On Covid-19 for DRH Emergency Medicine Residents

From Blaine White, M.D.

You are all in an exhausting clinical battle, and I'm just an old retired physician-scientist sitting at home with a computer. I can no longer serve with you in modules, but you all are very much on my mind. I do have time and the background to run searches and read. So perhaps I can help your understanding by putting together in one place the pieces regarding (1) the molecular biology of the virus, (2) the process by which it gets into our cells, and (3) potential antiviral strategies including hydroxychloroquine, a recombinant chimera protein to block the virus, a viral polymerase inhibitor, and transplant immunosuppressants. Conventional vaccine development is important but likely to require longer and so won't be reviewed here. I will shamelessly plagiarize other authors to put this together, although I will reference at the bottom of pages.

Molecular biology of the virus

The Covid-19 virus has a single-stranded RNA genome that contains 29,891 nucleotides, encoding for 9,860 amino acids[1]. Bats carry a number of coronaviruses, and Covid-19 shares 96% nucleotide sequence identity with a coronavirus carried by a common Asian bat - *Rhinolophus affinis*[2]. It also shares up to 80% of its sequence with the coronaviruses that were responsible last decade for the SARS (Severe Acute Respiratory Syndrome) and MERS (Middle-East-RS) diseases. Although the CoVs' genomes are the largest of the known RNA viruses, they are less than double the size of our small mitochondrial genome.

The Covid-19 genome does not contain catalytic RNA (a ribozymes) but rather encodes for translation by a host's ribosomes – first of a RNA-dependent RNA polymerase for its replication-transcription-complex[1]. Once translated into the component proteins, that complex works in part via the synthesis of subgenomic mRNAs from 6 transcriptional open reading frames (ORFs) in the viral genome. During translation by our ribosomes, reading frame shifts on these viral mRNAs and the post-translational activity of viral-encoded proteases result in production from the viral genome of 29 proteins that include structural proteins (spike, membrane, envelope, and nucleocapsid proteins) and 16 non-structural proteins.

A crucial issue is interaction of viral proteins with our proteins. This has been approached with yeast-2-hybrid studies[3] using human and viral transcripts together with artificial intelligence modeling of these interactions[4]. Of particular importance is (1) the spike protein binds strongly with cell-surface angiotensin-converting enzyme-2 (ACE2) as a receptor, and (2) non-structural-protein-1 (Nsp1) binds strongly with cellular protein immunophilins that have long been known to interact with tacrolimus (FK-506) and cyclosporine A. In rodent experiments over expression of Nsp1 derived from the SARS virus inhibited immune response and caused prolonged cytokine dysregulation[3], as has been seen clinically in both SARS and Covid-19 cases. Active Nsp1 is

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1 Marco Cascella; Michael Rajnik; Arturo Cuomo; Scott C. Dulebohn; Raffaela Di Napoli. Features, Evaluation and Treatment Coronavirus (COVID-19). StatPearls https://www.ncbi.nlm.nih.gov/books/NBK554776/.

2 Perico L, *et al.* Should COVID-19 Concern Nephrologists? Why and to What Extent? The Emerging Impasse of Angiotensin Blockade. Nephron 2020; 23:1-9. doi: 10.1159/000507305.

3 Pfefferle S, *et al*. The SARS-Coronavirus-Host Interactome: Identification of Cyclophilins as Target for Pan-Coronavirus Inhibitors. PLoS Pathog 2011; 7(10): e1002331. doi:10.1371/journal.ppat.1002331.

4 Srinivasen S, *et al*. Structural Genomics of SARS-CoV-2 Indicates Evolutionary Conserved Functional Regions of Viral Proteins. Viruses 2020, 12, 360. doi:10.3390/v12040360.

essential for CoV replication, and counter-intuitively CoV replication is blocked by tacrolimus and cyclosporine A[5], which are commonly used to inhibit transplant rejection.

Other than vaccine production against the spike protein, 3 potential therapeutic targets emerge from these molecular insights. They are

1. the viral RNA polymerase,
2. the interaction of the spike protein with ACE2 as its receptor, and
3. the interaction of Nsp1 with our protein immunophilins.

We will see that obstructing any of these 3 targets could stop the virus.

I also want to notice here that like all CoVs, Covid-19 is inactivated by ultraviolet light, heat, lipid solvents including soaps, 50% ethanol, chlorine-containing disinfectants, and peracetic acid - BUT NOT chlorhexidine.

