Repurposing Ivermectin to inhibit the activity of SARS CoV2 helicase: possible implications for COVID 19 therapeutics

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Abstract:

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has infected more than 3 million people around the globe and has caused severe socioeconomic impact. There are no approved drugs or vaccine. Ivermectin, an FDA-approved anti-parasitic, was shown to inhibit action against SARS-CoV-2 *in vitro.* Of the several drugs under investigation Ivermectin was shown to inhibit JEV by targeting it's helicase. To understand the mechanism of inhibition, we used an *in silico* structure-based screening of Ivermectin and Ivermectin like drugs. The approach helped us in identifying Ivermectin and Nystatin as potential inhibitor of SARS-CoV-2 helicase. Combined with the above results, known safety profiles for oral doses and its wide availability, Ivermectin warrants further investigation in clinical settings.

Introduction

The coronavirus disease 2019 (COVID-19), an infectious disease, is caused by a novel virus severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). The COVID-19 outbreak has infected more than 3 million people and claimed more than 200,000 lives (as of 02-05-2020). Though several clinical trials are underway to identify drugs against SARS-CoV-2, currently there are no approved drugs or vaccines available. Rapid identification of drugs to control the outbreak is urgently required. Drug repurposing, use of a preapproved drug for a different disease, drastically cuts down on both cost and time compared to *de novo* drug discovery. To make timely inroads in treatment of COVID-19 efficiently and reducing mortality, drug repurposing can be an effective drug development strategy.

Recently, Ivermectin was shown to reduce SARS-CoV-2 RNA by approximately 5000 folds at 48 hrs (Caly, et al. 2020). The exact mechanism of action of Ivermectin against SARS-CoV-2 is not known. Caly L. *et al*. proposed Ivermectin asserts its antiviral activity by inhibiting nuclear import of coronavirus protein. Ivermectin is an FDA-approved anti-parasitic drug (Canga, et al. 2008). It was shown to have an inhibitory effect on a broad range of viruses. Ivermectin was shown to hinder nuclear import of viral proteins like HIV-1 integrase protein and Dengue virus non-structural protein 5 (NS5) (K. M. Wagstaff 2012). However, SARS-Cov-2 being an RNA virus with RNA-dependent RNA polymerase, complete their life cycle in cytoplasm of the host cell. Nucleocapsid proteins of beta-coronaviruses like Mouse Hepatitis Virus (MHV) are known to transiently localize in nucleus and delay cell cycle (Wulan, et al. 2015). Unlike MHV, nucleocapsid protein of SARS-CoV predominantly remains in cytoplasm. Hence, inhibition of nucleus import of viral proteins might not be operational in case of SARS-CoV and CoV-2. CoV2SARS-CoV-2. (Wulan, et al. 2015).

In another study, Ivermectin was shown to inhibit replication of yellow fever virus (YFV) and Japanese encephalitis virus (JEV) by specifically targeting its NS3 helicase (Mastrangelo, et al. 2012), (Li, et al. 2016). Given the structural similarity between coronavirus helicase and helicase domain of JEV NS3, the role of SARS-CoV-2 helicase as potential target of Ivermectin entails further investigation.

SARS-CoV-2 is a single stranded positive sense RNA virus. SARS-CoV-2 has one of the longest genomes (29.9kb) among RNA viruses (true for coronaviruses in general). Multiple genomes of SARS-CoV-2 have been sequenced and phylogenetic analysis has revealed that it shares 79.7% nucleotide identity with SARS-CoV and 54.05% with MERS-CoV (Zhou, et al. 2020). A comparison of SARS-CoV and SARS-CoV-2 amino acid sequences revealed helicase gene (nsp13) to be the most conserved. Interestingly, helicase protein is well conserved across α, β and γ coronaviruses (Adedeji, et al. 2014). Helicase plays an indispensable role in the replication of viruses by unwinding double stranded oligonucleotides into single strand in an ATP dependent manner. Recently a study characterized human proteins that interact with SARS-CoV-2 proteins (D. J. Gordon 2020). They found SARS-CoV-2 helicase to be one of the proteins with large number of host interacting partners. Hence, inhibiting helicase might have wider disruption than inhibiting other proteins like protease. Taken together because of its

vital role in viral replication and its conserved nature, helicase was identified as an important drug target (Jia, et al. 2019).

