Are losartan and Imatinib Effective Against SARS-CoV2 Pathogenesis? A Pathophysiologic-Based in Silico Study

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WARNING: *As to the nature of in silico modeling studies which are a sort of theoretical swift investigation to determine the best way to start in vivo investigations negating the need for cumbersome time- and budget-consuming other sorts of in vitro studies, we declare that this study does not mean that the proposed drugs are certainly effective in the treatment of COVID 19. Unless robust subclinical studies are conducted all the conclusions might be assumptive.*

Abstract

Introduction: A novel virus called SARS-CoV2, of Coronaviridae family, has distributed all around the globe since December in 2019. COVID 19 the disease caused by this virus results in an atypical pneumonia which in some patients eventuates in acute respiratory distress syndrome (ARDS) with high morbidity and mortality. ARDS in COVID 19 has been linked to a sort of cytokine storm inexplicable only through activation of an immunopathological response to the virus but might probably be rather due to a hyperacute destructive surge of angiotensin II. In this context, losartan, an angiotensin receptor blockers (ARBs), has been proposed as a drug with the ability to restore the balance in ACE2/angiotensin (1-7)/Mas and ACE/Ang II/AT1R pathways in tissues to ameliorate lung inflammation. Besides, imatinib, a tyrosine kinase inhibitor, studied priorly in SARS and MERS may be suggested to block the fusion of the virus with the host cell or modulate the immunologic dysregulation in ARDS. In this article we propose a novel insight to the probable pathophysiology of cytokine storm in COVID19 and then we report the results of our in Silico theoretical study on the effects of losartan and imatinib against the virus pathogenesis.

Method: The required protein structures were obtained from Protein Data Bank, the structures were evaluated and purified selectively to achieve the most desirable structure.

The required drugs and small molecules were fully optimized for geometry, electrical and structural properties. Docking study was performed by AutoDock 4 to find the suitable orientation of the molecules in the active site of the protein structures. MD simulations of the protein−small molecule complexes following docking processes were performed with the GROMACS 2018 package. Through redocking, the binding energies and the binding status of the small molecules with the protein structures were evaluated. After performing MD simulation, we analyzed exported RMSD, RMSF, hydrogen bonding and Radius of gyration diagrams.

Results: According to our bioinformatic study losartan and imatinib could occupy and distort the binding of RBD with ACE2 through changing the conformational shape of its N-terminal α helix where RBD binds. This results in reduction of the affinity of the virus for ACE2. In addition, losartan and imatinib could pose in the structure of main and papin-like proteases as well as in p38MAPK with high affinity and may affect the behavior of these proteins. Losartan due to disturbing of papin-like protease structure may change the function of this protease with high probability, yet it loses its affinity to this protein to some degree. Binding of furin to imatinib revealed to be of high affinity. Imatinib may prevent S'2 cleavage through inhibiting furin. Losartan due to disturbing of papin-like protease structure may change the function of this protease with high probability. Losartan, vs other ARBs had higher affinity to bind with the protein structures.

Conclusion: Implied from reviewing the literature, the proposed novel theory about the pathophysiology of ARDS in COVID19 in this article seems to be legitimate. Considering this theory with our knowledge of losartan and imatinib pharmacokinetic and pharmacodynamic characterizations, and according to the bioinformatic data in our modeling study, it might be concluded that both losartan and imatinib are effective, yet to be approved, in alleviating ARDS in COVID 19 through:

1- their immunomodulatory effects,
2- (in case of losartan) restoring of the balance in physiologic responses in ACE2/angiotensin (1-7)/Mas and ACE/Ang II/AT1R pathways by reducing AT1R-activated downstream cascade in the context of Ang II excess,
3- probable changing the conformational structure of ACE2 and consequently lowering the ability and affinity of the virus to bind to ACE2,
4- (in case of imatinib) probable inhibiting of priming of S protein with furin,
5- probable inhibiting main and papin-like proteases,
6- probable inhibiting p38MAPK and the downstream inflammatory responses,

In this study the effects of other small molecules, such as cholecalciferol and some herbal extracts in few aspects of the virus life cycle were also evaluated. All the results are theoretical and must be validated in subclinical and clinical studies.

KeyWords: SARS-CoV2, losartan, imatinib, Ang II, ACE2, FURIN, TMPRSS2, papain-like protease, cytokine storm, ARDS, clinical bioinformatics, in silico study, IFN, cholecalciferol, herbal extracts

Abbreviation: ACE: angiotensin converting enzyme; ACE2: type 2 angiotensin converting enzyme; AT1R: angiotensin II type 1 receptor; AT2R: angiotensin II type 2 receptor; Ang II: angiotensin II; Im,nACE2: new ACE2 new ACE2 under the influence of imatinib after 100ns MD simulation; Lo,nACE2: new ACE2 under the influence of losartan after 100ns MD simulation; Mpro: Main protease; Pre-inh: predefined inhibitor in data bank; PLpro: papain-like protease; PRR: pattern recognition receptor

Introduction

Since December 2019, a viral disease called COVID19 has hunted thousands of people savagely all around the world and the death toll increases in a skyrocketing manner every day. It was elucidated through a breathtaking study that the disease was caused by a coronavirus named 2019-nCoV.[1] Thereafter this virus was named SARS-CoV2 and the disease, COVID 19.[2] Coronaviruses are classified in Coronavirinae subfamily, the family Coronaviridae and the order Nidovirales. The subfamily Coronaviridae is composed of four genera: Alphacoronavirus (αCoV), Betacoronavirus (βCoV), Deltacoronavirus (δCoV), and Gammacoronavirus (γCoV). SARS-CoV2 belongs to β-genus.[2,3] Other members of β-genus of this family, SARS-CoV and MERS-CoV, are the causes of severe acute respiratory syndrome (SARS) and the Middle East Respiratory Syndrome (MERS), respectively.

These pathogens are large, enveloped and positive-sense single stranded RNA viruses.[3, 4] SARS and COVID 19 are very similar in terms of clinical, pathological and radiological features, yet SARS-CoV2 and SARS-CoV are sufficiently divergent in their genomic characteristics to be considered as the causes of two distinct diseases.[5, 6, 7, 8] However, structurally they are 82% homologous despite a few mutations in their amino acid sequences.[3] The genome of SARS-CoV2 consists of 29891 nucleotides encoding 9860 amino acids. Its genomic sequence is:[3]

5′-replicase (orf1/ab)-structural proteins [Spike (S)-Envelope (E)- Membrane (M)-Nucleocapsid (N)]-3′

The nucleotide sequence of the genome of the virus contains two main open-reading frames (ORFs), ORF1a and ORF1b, the translation of which results in the expression of two co-terminal replicase polyproteins ppl1a (ORF1a) and ppl1ab (ORF1a & ORF1b together). These polyproteins are cleaved into non-strucrtural proteins (NSPs) by two proteases: a papain-like protease (PLpro) and the main serine type protease (Mpro).[3, 9, 10, 11]

The difference in the ORFs of SARS-CoV2 and SARS-CoV is not remarkable. It is implicated that the similarity of the polyproteins is high in these two viruses.[3] Replication and transcription of the virus are attributed to the assembly and proper functions of nsps, specially nsp3.[3, 9, 12] The amino acid sequence of these replicating proteins in SARS-C0V2 and SARS-CoV are 94.4% identical.[13] In addition, PLpro as well as some nsps (nsp3) suppress innate immunity through interfering with the production of type I interferon and inhibiting of Toll-like receptor3 (TLR3) and Toll-like receptor 7 (TLR7).[9, 10, 14] In vivo, it has been demonstrated that type I and type III interferon response against SARS-CoV2 is lower than that of seen in respiratory syncytial virus (RSV) and influenza A virus. It seems that SARS-CoV2 like SARS-CoV evade innate immunity successfully.[15, 16]

Viral RNA synthesis leads to genomic and subgenomic RNAs production, the latter serves as mRNA to encode for structural proteins.[11] Nucleocapsid (N), membrane (M) and envelop (E) proteins are structural proteins which determine the different compartments and shape of the virus. Furthermore, a glycoprotein structure called spike (S) protein protruding from the surface of the virus facilitates its entry into the host cells and

determines the host range, tissue tropism and the host immune responses.[17] As a class 1 fusion protein the characteristic clove-shape viral S protein of coronaviruses is composed of two trimeric subunits, S1 and S2. S1, containing the receptor binding domain (RBD), mediates the attachment of the virus to its receptor on the host cell and S2, the stalk of this glycoprotein, is responsible for virus-cell fusion.[13, 18, 19] Protein S of SARS-CoV2 has 76% homology to that of SARS-CoV; their RBDs' similarity relevant to their amino acid sequence is 74%.[18]

The critical point of the virus replication and pathogenesis is entry of the virus into the host cell. In this context, S1 and S2 subunits should be cleaved in a priming process through which, as S1 attaches to the receptor, S2 is exposed to fuse with the host cell membrane. Host cell proteases like furin, transmembrane protease serine protease-2 (TMPRSS2) and cathepsin L are responsible for this cleavage. Furin is highly found in the lungs.[20] It has been demonstrated that SARS-CoV2, to the contrary of SARS and other batcoronaviruses, has achieved a furin cleavage site in S'2 of protein S, which has resembled its infectivity rather to those of HIV, Ebola virus (EBoV) and some avian influenza viruses.[21]

The receptor of SARS-CoV2 on the host cells, like that of SARS-CoV, is angiotensin converting enzyme 2 (ACE2).[22, 23, 24] Apart from some few novel mutations in the SARS-COV2, the complex of (SARS-CoV2)-ACE2 is highly similar to (SARS-CoV)-ACE2. However, the binding affinity of SARS-CoV2 to its receptor, ACE2, is 10 to 20-fold higher than that of SARS-CoV.[25]

ACE2, a metallopeptidase and homologue of angiotensin converting enzyme (ACE), is a member of renin-angiotensin system (RAS). RAS is a very complicate network of ligands and receptors which not only exert their effects in a systemic counter/cross regulated signaling pathways but also engage in a set called local RAS that acts in an autocrine and intracrine manner in some organs.[26] These two collaborative systems contribute to the regulation of cardiovascular system, metabolism, cell growth, salt and electrolyte homeostasis and vascular resistance.[27, 28]

ACE2 is a mono-carboxypeptidase which removes single amino acids from peptides of RAS. ACE2 is not inhibited by ACE inhibitors like captopril or lisinopril.[29,30,31] It converts angiotensin I [1-10] and angiotensin II [1-8] to angiotensin [1-9] and angiotensin [1-7], respectively.[31,32] (31, 32). ACE2 is a functional competitor of ACE as the former converts angiotensin I [1-10] to a less active metabolite, angiotensin [1-9], so that less angiotensin I [1-10] remains available for ACE to be converted to Ang II. Besides, ACE2 converts Ang II [1-8] to angiotensin [1-7]. Opposing to ACE2, ACE degrades angiotensin [1-7] to inactive products like angiotensin [1-5].[33,34] Angiotensin [1-7] is considered as an active peptide in RAS with antioxidative, anti-inflammatory, antiproliferative/antifibrotic, potent vasodilatory, and anti-thrombotic properties which exerts most of its effect via Mas receptor.[34,35,36,37] On the other side of this intricate-regulatory RAS, ACE increases Ang II with its oxidative, proinflammatory, proliferative/fibrotic, vasoconstrictive and thrombotic effects which is mostly exerted through angiotensin II type 1 receptor (AT1R). Another receptor for Ang II called angiotensin II type 2 receptor (AT2R) with cell protective and some opposing post-receptor effects to AT1R is distribute in a limited number of organs.[37,38,39] Stimulating AT1R, Ang II induces mitochondrial dysfunction, ROS

generation through activating NADPH oxidase, production of cytokines such as TNF-α, IL-6 and IL-8 and activation of p38-MAPK and NF-kB pathways. AT1R resides on adipose and many other tissues such as cardiomyocytes, pulmonary vascular endothelial and bronchial epithelial as well as alveolar cells and specially on monocytes and macrophages.[40,41,42,43] It is implicated that ACE2/Ang [1–7]/Mas axis plays a counter-regulatory role against ACE/Ang II [1-8]/AT1R signaling pathway.[44,45,46]

ACE2, besides to its expression on cardiovascular system, kidney, small intestine and adrenal, is abundantly distributed in the apical portion of the ciliated nasal and tracheobronchial cells as well as pneumocytes type 1 and type 2.[47,48,49] Epithelial cells of the respiratory system including pneumocytes type II are immune competent cells. They contribute to playing an integral part in innate immunity of the lungs through keeping the integrity of the alveoli as a barrier, repairing any damaging insult to type I pneumocytes, enhancing the functions of dendritic cells and macrophages, secreting cytokines and chemokines and even presenting antigens and consequently facilitating the shifting of innate to adaptive immunity.[50,51]

Adsorption of recombinant protein S of SARS-CoV to ACE2 was shown to result in the downregulation of ACE2.[48,51] Internalization of this receptor with the virus into the host cell or shedding of ACE2 in the airways are potential causes of this phenomenon. Releasing of ACE2 from the epithelial cells of the airways is a dynamic phenomenon that occurs constitutively and may upregulate in response to various stimuli.[48] Intriguingly, protein S in binding with ACE2 induces ADAM-17/TACE as a sheddase to separate the ectodomain subunit of ACE2. Shedding of ACE2 is associated with the production of TNF-α which was argued to be the initiating cause in inflammation of the lung in SARS. Cytoplasmic domain of ACE2 plays an important role in this process as its mutation reduces SARS-CoV entry into target cells and release of TNF-α is abolished as well.[51,52] Some experts believe that ADAM17 is not involved in entry of SARS-CoV2 into the host cells and TMPRSS2 and cathepsin-L share in this process. As a debating subject to be studied more, it is also suggested that TMPRSS2 and ADAM17/TACE compete with each other in facilitating the viral-cell entry.[53,54] Anyhow, binding of S protein to ACE2 results in hyperacute downregulation of ACE2 which deregulates the balance in local RAS pathways in favor of ACE/Ang II [1-8]/AT1R in the lungs. In this context, hyperacute upregulation of local intracrine Ang II [1-8]/AT1R in the setting of invasion of huge number of SARS-CoV2 is not encountered with appropriate negative physiological feedback with ACE2. Furthermore, Ang II has been demonstrated to decline ACE2 expression and function via lysosomal degradation mediated by AT1R.[55] Henceforth, Ang II/AT1R sets on fire locally to provoke lung inflammation through pro-inflammatory, cytokine inducing, proliferative, thrombotic and tissue destructive effect as well as activating platelet derived growth factor receptor (PDGFR) which might spread to other organs like kidneys.

