Repurposing drugs for treatment of SARS-CoV-2 infection: Computational design insights into mechanisms of action

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Research Article

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Abstract

The COVID-19 pandemic has negatively affected human life globally. It has led to economic crises and health emergencies across the world, spreading rapidly among the human population and has caused many deaths. Currently, there are no treatments available for COVID-19 so there is an urgent need to develop therapeutic interventions that could be used against the novel coronavirus infection. In this research, we used computational drug design technologies to repurpose existing drugs as inhibitors of SARS-CoV-2 viral proteins. The Broad Institute's Drug Repurposing Hub consists of in-development/approved drugs and was computationally screened to identify potential hits which could inhibit protein targets encoded by the SARS-CoV-2 genome. By virtually screening the Broad collection, using rationally designed pharmacophore features, we identified molecules which may be repurposed against viral nucleocapsid and non-structural proteins. The pharmacophore features were generated after careful visualisation of the interactions between co-crystallised ligands and the protein binding site. The ChEMBL database was used to determine the compound's level of inhibition of SARS-CoV-2 and correlate the predicted viral protein target with whole virus *in vitro* data. The results from this study may help to accelerate drug development against COVID-19 and the hit compounds should be progressed through further *in vitro* and *in vivo* studies on SARS-CoV-2.

Introduction

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), is the causative agent of the ongoing COVID-19 pandemic which has become a global public health emergency (WHO, 2020). The human-to-human transmission is rapid, making the virus highly contagious and at present, there are approximately 18.7 million cases reported worldwide (as of 5th August 2020, Worldometer, 2020). Currently, there are no drug treatments or vaccinations and the global research community has united in the search for a cure of this infectious disease.

Structural considerations of SARS-CoV-2

Phylogenetic relationships and genomic structures indicate that the virus causing COVID-19 disease (belonging to the *Betacoronavirus* genus) has a close sequence similarity to severe acute respiratory syndrome-related coronaviruses (SARS-CoV) (Mousavizadeh and Ghasemi, 2020). Due to these similarities, the coronavirus study experts of the International Committee on Taxonomy of Viruses (ICTV) named the new virus as SARS-CoV-2 (Cascella *et al.*, 2020). Severe acute respiratory syndrome (SARS) and the Middle East respiratory syndrome (MERS) which also belong to the *Betacoronavirus* genus are associated mainly with nosocomial spread, but the SARS-CoV-2 is more widely transmitted in the community (Munster *et al.*, 2020).

The SARS-CoV viruses have a positive-sense, single-stranded RNA genome (ranging from 27-32 kilobase pairs) in an enveloped structure with a 5'cap and 3'-poly-A-tail (Fehr and Perlman, 2015; Astuti, and Ysrafil, 2020). The CoV genome has at least six open reading frames (ORFs), among these, there are two

large overlapping ORFs, *ORF 1a* and *ORF 1b*, which guide the synthesis of polyproteins 1a/1ab (pp1a, pp1b). These polyproteins are then processed by the viral proteases, chymotrypsin-like protease (3CL^{Pro}) or main protease (M^{Pro}) (Cascella *et al.*, 2020). In addition to 3CL^{Pro}, the viral papain-like protease (PL^{Pro}) is responsible for producing 16 non-structural proteins (nsps) by cleaving polyproteins which are encoded by the large *ORF1ab* replicase gene (Cascella *et al.*, 2020; Fehr and Perlman, 2015). The remaining ORFs encode for viral structural proteins, such as spike, membrane, envelope and nucleocapsid proteins (S, E, M, and N) (Perlman & Netland, 2009). In addition to these proteins, there are certain genes in the 3' region between *S-E-M-N* which are known to encode for accessory proteins such as 3a, 3b, 6, 7a, 7b, 8a, 8b and 9b (Marra *et al.*, 2003; Snijder *et al.*, 2003). The genomic RNA is packed inside a bead-like structure which is known as a capsid and is formed by the N protein, while the remaining structural proteins form an envelope surrounding the capsid.

The remaining proteins such as several non-structural proteins contribute to viral RNA replication or transcription (Snijder *et al.*, 2016) whereas, the accessory proteins interact with host cells to help the virus evade the host immune system thereby increasing their virulence (Menachery *et al.*, 2017). The viral cycle begins with binding of the spike glycoprotein to the ACE2 host receptor (angiotensin-converting enzyme 2) with the help of the TMPRSS2 protease (Hoffmann *et al.*, 2020). After viral entry into the host cells, the virus un-coats and releases the genome which is then transcribed and translated (Mousavizadeh and Ghasemi, 2020). The coronavirus replicase is reported to employ many RNA processing enzymes such as endoribonuclease, 3'-to-5' exoribonuclease, ADP ribose 1'-phosphatase and 2'-O-ribose methyltransferase (Zeibuhr, 2005).

Scientists all over the world are in a race to find a vaccine for SARS-CoV-2 but until then there is an urgent need to discover potent anti-COVID-19 agents which can be used for treating the viral infection. Spike protein and viral proteases have been the major targets of investigation for researchers around the world but in this paper, we have focused on non-structural proteins and the nucleocapsid protein.

