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In-silico screening and *in-vitro* assay show the antiviral effect of Indomethacin against SARS-CoV-2

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ABSTRACT

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has caused the worldwide spread of coronavirus disease 19 (COVID-19), and till now, it has caused death to more than 6.2 million people. Although various vaccines and drug candidates are being tested globally with limited to moderate success, a comprehensive therapeutic cure is yet to be achieved. In this study, we applied computational drug repurposing methods complemented with the analyses of the already existing gene expression data to find better therapeutics in treatment and recovery. Primarily, we identified the most crucial proteins of SARS-CoV-2 and host human cells responsible for viral infection and host response. An *in-silico* screening of the existing drugs was performed against the crucial proteins for SARS-CoV-2 infection, and a few existing drugs were shortlisted. Further, we analyzed the gene expression data of SARS-CoV-2 in human lung epithelial cells and investigated the molecules that can reverse the cellular mRNA expression profiles in the diseased state. LINCS L1000 and Comparative Toxicogenomics Database (CTD) were utilized to obtain two sets of compounds that can be used to counter SARS-CoV-2 infection from the gene expression perspective. Indomethacin, a nonsteroidal anti-inflammatory drug (NSAID), and Vitamin-A were found in two sets of compounds, and in the *in-silico* screening of existing drugs to treat SARS-CoV-2. Our *in-silico* findings on Indomethacin were further successfully validated by *in-vitro* testing in Vero CCL-81 cells with an IC_{50} of 12 μM. Along with these findings, we briefly discuss the possible roles of Indomethacin and Vitamin-A to counter the SARS-CoV-2 infection in humans.

1. Introduction

Coronavirus disease 2019 (COVID-19) has so far infected more than 525.2 million people worldwide and caused more than 6.2 million deaths (www.worldometers.info/coronavirus). Despite significant advancements in designing and dispersing multiple vaccines [18,49,80,109] against SARS-CoV-2, the arrival of newer strains [35,108] had prompted us finding more effective vaccines [110]. Using various methods of drug repurposing, many existing medicines had been tried and tested against SARS-CoV-2 with limited success [79,91,103]. Many vitamins and minerals were also suggested as immunity boosters to prevent or reduce the severity of SARS-CoV-2 infection [44]. Despite significant research, we are yet to achieve any widely accepted drug regimen to treat COVID-19 [67].

Abbreviations: COVID-19, Coronavirus disease 2019; SARS-CoV-2, Severe accute respiratory syndrome coronavirus 2; ORF, Open reading frame; NSP, Nonstructural protein; PDB, Protein Data Bank; RdRp, RNA-dependent RNA polymerase.

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As per our present knowledge, SARS-CoV-2 viruses mediate host cell infection and viral replication through a few key proteins to invade and sabotage the host cell cellular machinery [111]. Among these proteins, four structural proteins, *i.e.*, spike protein, membrane protein, nucleocapsid protein, and envelope protein are common to all coronaviruses and appeared to be attractive targets for therapeutic drug development. In addition, eleven accessory proteins (ORF- 3a, 3b, 3c, 3d, 6, 7a, 7b, 8, 9b, 9c, and 10) and sixteen nonstructural proteins (NSPs 1-16) are also being investigated to understand their role in replicating SARS-CoV-2 genome inside the host cell [75]. Among the nonstructural proteins, main protease (NSP5), papain-like protease (a domain of NSP3), RNA-dependent RNA polymerase (RdRp: NSP12 in complex with NSP7 and NSP8), NSP15, and NSP16 had been targeted by many drug developers due to their critical roles in SARS-CoV-2 genome replication. Attempts to find effective inhibitors from already existing drugs [12,61], chemical analogs of antiviral drugs [68], bioactive molecules available from natural sources [2,13,14,45,82,87], and many other approaches resulted in limited success. It is interesting to note that few recent works have identified the interacting partners of these SARS-CoV-2 proteins in human host cells [33]. Identifying the viral protein targets and their interacting host partners are of grave importance to combat SARS-CoV-2 infection. Computational screening is an *in-silico* method to identify potential lead compounds from a library of chemical compounds against a target molecule [50]. Various small molecule pharmaceutical compound libraries are available, for example, TTD [48,104], IUPHAR/BPS guide to pharmacology [11], VARIDT [28], TCDB [78], INTEDE [114], and many more. Though the use of these diverse pharmaceutical databases might increase the search space for identifying potential inhibitor compounds, drug repurposing strategy could be an interesting option to target the proteins crucial for SARS-CoV-2 infection. Among the various available libraries of chemical compounds, Drugbank [25] is a database often used to screen the chemical lead compounds of FDA-approved drugs against various target receptors.

Data of differentially expressed genes of SARS-CoV-2 infected primary human lung epithelium (NHBE) cells [34] are available. In addition, LINCS L1000 contains gene expression data on the effect of thousands of molecules (http://www.lincsproject.org/). Many useful databases and resources are extracted from L1000-based LINCS data and the search engine 'L1000 Characteristic Direction Signature' is one of them. It is designed to search for gene expression signatures against LINCS data to detect and prioritize small molecules that reverse or mimic the input gene expression signature. Therefore, this tool can be used to check the reversibility of SARS-CoV-2 infected gene expression with the candidate drug molecules. Similarly, another database, Comparative Toxicogenomics Database (CTD) (http://ctdbase.org/), can be used to investigate the molecules that influence a list of genes and this analysis may be compared to the list of genes obtained from the NHBE cells.

Our primary focus of this study was to identify the crucial host-pathogen interaction partners by extensive literature survey and data mining and to prepare a list of viral and host target proteins for virtual screening of the already existing drugs. We further aimed to apply computational screening methods of the existing FDA approved drug molecules to find out their interactions with these identified crucial proteins for SARS-CoV-2 infection. In order to investigate if any of the existing drug molecules can bind to important host and viral proteins of SARS-CoV-2 infection, we obtained the chemical structure files of the existing drugs from the DrugBank. Based on the available databases of various compounds and gene expression databases, we also wanted to investigate if any of the available compounds may reverse the genetic changes incurred by SARS-CoV-2. We found that two of the existing drugs, Indomethacin and Vitamin-A, might be effective to target the crucial host-viral interactions and compensate for the gene expression change caused by SARS-CoV-2. Between Indomethacin and Vitamin A, Indomethacin was found to be more involved with the changes in gene expression data in hosts. It motivated us to test the efficacy of Indomethacin against SARS-CoV-2 *in-vitro* and we observed a moderate efficacy (IC_{50} = 12 μm) of this drug against SARS-CoV-2. Our work corroborates the growing notion that Indomethacin has potential antiviral properties against SARS-CoV-2 and may be considered in treating COVID-19.

