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First inventor	Huang
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Assignee (if any)	--
Contact information	Zuyi Huang Villanova University 800 Lancaster Avenue Villanova, PA 19805

ABSTRACT

A method is provided for treating, curing, preventing, providing symptomatic relief, reducing the severity of, or reducing complications of, a viral infection. The method includes administering a pharmaceutical composition to a subject in need thereof. The pharmaceutical composition includes an effective amount of a 3CL protease inhibitor selected from 5-((1-([1,1'-biphenyl]-4-yl)-2,5-dimethyl-1H-pyrrol-3-yl)methylene)pyrimidine-2,4,6(1H,3H,5H)-trione (PMPT), N-(4-((3-(4-chlorophenylsulfonamido)quinoxalin-2-yl)amino)phenyl)acetamide (CPSQPA), a substituted derivative of each compound, a pharmaceutically-acceptable salt of each compound or derivative, and any combination thereof. The present disclosure also provides an antiviral composition comprising at least one active ingredient described above.

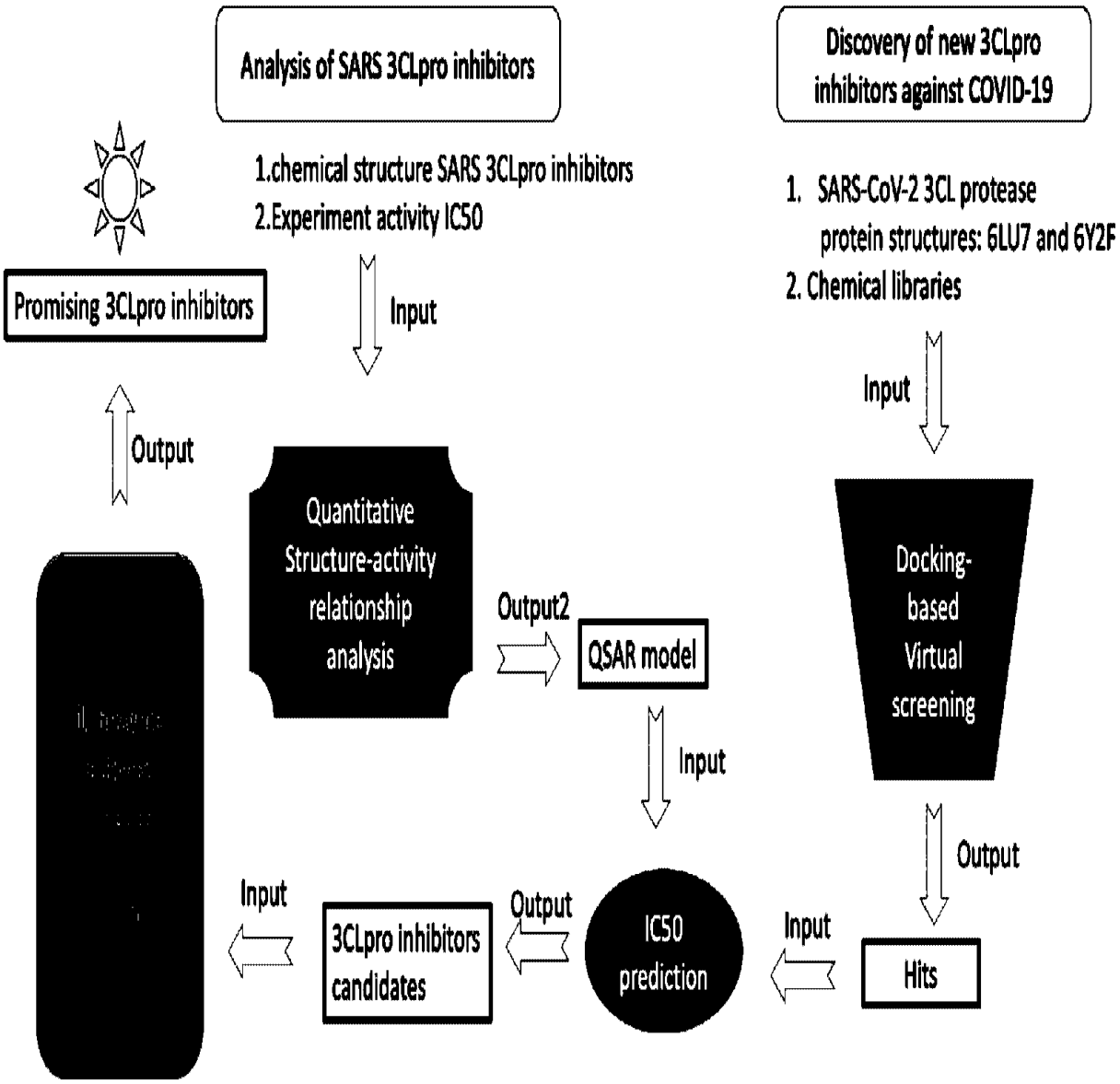

Analysis of SARS 3CLpro inhibitors
Discovery of new 3CLpro inhibitors against COVID-19
1.chemical structure SARS 3CLpro inhibitors
2.Experiment activity IC50
1. SARS-CoV-2 3CL protease protein structures: 6LU7 and 6Y2F
2. Chemical libraries
Promising 3CLpro inhibitors
Input
Input
Output
Quantitative Structure-activity relationship analysis
Docking-based Virtual screening
Output2
QSAR model
Input
Output
Input
3CLpro inhibitors candidates
Output
IC50 prediction
Input
Hits

FIG. 1

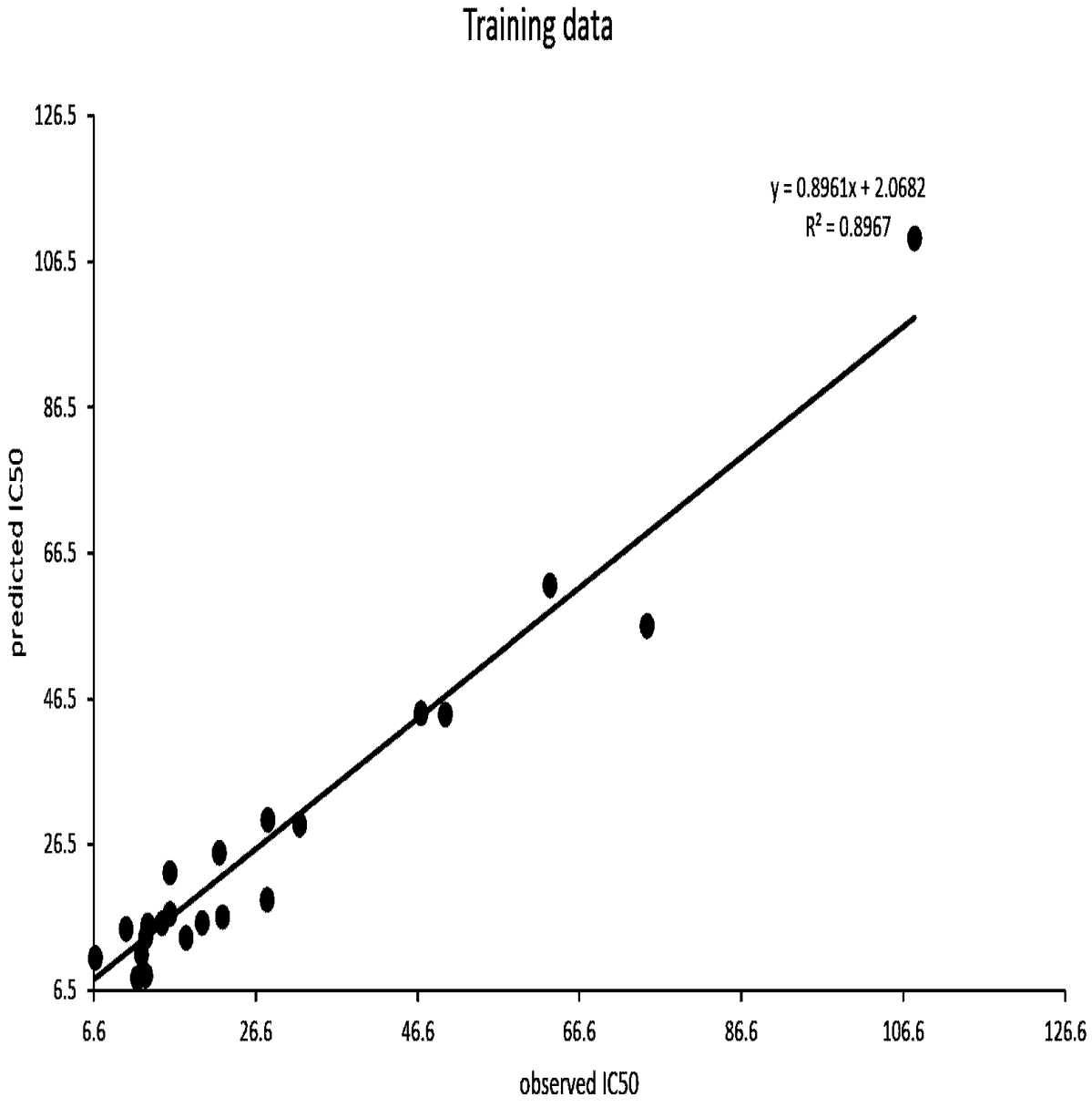

Training data
y = 0.8961x + 2.0682
R² = 0.8967
predicted IC50
observed IC50
126.5
106.5
86.5
66.5
46.5
26.5
6.5
6.6
26.6
46.6
66.6
86.6
106.6
126.6

