Prevention and Treatment of SARS-CoV-2, A Clinical Review

Abstract

We have learnt that although most human coronaviruses cause mild upper respiratory tract disease, they may be associated with severe multisystemic disease in all ages, particularly in immune-compromised individuals. The clinical experience with SARS-CoV-2 shows that CoV2 can present with multiple disease patterns ranging from mild upper respiratory tract illness to Severe Acute Respiratory Syndrome and Acute Respiratory Distress Syndrome in nearly 10% mortality; as a Systemic Coagulopathy, as a *Cytokine Storm* and as a Multisystem Organ Failure. In the absence of an effective medication or vaccine only those who have a strong natural resistance are expected to navigate the current pandemia. Further, a diminished immune system integrity and response caused by age, chronic metabolic, immunological, environmental and infectious illnesses greatly increase the risk of a severe disease and dismal outcome.

Developing therapeutic regimes for SARS-CoV-2 requires proper understanding of the disease(s) it causes. The pathogenesis of the virus, its genomics and structural biology and the host immune response pattern after infection. In addition to the knowledge on the structural makeup, a critical understanding of the life cycle and pathogenesis of SARS-CoV-2, is essential. Finally, the biochemistry and molecular pharmacology of the proposed drug.

The current challenge is conducting clinical trials on an urgent basis and determining the effectiveness and safety of different drugs alone or in combination.

The objectives of this clinical review are:

1.- Present to the medical community and the public a large another approach that should reverse the current perceived nihilism. The proposed solution invokes: a) the prevention of the latching of SARS-CoV-2 to the nasal epithelium and b) incrementing the immunity of the population at large, especially those most disadvantaged from the immunological point of view by the administration of Inosine Pranobex, that has along tract record of being both effective and safe.

2.- Suggest a comparative trial between two remarkable easy to obtain and inexpensive medications: Hydroxychloroquine and Inosine Pranobex.

3.- Present Zinc Gluconate as the ideal and very inexpensive prophylactic.

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BACKGROUND

Most human coronaviruses are responsible for about 5% to 10% of acute respiratory infections [**Chen Y** et al. ***Emerging coronaviruses: Genome structure, replication, and pathogenesis***. J. Med. Virol. 2020 Apr;92(4):418-423] usually mild (80%) but can be associated with more severe pulmonary disease especially in immuno-compromised individuals. There is ample evidence that viruses can mutate spontaneously imitating the biology of the host to ensure its propagation. This knowledge of viral evolution and the rates of divergence parallels those of the coronaviruses that preceded the current pandemic of SARS-CoV-2. Phylogenetic analysis of 2019-nCoV, the Severe Acute Respiratory Syndrome Corona Virus 2 (SARS-CoV-2), indicated that it is different but related to SARS- CoV-1 (~80% nucleotide identity) that appeared during the autumn of 2002 in the province of Guangdong, China spreading itself into 29 countries infecting 8,422 and killing 916 individuals. Despite their strong homology, the two coronaviruses have many differences at the Surface Glyco-Protein Region that interact with the cell receptor. The Severe Acute Respiratory Syndrome (SARS) coronavirus causes severe lower respiratory disease with nearly 10% mortality, additionally, it can cause multi-systemic organ dysfunction or failure. Deep sequencing revealed that this novel coronavirus isolated from lower respiratory tract samples of patients with SARS CoV-2 belongs to β-coronavirus (β-CoV) [**Zhu N** et al. ***China Novel Coronavirus Investigating and Research Team. A novel coronavirus from patients with pneumonia in China, 2019***. N Engl J Med 2020; 382(8): 727-733].

STRUCTURE AND LIFE CYCLE OF BETA CORONA VIRUS

SARS-CoV-2 is a member of the beta-corona virus genera and possesses 16 Non-Structural Proteins (NSP) among them Helicase, Main Protease (Mpro), Papain-Like Protease (PLpro), RNA Dependent RNA Polymerase (RdRp) that are critical for viral replication. [**Forster** et al., ***Phylogenetic network analysis of SARS-CoV-2 genomes.*** Proceedings of the National Academy of Sciences of the United States of America, 117, 9241-9243; **Siu** et al, ***The M, E, and N structural proteins of the severe acute respiratory syndrome coronavirus are required for efficient assembly, trafficking, and release of virus-like particles***. Journal of Virology, 82(22), 11318-11330]. Four Structural Proteins: Spike (S) Glyco-Proteins facilitate the attachment of the virus to their host cells, Envelope (E), Membrane (M), and Nucleocapsid (N) proteins that are implicated in crucial host cell functions [**Ashour**, et al. ***Insights into the recent 2019 novel coronavirus (SARS-CoV-2) in light of past human coronavirus outbreaks.*** Pathogens, 9(3), E186] The Nucleocapsid (N) protein clamps to the Ribo-Nucleic Acid (RNA) genome, whereas the S, E, and M proteins together shape the viral envelope, and the Glyco-Protein Spikes causes coronaviruses to form their unique crown-like appearance under electron microscopy [**Anthony** et al., ***Global patterns in coronavirus diversity.*** Virus Evolution, 3(1), vex012].

The coronaviruses are enveloped viruses with a single strand, positive-sense RNA genome, which is the largest known genome for an RNA virus (with an average diameter of 60 to 140 nm). Its single-stranded RNA genome contains 29,891 nucleotides, encoding for 9,860 amino acids. All coronaviruses share the same genome organization and expression pattern, with two large Overlapping Reading Frames (ORF1a) and (ORF1b) which encode 16 Non-Structural Proteins, (NSPs) followed by Overlapping Reading Frames for four major Structural Proteins: Spike (S), Envelope (E), Membrane (M), and Nucleocapsid (N) [**Forni D** et al. ***Molecular evolution of human***

coronavirus genomes. Trends Microbiol 2017; 25(1): 35-48]. It was found that Angiotensin-Converting Enzyme 2 (ACE2) was the main receptor for SARS-CoV-2.

In normal human lung, ACE2 is expressed on type I and II alveolar epithelial cells. Among them, 83% of the type II alveolar cells have ACE2 expression. Men had a higher ACE2 level in their alveolar cells than women. [**Zhao Y**, et al. ***Single-cell RNA expression profiling of ACE2, the putative receptor of Wuhan 2019-nCov***. bioRxiv. 2020. 10.1101/2020.01.26.919985] ACE2, a member of the Angiotensin-Converting Enzyme (ACE) family of dipeptidyl-carboxy-dipeptidase, is highly homologous to ACE1. ACE1 and ACE2 convert Angiotensin 1 into Angiotensin (Ang 1-9) and Angiotensin 2 into (Ang 1-7). ACE2 has a high affinity to Ang II type 1 and type 2 receptors and plays an important role in cell proliferation and hypertrophy, inflammatory response, blood pressure, and fluid balance. ACE2 is highly expressed in certain organs and tissues, suggesting that it plays an important role in regulating cardiovascular, renal, and reproductive functions. [**Turner A.J.** Chapter 25 - ***ACE2 Cell Biology, Regulation, and Physiological Functions;*** **Unger T**, Steckelings UM, dos Santos RAS, editors. ***The Protective Arm of the Renin-Angiotensin System (RAS)*** Academic Press; Boston: 2015. pp. 185-189; **Richards E.M.** ***ACE2 and pACE2: A Pair of Aces for Pulmonary Arterial Hypertension Treatment?*** Am J Resp Crit Care. 2018;198(4):422-423] In a phylogenetic analysis of 103 strains of SARS-CoV-2 from China, two different types of SARS-CoV-2 were identified, designated type L (accounting for 70 percent of the strains) and type S (accounting for 30 percent). The L type predominated during the early days of the epidemic in China but accounted for a lower proportion of strains outside of Wuhan. [**Tang X*, et al. On the origin and continuing evolution of SARS-CoV-2***.]

Crucial Steps of SARS-CoV-2 Life Cycle

1. Receptor Recognition is the initial step of SARS-CoV-2 virus entry into the host cell that occurs by the recognition of the receptor by the Spike Protein of the virus. The Receptor-Binding Domain (RBD) of S Protein attaches with the host cell receptor: The Angiotensin-Converting Enzyme 2 (ACE2). Fusion with, and subsequent entry into, the host cell is one of the critical steps in the life cycle of enveloped viruses. Proteolytic cleavage of the S protein exposes its Fusion Peptide, which initiates the process of membrane fusion. Next, the S Protein is proteolytically cleaved by a Type II Trans-Membrane Protease (TMPRSS2) resulting into two subunits called S1 and S2 where S2 is crucial for membrane fusion and virus entry [**Glowacka** et al. ***Evidence that TMPRSS2 activates the Severe Acute Respiratory Syndrome coronavirus Spike protein for membrane fusion and reduces viral control by the humoral immune response.*** Journal of Virology, 85(9), 4122-4134; **Holmes,** ***SARS-associated coronavirus.*** The New England Journal of Medicine, 348(20), 1948-1951; **Li.** R***eceptor recognition mechanisms of coronaviruses: A decade of structural studies***. Journal of Virology, 89(4), 1954-1964; **Li** et al. ***Conformational states of the Severe Acute Respiratory Syndrome coronavirus spike protein ectodomain.*** Journal of Virology, 80(14), 6794-6800; **Wan**, et al. ***Receptor recognition by the novel coronavirus from Wuhan: An analysis based on decade-long structural studies of SARS coronavirus.*** Journal of Virology, 94(7); **Wong,** et al. ***A 193-amino acid fragment of the SARS coronavirus S protein efficiently binds angiotensin-converting enzyme 2***. The Journal of Biological Chemistry, 279(5), 3197-3201]. Splitting of the S protein by proteases is essential for the viral entry to the host cells in a pH-dependent manner [**Simmons** et al. ***Characterization of Severe Acute Respiratory Syndrome-associated coronavirus (SARS-CoV) spike glycoprotein-mediated viral entry***. Proceedings of the National Academy of Sciences of the United States of America, 101(12), 4240-4245; **Yang & Shen**, ***Targeting the Endocytic pathway and autophagy process as a novel therapeutic strategy***

in COVID-19. International Journal of Biological Sciences, 16(10), 1724-1731). (SARS-CoV] It was shown that calcium ions (Ca2+) play an important role in fusogenic activity of the Fusion Peptide via a Ca2+ binding pocket with conserved glutamic acid (E) and aspartic acid (D) residues. [**Straus MR**. ***Ca ions promote fusion of Middle East Respiratory Syndrome Coronavirus with host cells and increase infectivity***. J Virol 2020 Ju 16; 94 (13)]

2. After uncoating, the coronavirus then releases its RNA (5′ methylated cap and a 3′ polyadenylated tail) into the host cell cytoplasm for replication [**Fehr & Perlman**, ***Coronaviruses: An overview of their replication and pathogenesis***. Methods in Molecular Biology, 1282, 1-23; **Kim** et al., ***The architecture of SARS-CoV-2 transcriptome***. bioRxiv 2020.2003.2012.988865; **Li**, ***Receptor recognition mechanisms of coronaviruses: A decade of structural studies***. Journal of Virology, 89(4), 1954-1964].

3. Genomic RNA of the coronavirus acts as a Messenger RNA (mRNA) for translation of the Replicase Poly-Proteins 1a (pp1a) and 1ab (pp1ab) using the translation machinery of the host cell. Then the Poly-Proteins are split into effector proteins by viral proteinases (3CLpro and PLpro), resulting in a number of Non-Structural Proteins (NSPs) including RNA Dependent RNA Polymerase (RdRp) and Helicase that form the Replicase-Transcriptase Complex [**Báez-Santos**, et al. ***The SARS-coronavirus papain-like protease: Structure, function, and inhibition by designed antiviral compounds.*** Antiviral Research, 115, 21-38; **Fehr & Perlman**, ***Coronaviruses: An overview of their replication and pathogenesis***. Methods in Molecular Biology, 1282, 1-23; **Gorbalenya** et al., S***evere acute respiratory syndrome-related coronavirus: The species and its viruses-A statement of the Coronavirus Study Group.*** bioRxiv 2020.2002.2007.937862; **Kim** et al. ***The architecture of SARS-CoV-2 transcriptome***. bioRxiv 2020.2003.2012.988865; **Ratia** et al., ***Severe acute respiratory syndrome coronavirus papain-like protease: Structure of a viral deubiquitinating enzyme***. Proceedings of the National Academy of Sciences of the United States of America, 103(15), 5717-5722; **Sola, Almazán**, et al. ***Continuous and discontinuous RNA synthesis in coronaviruses***. Annual Review of Virology, 2(1), 265-288].

4. RNA Dependent RNA Polymerase (RdRp) and Helicase localize to double-membrane vesicles and produce sub-genomic RNA (sgRNA), from which other proteins such as Structural Proteins are produced by translation. At the same time, the full-length positive-strand RNA is synthesized as genomic RNA [**Fehr & Perlman**, ***Coronaviruses: An overview of their replication and pathogenesis.*** Methods in Molecular Biology, 1282, 1-23; **Gordon CJ.** et al., ***Remdesivir is a direct-acting antiviral that inhibits RNA-dependent RNA polymerase from severe acute respiratory syndrome coronavirus 2 with high potency***. Journal of Biological Chemistry; **Sola** et al., ***Continuous and discontinuous RNA synthesis in coronaviruses.*** Annual Review of Virology, 2(1), 265-288; **Kim** et al., ***The architecture of SARS-CoV-2 transcriptome.*** bioRxiv 2020.2003.2012.988865].

5. Non-Structural Proteins 3, 4, and 6 (NSP3, NSP4 & NSP6) are responsible for anchoring the coronavirus replication and transcription complex through recruitment of intracellular Endoplasmic Reticulum (ER) Membrane to form double-membrane vesicles [**Fehr & Perlman**, ***Coronaviruses: An overview of their replication and pathogenesis***. Methods in Molecular Biology, 1282, 1-23; **Sola** et al. ***Continuous and discontinuous RNA synthesis in coronaviruses.*** Annual Review of Virology, 2(1), 265-288; **Stertz** et al., ***The intracellular sites of early replication and budding of SARS-corona virus.*** Virology, 361(2), 304-315].

6. Viral Nucleocapsids are assembled with genomic RNA and N protein in the cytoplasm, followed by budding into the lumen of the Endoplasmic Reticulum-Golgi Intermediate Compartment (ERGIC) [**Sola** et al., ***Continuous and discontinuous RNA synthesis in coronaviruses***. Annual Review

of Virology, 2(1), 265-288; **Stertz** et al. ***The intracellular sites of early replication and budding of SARS-corona virus.*** Virology, 361(2), 304-315].

7. The virions are then released from the cell through exocytosis (**Sola** et al., ***Continuous and discontinuous RNA synthesis in coronaviruses.*** Annual Review of Virology, 2(1), 265-288; **Stertz** et al., ***The intracellular sites of early replication and budding of SARS-corona virus.*** Virology, 361(2), 304-315).

SARS-CoV-2 VIRAL COMPONENTS

NON-STRUCRAL PROTEINS

1. Main Protease (Mpro)

Two large overlapping Replicase Poly-Proteins (pp1a and pp1ab), encoded by two linked ribosomal frameshift, are hydrolyzed by an extensive proteolytic action of viral proteases [**Fehr & Perlman.** ***Coronaviruses: An overview of their replication and pathogenesis.*** Methods in Molecular Biology, 1282, 1-23; **Graham,** et al. ***SARS coronavirus replicase proteins in pathogenesis***. Virus Research, 133(1), 88-100; **Pillaiyar**, et al. ***An overview of severe acute respiratory syndrome-corona virus (SARS-CoV) 3CL protease inhibitors: Peptidomimetics and small molecule chemotherapy***. Journal of Medicinal Chemistry, 59(14), 6595-6628]. One of the crucial viral proteases is the Mpro, also known as 3C-like Proteinase (3Clpro) or NSP5, which is a homodimeric cysteine protease belonging to the coronavirus Poly-Protein group [**Fan** et al. ***Biosynthesis, purification, and substrate specificity of severe acute respiratory syndrome coronavirus 3C-like proteinase.*** The Journal of Biological Chemistry, 279(3), 1637-1642]. Automatic cleavage of Poly-Proteins leads to the generation of Mpro, which cleaves the large Poly-Protein 1ab at 11 cleavage sites to generate Non-Structural Proteins (NSPs). The identification of cleaving sequence at most sites is Leu-Gln↓(Ser, Ala, Gly) to release Non-Structural Proteins 4-16 (NSP4-NSP16) [**Pillaiyar** et al. ***An overview of Severe Acute Respiratory Syndrome-coronavirus (SARS-CoV) 3CL protease inhibitors: Peptidomimetics and small molecule chemotherapy.*** Journal of Medicinal Chemistry, 59(14); **Yang** et al., ***Design of wide-spectrum inhibitors targeting coronavirus main proteases***. PLoS Biology, 3(10); **Zhang**, et al. ***Angiotensin-converting enzyme 2 (ACE2) as a SARS-CoV-2 receptor: Molecular mechanisms and potential therapeutic target.*** Intensive Care Medicine, 46(4), 586-590]. Mpro directly mediates the maturation of Non-Structural Proteins (NSPs), which is critical for the initiation of the SARS-CoV-2 replication cycle. [**zówka** et al. ***Molecular dynamics simulations indicate the COVID-19 Mpro is not a viable target for small-molecule inhibitors design***. bioRxiv 2020.2002.2027.968008].

2. Papain like Protease

Proteolytic enzymes play an essential role in viral replication. Papain like Protease (PLpro), also termed as Non-Structural Protein 3 (NSP3) cleaves the N-terminal part of the Poly-Proteins producing mature Non-Structural Proteins 1, 2 & 3 (NSP1, NSP2, and NSP3), a process that is crucial for viral replication. The cleavage specificity of Papain like Protease (PLpro), corresponds to the pattern (R/K) L(R/K)GG↓X. Moreover, the enzyme acts as deubiquitinase that removes (poly) ubiquitin units from proteins tagged with them [**Barretto** et al. ***The papain-like protease of Severe Acute Respiratory Syndrome coronavirus has deubiquitinating activity.*** Journal of Virology, 79(24), 15189-15198; **Lindner** et al. ***The papain-like protease from the Severe Acute Respiratory Syndrome coronavirus is a deubiquitinating enzyme.*** Journal of Virology, 79(24), 15199-15208]. The deubiquitinase activity of Papain like Protease (PLpro) interferes nuclear import of interferon-

regulatory factor 3 and the phosphorylation, thereby preventing the production of type-I interferons, such as IFN-α and IFN-β by the infected host cell and also helps in peptide substrate recognition [**Barretto** et al. ***The papain-like protease of Severe Acute Respiratory Syndrome coronavirus has deubiquitinating activity.*** Journal of Virology, 79(24), 15189-15198; **Clementz** et al. ***Deubiquitinating and interferon antagonism activities of coronavirus papain-like proteases.*** Journal of Virology, 84(9), 4619-4629; **Devaraj** et al. ***Regulation of IRF-3-dependent innate immunity by the papain-like protease domain of the Severe Acute Respiratory Syndrome coronavirus.*** The Journal of Biological Chemistry, 282(44), 32208-32221; **Frieman**, et al. ***Severe Acute Respiratory Syndrome coronavirus papain-like protease ubiquitin-like domain and catalytic domain regulate antagonism of IRF3 and NF-kappaB signaling***. Journal of Virology, 83(13), 6689-6705; **Ratia** et al. ***Severe Acute Respiratory Syndrome coronavirus papain-like protease: Structure of a viral deubiquitinating enzyme***. Proceedings of the National Academy of Sciences of the United States of America, 103(15), 5717-5722]. Additionally, Papain like Protease (PLpro), clears ubiquitin, and interferon-sensitive gene 15 from host-cell proteins, a mechanism that aids coronaviruses to evade host innate immune responses. Finally, the Papain like Protease (PLpro), has been reported to interfere with the nuclear factor κB pathway, which further assists SARS-CoV to counteract the innate immune response of the infected cells [**Clementz** et al. ***Deubiquitinating and interferon antagonism activities of coronavirus papain-like proteases.*** Journal of Virology, 84(9), 4619-4629].

