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Coronavirus (2019-nCoV) Deactivation via Spike Glycoprotein Shielding by Old Drugs, Bioinformatic Study

Mohammad Reza Dayer*

Department of Biology, Faculty of Science, Shahid Chamran University of Ahvaz, Ahvaz, Iran

***Corresponding author:**

Mohammad Reza Dayer, Department of Biology, Faculty of Sciences, Shahid Chamran University of Ahvaz, Ahvaz, Iran. Tel/Fax: +98-6113331045, E-mail: mrdayer@scu.ac.ir

Abstract:

The disease of COVID-19 comprises the most serious against human health worldwide with a high rate of virulence and mortality. The disease is caused by the 2019-nCoV virus from the beta coronavirus family. The virus makes use of its surface glycoprotein named S protein or spike to enter the human cells. The virus attached to its receptor named angiotensin-converting enzyme 2 on host cells surface via its receptor-binding domain and its fusion is mediated by cleavage at S2' site that is carried out by surface protease. Vaccines or drugs interfering with S protein binding or cleavage sites could be considered as drugs to get rid of the infection. In the current work and though docking and molecular dynamic experiments we have checked more than 100 drugs with high enough molecular weights for their shielding potency toward S protein binding sites and processing S2' sites. Our results indicate the shielding potency of:

fidaxomicin>ivermectin>heparin>azithromycin>clarithromycin>eryhthromycin>niclosamide>ritonavir.

Considering affluent reports regarding the complex disturbance in the immune system and multi-organ

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involvement in the disease there is no single or binary drug regime for cure expectedly and instead, we claim the multi-drug regime should be the choice in this context. Accordingly, we suggest our extracted drugs as an adjuvant for clinical trials.

Keywords: COVID-19, 2019-nCoV, Heparin, Ivermectin, Spike Shielding

Introduction:

Atypical pneumonia outbreak in 2019-2020 identified first in Wuhan in China's Hubei province is caused by a novel enveloped, positive-stranded RNA virus [1-3]. The virus is a new member of betacoronavirus family, including Severe Acute Respiratory Syndrome (SARS-CoV) and Middle East Respiratory Syndrome (MERS) coronaviruses called 2019-nCoV [4-5]. The disease is now called COVID-19 and accompanied by fever, cough and in advanced cases with severe respiratory distress with not well-characterized rate of mortality [6-7]. The virus, like other coronaviruses family, makes use of highly glycosylated S protein (spike) to enter host cells. The spike binds to angiotensin-converting enzyme 2 (ACE2) receptors in target cells with 10-20 fold higher affinity than SARS or MERS coronaviruses, the fact that underlies the high rate of virus spread between human cells as well as between individuals that leads to pandemic threat for worldwide safety [8-10]. Conformationally, spike glycoprotein is a trimeric protein belongs to class I fusion proteins. Each monomer or protomer contains 1288 amino acids in its primary structure. There are two major subunits called S1 and S2 subunits formed by cleavage of maternal string at Arg667-X668 residues (S1/S2 site) by membrane-bound furin protease to produce in mature prefusion form of the spike. In humans, the protease is expressed in multiple tissues, especially with high concentrations in alveolar cells. Protease process is essential for virus infiltration to host cells. Unlike 2019-nCoV, the SARS virus does not carry this cleavage site and so it is not dangerous as what 2019-nCoV is [11-15].

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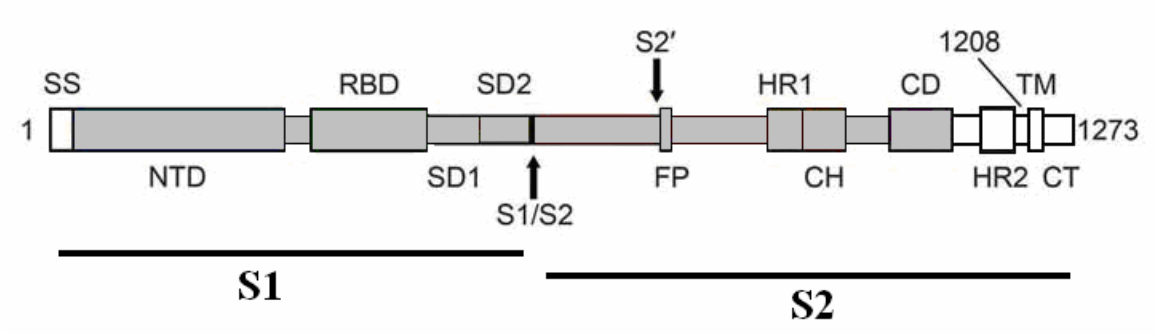