So far, we have a deadly virus with distinct replication and pathogenesis which:

1- evades innate immunity via avoiding IRF3, TLR3 and TLR7 pathways by its nsp3 and papin-like proteases and elicit a moderate immune response,

2- contains protein S requiring to be primed by furin and host cell proteases to facilitate its fusion with the host cell
3- downregulates its receptor (ACE2) after attachment
4- dysregulates the balance between two opposing axes of local RAS in the lungs in favor of Ang II with all the destructive and fibrotic properties in the lungs

As there is not any available anti-viral drug or an imminent potential vaccine against this deadly virus, it seems wise to find a way to reduce the death toll by preventing or mitigating the respiratory failure or through breaking the life cycle of the virus itself till a comprehensive preventive or curing measure is introduced.

We hypothesized that re-balancing Ang II/ACE2 with angiotensin receptor blockers and subsiding immunopathological changes by immunomodulators might alleviate the severity of the disease and may reduce the morbidity and mortality rates in COVID 19. To prove our hypothesis losartan (an ARB) and imatinib (a tyrosine kinase inhibitor) were chosen to be studied in an in silico study. It is noteworthy that imatinib had been introduced in the treatment of SARS and MERS.[56] Besides, low doses of imatinib was demonstrated to have the ability to enhance innate immune responses.[57] Intriguingly, imatinib also was effective in attenuating LPS-induced acute lung injury in an animal study.[58]

Method:

Preparation of the Protein Structures

The required protein structures were obtained from Protein Data Bank[59] (PDB) according to Table 1:

Table 1. The protein crystal structures used in this study

Macromolecule	Sequence Length	Organism	ID
SARS-CoV-2 spike receptor-RBD bound to ACE2	603/229	Homosapiens/SARS-Cov2	6m0j [60]
Angiotensin Converting Enzyme 2 (ACE2)	615	Homo sapiens	1r4l [61]
COVID-19 main protease	306	SARS-Cov2	6lu7 [62]
MAP Kinase p38	379	Homo sapiens	1a9u [63]
Furin	482	Homo sapiens	6hzb [64]
Papain-like protease	316	SARS-CoV	3mj5 [65]
Angiotensin II type 1 receptor & Angiotensin II	425/8	Homo sapiens	6os0 [66]

Proteins were studied for the date of publishing, crystallography techniques, the resolution, accompaniment of predefined inhibitor (“Pre-inh”) and any required reconstruction due to probable missing of amino acids in their sequence vs the sequence of reference protein. The structures were observed by visualizing softwares (UCSF

chimera[67], Pymol[68], Swiss-PdbViewer[69] to determine their unique protein chains and whether the structure is accompanied by other undesired molecules like (water, ions….) and to purify selectively to achieve the most desirable structure.

Preparation of Small Molecules

The required drug and small molecules were obtained from Structure Data Bank such as Pubchem database[70] and Drug bank database[71] according to the (Table 2 and Table 3). The structures of all the drugs and small molecules were imported through gauss view, and then fully optimized geometries and properties of the electronic and structural properties of all molecoules were derived by means of the density functional theory (DFT) method[72] with B3LYP functional[73]. For all systems, a geometrical optimization and calculation were performed using the STO-3G[74] based set. The calculations were carried out using the Gaussian 03 package[75]. The program Open Babel[76] was used to generate SMILES strings from the optimized structure representation, using them for a similarity study by drug bank Chemical Structure Search with 0.5 to 0.7 Similarity threshold.

Table 2. the list of required drugs and small molecules

Small Molecules	Pubchem CID	Approved drug	Plant extract
Azadirachtin	5281303		✓
Caftaric acid	6440397		✓
Chicoric acid	5281764		✓
Chlorogenic acid	1794427		✓
Cholecalciferol	5280795	✓	
Curcumin	969516		✓
Curcumin Solfate	66645351		✓
Dasatinib	3062316	✓	
Hesperidin	10621	✓	
Hydroxychloroquine	3652	✓	
Imatinib	5291	✓	
Milk Thistle	1548994		✓
Ramipril	5362129	✓	
Riluzole	5070		✓
Silibinin	31553		✓
Trandolapril	5484727	✓	

Docking Simulation

Docking study was performed by AutoDock4[77] to find the suitable orientation of the molecules in the active site of the protein structures. AutoDockTools 1.5.4 (ADT) was used to prepare input PDBQT files and to calculate a grid box. A special grid map appropriate for each structural size (table 4) around the active site of proteins were used. The center of the grid box was aligned to the coordinates of the “Pre-inh””. A Lamarckian genetic algorithm (LGA) was used for the searching of the status of binding sites. Every Lamarckian job was comprised of 250 runs. The final structures were grouped and

classified according to the most favorable binding energy. This procedure was applied to all small molecules in a similar manner. A more negative score determines which of these small molecules are more likely to dock with a protein structure (target protein) and enter into more favorable interactions. The docking model of each protein (table) complex with its "Pre-inh" was generated by AutoDock 4. The reliability of the applied docking protocol was assessed by redocking each "Pre-inh" into the active site of its protein structure.

Table 3. List of angiotensin II receptor blocker (ARBs)

Drug molecules	Pubchem CID
Pratosartan	9802561
Tasosartan	60919
Losartan	3961
Candesartan	2541
Irbesartan	3749
Fimasartan	9870652
Telmisartan	65999
Forasartan	132706
Azilsartan	135415867
Saprisartan	60921
Olmesartan	158781
Valsartan	60846
Eprosartan	5281037
Saralasin	6324663

Table 4 grid box dimensions for each structure

Macrmolcules	Steps	Grid points	Spacing Å	Grid Center
ACE2	1st Docking	$80 \times 100 \times 80$	0.375	$40 \times 6.0 \times 29$
ACE2	2nd Docking	$75 \times 70 \times 80$	0.375	$13 \times 15 \times 20$
ACE2	3rd Docking	$120 \times 85 \times 100$	0.600	$39 \times 3.0 \times 22$
Mpro		$80 \times 100 \times 100$	0.375	$-15 \times 13 \times 70$
p38MAPK		$70 \times 70 \times 70$	0.375	$4 \times 16 \times 29$
PLpro	Before MD	$80 \times 80 \times 80$	0.375	$-13 \times 45 \times -36$
PLpro	After MD	$80 \times 80 \times 80$	0.375	$40 \times 45 \times 47$
Furin		$80 \times 100 \times 80$	0.375	$40 \times 45 \times 47$

After exposing of the small molecules with the structures, we obtained docking energy for each [structure-small molecule] complex. The clusters of docking energies were determined for 250 posing status and the relevant numerical tables for each complex was studied.

MD Simulation

Molecular dynamic (MD) simulation is a method to study the dynamicity of the protein structure during a defined time period to characterize the behavior and stability of the structure. Gromacs software executed MD simulation in this study for 100ns.

MD simulations of the protein-small molecules complexes following docking process were performed with the GROMACS 2018 package using the GROMOS96 43a1 force field.[78] The conformation status for ACE2 and PLpro complexes with theirs ligands with the highest affinity were selected as the initial conformation for MD simulations. First the topology parameters of protein were created and the complex was immersed in a cubic box of simple point charge (SPC) water molecules.[79] The "solvated system" (protein, ions, small molecule and water) was neutralized by adding required counterions Na or Cl. To equilibrate the system, the solutes (Proteins, counterions, and small milecules) were subjected to the position-restrained dynamics simulation (NVT and NPT) at 299.177 K for 1000 ps. Finally, the full system was subjected to an MD production run for 100 ns at 300 K temperature and 1 bar pressure.

MD simulation was performed for ACE2 crystal, ACE2-SARS-CoV mutated RBD (refer to results), imatinib-ACE2, losartan-ACE2 and imatinib-PLpro, losartan-PLpro complexes.

Redocking study after MD simulation

After MD simulation of complexes for 100ns, the ligands were separated. Through redocking, the binding energies and the binding status of the small molecules with the protein structures was evaluated.[80]

Analysis

RMSD, RMSF, hydrogen bonding and radius of gyration diagrams exported after performing MD simulation were analyzed by qtgrace.[81] Ligplot[82] and poseview[83] were used for determining the hydrogen bonding, hydrophobic and pi-pi interactions after docking and MD simulation. Visual analyzing was done with ucsf chimera and pymol.

Configurations of computational systems:

In this study we used multiple computational systems by different configurations as Table 5:

Table 5. Configurations of computational systems

System	Operating Systems	CPU	GPU
Server 1	Linux 3.10	32 logical cores E5-2697	CUDA
Server 2	Linux 4.15	32 logical cores E5-2650	×

Results:

Losartan and imatinib bind to ACE2 with low energy (high affinity).

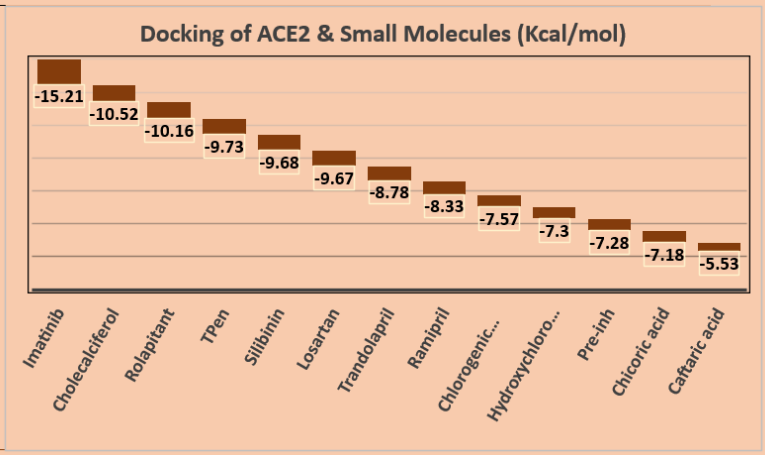
We exposed losartan and imatinib molecules to ACE2 (before MD simulation). Docking of imatinib and losartan in association with other selected small molecules with ACE2 were performed in three grid boxes with three distinct dimensions (Table 4).

In addition, docking energies for all the small molecules selected based on LBDD and SBDD for binding with ACE2 were assessed based on coordinates (grid box 1) of predefined inhibitor in crystal structure of ACE2. Among the molecules with the highest affinities to ACE2, small molecules with more tendency to contact with N-terminal α helix were selected to perform docking in grid box (grid box 2) relevant to the binding site of ACE2 with RBD. Grid box 3 for the whole structure of ACE2 was set only for imatinib and losartan to check the compatibility and reliability of hotspots and docking energies with the previous results in other grid boxes. The binding energies for all grid boxes and small molecules under study are mentioned in Table 6.

The positions of losartan and imatinib with the most affinity for ACE2 in grid box 1 vs the position of predefined inhibitor were determined (Figure 1).

