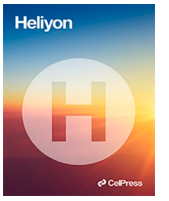
Heliyon 11 (2025) e41894

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon



Research article

Drug repurposing of argatroban, glimepiride and ranolazine shows anti-SARS-CoV-2 activity via diverse mechanisms

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ARTICLE INFO

Keywords:
COVID-19
ACE2
Drug-repurposing
Argatroban
Glimepiride
Ranolazine

ABSTRACT

Despite the vast vaccination campaigns against SARS-CoV-2, vaccine-resistant variants have emerged, and COVID-19 is continuing to spread with the fear of emergence of new variants that are resistant to the currently available anti-viral drugs. Hence, there is an urgent need to discover potential host-directed – rather than virus-directed – therapies against COVID-19. SARS-CoV-2 enters host cells through binding of the viral spike (S)-protein to the host angiotensin-converting enzyme 2 (ACE2) receptor, rendering the viral port of entry an attractive therapeutic target. Accordingly, this study aimed to investigate FDA-approved drugs for their potential repurposing to inhibit the entry point of SARS-CoV-2.

Accordingly, the FDA-approved drugs library was enrolled in docking simulations to identify drugs that bind to the Spike-ACE2 interface. The drugs list retrieved by the docking simulations was shortlisted to 19 drugs based on docking scores and safety profiles. These drugs were screened for their ability to prevent binding between ACE2 and S-protein using an ELISA-based Spike-ACE2 binding assay. Five drugs showed statistically significant inhibition of binding between ACE2 and S-protein, ranging from 4 % to 37 %. Of those five, argatroban, glimepiride and ranolazine showed potential antiviral activity at IC_{50} concentrations well below their CC_{50} assessed by the plaque assay. Their mode of antiviral action was then determined using the plaque assay with some modifications, which revealed that argatroban acted mainly through a direct virucidal mechanism, while glimepiride largely inhibited viral replication, and ranolazine exerted its antiviral impact primarily through inhibiting viral adsorption.

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https://doi.org/10.1016/j.heliyon.2025.e41894
Received 21 January 2024; Received in revised form 26 December 2024; Accepted 9 January 2025
Available online 10 January 2025

In conclusion, this study has identified three FDA-approved drugs – argatroban, glimepiride and ranolazine – which could potentially be repurposed and used for the management of COVID-19.

1. Introduction

The coronavirus disease 2019 (COVID-19) global pandemic caused by the novel severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), has posed a significant threat to human health, infecting over 600 million people with more than 6 million reported deaths [1,2]. Despite widespread vaccination efforts, new viral variants are continue to emerge, raising concerns about vaccine efficacy against these variants [3]. Many patients still experience severe symptoms and complications requiring hospitalization, especially those with comorbidities such as hypertension, diabetes, heart failure and cancer [4–7]. This highlights the need for new treatments for COVID-19. Therefore, repurposing existing FDA-approved drugs may be the fastest and most cost-effective approach to combatting COVID-19.

According to the CDC and NIH, approved drugs for the management of COVID-19 fall into two categories: those targeting viral proteins and those modulating the host immune response. Drugs targeting viral proteins include ritonavir-boosted nirmatrelvir, remdesivir, and molnupiravir [8]. Drugs modulating the host immune response include dexamethasone, baricitinib and tocilizumab, which help manage the cytokine storm during SARS-CoV-2 infection [8]. A general concern with viral protein-targeting drugs is the development of resistant strains. In fact, all previously approved neutralizing anti-spike antibody therapies, such as etesevimab, bebtelovimab, casirivimab, imdevimab, and sotrovimab, have shown decreased effectiveness against the Omicron sub-lineages BQ.1,

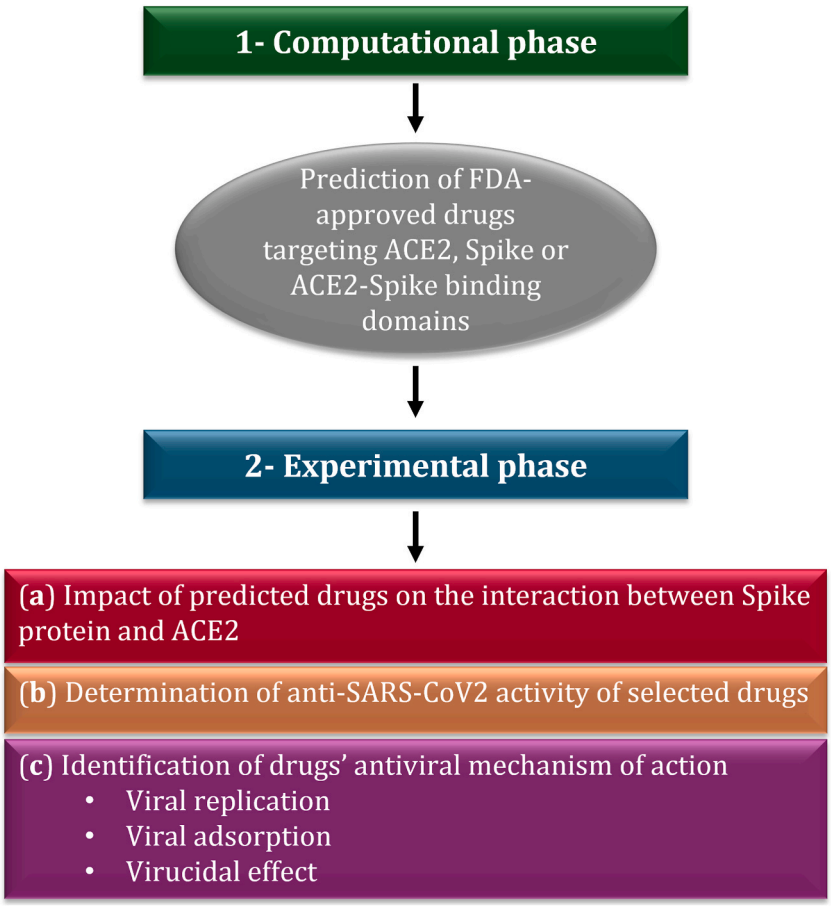

Fig. 1. A Schematic illustration of the workflow of the study. (1) An initial phase of computational analysis to screen for the FDA-approved drugs targeting ACE2, followed by primary selection of drugs. (2) Then an experimental validation phase for the selected drugs achieved by (a) evaluating the ability of the selected drugs to interrupt the binding between the viral S-protein and the host ACE2 receptor, (b) assessing the antiviral activity and cytotoxicity of the promising candidates to determine the IC_{50} and CC_{50} values and selectivity indices of the drugs followed by (c) exploring the mechanism of action of the chosen drugs.