Repurposing of existing drugs against SARS-CoV-2

At present, there are no drug treatments or a vaccination available for COVID-19. The patients who develop severe symptoms with respiratory failure are given oxygen therapy and artificial ventilator support (Cascella *et al.*, 2020). There is a need to find therapeutic interventions and to achieve this existing drugs can be repurposed (Schneider and Schneider, 2018; Cavalla *et al.*, 2017) for treating the SARS-CoV-2 infection (Pandey, *et al.* 2020) (Figure 1). Approximately one-third of drug approvals involve repurposed drugs (Talevi and Bellera, 2020). There is a broad range of choices which can curb the effects of viral infection and also prevent it. These choices include the use of small molecule medicines, monoclonal antibodies, vaccines, interferon treatments and oligonucleotide-based drugs (Li & Clercq, 2020) but the development process of a vaccine or a novel drug takes at least 10 years. The repurposing of existing vaccines has recently been proposed (O'Neill and Neta, 2020) but, considering the severity of the pandemic, we propose examining currently available drugs belonging to diverse therapeutic areas for

possible treatment of COVID-19. Drug repurposing involves redeveloping a drug (can be an approved, shelved, discontinued or investigational drug) for a different use than originally intended (Ashburn and Thor, 2004). The main SARS-CoV-2 protein targets along with some proposed therapies are (Wu *et al.*, 2020):

1. Therapies which act on viral enzymes or functional proteins, RNA synthesis and replication. (Targets – PL^{Pro}, RdRp (RNA-dependent RNA polymerase), 3CL^{Pro}, Helicase-nsp13).
2. Therapies which block the virus from binding to human cell receptors, acting on structural proteins. (Targets – Spike, nucleocapsid and envelope proteins).
3. Therapies which restore the host's innate immunity, inhibiting virulence factor. (Targets – nsp1, nsp3c, ORF7a).
4. Therapies acting on hosts' specific receptors thereby preventing viral entry. (Target – ACE2 receptor).

Materials And Methods

The research methodology conducted in this study involved generating pharmacophore features around the co-crystallized ligand in the 3D crystal structure of viral proteins followed by the VS for the hit compounds.

Websites, databases and software

The Broad Institute Drug Repurposing Hub was used for extracting the repurposing drug datasets. The database contained 5,836 molecules that were used for performing the VS step.

(<https://www.broadinstitute.org/drug-repurposing-hub>). OMEGA 3.1.1.2 (OMEGA, 2018; Hawkins et al., 2010) was used to generate 50 conformers of each molecule. The ChEMBL website (<https://www.ebi.ac.uk/chembl/>) was accessed for compound inhibition data on SARS-CoV-2. The Protein Data Bank (PDB) (Berman, 2000) website was used for downloading 3D crystal structures of both holo or apo viral proteins. MOE 2019.0101 (Chemical Computing Group, 2019) was used to generate pharmacophore features from the visualised interactions, for VS of the Broad collection and analysing the results.

Protein preparation

The crystal structures of the SARS-CoV-2 proteins were downloaded from the PDB. In this study, the 3D crystal structures of non-structural proteins (nsp3, nsp7-nsp8-nsp12 complex, nsp16-nsp10 complex and nsp15) and the nucleocapsid protein of SARS-CoV-2 were studied as holo structures were available. Each protein was loaded in MOE and the Quick Preparation function was used with the default Amber10 force field in order to consider explicit hydrogen atoms, tautomeric states and possible breaks in protein

structure prior to conducting restrained all atom molecular mechanics minimisation and electrostatics calculations.

3D protein structure alignment/superposition

The PDB had multiple crystal structures of a single protein with different co-crystallised ligands. Hence, it becomes necessary to analyse whether there were any structural differences among these protein structures. The protein alignment/superposition step was performed in MOE on different structures of the same protein (Table 1). First, the sequence alignment step was performed followed by 3D structural superposition using default parameters.

S. No.	Protein	PDB IDs of the superposed proteins	RMSD values (Å) after protein superposition
1.	Nsp 3	6YWL, 6YWK	1.433
2	Nsp 10-Nsp 16 complex	6W4H, 7BQ7	0.478
3.	Nsp 7-Nsp 8- Nsp 12 complex	7BV2, 7BW4	0.496
4.	Nsp 15	6WXC, 6WLC, 6X4I	0.332
5.	Nucleocapsid protein	6WKP, 6M3M	1.092

Table 1. Proteins with their respective PDB IDs that were used for the superposition step with resulting RMSD values.

After superposition of the proteins, the RMSD values were calculated. The holo protein structures were visually inspected to determine if there were any changes in the position of the binding site amino acids due to the ligand.

Pharmacophore generation

After studying the ligand-binding site interactions, pharmacophores were created in MOE using the unified scheme on the co-crystalised ligand. The pharmacophore features used were hydrogen bond acceptor (Acc – cyan colour), hydrogen bond donor (Don – purple colour), hydrophobic (Hyd – green colour), aromatic (Aro – orange colour) and charged groups such as cation (Cat – blue colour) or anion (Ani – red colour). The features were selected by carefully considering the ligand atoms responsible for binding interactions with the amino acid residues in the binding pocket and the default feature radius was used.

