Phenylethanoid glycosides as a possible COVID-19 protease inhibitor: a virtual screening approach

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Research Article

Keywords: Natural products, Protease inhibitors, Kinase inhibitors, MMPBSA, Molecular dynamics

DOI: https://doi.org/10.21203/rs.3.rs-165614/v1

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Abstract

From the beginning of pandemic more than 100 million people have been infected with a death rate higher than 2%. Indeed, the current exit strategy involving the spreading of vaccines must be combined with progress in effective treatments development. This scenario is sadly supported by the vaccine’s immune activation time and the inequalities in the global immunization schedule. Bringing the crises under control means providing the world population with accessible and impactful new therapeutics. We screened a natural product library that contains a unique collection of 2370 natural products into the binding site of the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) main protease (M\textsuperscript{pro}). According to the docking score and to the interaction at the active site, three phenylethanoid glycosides (forsythiaside A, isoacteoside and verbascoside) were selected. In order to provide better insight into the atomistic interaction and test the impact of the three selected compounds at the binding site, we resorted to a half microsecond-long molecular dynamics simulation. As a result, we are showing that forsythiaside A is the most stable molecule and it is likely to possess the highest inhibitory effect against SARS-CoV-2 M\textsuperscript{pro}. Phenylethanoid glycosides also have been reported to have both protease and kinase activity. This kinase inhibitory activity is very beneficial in fighting viruses inside the body as kinases are required for viral entry, metabolism, and/or reproduction. The dual activity (kinase/protease) of phenylethanoid glycosides makes them very promising anit-COVID-19 agents.

Introduction

The coronavirus disease (COVID-19) was declared a global pandemic by the World Health Organization (WHO) in March 2020. Of notice, the virus can infect humans and animal causing a wide range of diseases. The current exit strategy involving the spreading of vaccines must be combined with effective treatments. Bringing the crises under control means to provide the world population with accessible and impactful new therapeutics. The WHO solidarity trial of October 2020 reports remdesivir, hydroxychloroquine, lopinavir/ritonavir and interferon to have little to null effect on mortality and duration of the patient’s hospitalization. In addition, reinfection cases have been recorded and the common prevention rules were not successful to avoid COVID-19 second wave. Yet vaccines will not be sufficient to save lives on their own. Moderna and Pfizer two-dose vaccines imply the need for a second booster shot, whereas the J&J vaccine is expected to take approximately 29 days to build enough immunity [1]. Because the world-wide distribution will not be globally synchronized and equally organized the virus is expected to be defeated not earlier than one year from now at the very least. The lack of “herd immunity” translates in thousands of people risking COVID-19 associated death causes.

Even more importantly that time has come to face coronaviruses once and for all. Since the 1960s it is known that humans can be infected by alpha and beta coronaviruses [2]. SARS-CoV, MERS-CoV and, of course, SARS-CoV-2 all followed the same path involving animal-to-human transition. A virus mutating directly to humans through intermediary species is an event we won’t ever be able to get rid of. This underlines the need for therapeutics to indefinitely support our immune system against coronaviruses. In this context, the mechanism of choice for COVID-19 virus degradation is proteolysis. SARS-CoV-2 virus
has two proteases; main protease ($M^{\text{pro}}$) and papain-like protease ($PL^{\text{pro}}$). $M^{\text{pro}}$ plays a crucial role in viral replication which makes it an attractive target for anti-COVID-19 drug design [3].

