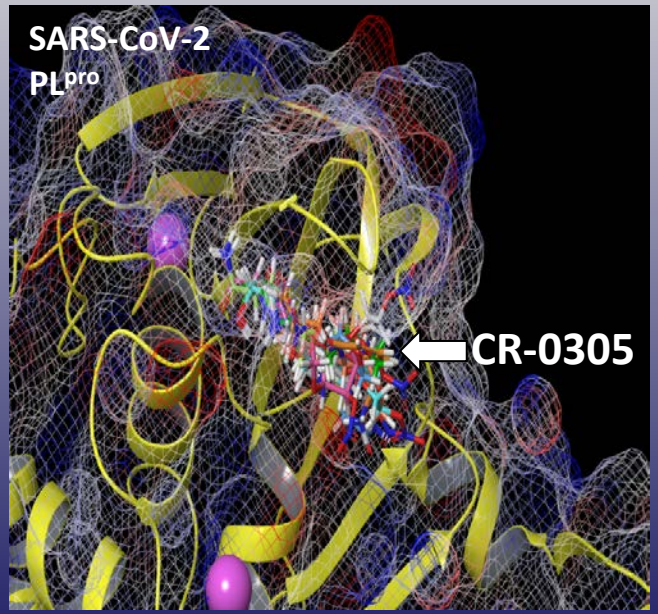
Novel Experimental Therapeutics For COVID-19 Derived From A Nitric Oxide Donor



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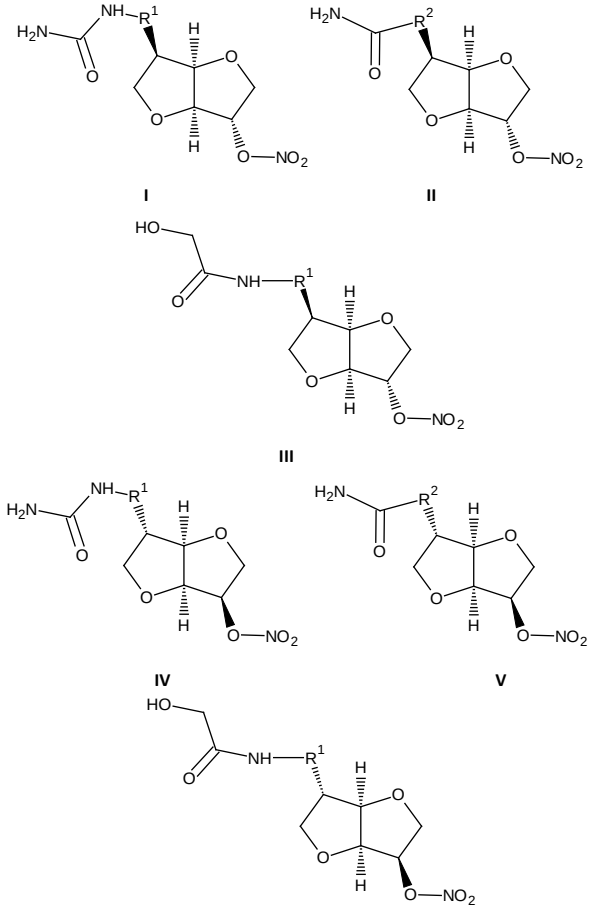
BACKGROUND

