Structure Based Screening Of Fungal Bioactive Metabolites As Potential Inhibitors Of Main Protease (M^{pro}) Of SARS-CoV-2

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Abstract

The pandemic disease COVID-19 has put the world into a massive threat. Till now no effective treatment has been established and the world is applying lockdowns as a preventive measure against this. Recent studies have identified that COVID-19 main protease (M^{Pro}) is essential for its life cycle and is responsible for the proteolytic mutation of this virus. Thus, destruction of the complete viral particle can be achieved through inhibition of this main protease. The fungal metabolites have already been proved as potential antimicrobial agents hence were selected for in-silico molecular docking, to check their affinity towards COVID-19 main protease. Metabolites like 2H-oxecin-3-one,3,4,7,8,9,10hexahydro-4-methoxy-10-methyl, Eicosanoic acid phenylmethyl ester, HEPES, 2,4-dihydroxy,2,5-dimethyl-furan(3)one and Dodecanoic acid-3-hydroxy have revealed strong binding affinity towards the main protease. Thus, the present study enlightens a path to combat COVID-19 by the use of bioactive metabolites.

Introduction

In 1965, two scientists Tyrell and Bynoe discovered the first human coronavirus named B814 (Tyrell and Bynoe, 1966). With the onset of the 21st century as many as 5 new types of human coronavirus were identified with SARS (Severe Acute Respiratory Syndrome) coronavirus considered as the most harmful till the end of 2019 (Khan et al,2020). The end of 2019 witnessed the emergence of the deadliest and extremely contagious new coronavirus, officially termed as SARS-CoV-2 or Novel Coronavirus (Mittal et al, 2020). A rapid outbreak of pneumonia like illness was identified in Wuhan city, Hubei province, China (Anderson et al,2020, Wu et al, 2020), later coined as COVID-19(Corona Virus like Disease-19) (Al-Khafaji et al,2020). The community transmission was first confirmed in Guangdong province, China (Chen et al, 2020). World Health Organization has declared this as global pandemic with more than 150 countries being affected. The progressing lockdown in most of the countries and the extreme contagious nature of the virus has left the scientific community to rely on the in-silico approach as this is the most feasible and possible way which can save precious time and also give some insights and hope for a novel drug discovery effective against this deadly virus.

SARS-CoV-2 belongs to Sarbecovirus genus or β -coronavirus subgenus, generally identified as enveloped virus containing single positive stranded RNA (Zhou et al 2020, Chan et al 2020, Licastro et al 2020, Lu et al, 2020). The genome of SARS-CoV-2 is the largest among all other RNA virus reported so far (Elfiky, 2020). The principle function of the genome begins after its infection into the host cell. The host infection is performed by the angiotensin converting enzyme 2 ie ACE2 receptors (Babadei Nejadi et al,2020). The single stranded RNA acts as messenger RNA and synthesizes two long polypeptides, polyprotein1a and polyprotein 1ab. These translated polypeptides then in turn code for new viral particles (Boopathi et al, 2020). The polypeptides also code for several mRNAs, structural proteins and two essential proteases that perform the cutting of polypeptides into other more important functional slices (John et al,2015, Cui et al,2019).

Recently, various strategies have been designed for the drug development for SARS-CoV-2, structure based virtual screening being the most likely (Krishnan et al,2020). Many researchers have already screened the already available databases for potential compounds. Docking studies have also been effectively employed for screening small molecules against the major proteins of COVID-19 (Benitez- Cardova et al, 2020, Enmozhi et al,2020). Scientists worldwide have analysed through experimental findings three main drug targets for SARS-CoV-2 which includes the RNA dependant RNA Polymerase (RdRp), the Angiotensin -converting enzyme 2 entry receptor (ACE2) and the main protease (M^{Pro}) (Borgio et al,2020). The COVID-19 main protease (M^{Pro}) is an essential protease and plays key role in viral proteolytic mutation (Jin et al, 2020). Thus, disruption or blocking of this main protease can led us towards effective drug development for this deadly virus (Gupta et al,2020). The drug molecules targeted towards ACE2 and RdRp have exhibited lower potency and significant side effects (Cameron and Castro, 2001). The M^{Pro} has emerged as the most promising drug targets for SARS-CoV-2 (Blanchard et al,2004).

In recent years fungal world has gained worldwide attention as producers of some immensely valuable metabolites that have also been found effective against variety of pathogens (Shrivastava and Saraf, 2019). Our study thus focuses on employing these metabolites for drug designing against SARS-CoV-2 through structure based virtual screening.