The main function of protease –also known as proteinase or peptidase– enzyme is to breakdown the peptide bonds of proteins to form smaller polypeptides [4]. Protease enzymes have several functions ranging from food digestion to cancer signaling cascades. In addition, they are also vital for virus’s replication and the spread of infectious diseases such as COVID-19 [5]. Based on the protease enzyme active site mechanism it is possible to list up to 5 different proteases groups: aspartyl, cysteine, metalloprotease, serine and threonine. Their inhibitors can be small molecules or compounds containing one or more peptide chains, some of the inhibitors can interact with more than a single type of protease [4]. COVID-19 main protease is a cysteine protease, characterized by three domains, that catalyzes the breakdown of peptide bonds using histidine (His-41) and cysteine (Cys-145) residues [6]. The catalytic site that is located between domain I and domain II [7]. The main protease of SARS-CoV-2 was crystallized with a peptide inhibitor called N3. This peptide forms a covalent bond with the Cys-145 and inhibit the protease with half-maximal inhibitory concentration ($IC_{50}$) of 16.77 µM [8]. However, the non-covalent bond formed by non-peptide small molecules can have less off-targets side effects and toxicity. The most potent $M^{\text{pro}}$ inhibitor is ML188 (non-covalent inhibitor, $IC_{50} = 12.9$ µM) [9]. Due to the reason that there is no specific drugs against SARS-CoV-2, currently there are 16 antiviral drugs that are used against COVID-19, five of these drugs (lopinavir, ritonavir, darunavir, velpatasvir and ledipasvi) are HIV and hepatitis C protease inhibitors [10]. On the other hand, Weisberg et al. characterized more than 30 approved kinase inhibitors as potential antiviral agents by inhibiting important kinases required for viral entry, metabolism, and/or reproduction [11] and recently, a combination of baricitinib (kinase inhibitor) and remdesivir (antiviral) have been approved by the U.S. Food and Drug Administration (FDA) for hospitalized adults with Covid-19 [12]. Computer-aided drug design and in silico virtual screening are widely applied in the drug discovery field to rapidly identify a therapeutic solution. Virtual screening approach already proved to be a suitable tool to identify molecules against COVID-19. Mittal et al. identified small molecules (nelfinavir, and birinapant) and peptides (pepstatin A and Leupeptin Hemisulphate) as potential inhibitors of SARS-CoV-2 $M^{\text{pro}}$ [13]. Most of the previous in silico studies have focused mainly on repurposing approved drugs or screening synthetic libraries to target several important SARS-CoV-2 proteins. Our aim is to identify novel natural product scaffolds that can bind to the active site of SARS-CoV-2 $M^{\text{pro}}$.

In drug discovery, natural products represent a profitable source to find novel small molecules that can act against a series of biological targets. Thus, we screened a natural product library —containing unique collection of 2370 natural products— into the crystal structure of COVID-19 main protease. The result of this diversified screening batch highlighted only three potential molecules to target the COVID-19 virus. All these compounds are Phenylethanoid Glycosides (PGs). PGs are naturally occurring water-soluble small molecules. Their structure is characterized by a phenethyl alcohol (C6-C2) moiety attached to a $\beta$-glucopyranose/$\beta$-allopyparanose through a glycosidic bond. Therefore, PGs are associated to a wide range of biological activities, in fact kinase inhibitory activity, HIV and respiratory syncytial virus [14–17] are
among the most important targets of PGs. Isoacteoside and verbascoside are renown for potent antiviral activity against respiratory syncytial virus [14, 18].

In closing, the research findings presented herein are a combination of drug discovery and a half microsecond-long Molecular Dynamics simulation. We exploit atomistic simulations as a standard tool to investigate protein-ligand interactions and predict the binding free energy of each individual selected compounds with SARS-CoV-2 M\textsuperscript{pro}. The outcome of our extensive analysis highlights the differences and similarities of three potential PGs natural small molecules that can ultimately act as anti-COVID-19 therapeutics.

**Experimental**

**Molecular docking**

The 3D structures of COVID-19 main protease (6LU7) in complex with an inhibitor N3 was downloaded from the Protein Data Bank (www.rcsb.org) [8]. Molecular docking was performed using MOE program (2014.09). Hydrogen atoms and partial charges were added to the protein. Protein minimization was performed with the side chains kept rigid and the ligand flexible. The selected site was isolated and minimized followed by protonating the protein [19]. The natural product library (Catalog No.L1400) which is a collection of unique collection of 2370 natural products was used for docking. The 2D library was convert to a 3D database by energy minimization; forcefield (MMFF94x), gradient 0.001 RMS kcal/mol/Å2. The library was docked to the active site of COVID-19 main protease (6LU7.PDB) with scoring affinity London dG and GBVI/WSA dG.