2. Methods

2.1. Data mining

Extensive literature surveys and text mining were performed with the help of the PubTator central tool [106]. The interactome dataset for Cov-2–Human proteins was obtained from the BioGRID database [88]. The interacting host-pathogen partners acquired from the literature as well as from the BioGRID repository were compiled together, and the interactions were shortlisted following various parameters (represented as a flowchart in Fig. 1).

Initially, only the experimentally validated SARS-CoV-2-human interactions were selected from the compiled dataset. Since the experimental validation (from the literature) for majority of the interactions was performed by affinity purification and mass spectrometry (AP-MS), the shortlisting in this level was done by keeping the average spectral count as the deterministic parameter for high confidence interactions [53]. To determine an interaction to be of high confidence, the average spectral count cut-off was kept at 10. Next, the high confidence interacting proteins were screened and selected according to the availability of three-dimensional structures deposited in the Protein Data Bank (PDB, www.rcsb.org). Finally, the active sites for the short-listed interacting partners were identified with the help of UniProt [20] or predicted using PROSITE [86] server, for further analyses.

2.2. Virtual screening

For the Virtual screening, a total of 1918 FDA-approved drugs were obtained from the [25] (www.drugbank.ca) in 3D SDF format. The SDF files were processed to assign Gasteiger partial charge to each ligand atom and generate the corresponding PDBQT files using Open Babel [65]. Besides, all the target proteins were obtained from the Protein Data Bank (www.rcsb.org). The protein atoms were treated with Kollman partial charges using AutoDockTools [63]. All the virtual screenings were performed using AutoDock Vina [97]. The search space for each target protein was defined according to its active site by using a grid box with a spacing of 3.5 Å.

2.3. Gene expression data analysis

RNAseq data of primary human lung epithelium (NHBE) of control vs infected with SARS-CoV-2 (USA-WA1/2020) for 24 h were selected from GSE147507 [15]. The control and treated groups contained independent biological triplets of each kind. The GSE sample IDs are given below in Table 1.

The expression dataset was analyzed using GREIN [55]: GEO RNA-seq Experiments Interactive Navigator. GREIN is an interactive web platform that offers easy-to-use solutions for GEO RNA-seq data exploration and analysis. GREIN is driven by a back-end code pipeline to process RNA-seq data reliably and a large number (>6000) of data sets already processed. There have been many algorithms which have performed excellently in processing OMICS data [27,89,90,112,113]. GREIN automates all Quality Control checks and Data preparation for its users which included data normalization using the trimmed mean of M-values (TMM) approach, where M-values represent empirical fold changes between two samples. GREIN handles all the standard pipelines for identifying differentially expressed genes in RNAseq data.

Simultaneously, the L1000 Fireworks Display database, L1000FWD [105] was used for searching and visualizing drug-induced gene signatures. Signature similarity search is used to retrieve signatures that

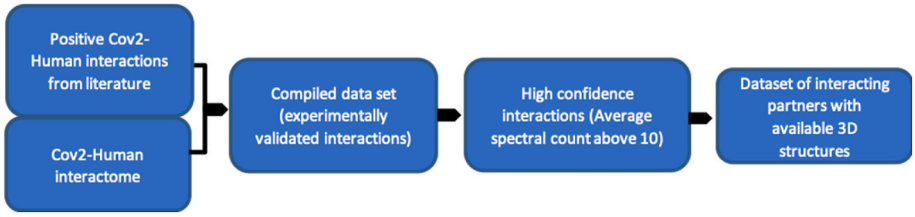

Fig. 1. Flow chart representing steps followed in shortlisting available SARS-Cov2-Human interacting partners.

Table 1
GSE sample IDs for control and infected sets.

Mock Treated/ Control	Infected with SARS-CoV-2 (USA-WA1/2020) for 24 h treatment
GSM4432378	GSM4432381
GSM4432379	GSM4432382
GSM4432380	GSM4432383

mimic or oppose the queried up and down-regulated COVID-signature gene set through a trained unsupervised clustering algorithm called k-means on 16,000 small drug molecules induced gene signatures. The Comparative Toxicogenomics Database, CTD [22] was used to retrieve Chemical-Gene interactions and mapped with the COVID-signature gene set (graphically represented in Fig. 2). CTD is a repository, curated and manually verified from scientific literature, contains interaction data among genes, chemicals, diseases, phenotypes, and pathways. We have used "Calculate and draw custom Venn diagrams" tool available at https://bioinformatics.psb.ugent.be/webtools/Venn/to draw Fig. 5.

