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RESEARCH ARTICLE

Repurposable drugs for SARS-CoV-2 and influenza sepsis with scRNA-seq data targeting post-transcription modifications

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Abstract

Coronavirus disease 2019 (COVID-19) has impacted almost every part of human life worldwide, posing a massive threat to human health. The lack of time for new drug discovery and the urgent need for rapid disease control to reduce mortality have led to a search for quick and effective alternatives to novel therapeutics, for example drug repurposing. To identify potentially repurposable drugs, we employed a systematic approach to mine candidates from U.S. FDA-approved drugs and preclinical small-molecule compounds by integrating gene expression perturbation data for chemicals from the Library of Integrated Network-Based Cellular Signatures project with a publicly available single-cell RNA sequencing dataset from patients with mild and severe COVID-19 (GEO: GSE145926, public data available and accessed on 22 April 2020). We identified 281 FDA-approved drugs that have the potential to be effective against severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection, 16 of which are currently undergoing clinical trials to evaluate their efficacy against COVID-19. We experimentally tested and demonstrated the inhibitory effects of tyrphostin-AG-1478 and brefeldin-a, two chemical inhibitors of glycosylation (a post-translational modification) on the replication of the single-stranded ribonucleic acid (ssRNA) virus influenza A virus as well as on the transcription and translation of host cell cytokines and their regulators (IFNs and ISGs). In conclusion, we have identified and experimentally validated repurposable anti-SARS-CoV-2 and IAV drugs using a systems biology approach, which may have the potential for treating these viral infections and their complications (sepsis).

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Key words: COVID-19; drug repurposing; SARS-CoV-2; influenza; single-cell RNA sequencing; adverse drug reaction; Library of Integrated Network-Based Cellular Signatures; sepsis; post-transcription modifications

Introduction

Coronavirus disease 2019 (COVID-19) is a highly contagious respiratory disease resulting from a life-threatening novel coronavirus, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). It has spread rapidly across the globe, resulting in over 198 million confirmed cases and 4 million deaths as of 2 August 2021.[1,2] SARS-CoV-2 is an enveloped RNA virus that belongs to the genus *Betacoronavirus* of the family *Coronaviridae*, which includes well-known severe acute respiratory syndrome coronavirus (SARS-CoV) and Middle East respiratory syndrome coronavirus (MERS-CoV).[3] Advancement in the management of these coronaviruses and other viruses, such as influenza virus H1N1 and Ebola infections, has provided insight into treating COVID-19. However, a portion of patients develop serious disease and subsequent complications including sepsis and septic shock, largely as a result of aberrant host inflammatory responses.[4,5] Sepsis and its related conditions are associated with 20% of total deaths and 20 deaths every minute globally.[6] Of patients with COVID-19, 6.8%–100% are diagnosed with sepsis.[7–9] A retrospective study of hospitalized patients with COVID-19 showed that, of the non-survivors, 100% of patients had sepsis and 70% were noted to have septic shock.[10] Unfortunately, there is a lack of mechanistic understanding of septic progression linked to pathogen-mediated pathogenesis and host immune response, impeding the discovery of effective treatments.

More than 3600 active clinical trials for COVID-19 are under way.[11,12] According to the World Health Organization (WHO), there are more than 154 candidate vaccines in preclinical evaluation and 44 candidate vaccines in phase 1 trials or beyond (last updated: 25 October 2020), several of which have entered phase 3 large-scale randomized clinical trials and shown benefits.[13] Unexpected side effects and immune-escape mutations pose a great challenge for the safety and efficacy of vaccines.[14] Chloroquine[15,16] and its hydroxyl analog hydroxychloroquine,[17] lopinavir/ritonavir,[18–20] and remdesivir,[16,21] developed for treating malaria, human immunodeficiency virus (HIV), and Ebola virus, respectively, have been suggested as treatments for COVID-19 and are being tested in clinical trials. However, some clinical trials have shown that these drugs have little or no effect on the treatment of patients hospitalized with COVID-19.[22–26] Drug repurposing is a fast and cost-efficient approach to identifying new potential uses for existing drugs to treat different diseases.[27] Hence, there is an urgent need to search for repurposed drugs.

COVID-19 is not the first outbreak of zoonotic coronaviruses. The severe acute respiratory syndrome (SARS) outbreak, first identified in the Guangdong Province of southern China in 2002, lasted for eight months, resulting in 8098 confirmed human cases in 29 countries, with 774 deaths (case fatality rate of 9.6%).[28,29] Approximately 10 years later, in 2012, Saudi Arabia isolated another highly pathogenic coronavirus, MERS-CoV, from the sputum of a male patient who died of acute pneumonia and renal failure.[30] MERS-CoV caused an outbreak with 2260 cases and 803 deaths (case fatality rate of 35.5%).[31,32] Although COVID-19 has caused fewer fatalities than SARS and MERS, older patients with comorbidities tend to experience more severe symptoms, making them more vulnerable. Most patients infected with SARS-CoV-2 display mild symptoms and generally have a good prognosis, classified as mild COVID-19.[18,33] However, a large proportion of patients, especially older men with underlying chronic diseases, have rapidly progressed to severe COVID-19 and suffered from respiratory distress requiring emergent medical interventions.[34] Unfortunately, as yet, there are no specific and effective drugs for COVID-19.[11,12]

Recent studies have shown the critical roles of host immune responses in protection and the pathogenesis of respiratory viral infections, for instance, SARS-CoV, MERS-CoV, and influenza A viruses.[35,36] Liao *et al.*[37] reported that an increase in $CD8^+$ T cells in patients with COVID-19 correlates with an improved outcome. They also proposed therapeutic strategies by targeting the myeloid cell compartment to treat COVID-19-associated inflammation.

There is an urgent need to find potentially useful drugs for COVID-19 among currently available drugs. Drug repurposing is an essential and universal strategy in developing new drugs and a potentially important strategy for discovering existing medicines to tackle COVID-19.[38] It may facilitate the discovery of new mechanisms of action for existing drugs, which would be less time-consuming and more cost-effective than discovery of novel drugs, and the pharmaceutical supply chains for formulation and distribution already exist.[39,40] Gordon *et al.*[41] also reported 69 repurposable known compounds targeting 66 human proteins based on 332 high-confidence SARS-CoV-2-human protein-protein interactions, which were physically associated using affinity-purification mass spectrometry. Another study[42] presented data on the antiviral activity of 20 FDA-approved drugs against SARS-CoV-2 that have previously been shown to inhibit SARS-CoV and MERS-CoV. Another research team[43] conducted a high-throughput analysis of the ReFRAME library to identify 30 candidate existing drugs that prevent SARS-CoV-2 from replicating in mammalian cells. In a study[44] based on public data of patients with pulmonary fibrosis and the Library of Integrated Network-Based Cellular Signatures (LINCS),[45]