Materials And Methods

Fungi are repertoires of a large variety of secondary metabolites, the most important being penicillin, lovastatin, β -lactam antibiotics and a cholesterol lowering drug (Boruta, 2018). However, production of pharmaceutically important lead compounds can be better achieved by altering the standard growth conditions which in turn trigger the associated biosynthetic genes. Scientists have already studied the effect of co-cultivation of fungi with *Lactobacillus* (Strom, 2005). Similar strategy has been applied in our work.

Production and characterization of fungal secondary metabolites- The production of fungal secondary metabolites was done according to the steps described by Shrivastava et al (2017). The media was supplemented with milk, hence providing the altered growth conditions in presence of *Lactobacillus*. Two fungal strains were procured from MTCC, IMTECH, Chandigarh, India to serve as control, namely *Aspergillus flavus* (MTCC 2725) and *Penicillium chrysogenum* (MTCC 3380). The GC-MS analysis was used for the identification of bioactive molecules. A total of 16 molecules were identified as potential lead compounds and were then employed for molecular docking.

Target Selection- The three-dimensional structure of SARS-CoV-2 main protease (6LU7) with resolution 2.16 Å was collected from Protein data bank (Jin et al, 2020). The target protein was pre-prepared with Autodock v4.2.6. The non-bonded and water atoms were deleted and partial charges were added.

Ligand Preparation: The GC-MS analysis of fungal secondary metabolites revealed 9 druggable compounds. The SDF files were converted to mol and mol2 files and were prepared for docking. The 3d conformations were determined through ChemSketch (ACD Labs).

Drug Likeness Prediction:- The drug likeness property is predicted to check the pharmacological properties and their ability to form a standard drug. The druggability of the compounds were checked through DruLito software (an open access software developed by the Department of Pharmacoinformatics, NIPER, India) and were confirmed through SwissADME Predictions. In addition to this, for drug designing PAINS Alert consideration is necessary. Pan-assay interference compounds (PAINS) are chemical compounds that during high throughput screening, frequently give false positive results (Dahlin et al, 2015).

Molecular Docking:- AutoDock v4.2.6 (Trott and Olsen, 2010) tools have been used for the virtual docking of various ligands to the target protein. The protein was prepared by deleting the water molecules and adding partial charges. The active site was determined by deleting the ligand N3. A grid box with co-ordinates X = -19.09, Y = 21.93 and Z = 67.924 was generated and all the compounds were docked onto this active site of the target protein.

Molecular Dynamics Simulations: MD simulation was performed for the analysis of the dynamic properties and conformational flexibility of protein and selected docked ligand into the target site. The protein-ligand complex giving the best docked score was selected for the MD simulation run. GROMACS 2018.1 package was used to run and analyze 100 ns MD simulation. GROMOS96 43a1 (force field) in single point charge (SPC) water models was used to generate protease and ligand force field and parameter files for the protein and PRODRG server for ligand respectively. The system was then solvated with water molecules in separated cubic boxes with 10 Å distance from the edge of the box. The system was neutralized by adding 4 Na^+ counter ions. The energy minimization the system was performed through running the steepest descent minimization algorithm with 50000 steps to achieve stable system with maximum force $< 1000 \text{ kJ mol}^{-1} \text{ nm}^{-1}$. Prior to the running of real dynamics, the solvent and ions of the system was equilibrated by NVT (constant under number of particles, volume, and temperature) and NPT (constant number of particles, pressure, and temperature) ensemble. After the completion of the MD simulation run, the trajectories were used for various dynamics analysis such as root mean square deviation (RMSD), root mean square fluctuation (RMSF), radius of gyration (Rg), number of hydrogen bonds, etc.

MM-PBSA Calculations: Docking and scoring are the most widely used computational methods for drug designing estimating the binding affinity followed by the binding energy between the receptor and ligand. This approach sometimes cannot be particularly accurate (Genheden and Ryde, 2015). Hence MD Simulations combined with MM-PBSA (Molecular Mechanics/Poisson-Boltzmann Surface Area) calculations is performed to compute interaction energies and to study biomolecular complexes

(Kumari et al, 2014). The MM-PBSA binding free energy, the change in potential energy in vacuum, Vander-waals and electrostatic interaction and polar and non-polar solvation energies of receptor-ligand docked complex was estimated by using this technique.

Results

Identification of bioactive metabolites isolated from fungal colonies: The majority of bioactive metabolites were isolated from 2 main fungal colonies namely *Acremonium cellulolyticus* and *Aspergillus chevaleri*. In addition the positive control fungal species also produced metabolites. The individual metabolites revealed via GC-MS analysis are as follows:-

From *Acremonium cellulolyticus*- 6-Acetyl- β -D-mannose, 2-Furancarboxaldehyde-5 methyl, HEPES, Desulphosinigrin.