**Ligand properties analysis**

SwissADME was used to analyze the properties of the three selected compounds. Canonical SMILES of the phenylethanoid glycosides were obtained from PubChem. SMILES were entered into SwissADME prediction server.

**Target identification**

Swiss Target Identification method was used to reveal the possible molecular targets of the three selected compounds [20, 21]. Canonical SMILES of the phenylethanoid glycosides were obtained from PubChem. SMILES were entered into Swiss target prediction server to predict their molecular targets and Homo sapiens was selected as a specie.

**Molecular dynamic**

The three selected protein/ligand complexes were subjected to molecular dynamics simulation for 500 ns using Gromacs 2020.2 [22]. Atomic charges of each ligand were assigned based on the AM1-BCC method of the AmberTools 19 (44) antechamber program. parmchk2 and tleap tools of the AmberTools19 package was also used to prepare the topology file of the ligands which was converted to gromacs readable format using ACPYPE tool. Gromacs tool (pdb2gmx) was employed to prepare the protein
topology parameters based on amber99sb force field. All the complexes were placed in the 96.31×96.31×96.31 Å cubic simulation box and solvated with 28662 molecules of tip4p water. Counter ions were also added to neutralize the systems. An energy minimisation step was done for each system using a steepest descent integrator for 2000 steps. The NPT ensemble was employed to run the simulations at 300K and 1 bar using the Velocity rescaling thermostat (0.1 ps time step) [23] and the Parrinello-Rahman barostat (2.0 ps time constant) [24]. leap-frog integrator was used with a time step of 2fs [25]. For short range coulombic interactions, a 10.0 Å cut-off was considered, while long-distance electrostatic interactions were calculated using the Particle-Mesh Ewald (PME) algorithm [26] with a Fourier grid spacing of 1.6 Å. Bonds to hydrogen were constrained using the Lincs algorithm [27]. Plumed v2.6.2 [28] and Gromacs 2020.2 [22] and packages were employed for the trajectory analysis. The molecular mechanics Poisson–Boltzmann surface area (MM/PBSA) method was employed to calculate the binding free energies of the protein–ligand complexes using g_mmpbsa software tool [29, 30]. The overall binding free energy of a protein–ligand complex is defined as follows:

$$\Delta G_{binding} = \Delta G_{elec} + \Delta G_{polar} + \Delta G_{vdw} + \Delta G_{non-polar}$$

where $\Delta G_{elec}$, $\Delta G_{polar}$, $\Delta G_{vdw}$, and $\Delta G_{non-polar}$ are the electrostatic energy, polar solvation energy, van der Waals energy, and nonpolar solvation energy, respectively.

Results And Discussion

PGs are biological active molecules with wide range of activities and they exist in many plant genus such as Veronica, Magnolia and Forsythia [15]. Out of 2370 natural products, we identify 3 PGs (Fig. 1) with high docking score which interact with amino acids at the binding site, including the catalytic residue His-41. Verbascoside had the highest docking score (-9.1317, RMSD = 1.51) followed by forsythiaside (-8.0889, RMSD = 2.10) and isoacteoside (-7.7302, RMSD = 1.68). The sugar part of forsythiaside A was able to bind to the catalytic residue His-41 and Asp-187 while one aromatic ring formed a hydrogen bond with Thr-190 and arene-H bond with Pro-168. Isoacteoside's sugars were able to form many hydrogen bonds at the binding site and one aromatic ring formed a hydrogen bond with Glu-166 and the second aromatic ring formed arene-H bond with Gln-189. Verbascoside formed 3 hydrogen bonds with the Thr-24, Asn-142 and Phe-140 and one arene-H bond with Gln-189 (Fig. 2).