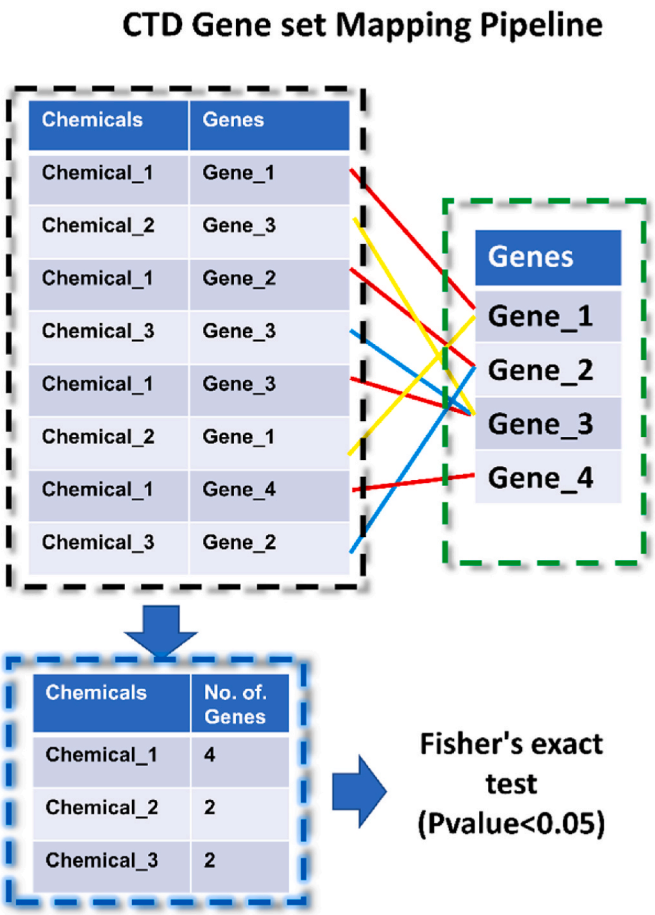

Fig. 2. CTD-Gene set data mapping pipeline.

2.4. Molecular docking

Common hits from the virtual screening, compounds from L1000 data that complement the gene signature of SARS-infected cells, and chemicals obtained from CTD linked with selected host genes were chosen for additional molecular docking investigations. AutoDock Tools 1.5.6 for AutoDock 4.2 [63] was used to import each protein independently. Afterward, water molecules and hetero-atoms were eliminated, followed by the addition of polar hydrogen and the computation of Gasteiger and Kollman charges. Finally, the proteins were saved in the pdbqt format. The grid size of the receptors was determined by selecting the active site amino acid residue data gathered from literature and Computed Atlas of Surface Topography of proteins (CASTp 3.0) [93]. The grid center and dimensions for each receptor are provided in supplementary file 1. To continue with the Genetic Algorithm, we select 50 runs, with a population size of 300, a number of evals of 2500000, and a number of generations of 27000. All other parameters were left at their default levels.

2.5. In-vitro drug testing of Indomethacin

The *in-vitro* testing and anti-viral assay for Indomethacin against SARS-CoV-2 (NIV2020-770 isolate) infected Vero CCL-81 cell line were performed at the ICMR-National Institute of Virology (ICMR-NIV), Pune-411001, India.

2.5.1. Preparation of the drug

A stock solution of Indomethacin (Sigma Aldrich, Israel; cat #17378) was prepared by dissolving in absolute ethanol at a concentration of 40 mM. Further, it was diluted to the desired concentrations (0.1 μM, 1 μM, 5 μM, 10 μM, 50 μM, 100 μM, 250 μM, 500 μM, 750 μM and 1000 μM) in Dulbecco's modified Eagle's medium (DMEM, Gibco, Grand Island, USA) culture medium [8].

2.5.2. Cell culture

Vero CCL-81 cell line (ATCC, CCL-81) were cultured at 37 °C in Dulbecco's modified Eagle's medium (DMEM), supplemented with 2% fetal bovine serum (FBS, Gibco) in the atmosphere with 5% CO_2. Cells were digested with 0.25% trypsin and uniformly seeded in 96-well plates.

2.5.3. Cytotoxicity assay

Vero CCL-81 cells (100 μL per well) were seeded onto a 96-well plate at a density of 3×10^6 cells/mL and grown for 24 h before adding the drug. Vero CCL-81 cells were treated with different concentrations of the drug Indomethacin (0.1 μM, 1 μM, 5 μM, 10 μM, 50 μM, 100 μM, 250 μM, 500 μM, 750 μM, and 1000 μM) for 72 h at 37 °C with 5% CO_2. Vehicle controls were treated with an equal volume of the vehicle. Cell control (CC) was not treated with the drug. After 72 h, MTT (3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide, 2 mg/ml concentration) was added to each well and incubated for 4 h at 37 °C in 5% CO_2 [59]. The cell supernatant was discarded and 200 μl of Dimethyl Sulphoxide (DMSO, Sigma) was added to each well. The plates were incubated for 30 min at room temperature and the OD was measured at 570 nm using an ELISA plate reader. CC_{50} (Concentration showing 50%

viability) was calculated using the Graph Pad Prism software version 9.

2.5.4. Antiviral assay post drug-treatment

All experiments involving handling of infectious materials were performed in the Biosafety Level-3 (BSL-3) laboratory with appropriate biosafety practices. The Vero CCL-81 cells were seeded in 96 well plates at a density of 2×10^4 cells/well and then grown for 24 h before adding the drug. Vero CCL-81 cells were infected with SARS-CoV-2 strain at an MOI (Multiplicity of Infection) of 0.001 for 2 h at 37 °C. The cells were washed with 1XPBS and treated with the drug (1 μM, 10 μM, 50 μM, 100 μM, 250 μM, and 500 μM concentration based on the CC_{50}) in triplicates and incubated for 72 h. Vehicle control had an equal concentration of the vehicle, drug controls had only the respective concentration of the drug without the virus, and cell control (CC) had no virus and drugs. Cells were observed at 48 h and 72 h for CPE (Cytopathic effect). The supernatant was collected after 72 h of treatment of Indomethacin.

Viral RNA was extracted from the supernatants of infected cells using the automated nucleic acid extraction system (Magmax, Thermo Scientific), following the manufacturer's recommendations. The extracted viral RNA was analyzed by relative quantification of the RdRp-2 gene by real-time RT-PCR using the SSIII qRT-PCR kit (Invitrogen) on the ABI 7300 Real-Time PCR system. A standard curve was generated by determining the copy numbers from serial dilutions (10^3-10^9 copies) of *in-vitro* transcribed RNA for RdRp-2 gene [19]. IC_{50} (Concentration showing 50% inhibition) was calculated by using Graph Pad Prism software version 9.

3. Results

3.1. Data mining/Literature survey

The compiled SARS-CoV-2-Human interaction data set from literature and the BioGRID repository consisted of more than 700 protein-protein interactions with more than 300 experimentally validated interactions (AP-MS validation). On further screening for high confidence interactions, 57 host-viral interactions were identified, among which 6 viral and 12 host proteins were identified (Table 2) based on the available 3D structural data (RCSB PDB).