Scheme 1: graphic representation of different domains for 2019-nCoV S protein

As it is indicated in scheme 1, S1 subunit contains different functional domains beginning from N-terminus with SS (small signal sequence), NTD (N-terminal domain, residues 14-305), RBD (receptor binding domain, residues 319-541) and subdomains of 1 and 2 (SD1/SD2, residues 542-685). Considering the overall mushroom-like shape of the spike, this subunit places in the mushroom head with the RBD domain faced in such a way to interact with the cellular receptor of ACE2 [16-18]. The RBD domains of the spike trimer which are responsible for ACE2 binding adjust one of two conformations of up and down conformations. Up conformation corresponds to the receptor accessible state, while down conformation is inaccessible conformation [19-22].

The next subunit, S2 contains a signal sequence, a next cleavage site called S2′ for protease. This cleavage site becomes accessible for protease action upon receptor binding to the receptor and its consequent dissociation in the prefusion state. Fusion peptide (FP, residues 788-806) domain helps the virus to fuse host cells membrane and to form the post-fusion complex. Heptad repeat 1 (HR1, residues 912-984) central helix (CH), connector domain (CD), heptad repeat 2 (HR2, residues 1163-1213), transmembrane domain (TM, residues 124-1237) and cytoplasmic tail (CT, residues 1238-1273) are the rest domains of subunit S2 [16-17].

Upon S protein binding to ACE2 receptor and cleavage at S1/S2, subunit S1 undergoes vast structural rearrangements that eventually lead to its release from prefusion complex and ultimate fusion of the virus

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with host cell [10-18]. The detailed scenario for the 2019-nCoV attack is as follows: RBD domain with up conformation preferentially binds to the ACE2 receptor. Simultaneous cleavage of S1/S2 site by protease triggers structural alterations in the S1 subunit destabilized the prefusion trimeric structure of S glycoprotein that leads to dissociation of S1 subunit and refolds S2 subunit to postfusion conformation [23-25]. Successful infection of host cells accomplished by S2' cleavage by furin protease and release of fusion peptide that is essential for postfusion state and virus entrance. The S2' site in the prefusion state is buried and inaccessible for furin but upon shedding of S1 in postfusion conformation become accessible for hydrolysis [26]. During this phenomenon heptad repeat, 1 (HR1) and heptad repeat 2 (HR2) interact with each other to form fusion core of a six-helical bundle which bringing viral and cellular membranes in close proximity for fusion. Currently, this hydrophobic core is considered as an ideal target for vaccine design or ligand interaction as effective tools to combat 2019-nCoV and COVID-19 treatment [10-18].

In the current work and through molecular dynamic/docking experiments we tried to enrollee different approved drugs to see if the can bind to RBD domain of spike protein in competition with ACE2 receptor or if they can bind to S2' region to mask it against hydrolysis by host cell protease and prevent human infections by this virus.

Methods and Materials:

Spike Coordinate: Coordinate structures of 2019-nCoV and SARS S protein with PDB ID 6VYB and 6CRZ as well as coordinate structure of ACE2 receptor with PDB ID 1O8A were retrieved from protein data bank (https://www.rcsb.org/). The structures were obtained by the X-ray diffraction and refined at the resolutions of 3.46Å, 3.30Å and 2.0Å respectively. The structures were energy-minimized in 12.85×13.13×17.12 nm, 14.68×14.28×17.72nm and 7.21×8.30×7.75nm separate rectangular boxes. The simulated boxes were filled with SPCE water with shells of 1.0-nm thickness. Energy minimization algorithm of Steepest descent was used to minimize the system energy to lower than 100 kJ/mol. Neutral

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pH (given Asp, Glu, Arg and Lys ionized), temperature of 37°C and one atmospheric pressure were used as energy minimization conditions [27-28].