The process by which Covid-19 gets into our cells

In general all pathological viruses enter our cells by a specific interaction with the cell surface. This interaction is highly conserved in families of viruses and reflects a long period of coevolution of viruses with hosts - in this case coronaviruses with mammals. So for example, influenza viruses bind to cell-surface sialic acids. Covid-19 in particular and the coronaviruses in general use their spike protein to bind to ACE2 as their receptor on the surface of cells. Hosts often make antibodies against the binding site on a viral surface. Viruses in turn mutate their binding site to escape antibodies, and this appears to have occurred with the Covid-19 spike protein as compared with that of the SARS and MERS coronaviruses[4]. However, these viral mutations do not suddenly leap to bind an alternative host surface receptor; the spike proteins of SARS, MERS, and Covid-19 all dock with the cell-surface ACE2 as their receptor. We will see later that this is an important aspect of Sorrento's "CovidTrap^{TM}" (designated STI-4398) therapeutic and passive-immunization approach that is entering clinical trial.

ACE2 is highly expressed in the mouth and tongue, facilitating viral entry in the host[2]. It is also expressed in the gut and kidneys, which may explain some of the clinical involvement of these organs. In our lower lungs, ACE2 is expressed on type I and II alveolar epithelial cells, which normally make surfactant protecting alveoli from collapse. After infection, Covid-19 entry starts by binding of the spike protein to ACE2 on the alveolar surface. This stimulates fusion at the cell membrane and clathrin-dependent endocytosis of the whole Covid-19-ACE2 complex in endosomes. This is facilitated by endosomal cysteine proteases that are dependent on a low pH, and raising endosomal pH can block the endocytosis of the virus. Once inside the cells, Covid-19 exploits the endogenous translational machinery to replicate.

Potential antiviral strategies

1. Block the virus from entering cells

a. Hydroxychloroquine

Chloroquine and hydroxychloroquine are well known to have *in vitro* and *in vivo* antiviral activity against a broad diversity of viruses, including coronaviruses[6]. It is thought that these antimalarial drugs exert a potent antiviral effect by virtue of ability to increase endosomal pH. Inside cells, hydroxychloroquine is rapidly protonated and concentrated in endosomes. The positive charge increases the organelle's pH and blocks virus-endosome fusion. Hydroxychloroquine, at

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[5] Carbajo-Lozoya J, *et al*. Replication of human coronaviruses SARS-CoV, HCoV-NL63 and HCoV-229E is inhibited by the drug FK506. Virus Res. 2012; 165:112-7. doi: 10.1016/j.virusres.2012.02.002.

[6] Devaux CA, *et al.* New insights on the antiviral effects of chloroquine against coronavirus: What to expect for COVID-19? Internat J Antimicrobial Agents 2020, doi.org/10.1016/j.ijantimicag.2020.105938.

concentrations consistent with those achieved clinically, inhibits Covid-19 replication in Vero cells[7,8] (derived from African green monkeys and commonly used in mammalian cellular research). These experimental findings are consistent with the very recent clinical report from Gautret *et al*[9] showing more rapid reduction of RT-PCR detectable Covid-19 in patients treated with hydroxychloroquine. Chen *et al.*[10] have also just published results of a controlled clinical trial involving 62 patients with ½ receiving 400 mg hydroxychloroquine daily. In this trial, time to clinical recovery and radiologic recovery from pneumonia were both significantly better in the treated group.

This treatment has become unfortunately politicized in the U.S. I am no Trump fan, and I view an unqualified politician making statements about medication use as combining ignorance with arrogance. Nevertheless, it is also true that bureaucratic hand-wringing in a pandemic emergency is at least as undesirable. There is a good and mature basic science rationale for this approach with an available, cheap, and relatively safe drug that we have considerable experience with. The basic science evidence is consistent with the early positive clinical reports, and we wouldn't be using hydroxychloroquine for Lupus patients if they were all dropping dead with Torsades. The objections are unconvincing, and several European countries and China have incorporated this approach into national guidelines. It is appropriate to continue using hydroxychloroquine as a treatment early in this disease.

b. ACE2-Fc

As discussed above, the slow course of coevolution has coronaviruses including Covid-19 committed to and stuck with their spike docking with ACE2 as their receptor on cells. This has led to the idea of administering a recombinant docking peptide derived from ACE2 to saturate the coronavirus spike and thereby prevent it from docking on cells[11]. Monteil *et al.*[12] have just reported from the prestigious Karolinska Institute that recombinant ACE2 can block Covid-19 infection in Vero cells and also in human vascular and kidney cells. There has also already been a Phase II clinical trial of recombinant ACE2 for ARDS[13] not involving CoV infections; the recombinant peptide was well tolerated but did not improve oxygenation. So we have the recombinant ACE2 peptide and evidence it can block Covid-19 infection and is well tolerated upon systemic administration. Furthermore, the affinity of recombinant ACE2 for the spike protein is ~1 nM, which is similar to the affinity of monoclonal antibodies[11].