3.2. Virtual screening

From the virtual screening (VS) results, we focused to identify the set of drug molecules that exhibited binding affinity less than −5 kcal/mol with possible interaction with selected viral and host targets. Such common drug molecules were much of interest as they could be potential candidates for multi-target interaction. The list of drug molecules along with their binding affinity with the corresponding targets are provided in supplementary files 2 and 3, and represented in Fig. 3. The total number of such common drug molecules were 1287 and 1381 for the host proteins and viral proteins respectively (Supplementary files 2 and 3). As we obtained a large number of hits, we investigated if any of these drug molecules could perturb the gene expression pattern on the SARS-CoV-2 infected cells, thereby cross-validating the potential of the identified drugs.

Table 2
List of selected host and viral proteins.

SARS-CoV-2 Targets (PDB ID)	Host Targets (PDB ID)	
Main Protease (6YB7) [74]	Angiotensin-converting enzyme 2 (6M0J: Chain A) [47]	Kinase and Ubiquitin-associated domains of MARK3/Par-1 (2QNJ) [116]
Spike S1 (6W41) [115]	Human glutathione peroxidase 1 (2F8A) [70]	Catalytic and ubiquitin-associated domains of MARK1/PAR-1 (2HAK) [56]
Papain like protease (6W9C) [73]	NTF2 domain of Ras GTPase-activating protein-binding protein (3Q90) [72]	Human Heme Oxygenase-1 (1N45) [117]
NSP-16/10 (6W4H) [76]	G3BP2 NTF2-like domain (5DRV) [41]	SmgGDS-558 (5XGC) [85]
NSP-15 (6VWW) [39]	Human plakophilin 2 (3TT9) [40]	Human insulin-degrading enzyme (2G47) [84]
NSP12-7-8/ RDRP (7BTF) [29]	Ubiquitin-protein ligase MIB1 (4XI6) [58]	Human Sirtuin homolog 5 (2B4Y) [71]

3.3. Gene expression data analysis

3.3.1. Analysis of GSE147507 GEO dataset

A total of 317 genes with p-values < 0.05 were found to be differentially expressed (Supplementary file 4). Of these, 90 mRNAs were down-regulated and 227 mRNAs were up-regulated, contributing as a signature of gene expression named 'COVID-signature', represented in Fig. 4. This expression signature was fed to the L1000 Firework Display (L1000FWD) to scan for the reverse gene expression signatures associated with small drug molecules. Through this step, 670 potential small drug molecules were identified (Supplementary file 5), which can significantly reverse the gene expression signature of the control vs SARS-CoV-2 infected gene set.

Mapping was done to retrieve chemicals that have a known interaction with the COVID signature gene set. Fisher's exact test was then applied to each chemical, as it was found that the chemical may interact with multiple genes from the signature gene set. The Fisher's exact test was used to detect non-significant chemical and gene associations. Two hundred and thirty-one chemicals were found to have significant interactions with the COVID signature gene set (Supplementary file 5). Finally, three molecular data sets were created (graphically represented in Fig. 5):

1. Approved drug molecules were retrieved from the Drugbank and screened through virtual screening. One thousand two hundred fifty-one approved drug molecules were analyzed based on the free energy values described in the above sections.
2. Six hundred and seventy drug molecules that have a significantly reversed signature expression compared to the COVID signature gene set in cell lines were considered.
3. Two hundred and thirty-one molecules with significant interactions with the COVID signature gene set were retrieved from CTD.

All the three data sets were subjected to the Venn diagram (Fig. 5) to find the common molecules among them. Two molecules, Indomethacin and Vitamin-A, were found common in all the three data sets. Indomethacin showed interactions with 85 genes of the COVID signature gene set, which were mapped from CTD data, whereas Vitamin-A had interactions with 4 genes form the COVID signature gene set, and a total number of reported Vitamin-A and gene interactions was 6. Docking analyses of Indomethacin revealed potential binding mechanisms in the active sites of the target proteins. The total docked energy/binding energy of the ligand and protein considered both intramolecular and intermolecular energy. In Tables 3 and 4, the host and the viral receptors are presented in ascending order of binding energies with their possible interaction types between the receptors and Indomethacin respectively. Figs. 6 and 7 illustrate the estimated postures of Indomethacin in the active site of the host and viral receptors with amino acids involved in interaction respectively. These figures show that Indomethacin can bind favorably to these target proteins which can modulate SARS-CoV-2 interactions with the host cells. In addition, as Indomethacin was shown to be involved with a significantly greater number of genes in the COVID signature gene set, it was further tested *in-vitro*.

3.4. In-vitro drug testing of Indomethacin

To check the efficacy of the computationally predicted drugs *in-vitro*,

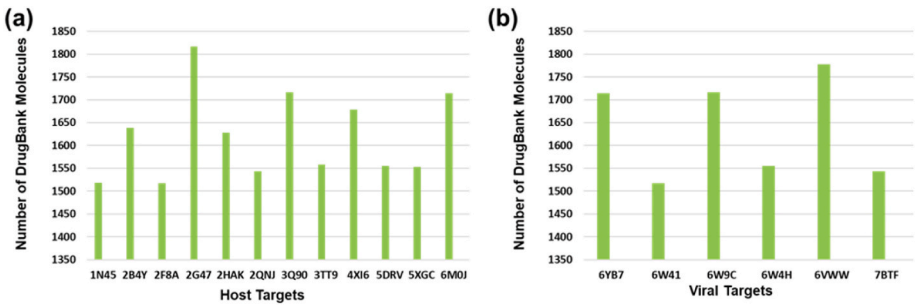

Fig. 3. The number of total drug molecules with binding energy < -5 kcal/mol for (a) Host targets and (b) Viral targets.