3. RNA Dependent RNA Polymerase

RNA dependent RNA polymerase (RdRp), also termed NSP12, is a pivotal enzyme of RNA viruses, including coronavirus, that catalyzes the replication of RNA from an RNA template, probably with the assistance of NSP7 and NSP8 as co-factors [**Guo**, et al. ***Coronavirus disease 2019 (COVID-19) and cardiovascular disease: A viewpoint on the potential influence of angiotensin-converting enzyme inhibitors/angiotensin receptor blockers on onset and severity of Severe Acute Respiratory Syndrome coronavirus 2 infection.*** Journal of the American Heart Association, 9(7); **Kirchdoerfer & Ward**. *Structure of the SARS-CoV NSP12 polymerase bound to NSP7 and NSP8 co-factors.* Nature Communications, 10(1), 2342 ; **Subissi** et al. ***SARS-CoV ORF1b-encoded non structural proteins 12-16 : Replicative enzymes as antiviral targets.*** Antiviral Research, 101, 122-130; **Venkataraman**, et al. ***RNA dependent RNA polymerases: Insights from structure, function, and evolution***. Viruses, 10(2)].

A protein complex consisting of NSP7, NSP8, and NSP12 of SARS-CoV-2 is related to that of SARS-CoV with a root-mean-square deviation value of 0.82 for 1,078 Cα atoms [**Gao** et al. ***Structure of the RNA-dependent RNA polymerase from COVID-19 virus***. Science, 368, 779-782]. RNA dependent RNA polymerase (RdRp) protein of SARS-CoV-2 and SARS-CoV share 96% amino acid sequence identity and 82% sequence identity at their genomic RNA level [**Morse**, et al. ***Learning from the past: Possible urgent prevention and treatment options for severe acute respiratory infections caused by 2019-nCoV.*** ChemBioChem, 21(5), 730-738]. The RNA dependent RNA polymerase (RdRp) of SARS-CoV-2 contains a large and deep groove as an active site for the polymerization of RNA. An Adenosine analog can be incorporated into nascent viral RNA, and subsequently inhibit the tRNA dependent RNA polymerase (RdRp), [**Siegel** et al. ***Discovery and synthesis of a Phosphoramidate Prodrug of a Pyrrolo[2,1-f][triazin-4-amino] adenine C-nucleoside (GS-5734) for the treatment of Ebola and emerging viruses.*** Journal of Medicinal Chemistry, 60(5), 1648-1661].

4. Helicase

SARS-CoV-2 Helicase (NSP13) is generated after the proteolytic cleavage of two large polyproteins (pp1a and pp1ab). This Helicase, being one of the components of the Replicase-Transcriptase Complex, plays significant roles in the life cycle of SARS-CoV-2 [**Marra** et al. ***The genome sequence of the SARS-associated coronavirus.*** Science, 300(5624), 1399-1404; **Subissi** et al. ***SARS-CoV ORF1b-encoded nonstructural proteins 12-16: Replicative enzymes as antiviral targets***. Antiviral Research, 101, 122-130]. Helicases are motor proteins that unwind double-stranded nucleic acids into two single-strands during replication [**Adedeji** et al. ***Evaluation of SSYA10-001 as a replication inhibitor of Severe Acute Respiratory Syndrome, mouse hepatitis, and Middle East respiratory syndrome coronaviruses.*** Antimicrobial Agents and Chemotherapy, 58(8), 4894-4898; **Shum & Tanner.** ***Differential inhibitory activities and stabilization of DNA aptamers against the SARS coronavirus helicase***. ChemBioChem, 9(18), 3037-3045]. Moreover, NSP13 N-terminus carries 26 cysteines where 14 positions are highly conserved and assumed to possess a binuclear Zn2+-binding cluster [**Seybert** et al. ***A complex zinc finger controls the enzymatic activities of nidovirus helicases.*** Journal of Virology, 79(2), 696-704].

STRUCTURAL PROTEINS

1. Spike Glycoprotein

The Spike Protein (S), giving a crown-like appearance to virion surface, render diverse molecular actions that usually mediates the coronavirus entry into the specific host [**Belouzard**, et al. ***Activation of the SARS coronavirus spike protein via sequential proteolytic cleavage at two distinct sites***. Proceedings of the National Academy of Sciences of the United States of America, 106(14), 5871-5876]. The Spike Protein of SARS-CoV and SARS-CoV-2 shares ~76% amino acid identity; however, SARS-CoV-2 S Protein binds 10 times more strongly than SARS-CoV S protein to their common host cell receptor [**Du** et al. ***The Spike protein of SARS-CoV-A target for vaccine and therapeutic development.*** Nature Reviews. Microbiology, 7(3), 226-236; **Hoffmann** et al. ***SARS-CoV-2 cell entry depends on ACE2 and TMPRSS2 and is blocked by a clinically proven protease inhibitor***. Cell, 181(2), 271-280; **Li.** ***Structure, function, and evolution of coronavirus spike proteins.*** Annual Review of Virology, 3(1), 237-261; **Tortorici** et al. ***Structural basis for human coronavirus attachment to sialic acid receptors.*** Nature Structural & Molecular Biology, 26(6), 481-489; **Wrapp** et al. ***Cryo-EM structure of the 2019-nCoV spike in the prefusion conformation.*** Science, 367(6483), 1260-1263]. Heavily glycosylated, the Spike Protein possesses a large ectodomain, a single-pass transmembrane anchor, and a short intracellular tail [**Li** ***Structure, function, and evolution of coronavirus spike proteins***. Annual Review of Virology, 3(1), 237-261). The ectodomain consists of a receptor-binding subunit (S1) and a membrane-fusion subunit (S2) (**Broer**, et al. ***Important role for the transmembrane domain of Severe Acute Respiratory Syndrome coronavirus Spike protein during entry.*** Journal of Virology, 80(3), 1302-1310; **Li.** Annual Review of Virology, 3(1), 237-261]. During virus entry, the S1 subunit recognizes and interacts to host receptors, and further conformational changes in the S2 subunit assist the fusion between the viral envelope protein and the host cell membrane [**Du** et al. Nature Reviews. Microbiology, 7(3), 226-236; **Li**. Annual Review of Virology, 3(1), 237-261; **Tortorici** et al. Nature Structural & Molecular Biology, 26(6), 481-489].

The S1 subunit has two major domains: The N-Terminal Domain (NTD) and the C-Terminal Domain (CTD). Depending on the coronavirus type, either C Terminal Domain or N Terminal Domain can function as the Receptor Binding Domain and bind with diverse proteins and sugars

[**Li.** Annual Review of Virology, 3(1), 237-261]. Notably, SARS-CoV-2 and MERS-CoV-1 utilize C Terminal Domain to bind their receptors [**Kubo** et al. ***Localization of neutralizing epitopes and the receptor-binding site within the amino-terminal 330 amino acids of the murine coronavirus Spike protein***. Journal of Virology, 68(9), 5403-5410; **Li** et al. ***Receptor and viral determinants of SARS-coronavirus adaptation to human ACE2***. The EMBO Journal, 24(8), 1634-1643; **Ou** et al., ***Characterization of spike glycoprotein of SARS-CoV-2 on virus entry and its immune cross-reactivity with SARS-CoV.*** Nature Communications, 11(1), 1620; **Wang N**. et al. ***Structure of MERS-CoV spike receptor-binding domain complexed with human receptor DPP4***. Cell Research, 23(8), 986-993]. Then, the proteases found in the cellular environment cause the cleavage of S Proteins, which ensures viral fusion. SARS-CoV-2 possesses a furin cleavage site between S1 and S2 subunit that permits efficient cleavage by proteases, including furin that determines viral infectivity and host range [**Andersen** et al. ***The proximal origin of SARS-CoV-2.*** Nature Medicine, 26(4), 450-452; **Coutard** et al. ***The spike glycoprotein of the new coronavirus 2019-nCoV contains a furin-like cleavage site absent in CoV of the same clade***. Antiviral Research, 176, 104742; **Ou** et al. ***Characterization of spike glycoprotein of SARS-CoV-2 on virus entry and its immune cross-reactivity with SARS-CoV.*** Nature Communications, 11(1), 1620; **Walls** et al. ***Structure, function, and antigenicity of the SARS-CoV-2 spike glycoprotein.*** Cell, 181(2), 281-292) coronaviruses of the same clade **(Coutard** et al. Antiviral Research, 176, 104742].

The S Protein also activates the immune response of the host cell toward coronaviruses [**Dosch** et al. ***SARS coronavirus spike protein-induced innate immune response occurs via activation of the NF-kappaB pathway in human monocyte macrophages in vitro.*** Virus Research, 142(1-2), 19-27; **Walls** et al. ***Structure, function, and antigenicity of the SARS-CoV-2 Spike glycoprotein.*** Cell, 181(2), 281-292]. Prior studies revealed that peptide inhibitors of SARS-CoV-2 S Protein block the S protein interaction with ACE2 and thus efficiently preclude the virus entry into human cells. Moreover, these inhibitors can be used as inhaled therapeutics, preventing the virus activation in the lungs [**Ou** et al. ***Characterization of spike glycoprotein of SARS-CoV-2 on virus entry and its immune cross-reactivity with SARS-CoV.*** Nature Communications, 11(1), 1620; **Walls** et al. ***Structure, function, and antigenicity of the SARS-CoV-2 Spike glycoprotein.*** Cell, 181(2), 281-292]. Recently, **Wan** [et al. ***Receptor recognition by the novel coronavirus from Wuhan: An analysis based on decade-long structural studies of SARS coronavirus.*** Journal of Virology, 94(7)] and **Tai** [et al. ***Characterization of the receptor-binding domain (RBD) of 2019 novel coronavirus: Implication for development of RBD protein as a viral attachment inhibitor and vaccine.*** Cellular & Molecular Immunology, 17, 613-620] sequenced and characterized Receptor Binding Domain of SARS-CoV-2, including its Receptor-Binding Motif (RBM) that directly associates with ACE2.

2. Envelope Protein

Envelope Protein (E) of coronavirus is a short hydrophobic membrane protein (a polypeptide integral membrane protein) involved in the assembly, budding, envelope formation, and pathogenesis [**Masters**. ***The molecular biology of coronaviruses.*** Advances in Virus Research, 66, 193-292]. It has been reported that the absence or inactivation of E protein could impact either virion morphology or tropism [**Khattari** et al. ***SARS coronavirus E protein in phospholipid bilayers: An x-ray study.*** Biophysical Journal, 90(6), 2038-2050; **Pervushin** et al., ***Structure and inhibition of the SARS coronavirus envelope protein ion channel.*** PLoS Pathogens, 5(7), e1000511]. The SARS-CoV E protein is more selective for Na+ than for K+ ions, and 90 times more selective for Na+ than Cl− ions and thus have membrane permeabilizing property [**Wilson,** et al. ***SARS coronavirus E protein forms***

cation-selective ion channels. Virology, 330(1), 322-331]. SARS-CoVs lacking E protein show lower viral titer, immature, and inefficient progenies [**Kuo**, et al. ***Exceptional flexibility in the sequence requirements for coronavirus small envelope protein function.*** Journal of Virology, 81(5), 2249-2262].

SUMMARY OF SARS-CoV-2 INFECTIVE MECHANISMS

Starting from the viral RNA, the synthesis of polyprotein 1a/1ab (pp1a/pp1ab) in the host is achieved. The transcription works through the Replication-Transcription Complex (RCT) organized in double-membrane vesicles via the synthesis of sub-genomic RNAs (sgRNAs) sequences. Transcription termination occurs at Transcription Regulatory Sequences, located between the Open Reading Frames that work as templates for the production of sub-genomic mRNAs. A frameshift between ORF1a and ORF1b guides the production of both polyproteins 1a/1ab (pp1a/pp1ab), polypeptides that are processed by virally encoded Chymotrypsin-Like protease (3CLpro) or Main protease (Mpro), as well as one or two Papain-like Proteases for producing 16 Non-Structural Proteins (NSPs). Other Open Reading Frames encode for structural proteins, including Spike, Membrane, Envelope, and Nucleocapsid proteins and accessory proteinic chains. Different coronaviruses present special structural and accessory proteins translated by dedicated sub-genomic RNAs. [**Perlman S** et al. ***Coronaviruses post-SARS: update on replication and pathogenesis.*** Nat. Rev. Microbiol. 2009 Jun;7(6):439-50]

Pathophysiology and virulence mechanisms of coronaviruses and of SARS-CoV-2 have links to the function of the Non-Structural Proteins and Structural Proteins. Non-Structural Proteins are able to block the host innate immune response. Among the functions of structural proteins, the Envelope has a crucial role in virus pathogenicity as it promotes viral assembly and release. [**Lei J**, et al. ***NSP3 of coronaviruses: Structures and functions of a large multi-domain protein.*** Antiviral Res. 2018 Jan; 149:58-74]. Among the structural elements of coronaviruses, the Spike Glyco-Proteins composed of two subunits (S1 and S2) on the viral surface, guide the link to host receptors. [**Song W,** et al. ***Cryo-EM structure of the SARS coronavirus spike glycoprotein in complex with its host cell receptor ACE2.*** PLoS Pathog. 2018 Aug;14(8): e1007236] The S-glycoprotein functional receptors for SARS-CoV are encoded by the ACE2 gene. In SARS-CoV-2, the S2 subunit - containing a Fusion Peptide, a Trans-Membrane Domain, and Cytoplasmic Domain - is highly conserved. On the other hand, the Spike Receptor-Binding Domain presents only a 40% amino acid identity with other SARS- CoVs.

According to recent research, a Spike mutation, which probably occurred in late November 2019, triggered jumping to humans. Angeletti et al. compared the SARS-CoV -2 gene sequence with that of SARS-CoV. They analyzed the transmembrane helical segments in the ORF1ab encoded Non-Structural Proteins 2 (NSP2) and Non-Structural Proteins 3 (NSP3) and found that position 723 presents a serine instead of a glycine residue, while the position 1010 is occupied by proline instead of isoleucine.[**Angeletti S** et al. ***COVID-2019: The role of the NSP2 and NSP3 in its pathogenesis.*** J. Med. Virol. 2020 Feb 21] The matter of viral mutations is key for explaining potential disease relapses.

PATHOPHYSIOLOGY

SARS-CoV-2 binds to ACE2 expressed on the nasal mucosal epithelial cell, and in approximately 96 hours enters human lower respiratory epithelial cells. ACE2 is abundantly expressed in the

lungs (males > females; adults > children 10 years of age and below) and small intestine and is highly expressed in endothelial cells and smooth muscle cells of virtually all organs (stomach, colon, skin, lymph nodes, thymus, bone marrow, spleen, liver, kidney, and brain).Therefore, once in the circulatory system, SARS- CoV-2 is likely to spread via blood flow. Further, ACE2 is highly expressed in renal tubular cells, mesenchymal cells, and testicular and vas deferens cells. Surprisingly, ACE2 mRNA and protein levels are higher in testis than in any other organ. [**Fan C.** et al. ***ACE2 Expression in Kidney and Testis May Cause Kidney and Testis Damage After 2019-nCoV Infection.*** 2020.02.12.20022418] SARS-CoV-2 does not attack T cells, nor CD4+ cells. SARS-CoV-2 can migrate after infecting sensory or motor nerve endings under the action of motor proteins, Dynein and Kinesins, can achieve neuronal transport retrograde or anterograde. Based on the unique anatomical structure of olfactory nerves and olfactory bulb, it becomes a channel between the nasal epithelium and the CNS. [**Wu Y**. et al. ***Nervous system involvement after infection with COVID-19 and other coronaviruses.*** Brain Behav Immun. 2020]

Once latched onto the nasal respiratory cell receptors the SARS-CoV2 engineers its fusion with the cell membranes and the viral RN is released intracellularly, followed by its translation and formation of a replication complex that produce more viral particles, which are then packaged in the Golgi Apparatus of the cell and finally released to the intracellular space to renew its attack on other cells.

Based on current clinical and epidemiological data, the clinical symptoms of SARS-CoV-2 infection vary a great deal from patient to patient. The virus first affects the respiratory epithelial cells and alveolar cells, followed by the digestive system, [**Yuhao Zhang**, et al. ***New Understanding of the Damage of SARS-CoV-2 Infection Outside the Respiratory System***. Biomed Pharmacother. 2020 Jul; 127:110195. Epub 2020 Apr 28.]

The data available indicate that viral infections are capable of producing, in some, an excessive immune reaction in the host labeled a Cytokine Storm, that causes an acute systemic inflammatory syndrome characterized by fever and multiple organ dysfunction. The Macrophage Activating Syndrome comprises a heterogeneous group of life-threatening disorders featuring excessive activation of T cells and macrophages, leading to a Cytokine Storm. It is the Neutrophil Extracellular Traps that consistently stimulate IL-8, TNF-α, and IL-1α secretion by human bronchial epithelia [**Hudock KM** et al. ***Neutrophil extracellular traps activate IL-8 and IL-1 expression in human bronchial epithelia.*** Am J Physiol Lung Cell Mol Physiol. 2020 Mar 11]. Although the protagonist of this Cytokine Storm initially seemed to be Inter-Leukin 6 (IL-6), produced by activated leukocytes in response to inflammatory diseases, infections, autoimmune disorders, cardiovascular diseases and some types of cancer. IL-1 is secreted hours before IL-6 is capable of inducing both IL-6 secretion and soluble IL-6 receptor the release of TNF-α and although shown to play a role in the pathogenesis of Cytokine Release Syndrome, it has been associated with exceptionally high serum levels of free IL-18 with concomitant hyper-ferritinemia, reflecting an IL-18/IL-18BP imbalance [**Jeroen Slaats,** et al. ***IL-1β/IL-6/CRP and IL-18/ferritin: Distinct Inflammatory Programs in Infections*** PLoS Pathog. 2016 Dec; 12(12): e1005973] Although the main effects of IL-6 are pro-inflammatory, acting on a large number of cells and tissues promoting the differentiation of B lymphocytes, stimulating the production of acute-phase proteins and affecting thermoregulation, bone maintenance and the functionality of the central nervous system, IL-6 can also have anti-inflammatory effects.

Jie-ying Liao et al. in 2011 showed that during viral infections, single-stranded RNA (ssRNA) and double-stranded RNA (dsRNA) are recognized by the host and induce innate immune responses.

