RESEARCH ARTICLE

Molecular Studies of Antiviral Drug Atazanavir and Hyaluronic Acid-Atazanavir conjugate as Novel Drugs to Target SARS-CoV-2 Viral Proteins

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ABSTRACT Several antiviral drugs and their inhibitory effects against Severe Acute Respiratory Syndrome Coronavirus - 2 (SARS CoV-2) were studied by using *in silico* studies. Atazanavir (ATZ) and its Hyaluronic Acid conjugated drug (HA-ATZ) are potentially inhibiting the viral replication and the present study elucidates the binding efficiency of the selected drugs on the Mpro (6M03), PLpro (6W9C), and ACE-2 (1R42) of SARS CoV-2. The crystallographic structure of Mpro, PLpro and ACE-2 available in the Protein Data Bank are the promising targets used for the present investigation. Autodock and cygwin tools are used to perform these docking experiments. The mean binding values observed in the ATA-HA trial with PLpro showed a minimum binding energy of -9.3 kcal/mol. Hyaluronic acid, an anionic polysaccharide appeared with CD44 receptors used to form conjugate the drug Atazanavir and specifically targeted drug delivery. Pharmacokinetic properties and docking scores were found to be favorable for the conjugated drugs. It is evidenced that the newly proposed conjugated drug can be used for further studies to improve the potential targets to control SARS-CoV-2.

KEYWORDS: SARS CoV-2, Mpro, PLpro and ACE-2, Atazanavir, Atazanavir conjugated with Hyaluronic acid, Molecular Docking, drug delivery

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1. INTRODUCTION

In early December 2019, several local health facilities first reported pneumonia cases of unknown origin in Wuhan, China. This new coronavirus infectious disease (COVID-19) caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), was first reported on December 1, 2020, and identified as a previously unknown beta coronavirus [1]. COVID-19, a viral disease was initiated in February 2020 and accelerated in the beginning of March 2020. Globally, COVID-19 has caused serious health, social, trade, and economic setbacks. In response to this severe condition, it is a vital challenge for scientists to invent appropriate drugs and vaccines to keep the pandemic under control. They have produced several COVID-19 drugs and vaccines, but there is no definite prescription of drugs for the disease. Coronaviruses (CoVs) are a large group of viruses enveloped with positive-sense, single-stranded RNA genomes. SARS-CoV-2 is the seventh type of coronavirus [2]. The most common symptoms reported were fever (83%), cough (82%), and shortness of breath (31%). Other symptoms include fatigue, muscle or body aches, a new loss of taste or smell, and nausea [3].

The coronaviruses are divided into four genera: alpha, beta, gamma, and delta. The alpha and beta coronaviruses are causing only common cold-like illnesses [4]. But in contrast, SARS-CoV and MERS-CoV are posing life-threatening diseases. The World Health Organization (WHO) has reported that SARS-CoV emerged during 2002-2003 in China and

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rapidly extended around the world, causing more than 8,000 infections and nearly 800 deaths. Initially, the virus infection starts with the binding of viral particles to the cellular receptors of the host surface. The receptors of the human coronaviruses are proteinaceous peptidases. Specifically, human aminopeptidase N (hAPN) is the receptor for HCoV-229E and human dipeptidyl peptidase 4 (hDPP4) for MERS-CoV [5]. SARS-CoV and SARS-CoV-2 enter the cell through the interaction of human angiotensin-converting enzyme 2 (hACE2). The phylogenetic analysis revealed that the bats might be the source of SARS-CoV-2; CoVs belong to the order Nidovirales and the family Coronaviridae, and the bat coronavirus sequence is 96.2% similar to the human coronavirus. In the genomes of SARS-Cov-2, it was found that there are three variants by distinguished amino acid changes, of which two types are significant outside East Asia and one other type is common in East Asia [6].

Alpha variant, or B.1.1.7 was found in the United Kingdom, and it comprises seventeen mutations, of which the maximum number occurred in spike protein [7]. A beta variant or B.1.351 with multiple mutations in the spike protein was responsible for the second wave of the pandemic situation observed in South Africa. The next variety is P.1, or gamma variant noted in Brazil, which also has spike protein mutations [8]. The delta variant or B.1.617.2 was detected in India and found to be the reason for the deadly second wave of the pandemic during April 2021 in India [9]. The variant B.1.1.529, or omicron, was initially recorded in South Africa. The spiky protein of omicron has more than thirty changes, and it increases the spreading rate [10].