BQ.1.1 and XBB [9]. Mutations in the viral RNA-dependent-RNA polymerase targeted by remdesivir and molnupiravir, as well as in the viral main protease (M-protease or M^{pro}) targeted by ritonavir and nirmatrelvir, have also been reported [10]. Therefore, the search for alternative therapies remains urgent, with a focus on targeting host factors that are essential for the SARS-CoV-2 life cycle.

SARS-CoV-2 is an enveloped, single-stranded positive-sense RNA virus in the beta genera of the *Coronaviridae* family [11,12]. Its genome encodes structural proteins, namely spike (S), envelope (E), nucleocapsid (N) and membrane (M) proteins, along with non-structural and accessory proteins [13]. The S-protein plays a crucial role in viral attachment, fusion, and entry into host cells. It consists of two subunits, S1 and S2. S1 subunit contains the receptor binding domain (RBD), which binds to Angiotensin Converting Enzyme 2 (ACE2) receptor on the surface of the host cell [11]. Their binding is followed by cleavage of the viral S-protein thereby facilitating viral fusion and entry [14]. The utilization of ACE2 by SARS-CoV-2 to enter host cells makes it an appealing therapeutic target. In fact, a recent study has shown that ACE2 could be blocked by a recombinant RBD fragment, thus inhibiting SARS-CoV-2 entry into ACE2-expressing cells [15].

Hence, our primary goal was to identify drugs that could potentially be repurposed to impede the entry point of SARS-CoV-2, the ACE2 receptor. Our approach involved an initial phase of computational screening of FDA-approved drugs, followed by experimental validation to assess their ability to disrupt the binding between the viral S-protein and the host ACE2 receptor. For promising candidates we proceeded to conduct functional assessments to understand their anti-SARS-CoV-2 mechanisms (Fig. 1).

2. Methodology

2.1. Structure based in-silico screening and scoring

The FDA-approved drugs library (Drug Bank, Canada), which contained around 2500 drugs, was enrolled in molecular docking simulations using Molecular Operating Environment (MOE) software package against resolved crystal structure of SARS-CoV-2 spike receptor-binding domain bound with ACE2 (PDB ID: 6M0J) [16]. The drugs were prepared by adding hydrogen addition/partial charges and energy-minimized using MMFF94 Force Field with root mean square (RMSD) gradient of 0.01 kcal/mol and RMS (Root Mean Square) distance of 0.1 Å. In addition, protein structures were prepared by removing water molecules and repeated chains. MOE QuickPrep protocol, 3D protonation, and calculation of partial charges were executed to optimize structural issues. The default procedures were applied with MOE Docking protocol parameters to select the best binding poses for the docked compounds, as previously reported [17]. To estimate energy profile based on the binding affinity with respect to hydrophobic-hydrophobic interactions, the obtained docking poses were estimated and interactions with the active site were considered based on London dG scoring method [18–20]. Finally, to choose the best fitting poses into the active site expressing the best S score correlated with the best interactions with the protein, visual inspection was performed. Drugs with S scores <10 kcal/mol were selected for experimental validation. Drugs with low safety profile, such as drugs known to have a wide range of side effects or narrow therapeutic indices were excluded.

2.2. Drugs reconstitution

The following list of drugs were purchased: tirofiban hydrochloride monohydrate, atovaquone, acenocoumarol, resveratrol, argatroban monohydrate, glimepiride, zanamivir, tamsulosin hydrochloride, gefitinib, ranolazine dihydrochloride, topiramate, terbutaline hemisulfate salt, leucovorin folinic acid calcium salt hydrate, leucovorin calcium salt, tiagabine hydrochloride, cromoglicic acid (all purchased from Sigma-Aldrich), arbutamine, oxitriptan (both purchased from Santa Cruz), glutathione (TOCRIS), and ibandronate sodium (USP).

Drugs were reconstituted in the recommended amount of solvent (water or DMSO) as per the manufacturer's instructions.

2.3. COVID-19 Spike-ACE2 binding assay

The potential of shortlisted drugs to inhib the binding between the SARS-CoV-2 S-protein and ACE2 receptor was evaluated using the COVID-19 Spike-ACE2 binding assay kit (RayBiotech) according to the manufacturer's instructions [21]. Two versions of the kit were utilized:

1. A 96-well plate coated with recombinantly-expressed spike protein receptor binding domain (S-RBD); the drugs being tested are added to the wells along with recombinant human ACE2 protein.
2. A 96-well plate coated with ACE2; the drugs are added to the wells in the presence of S-RBD protein.

Both versions of the kit follow the same principle and were used to confirm the results. Briefly, the S-protein receptor binding domain (RBD) or the ACE2 protein was diluted and combined with each drug to yield a final concentration of 2 mM. These mixtures were added to the respective 96-well plates and incubated overnight at 4 °C. After washing, a detection antibody was added, followed by an HRP-conjugated antibody for 1 h, then incubated with the TMB-substrate reagent for 30 min. Absorbance was read at 450 nm using a Hidex Sense multimode plate reader. All experiments contained a positive binding control (ACE2-test reagent or RBD-test reagent without the drug) and a vehicle negative control. The positive control signifies 100 % binding between ACE2 receptor and the S- protein, therefore it can verify whether the test reagent inhibits the binding. The vehicle blankis the solution in which the test reagent is dissolved and serves as the negative control that detects any unintended vehicle effects. Four replicates of the assay were

performed.

2.4. Anti-viral impact of the shortlisted drugs on SARS-CoV-2

2.4.1. Cell line

Vero E6 cells (African green monkey, kidney; CRL-1586, ATCC) were chosen for the antiviral assays due to their high support for SARS-CoV-2 replication. Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10 % fetal bovine serum (FBS) and 1 % antibiotic solution and incubated at 37 °C in a humidified 5 % CO_2 incubator.

2.4.2. Determination of cytotoxic concentration (CC_{50})

Cytotoxic activity of drugs was tested using a crystal violet assay as previously described [22] with minor modifications. 3×10^4 cells were seeded in 96-well plates. After 24 h, the cells were treated with various concentrations of each compound in triplicates (1 nM–10 mM). After 72 h, cell monolayers were fixed with 10 % formaldehyde and stained with of 0.1 % crystal violet. Cells were washed and dried overnight, followed by the addition of methanol. Absorbance was measured at λ_{max} 570 nm using Anthos Zenyth 200rt plate reader (Anthos Labtec Instruments, Heerhugowaard, Netherlands). The CC_{50} value was calculated using nonlinear regression analysis using GraphPad Prism software by plotting log concentrations of the compound versus normalized response (variable slope).

$$cytotoxicity\ \% = \frac{absorbance\ of\ cells\ without\ treatment - absorbance\ of\ cells\ with\ treatment}{absorbance\ of\ cells\ without\ treatment}\ X\ 100 [23]$$

The concentration that displayed 50 % cytotoxicity (CC_{50}) was calculated using a plot of percent cytotoxicity versus sample concentration.