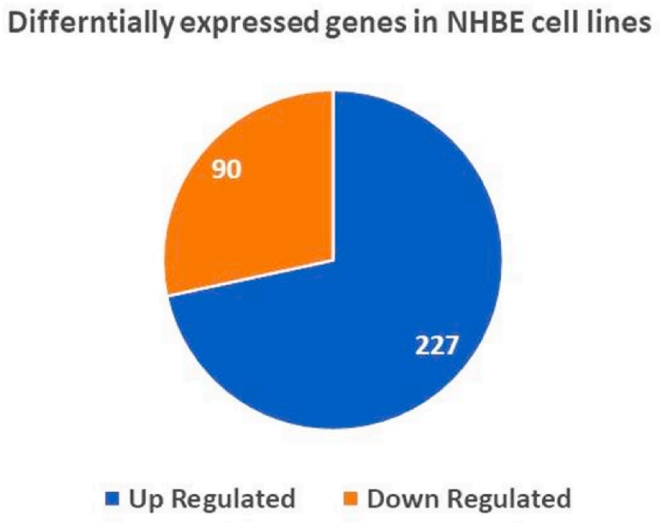

Fig. 4. Differentially expressed genes in NHBE cell lines.

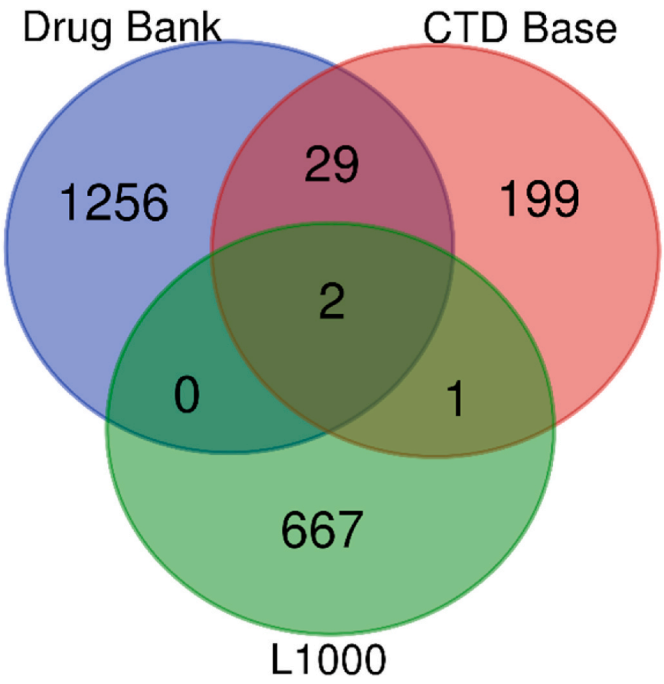

Fig. 5. Number of molecules retrieved from various data sets.

we tested Indomethacin on Vero CCL-81 cells infected with SARS-CoV-2. Indomethacin showed dose-dependent cytotoxicity in Vero CCL-81 cells when tested at the following concentrations - 1 μM, 5 μM, 10 μM, 50 μM, 100 μM, 250 μM, and 500 μM (Fig. 8). The CC_{50} of Indomethacin was found to be ~490 μM. The IC_{50} of 12 μM was observed from the antiviral efficacy assay. The selectivity index of Indomethacin was calculated to be ~40. One of the widely used drugs used against SARS-CoV-2 is Remdesivir and its IC_{50} was found to be 11.41 μM in previous *in-vitro* studies in Vero cells [42]. This result suggests that Indomethacin has antiviral activity against SARS-CoV-2 as shown by reduced RdRp-2 gene copy numbers.

4. Discussion

In the present study, using *in-silico* screening of the already existing drugs against crucial viral and host proteins, we have identified Indomethacin and Vitamin-A as potential drug candidates against SARS-CoV-2. Vitamin-A, an important molecule that supports human life, is required for cell growth, differentiation, immune response, and epithelial integrity. A lower level of Vitamin-A was previously observed in the TB patients compared to the healthy patients, indicating the association between lack of Vitamin-A and the occurrence of TB [4,64]. We have observed that Vitamin-A can also be effective in binding both the viral and the host proteins crucial for SARS-CoV-2 infection. It has also been speculated that the epithelial cells lacking Vitamin-A may be more prone to pathogen infection than others [77]. Vitamin-A is also important for the development and regulation of macrophages and neutrophils, migration and homeostasis of T-cells, immunoglobulin production, and B-cell activity [37]. Vitamin-A plays an important role in the formation of epithelial and mucous cells, whereas, coughing had long back been associated with the loss of epithelial cells [107]. In the case of COVID-19, 71.7% of patients were detected with low levels of Vitamin A [95]. Decreased levels of Vitamin-A were associated with increased severity of COVID-19 infection [92]. Analyses of deficiency in micronutrients showed that Vitamin-A was an important element missing from the COVID-19 patients and a lower risk of disease progression was observed with a higher level of Vitamin-A [102]. In another study, it was shown that 37% of COVID-19 patients were Vitamin-A deficient, whereas a high level of Vitamin-A was associated with asymptomatic COVID-19 cases [7]. The same study also showed that 23% decrease in the levels of Vitamin-A in severe cases compared to the asymptomatic COVID-19 patients [7]. Low levels of Vitamin- A in serum is often associated with liver damage, a marker of COVID-19 [57]. It has been speculated that Vitamin-A plays a crucial role in immunomodulatory functions by secreting IgA, which might be crucial in preventing SARS-CoV-2 infection [98]. In another hypothesis, retinol depletion and retinoid signaling pathway have been considered to play a critical role in the COVID-19 pathogenesis [81]. All-trans retinoic acid, a derivative of Vitamin-A, was shown to have an antiviral effect by inhibiting the main protease of SARS-CoV-2 [62]. There is a growing discussion if Vitamin-A can be used as a potential therapeutic/supplement [30,96] or as a nutrient supplementation [44,101]. Our study supports this notion

Table 3
This table lists the binding energies and inhibition constants calculated during docking with types of interactions involved between host receptors and Indomethacin.