Virtual screening

The Broad Institute's Drug Repurposing Hub Database was downloaded, the molecules were washed in MOE and the MMFF94x force field was used to generate 3D structures of each molecule. For each molecule, fifty conformers were generated using OMEGA (Hawkins *et al.*, 2010, OMEGA, 2019) software. This database was used for all pharmacophore searches. In MOE, hit compounds were viewed in the binding site to observe the interactions that these molecules made with the binding pocket of the target protein. The hits obtained from VS were then combined with the washed Broad database to obtain other relevant information such as clinical phase, disease area, indication, mechanism of action and target for these hit molecules.

ChEMBL database SARS-CoV-2 activity data

The ChEMBL database was used to determine if the *in silico* hit molecules were active or inactive against SARS-CoV-2 by referring to inhibition assay data. The ChEMBL website had a separate link to only SARS-CoV-2 related data and search filters such as type of bioassay result. The data used was generated by Ellinger *et al.*, 2020): Antiviral activity was determined as inhibition of SARS-CoV-2 induced cytotoxicity of Caco-2 cells at 10 μ M after 48 hours by high content imaging. The database file was downloaded to aid interrogation. These assay results were used to differentiate between active and inactive *in silico* hits against SARS-CoV-2.

Results

The PBVS CADD approach in our study resulted in multiple hit molecules predicted to bind to a variety of viral proteins. A number of the predicted active compounds have been previously validated *in vitro* to inhibit the activity of SARS-CoV-2 so our results indicate a possible mechanism of action of these compounds. The next step should be to conduct direct assay against these viral proteins to confirm on-target activity. For confirmation of inactivity, *in silico* hit compounds which displayed no response following *in vitro* studies should be re- assayed.

Non-structural protein 3

The SARS-CoV-2 non-structural protein 3 (nsp3) is known to cleave polypeptides, promote cytokine expression and block host innate immune response (Chen *et al.*, 2020; Lei *et al.*, 2018). The crystal structures of nsp3 (PDB IDs: 6YWL, 6YWK) were aligned and superposed which resulted in an RMSD of 1.433 Å after protein preparation. The two structures were similar and were properly superposed on each other. The co-crystallised ligands in the binding pocket of the two proteins were different. The 6YWL had adenosine-5-diphosphoribose (APR) as the main ligand in the binding site and 6YWK had 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (EPE). The ligand present in the binding site caused slight changes in the amino acid position. The pharmacophore was generated using the 6YWL crystal structure. Figure 2 shows the ligand interactions (2D) and site view (3D) of the ligand APR which is surrounded by hydrophilic and hydrophobic amino acid residues. Among them Asp22, Asp157 are polar acidic residues

that interact with the ligand. There is also the presence of water molecules which form bridges between the ligand and protein. Other residues such as Gly46, Asn40, Gly48, Ser128, Gly130 are polar in nature whereas, Ile23, Val49, Phe132 are hydrophobic residues which contribute to the ligand interactions.

The pharmacophore features (5 in total) on the ligand were three acceptor, one donor and one dual donor & acceptor features. After completing the pharmacophore search, six hit molecules were identified. The six hits, with results for ChEMBL reported *in vitro* SARS-CoV-2 inhibition in brackets, were dihydrostreptomycin (- 8.94%), hesperidin (-3.03%), isepamicin (20.73%), neohesperidin (5.55%), streptomycin (-5.98%) and tannic acid (28.53%) (Figure 3). These hit molecules had pharmacophore rmsdx values ranging from 0.5444 to 0.7203.

By visually analysing the interactions it was observed that dihydrostreptomycin, streptomycin neohesperidin and isepamicin fitted well inside the binding pocket of the protein. In addition to mapping to the pharmacophore, these molecules made extra hydrogen bonding interactions with the protein. Isepamicin and tannic acid are launched drugs and were active against SAR-CoV-2. Isepamicin fitted better inside the binding pocket than tannic acid as it made extra interactions with the Asn37 and Ala154 residues. Whereas, tannic acid was observed to clash with amino acid residues of the binding pocket due to its large size. In addition to isepamicin, streptomycin also made good interactions with the binding pocket (Figure 4) but *in vitro* assay showed no inhibition of SARS-CoV-2.

Non-structural protein 7, non-structural protein 8, non-structural protein 12 complex

The non-structural protein 7 (nsp7) and non-structural protein 8 (nsp8) complex along with co-factors is known to stimulate RNA-dependent RNA polymerase (RdRp) activity together with non-structural protein 12 (nsp12). Nsp12 alone also possesses minimal RdRp activity (Subissi *et al.*, 2014). The crystal structures of nsp7-nsp8- nsp12 complexes (PDB IDs: 7BV2, 7BW4) were aligned and superposed which resulted in an RMSD of 0.496 Å. The two structures were similar and were properly superposed on each other. The 7BV2 structure had F86 as the main ligand in the binding site and 7BW4 had only a zinc metal ion. The ligand present in the binding site caused slight changes in the amino acid position. The pharmacophore was generated using the 7BV2 crystal structure. Figure S1 shows the ligand interactions (2D) and site view (3D) of the ligand F86 which is surrounded by hydrophilic amino acid residues. Among them, AspA760, AspA761 and AspA623 are polar acidic residues in the binding pocket. There is also the presence of magnesium ion which forms a bridge between the ligand and protein. Other residues such as SerA759, AsnA691 and SecB20 are polar in nature which contributes to the ligand interactions.