Three replicates of the assay were performed.

2.4.3. Determination of inhibitory concentration 50 (IC_{50})

The IC_{50} values were determined as previously described [24], with slight modifications. Briefly, 2.4×10^4 Vero E6 cells were cultured overnight in 96-well plates. An aliquot containing 100 $TCID_{50}$ of the SARS-CoV-2 "NRC-03-nhCoV" virus, a strain isolated in Egypt in March 2020 sharing 99.9 % identity with the Wuhan isolate (2019-nCoV WHU01) [25], was incubated with serially diluted concentrations (1 nm–10 mM) of the drugs at 37 °C for 1h. Cells were incubated with virus/compound mixture for 72h, fixed with 10 % paraformaldehyde and stained with 0.5 % crystal violet at room temperature. Crystal violet dye was dissolved using absolute methanol and absorbance was measured at λ_{max} 570 nm. Untreated cells infected with the virus represent virus control. Uninfected cells treated only with the drug dissolution vehicle (DMSO/water) were used as negative control. The IC_{50} of the compound is the dose required to reduce the virus-induced cytopathic effect (CPE) by 50 %, relative to the virus control [26]. The IC_{50} value was calculated using nonlinear regression analysis by plotting log concentrations of the compounds versus normalized response (variable slope).

Three replicates of the assay were performed.

2.4.4. Potential stage of antiviral action

To determine which stage(s) of the viral replication were affected following treatment with argatroban, ranolazine, and glimepiride, the three drugs were tested for their impact on viral replication, adsorption and cell-free virucidal effect, as previously described [23]. In all the assays described below three replicates were performed and the following controls were used:

1. Cell control: uninfected and untreated cells used to ensure the validity of the assay.
2. Virus control: infected cells that were not treated with the drugs, used to calculate the percentage of viral inhibition following drug treatment.

2.4.4.1. Viral replication inhibition. Vero E6 cells were cultured at a density of 1.2×10^6 cells/well in a 6-well plate overnight. Cells were treated with SARS-CoV-2 inoculum for 1h. Cell monolayers were washed with 1X PBS and subsequently treated with predetermined non-cytotoxic concentrations of each compound then incubated at 37 °C for 1h. Cells were washed followed by the addition of 2 % agarose overlayers. Plates were left to solidify at 37 °C in 5 % CO_2 for 72 h. The cell monolayers were then fixed and stained using 0.1 % crystal violet solution. Viral plaques were counted and the percentage reduction in plaque formation compared to the control wells was calculated.

2.4.4.2. Viral adsorption inhibition. Vero E6 cells were cultured in 6 well plates (1.2×10^6 cells/well) and incubated with the drugs at 4 °C for 1 h to allow chemical adsorption onto cell receptors without active penetration. The plates were then washed with 1X PBS to remove the residual compounds. Subsequently, viral dilution of SARS-CoV-2 virus was applied to allow viral adsorption/infection and another incubation period commenced at 37 °C in 5 % CO_2 for 1 h. To remove the residual virus, cell monolayers were washed with 1X PBS and then overlayed with 2 % agarose/DMEM, and incubated at 37 °C in humidified 5 % CO_2 for 72 h. The cell monolayers were finally fixed and stained, and plaque reduction was assessed as described above.

2.4.4.3. Virucidal effect. A simple plaque reduction assay was performed where effective concentrations of the compounds were mixed with concentrated SARS-CoV-2 virus (3–4 folds higher than the countable virus dilution). This was followed by incubation of the virus/compound mixture at room temperature for 1 h. Subsequently, ten-fold serial dilutions of the virus/compound mixture (3 or 4 times) were performed to reach a countable viral titer. The mixture dilution with countable viral titer was applied to the Vero E6 monolayers (1.2×10^6 cells/well) including cell and virus control wells. The plates were then incubated at 37 °C in a humidified 5 % CO_2 incubator for 1 h. Cells were washed with 1X PBS then overlayed with 2 % agarose/DMEM and incubated at 37 °C in a 5 % CO_2 incubator for 72 h. Cell monolayers were fixed and stained, and plaque reduction was assessed as described above.

2.5. Statistical analysis

Statistical tests were performed using GraphPad Prism 10. Results are presented as mean ± standard deviation. Since the data analysis involved comparing two groups of unpaired outcome data, the unpaired student t-test was used. A p-value <0.05 was considered statistically significant, where * = $p < 0.05$, **$p < 0.01$ and ***$p < 0.001$. A minimum of three replicates were performed for each experiment.

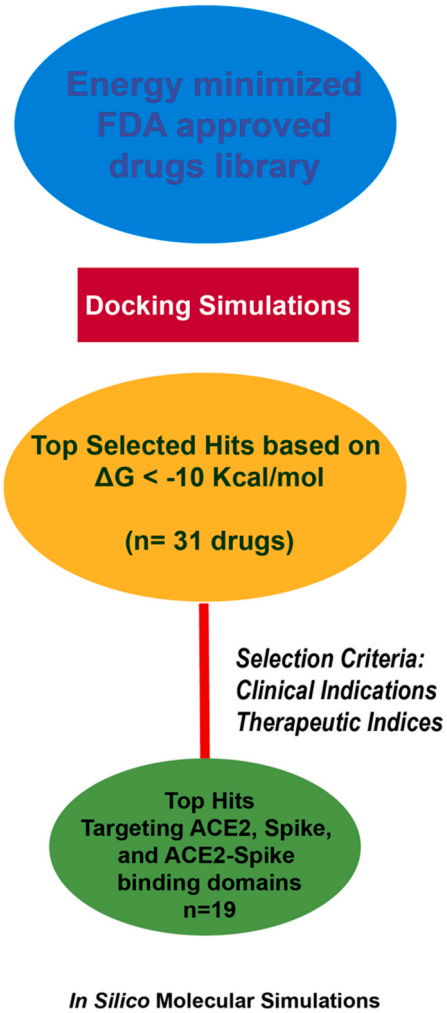

Fig. 2. Computational screening of drugs. Drugs that were computationally predicted to inhibit Spike-ACE2 interaction were first designated based on their docking scores. Out of 31 drugs showing docking scores < -10 kcal/mol, those with unfavorable safety profiles were excluded giving a final list of 19 drugs.