To analyze the selected 3 phenylethanoid glycosides properties, SwissADME was used (Table 1) [31–33]. All the three molecules are soluble in water and have low gastrointestinal absorption. We used Swiss Model (TargetPrediction) to predict the biological targets of the selected 3 PGs. Swiss model predicted the possible molecular targets along with the actual targets. Targets are ranked according to a score that combines both 2D and 3D similarity values with the most similar known active molecules to the query molecule [20, 21]. As shown in Fig. 3, PGs exhibit kinase and protease inhibitor activity according to the Swiss model. It has been reported that isoacteoside inhibits the expression of protease protein; matrix
metalloproteinase 2, 12 and 13 with IC$_{50}$ around 10 µM [34]. Additionally, forsythiaside and verbascoside inhibit protein kinase C alpha with IC$_{50}$ 1.9 µM and 68 nM respectively [35, 36].

A recent study reported that protein kinase C alpha and beta inhibitors have a pro-apoptotic effect in nucleated cells, and this creates a hostile environment for intracellular parasites including viruses. This can enhance the antiviral activity of antivirus drugs [37]. The dual activity (kinase/protease) of PGs could make them very promising as anit-COVID-19 agents.

From molecular dynamic simulations we assessed the stability of the protein-ligand bond. A label was associated to each different natural small molecule to improve the readability of the data analysis. Table 2 describes the PGs nomenclature adopted herein.

| Compound Names | Clinical Trail | Lipinski's Rules | Structural Alerts | Pharmacokinetics | Water Solubility |
|----------------|----------------|------------------|-------------------|------------------|------------------|
| Forsythiaside A (CHEMBL504363) | - | 3 violations MW $>$ 500, N/O $>$ 10, NH/OH $>$ 5 | PAINS$^1$: 1 alert (Catechol A), Brenk$^2$: 2 alerts (catechol, Michael acceptor1) | Low GI absorption | Soluble; $i$LOGP$^3$ 2.15 |
| Isoacteoside (CHEMBL504873) | - | 3 violations MW $>$ 500, N/O $>$ 10, NH/OH $>$ 5 | PAINS: 1 alert(Catechol A), Brenk: 2 alerts (catechol, Michael acceptor 1) | Low GI absorption | Soluble; $i$LOGP 2.15 |
| Verbascoside (CHEMBL444478) | Phase II | 3 violations MW $>$ 500, N/O $>$ 10, NH/OH $>$ 5 | PAINS: 1 alert(Catechol A), Brenk: 2 alerts (catechol, Michael acceptor 1) | Low GI absorption | Soluble; $i$LOGP 2.15 |

$^1$Pan-assay interference compounds, $^2$Brenk alerts to toxic groups, $^3$Octanol/water partition coefficient.
As shown in Fig. 4, the root-mean-square deviation (RMSD) measurement was carried out on each of the three ligands. Interestingly each ligand reaches a steady configuration at different times within approximately 50 ns. However, L1 stands out for reaching a very steady configuration with respect to the other ligands. This implies a lower noise and mean value of the L1 RMSD. In the Supporting information we are showing that also the protein’s RMSD is associated to lower noise when interacting with L1 (Fig. S1).

To further characterize the system, we decided to compute the root mean square fluctuation (RMSF) of atomic positions in the trajectory. In Fig. 5 we consider a subset of residues in the active site corresponding to the “pocket” of interaction. Here, it is evident that different ligands interact in different characteristic ways. There are different moieties of the pocket, highlighted in Fig. 5, where the fluctuation of the atoms depends strongly on the binding ligand. However, the fluctuations in the backbone of the active sites are always limited to less than one Angstrom. Which means that none of the three scenarios implies a detrimental ligand-protease interaction.

The ligand themselves interact with the active site mainly by means of their aromatic moieties. In order to distinguish the two aromatic rings, characteristic of each ligand, we considered their bonding to the central THF heterocyclic ring of Fig. 1. Ring A is defined as the one connected to the central THF heterocyclic ring via a carboxylic group; whereas ring B is not connected via a carboxylic group. Recording the RMSF of the aromatic moieties, reported in Fig. 6, L1 turns out to be the most stable compound of the three. In Fact, L2 and L3 both show one of the aromatic rings to be fluctuating to twice the extent of the other which allows L2 and L3 to explore different configurations.