The pharmacophore features (5 in total) around the ligand were two anionic acceptor, one donor, one acceptor and one dual donor & acceptor features. Six hit molecules were identified to have the assigned pharmacophore features. The six hits, with results for ChEMBL reported *in vitro* SARS-CoV-2 inhibition in brackets, were NADPH (-9.07%), adenosine triphosphate (2.34%), Coenzyme-I (29.03%), Coenzyme-A

(6.97%), nadide (- 1.83%) and Hydroxysafflor Yellow A (-7.05%) (Figure S2). These hit molecules had rmsdx value ranging from 0.4004 to 0.6174.

By visually analysing the interactions we observed that NADPH, adenosine-triphosphate, Coenzyme-I and nadide fitted well inside the binding pocket of the protein. These molecules also had moieties that made hydrogen bond interactions in addition to the pharmacophore query features. Coenzyme-I is in phase 2 clinical trials and was active against SAR-CoV-2. Overall, these hit molecules were large in size and were observed to clash with other amino acid residues of the binding pocket.

Therefore, a second pharmacophore query was generated having five features (three acceptors and two donors) with modified positions and types (Figure S3). The search resulted in 44 hit molecules. Among these molecules, epigallocatechin-gallate(-) (6.28%), kuromanin (3.66%), procyanidin-b-2 (3.96%) and rutin (40.82%) were the top four hits which fitted well to the specified pharmacophore query (Figure S4). Rutin is a launched drug and demonstrated the largest inhibition of SARS-CoV-2 whereas epigallocatechin-gallate(-) and procyanidin-b-2 are in clinical trials while kuromanin is in the preclinical phase.

Non-structural protein 10 – non-structural protein 16 complex

The SARS-CoV-2 nsp10 protein interacts with nsp14 and nsp16 (Pan *et al.*, 2008; von Brunn *et al.*, 2007). The nsp16 in complex with nsp10 acts as a 2'-O-methyltransferase (2'-O-Mtase) to selectively 2'-O-methylate the cap-0 structure to cap-1 structure (m7GpppAm-RNA) (Bouvet *et al.*, 2010; Chen *et al.*, 2011). The Mtase activity is crucial for the life-cycle of the coronavirus and its inhibition could suppress viral replication (Ke *et al.*, 2012). The crystal structures of the nsp10-nsp16 complexes (PDB IDs: 6W4H, 7BQ7) were aligned and superposed which resulted in an RMSD of 0.478 Å after protein preparation. The two structures were similar and were properly superposed on each other. The co-crystallised ligand in the binding pocket of both proteins was the endogenous metabolite S-adenosyl methionine (SAM). The ligand present in the binding site caused slight changes in the amino acid position. The pharmacophore was generated using the 6W4H crystal structure. Figure S5 shows the ligand interactions (2D) and site view (3D) of the ligand SAM which is surrounded by hydrophilic and hydrophobic amino acids. Among them, Asp6897, Asp6928 and Asp6912 are polar acidic residues that interact with the ligand. There is also the presence of water molecules which form bridges between the ligand and the protein. Other residues such as Gly6869, Asn6841, Gly6879, Cys6913 and Gly6871 are polar in nature whereas, Leu6898 and Ala6870 are hydrophobic residues which contribute to the ligand interactions.

The pharmacophore features (5 in total) around the ligand were three donor and two acceptor features. Fifteen hit molecules were identified to have the assigned pharmacophore features. Key among the fifteen hits were adaptavir, dihydrostreptomycin, ATN-161, alarelin, gramicidin and exenatide (Figure S6). These hit molecules had rmsdx values ranging from 0.3747 to 0.66. By visually analysing the interactions

it was found that dihydrostreptomycin (-8.94%), ATN-161 (2.44%) and adaptavir (-1.32%) (*in vitro* SARS-CoV-2 inhibition in brackets) fitted well inside the binding pocket of the protein. In addition to mapping to the pharmacophore, these molecules made extra hydrogen bonding interactions with the protein.

All molecules had minimal *in vitro* activity which may be due to their large size and they were observed to clash with amino acid residues in the binding pocket. Therefore, a second pharmacophore query was generated having five features (four donors and one acceptor) with different positions and types (Figure S7). The second pharmacophore search resulted in eighteen hit molecules. Among these molecules, TMC-353121 (3.28%) and paromomycin (4.7%) were the top hit molecules (*in vitro* SARS-CoV-2 inhibition in brackets) which fitted well to the specified pharmacophore query (Figure S8). These are still very low activity values which may be due to using the endogenous metabolite SAM for creating the pharmacophore. Paromomycin is a launched drug whereas TMC-353121 is in phase 2 clinical trials.