The genome structure of coronaviruses is well known among RNA viruses. Two-thirds of the viral genome has ORF1ab encoded with replicase polyproteins, and the remaining has genes encoded with structural proteins like spike (S), envelope (E), membrane (M), and nucleocapsid (N) proteins. ORF1ab has enzymes like papain-like protease (PLpro), 3-chymotrypsin-like protease (3CLpro), RNA-dependent RNA polymerase (RdRp), and helicase (Hel). There is a huge variation observed in the genome by four nonstructural proteins present in ORF1a and ORF1b [11].

Hyaluronan also known as Hyaluronic Acid (HA) is a naturally occurring polysaccharide of a linear repeating disaccharide unit consisting of β-(1→4)-linked D-glucopyranuronic acid and β-(1→3)-linked 2-acetamido-2-deoxy-D-glucopyranose, which is present in extracellular matrices, the synovial fluid of joints, and scaffolding that comprises cartilage. Despite the simplistic structure of hyaluronan, it behaves quite differently from other glycosaminoglycans in its mechanism of synthesis, its size, its physico-chemical properties such as the network-forming, viscoelasticity, and its charge characteristics, important to many biochemical properties of living tissues. It mediates its biological functions through specific protein receptors present on the different cell surfaces, which include CD44 (12), HARE (13), RHAMM (14), and LYVE (15).

Low molecular weight drug Atazanavir, suffer from some limitations, for example, they have limited solubility in water, lack in specificity, and exhibit side effects due to their indiscriminate distribution in the body and short in vivo half-life, which could limit their potentials in the clinic. With a view to overcome these limitations, we propose to covalently link it with hyaluronic acid (HA), which is ubiquitous in our body and is biocompatible and plays important role in our biological system. The proposed new conjugates (HA-ATZ) would overcome most of the above mentioned problems that are associated with the respective free drug molecules.

In this scenario, the proposed HA-ATZ conjugate and Atazanavir are carried out separately through molecular docking studies to understand their inhibition against three viral proteins Mpro, PLpro and ACE-2. Further, the efficiency of hyaluronic acid- Atazanavir drug conjugate and free drug for the novel treatment of COVID-19 is discussed.

2.0 MATERIALS AND METHODS

Materials and Methods
Ligand Generation

Atazanavir is a Food and Drug Administration (FDA)-approved antiviral drug for the HIV-1 treatment. It is a potential azapeptide proteinase inhibitor that prevents the generation and multiplication of virions. Anti-HIV activity of Atazanavir has been observed in the range of 2–5nM with MIC50 during cell culture studies. Oral administration of Atazanavir revealed rapid absorption, increased bioavailability, and reduced pharmacokinetic variability [16]. Atazanavir showed binding

conformations in X-ray crystallographic studies with respect to proteases by means of its molecular structure flexibility [17]. Atazanavir (ATZ), an antiviral drug, and its Hyaluronic acid conjugate, Hyaluronic acid-Atazanavir (HA-ATZ), were drawn in ACD/ChemSketch and the structure saved in mol file format (Figure 1a&b). The 2D mol file format of the ligand structures retrieved was submitted to the "Online SMILES Converter and Structure File Generator," and the same was converted into 3D PDB format and used for *in silico* analysis.