FIG. 2(A)

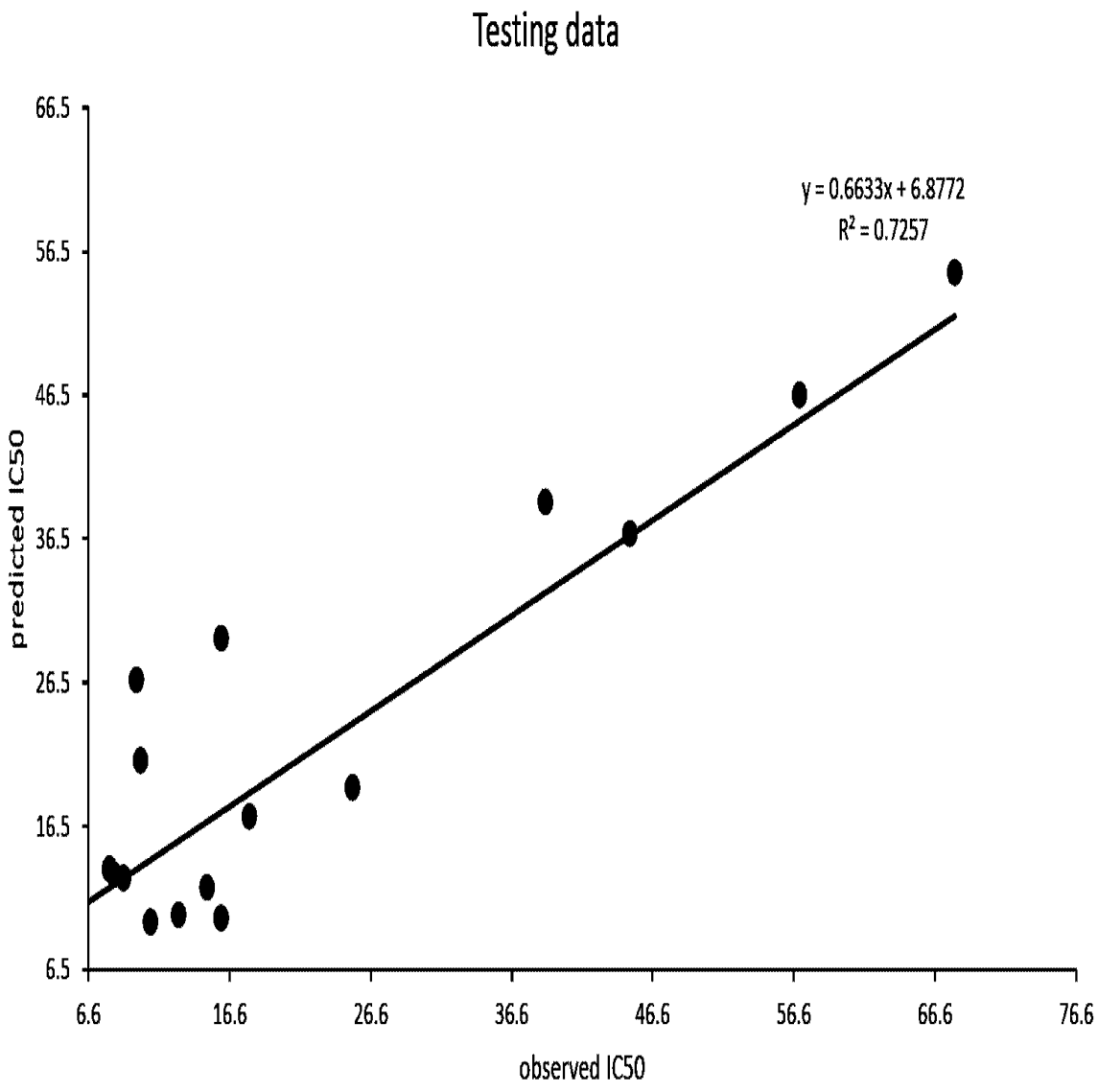

Testing data
y = 0.6633x + 6.8772
R² = 0.7257
predicted IC50
observed IC50
66.5
56.5
46.5
36.5
26.5
16.5
6.5
6.6
16.6
26.6
36.6
46.6
56.6
66.6
76.6

FIG. 2(B)

