Combining SARS-CoV-2 proofreading exonuclease and RNA-dependent RNA polymerase inhibitors as a strategy to combat COVID-19: a high-throughput *in silico* screen

Dr. Shradha Khater¹, Dr. Nandini Dasgupta² and Dr. Gautam Das²

¹Department of Biosciences and Bioengineering, Indian Institute of Technology Bombay, Mumbai, India

²miBiome Therapeutics LLP, Mumbai

Contact: shradha.khater@iitb.ac.in, gautam@microbiome.in

ABSTRACT:

SARS-CoV-2 has infected millions of people worldwide. Currently, many clinical trials for drugs against COVID-19 are underway. Viral RNA-dependent RNA polymerase (RdRp) remains the target of choice for prophylactic or curative treatment of COVID-19. Nucleoside analogs are the most promising RdRp inhibitors and have shown effectiveness *in vitro* as well as in clinical settings. One limitation of such RdRp inhibitors is removal of incorporated nucleoside analogs by SARS-CoV-2 exonuclease (ExoN). Thus, it accomplishes resistance to many of the RdRp inhibitors. We hypothesize that in the absence of highly efficient antivirals to treat COVID-19, combination drugs with RdRp and ExoN inhibitors will be a promising strategy to combat the disease. To repurpose drugs for COVID-19 treatment, 10,397 conformers of 2,240 approved drugs were screened against ExoN domain of nsp14. The molecular docking approach helped us to identify Dexamethasone metasulfobenzoate, Conivaptan, Hesperidin and Glycyrrhizic acid as potential inhibitors of ExoN activity. We recommend further investigation of a combinational therapy using RdRp inhibitors with a repurposed ExoN inhibitor, deciphered through this study, as a potential COVID-19 treatment.

INTRODUCTION:

On December 31, 2019, the World Health Organization (WHO) Office in China was informed that cases of pneumonia of an unknown cause had been detected in Wuhan City, in the Hubei Province of China. The Chinese authorities identified this to be a previously unknown type of coronavirus, Severe acute respiratory syndrome coronavirus 2 (SARS-Cov-2), causing the disease COVID-19. Since the outbreak, the number of confirmed cases of COVID-19 increased rapidly resulting in WHO declaring the disease a pandemic on March 11, 2020. Almost all countries have been affected by this pandemic with millions of confirmed cases and hundreds of thousands of deaths worldwide. Currently there are no approved drugs. Repurposed antivirals are showing good promise in clinical trials. However, there is an urgent need to have a repertoire of repurposed drugs to improve the efficacy and in addition be prepared for drug resistance. Synergistic action of a combination of drugs against SARS-CoV-2 can enhance the effectiveness of existing drugs which have shown partial success in clinical

trials. The class of antivirals which are RNA-dependent RNA polymerase (RdRp) inhibitors like Favirpiravir, Remdesivir, Ribavirin and Galidesivir have been on high priority since the beginning of COVID-19 trials. Trials have been completed/are in progress in many countries. Among these, Remdesivir and Favirpiravir has shown promise in different countries. These drugs being nucleoside analogs act either by introducing mutations in the viral RNA or by chain termination during replication. The action of these drugs on viruses which do not have proofreading enzymes have been good [1]. However, SARS-Cov-2 possesses a non-structural protein nsp14, with amino terminal domain coding for a proofreading exonuclease (ExoN) [2]. ExoN is capable of excising incorporated nucleoside analogs by virtue of its 3'-5' exonuclease proofreading activity. This results in negating the action of these drugs, to varying extents, depending on the type of nucleoside analog chemistry (Ribavirin, 5FU, Remdesivir) (Figure 1) [3, 4]. Hence, in case of repurposed drugs for COVID-19, limitation of efficacy exists. The new generation RdRp inhibitors like Remdesivir have been more effective than Ribavirin and 5FU, in that the excision of these nucleosides by viruses harbouring exoribonuclease has been weaker than Ribavirin and 5FU [5]. The delicate balance between incorporation and excision properties of nucleoside analogs between the RdRp and ExoN decides the fate of the action of RdRp-based antivirals.