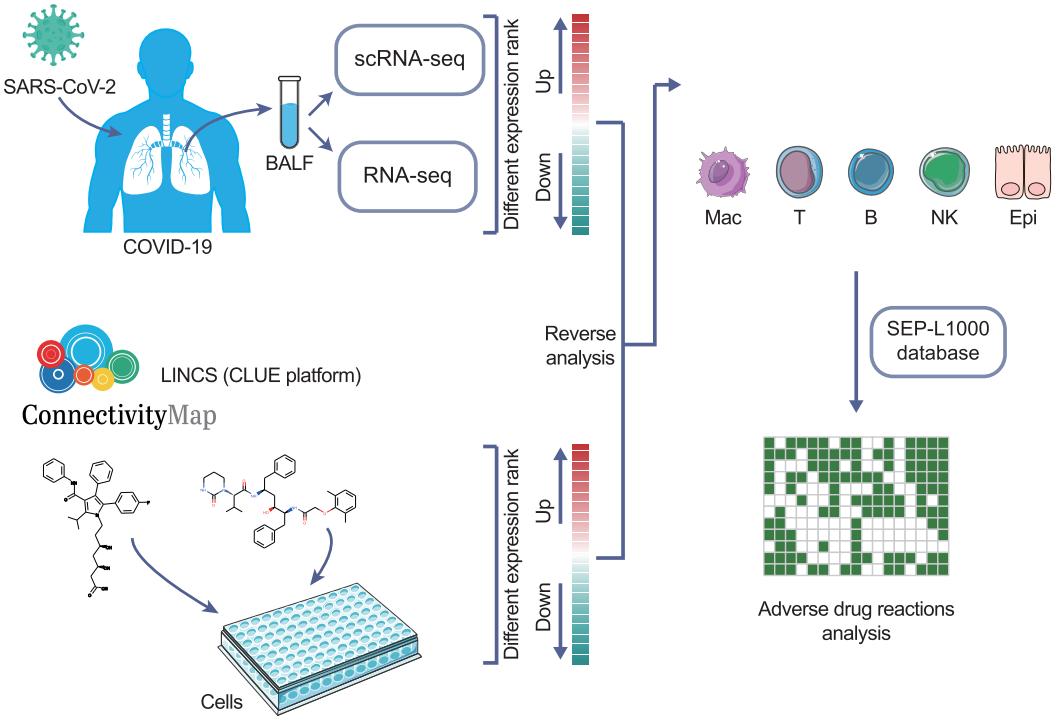

Figure 1. Workflow of drug repurposing for treating different durations of COVID-19. Publicly available scRNA-seq data and transcriptomic data of BALF from patients with COVID-19 were input against the LINCS database using the CLUE platform. Candidates were selected to have the potential to reverse the expression of upregulated differentially expressed genes (DEGs) upon drug treatment and were compared by connectivity score and the number of major cell subtypes across healthy, mild, and severe groups. This figure was created by modifying illustrations provided by Servier Medical Art (SMART, smart.servier.com) and Vecteezy.com.

several drugs targeting ACE2 were identified to be repurposable against COVID-19.

Considering that an RNA virus exhibits a considerable degree of sequence variation, drugs targeting host factors may cause less mutational resistance with more significant and broader antiviral spectrum potential.[46] Hence, there is an urgent need to identify potential therapeutics with new strategies for emerging infectious diseases. Repurposing clinically assessed drugs represents one of the most practical strategies for rapidly identifying treatments to combat COVID-19.

In this study, we analyzed a publicly available single-cell RNA sequencing (scRNA-seq) dataset of bronchoalveolar lavage fluid (BALF) collected from patients with mild and severe COVID-19 as well as separate bulk RNA-seq data of BALF from patients with COVID-19 (Fig. 1). Data mining was performed using the drug perturbation database LINCS to identify potential therapies for COVID-19. A total of 281 candidates with different courses of COVID-19 independent of cell subtypes were identified. Additionally, we identified and validated two candidates without adverse drug reactions (ADRs), tyrphostin-AG-1478 and brefeldin-a, exhibiting antiviral activity against the single-stranded ribonucleic acid (ssRNA) virus influenza A virus (IAV), as we do not have the experimental resources to perform live SARS-CoV-2 infection on campus (lacking BSL3 facility). These drug candidates may function at inhibiting viral replication and spread through a number of mechanisms, including targeting nucleic acid metabolisms and protein synthesis of pathogens as well as the transcription, translation, and post-translational modifications (PTM) of the host cytokines.[47,48] Our findings may aid in the rapid preclinical and clinical evaluation of these therapeutics and provide an important drug discovery pipeline to accelerate and facilitate the development of potential treatments for COVID-19 and other viral infections as well as the associated sepsis.

Materials and methods

ScRNA-seq data analysis and sample aggregation

The gene-barcode matrix files of all six COVID-19 donors including three mild cases and three severe cases (lung BALF), and of three healthy controls (lung tissues) were downloaded from the NCBI Gene Expression Omnibus database (accession ID: GSE145926).[37] The expression matrices were loaded into the R statistical analysis platform using Seurat v3,[49] keeping only the cells with gene numbers between 200 and 6 000, unique molecular identifier (UMI) count above 1 000, and mitochondrial gene percentage below 0.1. A total of 43 914 cells from nine samples were used for our further analyses. In addition to these scRNA-seq data, we also collected a list of differentially expressed genes (DEGs) in a separate cohort of SARS-CoV-2-infected lung BALF using a bulk RNA-seq

analysis to compare against the single cell-based data. This DEG list was obtained from the Chinese National Genomics Data Center (https://bigd.big.ac.cn/; accession ID: CRA002390).[50]

Dimensionality reduction and clustering

The LogNormalize method in Seurat was used for normalizing the filtered gene-barcode matrix. Principal component analysis (PCA) was performed using the top 2 000 most variable genes. Uniform Manifold Approximation and Projection (UMAP) was performed on the top 50 principal components for visualizing the cells. Graph-based clustering was performed on the PCA-reduced data with Seurat.

Differential analysis for clusters among the three groups

MAST, a generalized linear model framework treating the cellular detection rate as a covariate, was used to perform differential analysis. DEGs were identified by comparing each cluster between all three groups. Genes with average $|\log2FC| > 0.25$ and adjusted P value < 0.05 were deemed DEGs.

Drug repurposing using the LINCS drug-perturbation data

DEGs were first sorted by the log2FC values. The upregulated and downregulated genes were then chosen to identify drugs and compounds against the LINCS database using the Connectivity Map Linked User Environment (CLUE: https://clue.io) platform.[45] The drug connectivity score (CS) with a negative value smaller than -90 was used to determine candidate drugs and compounds. The COVID-19 database from the International Clinical Trials Registry Platform (ICTRP) (https://www.who.int/ictrp/en/, updated on 25 October 2020) was searched for clinical trial information associated with these drugs.

Adverse drug reaction analysis

Both on-label and off-label adverse drug reactions (ADRs) of all candidate drugs with CS values smaller than -90 were collected from the Side Effect Prediction based on L1000 (SEP-L1000) database (https://maayanlab.net/SEP-L1000/). The SEP-L1000 data include on-label ADRs of FDA-approved drugs collected from SIDER[51] and off-label ADRs from the PharmGKB database,[52] based on the post-marketing ADR reports in the FDA Adverse Event Report System (FAERS).

Cells and virus

Calu-3 and Vero E6 cells were maintained in Eagle's minimum essential medium (EMEM, Quality Biological Inc) supplemented with 10% heated-inactivated fetal bovine serum (FBS, Gibco) and penicillin and streptomycin (100 U/ml; Gibco) at 37°C with 5% CO_2. Influenza A virus (IAV, Puerto Rico/8/1934(H_1N_1)) viral stocks were titered by $TCID_{50}$, as described previously.[53] These experiments were performed under biosafety level 2 (BSL-2) conditions.