3. Results

3.1. Computational analysis

3.1.1. Computational prediction of FDA-approved drugs that potentially target ACE2 receptor

The U.S. FDA-approved drug database was downloaded (drugbank.ca) and 3D structures were energy minimized using MMFF94 forcefield. Computational analysis was performed to predict drugs that are expected to target either the ACE2 receptor, the S-protein of the virus, or ACE2-Spike interactions leading to competitive inhibition. The drugs with the highest docking scores were selected resulting in a list of 31 drugs. Drugs with a low safety profile were excluded, such as antineoplastic agents, like idarubicin and topotecan, known to have a wide range of side effects. Additionally, cardiotonic agents with narrow therapeutic indices like digoxin and ouabain, as well as drugs like cisapride withdrawn from the market in some countries due to potential serious risks were excluded. This process yielded a final list of 19 drugs to be experimentally tested (Fig. 2 and Table 1).

3.2. Experimental validation

3.2.1. Impact of the selected FDA-approved drugs on the binding between SARS-CoV-2 spike protein and ACE2 receptor

We used the COVID-19 Spike-ACE2 binding assay kit to experimentally validate whether the computationally predicted drugs could empirically inhibit the binding between the SARS-CoV-2 S-protein and ACE2 receptor. This assay served as an initial screening step for more accurate selection of the predicted drugs based on their ability to inhibit the binding between S-protein and ACE2 receptor. All 19 drugs were tested at a concentration of 2 mM using the two forms of the COVID-19 Spike-ACE2 binding assay kit. For each drug the assays were repeated twice. Out of the 19 tested drugs, five showed a statistically significant and consistent inhibition in at least one of the two binding assay formats. These five drugs are argatroban, glimepiride, ranolazine, cromoglicic acid, and resveratrol, with average binding inhibitions of 6.3 % ($p = 0.0094$), 4 % ($p = 0.0017$), 7 % ($p = 0.0072$), 7.1 % ($p = 0.0003$) and 37 % ($p = 0.0456$), respectively, compared to the positive binding control (Fig. 3A and B).

3.2.2. Anti-viral impact of argatroban, glimepiride, ranolazine, cromoglicic acid, and resveratrol on SARS-CoV-2

Initially, we assessed the impact of the five drugs (argatroban, glimepiride, ranolazine, cromoglicic acid, and resveratrol) on the viability of Vero E6 cells. The percentage of cell viability was calculated and plotted against log drug concentration to determine the cytotoxic concentration (CC_{50}) of each drug as shown in Table 2. Subsequently, Vero E6 cells were infected with SARS-CoV-2 "NRC-03-nhCoV" virus and incubated with serial dilutions of the drugs to evaluate their antiviral activity. The percentage of viral inhibition was calculated and plotted against log drug concentration to determine the viral inhibitory concentration (IC_{50}) of each compound (Table 2).

For resveratrol and cromoglicic acid, the calculated IC_{50} values exceeded their CC_{50} values, resulting in a selectivity index less than 1, indicating they are not recommended for use as antivirals against SARS-CoV-2. However, argatroban (Fig. 4A), ranolazine (Fig. 4B), and glimepiride (Fig. 4C) showed a selectivity index greater than 1 (95, 10, and 61 respectively), making them promising candidates for potential use against SARS-CoV-2.

Table 1
List of selected drugs to be experimentally tested.

ACE2 binding domain

Drug	S score (Kcal / mol)
Glutathione	-13.8193
Tirofiban	-13.1463
Arbutamine	-11.6225
Atovaquone	-10.881
Oxitriptan	-10.8773
Cromoglicic acid	-10.7614
Acenocoumarol	-10.7361
Resveratrol	-10.7308
Atorvastatin	-10.7281
Argatroban	-10.6904
Glimepiride	-10.3804
Zanamivir	-10.0817

Spike binding domain

Drug	S score (Kcal / mol)
Glutathione	-13.4855
Tamsulosin	-12.9337
Cromoglicic acid	-12.1031
Gefitinib	-11.795
Ranolazine	-11.305

ACE2-spike domain

Drug	S score (Kcal / mol)
Ibandronate	-15.1065
Glutathione	-14.2799
Topiramate	-14.2372
Zanamivir	-13.2225
Glimepride	-12.6293
Cromoglicic acid	-12.382
Terbutaline	-12.3007
Leucovorin	-12.2524
Tiagabine	-12.1668

*Drugs highlighted in blue were predicted to target more than one domain.

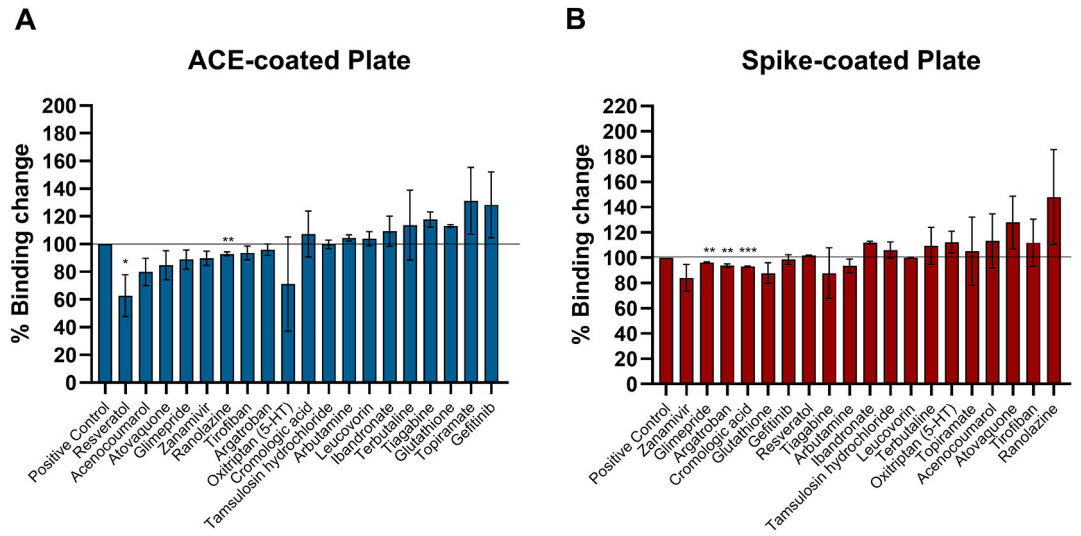

Fig. 3. Impact of drugs on binding of Spike protein to ACE2 receptor (A) Impact of drugs on binding of Spike protein to ACE2 receptor using the COVID-19 Spike-ACE2 binding assay kit with Spike-coated plates. (B) Impact of drugs on binding of Spike protein to ACE2 receptor using the COVID-19 Spike-ACE2 binding assay kit with ACE2-coated plates. The positive control represents 100 % binding between ACE2 receptor and Spike protein. Results are presented as mean ± SD (n = 4). Asterix (*) indicate statistical significance, where * = $p < 0.05$, **$p < 0.01$ and ***$p < 0.001$.