The cellular enzyme ADAR-1 (Adenosine Deaminase Acting on RNA-1) activation in virally infected cells leads to the presence of Inosine-containing RNA (Ino-RNA). In primary human cells, 10% of Inosine-containing RNA (Ino-RNA) rapidly and potently induced a significant increase in inflammatory cytokines, such as InterFeron (IFN)-β (35 fold), Tumor Necrosis Factor (TNF)-α (9.7 fold), and interleukin (IL)-6 (11.3 fold) ($p<0.01$). A corresponding 4-fold increase in the influx of neutrophils into the lungs by Inosine-containing RNA (Ino-RNA). Further, the extracellular of Inosine-containing RNA (Ino-RNA) was taken up by Scavenger Receptor class-A (SR-A) which activated downstream MAP Kinase pathways through Toll-Like Receptor 3 (TLR3) and dsRNA-activated Protein Kinase (PKR). [**Jie-ying Liao** et al. ***Inosine-Containing RNA Is a Novel Innate Immune Recognition Element and Reduces RSV Infection.*** PLOS ONE, Oct 2011]

HOST CELL ELEMENTS

1. Angiotensin-Converting Enzyme 2

ACE2 is a type I membrane protein, which is expressed in the heart, lungs, kidneys, and intestine. An N-terminal Peptidase Domain (PD) and a C terminal Collectrin-Like Domain (CLD) are usually found in a full-length ACE2 [**Donoghue** et al. ***A novel angiotensin-converting enzyme-related carboxypeptidase (ACE2) converts angiotensin I to angiotensin 1-9.*** Circ Res. 2000 Sep 1;87(5): E1-9. PMID: 10969042 2000]. A recent report demonstrated that the ectodomain of the SARS-CoV-2S protein interacts with the Peptidase Domain of ACE2 [**Wrapp** et al. PMID: 32075877, 2020]. SARS-CoV-2 virus exploits the ACE2 receptor to gain entry into host cells. Human cell-derived proteases cleave SARS-CoV-2 S Protein into S1 and S2, where S1 initially interacts its receptor molecule ACE2, and the other fragment, S2, further leads to the membrane fusion after the cleavage by a human cell surface serine protease (TMPRSS2) [**Hoffmann** et al., ***SARS-CoV-2 Cell Entry Depends on ACE2 and TMPRSS2 and Is Blocked by a Clinically Proven Protease Inhibitor*** Cell. 2020 Apr 16;181(2):271-280.e8. PMID: 32142651 2020]. These findings provide valuable insights into the molecular basis for coronavirus recognition and infection. The S protein of SARS-CoV-2 is approximately 10- to 20-fold more likely to bind to human ACE2 protein the S protein of SARS-CoV-2 [**Wrapp** et al., 2020]. This increase in affinity might have facilitated a more favorable person-to-person transmission of the SARS-CoV-2 infection than the SARS-CoV-1 [**Hoffmann** et al., 2020]. ACE2 can thus act as a unique adhesion protein molecule for SARS-CoV-2 infection.

Despite the role of ACE2 in viral entry, targeting interruption of ACE2 may not be the best strategy to prevent SARS-CoV-2 because ACE2 also maintains blood pressure. So, the entry inhibition strategy must be pathogen-specific

2. Transmembrane Serine Protease 2

Although the ACE2 is available in the vascular endothelial cells of all organs, the lungs are more susceptible to SARS-CoV infection [**Hamming** et al. ***Tissue distribution of ACE2 protein, the functional receptor for SARS coronavirus. A first step in understanding SARS pathogenesis.*** J Pathol. 2004 Jun;203(2):631-7. PMID: 15141377 2004]. Thus, other factors are associated with the actual pathogenesis of SARS-CoV-2. One such factor which accounts for the viral entry into host cells is TMPRSS2, a well-known human alveolar and airway protease [**Shulla** et al. ***A transmembrane serine protease is linked to the Severe Acute Respiratory Syndrome coronavirus receptor and activates virus entry.*** J Virol. 2011 Jan;85(2):873-82. PMID: 21068237 2011]. TMPRSS11a, a TMPRSS2 related protein,

has been found to cleave SARS-CoV S protein and to moderately accelerate viral infections [**Yiu-Wing Kam** et al. ***Cleavage of the SARS coronavirus spike glycoprotein by airway proteases enhances virus entry into human bronchial epithelial cells in vitro.*** PLoS One. 2009 Nov 17;4(11): e7870. PMID: 19924243 2009]. TMPRSS2 probably facilitates viral pathogenesis and transmission in two ways: 1. TMPRSS2 might activate SARS-CoV S protein for virus-cell and cell-cell fusion. 2. TMPRSS2 might protect viral recognition by different neutralizing antibodies of the host [**Glowacka** et al., PMID: 21325420 2011]. Evidence suggests that the entry of SARS-CoV-2 into host cells requires both ACE2 and TMPRSS2 [**Hoffmann** et al., 2020; **Matsuyama** et al. ***Enhanced isolation of SARS-CoV-2 by TMPRSS2-expressing cells.*** Proc Natl Acad Sci U S A. 2020 Mar 31:117(13) PMID: 32165541 2020].

HOST IMMUNE SYSTEM

1. Cytokines

Cytokines are small proteins secreted for intercellular signaling and communication. have autocrine, paracrine, and/or endocrine activity and, through receptor binding, can elicit control of cell proliferation and differentiation, and the regulation of angiogenesis and immune and inflammatory responses. The production of IFN-I or IFN-α/β is the key natural immune defense response against viral infections, and IFN-I is the key molecule that plays an antiviral role in the early stages of viral infection. The interferons (IFNs) are cytokines that play a central role in innate immunity to viruses and other pathogens. The interleukin refers to cytokines produced by leukocytes; however, they are known to be produced by a wide variety of cell types. IL-1α and IL-1β are proinflammatory cytokines that enhance IgM antibody responses and recruit $CD4^+$ T cells neutrophils, monocytes/macrophages to the site of infection. The majority of chemokines are proinflammatory, released by a variety of cells in response to virus (or other microbial) infection.

Characteristic plasma cytokine profiles change over time. The acute-response cytokines TNF and IL-1β and the chemotactic cytokines IL-8 and MCP-1 appear in the early minutes to hours after infection, followed by a more sustained increase in IL-6. The anti-inflammatory cytokine IL-10 appears somewhat later, as the body attempts to control the acute systemic inflammatory response. IL-6 concentrations in peripheral blood are used to assess the intensity of systemic cytokine responses in patients with sepsis, because IL-6 production is stimulated by TNF and IL-1β, providing an integrated signal of these two early-response cytokines. Systemic production of IL-10 following the onset of a cytokine storm is a marker of a counter-anti-inflammatory response. Patients with persistent downregulation of HLA-DR (a marker of immunosuppression) on monocytes 3 to 4 days after the onset of severe sepsis and cytokine storm have a high mortality rate.

The intensity of the inflammatory response in the lungs reflects a balance between proinflammatory cytokines (TNF and IL-1β) and their cognate soluble receptors or inhibitors (TNFR1, TNFR2, and IL-1RA), which inhibit the activity of these inflammatory cytokines in the aqueous phase of alveolar fluid. Zinc finger protein A20, prevents aberrant TLR activation. The production of anti-inflammatory cytokines, mainly IL-10 by macrophages, certain types of T cells (Th2 and regulatory T cells) and B cells.

SARS-CoV-2 infects human upper and lower airway epithelial cells, ThP-1 cells (a monocyte cell line), human peripheral blood monocyte-derived macrophages, and Dendritic cells and induces delayed but elevated levels of proinflammatory cytokines and chemokines. A delayed release of cytokines and chemokines occurs in respiratory epithelial cells, dendritic cells, and macrophages

at the early stage of SARS-CoV-2 infection. High levels of expression of IL-1β, IFN-γ, IP-10, and monocyte chemoattractant protein 1 (MCP-1) later activate T Helper type 1 (Th1) cell response a key event in the activation of specific immunity. Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF), triggers Monocytes to further secrete low levels of the antiviral factors Interferons (IFNs) and high levels of proinflammatory cytokines (Interleukin (IL)-1β, IL-6, and Tumor Necrosis Factor (TNF)) and Chemokines (C-C motif Chemokine Ligand (CCL)-2, CCL-3, and CCL-5). However, also have elevated levels of Th2 cell-secreted cytokines (IL-4 and IL-10), which inhibit the inflammatory response. The elevated serum cytokine and chemokine levels are related to the high number of neutrophils and monocytes in the patients' lung tissues and peripheral blood.

The delayed release of IFNs in the early stages of SARS-CoV-2 infection hinders the body's antiviral response. Afterward, the rapidly increased cytokines and chemokines attract many inflammatory cells, such as neutrophils and monocytes, resulting in excessive infiltration of the inflammatory cells into lung tissue and thus lung injury resulting in the generation of a Cytokine Storm the loss of regulatory control of pro-inflammatory cytokine production, both at local and systemic levels. This Cytokine Storm results in Acute Respiratory Distress Syndrome, Multiple Organ Failure, and even death [**Chen**, et al., PMID: 32157233 2020; **Zhou** et al. ***Sars-Cov-2: Underestimated damage to nervous system.*** Travel Med Infect Dis. 2020 Mar 24:101642. PMID: 32220634 2020].

In humans, pathogen-induced lung injury can progress into Acute Lung Injury a common consequence of a Cytokine Storm in the lung alveolar environment characterized by an acute mononuclear neutrophilic inflammatory response followed by a chronic fibroproliferative phase marked by progressive collagen deposition in the lung; or into its more severe form, Acute Respiratory Distress Syndrome (ARDS). IL-1β is a key cytokine driving proinflammatory activity in bronchoalveolar fluid. Thus, a Cytokine Storm is best exemplified by severe lung infections, in which local inflammation spills over into the systemic circulation, producing systemic sepsis, as defined by persistent hypotension, hyper- or hypothermia, leukocytosis or leukopenia, and often thrombocytopenia. The Cytokine Storm can also be a consequence of severe infections in the gastrointestinal tract, urinary tract, central nervous system, skin, joint spaces, and other sites. Increased cytokine levels (IL-6, IL-10, and TNF-α), lymphopenia (in CD4+ and CD8+ T cells), and decreased IFN-γ expression in CD4+ T cells are associated with severe COVID-19 [**Pedersen SF.** ***SARS-CoV-2: a Storm is raging.*** J Clin Invest. 2020 May 1;130(5):2202-2205 PMID: 32217834, 2020].

A severe pneumonia required mechanical ventilation in 71% and 67%) suffered Acute Respiratory Distress Syndrome. The core pathological change in Acute Respiratory Distress Syndrome [ARDS] is the pulmonary and interstitial tissue damage caused by nonspecific inflammatory cell infiltration. Local excessive release of cytokines is the decisive factor that induces this pathological change and clinical manifestation. the inflammatory cytokine storm is closely related to the development and progression of ARDS. The serum levels of IL-2R and IL-6 are positively correlated with the severity of the disease patients in the intensive care unit (ICU) display increased serum levels of Granulocyte Colony-Stimulating Factor, IP-10, MCP-1, macrophage inflammatory protein-1A, and TNF-α. [**Tisoncik JR.** et al. ***Into the eye of the Cytokine Storm.*** Microbiol Mol Biol Rev. 2012 Mar;76(1):16-32 PMID: 22390970 2012; **Qing Ye.** ***The pathogenesis and treatment of the Cytokine Storm in COVID-19***. J Infect. 2020 Jun;80(6):607-613].

Another consequence of the Cytokine Storm is an increased Capillary Permeability Syndrome.

Immunomodulation decreases the Cytokine Storm reduces pulmonary inflammation and mortality.

2. Natural Killer Cells

The innate immune response itself, without the association of CD8+ T cells and antibodies, is capable of controlling SARS-CoV [**Frieman,** et al. ***SARS coronavirus and innate immunity.*** Virus Res. 2008 Apr;133(1):101-12. PMID: 17451827 2008]. Natural Killer cells are the part of innate lymphocyte subsets that mediate anti-tumor and antiviral responses, and therefore have the potential for clinical use **[Abel AM**, et al. ***Natural Killer Cells: Development, Maturation, and Clinical Utilization.*** Frontiers in Immunology, 12 Aug 2018, 9:1869 PMID: 30150991, 2018]. Previous studies showed that Natural Killer cells showed significant roles in mitigating SARS-CoV-2 (National Research Project for SARS (NSPS), 2004). Recent epidemiological studies showed that SARS-CoV-2 infection in children is less frequent and lethal compared to that of adults, which might be due to the increased lymphocyte count, especially Natural Killer cells and trained immunity in children [**Cristiani** et al. ***Will children reveal their secret? The coronavirus dilemma.*** Eur Respir J. 2020 Apr 23 ;55(4) :2000749. PMID: 32241833 2020].

3. Autophagy

Autophagy is a natural cellular mechanism which ensures the maintenance of cellular homeostasis by lysosomal catabolic action, leading the degradation and recycling of intracellular endogenous (macromolecules, abnormal proteins, and damaged organelles) and exogenous (viruses and bacteria) particles [**Galluzzi L** et al. ***Molecular definitions of autophagy and related processes.*** EMBO J. 2017 Jul 3;36(13):1811-1836. PMID: 28596378; **Levine B**, et al. ***Development by self-digestion: molecular mechanisms and biological functions of autophagy.*** Dev Cell. 2004 Apr;6(4):463-77. PMID: 15068787]. Some viruses have developed strategies to escape autophagic degradation and use host autophagy machinery for their self-replication [**Dong**, et al. ***Autophagy and viruses: adversaries or allies?*** Journal of Innate Immunity, 30 Jan 2013, 5(5):480-493 PMID: 23391695 2013]. The significance of the autophagy process and its therapeutic development for MERS-CoV-1, and SARS-CoV-2 are reviewed by **Yang N**, et al. [***Targeting the Endocytic Pathway and Autophagy Process as a Novel Therapeutic Strategy in COVID-19.*** International Journal of Biological Sciences, 14 Mar 2020, 16(10):1724-1731 PMID: 32226290]. A recent study showed that MERS-CoV-1 halts the fusion of autophagosomes and lysosomes, whereas inhibitors like S-phase kinase-associated protein 2 induced autophagy, which leads to the reduction of the replication of MERS-CoV-1 [**Gassen** et al. ***SKP2 attenuates autophagy through Beclin1-ubiquitination and its inhibition reduces MERS-Coronavirus infection.*** Nat Commun. 2019 Dec 18;10(1):5770. PMID: 31852899]

The battleground between SARS-CoV-2 and human host is at the molecular level of the synthesis of nucleotides and ribonucleosides and the center of their interaction is the synthesis and actions of Inosine Mono-Phosphate (IMP).

THE STRUCTURE AND BIOSYNTHESIS OF PURINES

Purines are molecules formed by the fusion of two rings: a pyrimidine of six atoms and an imidazole of five. The principal molecules that include purines are nucleotides, part of the nucleic acids. In a nucleotide there are three components: (1) a phosphate group, (2) a sugar of five carbons (ribose), and (3) a nitrogenated purine or pyrimidine base; being the sugar the central component

of the molecule. The purines found in the nucleic acids are Guanine and Adenine. Both are rings of nine atoms. The purines form glycosidic ligands with ribose through nitrogen in position 9 and carbon 1 of the sugar). Both Adenine and Guanine are derived from the nucleotide Inosine Mono Phosphate (IMP), which is the first compound in the pathway to have a completely formed purine ring system. Adenine is converted to Adenosine or Inosine Mono Phosphate (IMP), either of which, in turn, is converted into Inosine. Purine nucleoside phosphorylase interconverts in Inosine and Hypoxanthine.

The uptake of a purine by actively dividing B cells (which cannot operate purine salvage pathways) can exceed 8 times that of normal body cells, and, therefore, this set of white cells is selectively targeted by the purine deficiency resulting from Inosine Mono Phosphate Dehydrogenase inhibition.

Inosine Mono Phosphate

Inosinic Acid or Inosine Mono Phosphate (IMP) is a ribonucleoside monophosphate important as an intermediate ribonucleoside monophosphate in purine metabolism. Inosine Mono Phosphate is synthesized on a pre-existing ribose-phosphate through a complex pathway. A key regulatory step is the production of 5-Phospho-α-D-Ribosyl 1-Pyro-Phosphate (PRPP) by Ribose-Phosphate-Pyro- Phospho Kinase, which is activated by inorganic phosphate and inactivated by purine ribonucleotides. It is not the committed step to purine synthesis (PRPP is also used in pyrimidine synthesis and salvage pathways). The first committed step is the reaction of PRPP, glutamine, and water to 5'-PhosphoRibosylAmine (PRA), glutamate, and pyrophosphate-catalyzed by Amido-Phospho-Ribosyl-Transferase, which is activated by PRPP and inhibited by AMP, GMP, and IMP.

Inosine, a ribonucleotide of Hypoxanthine is formed when Hypoxanthine is attached to a Ribose ring (known as a ribo-furanose) via a β-N9-glycosidic bond. It is the first nucleotide formed during the synthesis of purine. It is also formed by the deamination of Adenosine-Mono-Phosphate by AMP Deaminase and is hydrolyzed to Inosine. The latter, Inosine, is commonly found in tRNAs and is essential for proper translation of the genetic code. Important derivatives of Inosinic acid include purine nucleotides found in nucleic acids and Adenosine Tri-Phosphate (ATP), which is used to store chemical energy in all the cells.

Inosinate, and many other molecules, inhibit the synthesis of 5-PhosphoRibosylAmine from 5-phosphoribosyl-1-pyrophosphate (PRPP), disabling the enzyme that catalyzes the reaction: glutamine-5-phosphoribosyl-1-pyrophosphate-amidotransferase. There is a negative loop control of the levels of Inosinate and the synthesis of 5-phosphorybosilamine. As a consequence of a low level of Inosinate, Adenylate and Guanylate are not produced, which means that RNA synthesis cannot be completed because of the lack of these two important RNA nucleotides.

GUANOSINE MONOPHOSPHATE PATHWAYS

1. IMP Dehydrogenase (IMPDH) converts IMP into XMP
2. GMP Synthase converts XMP into GMP
3. GMP Reductase converts GMP back into IMP

ADENOSINE MONOPHOSPHATE PATHWAYS

1. Adenylo-Succinate Synthase converts IMP to Adenylo-Succinate
2. Adenylo-Succinate Lyase converts Adenylo-Succinate into AMP
3. AMP Deaminase converts AMP back into IMP

Adenine Degradation

□□ A nuclease frees the nucleotide
o A nucleotidase creates Adenosine, then Adenosine Deaminase creates Inosine
o Alternatively, AMP Deaminase creates Inosinic acid, then a nucleotidase creates Inosine
□□ Purine nucleoside phosphorylase acts upon Inosine to create Hypoxanthine
□□ Xanthine oxidase catalyzes the biotransformation of Hypoxanthine to Xanthine
□□ Xanthine oxidase acts upon Xanthine to create uric acid

Biosynthesis and Regulations of Purine Nucleotides

1. Biosynthesis

The biosynthesis of the purines starts with a ribose-5-phosphate skeleton. The enzyme Phospho-Ribosyl-Pyrophosphate synthetase catalyzes the addition of pyrophosphate. Later the enzyme Glutamine-PRPP Amido-Transferase o Amido-PhosPho-Ribosyl-Transferase, catalyzes the interaction between the Phospho-Ribosyl-Pyrophosphate (PRPP) and glutamine to form 5-Phospho- Ribosyl-Amino (PRA). This last compound serves as a skeleton to the addition of a series of molecules, whose end result is the formation of Inosine Mono-Phosphate, (IMP). This pathway consists of 10 enzymatic reactions. The whole process of purine synthesis is highly dependent on intracellular energy; thus, it consumes multiple molecules of ATP. The synthesis de novo of purines occurs in the cytoplasm of the liver cells. Inosine Mono-Phosphate can undergo conversion to AMP or GMP. These can be phosphorylated for the creation of high energy molecules ATP or GTP.