Antiviral assay

The drug-induced cytotoxicity of the test drugs (tyrphostin-AG-1478 and brefeldin-a, MedChemExpress, HY-13524 and HY-16592) on Calu-3 and Vero E6 cells was determined by a cell counting kit 8 (CCK-8, APExBIO). Cells were cultured overnight in 96-well cell-culture Petri dishes at a density of 1×10^4 cells/well. A 10 mM stock of drug was serially diluted in 100% DMSO to obtain a 10-point dilution series. Plates were cultured with fresh drug-containing medium at 37°C for 24 h. The DMSO concentration remained constant under all conditions at 0.05%. The absorbance of each well was determined using a 96-well multiscanner. After subtracting background absorption, the results were expressed as a percentage of viability relative to that of control cultures that received no drug. Drug concentrations that inhibited cell viability by 50% (IC_{50}) were determined using GraphPad Prism 8 software.

To evaluate the antiviral efficacy of these drugs, Calu-3 and Vero E6 cells were plated in 48-well cell culture Petri dishes at a density of 5×10^4 cells/well. Cells were inoculated with IAV (MOI of 1) for 1 h and rocked manually every 10 min to redistribute the inoculum. At 24 h post-infection (hpi), the cells were further treated with the same 10-point dilutions of drugs as described above. Supernatants were collected and processed by heat inactivation (10 min at 95°C) or stored at -80°C until use. Then, viral RNA levels in the supernatant of infected cells were quantified by quantitative reverse transcription PCR (RT-qPCR), representing the quantity of IAV replicated and secreted from cells.[54] A standard curve of 1:5 dilutions of IAV PCR target fragments from 1.5×10^{10} to 7.5×10^5 copies/ml was used to quantify the viral RNA with specific primers (fwd, CAAGCA GCAGAGGCCATGGA; rev, GACCAGCACTGGAGCTAGGA). Additionally, the infected cells were harvested with TRIzol, and total cellular RNA was extracted, reverse transcribed into cDNA and subjected to qPCR as previously described.[55] mRNA levels of MX1, ISG15, IFNB1, ACE2 and the housekeeping gene GAPDH were measured with specific primers (*MX1*: fwd, GTTTCCGAAGTGGACATC GCA, rev, CTGCACAGGTTGTTCTCAGC; *ISG15*: fwd, CG CAGATCACCCAGAAGATCG, rev, TTCGTCGCATTTGTCC ACCA; *IFNB1*: fwd, GCTTGGATTCCTACAAAGAAGCA, rev, ATAGATGGTCAATGCGGCGTC; *ACE2*: fwd, ACAGTCCACA CTTGCCCAAAT, rev, TGAGAGCACTGAAGACCCATT; and *GAPDH*: fwd, GGAGCGAGATCCCTCCAAAAT, rev, GGCT GTTGTCATACTTCTCATGG). Fold changes were calculated using the $\Delta\Delta CT$ method compared with untreated noninfected cells. Three technical replicates were used for each sample.

Results

Study design and analysis of single-cell data

Our study highlighted the identification of different therapeutic effects during various disease courses using publicly available single-cell RNA sequencing data. With the high variability of the cellular compartments underlying disease progression, our drug repurposing profiles from major cell subtypes included T, B, NK, epithelial cells, and macrophages. A total of nine scRNA-seq BALF samples, including three healthy cases, three mild cases, and three severe cases, were collected from publicly available scRNA-seq data (Supplementary Table 1). After quality filtering, approximately 250 000 gene expression values from 43 914 cells were obtained. The clustering analysis identified six major clusters of macrophages, NK cells, $CD4^+$ T cells, $CD8^+$ T cells, B cells, and epithelial cells (Supplementary Fig. 1), which were determined based on the unique signature genes CD68 (macrophage cell), IL7R, CD4 ($CD4^+$ T cell), CD8A ($CD8^+$ T cell), MS4A1 (B cells), and TPPP3 (epithelial cells), respectively (Supplementary Fig. 2). We then compared these six major clusters across the healthy, mild, and severe COVID-19 cases and identified differentially expressed genes (DEGs) between any of the two courses, as summarized in Supplementary Table 2A. Briefly, the mild-vs-healthy comparison (Supplementary Table 2B) had 439 ($CD8^+$ T cell) ~ 1 639 (B cell) DEGs, while the severe-vs-healthy comparison (Supplementary Table 2C) had 255 ($CD8^+$ T cell) ~ 1 127 (epithelial cell) and the severe-vs-mild comparison (Supplementary Table 2D) had 467 ($CD8^+$ T cell) ~ 1 132 (macrophage cell).

Overview of drug repurposing via the LINCS database

Connecting to the LINCS database of small-molecule perturbations of gene expression, we identified candidate drugs and compounds that can reverse the upregulation and downregulation of these genes via the Connectivity Map Linked User Environment (CLUE) platform. The closer the connectivity score (CS) is to -100, a score indicating a complete reversal, the higher the chance of identification of drug-adverse effect associations with upregulated or downregulated DEGs. In other words, drugs may show a better response to reverse expression of DEGs upregulated or downregulated in major cell subtypes in the BALF. A total of 281 candidates were selected by CLUE with a CS lower than -90 based on DEGs among all three comparisons between two courses (Supplementary Table 3). These candidates include potential anti-coronavirus agents, focusing on FDA-approved drugs and experimental agents that have already been tested in clinical trials. To prioritize known drugs for preclinical and clinical evaluation of the therapeutic effect of SARS-CoV-2, we selected candidates shared by at least three cell types and in ongoing COVID-19 clinical trials, as listed in Tables 1–3.

Repurposing analysis in patients with mild COVID-19

To search for therapeutic candidates for mild cases, we ranked drugs and compounds according to their CSs (Supplementary Table 4A). A total of 133 candidate drugs were identified as potential candidates in at least one cell type among the mild-vs-healthy group, and 53 of these were included in more than one cell subtype (Fig. 2A, Supplementary Table 3B). The 10 drugs identified in three or more cell types included tubulin inhibitors (flubendazole, mebendazole, nocodazole, and vincristine), a DNA methyltransferase inhibitor (azacytidine), BCL inhibitor (ABT-737), M5 modulator (VU-0365114–2), calcium channel blocker (calmidazolium), apoptosis stimulant (kinetin-riboside), and opioid receptor antagonist (JTC-801). Six additional drugs are currently undergoing COVID-19 clinical trials (Table 1), including the HIV protease inhibitor lopinavir/ritonavir[56] combination (phase 4), glucocorticoid receptor agonist dexamethasone (phase 3/4),[57] DNA replication inhibitor niclosamide (phase 2/3), antineoplastic agent lenalidomide (phase 4), and calcineurin inhibitor tacrolimus (phase 3).[58]

Repurposing analysis in patients with severe COVID-19

A total of 60 drugs were also found to have the potential to be effective in severe cases compared with controls (severe vs. healthy group), according to their average CS between the replicates, and 25 of these were involved in more than one cell subtype (Fig. 2B, Supplementary Table 3C, 4B). As listed in Table 2, nine drugs presented in at least three separate cell types, including ABT-737 (BCL inhibitor), brefeldin-a (protein synthesis inhibitor), indirubin (CDK inhibitor), TPCA-1 (IKK inhibitor), lopinavir (HIV protease inhibitor), GW-441756 (growth factor receptor inhibitor), treprostinil (prostacyclin analog), tyrphostin-AG-1478 (EGFR inhibitor), and epoxycholesterol (LXR agonist). In this group, lopinavir/ritonavir and hydrocortisone are ongoing in COVID-19 clinical trials.