Host Receptors	Binding Energy (kcal/mol)	Inhibition Constant (KI)	Interactions				
			No. of Hydrogen Bonds	No. of hydrophobic Interactions	Salt Bridges	Pi-Stacking	Pi-Cation Interaction
Human Insulin-Degrading Enzyme	−11.98	1.64 nM	5	4		3	
MZM-REP Domains of Mind bomb 1	−10.57	17.87 nM	1	7	1	1	1
Human Glutathione Peroxidase 1	−9.65	84.17 nM	4	3			
Catalytic and Ubiqutin-associated domains of MARK1/PAR-1	−9.53	103.34 nM	3	6		1	
Angiotensin Converting Enzyme-2	−9.22	173.61 nM	3	4	1	3	
Human Sirtuin homolog 5	−9.2	173.09 nM	2	7	2		
Kinase and Ubiquitin-associated domains of MARK3/Par-1	−9.18	187.71 nM	2	8	1		
NTF2 domain of Ras GTPase-activating protein-binding protein 1	−8.67	440.55 nM	3	5			
Human plakophilin 2 isoform a (PKP2a)	−8.32	792.90 nM	1	6	1		1
SmgGDS-558	−8.2	971.48 nM	2	8			
Human Heme Oxygenase-1	−8.1	1.16 uM	5	5			
G3BP2 NTF2-like domain in complex with a peptide	−4.18	4.82 mM	2	4			

Table 4
This table lists the binding energies and inhibition constants calculated during docking with types of interactions involved between viral receptors and Indomethacin.

Viral receptors	Binding Energy (kcal/mol)	Inhibition Constant (KI)	Interactions				
			No. of Hydrogen Bonds	No. of hydrophobic Interactions	Salt Bridges	Pi-Stacking	Pi-Cation Interaction
SARS-CoV-2 main protease	−11.52	3.62 nM	3	7	1		
SARS-CoV-2 RNA-dependent RNA polymerase	−10.9	10.18 nM	4	7			
SARS-CoV-2 receptor binding domain	−9.44	121.24 nM	3	2			
Papain-like Protease of SARS-CoV-2	−8.4	701.79 nM	5	1			
NSP16 from SARS-CoV-2	−8.36	740.66 nM	2	10		2	
NSP15 Endoribonuclease from SARS-CoV-2	−8.05	1.13 uM	3	4	1		

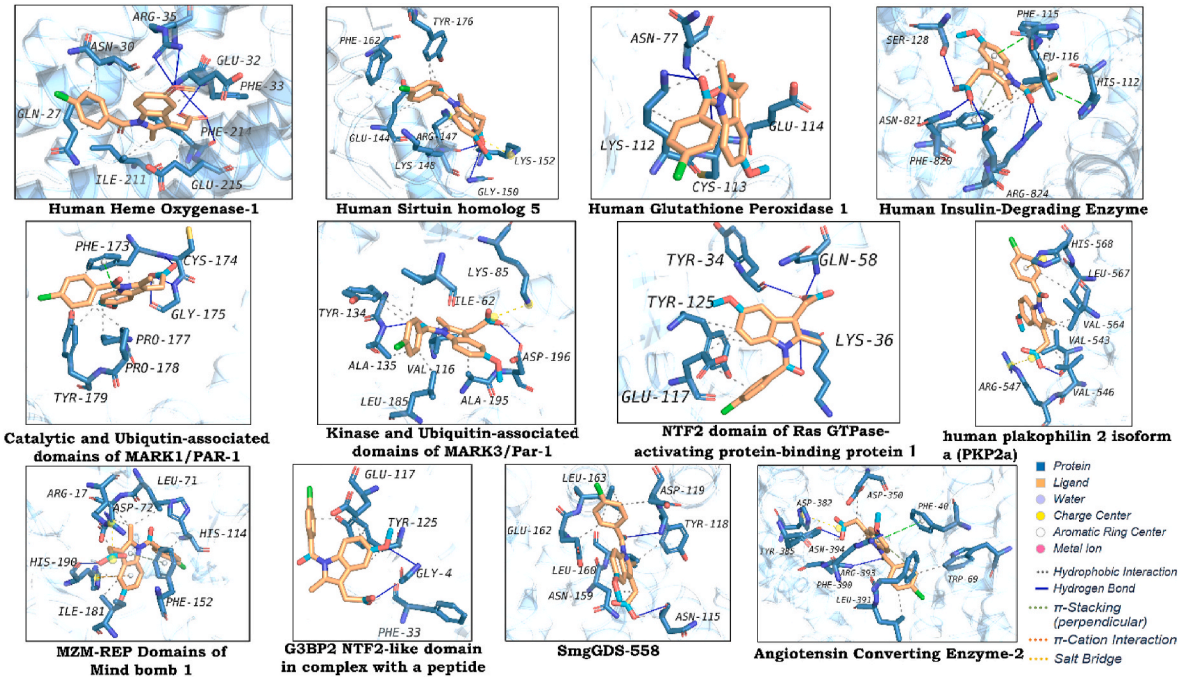

Fig. 6. Host targets human heme oxygenase-1, human sirtuin homolog 5, human glutathione peroxidase-1, human insulin degrading enzyme, catalytic and ubiquitin-associated domains of MARK-1/PAR-1, kinase and ubiquitin-associated domains of MARK3/PAR-1, NTF2 domains of Ras GTPase activating protein-binding domain, human plakophilin-2, Ubiquitin-protein ligase Mib1, G3BP2 NTF2-like domain, SmgGDS-558, and Angiotensin-converting enzyme 2 are docked with Indomethacin and their interactive residues at the active sites are shown in the images. Detailed interactions are listed in supplementary file 6.