ExoN inactivation was found to confer a “mutator phenotype,” as was evident from a 15- to 21-fold increase in mutation frequency – relative to the wild-type (wt) control - during replication and passaging in cell culture [6, 7]. There is evidence that in ExoN mutant background, Remdesivir has 4.5-fold more efficacy [8] and Ribavirin has 200-fold higher efficacy [3] when compared to a wild type ExoN viral genome. The mutagenesis results are supported by sequencing analyses too [6]. This provides a clear rationale to use a combination of antivirals Favirpiravir/Remdesivir/Ribavirin/Galidesivir and SARS-CoV-2 ExoN inhibitors. Currently there is no drug available to inhibit nsp14. Detailed molecular docking studies to find small molecules/peptides/natural molecules that have the potential to inhibit nsp14 ExoN, are required urgently [9]. This would potentially lead to improve the current efficacy of the RdRp inhibitors in COVID-19. It is interesting to note that coronaviruses lacking ExoN are susceptible to lethal mutagenesis [4]. Crystal structure of SARS-CoV nsp14-nsp10 [10] provides opportunities for molecular docking of the ExoN domain of nsp14 to different available drugs.

In this study, we propose that a combinatorial therapy with one drug from Favirpiravir/Remdesivir/Ribavirin/Galidesivir and an inhibitor of ExoN would be effective in increasing the efficacy of the above RdRp inhibitors (**Figure 1**). In an effort to repurpose drugs for COVID-19 treatments, we have performed molecular docking of 10,397 approved drug

conformers on ExoN domain of SARS-CoV-2 nsp14. Three known antivirals Conivaptan, Hesperidin and Glycyrrhizic acid show promise based on the docking results and their known inhibitory effects on beta coronaviruses *in vitro* [11] and in patients [12]. Molecular docking result indicated Dexamethasone metasulfobenzoate as lowest energy binding partner of ExoN domain. Interestingly, dexamethasone was recently shown to reduce fatality in COVID-19 patients [13]. Therefore, repurposing Dexamethasone metasulfobenzoate, Conivaptan, Hesperidin and Glycyrrhizic acid as potential inhibitors of proof-reading ExoN (nsp14) and using them in conjunction with RdRp inhibitors could lead to potentially high level of antiviral activity and a promising therapy for COVID-19.

RESULTS:

SARS-CoV-2 ExoN domain

SARS-CoV-2 nsp14 is a multidomain protein. The N-terminal domain functions as proofreading exoribonuclease and the C-terminal is a methyl transferase. SARS-CoV-2 nsp14 shares 95.07% amino acid sequence identity (over complete protein length) with SARS-CoV nsp14. ExoN domain of SARS-CoV nsp14 resembles DEDD-type exonucleases [10]. The DEDD superfamily members are defined by presence of three canonical motifs – DXE (Motif I), W(X)4EL (Motif II), and DAIMTR (Motif III) [8]. Presence of DEED instead of DEDD and an additional H makes the SARS-CoV ExoN a DEEDh type exonuclease [5]. In SARS-CoV-2, the catalytic residues - Asp90, Glu92, Glu191, His268, and Asp273, and the canonical motifs are conserved (**Supplementary Figure S1**). 3D model of SARS-CoV-2 nsp14 was built using SARS-CoV nsp14 (PDB ID: 5C8S) as template. A grid comprising of the three conserved motifs was used for docking.

Molecular Docking

10,397 conformers generated from 2,240 approved small molecule drugs were screened using AutoDOCK VINA. Based on binding affinity energy, top 20 binding poses were selected for further analysis (**Table 1, Figure 2**). All 20 poses interact with catalytic residues. Dexamethasone metasulfobenzoate binds to the catalytic site of ExoN with binding energy of -8.7 kCal/mol. Conivaptan, Dutasteride, Hesperidin, Lumacaftor and Glycyrrhizic acid bind ExoN active site with slightly higher energy of -8.6 kCal/mol. Interaction of ExoN domain with 12 unique drug molecules, corresponding to top 20 poses, was studied and are depicted in **Table 2**. Most of the analysed poses interact with at least 3 out of 5 catalytic residues (**Figures 3 & 4**). Hesperidin is the only drug that interacts with all five catalytic residues. Hesperidin and Glycyrrhizic acid has 4 and 3 ExoN catalytic residues respectively, within hydrogen bonding distance (**Figure 4**). Binding of these drugs to ExoN catalytic residues might potentially cause obstruction of substrate binding and catalysis.