Repurposing analysis in patients with severe COVID-19 compared with patients with mild COVID-19

A total of 111 candidate drugs were identified in severe cases compared with mild cases (severe-vs-mild group), 39 of which were involved in more than one cell subtype (Fig. 2C, Supplementary Table 3D, 4C). As listed in Table 3, nine drugs (those for which drugs were selected in three separate cell types or more), including fostamatinib (SYK inhibitor), VER-155008 (HSP inhibitor), KU-0063794 (MTOR inhibitor), PIK-90 (PI3K inhibitor), linsitinib (IGF-1 inhibitor), TAK-715 (p38 MAPK inhibitor), Y-27632 (Rho-associated kinase inhibitor), AZ-628 (RAF inhibitor), and lestaurtinib (FLT3 inhibitor), were identified. In this group, except lopinavir, we also assessed the eight listed drugs in clinical trials for the treatment of COVID-19 in Table 3, including the insulin sensitizer metformin (phase 3), HMGCR inhibitor atorvastatin (phase 2/3), phosphodiesterase inhibitor sildenafil (phase

Table 1. List of potential drugs for treating COVID-19 based on LINCS database and DEGs between mild and healthy samples in B, $CD4^+$ T, $CD8^+$ T, epithelial, NK cells, and macrophages.

Drug	LINCS ID	B cells	$CD4^+$ T cells	$CD8^+$ T cells	Epithelial cells	Macrophages	NK cells	SharedSets	Description	Phase*
Flubendazole	BRD-K86003836	+	+	+	+	−	+	5	Tubulin inhibitor	
Azacitidine	BRD-K03406345	−	+	+	+	+	−	4	DNA methyltransferase inhibitor	
ABT-737	BRD-K56301217	+	+	−	−	−	+	3	BCL inhibitor	
VU-0365114–2	BRD-K37456065	−	+	+	+	−	−	3	M5 modulator	
Calmidazolium	BRD-A98283014	−	+	−	+	+	−	3	Calcium channel blocker	
Mebendazole	BRD-K77987382	−	+	−	+	+	−	3	Tubulin inhibitor	
Kinetin-riboside	BRD-K94325918	−	+	−	+	+	−	3	Apoptosis stimulant	
Nocodazole	BRD-K12539581	−	+	−	+	+	−	3	Tubulin inhibitor	
JTC-801	BRD-K17705806	−	+	−	+	+	−	3	Opioid receptor antagonist	
Vincristine	BRD-K82109576	−	−	+	+	−	+	3	Tubulin inhibitor	
Lopinavir	BRD-K99451608	+	−	−	−	−	+	2	HIV protease inhibitor	Phase 4
Ritonavir	BRD-K51485625	+	−	−	−	−	+	2	HIV protease inhibitor	Phase 4
Dexamethasone	BRD-A35108200	−	+	−	−	−	−	1	Glucocorticoid receptor agonist	Phase 3/4
Niclosamide	BRD-K35960502	−	−	−	−	+	−	1	DNA replication inhibitor	Phase 2/3
Lenalidomide	BRD-K05926469	−	−	−	−	−	+	1	Antineoplastic	Phase 4
Tacrolimus	BRD-K69608737	−	−	+	−	−	−	1	Calcineurin inhibitor	Phase 3

Asterisk (*) represents a clinical trial for its efficacy in COVID-19 disease. (+) indicates drugs meeting the SC < -90 criteria, while (-) indicates drugs not meeting the criterion.

Table 2. List of potential drugs for treating COVID-19 based on LINCS database and DEGs between severe and healthy samples in B, $CD4^+$ T, $CD8^+$ T, epithelial, NK cells, and macrophages.

Drug	Description	B cells	$CD4^+$ T cells	$CD8^+$ T cells	Epithelial cells	Macrophages	NK cells	SharedSets	Description	Phase*
ABT-737	BRD-K56301217	+	+	+	–	+	+	5	BCL inhibitor	
Brefeldin-a	BRD-A17065207	+	+	+	–	+	+	5	Protein synthesis inhibitor	
Indirubin	BRD-K53959060	+	+	+	–	+	+	5	CDK inhibitor	
TPCA-1	BRD-K51575138	+	+	+	–	–	+	4	IKK inhibitor	
Lopinavir	BRD-K99451608	+	–	–	–	+	+	3	HIV protease inhibitor	Phase 4
GW-441756	BRD-K04146668	+	–	–	–	+	+	3	Growth factor receptor inhibitor	
Treprostinil	BRD-A67438293	+	–	+	–	–	+	3	Prostacyclin analog	
Tyrphostin-AG-1478	BRD-K68336408	–	+	+	–	+	–	3	EGFR inhibitor	
Epoxycholesterol	BRD-K61480498	–	+	+	+	–	–	3	LXR agonist	
Ritonavir	BRD-K51485625	–	–	–	–	+	+	2	HIV protease inhibitor	Phase 4
Hydrocortisone	BRD-A07000685	–	–	+	–	–	–	1	Glucocorticoid receptor agonist	Phase 3

Asterisk (*) represents a clinical trial for its efficacy in COVID-19 disease. (+) indicates drugs meeting the SC < -90 criteria, while (-) indicates drugs not meeting the criterion.

3), calcium channel blocker verapamil (phase 2/3), phosphodiesterase inhibitor sildenafil (phase 1/2/3), HMGCR inhibitor rosuvastatin (phase 3), CC chemokine receptor antagonist maraviroc (phase 1/2), and dipeptidyl peptidase inhibitor sitagliptin (phase 2/3).

Shared candidates based on all three comparisons

As shown in Fig. 3A,B and Supplementary Table 3A, there are 25 drug candidates, including "lopinavir" and "ritonavir" mentioned in the first sentence, and 23 additional drugs identified in the two comparisons, including SB-216763, ABT-737, JTE-907, brefeldin-a, PKCbeta-inhibitor, indirubin, GW-441756, flubendazole, tyrphostin-AG-1478, memantine, calyculin, kinetin-riboside, ascorbyl-palmitate, ON-01910, mirin, verrucarin A, emetine, EMF-bca1-57, TPCA-1, RHO-kinase-inhibitor-III[rockout], PD-158780, tipifarnib, and NVP-AUY922. For example, the glycogen synthase kinase (GSK) inhibitor SB-216763 acts as a neuroprotectant[59] and prevents cardiac ischemia.[60] JTE-907 is a cannabinoid receptor inverse agonist that produces anti-inflammatory effects.[61]

To further demonstrate the usefulness of the drug repurposing strategy, we identified potential therapeutic drugs based on the transcriptional changes in BALF of patients with COVID-19 obtained using bulk RNA-seq data.[50] Ten candidate drugs were identified using the same analysis pipeline, two of which, including the GSK inhibitor SB-216763 and PPAR receptor antagonist GW-6471, were also included in the single cell-based candidate lists (Supplementary Table 5).

Adverse drug reaction analysis

To prioritize the candidates for COVID-19 treatment, we characterized these candidates' known ADRs, which are a central consideration during drug development.[62] We conducted a computational approach using the SEP-L1000 database to predict relationships between drugs and the emergence of ADRs (Supplementary Tables 6 and 7). Figure 4 shows a heatmap of the top 50 drug-ADR associations for on-label (Fig. 4A) and off-label (Fig. 4B) ADRs. Interestingly, the majority of the candidate drugs were shown with few ADRs. Only lopinavir (associated with seven off-label ADRs), ritonavir (13 off-label ADRs), and memantine (unexpectedly, 44 on- and eight off-label ADRs) were included in two or more comparisons. In addition, for mild cases, mebendazole (six off-label), vincristine (31 on-label), dexamethasone (20 on-label and 37 off-label), and lenalidomide (18 off-label) showed drug-associated ADRs; for severe cases, there were two drugs, treprostinil (35 on- and six off-label) and valproic acid (44 on-label); and for comparison between mild and severe cases, metformin was associated with 31 on- and 25 off-label ADRs, atorvastatin 32 off-label, sildenafil 44 on-label, verapamil 32 on- and 27 off-label and dasatinib 45 on- and 22 off-label ADRs. These findings highlighted drug-ADR associations and may lead to informed clinical decisions regarding treatments for COVID-19.