Table 6. Docking Energies for Small molecules with ACE2 in 3 distinct grid boxes

Small Molecules	Grid Box1	Grid Box2	Grid Box3
Imatinib	-15.21	-11.79	-12.64
Cholecalciferol	-10.52	-8.87	
Rolapitant	-10.16	-9.21	
TPen	-9.73		
Silibinin	-9.68	-7.43	
Losartan	-9.67	-8.44	-9.6
Trandolapril	-8.78		
Ramipril	-8.33		
Chlorogenic acid	-7.57		
Hydroxychloroquine	-7.3		
Pre-inh	-7.28		
Chicoric acid	-7.18		
Caftaric acid	-5.53		



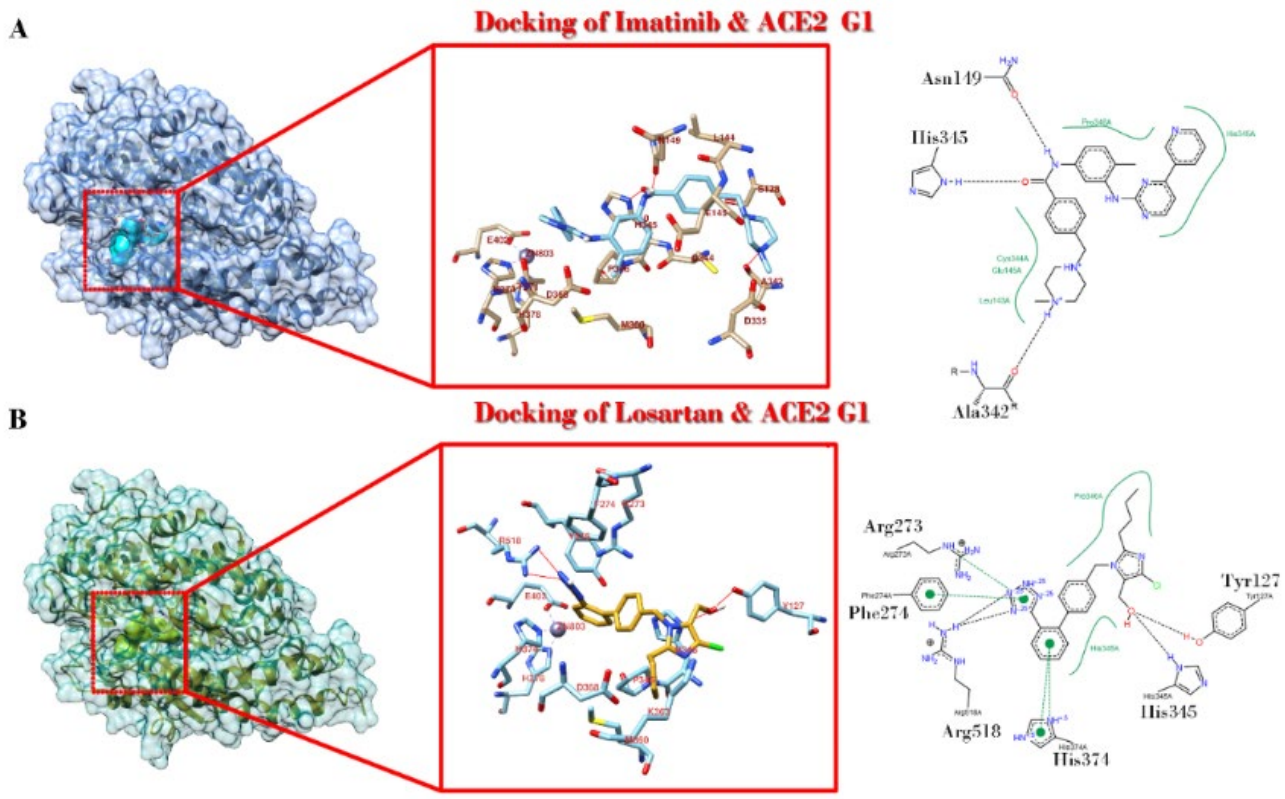


Figure 1. Docking of Imatinib and Losartan (before MD simulation); A: binding site of imatinib with ACE2 with hydrogen bonds, Asn149, Ala342, His345; B: binding site of losartan with ACE2 including hydrogen bonds, Tyr127, Arg273, Arg374, His345 and pi-pi interactions of Phe274 and His374

Losartan and imatinib could change the conformational structure of ACE2 persistently.

In order to study the persistency of losartan-ACE2 and imatinib-ACE2 complexes, we performed 100ns MD simulation for each complex. Two new ACE2 (nACE2) under the influence of each of these ligands were exported: losartan (Lo,nACE2) and imatinib (Im,nACE2). RMSD, RMSF, radius of gyration and h-bonding diagrams of each complex was obtained (Figure 2).

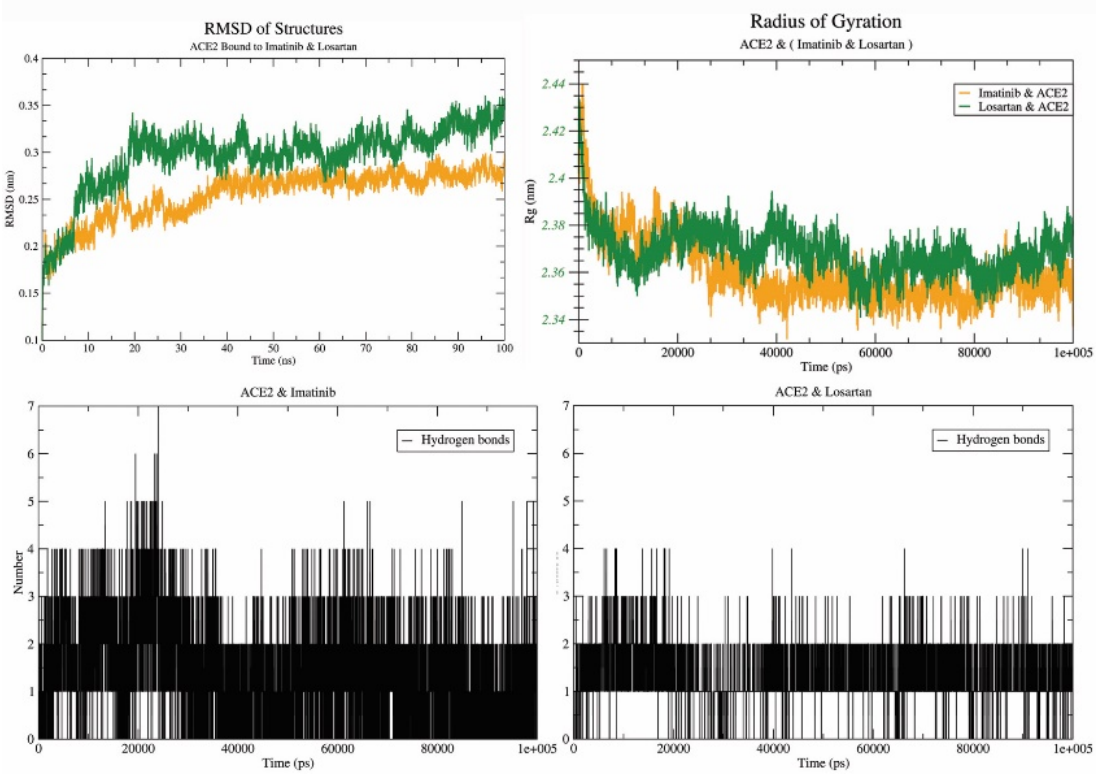


Figure 2. RMSD, Rg and H-bonding diagrams of losartan and imatinib complexes with ACE2

The structure of the two complexes were superimposed on the structure of ACE2 crystal to evaluate the degree of changes in conformational shape of each complex. The data showed that the conformational structures of ACE2 in binding with both losartan and imatinib changed significantly at the binding site of ACE2 to RBD (Figure 3). It is expected that losartan and imatinib lengthen the binding distance between ACE2 to SARS-CoV2 RBD due to relocation of contributing residues in ACE2 (Table 7).

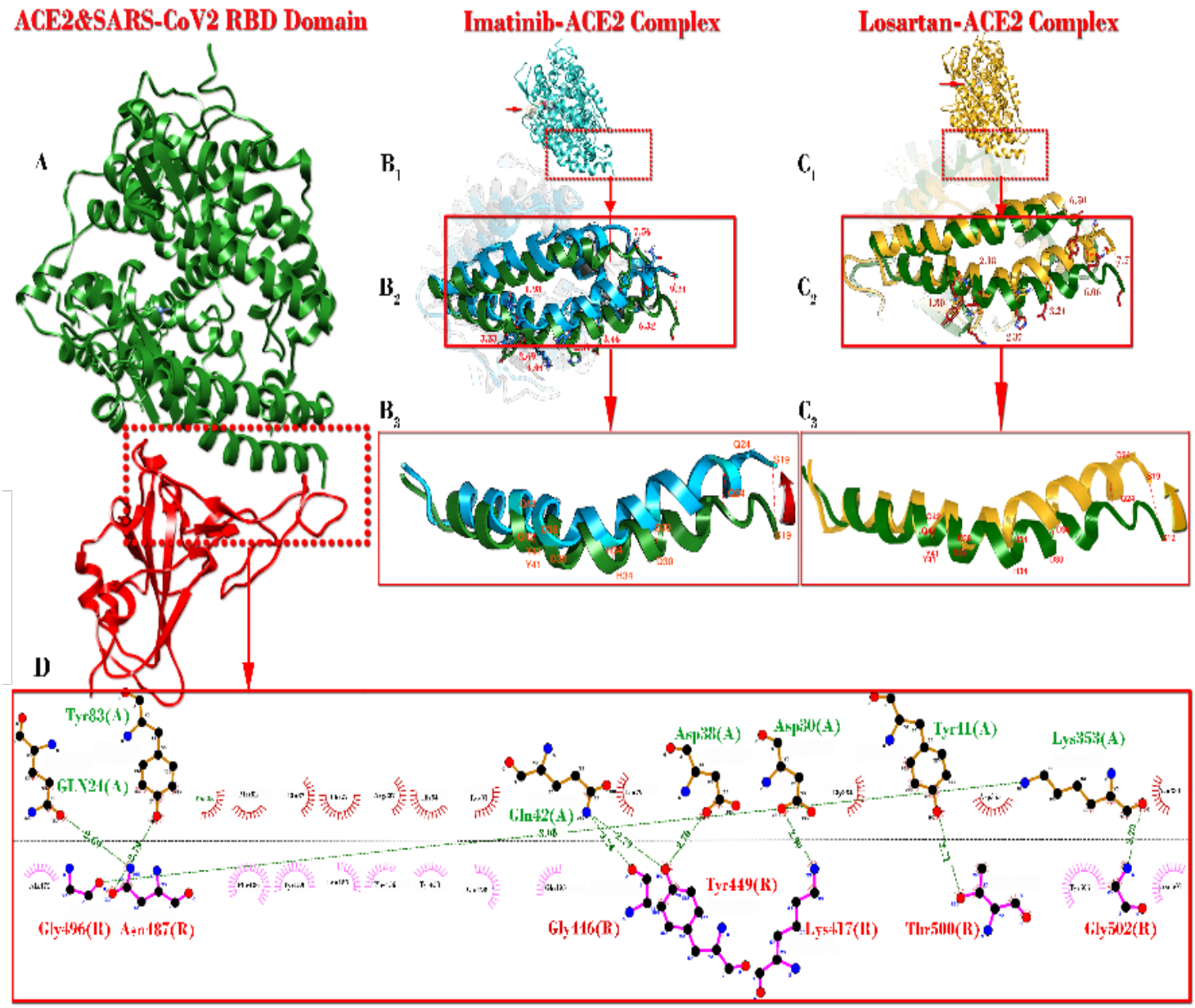


Figure 3. A:SARS-CoV2-ACE2 complex. B:1-Im,nACE2, 2-super-imposition of ACE2 and Im,nACE2, 3-super-imposition of α-helix of ACE2 and Im,nACE2. C:1-Lo,nACE2, 2-super-imposition of ACE2 and Lo.nACE2, 3-super-imposition of α-helix of ACE2 and Lo,nACE2. D: hydrophobic and hydrogen bonds of SARS-CoV2RBD-ACE2

Table 7. Relocation of Carbon alpha of N-terminal helix of ACE2

RBD	H-Bond length Å	Hydrophobic	Atom C(α)	ACE2 Residue Relocation Magnitudes Imatinib (Å)	Losartan (Å)
Asn487(R)	2.69		GLN 24.A	6.32	6.06
Lys417(R	2.90		ASP 30.A	3.46	3.24
	-	✓	HIS 34.A	2.59	2.37
Tyr449(R)	2.70		ASP 38.A	3.69	2.18
Thr500(R)	2.71		TYR 41.A	1.98	1.80
Tyr49(R)	2.79		GLN 42.A	3.33	2.05
Asn487(R)	2.69		Tyr 83.A	7.58	6.50
Gly502 (R)	2.78		Lys 353.A	4.04	3.99
N-Terminal			SER 19.A	9.24	7.71

The binding energy of losartan and imatinib with Lo,nACE2 and Im,nACE2 after performing 100ns MD simulation changed.