In order to study the dynamic behavior of spike proteins especially at RBD and fusion core we performed molecular dynamic simulations using double-precision MPI version of GROMACS 4.5.5 installed on UBUNTU version 16.04 with GROMOS force field for 20 ns at 37degrees centigrade and 1 atmosphere [29].

Sequence Alignment: given the binding property of RBD domains for SARS and 2019-nCoV is determined by their amino acid sequences we compared the RBD sequence with the same sequence of SARS-CoV through sequence alignment on EMBOSS Stretcher (www.ebi.ac.uk), scheme 2, to pick up the underling principles for their different pathogenesity comparatively [30-31].

```
6VYB---001          1 RVQPTESIVRFPNITNLCPFGEVFNATRFASVYAWNRKRISNCVADYSVL      50
                                |||||||||||||||:|.|||||.||:||||||||||||
6CRZ---001          1 -------------ITNLCPFGEVFNATKFPSVYAWERKKISNCVADYSVL      37

6VYB---001         51 YNSASF-STFKCYGVSPTKLNDLCFTNVYADSFVIRGDEVRQIAPGQTG-      98
                      ||| :| |||||||||.||||||||:||||||||::||:||||||||||
6CRZ---001         38 YNS-TFFSTFKCYGVSATKLNDLCFSNVYADSFVVKGDDVRQIAPGQTGV      86

6VYB---001         99 KIADYNYKLPDDFTGCVIAWNSNNLD--SKVGGNYNY-LYR-LFR--KSN     142
                       ||||||||||||.|||:|||:.|:|  | .| ||||  || | |  |
6CRZ---001         87 -IADYNYKLPDDFMGCVLAWNTRNIDATS-TG-NYNYK-YRYL-RHGK--     129

6VYB---001        143 LKPFERDISTEI-YQA--GSTPCN-GVEGFNCYFPLQSYGFQPTNGVGYQ     188
                      |:|||||||. : : :  |. ||.   ...|||:||..|||..|.|:|||
6CRZ---001        130 LRPFERDISN-VPF-SPDGK-PCTP--PALNCYWPLNDYGFYTTTGIGYQ     174

6VYB---001        189 PYRVVVLSFELLHAPATVCGPK-KSTNLVKNKCVNF--------------     223
                      ||||||||||||:|||||||||  ||:|:||:||||
6CRZ---001        175 PYRVVVLSFELLNAPATVCGPKL-STDLIKNQCVNFNFNGLTGTGVLTPS     223
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Scheme 2: sequence alignment result performed of EMBOSS Stretcher (www.ebi.ac.uk) for the RBD domains (residues 319-541) from FASTA files of protein structures with PDB IDs' of 6VYB and 6CRZ.

Ligands Coordinate structures: coordinate structures for fidaxomicin, ivermectin, heparin, azithromycin, clarithromycin, niclosamide, erythromycin and ritonavir were retrieved from PubChem database (https://pubchem.ncbi.nlm.nih.gov/) as SDF format, converted to PDB format in Open Babel server (http://openbabel.org/) and optimized in ArgusLab software (http://www.arguslab.com/) [32].

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Blind Docking experiments: to survey the potential binding potency of available drugs with high enough molecular weight and binding energy we carried out blind docking experiments in Hex 8.0.0 (http://www.loria.fr/~ritchied/hex/) using 2019-nCoV spike protein as receptor against enrolled drugs as lignds [33]. The mode of Sahpe+Electrostatic with macro sampling was used as docking parameters and the best 100 poses were analyzed accordingly.

Data Handling and Analysis: all the numerical data were exploited in Excel and SPSS software. P value under .05 was considered as the significance level.

Results and Discussion:

Studies on S proteins from SARS-CoV and 2019-nCoV origins indicated that despite large differences seen in their whole sequences and also at their domains including RBD which is determinant for receptor recognition and consequent virus infectivity, there seem to be structural similarities between these two proteins especially at their domains of NTD, RBD SD1 and SD2 from S1 subunit as well as domains of FP, HR1, HR2, and S2' cleavages site from S2 subunits with RMSD differences less than 4Å [11-15]. Henceforth it expected that these two proteins should behave similarly in their functions i.e. receptor recognition and host cell infection.

Given the much higher affinity of 2019-nCoV for ACE2 than SARS-CoV indicate that there should be detailed differences between these two S proteins that play a vital role in more severity of COVID-19 outbreak with high rates of virulence and mortality. Structural optimization and molecular dynamic simulations for S proteins from these two origins reveal detailed structural differences 2019-nCoV and SARS-CoV spike proteins. Figure 1-a, represents the root mean square fluctuations (RMSF) for alpha carbons of proteins during the 20ns period of simulation for S proteins. As it is clear S protein of 2019-nCoV expresses a lower average RMSF value of 0.52nm for the whole sequence while the average

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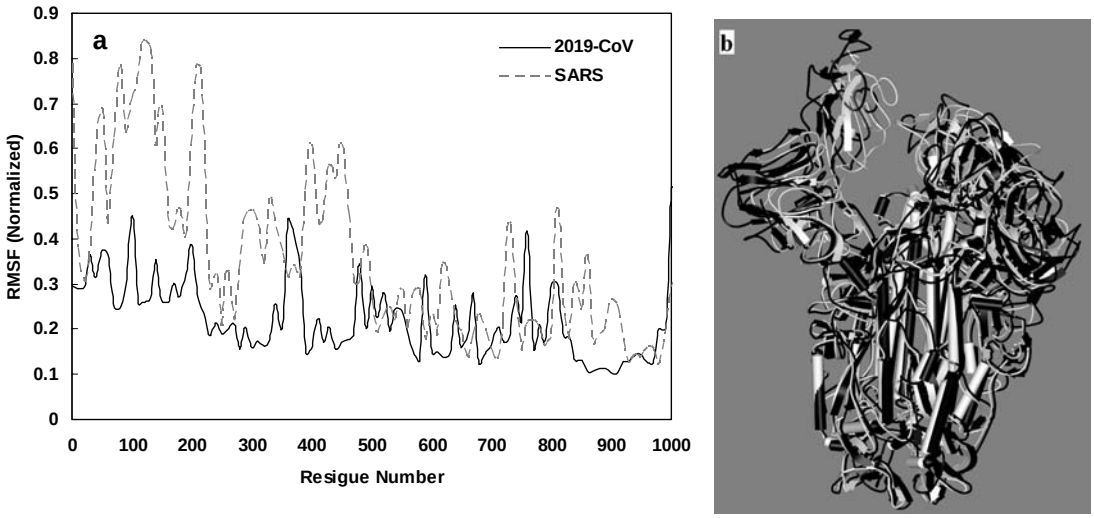
RMSF for SARS-CoV is 0.65nm and the curve of RMSF for 2019-nCoV places beneath SARS-CoV curve along the protein sequence. This means the vast alterations or mutations in the 2019-nCoV sequence lead to decreased flexibility and a more tightly folded structure for S protein of 2019-nCoV. This fact may play roles in the higher affinity of 2019-nCoV S protein for ACE2 receptor with higher infectivity. Calculations of RBD domains give 0.56nm and 0.83nm for 2019-nCoV and SARS-CoV respectively. This finding may be interpreted as the more constant and more effective binding interface for RBD in 2019-nCoV.

Figure 1-b represents the superposed for these two proteins. The protein of 2019-nCoV is shown in white color while the SARS-CoV one in black. It is evident that 2019-nCoV is surrounded by SARS-CoV protein indicating the more compacted and tightly folded structure for 2019-nCoV spike protein.



Figurt-1: a- root mean square fluctuations (RMSF) curve for 2019-nCoV and SARS-CoV spike protein extracted from trajectory file of 20ns simulation period at 37 degree centigrade, pH=7 and 1atmosphere pressure as mean fluctuation per alpha carbon. b- Superposed structures of 2019-nCoV (white) and SARS-CoV (black) spike proteins.

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To track the detailed mechanism of S protein binding to ACE2 receptor and to determine their probable sites of interactions we preformed blind docking experiments in Hex 8.0.0 software to statistically analyzed the binding pattern and their energies of RBD domains to ACE2 receptor for the best 100 poses. Our data in table 1 indicate that there are three kinds of binding patterns being between ACE2 and RBD domains. The RBD domains may be attached to the ACE2 receptor through up or down conformations of RBD or intervening regions of RBD domains. Table 1 indicates that the S protein of SARS-CoV in about 53 percent of the 100 poses is attached to ACE2 