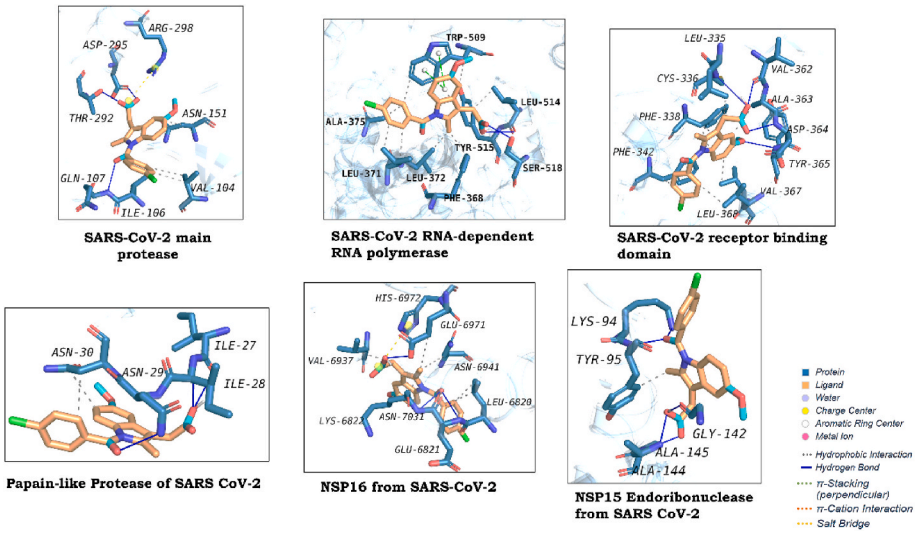

Fig. 7. Viral targets NSP-15, NSP-16/10, Papain like protease, Spike S1, Main protease, and RNA dependent RNA polymerase (RdRp) are docked with Indomethacin and their interactive residues at the active sites are shown in the images. Detailed interactions are listed in supplementary file 7.

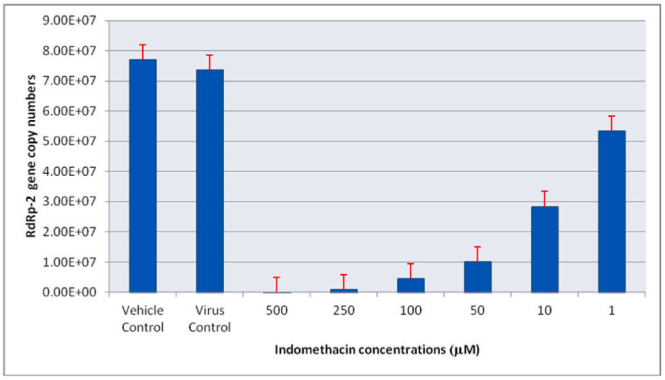

Fig. 8. Effect of Indomethacin treatment on Vero CCL-81 cells infected with SARS-CoV-2. Vero CCL-81 cells were infected with SARS-CoV-2 and treated with different concentrations of Indomethacin for 72 h. Virus control (VC) was not treated with the drug. RNA isolated from the respective cells after 72 h were subjected to qRT-PCR for the RdRp-2 gene target. All data are expressed as mean ± SD (standard deviation) for n = 3.

further by showing that Vitamin-A has the potential to bind the crucial proteins and slight ability to reverse the genetic changes brought upon by SARS-CoV-2.

As can be observed from Table 3, Indomethacin formed strong interactions with human insulin-degrading enzyme, MZM-REP Domains of Mind bomb 1, human glutathione peroxidase and the other host proteins studied here. Indomethacin showed strong hydrophobic interactions and significant H-bond formations with the host proteins. It can be observed (Fig. 6) that the residues His112, Phe115, Leu116, Ser128, Phe820, Asn821, and Arg824 of the Human insulin-degrading enzyme made crucial interactions with Indomethacin. Among these residues, only His112 is not included as it is slightly distant from the active site pocket as analyzed by CASTp 3.0 [93]. Similarly, residues Arg17, Leu71, Asp72, His114, Phe152, Ile181, and His190 from the active site of MZM-REP domains of Mind bomb 1 formed both hydrophobic and H-bonding interactions with Indomethacin. The other host receptors, such as Human Glutathione Peroxidase 1, Catalytic and Ubiquitin-associated domains of MARK1/PAR-1, Angiotensin-converting Enzyme-2, Human Sirtuin homolog 5, Kinase and Ubiquitin-associated domains of MARK3/Par-1, NTF2 domain of Ras GTPase-activating protein-binding protein 1, human plakophilin 2 isoform a (PKP2a), SmgGDS-558 and Human Heme Oxygenase-1 also showed strong affinity towards Indomethacin. Thus, Indomethacin can play a strong role in binding crucial host proteins involved in SARS-CoV-2 infection. Similarly, from Table 4, it can be observed that Indomethacin formed strong interactions with main protease, RNA-dependent RNA polymerase, receptor binding domain of the spike protein, Papain-like Protease, NSP16, and NSP15 Endoribonuclease of SARS-CoV-2 by employing both hydrophobic interactions and H-bonding interactions. Fig. 7 demonstrates that the residues Val104, Ile106, Gln107, Asn151, Thr292, Asp295, and Arg298 of the main protease interact with Indomethacin. His41 and Cys145 are the two main residues important for the proteolytic activity of this main protease [12,45,61]. However, it should be noted that the other potential inhibitors of the main protease interact with different sets of residues [12,45,61]. It can be further inspected if the interactions mentioned may cause any allosteric changes in the main protease of SARS-CoV-2. The residues Phe368, Leu371, Leu372, Ala375, Trp509, Leu514, Tyr515, and Ser518 of RNA dependent RNA polymerase (RdRp) help in binding Indomethacin. Previous studies showed that the residues important for binding Remdesivir and Favipiravir are K551, R553, and R555, and K545, K551, and R553, respectively [14]. The interacting residues with other potential inhibitors also vary slightly in a few other studies [14,68]. Similarly, detailed analyses should be performed for each host and viral protein to decipher the anti-viral mechanism of Indomethacin against SARS-CoV-2.