Non-structural protein 15

The SARS-CoV-2 non-structural protein 15 (nsp15) is characterised as an endoribonuclease that cleaves RNA at uridylates at the 3'-position (Bhardwaj *et al.*, 2006). Nsp15 targets and degrades the viral polyuridine sequences and double-stranded RNA (dsRNA) to prevent the host immune system from detecting the virus (Hackbart *et al.*, 2020; Deng and Baker, 2018). The crystal structures of nsp15 (PDB IDs: 6WXC, 6WLC) were aligned and superposed which resulted in an RMSD of 0.466 Å after protein preparation. The two structures were similar and were properly superposed on each other. The A and the B chains of these proteins were aligned and superposed separately. The co-crystallised ligands in the binding pocket of the two proteins were different; 6WXC had tipiracil (CMU) as the main ligand in the binding site and 6WLC had uridine-5'-monophosphate (U5P). The ligand present in the binding site caused slight changes in the amino acid position. The pharmacophores were generated using both crystal structures since they had different ligands with different interactions. Figures S9 and S10 illustrates ligand interactions (2D) and site view (3D) representations which shows the ligands tipiracil and U5P are surrounded by hydrophilic and hydrophobic amino acid residues. U5P interacts with Lys290 and Lys345 which are polar basic residues. There is also the presence of water molecules which form bridges between the ligand and protein. Other residues such as Tyr343 and Ser294 are polar in nature whereas, Leu346 and Val292 are hydrophobic residues which contribute to ligand interactions. Tipiracil interacts with Lys290, Lys345 and His250 which are polar basic residues. There is also the presence of a phosphate ion and water molecules which form bridges between the ligand and protein. Other residues such as Tyr343, Ser294, Gly247 and Gly248 are polar in nature whereas, Leu346 is a hydrophobic residue which contributes to ligand interactions.

The first pharmacophore (6 features) on the ligand tipiracil contained three donor, two acceptor and one aromatic features. Twelve hit molecules were identified to have the assigned pharmacophore features. Key among the twelve hits, with results for ChEMBL reported *in vitro* SARS-CoV-2 inhibition in brackets, were AT-9283 (2.8%), acadesine (-4.27%), olomoucine (4.05%), sapropterin (14.33%), gossypol (66.34%)

and tetrahydrofolic acid (28.84%) (Figure S11). These hit molecules had rmsdx values ranging from 0.6109 to 0.7910.

By visually analysing the interactions it was observed that acadesine, olomoucine, sapropterin and tetrahydrofolic acid fitted well inside the binding pocket of the protein. These molecules also made hydrogen bond interactions in addition to the pharmacophore query features. Of the active compounds, olomoucine is in preclinical stages while sapropterin and tetrahydrofolic acid are launched drugs.

A second pharmacophore query was generated on the U5P ligand of 6WLC having one donor, three acceptor, one anionic acceptor and one aromatic features (Figure S12). The pharmacophore search resulted in six hit molecules: uridine-5-triphosphate (1.07%), baicalin (-2.99%), cyclic-AMP (1.15%), INS316 (25.9%), adenosine phosphate (43.81%) and diadenosine tetraphosphate (2.33%). INS316 is in phase 2 clinical trials and adenosine phosphate is launched on the market. The pharmacophore query search around the two ligands resulted in different hits due to the co-crystallised ligands displaying different interaction patterns (Figure S13).

Another crystal structure (PDB ID: 6X4I) was also aligned with 6WLC and 6WXC which resulted in an RMSD of 0.332 Å. The three structures were similar and were properly superposed on each other. The ligand present in the binding site of 6X4I was 3'-uridinemonophosphate (U3P). The site view (3D) showed that the three ligands had different interactions within the binding pocket (Figure S14). VS with the pharmacophore generated around U3P found two hit molecules (uridine-5-triphosphate and adenosine phosphate) that were also found as hits in 6WLC pharmacophore search.

Nucleocapsid protein

The SARS-CoV-2 nucleocapsid protein packages the single-stranded positive sense viral genome into a ribonucleoprotein (RNP) complex called the capsid. It has also been shown to modulate the host cellular machinery and may have regulatory roles in the viral life cycle (Ababou and Ladbury, 2007; Hsieh *et al.*, 2005, Surjit *et al.*, 2006). The crystal structures of nucleocapsid proteins (PDB IDs: 6WKP, 6M3M) were aligned and superposed which resulted in an RMSD of 1.092 Å. 6WKP had 2-N-morpholino-ethanesulfonic acid (MES) as the main ligand in the binding site and 6M3M had no ligand so the pharmacophore was generated using the 6WKP structure. The ligand interactions (2D) and site view (3D) images show that the ligand MES is surrounded by hydrophilic and hydrophobic amino acid residues (Figure S15). Residues such as TyrA109, AsnB154, AsnB75 and ArgA107 are polar in nature whereas, AlaA55 is a hydrophobic residue which contributes to the ligand interactions. There is also the presence of water molecules which form bridges between the ligand and protein.