binds to RBD domain using up conformation with only in 1 percent to inter RBD domains region while in the rest 46 percent poses binds with far parts of sequences contrast to RBD domains. In contrast, the S protein of the 2019-nCoV virus binds to the ACE2 receptor using down conformation of RBD domain in 46%, 10% in up conformation, and 15% of inter domains regions. Unlike previous studies, our dockings indicate that in the trimeric structure of 2019-nCoV, S protein that alike SARS-CoV protein carries one RBD in up and two in down conformation, surprisingly, this protein can binds to ACE2 receptor by RBD domains both in up and down conformation and their intervening regions with much higher binding energy (-450.51kJ/mol) and affinity. Our data also indicates that S protein of SARS-CoV mainly binds to ACE2 receptor by up conformation of RBD domain with a much less binding energy of -379.66kJ/mol (p-value<0.01). This finding may be partially helpful in understanding the higher affinity of 2019-nCov for the ACE2 receptor and its more severe virulence [19-22].

Table 1: Binding pattern of S protein RBD domains to ACE2 receptor in accordance with their binding energies extracted from docking experiments performed on HEX 8.0.0

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	Up (%)	Down (%)	Inter domains region (%)	Binding Energy(kJ/mol)
SARS-CoV	53	0	1	-379.66
2019-nCoV	10	46	15	-450.51

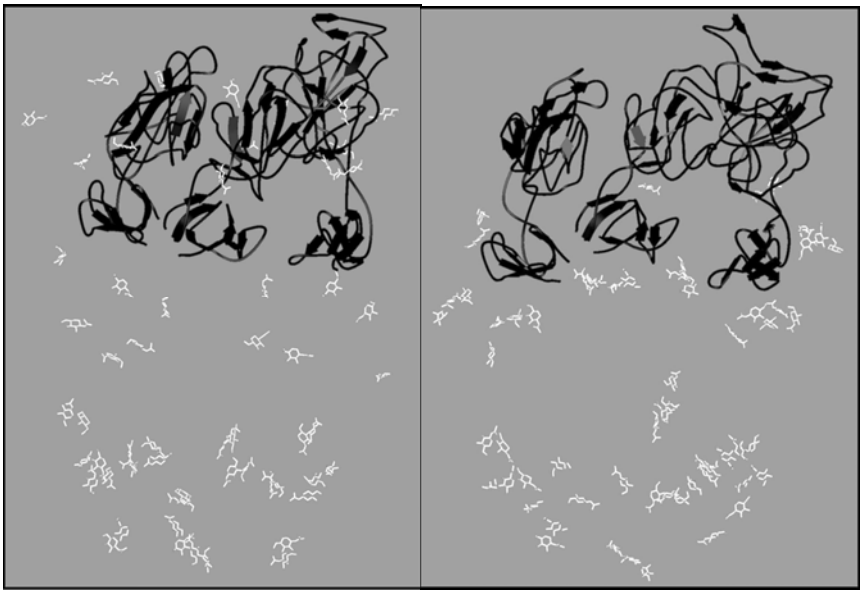

Figure 2: Schematic representation of N-acetyl glucosamine residues distribution through trimeric S protein.

The next parameter that may affect the potency of interactions between S protein and ACE2 receptor is the intervening interactions posed by sugar moieties of N-acetyl glucosamine (NAG) units that participate in hydrophobic interactions with receptor upon their interaction. Figure 2 graphically shows that contrast to SARS-CoV (right scheme), S protein of 2019-nCoV (left scheme) carries more quantities of NAG in its top side in near vicinity to RBD domains that can potentiate the interactions between RBD and ACE2 receptor upon binding [35-37]. This is the next factor that may interpret the higher affinity of 2019-nCoV S protein to host cell receptors we postulate. Isoelectric pH (pI) is the pH in which the protein has no net charge or the total charge of the protein is zero. Using protein sequence we have

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calculated the pI of proteins on www.web.expasy.org/compute_pi/ as 5.82 for ACE2 receptor than at blood pH of about 7.4 this protein like membrane phospholipids carries a negative charge. The calculated pI for RBD domains is 7.22 and 7.89 for S proteins of SARS-CoV and 2019-nCoV respectively. These calculations reveal that at blood pH S protein from SARS-CoV should carry negative while 2019-nCoV positive net charge and this means that the attractive electrostatic force between ACE2 and 2019-nCoV S protein fasten their binding and describe their higher binding affinity.