From *Aspergillus chevaleri* - 2,5-Dimethyl-4-hydroxy-3(2H)-furanone.

From *Aspergillus flavus* (MTCC 2725)- Eicosanoic acid- phenylmethyl ester and Tetraacetyl-d-xylonic nitrile.

From *Penicillium chrysogenum* (MTCC 3380)- Dodecanoic acid-3-hydroxy, 2-H-Oxecin-2-One,3,4,7,8,9,10hexahydro-4-methoxy-10-methyl.

Drug Likeness Properties: The metabolites were scanned for drug likeness properties through the Lipinski's Rule of 5. Although there are few violations but the compounds can be considered as potential candidates for drug designing. All the compounds listed have 0 PAINS alert and hence the chances of false positives can be considered nil. The detailed analysis is represented in Table 1.

Molecular Docking: A total of 9 bioactive metabolites were docked into the active site of SARS-CoV-2 main protease (M^{Pro}). The interactions between receptor and ligand showed that all the compounds have substantial affinity towards the target. The native ligand N3 interacted with His41, Thr 26, Ph3 40, Asn 142, cys 145, His 143, Met165, Glu166, Arg188, Ser144, Thr25, Ser146, Leu27 residues of the target. Majority of the fungal metabolites docked interactions matches with N3, hence the docking can be considered successful proving them to be potential inhibitors of the Main protease. The detailed analysis is depicted in table 2. The docked compounds were visualised using PyMol software (Shrodinger Ltd.) (Figure 3)

MD Simulations: The complex 6lu7-2H-oxecin-3-one,3,4,7,8,9,10hexahydro-4-methoxy-10-methyl was selected for the MD-simulations as the binding energy for this complex was found to the highest among all the analyzed complexes.

1. Root mean square deviation (RMSD)- The RMSD analysis denotes the system's dynamic stability and the conformational changes occurring during the simulations run. The compound 2H-oxecin-3-one,3,4,7,8,9,10hexahydro-4-methoxy-10-methyl's trajectory has got the equilibrium state after 80 ns. The average RMSD value was found to be 0.125 nm. The RMSD analysis of the protein in its apo form showed convergence after 75ns and an average RMSD value as 0.215 nm (Represented in figure 19 and 20).
2. Radius of gyration (Rg) analysis- The Rg analysis describes the compactness of receptor-ligand complex during the simulations. Less variation in the Rg value indicates the compact structure of the receptor-ligand while a high variation in the Rg value indicates the unstability or less compactness of the structure. In this study the Rg value was found to variate from 1.945 nm to 1.967 nm (Represented in figure 21).
3. Root mean square fluctuation (RMSF) analysis- The RMSF analysis determines the flexibility and mobility of the structure. In this study the RMSF value of protein and ligand complex was found to be around 0.1 to 0.4 nm reaching a peak at 0.397 nm.

From the above analysis it can be concluded that the protein ligand structure exhibited less conformational changes and can act as a stable complex.

MM/PBSA Binding Free Energy calculations- The binding energy, Vander-waals energy, Electrostatic energy, Potential energy and polar and non polar solvation energies of the complex are as represented in Table 3.

The binding energy calculated as -978.405 KJ/mol can be considered as a good score confirming a stable binding affinity of ligand towards the protein's active site. Other analyses like the Electrostatic energy and Vander waals energy contribute highly towards the stable binding. Also, among the two the contribution of electrostatic interaction is more than the Vander waals contribution.

Discussion

Compound 6acetyl β -d mannose has exhibited potential docking against the target protein with binding energy -4.29 kcal/mol. The compound is also reported from extracts of *Aspergillus fumigatus* and is also proved potent against various micro-organisms (Al-Jassaini et al, 2016).

Compounds like 2H-oxecin-3-one, Dodecanoic acid-3-hydroxy and Eicosanoic acid phenylmethyl ester have exhibited binding energies of -6.43 kcal/mol, -4.6 kcal/mol and -5.25 kcal/mol respectively. These compounds have also been identified as potent phytoconstituents of important medicinal plants like *Withania somnifera* and *Smilax china* (Vijaybaskar and Elango,2018).

The compound 2 Furancarboxyldehyde-5-methyl is also found in *Citrum annum*, brown algae and other plant sources but is not reported from fungi. Hence it can be considered that alteration in supplement media with *Lactobacillus* has resulted in extraction of production of some compounds that were previously not reported from fungi (source PubChem database). Similarly, the compound Tetracetyl-d-xylonic nitrile have also exhibited considerable docking against the target protein but has not yet been isolated from any fungi.