The pharmacophore (5 features) around MES of the B chain were two acceptor, two anionic acceptor and one hydrophobic features. 122 hit molecules were identified to have the assigned pharmacophore features. Key hits, with results for ChEMBL reported *in vitro* SARS-CoV-2 inhibition in brackets, were varespladib (68.26%), hexonic acid (37.6%), citric acid (46.04%), OSI-027 (104.83%), MK-5108 (77.92%),

stepronin (42.06%), calcium gluceptate (31.49%), sodium glucoheptonate (3.84%), CPP (33.36%), pirenoxine (13%), midafotel (19.88%), dexamethasone sodium phosphate (-1.11%) and maltobionic acid (13.12%) (Figure S16). These hit molecules had rmsdx values ranging from 0.3303 to 0.6789.

By visually analysing the interactions it was found that citric acid, varespladib, stepronin and calcium gluceptate fitted well to the specified pharmacophore features and the binding site. These molecules also had moieties that made hydrogen bond interactions in addition to the pharmacophore query features. Stepronin, calcium gluceptate, pirenoxine and maltobionic acid are already launched drugs. Varespladib and midafotel are in phase 3 clinical trials. CPP is in phase 2 clinical trials. OSI-027 and MK-5108 are in phase 1 clinical trials. Hexonic acid and citric acid are in the preclinical stage.

Discussion

Computational drug design approaches can speed up the process to discover urgently needed treatments for SARS-CoV-2 infection. Currently, there is no specific antiviral treatment or vaccine available for COVID-19. There is an urgent need to find therapeutic interventions by repurposing existing drugs for possible use against SARS-CoV-2 by targeting the proteins encoded by the viral genome. Several studies are ongoing, including clinical trials, on repurposing of approved drugs (Pacios *et al.*, 2020). The major focus of research interest has been on the spike glycoprotein and the proteases. So as to examine other therapeutic intervention options, in this paper, we have focussed on all X-ray structures published on the PDB (as of 9th July 2020) of non-structural proteins and the nucleocapsid protein. Non-structural proteins are known to participate in viral RNA replication or transcription (Snijder *et al.*, 2020). We have also examined the nucleocapsid protein which is known to bind to the viral RNA genome and package them into helical nucleocapsid structures (McBride *et al.*, 2014; Masters and Sturman, 1990). PBVS was used to create ligand interaction guided pharmacophores from the holo X-ray structures and VS the Broad Institute's Drug Repurposing collection for known drugs mapping to viral pharmacophores.

The SARS-CoV-2 nsp3 crystal structure (PDB ID: 6YWL) had five features that were assigned on the co-crystallized large ligand APR. PBVS resulted in eight hits and among them, the top hits were antibiotics (dihydrostreptomycin, streptomycin, isepamicin) and a flavanone glycoside (neohesperidin) as they fitted well inside the binding pocket of the protein and made extra interactions with surrounding amino acids. These extra interactions may contribute to improved inhibition of the target protein. Isepamicin and streptomycin are launched drugs used against infectious diseases and are known to treat pneumonia and tuberculosis respectively. Isepamicin was active against SARS-CoV-2 in an inhibition bioassay (Ellinger *et al.*, 2020) reported in the ChEMBL database. Streptomycin had better interactions within the active pocket and fitted well to the given pharmacophore query. Antibiotics such as Teicoplanin were reported to inhibit the growth of virus in human cells (Colson and Raoult, 2016), is used to treat staphylococci infections and was also efficacious in the first stage of MERS-CoV viral cycle (Colson and Raoult, 2016; Zhou *et al.*, 2016). Azithromycin is another antibiotic that was found to be effective in treating viral infections caused by Zika and Ebola viruses by inhibiting growth *in vitro* (Madrid *et al.*, 2015; Bosseboeuf *et al.*, 2018). Combination use of hydroxychloroquine and azithromycin has been reported to

treat COVID-19 patients (Gautret, 2020). These findings suggest that antibiotics found as VS hits can possibly inhibit SARS-CoV-2 and should be progressed to *in vitro* and *in vivo* studies.

Another SARS-CoV-2 target called the nsp7-nsp8-nsp12 complex, which is known to be involved with RNA- dependent-RNA polymerase activity (Subissi *et al.*, 2014), was used for pharmacophore generation. The superposition/alignment step of the two nsp7-nsp8 complex proteins (7BV2, 7BW4) gave a low RMSD, indicating structural similarity. It was observed that whenever a ligand binds in a protein binding pocket there can be a wide range of structural changes, ranging from hinge movement of domains to small side-chain rearrangements within the binding pocket (Najmanovich *et al.*, 2000). The ligand F86 was a large molecule and had many atoms making interactions with the protein but there was only a slight change within the binding pocket due to interaction with F86. The identification of compounds capable of inhibiting proteins involved in viral replication has proven to be successful in the drive to discover potential anti-viral drugs (Ashraf *et al.*, 2019; Par & Par, 2018; Qadir *et al.*, 2018). The hits found from PBVS can possibly inhibit RdRp activity thereby affecting the viral replication of SAR-CoV-2. The top hits from the pharmacophore search were NADPH, adenosine-triphosphate (ATP), Coenzyme-I and nadide. Coenzyme-I, NADPH and ATP are all coenzymes necessary for biological activities. It is interesting, that these biological molecules could be used as therapeutic interventions. It has been reported that ATP can act as an immune system modulator in the treatment of HIV/AIDS (Wagner, 2011). The vitamin A derivative, retinazone, was shown to inhibit growth of a range of viruses (Kesel et al., 2014). But the potential of these molecules to inhibit viral proteins is not fully explored. However, Coenzyme-I demonstrated a 29% inhibiting of SARS-CoV-2 *in vitro* which indicates anti-viral activity.