Is such a sophisticated highway of mechanistic studies different ways are now suggesting to combating or deactivating 2019-nCoV infections from S protein and ACE2 receptor interaction points of view. Among these ways shielding the S protein by small ligands, designing a vaccine against S protein, and/or injection of soluble forms of recombinant ACE2 to prevent or misled virion to attacks human cells receptors are more advised recently [10-18, 33-35]. Our docking results reveal that there is a significant correlation between drug molecular weights and their binding energies. Accordingly, we then have enrolled more than 100 candidates from approved drugs with high enough high molecular weights for docking and to chose drugs with considerable binding energy to S protein contrast to ACE2 receptor to suggest them as candidates to combat 2019-nCoV infections after clinical approval.

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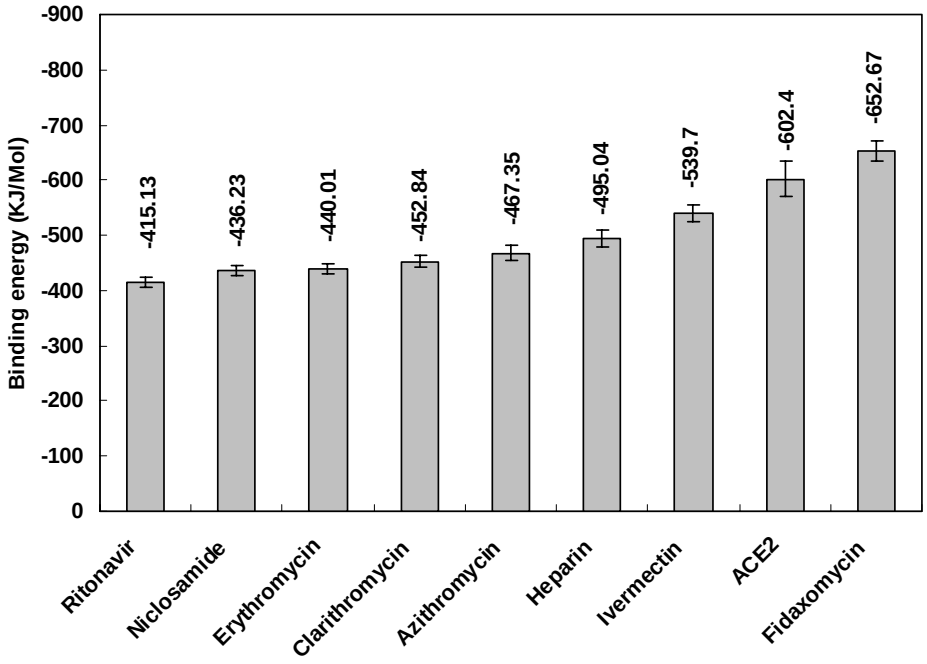

Figure 3: binding energies of selected nine drugs in accordance with to ACE2 to S protein extracted as Mean±SD from blond docking experiments performed on Hex 8.0.0

Finally and among the enrolled drugs, we have selected nine drugs with comparable potencies including fidaxomicin, ivermectin, heparin, azithromycin, clarithromycin, niclosamide, erythromycin, and ritonavir. Fidaxomicin represents the highest binding energy of -652.67±19.19 kJ/mol and the highest affinity for S protein. Unfortunately, we find that fidaxomicin does not bind via RBD domains to S protein, and instead, it binds merely to S2' site region and can prevent virus engagement to host cells in the late stage of activation. Ivermectin, is a medication used to treat parasite infestations, reveals binding energy of -539.7±16.19 kJ/mol that is significantly lower than that's of ACE2 receptor binding energy of -602.4±31.28 kJ/mol) in more than 45% of docking poses to RBD domains and in about 20% to S2' cleavage site and accordingly inhibits virus attachment to RBD domain in the first step and also prevents virus activation in late phase [38-39]. The next selected drug is heparin (MW=1134.9gr/Mol) which

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shows the binding energy of -495.04±14.94 kJ/mol. Our docking experiments indicate that heparin can extensively mask RBD and S2' regions against their accessibility for ACE2 binding and protease cleavage. Heparin is a medication used as an anticoagulant to treat heart attacks and unstable angina as intravenous or subcutaneous injections [40-41]. Bleeding, painful injected sites, decreased counts of platelets, and thrombocytopenia is the major drawbacks of heparin prescription. Nevertheless, there are reports showing that heparin beyond its anti-coagulation nature, shows the useful anti-inflammatory effect, decreasing immune cell recruitment, neutrophil activation, and degranulation [42-43]. Given the negative charge of heparin and previously mentioned positive charge of S protein, we hypothesize that the electrostatic binding forces between heparin and S protein are much higher than that undertaken in docking experiments performed by Hex software. Moreover, it is very important to mention that the heparin used in our experiments medicinally considered as ultra-low molecular weight heparin with 5 sugar residues. This kind of heparin is used primarily in acute coronary syndrome, pulmonary embolism, and deep venous thrombosis. In practice, low molecular weight heparin with