Table 2
Cytotoxic concentration (CC_{50}), viral inhibitory concentration (IC_{50}) and selectivity index of the selected drugs (n = 3).

Drug	CC_{50} (μM)	IC_{50} (μM)	Selectivity index
Resveratrol	600	900	<1
Argatroban	1900	20	95
Ranolazine	3010	300	10
Cromoglicic acid	>10,000	>10,000	<1
Glimepiride	2860	47	61

3.2.3. Mechanism of drug-mediated antiviral action

For the three drugs showing promising anti-viral impact against SARS-CoV-2 with a high selectivity index (argatroban, ranolazine, and glimepiride), we investigated the potential stage in the viral life cycle affected by each drug. According to Mostafa and coworkers [23], drugs exert their antiviral impact through hindering viral replication inside the host cell, hindering viral adsorption onto the host cell and/or direct virucidal effect against the virus via a cell-free mechanism. To identify which of these mechanisms were utilized by argatroban, ranolazine, and glimepiride, we conducted a plaque reduction assay [27] with some modifications as described in the methods section.

Argatroban primarily induced a direct virucidal effect showing up to 90 % viral inhibition, while it only showed mean inhibitions of 32 % and 27 % through the viral adsorption and replication stages, respectively (Fig. 5A–C). Glimepiride primarily inhibited viral replication, achieving a mean viral inhibition of 91 %, while a mean viral inhibition of 33 % and 20 % were achieved through direct virucidal impact and inhibition of viral adsorption, respectively (Fig. 5A–C). Finally, ranolazine exerted its antiviral impact mostly through inhibiting viral adsorption showing a mean viral inhibition of 61 %, followed by 47 % inhibition through the direct virucidal mechanism, and 35 % inhibition at the viral adsorption stage (Fig. 5A–C).

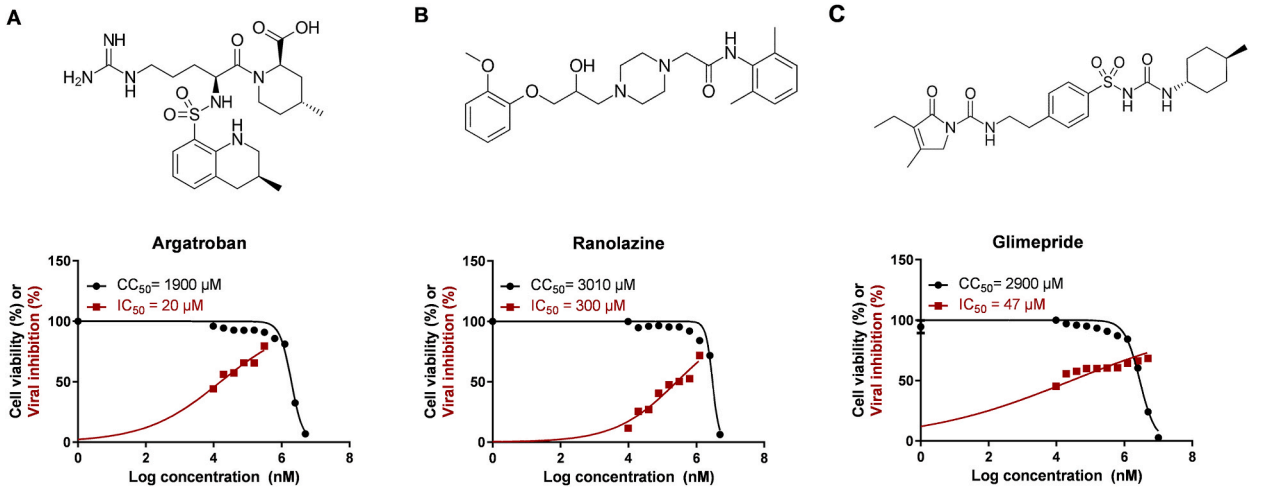

Fig. 4. Cytotoxicity and SARS-CoV-2 antiviral activity of selected compounds. The percentage of cell viability (black line) and SARS-CoV-2 viral inhibition (red line) were assessed in Vero E6 cells to determine the cytotoxic concentration (CC_{50}) and the viral inhibitory concentration (IC_{50}) using serial dilutions of (A) argatroban, (B) ranolazine, and (C) glimepiride (n = 3). The chemical structure of each drug is shown above each graph.

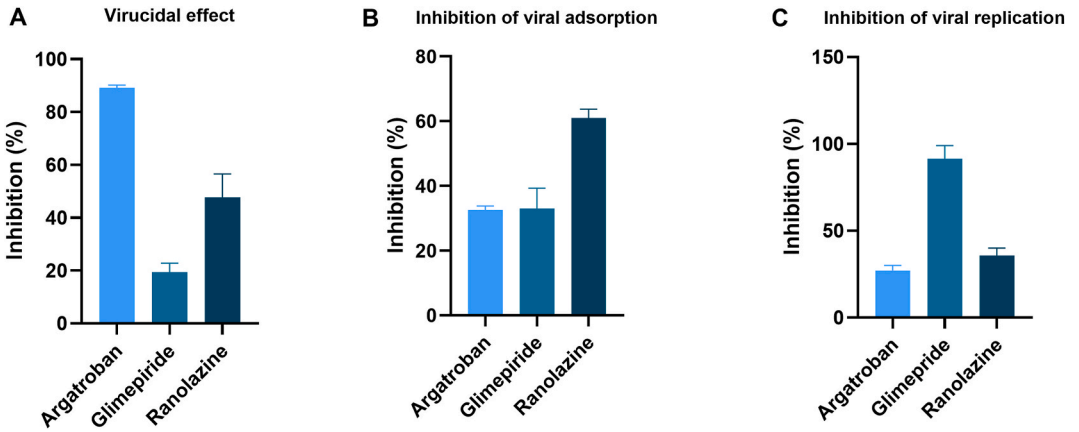

Fig. 5. Mechanism of antiviral activity of argatroban, ranolazine, and glimepiride. The mechanism of anti-SARS-CoV-2 activity of argatroban, ranolazine, and glimepiride was assessed in terms of (A) virucidal effect, (B) inhibition of viral adsorption to Vero E6 cells, and (C) inhibition of intracellular viral replication. Results are expressed as mean ± SD (n = 3).