Table 3. List of potential drugs for treating COVID-19 based on LINCS database and DEGs between mild and severe samples in B, $CD4^+$ T, $CD8^+$ T, epithelial, NK cells, and macrophages.

Drug	LINCS ID	B cells	$CD4^+$ T cells	$CD8^+$ T cells	Epithelial cells	Macrophages	NK cells	SharedSets	Description	Phase*
Fostamatinib	BRD-K20285085	+	+	+	+	+	+	6	SYK inhibitor	Phase 2
VER-155008	BRD-K32330832	+	+	+	+	+	+	6	HSP inhibitor	
KU-0063794	BRD-K67566344	+	−	−	−	+	+	3	MTOR inhibitor	
PIK-90	BRD-K99818283	+	+	−	−	−	+	3	PI3K inhibitor	
Linsitinib	BRD-K08589866	+	−	−	−	+	+	3	IGF-1 inhibitor	
TAK-715	BRD-K52751261	−	+	+	−	−	+	3	p38 MAPK inhibitor	
Y-27632	BRD-K44084986	−	+	+	−	−	+	3	Rho associated kinase inhibitor	
AZ-628	BRD-K05804044	−	−	+	+	−	+	3	RAF inhibitor	
Lestaurtinib	BRD-K23192422	−	−	−	+	+	+	3	FLT3 inhibitor	
Metformin	BRD-K79602928	−	−	+	−	−	−	1	Insulin sensitizer	Phase 3
Atorvastatin	BRD-U88459701	−	−	−	+	−	−	1	HMGCR inhibitor	Phase 2/3
Sildenafil	BRD-K50128260	−	−	−	+	−	−	1	Phosphodiesterase inhibitor	Phase 3
Verapamil	BRD-A09533288	−	−	−	+	−	−	1	Calcium channel blocker	Phase 2/3
Lopinavir	BRD-K99451608	−	−	−	+	−	−	1	HIV protease inhibitor	Phase 4
Sildenafil	BRD-K79759585	−	−	−	+	−	−	1	Phosphodiesterase inhibitor	Phase 1/2/3
Rosuvastatin	BRD-K82941592	−	−	−	+	−	−	1	HMGCR inhibitor	Phase 3
Maraviroc	BRD-A04352665	−	−	−	+	−	−	1	CC chemokine receptor antagonist	Phase 1/2
Sitagliptin	BRD-K19416115	−	−	−	−	+	−	1	Dipeptidyl peptidase inhibitor	Phase 2/3

Asterisk (*) represents a clinical trial for its efficacy in COVID-19 disease. (+) indicates drugs meeting the SC < -90 criteria, while (-) indicates drugs not meeting the criterion.

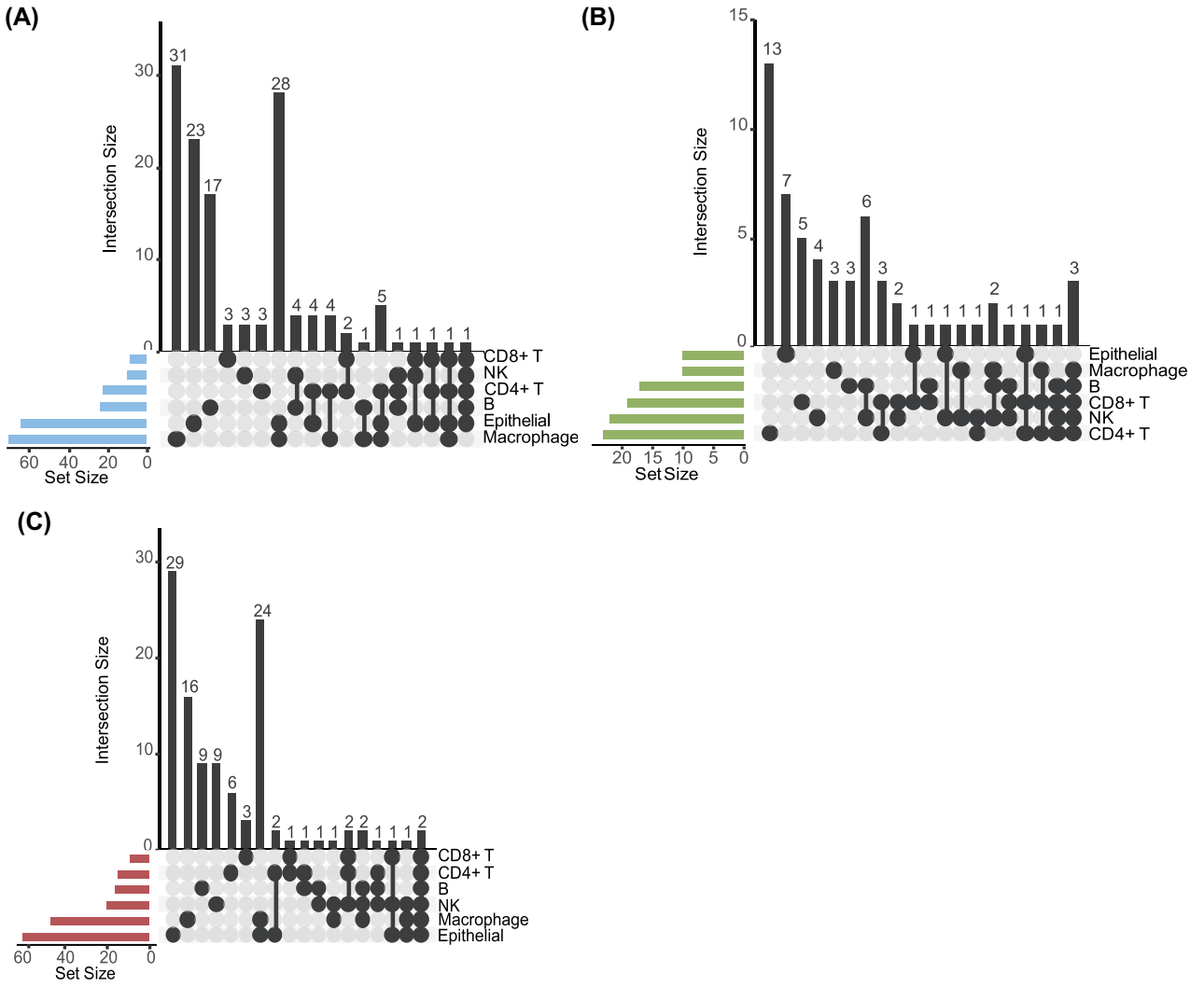

Figure 2. UpSet plots showing the overlap among the drug candidates for treating COVID-19 based on the LINCS database. DEGs between (A) mild and healthy, (B) severe and healthy, and (C) severe and mild samples in B cells, $CD4^+$ T cells, $CD8^+$ T cells, epithelial cells, NK cells, and macrophages.