higher molecular weight and more sugar residues range from 4 to 22 than what we used is the most favorable and more prescribed form of heparin in the clinic. This fact clearly indicates that such type of heparin expectedly will exert more shielding effects on S protein than what we claimed and can prevent virus entrance to human cells [44]. The macrolides antibiotics of azithromycin, clarithromycin, and erythromycin used in our experiments with binding energies of -468.35±13.88, -452.84±10.8 and -440.01±9.04 kJ/mol respectively comprises good anti-viral candidates. Taking into account that macrolide with confirmed immunomodulatory effects makes them valuable candidates for further studies in COVID-19 therapy [45-48]. Additionally, deliberate reviews on docking pose for macrolides indicate that they are capable to shield RBD domains as well as S2' cleavage sites. Niclosamide is an anthelmintics drug used to treat worm infections with broad antiviral properties is the penultimate candidate in this series [49]. Our

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docking experiment shows that niclosamide has a binding energy of -436.23±9.78 kJ/mol for S protein but like fidaxomicin can only bind to S2' region and can only prevent 2019-nCoV activation in late phase and host cells invasion. Among anti-HIV drugs only ritonavir with a molecular weight of 720.94 gr/Mol can bind to S protein with comparable energy of -415±10.09 kJ/mol and can act as shielding drugs in addition to its anti-protease activity. It is very important to mention that even though the binding energies of our shielding candidates except fidaxomicin are significantly lower than the binding energy for ACE2 receptor in 1:1 competition ratio but we should remember that in pharmacological dosage the ratio of drug/ACE2 receptor is far from unity and so we can expect that the total binding energies of shielding candidates are much higher than ACE2 receptor and hence they will comprise logic shield toward viral infections.

Conclusion:

To this end, the disease of COVID-19 with a high rate of virulence and fast spread in the human body with multi-organ involvement and high rate of mortality comprises the greatest problem since the Second World War with more than 3 millions infected cases and more than 218,000 deaths by April 2020 [50-52]. Decreased lymphocytes, increased C reactive protein (CRP), and pro-inflammatory cytokines as well as hypercoagulability with increased d-dimer lead to lung lesions with infiltrated immune [53-56]. It seems credulous to think that in a battle against COVID-19 that invades multi organs and disturbs the immune defensive system in a short period to be achieved by one or two drugs, especially at an advanced state. Based on plentiful reports in this context and considering our current and previous work [57] we imagine that a successful treatment regime should contain multi drugs of protease inhibitors, spike shielding drugs, and immunomodulatory drugs in early steps of the disease. Ivermectin>heparin (as intravenous or nebulized)>macrolides seem to be good adjuvant candidates in all anti 2019-nCov regimes to shield S protein even for prophylactic purposes.

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