4. Discussion

Over the past years, the COVID-19 pandemic has affected hundreds of millions of people imposing a great burden on the healthcare system and the economies of many countries worldwide. Despite the enormous efforts and widespread vaccination campaigns that have been conducted, there are still numerous cases involving severe disease symptoms and many complications requiring hospitalization. This highlights the necessity for the development of therapeutic agents for the treatment of patients with severe COVID-19 and its associated complications rather than relying solely on prevention through vaccination.

The currently approved anti-viral drugs recommended for managing COVID-19 are ritonavir-boosted nirmatrelvir, remdesivir, and molnupiravir, which are used for patients with mild to moderate symptoms at risk of progressing to severe disease [8]. These drugs target specific viral proteins, but with the continuous emergence of new viral variants, the virus is likely to develop resistance against them. In fact, mutations in the viral RNA-dependent-RNA polymerase targeted by remdesivir and molnupiravir, as well as in the viral M^{pro} targeted by ritonavir and nirmatrelvir, have already been reported [10]. Therefore, a potentially more reliable approach to circumvent resistance would be to target host factors essential for the SARS-CoV-2 life cycle, such as its entry route through the ACE2 receptor. A recent study showed that a commercially available compound, VE607, binds to the Spike-ACE2 receptor interface leading to a reduction in SARS-CoV-2 viral replication in mouse lungs [28]. These results provide proof-of-concept that inhibiting the binding between SARS-CoV-2 and the ACE2 receptor represents a promising approach for treating COVID-19.

Since the process of drug discovery can take decades, repurposing currently available FDA-approved drugs is considered one of the

most efficient approaches to finding potential ACE2 receptor inhibitors. This would allow for quick identification of drugs that may halt the spread of SARS-CoV-2, and their rapid use in treating COVID-19 patients, vastly shortening the time and reducing costs compared to *de novo* drug discovery. In this study the FDA-approved drugs library was enrolled in docking simulations to detect drugs that potentially bind to the ACE2 receptor or SARS-CoV-2 S-protein, or target ACE2-Spike interactions. The generated list was narrowed down to 19 drugs based on docking scores and safety profiles (Fig. 2 and Table 1).

To validate the computational findings with empirical data, the 19 shortlisted drugs were screened for their ability to prevent binding between the host ACE2 receptor and the SARS-CoV-2 S-protein using a cell-free ELISA-based COVID-19 Spike-ACE2 binding assay kit. Five drugs showed statistically significant inhibition between the binding of ACE2 receptor and S-protein, ranging from 4 % to 37 %: resveratrol, argatroban, ranolazine, cromoglicic acid, and glimepiride (Fig. 3A and B). Out of these, only three drugs showed promising results in antiviral activity against SARS-CoV-2 with a selectivity index (SI) greater than 10: glimepiride, argatroban and ranolazine (Fig. 4A–C).

Glimepiride, an anti-diabetic drug, was reported in a previous study using computational screening approaches to potentially target SARS-CoV-2 M^{pro}, a viral protease crucial for polyprotein processing [29]. Similarly, an *in silico* study suggested glimepiride to have very promising molecular docking scores against multiple SARS-CoV-2 critical proteins, including M^{pro}, papain-like protease, and the RNA-Dependent RNA Polymerase [30]. However, the impact of glimepiride against SARS-CoV-2 has never been experimentally validated. Our study empirically shows that glimepiride inhibits SARS-CoV-2 with an IC_{50} at 47 μM and a much higher CC_{50} at 2860 μM. For clinical applications, it is generally recommended that anti-viral drugs exhibit an IC_{50} at sub-micromolar concentrations to be considered potent and achieve the therapeutic effect at low doses. Hence, further optimizations are required to enhance the potency of glimepiride against SARS-CoV-2. The CC_{50}, however, is much higher than its IC_{50} with a SI of 61 implying its relative safety as drugs with SIs greater than 10 are considered safe. The antiviral activity of glimepiride was achieved through two mechanisms: mainly by inhibiting intracellular viral replication and, to a lesser extent, viral adsorption and thus entry into the cells (Figs. 5 and 6). Our finding that glimepiride inhibits SARS-CoV-2 replication goes in line with the reported computational predictions that glimepiride potentially targets M^{pro} and possibly other SARS-CoV-2 enzymes essential for viral replication. In addition, the inhibition of viral adsorption supports our findings that glimepiride targets ACE2 receptor, suggesting that it induces its antiviral activity through a dual mechanism. A previous study using docking and molecular dynamics simulations identified glimepiride as a potential drug that may bind to neuropilin-1 (NRP1), another host receptor utilized by SARS-CoV-2 for viral entry [31], indicating a possible combinatorial effect against multiple viral and/or host proteins.

Regarding argatroban, it is an anticoagulant that acts through competitive and selective inhibition of thrombin. It is administered parenterally in the prevention and/or treatment of heparin-induced thrombocytopenia as well as other thrombotic conditions [32]. Interestingly, thrombin itself has been implicated in the pathogenesis of multiple viruses, including influenza, human metapneumovirus and respiratory syncytial virus which cause respiratory infections [33]. Thrombin was shown to not only enhance the replication of these viruses, but also aggravate the associated inflammation of these infections [34,35]. Therefore, the anticoagulant, in addition to the anti-inflammatory and antiviral effects of thrombin inhibitors may prove to be of great use in other respiratory viral infections such as SARS-CoV-2. Among the currently well-established complications of COVID-19 infection are coagulopathies associated with heparin resistance [36,37]. In fact, two previous studies have reported the successful treatment of several COVID-19 patients for thrombotic complications using argatroban [38,39]. In the present study, we have first experimentally validated that argatroban can hinder the binding between ACE2 and the SARS-CoV-2 S-protein and have also revealed an anti-COVID-19 impact of argatroban mediated mainly through inducing a direct virucidal effect and to a lower extent through inhibiting viral adsorption and