Summarizing: Inosine Monophosphate is synthesized on a pre-existing ribose-phosphate through a complex pathway. In the first committed step the reaction of 5-Phospho-α-D-Ribosyl 1-PyroPhosphate (PRPP), glutamine and water form 5'-Phospho Ribosyl Amine (PRA), glutamate, and pyrophosphate-catalyzed by Amido Phospho Ribosyl Transferase, which is activated by PRPP and inhibited by AMP, GMP, and IMP. In the second step PRA, glycine and ATP react to create GAR, ADP, and pyrophosphate-catalyzed by Phospho Ribosyl Amine-Glycine Ligase (GAR synthetase), since PRA has a half-life of 38 seconds at PH 7.5 and 37 °C, it is channeled from Amido Phospho Ribosyl Transferase to GAR synthetase in vivo. [Antle VD et al. (April 1996). Substrate specificity of glycinamide ribonucleotide synthetase from chicken liver. The Journal of Biological Chemistry. 271 (14): 8192-5. PMID 8626510]

2. Regulations

The formation of 5'-Phospho Ribosyl Amine (PRA) from glutamine and PRPP catalyzed by PRPP Amido Phospho Ribosyl Transferase is the regulation point for purine synthesis. IMP, GMP, and AMP are inhibitors since in high concentration binds the enzyme to exert inhibition, while PRPP is an activator.

3. Recycling

Purines from the turnover of cellular nucleic acids

• The enzyme adenine phospho-ribosyl transferase (APRT) salvages Adenine.

• The enzyme hypoxanthine-guanine phospho-ribosyl transferase (HGPRT) salvages Guanine and Hypoxanthine. [Ansari MY et al. (February 2016). Establishment of correlation between in-silico and in- vitro test analysis against Leishmania HGPRT to inhibitors. International Journal of Biological Macromolecules. 83: 78-96. PMID 26616453.]

EFFECTS OF INOSINE

Inosine has a potent axon-promoting effect in vivo following unilateral transection of the corticospinal tract of rats. The mechanism of this action possibly includes serving as an agonist of a nerve growth factor-activated protein kinase (N-Kinase), conversion to cyclic nucleotides that enable advancing nerve endings to overcome the inhibitory effects of myelin, stimulation of differentiation in sympathetic neurons, augmentation of nerve growth factor-induced neurito-genesis and promotion of the survival of astrocytes, among others. The mechanism of the cardioprotective effects of Inosine is similarly unclear. Inosine has been reported to have a positive inotropic effect and to have mild coronary vasodilation activity. Exogenous inosine may contribute to the high-energy phosphate pool of cardiac muscle cells and favorably affect bioenergetics generally. Inosine has also been reported to enhance the myocardial uptake of carbohydrates relative to free fatty acids as well as glycolysis. In cell culture studies, inosine has been found to inhibit the production, in immune- stimulated macrophages and spleen cells, of the proinflammatory cytokines, tumor necrosis factor (TNF)-alpha, interleukin (IL)-1, interleukin (IL)-12, macrophage-inflammatory protein-1 alpha and interferon (IFN)-gamma. It also suppressed proinflammatory cytokine production and mortality in a mouse endo-toxemic model. These actions might account for the possible immunomodulatory, anti-inflammatory, and anti-ischemic actions of inosine. [http://www.drugbank.ca/drugs/DB04335]

- Inosine is an endogenous nucleoside that is produced by the metabolic deamination of Adenosine. Inosine is metabolically more stable (half-life 15h) than Adenosine (half-life <10 s). Inosine exerts anti-inflammatory and immunomodulatory effects similar to those observed with Adenosine. These effects are mediated in part through the Adenosine A2A Receptor (A2AR). Relative to Adenosine, Inosine exhibits a lower affinity towards the Adenosine A2A Receptor (A2AR). Yet, under conditions devoid of Adenosine, Inosine selectively and dose-dependently activated A2AR-mediated cAMP production and ERK1/2 phosphorylation in CHO cells stably expressing the human Adenosine A2A Receptor (A2AR). Inosine also inhibited LPS- stimulated TNF-α, CCL3, and CCL4 production by splenic monocytes in an A2AR-dependent manner. In addition, there is a Positive Allosteric Modulator (PAM) of the Adenosine A2A Receptor (A2AR) enhanced inosine-mediated cAMP production, ERK1/2 phosphorylation, and inhibition of pro-inflammatory cytokine and chemokine production. The cumulative effects of allosteric enhancement of adenosine-mediated and inosine-mediated A2AR activation may be the basis for the sustained anti-inflammatory and immunomodulatory effects observed in vivo. [**Ajith A**. et al. ***Enhancement of inosine-mediated A2AR signaling through positive allosteric modulation.*** Cell Signal. 2018 Jan; 42:227-235 PMID: 29126977]

Inosine modulates lung inflammation and regulates cytokine generation. Adenosine A2A Receptors (A2AR) mediate monocyte recruitment into the lungs. Histological analysis confirmed the effects of Inosine and Adenosine A2A Receptors (A2AR) on cell recruitment. Accordingly,

the treatment with Inosine reduced lung elastance, an effect related to A2 Receptors. Moreover, Inosine reduced the levels of Th2-cytokines, interleukin-4, and interleukin-5, and implicates Inosine as an endogenous modulator of inflammatory processes observed in the lungs of asthmatic patients. [**Fernanda da Rocha Lapa** et al. *Anti-inflammatory effects of inosine in allergic lung inflammation in mice: evidence for the participation of adenosine A2A and A3 receptors*. Purinergic Signal. 2013 Sep; 9(3): 325- 336. PMC3757142]

Neutrophil Extracellular Traps, an effective mechanism to fight against invading microorganisms, prevent microbial dissemination, and avoid overwhelming infections, arise from the release of granular and nuclear contents of neutrophils in the extracellular space in response to different classes of microorganisms, soluble factors, and host molecules. Composed by decondensed chromatin fibers coated with antimicrobial granular and cytoplasmic proteins, such as Myelo-PerOxidase (MPO), Neutrophil Elastase, and α-defensins. Yet, Neutrophil Elastase and Myelo-PerOxidase also regulate its formation. Furthermore, Histone deamination by peptidyl-arginine deiminase 4 (PAD4) is a central step to the formation of Neutrophil Extracellular Traps.

- However, there is a detrimental effect of excessive Neutrophil Extracellular Traps release, particularly important to lung diseases, because it can expand more easily in the pulmonary alveoli, causing lung injury. Moreover, Neutrophil Extracellular Traps and its associated molecules are able to directly induce epithelial and endothelial cell death. In this regard, its massive formation has been reported in several pulmonary diseases, including asthma, chronic obstructive pulmonary disease, cystic fibrosis, respiratory syncytial virus bronchiolitis, influenza, bacterial pneumonia, tuberculosis, and others. Thus, it must be tightly regulated in order to avoid Neutrophil Extracellular Traps mediated tissue damage. [**Porto BN** et al. ***Neutrophil Extracellular Traps in Pulmonary Diseases: Too Much of a Good Thing?*** Front Immunol. 2016 Aug 15; 7:311 PMID: 27574522]

  Cancer patients undergoing immunotherapy may develop Cytokine Release Syndrome (CRS), an inflammatory cytokine storm condition, followed by neurotoxic manifestations and may be life-threatening. It is defined as an inflammatory condition occurring when a large number of lymphocytes and/or myeloid cells are being activated, releasing high levels of inflammatory cytokines and manifests by high fever, nausea, headache, tachycardia, hypotension, cardiac dysfunction, rash, and shortness of breath with some patients experiencing severe inflammatory syndrome resulting in multiorgan failure which can lead to a life-threatening event. The main cytokines involved include tumor necrosis factor-α (TNF-α), interferon γ (IFN-γ), interleukin 1β (IL-1β), interleukin 2 (IL-2), interleukin 6 (IL- 6), interleukin 8 (IL-8), and interleukin 10 (IL-10), all known to be involved in the regulation of the innate and cellular immunity. The inflammatory cytokines are released, enhancing the immune response and activating the proliferation of immune cells to further secrete more inflammatory cytokines. This chain of events leads to a loop between the inflammatory cytokines and the immune cells, which may result in a cytokine storm. Cytokine Release Syndrome is at least partially IL-6 mediated. IL-6 is involved in promoting neutrophil trafficking, B-cell differentiation, and autoantibody production. It is associated with exceptionally high serum levels of free IL-18 with concomitant hyper-ferritinemia, reflecting an IL-18/IL-18BP imbalance. High Sensitivity C Reactive Protein (hsCPR) is an acute-phase reactant produced by the liver largely in response to IL- 6, and hsCRP levels serve as a reliable surrogate for IL-6 bioactivity. [**Lee DW**. et al. ***Current concepts in the diagnosis and management of cytokine release syndrome. Blood***. 2014 Jul 10; 124(2): 188- 195]

Adenosine, a ubiquitous purine nucleoside, induces a plethora of effects in the body via its binding to four Adenosine Receptors A1, A2a, A2b, and the A3. Highly selective agonists to the A3 Adenosine Receptor act as inhibitors of proinflammatory cytokines, possess robust anti-inflammatory and anticancer activity, and concomitantly, induce neuroprotective effects. Circulating monocytes secreting IL-1 are the primary cells responsible for the initiation of Cytokine Release Syndrome. IL-1 is secreted hours before IL-6 and is capable of inducing both IL-6 secretion and soluble IL-6 receptor the release of TNF-α has been shown to play a role in the pathogenesis of Cytokine Release Syndrome.

Adenosine is produced during inflammation, hypoxia, ischemia, or trauma and is released into the extracellular environment from metabolically active or stressed cells. Adenosine is known to regulate proliferation, differentiation, and cell death by binding to one of its four G protein-associated cell surface receptors A1, A2a, A2b, and A3. Adenosine induces inhibition of cyclic adenosine monophosphate (cAMP) upon binding to the A2a and A2b Adenosine Receptors (ARs), whereas Adenosine Receptors A1 and A3 activation inhibit adenylate cyclase and cAMP. Adenosine Receptors A3 activation also results in the inhibition of PI3K/Akt and subsequent deregulation of nuclear factor κB (NF- κB) and MAPK signaling pathways resulting in anti-inflammatory and anti-cancer effects. Yet, at the same time, Adenosine induces cardio-, neuro- and chemo-protective effects manifested by regulation of electrophysiological properties, suppressing neurotransmitter release, modulating dopaminergic motor activity, inhibiting cytokine release and platelet aggregation, inducing erythropoietin production, and modulating lymphocyte function. This differential effect of Adenosine depends on its extracellular concentration, receptor density on the cell surface, and the physiological state of the target cell, leading to apoptosis of pathological cells and the protective effects toward normal body cells.

Adenosine Receptors A3 is expressed on all types of immune cells with a broad distribution in inflammatory cells compared with a very low expression on normal cells. In addition, a direct correlation has been found between Adenosine Receptors A3 expression level and disease progression in inflammatory and cancer diseases in both experimental animal models and humans. [**Cohen S** et al. ***Targeting the A3 adenosine receptor to treat cytokine release syndrome in cancer immunotherapy.*** Drug Des Devel Ther. 2019 Jan 30; 13:491-497] The A3 Adenosine Receptor (A3 AR) subtype is coupled to inhibition of adenylyl cyclase and regulation of mitogen-activated protein kinase (MAPK) pathways, leading to modulation of transcription. Furthermore, A3 AR affects the functions of almost all immune cells. [**Jacobson KA** et al. ***A3 Adenosine Receptors as Modulators of Inflammation: From Medicinal Chemistry to Therapy***. Med Res Rev. 2018 Jul;38(4):1031-1072]

METABOLISM OF INOSINE

After ingestion, Inosine is metabolized into uric acid, which appears to be a natural antioxidant and peroxy-nitrite scavenger. Peroxy-nitrite has been correlated with axon degeneration [Neuhaus O et al. Immune-mediated injury, oxidative toxicity and excitotoxicity in multiple sclerosis. Possibilities for immune modulation and neuroprotection. 2007-03-11] Inosine induces axonal rewiring. [**Liu F** et al. (July 2006). ***Secondary degeneration reduced by inosine after spinal cord injury in rats.*** Spinal Cord. 44 (7): 421-6; **Chen P.** et al. (June 2002). ***Inosine induces axonal rewiring and improves behavioral outcome after stroke.*** Proceedings of the National Academy of Sciences of the United States of America. 99 (13): 9031-6. PMC 124418. PMID 12084941]

IMMUNOLOGIC EFFECTS OF INOSINE PRANOBEX

Inosine Pranobex (Inosine acedoben dimepranol or Methisoprinol) is the p-acetamidobenzoic acid salt of N,N-dimethylamino-2-propanol and β-inosine in a 3:1 molar ratio is a synthetic purine derivative with immunomodulatory, an analog of thymus hormones, and antiviral properties, which result from an apparent in vivo enhancement of host immune responses due to the drug. [American Cancer Society. Inosine Pranobex. 23 August 2010]

In clinical studies, it has been shown to normalize (to the patient's baseline) a deficient or dysfunctional cell-mediated immunity by evoking a Th1 type response which initiates T lymphocyte maturation and differentiation and potentiation of induced lymphoproliferative responses, in mitogen or antigen-activated cells. Similarly, the drug has been shown to modulate T lymphocyte and natural killer cell cytotoxicity, T8 suppressor, and T4 helper cell functions and to increase the number of IgG and complement surface markers. Increases cytokine IL-1 production and enhances IL-2 production, upregulating the expression of the IL-2 receptor in vitro. It significantly increases endogenous IFN -γ secretion and decreases the IL-4 production in vivo. It has also been shown to potentiate neutrophil, monocyte, and macrophage chemotaxis and phagocytosis.

In vivo, Inosine acedoben dimeparanol enhances potentiation of depressed lymphocytic mRNA protein synthesis and translational ability while inhibiting viral RNA synthesis achieved by yet-to-be-clarified degrees of
(1) incorporation of inosine-mediated orotic acid into polyribosomes;
(2) inhibition of poly-adenylic acid attachment to viral messenger RNA and
(3) molecular reorganization of lymphocyte intramembrane plasma particles (IMP) that results in a nearly threefold increase in density. Finally, it inhibits cGMP phosphodiesterase only at high concentrations in vitro and at levels not involved in the in vivo immunopharmacological effects.

Previous studies demonstrate that the immunomodulatory activity of Inosine Pranobex is characterized by enhanced lymphocyte proliferation, cytokine production, and NK cell cytotoxicity. The activation of NKG2D signaling on NK cells, CD8+ T cells, and γδ T cells also produces these outcomes. McCarthy et al. tested this hypothesis that Inosine Pranobex alters cellular immunity through the induction of NKG2 D ligand expression on target cells, thereby enhancing immune cell activation through the NKG2D receptor, and showed that exposure of target cells to Inosine Pranobex leads to increased expression of multiple NKG2D ligands. Using both targeted metabolic interventions and unbiased metabolomic studies, they found that Inosine Pranobex causes an increase in intracellular concentration of purine nucleotides and Tri-Carboxylic Acid cycle intermediates and NKG2D ligand induction. The degree of NKG2D ligand induction was functionally significant, leading to increased NKG2D-dependent target cell immunogenicity. These findings demonstrate that the immunomodulatory properties of Inosine Pranobex are due to metabolic activation with NKG2D ligand induction. [**McCarthy** et al. ***Inosine Pranobex enhances human NK cell cytotoxicity by inducing metabolic activation and NKG2D ligand expression.*** Eur. J. Immunol. 2020. 50: 130-137]

In a clinical trial by Rumel where multiple lymphocyte subsets - CD19 + B cells, CD3 + T cells, CD4 + T- helper cells, FoxP3hi/CD25hi/CD127lo regulatory T cells (Tregs), CD3 −/CD56 + NK cells, and CD3 +/CD56 + NKT cells - were, together with serum immunoglobulins and IgG subclasses, followed during 14 days of Inosine Acedoben Dimepranol administration to ten healthy volunteers; these selected from 27 individuals pre-screened in vitro for their capacity to respond

to Inosine Acedoben Dimepranol as gauged by increases in the percentage of T-reg and/or NKT cells arising in PHA-stimulated cultures. While a transient spike and dip in T-reg and T-helper fractions, respectively, were noted, the outstanding consequence of Inosine Acedoben Dimepranol administration (1 g po, qd) was an early and durable rise in NK cells. For half the cohort, NK cells increased as a percentage of total peripheral blood lymphocytes within 1.5 h of receiving drug. By Day 5, all but one of the volunteers displayed higher NK cell percentages, such elevation - effectively a doubling or greater - being maintained at the termination of the study. The Inosine Acedoben Dimepranol-induced populations were as replete in Granzyme A and Perforin as basal NK cells. [**Rumel A.** et al. ***Inosine Acedoben Dimepranol promotes an early and sustained increase in the natural killer cell component of circulating lymphocytes: A clinical trial supporting antiviral indications.*** International Immunopharmacology Volume 42, January 2017, Pages 108-114]

In Lasek's study, Isoprinosine (Inosine Pranobex) is an immunomodulatory and antiviral drug used in some viral infections, especially in patients with weakened immunity. In the present study, effects of Isoprinosine on the production of cytokines attributable to Th1 (IL- 2, IFN-g, and TNF-a) or Th2 cells (IL-4, IL-5, and IL-10) were tested in human peripheral blood lymphocyte cultures stimulated with phytohemagglutinin (PHA). Inosine Pranobex enhanced TNF-a secretion significantly (in short-term--24-hour, and prolonged-term--72- hour cultures) and IFN-g (in 72-hour cultures). Surprisingly, the production of IL-10 by PHA-stimulated lymphocytes was suppressed by Inosine Pranobex in a dose-dependent manner in both 24-hour and 72-hour cultures. **[Lasek W**. et al. ***Immunomodulatory effects of inosine Pranobex on cytokine production by human lymphocytes.*** Acta Pharm. 2015 Jun; 65(2):171-80]

To evaluate the serum levels of certain cytokines during and after Isoprinosine treatment, Petrova M et al. assigned 10 healthy volunteers to receive Isoprinosine 1 g, 3 times daily, 5 consecutive days weekly. Both treatment and follow-up phase last 3 weeks. Interferon-gamma (IFN-gamma), interleukin-2 (IL-2), IL-10, and tumor necrosis factor-alpha (TNF-alpha) were measured in serum using commercial ELISA kits at baseline, 7th, 10th, 14th, 21st, 28th, 35th, and 42nd day. They observed an increase in serum levels of all measured cytokines at 7th to 10th day. The levels of IL-2 had another raise at 42nd day after a drop to initial values ($P < 0.05$; $P < 0.001$, respectively). Those of IL-10 held up enhanced from 7th to 28th day of measurement ($P < 0.01$). There was a nearly flat line of values of TNF-alpha after an initial slight increase at 10th day, and found a moderate negative correlation between IFN-gamma and IL-2, IL-10, and TNF-alpha (Spearman's r: -0.63, -0.62, - 0.63; $P < 0.05$, respectively). [**Petrova M** et al. ***Isoprinosine affects serum cytokine levels in healthy adults.*** J Interferon Cytokine Res. 2010 Apr; 30 (4):223-8]

PHARMACOLOGY AND METABOLISM

When administered orally in man, it is rapidly and completely absorbed (≥ 90%) from the gastrointestinal tract and appears in the blood. Similarly, 94-100% of IV values of N, N-dimethylamino-2-propanol] and p-acetamido-benzoic acid components are recovered in the urine after oral administration. The radiolabeled distribution was found in the following tissues, in order of decreasing specific activity: kidneys, lung, liver, heart, spleen, testes, pancreas, brain, and skeletal muscle.