Remdesivir, an investigational drug for treatment of Ebola, was shown to inhibit SARS-CoV-2 RNA-dependent RNA polymerase, inhibiting RNA synthesis[14]. As Remdesivir is a drug without anti-ExoN activity, it was used as a negative control. The binding energy of Remdesivir with ExoN is -6.0 kCal/mol. Hence, the binding energy of Remdesivir is much higher than the top 20 poses obtained using approved drugs (**Table 1**).

DISCUSSION:

COVID-19, an infectious respiratory illness, is caused by a novel strain of coronavirus SARS-CoV-2. Currently, there are no approved drugs or vaccines. Drugs targeting RdRp like Favipiravir, Remdesivir, Ribavirin and Galidesivir have shown promise in SARS-CoV-2 and few other strains of coronavirus. Nucleoside analogs - Remdesivir and Favipiravir have been authorized for emergency use in the treatment of COVID-19 in different countries. These RdRp inhibitors act by competing with host nucleoside substrates for incorporation in nascent RNA being synthesized [15]. The mis-incorporated analogs either cause a premature chain termination or mutation in RNA (**Figure 1**). ExoN domain of nsp14 in CoVs is known to excise mis-incorporated analogs [3]. The excision action of ExoN decreases the efficacy of nucleoside analog like Ribavirin *in vivo* [3]. Decrease in efficacy of Remdesivir has been speculated due to ExoN activity [14]. A 100-fold increase in Remdesivir efficacy was seen in ExoN mutant of murine hepatitis virus (MHV), a betacoronavirus [16]. It's been hypothesized that simultaneous inhibition of RdRp and ExoN in CoVs could be an effective therapeutic strategy [17].

Hence, *in silico* drug screening method was used to search for potential inhibitor of ExoN. 10,397 conformers from 2,240 approved drugs were screened against SARS-CoV-2 nsp14 containing the ExoN domain. AutoDOCK VINA screening results indicated Dexamethasone metasulphobenzoate to be the top binder, and Conivaptan, Dutasteride, Hesperidin, Lumacaftor and Glycyrrhizic acid to be close second. All six compounds showed extensive interaction with nsp14 residues, especially the catalytic residues of ExoN domain (**Table 2**). Hence, we hypothesize that these drugs might occlude ExoN catalytic site, thereby inhibiting the proof-reading activity (**Figure 1**).

Interestingly, few of the approved drugs that bind with ExoN catalytic site *in silico*— Conivaptan, Hesperidin and Glycyrrhizic acid – have shown anti-viral activity in earlier studies. Conivaptan, a non-peptide inhibitor of vasopressin, has shown *in vitro* efficacy against feline infectious peritonitis coronavirus (FIPV) and human coronavirus OC43 (HCoV-OC43) [18]. Conivaptan showed inhibitory effect against Dengue and Zika virus at high concentrations. Inhibitory activity of Hesperidin against Influenza A virus was reported by Dong *et al* [19]. Its antiviral response was linked to increase in cell-autonomous immune responses (enhanced

expression of primary and secondary genes). In addition, Hesperidin inhibited export of viral ribonucleoproteins. Hesperitin, aglycone form of hesperidin, was shown to inhibit cleavage activity of SARS-CoV 3C-like protease.

Glycyrrhizic acid, an anti-tumoral, anti-inflammatory drug, has *in vitro* inhibitory effects on a broad range of viruses like flaviviruses [20], herpes viruses and human immunodeficiency virus [21]. Glycyrrhizic acid has been used for treatment of SARS-CoV [12] and chronic hepatitis virus in patients [22]. Hence, there are varied degree of evidence in support of antiviral use of Conivaptan, Hesperidin and Glycyrrhizic acid as antiviral. Our docking result combined with evidence in support of antiviral use of Conivaptan, Hesperidin and Glycyrrhizic acid, indicate their potential as SARS-CoV-2 ExoN inhibitor. When used in a combination with RdRp inhibitors, the higher concentrations reported for the repurposed ExoN inhibitors to exert antiviral activity could be minimised. In addition, the higher concentrations required for RdRp inhibitors to exert their action in the presence of exonuclease activity like in SARS-Cov-2 can also be reduced as dual action of RdRp and ExoN inhibition in parallel should ideally require lower concentrations of the respective drugs.