We need to digress a moment to be reminded about the antibody structure of IgG. Please don't be insulted if you know this in your sleep. IgG is a dimmer shaped like a **Y**, with the antigen-binding sites at the ends of the 2 upper arms. Because even 50 years ago we knew that the

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7 Yao X, *et al.* In Vitro Antiviral Activity and Projection of Optimized Dosing Design of Hydroxychloroquine for the Treatment of Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2). Clin Infect Dis. 2020 Mar 9. pii: ciaa237. doi: 10.1093/cid/ciaa237.

8 Liu J, *et al.* Hydroxychloroquine, a less toxic derivative of chloroquine, is effective in inhibiting SARS-CoV-2 infection *in vitro*. Cell Discovery 2020; 6:16 doi.org/10.1038/s41421-020-0156-0.

9 Gautret *et al*. Hydroxychloroquine and azithromycin as a treatment of COVID-19: Results of an open-label non-randomized clinical trial. International J Antimicrobial Agents 2020; doi:10.1016/j.ijantimicag.2020.105949.

10 Chen Z et al. Efficacy of hydroxychloroquine in patients with COVID-19: results of a randomized clinical trial. https://www.medrxiv.org/content/10.1101/2020.03.22.20040758v2.

11 Kruse R. Therapeutic strategies in an outbreak scenario to treat the novel coronavirus originating in Wuhan, China. F1000Res. 2020; 9:72. doi: 10.12688/f1000research.22211.2.

12 Monteil V et al. Inhibition of SARS-CoV-2 infections in engineered human tissues using clinical-grade soluble human ACE2. DOI: 10.1016/j.cell.2020.04.004.

13 Khan A *et al*. A pilot clinical trial of recombinant human angiotensin-converting enzyme 2 in acute respiratory distress syndrome. Crit Care 2017; 21, 234. doi: 10.1186/s13054-017-1823-x.

complement system bound to the bottom single arm and that the lower arm could be removed by a protease, the proteolytic fragment constituted by the bottom arm got named the "Fc-fragment" for "fragment complement-binding."

Fusing an Fc to the recombinant ACE2 substantially extends its circulating ½ life; in mice this fusion extends the ½ life from ~2 hours to over 1 week. And an ACE2-Fc fusion still neutralizes SARS-CoV *in vitro* with an affinity in the 1 nM range[11]. Furthermore, the effector functions of the Fc domain can be retained in this chimerical molecule, allowing recruitment against viral particles of dendritic cells, macrophages, and natural killer cells through the CD16 receptor.

I love this approach. It's like Patton said about an enemy army, "We hold it by the nose and kick it in the ass." We know the fusion chimera doesn't kill mice from the ½ life study. The ACE2-Fc fusion uses all human proteins, so there's no reason it should elicit an immune response itself. The virus can't just mutate the spike and escape because it's committed to using ACE2 as its receptor. The ACE2-spike binding is really tight, and the stuff the Fc recruits is going to eat the bound virus and present the pieces with dendritic cells for antibody production.

This will confer immediate passive immunity and also bring about vaccination with dead virus fragments. And because it leverages the coronavirus commitment to ACE2 as a viral receptor, it should work for all the coronaviruses. Finally, because it's a recombinant, we can make it fast in buckets in whatever cells they have put the expression vectors in. There's the prophylaxis for the health care team. Go Sorrento "CovidTrap***TM***" (STI-4398) clinical trials! We could have this as soon as it passes a Phase 1 trial – maybe by the end of this summer.

2. *Target the viral RNA-dependent-RNA- polymerase*

Although some primitive eukaryotes (like *C. elegans*) retain RNA-dependent-RNA-polymerases, animals appear to have discarded the genes for these proteins. Thus, the coronavirus RNA-dependent-RNA-polymerase function is a tempting target with no human analog to worry about. Unfortunately this approach has been historically difficult with limited clinical effectiveness against HIV and Ebola.

