2007. Induction of apoptosis by the severe acute respiratory syndrome coronavirus 7a protein is dependent on its interaction with the bcl-XL protein. J. Virol. 81 (12), 6346–6355. https://doi.org/10.1128/JVI.00990-07.

Te Velthuis, A.J.W., Van Den Worm, S.H.E., Snijder, E.J., 2012. The SARS-coronavirus nsp7–nsp8 complex is a unique multimeric RNA polymerase capable of both de novo initiation and primer extension. Nucleic Acids Res. 40 (4), 1737–1747. https://doi.org/10.1093/nar/gkr993.

Thurakkal, L., Singh, S., Roy, R., Kar, P., Sadhukhan, S., Porel, M., 2021. An in-silico study on selected organosulfur compounds as potential drugs for SARS-CoV-2 infection via binding multiple drug targets. Chem. Phys. Lett. 763 https://doi.org/10.1016/j.cplett.2020.138195.

Trott, O., Olson, A.J., 2009. AutoDock Vina: improving the speed and accuracy of docking with a new scoring function, efficient optimization, and multithreading. J. Comput. Chem. 31 (2) https://doi.org/10.1002/jcc.21324. NA-NA.

Vijgen, L., Keyaerts, E., Moes, E., Thoelen, I., Wollants, E., Lemey, P., Vandamme, A.-M., Van Ranst, M., 2005. Complete genomic sequence of human coronavirus OC43: molecular clock analysis suggests a relatively recent zoonotic coronavirus transmission event. J. Virol. 79 (3), 1595–1604. https://doi.org/10.1126/jvi.79.3.1595-1604.2005.

Wallace, A.C., Laskowski, R.A., Thornton, J.M., 1995. Ligplot: a program to generate schematic diagrams of protein-ligand interactions. Protein Eng. Des. Sel. 8 (2), 127–134. https://doi.org/10.1093/protein/8.2.127.

Wang, M., Cao, R., Zhang, L., Yang, X., Liu, J., Xu, M., Shi, Z., Hu, Z., Zhong, W., Xiao, G., 2020. Remdesivir and chloroquine effectively inhibit the recently emerged novel coronavirus (2019-nCoV) in vitro. Cell Res. 30 (3), 269–271. https://doi.org/10.1038/s41422-020-01276. Springer Nature.

Weiss, S.R., Leibowitz, J.L., 2011. Coronavirus pathogenesis. Adv. Virus Res. 79, 85–164. https://doi.org/10.1016/B978-0-12-385885-6.00009-2. Academic Press Inc.

Yaron, T.M., Heaton, B.E., Levy, T.M., Johnson, J.L., Jordan, T.X., Cohen, B.M., Kerlaky, A., Lin, T.-Y., Liberatorre, K.M., Bulson, D.K., Kastenhuber, E.R., Mercadante, M.N., Shobana-Ganesh, K., He, L., Schwartz, B.E., Chen, S., Weinstein, H., Elements, O., Piskounova, E., Heaton, N.S., 2020. The FDA-approved drug Alectinib compromises SARS-CoV-2 nucleocapsid phosphorylation and inhibits viral infection in vitro. BioXiv. https://doi.org/10.1101/2020.08.14.251207.