- Severe acute respiratory syndrome due to novel coronavirus (SARS-CoV-2) infection (COVID-19) is difficult to control. The pharmaceutical treatments are suboptimal and vaccines are not available as of yet. However, we know that nitric oxide acts as a pulmonary vasodilator and can be of therapeutic value in low oxygen states secondary to acute respiratory distress syndrome. Nitric oxide (NO) gas has antiviral activity against other strains of coronavirus in clinical and experimental models. Novel delivery systems that facilitate NO release in systemic and pulmonary blood vessels could reduce morbidity and mortality related to COVID-19.
- It would be desirable to develop compounds that potentiate vasodilatory release of NO in hypoxia, overcoming the inhibition observed. Given the fact that NO also has antiviral activity by inhibiting viral proteases (Saura et al., 1999) and that inhibition of SARS-CoV infection *in vitro* can be achieved using an NO donor (Keyaerts et al., 2004) this effort assumes a new and increased relevance to experimental therapeutics in the COVID-19 pandemic.
- Urea transport protein B, the product of the gene SLC14A1, facilitates transport of urea, water and urea analogues across cell membranes. (Shayakul et al., 2013) Intracellular urea concentrations are maintained by the action of UT-B and active expulsion of urea across cell membranes.
- SLC14A1 mRNA was markedly overexpressed in human vascular endothelial cells in culture under hypoxic (1% oxygen) conditions, vs. normoxia, in overexpression libraries derived from human vascular endothelium. The upregulation of expression of UT-B in hypoxia should pump urea out of the endothelial cell, pushing down endothelial urea concentrations. This is likely a factor in the previously documented reduction in eNOS-NO pathway activity in hypoxia. (Schmedtje et al.,1997)
- Arginine is metabolized to form urea via the enzyme arginase. If urea is in oversupply, arginine will be metabolized into NO via endothelial nitric oxide synthase instead of arginase-mediated metabolism into urea. If extracellular urea increases or UTB is inhibited, endothelial intracellular accumulation of urea leads to feedback inhibition of arginase and this leads to increased eNOS activity via an alternate pathway of arginine metabolism. (Sun et al., 2016)
- The findings herein suggest that at least one of the compounds studied, CR-0305, can bind to the catalytic site of PLpro so as to block protease activity essential for viral replication. We hypothesize that, while targeting delivery of antiviral NO to the SARS-CoV-2 virus, this same compound can modulate arginine metabolism in hypoxic vascular endothelium so as to indirectly facilitate NO formation.

METHODS

- The novel compounds were subjected to screening against SARS-CoV-2 proteins, through *in silico* modelling against nine SARS-CoV-2 targets using Maestro Schrödinger Suite software with Glide docking. Criteria for selection of virtual hits included docking scores and intermolecular interactions within the target's key binding pocket's amino acid residues. Compound characteristics and predicted physicochemical ADME/Tox properties were also calculated using Qikrprop, Schrödinger software. SARS-CoV-2 targets included: Main protease, 3CLpro (Nsp5), Spike Glycoprotein, Angiotensin Converting Enzyme 2, ACE2 (human), RNA-Dependent RNA Polymerase, RdRp (Nsp12), Endoribonuclease (Nsp15), Guanine-N7 methyltransferase (Nsp14), Papain-Like proteinase, PLpro (Nsp3), ADP-ribose phosphatase of Nsp3, and Bromodomain 2, BRD2 (human.)
- These targets were matched against 53 compound structures including 48 compound structures from U.S. Non-Provisional Patent Application No.16/600,476 filed Oct. 12, 2019, as well as isosorbide 2-nitrate, isosorbide 5-nitrate, 1,4:3,6-dianhydro-D-glucitol, 1,4:3,6-dianhydro-L-iditol and (3S,3aS,6R,6aR)-6-aminohexahydrofuro [3,2-b] furan-3-yl nitrate. Remdesivir, GS-441524, GS-441524 triphosphate and GRL-0617 were also screened against all 9 protein targets as control compounds. Control compounds were included in the Glide docking jobs for each of the SARS-CoV-2 targets.
- Focusing on high-performance ligand-receptor docking, Glide (Schrödinger Suite) was chosen as the screening model of compounds for affinity towards 9 SARS-CoV-2 protein targets. No constraints were considered in these docking screens (less-biased model) based on the recent reported structures for these targets. Crucial intermolecular hydrogen bonds within the amino acid residues of the binding pocket and compound as well as docking scores were main drivers in rank-ordering compounds in this *in silico* screening program.
- Upon successful completion of the Glide job, docking experiments poses for each compound were assessed by Glide score, a prediction of ligand affinity. As it simulates a binding free energy, more negative values represented tighter binders. Secondly, they were assessed by Emodel score which is a measure of pose strength and validity. Molecular Mechanics-Generalized Born Surface Area (MM-GBSA) method in Prime was used to rank order the docked pose of compounds (Hayes and Archontis, 2012)
- The Molecular Mechanics-Generalized Born Surface Area (MM-GBSA) method in the Schrödinger Suite Prime software package was used to predict binding free energies of hit compounds and provide better enrichment. Criteria for selection of hit compounds included docking scores, MM/GBSA scores and intermolecular interactions within the amino acid residues of the binding pocket of the target. Molecular Dynamics simulations were prepared using the classical simulation system set-up. (Bowers et al., 2006) A monomeric model was generated to reduce computation cost. SPC solvent model was used to solvate structure. The box volume was minimized automatically using a cubic shape. The model was neutralized by addition of Cl- ions from 0.15 M sodium chloride and solvated using an OLPS3 force field. (Harder et al., 2016) Relaxation of protein was first performed using standard Desmond relaxation protocol and then simulated for 10 ns with 1000 frames to ensure stable state. Temperature and pressure were 300 K and 1.01325 bar. Output of the stabilized model was subjected to 20 ns simulation using the same parameters. The simulation was analyzed using Schrödinger Maestro Simulation Event Analysis wizard.