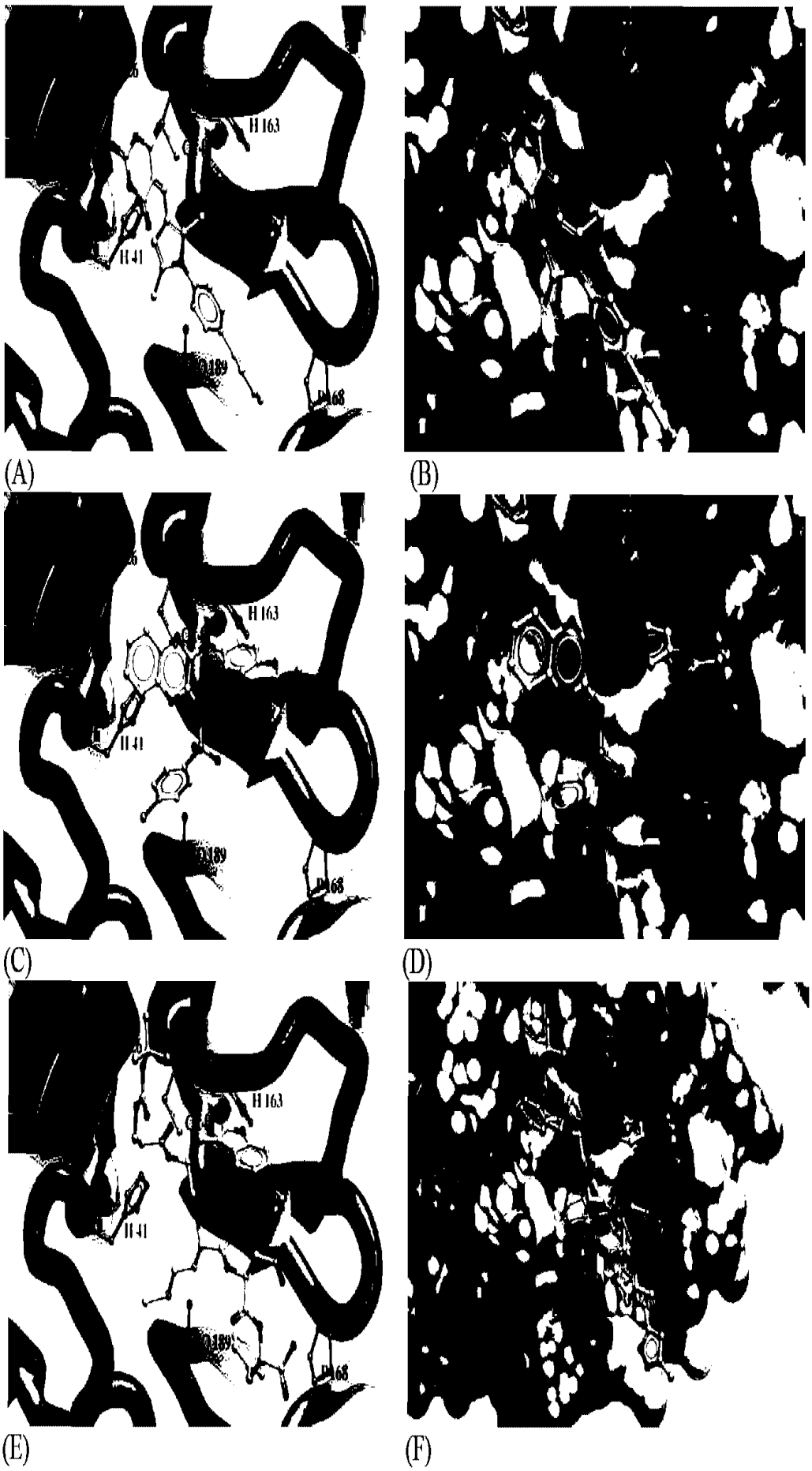


FIG. 3

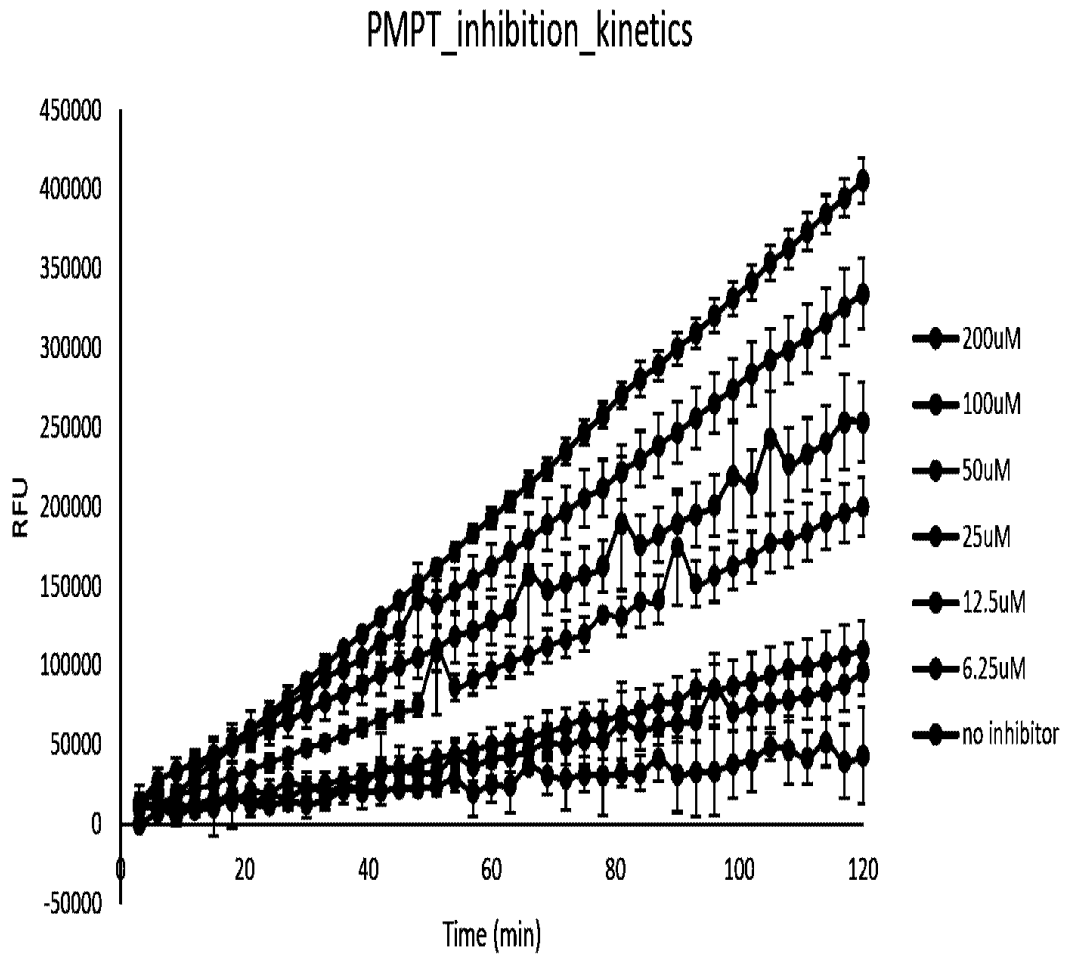

PMPT_inhibition_kinetics
450000
400000
350000
300000
250000
200000
150000
100000
50000
0
-50000
RFU
0
20
40
60
80
100
120
Time (min)
200uM
100uM
50uM
25uM
12.5uM
6.25uM
no inhibitor

FIG. 4A

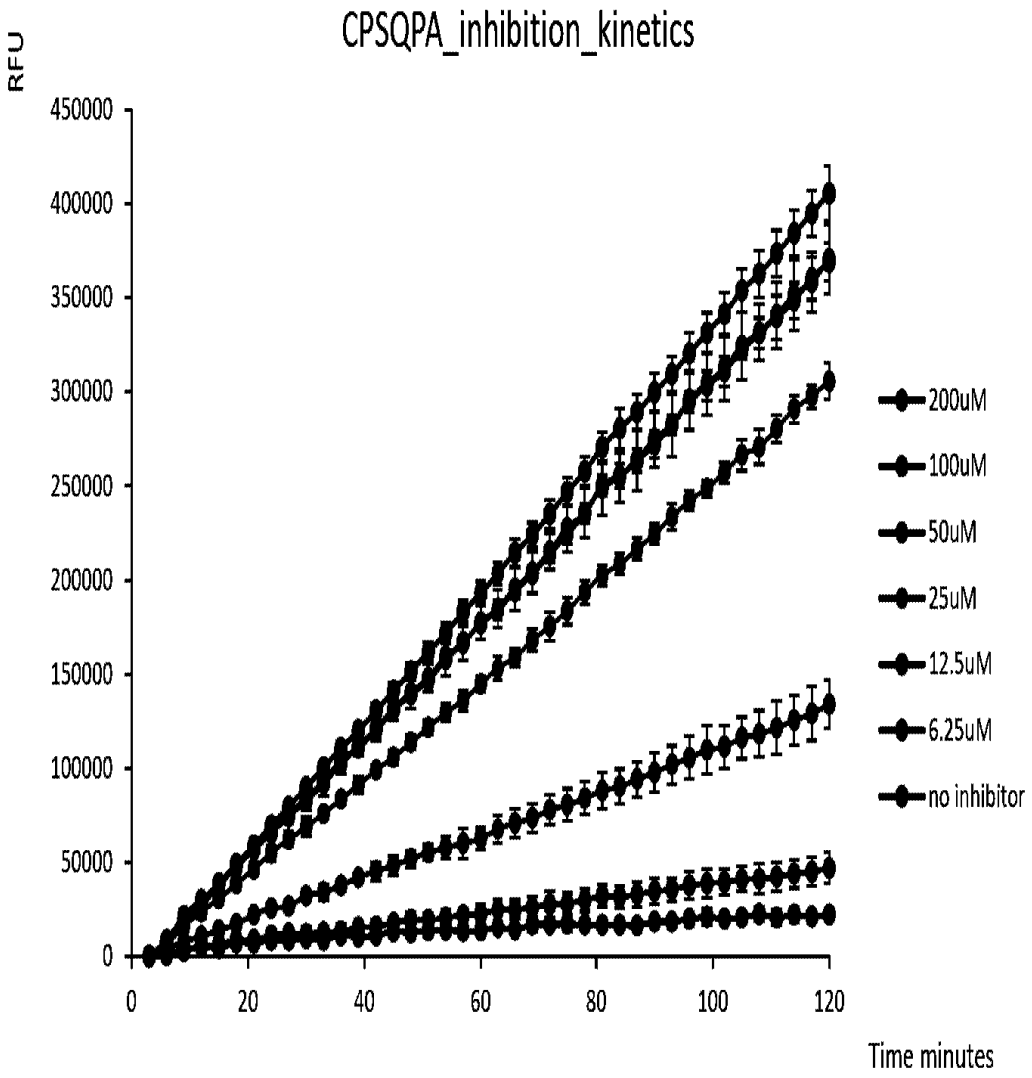

CPSQPA_inhibition_kinetics
RFU
450000
400000
350000
300000
250000
200000
150000
100000
50000
0
0
20
40
60
80
100
120
Time minutes
200uM
100uM
50uM
25uM
12.5uM
6.25uM
no inhibitor