Redocking for losartan with Lo,nACE2, imatinib with Im,nACE2, losartan with Im,nACE2 and imatinib with Lo,nACE2 were performed. The binding energies are as the followings (Table 8):

Table 8. Docking Energies for Lo,nACE2 and Im,nACE2 bound to imatinib and losartan after 100ns MD simulation

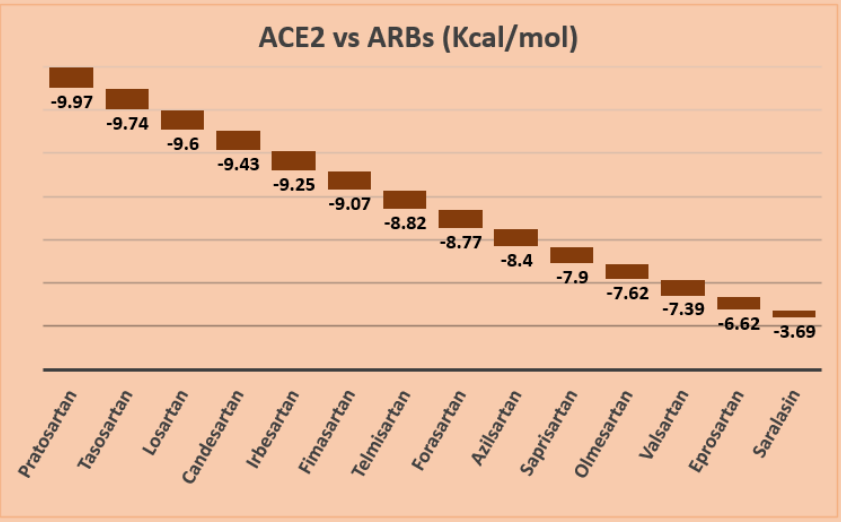
	Binding energy (Kcal/mole)	
nACE2	Losartan	Imatinib
Lo,nACE2	-11.99	-14.25
Im,nACE2	-8.47	-17.78

Available ARBs vs losartan could only bind to ACE2 with reasonable but lower affinity

ARBs were searched for on Kyoto Encyclopedia of Genes and Genomes (84). Docking was done for each member of ARBs with ACE2 crystal structure. The energy binding for each item was obtained. Losartan in association with two unavailable ARBs, pratosartan and tasosartan, was in the upper three ranking of binding energy.[71] (Table 9)

Table 9. Docking Energy for binding ARBs to ACE2

ARBs	Binding Energy
Pratosartan	-9.97
Tasosartan	-9.74
Losartan	-9.6
Candesartan	-9.43
Irbesartan	-9.25
Fimasartan	-9.07
Telmisartan	-8.82
Forasartan	-8.77
Azilsartan	-8.4
Saprisartan	-7.9
Olmesartan	-7.62
Valsartan	-7.39
Eprosartan	-6.62
Saralasin	-3.69



The mutated new amino acids in the sequence of SARS-CoV2 RBD were replaced on SARS-CoV RBD; MD-simulation was performed for new structure

In the beginning of our study (mid of February 2020) due to the lack of crystasl structure of SARS-CoV2 RBD we replaced 22 defined mutated amino acids in SARS-CoV2 RBD on the corresponding place on SARS-CoV RBD to achieve a RBD structure with the most similarity to the real crystal structure of SARS-CoV2 RBD, assuming that S proteins in these viruses are 76% homologous. MD simulation of 100ns was performed after accomplishing this replacement to achieve a persistently stable structure with the most homology to RBD-ACE2 complex of SARS-CoV2. RMSD, RMSF, H-bonding and radius of gyration diagrams are available. Due to the publishing of crystal structure of RBD-ACE2 complex of SARS-CoV2 by X-ray defraction (resolution of 2.45 Å) we quitted using the achieved RBD-ACE2 complex and continued our bioinformatic study on the new published one (Figure 4).

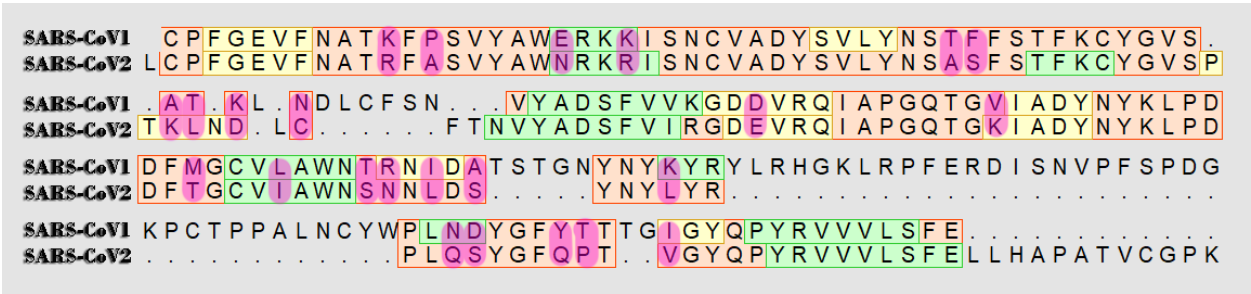


Figure 4. Sequence alignment of RBD of SARS-CoV and SARS-CoV2 for crystal structures by PDBID:3sci and PDBID: 6vsb

Imatinib and losartan could occupy the space where the predefined inhibitor ("Pre-inh") in crystal structure of the main protease (Mpro) of SARS-CoV2 poses; they might act as an inhibitor of Mpro.

We exposed imatinib, dasatinib, losartan, cholecalciferol, silibinin, hyodroxychloroquin and the other small molecules to crystal structure of Mpro. It was elucidated that imatinib in association with dasatinib, cholecalciferol and losartan had higher affinity to Mpro. It shows that these ligands based on their affinity probably behave as inhibitors of Mpro, considering CADD theories. (Figure 5) (Table 10)

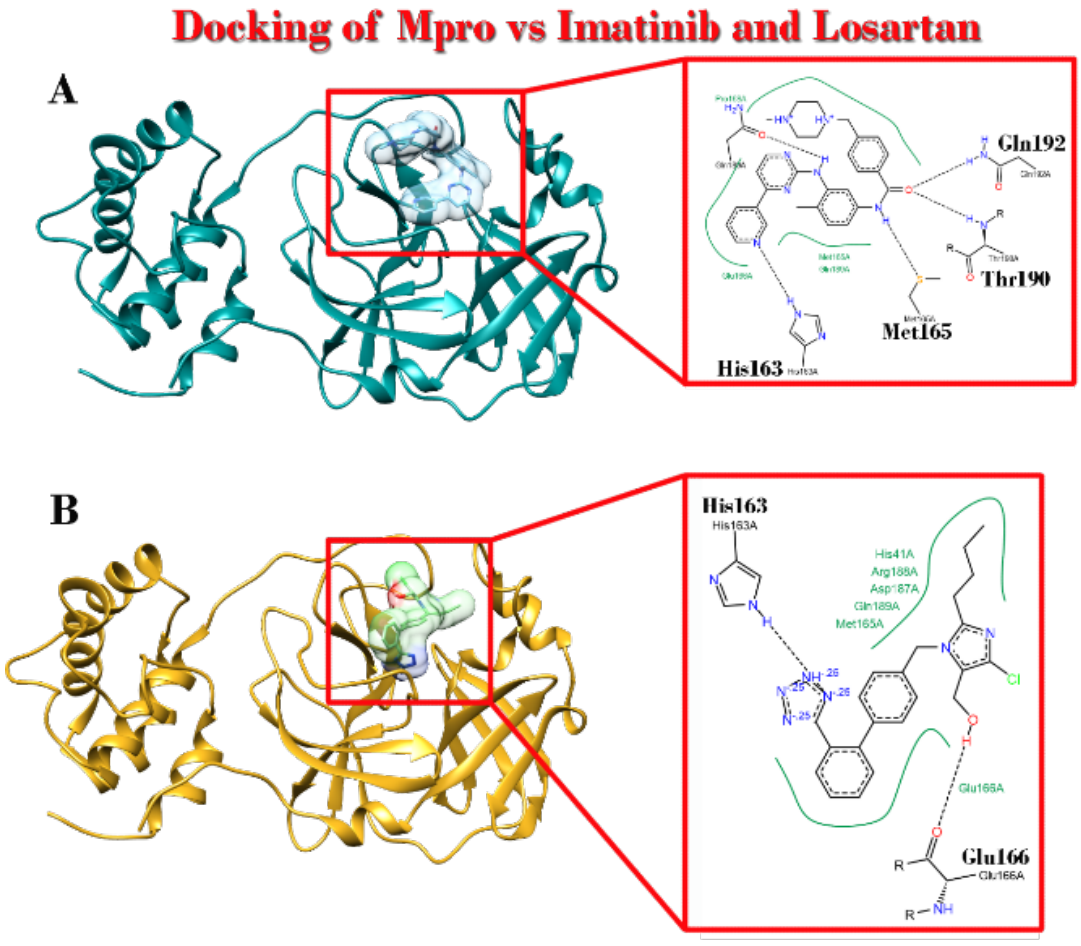
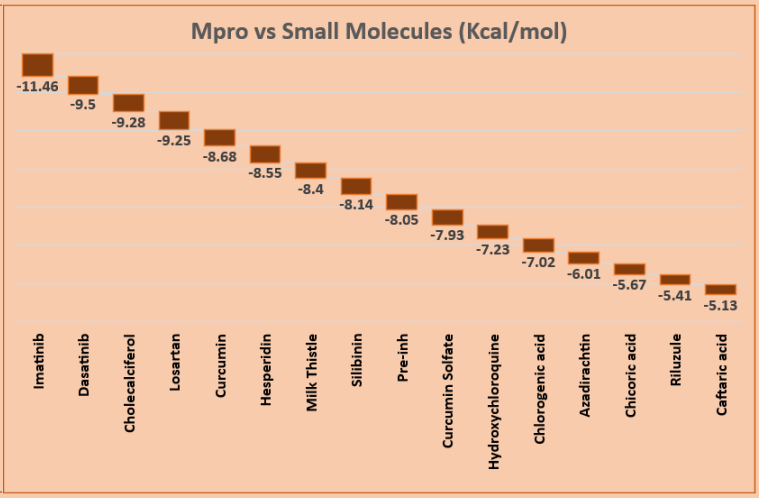


Figure 5. Main Protease (Mpro) of SARS-CoV2 complex with imatinib and Losartan.A:Ribbon view of complex (left) and Poseview of interaction with imatinib (Right) B: Ribbon view of complex (left) and Poseview of interaction with Losartan (Right)

Table 10. Docking Energies for binding of Small Molecules with Mpro (Kcal/Mol)

Small Molecoules	Binding Energy
Imatinib	-11.46
Dasatinib	-9.5
Cholecalciferol	-9.28
Losartan	-9.25
Curcumin	-8.68
Hesperidin	-8.55
Milk Thistle	-8.4
Silibinin	-8.14
Pre-inh	-8.05
Curcumin Solfate	-7.93
Hydroxychloroquine	-7.23
Chlorogenic acid	-7.02
Azadirachtin	-6.01
Chicoric acid	-5.67
Riluzule	-5.41
Caftaric acid	-5.13

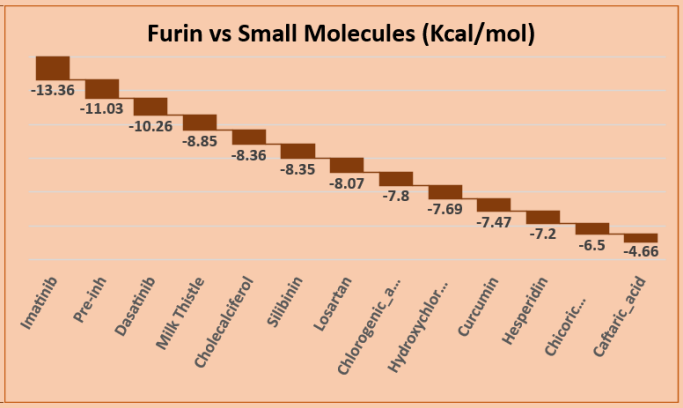


Imatinib and losartan could occupy the space where the “Pre-inh” in crystal structure of the furin of SARS-CoV2 poses. Imatinib shows higher affinity but losartan has lower affinity vs the inhibitor; imatinib might act as an inhibitor of furin.

We exposed imatinib, dasatinib, losartan, cholecalciferol, silibinin, hyodroxychloroquin and the other small molecules to crystal structure of furin. It was demonstrated that imatinib had higher affinity to furin. But dasatinib, cholecalciferol and losartan and the other molecules showed up with lower but reasonable affinity to furin. Considering CADD theories, it shows that imatinib based on its affinity will probably inhibit furin function. (Figure 6) (table 11)

Table 11. Docking Energies of Small Molecules for binding with Furin (Kcal/Mol)

Small Molecules	Binding Energy
Imatinib	-13.36
Pre-inh	-11.03
Dasatinib	-10.26
Milk Thistle	-8.85
Cholecalciferol	-8.36
Silibinin	-8.35
Losartan	-8.07
Chlorogenic_acid	-7.8
Hydroxychloroquine	-7.69
Curcumin	-7.47
Hesperidin	-7.2
Chicoric acid_opt	-6.5
Caftaric_acid	-4.66



It is worth mentioning that redocking of predefined inhibitor of furin in its crystal structure obtained from PDB was disturbed and showed error in the process. We optimized the structure and performed docking for the second round to earn a reliable reference for binding energy.