Targeting helicase to interfere with viral replication was shown to be successful in flaviviruses (Mastrangelo, et al. 2012), (Li, et al. 2016), picornaviruses (De Palma, et al. 2008) and Hepatitis C Virus (Manfroni, et al. 2009)(De Palma, et al. 2008) and Hepatitis C Virus (Manfroni, et al. 2009). In some of the above studies, Ivermectin, was used as potential inhibitors. With *in vitro* success of Ivermectin on SARS-CoV-2 in mind, and its proven inhibitory effect on other viruses, prompted us to analyse Ivermectin's mechanism of action on SARS-CoV-2.

The aim of this study is to identify FDA-approved molecules as candidate drug to treat COVID-19 patients. Molecular docking was employed to screen for potential inhibitors of SARS-CoV-2 helicase. Ivermectin and Ivermectin like drugs were used as probable inhibitors.

Materials and Methods

Amino acid similarity

Protein sequences from SARS-CoV and CoV2SARS-CoV-2 were aligned using blastp algorithm (Altschul, et al. 1990) to get the similarity between respective protein sequences.

Homology modelling of Helicase

Amino acid sequence of SARS-CoV-2 helicase (YP_009725308.1) was used as target sequence to build 3D-models using SWISS-MODEL webserver (Schwede, et al. 2003). Helicase from SARS-CoV and MERS-CoV (6JYT and 5WWP respectively) had high sequence identity to target sequence. These two 3D structures were used as templates for building SARS-CoV-2 helicase model. Though 6JYT sequence shared higher sequence identity to SARS-CoV-2 helicase compared to 5WWP sequence, the model quality score (QMEAN) was much better for MERS-CoV structure 5WWP. Hence, 3D model was built using 5WWP as template. (**Supplementary Table S2**)

Putative functional sites in helicase

Helicase catalyses the unwinding of double stranded oligonucleotide in an NTP dependent manner. Hence, the two substrates of helicase are NTP and double stranded oligonucleotide. Active site of ATP hydrolysis was recently determined by Jia Z *et al*., in SARS-CoV helicase (Jia, et al. 2019). Amino acid sequence of SARS-CoV-2 and SARS-CoV helicase are 99.83% identical

with one substitution at position 570. Hence, all the six residues (Lys288, Ser289, Asp374, Glu375, Gln404 & Arg567) are conserved in SARS-CoV-2 helicase. Hence, all the six residues (Lys288, Ser289, Asp374, Glu375, Gln404 & Arg567) are conserved in SARS-CoV-2 helicase. The same article also determined dsDNA binding site. The four DNA binding segments (153-179, 209-224, 331-357 and 523- 542) and additional residues contributing to unwinding (R507 and K508) are conserved in SARS-CoV-2 helicase too. A grid encompassing ATP hydrolysis site and DNA binding segments were used as docking sites **(Supplementary figure S1).**

Ligand selection

Ivermectin was chosen as a probable inhibitor of helicase. Ivermectin-like drugs were mined from DrugBank repository of approved, experimental and investigational drugs. 11 hits (including Ivermectin) with similarity score more than 0.7 were considered for virtual screening step. Remdesivir was used as negative control. The 3D structure of these 12 molecules were either obtained from PDB structures or minimized 3D conformer was generated using Frog2 webserver (Miteva, Guyon and Tufféry 2010) (**Supplementary Table S3**).

Virtual screening using AutoDock VINA

Docking grid was defined encompassing ATP hydrolysis and DNA binding sites. The 11 compounds were subjected to virtual screening using the AutoDOCK VINA (Trott and Olson 2010) tool on SARS-CoV-2 helicase. Compound-helicase interactions were visualized using LigPlot^{+} v2.1 (Laskowski and Swindells 2011).