FIG. 4B

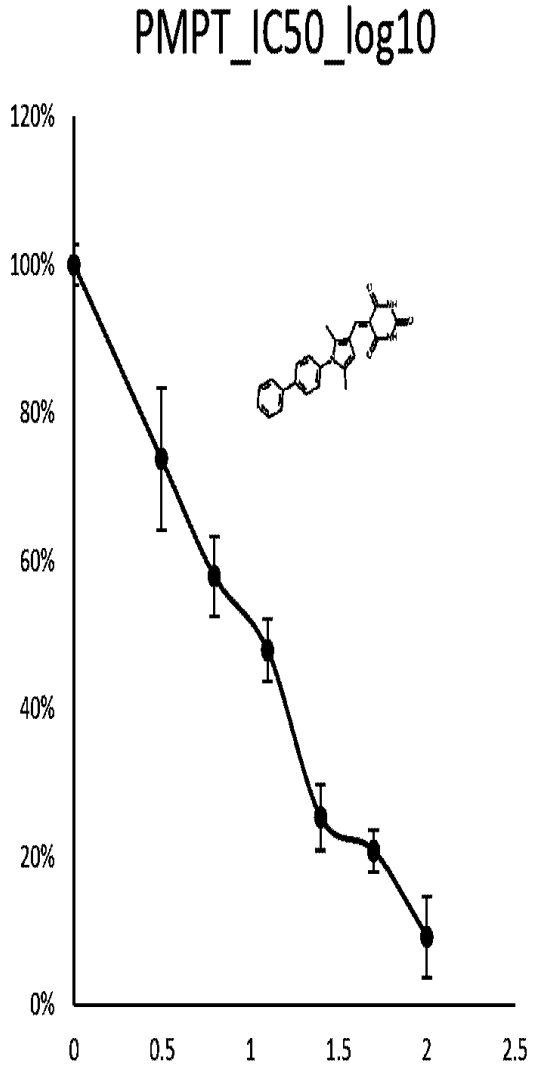

PMPT_IC50_log10
120%
100%
80%
60%
40%
20%
0%
0
0.5
1
1.5
2
2.5

FIG. 4C

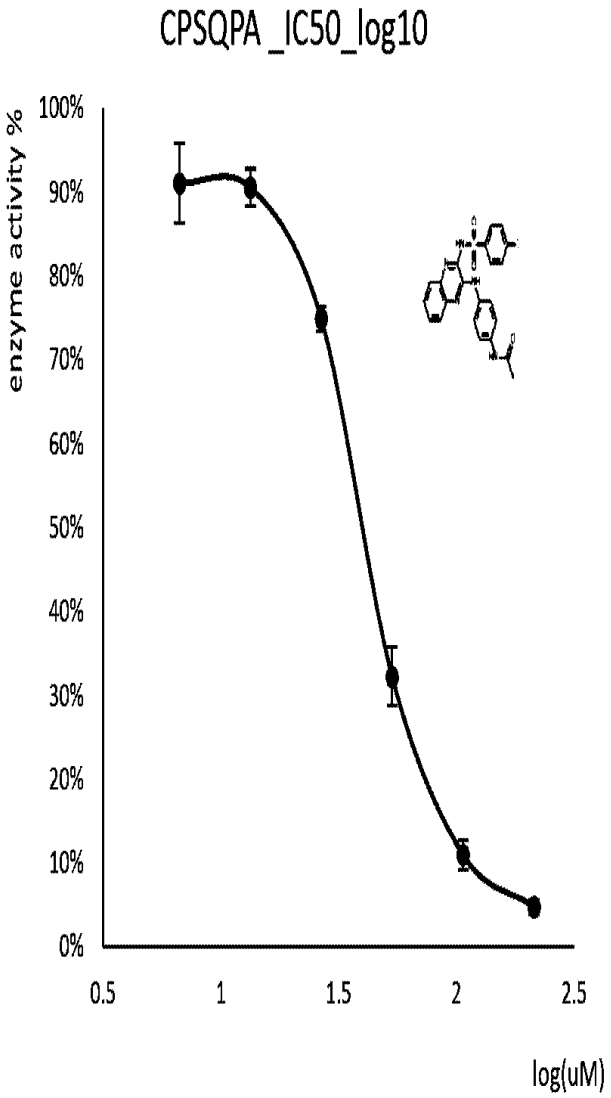
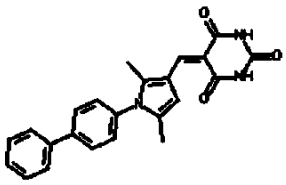
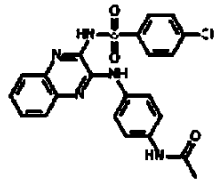
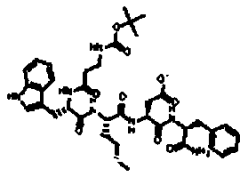
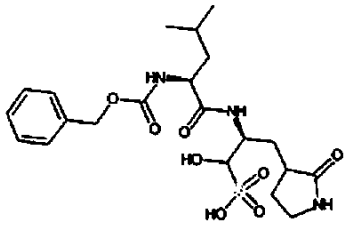

CPSQPA _IC50_log10
enzyme activity %
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
0.5
1
1.5
2
2.5
log(uM)

FIG. 4B

Table 1

Chemical	Pubchem CID	IBScreen ID	Name	IC50	Inhibition effect in 100uM
	3115780	STOCK2S-31334	5-((1-([1,1'-biphenyl]-4-yl)-2,5-dimethyl-1H-pyrrol-3-yl)methylene)pyrimidine-2,4,6(1H,3H,5H)-trione (PMPT)	29.8μM	79%
	1409854	STOCK2S-63827	N-(4-((3-(4-chlorophenylsulfonamido)quinoxalin-2-yl)amino)phenyl)acetamide (CPSQPA)	39.8μM	89%
	9853654	-	Pentagastrin	-	69%
		-	GC-376 (inhibitor control)	-	95%

INHIBITORS FOR SARS-COV-2 3CL PROTEASE AND METHOD FOR PREVENTING OR TREATING COVID-19

PRIORITY CLAIM AND CROSS-REFERENCE

[0001] None

FIELD OF THE INVENTION

[0002] The disclosure relates to a composition and a method for treating or preventing viral infections generally. More particularly, the disclosed subject matter relates to a pharmaceutical composition and a method for treating or preventing coronavirus infections such as those caused by SARS-CoV-2.

BACKGROUND

[0003] The emergence of the new coronaviruses, which are difficult to prevent and treat, highlights the need for the development of novel antiviral strategies. For example, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) causes a severe acute respiratory disease, COVID-19, and an ongoing pandemic all over the world. Clinical features of COVID-19 include fever, dry cough, and fatigue, and the disease can cause respiratory failure resulting in death. No effective therapeutic agent is available to prevent or treat SARS-CoV-2 infection. In view of the continuing threat to human health, there is an urgent need for preventive and therapeutic antiviral therapies for SARS-CoV-2 control.

[0004] Coronavirus is an enveloped RNA virus, which would infect respiratory tract of human and animals. Severe acute respiratory syndrome (SARS) and Middle East Respiratory Syndrome (MERS) are representative coronaviruses that have caused lots of human deaths in the 21st century. A new coronavirus, named as SARS-CoV-2, was detected in December 2019 in Wuhan, China. It was then quickly discovered in other countries. The disease caused by SARS-CoV-2, i.e., COVID-19, has been considered as a severe health problem, not only because of its rapid spread worldwide, but also due to its high fatality rate. In particular, COVID-19 breakout has caused more than 1.67 million deaths worldwide as on December 19, 2020. Effective anti-virus agents or vaccines and treatments are highly demanding now. Although a couple of

vaccines have been authorized for emergency use to control the spread of COVID-19, exploring effective anti-COVID-19 agents is still valuable. For example, the structure of the 3CLpro of SARS-CoV-2 is highly conserved in coronavirus. Identifying therapeutic agents to inhibit 3CLpro is not only meaningful for the treatment of COVID-19 patients, but also valuable for the treatment of infections caused by mutated SARS-CoV-2 in the future.

[0005] One of the efficient ways to identify effective anti-COVID-19 agents is trying those existing drugs that have been previously used to treat malaria, HIV, Ebola and bacteria. However, the efficacies of those drugs are not as good as expected. For example, although Remdesivir, which was created by Gilead, was able to shorten the recovery time and decrease the mortality rate, various side effects were reported. Sciences to target viral RNA polymerases of SARS coronavirus and MERS coronavirus, returned a mean half-maximal effective concentration (EC50) of 0.77μM towards the SARS-CoV-2 virus *in vitro*. This affinity (EC50) is not good enough for anti-viral drugs.

[0006] Another anti-COVID-19 therapeutic option is a combination of the HIV protease inhibitors lopinavir and ritonavir. Lopinavir, which acts against the 3-Chymotrypsin like protease (3CLpro) associated with HIV, is not a particularly potent therapeutic agent against SARS-CoV-2. The concentration necessary to inhibit viral replication is relatively high as compared with the serum levels found in patients treated with lopinavir–ritonavir. It is thus not surprising to find that no benefit was observed with lopinavir–ritonavir treatment when compared to the standard care protocol.

[0007] In addition to Remdesivir and lopinavir–ritonavir, chloroquine was found in the early *in vitro* studies to block COVID-19 infection with EC50 of 1.13 μM and a half-cytotoxic concentration (CC50) greater than 100 μM. Hydroxychloroquine, a less toxic derivative of chloroquine, is effective in inhibiting SARS-CoV-2 infection *in vitro*. The anti-viral and anti-inflammatory activities of chloroquine and hydroxychloroquine may account for its potent efficacy in treating patients with COVID-19 pneumonia. A study with six patients indicated that hydroxychloroquine treatment is significantly associated with viral load reduction/disappearance in COVID-19 patients and its effect is reinforced by azithromycin. However, WHO temporarily suspended in the solidarity trial of hydroxychloroquine due to a high mortality issue and an increased frequency of side effects like ventricular arrhythmias found in the hydroxychloroquine

treatment in around 10,000 COVID-19 patients. Therefore, effective anti-COVID-19 agents are still demanded to be developed.

SUMMARY OF THE INVENTION

[0008] The present disclosure provides a method for treating, curing, preventing, providing symptomatic relief, reducing the severity of, or reducing complications of, a viral infection.

[0009] The newly evolved SARS-CoV-2 has caused the COVID-19 pandemic in the world. While extensive studies have been conducted to develop effective anti-COVID-19 agents, no agent has been implemented widely in the clinical setting yet. The protease 3CLpro is essential for the rapid replication of SARS-CoV-2. Inhibiting this protease may open an avenue to combat the COVID-19 pandemic.

[0010] In the present disclosure, a computational docking approach was developed to identify potential small-molecule inhibitors for SARS-CoV-2 3CLpro. A total of 288 potential 3CLpro inhibitors were identified from half-million of bioactive chemicals through the protein-ligand docking platform in Molsoft ICM-Pro. To further evaluate the docking results, a quantitative structure activity relationship (QSAR) model of 3CLpro inhibitors was first developed based on existing small molecule inhibitors of the SARS-CoV 3CLpro and the IC50 data. The QSAR model would assess physicochemical properties of identified compounds and estimate their inhibitory effects on SARS-CoV 3CLpro. Seventy-one potential inhibitors of 3CLpro have been selected through the computational approaches, subsequently protease activity assay was conducted to validate effectiveness. The results show that Pentagastrin, an approved drug, inhibits the activity of 3CL protease by 69% in the concentration of 100 μM. Furthermore, two compounds, i.e., 5-((1-([1,1'-biphenyl]-4-yl)-2,5-dimethyl-1H-pyrrol-3-yl)methylene)pyrimidine-2,4,6(1H,3H,5H)-trione, and N-(4-((3-(4-chlorophenylsulfonamido)quinoxalin-2-yl)amino)phenyl)acetamide, were newly discovered having more effective inhibition on SARS-CoV-2 3CLpro with IC50 as 29.8 μM and 39.8μM, respectively. The findings, such as 3CLpro inhibitor candidates and the QSAR model, can be

helpful to accelerate the discovery of inhibitors for future coronavirus that may carry proteases with a similar structure of SARS-CoV-2 3CLpro.