This speculation is backed up by the gyration radius data (Figure S2) of the ligands and the distance between the center of mass of ring A and B. Such a distance, shown in Fig. 7, is indicative of the L1 aromatic rings constraining. Nonetheless, Fig. 7 shows the relative angle between the aromatic rings. This clarifies that L1 does not explore as many configurations as the other ligands, owing to its enhanced bond stability. At this point, a mere analysis of the average number of contacts between the ligands and the active site. Figure S3, is not sufficient to determine which structure would be more reliable. Detailed contact map is presented in Figure S4. Binding free energy calculations allow the measurement of binding strength in protein–ligand or protein–protein complexes [38, 39].

| Compounds     | Associated Label |
|---------------|------------------|
| Forsythiaside A | L1               |
| Isoacteoside  | L2               |
| Verbascoside  | L3               |
Table 3
MMPBSA energy calculated from the last 400 ns of MD simulation.

|       | Van der Waal energy (kJ/mol) | Electrostatic energy (kJ/mol) | Polar solvation energy (kJ/mol) | SASA energy (kJ/mol) | Binding energy (kJ/mol) |
|-------|-----------------------------|-------------------------------|---------------------------------|----------------------|------------------------|
| L1    | -253 ± 24                   | -64 ± 25                      | 221 ± 27                        | -25 ± 1              | -121 ± 19              |
| L2    | -246 ± 24                   | -109 ± 22                     | 279 ± 23                        | -25 ± 2              | -101 ± 22              |
| L3    | -174 ± 25                   | -69 ± 35                      | 211 ± 45                        | -21 ± 2              | -53 ± 18               |

The molecular mechanics of the MM/PBSA method, even though is not as accurate as more computationally intensive methods such as thermodynamic integration [40], has been shown to be satisfactory for the work of both computational and experimental researchers. Last 100 ns of the MD trajectories was used for the binding free energy calculations for protein/ligand complexes and the result reported in Table 3. According to the result, L1 has a statistically significant lower binding energy in compare with other ligands.

Conclusion

COVID-19 protease inhibitors should be able either to form hydrogen bonds or have a hydrophobic interaction with one of the two catalytic residues; Cys-145 or His-41. Out of 2370 unique natural products library we identified three PGs as a SARS-CoV-2 M<sub>pro</sub> inhibitors by means of molecular docking. Forsythiaside A was the only molecules among the three PGS to be able to interact with the catalytic residue His-41. Analyzing molecular dynamic trajectories of the selected molecules at the binding site, we identified forsythiaside A as the most stable one. In fact, this compound is characterized by the steadiest gyration radius and RMSD. The constant relative angle and distance between the aromatic moieties of forsythiaside A represent further evidence of indisputable stability. According to our extensive MD analysis and the resulting binding free energy this molecule is likely to possess the highest inhibitory effect against SARS-CoV-2 M<sub>pro</sub>. Of notice, PGs have been reported to show both protease and kinase activity. Because kinases are required for viral entry, metabolism, and/or reproduction the inhibitory activity is very beneficial in fighting viruses inside the human body. In fact, the dual activity (kinase/protease) of PGs shall then make them very promising anti-COVID-19 agents.

Declarations

Funding: N/A

Conflicts of interest/Competing interests: N/A

Availability of data and material: N/A

Code availability: N/A
Authors’ contributions: O.B. conceived the project. O.B. performed the virtual screening, target prediction and ADME analysis. M.R.G. performed the Molecular dynamic. M.B. analysed the molecular dynamic data. O.B., M.R.G and M.B wrote and subsequently revised the manuscript.

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Figures

![Forsythiaside A](image1)
![Isoacteoside](image2)
![Verbascoside](image3)

**Figure 1**

Two-dimensional structures of the selected phenylethanoid glycosides.
Figure 2

Two-dimensional ligands interaction in the binding site of COVID-19 main protease (Mpro) (6LU7.PDB).
Figure 3

Consensus molecular targets of the selected 3 phenylethanoid glycosides
Figure 4

RMSD of the ligands during the 0.5 microsecond molecular dynamics simulation

Figure 5

RMSF of the protein's backbone in pocket site
Figure 6

RMSF of the characteristic aromatic rings (ring A and ring B)

Figure 7

Distance and angle between the center of mass of the aromatic rings.
Supplementary Files

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