METHODS: COMPOUNDS



- Compounds of formula I, II, III, IV, V and/or VI were created, wherein:
- R1 is either 1: absent or 2: (CH2)2O, 3: (CH2)2NH, 4: (CH2)3O, 5: (CH2)3NH, 6: CH2C(=O)O, and 7: CH2C(=O)NH.
- R2 is selected from 1: (CH2)2O, 2: (CH2)2NH, 3: (CH2)3O, 4: (CH2)3NH, 5: CH2C(=O)O, 6: CH2C(=O)NH, 7: CH2OC(=O)O, 8: CH2OC(=O)NH, 9:CH2NHC(=O)O, and 10: CH2NHC(=O)NH.
- The present compounds are designed to release nitrate in addition to urea or a urea analogue (e.g., amide or glycolamide) at the point of therapy. They are small molecules with a central isosorbide moiety and an opposing nitrate group (-NO3). Glycolamide has the benefit of relatively high permeability via UT-B and low toxicity. (Zhao et al., 2007) The present compounds are expected to take advantage of the passive movement of urea and urea analogues across the cell membrane into the vascular endothelial cell.
- CR-XXYY number scheme is based on XX=formula number and YY= number R1 or R2 group associated with the formula number.

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RESULTS

- Some of the 53 submitted compounds measured well in binding to PL^{pro} and outperformed the control compounds and existing approved drugs. For the remaining targets, top hits either scored very similar to control compounds (such as $3CL^{pro}$, Spike glycoprotein, RdRp, Nsp15 and BRD2) or slightly lower than the control compounds (such as ACE2 and Guanine-N7 Methyltransferase of Nsp3). Lower Glide and Emodel scores were mostly due to the smaller size of some compounds compared to the controls. For instance, 3CLpro and Spike Receptor-Binding-Domain (RBD) binding sites are large, near surface and solvent exposed which require larger compounds to fit thoroughly in the pocket.
- PL^{pro} regulates IFN and NF-κB pathways and plays an important role in the virus RNA synthesis and replication in SARS-CoV-2. Therefore, inhibitors against this target could be the potential therapeutics against COVID-19.
- Shown in Results: Glide Scores and MM/GBSA, about 10 compounds which scored higher and/or equal to control compounds (GSK2251052 hydrochloride, Remdesivir, Ribavirin, RTP, GRL-0617) were identified as strong hits: such as III-5, VI-7, V-10 and II-1. These hits were ranked order via Molecular Mechanics/Generalized Born Surface Area (MM/GBSA) calculations on top poses to predict binding free energies of hit compounds and provide better enrichment. They further were evaluated for their binding interactions within the catalytic pocket.
- Using the crystal structure of SARS-CoV-2 PL^{pro} (6WUU PDB ID), we performed a 20+ nsec simulation to confirm the stability of the small molecules and protein binding pocket contacts and water networks. Based on the MD simulation results for CR-0305 compound, additional contacts were observed between compound/protein around residues 110-113 compared to the references. As seen in Results: Molecular Dynamics, there is water bridging (a hydrogen-bonded protein-ligand interaction mediated by a water molecule) between -OH and the active site cys (111) with over 87% contact strength (not seen for CR-0605, CR-0202 or GRL-0617) that defines CR-0305 as the compound that provides the highest energy and most stable PL^{pro} binding of the compounds studied. Our MD simulation did support binding for GRL-0617 at the catalytic site, but CR-0305 binding was more stable.