In human subjects following a 1 g oral dose, the plasma levels found for N, N-dimethylamino- 2-propanol, and p-acetamido-benzoic acid, respectively were found to be: 3.7μg/ml (2 hours) and

9.4μg/ml (1 hour). In human dose tolerance studies, peak post-dose elevation of uric acid levels as a measurement of drug-derived inosine is not linear and can vary + 10% between 1-3 hours.

The 24-hour urinary excretion of p-acetamido-benzoic acid and its major metabolite under steady-state conditions at 4g per day amounted to approximately 85% of the administered dose. 95% of the N, N-dimethylamino-2-propanol-derived radioactivity in urine was recovered as unchanged N, N- dimethylamino-2-propanol and N, N-dimethylamino-2-propanol N-oxide. The elimination half-life is 3.5 hours for N, N-dimethylamino-2-propanol, and 50 minutes for p-acetamido-benzoic acid. The major metabolites in humans are the N-oxide for N, N-dimethylamino-2-propanol, and the o- acyl- glucuronide for p-acetamido-benzoic acid. Because the inosine moiety is degraded by the purine degradation pathway to uric acid, radiolabeled experiments in humans are inappropriate. In animals up to about 70% of the administered inosine can be recovered as urinary uric acid following oral tablet administration and the remainder as the normal metabolites, xanthine, and hypoxanthine.

Urinary recoveries under steady-state conditions of the p-acetamido-benzoic acid moiety and its metabolite were found to be > 90% of the expected value from the solution. The recovery of the N, N- dimethylamino-2-propanol moiety and its metabolite was >76%. The plasma area under the curve was >88% for N, N-dimethylamino-2-propanol, and > 77% for p-acetamido-benzoic acid.

Inosine Acedoben Dimepranol has a low toxicity profile in multivariate acute, subacute, and chronic toxicology and produced the lowest acute oral LD50 at 50 times the maximum therapeutic dosage level of 100mg/kg/day. No evidence of perinatal toxicity, embryotoxicity, teratogenicity or impaired reproductive function with continuous parental dosing of up to 20 times the maximum therapeutically recommended human dose (100 mg/kg/day)

HYDROXYCHLOQUINE

In the previous months, there has been an increased interest in Hydroxychloroquine as a treatment for SARS-CoV-2 has arisen since Coronavirus cell entry occurs through the endo-lysosomal pathway [**Burkard C** et al. ***Coronavirus cell entry occurs through the endo-/lysosomal pathway in a proteolysis-dependent manner.*** PLoS Pathog. 2014;10] and it is known that Chloroquine and Hydroxychloroquine inhibits a pre-entry step of the viral cycle by interfering with viral particles binding to their cellular cell surface receptor. Chloroquine and Hydroxychloroquine inhibit quinone oxidoreductase 2 [**Kwiek J** et al. ***Kinetic mechanism of quinone oxidoreductase 2 and its inhibition by the antimalarial quinolines***. Biochemistry. 2004;43:4538-4547], The potent anti-SARS-CoV-1 effects of Chloroquine and Hydroxychloroquine in vitro were considered attributable to a deficit in the glycosylation of a virus cell surface receptor, the Angiotensin-Converting Enzyme 2 (ACE2) on Vero cells [**Vincent MJ** et al. ***Chloroquine is a potent inhibitor of SARS coronavirus infection and spread.*** Virol J. 2005; 2:69], A pH-dependent mechanism of entry of SARS-CoV-1 after binding of the DC-SIGN receptor of target cells was reported [**Yang ZY** et al. ***pH-dependent entry of Severe Acute Respiratory Syndrome coronavirus is mediated by the spike glycoprotein and enhanced by dendritic cell transfer through DC-SIGN.*** J Virol. 2004; 78:5642-5650] Chloroquine has a tendency to accumulate in lysosomes, where it sequesters protons and increases the pH. In addition, it interacts with many different proteins and cellular processes, resulting in the modulation of autophagy. The activation step that occurs in endosomes at acidic pH results in the fusion of the viral and endosomal membranes leading to the release of the viral SARS-CoV-1 genome into the cytosol [**Wang H** et al. ***SARS coronavirus entry into host cells through a novel***

clathrin- and caveolae-independent endocytic pathway. Cell Res. 2008; 18:290-301] These are sufficient reasons to consider it in a trial. [**Devaux C** et al. ***New insights on the antiviral effects of chloroquine against coronavirus: what to expect for COVID-19?*** Int J Antimicrob Agents. 2020 Mar 12: 105938. PMID: 32171740] However, it is also known that the toxicity of Chloroquine is greater than that of Hydroxychloroquine.

ZINC

Data from all countries indicate that the case fatality and morbidity rates from SARS-CoV-2 increases with age and for those with non-communicable chronic disease co-morbidities, both of which are associated with lower zinc status. Populations with confirmed zinc deficiency, people with insufficient dietary intake of zinc (e.g. people with limited access to animal foods, [consume plant-based diets high in phytic acids e.g. cereals, starchy roots, tubers and legumes, and older adults and people with an increased biological need for zinc (e.g. pregnant and breastfeeding women,] early post-natal infants, children, people with chronic diseases, and people with alcohol dependency. The most important sub-group populations are those experiencing more severe illness and at a higher risk of mortality from SARS CoV-2 infections and with a coinciding high risk for zinc deficiency/insufficiency. Known risk factors include older adults, people with chronic diseases, residents of aged care facilities, obesity, and possibly some Chronic Alcoholic Liver Disease, socioeconomic, or restrictive dietary groups.

Zinc can inhibit the enzymatic activity and replication of SARS-CoV RNA polymerase and may inhibit angiotensin-converting enzyme 2 (ACE2) activity. Zinc may also modify the host's response to an infection as it is an essential co-factor element with a broad range of functions in the body. Many of the beneficial effects of zinc appear to take place at the cell membrane. Zinc (Zn^{2+}) reduces the permeability of the cell membrane without penetration into or damage to the cell. Like other astringents, zinc alters the capillary epithelium, thus inhibiting the transcapillary movement of plasma protein that in turn may reduce local edema, inflammation, exudation, and mucus secretion.

Zinc insufficiency/deficiency is known to diminish antibody and cell-mediated immunity in humans is associated with an increased risk of infections and may only become apparent upon immune system provocation. Zinc has an essential role in immune and airways function, wound healing, and tissue repair that in turn, may delay or prevent recovery from viral respiratory illnesses. Other consequences of zinc deficiency include an increased risk of vitamin A deficiency that is also critical for immune function, due to carrier proteins and activation enzymes being dependent on sufficient zinc status.

Viral proteases are essential for pathogenesis and virulence of Severe Acute Respiratory Syndrome coronavirus (SARS-CoV). ARS-CoV PLP2 by itself differentially cleaves between the amino acids Gly180 and Ala181, Gly818 and Ala819, and Gly2740 and Lys2741 of the viral polypeptide pp1a. This protease is especially selective for the P1, P4, and P6 sites of the substrate. The reaction mechanism of SARS-CoV PLP2 is characteristic of papain and compatible with the involvement of the catalytic dyad (Cys)-S-/(His)-Im+H ion pair. Zinc ion and its conjugates inhibit the enzymatic activity of SARS-CoV PLP2. [**Yu-San Han** ***Papain-Like Protease 2 (PLP2) from Severe Acute Respiratory Syndrome Coronavirus (SARS-CoV-2): Expression, Purification, Characterization, and Inhibition.*** Biochemistry. 2005 Aug 2; 44(30): 10349-59]

Zinc ions are proven crucial for the proper folding and activity of various cellular enzymes and transcription factors. Zn2+ is probably an important cofactor for numerous viral proteins as well. The intracellular concentration of free Zn2+ is maintained at a relatively low level by metallothioneins, likely due to the fact that Zn2+ can serve as an intracellular second messenger and may trigger apoptosis or a decrease in protein synthesis at elevated concentrations [**Lazarczyk M**, et al.. ***Role of Zn2+ ions in host-virus interactions***. J Virol. 2008; 82:11486-11494].

3C proteases activity was inhibited by Zn2+ [**Cordingley MG**, et al. ***Cleavage of small peptides in vitro by human rhinovirus 14 3C protease expressed in Escherichia coli***. J Virol. 1989; 63:5037-50453], which is in line with the inhibition of polyprotein processing by zinc ions in cells infected with human rhinovirus and coxsackievirus B3 [**Krenn BM**, et al. ***Antiviral Activity of the Zinc Ionophores Pyrithione and Hinokitiol against Picornavirus Infections.*** J Virol. 2009; 83:58-64]. Inhibition of the viral RNA-dependent RNA polymerase (RdRp) and cellular cofactors. An inhibitory effect of Zn2+ on the activity of purified RdRps from rhinoviruses and hepatitis C virus was noted [**Ferrari E**, et al. ***Characterization of soluble hepatitis C virus RNA-dependent RNA polymerase expressed in Escherichia coli.*** J Virol. 1999; 73:1649-1654; **Hung M**, et al. ***Biochemical characterization of rhinovirus RNA-dependent RNA polymerase.*** Antiviral Res. 2002; 56:99-114]. A hallmark of the coronavirus replicative cycle is the transcription of a 5′- and 3′-coterminal nested set of subgenomic (sg) mRNAs from which the viral structural and accessory protein genes are expressed [**Pasternak AO**, et al. ***Nidovirus transcription: how to make sense…?*** J Gen Virol. 2006; 80:1403-1421; **Sawicki SG**, et al. ***A Contemporary View of Coronavirus Transcription.*** J Virol. 2007; 81:20-29]. Zinc ions were demonstrated to inhibit certain proteolytic cleavages in the processing of the coronavirus replicase polyproteins in infected cells and cell-free systems [**Denison MR**, et al. ***Intracellular processing of the N-terminal ORF 1a proteins of the coronavirus MHV-A59 requires multiple proteolytic events.*** Virology. 1992; 189:274-284]. The zinc-ionophore Pyrithione (PT) in combination with Zn2+ is a potent inhibitor of the replication of SARS-coronavirus (SARS-CoV-2) Zn2+ directly impairs SARS-CoV-2 RNA synthesis, that are based on membrane-associated RTCs isolated from infected cells (RTC assays) [**van Hemert MJ**, *et al.* ***SARS-Coronavirus Replication/Transcription Complexes Are Membrane-Protected and Need a Host Factor for Activity In Vitro***. PLoS Pathog. 2008; 4: e1000054] since it had a strong inhibitory effect in both RTC and RdRp.

The daily recommended dietary intake of elemental Zinc is around 2 mg for infants up to 6 months of age. Tolerable upper limits for zinc are estimated to be 7 mg for children aged 1-3 years of age, increasing up to 25 mg for adults and females of any age who are pregnant or lactating. The no observed adverse effect level for adults is around 50 mg/day.

Age (years)	Tolerable Upper Intake Level (UL) for Zinc
1-3	5 (mg per day)
4-8	10 (mg per day)
9-14	15 (mg per day)
15-19	20 (mg per day)
>20	25 (mg per day)
>35	50-100 (mg per day)

Tolerable Upper Intake Level (UL) for Zinc

SUMMARY OF SARS-CoV-2, HOST & DRUG INTERACTIONS

VIRAL	ENTRY TMPRSS2 ACE2	TRANSCRIPTION & REPLICATION	RELEASE
SPIKE GLYCOPROTEIN	**HQ, Zn**	**Zn**	
ACE2 & SPIKE BINDING COMPLEX	**HQ, Zn**		
LYPOSOME		**HQ**	
ENDOSOME ACIDIFICATION		**HQ, Colchicine, Bromhexine Hydrochloride**	
RNA dependent RNA polymerase **[RdRp]**		**HQ, Zn, IMP**	
3Chymotrypsin-Like protease **[3CLpro],**		**HQ, Zn, Famotidine**	**HQ, Colchicine**
Papain-Like Protease 2 **[PLP2]** *Papain-Like Protease* **[PLpro]**		**Zn, HQ**	
HUMAN HOST	**ACTIVATION**	**INHIBITION**	**MODULATION**
CYTOKINE	**IMP**	**Dexamethasone, Methylprednisolone**	**IMP, HQ, Famotidine**
NATURAL KILLER CELLS	**Zn, IMP**		**IMP**
AUTOPHAGY	**Zn**		**IMP**

HQ: Hydroxychloroquine. **IMP:** Inosine Mono-Phosphate **Zn**: Zinc

MAGNITUD OF THE SARS- CoV-2 EPIDEMIC

Presently (**August 21, 2020**) **there are in the world 23,043,507 CONFIRMED CASES OF SARS- CoV-2 WITH A FATALITY OF 801,196 and a MORTALITY RATE OF 5%. An increment of 22,630,040 cases since March 25, 2020** [WORLD CONFIRMED CASES 413,467 WITH A FATALITY OF 18,433 and a MORTALITY RATE OF 4.46%].

Although several combinations of medications have been proposed, these have apparently produced up to now little impact and the effects of a vaccine(s) appear likely to be short lived (about 2-3 months) or partially effective due to the rapid mutation rate of **SARS-CoV-2**. **The end result is that only those who have natural resistance are expected to navigate the current pandemic. Further, a diminished immune system integrity and response caused by age, chronic metabolic, immunological, environmental and infectious illnesses greatly increase the risk of a severe disease and dismal outcome.**

The objectives of this review paper are to bring the attention of researches and the public at large to another approach that should reverse the current perceived nihilism. **The tentative solution proposed is threefold.**

1. Fortify the immune system of the population at large, especially those most disadvantaged from the immunological point of view.

2. Prevent the latching of SARS-CoV-2 to the nasal mucosa since SARS-CoV-2 replicates efficiently in ciliated cells isolated from nasal and tracheobronchial airway regions (**Sims AC** et al. ***SARS-CoV-2 replication and pathogenesis in an in vitro human conducting airway epithelium*** Virus Res 2008 Apr; 133(10: 33-44) **by taking Zinc Gluconate 50 mg – 100 mg orally on a daily basis until the epidemic is under complete control.**

3. Proactive stratification of the entire population, thus, preventing or controlling the severity of the illness caused by SARS- CoV-2

CONCLUSION

Herein there is a convincing amount of scientific evidence concerning the mechanisms involved in SARS-CoV-2 itself, the interactions of the virus with the human host at the molecular level and the actions of Inosine Pranobex, those of Hydroxychloroquine, Zinc and Famotidine on those infected cells.

It is my hope that this paper stimulates the interest of researches throughout the planet to consider these clinically applicable observations and perform the necessary validating clinical trials as proposed as soon as it is humanly possible.

In the clinical trials already done it appears that it is more valuable the cost of the product that its effectiveness.

Costs as of May 20, 2020:
Remdesivir: $950 100 mg. «The drug is *cost-effective* at $4,460 per course of treatment»?
Tocilizumab: $1,100.16 vial 162 mg
Ruxolitinib: $14,108 tablet 10 mg (60 tablets)
Canakinumab: $115,596.06
Clazakizumab: $16,071 10 mg (6 vials)
Inosinoprine: $50 500mg (50 tablets)
Plaquenil: $156 200mg (60 tablets)

Yet a report in Bloomberg on April 10, 2020 estimated the manufacturing costs per treatment. (Minimum costs of production were estimated from the prices of active pharmaceutical ingredients using established methodology)
Remdesivir: $9
Hydroxychloroquine: $1

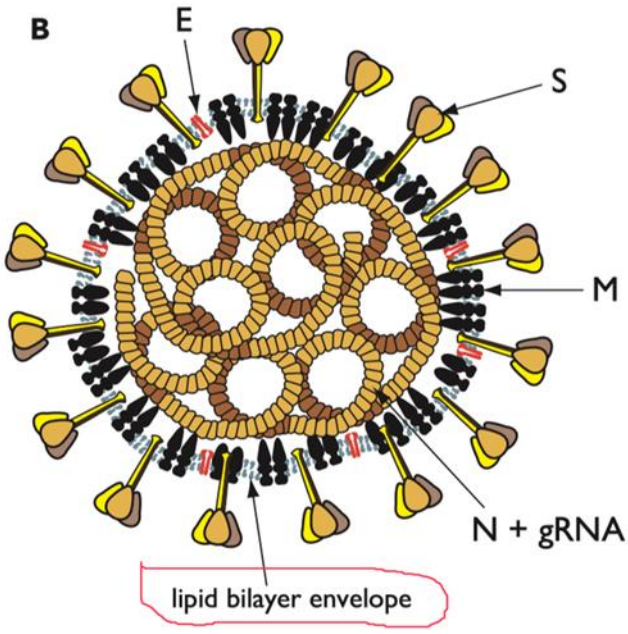
Since we cannot continue to afford the physical and psychological communal devastation that this pandemic is causing throughout the world, or its use for political control and manipulation; it is imperative that a simple interim solution be used. In words famously uttered during the Presidential Campaign of 2016: ***What do we have to lose!***

Preventing the latching of SARS-CoV-2 to the nasal epithelium requires ZINC GLUCONATE 50 mgs to 100 mgs a day for adults of the entire population for 2 months, since infection could appear up to 28 days post exposure. If a second layer of protective prophylaxis is needed or sought, then add Plaquenil 200 mg twice or trice a week or Inosine Pranobex 1 gm once or twice a day once it is approved by the FDA. In the rest of the world the latter is not an issue.

I am including **A CLINICAL STUDY PROTOCOL TO COMPARE THE EFFICACY AND SAFETY OF INOSINE PRANOBEX AND HYDROXYCHLOROQUINE COMBATING THE CURRENT SARS- CoV-2 PANDEMIC**

Declaration of interests: I declare no competing interests

Milton L. Pozo, MD, FACP.



ENVELOPE PROTEIN [E]

SPIKE GLYCO PROTEINE [S]

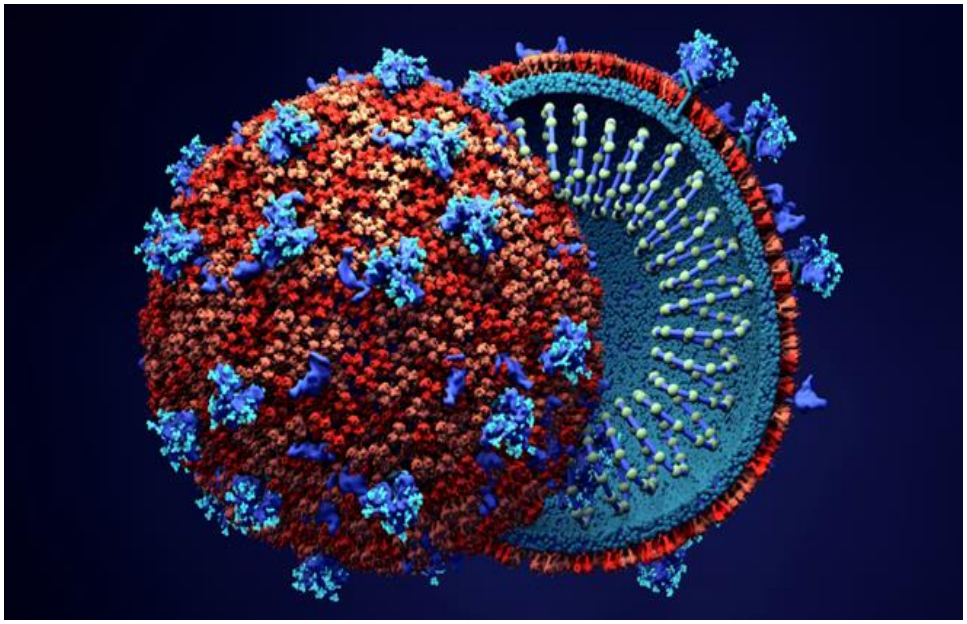
MEMBRANE PROTEINE [M]
NUCLEOCAPSID PROTEIN [N]

GENOMIC RNA [gRNA]

Ian M. MacKay, PhD.