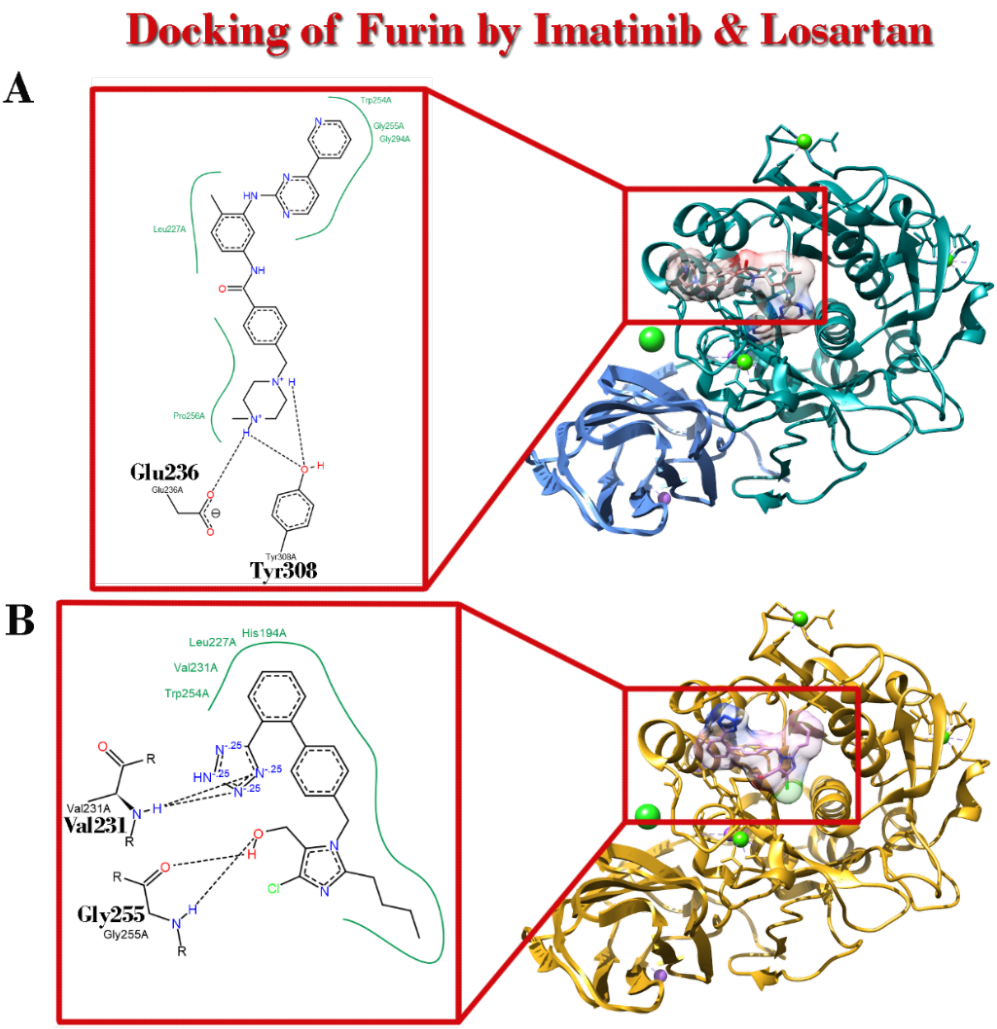


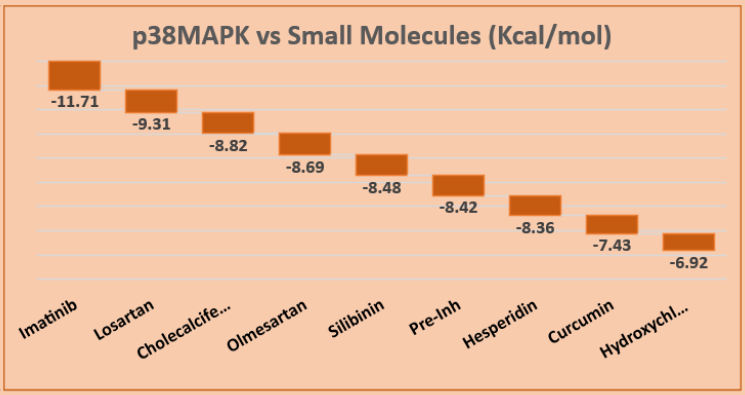
Figure 6. Position of Imatinib and Losartan in complex with furin with PDBID: 6hzb after Docking simulation. A:Ribbon view of complex (right) and Poseview of interaction with imatinib (left) B: Ribbon view of complex (right) and Poseview of interaction with Losartan (left)

Imatinib and losartan could occupy with higher affinity the space where the "Pre-inh" in crystal structure of p38MAPK poses; they might act as an inhibitor of p38MAPK.

We exposed imatinib, losartan, olmesartan, cholecalciferol, silibinin and the other small molecules to crystal structure of p38 MAPK. It was implicated that imatinib, losartan, olmesartan, cholecalciferol and silibinin had higher affinity to p38 MAPK. Considering CADD theories, it shows that imatinib, losartan, olmesartan, cholecalciferol and silibinin based on their affinity will probably inhibit p38MAPK function. (Table 12)

Table 12. Docking Energies for the Small Molecules with p38MAPK (Kcal/Mol)

Small Molecules	Biniding Energy
Imatinib	-11.71
Losartan	-9.31
Cholecalciferol	-8.82
Olmesartan	-8.69
Silibinin	-8.48
Pre-Inh	-8.42
Hesperidin	-8.36
Curcumin	-7.43
Hydroxychloroquine	-6.92



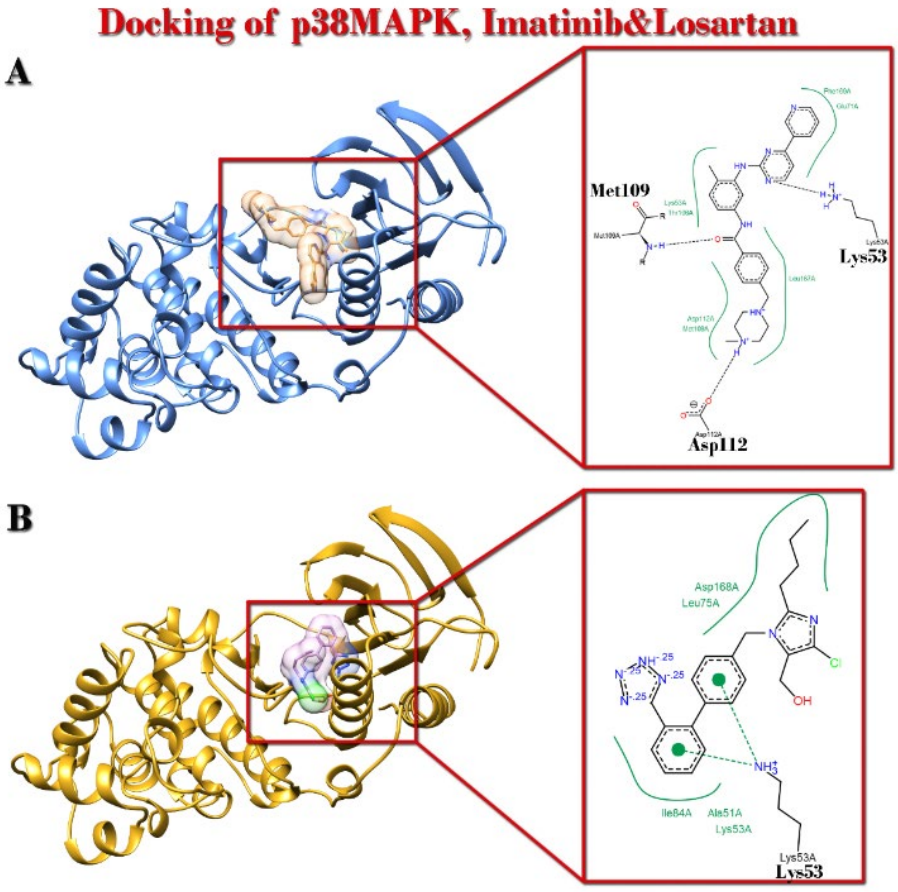


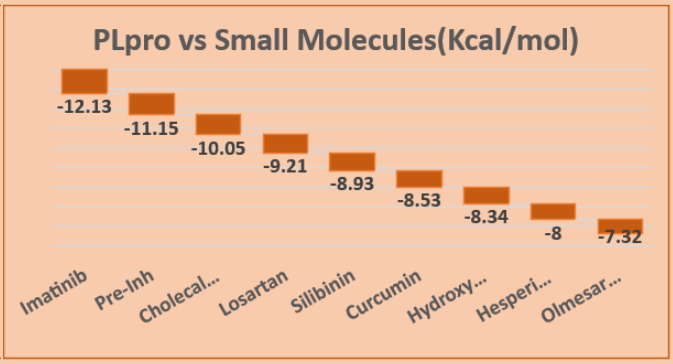
Figure 7. Position of Imatinib and Losartan in complex with p38MAPK with PDBID: 1a9u after Docking simulation. A:Ribbon view of complex (left) and Poseview of interaction with imatinib (Right) B: Ribbon view of complex (left) and Poseview of interaction with Losartan (right)

Imatinib could occupy with higher affinity the space where "Pre-inh" in crystal structure of papin-like protease (PLpro) poses; imatinib might act an inhibitor of PLpro.

We exposed imatinib, cholecalciferol, losartan, silibinin, curcumin, hydroxychloroquine and even olmesartan to crystal structure of PLpro. All the compounds showed high affinity to PLpro but lower than imatinib and “Pre-inh”; Considering CADD theories (R) imatinib might act as an inhibitor of PLpro function. (Table 13)(Figure 8)

Table 13. Docking Energies of Small Molecules for binding with PLpro (Kcal/Mol), before and after 100ns

Small Molecules	Before 100ns	After100ns
Imatinib	-12.13	-16.24
Pre-Inh	-11.15	
Cholecalciferol	-10.05	
Losartan	-9.21	-7.83
Silibinin	-8.93	
Curcumin	-8.53	
Hydroxychloroquine	-8.34	
Hesperidin	-8	
Olmesartan	-7.32	



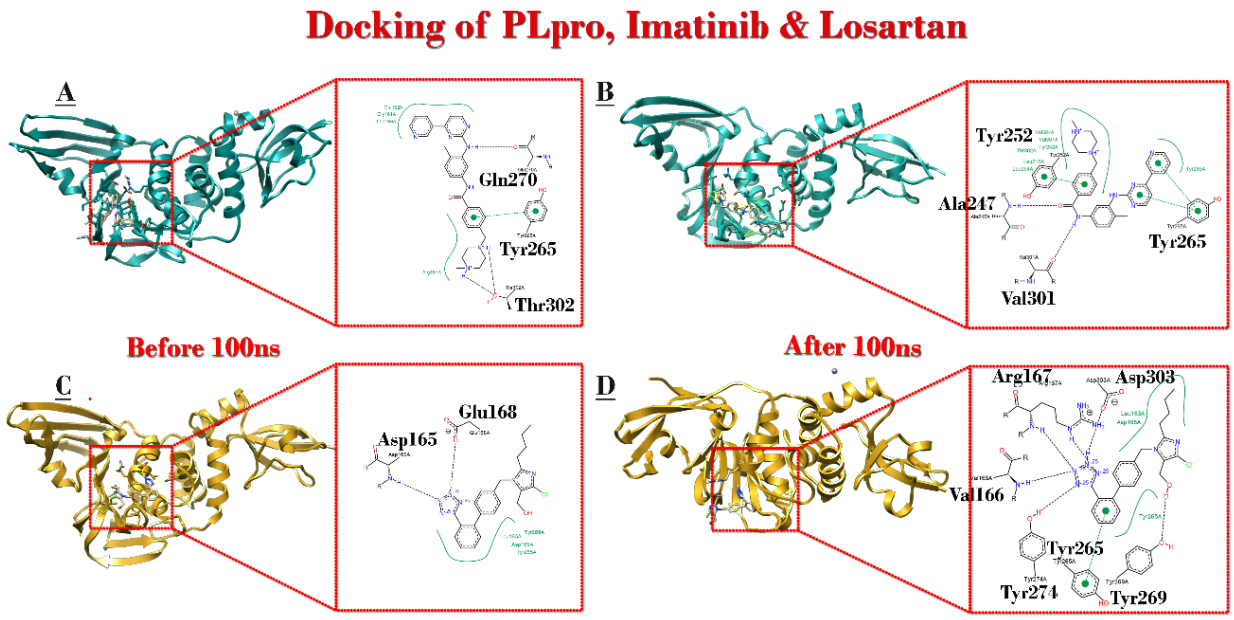


Figure 8. Position of Imatinib and Losartan in complex with PLpro with PDBID: 3mj5 , before and after 100ns MD simulation and redocking. A: Ribbon and poseview of complex of PLpro with imatinib before 100ns MD simulation, B: Ribbon and poseview of complex PLpro with imatinib after 100ns MD simulation C: Ribbon and poseview of complex of PLpro with losartan before 100ns MD simulation, D: Ribbon and poseview of complex PLpro with losartan after 100ns MD simulation