Remdesivir was developed in the Ebola epidemic and interferes with viral RNA polymerases. It has shown efficacy against the MERS coronavirus in mouse models[11]. Remdesivir is an antiviral nucleotide analog with *in vitro* antiviral activity against a diverse panel of RNA viruses such as Ebola, Marburg, MERS-CoV, SARS-CoV, respiratory syncytial virus, and Hendra virus. The mechanism of Remdesivir action is competition with the adenosine nucleotide for incorporation into RNA transcripts produced by the RNA-dependent-RNA-polymerase[14], thereby causing premature termination of viral RNA transcripts. For Remdesivir to be effective against the aggressive coronaviruses in animal experiments, it needed to be given early well before viral titers reached their peak[15]. Although Remdesivir showed laboratory promise against Ebola, it failed to improve mortality in a clinical trial. Similarly, a Phase 2 Chinese clinical trial during their Covid-19 epidemic appears to have been terminated because other treatments were more effective[15].

Remdesivir is again in clinical trials against Covid-19, but there is still no published evidence it is clinically effective.

3. *Block interaction of Nsp1 with our protein immunophilins*

In this review I was surprised to learn that Nsp1 interaction with our protein immunophilins is necessary for efficient replication of Covid-19. A brief story behind that is that in 1997 our research group was working to understand failure of protein synthesis in vulnerable neurons during

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[14] Zhang L, and Zhou R. Binding Mechanism of Remdesivir to SARS-CoV-2 RNA Dependent RNA Polymerase. Preprints **2020**, 2020030267. doi: 10.20944/preprints202003.0267.v1.

[15] Cao Y *et al.* Remdesivir for severe acute respiratory syndrome coronavirus 2 causing COVID-19: An evaluation of the evidence. Travel Med Infectious Dis https://doi.org/10.1016/j.tmaid.2020.101647.

brain reperfusion after resuscitation from cardiac arrest. As part of that we presented 2 key papers at the 1997 Copenhagen meeting of the Society for Cerebral Blood Flow and Metabolism. Those papers showed the very early activation of the calpain-1 protease and identified the molecular alteration in translation initiation that obstructed reperfused neurons' protein synthesis. At that meeting another group presented evidence that treatment with FK-506 during reperfusion facilitated restoration of neuronal protein synthesis. That led us to go literature digging, and we learned that there are a group of proteins that bind FK-506 (aka tacrolimus); inevitably they were called FKBPs and are now known to be part of the group of immunophilins that also interact with cyclosporine-A. FKBP-12 interacted with tacrolimus to inhibit loss of the normal high calcium concentration in the endoplasmic reticulum (ER). By 2001 we had discovered that it was persistent ER calcium loss into neuronal cytoplasm that both promoted activation of the calpain-1 protease and also caused activation of an enzyme called PERK that shut off normal protein synthesis. So we had met the immunophilins.

Now it's been discovered that Nsp1 from Covid-19 (1) binds to immunophilins, (2) obstructs normal immune responses, and (3) binding of Nsp1 to immunophilins is essential for efficient Covid-19 replication[3-5]. Although all of this work has been done in cell cultures, these results probably provide the molecular explanation for the lymphopenia seen in Covid-19 patients and for the prognostic association of that lymphopenia with case outcomes. Furthermore, the dysregulation of cytokine response seen with vector-directed over-expression of Nsp1[3] is probably the explanation for the "cytokine storm" seen in dying Covid-19 patients.

Of course the main use of tacrolimus or cyclosporine-A is to suppress transplant rejection, and they do that through their interactions with the immunophilins. But it now also looks like tacrolimus and/or cyclosporine can probably knock Nsp1 off the immunophilins, and it is clear these drugs block "the replication of CoVs of all genera, including SARS-CoV, human CoV-229E and NL-63, and feline CoV[3]."[5]

Thus it is frustrating that in spite of hours of search effort, I can find no systematic reports of either laboratory or clinical intervention using tacrolimus or cyclosporine for coronavirus infections in experimental animals or patients. We might want to ask our friends on transplant and rheumatology services if any of their patients have gotten sick with Covid-19 while on either of these drugs.

I hope this review is not "research-opaque" and helps to expand your underlying understanding of this very dangerous epidemic that you are providing care for.