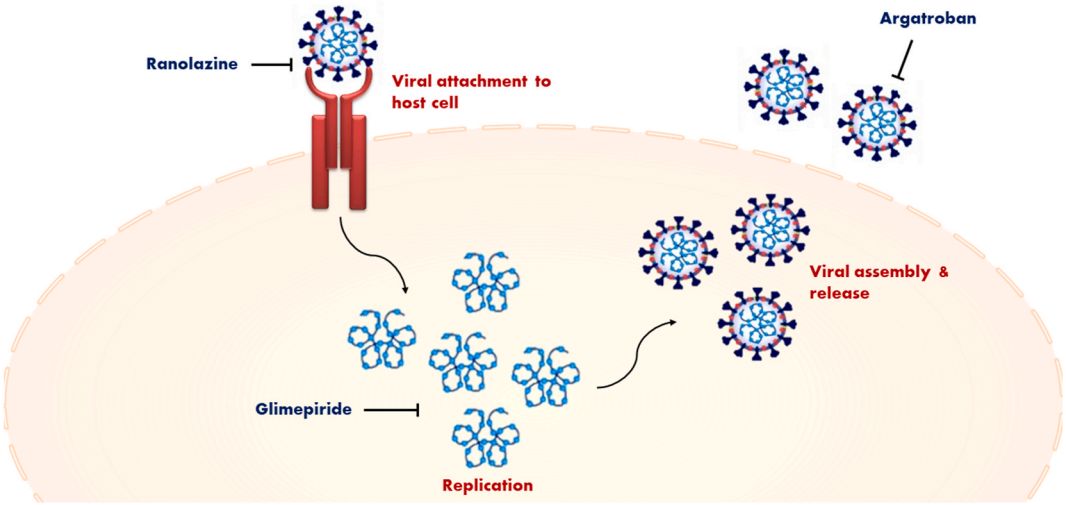

Fig. 6. Schematic representation of the mechanism of action of argatroban, ranolazine, and glimepiride. The figure shows the main site of action of each drug in the SARS-Cov-2 lifecycle, where argatroban acts directly on the viral particle inducing a virucidal effect. Ranolazine acts mainly by preventing the adsorption of the viral particles to the host cells, while glimepiride's main activity is via inhibiting viral replication.

replication (Fig. 5A–C and 6). Argatroban showed an IC_{50} of 20 μM and a CC_{50} of 1900 μM and subsequently a SI of 95. Similar to glimepiride, the drug is considered safe and allows for *in-vivo* testing owing to its high SI, however, it would be considered of moderate potency as its IC_{50} is in the micromolar range necessitating further optimizations to enhance its potency. Our findings confirm a previous bioinformatic study by Kim et al. which identified argatroban as a potential blocker of ACE2, however their finding was not experimentally verified [40]. Interestingly, another study utilizing a purely computational approach suggested that argatroban could also potentially inhibit the transmembrane serine protease TMPRSS2 which has been reported to aid in SARS-CoV-2 viral fusion and entry into the host cell [41]. Moreover, a virtual screening study proposed argatroban to be a possible inhibitor of the SARS-CoV-2 M^{pro} proposing an additional – albeit theoretical – antiviral mechanism for this drug [42] When taken together with our current findings, these data highlight the likely advantage of using argatroban in COVID-19 patients with coagulopathies to both combat the infection and manage the associated thrombotic and inflammatory complications of the disease.

Finally, ranolazine is an orally administered drug used for the management of chronic angina. In addition, ranolazine has anti-arrhythmic properties and is used off-label in the treatment of arrhythmia [43]. Among the COVID-19-associated complications are cardiovascular diseases, including arrythmias [44]. Interestingly, a few studies indicate that ranolazine might have beneficial effects in the management of COVID-19-related arrhythmias [45,46]. We have shown here that ranolazine has an antiviral activity against SARS-CoV-2 with an IC_{50} of 300 μM, a CC_{50} of 3000 μM and a SI of 10, and that this activity is mainly mediated through inhibiting viral adsorption (Figs. 5 and 6). This emphasizes that ranolazine induces its antiviral activity partly through interfering with ACE2-Spike interaction. The SI indicates that ranolazine use in vivo is applicable, however, its high IC_{50} suggests that it would probably be of low potency requiring high doses to achieve the desired antiviral effect. Accordingly, the potential anti-COVID-19 effect we have shown here, along with the reported antiarrhythmic effects shed light on a potential dual benefit of ranolazine, both as anti-viral and antiarrhythmic, in the management of COVID-19 patients with cardiovascular complications.

It is worth mentioning that despite their non-toxic impact on the cell lines, the obtained antiviral IC_{50} values for argatroban, glimepiride and ranolazine are higher than the therapeutically used plasma concentrations of each of the drugs for their currently approved indications (Table 3) [47–49]; hence, more studies may be required to determine the long-term safety and antiviral efficacy of these drugs at higher doses. We predicted the acute oral toxicity (LD_{50}) of the three drugs using the computational tool Pro-Tox 3.0 [50], where the predicted LD_{50} of each of the drugs was found to be much higher than the currently used therapeutic doses as shown in Table 3. This might indicate that the use of those drugs for the management of COVID-19 at doses higher than the currently used doses might still be safe. However, this does not negate the necessity to conduct further in-vivo studies to validate those findings. Alternatively, additional research could be performed to enhance the efficiency of the drugs or their derivatives against SARS-CoV-2. For instance, the mode of administration may be changed to local pulmonary delivery as aerosols using appropriate devices, which can enhance their concentration in the lungs, improve their efficacy, and limit their exposure to other tissues thereby lowering their side effects. Reformulation considerations are specifically important for argatroban which is administered by intravenous infusion, limiting patient compliance and its use outside of hospital settings. It would also prevent unwanted anticoagulant impact or bleeding tendencies that might be caused by systemic delivery of argatroban in COVID-19 patients that are not suffering from coagulopathies. Moreover, precautionary measures may need to be implemented for the use of the antidiabetic drug glimepiride, since it may cause hypoglycemia in non-diabetic COVID-19 patients. Therefore, administration of glimepiride via local pulmonary delivery may help overcome this side effect. Concerning ranolazine, it was reported to cause dizziness, nausea and headache when administered at high doses [51]. Since the obtained antiviral IC_{50} value for ranolazine was 300 μM, which is relatively higher than the therapeutically used plasma concentrations (3.40–13.35 μM) [49], those side effects would be expected and might lead to treatment discontinuation. Thus, administering ranolazine via inhalation might prevent the occurrence of its reported adverse effects.

Besides reformulation considerations, combining these drugs with the drugs currently approved for the management of COVID-19 could provide a synergistic effect through simultaneously targeting both viral and host proteins, and may allow for their use at lower doses, thereby reducing the risk of potential adverse effects of each drug. However, ranolazine has several potential drug interactions, including ritonavir/nirmatrelvir, which an is anti-viral drug combination approved for management of COVID-19; therefore, caution should be taken not to combine these drugs [52].