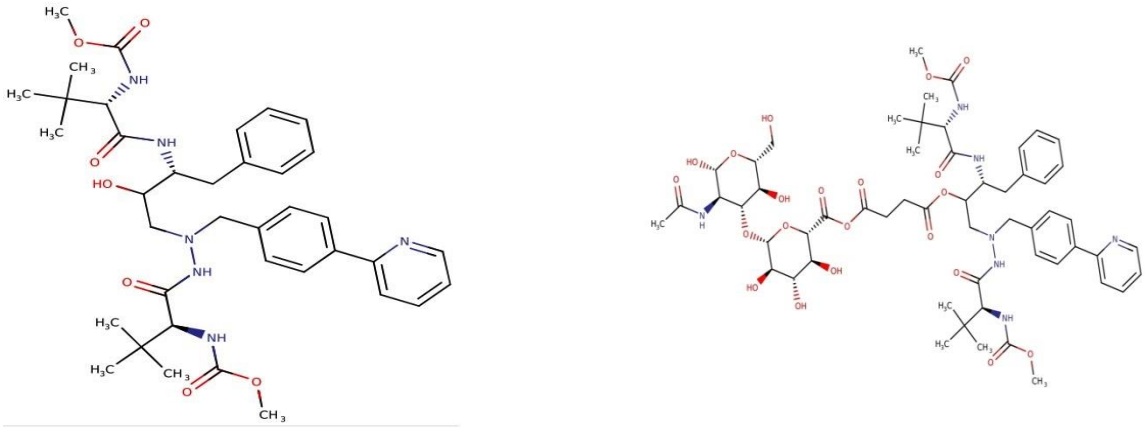

Figure 1 Structure of (a) Atazanavir and (b) HA-Atazanavir Conjugation

2.1. Analysis of Pharmacokinetic Profile

The prediction of small-molecule pharmacokinetic properties (pkCSM) online server was utilized to calculate the metabolic, excretion, and toxicity profile scores of the antiviral drug, and its hyaluronic acid-conjugated drug is exhibiting the importance of "Hyaluronan Drug Delivery (HDD)" technology in the discovery of new drugs. Hyaluronic acid is found to be one of the important components noted in the extracellular matrix (ECM), recorded as RHAMM (receptor for hyaluronan-mediated motility) and the ligand of the glycoprotein CD44 receptor (cluster of differentiation 44). In the present study, hyaluronic acid as a carrier of the drug was used in the new drug development. Atazanavir - Hyaluronic acid (ATZ-HA), a drug-carrier conjugate form, is bioavailable, stable, and less toxic, as confirmed by its pharmacokinetic properties. The drug and the conjugate form of the drug show many differences in structure and molecular weight and give rise to different computational results.

2.2. Preparation of Receptor and its Binding Site

Three target proteins important for the life cycle of SARS-CoV-2, including Mpro (PDB ID 6M03), PLpro (PDB ID 6W9C), and ACE-2 (1R42), play a crucial role in the viral replication and multiplication of SAR-CoV-2 viruses. These protein targets were found to be a new target to control the life cycle of SARS-CoV-2 in host cells. The 3D crystal structures of the target proteins Mpro, Plpro, and ACE-2 were retrieved from the RCSB PDB database (https://www.rcsb.org/).

2.3. Molecular docking

Docking studies were performed by AutodockVina (version 4), and the calculations were performed by Auto-dock tools. Three potential target proteins of SARS-CoV-2 and two compounds, viz., Atazanavir (ATZ) and Atazanavir - Hyaluronic acid (ATZ-HA), were used for the docking studies. Auto-grid is used to calculate the grid map for docking protein-binding pockets. The size of the grid for points x, y, and z was set for the protein targets of the virus. Other parameters of docking, such as population size (150), docking assessment (~100 times), evaluation of energy (maximum of 250,000) generations (maximum of 27,000), mutation rate (0.02), cross-over rate (0.8), and other default parameters of docking, were set by using the Auto Tor utility present in the auto dock tool. The docking results and 2D interaction plot of the target protein of the virus with the ligands were observed using the receptor-ligand interaction noted in Discovery Studio visualizer 2.5.

Density Functional Theory (DFT) Analysis

The DFT analysis performed using the Becke-3 Parameter-Lee-Yang-Parr (B3LYP) and 6-311** basis sets [18], was employed to optimize the molecule using the program package Gaussian 09 [19]. The output data, like the optimized geometry, HOMO-LUMO, and MEP, were visualized through the Gauss View 6 graphical interface. Natural bond orbitals (NBOs) were calculated by utilizing all interactions observed between filled donor and empty acceptor natural orbits, and their importance in terms of energy was predicted using second-order perturbation theory. The NBO program available in the Gaussian 09 package was utilized for the calculation of NBO.