Potent antiviral activity of candidates against ssRNA viruses in cell culture

Two candidates, tyrphostin-AG-1478 (AG-1478) and brefeldin-a (BFA), were selected as our top candidates and examined for potent activity against the ssRNA virus influenza A virus (IAV; strain Puerto Rico/8/1934 (H1N1)). IAV is a negative-sense ssRNA virus with eight genomic segments of different lengths (ranging from 0.89 to 2.3 kb). IAV was chosen because both SARS-CoV-2 and IAV are contagious ssRNA viruses that cause respiratory tract infections and their disease symptoms are quite similar.[63] Additionally, SARS-CoV-2 preferentially infects type II pneumocytes, which is the primary site of IAV replication.[64,65] AG-1478, an epidermal growth factor receptor (EGFR) inhibitor and cancer chemotherapy agent inhibits hepatitis C virus and encephalomyocarditis virus in cells.[66] BFA blocks the envelopment and egress of a mature viral particle by inhibiting protein transfer from the endoplasmic reticulum to the cis-Golgi.[67] BFA treatment inhibits the egress of both DNA viruses (herpes simplex virus)[67] and RNA viruses (Newcastle disease virus),[67] the entry of human papillomavirus and polyomavirus,[68] and the replication of mouse hepatitis coronavirus[69] and human immunodeficiency virus type 1.[70]

We tested whether these two drugs reduce viral RNA levels in Calu-3 and Vero E6 cells after infection with IAV by measuring IAV viral RNA levels in the cell culture supernatant by RT-qPCR. Upon treatment, both AG-1478 and BFA reduced IAV viral RNA levels dose-dependently at 24 h post-infection (Fig. 5A, Supplementary Fig. 3A–C). Neither compound caused significant cytotoxicity, but BFA slightly reduced viability in Vero E6 cells at high concentrations. Consistent with the reduction in viral replication, we observed that AG-1478 treatment elevated the mRNA levels of interferon-stimulated genes (ISGs) (ISG15 and MX1) and IFNβ in Calu-3 cells (Fig. 5B). The cellular receptor angiotensin-converting enzyme 2 (ACE2) is a key mediator of SARS-CoV-2 host cell entry.[71] We found

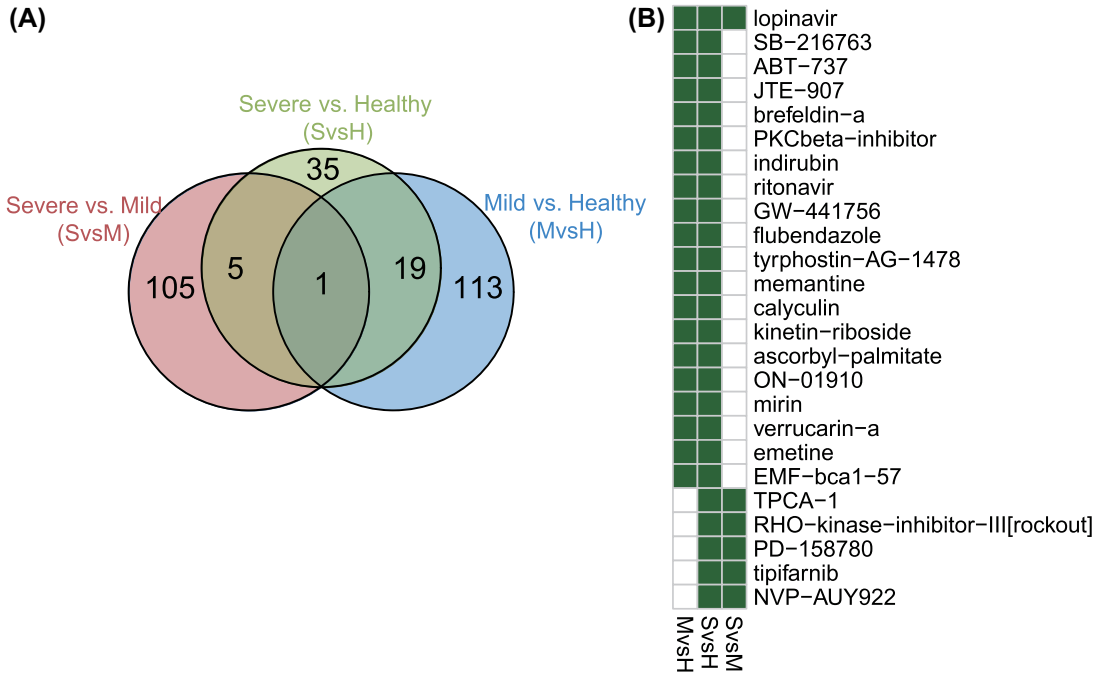

Figure 3. Common drug candidates. Venn diagram showing the overlap among the drug candidates for treating COVID-19 between three sets across the control, mild, and severe COVID-19 groups (A) and heatmap showing the 25 drugs shared by at least two sets (B). MvsH: mild-vs-healthy; SvsH: severe-vs-healthy; SvsM: severe-vs-mild.

that cellular mRNA levels of ACE2 increased in AG-1478-treated Calu-3 cells. In contrast, BFA treatment significantly reduced the expression of ACE2, MX1, ISG15, and IFNβ (Supplementary Fig. 3D). These data offer the possibility to further assess whether additional effects such as PTMs (glycosylation and phosphorylation) may be impacted by these inhibitors. Collectively, these results showed that AG-1478 and BFA exhibit a good antiviral effect on IAV, which provided novel possibilities for further preclinical development research.

Discussion

COVID-19 has spread rapidly worldwide, highlighting the urgent need to develop effective therapies. Various types of drugs, including antivirals, small-molecule drugs, biologics, and vaccines,[11,12] could potentially be used to control or prevent emerging coronavirus disease. However, because of the lack of effective therapeutic agents and long development cycles of vaccines, it is reasonable to consider repurposing existing drugs and compounds for COVID-19 as an alternative approach.

Both IAV and CoVs are common respiratory viruses. The flu season occurs annually, and its symptoms are similar to the respiratory symptoms caused by coronaviruses. In the United States, IAV has caused approximately 9.2–35.6 million illnesses with a mortality rate of 0.04%–0.83%,[72] while COVID-19 has caused more than 8 million positive cases and 220 000 deaths, with mortality rates of approximately 2.7%.[1] Although COVID-19 has higher morbidity and mortality than IAV infections, plasma cells were increased significantly in both patients with COVID-19 and IAV.[73] T cells and NK cells also were activated in both types of patients, which may contribute to the defense against the viruses.[73] SARS-CoV-2 needs to be manipulated in BSL-3 conditions for biosafety considerations, while certain IAV strains allow rapid assessment of antiviral activity *in vitro* in a BSL-2 laboratory.

In silico approaches to systematically predicting COVID-19 drug repurposing candidates are rapidly growing. In general, there are several computational strategies, including structure-based and deep-learning-based methods targeting virus, and signature-based and network-based approaches targeting host.[74] Our approach relied on a signature-based host-targeting method to identify potential drugs that can induce expression signatures that reversely correlate with the disease development and progression by altering the host immune response against viral pathogenesis.[45] Our approach is different from previous methods[41–44] for drug repurposing for coronavirus as it does not merely rapidly identify likely effective therapeutic agents in preventing or treating COVID-19, but tries to filter specific medications targeting immune reactions and specific modulators during the patients' disease courses. Furthermore, the scRNA-seq and transcriptome data were derived from human patients with COVID-19 at different disease states (mild vs severe), and two independent publicly available datasets were used for this study.