In a randomized controlled trial at Oxford University (Recovery Trial), Dexamethasone, a glucocorticoid, has been recently shown to reduce fatality by third in critically ill COVID-19 patients requiring ventilator support [13]. Dexamethasone acts by reducing inflammation and countering body's overreaction called cytokine storm. Here we propose use of Dexamethasone metasulfobenzoate as an inhibitor of ExoN. Dexamethasone's antiviral activity is predicted to be pronounced in combination with RdRp inhibitors, according to our model (**Figure 1**). Use of these potential ExoN inhibitors in combination with nucleoside analog targeting RdRp, warrants further investigation in clinical settings.

A combination of RdRp inhibitors and ExoN inhibitors, in addition to the increased efficacy, would also possibly avert drug resistance generated from mutations in ExoN to enhance proof-reading of nucleoside analogs in RdRp inhibition.

Strategy to use combination of RdRp inhibitor and antisense/RNAi technology: In addition to drug candidates for nsp14, it is conceivable that a combination of RdRp inhibitor and an antisense/RNAi strategy to inhibit nsp14 can lead to enhanced success in clinical trials. Combined with nasal delivery systems, this can hold promise in developing new combination antiviral therapies for COVID-19. An FDA-approved technology for antisense antiviral technology exists: Fomivirsen (Vitravene®, ISIS-2922), ASO antiviral drug with phosphodiester linkages that are modified by replacing a non-linking oxygen with a sulfur atom (phosphorothioate modification). An FDA-approved technology for RNAi also exists: Onpattro, an RNAi therapeutic for people with a rare and deadly genetic disorder called hereditary transthyretin-mediated amyloidosis. Morpholino oligos for coronavirus have been used experimentally [23].

We conclude that even if the repurposed ExoN inhibitors identified in this study are incomplete in isolation, the synergistic effect with RdRp inhibitors will make it a worthwhile case for clinical trials and treatments for COVID-19. These drugs have been extensively used on patients and their safety profiles are well known. Any incremental value in the efficacy of antivirals like Remdesivir, Favipiravir and Ribavirin would lead to huge implications for COVID-19-related healthcare burden globally.

METHODS:

Homology modelling of ExoN

Amino acid sequence of SARS-CoV-2 ExoN/nsp14 (P0DTD1) was used as a target sequence to build 3D-model using SWISS-MODEL webserver [24]. Nsp14 from SARS-CoV (PDB ID: 5C8S, chain B) had high sequence identity to target sequence – 95.07%. This 3D structure was used as a template for building SARS-CoV-2 nsp14 model. The model quality was assessed using QMEAN score. The QMEAN score for generated model was -3.14. Scores above -4 indicates a good model.

Virtual screening using AutoDock VINA

2D structures of approved small molecule drugs were downloaded from DrugBank in sdf format (2454 structures) [25]. The structures were converted to 3D format using OpenBabel -gen3d option [26, 27]. Few of the drug molecule structures showed error at this stage. 3D structures of these molecules were downloaded from other sources. Multiple conformers for each 3D structure were generated using OpenBabel.

Docking grid was defined encompassing the conserved motives of ExoN domain (see result). A total of 10,397 conformers from 2,240 compounds were subjected to virtual screening using the AutoDOCK VINA tool on SARS-CoV-2 nsp14 [28]. 20 poses were generated for each round. Compound-nsp14 interactions were visualized using LigPlot⁺ v2.1 [29].