[0011] In accordance with some embodiments, the method includes administering a pharmaceutical composition to a subject in need thereof. The pharmaceutical composition includes an effective amount of a 3CL protease inhibitor selected from 5-((1-([1,1'-biphenyl]-4-yl)-2,5-dimethyl-1H-pyrrol-3-yl)methylene)pyrimidine-2,4,6(1H,3H,5H)-trione (PMPT), N-(4-((3-(4-chlorophenylsulfonamido)quinoxalin-2-yl)amino)phenyl)acetamide (CPSQPA), a substituted derivative of each compound, a pharmaceutically-acceptable salt of each compound or derivative, and any combination thereof. The present disclosure also provides an antiviral composition comprising at least one active ingredient described above.

[0012] In some embodiments, wherein the viral infection is caused by a coronavirus. For example, the viral infection is COVID-19 infection caused by SARS-CoV-2 coronavirus. The pharmaceutical composition may further comprise a pharmaceutically acceptable carrier or excipient. The pharmaceutical composition may be administrated orally or parenterally. The subject is a mammal, for example, human as a patient.

[0013] In another aspect, the present disclosure also provides an antiviral composition (or formulation), which comprises an effective amount of a 3CL protease inhibitor selected from 5-((1-([1,1'-biphenyl]-4-yl)-2,5-dimethyl-1H-pyrrol-3-yl)methylene)pyrimidine-2,4,6(1H,3H,5H)-trione (PMPT), N-(4-((3-(4-chlorophenylsulfonamido)quinoxalin-2-yl)amino)phenyl)acetamide (CPSQPA), a substituted derivative thereof, a pharmaceutically-acceptable salt thereof, or a combination thereof, and a pharmaceutically acceptable carrier or excipient. The antiviral composition is a dosage for oral or parenteral administration. The 3CL protease inhibitor has an IC50 value in a range of from 30 μM to 40 μM in some embodiments.

BRIEF DESCRIPTION OF THE DRAWINGS

[0014] The present disclosure is best understood from the following detailed description when read in conjunction with the accompanying drawings. It is emphasized that, according to common practice, the various features of the drawings are not necessarily to scale. On the

contrary, the dimensions of the various features are arbitrarily expanded or reduced for clarity. Like reference numerals denote like features throughout specification and drawings.

[0015] FIG. 1 shows an overview of the proposed workflow to identify inhibitors of SARS-CoV-2 3CLpro from existing compound databases in accordance with some embodiments.

[0016] FIG. 2 (A) shows the QSAR model generated by the training data suggests a good fitting ($R^2 = 0.9$). FIG. 2 (B) shows that a strong correlation ($R^2 = 0.72$) between actual IC50 and predicted activities for the testing data suggests the QSAR model is good for predicting the half-inhibition concentration of potential SARS-CoV-2 3CLpro inhibitors against COVID-19.

[0017] FIG. 3 shows docking conformation of SARS-CoV-2 3CL protease inhibitors: (A, B) 5-((1-([1,1'-biphenyl]-4-yl)-2,5-dimethyl-1H-pyrrol-3-yl)methylene)pyrimidine-2,4,6(1H,3H,5H)-trione; (C, D), N-(4-((3-(4-chlorophenylsulfonamido)quinoxalin-2-yl)amino)phenyl)acetamide; and (E, F) Pentagastrin. Receptor was modified from 6LU7 structure by removing ligand and deleting of water, which was displayed in protein worm mode and colored dark grey. Surface Hydrophobicity of receptor was calculated by ICM and displayed. Compounds were shown in yellow and crystal ligand N3 were maintained conform in crystal and displayed in light blue. Amino acid residues interacting with the ligands were labelled, particularly catalytic residues H41 and C145 were in red label.

[0018] FIGS 4A-4D show enzyme inhibition kinetics (A,B) and IC50 (C,D) of newly identified inhibitors, PMPT and CPSQPA.

DETAILED DESCRIPTION

[0019] This description of the exemplary embodiments is intended to be read in connection with the accompanying drawings, which are to be considered part of the entire written description.

[0020] For purposes of the description hereinafter, it is to be understood that the embodiments described below may assume alternative variations and embodiments. It is also to be understood that the specific articles, compositions, and/or processes described herein are exemplary and should not be considered as limiting.

[0021] In the present disclosure the singular forms "a," "an," and "the" include the plural reference, and reference to a particular numerical value includes at least that particular value, unless the context clearly indicates otherwise. When values are expressed as approximations, by use of the antecedent "about," it will be understood that the particular value forms another embodiment. As used herein, "about X" (where X is a numerical value) preferably refers to ±10% of the recited value, inclusive. For example, the phrase "about 8" preferably refers to a value of 7.2 to 8.8, inclusive; as another example, the phrase "about 8%" preferably (but not always) refers to a value of 7.2% to 8.8%, inclusive. Where present, all ranges are inclusive and combinable. For example, when a range of "1 to 5" is recited, the recited range should be construed as including ranges "1 to 4", "1 to 3", "1-2", "1-2 & 4-5", "1-3 & 5", "2-5", and the like. In addition, when a list of alternatives is positively provided, such listing can be interpreted to mean that any of the alternatives may be excluded, e.g., by a negative limitation in the claims. For example, when a range of "1 to 5" is recited, the recited range may be construed as including situations whereby any of 1, 2, 3, 4, or 5 are negatively excluded; thus, a recitation of "1 to 5" may be construed as "1 and 3-5, but not 2", or simply "wherein 2 is not included." It is intended that any component, element, attribute, or step that is positively recited herein may be explicitly excluded in the claims, whether such components, elements, attributes, or steps are listed as alternatives or whether they are recited in isolation.

[0022] Understanding how SARS-CoV-2 invaded human body is helpful to develop effective anti-COVID-19 agents. The SARS-CoV-2 was found to attack lower respiratory system and the gastrointestinal system. Before entering the host cells, the spike (S) protein on coronavirus would bind with the angiotensin converting enzyme 2 (ACE2) on the surface of host cell. After the viral RNA entering the host cell, the replication of viral RNA would happen in double membrane vesicles (DMV). 3C-like protease (3CLpro) is important for the viral replication, which catalyzes the release process of 3CLpro, cleaves the transcript polyprotein, and releases other functional polypeptides of the coronavirus. Similarly, PLpro is another important protease in SARS-CoV-2 replication, which would assist the virus evade the immune response by deubiquitinating. Compared to PLpro, both the structure of 3CLpro and the inhibitors of SARS-CoV 3CLpro have been extensively studied. This motivates us to make use

of existing EC50 data for the inhibitors of SARS-CoV 3CLpro to accelerate the identification of effective inhibitors of SARS-CoV-2 3CLpro to combat the notorious COVID-19.

[0023] Existing studies on the structure and inhibitors of the SARS-CoV main proteinase 3CLpro pave the way to investigate effective inhibitors for SARS-CoV-2 3CLpro. Specifically, SARS-CoV 3CLpro is enzymatically active as a homodimer and its catalysis is under extensive regulation by the unique extra domain. The monomeric enzyme is irreversibly inactivated because its catalytic machinery is frozen in the collapsed state, characteristic of the formation of a short 3_{10}-helix from an active-site loop. Inhibiting dimerization of 3CLpro monomer is thus one way to inhibit 3CLpro. However, dimerization inhibitors are usually targeted to the dimerization interface and need to compete with the attractive forces between subunits to be effective. Previous study suggested covalent inhibitors of 3CLpro targeting residue C145 cysteine in the active pocket which functions as a common nucleophile in the proteolytic process eliminated the enzyme activity. On the other hand, a cluster of serine residues (Ser139, Ser144, and Ser147) was identified near the active site cavity and was susceptible to being targeted by compounds containing boronic acid compounds, which are particularly effective at inhibiting the active site of 3CLpro with inhibition constants as strong as 40 nM. Targeting the active site is thus preferred for 3CLpro inhibitors.

[0024] Attempts have been made to provide a complete description of the structural features and detailed mechanisms of action of existing SARS 3CLpro inhibitors. Many peptide inhibitors were designed to mimic the natural viral polypeptides and covalently bind to the active site C145 of 3CLpro protease to inhibit its activity. Despite their potent inhibition of SARS-CoV 3CLpro and relatively long half-life in buffer at neutral pH values, these peptide inhibitors are likely to be problematic, because of their high propensity to be rapidly hydrolyzed by lipase, esterase, and other enzymes in mammalian cells. Moreover, these compounds can potentially react nonspecifically with other thiols or nucleophiles in mammalian cells, thereby leading to toxicity. The other category of inhibitors against SARS 3CLpro includes nonpeptidic small molecules. In general, small molecules have been found to be noncovalent or reversible covalent inhibitors, which have advantages regarding side effects and toxicity, which often arise with covalent inhibitors. These inhibitors were discovered by high throughput screening of synthetic

compounds and natural products, such as etacrynic acid derivatives, isatin, flavonoid derivatives, terpenoid, active heterocyclic ester analogues, pyrazolone and pyrimidines.