The present structure based screening and molecular docking of fungal secondary metabolites have proved them to be effective inhibitors of proteins causing COVID-19. Some of the metabolites can also be isolated from various other sources and few have been isolated only in this study. This confirms that altering the growth conditions can result in production of some bizzare compounds. These compounds also showed a stable conformation during the MD simulations run. The values obtained were better than that obtained through the native N3 ligand complex as observed by Das et al (Das et al, 2020). The ADME calculations of all the predicted ligands have revealed that they are non- toxic and can be used for medicinal purposes against this deadly Novel Corona virus after proper preclinical trials.

Declarations

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Competing interests- Author 1 declares that he/she has no competing interest. Author 2 declares that he/she has no competing interest. Author 3 declares that he/she has no competing interest.

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Tables

Table 1: Drug Likeness Properties of fungal secondary metabolites.

Name	Molecular formula	Molecular weight (<500Da)	logP(<5)	No. of Hydrogen bond Acceptor(<10)	No. of Hydrogen bond donors (<5)	Lipinski's violation	PAINS Alert
6-acetyl-β-d-mannose	C8H14O7	222.19	0.87	7	4	0	0 alerts
2,4-dihydroxy,2,5-dimethyl-furan(3)one	C6H8O4	144.13	1.2	4	2	0	0 alerts
2H-oxecin-3-one,3,4,7,8,9,10hexahydro-4-methoxy-10-methyl	C10H16O3	184.23	2.02	3	1	0	0 alerts
Desulphosingrin	C10H19NO6S	281.33	0.79	7	6	1	0 alerts
Dodecanoic acid-3-hydroxy	C12H24O3	216.32	2.68	3	2	0	0 alerts
Eicosanoic acid phenylmethylester	C27H46O2	402.65	5.98	2	0	1	0 alerts
HEPES	C8H18N2O4S	238.3	0.79	6	2	0	0 alerts
2H-furancaboxyldehyde5methyl	C44H62N9O9P	891.99	5.86	15	8	3	0 alerts
Tetra-acetyl-d-xylonic nitrile	C14H19NO9	345.3	2.19	10	0	0	0

Table 2: Binding Energies and Ligand Interactions of Fungal Metabolites.

Name	Binding Energy (kcal/mol)	Interacting amino acid residues
2H-furancaboxyldehyde5methyl	-4.42	Asn142, Leu141, Gly143, Phe140, Ser144, Gly145
Desulphosingrin	-3.48	Leu141, Glu166, Asn142, Phe140, Met165, His172, His163, Val3
2H-oxecin-3-one,3,4,7,8,9,10hexahydro-4-methoxy-10-methyl	-6.43	Cys145, Ser144, Asn142, Phe140, Leu141, His172, Glu166
Dodecanoic acid-3-hydroxy	-4.6	Asn142, Gly143, His163, Leu27, Thr26, His41, Leu4
Tetra-acetyl-d-xylonic nitrile	-3.27	Asp153, Asn151, Gln110, Thr111, Gln107, Val104, Ser158
HEPES	-5.14	Glu166, Ser144, Phe140, His164, Leu4
Eicosanoic acid phenylmethylester	-5.25	Thr45, Thr24, Gly143, Ser144, Asn142, Met165
6-acetyl-β-d-mannose	-4.29	Pro122, Cys69, Met17, Cys16, Asn119, Gly71, Gln69, Ser121, Trp31, Val18
2,4-dihydroxy,2,5-dimethyl-furan(3)one	-4.97	Gly143, Met165, Glu166, Ser144, Cys145, Asn142, Leu141, Phe140.

Table 3: MM-PBSA calculations

Vander Waals energy	:	-236.240±7.230 KJ/mol
Electrostatic energy	:	-2150.392±36.690 KJ/mol
Polar solvation energy	:	1653.312±7.636 KJ/mol
SAV energy	:	-245.086±14.246 KJ/mol
Binding energy	:	-978.405±32.491 KJ/mol

Figures

Figure 1

SARS-CoV-2 Main protease bound with ligand N3 (Apo form). (PDB id: 6LU7)

Figure 2

Photo plate showing different secondary metabolites isolated from fungi

Figure 3

Photo plate showing the docking patterns of the isolated metabolites with 6LU7

RMSD analysis of the docked system

RMSD analysis of protein in its apo form

Radius of gyration analysis (Rg)

Root mean square fluctuation analysis (RMSF)

Figure 4

Photo plate showing the MD simulations result