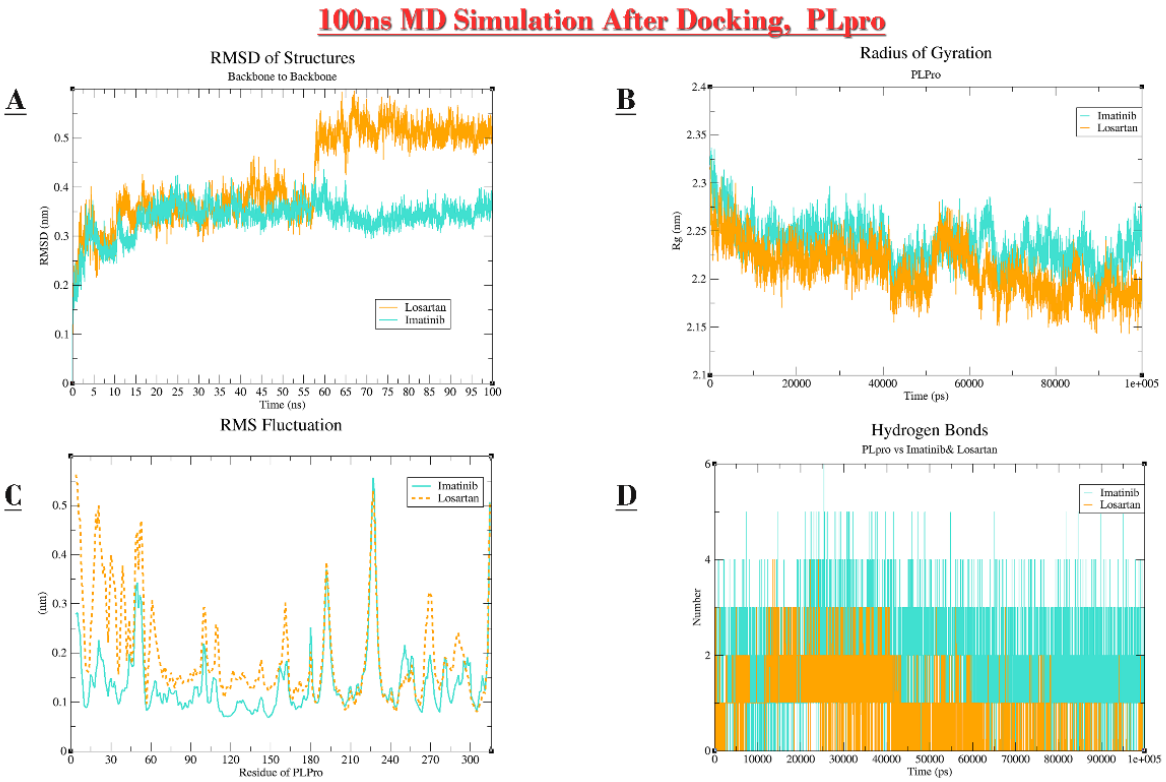


Figure 9. Diagrams of Losartan-PLpro and Imatinib-PLpro complexes after 100ns MD simulation; A: RMSD; B: Rg; C:RMSF; D: H-Bonding

Angiotensin II (Ang II) – Imatinib Interaction

We exposed imatinib and Ang II. The result shows that imatinib binds with Ang II with binding energy of $-8.64\ Kcal/mol$.

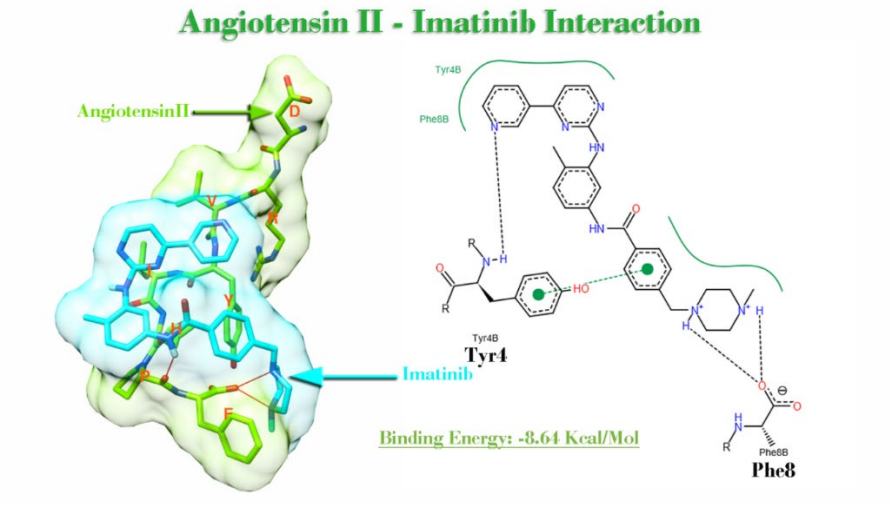


Figure 10. Angiotensin II – Imatinib Interaction in Surface view (Left) and poseview of interaction with two hydrogen bonds with Phe8 and pi-pi interaction with Tyr4 (right)

Discussion

A new insight to pathophysiology of ARDS in COVID19

The fatality and the change of the life style imposed by COVID19 have resulted in uprising death and economic burden in all countries, respectively. Despite the breathtaking efforts done, the development of acute respiratory distress syndrome (ARDS) as the culprit of high morbidity and mortality in infection with SARS-CoV2 has remained elusive. The replication of the virus starts soon after it incubates in the upper respiratory tract. Thereafter the replicas are released and pour into the lower airways and alveoli in huge number (about 10-1000 virion/µl in its peak on day 5-6 of infection).[85] 59(85). Type II pneumocytes and bronchial epithelial cells are immune competent cells and possess high amount of ACE2.[86,87,88] With the knowledge that the affinity of SARS-CoV2 to ACE2 is 10-20 times more than that of SARS-CoV it is legitimate that these sentinel cells get infected easily and in great number.[89]

Penetrating into the host cells, the viruses introduce their genome to the cytoplasm to replicate more numbers of complete viruses or their fragments. Using a conserved pathway led by papain-like protease and nsp3, SARS-CoV2 evade immunologic recognition by pattern recognition receptor (PRR) and avoid triggering interferon (IFN) response (significant lack of IFN I and III expression in covid19) as well as activating dendritic cells. Consequently, the innate immunity becomes inefficient in ensuing an adaptive response.[10,11,14,90] Apart from ARDS, COVID19 compared to influenza and respiratory syncytial disease, is a moderate illness, since SARS-CoV2 elicits a limited antiviral response.[15] In COVID 19, dendritic cells fail to process the antigens properly and to migrate to prime naïve T cells in the local lymph nodes.[91,92] Lung CT-scans of patients approve this phenomenon as lymphadenopathy in the lungs is scarce or absent on the images.[93,94,95] Recently, ARDS in COVID19 has been attributed to a kind of cytokine storm phenomenon. If this virus evades innate immunity and spread insidiously how come the destructive cytokine storm involving the lungs or other organs ensues?! What is the triggering factor?

Relying too much on the clinical studies has resulted in ignorance of some physiological local tissue responses that may convert to systemic harmful reactions if not dealt with immediately. Cytokine storm is triggered from some deregulations in immunological responses which may eventuate into sudden outpouring of pro-inflammatory and tissue destructive cytokines. The origin of this devastating process might be infectious or non-infectious.[96] Whenever innate immunity fails to switch to adaptive responses with proper priming of T cells, the pathogen is not cleared out in a well-orchestrated manner. Instead, unrestrained sequential release of cytokines ensues. Amplifying this response, if not halted soon, eventuates to this storm.[97,98] The fragile texture of lung tissue with its high exposure to different antigens requires that suppressive responses (immunomodulators) be meticulously coordinated with promoting responses (immunosensors). Otherwise, dysregulation of modulators and sensors will be hazardous.[99]

SARS-CoV2 and SARS-CoV replicate rapidly in host cells with early high viral load.[57,100] It seems that replicating of CoVs is abortive and delayed in dendritic, monocyte/macrophages and lymphocytes.[11,101,102] SARS-CoV2 infects type II alveolar and bronchial epithelial cells which contribute to innate immunity. SARS-CoV and SARS-CoV2, in entry to the host cells, downregulate ACE2 and induce imbalance in ACE2/angiotensin[1-7]/Mas and ACE/Ang II/AT1R pathways in favor of the latter.[51,103] As

the virus evades the immune system, hyperacute imbalance (low ACE2/ACE ratio) of the two opposing RAS pathways in favor of pro-inflammatory, proliferative, prothrombotic, tissue destructive and pro-apoptotic Ang II might be the igniting or at least a robust major co-stimulatory factor in inducing cytokine storm. Preliminary clues to this theory are:

1- Ang II level has been reported to be higher in infected patients with SARS-CoV2 than in non-infected healthy people,[104]
2- ACE2 is tissue protective for the lungs in acid- or sepsis-induced ARDS in mice,[105]
3- Mechanical-stress induced ARDS is correlated to activation of [Nox1(NADPH-oxidase1)-MK(midkine)-Notch2-ACE] pathway.[106]

Although probable contribution of RAS to evolving ARDS was suggested in SARS previously [103] it seems that many latent aspects of the involving pathways and the role they may play to solve this puzzle have recently been elucidated. There are three orders of RAS in our body: systemic-hormonal, tissue-local (with paracrine and autocrine effects) and cellular-subcellular (with intracrine effects).[26,107,108] It is noteworthy that while ACE is detectable in 20% of capillary endothelium of non-respiratory organs this peptidase is expressed on the entire endothelial cells of the alveolar capillaries.[109] Thence, lungs can be considered as the major organ producing Ang II.

Invasion of SARS-CoV2 in huge number downregulates ACE2 in the host cells outrageously so that the balance in tissue-local and cellular-subcellular RAS disrupts hyper-acutely. Accordingly, the host cells lose their ability to adapt to or defeat against the consequences of sudden gush of Ang II with adequate negative feedback responses by ACE2. It is worth mentioning that in healthy people, intra-cellular Ang II content in some tissues may reach up to 1000 times higher than that of plasma.[110] In ACE2 deficiency, Ang II is not hydrolized to angiotensin[1-7] (with its cytoprotective effect) or to other less active metabolites. Consequently, hyperacute excessive content of Ang II exerts rather untoward chaotic pathological effects in an intracrine (intracellular) and autocrine (cell to the same cell) manner and even through spilling over extracellularly, in a paracrine (cell to different neighboring endothelial, macrophages, monocytes, vascular smooth muscle cells or fibroblasts) and endocrine (cell to circulation) fashion. On the other hand, some studies demonstrated that Ang II itself downregulates ACE2 expression through internalization, lysosomal degradation and AT1R-mediated ROS activated ERK/p38 MAPK pathway which promotes TACE/ADAM17 activity as well.[53,111,112]

Ang II increases reactive oxygen species (ROS) through AT1R-dependent induction of NADPH oxidase (Nox), mostly Nox2 and Nox4.[109,113] In this pathway which is dependent on intra- and extra-cellular calcium, phospholipase C (PLC) and protein kinase C (PKC) are involved.[109] Even though ROS as a signaling molecule contributes to cell homeostasis, its overproduction may lead to cell damage.[114] Intriguingly, ROS upregulates the production of Ang II in a positive feedback response. In the absence of ACE2, this amplifies the production of ROS dramatically.[115,116] ROS causes DNA damage and mitochondrial dysfunction.[117,118] It has been reported in animal studies that Ang II through AT1R reduces mitochondrial number and prosurvival genes (*Nampt* and *sirtuin3*).[119] ROS, in turn, through opening mitochondrial K-ATP channels and disturbing mitochondrial membrane potential, upregulates mitochondrial ROS (mtROS) production in a positive feedback response.[120] Ang II, directly, through activating of type 2 Ang II receptor (AT2R) residing on mitochondrial membrane inhibits mitochondrial respiration (NO-dependent) and increases mtROS.[106] mtROS functions as a triggering signaling molecule for production of pro-inflammatory cytokines.[121] It was reported in influenza that

regulated amount of mtROS induces interferon γ (IFNγ) to restrain infection.[122] But when mtROS rises up excessively, striking upregulation of pro-inflammatory cytokines must be expected. mtROS was shown to activate NLRP3 which induces IL-1 and IL-18 production.[123] Furthermore, ROS activates inflammatory responses by inducing redox-sensitive transcriptional factors like NF-kB and activator protein 1 (AP1).[107,124]

As an inconclusive subject, over-expression of TNF-α during hyper-acute shedding of ACE2 by ADAM17 through synergism with Ang II may aggravate the situation by inducing oxidative stress via NF-kB and p38 MAPK dependent pathways.[125,126]

In addition, Ang II through AT1R was reported to amplify oxidative stress by distorting iron homeostasis, increasing labile ferrous iron and expression of ferritin in endothelial cells.[127,128] Even though ferritin may show an antioxidative effect, it has been described in mice that ferritin may act as a local cytokine and activate NF-kB through MAPK-mediated pathway. This response results in rise of inducible NO synthase (iNOS) of about 100-fold and IL-1β and RANTES 50-fold with a small increase in intercellular adhesion molecule (ICAM). Ferritin may suppress adaptive immune response, as well.[129]

Ang II by activating AT1R induces expression of TNF-α (presented already in the scene), IL-1β, IL-6, IL-8, MCP-1 and even IL-10 through a NF-kB and activating protein 1 (AP-1) transcriptional factors.[130,131] Aggravating to these pro-inflammatory effects, Ang II by activating AT1R increases vascular permeability in the lung by release of prostaglandins and vascular endothelial growth factor (VEGF).[132] Disruption of endothelial-epithelial barrier in alveoli and increase in permeability of endothelium rises the fluid in the alveolar sacs that should be cleared out by epithelial Na channels (ENaCs). In rats, endogenous activation of AT1R by Ang II downregulates ENaC expression and disturbs pouring out the extra fluid.[133]

Of these cytokines induced by Ang II, IL-6 plays a more special role in immunopathological effect of Ang II. It induces signaling processes associated with JAK2/STAT1/3 activating pathway which promotes many genes contributing to the production of signaling molecules like cytokines, adaptors, receptors and protein kinases.[132,134] In this context regulation of differentiation of monocytes into macrophages mediated by the expression of macrophage colony-stimulating factor, upregulation of B-cell IgG production, downregulation of dendritic cell maturation by activation of the STAT3 signaling pathway and the promotion of the Th2 response by inhibiting Th1 polarization are attributed to IL-6.[132] Tocilizumab (atemra) achieved its fame in COVID19 through its inhibitory effect against the receptor of IL-6.