[0025] On the basis of the aforementioned SARS 3CLpro inhibitors and IC50 data, we implemented a protein-ligand docking approach to narrow down 3CLpro inhibitor candidates and then developed a quantitative structure–activity relationship (QSAR) model to identify effect inhibitors (i.e., with low IC50) for SARS-CoV-2 3CLpro. The three-dimensional QSAR model attempts to correlate 3D molecular structure to biological activity, often using a variety of molecular descriptors such as physicochemical, topological, electronic and steric properties. In particular, 3D Atomic Property Fields (APF) QSAR methods calculate physico-chemical properties of superimposed chemicals and utilize their half-inhibition data to weight contributions for each property through Partial-Least-Squares (PLS) regression modeling. The QSAR model allows quantitatively predict pharmacological activities of congeneric unknown compounds and directs the design of novel derivatives with enhanced activity. Since hundreds of compounds exist for repurposing and testing, automated molecular docking, which has been widely implemented in drug discovery research for hit identification, was implemented to evaluates the binding of compounds from FDA-approved drugs and IBScreen database to SARS-CoV2 3CL protease. Only those inhibitors with high-binding affinities in the docking program will be fed into the 3D QSAR model to estimate their IC50. The inhibitors with good IC50 are regarded as good candidates for further enzyme activity assay and IC50 experiment evaluation. One hypothesis in our work is that the 3D QSAR model that links structures of SARS 3CLpro inhibitors to IC50 can be applied to the inhibitors of SARS-CoV2 3CLpro identified from the docking program. The rationale behind this is that the crystal structure of SARS-CoV2 3CLpro is similar to that of SARS 3CLpro and the active pockets are conserved.

[0026] The present disclosure provides a method and a composition for treating, curing, preventing, providing symptomatic relief, reducing the severity of, or reducing complications of, a viral infection. The present disclosure provides an integrated computational and experimental approach to identifying inhibitors for SARS-CoV-2 3CL Protease.

[0027] The term “effective amount” as used herein means an amount of a compound sufficient to inhibit 3CL protease so as to treat, cure, prevent, or lessen the viral infection,

particularly the viral infection caused by such as a coronavirus. In some embodiments, such a coronavirus is SARS-CoV-2, which can cause COVID-19 infection.

[0028] The term “subject” or “patient” as used herein refers to an animal, preferably a mammal, most preferably a human, who has been the object of treatment, observation or experiment.

[0029] The term "alkyl" as used herein refers to a straight chain, cyclic, branched or unbranched saturated or unsaturated hydrocarbon chain containing 1-10 carbon atoms, such as methyl, ethyl, propyl, tert-butyl, n-hexyl and the like. “A C_{1-6} alkyl” as used herein refers to an alkyl group having a number of carbon atoms selected from 1 to 6.

[0030] The term "optionally substituted" means that group in question may be unsubstituted or it may be substituted one or several times, such as 1 to 3 times or 1 to 5 times. For example, an alkyl group that is "optionally substituted" with 1 to 5 chloro atoms, may be unsubstituted, or it may contain 1, 2, 3, 4, or 5 chlorine atoms. Substituted chemical moieties include one or more substituents that replace hydrogen.

[0031] In accordance with some embodiments, the method includes administering a pharmaceutical composition to a subject in need thereof. The pharmaceutical composition includes an effective amount of a 3CL protease inhibitor selected from 5-((1-([1,1'-biphenyl]-4-yl)-2,5-dimethyl-1H-pyrrol-3-yl)methylene)pyrimidine-2,4,6(1H,3H,5H)-trione (PMPT), N-(4-((3-(4-chlorophenylsulfonamido)quinoxalin-2-yl)amino)phenyl)acetamide (CPSQPA), a substituted derivative of each compound, a pharmaceutically-acceptable salt of each compound or derivative, and any combination thereof. The present disclosure also provides an antiviral composition comprising at least one active ingredient described above.

[0032] The substituted derivatives are based on each of these two compounds, in which one or more substitution groups are bonded onto one or more ring structures. Examples of a suitable substitution group include, but are not limited to, fluoro, chloro, amino, carboxyl, alkyl, or other suitable groups or a combination thereof.

[0033] In some embodiments, wherein the viral infection is caused by a coronavirus. For example, the viral infection is COVID-19 infection caused by SARS-CoV-2 coronavirus. The pharmaceutical composition may further comprise a pharmaceutically acceptable carrier or

excipient. The pharmaceutical composition may be administrated orally or parenterally. The subject is a mammal, for example, human as a patient.

[0034] The compositions including the active ingredient(s) are administered with a pharmaceutical carrier selected on the basis of the chosen route of administration and standard pharmaceutical practice. The compounds of the invention can, for example, be administered orally (e.g., via tablet or capsule), parenterally (including subcutaneous injections, intravenous, intramuscular or intrasternal injection, or infusion techniques), by inhalation spray, or rectally, in the form of a unit dosage of a pharmaceutical composition containing an effective amount of the compound and conventional non-toxic pharmaceutically acceptable carriers, adjuvants and vehicles.

[0035] The antiviral composition may further comprise a carrier. Such a carrier can be a solvent or any pharmaceutically acceptable carrier. The carrier may be a liquid such as water and alcohol, or a combination thereof. The carrier may also be a solid ingredient.

[0036] The antiviral composition is in a solid dosage form for oral administration, or a liquid form for *in vivo* administration or *in vitro* application.

[0037] EXPERIMENTAL:

[0038] 1. Materials and Methods:

[0039] While the detailed methods are introduced in each of the following subsections, FIG. 1 provides an overview of the proposed workflow for identifying inhibitors of SARS-CoV-2 3CLpro via docking-based virtual screening and QSAR modeling and validating through bioassay, through the following steps: 1) FDA-approved drugs and IBScreen compounds libraries were docked into crystal structures of SARS-CoV-2 3CLpro (6LU7 and 6Y2F) to evaluate strong binders; 2) Half-inhibition concentration (IC50) data along with the structures of existing SARS 3CLpro inhibitors were first used to develop a QSAR model to predict IC50 of new SARS-CoV-2 3CLpro inhibitors; 3) the top inhibitor candidates were tested by enzyme activity assay; 4) the inhibitor candidates with the best performance in enzyme activity assay were tested in the IC50 experiment As for the QSAR modeling, 50% of compounds were used for training the QSAR model, while the other compounds were reserved to validate the model. Molsoft ICM was applied to identify compounds with good binding affinities with SARS-CoV-2 3CLpro in the first step. Molsoft ICM is also the tool for developing both the QSAR model to

predict their IC50 when acting on SARS-CoV-2 3CLpro. The compounds with relative low docking scores and IC50 values are regarded as good inhibitor candidates for IC50 experiment evaluation.

[0040] 2. Virtual screening: identify of compounds with high binding affinity with SARS-CoV-2 3CL protease

[0041] Structures of SARS-CoV-2 3CL proteases (PDB code 6LU7 and 6Y2F) bound with inhibitor were obtained from Protein Data Bank (PDB) and fed into ICM-Pro. The structure 6LU7 was converted into the format used in ICM-Pro and modified by removing ligand, deleting of water, and adding hydrogens. The following residues were further optimized: three protonation states and two rotations of His were tried. A 180-degree flip of Asn and Gln were tried. Cys residues were adjusted in the vicinity of Zn, Cu, Fe and Co to Cym. Ligand binding pocket was predicted by icmPocketFinder with tolerance 4.6 and selected the largest one covering crystal ligands. Docking box was generated in size of 27.6×18.0×24.5 with the probe at the center. Structure 6Y2F was modified following same steps. Crystal ligands from the two structures were extracted and redocked into the receptor getting scores -29.02 and -24.61, which was set to be score threshold in virtual screening.

[0042] In terms of chemical library, FDA approved drugs (2305 compounds) is the first option and InterBioScreen provide worldwide most reliable high-quality compound database (>550,000 compounds) for drug screening. Compounds that were filtered by “Lipinski's rules of five” were then screened using ICM scoring function 2005 version with docking effort 1. The first docking scores were recorded as score1 and the compounds with docking score1 lower than that of crystal ligand N3 (-29.02) would be collected. The compounds were further docked into the second structure 6Y2F with effort 10 and scoring function 2016 version. Docking conformations of each compound generated from the two receptors were manually checked. Compounds binding at the same position with similar conformations were put into list of hits. Since the SARS-CoV-2 3CLpro structure 6LU7 of was firstly released, the virtual screening was conducted first based on this. After first screening was finished, more 3CLpro structures were posted onto PDB including 6Y2F. As the two structures are sequence identical and highly conserved, re-docking hit into the second target 6Y2F could double check conformations and scores. The docking program calculated binding free energy ΔG including hydrogen bond

energy, hydrophobic energy in exposing a surface to water, van der Waals interaction energy, internal conformation energy of the ligand, desolvation of exposed h-bond donors and acceptors, solvation electrostatics energy change upon binding, loss of entropy, and potential of mean force score. A second scoring function (2016 version) adjusted parameters of each energy component. Either of the two ICM scoring functions is widely used in docking and virtual screening in recent studies, thereby results calculated by two version scoring functions would be more reliable. Additionally, molLogP, log of the octanol/water ratio, was calculated by Molsoft ICM to consider the water solubility and bioavailability of each drug. Consequently, selected compounds (score1< -29.02 and score2 < -24.61) would enter next QSAR prediction steps.

[0043] 3. 3D QSAR analysis: filtering drug candidates with relative high potency.