RESULTS AND DISCUSSION

Coronaviruses are a group of viruses that pose a potential threat to the human population. They can infect the respiratory, digestive, and nervous systems of human beings and animals [20]. The present molecular study gives scope to find out a new target that potentially binds to the SARS CoV-2 main protease. The CoV Mpro is vital for the virus during the proteolytic maturation cycle, and it is found to be an appropriate target to prevent the infection caused by the virus and inhibit its polyprotein cleavage. The replication of coronaviruses is mediated by the presence of proteases, and it has been identified that the amino acids His 41, Cys 145, Glu 166, and Asn 142 are involved in the drug interactions. In the majority of viruses, proteases play a prominent role in the replication of viruses, and proteases are the main target during the development of antiviral drugs and therapeutic processes [21].

Molecule Transformer-Drug Target Interaction (MT-DTI) is a drug-target interaction model trained by deep learning methods that results in the finding that Atazanavir, the antiretroviral drug, shows maximum inhibitory potential against the main protease of SARS CoV. We provide supporting evidence based on our molecular studies for Atazanavir, a progressive protease inhibitor, to control the activity of the main protease of SARS-CoV-2. The inhibition potential of this drug is to disrupt viral replication and cytokine storm-associated mediators linked to impaired immune function during viral infection.

Analysis of pharmacokinetic profile

Drug design and discovery can be performed based on the results of ADMET (Absorption, Distribution, Metabolism, Excretion, and Toxicity), which support identifying the dosage and proper use of drugs. ADMET properties of drug molecules are predicted using systems and software. The application and usage of drugs to study drug likeliness are important in the drug discovery steps. Intestinal absorption of drug molecules and finding bioavailability are involved with the ADMET analysis [22]. The gut-blood barrier is described by Caco-2 cell permeability [23]. Table 1 shows the ADMET results recorded in the present pharmacokinetic study. The Caco-2 cell permeability observed in the present study was 0.286 x 106 cm/sec. and 2.258 x 106 cm/sec for the drugs Atazanavir (ATZ) and HA-Atazanavir (HA-ATZ), respectively. P-glycoprotein I and II inhibitor screening resulted in inhibition against the proteins by the drug and its drug conjugate. Skin permeability tests of the compounds showed -2.327 and -2.121 kg log Kp values for the drugs ATZ and HA-ATZ, respectively. Human intestinal absorption (HIA) is an important parameter studied for drug formulation. In the present observation, HIA values were recorded as 47.458 and 64.521 for the drug ATZ and its conjugate form, HA-ATZ, respectively. CNS permeability of 5.411 and blood-brain barrier penetration of 3.314 log BB resulted from the drug conjugate ATZ-HA. HIA and CaCO2 permeability can be used to calculate absorption properties. CYP proteins 1A2, 2C9, 2C19, 2D6, and 3A4 are the reactions that cover ninety percent of the metabolic reactions [24]. The metabolism profile analysis of the drugs resulted in no inhibition against the metabolizing enzymes except CYP239 and CYP3A4 with the drug Atazanavir. The total clearance value of 1.321 log ml/min/kg was found to be highest with the drug conjugate ATZ-HA. No significant value of renal OCT2 was found with the experimental drugs. The drugs were recorded with no AMES toxicity, hERG toxicity, or skin sensitization tests. It resulted in hepatotoxicity with both drugs. The maximum LD50 value was 6.231 mol/kg with the drug conjugate ATZ-HA, and the LD50 for the drug ATZ was 2.652 mol/kg. Recently, conjugated drugs were efficiently used for the treatment of tuberculosis [25] and COVID-19 [26]. The determination of the ADMET profile caters to finding out the important features required for

the drug molecule and extends the way to design a drug with high safety. In the present observation, it was found that there was no AMES activity. The drugs recorded favourable pharmacokinetic properties, which support their activity against the targeted protein molecules [27,28]. The dosage, metabolic rate, rate of excretion, and bioavailability of the drug can be studied by total clearance. This can be used to understand the pharmacokinetic properties of the drug and make it easy to find the drug molecule [29]. The hERG (human ether-a-go-go-related gene) is noted as an important model that inhibits the hERG potassium channel. It supports finding QT intervals and is vital for understanding heart-related health issues [30].