Brisbane, Australia

virologydownunder.com

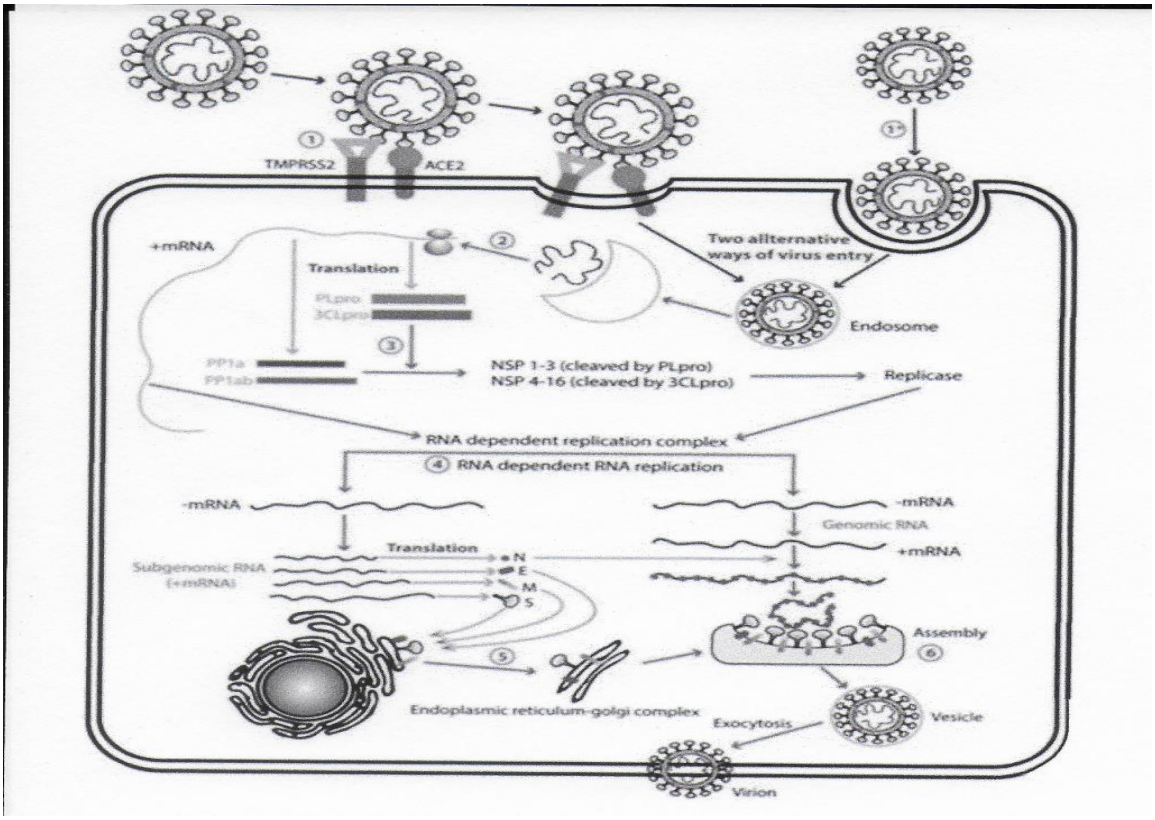


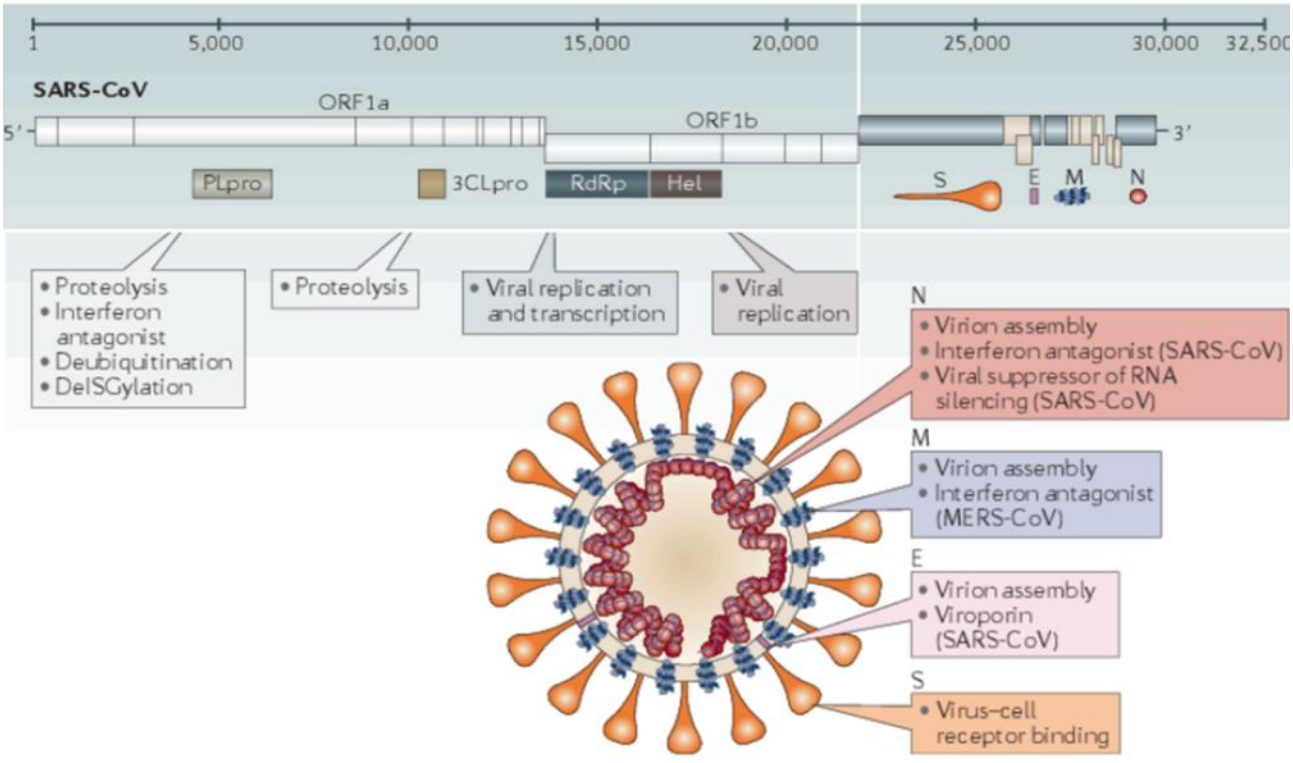
(Image: © Shutterstock)

Stephanie Pappas

Live Science

March 11, 2020


TMPRSS2
ACE2
+mRNA
Translation
PLpro
3CLpro
PP1a
PP1ab
NSP 1-3 (cleaved by PLpro)
NSP 4-16 (cleaved by 3CLpro)
Replicase
Two alternative ways of virus entry
Endosome
RNA dependent replication complex
RNA dependent RNA replication
-mRNA
Genomic RNA
+mRNA
Subgenomic RNA (+mRNA)
N
E
M
S
Assembly
Endoplasmic reticulum-golgi complex
Exocytosis
Vesicle
Virion


1
5,000
10,000
15,000
20,000
25,000
30,000
32,500
SARS-CoV
ORF1a
ORF1b
5'
3'
PLpro
3CLpro
RdRp
Hel
S
E
M
N
• Proteolysis
• Interferon antagonist
• Deubiquitination
• DelSGylation
• Proteolysis
• Viral replication and transcription
• Viral replication
N
• Virion assembly
• Interferon antagonist (SARS-CoV)
• Viral suppressor of RNA silencing (SARS-CoV)
M
• Virion assembly
• Interferon antagonist (MERS-CoV)
E
• Virion assembly
• Viroporin (SARS-CoV)
S
• Virus–cell receptor binding

A CLINICAL PROTOCOL COMPARING THE EFFICACY OF INOSINE PRANOBEX AND CHLOROQUINE PHOSPHATE IN COMBATING THE CURRENT SARS-CoV-2 PANDEMIC

Milton L. Pozo, MD., FACP.

Study population and Methods

Patients should be excluded if they have a known allergy to any of the components of the medications included in the clinical trial **or** have another known contraindication to treatment with the study drugs. Breastfeeding and pregnant patients should not be excluded, and pregnancy test results are not required because of the minimal teratogenicity of these medications at the doses promulgated.

Informed consent

Written informed signed consent is to be obtained from adult participants (≥ 18 years) or from parents or legal guardians for minors (<18 years). An information document that clearly indicates the risks [ineffectiveness and potential death from the illness] and the benefits associated with the participation to the study is to be given to each patient. Patients must receive information about their clinical status during care regardless of whether they participate in the study or not. Regarding patient identification, a study number is to be assigned sequentially to included participants, according to the range of patient numbers allocated to each study center. The study is to be conducted in accordance with the guidelines of the International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use, and applicable standard operating procedures.

The protocol, appendices and any other relevant documentation are to be submitted to Ministry of Health

Procedure

Patients are seen at baseline for enrolment, initial data collection and treatment: Day - 0, and again for daily follow-up for 14 days. Each day, patients receive a standardized clinical examination and a nasopharyngeal sample is collected weekly. All clinical data is to be collected using standardized questionnaires.

Patients who refuse the treatment or have an exclusion criterion, should serve as controls. Symptomatic treatment and antibiotics as a measure to prevent bacterial super-infection is provided by investigators based on clinical judgment.

Classification and Clinical Stratification

Patients are assigned in the following categories:

General Population: Medical, Hospital and Health Personnel should receive **prophylactic treatment.** They do not require initial testing but can grouped as personal point of reference.

They should be stratified into **four subgroups of INTERVENTION**: **Group 0: Zinc Gluconate 50 mg/d - 100 mg/d. Group 1. Inosine Pranobex 1 gm/d. Group 2. Hydroxychloroquine (Plaquenil) 200 mg/3d** per **week (L, M, V). Group 3. Refused intervention. Subgroups are to be evaluated every Friday during the study period.**

Diagnostics: Positive of SARS-CoVID2 (carriage in nasopharyngeal sample at admission to the study whatever their clinical status) **via Reverse Transcription- PCR.**

Asymptomatic Disease: SARS-CoVID2 positives via RT- PCR**.** They can make up 15% of cases. Their Recuperation is expected in two weeks (average)

They should be stratified into **three subgroups of INTERVENTIO**N: **Group 1. Inosine Pranobex 2 gm/d. plus Zinc Gluconate 50 mg/d - 100 mg/d. Group 2. Plaquenil 200 mg /5d** x **week plus Zinc Gluconate 50 mg/d - 100 mg/d. Group 3. Refused intervention. Subgroups are to be evaluated every Friday during the study period. Weekly documentation of: digital oximetry, temperature, breathing pattern & frequency, pulse, and symptomatology.**

Mild Disease: mild fever (38 C), dry cough, general discomfort, headache, muscle pain; (nasal congestion, rhinorrhea, sneezing, sore throat ((5%) o mild bronchitis (10%). They can constitute 25% of cases. They are expected to recover in three weeks (average). Both patients and those who care for them should receive specific information about monitoring and daily documentation of: **digital oximetry, temperature, breathing pattern & frequency, pulse, and symptomatology. Monitor the prognostic tests values** (CPR, Ferritin, D dimer, LDH, Lymphocytes**)**.

They are stratified into **three subgroups of INTERVENTIO**N: **Group 1. Inosine Pranobex 2 gm/d. plus Zinc Gluconate 100 mg/d, Budesonide inhalations** and **Famotidine 40 mg/d Group 2. Hydroxychloroquine (Plaquenil) 400 mg/d. plus Zinc Gluconate 100 mg/d, Budesonide inhalations** and **Famotidine 40 mg/d. Group 3. Refuse treatment**.

Moderate Disease: Nausea, vomiting and diarrhea. Fever that responds to antipyretics, **anosmia, ageusia,** moderate general discomfort associated with progressive dyspnea ([light shortness of breath & slight tachypnea (> 20 breaths/min), and slight hypoxia (SpO2 >/93% ambient air)], and/or Bronchopneumonia or new Infiltrates < 50% of a lung within 24 to 48 hours of symptomatology. Cyanosis in children/children. They can constitute 20% of cases. It is expected to recuperate in four weeks (average). Avoid dehydration. **Critical laboratories: <30% increase in hs-CRP within 36 hours, LDH, Ferritin, D dimer. Mandatory monitoring and daily documentation of: digital oximetry, temperature, breaths, pulse, and symptomatology.**

They are stratified into **three subgroups of INTERVENTION**: **Group 1. Inosine Pranobex 3 gm/d. (**The ideal proposed dose of **Inosine Pranobex** is 50 mg/kg/day orally for ten days. The dose of the subjects is calculated by the formula [height in cm x weight in pounds x 0.44]) plus **Zinc Gluconate 100 mg/d, Budesonide inhalations** or **Dexamethasone 0.75 mg/d** oral, **Aspirin 162md/d** and **Famotidine 80 mg/d. Group 2. Hydroxychloroquine (Plaquenil) 600 mg/d. plus Zinc Gluconate 100 mg/d, Famotidine 80 mg/d, Aspirin 162md/d,** and **Dexamethasone 0.75 mg/d** oral **or Budesonide inhalations. To those with Bronchopneumonia add Doxycycline 200 mg/d. Group 3. Refuse intervention or receive** an alternative **treatment**. **Prophylactic hospitalization for Patients with Chronic** or **Immune Diseases. Elective hospitalization for others.**

Severe Disease: Antipyretic resistant fever, oxygenation decline. **Saturation (<93%) marks the crucial phase of the disease by characterizing it as** Severe. These patients are experiencing marked inflammation of the lungs and rapidly deteriorating. Chest Computerized Tomography or Plain Chest X-rays show bilateral **infiltrates > 50%).** They can constitute 20% of cases. **All Critical laboratories are abnormal.**

They are stratified into **three subgroups of INTERVENTION**: **Group 1. Inosine Pranobex 4 gm/d. (use liquid formulation via nasogastric tube if necessary), Group 2. Hydroxychloroquine (Plaquenil) 600 mg/d. Add to Groups 1 and 2** add **Zinc Gluconate 100 mg/d**, **Famotidine 80 mg/d, Doxycycline 200 mg/d, nebulization of Albuterol q4-6 hrs. and inhalations of Budesonide or Dexamethasone 1.5 mg/d oral. Prophylactic use of high-volume nasal cannula via facial mask with**

positive and continuous airway pressure (CPAP)**. Group 3. Alternate treatment. Hospitalization is mandatory.**

Pulmonary Pathology of Severe Disease:

Lungs show edema and protein exudates that resemble large protein blood cells, combined vascular congestion groups of fibrinous material with giant multinucleated cells and pneumocyte hyperplasia [**Tian S**, et al. ***Pulmonary Pathology of Early-Phase 2019 Novel Coronavirus (COVID-19) Pneumonia in Two Patients with Lung Cancer***. J Thorac Oncol. 2020 Feb 28]. **The dominant pathological process in all the lungs examined was diffuse alveolar damage, accompanied by small vessel thrombosis with significant bleeding.** [**Fox, SE.,** et al. ***Pulmonary and cardiac pathology in African American patients with COVID-19: an autopsy series from New Orleans.*** Lancet Respir Med.2020 May 27. PMCID: PMC7255143 PMID: 32473124]

Admission to Intensive Care:

Critical disease:

1. Respiratory Failure: Respiratory function and oxygenation deteriorate by initially requiring high volume nasal cannulas via face mask with positive and continuous airway pressure (CPAP) or tracheal intubation and mechanical ventilation with volume de 5-6 ml/kg and inspiring pressure of 30 cm H2O or less, increasing the ventilatory frequency and administering Sodium Bicarbonate to keep the arterial pH at 7.3. **The goal is to keep oxygen saturation around 90%, and the fraction of oxygen inspired (FiO2) less than 65% within the first 24-48 hours.** 60-75% of patients improve their oxygenation in a prone position for at least 16 hours per day. They can constitute 5%of cases.

They are stratified into **three subgroups of INTERVENTION**: **Group 1. Inosine Pranobex 4 gm/d. (liquid formulation via nasogastric tube), Zinc Gluconate 100 mg/d., Dexamethasone 4 mg/d decreasing at 1mg/d on the fifth day and Dipyridamole (Persantine) 75 mg/d. Group 2. Hydroxychloroquine (Plaquenil) 600 mg/d, Zinc Gluconate 100 mg/d., Dexamethasone 4 mg/d decreasing to 1mg/d on the fifth day. Dipyridamole (Persantine) 75 mg/d. Hyperalimentation.**

- **Mild ARDS**: 200 mmHg < PaO2/FiO2 ≤ 300 mmHg. In not-ventilated patients or in those managed through non-invasive ventilation by using positive end-expiratory pressure (PEEP) or a continuous positive airway pressure (CPAP) ≥ 5 cmH2O.
- **Moderate ARDS**: 100 mmHg < PaO2/FiO2 ≤ 200 mmHg.
- **Severe ARDS**: PaO2/FiO2 ≤ 100 mmHg.

2. Systemic Inflammatory Response: Thermal extremes (<36C (96.8F) or 38C (100.4F), confusion, hypoxia (saturation <80%), tachypnea (breaths >30 rpm), or PaCO2 <32 mm Hg, hypotension (systolic pressure <90 mm Hg), tachycardia (pulse >100 lpm) and/or insufficiency or failure of multiple organs by micro-thrombosis [**hyperbilirubinemia, acidemia (arterial pH <7), lactate >2 mmol/L (18 mg/dL), coagulopathy (abnormal PT, PTT, Fibrinogen), leukocytosis (>12 K/mm^3), bands 10% or leukopenia (<4 K/mm^3) with lymphopenia, and thrombocytopenia (platelets 110 K/mm^3)**]; **5% of cases** [**Wu Z**, **McGoogan JM**. ***Characteristics of and Important Lessons From the Coronavirus Disease 2019 (COVID-19) Outbreak in China: Summary of a Report of 72 314 Cases From the Chinese Center for Disease Control and Prevention***. JAMA. 2020 Feb 24] They can constitute 5% of cases.

They are stratified into **three subgroups of INTERVENTION**: **Group 1. Inosine Pranobex 4 gm/d. (liquid formulation via nasogastric tube), Zinc Gluconate 100 mg/d., Dexamethasone 10 mg/d**

decreasing to 2mg/d on the fifth day. Dipyridamole (Persantine) 150 mg/d Group 2. Hydroxychloroquine (Plaquenil) 600 mg/d., Zinc Gluconate 100 mg/d., Dexamethasone 10 mg/d decreasing at 2mg/d on the fifth day and Dipyridamole (Persantine) 150 mg/d. Hyperalimentation.