Results

SARS-CoV-2's genome encodes 11 open reading frames (ORFs) (Wu, et al. 2020). The first ORF, ORF1ab, occupies approximately 2/3rd of the genome (Khailany, Safdar and Ozaslan 2020). It is a polyprotein, which is cleaved into 16 non-structural proteins (NSPs). At nucleotide level, its genome shows high similarity to SARS-CoV's genome. At amino acid level the percentage identity varies, but most sequences share high similarity (**Supplementary Table S1**). The helicase protein sequences from the two viruses are nearly identical, sharing 99.83 % identity over complete length of sequence. Helicase protein sequences from α, β and γ coronaviruses (Adedeji, et al. 2014) was shown to be highly conserved. The ATP hydrolysis active sites were shown to be well conserved too. The high conservation of helicase across coronavirus family,

makes it an ideal target for a broad spectrum anticoronaviral drug. It also reduces the chances of selection of inhibitor-resistance strains.

Homology model of SARS-CoV-2 helicase was built based on MERS-CoV helicase 3D structure 5WWP. Docking site comprised of the oligonucleotide binding site and ADP binding site (see Methods). The model was used to screen Ivermectin and 10 Ivermectin like drugs using AutoDock VINA. Based on the binding affinities to SARS-CoV-2 helicase, top 10 docking models were selected for further analysis (**Table 1**). In 9 out of the top 10 models, belonging to 5 compounds, the compound binds to the DNA binding region **(Figure 1a)**. Both Ivermectin and natamycin binds helicase with equal affinities (8.8 kcal/mol) in different but adjacent sites (**Figure 1b**).

Lowest energy model of Nystatin makes an extensive hydrogen bond interaction with helicase (**Figure 2a** and **Table 2**). Of the 5 helicase residues with which Nystatin forms hydrogen bonds, 2 are involved in oligonucleotide binding. Of the 3 hydrophobic contacts of natamycin, 1 are involved in oligonucleotide binding. Hence, Nystatin partially blocks the oligonucleotide binding pocket.

Lowest energy model of Ivermectin's interaction with helicase is depicted in **Figure 2a** and **Table 2.** Arg178, Asn179 and T532 (pink sticks) from the oligonucleotide binding pocket is involved in hydrogen bonding with Ivermectin. 4 out of 13 hydrophobic contacts of Ivermectin are part of oligonucleotide binding pocket. Hence, the lowest energy model of Ivermectin occludes the oligonucleotide binding pocket.

Remdesivir, an investigational drug for treatment of Ebola, was shown to inhibit SARS-CoV-2 RNA-dependent RNA polymerase inhibiting RNA synthesis (Gordon, et al. 2020). As Remdesivir is a drug without anti-helicase activity, it was used as a negative control. Though the lowest energy poses of Remdesivir are in the vicinity of nucleotide binding pocket, their binding energy are much higher than the ten lowest energy poses of Ivermectin and Ivermectin like drugs (**Supplementary Figure S2 and Supplementary Table S4**). This indicates that Ivermectin and some of the Ivermectin like drugs binds to helicase better than Remdesivir.

Recently, González-Paz *et al*. demonstrated *in silico* binding of Ivermectin to 3CL-protease (PDB ID: 6LU7) and HR2 domain of S glycoprotein (6LVN) (Gonzalez Paz, et al. 2020). The

authors considered those SARS-CoV-2 proteins which had crystal structure solved. Hence, Ivermectin docking on Helicase was not done. To compare the binding affinities of Ivermectin to helicase and reported binding partners (3CL-protease and HR2 domain) we did a blind docking. The HR2 domain was not considered as it is a small part of the S protein (36 amino acid out of 1273 amino acids). Considering the HR2 domain in isolation of other domain would have less biological meaning. Therefore, blind docking of Ivermectin using AutoDock VINA was done on free form of 3CL-protease (PDB ID: 6Y2E). The lowest energy poses of Ivermectin on 3CL-protease had a binding affinity of -8.4 kcal/mole or more **(Supplementary Table S5).** Hence, the lowest binding energy of 3CL-protease to Ivermectin is less than that of helicase.