RESULTS: GLIDE SCORES AND MM/GBSA FOR PL^{PRO} CATALYTIC SITE

Name	prime MM/GBSA dG	Glide Score (kcal/mol)	Emodel Score
CR-0305	-57.51	-7.329	-56.858
CR-0607	-44.38	-6.882	-56.269
CR-0307	-41.79	-6.827	-45.819
CR-0510	**-42.09**	**-6.449**	**-54.627**
CR-0605	**-49.69**	**-6.263**	**-56.804**
GS-441524-TP	**-32.5**	**-6.147**	**-53.27**
CR-0201	**-33.2**	**-6.064**	**-51.058**
alpha-ketoamide		**-5.903**	**-70.513**
Remdesivir	**-36.84**	**-5.871**	**-61.628**
Ribavirin	**-30.49**	**-5.820**	**-46.403**
CR-0103	-43.88	-5.830	-55.421
CR-0204	-46.52	-5.747	-54.92
CR-0101	-33.09	-5.619	-46.491
CR-0506	-35.28	-5.607	-55.199
CR-0508	-41.17	-5.594	-53.101
CR-0206	-40.25	-5.560	-54.239
CR-0301	-34.17	-5.487	-48.697
CR-0403	-34.22	-5.453	-49.416
CR-0107	-37.63	-5.374	-52.948
CR-0606	-35.28	-5.355	-48.416
CR-0302		-5.332	-53.024
CR-0601		-5.273	-47.677
CR-0210		-5.255	-51.841
CR-0401		-5.205	-45.562
CR-0303		-5.203	-51.092
CR-0504		-5.198	-43.504
CR-0203		-5.172	-48.871
GRL0617	**-26.3**	**-5.152**	**-43.591**
CR-0503		-5.150	-47.168
CR-0402		-5.127	-44.861
CR-0306		-5.107	-53.021
CR-0406		-5.091	-44.582

Name	prime MM/GBSA dG	Glide Score (kcal/mol)	Emodel Score
CR-0209		-5.069	-51.846
CR-0502		-5.047	-46.083
CR-0106		-5.004	-50.705
CR-0304		-4.993	-48.802
GS-441524	**-30.15**	**-4.950**	**-45.88**
CR-0205		-4.936	-50.937
CR-0208		-4.919	-50.588
CR-0102		-4.909	-48.039
CR-0202		-4.893	-44.644
CR-0501		-4.890	-48.118
1,4:3,6-dianhydro-D-glucitol		**-4.879**	**-29.831**
Lopinavir		**-4.822**	**-48.575**
1,4:3,6-dianhydro-L-iditol		**-4.812**	**-30.411**
CR-0407		-4.809	-51.083
CR-0507		-4.751	-49.661
CR-0509		-4.614	-44.742
CR-0604		-4.558	-49.034
JQ1		**-4.553**	**-52.148**
(3S,3aS,6R,6aR)-6-aminohexahydrofuro[3,2-b]furan-3-yl nitrate		**-4.546**	**-33.765**
CR-0505		-4.540	-45.489
CR-0207		-4.502	-44.971
Isosorbide 5-Nitrate		**-4.342**	**-35.096**
GSK2251052 HCl		**-4.307**	**-38.976**
Isosorbide 2-Nitrate		**-4.306**	**-32.169**
CR-0105		-4.159	-49.024
Boceprevir		**-4.105**	**-44.717**
Isosorbide Dinitrate	**-22.01**	**-3.970**	**-39.446**
CR-0104		-3.821	-42.205
CR-0405		-3.803	-44.337
CR-0404		-3.113	-42.918
CR-0602		-3.100	-45.633
CR-0603		-2.994	-40.359