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Conflict of Interest:

Authors declare no conflict of interest

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Figures and Tables:

Table 1: Screening results of twenty conformers with lowest binding energies. Binding energy is expressed as kcal/mol

Drug Bank ID	Conf ID	Name	Affinity (kCal/mol)
DB14703	1	Dexamethasone metasulfobenzoate	-8.7
DB00872	3	Conivaptan	-8.6
DB01126	1	Dutasteride	-8.6
DB01126	3	Dutasteride	-8.6
DB04703	1	Hesperidin	-8.6
DB09280	1	Lumacaftor	-8.6
DB13751	3	Glycyrrhizic acid	-8.6
DB14703	0	Dexamethasone metasulfobenzoate	-8.6
DB14703	2	Dexamethasone metasulfobenzoate	-8.6
DB14703	3	Dexamethasone metasulfobenzoate	-8.6
DB14703	4	Dexamethasone metasulfobenzoate	-8.6
DB00696	2	Ergotamine	-8.5
DB01126	0	Dutasteride	-8.5
DB03147	3	Flavin adenine dinucleotide (FAD)	-8.5
DB06210	1	Eltrombopag	-8.5
DB00637	3	Astemizole	-8.4
DB00696	1	Ergotamine	-8.4
DB00696	3	Ergotamine	-8.4
DB00878	0	Chlorhexidine	-8.4
DB01251	1	Gliquidone	-8.4

Table 2: Residues involved in hydrogen bond and hydrophobic interaction

Drug Bank ID	Name	H-bond contacts	Hydrophobic Contacts
DB14703	Dexamethasone metasulfobenzoate	D90 , N104, N252, L253	V91, E92 , G93, Q145, F146, W186, F190, Q254, N266, H268 , D273 ,
DB00872	Conivaptan		M58, D90 , V91, E92 , G93, N104, F146, W186, A187, F190, N252, L253, Q254, N266, H268 , D273 ,
DB01126	Dutasteride	G93, N104, Q254	E92 , F190, N252, N266, H268 , D273 ,
DB04703	Hesperidin	D90 , V91, E92 , Q145, E191 , N252, D273(3)	H148, F146, W186, A187, F190, L253, N266, H268 , A267
DB09280	Lumacaftor	D90 , G93(2), N104, N252, Q254, N266,	V91, P141, F146, W186, A187, F190, H268
DB13751*	Glycyrrhizic acid	D90 , E92 , G93(2), N104, E191	V91, Q145, F146, F190, N252, L253, Q254, H268 , N266
DB00696	Ergotamine		D90 , V91, E92 , G93, N104, P141, Q145, F146, W186, F190, E191 , N252, L253, Q254, N266, D273 ,
DB03147	Flavin adenine dinucleotide	V91, N104, E191 , N252, L253, Q254,	D90 , G93, H95, P141, F146, A187, F190, D273 ,
DB06210	Eltrombopag	D90 , G93(2), N104	H95, P141, F146, W186, A187, F190, E191 , N252, Q254, N266, H268 , D273 ,
DB00637	Astemizole	D90 , N266	V91, E92 , G93, N104, P141, F146, W186, A187, F190, N252, Q254, L253, H268 , D273
DB00878	Chlorhexidine	N266	W186, Q145, N252, L253, H268 , D273
DB01251	Gliquidone	N252, N266	D90 , E92 , H95, N104, P141, Q145, F146, W186, A187, F190, H268 , D273 ,

* LigPlus did not work, manually done

Blue - Catalytic residues

Number in bracket indicates number of hydrogen bonds formed with the particular amino acid

Figures:

Figure 1: Schematic describing ExoN proofreading activity and mode of action of inhibitors (Left Panel) Replication in viruses like Hepatitis C Virus (HCV), with no proofreading mechanism. Replication by low fidelity RdRp in absence of ExoN is error prone. Incorporated nucleoside analogs (NA) is not excised resulting in either premature replication termination or incorporation of mutation. (Mid panel) Replication in viruses like SARS-CoV with proofreading Exonuclease. Like HCV, in SARS-CoV-2 too replication is by low fidelity RdRp but errors and NA are excised by proofreading ExoN, decreasing the efficacy of antivirals. (Right panel) Same as mid panel but in presence of ExoN inhibitor. In presence of ExoN inhibitor NA might not be excised, resulting in premature replication termination.

Figure 2: 20 lowest binding energy conformations from the molecular screen

(A) SARS-CoV-2 nsp14 is depicted as surface representation and the 20 lowest binding energy poses are depicted as sticks. The ExoN domain is coloured in green and MTase domain is coloured in blue. **(B)** Zoomed in version depicting bound conformers of drug molecules.