Discussion

An ongoing break of severe respiratory illness termed COVID-19 is caused by a novel strain of coronavirus Sars-CoV-2. The World Health Organization declared COVID-19 outbreak as a pandemic. Currently no effective drug exists. Of the several drugs under investigation, Ivermectin was shown to be effective *in vitro* (Caly, et al. 2020).

Ivermectin is a widely used antiparasitary drug with established safety profile (Muñoz, et al. 2018). Recent research has shown its anti-viral activity on a broad range of viruses. Its first anti-viral activity was demonstrated against import of human immunodeficiency virus (HIV-1) integrase protein (IN) into nucleus of infected cell (Wagstaff, et al. 2011). Ivermectin specifically inhibits IN-importin α/β interaction. It has since been shown to be effective against a broad range of RNA viruses like Dengu Virus 1-4, West Nile Virus, Venezuelan equine encephalitis virus (VEEV) and influenza. The success can be attributed to dependence of RNA viruses on IMP α/β1 for transport of nucleocapsid protein to nucleus resulting in effective viral replication. Inhibition of YFV NS3 helicase by Ivermectin was reported as a new mechanism of action (Mastrangelo, et al. 2012). Ivermectin exerts its antiviral activity at the onset of viral RNA replication, the only phase when helicase is active. The former mechanism of action is less likely in SARS-CoVs as its nucleocapsid protein was demonstrated to be predominantly cytoplasmic (Wulan, et al. 2015). In SARS-CoV-2 the latter mechanism necessitates further investigation. Hence, *in silico* drug screening method was used in this study to determine whether SARS-CoV-2 helicase is a potential target of Ivermectin. Drugs structurally similar to Ivermectin were included in the screening process too.

AutoDock VINA screening results indicated Nystatin and Ivermectin to be the lowest energy binding partners of SARS-CoV-2 helicase (binding affinity of -11.1 and -10.7 kcal/mol respectively) (**Table 1**). Both compounds show extensive interaction with residues of helicase, specifically with the oligonucleotide binding regions (**Figure 2 and Table 2**). Okadaic acid and Natamycin had binding energy of -10.5 and 10.3 kcal/mol respectively, which was marginally more than Ivermectin. Natamycin and nystatin are polyene anti-fungals with predominantly fungicidal activity. Natamycin is approved primarily for topical use whereas administration of nystatin is approved for topical use and in gastrointestinal fungal infections (Ashbeea and Gilleeceb 2014). Both show extremely low gastrointestinal absorption. Nystatin's systemic administration has shown high toxicity (Dos Santos, et al. 2017). Okadaic acid is an experimental drug and has been used in studies as cancer inhibitors but it was shown to have neurotoxic, immunotoxic, and embryotoxic effects (Vanessa Valdiglesias 2013). Hence, even though Nystatin, Natamycin and Okadaic Acid might bind helicase with high affinity, their suitability in control of SARS-CoV-2 infection is limited.

Remdesivir, which was shown to interact with CoV2SARS-CoV-2 polymerase, was included in the screening process as a negative control. The lowest binding energy of Remdesivir was -7.5 kcal/mol, much higher than that of Nystatin and Ivermectin (**Supplementary Table S4**). The binding energy suggests Nystatin and Ivermectin bind to helicase better than Remdesivir, a helicase non-binder. Additionally, Ivermectin showed poorer binding to 3CL-protease compared to helicase.

Taken together these results demonstrate Ivermectin as potent inhibitor of SARS-CoV-2 helicase with established safety profile. Use of Ivermectin as SARS-CoV-2 antiviral warrants further investigation and tests in clinical setting.