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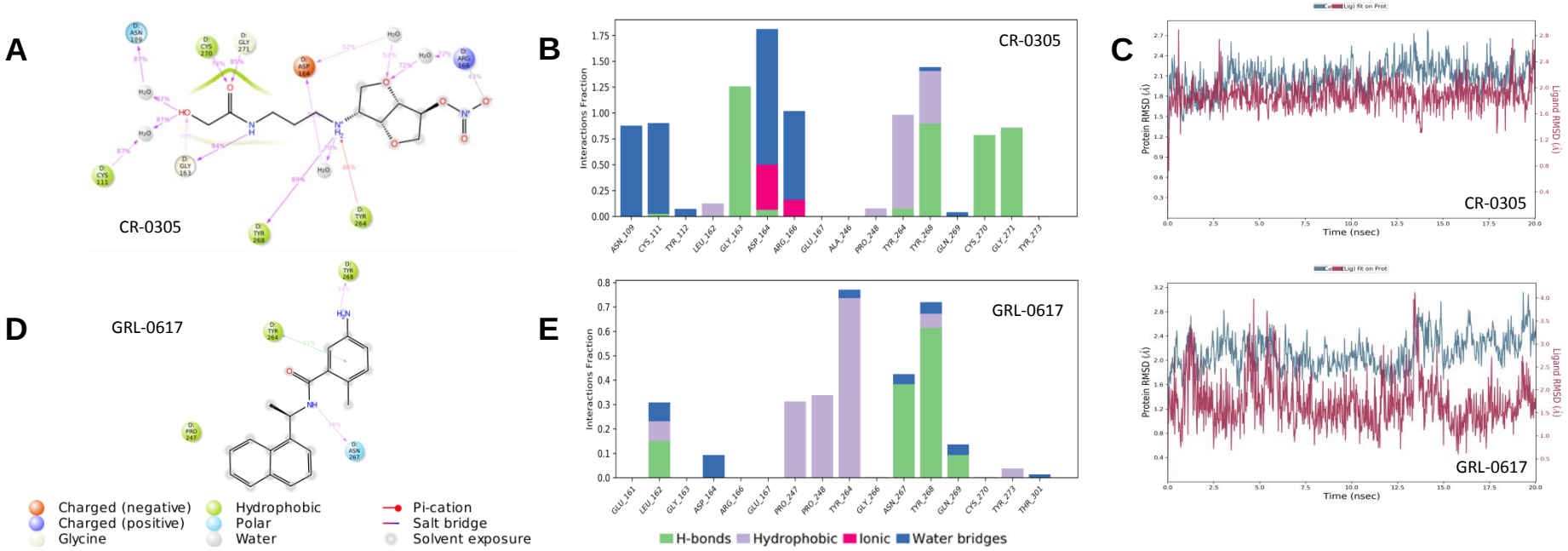
RESULTS: MOLECULAR DYNAMICS



Legend: (A) and (D): A schematic of detailed ligand atom interactions of CR-0305 (A) and GRL-0617 (D) with the PL^{pro} protein residues. Interactions that occur more than 30% of the simulation time in the selected trajectory (0 through 20 nanoseconds), are shown. **(B) and (E):** Protein-ligand interactions with PL^{pro} active site residues (or 'contacts') are categorized into four types: Hydrogen Bonds, Hydrophobic, Ionic and Water Bridges. Interactions at the catalytic site of PL^{pro} hinge critically on Cysteine-111 and the water bridge between CR-0305 (B) and PL^{pro} proved to be the distinguishing feature when comparing with (E) GRL-0617. **(C) and (F):** The Root Mean Square Deviation (RMSD) is used to measure the average change in displacement of a selection of atoms for a particular frame with respect to a reference frame. Changes larger than 1-3 Å indicate that the protein is undergoing a large conformational change during the simulation. Binding of CR-0305 over time (C) to the catalytic site of PL^{pro} is more stable than binding of GRL-0617 (F) over time.

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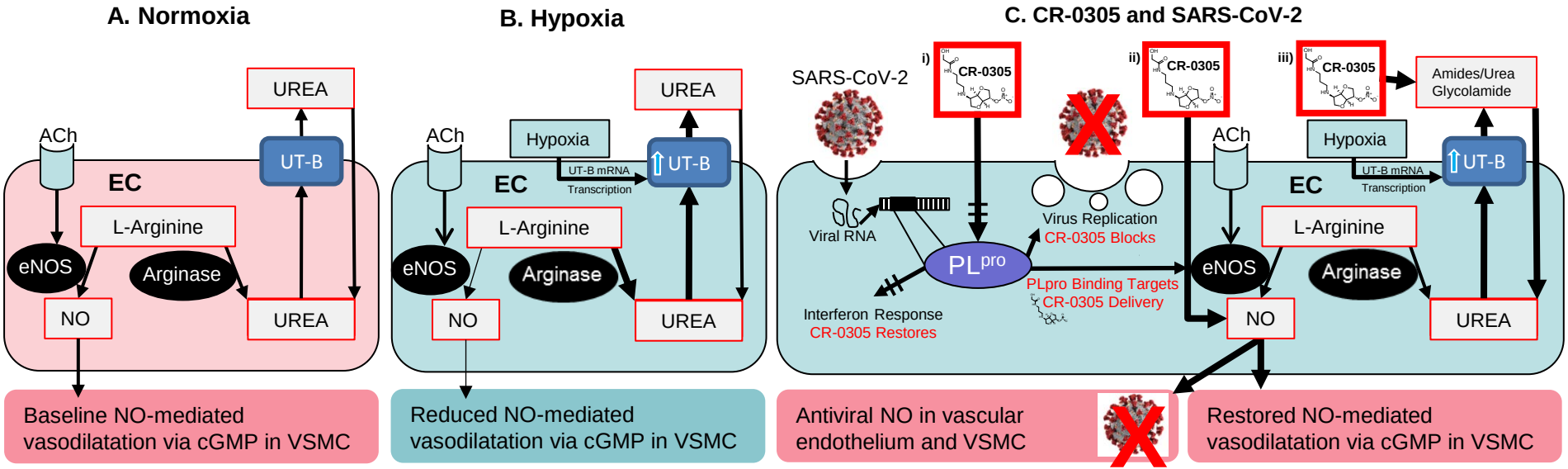
CR-0305 Proposed Mechanisms of Action