[0044] QSAR analysis includes two steps: the first step deals with the generation of QSAR model by using known 3CLpro inhibitors, while the second step is focused on the prediction of inhibitory activity of new compounds. The non-covalent inhibitors of SARS 3CLpro and their activity data were obtained from literatures, including Decahydroisoquinoline derivatives, octahydro-isochromene derivatives, pyrazolone and pyrimidines, 3CLpro inhibitors with 3-pyridyl or triazole or piperidine moiety and natural product derivatives. 3D structures of the inhibitors were converted from SMILES based on Merck Molecular Force Field (MMFF) atom type and force field optimization. A 3CLpro inhibitor ML188 extracted from 3CL protease structure (PDB ID 3V3M) was used as a template for 3D alignment. Totally 65 inhibitors were aligned to the template through flexible Atomic Property Fields (APF) superimposition method. Consequently, 35 compounds were used as the training set to build a 3D QSAR model and 30 compounds was grouped as testing set for validation.

[0045] For each of the aligned compounds, seven physicochemical properties were calculated and pooled together by APF. The APF method, designed by MolSoft, uses the assignment of a 3D pharmacophore potential on a continuously distributed grid using physio-chemical properties of the selected compound(s) to classify or superimpose compounds. These properties include hydrogen bond donors, acceptors, Sp2 hybridisation, lipophilicity, size, electropositivity/negativity and charge. Based on the half-inhibition data obtained from literature and the 3D aligned structures for the known compounds, weighted contributions for each APF component were obtained to allow quantitative activity predictions for unknown compounds.

The optimal weight distributions were assigned by partial least-squares (PLS) methodology, where the optimal number of latent vectors for PLS was established by leave-one-out cross-validation on the training set. Then the weighted contributions were added together. All potential 3CLpro inhibitors were subjected to the conversion and alignment protocol using Molsoft's ICM Pro software. Finally, compounds were further narrowed down into 71 to satisfy bioassay requirement.

[0046] 4. Experimental verification: the IC50 value of potential 3CLpro inhibitors.

[0047] 4.1. Potential inhibitors tested and stock solution preparation

[0048] 71 potential inhibitors were tested. Among them, 70 compounds were purchased from InterBioScreen Itd (IBS, Russia). The remaining one potential inhibitor was pentagastrin, which is an FDA-approved drug (MedChemExpress Inc., NJ). The stock solution of the predicted inhibitors to be tested was dissolved in DMSO (Sigma-Aldrich Inc., St. Louis, MO) to reach the final concentration of 10g/L. The stock solutions were stored under -20 degree C until further use.

[0049] 4.2. Enzyme activity test

[0050] In each experimental group, 30μL of 15nM purified recombinant 3CL-pro (BPS Bioscience Inc., CA) and 10 μL of 5 00μM prepared inhibitor solution was added into black 96-well plate (Nunc U96). 30μL of 15nM purified recombinant 3CL-pro and 10 μL of a known inhibitor GC376 was added into inhibitor control group. 30μL of 15nM purified recombinant 3CL-pro and 10 μL of 5% DMSO in water was added into positive control group. After preincubation at room temperature with slow shaking for 30min, 10μL of 200μM substrate solution DABCYL-KTSAVLQSGFRKME-EDANS (BPS Bioscience Inc., CA) was added into each group. Incubated the plate in room temperature with slow shaking for four hours. The fluorescent value was measured by CLARIOstar plus (BMG labtech, Germany) at 25 degree C with excitation wavelength of 360nm and the detection emission wavelength. Duplicate experiments were performed and the enzyme activity in the inhibitor group was used as a standard to select effective inhibitors of 3CL pro.

[0051] 4.3 IC50 test

[0052] The IC50 value of the two most effective inhibitors selected from the enzyme activity test was evaluated. The chemical stock solution of the inhibitors to be tested were diluted

with DMSO using a 2-fold serial dilution method. All the groups were further diluted 20 times with dH2O. In each experimental group, 30μL of 15nM purified recombinant 3CL-pro and 10 μL of diluted predicted inhibitor solution was added into black 96-well plate. In the positive control group, 30μL of 15nM purified recombinant 3CL-pro and 10 μL of 5% DMSO in dH2O was added into black 96-well plate. The plate was preincubated in room temperature with a slow shake for 30 min. Then 10μL of 200μM substrate solution DABCYL-KTSAVLQSGFRKME-EDANS was added into each group. The fluorescent value was measured by CLARIOstar plus (BMG labtech, Germany) at 25 degree C with excitation wavelength of 360nm and detection emission wavelength at 460nm every 3 minutes for 2 hours. Experiments were performed in triplicate.

[0053] 5. Results

[0054] 5.1 Potential SARS-CoV-2 3CLpro inhibitors identified through virtual screening and 3D QSAR modeling

[0055] 288 compounds were identified through structure-based virtual screening in ICM-Pro from half million of compounds from FDA-approved compound library and the IBScreen database. Docking scores estimated ligands binding affinity within structure 6LU7 and structure 6Y2F respectively. Score1 values of the strong binders were ranged from -41.3 to -30, which were lower than crystal ligands N3 score -29 in protein receptor (6LU7). Score2 values were ranged from -38.1 to -24.8, lower than ligand score -24.6 in receptor (6Y2F). The lower docking scores indicated relatively higher binding affinity and stronger ligand-receptor interaction of identified compounds. In term of conformation, the compounds were predicted bound with active site of 3CLprotease by occupying the ligand position. Furthermore, each compound had similar docking poses within the two receptor structures (Å < 2), which indicated acceptable docking simulations.

[0056] The 288 compounds were further screened by the QSAR model. Regards to QSAR modeling, the training dataset showed a good fitting quality (R2= 0.8967) (FIG. 2A), while the testing dataset suggested the predicted IC50 was correlated to actual IC50 (R2= 0.7257) (FIG. 2B). QSAR model generated by SARS-1 3CLpro inhibitors was then used to evaluate IC50 of new 3CLpro inhibitors SARS-CoV-2. The identified 288 hits were input into the developed QASR model to estimate half-inhibition values. The predicted IC50 for each

compound was mainly used to evaluate which compound might have better inhibitory effect than others.

[0057] Consequently, the QSAR model is useful to further sort and narrow down the potential 288 inhibitors. Seventy-one compounds, were selected for further enzyme activity assay, IC50 of which were ranged from 0.35μM to 19.86μM.

[0058] 5.2 Binding conformations of compounds predicted by Docking

[0059] Three new SARS-CoV-2 3CLpro inhibitors were identified by the protein-ligand docking program and the QSAR model. Seventy-one compounds were found in this approach. Example docking conformers and ligand-receptor interactions were presented in FIG. 3 for the purpose of illustration. In particular, pentagastrin was found to noncovalently bind to the active site of the SARS-CoV-2 3CL protease so that the catalytic residues H41 and C145 acting on breaking viral polypeptide substrate were blocked. Interestingly, pentagastrin might have a very similar binding mode as crystal ligand N3 inhibitor does, shown in FIG. 3F. Similar with Pentagastrin, two IBS hits, i.e., N-(4-((3-(4-chlorophenylsulfonamido)quinoxalin-2-yl)amino)phenyl)acetamide and 5-((1-([1,1'-biphenyl]-4-yl)-2,5-dimethyl-1H-pyrrol-3-yl)methylene)pyrimidine-2,4,6(1H,3H,5H)-trione, non-covalently bind to the substrate binding site of 3CLpro and probably occupy sub-pocket S1, S1' and S2/S3 at the active site (FIGS. 3B-3D). The identified molecules might form hydrogen bonds with some of the residues including E166, H41, C145, G143, and T26. They have hydrophobic interaction with the deep lipophilic pocket. Interrupting C145, the key catalytic residue, would eliminate the function of the viral main protease, thereby has potential to prevent viral replication and infection. Therefore, the two IBS compounds were considered as non-covalent 3CLpro inhibitors which competitively interrupt substrate binding, and subsequently validated by experiments.

[0060] 5.3 Inhibitory activity and IC50 of SARA-CoV-2 3CLpro inhibitor

[0061] Before testing the IC50 value of the predicted inhibitors, we further investigated the inhibition effect of those 71 lead compounds identified from the QSAR model. For this purpose, *in vitro* fluorescence resonance energy transfer (FRET) enzymatic assays were conducted at the concentration of 100μM. Three compounds listed in Table 1, including 5-((1-([1,1'-biphenyl]-4-yl)-2,5-dimethyl-1H-pyrrol-3-yl)methylene)pyrimidine-2,4,6(1H,3H,5H)-trione (PMPT) , N-(4-((3-(4-chlorophenylsulfonamido)quinoxalin-2-

yl)amino)phenyl)acetamide(CPSQPA) and Pentagastrin, were observed apparently decreased enzyme activity in the primary screening. Particularly, with the treatment of PMPT, the relative 3CLpro enzyme activity remains 21% comparing to the positive control group. With the treatment of CPSQPA, the relative 3CLpro enzyme activity remains 11% comparing to the positive control group. Although pentagastrin shows 69% inhibiting effect on 3CLpro, considering the 95%inhibitory effect of the known inhibitor GC-376, we only measured the IC50 value of the two compounds CPSQPA and PMPT.