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Conflict of Interest:

Authors declare no conflict of interest

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Figures and Tables

Table 1: Molecular docking results of ten compounds with lowest binding energies. Binding energy is expressed as kcal/mol

Drug Name	Model ID	Binding Affinity (kcal/mol)
Nystatin	Model 1	-11.1
Ivermectin	Model 1	-10.7
Okadaic Acid	Model 1	-10.5
Okadaic Acid	Model 2	-10.4
Natamycin	Model 1	-10.3
Nystatin	Model 2	-10.3
Okadaic Acid	Model 3	-10.3
Okadaic Acid	Model 4	-10.3
Amphotericin	Model 1	-10.2
Ivermectin	Model 2	-10.1

Table 2: Hydrogen bond and hydrophobic interaction in top five lowest energy models

Compound Name	Hydrogen Bond	Hydrophobic Interactions
Nystatin	N177, D534, L512, N516, H554,	P175, T413, Y515
Ivermectin	R178, N179, K202, T532	P175, L176, E201, Y205, S310, M378, P408, V484, S486, N516, D534, S535, R560
Okadaic Acid	R409, N516	P175, N177, P406, P408, T410, L412, G415, L417, Y515, T532, D534
Okadaic Acid	Y180, H554, R560	P175, L176, N177, Y205, P408, R409, T410, L412, P514, N516, T532, D534
Natamycin	N177	N179, M378, L405, P406, P408, R409, T410, L412, N516, T532, D534, S535, H554, R560

***Red coloured residues are oligonucleotide interacting residues of helicase.**

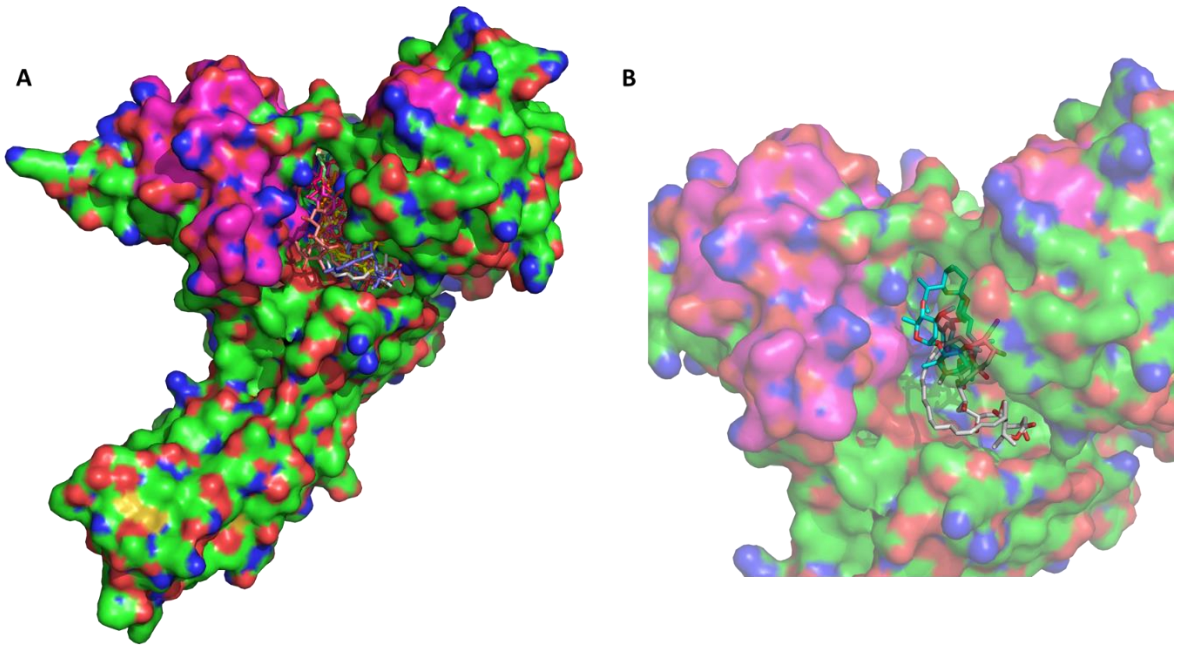


Figure 1: Docking poses of lowest binding energy conformer from the molecular screen. SARS-CoV-2 helicase is shown as surface representation. Region colored in pink represent nucleotide binding site. A) Ivermectin and Ivermectin like compounds are represented as sticks. B) Top two docking poses – Nystatin (grey colored stick) and Ivermectin (cyan colored stick)