Figure 3: Putative binding pockets of docked compounds

Interaction of ExoN residues (grey colored) with docked conformer of Dexamestasone metasulfobenzoate (yellow sticks) (A) and Conivaptan (pink sticks) (B). H-bonded residues are depicted as sticks. Yellow dotted lines represent hydrogen bond and the distance is in Angstroms. Residues in hydrophobic contact are depicted as surface representation.

Figure 4: Putative binding pockets of docked compounds:

Interaction of ExoN residues (grey colored) with docked conformer of Hesperidin (green sticks) (A) and Glycyrrhizic acid (purple sticks) (B). H-bonded residues are depicted as sticks. Yellow dotted lines represent hydrogen bond and the distance is in Angstroms. Residues in hydrophobic contact are depicted as surface representation.

Supplementary Figure:

Expect		Identities	Positives	
0.0		501/527 (95%)	520/527 (98%)	
Query	1	AENV TGLFKDCSKIITGLHPTQAPTHLSVDIKFKTEGLCVDIPGIPKDMTYRRLISMGMF	60	
Sbjct	1	AENV TGLFKDCSK+ITGLHPTQAPTHLSVD KFKTEGLCVDIPGIPKDMTYRRLISMGMF	60	
Query	61	KMNYQVNGYPNMFITREEAIRHVRWIGFIVEGCHATRDVGTNLPLQLGFSTGVNLVAV	120	
Sbjct	61	KMNYQVNGYPNMFITREEAIRHVRWIGFIVEGCHATREAVGTNLPLQLGFSTGVNLVAV	120	
Query	121	PTGYVDTENNTFTRVNAKPPPGDQFKHLIPLMYKGLPWNVVRKIVQMLSDTLKGLSDR	180	
Sbjct	121	PTGYVDT NNT+F+RV+AKPPPGDQFKHLIPLMYKGLPWNVVRKIVQMLSDTLK LSDR	180	
Query	181	VVFVLWAHGFELTSMKYFVKIGPERTCCLCDKRATCFSTSSDTYACWNHSGFDYVYNPF	240	
Sbjct	181	VVFVLWAHGFELTSMKYFVKIGPERTCCLCDRATCFSTASDTYACWHHSIGFDYVYNPF	240	
Query	241	MIDVQQWGFTGNLQSNHDQHCQVHGNAIVASCDAIMTRCLAVHECFVKRVDWSVEYPIIG	300	
Sbjct	241	MIDVQQWGFTGNLQSNHDLYCQVHGNAIVASCDAIMTRCLAVHECFVKRVDWTIEYPIIG	300	
Query	301	DELRVNSACRKVQHMOVKSALLADKFPVLHDIGNPKAIKCVPAEVEWKFYDAQPCSDKA	360	
Sbjct	301	DEL++N+ACRKVQHMOVK+ALLADKFPVLHDIGNPKAIKCVPA+VEWKFYDAQPCSDKA	360	
Query	361	YKIEELFYSYATHHDKFTDGVCLFWNCNVD RYPANAIVCRFDTRVLSNLNLP GCDGGS LY	420	
Sbjct	361	YKIEELFYSYATHDKFTDGVCLFWNCNVD RYPANSIVCRFDTRVLSNLNLP GCDGGS LY	420	
Query	421	VNKHAFHTPAFDKSAFTNLKQLPFFYYSDSPCESHGKQVVS D IDYVPLKSATCITRCNLG	480	
Sbjct	421	VNKHAFHTPAFDKSAFVNLKQLPFFYYSDSPCESHGKQVVS D IDYVPLKSATCITRCNLG	480	
Query	481	GAVCRHHANEYRQYLDAYNMMISAGFSLWIYKQFDTYNLWNTFTRLQ	527	
Sbjct	481	GAVCRHHANEYR YLDAYNMMISAGFSLW+YKQFDTYNLWNTFTRLQ	527	

Supplementary Figure S1: Pairwise alignment of nsp14 from SARS-CoV and SARS-CoV-2 Alignment of SARS-CoV nsp14 (Query) and SARS-CoV-2 nsp14 (Subject) to depict conservation of catalytic residues (highlighted in green). Arrow depicts end of ExoN domain and start of methyl transferase domain.