Table 1 ADMET properties of Atazanavir, Hyaluronic acid-Atazanavir conjugates

ADMET	Atazanavir	HA Atazananvir
ABSORPTION		
Caco-2 cell permeability (10^6 cm/sec)	0.286	2.258
P-glycoprotein I Inhibitor	Yes	Yes
P-glycoprotein II Inhibitor	Yes	Yes
Skin Permeability log Kp	-2.327	-2.121
HIA	47.458	64.521
DISTRIBUTION		
CNS Permeability	3.134	5.411
Blood-brain barrier penetration (log BB)	1.322	3.314
METABOLISM		
CYP1A2 Inhibition	No	No
CYP2C9 Inhibition	Yes	No
CYP2C19 Inhibition	No	No
CYP2D6 Inhibition	No	No
CYP3A4 Inhibition	Yes	No
EXCRETION		
Total Clearance (log ml/min/kg)	0.612	1.321
Renal OCT2 Substrate	No	No
TOXICITY		
AMES Toxicity	No	No
hERG Toxicity	No	No
Oral Rat Acute Toxicity (LD50) (mol/kg)	2.652	6.231
Hepatotoxicity	Yes	Yes
Skin Sensitization	No	No

Molecular docking studies

Molecular docking study is useful to predict interaction between two structures (protein-ligand or protein-protein). In the present study, the drug molecule Atazanavir (ATZ) and its conjugate Hyaluronic acid-Atazanavir (HA-ATZ) are used to bind the target protein molecules

Mpro, PLpro, and ACE-2. The Mpro of SARS-CoV-2 is essential for the replication of viruses and for the multiplication and reproduction of viral particles. The papain-like protease (PL-pro) is an effective target because it is responsible for the cleavage, multiplication of viral proteins, and replicase-transcriptase complex assembly. Angiotensin-converting enzyme-2 (ACE-2) is a highly effective therapeutic target that is responsible for the process of viral infection and its translation.

The docking scores and interaction plots are presented in Table 2. The two dimensional interaction plots for Mpro, PLpro, and ACE-2 against the drug and its conjugate are displayed in the Figures 2a-2e. The minimum docking score of -9.3 kcal/mol was calculated with the binding of PLpro and drug conjugate HA-ATZ. It was followed by the minimum docking score of -8.9 kcal/mol with ACE-2 and HA-ATZ, and it was showing -6.8 kcal/mol for the same viral protein ACE-2 with the drug Atazanavir (ATZ). There were three bonded amino acid residues (Asp153, Tyr154, and Thr304) and seven non-bonded interactions of residues (Asn151, Phe294, Asp295, Val297, Arg298, Val303, and Phe305) with the interaction of Mpro and the drug Atazanavir (ATZ), whereas the Hyaluronic acid conjugated drug Atazanavir (HA-ATZ) with the enzyme Mpro resulted in eight bonded interactions of the amino acid residues (Thr24, Asn119, Asn142, Gly143, Cys145, His143, His163, Glu166, and Gly189), and there were no non-bonded interactions were observed. PLpro and ATZ showed one bonded interaction with the residue Phe258 and five non-bonded interactions with Gly256, Thr257, Thr259, Lys279, and Lys306. The conjugated drugs HA-ATZ and PLpro exhibited four bonded interactions with the amino acid residues Glu214, Thr257, Ser278, and Lys306 and two non-bonded interactions with the residues Phe258 and Thr259. The bonded interaction with four residues and the non-bonded interaction with six residues in the binding were observed in ACE-2 and ATZ. Five bonded interactions and five non-bonded interactions were noted in the binding of the conjugated drug ATZ-Ha with the ACE-2 enzyme. The catalytic triad consists of Cys112, His273, and Asp287, observed in the PLpro enzyme of SARS-CoV and important to the replication of the virus and pathogenesis [31]. PLpro has been found to have cysteine protease catalytic activity, in which Histidine 273 acts as a universal acid base, Cysteine 112 acts as a nucleophile, and Aspargine 287 is attached to histidine to perform the incorporation and deprotonation of Cysteine 112 [32]. Biological conjugation of drugs with natural polymers like PEG (Poly Ethylene Glycol) and HA (Hyaluronic Acid) maximizes properties like pharmacokinetics to find more biologically active pharmaceutical materials [33].