Indomethacin is a non-steroidal anti-inflammatory medication (NSAID) that is frequently used in the treatment of rheumatoid arthritis and gout. Indomethacin acts similarly to other NSAIDs, such as aspirin and ibuprofen, by decreasing the activity of cyclooxygenase-1 and 2 (COXs) and inhibiting pro-inflammatory prostaglandin formation [99,100]. In comparison to steroidal drugs such as betamethasone and hydrocortisone, NSAID Indomethacin inhibited phospholipase A2 significantly and more effectively [54]. The host's active response to viral infection, such as SARS-CoV-2, results in the accumulation of mucus and inflammation, particularly in the lungs, where patients frequently exhibit profuse phlegm, resulting in severe dyspnea [36]. NSAIDs such as Indomethacin can suppress such responses and alleviate respiratory distress in the patient [17]. Indomethacin had also been demonstrated to activate eIF2 double-stranded RNA (dsRNA) dependent protein kinase R (eIF2 kinase PKR) and limit viral multiplication and translation directly, without impairing the host cell translation machinery [9]. Various previous studies had shown that Indomethacin possesses potential anti-viral effects against viruses such as Epstein-Barr virus [21], HIV

[16], SARS-CoV [8], HSV-1 [51], and vesicular stomatitis virus [9]. Apart from its anti-inflammatory properties, Indomethacin had been shown *in-vitro* and *in-vivo* to decrease viral replication in SARS-CoV [8] and SARS-CoV-2 [94]. Notably, in this study, we have observed Indomethacin influences 85 genes (Supplementary file 4) associated with the COVID signature, indicating a significant role it may play in host responses. We also identified many host and viral proteins that Indomethacin can bind. Our analyses of gene expression data indicate that Indomethacin may be critical in reversing the effects of SARS-CoV-2. Our *in-vitro* data on antiviral studies suggested that dose-dependent administration of Indomethacin has an anti-SARS-CoV-2 impact, as determined by RdRp-2 gene copy numbers. Various possible mechanisms explaining how Indomethacin can be effective against COVID-19 had been hypothesized [83]. One such hypothesis involves the biosynthesis pathway of prostaglandin. Indomethacin had been known to interact with PGES2 (human prostaglandin E synthase type 2) [5], which had been shown to interact with the NSP-7 protein of SARS-CoV-2 [33]. A study of various NSAIDs using a network pharmacology approach revealed a potential role of Indomethacin by inhibiting crucial hub proteins of the RAS signaling pathways, thus reducing SARS-CoV-2 induced excessive inflammation [66]. In our study, we observed that Indomethacin could bind to the NTF2 domain of Ras GTPase-activating protein-binding protein 1 (Table 3, Fig. 6), which calls for further investigation in this direction.

The combination of Indomethacin with other lead molecules showed to enhance the antiviral efficacy significantly in another *in-vitro* study [38]. Indomethacin showed a prominent binding affinity with the main protease of SARS-CoV-2 and had better binding than many other NSAIDs [3]. Derivatives of Indomethacin had been shown to inhibit the main protease of SARS-CoV-2 [31]. It had also been hypothesized whether the use of Indomethacin helps in the recovery from SARS-CoV-2 induced dry cough [6]. A few proteolysis targeting chimeras (PROTAC) derived from Indomethacin were shown to possess better antiviral efficacy than Indomethacin against some coronavirus strains [23]. Our findings collectively support the hypothesis that Indomethacin may be considered as a possible treatment for SARS-CoV-2. When there are no contraindications for its use, Indomethacin may be beneficial to the patient. Clinically, Indomethacin treatment had been shown to alleviate headache in SARS-CoV-2 patients in a recent study [43]. In another clinical study, it had been suggested to treat mild and moderate COVID-19 with Indomethacin [69]. Another *in-vitro*, animal and model-based simulation study showed Indomethacin can be used against SARS-CoV-2 [32].

Hydroxychloroquine, Remdesivir, and Lopinavir were initially tested against SARS-CoV-2 infection in Vero cells. Antiviral efficacy of Indomethacin (IC_{50} = 12 μM) was observed to be in the similar ranges of Remdesivir (IC_{50} = 11.41 μm), Hydroxychloroquine (IC_{50} = 7.28 μm), and Lopinavir (IC_{50} = 9.12 μm) [42]. It was also shown that Remdesivir was more effective in a human cell line (IC_{50} = 1.3 μm) and the selectivity index was 38.5, which is also in the similar range of Indomethacin in this present study (~40). In another study, PF-00835231 showed better antiviral efficacy than Remdesivir in two different cell lines after 24 and 48 h, and had been hypothesized to be a more effective drug against SARS-CoV-2 [24]. IC_{50} values of the drugs Remdesivir, Lopinavir, and Chloroquine varied in different cell lines [42]. Additional *in-vitro* studies in other relevant cell lines could be performed to further confirm the antiviral efficacy of Indomethacin. In this study, we showed how Indomethacin may bind to the critical receptor proteins. However, whether these bindings are stable or not could be further checked by molecular dynamics simulations. Our study shows Indomethacin and Vitamin-A can bind to crucial host and viral proteins for the SARS-CoV-2 interaction. However, which precise interaction these drugs are effectively targeting should be further investigated in future studies. Despite the limitations, our study shows that Indomethacin contains antiviral efficacy probably by either blocking the viral receptors/host proteins, and/or altering the gene expression of the infected host cells. New Indomethacin analogs were shown to exhibit better inhibition against cyclooxygenase enzymes to reduce prostaglandin synthesis, which in turn would reduce inflammation [1]. Similarly, antiviral efficacy of various Indomethacin analogs should be tested against SARS-CoV-2 infection.

5. Conclusion

Our initial research focused on finding out the viral and host proteins critical for SARS-CoV-2 infection by conducting a review of the literature. We attempted a computational drug repurposing study to discover which existing drugs can target these essential proteins. In addition, based on the existing gene expression data, we evaluated whether certain compounds may reverse the genetic alteration brought about by SARS-CoV-2 infection in the host cells. Combining these two research analyses revealed that Indomethacin and Vitamin-A are two major existing medications that possess the ability to counter and reverse the genetic alterations caused by SARS-CoV-2. As Indomethacin demonstrated more encouraging results in reversing the effects of SARS-CoV-2, we conducted additional *in-vitro* tests with moderate success. We describe briefly why Vitamin-A may be considered as a supplement in the event of SARS-CoV-2 infection. We also discuss recent findings on Indomethacin against SARS-CoV-2. More *in-vitro* studies of various Indomethacin analogs in combination with Vitamin-A may be performed in the future to find out better therapeutics against SARS-CoV-2.

Declaration of competing interest

The authors declare no competing financial interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.compbiomed.2022.105788.

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