Legend: CR-0305 inhibits SARS-CoV-2 PL^{pro}, restoring interferon protection against SARS-CoV-2 and blocking viral replication, while as an NO donor it mediates endothelium-dependent vasodilatation and has a direct antiviral effect on SARS-CoV-2. **A. Normoxia:** L-arginine is catalyzed by endothelial NO Synthase and arginase to generate NO and urea, respectively. Urea is excreted though the UT-B urea transporter. **B. Hypoxia:** UT-B mRNA expression is increased in hypoxia, leading to increased expulsion of urea and a compensatory increase in arginase activity. This decreases availability of L-Arginine for conversion to NO . **C. CR-0305 and SARS-CoV-2:** PL^{pro} mediates inhibition of host immune responses via blocking of endogenous interferon expression while enabling viral replication via polypeptide processing. **i)** PL^{pro} inhibition by CR-0305 restores cellular interferon antiviral responses and blocks viral replication. **ii)** PL^{pro} binding targets delivery of the nitrate moiety in CR-0305 to infected cells as a donor of NO. **iii)** The urea analogue glycolamide, a constituent attached to the trans aspect of the isosorbide moiety of CR-0305, may have a feedback effect on intracellular arginase. Abbreviations: CR-0305, Coeurative Compound 0305; NO, nitric oxide; UT-B, urea transporter B; EC, endothelial cell; VSMC, vascular smooth muscle cell.

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DISCUSSION

The compounds discussed herein were designed originally to augment nitrate delivery in the vascular endothelium in the setting of arterial insufficiency. However, given the well-known direct antiviral action of NO, direct anti-viral action of these compounds against SARS-CoV-2 was proposed.

The papain-like protease (PL^{pro}) was considered as a primary target for therapeutic inhibition of the first SARS-CoV. (Baez-Santos et al., 2015) PL^{pro} was judged to have a high affinity for ribavirin in an *in silico* study of SARS-CoV-2 and ribavirin was at first believed to be a potential therapeutic agent. (Wu et al., 2020)

GRL-0617 has also shown some promise as a antiviral and has been proposed as part of dual therapeutic strategy in which targeting of PL^{pro} suppresses SARS-CoV-2 infection and promotes anti viral immunity. GRL-0617 appears to lead to maintenance of the antiviral interferon pathway and reduced viral replication in infected cells. (Ratia et al., 2008 and Shin et al., 2020) The proposed mechanism of action of CR-0305 involves binding of the compound to PL^{pro} at the same location as GRL-0617 while using affinity to PL^{pro} to target delivery of NO to SARS-CoV-2 in the form of the nitrate group attached to isosorbide at the 2-carbon.

CR-0305 was superior to GRL-0617, Remdesivir, GS-441524, Lopinavir, Boceprevir and Ribavirin in binding PL^{pro}. CR-0305 appears to have a higher affinity to SARS-CoV-2 than other antivirals as it sits firmly in the PL^{pro} catalytic pocket and makes the most of critical interactions with the key catalytic pocket amino acids Gly163, Asp164, Gly271 and Tyr264.

CR-0305 could prove superior to NO and other antivirals because, *in silico*, CR-305 binds to the catalytic site of PL^{pro} so as to block protease activity essential for viral replication while targeting delivery of antiviral NO to the SARS-CoV-2 virus in COVID-19. The findings herein inform the attached CR-0305 Proposed Mechanisms of Action.

These findings and the hypotheses generated call for further research on the novel antiviral agent CR-0305 as it appears to bind with increased avidity to PL^{pro} compared with other known antivirals while targeting delivery of NO to the SARS-CoV-2 virus. As such, it could prove to be a useful pharmaceutical component of a comprehensive strategy for treatment as well as prevention of COVID-19.