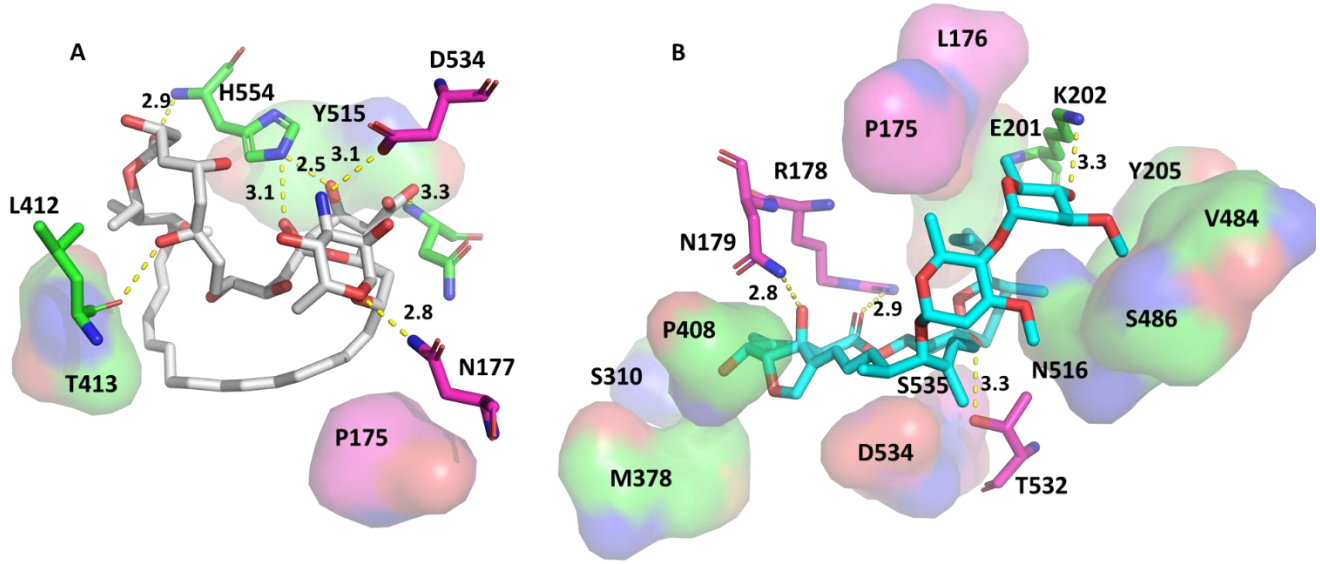


Figure 2: Putative binding pockets of docked compounds: Hydrogen Bond and hydrophobic interactions with docked conformer of Ivermectin (A) and Natamycin (B). H-bonded residues are depicted as sticks. Yellow dotted lines represent hydrogen bond and the distance is in Angstroms. Residues in hydrophobic contact are depicted as surface representation. Carbon atom of helicase residues that are involved in oligonucleotide binding are colored pink and the ones involved in ATP hydrolysis are colored in peach color.