HA have favorable properties to conjugate with many molecules including peptide drugs that show positive results when compared to PEG [34]. Hyaluronic acid (HA) is a mucosal polysaccharide that is naturally observed in connective tissues and is anionic in nature. The extracellular matrix participates in the signaling pathway to activate, regulate, and differentiate cell types. HA is found to be a receptor noted on the cell surface that has more binding efficiency. It is actively involved in the drug delivery process. HA noted its available biocompatibility and biodegradability, and its extensively used in conjugation methods for carrying drug materials. HA-conjugated drugs are efficient at targeting many drug molecules and highly specific to the pathologically important ones [35].

DFT Analysis

Frontier molecular orbital's (FMOs) are utilized for the prediction of effective reactive regions in molecular systems and to depict all the available types of reactions. The prediction of FMO properties can be performed by using HOMO and LUMO orbital's, which are elaborately used in the characterization of materials. Entire molecules' characteristics were obtained with the gap of HOMO-LUMO. Especially, they engaged to identify the molecule's chemical reactivity. The HOMO is a rich electron orbital, and it tends to transfer the electrons to unoccupied orbitals. LUMO is lacking electrons, but it can easily accept electrons from other occupied orbitals. The maximum HOMO and LUMO gap is responsible for high kinetic stability, and it is more important for the stability of the chemical molecule [36]. In the present study, the HOMO-LUMO gap for the drug ATZ is -0.17974 eV, whereas its conjugate HA-ATZ gap is -0.14055 eV. The HOMO – LUMO values are given in the Table 3 and shown in the Figure 3a-3d. The lowest chemical potential of -0.1420 μ eV was recorded with the conjugated drug HA-ATZ.

Table 2 Docking scores and interaction plots related to the drug Atazanavir and its HA conjugated drug

Compounds	Docking Score (kcal/mol)	Bonded Interaction	Non Bonded Interaction
Mpro (6M03) -Atazanavir	-6.9	Asp153,Tyr154,Thr304	Asn151,Phe294,Asp295,Val297, Arg298, Val303,Phe305
PLpro (6W9C) – Atazanavir	-7.2	Phe258	Gly256,Thr257,Thr259,Lys279, Lys306
ACE-2 (1R42) - Atazanavir	-6.8	Arg98,Gln102,Tyr196, Arg219	Leu95,Ala99,Trp203,Gly205, Asp206,Glu208
Mpro (6M03) -Atazanavir	-8.1	Thr24,Asn119,Asn142, Gly143,Cys145,His163, Glu166,Gln189,	-
PLpro (6W9C) – Atazanavir	-9.3	Glu214,Thr257,Ser278, Lys306	Phe258,Thr259,
ACE-2 (1R42) - Atazanavir	-8.9	Gln110,Thr111,Ser158, Phe294,Thr292	Arg105,Val202,Val297,Pro293, Ile249

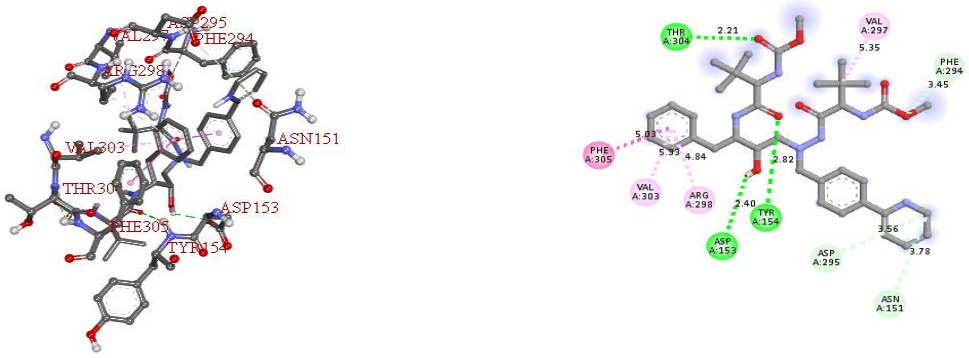

Figure 2 (a) Two dimensional interaction plot for Atazanavir against Mpro (PDB ID 6M03)