Notes

***Levy MM**., et al. (April 2003). **2001 SCCM/ESICM/ACCP/ATS/SIS International Sepsis Definitions Conference** (PDF). Critical Care Medicine. **31** (4): 1250–6. doi:10.1097/01.CCM.0000050454.01978.3B. PMID 12682500.*

***Bone**RC., et al. (The ACCP/SCCM Consensus Conference. American College of Chest Physicians/*Society of Critical *Care Medicine*(June*1992). **Definitions for sepsis and organ failure and guidelines for the use of innovative therapies in sepsis**. Chest. **101** (6): 1644–55. doi:10.1378/chest.101.6.1644. PMID 1303622.*

Guerin C., et al. ***Prone positioning in severe Acute Respiratory Distress Syndrome*** NEJM 2013 June 6 368 (23) 2159-68

SPECIFIC LABORATORY EVALUATIONS

LABORATORY DETERMINATIONS

CBC, CMP, PT, PTT, CRP, D DIMER, FIBRINOGEN, FERRITIN, ARTERIAL BLOOD GASES

PCR assay

SARS-CoV-2 RNA was assessed by real-time Reverse Transcription-PCR. (Detects 5 RNA copies per reaction, which reflects high sensitivity so it is suitable for worldwide use) Muenchhoff, M.et al. ***Multicentre comparison of quantitative PCR-based assays to detect SARS-CoV-2, Germany, March 2020***. Euro Surveill. 2020 Jun 18; 25(24): 2001057. PMCID: PMC7315722 PMID: 32583765.

Culture

For all patients, 500 µL of the liquid collected from the nasopharyngeal swab should be passed through 0.22-µm pore sized centrifugal filter (Merck millipore, Darmstadt, Germany), then inoculated in wells of 96-well culture microplates, of which 4 wells contained Vero E6 cells (ATCC CRL-1586) in Minimum Essential Medium culture medium with 4% fetal calf serum and 1% glutamine. After centrifugation at 4,000 g, microplates incubated at 37°C. Plates are to be observed daily for evidence of cytopathogenic effect. Presumptive detection of virus in supernatant is done using SU5000 SEM (Hitachi) then confirmed by specific RT-PCR.

END OF THE PROPOSED PROTOCOL. CLINICAL INVESTIGATORS ARE FREE TO MODIFY THIS PROTOCOL TO SUIT THEIR NEEDS

Relevant Clinical Studies

Famotidine is a widely available histamine-2 receptor antagonist that is used to suppress gastric acid production in a wide range of doses from 20mg daily to 160mg four times daily. In computational simulations, Famotidine has been identified as a probable **3-chymotripsin protease inhibitor (3CLpro)**). **A retrospective cohort study identified a significantly reduced risk of death or intubation (adjusted risk ratio 0.43; 95% confidence interval 0.21-0.88) in COVID-19 patients taking Famotidine before or at the time of admission to the hospital.** Some people have self-

medicated with Famotidine while outpatients with COVID-19. *ClinicalTrials.gov identifier (NCT number):* ***NCT04389567***

Freedberg DE., et al. ***The use of famotidine is associated with better clinical outcomes in patients Hospitalized by COVID-19: A retrospective cohort study,*** gastroenterology (2020), doi: https://doi.org/10.1053/ j. gastro.2020.05.053. **A lower maximum ferritin value was observed among users of Famotidine, supporting the hypothesis that the use of Famotidine may decrease Cytokine Release during a SARS-CoV-2 infection. In short, in patients hospitalized with COVID-19 and not initially intubated, the use of Famotidine was associated with a double reduction in clinical deterioration leading to intubation or death. Dosage 10, 20, 40 mg/d.**
https://clinicaltrials.gov/ct2/show/NCT04389567?recrs=e&cond=covid-19&cntry=US&draw=2&rank=2

Dexamethasone 4mg three times a day for 2 days, 2 times a day for 2 days, once a day for 2 days. Moderate Disease with at least a 30% increase in hs-CRP within 36 hours of admission, and with increased oxygen requirements. All patients with Severe Disease have evidence of increased oxygen requirements. Exclusion of the presence of secondary bacterial infections as a likely cause of increased CRP levels. Pulmonary embolism and/or cardiac dysfunction were excluded as likely causes of worsening hypoxia in all selected patients. Effect on transfers to the Intensive Care Unit and the escalation of care that needs mechanical ventilation. Change in CRP levels. Effect on the duration of hospitalization. *ClinicalTrials.gov identifier (NCT number):* ***NCT04445506***
https://clinicaltrials.gov/ct2/show/NCT04445506?recrs=e&cond=covid-19&cntry=US&draw=2&rank=9

Front Line Covid-19 Critical Care Alliance. The clinical study: Recovery trial, conducted by the University of Oxford, reported that Dexamethasone increased survival in 30% of patients in respiratory failure ventilator and 20% of patients requiring oxygen. 1) Methylprednisolone penetrates lung tissue into high concentrations and 2) Based on analysis of activation patterns of inflammation genes induced by SARS-CoV-2, the suppression of activation of these genes by Methylprednisolone suggests greater efficacy than Dexamethasone when used in Covid-19 especially if used longer. So, it is suggested escalation/reduction according to the clinical condition of each patient.

HOSPITAL PROTOCOL OF SARS-CoV - 2: MATH+. [MODIFIED]

CONTROL THE INFLAMATION & EXCESSIVE COAGULATION

In all hospitalized patients, the therapeutic approach should be early intervention utilizing therapies based on scientific evidence to arrest:

— Widespread damage of the inflammatory response
— The severe systemic hypercoagulable state that cause organ damage
This Protocol should **be initiated immediately that the patient complies with the criteria of oxygen supplementation, or has need** of **mechanical ventilation**
The admission to the Intensive Care **Unit decreases dramatically.**

PROTOCOL MATH+ [Used in hospitals for the treatment of SARS-CoV-2]

1. Methylprednisolone [Intravenous]
—A. Mild hypoxia (<4L O2): 40mg/d [up to the time oxygen is not needed]
— B. Disease Moderate–Severe: 80mg bolus, followed of 20mg q6h IV by 7d*
• Alternative:40mg q12h for7d*
— Day 8: Switch to oral Dexamethasone, and eliminate by day 6
***Consider high doses for patients that do not improve their ARDS/oxygenation and/or with persistent, increment, or elevation** several of **marks** of **inflammation (Cytokine** Storm),**i.e. 60–125mg q6h–q8h, or 1,000mg/d** for **3 days.**

2. Ascorbic Acid [infusion]
— 3 grams/100ml q6h
— Continue for 7 days or until hospital discharge

3. Thiamine
— 200mg IV q12h until hospital discharge

4. Levonox [Low Molecular Weight Heparin/LMWH)]]
— A. Patient Stable in medical floor: 0.5mg/kg q12h; If CrCl s 30ml/min, 0.5mg/kg/d
— B. Patient Critical or ICU: 1mg/kg q12h unless contraindicated. Reduce the dose for CrCl by 15–30ml/min
• If CrCl is 15ml/min, use unfractionated heparin [UFH]
• Monitor anti-Xa activity, keep between 0.6–1.1 unit/ml/ml
- Continue to hospital dismissal

5. PLUS optional interventions: Melatonin (6–12mg at night), Zinc (100mg/d), Vitamin D3 (2,000–4,000 units/d), Famotidine (80mg/d), and Magnesium (2g IV in ICU patients, keep the Mg level between 2.0–2.4mmol/l).

HYPOXIA TREATMENT
— If the patient has a digital saturation <88 % with nasal cannula, initiate high volume tempered oxygenation via facial mask with positive and continuous airway pressure (CPAP) **— Do not hesitate to increase the volume if necessary.**
— Avoid early intubation based only on oxygen requirements. Use *permissible hypoxemia* if tolerated.
— Intubate only if the patient shows excessive respiratory work.
— **Use *ventral position* to improve oxygen saturation.**
https://covid19criticalcare.com/

1 McCarthy M et al. ***Inosine Pranobex enhances human Natural Killer cell cytotoxicity by inducing metabolic activation and NKG2D ligand expression.*** Eur. J. Immunol. 2020. 50: 130–137.

Inosine Pranobex is a synthetic immunomodulating compound, indicated for use in the treatment of human papilloma virus-associated warts and subacute sclerosing panencephalitis. Previous studies demonstrate that the immunomodulatory activity of **Inosine Pranobex** is characterized by enhanced lymphocyte proliferation, cytokine production, and NK cell cytotoxicity. The activation of NKG2D signaling on NK cells, CD8+ T cells, and γδ T cells also produces these outcomes. We hypothesized that **Inosine Pranobex** alters cellular immunity through the induction of NKG2 D ligand expression on target cells, hereby enhancing immune cell activation through the NKG2D receptor. We tested this hypothesis and show that exposure of target cells to **Inosine Pranobex** leads to increased expression of multiple NKG2D ligands. Using both targeted metabolic interventions and unbiased metabolomic studies, we **found that Inosine Pranobex causes an increase in intracellular concentration of purine nucleotides and tricarboxylic acid (TCA) cycle intermediates and NKG2D ligand induction**. **The degree of NKG2D ligand induction was functionally significant, leading to increased NKG2D-dependent target cell immunogenicity. These findings demonstrate that the immunomodulatory properties of Inosine Pranobex are due to metabolic activation with NKG2D ligand induction**.

2 Rumel A et al**.** ***Inosine Acedoben Dimepranol promotes an early and sustained increase in the natural killer cell component of circulating lymphocytes: A clinical trial supporting anti-viral indications.***
International Immunopharmacology Volume 42, January 2017, Pages 108-114

Inosine Acedoben Dimepranol, licensed for the treatment of cell-mediated immune deficiencies associated with viral infections, has been reported to impact a variety of immune parameters both *in vitro* and *in vivo*. Here we report the results from a clinical trial where multiple lymphocyte subsets – CD19 + B cells, CD3 + T cells, CD4 + T- helper cells, FoxP3^{hi}/CD25^{hi}/CD127^{lo} regulatory T cells (Tregs), CD3 −/CD56 + NK cells, and CD3 +/CD56 + NKT cells – were, together with serum immunoglobulins and IgG subclasses, followed during 14 days of Inosine Acedoben Dimepranol

administration to ten healthy volunteers; these selected from 27 individuals pre-screened *in vitro* for their capacity to respond to Inosine Acedoben Dimepranol as gauged by increases in the percentage of Treg and/or NKT cells arising in PHA-stimulated cultures. While a transient spike and dip in Treg and T-helper fractions, respectively, was noted, the outstanding consequence of Inosine Acedoben Dimepranol administration (1 g po, qds) was an **early and durable rise in NK cells**. **For half the cohort, NK cells increased as a percentage of total peripheral blood lymphocytes within 1.5 h of receiving drug. By Day 5, all but one of the volunteers displayed higher NK cell percentages, such elevation – effectively a doubling or greater – being maintained at termination of study**. The Inosine Acedoben Dimepranol-induced populations were as replete in Granzyme A and Perforin as basal NK cells. The novel finding of Inosine Acedoben Dimepranol boosting phenotypically competent NK numbers in healthy individuals supports the drug's indicated benefit in conditions associated with viral infection and reinforces the potential for uplift where immune performance may be compromised.

3 Lasek W et al. ***Immunomodulatory effects of inosine Pranobex on cytokine production by human lymphocytes.*** Acta Pharm. 2015 Jun; 65(2):171-80. doi: 10.1515/acph-2015-0015.

Inosine Pranobex (Inosine Dimepranol Acedoben, **Isoprinosine) is an immunomodulatory and antiviral drug used in some viral infections, especially in patients with weakened immunity**. In the present study, **effects of Isoprinosine on the production of cytokines attributable to Th1 (IL-2, IFN-g, and TNF-a) or Th2 cells (IL-4, IL-5, and IL-10) were tested in human peripheral blood lymphocyte cultures stimulated with phytohemagglutinin (PHA). Inosine enhanced TNF-a secretion significantly (in short-term--24-hour, and prolonged term--72-hour cultures) and IFN-g (in 72-hour cultures). Surprisingly, production of IL-10 by PHA- stimulated lymphocytes was suppressed by Isoprinosine in a dose-dependent manner in both 24-hour and 72- hour cultures.** These results shed some light on immunomodulatory properties of Isoprinosine and suggest applicability of this agent in patients with a depressed function of the immune system.

4 Petrova M et al. ***Isoprinosine affects serum cytokine levels in healthy adults.*** J Interferon Cytokine Res. 2010 Apr; 30 (4):223-8. doi: 10.1089/jir.2009.0057.

Isoprinosine is a synthetic purine derivative with immunomodulatory and antiviral properties, which result from an apparent in vivo enhancement of host immune responses. To evaluate the serum levels of certain cytokines during and after Isoprinosine treatment, we assigned 10 healthy volunteers to receive **Isoprinosine 1 g, 3 times daily, 5 consecutive days weekly**. Both treatment and follow-up phase last 3 weeks. Interferon-gamma (IFN-gamma), interleukin-2 (IL-2), IL-10, and tumor necrosis factor-alpha (TNF-alpha) were measured in serum using commercial ELISA kits at baseline, 7th, 10th, 14th, 21st, 28th, 35th, and 42nd day. We observed an **increase in serum levels of all measured cytokines at 7th to 10th day. The levels of IL-2 had another raise at 42nd day after drop to initial values ($P < 0.05$; $P < 0.001$, respectively). Those of IL-10 held up enhanced from 7th to 28th day of measurement ($P < 0.01$). There was a nearly flat line of values of TNF-alpha after initial slight increase at 10th day.** We found a moderate negative correlation between IFN-gamma and IL-2, IL-10, and TNF-alpha (Spearman's r: -0.63, -0.62, - 0.63; $P < 0.05$, respectively). We have demonstrated the immunomodulating properties of Isoprinosine in healthy adults. It suggests resumption of the research with up-to-date methods to elucidate the mechanisms of action of Inosine Pranobex and maybe the other inosine compounds in different clinical settings.

5 Gołebiowska-Wawrzyniak M et al. ***Immunological and clinical study on therapeutic efficacy of Inosine Pranobex.*** Pol Merkur Lekarski. 2005 Sep;19 (111):379-82.

Many studies in vitro and in vivo have shown immunomodulating and antiviral activities of Inosine Pranobex. The object of this research was to examine the potential **beneficial effects of Inosine Pranobex (Groprinosin) on immune system in children with cellular immunodeficiency as a prophylaxis of recurrent infections, mainly of viral origin. 50 mg/kg bw/day of Inosine Pranobex**

in divided doses was given to the group of 30 children aged 3-15 years for 10 days in 3 following months. Clinical and immunological investigations were done before and after the treatment. Statistically significant rise of CD3T lymphocytes number (p = 0.02) and in this CD4T lymphocytes number (p = 0.02) as well as statistically significant improvement of their function (p = 0.005) evaluated with blastic transformation method were found. These laboratory findings were parallel to clinical benefits.

6 Tsang KY et al. ***In vitro restoration of immune responses in aging humans by Isoprinosine.*** Int J Immunopharmacol. 1985;7(2):199-206

The in vitro effects of Isoprinosine (ISO) on the immune responses of aging humans were investigated. **64 healthy elderly humans (65 yr of age or over)** were included in this study. **Four immune parameters were measured, namely, Concanavalin A (ConA)-induced lymphocyte proliferation, natural killer cell (NK) activity, neutrophil chemotaxis, and interleukin-2 (IL-2) production. The ConA-induced lymphocyte proliferation was depressed in 55 of the 64 individuals (85.9%%), while the NK activity was depressed in 41 of the 64 individuals (64%). Neutrophil chemotaxis was depressed in 52 of the 64 individuals (81.1%) and IL-2 production was depressed in 35 of the 64 individuals (54.6%). In the presence of Isoprinosine, ConA-induced lymphocyte proliferation, NK activity, neutrophil chemotaxis, and IL-2 production were restored to normal or near normal levels in 50 of the 55 (90.0%), 35 of the 41 (85.3%), 44 of the 52 (84.6%), and 25 of the 35 (71.4%) aging humans, respectively.** Our results indicate that ISO acts as an immune potentiator in these in vitro immune assays.

7 Balestrino C et al. ***Augmentation of human peripheral blood natural killer activity by Methisoprinol.*** Journal of Biological Response Modifiers, 31 Dec 1982, 2(6):577-585

Methisoprinol was found to augment natural killer (NK) **activity of peripheral blood mononuclear cells** (PBMC), as measured in a 4-h 51Cr release assay using K562 cells as targets. Overnight incubation of PBMC with 0.1 microgram/ml **Methisoprinol**, followed by removal of the drug, resulted in significant increases in the NK activity of all 17 donors studied. Augmentation of NK, expressed as lytic units (LU)/10(7) effector cells, was generally two- to fourfold, and was manifest as early as 1 h after incubation with the drug, but was maximal after 4 h. **The effect of Methisoprinol was dose dependent up to 0.1 microgram/ml and remained at a plateau up to 10 micrograms/ml, where cytotoxicity to the effectors was observed**. The effect of **Methisoprinol** was exerted on the effector cells and was not due to an increased susceptibility of target cells to lysis. In addition, this phenomenon was probably independent of interferon (IFN) production. Binding and killing at the single-cell level were shown to be unaffected by prior treatment with **Methisoprinol**. Rather, the analysis of the kinetics of NK activity indicated that **Methisoprinol** increased the recycling ability of NK effector cells. Augmentation of NK by **Methisoprinol** was dependent on the presence of adherent cells in PBMC fractions. Populations depleted of plastic adherent cells or populations enriched for adherent cells themselves could not undergo boosting by **Methisoprinol**, indicating that **Methisoprinol** did not act to recruit functional cells from inactive precursors. These studies suggest that the observed antiviral and/or immunoregulatory actions of **Methisoprinol** might be mediated through an increase NK cell activity.

8 Sliva J et al. ***Inosine Pranobex: A Key Player in the Game Against a Wide Range of Viral Infections and Non- Infectious Diseases.*** Adv Ther. 2019 Aug;36(8):1878-1905.

Inosine Pranobex, commonly known as Inosine Acedoben Dimepranol, Isoprinosine and Methisoprinol, has been proven to **positively impact the host's immune system, by enhancing T-cell lymphocyte proliferation and activity of natural killer cells, increasing levels of pro-inflammatory cytokines, and thereby restoring deficient responses in immunosuppressed patients. At the same time, it has been shown that it can affect viral RNA levels and hence inhibit growth of several viruses**. Due to its immunomodulatory and antiviral properties, and its safety profile, it has been widely used since 1971 against viral infections and diseases, among which subacute sclerosis panencephalitis, herpes simplex

virus, human papilloma virus, human immunodeficiency virus, influenza and acute respiratory infections, cytomegalovirus and Epstein-Barr virus infections. Following an analysis of almost five decades of scientific literature since its original approval, we here summarize in vivo and in vitro studies manifesting the means in which Inosine Pranobex impacts the host's immune system. We also provide a synopsis of therapeutic trials in the majority of which Inosine Pranobex was found to have a beneficial effect. Lastly, positive results from limited studies, suggesting the putative future use of Inosine Pranobex in new therapeutic indications are briefly described. In order to support use of Inosine Pranobex against viral infections apart from those already approved, and to establish its use in clinical practice, further well-designed and executed trials are warranted.