Supplementary Tables and Figures

Supplementary Table S1

Attached Excel file

Supplementary Table S2: Homology modelling result for SARS-CoV-2 helicase

PDB ID	Source organism	Sequence Identity	Query coverage	GMQE	QMEAN score*
6JYT	Human SARS coronavirus	99.83%	99.16%	0.98	-5.63
5WWP	MERS-CoV	72.20%	98.16%	0.83	**-1.72**

Supplementary Table S3: Ivermectin and Ivermectin-like drugs from Drug Bank

Drug Name	Drug Bank ID	Similarity Score	Approved status	Synonym	Ligand 3D conformer (PDB ID/Frog2)
Ivermectin	DB00602	1.00	Approved		5VDH
Moxidectin	DB11431	0.812	approved, investigational		Frog2
9,10-Deepithio-9,10-Didehydroacanthifolicin	DB02169	0.793	experimental	Okadaic acid	4WRI
Fusicoccin	DB01780	0.758	experimental		5NWK
P-57AS3	DB06569	0.742	investigational		Frog2
Oleandrin	DB12843	0.721	investigational, experimental		Frog2
Nystatin	DB00646	0.72	approved		Frog2
Gitoformate	DB13537	0.714	experimental		Frog2
Amphotericin B	DB00681	0.71	approved, investigational		Frog2
Natamycin	DB00826	0.706	approved	Pimaricin	Frog2
Concanamycin A	DB14062	0.701	experimental		Frog2

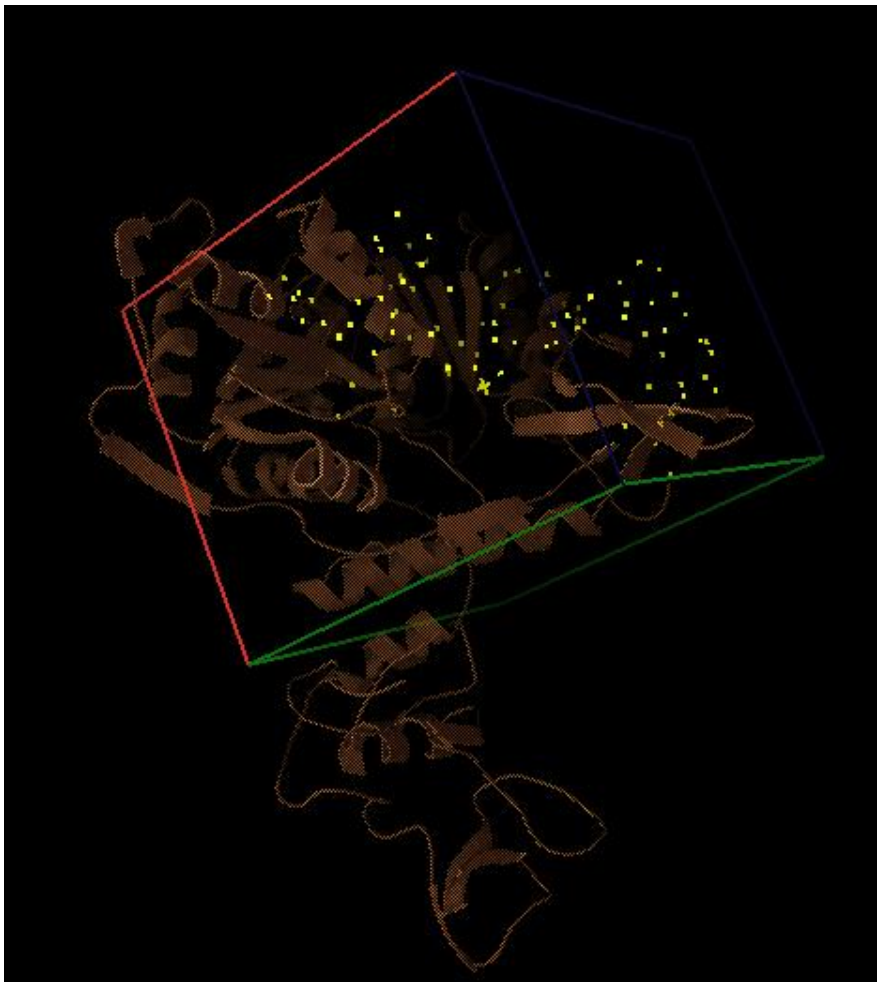
Supplementary Table S4: Top ten poses of Remdesivir – negative control