During analysis of the VS results, we observed that the majority of the hit molecules clashed with the binding pocket amino acids due to their large size. Often molecules that are large in size tend to form the required interactions with amino acid residues, but a significant portion of the molecule is outside of the binding site or clashing with nearby amino acid residues. A second pharmacophore was generated with new features to search for compounds displaying fewer binding site clashes. PBVS found four top hit compounds: epigallocatechin- gallate(-) (EGCg), kuromanin, procyanidin-b-2 and rutin. These hit molecules are naturally occurring compounds and displayed varying active against SARS-CoV-2 *in vitro*. Among these, EGCg (6.28% inhibition) and rutin (40.82% inhibition) possess known anti-viral properties. EGCg is a catechin component of green tea and was reported to have antiviral activity against human immunodeficiency virus (HIV) type 1 as it impinges on each step of the viral life cycle (Yamaguchi et al., 2002). Rutin is a flavonoid glycoside which not only has antiviral activity against various infections (hepatitis B, herpes virus and retrovirus) but also possesses anti- asthmatic activity (Ganeshpurkar and Saluja, 2017). The use of natural compounds like lycorine was reported to show potent antiviral activity against SARS-CoV (Li et al., 2005). These studies indicate the potential utility of natural compounds against viral infections and that they should be considered as possible therapeutic interventions.

The nsp10-nsp16 complex is known to have the methyltransferase activity. Two nsp10-nsp16 crystal structures were aligned, superposed and had no structural differences. The ligand SAM was co-crystallised in the binding pocket, made several interactions with the amino acids in addition to bridging

water molecules which assisted with ligand binding. The presence of bridging water molecules can be used to increase ligand specificity and affinity so has proven useful in drug design (Ladbury, 1996; Schiebel, *et al.* 2018). Due to the large size of the ligand, two pharmacophore queries were generated to examine the effect of different feature combinations. Large ligands are likely to have multiple protein interactions which makes the query search more precise but results in fewer hits. The first pharmacophore search found dihydrostreptomycin, ATN-161 and adaptavir to fit well inside the protein binding pocket but none had appreciable *in vitro* inhibition. Adaptavir is in phase 2 clinical trials for the treatment of HIV and hepatitis B virus (HBV) infections whereas, dihydrostreptomycin is an antibiotic used for treating tuberculosis. The second pharmacophore search found TMC-353121 and paromomycin as hits. TMC-353121 is a potent respiratory syncytial virus fusion inhibitor (Ispas *et al.*, 2015) and *in vitro* had 3.28% inhibition of SARS-CoV-2. Paromomycin is an antimicrobial used to treat parasitic infections which had 4.7% inhibition of SARS-CoV-2. A recent study indicated the potential of an anti-parasitic drug called Ivermectin to have anti-viral activity *in vitro* against the SARS-CoV-2 (Caly *et al.*, 2020). Repurposing of these drugs which belong to diverse therapeutic areas (anti-viral, anti-parasitic and anti-bacterial) against the novel coronavirus is an important approach to finding a quick therapeutic alternative.

The two structures (6WXC, 6WLC) of SARS-CoV-2 nsp15 contained different ligands (tipiracil and U5P), were involved in dissimilar interactions, so separate pharmacophore searches were undertaken. Tipiracil made good interactions within the binding pocket and a phosphate ion was also involved in the network of hydrogen bonds. A recent study indicated that tipiracil inhibits the nsp15 endoribonuclease (Kim *et al.*, 2020) hence generating pharmacophore features from it can help to rapidly identify other possible drugs against SARS-CoV-2. The pharmacophore search generated hits such as AT-9283, acadesine, sapropterin, tetrahydrofolic acid, gossypol and olomoucine. Among them gossypol was found to be active *in vitro* against SARS-CoV-2 with inhibition of 66.34%. Gossypol is known to have various therapeutic effects and is often found as a hit molecule in computational drug design and follow-on *in vitro* assays so we do not recommend progressing this compound further. Sapropterin (14.33% inhibition), a phenylalanine-4-hydroxylase stimulant and tetrahydrofolic acid (28.84% inhibition) were also confirmed *in vitro* to be SARS-CoV-2 inhibitors. Folic acid and its derivatives (tetrahydrofolic acid and 5-methyl tetrahydrofolic acid) have been reported to have good binding affinity to viral proteins in various VS studies (Kumar & Jena, 2020). The second pharmacophore search based on the U5P ligand resulted in uridine-5-triphosphate, baicalin, cyclic-AMP, INS316, adenosine phosphate and diadenosine tetraphosphate. Baicalin was found to be inactive against SARS-CoV-2 *in vitro*, but we cannot fully ignore the potential of baicalin as it was previously reported to inhibit SARS-CoV-2 3CL^{Pro} *in vitro* (Su *et al.*, 2020). INS316 (25.9% inhibition) and adenosine phosphate (43.81% inhibition) are promising candidates for further *in vitro* and *in vivo* studies. To analyse the binding site interaction with different ligands (U5P, tipiracil and U3P) and to observe the variation in the position of the amino acid residues we performed an alignment/superposition step with three different nsp15 crystal structures (6X4I, 6WLC and 6WXC). The three ligands made different interactions within the binding site, however, the pharmacophore generated around U3P had two common hit molecules (uridine-5-triphosphate, adenosine phosphate) with the U5P

pharmacophore search which is unsurprising due to the close similarity between the co-crystallised ligands.