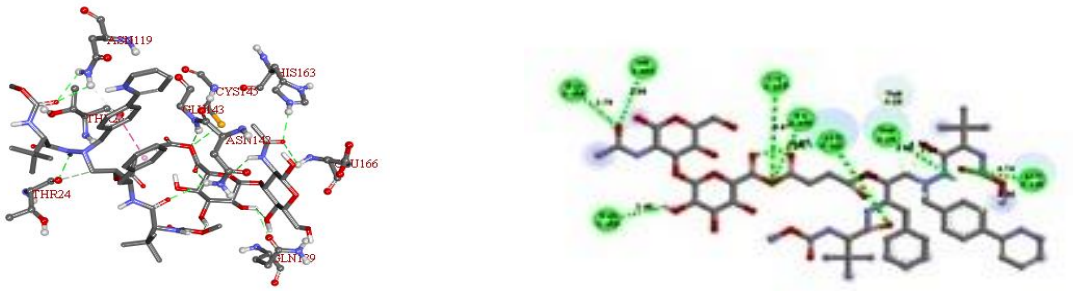

Figure 2 (b) Two dimensional interaction plot for HA-Atazanavir against Mpro (PDB ID 6M03)

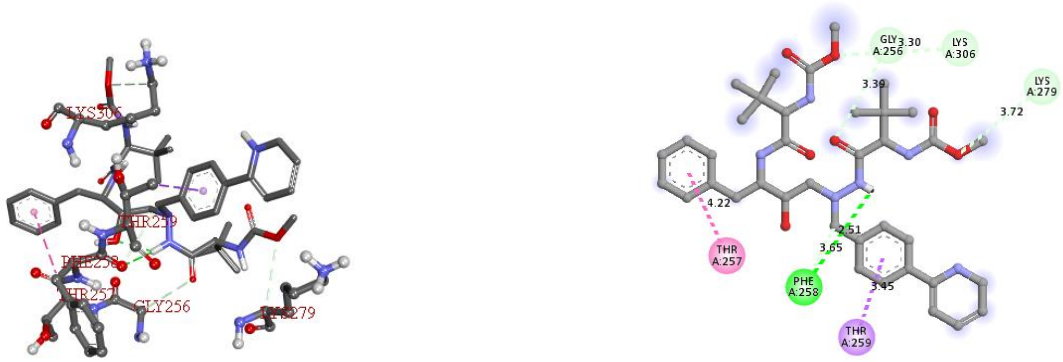

Figure 2 (c) Two dimensional interaction plot for Atazanavir against PLpro (PDB ID 6W9C)

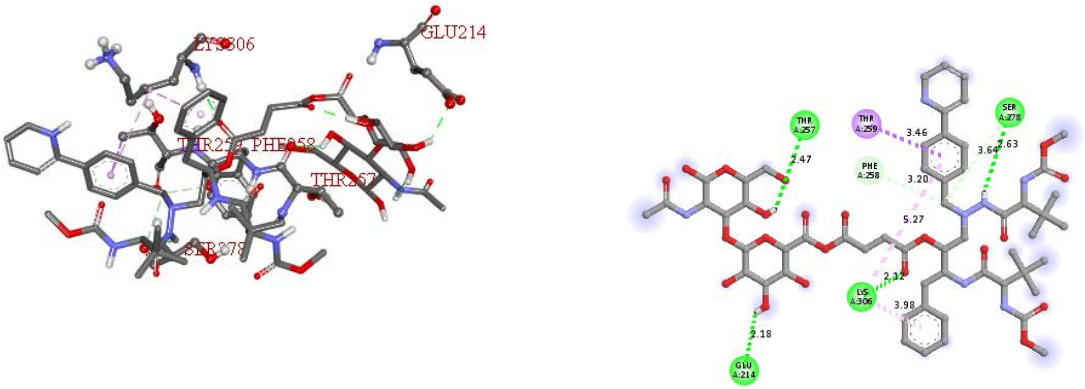

Figure 2 (d) Two dimensional interaction plot for HA-Atazanavir against PLpro (PDB ID 6W9C)