[0062] As for enzyme activity kinetics (FIGS. 4A & 4B), the two inhibitors (i.e., PMPT and CPSQPA) show good inhibitory effect on 3CL protease, with the IC50 between 50uM to 200uM. Enzyme without being inhibited would catalyze the generation of florescent product in rate of 3479 RFU/min. Nonetheless, the reaction rate of the group with PMPT and CPSQPA in the concentration of 100uM were 724 and 383 RFU/min. Therefore, the two compounds significantly inhibited enzyme activity by 79% and 89%, respectively. Furthermore, the IC50 of PMPT and CPSQPA were quantified by plotting slope of enzyme kinetics and inhibitor concentration gradient, with the value of 29.8 and 39.8 μM respectively. The IC50 values were at the acceptable range of known none-covalent 3CLpro inhibitors. Therefore, the two newly discovered 3CLpro inhibitors effectively inhibit activities of the protease *in vitro* and serve as a good starting point for lead optimization.

[0063] Table 1. Newly discovered SARS-CoV-2 3CLprotease inhibitors. (Table 1 has been moved into the Drawing Section because the USPTO's Patent Center cannot fully recognize the table).

[0064] Structure-based virtual screen method is effective to identify 3CLpro inhibitors. The two structures of 3CLpro proteins (PDB ID 6LY7 and 6Y2F) are homology and the residues around crystal ligand are highly conserved, which suggests that various inhibitors bound at the active site might not significantly initiate conformational change of the protease. Therefore, virtual screening based on rigid receptor is rational and possible to find new inhibitors. The identified compounds e.g. PMPT, CPSQPA and Pentagastrin is expected competitively inhibit activity of the viral protease and thereby inhibit replication and invasion of the SARS-CoV-2 coronavirus. Pentagastrin (trade name Peptavlon) is a FDA-approved synthetic polypeptide

which stimulates the secretion of gastric acid, pepsin, and intrinsic factor, and has been used as a diagnostic aid as the Pentagastrin-stimulated calcitonin test. Drug repurposing is an effective strategy against SARS-CoV-2, thereby repurposing development of Pentagastrin could be a possible approach to combat COVID-19. Additionally, Pentagastrin has also confirmed by another *in-silico* drug discovery study. CG-376 used as an inhibitor control in experiment has been found inhibiting SARS-CoV-2 viral replication by targeting the 3CL protease with IC50 0.15μM. However, GC-376 which covalently links with C145 of 3CLpro might have off-target binding problems. PMPT and CPSQPA are small-molecule reversible inhibitor of 3CLpro, they might have lower chance of off-target side effects. By searching PubChem, we did not find toxicity record related to the two compounds.

[0065] Molecular docking indicated as an efficient approach to identify potential 3CLpro inhibitors based on intermolecular interactions. All the docking and 3D QSAR modeling were conducted in Molsoft ICM, which is regarded as one of the best docking platform and provide a superior docking and 3D QSAR method. ICM Docking score, to some degree, suggested the ligand binding affinity; however, it was designed to evaluate all ligand-receptor interaction. Due to specificity of 3CL protease, it would be limited to predict selective inhibitors. Therefore, using QSAR modeling to double check the top compounds screened by docking is necessary. The model generated by data of SARS 3CLpro inhibitors would quantitively estimate IC50 of new inhibitor for SARS-CoV-2. Compounds filtered by QSAR would be more similar to known inhibitors in terms of physicochemical properties. Our computational results significantly narrow down the drug candidates for high through-put screening and thus accelerate the discovery of COVID-19 cure. Therefore, we could expect identified new 3CLpro inhibitors could provide more therapeutic options in treatment of COVID-19.

[0066] Vaccine is an efficient way to end COVID-19 pandemic. However, safety issue for a certain people e.g., allergenic, pregnant, and immune disorder, is still unknown. Vaccine is for healthy people to gain immunity to SARS-CoV-2, while 3CLpro inhibitors could be a potential therapeutic to infected patients. We still do not know whether it deserves to let all people expose under risk of vaccination. An alternative way is development of therapeutic drugs for coronavirus which is only for infected people. Additionally, manufacture, delivery and injection could challenge every country in the world. Coronavirus mutates rapidly, we have

observed a new variant speared from UK recently, which may be up to 70 per cent more transmissible. It is possible that COVID outbreak become a seasonal, but vaccine development takes years.

[0067] In order to inhibit the replication of SARS-CoV-2, an integrated computational and experimental approach was developed in this work. A total of 288 potential inhibitors of main protease (3CLpro) of SARS-CoV-2 were identified through virtual screening of half of millions of compounds from existing databases. Inhibitory activities of the compounds are predicted from a QSAR model developed from existing data for the inhibitors of SARS-CoV 3CLpro. Among these potentil inhibitors, seven-one compounds were further selected for enzyme activity assay. The predicted IC50 of these inhibitors were in an acceptable range by the experimental data. The IC50 of two compounds (i.e., 5-((1-([1,1'-biphenyl]-4-yl)-2,5-dimethyl-1H-pyrrol-3-yl)methylene)pyrimidine-2,4,6(1H,3H,5H)-trione, N-(4-((3-(4-chlorophenylsulfonamido)quinoxalin-2-yl)amino)phenyl)acetamide) were in the range of 30 to 40 μM. These compounds are of high potential for treating COVID-19 patients. In future study, cellular infection and animal test should be conducted to validate efficacy and safety.

[0068] Although the subject matter has been described in terms of exemplary embodiments, it is not limited thereto. Rather, the appended claims should be construed broadly, to include other variants and embodiments, which may be made by those skilled in the art.

What is claimed is:

1. A method for treating, curing, preventing, providing symptomatic relief, reducing the severity of, or reducing complications of, a viral infection, comprising:

Administering a pharmaceutical composition to a subject in need thereof, the pharmaceutical composition comprising an effective amount of a 3CL protease inhibitor selected from 5-((1-([1,1'-biphenyl]-4-yl)-2,5-dimethyl-1H-pyrrol-3-yl)methylene)pyrimidine-2,4,6(1H,3H,5H)-trione (PMPT), N-(4-((3-(4-chlorophenylsulfonamido)quinoxalin-2-yl)amino)phenyl)acetamide (CPSQPA), a substituted derivative thereof, a pharmaceutically-acceptable salt thereof, or a combination thereof.

2. The method of claim 1, wherein the viral infection is caused by a coronavirus.

3. The method of claim 1, wherein the viral infection is COVID-19 infection caused by SARS-CoV-2 coronavirus.

4. The method of claim 1, wherein the pharmaceutical composition comprises a pharmaceutically acceptable carrier or excipient.

5. The method of claim 1, wherein the pharmaceutical composition is administrated orally or parenterally.

6. The method of claim 1, wherein the subject is a mammal.

7. The method of claim 1, wherein the subject is human.

8. An antiviral composition, comprising:

an effective amount of a 3CL protease inhibitor selected from 5-((1-([1,1'-biphenyl]-4-yl)-2,5-dimethyl-1H-pyrrol-3-yl)methylene)pyrimidine-2,4,6(1H,3H,5H)-trione (PMPT), N-(4-((3-(4-chlorophenylsulfonamido)quinoxalin-2-yl)amino)phenyl)acetamide (CPSQPA), a

substituted derivative thereof, a pharmaceutically-acceptable salt thereof, or a combination thereof; and

a pharmaceutically acceptable carrier or excipient.

9. The antiviral composition of claim 8, wherein the antiviral composition is a dosage for oral or parenteral administration.

10. The antiviral composition of claim 8, wherein the 3CL protease inhibitor has an IC50 value in a range of from 30 μM to 40 μM.

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PTO/SB/452 (09-20)

CERTIFICATION AND REQUEST FOR COVID-19 PROVISIONAL PATENT APPLICATION PROGRAM

(Page 1 of 1)

First Named Inventor:	Zuyi Huang
Title of Invention:	INHIBITORS FOR SARS-COV-2 3CL PROTEASE AND METHOD FOR PREVENTING OR TREATING COVID-19
Contact information to include in database (optional)	Villanova University, 800 Lancaster Avenue, Villanova, PA 19085

APPLICANT HEREBY MAKES THE FOLLOWING CERTIFICATIONS AND REQUESTS THAT THE USPTO INCLUDE THE DESCRIPTION OF THE ACCOMPANYING PROVISIONAL PATENT APPLICATION IN A PUBLIC DATABASE.

1. The description of the accompanying provisional patent application concerns a product or process relating to COVID-19 and such product or process is subject to an applicable FDA approval for COVID-19 use.

2. The accompanying application is in the English language.

3. The accompanying application is being filed in DOCX format via the USPTO's Patent Center filing system, together with this form.

4. The applicant understands that while the required filing fee for the accompanying provisional application may be deferred by acceptance into this program, the appropriate filing fee must be paid in order for a subsequent U.S. nonprovisional application to claim the benefit of the filing date of the accompanying provisional application. Applicant recognizes that the filing fee due in the future may be more than the current fee due and that by deferring payment of the filing fee, there may be an increase in the total fee due.

5. Applicant authorizes and requests that the description, including the specification and any drawings, claims and/or abstract of the accompanying provisional patent application, as well as this form, be included in a searchable online public database.

6. Applicant understands that inclusion in the public database is a publication of the description and this form.

Signature /Jiazhong Luo/	Date 2021-01-27
Name (Print/Typed) Jiazhong Luo	Practitioner Registration Number 59115

Note: *This form must be signed in accordance with 37 CFR 1.33. See 37 CFR 1.4(d) for signature requirements and certifications. Submit multiple forms if more than one signature is required.**

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