The majority of the crystal structures of the nucleocapsid protein had no co-crystallized ligand. 6WKP had MES as the ligand which made a number of interactions with the amino acid residues of the protein. Buffers like MES are used for NMR and X-ray crystallography and it is generally believed that the interactions between the protein and buffer agents have no significant influence on protein structure. But it is important to understand the effects of buffer agents on protein dynamics. One such study indicated a change in protein dynamics due to the interactions between MES buffer and human liver fatty acid-binding protein (Long and Yang., 2009). We assumed that MES was located in the active site of the protein and that the interactions made were relevant for generation of pharmacophore features. The first pharmacophore resulted in multiple hits also displaying good *in vitro* inhibition of SARS-CoV-2. The hits belonged to diverse therapeutic areas: Varespladib has anti-inflammatory properties, stepronin is a mucolytic drug, calcium gluceptate is a calcium supplement and citric acid has antioxidant properties. Nearly all of the VS predicted inhibitors of the nucleocapsid protein also display promising *in vitro* inhibition of SARS-CoV-2 indicating that, if on-target binding is subsequently confirmed, this protein is a promising anti-viral target.

Conclusion

The COVID-19 pandemic has negatively affected normal human life. It has been estimated that due to underlying health conditions, about one in five individuals worldwide could be at greater risk of developing severe COVID-19 after they become infected (Clark *et al.*, 2020). When time is a critical factor, repurposing of existing drugs can be a successful strategy leading to the discovery of therapeutic interventions which have already proven to be sufficiently safe in human trials.

To the best of our knowledge, this is the first comprehensive PBVS campaign against all publically available holo SARS-CoV-2 nsp and nucleocapsid proteins which has discovered multiple existing drugs that are predicted to inhibit SARS-CoV-2 proteins. The following compounds are proposed to inhibit the activity of viral proteins, all except streptomycin display *in vitro* inhibition of SARS-CoV-2 and are recommended for biological follow-up: isepamicin and streptomycin (nsp3); Coenzyme-I, rutin, epigallocatechin-gallate(-) and procyanidin- b-2 (nsp7/nsp8/nsp12); paromomycin (nsp10/nsp16); olomoucine, sapropterin, tetrahydrofolic acid, INS316 and adenosine phosphate (nsp15); varespladib, hexonic acid, citric acid, OSI-027, MK-5108, stepronin, calcium gluceptate, CPP, pirenexine, midafotel and maltobionic acid (Nucleocapsid).

By undertaking computational studies, we analysed key protein binding site amino acid residues, generated bespoke pharmacophores and obtained hits predicted to inhibit viral proteins. All excluding one of these hit molecules were previously validated *in vitro* to inhibit the activity of SARS-CoV-2. Our results indicate the underlying mechanism of action for the viral inhibitory activity of these molecules. We recommend conducting confirmatory SARS-CoV-2 biological assays of the *in silico* hit molecules which

were reported inactive in the bioassay conducted by Ellinger *et al.*, 2020. For hit compounds with *in vitro* activity, we recommend that confirmatory on target assays are undertaken, followed by detailed SARS-CoV-2 *in vitro* and *in vivo* studies. These hit compounds may have utility in fighting SARS-CoV-2 infection after further validation work, which will be particularly important if there are mutations of the spike or protease proteins which render future developed vaccine or small molecule treatments

Declarations

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Disclosure statement

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Figures

Natural, semi-synthetic or synthetic compounds with known formulation, dosage, combination, delivery, safety and efficacy for a disease

Figure 1

The flow chart depicts the process of drug repurposing where a marketed drug or a drug which has already been tested safe in preclinical and clinical settings is repurposed for treating a new disease. These drugs can either be natural, semi-synthetic or synthetic compounds.

Figure 2

(A) A 2D representation of the ligand-binding pocket showing attractive interactions between APR and amino acid residues. (B) A 3D representation of ligand APR (pink colour) interactions with amino acid residues. (C) Ligand inside the binding pocket with a surface representation. (D) Superposition of 6YWL and 6YWK showing structural similarity (Green colour – 6YWL, Orange colour – 6YWK).

Name of hit molecule	Structure of hit molecule
1. Isepamicin
2. Neobesperidin
3. Tannic acid
4. Streptomycin

(A) Pharmacophore features on APR (pink colour) from the 6YWL protein structure. (B) Structures of selected hit molecules (isepamicin, neohesperidin, tannic acid and streptomycin) obtained after VS.

Figure 4

(A) The pharmacophore features around the hit molecule Isepamicinn (light green colour) and its interactions with binding pocket amino acids. (B) The pharmacophore features around the hit molecule streptomycin (dark green colour) and its interactions with binding pocket amino acids.

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