Drug Name	Model ID	Binding Affinity (kcal/mole)
Remdesivir	1	-7.5
	2	-7.5
	3	-7.4
	4	-7.3
	5	-7.3
	6	-7.1
	7	-7.1
	8	-7.1
	9	-7.1
	10	-7

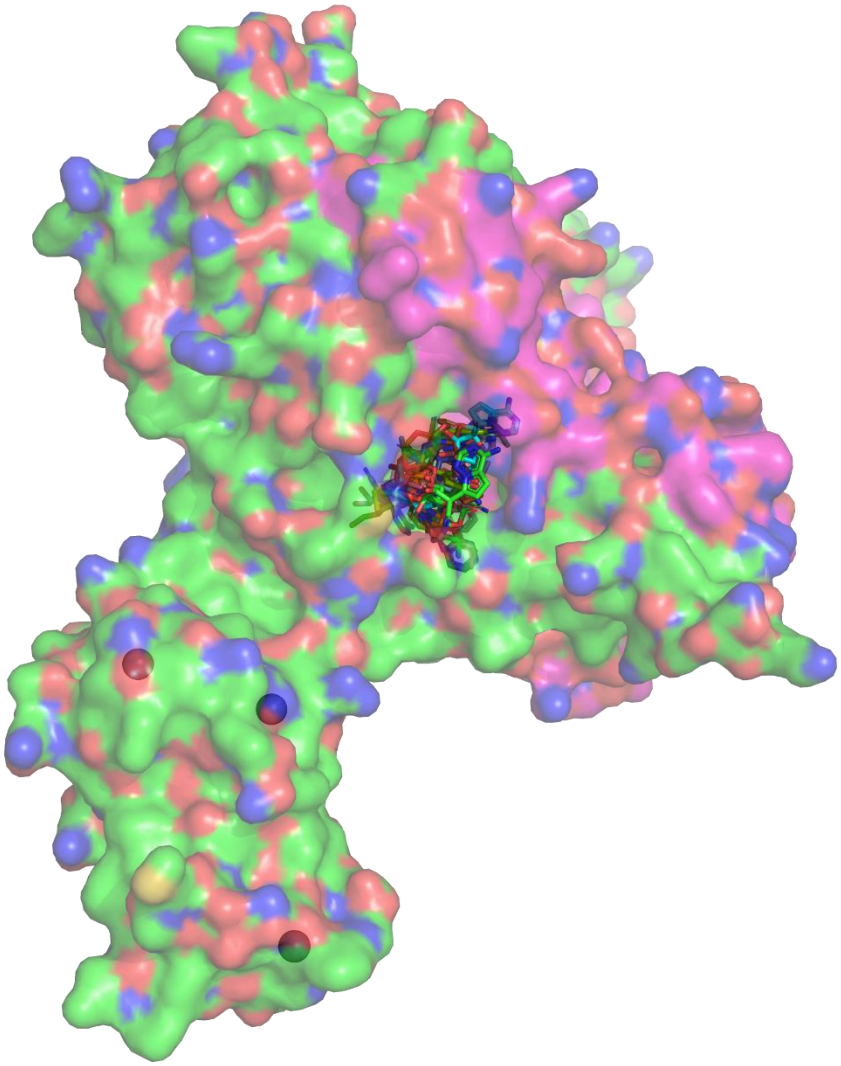
Supplementary Table S5: Top ten poses of Ivermectin on 3CL-protease

Drug Name	Model ID	Binding Affinity (kcal/mole)
Ivermectin	1	-8.4
	2	-8
	3	-7.8
	4	-7.6
	5	-7.5
	6	-7.5
	7	-7.4
	8	-7.3
	9	-7.2
	10	-7.2

Supplementary Figures



Supplementary Figure S1: Docking grid marked as box on SARS-Cov-2 helicase**.**



Supplementary Figure S2: Docking poses of lowest binding energy conformer from the Remdesivir molecular screen. SARS-CoV-2 helicase is shown as surface representation. Region colored in pink represent nucleotide binding site. Various docking poses are represented as sticks.