Besides the previously mentioned precautions, therapeutic trials of our drugs might face other challenges since host-directed antivirals are known to have a higher potential of causing inflammatory side effects [53], therefore it is crucial to consider testing the inflammatory reactions of these drugs *in-vivo* before using them as therapeutic agents for COVID-19.

Accordingly, the clinical applicability of the repurposed drugs is dependent on the selection of the appropriate dose and mode of administration, as well as consideration of the safety profile of the drug, including its side effects and possible drug interactions; this necessitates the integration of pharmaceutical sciences as well as toxicology to create safer targeted drug delivery [54].

As demonstrated by the success of the anti-Ebola drug remdesivir in managing COVID-19, the initial step in drug repurposing requires showing promising antiviral activity *in vitro* [55], which is later confirmed in preclinical studies [56,57], followed by clinical trials [57–59]. In this study, we have identified three drugs – argatroban, glimepiride, and ranolazine – that exhibit potential antiviral activity against SARS-CoV-2, possibly by inhibiting ACE2 receptor *in vitro*.

Notably, the *in vitro* studies conducted on remdesivir's antiviral activity in Vero E6 cells showed a similar pattern to the antiviral action observed with our three drugs in the same cell line. The CC50 of remdesivir (>100 μM) was significantly higher than its inhibitory concentration on SARS-CoV-2 replication (23.15 μM) [60]. These findings suggest that our *in vitro* data is comparable to an existing treatment for SARS-CoV-2, thus supporting our findings and putting argatroban, glimepiride, and ranolazine on the right track towards being therapeutically repurposed as anti- SARS-CoV-2 medications. It is important to note, that remdesivir has several drawbacks, such as its administration via intravenous infusion limiting its use to hospitalized patients in addition to its high cost which can strain hospitals and healthcare providers [61,62]. This emphasizes that our findings may offer more suitable treatment alternatives

Table 3
Comparison of drugs obtained IC50 and predicted LD50 to currently used theraputic doses and plasma concentrations in humans.

Drug	IC_{50} (μM)	Peak plasma concentration (Cmax) in human (μM)	Oral predicted LD_{50} (mg/kg)	Current therapeutic dose
Argatroban	20	0.78-2.35[48]	300	2 mcg/kg/min IV continuous infusion over 1-3 hours
				~ 0.12 -0.360mg/kg
Ranolazine	300	3.40-13.35 [49]	1550	500mg twice daily
Glimepiride	47	0.40 [47]	4000	1-2mg once daily

if proven safe and effective in vivo. Additionally, those drugs may have added benefits over the current medications in the management of COVID-19 patients with coagulopathies and cardiovascular complications if used systemically, with argatroban providing anticoagulant effects in patients with thrombotic complications and ranolazine being effective in managing patients with arrhythmias.

In conclusion, the findings of this study provide evidence for the anti-SARS-CoV-2 activity of argatroban, glimepride and ranolazine potentially through blocking the ACE2 receptor, making the first step along their journey of drug repurposing.

4.1. Study limitations

Further research is needed to understand the exact mechanism of action of each drug, since although all three drugs affected viral adsorption and entry into the cells, they also showed virucidal and replication inhibitory effects indicating that they could have other targets besides the ACE2 receptor. In addition, *in-vivo* experiments are necessary to confirm the antiviral effects observed *in vitro*, assess dosing requirements and efficacy of argatroban, glimepride and ranolazine against COVID-19, determine pharmacokinetic/pharmacodynamic profiles and confirm their safety. Future *in-vivo* testing should include studying the impact of argatroban, glimepride and ranolazine on viral titers as well as respiratory symptoms, radiographic findings, as well as assessing their impact on reducing pneumonia and mortality [63]. Moreover, deep sequencing from animals treated with any of the three drugs might be of great importance to ensure the absence of resistance mutations [63]. While several animal models are available for testing anti-COVID-19 drugs such as mice and Syrian hamsters, we recommend performing the in-vivo studies on rhesus macaque, since they are phylogenetically closer to humans, and they develop similar symptoms to those observed in humans in response to SARS-CoV2-infection. Accordingly, the impact of the drugs observed in those animals in terms of clinical effectiveness as well as potential side effects would better resemble what would be expected in humans [64]. Clinical trials should follow before these drugs can be incorporated into COVID-19 management regimens. Phase II clinical trials could be performed, where the efficacy and safety of the three drugs alone and in-combination with currently approved COVID-19 treatment such as remdesivir would be assessed. Moreover, owing to their currently approved uses, argatroban and ranolazine could be tested in COVID-19 patients with thrombotic complications and arrythmias, respectively.

CRediT authorship contribution statement

Shereen A. El Sobky: Writing – review & editing, Supervision, Methodology, Investigation, Formal analysis, Data curation. **Injie O. Fawzy:** Writing – review & editing, Supervision, Project administration, Methodology, Investigation, Formal analysis, Data curation. **Mahmoud S. Ahmed:** Methodology, Investigation, Formal analysis, Data curation. **Manon Ragheb:** Methodology, Data curation. **Merna H.M. Hamad:** Methodology, Data curation. **Rowan Bahaaeldin:** Methodology, Data curation. **Salma A. Fahim:** Methodology, Data curation. **Rana Saad:** Methodology, Conceptualization. **Ziad A. Khalil:** Methodology, Data curation. **Sara H. Mahmoud:** Validation, Methodology, Formal analysis, Data curation. **Ahmed Mostafa:** Investigation, Formal analysis, Data curation. **Mohamed A. Ali:** Writing – review & editing, Supervision, Formal analysis. **Hesham A. Sadek:** Supervision, Funding acquisition, Formal analysis, Conceptualization. **Nada El-Ekiaby:** Writing – review & editing, Writing – original draft, Supervision, Investigation, Funding acquisition, Formal analysis, Conceptualization. **Ahmed I. Abdelaziz:** Writing – review & editing, Supervision, Investigation, Funding acquisition, Formal analysis, Conceptualization.

Data availability statement

Data associated with this study will be made available upon request.

Ethics statement

Review and/or approval by an ethics committee was not needed for this study because it does not include human or animal

participation.

Funding

This study was supported by the Egyptian Science, Technology & Innovation Funding Authority under Emergency Covid Call ID 43736. The funding agency was not involved in study design, the collection, analysis and interpretation of data or in the writing of the manuscript.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:Ahmed Ihab Abdelaziz reports financial support was provided by Science and Technology Development Fund. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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