Ang II promotes production and release of IL-6 and IL-8 from human cultured adipocytes by NF-kB-mediated pathway to which AT1R rather than AT2R contributes. It is demonstrated that in the obese IL-6 plasma level is closely correlated to body mass index (BMI).[40] Ang II-induced over expression of IL-6 in adipose tissue might be the reason why obesity is a risk factor in severity of COVID19.[135] This cytokine has been found to increase platelet and immune cell aggregation through a T-cell dependent mechanism by Ang II.[136] Compatible with the theory of Ang II-mediated immunopathology in COVID 19, this might be the reason why patients with COVID19 are prone to vascular thrombosis.[137] IL-6 level has been correlated to the severity of COVID19.[138] This cytokine, in association with IL-1 and TNF-α, is the major inducer of CRP production in the liver.[139,140] In addition, Ang II, induces CRP expression in hepatocytes in a time- and dose-dependent manner through

activation of AT1R and resulting from ROS-MAPK-(NF-kb) pathway independent of IL-1β and IL-6.[141]

During an immunologic reaction to a pathogen, switching from innate to adaptive immunity requires that naïve CD4+ T cells (Th0) be differentiated to effector T-helper cells: Th1 (producing IL-2, IFNγ, lymphotoxins) and Th2 (producing IL-4, -5, -13) which help to activate cytotoxic CD8+ T cells (CTLs) and B cells, respectively.[142] As a potent pyrogenic cytokine, IL-6 in synergism with IL-7 and IL-15 promotes the differentiation and cytolytic activity of CD8+ T cells.[132] Different cytokines may also promote Th0 cell to differentiate to two opposing classes of T cells: anti-inflammatory T-regulatory (Treg) cells and pro-inflammatory Th17.[142] Ang II through AT1R-PKA-proteosome pathway and activation of STAT1 and NF-kB promotes differentiation of Th0 to Th1. It has been demonstrated that in shifting from Th0 to Th1 or Th2, Ang II upregulates the production of IFNγ (10-fold), IL-2 (18-fold), IL-4 (3.5-fold) and IL-10 (1.5-fold). In addition, Ang II increases Tbox transcription factor mRNA (Tbet, marker for Th1) and GATA3 mRNA (marker for Th2) by 38 and 1.6-fold, respectively. Amazingly, losartan, AT1R blocker, has been shown to inhibit markers for Th1 differentiation without having any effect on that for Th2.[143] It is noteworthy that Th1 is differentiated to Th17 in the presence of IL-6 and TGF-β.[132,143] Th17 induces synthesis of IL-17, IL-21, IL-22 and TNF-α. TNF-α, itself in the presence of IL-6 and IL-1β may promote differentiation of T cells to Th17.[143,144] High level of IL-17 in patients with ARDS suggests its contribution to this syndrome. This cytokine in a model of influenza and LPS induced acute lung injury has been associated with neutrophil recruitment and increase in alveolar layer permeability.[145]

There seems to be a local RAS in DCs, T cells and NK cells with a complete enzymatic repertoire enabling them to synthesize and metabolize Ang II and even AT1R and AT2R. It has also been described that these cells not only respond to Ang II but they have tendency to migrate to this peptide. Thenceforth, Ang II may orchestrate recruitment of leukocytes to the site of inflammation. Ang II induces synthesis of CCL5/RANTES chemokine in T cells and NK cells. It shows that Ang II may direct chemotaxis of cells possessing CCR1, CCR3 and CCR5 and even regulate proliferation of T cells via CCR5.[146]

Activation of AT1R by Ang II has been associated with apoptosis in pneumocytes.[147] It seems that alveolar and bronchial cell death may restrain the distribution of the virus. But even low concentration of IL-6 (upregulated by Ang II to high levels in cytokine release syndrome) in synergism with IL-17 (secreted by Th17) is able to induce expression of prosurvival proteins Bcl-2 and Bcl-xl which inhibit cell destruction by CD8+ cytotoxic T cells and prevent apoptosis of pneumocytes through STAT3 and NF-kB signaling.[148] IL-22, a member of IL-10 anti-inflammatory cytokine family, secreted by Th17 also prevents apoptosis of pulmonary endothelial cells and may ameliorate ARDS through inducing JAK2/STAT3 pathway.[149] But it should be taken into account that over-expression of Ang II upregulates IFNγ (10-fold) and IL-2 (18 fold) (pro-inflammatory cytokines) much more than IL-4 (3.5-fold) and IL-10 (1.5-fold) (anti-inflammatory cytokines).[143] It seems that in this milieu of highly complicated set of pro-inflammatory cytokines some anti-inflammatory molecules prevent apoptosis and cells death of the respiratory epithelial and as a theory even help giant cells produced in COVID 19 to survive.

Destruction of the pulmonary tissue encompasses the interstitial and basal collagen-elastin structures as Ang II upregulates matrix metalloproteinases (MMPs) in vascular smooth muscle cells: MMP2 [through AT1R-mediated extra-cellular signal-regulated

kinase (ERK)1/2 activation], MMP1, MMP3 and MMP9 (via AT1R-ROS mediated NF-kB and AP-1 pathways). These proteinases regulate remodeling and turn-over of extra-cellular matrix (ECM) and promote smooth muscle and endothelial cell proliferation and migration resulting in vascular wall fibrosis which eventually may end up in pulmonary hypertension if lasts.[150,151,152,153] It has been reported in an animal study that ACE2 deficiency results in activation MMP and STAT3 pathway which may promote lung injuries.[154]

Macrophages are important in the healing process. These cells should switch from M1 (with more pro-inflammatory cytokines) to M2 (with more IL-4 and IL-13 dependent IL-10) phenotype to resolve the inflammation. In oxidative stress macrophages exhibit more AT1R. Activating AT1R impairs efferocytosis (clearance of apoptotic cells) and interferes with resolution of the inflammatory cascade.[155,156] MerTK (a tyrosine kinase) which shifts DCs from pro-inflammatory to anti-inflammatory status increases survival of macrophages in acidotic environment.[157] Ang II induces shedding of MerTK off the cell membrane through AT1R/ROS/p38MAPK/ADAM17 pathway.[158] Consequently, Ang II impairs switching M1 to M2. Continuation of pro-inflammatory status result in activation of MMPs and inducing of ECM remodeling processes. Failure of Tregs to show up due to the predominance of IL-6 promoting Th17 may prevent effective efferocytosis. In this milieu, pro-fibrotic IL-13 and TGF-β may lead to lung fibrosis. Ang II through AT1R induces fibrotic changes in the lungs with direct and indirect effects. It has been shown in transgenic mice that Ang II stimulates lung fibroblasts/myofibroblast proliferation and synthesis of ECM. It induces production of TGF-β and connective tissue growth factor (CTGF).[159,160] Ang II in synergism with TGF-β may promote fibrosis in many organs including the lungs.[161] On the other hand, angiotensin [1-7], the product of ACE2, has been considered an antifibrotic molecule.[36] Oxidative stress may promote pulmonary fibrosis through deregulation of sirtuin3.[162]

Losartan, an AT1R blocker, in COVID 19

Ang II was previously considered as a factor that might play a role in ARDS.[163] There is a reliable amount of evidence that losartan, an ARB, is effective in ameliorating ARDS.[164,165,166] Considering the above proposed pathophysiology of ARDS in COVID19 with the new insight that hyper-acute dysregulation of AngII/ACE2 due to the dramatic downregulation of ACE2 might be the cause of cytokine storm and ARDS, led us choose losartan, an AT1R blocker, to inhibit the post receptor effects of Ang II just to moderate the pathological effects of ACE2 deficiency in this disease. In this context, striking upregulation of AT1R by Ang II in a positive feedback manner which might occur in the lungs similar to other organs in oxidative stress could not be ignored, bearing in mind that dramatic downregulation of ACE2 in invasion of huge number of virions and the consequent drastic impairment of hydrolyzing of the intracellular content of Ang II occur in a very short time.[163] Reviewing the literature, it was clear that losartan, candesartan and olmesartan were used to modulate local RAS in previous studies.[131,143,150,159,167] But losartan probably due to its more hydrophobicity and availability might have been chosen more. Losartan was effective in many studies in suppressing pro-inflammatory effects of cytokines due to its immunomodulatory properties or even in preventing lung fibrosis; it can be considered as an ameliorating drug in ARDS in COVID 19.[39,153,157,168,169]

As a debating subject some of the experts according to some animal studies, *not proved in other animal or human studies,* suggested that ARBs might upregulate ACE2 expression on the cells and thus increase the viral load in the body.[170,171,172] In those studies, overexpression of ACE2 after administration of ARBs needed 25-28 days to appear. Furthermore, the impact of hyper-acute increase in the level of local Ang II in the lungs, heart and kidneys in COVID 19 within a short time is much more dangerous. In addition, it seems that the infectivity of the virus, like that of HIV might decrease with the increase of ACE2 content on the cell membrane. Entry of the virus to the cell is a complicated multifactorial subject. It is also dependent on the presence of cell membrane proteases. Upregulation of ACE2 content of the cell membrane is not necessarily associated with an increase in other engaging factors in cell entry such as TMPRSS2. Overexpression of ACE2 without concomitant increase in TMPRSS2 results in the presence of extra ACE2 unable to lead the entry of the virions but capable of entrapping and decreasing the infectivity of the virus. As ADAM17 induces shedding of ACE2, free extra ACE2 is released to the airways with the ability to trap the inhaled viruses. Consequently, upregulation of ACE2 increases entrapment of the virus.[173,174,175] There is not any reported adverse effect of ARBs in COVID 19 and due to favorable effects of these drugs scientific societies in Europe and the USA recommended that ARBs be continued in patients with hypertension or heart diseases. Even two official studies on losartan in the treatment of out and in-patients with COVID 19 have recently been conducted.[176]

Imatinib, an Abl tyrosine-protein kinase inhibitor

It is wise mentioning that patients with cytokine storm may experience hypotension in their extremes of severity of the disease. With the knowledge that losartan may aggravate hypotension and emphasizing that the initial release of TNF-α simultaneous with binding of RBD and ACE2 and consequent shedding of ACE2 may have co-stimulatory effect in starting of cytokine storm it seems to be rational to recommend another immunomodulator in association with losartan to stop the storm. This combination provides the possibility to reduce the required dosage of each drug. There is conflicting evidence that systemic corticosteroids may be hazardous to patients with COVID 19.[177] Recently, it has been described that glucocorticoids may increase Th17 in T cell culture of healthy human.[178,179] Reviewing the literature, tyrosine kinase inhibitors were previously introduced in the treatment of SARS, MERS and ARDS. Abelson tyrosine-protein kinase 2 (Abl 2) is needed in replication of SARS-CoV and MERS-CoV. In this family of viruses, imatinib hinders the initial phases of the virion replication by inhibiting fusion of the virion at the endosomal membrane.[58,180,181] Imatinib, with immunomodulatory effects, has also been suggested to have subsiding effects specially against vascular leak in ARDS.[182,183,184] There is also a report that low dose imatinib was effective in reducing pulmonary blood pressure in dogs.[185] In another report inhaled imatinib was also used as a drug to subside pulmonary hypertension.[186]