Several top-scoring drugs out of the 281 drugs we identified have already shown antiviral potential and are even undergoing clinical trials. The tubulin inhibitor flubendazole, widely used in treating intestinal parasites, is a potent inducer of autophagy initiation and can block

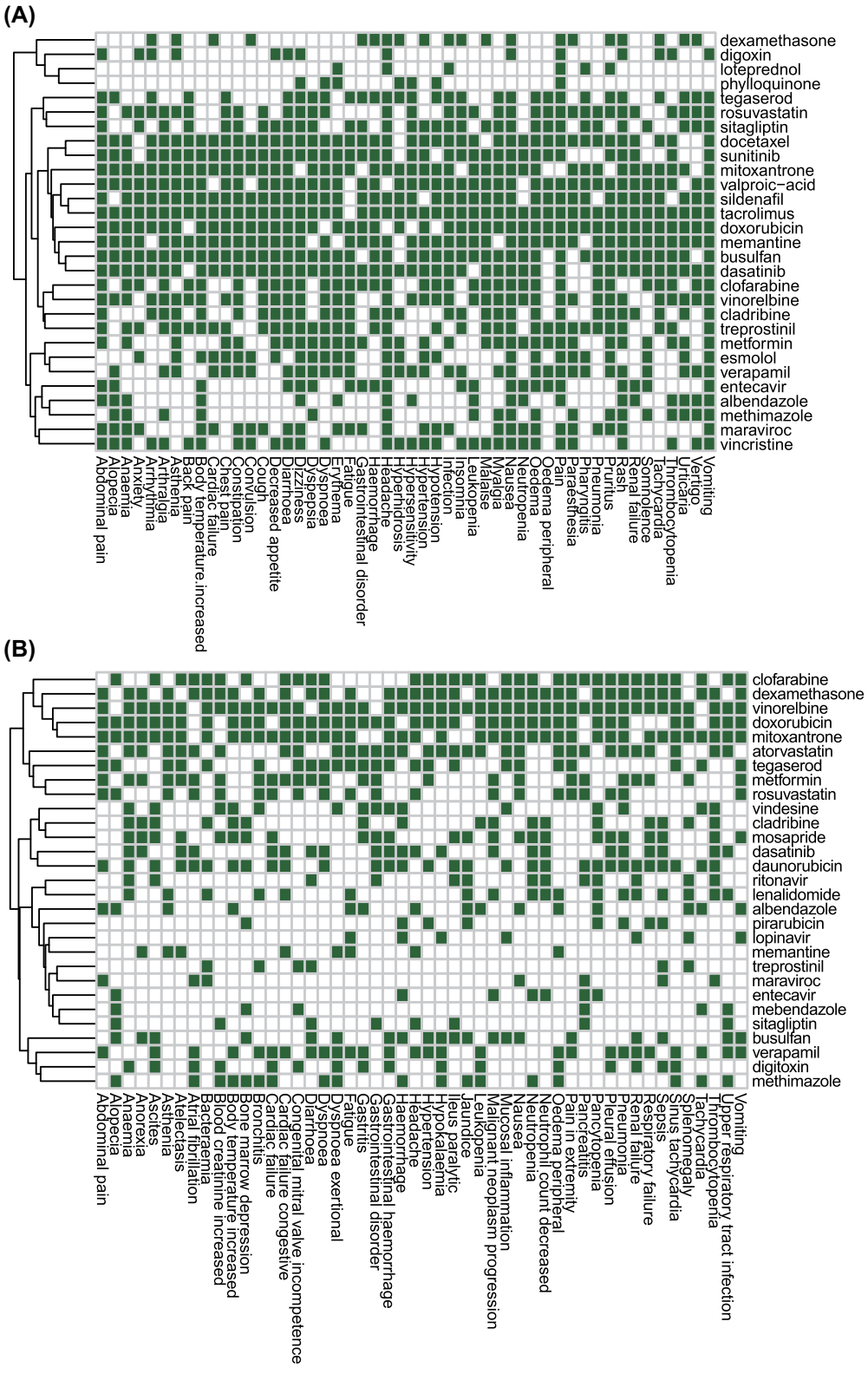

Figure 4. Heatmap of drug-ADR association. On-label (A) and off-label (B) ADRs are illustrated in heatmaps. White color indicates no association between drug and ADRs.