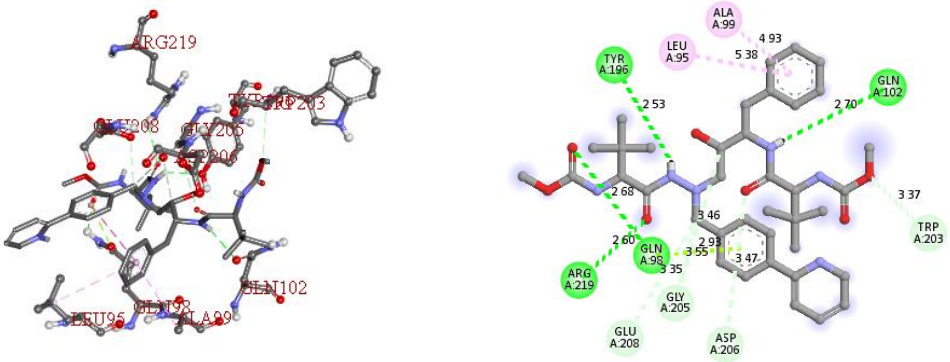

Figure 2 (e) Two dimensional interaction plot for Atazanavir against ACE-2 (PDB ID 1R42)

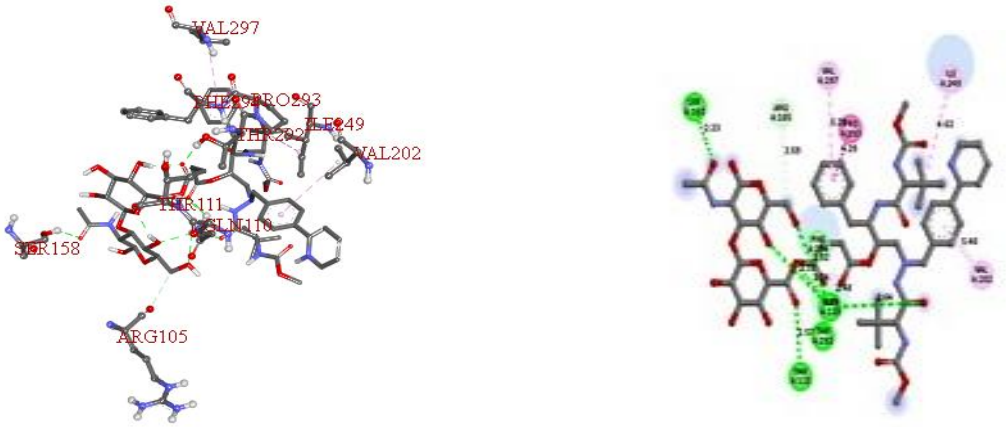

Figure 2 (e) Two dimensional interaction plot for HA-Atazanavir against ACE-2 (PDB ID 1R42)

Figure 3 (a) Atazanavir HOMO orbital (b) Atazanavir LUMO orbital

Figure 3 (c) HA-Atazanavir HOMO orbital (d) HA-Atazanavir LUMO orbital

Table 3 Molecular orbital properties of Atazanavir and its HA-Atazanavir

Compounds	HOMO	LUMO	Energy Gap	Ionization potential (IE) (eV)	Electron affinity (EA)(eV)	Electro negativity (χ)(eV)	Electro chemical potential (μ)(eV)	Hardness (η) (eV)	Softness (σ) (eV)
Atazanavir	-0.21277	-0.0330	-0.1797	0.21277	0.03303	0.1229	-0.1229	0.0898	11.127
HA-Atazanavir	-0.21480	-0.0692	-0.1405	0.21480	0.06925	0.1420	-0.1420	0.0727	13.740

CONCLUSION

The results of the present study revealed that the Atazanavir conjugated with Hyaluronic acid (HA-ATZ) showed better interaction with the viral protein target than the free ATZ drug. The drug conjugate may be used to treat COVID-19 when compared to the pure form of drugs. Further studies are in progress to synthesis HA-ATZ conjugates with different degree of the substitution of the drugs and to evaluate the antiviral activity of both *in-vivo* and *in-vitro* towards SAR-CoV-2 viral protein target.

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