Amazingly, Imatinib was found to have an inhibitory effect against Ang II impact on vascular smooth muscle cells in dissection of the aorta in mice.[187] Furthermore, expression of MHC class I and II, production of co-stimulatory molecules and secretion of cytokines and chemokines in monocyte-derived dendritic cells decrease in the presence of imatinib. This tyrosine kinase inhibitor subsides phosphatidylinositol 3-kinase/Akt pathways and downregulates exhibition of NF-kB in the nucleus.[188] Cultured

human monocytes are morphologically and functionally suppressed in the presence on imatinib which reduces the ability of these cells to synthesize IL-6 and TNF-α and to respond efficiently to M-CSF and GM-CSF stimulation.[189] In an in vitro study, imatinib could inhibit expression of TNF-α, IL-6, IFNγ and IL-17 in cultured splenocyte of mice with arthritis in a dose dependent manner.[190] In monocytes and macrophages, TNF-α production was reduced by imatinib while IL-10 expression did not change.[191] Imatinib in mice with hyper-reactive airway disease could subside peri-bronchial eosinophil cell accumulation and decrease secretion of IL-4 and IL-13 by Th2 as well as CCL2, CCL5 and CCL6 chemokines.[192] In vitro, imatinib could impair immunosuppressive ability and expression of FoxP+ in Tregs along with the subsiding of STAT3 and STAT5 pathways without affecting IL-10 and TGF-β in these cells.[193] In some pathogens it has been reported that imatinib exhibits negative effects against the pathogen's entry (polyomaviruses), intracellular transit (Mycobacteria) and exit (poxviruses and filoviruses). Besides, in low dosage imatinib may elicit a physiologic innate immune response to infection called "emergency response" in which myelopoiesis, but not lymphopoiesis, and the immune response to infections are potentiated.[194] S protein of coronavirus directs the virus entry into the cell and virus-cell as well as cell-cell fusion and syncytia formation (giant cells) in the epithelium of the lungs. Abl kinases are involved in S protein-dependent virus-cell or cell-cell fusion. Imatinib inhibits not only virus-cell fusion but also prevents syncytia and giant cell formation in the lungs.[195]

In Silico Study

We conducted an *In silico* modeling study to investigate the probable inhibitory or modulatory effect of losartan and imatinib in some critical points of the life cycle of SARS-CoV2. In this study we used bioinformatic tools (refer to the method) to assess the effect of the suggested drugs on important points found in the proposed theoretical pathophysiology, as well. It was elucidated that both losartan and imatinib could bind to ACE2 with lower docking energy (higher affinity) relative to the "Pre-inh"" (reference ligand, table 2). It does not mean that these drugs could inhibit the catalytic property of this carboxypeptidase: neither of these two drugs has been reported to exhibit any inhibitory property against ACE2, yet. Intriguingly, cholecalciferol showed similar behavior.

As a novel finding, we have demonstrated that losartan and imatinib could distort the binding site of SARS-CoV2 RBD to ACE2. According to our study there are seven points on the α-helix arm of ACE2 molecule located between glycine 24 and lysine 353 (fig 2A and 2D, table 3) where SARS-CoV2 RBD and ACE2 (acceptor-donor) may establish hydrogen (H) bonds (refer to the method and results). The distance between the acceptor-donor residues at these points are between 2.69-2.90Å (table 3). It means that hydrogen bond energies between SARS-CoV2 RBD and ACE2 based on the distance of acceptor and donor residues, without considering the hydrophobic contacts, are of moderate magnitude and mostly electrostatic.[196] Considering the low docking energy (high affinity) of losartan and imatinib to ACE2 which even dropped more (resulted in more affinity) after 100ns of exposure of these two drugs to ACE2 in MD simulation, it is obvious that the bond between losartan and imatinib with ACE2 is stable enough. In our modeling study, ACE2, after 100ns, exhibited significant translocation of the α-helix in its structure where RBD binds. According to table 3 this change in conformational shape of ACE2 made relocation of all 7 points of binding of ACE2 to SARS-CoV2 RBD for at least 1.80 Å. But

in some binding residues the change in location was more significant (more than 3.00 Å) which means that H-bonds at these points after the relocation might not be strong and stable enough:

1- for losartan in four out of seven binding points,
2- for imatinib in six out of seven binding points.

According to this modeling it is expected that the affinity of the virus to its receptor might decrease in the presence of these two drugs. In addition, our study showed that losartan among other available ARBs in the market has the highest affinity to ACE2 (table 5). It is implicated that other ARBs might not efficacious in docking to ACE2 to make an effective and stable similar conformational structural change in the receptor of the virus. In addition, in COVID 19 we aimed at inhibiting local intra-cellular rather than systemic RAS. Except telmisartan, other ARBs compared to losartan are more soluble in water. Therefore, losartan may penetrate into the cell more efficiently.

Furthermore, we could find that both losartan and imatinib as well as cholecalciferol could pose in Mpro, PLpro and MAPK molecules in the position of their “Pre-inh”” with higher affinity. These docking energies in our model are important because they determine how these ligands may probably affect the behavior of the proteases.

Of all the proposed ligands, imatinib could pose favorably in furin structure with higher affinity relative to its “Pre-inh”. Considering high expression of furin in the lungs[21] and importance of S’2 cleavage[21] in the entry of SARS-CoV2 to the target cell, if the inhibitory effect of imatinib against furin is approved in in vivo studies it can be regarded as an inhibitor that hinders entry of SARS-CoV2 to the target cell. Imatinib, cholecalciferol and losartan due to their effective docking to PLpro and Mpro with higher affinity relative to “Pre-inh” might be successful in preventing those proteases from letting the virus evade innate immunity or start replication. Although in the case of PLpro the docking energy for losartan and cholecalciferol were higher (lower affinity) than that of “Pre-inh”, the magnitude of energies (<-9.21) were low enough to consider them as probable inhibitor of PLpro. Studying RMSD diagram for 100ns MD simulation of losartan-PLpro complex, we could find that a sudden change of about 0.25 nm (2.5 Å) in the mean position of its molecules occurred in about 60ns that continued till the end of MD simulation for 100ns with a rise (fall in affinity) in the docking energy [from -9.21 (Kcal/mole) before MD simulation to -7.83 (Kcal/mole) post-hoc]. According to RMSF diagram almost the first 30 residues of PLpro showed the most fluctuations during 100ns simulation. These changes may indicate that losartan might affect the conformational shape and probably the function of PLpro at the expense of losing its affinity to the protein to some degree.[190,191] Perhaps longer dynamic study is needed to explain the real behavior of PLpro in the presence of losartan.

Losartan increases bradykinin concentration up to 2-fold but the risk of angioedema due to losartan is very low (0.1-0.4%).[199] Bradykinin induces allergic inflammatory responses to which p38 MAPK may contribute. Besides, bradykinin activates airway fibroblast/myofibroblasts through MAPK pathway.[200,201] In many of the pathophysiological destructive pathways in COVID 19 the signature of MAPK is evident. Surprisingly, in this modeling in silico study losartan and imatinib showed to have significant tendency to bind with p38 MAPK. This might indicate that these drug ligands may have inhibitory effect against p38MAPK and its downstream pathways and may inhibit untoward bradykinin-

dependent or responses, as well. In our *In silico* study, imatinib also showed its tendency to bind to Ang II. It should be investigated if imatinib could change the function of Ang II.

We, at Bazarganan Hospital, Tehran, Iran, in some patients (who accepted to be given losartan after being informed and written consent was taken) administered low doses of losartan (6.25-12.5 mg twice a day in non-hypertensive patients for 14-20 days) along with the anti-viral drugs (lopinavir/ritonavir) according to the approved national therapeutic protocols in patients with COVID19. Below (Figure 11) two slices (the same level and condition) of spiral lung CT-scan belonging to one patient taken on the day of admission and 4 days later, show significant clearing of ground glass opacities after administration of losartan. The patient's dyspnea subsided significantly after three doses of 6.25 mg of losartan.

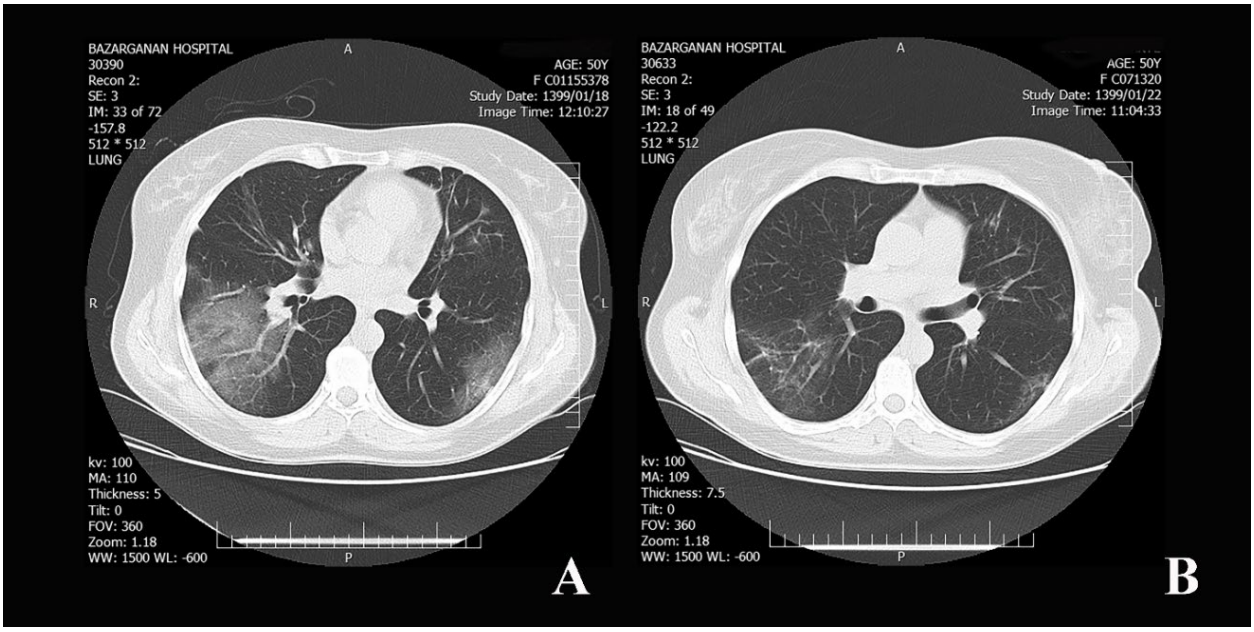


Figure 11. Spiral lung CT-scan of a 50 years old patient with COVID19; A: Apr 6, 2020; B: Apr 10, 2020

There comes an argument regarding increased mortality in hypertensive patients with COVID19 who have already been using ARBs for a long time. How if losartan can be protective against COVID19 these patients are more at risk of being infected by SARS-CoV2. Surprisingly, in an animal study it was demonstrated that chronic administration of losartan upregulates AT1R and PKCσ, both the latters increase the vulnerability of myocardium to ischemia and ischemia/reperfusion injuries.[202] Considering the above proposed hypothetical pathophysiology for the cytokine storm, hyperacute increase in Ang II in hypertensive patients with COVID19 who have been using ARBs may pose them to increased downstream effects of AT1R activation more than that seen in normotensive patients who has not used losartan.

We did not prescribe imatinib because its aerosolized form should be manufactured then be allowed to administer after successful subclinical studies. In addition, our study should be validated in clinical studies which at least for losartan it is under way in the USA.[175]

Conclusion

COVID 19 is a viral disease which involves the lung with an ARDS-like pathology, the major cause of death in this disorder. As the receptor of this virus is ACE2, a member of RAS family, which in entry of the virus to target cell downregulates in favor of pro-inflammatory ACE/Ang II/AT1R pathway it seems that imbalance of two opposing limbs of RAS contributes to the immunopathological features of this disorder. Shedding of ACE2 after contact with virus S protein is one the causes of downregulation of ACE2. As a debating theory, TNF-α is released in this process which as a pro-inflammatory cytokine aggravates the ignited inflammation. As there is not any efficient anti-viral drug or imminent vaccine against SARS-CoV2 it seems logic that treatment of ARDS in COVID 19 will be an effective measure in reducing the death toll of this infection.

In order to regulate RAS, which according to the above discussed pathophysiology, results definitely in modulation of the imbalance in pro- and anti-inflammatory responses and to boost anti-inflammatory potentials to subside the cytokine storm we selected losartan and imatinib to be investigated as probably efficient therapy in COVID 19 in an in Silico modeling study.

According to the findings in this study and the preliminary clinical evidences, we suggest low dose systemic losartan and inhaled aerosolized low dose imatinib be studied in a subclinical setting in treating ARDS in patients with COVID 19. In this manner while losartan by blocking AT1R antagonizes Ang II effects, aerosolized imatinib may modulate the local immunological responses. Based on our study both losartan and imatinib may change the behavior of PLpro. If this property of these drugs is approved in further subclinical studies it means that PLpro may not be able to let the virus evade innate immunity. This may provide the opportunity that PRRs recognize and present the virus meticulously to adaptive immunity apparatus. Furthermore, this combination may reduce the probability of binding of the virus to ACE2.

This modeling in silico study does not mean with certainty that these drugs would treat ARDS in COVID19. According to the proposed theory and these findings we were to shed a light on a novel route that might decline the fatality rate of this disease. Besides, these therapeutic measures do not obviate any need for future investigations in finding immediate novel anti-viral drugs or vaccine.

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c. Dr. Maryam Afkar, Radiologist,
d. Emergency Medicine Specialists,
e. All the personnel of ICU (specially the respectable nurses), Imaging Department and Emergency Room,

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