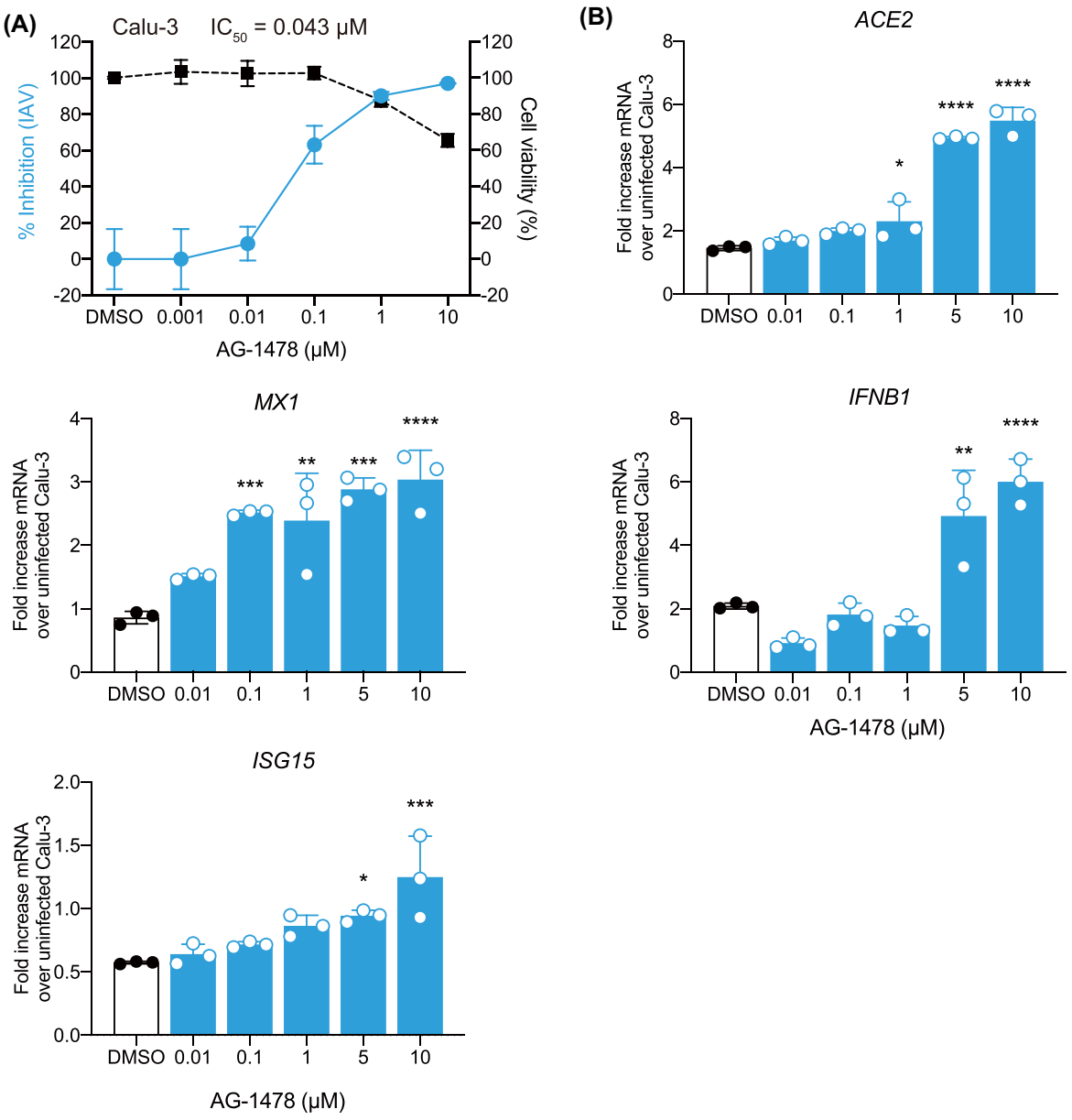

Figure 5. Tyrphostin-AG-1478 is effective against IAV in Calu-3 cell cultures. (A) Antiviral activity of tyrphostin-AG-1478 (AG-1478, 0 to 10 μM, IC_{50} = 0.043 μM) was assessed in Calu-3 cells infected with IAV PR8 (H1N1, MOI of 1) at 24 hpi. Inhibition of IAV, blue; cell viability, black. IC_{50} was calculated based on normalization to the control and fitted in GraphPad Prism. (B) Bar plots showing mRNA levels of cellular ACE2, MX1, ISG15, and IFNB1, calculated with ΔΔCT over noninfected Calu-3 cells. Calu-3 cells were treated with AG-1478 (0 to 10 μM) and infected with IAV (MOI of 1). Data are represented as the mean ± SD with three technical replicates each. Statistical significance was determined using one-way ANOVA. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

HIV transfer from dendritic cells to T cells.[75] Azacytidine partially reversed aberrant DNA methylation. Azacytidine combined with chemotherapy (fludarabine and cytarabine) in treating childhood leukemia is in a phase 1 clinical trial, and azacytidine in conjunction with APR-246 for myelodysplastic syndrome is in a phase 3 clinical trial.[76] The BCL inhibitor ABT-737 exhibits potential pro-apoptotic and antineoplastic activities.[77,78] The protein synthesis inhibitor brefeldin-a has been widely used to inhibit the entry of some viruses, such as human papillomavirus and polyomavirus,[79] and egress of others, such as herpesviruses and paramyxoviruses.[80] Indirubin, an active ingredient of the traditional Chinese medicine "Danggui Longhui Wan", has potent activity against myelocytic leukemia[81] and therapeutic potential as an antiviral agent against IAV.[82] The SYK inhibitor fostamatinib produced clinically meaningful responses for adult persistent and chronic immune thrombocytopenia in two parallel, phase 3 randomized trials.[83] Fostamatinib is now in a phase 2 COVID-19 trial as it reduces the protein abundance of mucin-1, a biomarker of acute lung injury and respiratory distress syndrome.[84] The HSP inhibitor VER-155008 could regulate Kaposi's sarcoma-associated herpesvirus lytic replication, highlighting its potential as

a novel antiviral agent.[85] The FLT3 inhibitor lestaurtinib has secured orphan drug approval from the FDA for acute myeloid leukemia[86] and is in a phase 2 trial for advanced multiple myeloma and phase 1 trials for prostate cancer.

We also explored the underlying risk factors associated with some side effects of the candidates. Understanding toxic on- and off-label ADRs linked to drugs affecting vital organs can improve patient safety and reduce financial costs.[87] Although the mechanisms of ADRs are complicated and not well understood, SEP-L1000 provides insights into the connection between general structural information and ADRs.[88] Notably, our candidate drugs did not show many ADRs.

The selection and validation of AG-1478 and BFA were performed because 1) both were shared in the drug repurposing analysis of patients with mild and severe COVID-19; 2) both were expected to be effective in multiple immune cell types (Fig. 3b); 3) they can inhibit viruses according to recent reports;[66–70] and 4) no adverse side effects were found in our analyses. These two candidates also showed robust antiviral activities against IAV *in vitro* in cell lines. Interestingly, AG-1478 or BFA treatment enhanced or inhibited the expression of IFNβ and ISGs, respectively. It is known that type I interferons (IFN-Is) and ISGs confer antiviral activities to host cells. However, inappropriate amounts of IFN-Is and ISGs at the wrong time and/or wrong places result in excessive inflammation, tissue damage and sepsis, especially in severe or critical COVID-19.[89] Although ACE2 mediates the cell entry of coronavirus, it also offers protection in acute lung injury against SARS-CoV-2.[90] Recombinant human ACE2 therapy to prevent S-protein interactions with endogenous ACE2 is currently in a phase 2 clinical trial in Europe.[91] Thus, these candidates have therapeutic potential for rational selection to target patients with mild or severe COVID-19. Overall, our data serve as a basis for future pharmacological studies in the treatment of SARS-CoV-2 and will guide the future development of therapies for the different severities of COVID-19 and other viral respiratory infections.

As PTMs regulate host-pathogen interaction, immune response, and virion packaging and budding, several studies have focused on SARS-CoV-2 Spike protein modifications.[92–94] Glycosylation is one of the most prominent PTMs, and Spike glycans are essential for SARS-CoV-2 virus binding, fusion, and entry into host cells.[95] These findings highlight the potential of chemical inhibitors of glycosylation for treatment of COVID-19. Interestingly, the candidate BFA may indirectly block glycosylation by interfering with protein transport between the endoplasmic reticulum and Golgi.[48] AG-1478 exhibits BFA-like activity by inducing reversible Golgi disruption and inhibiting the secretory transport in human cells.[47] Therefore, AG-1478 and BFA potentially provide a new way to treat COVID-19.

This study has several limitations. First, the public scRNA-seq data had a small number of clinical samples ($n = 9$) without detailed patient information available at the time of our analysis, making comparisons between studies difficult and making it impossible to dissect the patient's clinical features with the particular cell type-mediated immune responses. Given the relatively small number of individuals and all data based on the same race/ethnicity and single virus strain, the inclusion of more ethnically diverse populations and multiple strains of SARS-CoV-2 virus is recommended for future studies to rule out possible treatment effect heterogeneity. Second, IAV was chosen to test candidate drugs antiviral activity. Although both SARS-CoV-2 and IAV are enveloped ssRNA viruses, their genomes are different in genetic polarity and segmentation as well as surface proteins, which may result in different immune responses and gene expression patterns during viral infection.[64,65] We tested the transcriptional and translational levels of some critical cytokines and their regulators (e.g. IFN-Is and ISGs) after infection. It would be beneficial to further test their alterations and regulations at PTMs (glycosylation and phosphorylation) levels. Future work on large-scale international data mining and experimental validation in COVID-19 animal models and human subjects would help us better identify antiviral drugs to combat the disastrous pandemic.

Conclusions

The COVID-19 pandemic represents a great global public health crisis. Repurposing existing drugs is a fast and cost-efficient way to accelerate and optimize drug development. We investigated potentially repurposable candidates for treatment of COVID-19 progression. The findings can guide additional repurposing studies tailored to different stages of disease progression.

Supplementary data

Supplementary data are available at *PCMEDI* online.

Data availability

The scRNA-seq and bulk-RNA-seq data are available from the NCBI Gene Expression Omnibus database (https://www.ncbi.nlm.nih.gov/geo/; accession ID: GSE145926) and the Chinese National Genomics Data Center (https://bigd.big.ac.cn/; accession ID: CRA002390), respectively. All the codes used in this study are available at https://github.com/guokai8/COVID19/.

Author contributions

K.G., M.W., C.L., and J.H. designed the project and revised the manuscript. Z.W. and K.G. collected data, performed the analyses, prepared figures, and wrote the manuscript. P.G. performed analyses, prepared tables, and wrote the manuscript. Q.P. revised the manuscript.

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Conflict of interest

As an Editorial Board Member of *Precision Clinical Medicine*, the corresponding author Min Wu was blinded from reviewing and making decision on this manuscript.

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