9 Beran J et al. ***Inosine Pranobex is safe and effective for the treatment of subjects with confirmed acute respiratory viral infections: analysis and subgroup analysis from a Phase 4, randomized, placebo-controlled, double- blind study*****.** BMC Infect Dis. 2016 Nov 7;16(1):648.

Inosine Pranobex (Isoprinosine®) is an immunomodulatory drug approved in several countries for the treatment of viral infections. This study compared the efficacy and safety of Inosine Pranobex versus placebo **in subjects with clinically diagnosed influenza-like illness, including subjects with laboratory-confirmed acute respiratory viral infections. Subgroup analyses evaluated the efficacy of Inosine Pranobex compared to placebo in otherwise healthy (without related ongoing disease) subjects that were less than 50 years of age** and healthy subjects that were at least 50 years of age. The effect of Body Mass Index (BMI) was evaluated in subjects less than 50 years of age. **The study results indicate the safety of Inosine Pranobex for the treatment of subjects with confirmed acute respiratory viral infections and confirm the efficacy of Inosine Pranobex versus placebo in healthy non-obese subjects less than 50 years of age with clinically diagnosed influenza-like illnesses.**

10 Porto BN et al. ***Neutrophil Extracellular Traps in Pulmonary Diseases: Too Much of a Good Thing?*** Front Immunol. 2016 Aug 15; 7:311. doi: 10.3389/fimmu.2016.00311. e Collection 2016.

Neutrophil Extracellular Traps arise from the release of granular and nuclear contents of neutrophils in the extracellular space in response to different classes of microorganisms, soluble factors, and host molecules. Neutrophil Extracellular Traps are composed by decondensed chromatin fibers coated with antimicrobial granular and cytoplasmic proteins, such as myeloperoxidase, Neutrophil Elastase, and α-defensins. Besides being expressed on Neutrophil extracellular traps fibers, Neutrophil Elastase and MPO also regulate Neutrophil Extracellular Traps formation. Furthermore, histone deimination by peptidyl-arginine deiminase 4 (PAD4) is a central step to Neutrophil Extracellular Traps formation. Neutrophil Extracellular Traps formation has been widely demonstrated to be an effective mechanism to fight against invading microorganisms, as deficiency in Neutrophil Extracellular Traps release or dismantling Neutrophil Extracellular Traps backbone by bacterial DNases renders the host susceptible to infections. Therefore, the primary role of Neutrophil Extracellular Traps is to prevent microbial dissemination, avoiding overwhelming infections. However, an excess of Neutrophil Extracellular Traps formation has a dark side. The pathogenic role of Neutrophil Extracellular Traps has been described for many human diseases, infectious and non- infectious. The detrimental effect of excessive Neutrophil Extracellular Traps release is particularly important to lung diseases, because Neutrophil Extracellular Traps can expand more easily in the pulmonary alveoli, causing lung injury. Moreover, Neutrophil Extracellular Traps and its associated molecules are able to directly induce epithelial and endothelial cell death. In this regard, massive Neutrophil Extracellular Traps formation has been reported in several pulmonary diseases, including asthma, chronic obstructive pulmonary disease, cystic fibrosis, respiratory syncytial virus bronchiolitis, influenza, bacterial pneumonia, and tuberculosis, among others. Thus, Neutrophil Extracellular Traps formation must be tightly regulated in order to avoid Neutrophil Extracellular Traps mediated tissue damage. Recent development of therapies targeting Neutrophil Extracellular Traps in pulmonary diseases includes DNA disintegration with recombinant human DNase, neutralization of Neutrophil Extracellular Traps proteins, with anti- histone antibodies

and protease inhibitors. In this review, we summarize the recent knowledge on the pathophysiological role of Neutrophil Extracellular Traps in pulmonary diseases as well as some experimental and clinical approaches to modulate their detrimental effects.

11 Mihara M ***IL-6/IL-6 receptor system and its role in physiological and pathological conditions.*** Clin Sci (Lond). 2012 Feb;122(4):143-59. PMID: 22029668

Inter-Leukin 6 (IL-6), **originally identified as a B-cell differentiation factor, is a multifunctional cytokine that regulates the immune response, haemopoiesis, the acute phase response and inflammation**. IL-6 is produced by various types of cell and influences various cell types and has multiple biological activities through its unique receptor system. **IL-6 exerts its biological activities through two molecules: IL-6R (IL-6 Receptor) and gp130. When IL- 6 binds to mIL-6R (membrane-bound form of IL-6R), homodimerization of gp130 is induced and a high-affinity functional receptor complex of IL-6, IL-6R and gp130 is formed. Interestingly, sIL-6R (soluble form of IL-6R) also binds with IL-6, and the IL-6-sIL-6R complex can then form a complex with gp130. The homodimerization of receptor complex activates JAKs (Janus kinases) that then phosphorylate tyrosine residues in the cytoplasmic domain of gp130. The gp130-mediated JAK activation by IL-6 triggers two main signaling pathways: the gp130 Tyr759-derived SHP-2 (Src homology 2 domain-containing protein tyrosine phosphatase- 2)/ERK (extracellular- signal-regulated kinase) MAPK (mitogen-activated protein kinase) pathway and the gp130 YXXQ-mediated JAK/STAT (signal transducer and activator of transcription) pathway.** Increased IL-6 levels are observed in several human inflammatory diseases, such as rheumatoid arthritis, Castleman's disease and systemic juvenile idiopathic arthritis. IL-6 is also critically involved in experimentally induced autoimmune diseases. All clinical findings and animal models suggest that IL-6 plays a number of critical roles in the pathogenesis of autoimmune diseases. In the present review, we first summarize the IL-6/IL-6R system and IL-6 signal transduction, and then go on to discuss the physiological and pathological roles of IL-6.

12 Jiří Beran et al. ***Inosine Pranobex is safe and effective for the treatment of subjects with confirmed acute respiratory viral infections: analysis and subgroup analysis from a Phase 4, randomized, placebo-controlled, double- blind study.*** BMC Infect Dis. 2016; 16: 648. PMCID: PMC5100179

Acute respiratory infections are categorized as either upper or lower respiratory infections and are caused by well- recognized viral pathogens, including but not limited to influenza virus (types A and B), parainfluenza virus, respiratory syncytial virus (RSV), metapneumovirus (types A and B), **coronavirus**, rhinovirus, enterovirus, reovirus, bocavirus, and adenovirus, and bacterial pathogens, primarily *Streptococcus pneumoniae* and *Haemophilus influenza* [**Osidak LV** et al. ***The results of studying of inclusion of Inosine Pranobex into the therapy of acute respiratory viral infections in children*** [in Russian]. Health Prof. 2012;(10) **Chavan RD** et al. ***Surveillance of acute respiratory infections in Mumbai during 2011-12***. Indian J Med Microbiol. 2015; 33(1):43– 50].

Influenza-like illnesses are considered a subset of acute respiratory infections and result in the sudden onset of symptoms such as fever (body temperature greater than 38 °C), cough, and sore throat in patients [**Otomaru H** et al. ***Influenza and other respiratory viruses detected by influenza-like illness surveillance in Leyte Island, the Philippines, 2010-2013***. PLoS One. 2015;10(4): e0123755. doi: 10.1371/journal.pone.0123755]. Physicians have difficulty with the treatment of Influenza-like illnesses because determining the etiology is generally not possible solely on a clinical basis, Available antiviral treatments for influenza infection are M2 ion channel inhibitors (amantadine and rimantadine) and neuraminidase inhibitors (oseltamivir and zanamivir) The first 36 h is the period of maximal viral replication and antiviral medication is expected to have the most benefit during this time.

Inosine Pranobex stimulates a nonspecific immune response that is independent of the specific viral antigen responsible for the Influenza-like illnesses In clinical studies, Inosine Pranobex has been shown

to induce a type 1 T helper cell-type response in mitogen- or antigen-activated cells, and this response initiates T-lymphocyte maturation and differentiation and potentiates induced lymphoproliferative responses (**Petrova M** et al. ***Isoprinosine affects serum cytokine levels in healthy adults.*** J Interferon Cytokine Res. 2010; 30(4) :223–8; **Lasek W** et al. ***Immunomodulatory effects of Inosine Pranobex on cytokine production by human lymphocytes.*** Acta Pharm. 2015;65(2):171–80; **Yakupova RS** et al. ***Efficacy of immunomodulators in children with respiratory diseases in environmentally poor areas.*** Gig Sanit. 2012; 3:33–4). Similarly, the drug modulates T- lymphocyte and natural killer cell cytotoxicity and CD8+ suppressor and CD4 + -helper cell functions and increases the number of immunoglobulin G and complement surface markers. Inosine Pranobex also increases cytokine interleukin (IL)-1 production and IL-2 production and upregulates the expression of the IL-2 receptor in vitro. The safety profile of Inosine Pranobex has been established through clinical trials for several indications and populations [**Majewska A** et al. ***Inosine Pranobex - cytotoxic activities and effect of on replication of human parainfluenza viruses (HPIV-2, HPIV-4), enteroviruses (CA16, EV71) and adenoviruses (HAdV-2, HAdV-5) in vitro*** [in Polish] Med Dosw Mikrobiol. 2015;67(2):107–13]. A rapid increase in the number of mononuclear cells after the first dose of Inosine Pranobex was observed in 75 % of the subjects, and this increase was consistent with clinical observations of rapid resolution of common cold symptoms [**Krastev Z** et al. ***Isoprinosine induces a rapid lympho-mononuclear response in adult participants.*** Med Inform. 2015;2(1):80–5].

Influenza-like illnesses were defined as an oral temperature of at least 38 °C observed at the study site with at least 1 respiratory symptom of cough, sore throat, or nasal obstruction and at least 1 constitutional symptom of fatigue, headache, myalgia, or feverishness. The respiratory and constitutional symptoms were required to be considered by the subject as moderate or severe in intensity (a score of more than 1 on the 4-point influenza-like symptoms assessment scale). The subjects were required to have experienced the onset of Influenza-like illnesses no more than 36 h prior to screening, where onset is defined as the time when the subject experienced fever and at least 1 respiratory symptom and at least 1 constitutional symptom. Inosine Pranobex or placebo 500-mg tablets were self-administered by the subjects for 7 days (2 tablets orally 3 times daily). Efficacy of Inosine Pranobex in subjects with clinically diagnosed ILI, including subjects with laboratory- confirmed acute respiratory viral infections due to influenza A or B virus, RSV, adenovirus, or parainfluenza virus 1 or 3.

Immuno-senescence, ie, the age-related decline of the immune system, and obesity play an important role in the efficacy of the immune response to pathogens [**Stervbo U** et al. ***Effects of aging on human leukocytes (part II): immunophenotyping of adaptive immune B and T cell subsets. Age*** (Dordr) 2015;37(5):93. doi: 10.1007/s11357-015- 9829-2.

13. Sheridan PA et al. ***Obesity is associated with impaired immune response to influenza vaccination in humans.*** Int J Obes (Lond) 2012;36(8):1072–7].

Older subjects show a diminished immune response to pathogens, which increases their risk for severe infection and compromises their ability to adequately combat viral infections. Low response resulted in increased susceptibility to influenza and associated complications in older adults compared to younger adults who typically benefit from a higher response [**Pera A** et al. ***Immunosenescence: implications for response to infection and vaccination in older people***. Maturitas. 2015; 82(1):50–5.; **Stervbo U** et al. ***Effects of aging on human leukocytes (part I): immunophenotyping of innate immune cells. Age*** (Dordr) 2015;37(5):92]. Obesity has also been identified as an independent risk factor for increased susceptibility to influenza virus infection; this susceptibility results from diminished CD4+ and CD8+ T-cell responses and lower influenza vaccine antibody levels [**Paich HA** et al. ***Overweight and obese adult humans have a defective cellular immune response to pandemic H1N1 influenza A virus***. Obesity (Silver Spring) 2013;21(11):2377–86; **Milner JJ** et al. ***Obesity and influenza infection severity***. Future Virol. 2014;9(3):223–5]. Obesity may also increase the risk of pneumonia or other infections by restricting lung volume [**Milner JJ** et al. ***The impact of obesity on the immune response to infection***. Proc Nutr

Soc. 2012;71(2):298–306]. They may take longer to recover from illnesses such as influenza and anti-influenza drugs may not be as effective. non-obese (BMI <30 kg/m^2).

14 Jie-ying Liao et al. ***Inosine-Containing RNA Is a Novel Innate Immune Recognition Element and Reduces RSV Infection***. PLOS ONE, **Oct 2011**

During viral infections, **single- stranded RNA** (**ssRNA**) and **double-stranded RNA** (**dsRNA**) **are recognized by the host and induce innate immune responses. The cellular enzyme ADAR-1 (adenosine deaminase acting on RNA-1) activation in virally infected cells leads to presence of Inosine-containing RNA (Ino-RNA).** Here we report that **single- stranded Inosine RNA (ss-Ino-RNA**) **is a novel viral recognition element**. We synthesized unmodified ssRNA and ssRNA that had 6% to16% inosine residues. **The results showed that in primary human cell**s, or in mice, **10% ss- Ino-RNA rapidly and potently induced a significant increase in inflammatory cytokines, such as interferon (IFN)-β (35 fold), tumor necrosis factor (TNF)-α (9.7 fold), and interleukin (IL)-6 (11.3 fold)** ($p<0.01$). Flow cytometry data revealed a corresponding **4-fold increase in influx of neutrophils into the lungs** by ss-Ino-RNA treatment. In our in vitro experiments, **treatment of epithelial cells with ss-Ino-RNA reduced replication** of respiratory syncytial virus (RSV). Interestingly, RNA structural analysis showed that ss-Ino-RNA had increased formation of secondary structures. Our data further revealed that **extracellular ss-Ino-RNA was taken up by scavenger receptor class-A (SR-A) which activated downstream MAP Kinase pathways through Toll-like receptor 3 (TLR3) and dsRNA-activated protein kinase (PKR)**. Our data suggests that ss-Ino-RNA is an as yet undescribed virus-associated innate immune stimulus.

15 Hudock KM et al. ***Neutrophil extracellular traps activate IL-8 and IL-1 expression in human bronchial epithelia.*** 11 MAR 2020 https://doi.org/10.1152/ajplung.00144.2019

Neutrophil Extracellular Traps provide host defense but can contribute to the pathobiology of diverse human diseases. We sought to determine the extent and mechanism by which **Neutrophil Extracellular Traps** contribute to human airway cell inflammation. **Methods**: Primary **normal Human Bronchial Epithelia** grown at air liquid interface and wtCFBE41o- cells (expressing wtCFTR) were exposed to cell-free **Neutrophil Extracellular Traps** from unrelated healthy volunteers for 18 hours in vitro. Cytokines were measured in the apical supernatant by Luminex and the effect on the **Human Bronchial Epithelia** transcriptome was assessed by RNA sequencing. **Results: Neutrophil Extracellular Traps consistently stimulated IL-8, TNF-α and IL-1α secretion by Human Bronchial Epithelia** from multiple donors, with variable effects on other cytokines (IL-6, G-CSF, GM-CSF). **Human Bronchial Epithelia RNAs encoding IL-1 family cytokines, particularly IL-36 subfamily members, were increased in response to Neutrophil Extracellular Traps**. **Neutrophil Extracellular Trap** exposure in the presence of anakinra (recombinant human IL- 1RA) dampened **Neutrophil Extracellular Trap**-induced changes in IL-8 and TNF-α proteins as well as IL-36α RNA. RhIL-36RA limited the increase in proinflammatory cytokine RNAs in **Human Bronchial Epithelia** exposed to **Neutrophil Extracellular Traps**. **Conclusion**: **Neutrophil Extracellular Traps selectively upregulate an IL-1 family cytokine response in Human Bronchial Epithelia, which enhances IL-8 production** and is limited by rhIL-1RA. Present findings describe a unique mechanism by which **Neutrophil Extracellular Traps** may contribute to inflammation in human lung disease in vivo. **Neutrophil Extracellular Trap**-driven IL-1 signaling may represent a novel target for modulating inflammation in diseases characterized by a substantial **Neutrophil Extracellular Trap** burden.

RESOURCES FOR THE GENERAL PUBLIC

Chemical Name Inosine, compd. wit 1-(dimetylamino)-2-propanol 4-(acetylamino)benzoate (salt) (1:3)
Foreign Names Inosiplex (German) Metisoprinol (Spanish)
Generic Names Inosine Pranobex, Inosine acedobene dimepranol, Inosiplex, Isoprinosin, Methisoprinol (IS)

Brand Names

- **Delimmun 120, 25mg**; **375, 75mg**: KoRa Healthcare, Germany
- **Eloprine**: Polfarmex, Latvia; Polfarmex, Poland
- **Groprinosin**; **Groprinosin Baby**: Gedeon Richter, Georgia and, Poland; Gedeon Richter, Russian Federation
- **Imin**: Yung Shin, Taiwan
- **Immunosin**: Westmont, Philippines
- **Imunovir**: Kora Healthcare, Canada; Kora Healthcare, United Kingdom; Kora Healthcare, Ireland; Farmayala, Ecuador
- **Inoseda**: World Medicine, Georgia
- **Inosine Health Chemical**: Health Chemical, Taiwan
- **Ishutin**: Panion & BF, Taiwan
- **Isolin**: Johnson, Taiwan
- **Isoprinosine**: **Armstrong Laboratorios de Mexico, Mexico**; Darya-Varia, Indonesia; Ewopharma, Czech Republic; Ewopharma, Lithuania; Ewopharma, Latvia; Ewopharma, Poland; Ewopharma, Slovakia; **Laboratorios Andromaco, Chile; Lukoll, Peru; Lusomedicamenta,** Macedonia; Lusomedicamenta Sociedade Tecnica Farmaceutica, Serbia; Mochida Pharmaceutical, Japan; MUP, Egypt; Newport, Kuwait; Newport, Philippines; Panbiotic, Taiwan; Pediatrica, Indonesia; Sanofi Belgium, Belgium; Teva Pharmaceutical Industries, Russian Federation
- **Isoprinosine 500mg; 50 mg/ml**: Ewopharma, Lusomedicamenta Bulgaria; Ewopharma, Hungary; Sanofi Belgium, Luxembourg
- **Isoprinosine Ewopharma**: Ewopharma, Romania
- **Isprinol**: Novell Pharmaceutical, Indonesia
- **Izoreks 500mg**; **50 mg/ml;** ABC, Bulgaria
- **Izzoprin 1000mg; 500 mg, 50 mg/ml**: Adipharm, Bulgaria
- **Laprosin**: Lapi Laboratories, Indonesia
- **Methisine**: Union, Taiwan
- **Methisoprinol Phapros**: Phapros, Indonesia
- **Neosine**, **Neosine forte**: Aflofarm Farmacja, Poland
- **Normomed**: ABC Farmaceutici, Russian Federation
- **Pranosine**: **Sanfer, Mexico**
- **Pronovir**: Meprofarm, Indonesia
- **Qualiprinol**: Quality Pharm, Hong Kong
- **Viridis**: Pharos, Indonesia
- **Virux**: Royal, Taiwan
- **Viruxan**: **Far-Med, Paraguay; Sigma-Tau Industrie Farmaceutiche Riunite, Italy**
- **Visoprine**: Phapros, Indonesia